MONASH University

Bioprocessing and immobilization of cell envelope proteinases from *Lactobacillus delbrueckii* subsp. *lactis* 313, for protein degradation

By

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A thesis submitted for the degree of Doctor of Philosophy

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GENERAL DECLARATION

Monash University Institute of Graduate Research

Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 6 original papers and book chapters published in peer reviewed journals and edited books and 2 manuscripts under review. The core theme of the thesis is "**Bioprocessing and immobilization of lactobacilli proteinases**". The ideas, development and writing up of all the papers in the thesis were the principal responsibility of me, the candidate, working within the Department of Chemical Engineering under the supervision of Dr. Lizhong He, Dr. Ravichandra Potumarthi, Dr. Michael Kobina Danquah, and Prof Michael James.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

The following international peer-reviewed academic journal articles are presented in this thesis:

Item No.	Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
1	2	Production of lactobacilli proteinases for the manufacture of bioactive peptides: Part I—upstream processes	Published *Marine proteins and peptides*, Kim, S.-K., Ed. John Wiley & Sons, Ltd: 2013; pp 207-229	Initiation and Writing up [80%]
2	2	Production of lactobacilli proteinases for the manufacture of bioactive peptides: Part I—downstream processes	Published *Marine proteins and peptides*, Kim, S.-K., Ed. John Wiley & Sons, Ltd: 2013; pp 207-229	Initiation and Writing up [80%]
3	3	In-depth characterisation of *Lactobacillus delbrueckii* subsp. *lactis* 313 for growth and cell-envelope-associated proteinase production	Published *Biochemical Engineering Journal* **2012**, *64*, 61-68	Key ideas, Experimental, Development, Results interpretations, Writing up [80%]
4	3	Optimisation of batch culture conditions for cell-envelope-associated proteinase production from *Lactobacillus delbrueckii* subsp. *lactis* ATCC® 7830™	Published *Applied Biochemistry and Biotechnology* **2012**, *168*, 1035-1050.	Initiation, Key ideas, Experimental, Development, Results interpretations, Writing up [80%]
5	4	Bioanalytical evaluation of *lactobacillus delbrueckii* subsp. *Lactis* 313 cell-envelope proteinase extraction.	Published *Chemical Engineering Science* **2013**, *95*, 323–330	Initiation, Key ideas, Experimental, Development, Results interpretations, Writing up [80%]

The following manuscripts are under review and also presented in this thesis:

Item No.	Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
6	5	Quick and low cost immobilization of proteinases on polyesters: comparison of lactobacilli cell-envelope proteinase and trypsin for protein degradation	Submitted *Journal of Biotechnology (2013)* (manuscript number: JBIOTEC-D-13-01446)	Key ideas, Experimental Development, Results interpretations, Writing up [65%]
7	6	Evaluation of cross-linked enzyme aggregates from *Lactobacillus delbrueckii* subsp. *lactis* 313 cell-envelope proteinases, for protein degradation	Submitted *Food and Bioprocess Technology (2013)* (manuscript number: FABT-S-13-01918)	Initiation, Key ideas, Experimental, Development, Results interpretations, Writing up [70%]

The following related peer-reviewed academic journal articles are also presented in this thesis:

Item No.	Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
8	APPENDIX – C	Carbohydrate utilization affects *lactobacillus delbrueckii* subsp. *Lactis* 313 cell-envelope-associated proteinase production.	Published *Biotechnology and Bioprocess Engineering* **2012**, *17*, 787-794	Initiation, Key ideas, Experimental, Development, Results interpretations, Writing up [90%]

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ___ [redacted] _____

Date: Monday 16th December 2013

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ACKNOWLEDGEMENTS

"*Magna opera Domini exquisita in omnes voluntates ejus*"

"The works of the Lord are great, sought out of all them that have pleasure therein"

[Psalm CXI: 2].

The intricacies of a biological system are intriguing and for me, the rational intelligibility of the Universe is the foundation and rationale why I study Science. The Psalmist is right when he states "the works of the Lord are great [and it is] studied by all who have pleasure in it". It is no wonder this same text was inscribed on the doors of the *Laboratory for Experimental Physics-Cambridge*, 1874, under the leadership of Professor James Clerk Maxwell! I therefore give special thanks beyond measure to Almighty God and Father our Lord Jesus Christ. His gifts of life, strength and wisdom have been my portion from dusk to dawn, bringing me thus far.

I also wish to express my deepest gratitude to all and sundry who have been undoubtedly very supportive through the writing, development and production of this work. My deep gratitude and appreciation to my supervisors: Drs. Lizhong He, Michael K. Danquah, Ravichandra Potumarthi and Prof Michael James who provides detailed guidance throughout this research project. I am also indebted to Ms. Lilyanne Price for the help she rendered me from the time I was applying for admission to the completion of my PhD. Special thanks also go to the entire membership of the BEL Research Group over the years, for their help and support. Many thanks to the Monash University and the Department of Chemical Engineering, for providing scholarship and an opportunity to study in this noble university. The slogan "*Ancora imparo*" will ever stay with me

and spur me on to higher levels of academic and research exploits for the benefit of mankind and out planet.

I also duly acknowledge the Australian Institute of Nuclear Science and Engineering (AINSE) for the Postgraduate Award offered me which allowed me access to advanced equipment and facilities, as well as high profile research staff at the Australian nuclear Science and Technology Organization.

I am also greatly indebted to family and friends for their unfailing support, love and prayers. These include the Café Care Clayton Committee, the Christian Fellowship Church – Melbourne, and the Monash Uniting Church. I could not do without you. Special thanks to my dear Mum, Auntie Agartha Arhin. You are the best. Due acknowledgement is also given to my mentor, Mr Mensah Fosuaba Banahene, for his immense help, support, advice and guidance in all matters of life. Lastly, my heartfelt appreciation is to my beloved Ellen Asare Bediako. Your encouragements, patience, support and love is precious. You are so sweet and shall ever be my *Saccharin!*

ABSTRACT

Proteolytic enzymes are a useful class of biomolecules due to their ubiquity and the plethora of physiological roles they play in living systems. These enzymes are responsible for the breakdown of proteins to peptides and have several applications in food, pharmaceuticals, diagnostics, photographic, waste treatments, bioremediation, and in the textile industry.

Cell-envelope proteinases (CEPs) are a special class of industrially relevant extracellular proteolytic enzymes obtained from lactic acid bacteria. In the food industry, CEPs have been known to improve the texture and organoleptic characteristics of dairy products and also have the potential to release bioactive peptides encrypted in dairy proteins. However, research is lacking on detailed optimisation of fermentation parameters essential for the generation of CEPs of organisms in the genera *Lactobacillus*. The proteolytic system and CEPs from lactobacilli are also not fully characterized and further; the use of free CEPs in industrial processes is currently suboptimal and presents certain drawbacks such as poor operational stability. Regulatory requirements such as that of the United Nations Food and Agriculture Organization demand the separation of enzymes from certain food and pharmaceutical products when enzymes are involved in the production process. This is difficult to do, if not impossible, for soluble enzymes. These make soluble CEP-based process economically unfeasible, especially when combined with the usual challenges associated with the use of most soluble enzymes, i.e. high cost, poor stability, and lack of multiple utility. This study therefore explored the production of CEPs expressed in *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) in a cost effective manner. Immobilization techniques were also deployed for the design and production of cheap, reusable and stable biocatalysts of LDL 313 CEPs, for use in protein degradation.

LDL 313 was an understudied bacterium, thus, initial work considered it's growth characterization for the purpose of CEP production. As expected, cell growth was dependent on fermentation conditions such as temperature and initial pH. However, cell growth rates under anaerobic conditions were markedly higher than growth under microaerophilic conditions. The caseinolytic specificity of LDL 313 CEPs was also identified. Being proteolytic for for β-casein and κ-casein, CEPs from LDL 313 were classified as class I CEP (CEP_I) of the lactococcal proteinase classification system. Studies were also done to optimize the batch culture conditions that enhance CEPs expression. Using a combination of conventional sequential techniques, the batch growth conditions (inoculum concentration, culture agitation speed, incubation temperature, starting pH, and carbon/nitrogen ratio of production medium) were optimized, for the first time, to ensure profuse CEP production in LDL 313.

Moreover, since CEPs are cell-envelope-bound enzymes they are relatively easy to extract from lactic acid bacteria cells. When CEP extraction was studied with different extraction agents, 5 M LiCl was observed to be the most suitable. Sub-cellular localization studies also showed that

about 95% of CEP activity was detected in cell-wall fractions implying that CEPs in LDL 313 are located in the peptidoglycan cell-wall. Together, these results provide insights into conditions and parameters that ensure optimum cell growth, high CEP yields and extraction protocol to release high levels of CEPs.

Following this, two different strategies for making stable biocatalysts from CEPs were explored, namely, the immobilization of enzymes onto a fabric carrier, and, cross-linking enzyme aggregates. Firstly, CEPs and trypsin (as a model enzyme in comparison with CEP) were immobilized in a simple, cheap and quick approach onto polyester via support functionalization with ethylene diamine and cross-linking with glutaraldehyde. Secondly, cross-linked enzyme aggregates were prepared from CEPs via coupled precipitation/cross-linking with ammonium sulphate and glutaraldehyde respectively. The immobilized biocatalysts had good activity characteristics and properties. For example, immobilized enzymes had good recovered activity (85% for CEP immobilized on polyester; and ~ 22% and ~ 41% respectively for CLCEPA prepared in the absence and presence of proteic feeders). They also had the ability to be used at other than usual conditions such as high temperatures (40 - 70 °C); and organic solvent conditions (CLCEPA still retained activity in 20 – 100 % ethanol/buffer mixtures). Both immobilized enzymes had the ability to be recycled (CLCEPA retained ~ 22% initial activity whereas CEPs immobilized on polyester retained ~ 41% when recycled 5 times. Additionally, both immobilized biocatalyst had proteolytic properties, effectively hydrolysing several macromolecular proteins substrates (casein, bovine serum albumin, whey protein isolate, β-lactoglobulin, skimmed-milk protein and chicken egg albumin). The immobilized CEPs biocatalysts could be utilized for protein degradation to obtain two streams of industrially relevant products namely protein/peptide-rich product mixture from casein; and whey protein/peptide-based surface active foams – both of which can be used for food applications.

In summary, the potential of *Lactobacillus delbrueckii* subsp. *lactis 313* to produce cell-envelope proteinases was explored and the development of stable forms of these enzymes was studied, optimized and tested with several food proteins. The outcome is a whole systems approach to developing and establishing an enzyme framework for protein degradation in a cheap cost-effective manner. This therefore has the potential for various industrial applications in protein degradation and/or peptide production.

Keywords: bioprocessing, cell-envelope proteinases, enzyme immobilization, protein degradation

DETAILS OF PUBLICATIONS FROM THESIS

Details of the findings in this dissertation are described in 9 published/submitted peer reviewed international journal papers, 1 invention disclosure (for patent application), 8 peer-reviewed symposia /conference proceedings and 2 book chapters. This constitutes the requirements based on Monash University guidelines for PhD thesis by publications.

Peer-Reviewed Journal Papers

1) **Agyei D**, Danquah MK (2012) In-depth characterisation of *Lactobacillus delbrueckii* subsp. *lactis 313* for growth and cell-envelope-associated proteinase production. *Biochemical Engineering Journal* 64 (2012):61-68. doi:10.1016/j.bej.2012.03.006 **[INCLUDED IN THESIS]**

2) **Agyei D**, Potumarthi R, Danquah MK (2012) Optimisation of batch culture conditions for cell-envelope-associated proteinase production from *Lactobacillus delbrueckii* subsp. *lactis* ATCC® 7830™. *Applied Biochemistry and Biotechnology.* doi:10.1007/s12010-012-9839-9 **[INCLUDED IN THESIS]**

3) **D Agyei**, W Lim, M Zass, D Tan, M K. Danquah. Bioanalytical Evaluation of *Lactobacillus delbrueckii* subsp. *lactis* 313 Cell-Envelope Proteinase Extraction. *Chemical Engineering Science* (2013), doi: 10.1016/j.ces.2013.03.049 **[INCLUDED IN THESIS]**

4) **D Agyei, S Tambimuttu, B Kasargod, Y Gao, LHe.** Quick and low cost immobilization of proteinases on polyesters: comparison of lactobacilli cell-envelope proteinase and trypsin for protein degradation. *Journal of Biotechnology*. Under review. **[INCLUDED IN THESIS**]

5) **D Agyei & LHe** Evaluation of cross-linked enzyme aggregates from *Lactobacillus delbrueckii* subsp. *lactis* 313 cell-envelope proteinases, for protein degradation. *Food Bioprocess Technology*. Under review. **[INCLUDED IN THESIS]**

6) **Agyei D**, Danquah M (2012) Carbohydrate utilization affects *Lactobacillus delbrueckii* subsp. *lactis* 313 cell-envelope-associated proteinase production. *Biotechnology and*

Bioprocess Engineering 17 (4):787-794. doi:10.1007/s12257-012-0106-2 **[INCLUDED IN THESIS - APPENDIX]**

7) **Agyei D**, Danquah MK (2012) Rethinking food-derived bioactive peptides for antimicrobial and immunomodulatory activities. *Trends in Food Science & Technology* 23 (2012):62-69. doi:10.1016/j.tifs.2011.08.010 (Review)

8) Danquah MK and **Agyei D** (2012). Pharmaceutical applications of bioactive peptides. *OA Biotechnology* 1(2):5 (Review)

9) **Agyei D**, Danquah MK (2011) Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. *Biotechnology Advances* 29 (3):272-277. doi:10.1016/j.biotechadv.2011.01.001 (Review)

Conferences / Symposia presentations (Peer reviewed)

1) **D. Agyei**, **L. He**. Abstract of Oral Presentation. 3rd Annual Monash University Chemical Engineering Conference, Monash University, Melbourne, Australia, 2013; (Bioprocessing and immobilization of cell envelope proteinases from *Lactobacillus delbrueckii* subsp. *lactis* 313, for protein degradation). *Oral Presentation*

2) **D. Agyei, L. He**. Abstract of Presentations. Humboldt Colloquium - Sydney, Australia, 2013. (Cross-linked enzyme aggregates from Lactobacilli cell-envelop proteinase: design and evaluation of peptide production properties); *Poster Presentation*

3) **D. Agyei**, S. Tambimuttu, Y. Gao, L. He. Peptide Production from Milk Proteins by Enzyme Immobilized on Polyester) – accepted for Oral Presentation at the 8th NIZO Conference 2013, Netherlands

4) **D. Agyei**, M. K. Danquah, R. Potumarthi. (Cell-envelope Proteinases of *Lactobacillus delbrueckii* subsp. *lactis* 313: Production and Use in (Bioactive) Peptide Preparation) – accepted for Poster Presentation at the 8th NIZO Conference 2013, Netherlands

5) **D. Agyei**, and L. He. Abstract of Poster Presentations. OCE Cutting Edge Symposium – CchemBiocat, CSIRO, Parkville 2012; (Enzyme-based bioprocess for production of bioactive peptides from milk proteins).

6) **D. Agyei**, R. Potumarthi, M. K Danquah. Abstract of Oral Presentation. 2nd Annual Monash University Chemical Engineering Conference, Monash University, Melbourne, Australia, 2012; (Process variables for enhancing cell-envelope proteinase yield in *Lactobacillus delbrueckii* subsp. *lactis* 313).

7) W. Lim, M. Zass, D. Tan, **D. Agyei**, M. K. Danquah, R. Potumarthi. Poster Presentation. 2nd Annual Monash University Chemical Engineering Conference, Monash University, Melbourne, Australia, 2012; (Extraction of cell-envelope proteinases from *Lactobacillus delbrueckii* subsp. *lactis* 313).

8) **Agyei D** and Danquah MK. Abstract of Papers, CHEMECA, Sydney, Australia, 2011; Abstract 431. (Effect of sugar on *Lactobacillus delbrueckii* subsp. *lactic* 313, growth and proteinase production).

Scholarly Book Chapters

1) **Agyei, D**., Potumarthi, R., & Danquah, M. K. (2013). Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part I—Upstream Processes. In S.-K. Kim (Ed.), *Marine Proteins and Peptides* (pp. 207-229): John Wiley & Sons, Ltd.

2) **Agyei, D**., Potumarthi, R., & Danquah, M. K. (2013). Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part II—Downstream Processes. In S.-K. Kim (Ed.), *Marine Proteins and Peptides* (pp. 231-251): John Wiley & Sons, Ltd.

CHAPTER 1

INTRODUCTION

1. CHAPTER 1: Introduction

1.1. Background

Proteolytic enzymes are ubiquitous biomolecules present in all life forms. This unique class of biomolecules play an important role in cellular metabolic processes and have therefore gained enormous industrial and research attention (Rao et al., 1998). Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially. The current estimated value of the worldwide sales of industrial enzymes is expected to reach $8.5 billion in 2015 (TMR, 2013). Hydrolases account for about 80% of the current industrial enzymes (Galante and Formantici, 2003) and proteases are among the most industrially exploited and well studied of the hydrolases (Gupta et al., 2002; Kirk et al., 2002). Proteases are among the three largest groups of industrial enzymes, accounting for close to 60% of the total global sale of enzymes (Rao et al., 1998). They find use in industrial market sectors such as the medical, diagnostics, food, cosmetic, pharmaceuticals, leather, detergent, textile, photographic, bioremediation, and waste management sectors (Gupta et al., 2002; Najafi et al., 2005; Subba Rao et al., 2009).

Cell-envelope proteinases (CEPs) are a class of proteases produced by lactic acid bacteria and have several applications (Tsakalidou et al., 1999; Sadat-Mekmene et al., 2011; Agyei and Danquah, 2012). They are known to improve the texture and organoleptic characteristics of dairy products (Espeche Turbay et al., 2009). Recently, the application of CEPs is the production of bioactive peptides has been highlighted and this role of CEPs in releasing physiologically active

peptide encrypted in food proteins is gaining research attention (Kitts and Weiler, 2003; Korhonen and Pihlanto, 2006; Möller et al., 2008; Korhonen, 2009; Danquah and Agyei, 2012). However, the full potential of CEPs in the aforementioned applications is not fully exploited. This is partly because the proteolytic system of many lactobacilli species has not been fully described (Tsakalidou et al., 1999; Espeche Turbay et al., 2009). For example, *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) is one of the three key lactobacilli that play an important role in the dairy industry (Forsman and Alatossava, 1991; Delley and Germond, 2002; Germond et al., 2003). However, despite this and other technological applications, scientific information is scant on the proteolytic properties of LDL 313 (Agyei and Danquah, 2012; Agyei et al., 2012).

Although CEPs have numerous potential, there exist some limitations to their production in large quantities to satisfy the growing market demands. Laboratory-scale processes for CEP production are not optimised and routinely results in low volumetric titres. Compared with alkaline proteases from *Bacillus* spp., the commercial development of fermentation processes for proteinase production by lactobacilli is almost non-existent. To meet market demands fermentation bioprocesses for producing CEPs must ensure production at large scale and at low cost. Research efforts aimed at improving microbial enzyme yields usually focuses on strain improvement and optimization of the fermentation medium and production conditions (Kumar and Takagi, 1999). Strain improvement is often achieved by the use of systems and synthetic biology techniques (Liu et al., 2013). Also, to be cost-effective, fermentation medium must be derived from readily available, renewable, and inexpensive raw materials (Gupta et al., 2002a; Gupta et al., 2002b; Posch et al., 2012). However, not much work has been done in these two areas for the lactobacilli in order to improve CEP production. There is therefore the need to

develop a scalable and economically feasible technology for the production of CEPs from lactobacilli.

Furthermore, a large variety of most enzymes catalyse bio-reactions under mild conditions of temperature and pH. However, for industrial applications, some degree of 'hardiness' in enzyme properties is often desired to allow the enzyme to survive 'other-than-usual' conditions that may be present in the industrial processes. Qualities such as good thermal and storage stability and sustained activity over the range of desired reaction conditions (e.g. extreme pH, elevated temperature, organic solvents, mechanical stress) are among the most desired (Chen et al., 2006; Brady and Jordaan, 2009). Recyclability of the same lot of enzyme is another useful property (Mateo et al., 2007), as well as the separation of products from biocatalyst (FAO/WHO, 2006; Opwis, 2010). These expectations have economic implications and in many cases are hard to be met by the use of most soluble enzymes including CEPs. CEPs have poor stability, and the currently existing CEP-based processes are not economically feasible (Exterkate, 2000). All these hamper the application of CEPs at industrial scale.

Several enzyme immobilization techniques have been used to enhance enzyme activity, improve operational stability and recyclability. The four most widely used immobilization techniques are adsorption, entrapment, cross-linking, and covalent binding. (Wang and Hsieh, 2004; Mateo et al., 2007; Sheldon, 2007; Brady and Jordaan, 2009; Opwis, 2010; Sen et al., 2012). However, the choice of immobilization method is dependent on the enzyme (e.g. biochemical and kinetic properties), the carrier materials (e.g. cost, chemical characteristics and mechanical properties) and the immobilization method (e.g. stability, toxicity, efficiency) (Tischer and Wedekind,

1999). The interplay of these factors determines the final recovered activity of the immobilized enzyme. For an enzyme like CEP, studies are needed to select the best immobilization method and to establish the optimum parameters and conditions that enhance the immobilization efficiency. This study therefore explored the production of CEPs expressed in *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) in a cost effective manner. Two immobilization technologies, namely, covalent attachment of enzyme onto polymer fabric, and cross-linking of enzyme aggregates were also deployed for the design and production of reusable and stable immobilized CEPs from LDL 313, for use in the degradation of food proteins.

1.2. Scope of research

A short running title for this project is "bioprocessing and immobilization of lactobacilli proteinases". Specifically, the key task was to explore the production and immobilization of cell-envelope proteinases (CEPs) expressed from *Lactobacillus delbrueckii* subsp. *lactis 313*, to be used for protein degradation.

As described above, CEPs are an important class of proteolytic enzymes. However, their full potential has not been exploited due to the dearth of scientific background information on lactobacilli CEPs. Additionally, biochemical characterisation of CEPs from some lactobacilli has not been fully described (Tsakalidou et al., 1999). As such, little has been done in the area of proteinase immobilization to improve enzyme performance and overcome the challenges faced with the use of soluble proteinases. Addressing these challenges will require detailed understanding and optimisation of the entire bioprocess (from bioreaction through to enzyme immobilization).

For this study, the wild type organism was used and the part of the study aimed at improving proteinase yield was done by optimizing certain fermentation parameters and cell growth conditions; recombinant DNA technology was not used.

The final expected outcome of this project was a complete and commercially viable bioprocess based on immobilized CEPs for the hydrolysis of proteins to hydrolysates. The degradation of proteins has numerous industrial applications. However, for this project, the focus of application is value-added ingredients to be used in preparing physiologically- or functionally-active food and pharmaceutical products. What is desired in these two areas of application is a complete cGMP compatible food-grade bioprocess which is cost effective and scalable. Food-grade materials and reagents were therefore chosen for the various aspects of this study, especially for the preparation of immobilized enzymes. This was necessary to ensure that the final desired outcome is a food-grade bioprocess.

1.3. Aim and objectives

This study was aimed at the development of an integrated bioprocess involving production and immobilization of cell envelope proteinases from *Lactobacillus delbrueckii* subsp. *lactis 313;* to yield stable, cheap, commercially viable biocatalyst for use in protein degradation. The specific objectives are as follows:

1. To study and establish the growth kinetics and metabolic processes responsible for profuse cell growth and enhanced proteinase production by *Lactobacillus delbrueckii* subsp. *lactis* 313, and partially characterize the proteolytic system of this lactobacillus species. The culture conditions necessary to improve the expression of cell-envelope-

associated proteinase from *Lactobacillus delbrueckii* subsp. *lactis* 313 will also be studied and optimized.

2. To study and evaluate the extraction of cell-envelope-associated proteinase from *Lactobacillus delbrueckii* subsp. *lactis* 313 by the use of different extraction agents and under different process conditions

3. To deploy enzyme immobilization techniques for the design and production of cheap, reusable and operationally stable cell-envelope proteinases biocatalysts from *Lactobacillus delbrueckii* subsp. *lactis* 313.

4. To utilize the immobilized enzymes for protein degradation in order to obtain industrially relevant products such as protein/peptide mixtures and food peptides, and preliminarily test their functions such as foaming properties.

1.4. Outline of the thesis

The thesis takes the format of "thesis by publication", as outlined in the Monash University Handbook for Doctoral and MPhil Degrees (MIGR, 2013). With the exception of Chapters 1 and 2, the experimental section of this dissertation (i.e. Chapters 3 to 6) is divided into two parts – I and II. Part I deals with the upstream and downstream processes for the production and extraction of CEPs from LDL 313. Part II highlights the development of stable biocatalysts from CEPs by the use of immobilization techniques. Various applications of immobilized CEPs for protein degradation are also presented in Part II.

Chapter 1 – General introduction

This chapter gives a general introduction and establishes the research background, project objectives and scope of the research. The organization and outline of the thesis is also given in this chapter.

Chapter 2 – Literature review

This chapter gives a comprehensive literature review on proteolytic enzymes, and highlights the classification, sources and key characteristics for the utilization of proteases in the major areas of applications. *Lactobacillus delbrueckii* subsp. *lactis* 313, the model bacterium for this study and the bioprocessing of lactobacilli cell-envelope proteinases (CEPs) are also described. Described in-depth are the genetic control of lactobacilli metabolism, the CEP protein domain structure, as well as the molecular underpinning for the attachment of CEPs onto the bacterial cell-envelope. The sub-sections that follow describe in-depth a complete bioprocess for obtaining CEPs from lactobacilli by fermentation. This part of the literature review is based on two published book chapters:

> Agyei D, Potumarthi R and Danquah MK (2013). Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part I—Upstream Processes. Marine Proteins and Peptides. Kim, John Wiley & Sons, Ltd: 207-29;

and

> Agyei D, Potumarthi R and Danquah MK (2013). Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part II—Downstream Processes. Marine Proteins and Peptides. Kim, John Wiley & Sons, Ltd: 231-51.

The sub-sections that follow describe key enzyme immobilization techniques, their advantages and their prospects of application for proteolytic enzymes. The chapter concludes with a summary where the knowledge gaps in the literature are identified.

PART I: UPSTREAM PROCESSES: PRODUCTION OF CELL-ENVELOPE PROTEINASES

Chapter 3 – Growth characterisation and cell-envelope proteinase production by *Lactobacillus delbrueckii* subsp. *lactis* 313

In this chapter, the fermentation and growth description and cell-envelope proteinase production by LDL 313 are described. Two published journal articles are included in this chapter, the findings of which collectively provide the necessary information regarding the basic fermentation profile and extent of LDL 313 growth under different conditions to improve CEP production. The two publications include:

Agyei D and Danquah MK. In-depth characterisation of *Lactobacillus delbrueckii* subsp. *lactis* 313 for growth and cell-envelope-associated proteinase production. *Biochemical Engineering Journal* 2012;64(2012):61-8;

and

Agyei D, Potumarthi R and Danquah MK. Optimisation of batch culture conditions for cell-envelope-associated proteinase production from *Lactobacillus delbrueckii* subsp. *lactis* ATCC® 7830™. *Applied Biochemistry and Biotechnology* 2012;168(1035-50)

Chapter 4 – Extraction of cell-envelope proteinases

In chapter 4, the effects of different extraction methods were investigated for their ability to efficiently release CEPs from LDL 313. In order to fully exploit these numerous biotechnological potential of CEPs, detailed understanding of the biosynthesis and metabolite accumulation is essential to develop optimal techniques for effective extraction and recovery of CEPs. Although several procedures exist for the extraction of CEPs from lactobacilli none is fully optimized for the extraction of structurally intact CEPs from different cellular species and under different metabolic and process conditions. This is because the sub-molecular localization attachment of CEPs on cell-envelope differs for the various bacteria species.

This chapter concludes Part I of the Thesis is published in *Chemical Engineering Science* as:

> Agyei D, Lim W, Zass M, Tan D and Danquah MK. Bioanalytical Evaluation of *Lactobacillus delbrueckii* subsp. *lactis* 313 Cell-Envelope Proteinase Extraction. Chemical Engineering Science 2013;95(2013):323–30

PART II: DEVELOPMENT AND ESTABLISHMENT OF STABLE BIOCATALYSTS FROM CELL-ENVELOPE PROTEINASES

Chapter 5 – Covalent immobilization of cell-envelope proteinases

This chapter opens Part II of the thesis and describes the development and establishment of stable biocatalysts from CEPs by covalently immobilizing them on synthetic polymer fabric.

Reported in this study is a simple, cheap and quick strategy for the immobilization of enzymes onto polyester fabric via support functionalization with ethylenediamine and cross-linking with

glutaraldehyde. This three-step process was carried out on CEPs of LDL 313 and also on trypsin (as a model of conventional proteinase). Optimisation of immobilization parameters (ethylenediamine activation conditions, cross-linker concentration, immobilization pH and temperature, use of spacer molecules) were done and the two immobilized proteinases were also compared for their protein hydrolysis performance using casein, skimmed milk proteins and bovine serum albumin. This chapter is based on the manuscript submitted to *Journal of Biotechnology* (manuscript number: JBIOTEC-D-13-01446).

Chapter 6 – Immobilization of cell-envelope proteinases through cross-linked enzyme aggregates (CLEA)

In this chapter, the preparation of cross-linked CEP aggregates from LDL 313 CEPs, for protein degradation is described. Cross-linked enzyme aggregates (CLEAs) are an attractive strategy for the immobilization of enzymes because they can be made without extensive protein purification, have low production costs, and offer the possibility to co-immobilize different enzymes. CLEA preparation is also affected by several parameters and conditions all of which can be fined-tuned to improve recovered activity. Due to its technological and economic advantages, CLEA was selected as one of techniques for the immobilization of CEPs to provide a cheap alternative to the production of stable biocatalysts from CEPs of LDL 313. This chapter is based on the manuscript submitted to in *Food and Bioprocess Technology* (manuscript number: FABT-S-13-01918). Chapters 5 and 6 form Part II of the Thesis.

Chapter 7 – Concluding Remarks and Future work

This chapter concludes the research work by reiterating the key findings and relevant applications. The chapter also proposes some future work which could expand the potential of immobilized CEPs for various protein degradation processes and identify further improvements relating to the process-economics and environmental impacts at large-scale production.

APPENDICES

Appendix A – Packed-bed enzyme bioreactor using immobilised cell-envelope proteinase: low-cost process for degradation of milk proteins

Appendix A describes the use of packed-bed immobilized enzyme reactor for the production of protein/peptide-rich product mixture for food applications. Here, a method is described for the *in situ* immobilization of CEPs from LDL 313 to prepare a packed-bed enzyme reactor for degradation of dairy proteins. The technological aspects and the economic implications of the production of peptides from milk proteins are explored.

Appendix B – Interfacial and foaming properties of protein hydrolysates prepared from immobilized proteinases from *Lactobacillus delbrueckii* subsp. *lactis* 313

Appendix B describes the production of surface active foams from various food proteins by the use of cross-linked enzyme aggregates (CLEA) from LDL 313 CEPs. Proteins tested for formability included casein, whey protein isolate and β-lactoglobulin where their foaming characteristics (foaming power and foam stability) were explored and correlated with the degree of hydrolysis.

Appendix C – Other related published articles

Another published journal article that is relevant but not central to the objectives of the thesis is presented in this section. It is **Agyei D** and Danquah M (2012) Carbohydrate utilization affects *Lactobacillus delbrueckii* subsp. *lactis* 313 cell-envelope-associated proteinase production. *Biotechnology and Bioprocess Engineering* 17 (4):787-794. doi:10.1007/s12257-012-0106-2.

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CHAPTER 2

LITERATURE REVIEW

2. Chapter 2: Literature Review

2.1. Introduction - Proteases: an overview

Enzymes are a ubiquitous class of biomolecules present in all life forms where they are responsible for cell growth, cell function and differentiation. These physiological roles of enzymes have been commercially exploited in many industrial processes (Rao et al., 1998; Gupta et al., 2002b). The opening of last century saw the use of enzymes in the synthesis of many fine chemicals. Currently, enzymology has evolved to become one of the major area of modern biotechnology (Galante and Formantici, 2003; Gutterres et al., 2009). In this era, most manufacturing processes are undertaken with the use of enzymes as catalyst. This is because; unlike chemical methods, enzymes are highly efficient, 'green' and biodegradable and are largely safe to handle since they are operated at normal environmental conditions. Moreover, the rapid increase in world energy prices has also fostered the demand for enzymes processes, being more cost effective approach than physico-chemical methods (Research & Markets, 2012; TMR, 2013).

About 80% of current major industrial enzymes are hydrolases (Galante and Formantici, 2003) and within this proportion proteases are among the most industrially exploited and well studied (Gupta et al., 2002b; Kirk et al., 2002). The global market for enzymes is estimated to reach $8.5 billion in 2015 (TMR, 2013). Of this, proteases alone account for well over 40% of enzyme sales worldwide and these enzymes find use in industrial market sectors such as medical, diagnostics, food, monogastric animal feed, cosmetic, pharmaceuticals, leather, pulp & paper, detergent,

textile, photographic, bioremediation, and waste management (Gupta et al., 2002b; Galante and Formantici, 2003; Najafi et al., 2005; Subba Rao et al., 2009).

The wide use of enzymes in industrial processes have been aided by the advances in recombinant DNA technology and protein/enzyme engineering which are used for the design and production of new enzymes with novel 'pliable' properties that are able to reach high production yields at competitive costs (Gupta et al., 2002b; Galante and Formantici, 2003). For the purpose of this review, a description of proteases and their industrial applications are highlighted. A description of *Lactobacillus delbrueckii* subsp. *lactis* 313 and its protease production potential is also mentioned, together with the bioprocess operations (fermentation and purification) employed in the production of proteases from lactic acid bacteria. The various immobilization techniques and their applicability to proteases have also been discussed. The chapter concludes with a summary where the knowledge gaps in the area of bioprocessing and immobilization of lactobacilli proteinases are identified.

2.2. Industrial applications of proteases

2.2.1. General applications

Proteolytic enzymes have several applications in many biotechnological industries. These areas include bioremediation, waste treatment, leather and textile processing, laundry and detergent manufacturing, as well as the food and pharmaceutical application (Kirk et al., 2002; Galante and Formantici, 2003; Turk, 2006; López-Otín and Bond, 2008; Vázquez and Murado, 2008; Queiroga et al., 2012). Cell envelope proteinases (CEPs) are the type of proteolytic enzymes chosen for this study. To date, CEPs are mostly used in the dairy industry (Siezen, 1999; Sadat-

Mekmene et al., 2011a). The other applications of proteases have not yet been exploited industrially for CEPs. Ground breaking research efforts are needed to unravel the prospects of applying CEPs in these areas. This will help improve the utility and commercial value of CEPs beyond the food and pharmaceutical applications. Captured in Table 2.2.1.1 is a spectrum of proteolytic enzymes applications. Application of CEPs in the food industry is therefore the focus of section 2.2.2 presented below.

Table 2.2.1.1: Some application of proteolytic enzymes in the major industries

Industry	**Application**
Food	Milk clotting Meat tenderization Preparation of Low allergenic infant formulas and pre-digested foods with high bioavailability Flavour enhancement Use in baked products (biscuits and cookies) Preparation of surface active foams Preparation of physiologically active protein hydrolysates Beer clarification and wine stabilization
Detergent	Protein stain removal
Leather	Unharing of hides and skins; bating of leather
Textile	Degumming of raw silk
Pulp and paper	Biofilm removal
Medical and diagnostics	Treatment of inflammations and some ulcers

Adapted from (Kirk et al., 2002); (Gurung et al., 2013); (Ogrydziak, 1993) and (Gupta et al., 2002b)

2.2.2. Applications in the food industry

The use of enzymes in food processing is the second largest industrial application of proteolytic enzyme (Kirk et al., 2002). Proteases are widely used in many sectors of the food industry, especially in the dairy products. Here, CEPs have been known to improve the texture and organoleptic characteristics of dairy products and also have the potential to release bioactive peptides encrypted in dairy proteins (Tsakalidou et al., 1999; Espeche Turbay et al., 2009; Sadat-Mekmene et al., 2011b) (See Figure 2.2.2.1). Indeed, enzymatic hydrolysis has been widely used for improving the functional, nutritional and physiological properties of natural food proteins (Rodríguez Patino et al., 2007). Some functional properties of proteins that are enhanced via enzymatic hydrolysis include fat- and water-holding capacity, emulsion capacity and stability, foaming capacity and stability, and textural properties (Rodríguez Patino et al., 2007). After extensive hydrolysis, some protein hydrolysates become suited for nutritional applications (such as enteral formulae and medical diets, sports and body building nutrition, and hypoallergenic infant formulae) (Foegeding et al., 2002).

Another important area where proteases are useful is in the production of biologically active proteins hydrolysates. These hydrolysates (also called bioactive peptides) are specific protein fragments, which, once ingested and absorbed, have a potential impact on body functions or conditions and thus ultimately influence health (Korhonen and Pihlanto, 2006). The potential for bioactive peptides to contribute to a healthier nutrition (e.g. by ingesting them with functional foods) has been widely discussed in the scientific community. The discovery of bioactive peptides with potential health benefits has therefore been the subject of growing research and commercial interest in the context of health-promoting functional foods

(Korhonen, 2009; Agyei and Danquah, 2012; Danquah and Agyei, 2012; Korhonen and Marnila, 2013). A large number of research studies have reported the use of lactobacilli cell-envelope proteinases in the production of bioactive peptides from several food proteins (Sütas et al., 1996; Yamamoto et al., 1999; Courtin et al., 2002; Minervini et al., 2003; Pan et al., 2005; Murray and FitzGerald, 2007; Hebert et al., 2008; Pescuma et al., 2013). As research continues to identify novel proteases from a wide range of sources, it can be postulated that their prospective applications is only limited by the imagination.

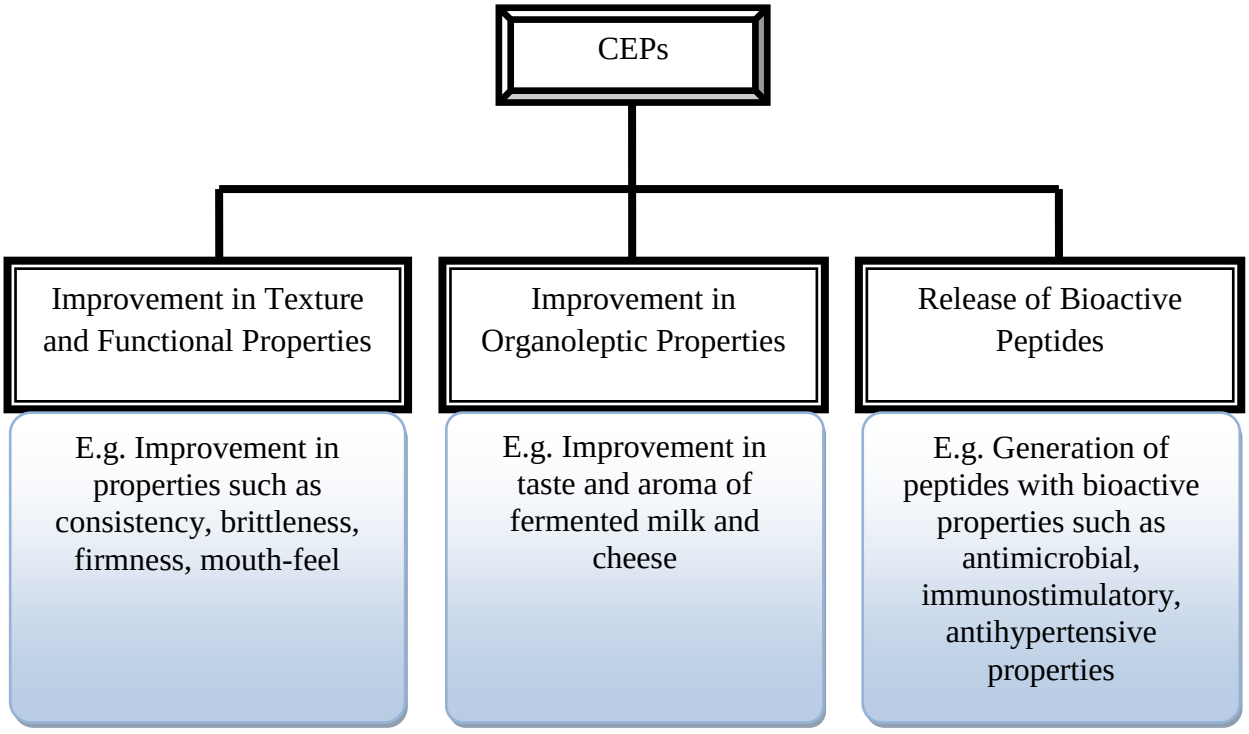

Fig 2.2.1.1: A schematic for the applications of cell-envelope proteinases (CEPs) in the dairy industry

2.3. Classification and sources of proteases

Proteolytic enzymes (also designated proteases, proteinases or peptidases), constitute one of the largest functional groups of proteins, with a large number of well described members (Supuran et al., 2001). Bioinformatics analysis of the mouse and human genomes alone have identified at least 500–600 proteases (Turk, 2006).

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases (EC. 3.4) are subdivided into exopeptidases (EC 3.4.11-19 which consists of amino- and carboxypeptidases by substrate specificity) and endopeptidases (or proteinases, EC 3.4.21-99), four (4) groups that are classified according to mechanism of action and affinity for specific inhibitors). The possibility of the existence of other classes of proteases is demonstrated by the fact that one class of proteinases (EC 3.4.99) has been shown in some instances to possess affinities for several standard inhibitors, whereas at other times no affinities are observed. They also have no known mechanism of action (Klimova and Chebotarev, 2000). Based on standard inhibitors, six (6) catalytic types of endopeptidases, namely: serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic (EC 3.4.23), metalloproteinases (EC 3.4.24), threonine (EC 3.4.25), and glutamic acid proteinases have been recognized so far (NC-IUBMB, 2013).

Extracellular proteases have been exploited commercially from those organisms that produce substantial amounts (Gupta et al., 2002a). These include plants, animals and microorganisms. However, mostly due to economic reasons, enzymes from microorganisms have come to dominate the scene and is the most widely exploited at industrial scale. Further, microbial proteases are largely derived from yeasts, molds, and bacteria, but because yeast proteases are mainly intracellular in nature they have not gained significant commercial interest (Kamini et al.,

1999). Thus, microbial proteases obtainable from the *Bacillus* spp., *Bifidobacterium* and the Lactic Acid Bacteria (LAB) account for the bulk of industrial enzymes.

The Lactic Acid Bacteria (LAB), or the ‘’Lactics’’, have been used for centuries in fermented food products such as dairy, vegetable and meat products. Due to their long history of use in cultured foods with no adverse effects, they have been assigned a ‘GRAS’ (Generally Regarded as Safe) status (Kaushik et al., 2009) and are non-toxic and non pathogenic (Gupta et al., 2002a). Their ability to improve the quality, safety and nutritional content of food is based on the reduction of carbohydrates and the subsequent production of many antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocins (Vesterlund et al., 2004). The lactic acid bacteria are equipped with proteinases and intracellular peptidases, the former being the most industrially exploited.

The genus Lactobacillus is by far the largest and has the widest industrial and technological scope of application (Axelsson, 2004). It is also very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. These properties account for the wide range of application of organisms in the lactobacilli group. The biochemical characterisation of *Lactobacillus delbrueckii* subsp. *lactis* 313, the organism of choice for this PhD work, is given in the next section. The rationale for choosing *Lactobacillus delbrueckii* subsp. *lactis* 313 as the source of CEP proteases is discussed in details in Section 2.4. CEPs from other microorganisms are reviewed in Section 2.5 of this thesis.

2.4. *Lactobacillus delbrueckii* subsp. *lactis* 313

Lactobacillus delbrueckii subsp. *lactis* 313 (ATCC® 7830™) is classified as cellular organisms → Bacteria → Firmicutes → Bacilli → Lactobacillales → Lactobacillaceae → Lactobacillus → *Lactobacillus delbrueckii* → *Lactobacillus delbrueckii* subsp. *lactis*. It is therefore among the homofermentative thermophilic lactic acid bacteria (LAB) (Espeche Turbay et al., 2009; Hebert et al., 2013). The Taxonomic Subcommittee of the Lactobacilli has reclassified *Lactobacillus leichmannii* as *Lactobacillus delbrueckii* (Carr et al., 2002; Taranto et al., 2003). *Lactobacillis delbrueckii* has three subspecies: *lactis*, *bulgaricus,* and *delbrueckii* and all these species are important for the dairy industry (Forsman and Alatossava, 1991; Delley and Germond, 2002; Germond et al., 2003b).

Lactobacillus delbrueckii subsp. *lactis* 313 (LDL 313) is closely related to another strain of the same species, i.e. strain CRL 581 which in the dairy industry is used for the production of hard cheeses, such as Emmenthal, Provolone and Grana (Espeche Turbay et al., 2009; Hebert et al., 2013). Moreover, due to its ability to produce organic acids, LDL 313 is used in the production of sour bread such as the German sour rye (Vogel and Ehrmann, 2008), grape wines (Edwards et al., 1993), and also for microbiological assays of the B_{12} and amino acid content of food products (Taranto et al., 2003). The organism therefore has a spectrum of industrial application. Despite these applications prospects, literature is scarce on the cell wall/membrane characteristics and their effect on proteolytic properties of LDL 313. As discussed in subsequent sections, some biochemical data have been reported on proteinases of species *Lactobacillus delbrueckii* and some similarities exist among members of the species but these do not necessarily hold true for the sub-species and strain *lactis* 313.

2.4.1. Cell metabolism and proteinase domain structure

Due to the homofermentative properties of *L. delbrueckii* the metabolism of casein and sugars are the two key biochemical processes coded by genes *prtB* and the *lac* operon respectively. About 1,562 genes code for proteins PrtB and the *lac* operon (Germond et al., 2003b). Within the *lac* operon is the *lac*S, *lac*Z, and *lac*R genes that encodes for the uptake and breakdown of lactose. The *lac*S gene codes for lactose permease responsible for the ability to transport lactose through the cell membrane. The important enzyme β-galactosidase necessary for lactose metabolism is encoded in the *lac*Z gene. Downstream of *lac*Z is the regulatory gene *lac*R. *L. delbrueckii* subspecies *lactis* produces exclusively the D-isomer of lactic acid (Delley and Germond, 2002).

The ability of *L. delbrueckii* subspecies to grow in dairy products is owed to the enzymatic activity that results in casein breakdown to release essential amino acids and peptides (Gilbert et al., 1996; Kitazawa et al., 2000). This enzyme, the cell-envelope proteinase (PrtB or CEP), is responsible for the digestion of casein. CEPs are extracellular, cell-envelope-bound proteinases of lactic acid bacteria and are also referred to as cell-surface proteinases, cell wall-bound proteinases or lactocepins (Siezen, 1999; Savijoki et al., 2006). It has been shown that a proteinase-negative strain of *L. delbrueckii* reaches only 22% of the final biomass of a proteinase-positive strain when grown in milk (Gilbert et al., 1997). Its genome encoded gene (*prtB*), located downstream of the *lac* operon, has been isolated and characterized from *L. delbrueckii* subsp. *bulgaricus* (Gilbert et al., 1996). *prtB* gene has been sequenced from *L. delbrueckii* subsp. *bulgaricus* strain NCDO1489 and encodes a protein of 1946 amino acid residues (Genebank Accession Number L48487) (Siezen, 1999; Sadat-Mekmene et al., 2011).

The expression of *prtB* was recently shown to be differently regulated among the *L. delbrueckii* species. In the subsp. *bulgaricus*, the protease is largely expressed constitutively, whereas in the subspecies *lactis* it is tightly repressed by the presence of peptides in the growth media (Gilbert et al., 1997; Hébert et al., 2002; Hebert et al., 2008). The complete genome sequence of *Lactobacillus delbrueckii* subsp. *bulgaricus* strain ND02 (Sun et al., 2011) and that of *Lactobacillus delbrueckii* subsp. *lactis* CRL 581 (Hebert et al., 2013) were recently sequenced.

To date, five (5) main CEP genes have been known. There are the genes *prtB* of *L. delbrueckii* subsp. *bulgaricus*, *prtH* of *L. helveticus*, *prtP* of *Lactococcus lactis*, scpA of *Streptococcus pyogenes* and *csp* of *Streptococcus agalactiae*. These genes code for the proteolytic enzymes products PrtB, PrtH, PrtP, ScpA, and Csp respectively. These are of significant importance in metabolism of casein by lactic acid bacteria, with the exception of ScpA, and Csp which are virulence factors in *Streptococcus* (Siezen, 1999; Pastar et al., 2003).

2.4.2. Attachment of cell-surface proteins in Gram-positive bacteria

CEPs of *L. bulgaricus* is bound to the cell wall at the C-terminal end (Savijoki et al., 2006). Deletion of the last 99 residues of PrtB is sufficient to allow the release of this truncated but active proteinase into the culture medium (Germond et al., 2003a; Germond et al., 2003b). CEPs are synthesized as inactive pre–pro-protein and the maturation process to active lactococcal proteinases (PrtP) requires the help of PrtM chaperone, a 299-residue envelope lipoprotein that is responsible for the autodegradation of the pre–pro-protein to give a mature PrtP (Pederson et al., 1999).

In *L. delbrueckii*, the C-terminal end of PrtB (amino acid residues 1743 to 1938) is characterized by a high content in lysine (Sadat-Mekmene et al., 2011). However, interestingly, unlike PrtP of *Lactococcus,* the maturation PrtB of *L. delbrueckii* subsp. *bulgaricus* does not require a PrtM-like chaperone (Sadat-Mekmene et al., 2011). The C-terminal region of PrtB has up to 25% identity to the C-terminal region of the S-layer protein from *L. acidophilus*. These results suggest that PrtB is anchored to the cell envelope in a manner similar to that of s-layer proteins (Pederson et al., 1999). The attachment of s-layer protein in Gram-positive bacteria has been described in detail by (Desvaux et al., 2006). There is a choline-binding domain, also called cell wall binding domain of Type 1 (CWBD1), which consists of several imperfect repeats of ~ 20 amino acids each and is also characterized by the presence of conserved aromatic residues. Binding of the CWBD1 are non-covalent and are essentially found within the *Clostridiales* and *Lactobacillales* orders. This design renders the cell wall of Gram-positive bacteria permeable so that proteins can interact with their environment without ever poking out in a jagged manner out of the cell wall (Desvaux et al., 2006).

2.4.3. Classification and the role of Ca^{2+} ions in the activity and extraction of CEPs

The cell-wall proteinases of LAB belong to the same multi-domain proteinase family and show significant homologies, even though differences in specificity exist (Siezen, 1999; Courtin et al., 2002). Proteinase of *L. delbrueckii* has been classified in the subfamily of cysteine subtilisins called the subtilases which are characterized by a catalytic triad, Asp-His-Ser (Pederson et al., 1999; Fira et al., 2001). The enzyme mechanism requires the use of sulfhydryl groups and thus, hydrolysis of casein by PrtB results in the release of bitter proline-rich oligopeptides (Morel et al., 1999; Tsakalidou et al., 1999).

Calcium (II) ions are important for proteinase synthesis in lactobacilli. Whereas Ca^{2+} content does not improve proteinase yield via metabolic mechanisms, it is however important for the stability of proteinases during cell growth (Rahman et al., 2003). Proteinases in lactobacilli are cell-envelope bound and are therefore susceptible to being degraded or shed into the growth medium. Research has shown that calcium ions are directly involved in the active moiety of the enzyme to the anchor sequence (Coolbear et al., 1992). Also, the correct functional proteinase domain requires the binding of at least two Ca^{2+} ions (Genov et al., 1995; Exterkate and Alting, 1999; Siezen, 1999). The functional involvement of calcium ions in the active conformation of the proteinase has been demonstrated and calcium ions have been shown to protect the enzyme against autoproteolytic release (Exterkate, 1995).

The release of these CEPs from the cell surface occurs spontaneously in a Ca^{2+}-free buffer via repeated washing of the cells in the buffer (Exterkate, 1990; Kojic et al., 1991; Tsakalidou et al., 1999; Fira et al., 2001). The removal of relatively weakly bound calcium in CEPs initiates a structural rearrangement in the proteinase domain and this causes an intramolecular autoproteolytic event which truncates the proteinase at the C-terminal end causing the release of the enzyme (Martín-Hernández et al., 1994; Kunji et al., 1996; Exterkate, 2000). This method is simple and gives low levels of cell lyses (Tsakalidou et al., 1999), however, it conduces to a decrease in the specific activity and thermal stability of the enzyme (Exterkate and Alting, 1999; Exterkate, 2000). Consequently, it has also been observed that the activity of crude CEPs extracted with Ca-free buffer is restored by the addition of Ca^{2+} ions to concentration. Further, the stability of enzyme activity is maintained when crude CEPs are supplemented with Ca^{2+}. This relation of enzyme activity and stability is proportional to the concentration of added Ca^{2+}

to about 10 mM (Exterkate and Alting, 1999). This role of Ca^{2+} ions in inducing and stabilizing active conformations of the bound proteinases on cell envelope surfaces (Coolbear et al., 1992; Siezen, 1999) has inspired many authors to supplement chemically defined growth media with low concentration of Ca^{2+} in CEP production/characterization studies of certain lactobacilli (Hebert et al., 2008).

However, it is worth mentioning that the effect of Ca^{2+} ions on the CEP is strain dependent. In another study, the activity, release, and stability of CEP from *L. delbrueckii* subsp. *lactis* CRL 581 were not affected by the presence of calcium ions (Espeche Turbay et al., 2009). Moreover, unless cells are treated with lysozyme, the use of Ca-free buffer is either unable to release CEPs or shows the release of a minor amount from certain lactobacilli species (Martín-Hernández et al., 1994). In their work, Atlan *et al*., observed that repeated washing of *L. bulgaricus* CNRZ 397 cells with Ca^{2+} buffer could not release the proteinase from the cell wall and they explained the observation to be due to the rather close and tight association of the proteinase with the peptidoglycan wall (Atlan et al., 1989; Laloi et al., 1991).

It can be observed from the ongoing discussion that scientific biochemical data is scant for CEPs of the strain LDL 313 used in this study. Thus, the proteolytic system of this strain needs to be studied in order to understand their role in proteolysis. This will help broaden its application in less explored areas. The genetic characterization of the proteolytic system of this strain is beyond the scope of this study, as such, only the biochemical aspects will be considered. Such studies need to address the following concerns, among others:

- Do fermentation conditions influence enzyme yield and/or enzyme activity? If so, what conditions, and by how much?
- Are there present more than one CEP? How are the enzymes expressed? Are they produced and bound on the cell-membrane or they are released into the culture medium? If the latter is true what conditions favours the release of enzyme into the culture medium?
- Which fraction exhibits a higher proteolytic potential - the whole microbial cells or the extracted enzymes, or enzymes purified from the culture supernatant?
- What is the appropriate method for harvesting the enzyme with minimum risk of denaturing or loss in enzyme activity?
- Do the enzyme(s) possess caseinolytic properties? What is the type of proteinase(s) and what are their caseinolytic specificities?
- What will be the most suitable method of immobilization in order to prepare stable enzyme forms from CEPs of LDL 313? And what is the proteolytic potential of immobilized CEPs on food proteins?

  These are among the questions that this research project seeks to address.

In subsequent sections of this review the bioprocess operations (both upstream and downstream) for the production of proteinases from wild-type lactobacilli is highlighted. The various nutritional and environmental parameters affecting the production of proteinases are discussed and purification and characterization of these proteinases are also delineated. Sections 2.5 and 2.6 were published as book chapters 10 and 11, respectively in

Agyei D, Potumarthi R and Danquah MK (2013). Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part I—Upstream Processes. Marine Proteins and Peptides. Kim, John Wiley & Sons, Ltd: 207-29;

and

Agyei D, Potumarthi R and Danquah MK (2013). Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part II—Downstream Processes. Marine Proteins and Peptides. Kim, John Wiley & Sons, Ltd: 231-51.

They have been reported here without any reformatting.

Section 2.5

2.5. Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part I—Upstream Processes

Monash University
Declaration for Thesis Chapter 2 Section 5

Declaration by candidate

In the case of Chapter 2 Section 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Initiation, key ideas and writing up	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Dr. Ravichandra Potumarthi	Initiation, and key ideas
Dr. Michael K Danquah	Initiation, key ideas and writing up

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12 December 2013
Main Supervisor's Signature	[redacted]	**Date** 12 December 2013

10 Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part I—Upstream Processes

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10.1 INTRODUCTION: BIOACTIVE PEPTIDES—PRODUCTION AND FUNCTIONALITIES

The use of food-grade microorganisms to enrich food with bioactive peptides via proteolysis has recently been highlighted and appraised by researchers, clinicians and food manufacturers (Agyei & Danquah, 2011, 2012c). Upon oral administration and/or absorption, bioactive peptides may induce several physiological effects, such as antioxidative, antimicrobial, antihypertensive, cytomodulatory and immunomodulatory effects, under *in vivo* and *in vitro* conditions (Gibbs, 2004; Hartmann & Meisel, 2007; Korhonen, 2009; Korhonen & Pihlanto, 2006; Yang *et al*., 2009). Thus, the biological potency of bioactive peptides in the major body systems continues to be a major research endeavour (Möller *et al*., 2008) and forms the basis of application in the consumer industries (see Table 10.1).

Despite the clinical and nutritional importance of bioactive peptides, bioprocesses and production methodologies are not fully optimised for industrial-scale titres (Agyei & Danquah, 2011). An economically feasible bioprocess for the manufacture of bioactive peptide requires that the necessary ingredients and processes should be economical and cost-effective. For example, enzymes for use in bioactive peptide manufacture should preferably satisfy such criteria as ease of availability, extraction and purification; variety in proteolytic specificity; and hardiness with use under unfavourable conditions. The lactobacilli are good sources of enzymes which meet these criteria, since they are equipped with a complex proteolytic system consisting of proteinases and peptidases with varied activities and specificities (Agyei & Danquah, 2011). Also, proteinases of lactobacilli are able to hydrolyse over 40% of the peptide bonds of α_{s1}- and β-casein, giving rise to oligopeptides with 4–40 amino acid residues (Kunji *et al*., 1996). Several of the known bioactive peptides have amino acid residues within this range (Korhonen & Pihlanto, 2006). Consequently, the lactobacilli are good candidates for the generation of bioactive peptides. Factors such as strain selection and the influence of nutritional and environmental parameters on lactobacilli proteolysis thus markedly influence the release of encrypted bioactive peptides from proteins (Minervini *et al*., 2003).

Bioactive peptides are produced via the proteolytic action of microorganisms on proteins or by *in vitro* enzymatic hydrolysis of proteins with enzymes of gastrointestinal or microbial origin (Fig. 10.1). With adequate control of hydrolyses, this method results in

Marine Proteins and Peptides: Biological Activities and Applications, First Edition. Edited by Se-Kwon Kim.

Table 10.1 Application of bioactive peptides in the major consumer industries.

Bioactivity	Area of application	Industry
Antimicrobial peptides	Natural preservatives	Food Nutraceuticals and functional foods Pharmaceuticals Cosmetics
Antimicrobial Angiotensin-converting enzyme (ACE)-inhibitory Immunomodulatory	Therapeutic products	Pharmaceuticals
Immunomodulatory	Immunonutrition Food-fortification programs	Nutraceuticals and functional foods

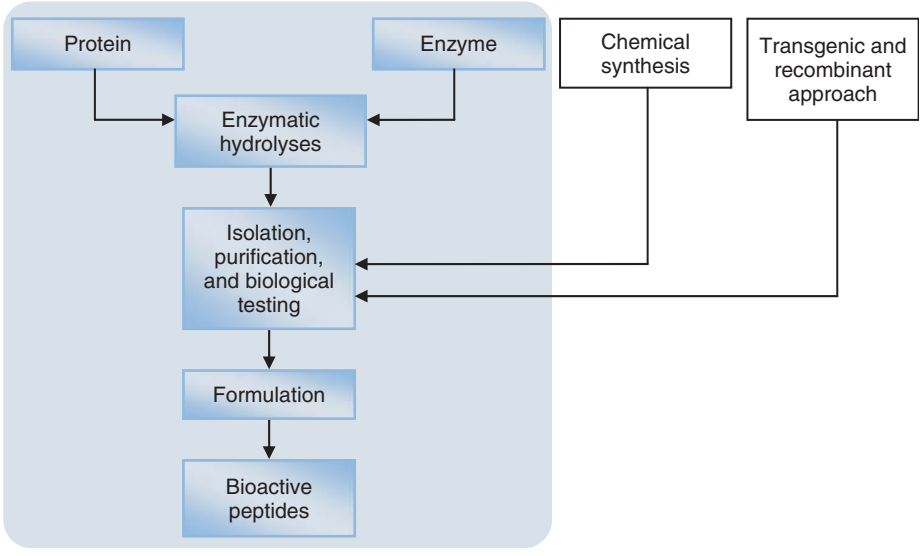

Fig. 10.1 Schematic for the production of bioactive peptides by enzyme hydrolyses *in vitro* (selected region).

breakage of the peptide bonds in proteins and subsequent generation of smaller peptides with little or no free amino acids. Although proteins can be hydrolysed by alkaline and acid treatments equally, these methods are not recommended, because, whereas alkaline hydrolysis causes the racemisation or destruction of certain amino acids at high pH (Neklyudov *et al*., 2000), acid treatment destroys tryptophan and hydrolyses asparagine and glutamine to their respective conjugate acids (Walker & Sweeney, 2002). The digestive and microbial proteases, including alcalase, trypsin, pepsin, chymotrypsin, pancreatin, pepsin, thermolysin and cell-envelope proteinases (CEPs), are among the most widely used enzymes (Korhonen & Pihlanto, 2006). It is important that enzymatic hydrolyses be carried under optimum pH and temperature conditions, in order to ensure maximum activity and reduce the formation of undesirable products (Kim & Wijesekara, 2010).

Other approaches to the production of bioactive peptides include transgenic, recombinant and synthetic methods (Marx, 2005). However, these production technologies are prohibitive for large-scale applications, due to cost (Hancock & Sahl, 2006). Bioactive peptides could also be produced naturally from dietary proteins during gastrointestinal transit, but production through such routes is uncontrolled and it might generate insufficient quantities to stimulate physiological responses in adult humans (Gauthier *et al*., 2006). The development of commercially viable processes capable of up-scaling bioactive peptide production is therefore crucial.

10.2 LACTOBACILLI METABOLISM

The lactic acid bacteria (LAB), or the 'lactics', have been used for centuries in fermented food products, such as dairy, vegetables and meat. Due to their long history of use in cultured foods with no adverse effects, they have been assigned a 'GRAS' (Generally Regarded as Safe) status (Kaushik *et al*., 2009). They are nontoxic and nonpathogenic (Gupta *et al*., 2002a). Their ability to improve the quality, safety and nutritional content of food is based on their reduction of carbohydrates and on their production of many antimicrobial agents, such as organic acids, hydrogen peroxide and proteinaceous low-molecular-weight-like bacteriocins (Vesterlund *et al*., 2004). The lactics are also important commercially in the processing of meats, alcoholic beverages and vegetables, including sausage, cured hams, wine, beer, fortified spirits, pickles and sauerkraut. However, although the LAB serve beneficial roles in the food industry, they can occasionally become a nuisance by producing off-flavours via contamination of products (Carr *et al*., 2002).

The LAB consist of a number of genera, with *Lactobacillus* being the largest and having the widest industrial and technological application (Axelsson, 2004). There is a high level of biochemical, physiological and phenotypic heterogeneity among the lactobacilli. The broadly interested reader is referred to Carr (2002), Axelsson (2004) and Vogel (2008) for a more comprehensive review of LAB and their metabolic processes. The focus of this chapter is on the metabolism of genus *Lactobacillus*, with emphasis on the proteolytic potential of the well-known species in order to highlight their potential use in the production of bioactive peptides.

10.3 THE PROTEOLYTIC SYSTEM OF THE LACTOBACILLI

It is generally believed that LAB have a very limited capacity to synthesise amino acids using inorganic nitrogen sources. They are therefore dependent on preformed small peptides and amino acids being present in the growth medium as a nitrogen source (Atlan *et al*., 1990; Axelsson, 2004; Kunji *et al*., 1996; Picon *et al*., 2010; Tsakalidou *et al*., 1999). Most LAB generally require complex or enriched media for growth. It is well established that most LAB are auxotrophic for between 4 and 14 amino acids and that the amino acid requirement is species- and strain-dependent (Kunji *et al*., 1996). The requirement for a particular amino acid may be the result of mutations in the genes for amino acid biosynthesis and/or the downregulation of these genes or the enzymes involved (Chopin, 1993). Moreover, the amino acid auxotrophy of some LAB species has been attributed to a lack of key fermentative enzymes necessary for amino acid metabolism. For

example, the obligately homofermentative lactobacilli lack the phosphoketolase enzyme, which explains their inability to synthesise the aromatic amino acid family and histidine from their precursors D-erythrose-4-phosphate and ribose-5-phosphate, respectively. This fermentative pattern could explain the degree of amino acid auxotrophy in the species *L. delbrueckii* subsp. *lactis*, which is prototrophic for glutamine, glycine, threonine, aspartic acid, asparagine, proline and alanine (Hebert *et al*., 2004).

Protease production is an inherent property of all organisms, but only those microbes that produce a substantial amount of extracellular protease have been exploited commercially. Of these, the lactobacilli have been the most widely exploited, owing to their biotransformation and caseinolytic properties (Gupta *et al*., 2002a). The basis of their use in the dairy industry stems from the fact that for optimal growth, these bacteria must be able to degrade milk proteins, since the concentration of free amino acids and peptides present in milk is not sufficient for the growth of LAB (Tsakalidou *et al*., 1999). Protein degradation and subsequent utilisation of the degradation products therefore requires a complex proteolytic system consisting of proteinases, peptidases and amino acid and peptide carriers. The structural components of the proteolytic systems are threefold, on the basis of their function: (1) proteinases that breakdown caseins to peptides, (2) peptidases that degrade peptides and (3) transport systems that translocate the breakdown products across the cytoplasmic membrane (Kunji *et al*., 1996; Liu *et al*., 2010) (Fig. 10.2). In addition to their role in bacterial growth in proteins such as milk, the proteinases also contribute to the development of texture and organoleptic characteristics of fermented milk products and may cause the release of bioactive peptides, which can contribute to health improvements beyond basic nutrition (Espeche Turbay *et al*., 2009). Research efforts aimed at understanding the proteolytic system of the lactics are therefore imperative, considering the numerous fields of technological application of lactobacilli proteases.

Our knowledge of the proteolytic system of the LAB is largely derived from studies with *Lactococcus* species. Among the LAB, the CEPs of *Lactococcus lactis*, subspecies

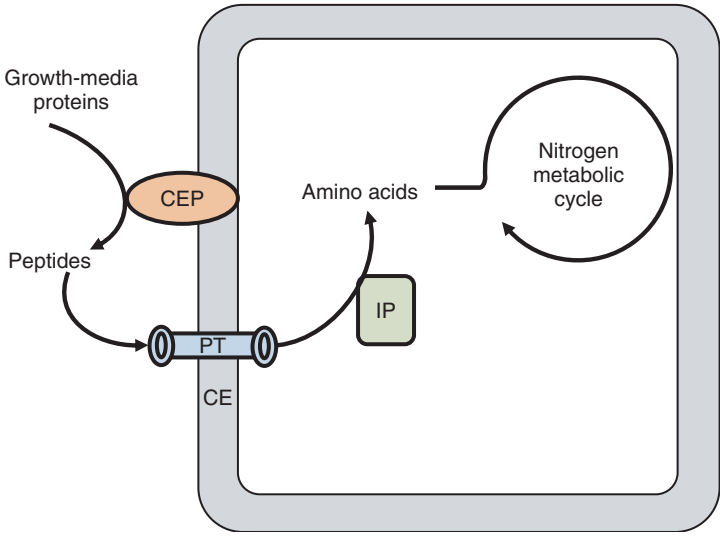

Fig. 10.2 Simplified structural schematic of the proteolytic system of lactobacilli. CEP, cell membrane-associated proteinase; PT, peptide transporters; IP, intracellular peptidases; CE, cell envelope.

SK11 and Wg2, have been the most extensively characterised, both genetically and biochemically, and are also the best documented (Kunji *et al*., 1996). These enzymes represent two of the several types of lactococcal CEP that have been distinguished on the basis of their specificity toward α-, β- and to a lesser extent κ-caseins. The primary substrates for CEP_I are β- and to a lesser extent κ-casein, while CEP_{III} uses αs_1-, β- and κ-caseins (Kunji *et al*., 1996; Oberg *et al*., 2002; Oommen *et al*., 2002). In addition to casein specificity, lactobacilli exhibit an extremely wide range of proteolytic activity, which is usually species- and/or strain-specific. Studies have also demonstrated that the proteinase enzymes from *Lactobacillus* spp. can be loosely grouped into a CEP_I/CEP_{III}-type classification (Kunji *et al*., 1996). A further, more detailed classification of CEPs into seven groups on the basis of their ability to cleave the chymosin-derived peptide α_{s1}-casein (f1–23) has also been proposed (Oberg *et al*., 2002).

10.4 SOURCES OF PROTEASES AND ADVANTAGES OF MICROBIAL PROTEASES

Enzymatic hydrolysis of proteins is the most common way of producing bioactive peptides (Korhonen & Pihlanto, 2006). This approach requires the two major raw materials: proteins and proteolytic enzymes. Byproducts of food protein processing, such as minced meat, fish meal, skins, bones, pulse cakes and protein wastewaters, are relatively cheap, thus their usage in the production of bioactive peptides will largely be conducive to a reduction in production cost (Agyei & Danquah, 2011). Alternatively, bioactive compounds may be formed from components present in the waste by microbial fermentation and/or enzyme hydrolysis, thus maximising profitability and providing an avenue for efficient waste disposal (Wilson *et al*., 2011; Yang *et al*., 2009). Proteolytic enzymes, on the other hand, are usually obtained from plants, gastrointestinal animals and microbial organisms. Microbial proteases obtainable from the *Bacillus* spp., *Bifidobacterium* and LAB are the most widely used for industrial processes (Ferrero, 2001) and present several advantages over proteases from other sources. The advantages of the lactobacilli proteinases include the following (Agyei & Danquah, 2011):

1. They are safe and nontoxic, which gives less concern for safety, especially when their products are intended for human food or drug-based application.
2. They have minimal nutritional requirements and a short maturation time for cells, which means a reduction in production costs.
3. Their production is influenced by culture growth conditions, making it possible to manipulate their yield and properties.
4. Their location in the lactobacilli cell, which is produced and borne on the cell-envelope surface, makes harvesting and enzyme purification relatively cheaper and less laborious.
5. They have a broad biochemical diversity of the microorganisms. Many of these can be subjected to genetic manipulation in order to improve enzyme yield. Recent developments in culturing procedures and taxonomic profiling of microorganisms have provided avenues to exploit different proteolytic enzymes produced by proteolytic starters and nonstarters.
6. Not only are the microorganisms diverse, so are their enzyme products. The plethora of proteases produced by lactobacilli offers varied enzymatic activities and specificities. There are the CEPs and a host of intracellular peptidases (endopeptidases,

aminopeptidases, tripeptidases and dipeptidases) (Khalid & Marth, 1990). Added to this, some *Lactobacillus helveticus* strains produce and express more than one CEP, with differences in protein cleavage patterns (Oberg *et al*., 2002). For the purposes of bioactive peptide production, the diversity in enzyme types and specificities ensures the generation of numerous peptides, each with unique potential bioactivities.

7. Microbial proteins have a longer shelf life and can keep longer under less than ideal conditions without significant loss of activity (Gupta *et al*., 2002a).

10.5 MARINE LACTOBACILLI

The marine environment is rich in nutrient and organic matter and thus supports microbial growth. Some lactobacilli are adapted to living in seawater, from which they can be isolated and subcultured (Rajaram *et al*., 2010). Others are usually found living in symbiotic relationships with other marine animals and are thus usually isolated from gut, muscles or shells. A large percentage of marine *Lactobacillus* species are detected in Pacific oysters (*Crassostrea gigas*) (Shiflett *et al*., 1966); the prevalent species include *L. paracasei*, *L. johnsonii*, *L. plantarum*, *L. pentosus*, *L. paraplantarum*, *L. parabuchneri* and *L. rhamnosus* (Lee *et al*., 2010). Marine-derived lactobacilli are a rich source of useful enzymes for food and pharmaceutical applications. Most are useful in the production of bacteriocins with antagonistic effect on fish pathogens (Lee *et al*., 2010). Captured in Table 10.2 are some bioactive peptides produced from purified proteinase or via lactobacilli fermentation. Among the lactobacilli, the proteinase system of *Lactobacillus casei* is the best studied (Khalid & Marth, 1990; Kojic *et al*., 1991; Tsakalidou *et al*., 1999). The proteolytic systems of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Laloi *et al*., 1991), *Lactobacillus sanfrancisco* CB1 (Gobbetti *et al*., 1996), *Lactobacillus helveticus* (Martín-Hernández *et al*., 1994) and *Lactobacillus delbrueckii* subsp. *lactis* ACA-DC 178 and CRL 581 (Espeche Turbay *et al*., 2009; Tsakalidou *et al*., 1999) have also been studied. Other strains, such as *Lactobacillus delbrueckii* subsp. *lactis* 313 ATCC 7830, have been studied to a lesser degree (Agyei & Danquah, 2012b). Research aimed at fully characterising the proteolytic systems of all known lactobacilli is a major endeavour, as the achievement of high yields of bioactive peptides, under process and economic optimum conditions, hinges upon it.

10.6 PROTEINASE PRODUCTION REQUIREMENTS

10.6.1 Cell-Line Acquisition

The screening of proteinase-producing microorganism is the first step in obtaining microbial proteinases for industrial purposes. Although there are many microorganisms that produce proteases in nature, for industrial purposes it is convenient to find highly proteolytic strains. Some *Lactobacillus* proteinases have relatively broad specificity, resulting in a large number of possible cleavage sites on proteins (Oberg *et al*., 2002). For example, *L. helveticus* CNRZ32 (Blanc *et al*., 1993; Gilbert *et al*., 1997; Pederson *et al*., 1999; Sadat-Mekmene *et al*., 2011; Yamamoto *et al*., 1998) and *L. delbrueckii* subsp. *bulgaricus* ACA DC235 (Stefanitsi *et al*., 1995) have each been found to express two different cell-surface proteinases. Highly proteolytic strains or strains with peculiar specificities can

Table 10.2 Bioactive peptides produced from purified proteinase or via lactobacilli fermentation.

Peptide sequence/name	Bioactivity	Fermentation by/ proteinase sourced from	Reference
Tyr-Lys-Val- Pro-Glu-Leu	ACE inhibitory	*Lb. helveticus* CP790	(Murray and FitzGerald, 2007)
Ile-Pro-Pro	Antihypertensive	*Lb. delbrueckii* ssp. *lactis* CRL 581	(Hebert *et al.*, 2008)
Uncharacterized peptides	Immunomodulatory	*Lb. casei* strain GG + pepsin/trypsin	(Sütas *et al.*, 1996)
Tyr-Pro	Antihypertensive	*Lb. helveticus* CPN4	(Yamamoto *et al.*, 1999)
Tyr-Pro-Phe-Pro, A la-Val-Pro-Tyr-Pro-Gln-Arg, Thr-Th r-Met-Pro-Leu-T rp	Opioid, Antihypertensive, Immunostimulatory	*Lactobacillus GG* enzymes + pepsin & trypsin	(Korhonen and Pihlanto, 2006)
Val-Pro-Pro and Ile-Pro-Pro	Antihypertensive	*Lb. helveticus JCM1004*	(Pan *et al.*, 2005)
Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile	Antihypertensive	*Lb. delbrueckii* ssp. *bulgaricus*	(Korhonen, 2009)
β-casein f184-210	Antibacterial activity	*Lb. helveticus* PR4	(Minervini *et al.*, 2003)
Ala-Arg-H is-Pro-His-Pro-H is-Leu-Ser-Phe-Met	Antioxidative	*Lb. delbrueckii* ssp. *bulgaricus* IFO13953	(Korhonen and Pihlanto, 2006)

f, fraction.

be identified by consulting the published literature. Strains for lactobacilli can be obtained from commercial organisations, academic groups and research institutes, or from a variety of culture collections such as the German Collection of Microorganisms and Cell Cultures (DSMZ), the American Type Culture Collection (ATCC), the National Collection of Industrial and Marine Bacteria (NCIMB) and the National Center for Agricultural Utilization Research (NCAUR), among others.

In microbial biotransformation, the production of bioproducts from wild strains of source organisms often does not support industrial-scale titres (Shirley, 1999). Moreover, the enzymes must have high catalytic activity and stability in an industrial environment. Therefore, molecular techniques may be required to improve proteinase production in the selected strains and thus engineer enzymes with the desired properties for all kinds of processes.

These are achieved by either conventional mutagenesis (ultraviolet (UV) or chemical exposure) or recombinant DNA technology (rDNA) to selectively generate mutants that exhibit higher proteinase yields (Gupta *et al*., 2002b). Over the past few decades, rDNA techniques have changed bulk enzyme production dramatically (Hodgson, 1994). The cloning of genes that encode key regulatory metabolic enzymes and proteinases should therefore be of special interest in attempts at genetic manipulation of lactobacilli. The main

objective of cloning bacterial enzyme genes has been the overproduction of enzymes for various commercial applications (Rao *et al*., 1998). The prospects for genetic manipulation of lactobacilli are bright, since some remarkable milestones have been achieved in developments of gene-exchange system via vector development, gene cloning, transfection, conjugation and transformation (Chassy, 1987). Other methods such as directed evolution are gaining popularity as biotechnological tools for the 'creation' of enzymes with useful desired properties (Hodgson, 1994; Otten & Quax, 2005).

10.6.2 Production (Growth) Media Selection

The expression of many extracellular products such as exopolysaccharides and proteinases, by lactobacilli is largely dependent on cell growth, which is directly linked with the composition of the growth medium. Most lactobacilli exhibit a complex nutritional requirement for growth and proteinase production, and usually consist of carbohydrate and nitrogen sources in the right ratio, vitamins, nucleotides, salts and other supplements. This nutrient requirement is usually satisfied during growth in complex growth media (containing peptone, meat and yeast extract, as well as other undefined compounds) or by the use of a chemically defined medium (CDM). A CDM is essential to the design of reproducible biochemical, physiological and genetic studies of cell growth kinetics, as well as the regulation of proteinases (Hebert *et al*., 2004). Despite these advantages, a typical CDM contains several constituents—usually over 20 nutrients—and has the added drawback of increased cost, since all the vitamins, minerals, bases and amino acids are used in extracted and purified forms. Thus, research aimed at studying the minimal growth requirements of lactobacilli is a worthy venture, as it will allow the development of lactobacilli growth media with small numbers of constituents and low costs.

10.6.2.1 Carbon Sources

The type and concentration of carbon in a growth medium are important variables that influences the proteinase activity of lactobacilli. Several authors have studied the utilisation of sugars by lactobacilli for numerous applications, and simultaneous carbohydrate utilisation has been demonstrated in a few different species (Chervaux *et al*., 2000; Kim *et al*., 2009, 2010; Schiraldi *et al*., 2003). However, little is known concerning the effect of the carbon source on the proteinase activity of lactobacilli, and the few studies that have been reported largely focus on genetic control mechanisms. Biochemical studies relevant to validating and confirming the link between carbohydrate metabolism and proteinase synthesis are scarce (Agyei & Danquah, 2012a).

When bacteria are exposed to a mixture of carbon sources they choose the substrate that yields the maximum profit for maximum survival (Titgemeyer & Hillen, 2002). The repression of secondary carbohydrate utilisation is achieved through several mechanisms, which are collectively termed carbon catabolite repression (CCR). This is controlled by inducer exclusion and genetic repression by the catabolite control protein (CcpA) and by repressor proteins (Kim *et al*., 2009). Genes encoding CcpA and CcpA-like proteins have been described from a number of lactobacilli and the genetic organisation of ccpA genes is always in the order of *pepQ-ccpA*, meaning that *pepQ*—which encodes a prolidase—and *ccpA* are always divergently transcribed (Mahr *et al*., 2000). The fact that *ccpA* in LAB is always linked to a divergently transcribed *pepQ* gene encoding a proline-specific peptidase suggests that expression of this and perhaps other *pep* or *prt* genes may be regulated by

CcpA and may therefore be coordinated with carbon regulation, thereby linking carbon utilisation to proteolysis (Titgemeyer & Hillen, 2002).

The amino acid auxotrophy of some *Lactobacillus* species is the basis of proteolysis in peptide-rich media and has been attributed to the lack of key fermentative enzymes in the sugar metabolic cycle. For example, the obligately homofermentative lactobacilli lack the phosphoketolase enzyme, which explains their inability to synthesise the aromatic amino acid family and histidine from their precursors D-erythrose-4-phosphate and ribose-5-phosphate, respectively (Hebert *et al*., 2004).

Catabolite control of PepQ has been demonstrated in *L. delbrueckii* subsp. *lactis* DSM 7290, where the enzyme activity was twofold higher with cells grown in lactose, as compared to in the presence of glucose (Schick *et al*., 1999). Agyei & Danquah (2012a) have also demonstrated in *L. delbrueckii* subsp. *lactis* 313 that different sugars stimulate the production of different cell-surface proteins, with a significant effect on cell proteinase activity. This shows that greater amounts of proteinase can be obtained from lactobacilli with minimal effort, simply by optimising medium carbon compositions.

10.6.2.2 Nitrogen Sources

The amino acid auxotrophy exhibited by most lactobacilli indicates that cell growth depends on an ability to produce enough proteinases by which to hydrolyse proteins in the growth medium to smaller units. Thus, by inference, profuse growth should be expected in a peptide-rich medium, with a subsequent increase in proteinase yields. However, studies have shown that this is not the case. For the lactobacilli, CEP activity levels are controlled by the peptide content of the growth medium. In their study with *Lactobacillus delbrueckii* subsp. *lactis* CRL 581, Hebert *et al*. (2008) observed that the maximum cell proteinase activity was observed in a minimal defined medium, whereas in the presence of casitone, casamino acids or yeast extract the synthesis of proteinase was inhibited 99-, 70- and 68-fold, respectively. Also, low-molecular-weight (<3 kDa) peptides extracted from casitone have been shown to significantly affect proteinase yields from *Lactobacillus helveticus* CRL 1062 (Hebert *et al*., 2000).

In another study, the proteinase activities of several strains of the thermophilic lactobacilli *L. delbrueckii* subsp. *lactis* and *L. helveticus* were remarkably reduced when cells were grown in peptide-rich medium MRS broth or in a CDM supplemented with casitone. However, when cells were grown in a synthetic medium containing free amino acids the proteinase activity was remarkably enhanced. Further, although peptides substantially affected the cell-envelope proteinase activities of thermophilic lactobacilli, they had no effect on the peptidase activity, and their effect on the proteinase was strain-dependent (Hébert *et al*., 2002). This observation was accounted for by the presence of uncharacterised repressing factors in MRS medium and casitone.

Several genetic control mechanisms have been studied using *Lactococcus lactis* as the model organism, in order to establish a link between nitrogen metabolisms and cell proteolyses in the LAB. The expression of the divergently transcribed genes (*prtP* and *prtM*) involved in proteinase production of *Lactococcus lactis* SK11 is controlled at the transcriptional level by the peptide content of the growth medium (Marugg *et al*., 1995). The genes *prtP* and *prtM* are required for the production of active serine proteases (Kok, 1990), and their level of expression was high in whey permeate growth medium containing relatively low concentrations of peptides (Marugg *et al*., 1995).

CodY is a pleiotropic transcriptional regulator conserved in lactobacilli and other low-G+C Gram-positive bacteria. Two distinct signals have been shown independently to

influence the activity of this regulator: the level of intracellular guanidine triphosphate, GTP (as in *Bacillus subtilis*), and the level of intracellular branched-chain amino acids (BCAA), isoleucine, leucine and valine (as in *Lactococcus lactis*). The difference in the function of CodY between *B. subtilis* and *L. lactis* seems to reflect the difference in the physiology between these two bacteria and thus their proteolytic properties (Petranovic *et al*., 2004). It has also been demonstrated that CodY is responsible for the repression of several transcriptional units of the lactococcal proteolytic system (including *prtP*), when cells are grown in the presence of rich nitrogen sources, such as casein hydrolysates, casitone or casamino acids (Hebert *et al*., 2008). The variation in activity of CodY as influenced by growth conditions such as nutrient availability demonstrates the effect of nitrogen source on cell proteolyses.

Further, in *Lactobacillus helveticus* the casein hydrolytic pattern (form polyacrilamide gel electrophoresis) differs between cells grown in peptide-rich media and those grown in milk, and this suggests that the biosynthesis of cell-surface proteinases with different cleavage specificities is medium-dependent and medium-induced (Gilbert *et al*., 1997). Thus, the type and concentration of nitrogen available in the growth medium has a significant effect on the genetic and biochemical pathways that lead to the production of proteinases in lactobacilli.

10.6.2.3 Carbon/Nitrogen Ratio

Because the type and concentration of nitrogen and carbon substrate in a growth medium influence the production of proteinases in lactobacilli, high productivities of proteinases require carbon and nitrogen to be present in the right ratio. The production of many other bioproducts in complex media has been shown to be dependent on the carbon/nitrogen (C/N) ratio, including exopolysaccharides (Degeest & De Vuyst, 1999), plasmids (Danquah & Forde, 2007), bacteriocins (Mataragas *et al*., 2004) and α-amylase (Singh *et al*., 2011). Usually, lower C/N ratios are beneficial for cell growth. An increase in C/N ratio increases proteinase production due to better cell growth, but this happens only until a certain plateau value is reached, after which proteinase yields begin to fall again. The decrease in proteinase yield at high C/N ratios is probably due to high osmotic stress, caused by a high carbon concentration. The C/N ratio and its effect on proteinase yield is an important parameter to consider in the design of growth media for proteinase production.

10.6.2.4 Metal-ion Requirement

Most proteolytic bacteria show variation in their requirement for salts for growth as well as in enzyme formation. Calcium (II) ions are important for proteinase synthesis in lactobacilli. Whereas Ca^{2+} content does not improve proteinase yield via metabolic mechanisms, it is important to the stability of proteinases during cell growth (Rahman *et al*., 2003). Proteinases in lactobacilli are cell-envelope bound and are therefore exposed to the susceptibility of degradation or shedding into the growth medium. Research has shown that calcium ions are directly involved in the active moiety of the enzyme to the anchor sequence (Coolbear *et al*., 1992). The functional involvement of calcium ions in the active conformation of the proteinase has been demonstrated and calcium ions have been shown to protect the enzyme against autoproteolytic release (Exterkate, 1995). The Ca^{2+} ions are weakly bound in proteinases and their removal initiates a structural rearrangement in the proteinase domain, causing an intramolecular autoproteolytic event which truncates the proteinase at the C-terminal end, leading to the release of the enzyme (Exterkate, 2000; Martín-Hernández *et al*., 1994; Kunji *et al*., 1996). Thus, the addition of

lower concentrations of Ca^{2+} in the growth medium of lactobacilli will inhibit proteinase degradation or autoproteolysis, since calcium plays a role in inducing and stabilising active conformations of the bound proteinases on cell surfaces (Coolbear *et al*., 1992; Exterkate, 1995; Siezen, 1999). Hebert *et al*., (2008) have used a minimal defined medium supplemented with 5 mM of $CaCl_2$ in characterisation studies of *Lactobacillus delbrueckii* subsp. *lactis* proteinases.

10.6.3 Process Optimisation for Growth and Proteinase Production

In addition to growth-media requirements, fermentation conditions such as incubation temperature, broth pH, agitation and the presence of oxygen also show a significant impact on proteinase synthesis.

10.6.3.1 Culture pH

As in most microorganisms, growth and enzyme production by lactobacilli are strongly dependent on extracellular pH (Espeche Turbay *et al*., 2009; Tsakalidou *et al*., 1999). Proteinase production by microbial strains is affected by extracellular pH via either pH-dependent control of protease gene expression (Young *et al*., 1996) or culture pH strongly controlling cell growth by influencing many enzymatic processes and transport of various components across the cell membranes (Ellaiah *et al*., 2002). The optimum pH range that promotes increased yields of proteinases is usually between 7.0 and 5.5, depending on whether fermentation occurred under controlled or uncontrolled pH conditions (Agyei & Danquah, 2012b; Espeche Turbay *et al*., 2009). The metabolic processes of most protease-producing microorganisms show a close relationship between proteinase syntheses, the utilisation of nitrogenous compounds and changes in pH. Thus, during fermentation, pH variation may indicate kinetic information about the enzyme production, such as the onset and consummation of the enzyme production (Kumar & Takagi, 1999). Moreover, there often exists a difference between the pH optimum for growth and that for proteinase production. This demonstrates that although bacterial growth and its enzyme production are greatly controlled by environmental conditions such as temperature and pH, the optimum conditions for enzyme synthesis and for optimum growth may differ (Das Mohapatra *et al*., 2009).

10.6.3.2 Incubation Temperature

Temperature affects all the physiological activities in a living cell and is an important environmental factor in the control of growth, microbial activities and the normal functioning of a cell's enzymes. The primary role temperature plays is to change the rates of the biochemical reactions necessary for cell growth; there is thus a link between enzyme synthesis and energy metabolism, which is controlled by temperature (Kumar & Takagi, 1999). The optimal temperature for growth of LAB varies between genera, from 20 to 45 °C (Panesar *et al*., 2010). For most bacteria there exists a linear relationship between the square root of the growth rate (μ_{max}) and the temperature (Fig. 10.3). This simple equation has been used successfully to predict the effect of temperature on the growth of a wide range of bacteria (Mackey & Kerridge, 1988; Membré *et al*., 2005). The temperature, obtained by extrapolation, at which the growth rate is zero is defined as T_o and may be a cardinal characteristic property of an organism growing under defined nutrient conditions (Mackey & Kerridge, 1988).

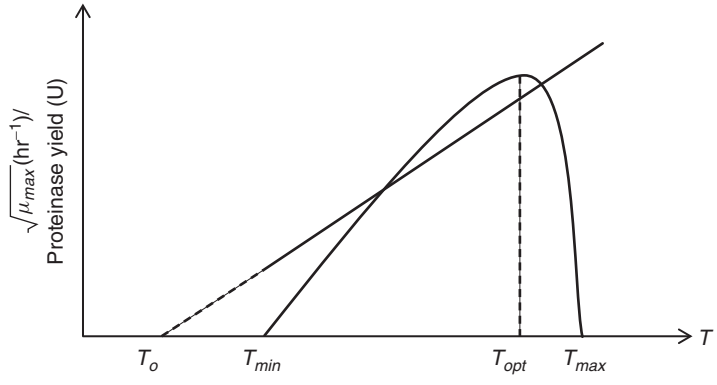

Fig. 10.3 Variation in the proteinase yield (parabola) and the square root of the growth rate, μ_{max} (straight line), as influenced by temperature of growth, T, for lactobacilli.

Further, proteinase activity increases linearly with temperature up to an allowed maximum and then decreases sharply (Fig. 10.3, parabola). Agyei *et al*. (2012) made this observation in their work with *L. delbrueckii* subsp. *lactis* 313. No proteinase activity was observed at temperatures 30 °C and below, whereas optimum proteinase yield was recorded at 45 °C. The decrease in proteinase activity beyond the optimum is likely due to thermal inactivation of biosystems at temperatures higher than the optimum; that is, the progressive enzyme distortion with respect to its biologically active conformation induced by thermal agitation (Perego *et al*., 2003; Sampaio *et al*., 2006). Therefore, although a high incubation temperature results in high cell growth rates, beyond the optimum temperature, T_{opt}, it does not necessarily give a higher protease yield. Because its effect on proteinases varies from organism to organism, critical points such as T_o, T_{opt} and T_{max} have to be elucidated for each organism in order to aid the control of proteinase via temperature manipulation.

The results of recent studies seem to suggest that inducing stress via fluctuating conditions such as osmosis, pH and temperature may improve bioproduct synthesis from bacteria. For example, temperature fluctuation has been used to improve the volumetric and specific yields of plasmid DNA by *E. coli* (Ongkudon *et al*., 2011). Proteinase synthesis, like plasmid synthesis, is induced and controlled by temperature, indicating that proteinase yields might also be improved via temperature-induction techniques. For example, heat-shock responses have been linked to the regulation of some peptidases in *E. coli* (Gottesman, 1996). Further, temperature fluctuation and its effect on the synthesis of inducible proteins have been demonstrated in lactobacilli such as *L. plantarum* (De Angelis *et al*., 2004) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Gouesbet *et al*., 2002). Consequently, temperature fluctuation and its effect on proteinase yield must be established for the different *Lactobacillus* species.

10.6.3.3 Gaseous Regime (Aeration)

LAB are generally phylogenetically intermixed with the aerobic and facultatively anaerobic genera of the of Gram-positive bacteria (Axelsson, 2004). The effect of a gaseous regime on proteinase production by lactobacilli is directly related to the bacterial growth at the indicated dissolved-oxygen level of the specified gaseous conditions. Usually, the dissolved-oxygen level of a fermentation culture is controlled by three mechanisms:

(1) varying the aeration rate; (2) varying the agitation speed of the bioreactor; or (3) using an oxygen-rich or oxygen-deficient gas phase as the oxygen source (Kumar & Takagi, 1999).

Differences in cell growth rate and cell density under different gaseous compositions have been demonstrated in several lactobacilli. For example, *L. plantarum* observes optimum growth under aerobic conditions (Murphy & Condon, 1984), whereas *L. delbrueckii* subsp. *lactis* 313 observes optimum growth under anaerobic conditions (Agyei & Danquah, 2012b).

During growth, lactobacilli adapt to various conditions and change their metabolism accordingly in order to favour pathways of efficient carbohydrate utilisation by altering pyruvate metabolism. During growth under microaerophilic and/or anaerobic conditions, the enzyme pyruvate-formate lyase catalyses the reaction of pyruvate and CoA to formate and acetyl CoA. This results in an increased overall ATP/glucose yield, as well as a change from the normal homolactic to a heterolactic-mixed acid fermentation, with consequent high culture acidification. This pathway is operational in several lactobacilli, such as *L. delbrueckii*, *L. plantarum* and *L. casei*, and results in high cell densities and high levels of organic acids, due to efficient glucose assimilation (Agyei & Danquah, 2012b; Kandler, 1983). However, in the presence of oxygen, reduced growth levels are observed for some lactobacilli. This has been attributed to the action of pyruvate oxidase, which is involved in the oxidation of pyruvate to acetyl phosphate, acetate and an equimolar amount of hydrogen peroxide (Hickey *et al*., 1983; Kandler, 1983). The low yields of ATP/glucose and the production and accumulation of toxic hydrogen peroxide in the culture medium lead to a slow growth rate and low cell density (Batdorj *et al*., 2007; Sakamoto & Komagata, 1996). Agyei & Danquah (2012b) have demonstrated that cell growth profile rate and proteinase-yield kinetics for cells grown under anaerobic conditions differ from those grown under microaerophilic conditions. The gaseous regime for the growth is therefore an important parameter to consider during proteinase production from lactobacilli.

10.6.3.4 Agitation Speed

For many lactobacilli, higher agitation speeds during growth generally result in higher biomass, due to generation of additional ATP and enhancement of fluid–particle mass transfer (Gupta *et al*., 2010). In addition to ensuring homogeneity of the fermentation broth, agitation also creates an aerobic milieu in the culture broth. Consequently, several studies have reported higher cell-biomass yields for lactobacilli grown at high agitation speeds or under micro-aeration conditions (Gupta *et al*., 2010; Murphy & Condon, 1984; Tango & Ghaly, 1999). However, Agyei & Danquah (2012b) have demonstrated that for *L. delbrueckii* subsp. *lactis* 313, vigorous agitation and other factors that promote aerobic conditions inhibit cell biomass yields. On the other hand, specific proteinase formation due to biomass (Yp/x) is significantly enhanced at higher agitation speeds of about 150 rpm. At high agitation speed, aeration of the culture medium is enhanced, which can lead to sufficient supply of dissolved oxygen in the media and/or enhanced nutrient uptake by bacteria, thus resulting in increased proteinase yields (Sepahy & Jabalameli, 2011). Thus, micro-aeration conditions favour proteinase synthesis, whereas inadequate aeration and nutrient uptake can be a cause of reduced proteinase yield. The effect of high culture agitation speeds on protease yields is well documented for several proteases that produce bacteria species (Potumarthi *et al*., 2007; Sepahy & Jabalameli, 2011), as well as some bacillus species (Saurabh *et al*., 2007). However, extremely high agitation speeds can be detrimental to proteinase yields, as seen in some studies (Agyei *et al*., 2012; Sepahy &

Jabalameli, 2011). Very high agitation rates can damage bacterial cells, thereby reducing proteinase yields. Also, at very high agitation speeds, CEPs are highly susceptible to autoproteolytic release into culture medium.

10.6.3.5 Inoculum Conditions

In food microbiology, the inoculum level significantly affects the changes in the physicochemical, biochemical and microbiological properties of food during fermentation (Visessanguan *et al*., 2006). In the same way, the proteinase yields of lactobacilli are greatly impacted by inoculum conditions such as density and age. Usually, proteinase yields increase with an increase in inoculum levels until a maximum point, beyond which they begin to decrease (Agyei *et al*., 2012). Seeding of cultures with low levels of inoculum often prolongs fermentation duration and achievement of high proteinase yields. On the other hand, very high inoculum levels also result in a decrease in the cell growth rate, perhaps due to the increasing limitation of key nutrients, and accumulation of greater amounts of growth-inhibitory metabolites, especially for cells at higher densities. A further explanation arises from reports that discuss cell–cell chemical signalling (quorum-sensing effects), which affects growth initiation under stressful conditions for cells at higher cell densities (Koutsoumanis & Sofos, 2005).

Moreover, inoculum can be conditioned to favour high proteinase yields either by using exponential phase cell as the inoculum or by preparing the inoculum in specialised media. Sakellaris & Gikas (1991) have reported that casein has the ability to stimulate proteinase production in pH-controlled fermentations of some *Lactobacillus* species. Thus, by preparing inoculum in casein, higher levels of proteinase yields can be induced from certain *Lactobacillus* species.

10.6.3.6 Time Course for Proteinase Production

For most microorganisms, the production of proteases exhibits a characteristic relationship with regards to the growth phase of the organism (Kumar & Takagi, 1999). Since lactobacilli are proteolytic for several amino acids, it follows that the synthesis of proteinase is largely constitutive (Gupta *et al*., 2002a). There are no or low proteinase yields during the lag phase. Proteinase production increases with cell growth until the middle-to-late exponential phase and then decreases gradually until the early stationary growth phase (Agyei & Danquah, 2012b; Tsakalidou *et al*., 1999). Some amount of enzyme activity is detected in the stationary phase, but it is usually low—the result of denaturation caused by prolonged enzyme exposure to very low pH (Espeche Turbay *et al*., 2009). Also, at the stationary phase, the protein machinery of a cell probably shifts from proteinase production to the production of stress-induced proteins such as surface-layer proteins (Agyei & Danquah, 2012b). Elucidating the proteinase production profile with cell growth is very important in identifying the ideal harvesting time for maximum proteinase activity.

10.7 EFFECT OF FERMENTATION MODES ON CELL GROWTH AND PROTEINASE PRODUCTION

The usefulness of lactobacilli in food processing and in the manufacture of bioproducts is largely dependent on the physiological state of the cells during fermentation, which is in turn influenced by the fermentation mode, growth conditions and growth parameters. It is therefore important to maintain proper control of starter production in order to prevent

poor fermentation yields and improve the product quality. This control may be achieved by studying the process parameters of the different fermentation modes (whether batch, fed-batch or continuous) and their effects on the growth kinetics of the bacteria and the cell physiological state during growth and product synthesis (Rault *et al*., 2009).

In batch fermentation, the materials required for fermentation are loaded on to the fermentor and sterilised before beginning the process in a partially closed system. The materials that enter and exit the system are the gas exchanges and pH control solutions. Products are removed at the end of the process. Economically, batch processes are easy to set up and maintain. If contamination occurs, only one batch is affected. Batch fermentation remains the most commonly used approach in most industrial-scale processes (Bouguettoucha *et al*., 2009). It is, however, characterised by substrate and product inhibition kinetics, which limits the growth rate and productivity of cultures (Boonmee *et al*., 2003; Rault *et al*., 2009). Also, product quality may vary with the different batches as conditions change with time and the fermentor is in an unsteady-state system.

With fed-batch fermentation, cells are first grown under a batch regime until a certain point in time (usually the attainment of the exponential growth phase), when the setup is fed with a solution of fresh substrates without removing the spent culture fluid. Fed-batch processes are useful in achieving a high product yield based on biomass over a relative large span of time.

For continuous fermentation, nutrients and substances (e.g. pH control solutions) are added and products and spent media or cells are removed continuously at rates at which organisms are held in the exponential growth phase. A steady state can be attained with a continuous fermentation mechanism which allows the determination of the relationship between microbial behaviour and the culture environmental conditions. The characteristic features of continuous systems—the continuous operation of the bioreactors at a dilution rate exceeding the maximum specific growth rate and the reduced inhibitory effects of the substrate, product or both—lead to improvements in the system's efficiency and increased productivity (Tyagi *et al*., 1992). The continuous fermentation mechanism is useful for reducing production costs and improving the process efficiency and product yield (Vasconcelos *et al*., 2004). Continuous production is more advantageous than the batch processes because the unproductive time employed in charging, discharging, cleaning, sterilisation and so on is avoided or minimised.

A number of studies have been published on improving lactobacilli biomass by exploiting novel bioreactor systems which permit media supplementation and/or the exchange of medium to prevent product or waste accumulation and therefore alleviate growth inhibition and achieve high cell density. However, these are largely optimised for products like lactic acid, and not for proteinases. Consequently, the literature is very scanty on the effects of different fermentation modes on proteinase production in the *Lactobacillus* species.

Fermentation activities for most of the literature references used in this chapter were conducted in batch mode (e.g. Agyei & Danquah, 2012b; Espeche Turbay *et al*., 2009; Hebert *et al*., 1997, 2004, 2008; Tsakalidou *et al*., 1999) but the focus of these studies was not on comparing fermentation modes and their effects on proteinase yields. On the other hand, some literature exists for *Lactococus lactis*, which can be approximated for the lactobacilli. Marugg *et al*. (1995) have reported that the expression of *prtP* gene promoter is only affected marginally by the growth rate for batch cultures of *Lactococus lactis* SK11. However, in continuous cultures at increasing dilution rates ($0.05 < D < 0.5\,h^{-1}$), a maximally threefold decrease in *prtP* expression levels was observed.

Laan *et al*. (1993), on the other hand, observed a different growth-rate dependency for *Lactococus lactis* Wg2 proteinase gene expression, where the expression level in continuous cultures was found to be maximal at a dilution rate of $0.23\,h^{-1}$ but decreased at higher dilution rates. This implies that the different fermentation modes trigger different responses in proteinase synthesis levels among the LAB. In order to scale up proteinase production from lactobacilli to the industrial level, biochemical engineering strategies need to be applied to obtain high yields of proteinases in a bioreactor. The literature is replete with bioreactor systems and fermentation modes that have been used to improve the yields of products other than proteinases. These include various stirred-tank reactors (STRs), cyclone reactors, gas-lift reactors, shaken ceramic flasks, compact, submerged membrane bioreactors (Ramchandran *et al*., 2012) and fed-batch, semi-batch and chemostat fermentations. Research studies are needed to establish the kinetics of proteinase synthesis by lactobacilli for these systems.

10.8 CELL SYSTEMS FOR PROTEINASE PRODUCTION

Microbial products are usually produced by either free or immobilised cells and the use of immobilised cells as industrial catalysts offers more advantages than batch fermentation processes (Adinarayana *et al*., 2005). The use of immobilised biocatalysts (whole cells or enzymes) is advantageous because such biocatalysts display better operational stability, higher catalysis efficiency and higher cell density, as well as allowing reusability, during continuous fermentation (Adinarayana *et al*., 2005; Norton *et al*., 1994). Research information is abundant on the use of immobilised systems for the production of *Bacillus* proteases (Gupta *et al*., 2002b), as well as for the production of lactic acid from lactobacilli (Norton *et al*., 1994). However, in spite of the considerable commercial interest, relatively little work has been carried out on this subject (Alekseiva *et al*., 1998). *Lactobacillus* cell morphology reveals that the proteinases are cell envelope-bound and not strictly 'extracellular' products, and thus do not lend themselves to production by immobilised cells. The merits of the use of immobilised cells ought therefore to be weighed against the demerits. The cell system which incorporates lower costs and higher proteinase yields would be preferred.

10.9 STATISTICAL METHODS AND MATHEMATICAL MODELS

The conventional sequential method used for optimisation studies is often cumbersome, tedious and time-consuming, and requires a large number of experiments when a large number of parameters are being studied. In addition, it does not consider the effect of different interactions of various parameters (Vellanki *et al*., 2009). The use of statistical designs, however, applies a mathematical framework that covers all experimental factors, with a minimum number of experiments required to achieve useful results (Beg *et al*., 2002). Using an appropriate statistical approach, optimisation can be carried out by the simultaneous control of many factors (fermentation conditions and process parameters). For example, the Plackett–Burman factorial design is often used in preliminary studies to select variables that can be fixed or eliminated in further optimisation processes. It allows for the screening of main factors from a large number of process variables

(Reddy *et al*., 2008). Response-surface methodology, on the other hand, is used in many biotechnology processes to evaluate and study the interactions between different process parameters (Vellanki *et al*., 2009). Mathematical models also exist to study microbial growth or the kinetic constants of microbial enzymes (Beg *et al*., 2002). These offer better understanding of the fermentation process and its optimisation. The Gompertz model is an efficient mathematical method of quickly estimating and describing the microbial growth parameters in easy-to-understand and technologically relevant terms (Tomás *et al*., 2010; Zwietering *et al*., 1990). Other models, such as Luedeking–Piret, provide both a 'growth-associated' and a 'non-growth-associated' term for product yield (Luedeking & Piret, 2000). They were originally designed for products such as lactic acid but can be modified and extended for proteinase production mechanisms in lactobacilli. The use of properly designed models with multifactor analyses will help the design and scale-up of proteinase yields.

10.10 CONCLUSION

Proteinases are an important biocatalyst for the production of biotechnological products such as bioactive peptides, and microbes represents the preferred source of these enzymes due to their rapid growth, the limited space required for their cultivation and their genetic pliability. The vast diversity of lactobacilli proteinases represents an industrially feasible avenue for exploitation in biotechnological applications. However, continued improvement of proteinase yields will depend on a number of factors: identification of highly proteolytic strains, improvement of proteinase yields via optimisation of media-component and process parameters and efficient product recovery. Successfully addressing these issues will require a multidisciplinary approach, encompassing fields such as microbiology, statistics and mathematical modelling and bioprocess engineering.

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Section 2.6

2.6. Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part II—Downstream Processes

Monash University
Declaration for Thesis Chapter 2 Section 6

Declaration by candidate

In the case of Chapter 2 Section 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Initiation, key ideas and writing up	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**
Dr. Ravichandra Potumarthi	Initiation, and key ideas
Dr. Michael K Danquah	Initiation, key ideas and writing up

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12 December 2013
Main Supervisor's Signature	[redacted]	**Date** 12 December 2013

11 Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part II — Downstream Processes

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11.1 INTRODUCTION: CELL RECOVERY

The various process engineering techniques necessary to increase proteinase synthesis by lactobacilli have been discussed in Chapter 10. This chapter deals with the remaining downstream processes: recovery, isolation and purification strategies.

After fermentation is complete, the cells can be separated from the culture medium via centrifugation using continuous disc centrifuges or filtration using vacuum rotary drum filters. In order to enhance the separation, the cells can be flocculated by flocculating agents, which neutralise the charges on the microbial cell surfaces and alter the ionic environment. This aids the formation of larger flocs or agglomerates, which in turn accelerate solid–liquid separation. The cell flocculants commonly used are organic polyelectrolytes, inorganic salts and mineral hydrocolloids (Kumar & Takagi, 1999). Following this, harvested cells are washed with appropriate buffer, such as sterile phosphate-buffered saline or 0.15 M saline supplemented with 10–20 mM $CaCl_2$. The resulting cell pellet is then ready for the subsequent stages of the process, in which proteinases are extracted.

It is important that the cell-recovery stages be undertaken under low-temperature conditions. The lowering of the temperature to below 5 °C helps prevents microbial contamination, as well as maintaining enzyme activity and stability (Kumar & Takagi, 1999).

11.2 ISOLATION: PROTEINASE-EXTRACTION METHODOLOGIES

Usually, no cell disruption is necessary to recover proteinases from lactobacilli. That is because during lactobacilli fermentation, the proteinases are expressed and anchored on to the cell membrane. This is why the proteinases are referred to as cell envelope-associated proteinases, or cell envelope-bound proteinases, cell wall-bound proteinases, cell-surface proteinases (generally abbreviated as CEPs) or lactocepins (Siezen, 1999). Their position on the cell surface makes harvesting and purification of the proteinases relatively less laborious and cheaper (Agyei & Danquah, 2011). The CEPs in bacteria have

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been shown to display five different functional multidomain structures on the cell surface; beginning at the N-terminus, these are PrtP (present in *Lactococcus lactis*), PrtB (present in *Lb. delbrueckii* subsp. *bulgaricus*), PrtH (present in *Lb. helveticus*), ScpA (present in *Streptococcus pyogenes*) and Csp (present in *Streptococcus agalactiae*) (Gilbert *et al*., 1997; Pastar *et al*., 2003; Siezen, 1999).

The prepro domain (PP domain) has been shown to be important for the secretion and activation of proteinase, whereas the PR domain is the catalytic serine protease domain, which comprises several sequences homologous to subtilases and an internal domain (I domain). The large A domain common to all CEPs immediately follows the PR domain. The B domain is found only in the lactococci and lactobacilli, and the helix spacer domain (H domain) is present in PrtP and PrtH following the A domain. The W domain (cell-wall spacer) is present in all five types of proteinase. The W domain of PrtP and streptococcal CEPs precedes a typical cell-wall anchor (AN domain), while PrtH and PrtB lack the C-terminally positioned AN domain and bind the cell wall by means of the W domain itself (Pastar *et al*., 2003; Siezen, 1999).

Generally the total cell envelope-associated proteins in the lactobacilli consist of ribosomal proteins; permeases of the glycolytic pathway (Sánchez *et al*., 2009); the crystalline surface-layer (S-layer) proteins, which are responsible for cell protection, adhesion and cell-surface recognition (Deepika *et al*., 2009; Lortal *et al*., 1992; Schar-Zammaretti *et al*., 2005); and the CEPs responsible for hydrolysis of proteins to peptides (Espeche Turbay *et al*., 2009; Tsakalidou *et al*., 1999).

Elucidating the most suitable method for the extraction of cell-envelope proteinases from lactobacilli is an important undertaking as the use of a particular extraction agent produces yields that are species- and/or strain-dependent, and no single method works for all lactobacilli. A suitable method should release large quantities of CEPs of high specific activity and a low degree of cell lysis, in order to avoid contamination of CEPs with intracellular enzymes. The mechanism of action of the different CEP-extracting agents is based on their ability to disrupt the molecular interactions anchoring cell-surface proteins (including proteinases) to the cell envelope, such as the S-layer, peptidoglycan layer and phosphlipid bilayer. Some of the methods used for the extraction of CEPs from lactic acid bacteria (LABs) are shown in Table 11.1. Broadly, the widely used extraction methods for LABs consist of: (1) Ca^{2+}-free buffers such as Tris–HCl or phosphate-buffered saline; (2) low-concentration detergents such as sodium dodecyl sulfate (SDS); (3) chaotropic agents such as LiCl and guanidine hydrochloride; and (4) enzymes (muramidases).

11.2.1 Ca^{2+}-Free Buffers

The most extensively used method for releasing CEPs from LAB cells is to wash or incubate the cells in a calcium-free buffer (Exterkate, 1990; Fira *et al*., 2001; Tsakalidou *et al*., 1999). It has been observed that the release of lactococcal CEPs from the cell surface occurs spontaneously in a Ca^{2+}-free buffer. The removal of relatively weakly bound calcium in CEP initiates a structural rearrangement in the proteinase domain. This causes an intramolecular autoproteolytic event, which truncates the proteinase at the C-terminal end, causing the release of the enzyme (Exterkate, 2000; Kunji *et al*., 1996; Martín-Hernández *et al*., 1994). However, although this method is simple and gives low levels of cell lysis (Tsakalidou *et al*., 1999), it leads to a reduction in the enzyme activity and thermal stability of the CEP released (Exterkate, 2000). Moreover, Martín-Hernández *et al*.

Table 11.1 Agents for the extraction of surface proteins from some lactobacilli species.

Agent	Conc.	Incubation		LAB species studied	Reference
		Time (min)	Temperature (°C)		
Guanidine-HCl	4 M	30	37	*L. acidophilus* W	Bhowmik *et al.* (1985)
Sodium dodecyl sulfate	0.2% (w/v)	30	37	*L. acidophilus* W	Bhowmik *et al.* (1985)
Urea	8 M	60	37	*L. acidophilus* W, *L. rhamnosus* (strains E/N, Oxy, and Pen)	Bhowmik *et al.* (1985), Jarocki *et al.* (2010)
Lithium Chloride	1 M, 5 M	60	30	*L. rhamnosus*	Jarocki *et al.* (2010)
Glycine (pH 2.2)	0.2 M	15	4	*L. fermentum* BR11	Turner *et al.* (1997)
Na-phosphate buffer (pH 7)	50 mM	120	30	*L. delbrueckii* subsp. *lactis* ACA-DC 178	Tsakalidou *et al.* (1999)
Lysozyme in buffer	800 U/ml			*L. rhamnosus* GG	Sánchez *et al.* (2009), Macedo *et al.* (2003)
NaOH	10 mM	30[a]	37	*L. rhamnosus* GG	Sánchez *et al.* (2009)
Mutanolysin (with or without lysozyme)	42 U/ml			*L. plantarum*	Macedo *et al.* (2003)
Lysozyme + sucrose in NH_4CO_3		30[a]	37	*L. rhamnosus* GG	Sánchez *et al.* (2009)

[a]Incubation done with gentle agitation.

(1994) observed that no CEPs are released via washing or incubation of *L. delbrueckii* and *L. casei* cells in calcium-free buffer, unless the cells were treated previously with lysozyme. In their work, Atlan *et al*. (1989) observed that repeated washing of *L. bulgaricus* CNRZ 397 cells with Ca^{2+} buffer could not release a metallo-enzyme from the cell wall, and they explain that this is due to the rather close and tight association of the proteinase with the peptidoglycan wall (Laloi *et al*., 1991). In another study, the activity, release and stability of CEP from *L. delbrueckii* subsp. *lactis* CRL 581 were not affected by the presence of calcium ions (Espeche Turbay *et al*., 2009). This indicates that the effect of Ca^{2+} on CEPs is strain-dependent. This property is probably dependent on the structure of the CEP domains on the cell surface (Siezen, 1999).

11.2.2 Chaotropic Agents

Chaotropic ions favour the transfer of apolar groups to water and thus provide a means for the resolution of proteins and for increasing the water solubility of particular proteins (Hatefi & Hanstein, 1969). The action of chaotropic agents is related to their effect on the structure and lipophilicity of water, since they dissociate aggregated proteins by increasing the solubility of hydrophobic regions of proteins in aqueous environments (Clinkenbeard *et al*., 1995). Thus chaotropic agents interfere with noncovalent interactions between surface proteins and bacterial surfaces.

11.2.2.1 Urea

Microbial cell-surface proteins can also be extracted by employing the chaotropic properties of detergents and salts. For example, Jarocki *et al*. (2010) observed that the use of urea releases large quantities of surface-associated proteins from *L rhamnosus*. Urea is a strong protein denaturant and disrupts the noncovalent bonds in the proteins, thus working through a solvation mechanism. Urea exerts its effect directly by binding to the protein and/or indirectly by altering the solvent environment (Bennion & Daggett, 2003). Direct urea interactions involve hydrogen bonding to the polar moieties of the protein; particularly peptide groups of the S-layer proteins and peptidoglycan layer. Subsequently, this leads to disruptions of intramolecular hydrogen bonds of the protein. Since urea acts on both the S-layer and the peptidoglycan layer, it has been demonstrated to exhibit the best results with the highest mean activity and specific activity (Hua *et al*., 2008). In comparison with other extraction agents, urea is known to release larger quantities of cell surface-associated proteins without significantly affecting the morphological structure or viability of cells (Jarocki *et al*., 2010).

11.2.2.2 Lithium Chloride

Lithium chloride is a mild extraction agent and predominantly attacks only the surface layer. The lithium ions attack the S-layer protein subunits, which are noncovalently linked to each other, and disintegrates them into monomers by cation substitution (Schär-Zammaretti & Ubbink, 2003). Sánchez *et al*. (2009) have shown treatment of *Lb. rhamnosus* GG (LGG) cells with 5 M LiCl to be the most suitable method for the extraction of surface proteins in LGG, since this allows the extraction of some additional cell surface-associated proteins compared to other extraction methods. The use of LiCl is an established method for selective surface-molecule solubilisation with maintained cell integrity (Hussain *et al*., 1999).

11.2.2.3 Guanidine Hydrochloride

Guanidine–HCl is a chaotropic agent that denatures proteins by disrupting their secondary structure, leading to the formation of random coils, which become further displaced by guanidine ions. The guanidinium ion is a strong base with a pKa of 13.6 (Perrin, 1972). Consequently, it is able to disrupt the noncovalent interactions between S-layer monomer units. Because it is such a strong base, the solution may also hydrolyse the glycoproteins of the S-layer monomer, peptidoglycan layer and phospholipid bilayer through hydroxide nucleophilic attack of the carbonyl group in the peptide linkages. Bowmik *et al*., (1985) have remarked that guanidine hydrochloride solution is the best extraction agent by which to obtain large quantities of cell-surface proteins from *L. acidophilus* strains. Guanidine–HCl is also used in medical microbiology extraction of indicator proteins from the cell walls of pathogenic microorganisms, due to its protein-dissolution properties (Russell & Facklam, 1975).

11.2.2.4 Glycine

Glycine is the smallest amino acid, with an isoelectric point of 6.06. At a pH of 2.2, the solution mostly contains the cationic acid ion of glycine (Brown & Poon, 2010). Since it is a polar cationic molecule, it is thought to disrupt the S-layer protein-monomer noncovalent interactions. Glycine at 0.2 M has been used in the preparation of a virtually pure 32 kDa protein from *Lb. fermentum* BR11 (Turner *et al*., 1997). However, glycine at the same concentration is unable to release cell-surface proteins from *Lb. fermentum* 104R (Rojas *et al*., 2002). This shows that some of the extraction agents are very strain-specific.

11.2.3 Low-Concentration Detergent

11.2.3.1 Sodium Dodecyl Sulfate

SDS is an anionic denaturing agent and is thus capable of disintegrating the S-layer protein subunits of lactobacilli cell-surface proteins into monomers (Schär-Zammaretti & Ubbink, 2003). It denatures secondary and non-disulfide-linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. This causes a breakdown of the cell membrane due to emulsification of surface lipids and proteins and the disruption of the polar interactions that hold it together (Brown & Poon, 2010). Based on its mechanism of action, SDS can be quite a harsh extraction agent and may irreversibly denature proteinases when used at higher concentrations. It is generally effective at concentrations between 0.1 (Bhowmik *et al*., 1985) and 2% (v/v) (Chagnaud *et al*., 1992). The use of SDS in proteinase extraction from lactobacilli cells may require an initial physical or chemical treatment to enhance extraction efficiency. For example, Chagnaud *et al*. (1992) have reported that extraction of cell-surface proteins from lactobacilli with SDS alone shows improved effectiveness if the cells are sonicated prior to SDS treatment.

11.2.3.2 Triton X-100, CHAPS and DTT

Triton X-100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) are milder detergents that are not effective in releasing large quantities of cell-surface protein but are however useful for retaining the protein's native conformation during subsequent purification steps (Bhowmik *et al*., 1985; Wu *et al*., 2009). This property makes them useful additives in mixtures of extraction agents.

11.2.4 Use of Enzymes (Muramidases)

Proteinase extraction has also been achieved via the hydrolytic properties of lysozyme and/or mutanolysin for the hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the peptidoglycan cell wall. This causes the release of all cell wall-bound proteins. Atlan *et al*. (1990) obtained an excellent yield of proteinases from *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 when they coupled lysozyme/mutanolysin treatment with cold-temperature shock, and the dual treatment did not cause significant cell lysis. However, based on the mechanism of their action, the use of muramidases without proper control of process conditions (such as incubation time and temperature) could result in significant levels of cell lysis. Another downside to the use of lysozyme is that lysozyme exhibits an antibacterial activity against some Gram-positive bacteria. This antimicrobial activity is independent of its enzymatic activity (McKenzie & White, 1991).

11.2.5 Use of a Cocktail of Extractant

Proteinases can also be solubilised with cocktails or combination of two or more extraction agents. The use of a combination of methods provides the advantage that the demerits of one component can be counterbalanced by the merits of another. There is also the possibility of synergistic action between the components, which can enhance activities and thus reduce overall cost. Rojas *et al*. (2002) have used 1 M lithium chloride–lysozyme solution (comprising 0.1 M Tris, 0.015 M NaCl, 0.05 M $MgCl_2$ and 40 µg/ml of lysozyme) to extract cell-surface proteins from *L. fermentum* 104R. Sánchez *et al*. (2009) have also used lysozyme buffer (containing 800 U/ml lysozyme and 0.5 M sucrose in 30 mM ammonium bicarbonate) to release cell surface-associated proteins from *L. rhamnosus* GG.

11.2.6 Factors Affecting Enzyme Extraction

11.2.6.1 Cell Lyses

During the extraction of proteinases, cell lyses leading to the release of intracellular substances is a nuisance and must be kept to the allowed minimum. This is because intracellular enzymes contaminate, and may also compromise, the activity of crude proteinases. They also increase the cost of proteinase purification. Assessment of the performance of an extraction agent should include its effect on cell lysis. The release of intracellular enzyme is characterised by perforation or lysis of the cell during enzyme extraction. Cell lysis or cell death is quantified by measuring the activity of one or more intracellular enzyme(s), such as lactate dehydrogenase, β-galactosidase or intracellular peptidases (such as lysyl-aminopeptidase). Cell lysis can also be verified by running and comparing the SDS-PAGE profile of cell-surface extracts and total cell protein extracts. Moreover, viable bacterial counts made before and after extraction treatments will give an indication of cell death.

11.2.6.2 Incubation Time, Temperature and pH

During extraction, the pH of extractant, the incubation temperature and the time are important factors to control, for economic reasons. For example, too long an incubation time will contribute to a high processing cost. Further, the efficiency of an extractant depends partly on these factors. The native chemical and functional properties of proteins

imply that interaction with solubilising agents is largely influenced by temperature and pH, which, if uncontrolled, could result in a compromise of the activity and stability of proteinases.

All extraction agents are used at an optimal temperature, which is usually equal to or less than the fermentation temperature. Other authors have reported the use of cold shock to aid the release of proteinase in *L. delbrueckii* subsp. *bulgaricus* (Atlan *et al*., 1990).

It has also been reported that the attachment of some cell-surface proteins to viable cells is pH-dependent, and release of surface proteins into the bacterial surroundings is pronounced at neutral or slightly alkaline pH (Antikainen *et al*., 2007; Sánchez *et al*., 2009). This pH dependency for the attachment/release of cell surface-bound proteins implies that extraction agents must be used at the optimal pH; that is, the pH at which protein solubilisation is enhanced and enzyme activity is maintained.

11.3 PURIFICATION OF ENZYMES

Captured in Table 11.2 are a number of proteinases from different lactobacilli and a summary of their purification techniques. There are challenges for the bioprocess industry in downstream processes for the separation and fractionation of proteins, which are due to the complexity of biosuspensions, the selectivity and low filtration velocity of targeted molecules and the low concentration of product (Saxena *et al*., 2010). Also, in protein purification, not only should a downstream purification scheme achieve the required purity and recovery levels of the desired product in a safe, reliable and reproducible manner, but this must be done in an economically viable and cost-effective manner (Desai *et al*., 2000). This requires the exploitation of several alternative purification routes (Fig. 11.1) and selection of the most optimal for the proteinase of interest.

Often, the enzyme activity for crude cell-free extract is low. It is therefore important to concentrate the extract down via the removal of water. This is achieved by membrane separation technologies such as ultrafiltration (UF), which is used largely for the recovery of enzymes (Kumar & Takagi, 1999). Other techniques for protein concentration include salting out with sodium sulfate or ammonium sulfate (Gupta *et al*., 2002a; Kumar & Takagi, 1999; Macedo *et al*., 2003) and the use of organic solvents such as acetone (Kumar & Takagi, 1999) and ethanol (Gupta *et al*., 2002a). Enzyme precipitation can also be achieved by the use of water-soluble, neutral polymers such as polyethylene glycol (Kumar & Takagi, 1999). Through these concentration steps, some level of purification is also achieved.

Primary and intermediate purification stages are performed on clarified and/or concentrated product for the purpose of removing impurities such as media components, DNA, viruses and endotoxins. The actual purification of the enzyme requires a combination of one or more techniques, usually high-performance tangential flow filtration (HPTFF), high-performance liquid chromatography (HPLC) and UF techniques. Other methods, including foam fractionation, aqueous two-phase systems and dye-ligand chromatography, have been employed on a small scale and are currently awaiting scale-up (Gupta *et al*., 2002a).

The purification process is finally completed by the polishing stage, in which trace impurities and contaminants are removed, leaving behind an active and safe product suitable for formulation or utilisation.

Table 11.2 Characterization of proteinases of lactobacilli.

Strain	Molecular weight	Structure/ proteinase type	CEP type	Purification technique(s)	Optimum pH	Optimum temperature (°C)	Metal-ion inducers	Reference
Lb. delbrueckii subsp. *lactis* ACA-DC 178	–	Serine- proteinase	Type I	No purification—crude proteinase used	6	40		Tsakalidou *et al.* (1999)
Lb. casei NCDO 151	–	Serine proteinase		DEAE-sephacel ion-exchange chromatography	7	40		Ezzat *et al.* (1988)
Lb. casei NCDO 151	–	Cysteine proteinase		DEAE-sephacel ion-exchange chromatography	7.5	45	–	Ezzat *et al.* (1988)
Lb. helveticus	–	Serine proteinase	–	Sephacryl gel-filtration chromatography	7	40		Ezzat *et al.* (1993)
Lb. casei HN14		Serine proteinase	Type I	No purification—crude proteinase used	–	–	–	Kojic *et al.* (1991)
Lb. paracasei subsp. *paracasei* NCDO 151	135 kDa and 110 kDa[a]	–	–	Anion-exchange; hydrophobic interaction; chromatofocusing; gel-filtration chromatography	–	–	–	Nas & Nissen-Meyer (1992)
Lb. casei NCDO 151	150 kDa	Serine proteinase	–	(Mono Q HR 5/5) Anion-exchange FPLC; chromatofocusing (Mono P, HR 5/20);	5.6 (with casein as substrate)	35–37	Ca^{2+}, Co^{2+}	Næs *et al.* (1991)
Lb. delbrueckii ssp. *bulgaricus* CNRZ 397	170 kDa	Cysteine proteinase		Ultrafiltration; ion-exchange chromatography	5.5	42		Laloi *et al.* (1991)
Lb. helveticus L89	180 kDa	Serine proteinase	Type I/III mixed-type variant	Hydrophobic interaction chromatograph; diafiltration	7	50	–	Martín-Hernández *et al.* (1994)
Lb. helveticus CP790	45 kDa	Serine proteinase	Type III	Ion-exchange chromatography	6.5	42	–	Yamamoto *et al.* (1993)
Lb. rhamnosus BGT10	154 kDa	Serine proteinase	–	No purification—crude proteinase used	6.5	–	–	Pastar *et al.* (2003)
		Serine proteinase		Ion-exchange gel filtration	7.5–8	42		Zevaco & Gripon (1988)

[a]As determined by SDS-PAGE.

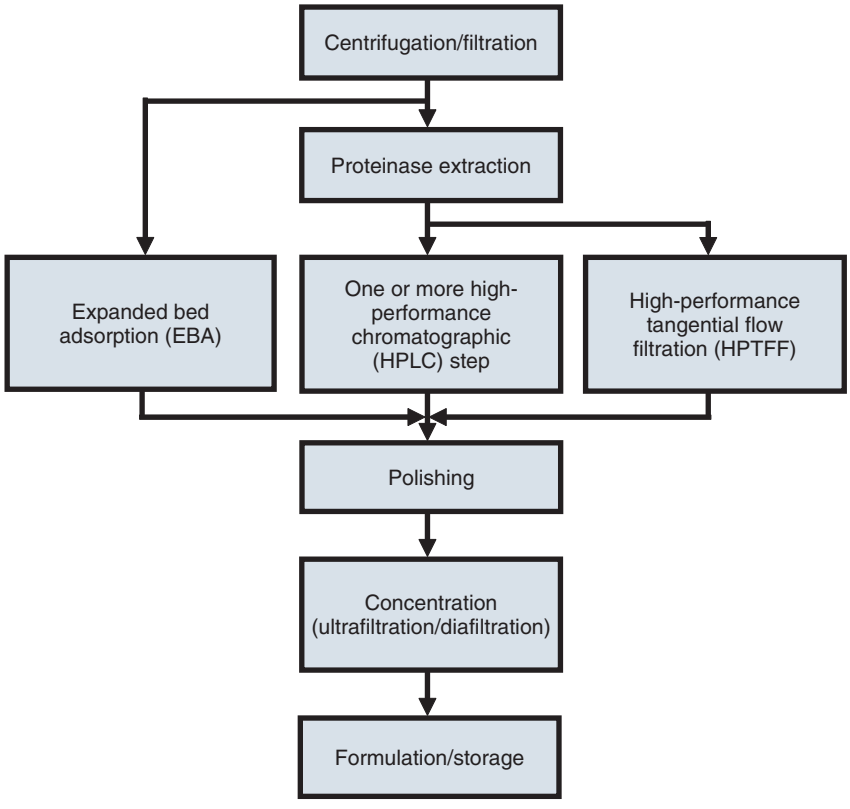

Fig. 11.1 Schematic of downstream purification operations.

11.3.1 High-Performance Tangential Flow Filtration

HPTFF is a highly selective technology that can be used to separate charged species of similar or different size using semipermeable membranes (van Reis *et al*., 1997, 1999). It is a two-dimensional purification method that exploits differences in both size and charge characteristics of biomolecules. HPTFF technology can provide protein concentration, buffer exchange and purification in a single-unit operation; processes that are performed using a combination of quite a number of separate chromatography and UF steps. Also, by the control of filtrate flux and device fluid mechanics, this technique eludes to a great degree the problem of fouling. The performance of HPTFF has been shown to be reproducible and robust. Usually, the charged membrane used in HPTFF can be regenerated and reused many times without loss of selectivity or throughput. There are also the added advantages of the elimination of one or more chromatographic steps, yield improvement and a significant reduction in the cost of product purification (Lebreton *et al*., 2008).

It has been demonstrated that significant improvements in performance and maximal protein selectivity in HPTFF can be achieved simply by controlling buffer pH and ionic strength. Saksena & Zydney (1994) have shown that selectivity for the filtration of bovine serum albumin (BSA) and immunoglobulin G (IgG) is increased from a value of 2 to more than 30 simply by adjusting the pH from 7 to 4.7 and lowering the solution ionic

strength. Van Reis *et al*. (1999) demonstrated the possibility of exploiting the membrane charge to further enhance performance. They reported, in a study in which the effects of membrane charge and buffer pH were explored, that purification factors up to 990-fold were obtained with yields of 94%. Further, Rao *et al*. (2007) have demonstrated the ability to use small charged affinity ligands with biospecific binding characteristics to enhance protein separation by HPTFF. HPTFF therefore lends itself as a promising technique to proteinase purification, based on its success rates in the purification of other protein systems. Further, based on the aforementioned advantages and high throughput values, HPTFF can be used in initial, intermediate and final purification stages and can easily be utilised in even industrial-scale systems with reduced production costs (van Reis *et al*., 1999).

11.3.2 High-Performance Liquid Chromatography

Although protein separations can be achieved by a variety of techniques, chromatographic separations are by far the most widely used (Jones & Baines, 2000; Kennedy, 2001). Liquid-column chromatographic techniques have been used for many decades in analytical biochemistry. The introduction of HPLC in the mid-1970s saw the use of extremely high pressures and smaller-particle packing materials. This development has completely revolutionalised liquid chromatography, improving efficiency, purification, separation, identification and quantification, as well as reducing analysis time and allowing for the separation of samples at the microscale (MacNair *et al*., 1997). In HPLC, the different interactions and/or binding mechanisms between a solid-phase resin and the target molecule in the feedstream affect the separation or purification of biomolecules (Desai *et al*., 2000). The well-known HPLC types and their potential in the separation of proteinases are described in this section.

11.3.3 Ion-exchange Chromatography

Ion-exchange chromatography (IEC) is one of the most commonly used HPLC modes. Due to its rapid separation, easy sanitisation, lack of organic-solvent requirements and wide selection of available stationary phases, IEC remains one of the most prominent methods in the separation and purification of most charged biomolecules that are soluble within an aqueous system (Ongkudon & Danquah, 2010). IEC separates on the basis of ionic interaction between molecules of different charge and charges of stationary matrix. Elution of bound molecules from the column is done by an increasing salt or pH gradient. IEC is ideal for proteinases because it offers binding and elution at relatively mild conditions, allowing the retention of biological activity (Desai *et al*., 2000).

The ion-exchange matrix for IEC can contain either anion exchanger (positively charged) or cation exchanger (negatively charged), determined by the presence and type of charged ligand groups. Some common functional groups used in ion-exchange matrices are diethylaminoethyl (DEAE), quarternary aminoethyl (QAE) and quarternary ammonium (Q) functional groups for anion-exchange chromatography; and carboxymethyl (CM), sulfopropyl (SP) and methyl sulfonate (S) functional groups for cation-exchange chromatography. The use of anion exchangers for proteinase purification is very common (Table 11.2).

11.3.4 Size-exclusion Chromatography

Size-exclusion chromatography (SEC) separates proteins according to their effective molecular size. It is also referred to as 'gel permeation' or 'gel filtration'. The resins used involve porous silica and polyacrylamide crosslinked with agarose gel matrices. These can be modified to give separation resins that possess specific surface and structural properties, and still maintain high mechanical strength and chromatographic properties.

The common applications of SEC include protein concentration, fractionation, desalting and buffer exchange. With SEC, the analyte does not interact with the chromatographic system. This aids the retention of biological activity, while separating the impurities (Desai *et al*., 2000). SEC is one of the most widely used chromatographic steps for proteinase purification (see Table 11.2).

11.3.5 Hydrophobic-interaction Chromatography

Hydrophobic-interaction chromatography (HIC) is a salt-mediated separation of proteins adsorbed on to a weakly hydrophobic support matrix. That is, HIC exploits the interaction between the hydrophobic moiety of proteins and the bulk aqueous environment, in the presence of a neutral salt, to facilitate the adsorption and elution of proteins of interest. Elution is usually achieved by reducing the ionic strength of the mobile phase, in a decreasing salt gradient (Queiroz *et al*., 2001). HIC is largely used in industrial operations and also as an analytical technique for the separation of several biotechnological products, such as homologous proteins, antibodies, recombinant proteins and nucleic acids (Desai *et al*., 2000; Mahn *et al*., 2009). It shows a high level of resolution and can be used as an orthogonal method for the purification of complex protein mixtures (Mahn *et al*., 2009). It is also considered the ideal next step after ammonium sulfate precipitation via elution at high ionic strength (Cramer & Jayaraman, 1993; Nfor *et al*., 2011). Further, since relatively weak van der Waals forces are the major contributing factor to the hydrophobic interactions in HIC, it follows that there is low structural damage to the protein, and that its biological activity is maintained, with HIC versus affinity chromatograypy (AC), IEC or reversed-phase HPLC (rHPLC) (Queiroz *et al*., 2001).

Because HIC exploits the surface hydrophobicity of proteins, it is affected by certain characteristics of the mobile phase: temperature, pH, salt concentration and salt type (Nfor *et al*., 2011). These are important parameters in improving the performance of HIC. Further, certain authors have increased protein hydrophobicity through the addition of short tags of hydrophobic peptides to the target protein, in order to enhance HIC selectivity (Lienqueo *et al*., 2007). Other parameters for consideration in optimising HIC are the temperature of the chromatographic system and the properties of the stationary phase (such as the chemical nature of the matrix, the hydrophobic ligand type and the degree of substitution of the resin) (Mahn *et al*., 2009). Preparative HIC has been used in the purification of several lactobacilli proteinases (Table 11.2)

11.3.6 Reversed-phase HPLC (rHPLC)

As the name suggests, in rHPLC the mobile phase is significantly more polar than the stationary phase. It is the most popular chromatographic technique, due to its speed, high resolution, robustness and reproducibility, and the stability of its microparticulate bonded

phase (Ballschmiter & Wößner, 1998; Desai *et al*., 2000; Dorsey & Dill, 1989). The actual mechanism of action and the binding interaction observed in rHPLC are not well understood (Dorsey & Cooper, 1994). However, it is usually thought that proteins bind to the matrix at a low organic concentration and elute at increasing organic concentrations, depending on the protein hydrophobicity (Desai *et al*., 2000). Gradient elution is often used because gradient elution achieves both concentration and purification in a single step, which is beneficial, especially for dilute proteinase samples (Gu & Zheng, 1999). It is also very powerful in the separation of proteins that differ at only one amino acid and in conformational isomers of peptides (McNay & Fernandez, 2001).

The key parameters that affect an RPC separation are the stationary phase, organic solvent, elution gradient, ion-pairing agents, pH and temperature (Desai *et al*., 2000; Melander *et al*., 1979).

rHPLC is a good chromatographic technique for final polishing of proteins. However, because it is particularly useful for small polypeptides and proteolytic fragments, it is not very efficient for proteinases with high molecular weights. Also, the use of rHPLC leads to protein denaturation, due to the effect of strong adsorption and the organic modifiers needed for desorption. rHPLC is also accompanied by solvent disposal issues (McNay & Fernandez, 2001), which make it unrecommendable for use in process-scale proteinase purification.

11.3.7 Affinity Chromatography (AC)

AC is a highly specific and selective technique which separates biomolecules based on their peculiar biological or chemical interaction with ligands (Desai *et al*., 2000). It is usually the method of choice for the purification of high-value proteins and biologics. It offers a means of separating and purifying any given protein in one step, directly from crude solution. Thus, this approach eludes the product loss (and its attendant cost) associated with multistep bioprocessing. Additionally, AC has simple scale-up procedures and the unique ability to stabilise analyte, and it performs a concentration role when the starting material is dilute (Jones & Baines, 2000).

The common ligands used in AC are usually natural high-molecular-weight biomolecules (such as peptides, saccharides) coupled on to adsorbents. Many of these natural ligands are plagued with limitations, such as high cost, poor chemical and biological stability and ligand leakage (which lead to product contamination). In addition to the fickle nature of some affinity ligands, AC is plagued with the high costs of enzyme supports (Kumar & Takagi, 1999). However, research continues to churn out designed synthetic ligands that are resistant to chemical and biological degradation (Jones & Baines, 2000). This will increase the popularity of AC invaluably in the downstream processing of important proteins such as proteinases.

For the isolation of proteinases, the substrate may serve as a ligand: a competitive reversible inhibitor or an allosteric proteinase activator. This approach is gaining momentum and has been applied in the isolation of certain enzymes other than proteinases (Eijsink *et al*., 1991; Govrin & Levine, 1999; Peters & Fittkau, 1990). For example, the synthetic inhibitor para-aminobenzamidine has been used as the affinity ligand for the removal of trypsin-like serine proteases (Nakamura *et al*., 2003). Other authors have also designed efficient and specific-affinity ligands for the isolation of serine proteases by preparing a peptide derivative which imitates the structure of natural substrates but contains a bond resistant to proteolysis (Kuznetsova *et al*., 1997). The isolation of cysteine

proteases by the use of a fungal cysteine protease inhibitor has also been reported (Sabotič *et al*., 2012).

11.3.8 Methacrylate Monoliths as Stationary Phase in Chromatography

In chromatography, a stationary phase or support is employed for ligand immobilisation (Kline, 1993). Support materials must have such properties as a large specific area, high rigidity, suitable particle form, hydrophilic character and high permeability, and they must also be insoluble in the system where the target molecule is found (Hermanson *et al*., 1992).

Although a significant number of bead-based particulate-separation sorbents have been in existence since the development of chromatographic techniques, there are many limitations to their use. For example, particle-based sorbents function by diffusion, which gives very low binding capacity for large biomolecules (Ongkudon & Danquah, 2010). There is also the problem of interparticular volume, slow mass transfer and decreasing in separation efficiency due to broadening of the chromatographic zones from the large void volume between the packed particles (Vlakh & Tennikova, 2007). The introduction of monolithic solid supports is a giant leap forward, since these are able to circumvent the setbacks of particle-based sorbents, becoming the most popular and most successfully explored sorbents.

A monolith is a continuous phase consisting of a piece of highly porous organic or inorganic solid material. The methacrylate-based monoliths are polymeric macroporous materials, made by radical co-polymerisation of glycidyl methacrylate and ethylene glycol dimethacrylate (GMA/EDMA). Additionally, the pore size of a monolith support can be optimised to allow all of the mobile phase to flow through. Consequently, mass transport is by convection (rather than diffusion). The pore size of the monolith also helps in providing anchorage for both ligand attachment and biomolecule mobility (Ongkudon & Danquah, 2010), which allow the separation of biomolecules at extremely high flow rates without the loss of efficiency and capacity and lead to very short operation times (Vlakh & Tennikova, 2007). Further, scaling up and scaling down of monolithic support is simple. However, the use of methacrylate monolithic support for enzyme purification is in its infancy and therefore requires urgent research attention.

11.3.9 Expanded-bed Adsorption

In most protein-purification systems, the liquids contain suspended particulates, as observed with whole cells in fermentation broth. Thus, downstream processing should include at least one unit operation for the removal of suspended particulates. In some cases, the removal of particulates is attended with high cost, reduction in product yield and technical difficulties (as in the case of particulate removal from viscous liquids and particles of submicron size). It is also near to impossible to apply particulate-containing materials to a fixed, packed bed of adsorbent, as in the case of HPLC adsorbents, since this results in an increase in the pressure drop across the bed, as well as the formation of a plug of trapped solids near the bed inlet. A promising solution to the problem of applying particulate-containing material to a packed bed is the use of a fluidised or expanded bed (Chase, 1994). Expanded-bed adsorption (EBA) combines the effects of centrifugation, filtration, concentration and purification into one step,

thereby increasing yield and cutting down processing time (Desai *et al*., 2000; Hubbuch *et al*., 2005; Kennedy, 2001; Thömmes, 1997). The use of fluidised beds for the direct extraction of proteins from whole fermentation broths has received much research attention in the past few decades (Chase, 1994; Gailliot *et al*., 1990; Hjorth, 1997).

EBA works in a five-step cycle: creation of bed sediment; expansion and equilibration of adsorbents; sample application and washing; elution; and cleaning-in-place. Factors that are critical for the successful operation of EBA are the choice of adsorbent and the design of the apparatus in which the separation is performed (Chang & Chase, 1996; Chase, 1994). Although EBA has made significant strides towards product recovery in large volumes of feedstock, there is still a need to develop new adsorbents with highly stable and specific ligands that give enhanced protein-binding capacities (Hjorth, 1997).

11.4 ENZYME CONCENTRATION AND STORAGE

The concentration and storage of proteinases are the likely steps following purification. Concentration of purified proteinases can be achieved by appropriate HPLC techniques, dialysis, UF and precipitation and/or freeze-drying, followed by reconstitution in a smaller volume of buffer. Notably, pressure-driven separation techniques such as UF are inexpensive, result in little loss of enzyme activity and offer both purification and concentration in the same step (Kumar & Takagi, 1999). The setback with the use of membrane-based separation kits is the fouling or membrane clogging due to the formation of precipitates by the final product. This can usually be alleviated or overcome by treatment with detergents, proteases or acids and alkalies (Kumar & Takagi, 1999).

The storage of proteinases must be accomplished in a suitable environment that will not compromise activity. Storage temperature and pH and the buffer are some parameters to consider. There might be a need for the addition of the necessary cofactors to maintain enzyme activity.

11.5 CHARACTERISATION OF PROTEINASE

11.5.1 ENZYME-ACTIVITY DETERMINATION

Quantitative determination of proteinase activity is achieved primarily by methods based on the enzyme's ability to hydrolyse casein or synthetic chromogenic substrates. Several synthetic chromogenic substrates (such as amino acyl 4-nitroanilide and β-naphthylamides derivatives) have been used for the estimation of proteinase activity (Exterkate, 1990). They are made of functional methoxy and/or succinyl groups bound to a chain of amino acid residues, with the whole bulk bound to an aniline group at the *para* position. The methoxy and/or succinyl functional groups help increase the solubility of the substrate in aqueous medium. Enzymatic cleavage of 4-nitroanilide substrates yields 4-nitroaniline, which gives a yellow colour and absorbs light at 410 nm.

11.5.1.1 Use of Casein in the Estimation of Proteinase Activity

The basis of using casein as a suitable substrate in the estimation of proteolytic activity can be classed into three groups. First, β-casein can be labelled with radioactive ^{14}C to produce ^{14}C-methylated β-casein, which is used as the substrate. The reaction mixture, comprising the substrate, enzyme and a suitable buffer such as TRIS-maleate, is allowed

to proceed for a fixed period of time, after which the reaction is stopped with an organic acid. The reaction mixture is allowed to incubate for some time, then organic acid-soluble aliquots are taken and their radioactivity is measured by liquid-scintillation counting. The number of counts per minute (cpm) or disintegrations per minute (dpm) obtained is proportional to the extent of hydrolysis. One unit of proteinase is usually defined as the amount of enzyme that releases 1 dpm/minute per millilitre (Laloi *et al*., 1991).

Second, casein and the enzyme extract are incubated in a suitable buffer. After incubation, the reaction mixture is centrifuged and the reaction is stopped by the addition of a chaotropic agent, such as organic acid (trichloroacetic acid) or ninhydrin. After centrifugation, the concentration of soluble products is quantified by an appropriate method, such as that of Lowry *et al*. (1951), from which an arbitrary unit (AU) of the proteolytic activity can be defined (Fira *et al*., 2001).

Third, the number of primary amino groups released by the enzyme is estimated with o-phtaldialdehyde (OPA). OPA reacts with primary amino groups and a thiol compound (e.g. dithiothreitol) to form a compound that will absorb light at 340 nm (Nielsen *et al*., 2001). The proteinases of some lactobacillus species do not hydrolyse 4-nitroanilide substrates (Laloi *et al*., 1991), thus the use of casein in the estimation of proteinase activity offers an advantage over the use of 4-nitroanilide substrates. Polyacrylamide-gel electrophoresis (PAGE) is also commonly used, qualitatively, for the estimation of proteinase activity. The disappearance of intact casein is used as an indicator of the extent of proteolysis. Newer methods used to monitor and quantify, respectively, the degradation of casein and the appearance of breakdown products include HPLC, free-solution capillary electrophoresis and micellar electrokinetic capillary chromatography (Oommen *et al*., 2002).

11.5.2 Proteinase Kinetic Parameters

In enzyme catalyses, key parameters such as V_{max} (maximum rate), K_m (the substrate concentration at half v_{max}), K_{cat} (turnover number) and E_a (activation energy) are enzyme-specific as well as substrate- and environment-specific. Studying the kinetics of proteinases can unravel their catalytic mechanism, how their activity can be controlled and how the enzyme can be inhibited. Information about kinetic parameters of the proteinase of interest is therefore of utmost importance in an enzyme-based process, since the knowledge thereof is essential to designing enzyme reactors or quantifying the applications of the enzyme under different conditions (Gupta *et al*., 2002a).

11.5.3 Optimum Conditions for Proteinase Activity

In order to fully characterise the proteinase obtainable from lactobacilli, it is important to elucidate the optimum conditions (such as pH, temperature, and ionic strength) of enzyme activity. Such information is needed to decide the best storage conditions by which to delay or avoid the loss of enzyme activity.

The optimum pH range of alkaline proteases is generally between 5.5 and 7.5. Some few lactobacillus proteinases have an optimum pH of 8 (Zevaco & Gripon, 1988). The optimum temperature of lactobacilli proteinases ranges from 35 to 50 °C. The release of some lactobacilli proteinases is more pronounced at higher temperatures (around 40 °C) but the enzymes are more stable at cold temperatures (4 °C) (Espeche Turbay *et al*., 2009).

The thermal stability of proteinase is another important property. It helps determine whether they will remain active at the temperature conditions found during the manufacture of a product of interest. For example, in the dairy industry an important characteristic of dairy starter is its ability to grow and produce acid(s) at high temperatures. Dairy products such as Cheddar cheese require higher-than-normal processing temperatures of 42 °C (Hickey *et al*., 1986). Lactobacilli and proteinases intended for use in such products must demonstrate their ability to survive the high temperature for a sustained period of time. Thermal stability is usually determined by diluting an enzyme into an assay buffer and pre-warming it at a fixed inactivation temperature. Fractions are then taken at different times of incubation and immediately chilled for proteinase-activity assay (Atlan *et al*., 1989). The gradient of a graph of residual activity with time will give an indication of how thermally stable the proteinase is.

11.5.4 Molecular-mass Estimation, Metal-ion Inducers and Inhibitors

The requirement for metal ions, inhibitors and the molecular weight of proteinase are also worth determining, as these help classification of the proteinases. The molecular mass of most proteinases ranges between 45 and 180 kDa. This range of values is higher than those observed for the *Bacillus* proteases, which are usually between 15 and 30 kDa. Further, most alkaline proteases require a divalent cation such as Ca^{2+}, Mg^{2+} or Mn^{2+}, or a combination, for maximum activity (Kumar & Takagi, 1999). The importance of Ca^{2+} in the stabilisation of proteinases during *Lactococcus* cell growth has been demonstrated (Rahman *et al*., 2003). However, for most lactobacilli, proteinase activity and stability have been shown to be independent of the presence of Ca^{2+} ions in the medium (Espeche Turbay *et al*., 2009; Laloi *et al*., 1991). In addition, most lactobacilli proteinases are inhibited by phenylmethylsulfonyl fluoride (PMSF) or diisopropylfluorophosphate, both of which are specific inhibitors of serine-type proteinases.

11.5.5 Substrate Specificity

Lactobacilli proteinases vary in their activity towards synthetic substrates and native proteins such as casein. Substrate specificity is elucidated via proteinase hydrolysis of specific synthetic dipetides or peptides with labelled amino acid residues. It can also be found via the hydrolysis of native proteins (such as casein) followed by liquid chromatography–mass spectrometry analysis of the peptides fractions.

In the *Lactococcus*, proteinase types have been distinguished on the basis of their specificity toward α-, β-caseins and to a lesser extent κ-casein. CEP_I proteinases predominantly hydrolyse β-casein, and to a lesser extent κ-casein, while CEP_{III} uses αs_1-, β-caseins and κ-casein (Kunji *et al*., 1996, Oberg *et al*., 2002; Oommen *et al*., 2002). A mixed-type $CEP_{I/III}$ classification also exists for some lactobacilli (Kunji *et al*., 1996). The proteolysis of α_{s1}-casein (f1–23) by the lactobacilli forms a basis for the classification of proteinases into seven groups (Oberg *et al*., 2002). Most lactobacilli proteinases are able to hydrolyse the Gln_9-Gly_{10}, Gln_{13}-Glu_{14} and Leu_{21}-Arg_{22} bonds in α_{s1}-casein (f1–23) and the Ser_{15}-Leu_{16}, Glu_{42}-Asp_{43}, Leu_{192}-Tyr_{193}, Pro_{206}-Ile_{207} Met_{156}-Phe_{157}, Ser_{161}-Val_{162} and Leu_{192}-Tyr_{193} bonds in β-casein (Hebert *et al*., 2008).

Knowledge of the substrate specificity of a proteinase helps in classification as well as its directed use.

11.6 SOLVENT AND ENZYME ENGINEERING FOR ENHANCED STABILITY AND SPECIFICITY

It is well known that the tertiary structure of an enzyme determines its catalytic role. Thus, manipulating the tertiary structure may be a useful tool for the improvement of enzyme stability and catalytic behaviour. The stability of enzymes under other-than-usual conditions is also a major factor in their industrial application, necessitating a consistent search for methods for the preparation of stable enzymes.

The study of protein structures and functions has shown that when enzymes are subjected to hostile environments (such as in organic solvents), they exhibit new behaviours, increase in stability and can catalyse reactions that were impossible in water (Gupta *et al*., 2002b). Some other methods used for stabilising enzymes are chemical modification, chemical crosslinking, immobilisation in hydrophobic solvents and polyelectrolyte complexes, use of lyoprotectants and protein engineering (Gupta *et al*., 2002b; López-Gallego *et al*., 2005; Wong & Wong, 1992; You & Arnold, 1996).

11.7 CONCLUSION

The production and downstream processing of lactobacilli proteinases is an ongoing research endeavour, and various types of proteinase have been characterised and have had their potential industrial applications explored. One of the most pressing bioprocess challenges in the area of production of pharmaceutical/biological materials is that of isolation and purification of the finished products. Although several isolation and purification methods exist for proteins, they must all be weighed out based on their merits and demerits. From the already established molecular and particle separations using membranes, centrifugation and phase-partitioning techniques, combinatoric techniques can be developed to ensure proteinase purification at the minimum allowed cost and high product purity. The industrial-scale use and commercialisation of lactobacilli proteinases will require the inputs of scientists, engineers, controls specialists, quality experts and economists. Further, with the important role and prospect of lactobacilli proteinases in industry, more targeted research is needed to aid in the development, purification and characterisation of robust enzymes with desired properties for wider industrial purposes.

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2.7. Immobilization technology in proteinases processing and application

In living organisms, most proteolytic enzymes catalyse bio-reactions under mild conditions of temperature and pH. However, for industrial applications, an improvement in enzyme properties is often desired to allow the enzyme to survive any 'other-than-usual' conditions that may prevail during industrial processes. For example, enzymes for industrial applications must possess qualities such as good stability and sustained activity over the range of desired reaction conditions (e.g. extreme pH, elevated temperature, organic solvents, mechanical stress) (Chen et al., 2006; Brady and Jordaan, 2009). As well, many cycles of high yield processes are desired from the same lot of enzymes (Mateo et al., 2007) and the separation of products from biocatalyst is also often desired or required (FAO/WHO, 2006; Opwis, 2010). These expectations have economic implications and in many cases are hard to be met in industrial processes which rely solely on the use of soluble enzymes. To overcome this challenge, research studies in the last decades has focused on the preparation and used of immobilized biocatalysts systems.

Indeed, it is proposed that isolated enzymes may work better when immobilized in a matrix or on a support material, since this is observed widely in nature as many biocatalysts are naturally attached to cell membranes or entrapped in the intracellular milieu (Svec and Gemeiner, 1996).

Several biotechnological advancements have been made in enzyme immobilization processes thus improving the economical feasibility of many enzyme systems. Very simple immobilization protocols have been used to improve enzyme functional properties such as activity enhancement, substrate selectivity and enantioselectivity modification, and the option of single-pot multi-enzyme reactions (Mateo et al., 2007; Sheldon, 2007; Brady and Jordaan, 2009).

The resulting properties of an immobilized enzyme system is dependent on both the enzyme and the carrier material, and the interaction between these two entities furnishes the biocatalyst with distinct kinetic, mechanical, chemical, and biochemical properties (Tischer and Wedekind, 1999) (Figure 2.7.1).

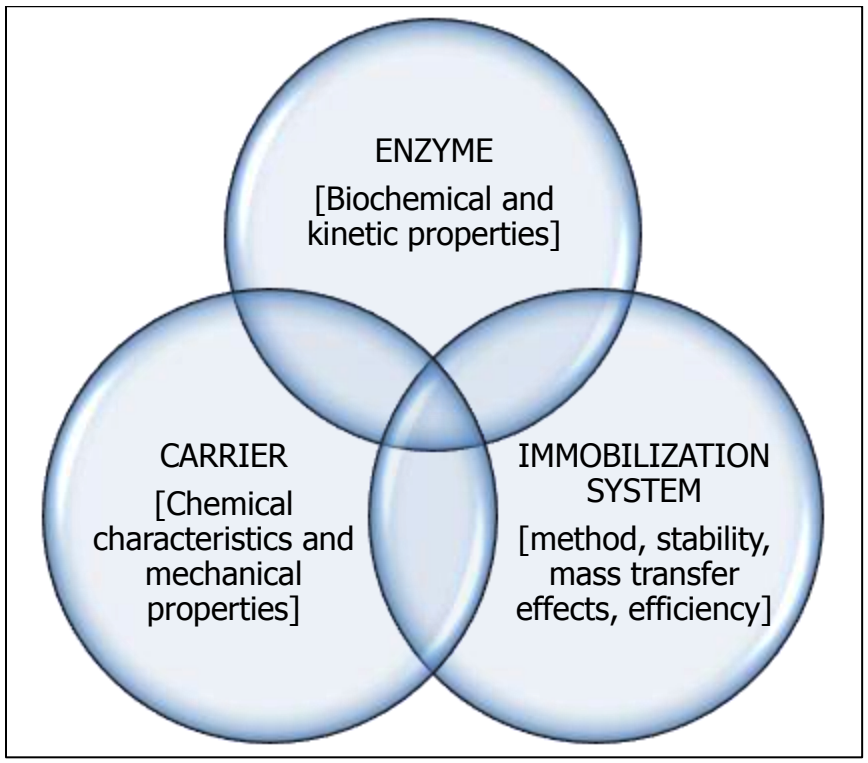

Figure 2.7.1: Parameters that affect and enzyme immobilization system; adapted from (Tischer and Wedekind, 1999).

2.7.1. Methods for enzyme immobilization

A wide range of strategies have been used for protein immobilization. Broadly, four approaches are employed to immobilize enzymes onto surfaces (Tischer and Wedekind, 1999):

a) Prior activation of the enzyme by a suitable reaction before immobilization
b) Support modification and activation before immobilization
c) Use of bi- or multifunctional coupling agent as a mediator between carrier support and enzyme functional groups
d) Modification of enzyme via recombinant DNA techniques to make the protein "(bio)specific" for a carrier via (bio)affinity binding

Thus, immobilization approaches that are used include adsorption, entrapment, affinity immobilization, covalent binding and cross-linking (Datta et al., 2013) (see Figure 2.7.1.1).

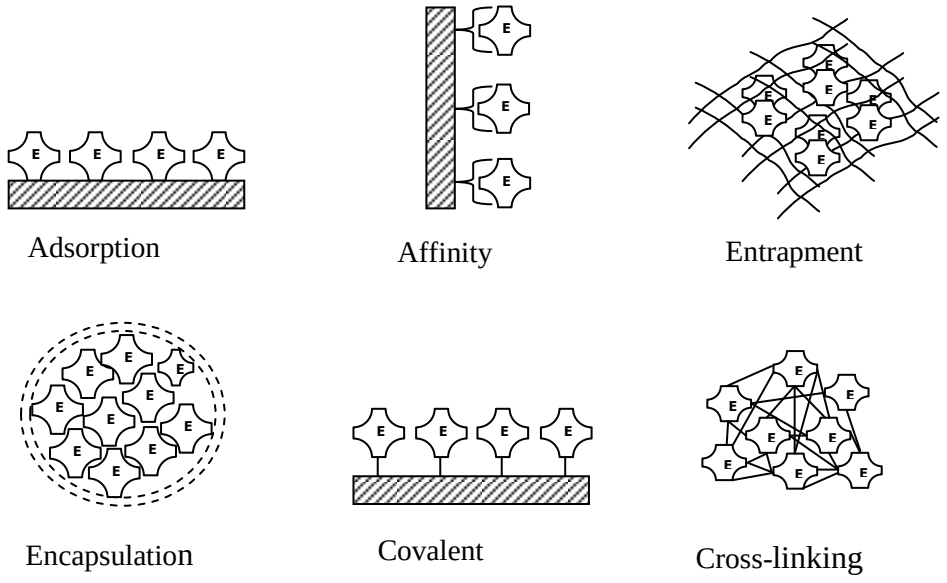

Figure 2.7.1.1 - Basic methods of enzyme immobilization

Adsorption

Adsorption of enzymes unto a support matrix relies on the 'bonding' of enzymes to a suitable material, upon mixing, as mediated by salt linkages, hydrogen bonds and Van der Waal's forces. This method of enzyme immobilization is simple, cheap, and less destructive on enzymes since it involves little or no extensive functionalization steps. Recovery of enzymes immobilized by adsorption is therefore simple and easy (Qi et al., 2011). In fact, the earliest reported study on enzyme immobilization was based on adsorption (Michaelis and Ehrenreich, 1908). However, this method of immobilization is very slow and because the bonds between enzyme and support are weak there is a high tendency for enzyme desorption, especially during changes in conditions and parameters such as temperature, pH and ionic strength. Immobilization by entrapment has been studied for some proteases, including neutrase from *Bacillus subtilis* on vermiculite (Chellapandian and Sastry, 1992), bovine pancreatic α-chymotrypsin on porous chitosan beads (Kise and Hayakawa, 1991); and trypsin on mesoporous silicates (Yiu et al., 2001b; Goradia et al., 2005).

Affinity immobilization

Affinity immobilization makes use of the "specificity of an enzyme to a suitable support under different physiological conditions" (Datta et al., 2013). It is usually achieved by the functionalization of enzyme or material or both with a ligand that is bio-specific for the other material. An adapted form of this immobilization method is bioaffinity layering where alternate layers of affinity ligand and the enzyme are deposited on the immobilization matrix (Sardar and Gupta, 2005). This strategy gives biocatalysts with very high stability and reusability (Haider

and Husain, 2008; Datta et al., 2013). Miladi et al., (2012) have studied the affinity immobilization of tobacco etch virus (TEV) protease on streptavidin-agarose matrix.

Entrapment

With immobilization by entrapment, the enzyme molecule is physically confined within a matrix that is insoluble and highly hydrophilic (Svec and Gemeiner, 1996). This is expected to allow the free diffusion of low molecular weight substrates and reaction products while hindering the leakage of enzyme. The enzyme is usually pre-mixed with the polymer/monomer solution, converted into a gel and then broken up to the desired particle size. Entrapment usually maintains the structural integrity of the enzyme structure. However, leaking of the enzyme is inevitable if uniform pore size of the gel is not obtained. One other disadvantage with this method is that since it relies on diffusion of species, it is unsuitable for high molecular weight substrates (such as proteins), thus limiting its use with proteases. Immobilization by entrapment has been studied for some proteolytic enzymes, including trypsin in sol-gel glass matrix (Lu et al., 2009); fish visceral protease in alginate beads (Geethanjali and Subash, 2013); trypsin on polysaccharide-cellulose gauze (Monteiro et al., 2007); and α-chymotrypsin in a composite cryogel beads made of poly(*N*-vinyl caprolactam)-calcium alginate covalently attached to poly(vinyl alcohol) (Kuptsova et al., 2000) and trypsin, α-chymotrypsin, carboxypeptidase B, and thrombin in poly(*N*-vinyl caprolactam)-based hydrogels (Markvicheva et al., 2000).

Covalent binding

Covalent binding of enzymes is the most well studied immobilization method. This is because it gives the strongest bonds and provides a more stable polymer–enzyme conjugates (Wang and Hsieh, 2004). There is a wide range of chemical binding mechanisms, and insoluble support materials with functional groups that can be used for covalent immobilization of enzymes. To ensure optimum enzyme recovery by this method, the binding reaction conditions should not compromise enzyme activity and neither should the enzyme active be affected by the reagents used.

Reactive groups used in immobilization

To improve the efficiency of any immobilization method, it is important to have some knowledge on surface properties of support materials and the chemical reaction mechanism between enzymes and activation reagents or linkers is necessary. The polyfunctional and multi-charged nature of amino acids in protein structures serve as native chemical properties that can be exploited for covalent immobilization. Native functional groups that can be used for covalent enzyme immobilization include active groups of amino acid side chains (amino groups of lysine and arginine, carboxyl groups of aspartic and glutamic acids, phenolic ring of tyrosine, thiol groups of cysteine, hydroxyl groups of serine and threonine, imidazile group of histidine, and indole group of tryptophan); and carbohydrate residues linked to special amino acids in the protein structure (Tischer and Wedekind, 1999). Synthetic functional groups can also be created via the introduction of reactive groups onto the enzyme structure (Tischer and Wedekind, 1999). These are however introduced onto the enzyme molecule and/or support matrix by the use of chemical reactive agents. When used on enzymes, these often alter the surface charge of the

enzyme making it more reactive, but they should not be inhibitive to the enzyme activity. Table 2.6.1 provides some examples of the key functional groups utilized in covalent immobilization of enzymes.

Table 2.7.1 Functional/reactive group exploited for enzyme immobilization

Native	**Synthetic**
Groups on amino acid side chains; e.g. amino, carboxyl, phenolic, thiol, hydroxyl, imidazile, indole	Transformation of carboxylic acids to amines
Carbohydrate residues in enzyme structure (e.g. lectins),	Transformation of thiols or amines into carboxylic acids
	Introduction of vinyl groups by alkylating or acylating the enzymes
	Use of fusion proteins with specific binding tags
	chemical modification of carbohydrate residues in enzyme structure to make them more reactive

Support (carrier) materials used in immobilization

The choice of carrier material (and functional groups on it) also has a significant effect on enzyme covalent immobilization of enzymes. Some useful properties worth considering are: functional group, surface area, hydrophobicity/hydrophilicity, insolubility, mechanical strength, permeability, form and size, and resistance to microbial attack (Tischer and Wedekind, 1999; Datta et al., 2013). Support material could be natural polymers (sepharose, pectin, starch, cellulose, gelatin, carrageenan, collagen, chitosan, alginates, etc); synthetic polymers (polyvinyl

chloride, polyurethane microparticles, methacrylated/fumaric acid-modified epoxy, polyaniline, nylon, polystyrene, polyethylene teraphthalate, etc.); or inorganic material (sand, bentonite, kaolinite, zeolite, ceramic, celite, silica, glass, activated carbon, charcoal, etc) (Svec and Gemeiner, 1996; Datta et al., 2013).

Several studies have been reported on the covalent immobilization of proteases. These include immobilization of trypsin on polyester fleece (Nouaimi et al., 2001; Nouaimi-Bachmann et al., 2007); trypsin on polyaniline (Purcena et al., 2009); trypsin on porous glass (Sears and Clark, 1993); trypsin on grapheme oxide (Xu et al., 2012); trypsin on macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolithic capillary column; trypsin on cellulosic exopolysaccharides membrane (Cavalcante et al., 2006); mixed proteases (trypsin and chymotrypsin) on modified polyvinyl chloride microspheres (Li et al., 2013) and lactobacilli cell-envelope proteinase on non-woven polyester (this study). It can be observed that most of these studies have been conducted using trypsin as a model enzyme. Trypsin is well studied because much is known about this enzyme (Yiu et al., 2001a) and this assists a rational optimization of the immobilization technique and conditions for improved performance.

Cross-linking of soluble and aggregated enzymes

The development of carrier-free immobilised enzymes has gained much research attention in the past few decades due to the cons of carrier-bound immobilized enzyme systems. The use of carrier materials in enzyme immobilization often leads to ‘dilution of activity’ of immobilised enzymes, owing to the fact that a large portion of the conjugate (99.9% – 90%) is non-catalytic (Tischer and Kasche, 1999; Cao et al., 2003).

Immobilization of enzymes via cross-linking is achieved by coupling the enzymes to other protein molecules or to functional groups on an insoluble support matrix. In recent years, there has been an increased interest in carrier-free immobilised enzymes. These have no need for carrier and are generally prepared by directly cross-linking different enzyme preparations such as dissolved enzyme (CLEs), crystalline enzyme (CLECs), spray-dried enzyme (CSDEs) and physically aggregated enzyme (CLEA)(Cao et al., 2003; Sheldon, 2011). Among these, CLECs and CLEA are the most widely studied. However, high cost and tedious crystallization procedures (mainly because very pure enzyme preparations are required for the crystallization step) are the drawback of CLECs (Wilson et al., 2004). CLEAs on the other hand are attractive because they do not require extensive protein purification (Shah et al., 2006) and result in a preparation that has a high concentration of enzyme per unit volume (López-Serrano et al., 2002; Wang et al., 2011).

Additionally, CLEAs offers the advantages of improved enzyme activity, high enzyme stability, low production costs owing to the exclusion of expensive carriers and the possibility to co-immobilize different enzymes (Cao et al., 2003; Chen et al., 2006). To prepare CLEA, the enzyme is precipitated from aqueous solution via the addition of a salt or a water-soluble organic solvent to form aggregates which are then cross-linked with a bi-functional cross-linker, such as glutaraldehyde or dextran polyaldehyde (Mateo et al., 2004; Hobbs et al., 2006). Cross-linked aggregates have been prepared and studied for a few proteases, including trypsin, (Chen et al., 2006), chymo-trypsin (Schoevaart et al., 2004), subtilisin (Skovgaard et al., 2010), and lactobacilli cell-envelope proteinase (this study).

2.7.2. Processing cost of immobilization

Key parameters that affect manufacturing cost of immobilized enzymes are the immobilization method and the amount of soluble enzyme used (Tischer and Wedekind, 1999). Further, enzyme immobilization is usually accompanied with a decrease in activity due to changes in binding procedure, loss in enzyme mobility, and reduced availability of enzyme molecules to product (Tischer and Wedekind, 1999; Opwis, 2010). If the cost of enzyme bioprocessing (from bioreaction through to immobilization) is high it can be inferred that immobilized enzymes do not provide much cost benefit, unless they allow for multiple reuse. In other words, the integral activity (i.e. relative activity x number of reuses, assuming relative activity is ~ constant) is always greater for immobilized enzyme than it is for soluble enzyme (Opwis, 2010).

2.8. Summary and Research Gaps

It has been established above that proteolytic enzymes are used in many useful products and processes. Further, the current demand of the world's biotechnological industries is enzymes with enhanced productivity, increased number of reusability, long shelf life and low cost. (Datta et al., 2013). However, several research challenges are still pertinent to the industrial application of some proteases (i.e. lactobacilli proteinases) and these are delineated below.

a) There is lack of efficient bioprocesses for CEP production

There is currently no completely optimized bioprocess, transferrable to a large-scale, for the production of CEPs. This challenge is of great concern to the process engineer and it is fuelled in part by the poor fundamental knowledge of cell growth characteristics and their effect on the proteolysis by lactobacilli CEPs. Compared with alkaline proteases from *Bacillus* spp., the

commercial development of fermentation processes for proteinase production by lactobacilli is almost non-existent.

As discussed earlier the proteolytic system of some lactic acid bacteria has been well characterized (Gobbetti et al., 1996). It consists of a plethora of proteases including cell-envelope proteinases (CEPs), endopeptidases, aminopeptidases, tripeptidases and dipeptidases (Khalid and Marth, 1990) all of which represent a wide range of enzymatic activities and specificities that can be exploited in many industrial processes. Despite this huge industrial potential, research information is lacking in characterization of lactobacilli proteolytic system (Tsakalidou et al., 1999; Espeche Turbay et al., 2009). Consequently, laboratory-scale processes for CEP production have not been optimised.

A bioprocess for CEP production must satisfy two requirements: production at large scale and at low cost. Strain improvement and optimization of the fermentation medium and production conditions are two areas where research efforts are concentrated in order to improve microbial enzyme yields (Kumar and Takagi, 1999). Fermentation medium must also be derived from readily available, renewable, and inexpensive raw materials (Gupta et al., 2002a; Gupta et al., 2002b; Posch et al., 2012) if a bioprocess for CEP production is to be cost-effective. Yet, not much work has been done in these two areas for the lactobacilli in order to improve CEP production (Agyei et al., 2012). There is therefore the need to develop an economically feasible bioprocess for the production of CEPs. Additionally, the model organism used in this study, *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) is an understudied organism despite its many uses. The fermentation growth kinetics and its effect on CEP synthesis, as well as the

proteolytic system of LDL 313 (ATCC 7830), has rarely been studied (Agyei and Danquah, 2012). This lack of knowledge is a bottle neck to the bioprocessing and application of CEPs. This thesis will address these issues but focus more on the engineering approach to CEP production by LDL 313.

b) The use of soluble CEPs has limitations

Lactobacilli CEPs have a poor stability, and this is another research challenge that precludes their industrial application. CEPs are membrane-bound enzymes and the most extensively used method of releasing them from lactobacilli is by washing or incubating the bacteria cells in a calcium-free buffer (Tsakalidou et al., 1999; Fira et al., 2001). However, this method compromises the storage and thermal stability of the CEPs so obtained since it results in an auto-proteolytic truncation of some subunits of the enzyme molecule (Laloi et al., 1991; Martín-Hernández et al., 1994; Hébert et al., 1997). The overall result is an enzyme molecule with reduced operational stability, reduced shelf-life and compromised pliability. This makes the industrial application of CEPs non-lucrative, especially when combined with the utter impossibility of reusing the enzyme in a soluble form. Further, when enzymes are used in the preparation of some products (such as food and pharmaceutical products), regulatory bodies such as the United Nations Food and Agriculture Organization (FAO) require separation of the enzymes from the products (FAO/WHO, 2006). This is a task that is very difficult to do, if not impossible, for soluble enzymes. The accumulative effect of all these factors makes soluble CEP-based processes economically unfeasible and as such research efforts are needed to address this challenge.

c) Scientific and technological information on immobilization of lactobacilli CEPs is lacking

The quest for optimum enzyme performance has made enzyme immobilization the preferred technology for designing industrially feasible and stable biocatalyst. However, information on the immobilization of lactobacilli proteinases is largely lacking. A literature search in the Scopus Database with the entry "immobilization of lactobacilli proteinases" yielded zero results! It is obvious therefore that little has been done on the use of immobilization for the preparation of stable biocatalysts from lactobacilli CEPs. Undoubtedly, this creates a bottleneck to the full exploitation of lactobacilli CEPs in industrial products and processes.

The achievement of high-yields of CEP for industrial application hinges on detailed optimisation of fermentation parameters essential for the generation of these proteolytic enzymes. After extraction, stable preparations of these enzymes have to be prepared via the use of immobilization techniques under technologically optimum and economically feasible conditions. This PhD project therefore seeks to address this challenge by seeking to produce, extract, and immobilize lactobacilli CEP in a cost-effective manner, to be applied in various food protein degradation processes.

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PART I: UPSTREAM PROCESSES: PRODUCTION OF CELL-ENVELOPE PROTEINASES

3. CHAPTER 3: Growth Characterisation and Proteinase Production

Section 3.1

3.1. In-depth characterisation of *Lactobacillus delbrueckii* subsp. *lactis* 313 for growth and cell-envelope-associated proteinase production

Monash University
Declaration for Thesis Chapter 3 Section 1

Declaration by candidate

In the case of Chapter 3 Section 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Initiation, key ideas and writing up	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**
Dr. Michael K Danquah	Initiation, key ideas and writing up

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12 December 2013
Main Supervisor's Signature	[redacted]	**Date** 12 December 2013

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In-depth characterization of *Lactobacillus delbrueckii* subsp. *lactis* 313 for growth and cell-envelope-associated proteinase production

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ABSTRACT

The effect of process conditions on the growth and production of cell-envelope-associated proteinase (PrtL) by *Lactobacillus delbrueckii* subsp. *lactis* ATCC® 7830™ (LDL 313) was studied. Cell growth was profuse under the temperature conditions of 37 °C, 40 °C and 45 °C, with cell growth rates, μ_{max} increasing with temperatures. Initial culture pH of 6 displayed the highest PrtL yield of 2.33 U. For each growth temperature the cell growth rates under anaerobic conditions were markedly higher than microaerophilic conditions, attributable to efficient sugar utilization under anaerobic fermentation. Overall, the proteolytic activities of cell-bound PrtL (bPrtL) were found to be higher than that of released PrtL (rPrtL). Higher rPrtL activities were displayed by anaerobic cultures over the entire fermentation period whereas the converse was true for bPrtL with microaerophilic cultures in the mid-late exponential phase displaying higher bPrtL activities than anaerobic cultures. Further, the proteinases had caseinolytic specificity for β-casein and κ-casein placing them in the cell-envelope-associated proteinases (CEPs) class I (CEP_I) of the lactococcal CEPs grouping. This study provides insights into conditions for profuse growth and proteinase production by LDL 313 for subsequent technological applications in fermented foods, the dairy industry and bioactive peptide production.

1. Introduction

Lactic acid bacteria (LAB) are ubiquitous and fastidious microorganisms with several technological, health and industrial significance. They are auxotrophic for several amino acids and their requirement for amino acids is not fully satisfied by the low concentration of free amino acids in milk; hence, they synthesize several proteolytic enzymes in order to grow and survive in milk [1–3]. The proteolytic system of several LAB species has been well characterized and is composed of cell wall-bound proteinases, responsible for the first step of casein hydrolysis, as well as several peptide- and amino acid-transport systems, and various intracellular peptidases (mainly aminopeptidases and dipeptidases) responsible for the release of single amino acids [4–6]. As a consequence of their proteolytic ability, LAB enhances the organoleptic properties of dairy foods, and facilitates the release of bioactive peptides from native proteins when used as starters in dairy foods [7,8].

However, unlike the genera *Lactococcus*, the proteolytic system of the genera *Lactobacillus* has not yet been characterized systematically [2,8]. Among the lactobacilli, the proteinase system of *Lactobacillus casei* is the best studied [2,5,9]. The proteolytic systems have also been studied each for *Lactobacillus delbrueckii* subsp. *bulgaricus* [10], *Lactobacillus sanfrancisco* CB1 [11], *Lactobacillus helveticus* [12] and *L. delbrueckii* subsp. *lactis* strain ACA-DC 178 and CRL 581 [2,8]. However, to the best of our knowledge the proteolytic system of *L. delbrueckii* subsp. *lactis* strain 313 (ATCC® 7830™) has not been studied.

L. delbrueckii subsp. *lactis* strain 313 (LDL 313) is employed as biological material in certain vitamin B_{12} assays [13,14] because it is auxotrophic for vitamin B_{12}. The species is also known for its ability to produce hydrogen peroxide [15] as well as its use in the production of sour bread such as the German sour rye due to its high acid tolerance [16]. Further, like other lactobacilli species, LDL 313 putatively produces proteolytic enzymes and finds use in the dairy industry for the production of hard cheeses, such as Emmenthal, Provolone and Grana [8] and yogurt [15]. Proteases produced by LDL 313 strains may also release bioactive peptides from proteins and thus represent new sources for biotechnological use. Despite the wideness in the utility of LDL 313, extensive studies on the growth and product formation, as influenced by process conditions, is largely lacking. The scope of technological and industrial application of LDL 313 is expansible considering the peculiarities of biochemical properties of this species. For example, extensive biochemical studies on its proteolytic system will help unravel the role of LDL 313 enzymes in proteolysis and subsequent use of the hydrolytic products. Once an application is established

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for the enzymes, the necessary process conditions (such as culture conditions) that enhance enzyme synthesis can be duly optimized to increase product yield. A major advantage of fermentation is that conditions such as media composition, temperature, pH, dissolved oxygen and build-up of waste metabolites, which influence cell growth and product synthesis, can be examined and controlled [17]. Elucidating the best conditions for the profuse synthesis of bioproducts is therefore necessary to improve fermentation processes for useful technological applications. This work seeks to understand the growth profile of LDL 313 under different fermentation conditions in order to optimize proteinase production and yields from this species. The Gompertz model was used to interpret the kinetic growth data obtained from microbial growth.

2. Materials and methods

2.1. Strain and growth condition

L. delbrueckii subsp. *lactis* ATCC® 7830™ was obtained from ATCC and propagated twice in deMan, Rogosa and Sharpe (MRS) Broth (Oxoid Pty Ltd., Australia) at 37 °C. Loopfulls of the culture were streaked on MRS Agar (Oxoid Pty Ltd., Australia), and incubated at 37 °C, 5% CO_2, for 2 days. To obtain a working culture, an inoculum was obtained from the streaked cells and recultured in MRS Broth at 37 °C and stored at −70 °C (Ultraflow freezer, Plymouth, USA). Revived culture was grown to early-stationary phase (optical density at 560 nm (OD_{560}) of 1.0 ± 0.2) in MRS broth (Acumedia, Michigan, USA) and was used to inoculate fresh MRS broth to an initial OD_{560} of 0.1 ± 0.5. Using a culture volume of 250 ml fermentation was carried out in a 500 ml Erlenmeyer anaerobic shake flask [18], in a gyratory shaker (100 rpm). Fermentation setup designated as 'anaerobic' was sparged with sterile nitrogen gas for ~1 min to displace the air present in the flask, whereas for conditions designated as 'microaerophilic' headspace gas was not altered in any way.

2.2. Bacteria growth

Bacteria growth was determined as described by Espeche Turbay et al. [8]. Samples were aseptically withdrawn with a disposable syringe at the said times to determine cell growth by optical density measurement at 560 nm (Shimazu UV–visible spectrophotometer, UV-2450). Consecutive samples were taken at intervals from the fermentation suspension and immediately cooled on ice. The samples were centrifuged, and the cell pellets resuspended in equal volume of distilled water for cell growth determination. An OD_{560nm} of 1.0 U corresponds to 200 μg/ml of bacterial dry weight. The pH of the culture was recorded with a pH meter (TPS Digital pH-mV-temperature meter, model 1852 mV).

2.3. Description of the bacterial growth curves

The experimental data of the growth curves are described by the Gompertz model parameters A, μ_{max}, λ, and ε based on Eq. (1) [19].

$$D_t = A \exp\left\{-\exp\left[\left(\frac{\mu_{max} e}{A}\right)(\lambda - t) + 1\right]\right\} \quad (1)$$

D_t is OD at time t; t is time of growth in hours; D_0 is OD at $t = 0$; A, the asymptotic value is the increase of OD between D_0 and OD_{max}; μ_{max} is maximum growth rate (h^{-1}), calculated from the slope of a semi-logarithmic plot of OD_{560} versus time (h); λ is duration time of lag phase in hours; ε is the exponential growth time, given by:

$$\varepsilon = \frac{A}{\mu_{max}} \quad (2)$$

The parameters were estimated by the application of the Levenberg–Marquardt algorithm (First Optimization Software, version 1.5, 7D-Soft High Technology Inc.).

2.4. Effect of initial pH on bacteria growth

To determine the effect of initial pH on bacteria growth and concomitant proteinase production LDL 313 was cultivated in 30 ml of MRS growth medium at different initial pH (i.e. 4, 5, 6, 6.5, 7, 7.5, and 8). The pH of media was adjusted by using appropriate buffer (0.2 M acetate buffer for pH 4, 5, and 6; and 0.2 M phosphate buffer for pH 6.5, 7, 7.5 and 8). Each setup was inoculated with 200 μl of exponential phase culture. Cell growth was determined by measuring OD_{560} with SpectraMax M2e Multi-Mode Microplate Reader (Molecular devices, Sunnyvale, USA). Cells were harvested after 12 h of cultivation and aliquots taken for proteinase production assay.

2.5. Determination of zeta potential

Zeta potential of bacteria cells was measured with a Zetasizer Nano ZS (Malvern Instruments, Malvern, United Kingdom). The zeta potential values were calculated according to the Smoluchowski equation. Disposable folded capillary cells (DTS 1060) were used. About 800 μl the bacterial suspensions were injected into the cells using a disposable syringe and three readings made [20]. Bacteria suspensions consisted of bacteria cells harvested (by centrifugation 4000 × *g*, 5 min, 4 °C) and washed in deionized water (MilliQ; 0.22 μM filtered, Millipore). The measurements were carried out in triplicate and at 25 °C. Before injection of the bacterial suspension, the measurement cell was flushed with ultrapure water (MilliQ; 0.22 μM filtered, Millipore).

2.6. Preparation of the cell wall extract and whole cell suspension

At the indicated time points, cultured cells were harvested by centrifugation at 4000 × *g* and 4 °C for 10 min, washed with an equal volume of 0.15 M sterile saline water supplemented with 10 mM $CaCl_2$, and resuspended to an OD of 3 in the extraction solution containing 5 M LiCl and 50 mM sodium phosphate buffer (pH 7) in a 1:1 (v/v) ratio. This system was incubated at 30 °C for 1 h with slight shaking, and then centrifuged at 4000 × *g* and 4 °C for 10 min. The supernatant was decanted and filtered through a 0.22 μm membrane (Acrodics, Pall Life Sciences). The filtrate was subjected to buffer-exchange and concentration in 50 mM sodium phosphate buffer using Ultracel 3K (3000MWCO, Amicon Ultra, Millipore) according to the manufacturer's instruction. Protein concentration was determined with a Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). For cell aliquots designated as whole-cell suspension cells were harvested by centrifugation (4000 × *g*, 10 min, 4 °C), washed twice in sterile 0.85% (w/v) saline, and resuspended in an equal volume of 50 mM sodium phosphate (pH 7.0) for use.

2.7. Proteinase activity assay

Proteinase activity was assayed according to the method of Exterkate [21] with slight modifications. The reaction mixture (284 μl) consisted of the buffered enzyme solution or whole cells (50 μl); 50 mM sodium phosphate buffer, pH 7.0; 37.5 mM imidazole; and 1 mM of the peptide succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide (S-ala, Sigma).

For cell extracts, the setup was incubated at 40 °C for 10 min by the thermostatically controlled cell compartment of the microplate reader after which absorbance was read immediately. For whole cells, setup was incubated at 40 °C in a water bath for 10 min after which the reaction was stopped by centrifugation (10,000 × *g*,

10 min, 4 °C). Aliquots were taken for optical density reading. The release of p-nitroaniline was followed at 410 nm ([E 410] = 8800 M^{-1} cm^{-1}) by using a SpectraMax M2e Multi-Mode Microplate Reader (Molecular devices, Sunnyvale, USA). One unit of proteinase was defined as the amount required to liberate 1 nmol of nitroaniline per minute.

2.8. Casein hydrolysis

A whole-cell suspension (20 μl) or cell wall extract (20 μl) was incubated with 20 μl of casein solution (4 mg/ml each of α-, β-, or κ-casein (Sigma)) in 40 μl of 50 mM phosphate buffer (pH 7.0) at 40 °C for 1, 8 and 24 h. Cell extracts for caseinolytic assay was obtained from 8 h old anaerobic cultures.

For the cell wall extract, the reaction was stopped by heating the setup in water bath at 90 °C for 5 min and immediate cooling by storing in −80 °C freezer. For the whole-cell suspensions, washed cells were suspended in 100 mM sodium phosphate buffer (pH 7.0) and allowed to utilize the residual intracellular amino acids for 30 min at 40 °C before casein degradation was carried out [7]. The reaction was stopped by centrifugation (12,000 × *g*, 4 °C, 5 min); the supernatant obtained was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).

2.9. Electrophoretic analysis

SDS PAGE was carried out with an Experion automated microfluidic electrophoresis system and Pro260 Assay Kit (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions [22]. This assay kit integrates protein separation, detection, and data analyses within a single platform and uses smaller sample and reagent quantities that the standard protocol. Briefly, the samples were mixed with sample buffer and heated at 95 °C for 5 min to denature the proteins, and 4 μl aliquot of each sample was loaded into each chip well.

3. Results and discussion

3.1. Effect of initial culture pH on microbial growth and proteinase activity

Fig. 1 shows the effect of initial culture pH on the growth of *L. delbrueckii* subsp. *lactis* ATCC® 7830™ in MRS broth. The use of the Gompertz model helped estimate quickly and describe the growth parameters in easy-to-understand terms that describe conditions to be applied in the technological uses of this lactobacillus species. Under culture pH conditions of 8, the cell growth was marginal and further decreased considerably within a short time of fermentation (results not shown). Cell growth was observed for pH between 5.0 and 7.5. Cultures of initial pH 6.5 and 6.0 completed exponential growth after about 9 and 10 h of fermentation respectively and cell growth rate was highest at pH 6.5 (0.23 h^{-1}). After 12 h, the growth profiles for cultures with initial pH 5.0 and 7.0 were in the exponential phase and showed indications of increasing further as the final pH of these cultures attained the optimal pH range for growth. Because of this the growth parameters based on the Gompertz model were giving some extremely large values. Thus, for pH 5.0 and 7.0, the Gompertz model was used to forecast growth readings that extend beyond 12 h growth for two more hours. For all experiments although high growth levels were observed, the acidification rate and organic acid production was low as MRS broth has a high buffering capacity due to the high ionic concentrations and the presence of peptides [23].

The effect of initial pH on cell wall proteinase (PrtL) yield is shown in Fig. 2. Proteinase was assayed from cell wall extract in the late exponential growth stage (after 12 h) and observed higher values for growth pH between 6.0 and 7.0. These results are similar to observations made in other studies with lactobacilli where growth and proteinase production were optimal for pH between 5.5 and 6.5 [2,8]. Although the cell growth rate was highest at pH 6.5, the optimum proteinase activity was rather observed for culture at initial pH 6. This shows that under slightly acidic conditions the enzymatic activity is enhanced, probably through enhanced activation of the ionic groups present at the enzyme active sites. Culture pH is known to affect strongly many enzymatic processes, transport of various components across the cell membrane and protease synthesis [24].

Further, the results demonstrate that although bacterial growth and its enzyme production is greatly controlled by environmental conditions such as temperature and pH, the optimum condition for enzyme synthesis and that for optimum growth may differ [25].

3.2. Effect of temperature on the growth and acidification rate

L. delbrueckii subsp. *lactis* ATCC® 7830™, (LDL 313), is a thermobacteria and thus grows at the normal thermophilic temperatures of most bioprocessing operations [26]. Some growth parameters of LDL 313 as influenced by three growth temperatures (37 °C, 40 °C and 45 °C) are presented in Table 1. For both anaerobic and microaerophilic cultures, estimated lag phase times (λ) were between 1.50 h and 1.80 h for cultivation temperatures of 40 °C and 37 °C whilst at 45 °C a longer lag time of ∼3.5 h and 3.8 h were observed for microaerophilic and anaerobic cultures respectively. Overall, short lag phase times were observed for microaerophilic cultures than for anaerobic.

Further, growth of LDL 313 under the different temperature conditions was profuse with growth rates varying with temperature. Growth rate and maximal cell density were in the order 45 °C > 40 °C > 37 °C, as expected (Table 1). High temperatures influence the activation energy of biomolecules in the fermentation stock which improves enzymatic reactions with concomitant effect on cell growth. During the growth of microorganisms for biotransformation purposes, a short overall fermentation time is a desired characteristic. In this study, length of lag phases was inversely related to temperature with the exception of growth at 45 °C which recorded a relatively longer lag phase of over 3 h even though the maximal growth rate (μ_{max}) at this temperature was highest among all the temperatures studied (0.36 and 0.86 h^{-1} for microaerophilic and anaerobic cultures respectively). This is explained by the phenomenon that at the early phase of growth the cells experienced a heat shock since the inocula used for the experiment had till then never been activated and grown at 45 °C. Thus the necessary adaptation mechanisms and the production of heat stable constitutive enzymes were necessary. Despite the prolonged lag phase time the effect of elevated temperatures on cell growth was exerted and the cells were able to attain a high growth levels. The cell growth profile at 45 °C indicated the possibility of further exponential growth (results not shown). The ability of LDL 313 to grow at high temperatures shows the plausibility of using this species in food processing operations that require higher-than-normal-processing temperatures such as in the manufacture of Cheddar cheese where a temperature of ca 42 °C is required.

The pH profile and acidification rate corroborated cell growth at all temperatures and the magnitude of pH decrease was higher for anaerobic cultures than for microaerophilic cultures as seen in Table 1. LDL 313 is a homofermentative lactobacilli already used in the production of sour bread such as the German sour rye [16] and Mongolian yogurt [15] due to its high acid tolerance. Acid tolerance is an importance factor in the selection of probiotic strains [27] making LDL 313 plausible for potential application in other fermented acid-based food products. Thus for the purposes of cell biomass and acid production by LDL 313, growth temperatures

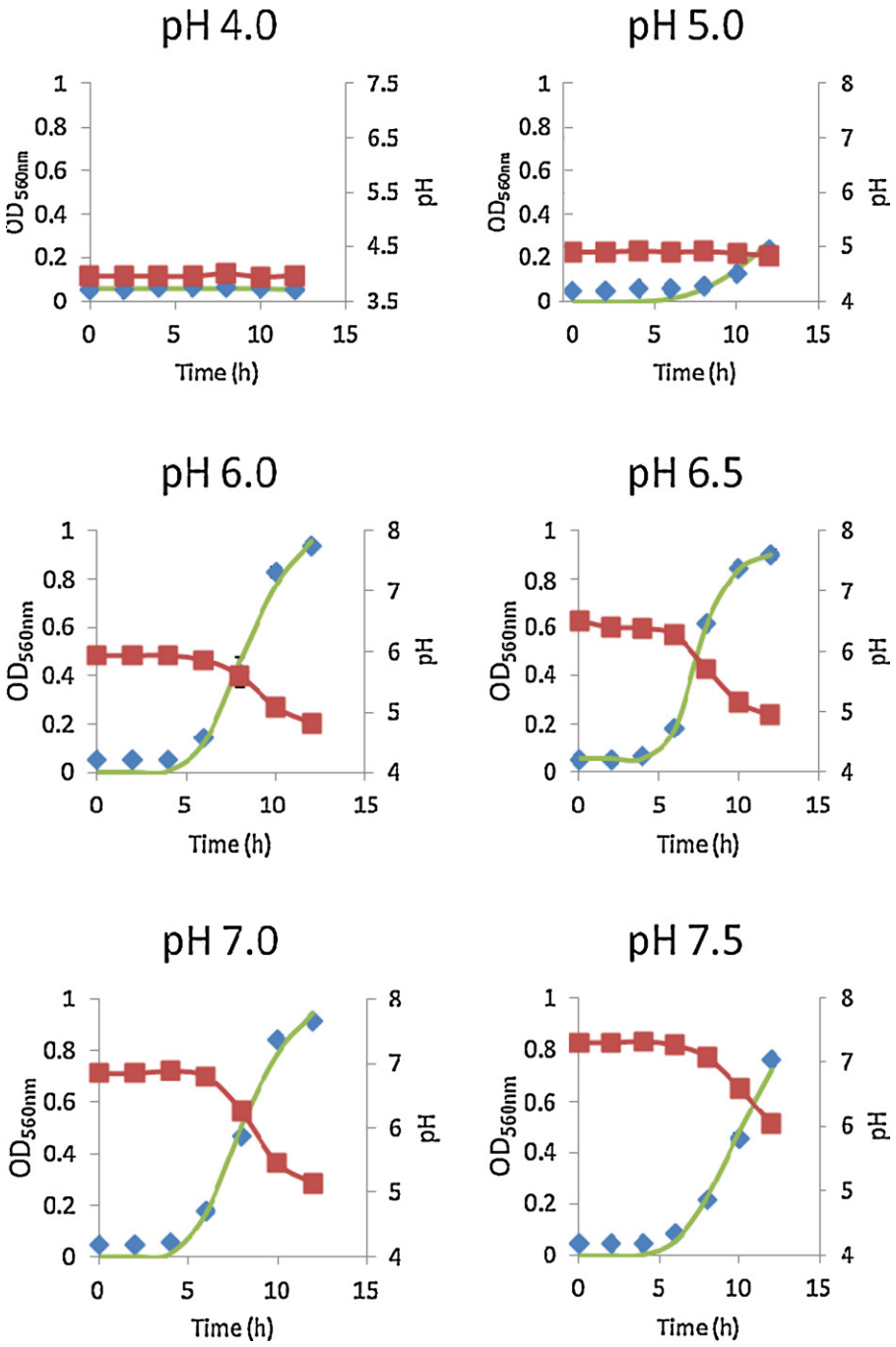

Fig. 1. Kinetics of growth (♦) and decrease in pH (■) of *L. delbrueckii* subsp. *lactis* 313 under different initial culture pH conditions. The symbols represent the mean of experimental data obtained ($n = 3$), while the lines are predicted plots from the Gompertz model estimate.

Table 1
Estimates of growth parameters based on the Gompertz model for *L. delbrueckii* subsp. *lactis* 313 under different growth temperature and culture conditions, *A*, increase between initial and maximum biomass; μ_{max}, maximum specific growth rate; λ, lag phase, ε, exponential phase time (see Eq. (1) in Section 2).

Conditions		μ_{max} (h^{-1})	*A*	λ (h)	ε (h)	pH decrease[a]	R^2 for model estimates
37 °C	MiAe	0.18	0. 71	1.54	5.15	0.61	0.96
	An	0.25	2.01	1.60	8.04	1.46	0.99
40 °C	MiAe	0.15	1.14	0.56	7.59	0.64	0.98
	An	0.38	2.22	1.79	5.84	1.29	0.99
45 °C	MiAe	0.36	1.47	3.49	4.08	1.21	0.99
	An	0.83	2.52	3.83	3.04	2.55	0.99

An, nitrogen or anaerobic cultures; MiAe, microaerophilic cultures; (the Gompertz model was applied to the mean optical density readings ($n = 2$) of experimental data).
[a] From initial pH, for 10 h of incubation.

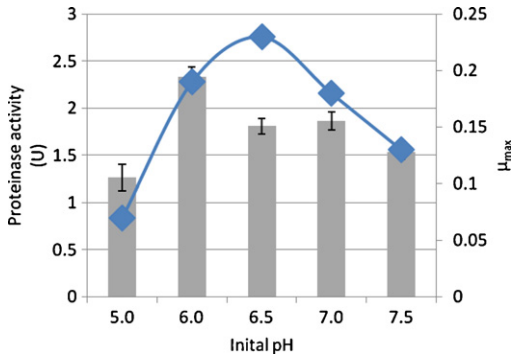

Fig. 2. Cell wall proteinase yield (bars) and growth rates (line) for *L. delbrueckii* subsp. *lactis* ATCC® 7830™ under different initial growth pH conditions; (cells harvested at $t = 12$ h; error bars are standard deviation values, $n = 3$).

of between 37 °C and 45 °C can be used, however, 45 °C gives the optimum yield.

3.3. Effect of culture gaseous composition on growth

The effect of the two culture gaseous compositions (i.e. anaerobic or microaerophilic) on cell growth and cell-envelope-associated proteinase yield were also studied in a repeated experiment at 37 °C for a prolonged fermentation time (i.e. 24 h). There were marked differences in growth pattern of LDL 313 in microaerophilic and anaerobic cultures at 37 °C, even a for prolonged fermentation time.

Microaerophilic cultures departed from exponential growth at a lower cell density compared with anaerobic cultures. Growth rates (μ_{max}) of exponential phase were respectively, 0.14 h^{-1} and 0.03 h^{-1} and a maximal cell biomass (dry cell weight) of 1.02 mg/ml and 0.24 mg/ml for anaerobic and microaerophilic cultures respectively (Fig. 3). It is observed from Fig. 1 that growth of LDL 313 under anaerobic conditions gives higher biomass and organic acids levels compared with microaerophilic growth.

The difference in growth rate and cell density observed with the two gaseous compositions have been demonstrated in several other lactobacilli species. LAB adapts to various conditions and change their metabolism accordingly in order to favor efficient carbohydrate utilization. Fig. 4 is a schematic of the alternative pathways observed for pyruvate metabolism. Pathways II and III

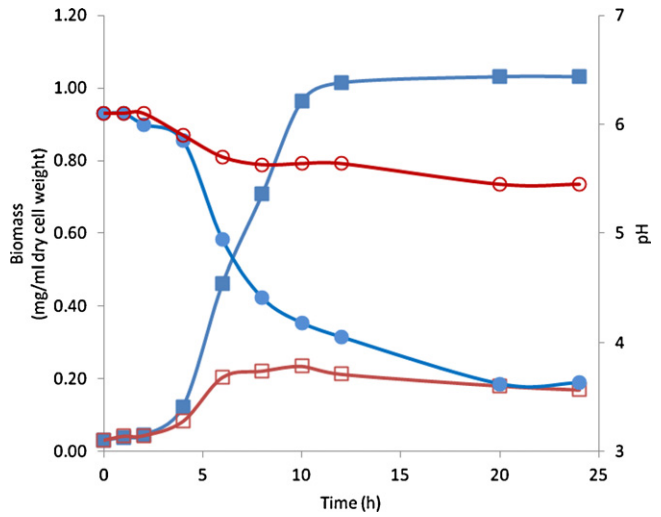

Fig. 3. Dry cell weight biomass (■, □), and pH profile (●, ○) of *L. delbrueckii* subsp. *lactis* ATCC® 7830™ grown at 37 °C in nitrogen or anaerobic (closed symbols) and microaerophilic (open symbols) culture; ($n = 3$ independent experiments).

are activated under aerobic and anaerobic conditions respectively and can occur in addition to pathway I, the normal homolactic fermentation, depending on the gaseous condition present. The pyruvate-formate lyase system (pathway III) has been shown to be operational in *Escherichia coli* [28], *Streptococcus mutans* [29], and *L. delbrueckii*, *Lactobacillus plantarum* and *Lactobacillus casei* [30]. The pathway ensures the activation of a substrate-level phosphorylation involving acetyl phosphate and acetate kinase which increases ATP/glucose yield [31,32]. With this pathway, a high culture acidification rate results as formic acid and acetic acid are produced. Moreover, the active pyruvate-formate lyase is extremely oxygen-sensitive: and its activity is lost irreversibly by exposure to air [29]. In this study with the growth under anaerobic conditions, high cell densities and high acidification rates were observed and thus metabolic pathway III is predominantly expected.

On the other hand, reduced growth levels of lactobacilli, under aeration conditions have been attributed to the pyruvate oxidase system (pathway II). Whereas the conversion of pyruvate to acetyl phosphate and acetate results in a low ATP/glucose yield [30,33],

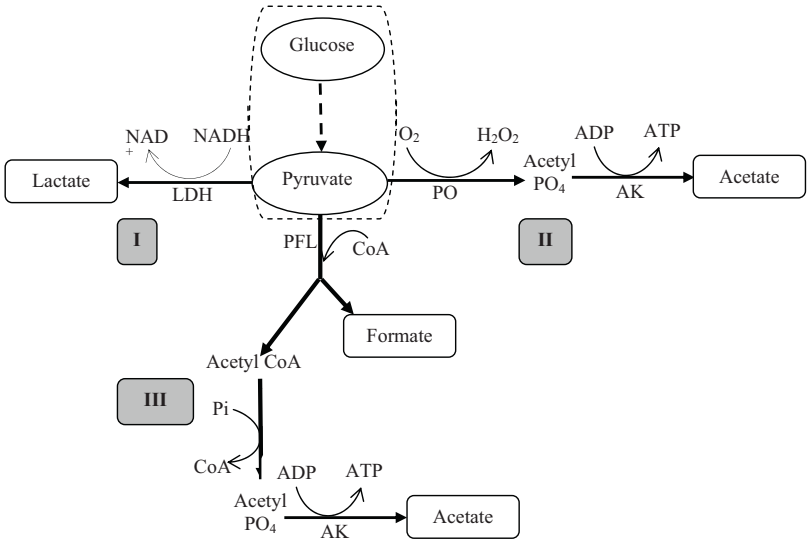

Fig. 4. Schematic of the fates of pyruvate metabolism; LDH: lactate dehydrogenase; PO: pyruvate oxidase; AK: acetate kinase; PFL: pyruvate-formate lyase; I, normal homolactic metabolism, II, microaerophilic metabolism, III, anaerobic metabolism.

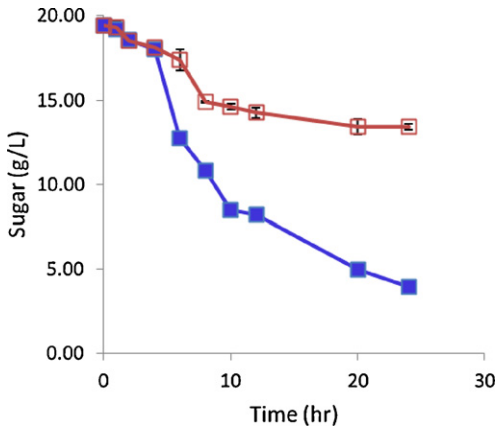

Fig. 5. Sugar consumption profile for *L. delbrueckii* subsp. *lactis* ATCC® 7830™ grown at 37 °C in nitrogen or anaerobic (closed symbols) and microaerophilic (open symbols) cultures. (Results of three independent experiments; error bars for anaerobic cultures are smaller than marker sizes.)

the accumulation of hydrogen peroxide could also be toxic to cells [15,34] which accounts for the low levels of cell growth. The rate of energy production from glucose is faster in the anaerobic pathway than in the microaerophilic pathway. Thus, the slowing of growth under microaerophilic conditions seen in this study reflects a switch in metabolism to a pathway which produces lesser amounts of energy.Consumption of reducing sugar by the two cultures showed that anaerobic cultures utilized more glucose over the entire fermentation period than microaerophilic culture (Fig. 5). Sugar consumption was same in the lag phase for both cultures; however at the end of the exponential phase about 56% and 25% of sugars had been consumed in aerobic and microaerophilic cultures respectively. Overall, 80% and 31% of sugar had been consumed for aerobic cultures and microaerophilic cultures respectively, by the end of the experiment (after 24 h) showing that sugar was efficiently utilized for cell growth in anaerobic than in microaerophilic cultures. This is in agreement with the explanation given above for the switch in metabolic activities under different gaseous conditions. The sugar consumption profile thus confirms the growth profile observed for the two fermentation conditions. This study establishes the growth of LDL 313 under anaerobic conditions where growth and acid production is maximal. Thus, LDL 313 could be applied in food processing and modified atmosphere food packaging operations that require anaerobic conditions.

3.4. Effect of gaseous conditions on extracted proteinase yield

The proteolytic properties of LDL 313 were determined by estimating the proteinase (PrtL) activity as measured in the cell-envelope extracts (released PrtL, [rPrtL]) and also in the cell pellet (bound cell-envelope-associated PrtL, [bPrtL]). As well, SDS-PAGE, profiles of α-, β-, and κ-caseins substrates were also used. The highest rPrtL activities were recorded when cells were at the exponential phase for both cultures, and the same observation was made in other studies [2,7,8]. Putatively, the genetic and metabolic activities of the cell at these stages favor the production of proteinases which are important for cell growth. The proteinase activities recorded for anaerobic and microaerophilic cultures differed with anaerobic culture giving an overall higher proteinase activity than microaerophilic cultures (Fig. 6). PrtL activity for anaerobic cultures was 32% higher than that for microaerophilic cultures at the optimum level.

Proteinase activity was detected by cells at the stationary phase, although the levels were low (0.007 and 0.008 U, respectively for anaerobic and microaerophilic cultures).

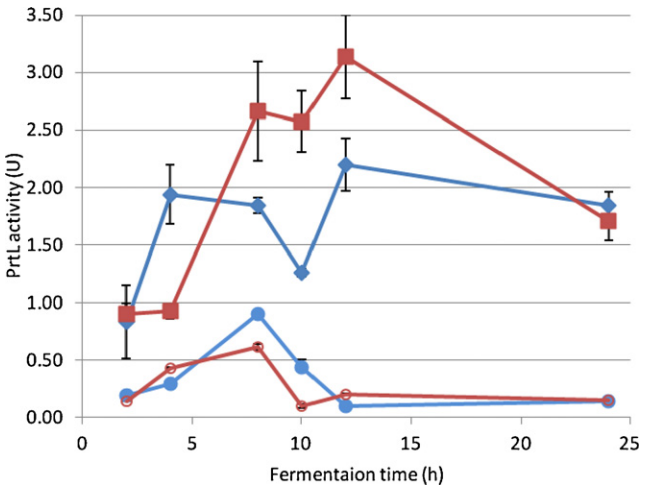

Fig. 6. Proteinase activities of cell-bound PrtL (squares) and released PrtL (ovals) from *L. delbrueckii* subsp. *lactis* ATCC® 7830™ grown at 37 °C in nitrogen or anaerobic (closed symbols) and microaerophilic (open symbols) culture. NOTE: in order to allow for comparison and maintain clarity in the figure, the values plotted for released PrtL (r PrtL) represent the observed values × 20 units. (Error bars, standard error of mean, $n = 2$.)

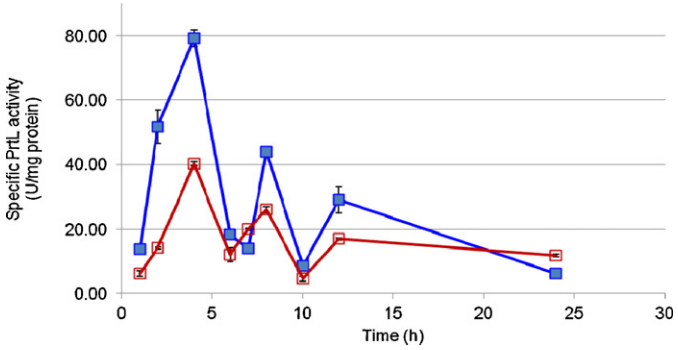

Fig. 7. Specific proteinase (rPrtL) activity profile for anaerobic cultures (closed symbols) and microaerophilic cultures (open symbols). (Error bars, standard error of mean, $n = 2$.)

This is because in the stationary phase, the cell metabolic processes shifts from product synthesis to the production of stress-induced proteins such as surface-layer proteins [20] in order for the cells to survive harsh conditions presented by the growth medium.

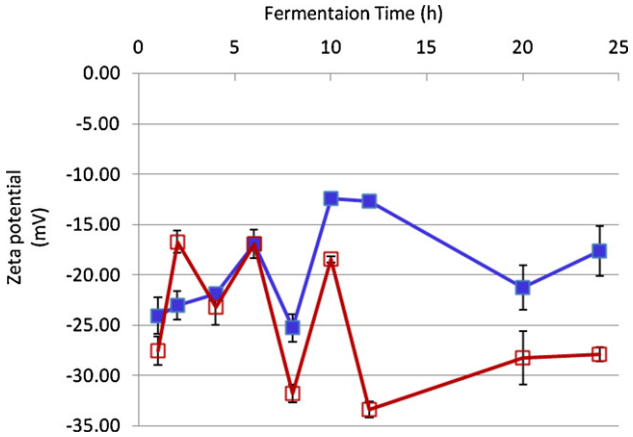

Fig. 8. Zeta potential for *L. delbrueckii* subsp. *lactis* ATCC® 7830™ cells grown at 37 °C for anaerobic cultures (closed symbols) and microaerophilic cultures (open symbols). (Error bars, standard deviation of distribution, $n = 3$.)

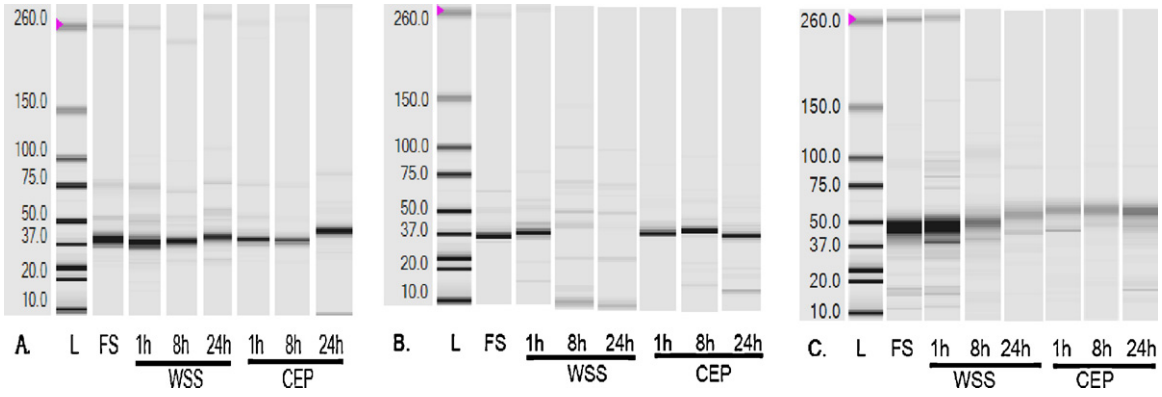

Fig. 9. Action of *L. delbrueckii* subsp. *lactis* ATCC® 7830™ whole cell suspension (WSS) and cell extract (CEP) on 4 mg/ml α-casein (A), β-casein (B) and κ-casein (C). Samples were taken at 1, 8 and 24 h for each setup. L, molecular weight ladder; FS, fresh casein samples.

Alternatively, as fermentation was carried out under uncontrolled pH the long exposure of enzymes to low pH condition compromised enzyme stability and activity.

The released CEP (rPrtL) specific activity profile is presented in Fig. 7. The results signify that significant quantities of the total cell wall proteins synthesized during early exponential phase are comprised of the CEPs. For most lactobacilli, PrtL production increases with cell growth until the middle of the exponential growth phase and then decreases gradually until early stationary growth phase. The maximum PrtL activity levels are therefore observed as one peak in the middle of the exponential growth phase [7,8]. However, PrtL activity levels from this study seem to deviate from that normal trend with the observation of more than one peak. To explain the observed trend in Fig. 7, it is maintained that the synthesis of PrtL in LDL 313 seems to happen in alternating cycles of production and utilization. That is, LDL 313 produces the CEPs in 'batches', and these CEPs are utilized until their activity is lowered by conditions such pH and/or until the CEPs are shed into the growth medium via prolonged culture agitation. Two alternative cycles are observed within the exponential phase (i.e. before 12 h) and the levels of production in both cycles decreases with time for both anaerobic and microaerophilic cultures. This is unusual and has not been reported for any lactobacilli. The metabolic reasoning behind the observed trend is therefore subject of further investigation.

It is observed from Fig. 6 that the observed profile in terms of origin of PrtL (whether cell-bound or released) differ for anaerobic and microaerophilic cultures; i.e., whereas rPrtL were high for anaerobic cultures the converse was true for bPrtL. This observation could be explained with the changes in cell surface protein distribution during fermentation under the two aeration regimes. During cell growth, proteinases are produced and distributed on the surface of the cell in an order which is dependent on the type and concentration of the limiting protein substrates. The strength of the cell membrane protein distribution is a function of protein–protein and protein–cell membrane intermolecular forces. Anaerobic cultures produce high biomass concentrations hence cell membrane protein distribution under anaerobic environment undergoes periodic redistribution of surface proteinases responsible for rapid metabolism of different growth-limiting proteins. This phenomenon is predominant in the exponential phase and produces a higher number of different enzymes to attack different protein molecules. Also, the production of numerous enzymes to support metabolic activities results in the less production of any specific enzyme capable of reducing specific protein substrates. The membrane proteinase redistribution mechanism is comparatively less prevalent in the case of the microaerophilic system which displays a lower biomass concentration; hence the rate of change in surface proteinase composition is relatively constant and results in high metabolic rate of specific protein molecules when the whole cells are applied. However, extracting the cell enveloped proteinases results in the release of all the different enzymes bound to the cell membrane of the anaerobic culture, thus increasing activity in the free enzyme space. The cell surface charge profile (as measured by zeta potential (Fig. 8) supports the observed trend in bPrtL (shown in Fig. 6) as well as the proposed phenomenon of changes in cell surface protein distribution used to explain the observation.

3.5. Caseinolytic properties of *L. delbrueckii subsp. lactis* ATCC® 7830™

The cell-envelope associated proteinases (CEPs) of *Lactococcus lactis*, subspecies SK11 and Wg2, have been characterized extensively both genetically and biochemically and is the best documented among LAB [35,36]. These enzymes represent two of the several types of lactococcal CEPs, which have been distinguished on the basis of their specificity toward α-, β-caseins and to a lesser extent κ-casein. The primary substrates for CEP_I are β-casein, and to a lesser extent κ-casein; while CEP_{III} use αs_1-, β-caseins and κ-casein [35,37,38]. Studies of proteinase enzymes from *Lactobacillus* spp. suggest that their enzymes can also be loosely grouped into a CEP_I/CEP_{III}-type classification [35]. It is also observed that cell-bound PrtL hydrolyzes completely caseins substrates while the breakdown of these substrates by the released PrtL was lower (Fig. 9). As in other similar studies [8], the proteolytic activities of cell-bound PrtL are therefore higher than that for released PrtL (also see Fig. 6). Further, the caseinolytic specificity of the species is predominantly for β-casein and to a lesser extent for κ-casein (Fig. 9). Whereas LDL 313 degraded β and κ-casein, Espeche Turbay et al. [8] reported *L. delbrueckii* subspecies *lactis* CRL 581, degraded α-and β-casein. The strain difference could account for this observation. The casein hysrolyses profile indicated that proteinases of *L. delbrueckii* subsp. *lactis* ATCC® 7830™ can be classed as a CEP_I type according to the lactococcal CEPs classification.

4. Conclusion

In this study, the effect of different growth conditions on the growth and the production of cell-envelope associated proteinases by *L. delbrueckii* subsp. *lactis* ATCC® 7830™ has been demonstrated. Significant in the findings of the study is the demonstrated effect of different gaseous composition on cell growth and cell-envelope-associated proteinase yield. Growth and biomass yields are very high under anaerobic growth compared to microaerophilic. For example, at 37 °C, μ_{max} and biomass yields for anaerobic cultures were respectively 28% and 75% higher than microaerophilic

cultures. This shows that the presence of air in the growth vessel results in low levels of cell growth. Thus, during cultivation of LDL 313 for the purposes of high cell densities, the gaseous composition should be altered to exclude oxygen, either by sparging with sterile nitrogen or by reducing the headspace volume to the allowed minimum.

Moreover, the production of cell-envelope-associated proteinases (PrtL) by LDL 313 is influenced by initial cell growth pH and gaseous composition of headspace (i.e. whether anaerobic or microaerophilic). Cell growth rate (0.23 h^{-1}) and PrtL yields (2.33 U) were optimum at pH 6.5 and 6 respectively; while released PrtL activities were higher by 32% for anaerobic cultures at the optimum level. Proteinases from LDL 313 can loosely be classified as a CEP_I type based on their ability to hydrolyse β-casein completely and κ-casein to a lesser extent. The results give useful information for further plausible areas of technological application for *L. delbrueckii* subsp. *lactis* ATCC® 7830™ especially in the fermented food and dairy industry. LDL 313 enzymes could also find use in bioactive peptide production.

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Section 3.2

3.2. Optimisation of batch culture conditions for cell-envelope-associated proteinase production from *Lactobacillus delbrueckii* subsp. *lactis* ATCC® 7830™

Monash University
Declaration for Thesis Chapter 3 Section 2

Declaration by candidate

In the case of Chapter 3 Section 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Initiation, key ideas and writing up	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**
Dr. Michael K Danquah	Initiation, key ideas and writing up
Dr. Ravichandra Potumarthi	Initiation, key ideas and writing up

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12 December 2013
Main Supervisor's Signature	[redacted]	**Date** 12 December 2013

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Optimisation of Batch Culture Conditions for Cell-Envelope-Associated Proteinase Production from *Lactobacillus delbrueckii* subsp. *lactis* ATCC® 7830™

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Abstract Using a combination of conventional sequential techniques, the batch growth conditions for the production of cell-envelope-associated proteinases have for the first time been studied and optimised in *Lactobacillus delbrueckii* subsp. *lactis* 313 (ATCC 7830; LDL 313). Concentrations of inoculum ($0.1<X<10$ % vol/vol), agitation speed ($0<S<200$ rpm), varying incubation temperature ($30<T<50$ °C), starting pH ($4.5<\text{pH}<7$) and carbon/nitrogen ratio of production medium ($0.2<r<5$) had an individual effect on proteinase yield ($p<0.01$). Optimal conditions for proteinase production included an initial pH of 6.0, 45 °C incubation temperature, 2 % (*v*/*v*) inoculum size of $OD_{560}=1$, 150 rpm agitation speed, and growth medium carbon/nitrogen ratio of 1.0. Maximum proteinase activity obtained for whole cells was 0.99 U/ml after 8 h of incubation. The variables studied are very relevant due to their significance in improving the productivity of proteinase synthesis from LDL 313, under process and, likely, economic optimum conditions.

Keywords Fermentation · *Lactobacilli delbrueckii* subsp. *lactis* 313 · Optimisation · Process variables · Cell-envelope proteinases

Introduction

The Lactobacilli are fastidious ubiquitous organisms that have been used for centuries in fermented food products such as dairy, vegetable and meat products. Due to their long history of use in cultured foods with no adverse effects, they have been assigned a 'Generally Regarded as Safe' status [1] and are non-toxic and non-pathogenic [2]. Their ability to improve the quality, safety and nutritional content of food is due to the reduction of

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certain food components and subsequent production of many bioactive and antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocins [3]. The production of useful bio-products by lactobacilli is the result of certain inherent genetic machinery which may confer or preclude specific metabolic abilities. For example, it is generally known that the lactobacilli have a very limited capacity to synthesise amino acids using inorganic nitrogen sources. Thus, during growth in milk, lactobacilli synthesise certain proteases to degrade casein into peptides and amino acids [4]. These proteases are the cell-envelope-associated proteinases (CEPs) and a host of intracellular peptidases such as endopeptidases, aminopeptidases, tripeptidases and dipeptidases [4, 5]. The CEPs are responsible for the initial stages of casein breakdown which contributes to the development of texture and organoleptic characteristics of fermented milk products and may also cause the release of bioactive peptides which can contribute to health improvement beyond basic nutrition [6]. The CEPs have numerous potentials and thus the production of large quantities thereof is needed to satisfy any growing market demands.

The production of lactobacilli CEP has been shown to be greatly influenced by fermentation conditions which can be controlled or 'finetuned' to positively influence the production and over-expression of proteases from lactobacilli [2, 7]. A major advantage of fermentation is that medium compositions and culture conditions such as temperature, pH, dissolved oxygen and build-up of waste metabolites, that influence cell growth and product synthesis—can be examined and controlled to enhance product syntheses [8, 9]. The optimisation of various fermentation parameters for maximising CEP production by lactobacilli is therefore a major research endeavour. However, studies on the effect of fermentation conditions on CEP production by lactobacilli is generally scarce, with the exception of few studies which have reported the effect of fermentation conditions (pH, temperature, nitrogen sources, osmolites and carbohydrates) on microbial growth kinetics and proteinase production for some lactobacilli [6, 10]. Literature findings indicate that there are more reports on process optimisation for protease production involving *Bacillus* species than there are for lactobacilli species. CEPs of thermophilic lactobacilli have been isolated and characterised, mainly from *Lactobacillus casei* [4, 11, 12], *Lactobacillus delbrueckii* subsp. *bulgaricus* [13], *Lactobacillus sanfrancisco* CB1 [5], *Lactobacillus helveticus* [14] and *L. delbrueckii* subsp. *lactis* strain ACA-DC 178 and CRL 581 [4, 6]. However, studies on the effect of fermentation conditions on CEP biosynthesis are scarce.

L. delbrueckii subsp. *lactis* 313 (ATCC 7830) is a less-studied species despite certain peculiar properties and some industrial applications. Aside their use in dairy products, the species plays certain analytical roles in vitamin B_{12} and amino acid autotrophy assays [15, 16]. It is also used in hydrogen peroxide production [17] and the production of sour bread such as the German sour rye, due to its high acid tolerance [18]. Furthermore, being a lactobacillus, the CEPs produced by *L. delbrueckii* subsp. *lactis* 313 (ATCC 7830) can be utilised to release bioactive peptides from food proteins [19]. However, research studies on the technological applications of CEPs obtainable from *L. delbrueckii* subsp. *lactis* 313 (ATCC 7830) is lacking. To the best of our knowledge, no detailed optimisation studies have been reported on the fermentative behaviour of this bacterium strain. Consequently, knowledge on the fermentation conditions and process optimisation to aid proteinase production by this species is scarce. This study seeks therefore to use a combination of conventional techniques to study and optimise the batch growth conditions for the production of CEPs by *L. delbrueckii* subsp. *lactis* 313 (ATCC 7830). In particular, we investigated the effects of certain condition including incubation temperature, initial pH, agitation speed, inoculum size and the carbon/nitrogen ratio of growth medium on growth kinetic parameters and

proteinase yields. To the best of our knowledge, this is the first detailed study of these five process variables (parameters and conditions) and their influence on proteinase production by *L. delbrueckii* subsp. *lactis* 313 (ATCC 7830).

Materials and Methods

List of Materials

The following were the chemicals or materials used in the study: deMan, Rogosa and Sharpe (MRS) Broth and agar (Oxoid Pty Ltd, Australia), MRS broth (Acumedia, Michigan), −70 °C Freezer (Ultraflow freezer, Plymouth), SpectraMax M2e Multi-Mode Microplate Reader, (Molecular devices, Sunnyvale), pH meter (TPS Digital pH-mV-temperature meter, model 1,852 mV), KOH (Merck), HCl (Sigma), K_2HPO_4 (Merck), Na acetate (Merck), NH_4 citrate (Sigma), $MgSO_4$, $MnSO_4$ (Sigma), Tween 80, (Sigma), Glucose, (Merck), Meat peptone (Sigma), Yeast extract (Sigma), NaCl (Sigma), $CaCl_2$ (Merck), and succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide (Sigma).

Microbial Strain Description and Method of Activation

L. delbrueckii subsp. *lactis* ATCC® 7830™ was obtained from ATCC and revived by propagating twice in deMan, Rogosa and Sharpe (MRS) Broth (Oxoid Pty Ltd, Australia) at 37 °C. Loopfulls of the culture were streaked on MRS Agar (Oxoid Pty Ltd, Australia) and incubated (Lishen HF151UV CO_2 Incubator) at 37 °C, 5 % CO_2 for 2 days. To obtain a working culture, loopfull inoculum was obtained from the streaked cells and recultured in MRS Broth (Acumedia, Michigan) at 37 °C and stored at −70 °C (Ultraflow freezer, Plymouth). Revived culture was grown to exponential phase (optical density at 560 nm (OD_{560}) of 1.0±0.2) in MRS broth (Acumedia, Michigan) and was used to inoculate fresh MRS broth as required.

Bacteria Growth and Description of the Bacterial Growth Curves

During cell growth, samples were withdrawn periodically to determine cell growth by optical density measurement (OD) at 560 nm with SpectraMax M2e Multi-Mode Microplate Reader (Molecular devices, Sunnyvale). Consecutive samples (200 μL) were taken at intervals from the fermentation suspension and immediately cooled on ice. The samples were centrifuged, and the cell pellets resuspended in distilled water for cell growth determination. From a previously prepared calibration curve relating optical density readings to biomass weight, an OD_{560} of 1.0 unit corresponds to 200 μg/ml of bacterial dry weight. The pH of the culture was recorded with a pH meter (TPS Digital pH-mV-temperature meter, model 1,852 mV). With the exception of experiments on the effect of agitation speed, all other experiments were conducted in a sterile 22-ml glass bottle with a working volume of 20 ml, and all experiments were conducted under 'microaerophilic' conditions.

The experimental data of the growth curves are described by the Gompertz model parameters A, μ_{max}, λ and ε based on Eq. 1 for the lag and exponential phases [20].

$$D_t = A\exp\left\{-\exp\left[\left(\frac{\mu_{max}e}{A}\right)(\lambda - t) + 1\right]\right\} \quad (1)$$

D_t is OD at time t; t is time of growth in hours; D_0 is OD at t=0; A, the asymptotic value is increase of OD between D_0 and OD_{max}; μ_{max} is maximum growth rate (in hours) calculated

from the slope of a semi-logarithmic plot of OD_{560} versus time (in hours); λ is duration time of lag phase in hours; and ε is the exponential growth time, given by:

$$\varepsilon = \frac{A}{\mu_{max}} \tag{2}$$

The parameters were estimated by the application of the Standard Simplex Method+ Universal Global Algorithm (SM1; Auto2Fit, Professional version 5.5, 7D-Soft High Technology Inc).

Effect of Inoculum Size

A volume of 20-ml sterile MRS broth (Acumedia, Michigan) was inoculated with exponential phase culture (optical density at 560 nm (OD_{560}) of 1.0±0.2) and incubated at 37 °C, initial pH 6.5 and 100 rpm agitation for 24 h. Innocula sizes of 0.1, 0.5, 1, 2, 5 and 10 % (*v*/*v*) were studied. Cells were harvested after 12 h of cultivation and aliquots (20 ml) taken for proteinase production assay.

Effect of Agitation

Experiments to study the effect of agitation on cell growth and proteinase production were conducted in 100-ml sterile conical shake flasks. A volume of 50 ml sterile MRS broth (Acumedia, Michigan) was inoculated with 1 % (*v*/*v*) of exponential phase culture (optical density at 560 nm (OD_{560}) of 1.0±0.2) and incubated at 37 °C and initial pH 6.5 for 24 h. Agitation speeds of 0, 25, 50, 75, 100, 150 and 200 rpm were studied. Cells were harvested after 12 h of cultivation and aliquots (20 ml) taken for proteinase production assay.

Effect of Incubation Temperature

Cells were cultivated in 20 ml of MRS broth (Acumedia, Michigan) at different temperatures (i.e. 30, 37, 40, 45 and 50 °C). Each setup was inoculated with 1 % exponential phase culture (optical density at 560 nm (OD_{560}) of 1.0±0.2) and grown at 100 rpm agitation speed and initial pH 6.5. Cells were harvested after 12 h of cultivation and aliquots (20 ml) taken for proteinase production assay.

Effect of Initial pH

Cells were cultivated in 20 ml of MRS broth (Acumedia, Michigan) at different initial pH (i.e. 4, 5, 6, 6.5, 7, 7.5 and 8). The pH of media was adjusted with 1 M KOH (Merck) or 1 M HCl (Sigma). Each setup was inoculated with 1 % (*v*/*v*) exponential phase culture (optical density at 560 nm (OD_{560}) of 1.0±0.2) and grown at 37 °C, 100 rpm agitation speed. Cells were harvested after 12 h of cultivation and aliquots (20 ml) taken for proteinase production assay.

Effect of C/N Ratio

For testing the effect the effect of carbon/nitrogen (C/N) ratio, cells were grown in 20 ml of modified MRS medium (% (*w*/*v*) composition being K_2HPO_4, 0.2; Na acetate, 0.5; NH_4 citrate, 0.2; $MgSO_4$, 0.01; $MnSO_4$, 0.005; Tween 80, 0.1; glucose, 2; peptone, 1; and yeast

extract, variable). C/N ratios were achieved by adjusting concentration of yeast extract. Each setup was inoculated with 2 % exponential phase culture (optical density at 560 nm (OD_{560}) of 1.0±0.2) and grown at 45 °C, initial pH 6.0 and 200 rpm agitation speed. Cells were harvested after 12 h of cultivation and aliquots (20 ml) taken for proteinase production assay.

Preparation of Whole Cell Suspension and Proteinase Activity Assay

Cultured cells were harvested by centrifugation at 4,000×*g* and 4 °C for 10 min, washed with an equal volume of 0.15 M sterile saline water supplemented with 20 mM $CaCl_2$ and resuspended to an OD of 4 in 50 mM sodium phosphate buffer (pH 7). This was designated as whole cell suspension. Proteinase activity was assayed according to the method of Exterkate [21] with slight modifications. The reaction mixture (284 μL) consisted of whole cell suspension (50 μL); 50 mM sodium phosphate buffer, pH 7.0; 37.5 mM imidazole; and 1 mM of the peptide succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide (Sigma). The setup was incubated at 40 °C in a water bath for 15 min after which the reaction was stopped by centrifugation (10,000×*g*, 10 min, 4 °C). Aliquots were taken for optical density reading. The release of *p*-nitroaniline was followed at 410 nm ((E 410)=8,800 $M^{-1}cm^{-1}$) by using a SpectraMax M2e Multi-Mode Microplate Reader (Molecular devices, Sunnyvale). One unit of proteinase was defined as the amount of enzyme required to liberate 1 nmol of nitroaniline per minute, under standard assay conditions.

Statistical Analysis

Data were analysed by Fisher's least significant difference (Statsgraphics Centurion version XV). Significant differences were declared at $p \leq 0.05$.

Results and Discussion

The effect of different fermentation conditions on cell-envelope-associated proteinase yield by *L. delbrueckii* subsp. *lactis* 313 (LDL 313) was studied and results are given below. The study was accomplished with the help of the Gompertz model predictive modelling tool because a large quantity of data was involved. The Gompertz model help describe the growth curves by reducing the experimental data to a limited number of biologically relevant parameters [20]. In this study, the modified Gompertz model [20] was used and in all cases the growth curve that resulted from plotting the experimental and predicted data has a sigmoid shape with good determination coefficient (R^2) and *F* values, thus justifying the suitability of the model. Furthermore, it was observed that all five process parameters studied had a significant effect on growth and proteinase yield. The significance of the study lies in the fact that, to the best of our knowledge, no such extensive study has been conducted for *L. delbrueckii* subsp. *lactis* 313.

Effect of Inoculum Size

The synthesis of useful bio-products by microorganisms is dependent on nutrient consumption which in turn is largely dependent on the density of bacteria in the finite volume of a culture medium. This requires that bacterial inoculum size should be controlled to ensure optimum nutrient uptake that conduces in a high product synthesis [22]. In addition, the initial biomass constitutes an important fraction of the process costs, which is a necessary factor in optimisation and/or bioprocess economic feasibility studies [23].

In this study, inoculum size resulted in a significant effect on proteinase yield ($p=1.49\times 10^{-07}$). At the given inoculum size range (0.1 % to 10 %, *v*/*v*), proteinase yields increased with increasing size of the inoculum, from 0.1 % to 2 % (*v*/*v*), and then dropped for 5 and 10 % (*v*/*v*) inocula size (see Fig. 1). Based on these results, the optimal inoculum size for proteinase production was determined to be 2 % (*v*/*v*) of exponential phase culture, which gave the highest specific proteinase yield (0.513±0.032 U/mg dry cell weight).

The effect of inoculum size on growth parameters (A, μ_{max}, λ and ε) is captured in Table 1. Inoculum size has been shown to affect bacterial growth [24]. Maximum cell growth rates (μ_{max}) were affected by inoculum size with μ_{max} increasing with inoculum size until a maximum of 0.16 h^{-1} (for 5 % (*v*/*v*) inoculum size) and then decreasing to 0.10 h^{-1} (for inoculum size 10 % (*v*/*v*)). The change of inoculum size had a decisive effect on the specific proteinase yield; therefore, the effect of inoculum size on proteinase yield was mainly a result of its effect on proteinase biosynthesis.

Among the *Lactococcus lactis,* cells growth rate has been known to influence to some extent, the expression of *prt* genes responsible for proteinase production [25]. The same observation could be true for *L. delbrueckii.* Culture at 10 % (*v*/*v*) observed a low μ_{max}, no lag phase and the highest pH decrease (Table 1). For many microbial species, an increase in inoculum density usually leads to higher biomass productivity but lower average specific growth rate [26] .The decrease in the growth rate for cells at higher densities (>5 % (*v*/*v*)) was due perhaps to the increasing limitation of key nutrients, and accumulation of greater amounts of growth inhibitory metabolites. A further explanation arises from reports that discuss cell-to-cell chemical signalling (quorum sensing effects) which affects growth initiation under stressful conditions for cells at higher cell densities [24]. On the other hand, a very small inoculum size means insufficient number of bacteria which leads to a reduced amount of secreted protease [23]. This accounts for the low proteinase yield for inoculum size less that 1 % (*v*/*v*).

Effect of Agitation

Culture agitation speed significantly affected cell biomass and specific proteinase yield ($p<0.001$). Higher agitation rates gave lower cell biomass yields. Increasing the agitation speed

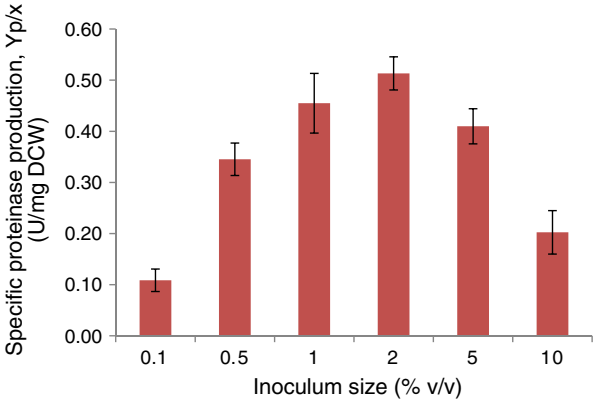

Fig. 1 Specific product formation ($Y_{p/x}$) of proteinase with respect to biomass (U/mg dry cell weight (*DCW*)); by *L. delbrueckii* subsp. *lactis*, 313 grown at different inoculum size (%); 100 rpm, 37 °C and initial pH 6.5; values are the mean±standard deviation (*error bars*) of three independent experiments; bacteria were grown during 12 h in MRS medium

Table 1 Estimates of growth parameters based on the Gompertz model for *Lactobacillus delbrueckii* subsp. *lactis* 313 under different inoculum size

Inoculum size (%, *v*/*v*)	μ_{max} (h^{-1})	*A*	λ (h)	ε (h)	pH decrease[a]	R^2 for model estimates	*F* statistic
0.1	0.10	0.59	5.44	5.67	1.55	0.99	99.07
0.5	0.10	0.57	3.65	5.48	1.50	0.99	116.00
1	0.12	0.58	3.51	4.95	1.51	0.99	103.27
2	0.12	0.68	2.87	5.51	1.64	0.96	46.22
5	0.16	0.67	2.43	4.31	1.78	0.99	92.89
10	0.10	0.71	−0.35	7.40	1.95	0.96	53.44

The Gompertz model was applied to the mean optical density readings (n=3) of experimental data

A increase between initial and maximum biomass, μ_{max} maximum specific growth rate, λ lag phase, ε exponential phase time (see Eq. (1) in 'Materials and Methods')

[a] After 24 h of incubation from initial pH of 6.50

from 0 to 200 rpm resulted in a 64 % decrease in biomass (in milligrammes per millilitre dry cell weight; see Fig. 3). On the contrary, specific proteinase production was higher at higher agitation speeds with agitation speed of 150 rpm giving the optimum specific proteinase yield of 1.66±0.067 U/mg dry cell weight (Fig. 2).

For many lactobacilli, higher agitation speeds during growth generally results in higher biomass due to generation of additional ATP and enhancement of fluid-to-particle mass transfer [27]. In addition to ensuring homogeneity of fermentation broth, agitation also creates an aerobic milieu in the culture broth. Consequently, several studies have reported higher cell biomass yields for lactobacilli grown at high agitation speeds or micro-aeration conditions [27–29]. However, the aforementioned studies are opposite to the results of the present study with LDL 313.

High speed of agitation has been shown to have a significant effect on bacterial growth for some lactobacilli (such as *Lactobacillus plantarum*) [27]. However, this and other studies we have conducted [30] have shown that vigorous agitation and other factors that promote aerobic conditions actually inhibit cell biomass yields for LDL 313. The metabolism of LDL

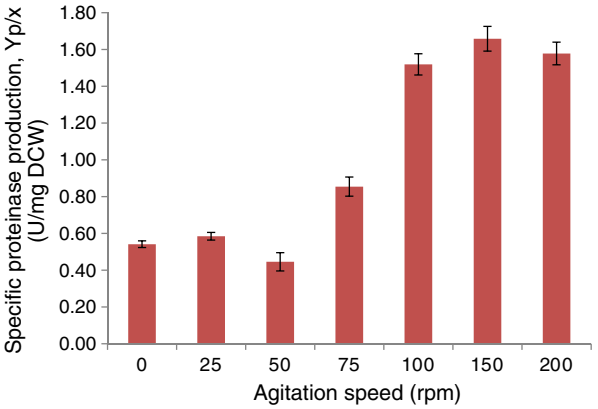

Fig. 2 Specific product formation ($Y_{p/x}$) of proteinase with respect to biomass (U/mg DCW) for growth at different agitation speeds; 1 % (*v*/*v*) inoculum, 37 °C and initial pH 6.5; values are the mean±standard deviation (*error bars*) of three independent experiments; bacteria were grown during 12 h in MRS medium

313 is obligately homofermentative [31] and is classified by ATCC as microaerophilic. Like other lactic acid bacteria, LDL 313 strain adapts to various conditions and change their metabolism accordingly in order to favour efficient carbohydrate utilisation. In the presence of oxygen, pyruvate is not converted exclusively to lactate but also to acetyl phosphate and acetate which results in a low ATP/glucose yield [32, 33]. Also, cell growth under aerobic condition is typically low due to oxygen stress or product inhibition by hydrogen peroxide, a by-product of the aerobic pathway [17, 34, 35]. Thus, high oxygen supply to the bacteria cells (as a result of the high aeration speeds) might have been the cause for the suppressed growth and low biomass yields for growth at higher agitation speed (Fig. 3). On the other hand, specific product formation due to biomass ($Y_{p/x}$) was significantly higher at higher agitation speeds (Fig. 2).

The higher microbial biomass seen when the flasks were rotated at low speed (50 rpm and below) could be accounted for by the action of pyruvate-formate lyase, an oxygen-sensitive enzyme that is activated during anaerobic growth for pyruvate metabolism to formate and acetyl CoA. The pathway ensures the activation of a substrate-level phosphorylation involving acetyl phosphate and acetate kinase which increases ATP/glucose yield and thus, biomass yield [29, 31].

An increment of 67.4 % in proteinase yield was observed between agitation speeds 0 and 150 rpm. In the present investigation, LDL 313 grown in MRS showed maximum proteinase activity at 150 rpm agitation speed after 12 h of incubation (Fig. 2). At high agitation speed, aeration of the culture medium is enhanced which could lead to sufficient supply of dissolved oxygen in the media and/or enhanced nutrient uptake by bacteria, thus resulting in increased proteinase yields [36]. These results therefore imply that micro-aeration conditions favour proteinase synthesis. At agitation speed of 75 rpm and below, inadequate aeration and nutrient uptake could be the cause of reduced proteinase yield. The effect of high culture agitation speeds on protease yields is well documented for several proteases producing bacteria species [7, 36, 37].

Effect of Incubation Temperature

Temperature affects all the physiological activities in a living cell and it is an important environmental factor to control the growth, microbial activities, and normal functioning of the cellular enzymes. The primary role of temperature is to change rates of biochemical

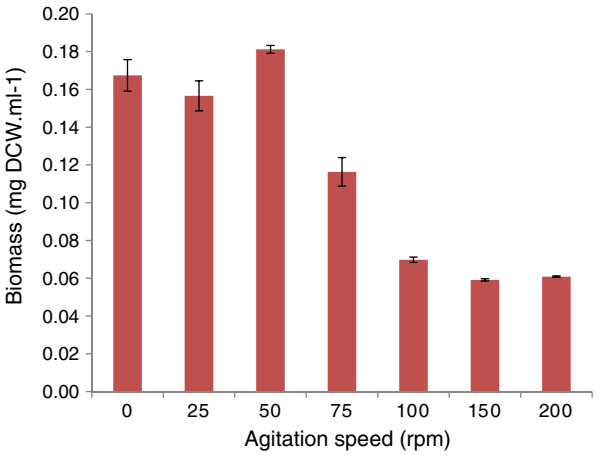

Fig. 3 Biomass yield in mg dry cell weight (*DCW*); by *L. delbrueckii* subsp. *lactis* 313, grown at different agitation speeds; 1 % (*v*/*v*) inoculum, 37 °C and initial pH 6.5; values are the mean±standard deviation (*error bars*) of three independent experiments; bacteria were grown during 12 h in MRS medium

reactions necessary for cell growth. The optimal temperature for growth of lactic acid bacteria varies between the genera from 20 to 45 °C [38]. In this study, cell maximum specific growth rate (μ_{max}) increased with temperature over the 30 to 50 °C range studied (Table 2).

It was also observed that a linear relationship between the square root of the growth rate (μ_{max}) and temperature existed (Fig. 4). This simple equation has been used successfully to predict the effect of temperature on growth of a wide range of bacteria [26, 39]. The temperature, obtained by extrapolation, at which growth rate is zero is defined as T_0, and may be a cardinal characteristic property of an organism growing under defined nutrient conditions [26]. In this case, T_0 was calculated to be 22.83 °C for LDL 313 and this value could be explored for significance during temperature based food process operations. Furthermore, the ability of LDL 313 to grow at relatively high temperatures enables this species to be used in processing operations that require higher-than-normal-processing temperatures such as in the manufacture of Cheddar cheese which is processed at 42 °C.

It should be borne in mind that this equation is only valid within the optimal growth temperature range for a typical bacteria species. This is because; beyond a critical maximum temperature, T_{max}, cell growth is inhibited by extreme heat leading to a decrease in cell growth rate [39]. Thus, the trend line in Fig. 4 cannot linearly protrude indefinitely to imply an increasing maximum growth rate beyond temperatures above 50 °C.

Although the mechanism of temperature control of enzyme production is not well understood, a relation has been shown to exist between enzyme synthesis and energy metabolism in bacilli and these observations are controlled by temperature and oxygen uptake [40]. In this study, proteinase activity increased linearly for temperatures 37 till 45 °C and no proteinase activity was observed at incubation temperature of 30 °C. Optimum proteinase production was obtained at 45 °C (Fig. 5). The sharp decrease in proteinase activity over 45 °C is likely due to thermal inactivation of biosystems at temperature higher than the optimum, i.e. the progressive enzyme distortion with respect to its biologically active conformation induced by thermal agitation [41, 42]. It can therefore be inferred that although high incubation temperature gives high cell growth rate, beyond 45 °C it does not necessarily give higher protease yield.

Effect of Initial pH

The results of this study indicate that proteinase production by LDL 313 is affected significantly (p=0.0052) by initial pH of growth medium. The effect of pH on the growth rates of LDL 313 is shown in Fig. 6 and the growth parameter estimates from the Gompertz

Table 2 Estimates of growth parameters based on the Gompertz model for *Lactobacillus delbrueckii* subsp. *lactis* 313 under different incubation temperatures

Temperature (°C)	μ_{max} (h^{-1})	A	λ (h)	ε (h)	pH decrease[a]	R^2 for model estimates	F statistic
30	0.02	0.19	8.17	9.38	0.20	0.99	96.68
37	0.06	0.74	11.54	11.58	1.19	0.99	1,017.99
40	0.06	1.10	7.93	19.66	0.81	0.99	213.21
45	0.16	0.73	7.81	4.48	1.35	0.99	1,575.19
50	0.23	0.84	7.83	3.62	1.82	0.99	1,131.74

The Gompertz model was applied to the mean optical density readings (n=3) of experimental data

A, increase between initial and maximum biomass, μ_{max} maximum specific growth rate, λ lag phase, ε exponential phase time (see Eq. (1) in 'Materials and Methods')

[a] After 24 h of incubation from initial pH of 6.55

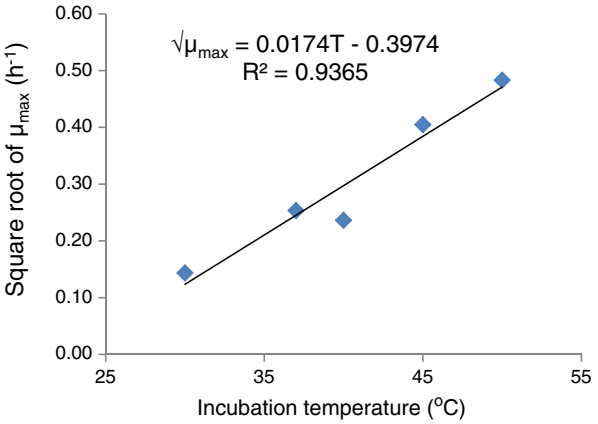

Fig. 4 Variation of μ_{max} according to the temperature of growth, T for *L. delbrueckii* subsp. *lactis* 313

model are given in Table 3. Protease production by microbial strains is affected by extracellular pH via either pH dependent control of protease gene expression [40, 43], or culture pH strongly controlling cell growth by influencing many enzymatic processes and transport of various components across the cell membranes [44]. In this study, proteinase activity was higher for growth pH between 5.5 and 6.5 with pH 6.0 giving the optimum proteinase yield of 0.233±0.005 U. These results are similar to that of other proteinase production studies involving lactobacilli [4]. Espeche Turbay et al. [6] have also shown that proteinase production and stability by *L. delbrueckii* subspecies *lactis* CRL 581 is influenced by growth culture pH. Their work also demonstrated that proteinase production for fermentation in uncontrolled pH does not differ significantly from that occurring in controlled pH conditions. Thus, for the purpose of proteinase production, growth at uncontrolled pH is recommended since the cost of process operations and consumables for restricting pH is eluded. Adjusting initial pH to 6.0 also ensures that proteinase yield is maximal.

From Table 3, cell concentration at pH 6.0 and 6.5 did not differ much. Respectively, an increase from 0.03 mg/ml DCW to 0.25 mg/ml DCW; and 0.03 to 0.22 mg/ml DCW were

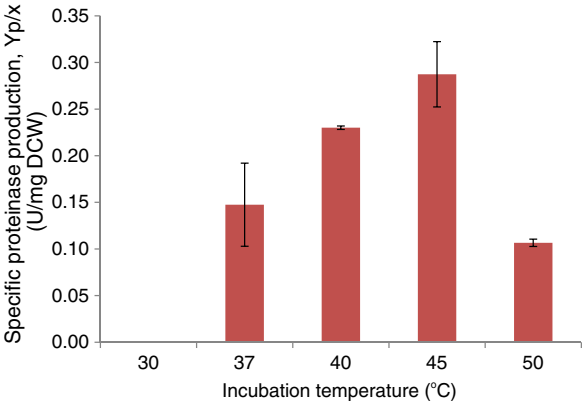

Fig. 5 Specific product formation ($Y_{p/x}$) of proteinase with respect to biomass (U/mg DCW) for growth at different incubation temperatures; 100 rpm, 1 % (*v*/*v*) inoculums and initial pH 6.5; values are the mean± standard deviation (*error bars*) of two independent experiments; bacteria were grown during 12 h in MRS medium

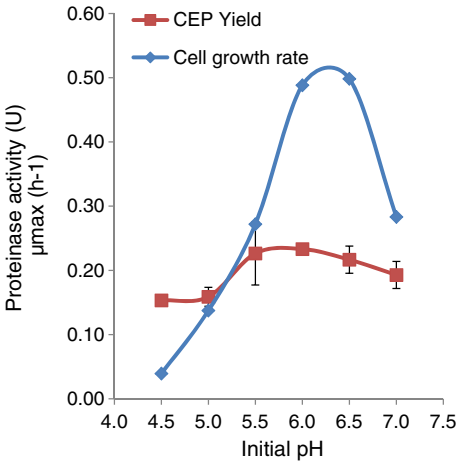

Fig. 6 Cell growth rates, μ_{max} (*filled diamonds*) and cell-envelope-associated proteinase (*CEP*) yield (*filled squares*) for growth at different initial pH; 150 rpm, 2 % (*v*/*v*) inoculum; 45 °C; values are the mean±standard deviation (*error bars*) of three independent experiments; bacteria were grown during 12 h in MRS

observed after 15 h of incubation. The kinetic profiles were very well described by the Gompertz equation (R^2=0.99). The cell growth rate (μ_{max}) was optimum at pH 6.5 whereas the optimum proteinase activity was observed for culture at initial pH 6.0. The difference in pH optima for growth and proteinase production shows that although bacterial growth and proteinase production is greatly controlled by culture pH, the optimum condition for enzyme synthesis and that for optimum growth may differ [45]. That optimum proteinase yield occurred at pH 6.0 could be accounted for by the fact that under slightly acidic conditions the enzymatic activity is enhanced, likely through improved activation of the ionic groups present at the enzyme active sites.

Effect of C/N Ratio

The type and concentration of nitrogen [25, 46] and carbon substrate [47] in growth medium have been known to influence the production of proteases in lactobacilli. Thus, high

Table 3 Estimates of growth parameters based on the Gompertz model for *Lactobacillus delbrueckii* subsp. *lactis* 313 under different initial growth pH conditions

Condition (initial pH)	μ_{max} (h^{-1})	*A*	λ (h)	ε (h)	pH decrease[a]	R^2	*F* statistic
4.5	0.04	0.56	0.38	14.23	0.12	0.90	19.07
5.0	0.14	0.72	2.62	5.24	1.25	0.97	30.76
5.5	0.27	0.79	3.02	2.89	0.43	0.98	37.08
6.0	0.49	1.24	3.20	2.55	1.17	0.99	90.94
6.5	0.50	1.05	3.30	2.11	1.20	0.99	75.40
7.0	0.28	1.01	2.81	3.56	1.32	0.98	47.66

The Gompertz model was applied to the mean optical density readings (n=3) of experimental data

A, increase between initial and maximum biomass, μ_{max} maximum specific growth rate, λ lag phase, ε exponential phase time (see Eq. (1) in 'Materials and Methods')

[a] After 24 h of incubation from initial pH

productivities of proteases in lactobacilli require the carbon and nitrogen in the correct ratio. The production of many other bio-products in complex media has been shown to be dependent on the carbon/nitrogen ratio, including exopolysaccharides [48], plasmids [9], bacteriocins [49] and α-amylase [50].

In this study, the ratio of glucose to yeast extract in modified MRS medium was adjusted between 0.2 and 5 by altering the concentration of yeast extract. The result (Fig. 7) showed that C/N ratio affects proteinase specific yield with a maximum specific yield of proteinase of 0.11 U/mg dry cell weight, obtained when C/N=1.0. Highest cell biomass yield of 1.03 mg/ml dry cell weight was obtained for culture of C/N ratio 0.5 (Fig. 8).

That the growth and production of proteinase by LDL 313 was influenced by the C/N ratio suggests nitrogen as a limiting factor for growth and proteinase synthesis and the optimal ratio between nitrogen and carbon source permits the increase of the proteinase activity. However, an extremely nitrogen-rich environment will not necessarily lead to a gain of proteinase activity. This is because whereas peptide-rich media (such as MRS) support profuse microbial growth, they also decrease expression of *prt* genes responsible for proteinase production in most lactobacilli [10, 51, 52]. An increase of the nitrogen availability will increase proteinase production due to better cell growth, but only until a certain plateau value is reached, after which proteinase yields begin to fall again. The C/N ratio and its effect in proteinase yield is an important parameter to consider in the design of production media chiefly for proteinase production.

Growth and CEP Production Profile

The time course for growth and proteinase production is shown in Fig. 9. Cell growth at the optimum conditions recorded maximum growth rate, μ_{max} of 0.36 h^{-1}, lag phase, λ of 1.7 h, and exponential phase time, ε of 1.37 h (R^2=0.97, F statistic=62.12). The strain took 12 to 14 h to reach maximum cell concentration, A=2.34). The parameter, exponential phase time, ε, is the length of time that the cells spend in the exponential phase [53]. This is significant for fermentations of growth–associated products where maximum yields are obtained in the log phase. The formation of proteinase significantly started from early exponential phase and

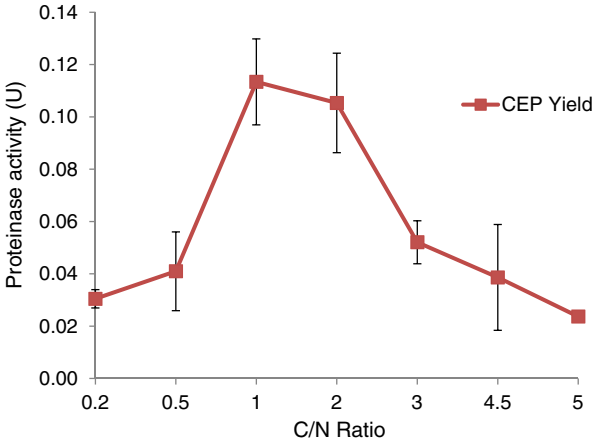

Fig. 7 Effect of C/N ratio on cell-envelope-associated proteinase (*CEP*) yield from modified MRS medium. C/N ratios were achieved by adjusting concentration of yeast extract. Bacteria were grown at initial pH 6.0; 150 rpm, 2 % (*v*/*v*) inoculum; 45 °C for 12 h

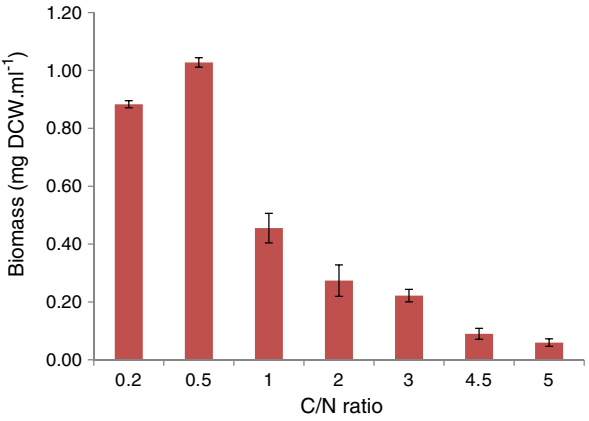

Fig. 8 Biomass yield in mg dry cell weight (*DCW*); by *L. delbrueckii* subsp. *lactis*, 313 grown at different C/N ratios; 2 % (*v*/*v*) inoculum, 45 °C and initial pH 6.0; values are the mean±standard deviation (*error bars*) of three independent experiments; bacteria were grown during 12 h in modified MRS medium

reached a maximum in 8 h, with levels of 0.99 U/ml and then began to fall. It was observed that the highest proteinase production was during exponential growth, which implies a correlation between cell growth and proteinase formation, in accordance with other reports [6, 10]. The optimum harvesting time for proteinases form LDL 313 is therefore at the late exponential phase (between 7 and 9 h of cultivation). After the exponential phase, the gradual decrease in enzyme units observed with increasing incubation period clearly shows the enzyme's role as a primary metabolite, being produced in the log phase of growth for utilisation of nutrients present in growth medium.

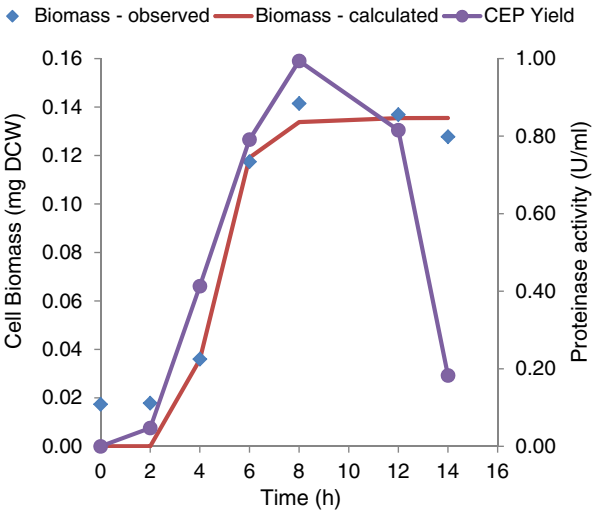

Fig. 9 Time course of growth and proteinase production by *L. delbrueckii* subsp. *lactis* 313; shake flask fermentation was performed at initial pH 6.0; 150 rpm, 2 % (*v*/*v*) inoculum; 45 °C; cell biomass (mg DCW; *filled diamonds*), *smooth line* is predicted plots from the Gompertz model estimate; Proteinase (*filled circles*); *symbols* represent the mean of experimental data obtained (n=3)

Conclusions

This work has shown, for the first time, that incubation temperature (45 °C), inoculum size (2 % (*v*/*v*) of OD_{560}=1), initial pH (6.0), agitation speed (150 rpm) and C/N ratio (1.0) of production medium are important variables determining the growth and production of cell-envelope-associated proteinases by *L. delbrueckii* subsp. *lactis* 313 (ATCC 7830) in MRS. The optimal conditions yielded 0.99 U/ml proteinase after 8 h of fermentation.

The usefulness and application of proteinases, in coming years, is likely to expand beyond the manufacture of products with nutritional, organoleptic and therapeutic properties. Such advances hinge on detailed optimisation of fermentation parameters essential for the generation of proteolytic enzymes at optimum process and economic conditions. The results of this study give useful information towards the achievement of such ends, in addition to providing other avenues of utility for *L. delbrueckii* subsp. *lactis* 313 (ATCC 7830) in the food industry.

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4. CHAPTER 4: Extraction of Cell-Envelope Proteinase

Section 4.1

4.1. Bioanalytical Evaluation of *Lactobacillus delbrueckii* subsp. *lactis* 313 Cell-Envelope Proteinase Extraction

Monash University
Declaration for Thesis Chapter 4 Section 1

Declaration by candidate

In the case of Chapter 4 Section 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Initiation, key ideas and writing up	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Wendy Lim	Key ideas, Experimental, Development, Results interpretations, writing up	5
Michael Zass	Key ideas, Experimental, Development, Results interpretations, writing up	5
Darren Tan	Key ideas, Experimental, Development, Results interpretations, writing up	5
Dr. Michael K Danquah	Initiation, key ideas and writing up	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12 December 2013
Main Supervisor's Signature	[redacted]	**Date** 12 December 2013

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Bioanalytical evaluation of *Lactobacillus delbrueckii* subsp. *lactis* 313 cell-envelope proteinase extraction

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HIGHLIGHTS

- Cell-envelope proteinases (CEPs) in *Lactobacillus delbrueckii* subsp. lactis 313 (LDL 313) are cell-wall anchored.
- The enzymes are subject to autoproteolytic release and ionic misfolding in calcium-free buffer.
- CEP localization and self-digestion properties affect the efficiencies of different CEP extraction methods.
- Incubation of LDL cells in 5M LiCl was the most effective method for releasing high levels of CEPs from LDL 313.

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ABSTRACT

Lactobacilli cell-envelope proteinases (CEPs) have demonstrated numerous biopharmaceutical applications in the development of new streams of blockbuster nutraceuticals; thus, the development of efficient and commercially viable methods for CEP extraction will promote their full-scale application. In this study, the sub-cellular location of CEPs in *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) was identified and the effects of different extraction methods were investigated for their ability to efficiently release CEPs from LDL 313. Significantly high relative proteinase activity of~95% was detected in cell-wall fractions and ~5% activity was observed for osmotic fluids, implying that proteinases in LDL 313 are cell-wall bound. CEPs were released from cell-wall via incubation in calcium-free buffer, indicating the enzyme is liable to self-digestion and ionic misfolding. Of the different extraction methods investigated, the use of 5 M LiCl was the most suitable, under the conditions of experimentation, for releasing high levels of CEPs from LDL 313.

1. Introduction

Cell-envelope proteinases (CEPs) are a class of industrially relevant proteolytic enzymes produced by some bacteria species such lactobacilli (Agyei and Danquah, 2012b; Sadat-Mekmene et al., 2011). In order to grow in milk media, lactobacilli synthesize CEPs to degrade casein into peptides and amino acids (Tsakalidou et al., 1999) to enable cellular uptake. CEPs also help improve the texture and organoleptic characteristics of fermented milk products (Espeche Turbay et al., 2009). CEPs have been shown to cause the release of bioactive peptides from food proteins, and these subunit biomolecules have found numerous therapeutic and health applications beyond basic nutrition (Danquah and Agyei, 2012; Korhonen, 2009). Proteolytic enzymes are relevant digestive catalysts for the production of bioactive peptides from different protein sources, and lactobacilli expression of extracellular CEPs is a metabolically effective source to facilitate proportional-integral-derivative (PID)-controlled microbial proteinase synthesis in bioreactors for bioactive peptides production with cost effectiveness, scalability and commercial viability (Agyei and Danquah, 2011).

In order to fully exploit these numerous biotechnological potentials of CEPs, detailed understanding of the biosynthesis and metabolite accumulation is essential to develop optimal techniques for effective extraction and recovery of CEPs. Although several procedures exist for the extraction of CEPs from lactobacilli (Jarocki et al., 2010; Sánchez et al., 2009; Tsakalidou et al., 1999), none is fully optimized for the extraction of structurally intact CEPs from different cellular species and under different metabolic and process conditions. Effective extraction of CEPs is a function of different biological and physicochemical characteristics such as the cell type; metabolic stage; CEP molecular structure; type, polarity, ionic strength and concentration of the diluent; and conditions of the extraction process, including pH, conductivity and temperature. The most extensively used method of releasing

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Table 1
Experimental methods for the extraction of surface proteins from some lactobacilli species.

Agent	Conc.	Incubation condition		Ref.
		Time (min)	Temperature (°C)	
Guanidine–HCl	4 M	30	37	(Bhowmik et al., 1985)
Glycine (pH 2.2)	0.2 M	15	4	(Turner et al., 1997)
Sodium dodecyl sulfate (SDS)	0.2% (w/v)	30	37	(Bhowmik et al., 1985)
Urea	8 M	60	37	(Jarocki et al., 2010)
NaOH	10 mM	30[d]	37	(Sánchez et al., 2009)
Lithium chloride	5 M	60	30	(Jarocki et al., 2010)
Na–phosphate buffer (pH 7)	50 mM	120	30	(Tsakalidou et al., 1999)
Lysozyme buffer[a]	–	30	37	(Sánchez et al., 2009),
Lysozyme (dissolved in 50 mM sodium phosphate buffer, pH 7)	0.2% (w/v)	30	37	(Sánchez et al., 2009)
Lysozyme with cold shock[c]	0.2% (w/v)	10	3	(Atlan et al., 1990)
Lithium chloride–lysozyme solution[b]	–	60	4	(Rojas et al., 2002)

[a] Lysozyme buffer: 0.3% lysozyme, 0.5 M sucrose, 0.03 M ammonium bicarbonate.
[b] Lithium chloride-lysozyme solution: 1 M lithium chloride, lysozyme solution (0.1 M Tris, 0.015 M NaCl, 0.05 M $MgCl_2$, 40 μg of lysozyme mL^{-1}.
[c] Treatment with cold distilled water followed by lysozyme treatment (37 °C, 30 min).
[d] Incubation done with gentle agitation.

CEPs from lactobacilli is by washing or incubating the cells in a calcium-free buffer (Exterkate, 1990; Fira et al., 2001; Tsakalidou et al., 1999). However, this method does not release CEP from some lactobacilli species (Atlan et al., 1989; Hébert et al., 1997; Laloi et al., 1991; Martín-Hernández et al., 1994) and also leads to low enzyme activity and thermal stability of the CEP released (Exterkate, 2000). Other methods for the extraction of cell surface proteins include the use of muramidases or chaotropic agents. Among several chaotropic agents, including lithium chloride, guanidine hydrochloride, glycine, and urea, used for cell-surface protein extraction, Jarocki et al. (2010) observed that the use of urea releases large quantities of cell surface-associated proteins from *L. rhamnosus* with no cell lysis. Bhowmik et al. (1985) also observed that guanidine hydrochloride solution was the best extraction agent to obtain large quantities of cell surface proteins from *L. acidophilus* strains. Muramidases, such as the lysozyme and/or mutanolysin, have been used for the hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the peptidoglycan cell wall to facilitate the release of cell surface bound proteins. Atlan et al. obtained an improved yield of proteinases from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 with no cell lysis when they coupled lysozyme/mutanolysin treatment with cold temperature shock (Atlan et al., 1990; Laloi et al., 1991). Hence for this lactobacilli species, lysozyme treatment and osmotic shock is a much more efficient procedure for releasing CEPs than washing cells with calcium-free buffer (Atlan et al., 1990; Laloi et al., 1991). The inability of some extraction protocols to release CEPs from certain lactobacilli is attributable to differences in the localization and/or attachment of CEPs on the microbial cell-envelope. It is therefore conclusive that the method for the release of surface proteins is species and/or strain dependent, and no single method works for all lactobacilli.

L. delbrueckii subsp. *lactis* (LDL 313) is a homofermenter that has wide analytical and industrial applications. It is utilized in microbiological assays of vitamin B_{12} and amino acid contents of food products (Taranto et al., 2003) as well as in the production of sour bread, grape wines, hard cheeses and yoghurt (Batdorj et al., 2007; Edwards et al., 1993; Espeche Turbay et al., 2009; Vogel and Ehrmann, 2008). However, biochemical and research data to substantiate the application of LDL 313 in the aforementioned areas is largely lacking, and LDL 313 still remains an understudied species. We have described in part the CEP production mechanics of this species in previous studies (Agyei and Danquah, 2012b) and it follows that LDL 313 putatively produces proteolytic enzymes that can find use in the nutraceutical industry. The scope of technological and industrial application of CEPs from LDL 313 is expansible, but these prospects largely hinge on extensive studies in understanding the cellular mechanism of CEP synthesis and accumulation, along with the development of feasible, efficient and commercially viable protocols for CEP extraction. To this end, the aim of this study is to identify the sub-cellular location of CEPs in *L. delbrueckii* subsp. *lactis* 313 and to ascertain the most suitable method for the extraction of CEPs from this lactobacilli species.

2. Materials and methods

2.1. Strain and growth condition

L. delbrueckii subsp. *lactis* 313 (ATCC® 7830™) was used in this work and the detailed propagation and the 'anaerobic' growth conditions employed are described elsewhere in our previous work (Agyei and Danquah, 2012b). Fermentation was carried out in 500 ml anaerobic shake flasks (Daniels and Zeikus, 1975), and setup designated as 'anaerobic' was sparged with sterile nitrogen gas for ~1 min to displace the air present in the flask. The specific growth rate (μ_{max}) was calculated from the slope of a semi-logarithmic plot of OD_{560} versus time (h). Microbial growth was expressed as $ln\ x/x_o$ versus time, where x is OD_{560} at time t and x_o is initial OD_{560}. The pH of the culture was monitored periodically with a pH meter (TPS Digital pH-mV-temperature meter, model 1852 mV).

2.2. Extraction of cell surface proteins

Cells were harvested by centrifugation (4000*g*, 10 min, 4 °C), washed once with an equal volume of sterile saline solution (0.15 M) supplemented with 10 mM $CaCl_2$, and resuspended to an OD of 5 in the extraction solution. The cell suspension was incubated under different specific conditions as detailed in Table 1 and centrifuged (4000*g*, 10 min, 4 °C) afterwards. The supernatant was decanted and filtered through a 0.22 μm membrane (Acrodics, Pall Life Sciences). The filtrate was subjected to buffer-exchange and concentration using Ultracel 3K (3000MWCO, Amicon Ultra, Millipore) according to the manufacturer's instruction and finally suspended in 50 mM phosphate buffer (pH 7.0). Protein concentration was determined with a Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Eleven extraction protocols were tested and evaluated based on their enzyme extraction performances. The extraction protocols were mostly obtained from literature and

pursued on *L. delbrueckii* subsp. *lactis* 313 cells with little or no modification as summarized in Table 1.

2.3. SDS-PAGE

Electrophoresis of the extracts was carried out with the Experion™ system and Pro260 Assay Kit (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions and as described in our previous work (Agyei and Danquah, 2012b). The cell extract samples were mixed with sample buffer and heated at 95 °C for 5 min to denature the proteins. A 4 μl aliquot was loaded into each well of the microchip for analysis.

2.4. Cell lysis estimation

The occurrence of cell lysis during incubation was determined by the detection of the intracellular enzyme lactate dehydrogenase (LDH). LDH was assayed by the method reported by Krishnan et al. (2000). The degree of cell lysis for an extraction method was estimated as a percentage of total LDH activity of cells. To induce cell lysis, a mid-exponential phase (7 h) culture immersed in ice was lysed using Ultrasonication (Vibra-Cell™ Ultrasonic Processor, Newtown, Connecticut) at 130 W, 40 kHz for ~1 min burst of 10 cycles each until cells were completely lysed. The cell lysis process during sonication was monitored by measuring particle size of broken cells with a Zetasizer Nano ZS (Malvern Instruments, Malvern, United Kingdom).

2.5. Proteinase activity assay of extract

Proteinase activity assay used for this work is described elsewhere (Agyei and Danquah, 2012a) with slight modification. The reaction mixture (284 μL) consisted of buffered enzyme solution (50 μL), 25 mM NaH_2PO_4, pH 7.0, 37.5 mM imidazole, and 1 mM of the peptide succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide (S-ala, Sigma), and was incubated at 40 °C for 10 min after which the absorbance was read immediately. The released nitroaniline was measured at 410 nm by using a SpectraMax M2e Multi-Mode Microplate Reader (Molecualr devices, Sunnyvale, USA) with a thermostatically controlled cell compartment. The release of p-nitroaniline was followed at 410 nm ([E 410] = 8800 M^{-1} cm^{-1}). One unit of proteinase was defined as the amount required to liberate 1 nmol of nitroaniline per minute.

2.6. Sub-cellular fractionation for localization of CEPs

Cellular extracts were obtained according to a method described by (Blanc et al., 1993) with slight modification. Culture at mid-exponential phase (7 h) were harvested by centrifugation (4000*g*, 10 min, 4 °C), washed once with an equal volume of sterile saline solution (0.15 M) supplemented with 10 mM $CaCl_2$, resuspended to an OD of 5 in 50 mM Na–phosphate buffer supplemented with 0.6 M-sucrose, and treated with 1 mg ml^{-1} lysozyme from chicken egg white (Sigma) for 30 min at 37 °C with gentle shaking. The suspensions were centrifuged (10,000*g*, 10 min, 4 °C) and the supernatants referred to as lysozyme fluids. Lysozyme-treated bacteria were subjected to osmotic shock by suspending them in ice cold distilled water for 10 min. Shocked cells were centrifuged (10,000 *g*, 10 min, 4 °C) and the supernatants referred to as osmotic fluids. The bacterial pellets were resuspended in cold Tris-HCl buffer and disrupted with by Ultrasonication (Vibra-Cell™ Ultrasonic Processor, Newtown, Connecticut) at 130 W; 40 kHz in a 4 cycle burst for ~30 s each. The supernatants obtained after centrifugation (25,000*g*, 20 min, 4 °C) of the broken cells are referred to as soluble cytoplasmic fluids. The soluble cytoplasmic fluids were also centrifuged (25,000*g*, 1 h, 4 °C) and the pellets, called membrane fractions, were suspended in 0.2 M Tris-HCl (pH 7.3).

3. Results and discussion

3.1. Growth profile and acidification rate

L. delbrueckii subsp. lactis 313 (LDL 313) was cultivated over a 12 h period under 'anaerobic' growth conditions. The semi-log growth profile plotted is shown in Fig. 1. The specific growth rate (μ_{max}) of cells was calculated to be 0.36 h^{-1}, giving doubling time of 1.93 h. The cells were harvested for crude proteinase extraction at the mid-to-late exponential phase (after 7 h of fermentation) with OD_{560nm} of 1.3 (i.e. about 270 μg ml^{-1} dry cell biomass). From preliminary results 7 h of fermentation was the optimal time point to harvest cells for CEPs extraction.

The pH of growth media changed from 6.3 to 4.61 (a decrease of~1.7) in the 12 h of cell growth (Fig. 1). This high acidification rate observed in the culture pH is attributable to the production of high amounts of organic acids, as observed when LDL 313 is cultured under 'anaerobic' growth conditions (Agyei and Danquah, 2012b). External growth pH is an important condition that affects cell biomass growth by influencing many enzymatic processes and the transport of various cellular components across the cell membranes (Ellaiah et al., 2002; Espeche Turbay et al., 2009; Tsakalidou et al., 1999). Our previous studies have shown that CEP production by LDL 313 is significantly affected by external pH of growth medium (Agyei and Danquah, 2012a; Agyei and Danquah, 2012b; Agyei et al., 2012).

Moreover, the distribution and attachment of cell surface CEPs can be influenced by the 'anaerobic' growth conditions and extracellular pH. This is because, during cell growth, the relevant enzymes and proteinases are synthesized to metabolize specific protein substrates present in the growth medium. The distribution of proteins and proteinases on the cell surface is therefore in an order dependant on the type and concentration of the limiting protein substrates. Also, the mechanical integrity of the cell membrane protein distribution is a function of protein–protein and protein-cell membrane intermolecular forces (Agyei and Danquah, 2012b). The high cell growth rate of 0.36 h^{-1} indicates that 'anaerobic' cultures undergo rapid periodic redistribution of surface proteinases in response to the rapid cell doubling and expansion. This phenomenon of rapid cell-surface changes (especially at the exponential phase) may influence the strength of CEP attachment and autoproteolytic release of CEPs. The efficiency of CEP extraction may therefore be affected by the culture growth conditions and the cell growth phase during which CEPs were extracted.

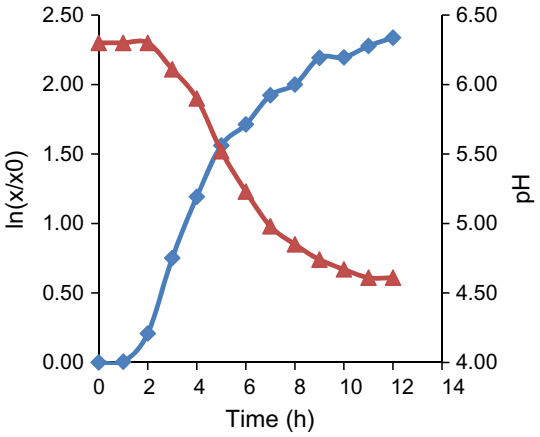

Fig. 1. Growth (♦) profile and pH (▲) profiles for *Lactobacillus delbrueckii* subsp. lactis 313 in MRS medium (bacteria were grown at initial pH 6.3; 100 rpm, 37 °C).

Table 2
Enzyme activity readings of the various sub-cellular fractions.

Sub-cellular fraction	Enzyme activity	
	(U)	**(%)**
Lysozyme fluids	0.009 ± 0.001	94.78 ± 6.04
Osmotic fluids	0.001 ± 0.000	5.22 ± 1.31
Soluble cytoplasmic fluids	ND	ND
Membranes	ND	ND
Total	*0.0096*	*100*

[#] Total activity was the sum of activities measured in the four extracts; Mean ± - standard deviation of triplicate experiments; ND, not detected.

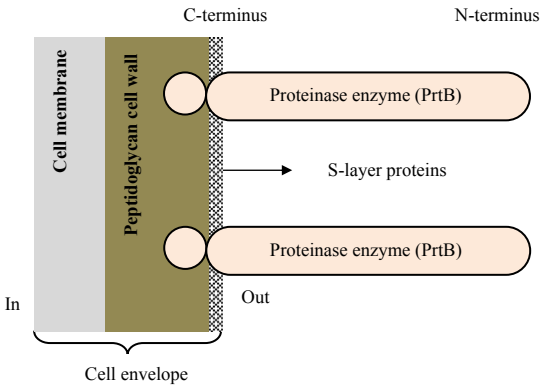

Fig. 2. A simplified schematic view of PrtB structural attachment to cell envelope in LDL 313.

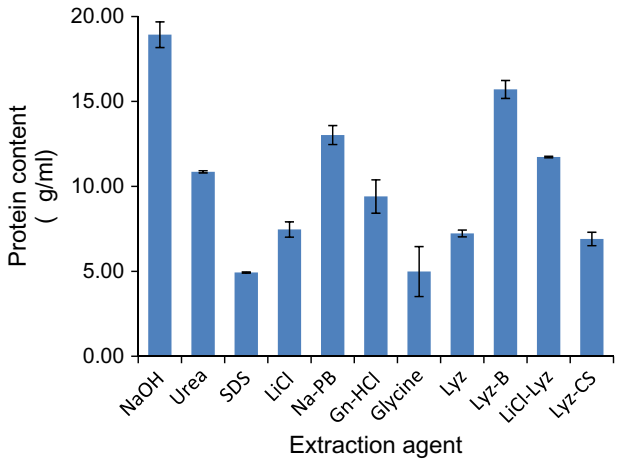

Fig. 3. Protein content of cell extracts from different extraction agents. Results of three independent experiments; (SDS: sodium dodecyl sulfate; Na-PB: sodium phosphate buffer; Gn-HCl, guanidine hydrochloride; Lyz: lysozyme; Lyz-B, lysozyme buffer; Lyz-CS: lysozyme-cold shock treatment; see Table 1 for buffer compositions and concentrations).

3.2. Cellular location of the cell-envelope proteinases

Cell-envelope proteinases from LDL 313 are able to hydrolyze β- and κ-caseins (Agyei and Danquah, 2012b) and they are also active on the chromophoric substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide. Proteolytic activity was demonstrated by the extract obtained from washing the cells with a calcium-free Na-phosphate buffer, suggesting that the release of proteinase is due to autoproteolysis as observed with most lactobacilli and with lactococci (Agyei and Danquah, 2012b; Atlan et al., 1989; Espeche Turbay et al., 2009; Exterkate, 2000; Laan and Konings, 1989).

The proteolytic activities of the different cellular fractions of LDL 313 are shown in Table 2. The enzyme was detected at high levels of ~95% in the lysozyme fluids whilst the relative proteinase activity in the osmotic fluids was only ~5%. No proteinase activities were detected in the cytoplasmic fluids and membrane fractions (Table 2). These results imply that CEPs in LDL 313 are mostly accumulated and bound to the cell wall during biosynthesis and this is corroborated by the structure and attachment of CEP protein domains in *L. delbrueckii* species. The cell wall–anchored protease operational in *L. delbrueckii* species was first isolated and characterized from *L. delbrueckii* subsp. *bulgaricus* and designated PrtB. PrtB is genome encoded by the *prtB* gene which is located downstream of the *lac* operon (Gilbert et al., 1996). The C-terminal region of PrtB (amino acid residues 1743–1938) has up to 25% identity to the C-terminal region of the s-layer protein from *L. acidophilus*, suggesting that PrtB is anchored to the cell envelope in a manner similar to that of s-layer proteins (Pederson et al., 1999). S-layer proteins and proteinases in Gram-positive bacteria have been described as cell-wall bound (in a non-covalent attachment) and consist of a choline-binding domain also called cell wall binding domain of Type 1 (CWBD1) (Desvaux et al., 2006; Siezen, 1999). A schematic view of the structural attachment of CEP in LDL 313 has been proposed in Fig. 2.

3.3. Effect of extraction agents

In lactobacilli, CEPs are responsible for the initial stages of casein breakdown and are situated extracellularly, making harvesting of these enzymes easy and less laborious. An appropriate enzyme extraction method should release significant quantities of cell surface proteins with minimal cell lysis. Methods investigated for CEPs extraction in this work can be categorized under (i) the use of Ca^{2+}-free buffer (ii) the use of detergent and chaotropic agents (iii) the use of enzymes (muramidases).

3.3.1. Ca^{2+}-free 50 mM sodium phosphate buffer

Proteinase extraction with sodium phosphate buffer displayed a mean concentration and specific activity of 13.02 μg ml^{-1} and 37.87 U mg^{-1} respectively as shown in Figs. 3 and 4. SDS PAGE profile shows several visible bands in the range of 25–100 kDa. The LDH assay test confirmed the presence of low levels of LDH (0.64% of total cytoplasmic LDH activity, Table 3), demonstrating that no significant levels of cell lysis occurred with this extraction technique, as observed in other studies (Tsakalidou et al., 1999). Phosphate ions are used as a buffer because there are two protonated forms ($H_2PO_4^-$, and HPO_4^{2-}) in the buffer solution with pKa 7 and 12 respectively. These are relatively weak acids species and their mechanism of extracting CEPs and proteins is due to acid hydrolysis mostly targeting s-layer modified N and O-glycosidic linkages. The modified N and O-glycosidic s-layer linkages are especially more susceptible to hydrolysis than C-glycosyl bonds (Schär-Zammaretti and Ubbink, 2003). Additionally, through diffusion, phosphate buffer solution causes the removal of relatively weakly bound calcium in CEPs and initiates a structural rearrangement in the proteinase domain. This causes an intramolecular autoproteolytic event which truncates the proteinase at the C-terminal end causing the release of the enzyme (Exterkate, 2000; Kunji et al., 1996; Martín-Hernández et al., 1994). Although this method is simple and gives low levels of cell lysis it conduces to a decrease in the specific activity of the proteinase (Exterkate, 2000; Exterkate and Alting, 1999) and this could account for the relatively low specific activity of extract obtained with sodium phosphate buffer (Fig. 4).

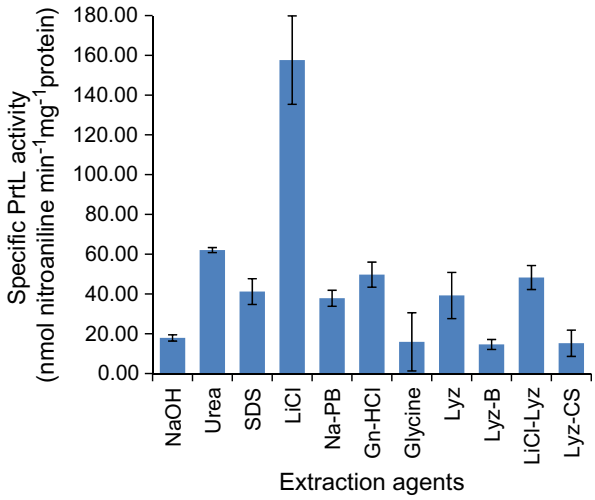

Fig. 4. Specific proteinase activities of cell extracts obtained from different extraction agents. The results represent the analyses of three independent experiments; (SDS: sodium dodecyl sulfate; Na-PB: sodium phosphate buffer; Gn-HCl, guanidine hydrochloride; Lyz: lysozyme; Lyz-B, lysozyme buffer; Lyz-CS: lysozyme-cold shock treatment; see Table 1 for buffer compositions and concentrations).

Table 3
Relative level of cell lyses assessed from LDH analysis.

Extract	Relative level of cell lyses[a]
NaOH	0.06
Urea	0.45
SDS	1.64
LiCl	0.00
Na–PB	0.63
Gn–HCl	0.00
Glycine	1.78
Lyz	27.31
Lyz-B	15.76
LiCl–Lyz	39.28
Lyz–CS	13.08

[a] calculated as the percentage of total LDH activity in each extract; (SDS: sodium dodecyl sulfate; Na–PB: sodium phosphate buffer; Gn–HCl, guanidine hydrochloride; Lyz: lysozyme; Lyz-B, lysozyme buffer; Lyz–CS: lysozyme-cold shock treatment.

3.3.2. Detergent treatment with 10 mM NaOH

From Figs. 3 and 4, NaOH treatment recorded the highest protein concentration of 18.93 μg ml^{-1} but a low mean specific activity of 17.93 U mg^{-1} proteins. The electrophoretic profile of the extract showed a significant release of cell-surface proteins with several of the bands between molecular weights 10 and 100 kDa (Fig. 5). A percentage relative activity of 0.06% was observed from the LDH assay according to Table 3, indicating that cell lysis with 10 mM NaOH was negligible. NaOH is a strong base with a pKa of 15.9 and interacts with the various glycoprotein units in the surface layer. NaOH hydrolyzes the s-layer by two mechanisms: (a) hydrolysis via hydroxide nucleophilic attack of the carbonyl group in the peptide linkages of the glycoproteins of the s-layer monomer unit, or (b) cation substitution of the sodium ion which can disintegrate the s-layer proteins subunits into monomers since they are non-covalently linked to each other and the supporting cell wall (Siezen, 1999). Hydroxyl ions are better nucleophiles than dipoles such as water and this explains the high protein concentration in the extract (Fig. 3) and SDS PAGE image (Fig. 5). The low specific enzyme activity displayed by NaOH treatment could be ascribed to high pH of NaOH, resulting in enzyme denaturation,

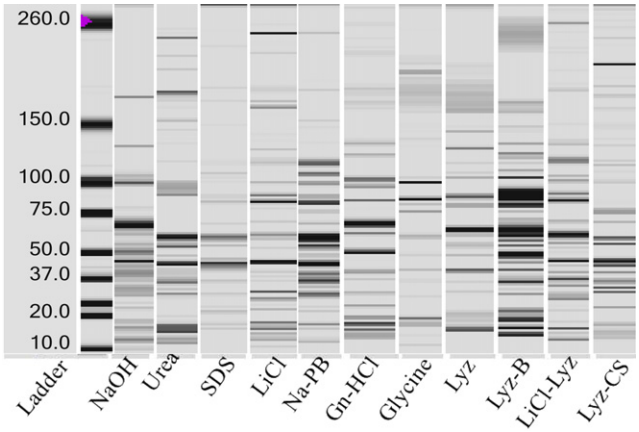

Fig. 5. SDS-PAGE image for cell extracts obtained from the different extraction agents; (SDS: sodium dodecyl sulfate; Na-PB: sodium phosphate buffer; Gn-HCL, guanidine hydrochloride; Lyz: lysozyme; Lyz-B, lysozyme buffer; Lyz-CS: lysozyme-cold shock treatment; see Table 1 for buffer compositions and concentrations).

distortion of enzyme tertiary structure due to possible ionic cleavage and inaccessible active groups; thus inhibiting the activities of CEPs.

3.3.3. Detergent treatment with 0.2% SDS

The use of 0.2% (w/v) SDS produced the lowest protein concentration of 4.93 μg ml^{-1} and a specific mean activity of 1.24 U mg^{-1} according to Figs. 3 and 4. The SDS PAGE results (Fig. 5) produced bands with the highest concentration of proteins within 37–50 kDa molecular weight range. The LDH assay recorded 1.64% of total cytoplasmic LDH activity, showing that the bacterial cells were not lysed significantly. SDS is a denaturing agent and thus capable of disintegrating the s-layer proteins subunits into monomers (Schär-Zammaretti and Ubbink, 2003). As an anionic detergent it denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass (charge density distribution). This causes a breakdown of the cell membrane due to emulsification of surface lipids and proteins, and the disruption of the polar interactions that hold the cell membrane together (Brown and Poon, 2010). In higher concentrations, SDS can be quite a harsh extraction agent, resulting from an increased level of charge distribution, and this can cause significant cell lysis to release intracellular components. However, it was observed that 0.2% SDS was mild and did not result in significant cell lysis. Consequently, it was quite specific in extracting s-layer proteins and CEPs without inhibiting enzyme activities.

3.3.4. Use of lysozyme and modified lysozyme mixtures

Lysozyme (muramidase) hydrolyzes preferentially the β-1,4 glycosidic linkages between the N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) of the peptidoglycan layer. The enzyme lysozyme cleaves the bonds between the peptidoglycan monomers and thus allows the surface proteins embedded in this layer to be released from the cell envelope.

Theoretically, the lysozyme containing buffers were expected to produce one of the highest protein concentrations readings since it attacks the peptidoglycan layer where the CEPs are anchored to the cell wall via the W-domain (Siezen, 1999), resulting in a highly specific extraction mechanism without compromising the structural integrity of the CEPs. However, the extraction performance was not as expected, and this is possibly due to the exclusivity of lysozyme in only disrupting the peptidoglycan layer and not the outer s-layer.

This also explains why relatively fewer bands are seen in the SDS PAGE image (Fig. 5).

The lysozyme buffer contained 0.03 M ammonium bicarbonate, 0.5 M sucrose and 0.3% lysozyme, and had the second highest protein concentration of 15.71 μg ml^{-1} compared to all other extraction agents containing lysozyme (Fig. 3). A low mean specific CEP activity of 14.65 U mg^{-1} protein was recorded (Fig. 4). The SDS PAGE results indicated that there were many dense bands produced with most of them in the molecular weight range of 10–150 kDa (Fig. 5).

The LDH assay test confirmed the presence of lactate dehydrogenase (a percentage relative activity of 15.76%), demonstrating the occurrence of cell lysis (Table 3) for this extraction technique. In comparison with the lysozyme buffer, the other lysozyme-containing extraction agents, namely, lysozyme-LiCl, lysozyme solution and lysozyme with cold shock treatment, recorded low protein yields of 11.73, 7.23 and 6.91 μg ml^{-1} respectively. However, specific proteinase activity was high for lysozyme–LiCl (48.29 U mg^{-1}) and lysozyme solution (39.27 U mg^{-1}) compared with the lysozyme buffer and lysozyme with cold shock treatments.

The difference between lysozyme and lysozyme buffer is the addition of 0.03 M ammonium bicarbonate and 0.5 M sucrose. Ammonium bicarbonate acts as an alkaline buffer to maintain the physiological pH (between 7 and 9) at which the lysozyme enzyme would be most stable. Moreover, the ammonium ion could possibly disrupt the s-layer protein subunits by cation substitution (Schär-Zammaretti and Ubbink, 2003). The coupled action of attacking the s-layer by the ammonium carbonate and lysozyme disrupting the peptidoglycan layer explains the higher protein concentration compared to the pure lysozyme agent. The presence of sucrose may have increased the stabilization of proteins in solution. Whilst the apparent activation energy of the unfolding process is increased by the addition of sucrose, it is preferentially excluded from the protein domain, and thus increasing the free energy of the system. Thermodynamically, this leads to protein stabilization since the unfolded state of the protein becomes thermodynamically less favorable in the presence of sucrose (Lee and Timasheff, 1981). The SDS PAGE image shows lysozyme buffer with many dense bands compared with that for pure lysozyme.

LiCl-lysozyme buffer produced a relatively high specific enzyme activity out of the lysozyme-based solutions. The addition of 0.1 M Tris kept the lysozyme at its optimal physiological pH since the buffer range is between pH 7 and 9 whilst the addition of the salts and metals, NaCl, $MgCl_2$, and LiCl aided in the degradation of the s-layer to release the proteins. The SDS PAGE image shows lysozyme buffer with the densest protein bands (Fig. 5).

The combined effect of the 1M LiCl and lysozyme meant that both the S-layer and phospholipid bilayer would have been disrupted. This dual action conduced into the greatest cell lysis (39.28% of total cytoplasmic LDH activity) in comparison to the other lysozyme extraction techniques (Table 3).

The follow up treatment of cold shock at 4 °C for 10 min for the lysozyme cold shock treatment changes the lipid phase of the cells. The membrane therefore develops hydrophobic holes and this results in enhanced permeability of the cell membrane and release of internal compounds (Tabatabaie and Mortazavi, 2008). Cell treatment with lysozyme will result in the production of protoplasts which can cause lysis under cold shock conditions to release intracellular biomolecules. Consequently, LDH activity assay showed that significant cell lysis was observed (13.08% of total LDH activity).

3.3.5. 5 M LiCl chaotropic agent

The use of 5 M LiCl resulted in a mean protein concentration of 7.47 μg ml^{-1} and the highest proteinase specific activity of 157.59 U mg^{-1} proteins. The SDS PAGE image shows bands that are widely distributed over the entire scale of virtual gel ladder. The highest concentrations of proteins were at 210 kDa, 150 kDa, and between 75 and 10 kDa (Fig. 5). No LDH was detected in LiCl extracts, demonstrating this extraction technique did not cause any detectable level of cell lysis (Table 3). The mechanism of action of LiCl is such that the lithium ions attacks the s-layer protein subunits which are non-covalently linked to each other and disintegrates it into monomers by cation substitution (Schär-Zammaretti and Ubbink, 2003). Thus LiCl extracts s-layer and other cell wall bound proteins from intact cells efficiently and selectively gives the highest specific proteinase activity (157.59 U mg^{-1}). The unique mechanism of LiCl in protein extraction makes it the ideal candidate for the extraction and solubilization of adhesins and other surface components of staphylococci while maintaining cell integrity (Hussain et al., 1999).

3.3.6. 8 M Urea chaotropic agent

As shown in Fig. 3, 8 M urea extract had a protein concentration of 10.86 μg ml^{-1} and recorded the second highest specific proteinase activity of 62.11 U mg^{-1}. The SDS PAGE results showed a widely distributed protein bands over the entire molecular weight ladder scale (Fig. 5). The LDH activity assay test were low (0.45% of total LDH activity) for the urea extracts (Table 3). As a chaotropic agent, urea works through a solvation mechanism and exerts its effect directly via binding to the protein, and/or indirectly by altering the solvent environment (Bennion and Daggett, 2003). Direct urea interactions involve hydrogen bonding to the polar moieties of the protein, particularly peptide groups of the S-layer proteins and peptidoglycan layer. Subsequently, this leads to disruptions of intramolecular hydrogen bonds of the protein. Urea is a strong protein denaturant and it disrupts the non-covalent bonds in the proteins. In comparison with other extraction agents urea is known to releases larger quantities of cell surface-associated proteins without significantly affecting morphological structure or viability of cells (Jarocki et al., 2010).

3.3.7. 4 M Guanidine hydrochloride chaotropic agent

This extraction agent gave a mean protein concentration and mean specific activity of 9.41 μg ml^{-1} and 49.55 U mg^{-1} respectively (Figs. 3 and 4). The SDS PAGE profile produced several peaks concentrated within the 20–100 kDa molecular weight range (Fig. 5). No cell lysis was observed from the LDH activity assay (Table 3). Guanidine–HCl is a chaotropic agent which denatures proteins by disrupting the secondary structure, leading to the formation of random coils which further become displaced by guanidine ions. The guanidinium ion is a strong base with a pKa of 13.6 (Perrin, 1972). Consequently, it is able to disrupt the non-covalent interactions between s-layer monomer units. Moreover, because it is such a strong base, the 4 M solution may also hydrolyze the glycoproteins of the s-layer monomer, peptidoglycan layer and phospholipid bi-layer through hydroxide nucleophilic attack of the carbonyl group in the peptide linkages. However, in this study, it seemed that the 4 M Guanidine–HCl solution was quite mild in its action, producing a relatively high specific activity with no enzyme inhibition.

3.3.8. 0.2 M Glycine treatment

Glycine (0.2 M, pH 2.2) produced a low mean protein concentration of 4.99 μg ml^{-1} and a specific activity of 15.96 U mg^{-1}. The LDH assay test concluded that it did not cause significant cell lysis (1.78% total LDH activity). Glycine is the smallest amino acid with an isoelectric point of 6.06, and at a pH of 2.2 the solution mostly contains the cationic acid ion of glycine (Brown and Poon, 2010). Since it is a polar cationic molecule, it is thought to disrupt the s-layer protein monomer non-covalent interactions. From Fig. 5, the

SDS PAGE image proved glycine to be an extremely mild extracting agent and did not release much protein bands.

3.3.9. Elucidating the potential proteinase from extraction profiles and genomic data

Lactobacillis delbrueckii has three subspecies namely, *lactis*, *bulgaricus*, and *delbrueckii* and all these species are important for the milk industry as fermenting lactic acid bacteria (Germond et al., 2003b). The cell wall-anchored protease (PrtB) responsible for the digestion of casein is genome encoded by the gene *prtB*, and located downstream of the *lac* operon. It has been isolated and characterized from *L. delbrueckii* subsp. *bulgaricus* (Gilbert et al., 1996), sequenced from *L. delbrueckii* subsp. *bulgaricus* strain NCDO1489 and shown to encode a pre-pro-protein of 1946 amino acid residues (Genebank Accession Number L48487) with a predicted molecular weight (MW) of 212 kDa (Gilbert et al., 1996; Sadat-Mekmene et al., 2011; Siezen, 1999).

CEPs are synthesized as inactive pre-pro-protein (Pederson et al., 1999), however, unlike PrtP of *Lactococcus*, the maturation PrtB of *L. delbrueckii* subsp. *bulgaricus* does not require a PrtM-like chaperon (Sadat-Mekmene et al., 2011). Deletion of the last 99 residues of PrtB is sufficient to allow the release of this truncated and active proteinase into the culture medium or extraction buffer (Germond et al., 2003a). The molecular weight (MW) of the remaining 1847 amino acids residues of the active proteinase is estimated to be 200.6 kDa with the aid of the ExPASy-ProtParam tool (Wilkins et al., 1999). In this study, a protein band of MW around 210 kDa was observed for Urea, Lyz–LiCl and especially LiCl extracts and this band could potentially be the active proteinase of LDL 313. However, the crude CEP needs to be further purified and characterized to confirm this predicted molecular weight, and this is a subject of our future work.

4. Conclusion

It is observed from the study that cell-envelope proteinases (CEPs) in *L. delbrueckii* subsp. lactis 313 (LDL 313) are cell-wall anchored. Also, the potency of the specific extraction methods significantly differed for the same aliquots of suspended cells. Further, although the methods investigated are generally reported as suitable for the extraction of CEPs from other lactobacillus species, it is evident from the studies that not all of them are suitable for *L. delbrueckii* subsp. lactis 313 (LDL 313). All the extraction agents were effective in releasing cellular proteins (including CEPs) from LDL 313. The use of protein concentration and enzymatic activity tests is not ideal for selecting the most suitable CEPs extraction agent since the extraction agents are not explicit to surface enzymes only. Hence specific enzymatic activity and cell lysis were used to assess the effectiveness of the extraction techniques toward CEPs extraction. The efficiency of each extraction agent varied and the most suitable extraction agent for LDL 313 was identified as 5 M LiCl, as determined by the high specific activity of extracts and low cell lysis. The screening model employed in this work presents an effective bioanalytical tool essential to optimize the extraction process for obtaining high concentration titers of CEPs from different cellular systems for subsequent protein purification steps.

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PART II: DEVELOPMENT AND ESTABLISHMENT OF STABLE BIOCATALYSTS FROM CELL-ENVELOPE PROTEINASES

5. CHAPTER 5: Covalent Immobilization of Cell-Envelope Proteinases

Section 5.1

5.1. Quick and low cost immobilization of proteinases on polyesters: comparison of lactobacilli cell-envelope proteinase and trypsin for protein degradation

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Monash University
Declaration for Thesis Chapter 5 Section 1

Declaration by candidate

In the case of Chapter 5 Section 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Key ideas, Experimental Development, Results interpretations, Writing up	65

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Shaun Tambimuttu	Experimental, Development, Results interpretations,	10
Bhuvana Kasargod	Experimental, Development, Results interpretations, Writing up	5
Dr. Yuan Gao	Key ideas and Writing up	
Dr. Lizhong He	Initiation, Key ideas, Results interpretations, Writing up	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12 December 2013
Main Supervisor's Signature	[redacted]	**Date** 12 December 2013

Quick and low cost immobilization of proteinases on polyesters: comparison of lactobacilli cell-envelope proteinase and trypsin for protein degradation

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5.2. Abstract

Cell-envelope proteinases (CEPs) are a class of proteolytic enzymes produced by lactic acid bacteria and these enzymes have several industrially relevant applications. However, soluble CEP-based processes are economically unfeasible due to their poor stability and lack of multiple utility. In a quest to prepare stable biocatalysts with improved optimum performance, CEPs and trypsin (as a model enzyme) were immobilized onto nonwoven polyester fabric in a three-step activation protocol including ethylenediamine activation and glutaraldehyde cross-linking. Immobilization gave protein loading yields of 21.9% (CEP) and 67.7% (trypsin) while residual activity yields of 85.6% (CEP) and 4.1% (trypsin) were observed. There was a strong dependence of immobilized enzyme activity on pH, and activity was retained at elevated temperatures (40 - 70 °C). An increase in K_m values was observed for both enzymes after immobilization. Immobilized CEP retained ~ 62% (lyophilized samples) and 96% (stored in buffer at 4 °C) of initial activity after 70 days of storage. Both immobilized enzymes were able to hydrolyze proteins such as casein, skimmed milk proteins and bovine serum albumin. This immobilization protocol is cheap and simple and has the potential for use in various protein degradation processes.

Keywords: Enzyme immobilization; cell-envelope proteinase; Lactobacilli; polyester fabric; protein degradation

5.3. Introduction

Enzymes are a ubiquitous and industrially relevant class of biomolecules used in the 'green' catalyses of diverse reactions with high activity and high degree of selectivity. However, the use of some soluble enzymes is often plagued by major limitations such as high costs and lack of multiple utility, hampering their implementation at industrial scale (Mateo et al., 2007; Opwis et al., 2007). Additionally, Food and Agriculture Organization (FAO) regulations stipulate that enzyme preparations used in the production of some consumables (such as food or pharmaceuticals) must be removed from products after processing (FAO/WHO, 2006). This is difficult to do, if not impossible, for soluble enzymes. These challenges have therefore made the use of immobilized enzymes an attractive alternative.

Enzyme immobilization has been shown to enhance enzyme activity, modify substrate selectivity and enantioselectivity, and allow single-pot multi-enzyme reactions (Brady and Jordaan, 2009; Mateo et al., 2007; Sheldon, 2007). The economical feasibility of enzymes has been improved significantly as a result of advancement in biotechnological immobilization processes that 'modify' and increase enzyme utility and pliability. Further, biocatalyst process economics largely depend on enzyme immobilization which promotes improvements in enzyme stability and reusability while enhancing flexibility in reactor design and product recovery without catalyst contamination of final product (Brady and Jordaan, 2009; Opwis, 2010; Sen et al., 2012). Among the four immobilization strategies (namely adsorption, entrapment, crosslinking, and covalent binding) covalent binding of enzymes is particularly attractive because it gives the strongest bonds and provides stable biocatalysts (Zhao et al., 2013a; Zhao et al., 2013b).

Immobilization of enzymes on fabric support has several advantages over the use of bead-based or gel-sol supports. Textile fabrics made of natural and synthetic fibres like cotton, polyamide or polyester have special properties such as flexibility and high surface area that makes them appropriate support materials for enzyme immobilization. The flexible, woven structure and open construction of fabrics present low mass transfer resistance while allowing high substrate throughput and turn-over of products (Freitas et al., 2011; Opwis et al., 2007). Additionally, whereas the use of immobilized enzymes on bead-based supports in reactors often require a filtration step, fabrics on the other hand can be quickly removed leaving the reaction mixture with no enzyme residues (Opwis, 2010). Polyester [poly(ethylene terephthalate), PET], is one of the most important and widely used synthetic fibers in the textile industry. Its structure can be described as partially intertwined mass of fibres adhering together and forming a flat fabric sheet which provides a rigid structure and mechanical tightness (Nouaimi-Bachmann et al., 2007; Nouaimi et al., 2001). PET fibers are cheap, have good chemical stability against some acids and oxidizing agents, and also have good resistance to sunlight and microbial attack (Yiğitoğlu and Temoçin, 2010). These make PET an excellent low-cost support for enzyme immobilization.

Cell-envelope proteinases (CEPs) are a class of proteolytic enzymes produced by lactic acid bacteria and these enzymes have several industrially relevant applications (Agyei and Danquah, 2012b; Sadat-Mekmene et al., 2011; Tsakalidou et al., 1999). They are known to improve the texture and organoleptic characteristics of dairy products (Espeche Turbay et al., 2009) and have also been shown to hydrolyze proteins releasing bioactive peptides with numerous biopharmaceutical and health applications beyond basic nutrition (Danquah and

Agyei, 2012; Korhonen, 2009; Korhonen and Pihlanto, 2006). The use of free CEPs for the production of bioactive peptides is currently suboptimal and presents further drawbacks such as poor stability (Exterkate, 2000) and lack of recycling. Also, to the best of our knowledge, no report exists in the literature on the preparation of stable forms of CEPs. Thus, studies on the design; development and establishment of stable CEPs conjugates are needed to expand their technological application in various protein degradation processes. CEPs of *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) were chosen for this study because LDL 313 is a useful lactobacillus with application in the food industry (Vogel and Ehrmann, 2008), but CEPs from this species have not been extensively studied (Agyei and Danquah, 2012b). Consequently, preparation and application of stable CEP biocatalysts from this species have also not been studied. Reported in this study is a simple, cheap and quick strategy for the immobilization of enzymes onto polyester fabric via support functionalization with ethylenediamine and cross-linking with glutaraldehyde. This three-step process was carried out on CEP of LDL 313and also on trypsin (as a model of conventional proteinase). These two immobilized proteinases were compared for their protein hydrolysis performance using casein, skimmed milk proteins and bovine serum albumin.

5.4. Materials and Methods

Materials

Nonwoven polyester material [poly(ethylene terephthalate)] (PET), produced at Materials Science and Engineering Division, Commonwealth Scientific and Industrial Research Organization (CSIRO), was used in this study. Ethylenediamine (99%), grade 1 glutaraldehyde solution (50%), and succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide (s-ala), porcine pancreas trypsin, bovine serum albumin (BSA) (98%), casein from bovine milk (technical grade) and Brilliant Blue G (90%) were all obtained from Sigma-Aldrich (Castle Hill, Australia). Skimmed milk proteins were obtained from Murray Goulburn (Melbourne, Australia). Protein assay kit and 4-15% Mini Protean TGX precast polyacrylamide gels were obtained from Bio-Rad Laboratories (Richmond, CA).

Cell biomass and preparation of crude cell envelope extract

Cell biomass and preparation of crude cell envelope extract is as described (Agyei and Danquah, 2012a). *L. delbrueckii* subsp. *lactis* 313 (ATCC® 7830™; LDL 313) was obtained from American Type Culture Collection (ATCC), propagated twice in deMan-Rogosa-Sharpe (MRS) Broth (Oxoid Pty Ltd, Australia) at 37 °C and stored at -70 °C. Frozen cells were thawed, plated, and cultured in fresh MRS Broth at 37 °C for 12 h, and aliquot taken to inoculate fresh MRS medium (Acumedia) to an initial optical density (OD_{560}) of 0.1 at 560 nm. Cells were allowed to grow (shake flask, 45 °C, 100 rpm), to late exponential phase (OD_{560} = 1.117). This OD corresponds to about 229 μg ml^{-1} dry cell weight, from a previously prepared calibration curve for converting OD 560 nm units to dry cell weight units. Cells were harvested by centrifugation (4,000×g, 4 °C, 10 min) and concentrated to 5 times the initial

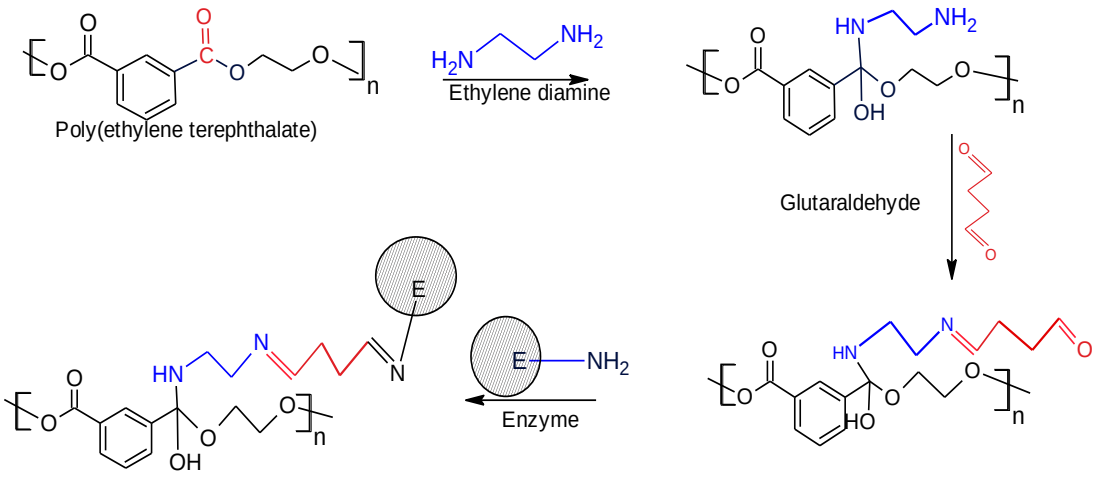
OD_{560} in the extraction solution containing 50 mM sodium phosphate buffer (pH 7). This system was incubated (28 °C, 2 h, 50 rpm) after which the setup was centrifuged (4,000×g, 4 °C, 10 min). The supernatant was designated crude cell-envelope proteinase (CEP). Protein concentration was determined with a Protein Assay Kit (Bio-Rad Laboratories).

Immobilization of enzymes on polyester, optimization of immobilization parameters and determination of kinetic parameters

Immobilization of enzymes on polyesters was based on the method previously described (Gao et al., 2013), and the experimental conditions were optimized for cell-envelope proteinases in this work. Briefly, polyester [poly(ethylene terephthalate)] fabrics were cut into circular pieces (1.0 cm diameter and ~ 2.5 mg each with a puncher. The polyester fabric was treated with 99% EDA (at a fabric liquor ratio of 3 discs to 1 mL) and incubated (60 °C, 1 h, 100 rpm on a shaker). The EDA activated fabrics were washed intensely with Mill-Q water (1 fabric to 5 mL water) three times via shaking (100 rpm, 10 min) each time, then reacted with 5% v/v glutaraldehyde (GA) in phosphate buffer under incubation (21 °C, 100 rpm, 2 h) at a fabric liquor ratio of 3 discs to 1 mL. The treated fabrics were then washed again intensely with Mill-Q water. The activated fabrics were treated with enzymes (15 mL of 1 mg ml^{-1} trypsin or 10 mL of 25 μg ml^{-1} crude CEP) via incubation overnight (21 °C, 100 rpm). Fabrics with immobilized enzymes were then washed three times via shaking (100 rpm, 10 min each) in buffer (50 mM sodium phosphate buffer, 500 mM NaCl, pH 7.0) at a fabric liquor ratio of 1 fabric to in 5 mL. These wash steps was able to remove most free enzymes as detected by $A_{280\ nm}$ measurements. Unreacted carbonyl groups that may have been present on polyester fabrics were quenched by treating fabric with 1 M Tris-HCl pH 7.6 (100 rpm, 30 min, 21 °C). The treated fabrics were then washed again thrice via shaking (100 rpm, 10 min each) in buffer (50

mM Na-phosphate buffer, 500 mM NaCl, pH 7.0) at a fabric liquor ratio of 1 discs to in 5 mL, with a change of buffer for each step. Finally, they were rinsed with fresh buffer (50 mM Na-phosphate buffer, pH 7.0).

The immobilization mechanism in this study involves amine functionalization of polyester followed by conjugation of enzyme through glutaraldehyde cross-linking. Alkyl amines react with polyester in a nucleophilic substitution of basic amine-group into the ester carbonyl. Glutaraldehyde, a *bis*-aldehyde homobifunctional cross-linker is then made to reacts via a nucleophilic attack of the exposed primary amine groups on the polyester–amine conjugate resulting in a Schiff base and leaving terminal carbonyl groups which are coupled to amine groups in the enzyme molecule by nucleophilic substitution (See Scheme 1). The resulting product is a strong covalently bonded enzyme molecule on polyester fabric.



Scheme 1: Schematic of reaction mechanism for the immobilization of enzyme on polyester via ethylene diamine linker and glutaraldehyde cross-linker

Enzyme immobilization parameters were optimized for concentration and temperature of EDA functionalization conditions; GA concentration (2, 5, and 10 % v/v in Na-phosphate buffer, pH 7.0); immobilization temperature (4°C and 20 °C); immobilization pH of enzymes (6.0, 7.0, 7.5, and 8.0); and effect of proteic spacer molecules (i.e. BSA). To test the effect of BSA as a spacer molecule, the procedure described above was followed with slight modification. After GA cross-linking step, the setup was made to react with 1 mg ml^{-1} of BSA in Na-phosphate buffer, pH 7.0 overnight, followed by another GA reaction step before trypsin enzyme was coupled. For EDA optimization, polyester fabrics were placed in 24-well plates and reacted with EDA at various concentrations (99% and 50%), temperature (20 °C, 40 °C and 60 °C) and incubation time (between 15 to 120 min). This was followed by washing two times (10 min, 100 rpm shaking) at 2 discs per 50 mL deionized water). After this, about 0.5 mL of 0.2% of the food dye, E 124 (Ponceau 4R or trisodium (8Z)-7-oxo-8-[(4-sulfonatonaphthalen-1-yl)hydrazinylidene]naphthalene-1,3-disulfonate) was added to fabric and incubated (room temperature, 5 min); after which dye was decanted off and fabrics washed with destaining buffer (40% acetic acid, 20% ethanol, 40% water) and with copious amount of water until supernatant was clear. The fabrics were then visualized and images taken. Due to the nature of the polyester fabric used (i.e. its flexible and open construction) the immobilized enzymes is expected to be thoroughly loaded into the fabric.

The kinetic parameters of the Michaelis–Menten equation for the free and immobilized enzymes were determined by measuring the initial rates of s-ala (0.25–20 mM) hydrolysis at 50 °C for trypsin and 30 °C for CEP and pH 7.0. The kinetic constants (K_m and V_{max}) were

obtained by fitting the data to the Michaelis–Menten equation using Auto2Fit, (Professional Version 5.5, 7D-Soft High Technology Inc).

Enzyme activity assay on chromogenic substrate

Enzyme activity of free and immobilized enzymes was measured (in duplicate for each assay) according to the method of Agyei & Danquah (2012b) with some modifications. Buffered enzyme solution (100 μL for crude CEP and 10 μL for trypsin) and immobilized enzyme on fabric were placed in a 24-well plate (1 piece of fabric per well), in an incubator shaker and equilibrate to 40 °C. It was observed that, during agitation, the enzyme-fabric complex did not sit at the bottom of the plate but was rather suspended within the reaction mixture. To this setup, 450 μL of buffered peptide (50 mM sodium phosphate buffer, pH 7.0; 37.5 mM imidazole; and 1 mM of the peptide succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide) was added to initiate reaction. The reaction was terminated either by the removal of immobilized enzyme-fabric with a clean forceps or by the addition of 100 μL of 10% (w/v) trichloroacetic acid (in case of soluble enzyme assay) and centrifuged (10,000×g, 5min, 25 °C). The release of *p*-nitroaniline was followed on a spectrophotometer at 410 nm (using the molar extinction coefficient ε, = 8800 M^{-1} cm^{-1}). One unit of proteinase was defined as the amount of enzymes required to liberate 1 μmol of nitroaniline per minute at 40 °C, pH 7.0.

Reusability and storage stability of immobilized enzymes

The reusability test was performed as follows: fabrics with immobilized enzymes were recovered from the incubation mixture, washed three times via shaking (100 rpm, 10 min each) in buffer (50 mM Na-phosphate buffer, 500 mM NaCl, pH 7.0) and made ready for the next assay after a final rinsing step with fresh buffer (50 mM Na-phosphate buffer, pH 7.0).

The storage stability of immobilized enzymes was estimated in dry ambient conditions (by air-drying and storage at room temperature), wet surroundings (by storage under 50 mM Na-phosphate buffer, pH 7.0) and freeze-dried conditions.

Enzyme immobilization efficiency

The efficiency of immobilized enzymes was evaluated in terms of retained activity (%) as follows:

$$\text{Retained activity (\%)} = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of free enzyme introduced}} \times 100\% \quad [1]$$

$$\text{Protein Loading yield (\%)} = \frac{\text{amount of protein loaded on support}}{\text{total amount of protein introduced}} \times 100\% \quad [2]$$

Estimation of optimum temperature and pH of immobilized enzymes

To study the effect of pH on the free and immobilized enzyme, the temperature of the reaction medium was maintained at 40 °C and the pH of the reaction medium varied by employing different buffer solutions (50 mM Na-acetate buffer for pH 4-5; 50 mM Na-phosphate buffer for pH 6-7.6; 50 mM Tris-HCl for pH 8.2-12.1). To study the effect of temperature, the pH of the reaction medium was maintained at 7.0 in 50 mM Na-phosphate buffer, and the temperature varied from 20 to 70 °C. The relative activities of free and immobilized enzymes were normalized to their highest activities within the pH or temperature ranges.

Effect of immobilization on thermodynamic parameters

Thermodynamic parameters were determined as described by Aryee & Simpson (2012). Data obtained from the optimum temperature estimation experiment were also used to determine the apparent Arrhenius energy of activation (E_a). E_a was estimated from the slope of the plot of log of reaction rate (k) versus the reciprocal of absolute temperature (T) in Kelvin according to Eq. [3], where A is the Arrhenius frequency factor and R is the ideal gas constant (8.314 J mol^{-1} K).

$$\ln k = \left(\frac{-E_a}{R}\right)\frac{1}{T} + \ln A \qquad [3]$$

Enthalpy of activation (ΔH^*), at the optimum assay temperatures of 30 °C (for CEP) and 50 °C for trypsin, were calculated using Eq. [4].

$$\Delta H^* = E_a - RT \qquad [4]$$

Hydrolysis of proteins by immobilized enzymes and peptide assay via o-phthaldialdehyde (OPA) reaction

In a 24-well plate, two pieces of fabric containing either immobilized trypsin or immobilized CEP (typically 0.33 U mL^{-1} and 0.006 U mL^{-1} respectively, by enzyme activity assay in section 2.4) were immersed in 2 mL of protein solution each, namely, 4 mg ml^{-1} each of BSA, casein and skimmed milk protein prepared in 50 mM Na-phosphate buffer pH 7.0. This was incubated (40 °C, 150 rpm) and peptides generated monitored with the o-phthaldialdehyde assay and SDS PAGE. The OPA (o-phtaldialdehyde) reaction was used to estimate proteolysis macromolecular protein substrates by immobilized enzymes via the measurement of free amino acids and peptides containing primary amino acids. OPA reagent was prepared

according to (Nielsen et al., 2001) and the OPA method used was according to (Alhaj et al., 2010) with slight modification. About 5 μL of unhydrolysed protein or protein hydrolyzed by immobilized enzyme were added to 50 μL of OPA reagent containing dithiothreitol (DTT), followed by 150 μL of deionized water was added. This was mixed briefly and left to stand at room temperature (21 °C) for 10 min. The absorbance of the solution was measured at 340 nm. The absorbance readings were discounted from the initial reading of unhydrolysed proteins. The degree of hydrolysis for each sample was determined according to the following equation:

$$\% \text{ Degree of Hydrolysis} = \frac{(S-C)}{D} \times 100 \quad [5]$$

where C is the reading for unhydrolysed protein (control), S is the sample reading of the protein hydrolysate formed by immobilized enzyme (samples), and D the difference between the sample reading after 48 h (taken as 100% degree of hydrolysis) and the unhydrolysed protein reading taken as 0%.

Protein hydrolysis estimation by SDS-polyacrylamide gel electrophoresis

Aliquots of protein samples described above were mixed in a 1:1 ratio with solubilisation buffer (Kojic et al., 1991), heated for 5 min at 100 °C, and analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (4-15% Mini Protean TGX Precast acrylamide gels) according to the standard method of Laemmli (1970) and stained with Brilliant Blue G.

Morphological studies by SEM

Scanning electron microscopy (SEM) images of fabrics were made using a Nova™ NanoSEM 430, FEI. Fabric samples were coated with Au.

Statistical analyses

Data were analyzed by Fisher's least significant difference (Statsgraphics Centurion version XV). Significant differences were declared at $p \leq 0.05$.

5.5. Results and Discussions

The preparation of a successfully immobilized enzyme depends on the support as well as on the immobilization mechanism and conditions used. Immobilization parameters such as temperature, pH, use of cross-linkers and spacer molecules are known to have effects on the resulting biocatalytic activity of immobilized enzymes (Mateo et al., 2007). In this study, enzyme immobilization yield was affected by ethylenediamine activation conditions, glutaraldehyde concentration, and immobilization temperature and pH.

Ethylenediamine activation conditions

To elucidate the optimum reaction conditions of ethylenediamine (EDA), polyester was treated with EDA at different concentration (v/v), temperature (°C) and time (minutes). The basic amino groups chemically attached onto PET fabric were then reacted with the food acidic dye, Ponceau 4R (E 124), which is a disodium salt of sulfonic acid. This acidic dye reacts in a neutralization reaction with the basic -NH_2 groups on EDA-treated polyester leaving off the characteristic red color. The intensity of the red color is a direct function of the amount of amino groups deposited on polyester by EDA. The results for this simple qualitative test are shown in Figure 1. It can be observed that all three conditions - concentration, temperature and reaction time, at higher values, improved EDA activation of polyester. However, extensive hydrolysis was observed for polyester fabric treated with 99% EDA at 60 °C for 120 min where the fabric disintegrated into a slurry thus losing all the mechanical integrity. Also, for PET fabric treated at 60 °C for 60 min, mechanical strength was lost and fabric was split into fibers during repeated washing in the subsequent enzyme immobilization steps. The optimum conditions for EDA activation were 99% v/v EDA at 40

°C for 120 min or 99% v/v EDA at 60 °C for 60 min. At these conditions sufficient amount of EDA are deposited on PET fabric without compromising fabric strength and mechanical properties.

Figure 1: Optimization of ethylenediamine (EDA) binding conditions: time, concentration and temperature. FP, fresh untreated polyester. The intensity of the red color reflects the amount of amino groups deposited on polyester by EDA

Glutaraldehyde concentration

Among others, glutaraldehyde (GA) remains a cheap and very versatile agent for protein cross-linking. In solution, GA exists in both monomeric and polymeric forms in equilibrium. Although the main reactive species that participates in the cross-linking process is unknown, it has been shown that GA mainly reacts with ε-amino groups, followed by α-amino, guanidinyl, secondary amino, and hydroxyl groups at near neutral pH (Migneault et al., 2004; Ortiz-Soto et al., 2009). Trypsin contains 14 lysine residues (Villalonga et al., 2000) and the ε-amino groups on these lysine molecules can react with GA to establish a multi-point covalent

enzyme-support attachment. However, it is important to optimize GA reaction conditions because some enzymes are inactivated by GA cross-linking. Optimization is also needed in order to avoid excessive cross-linking which can results in enzyme rigidification and compromise immobilized enzyme activity. In this study, GA concentration had significant effect on enzyme immobilization ($p = 0.0007$). The optimum GA concentration for enzyme immobilization was 5% vol/vol. The use of GA at low concentration resulted in insufficient cross-linking giving low levels of enzyme immobilization. On the other hand, if used at high concentrations, GA may deactivate the immobilized enzyme, leading to lower immobilized activity. Further, at high concentrations, GA gives rise to excessive and unselective cross-linking, which may affect the correct structural conformations necessary for substrate-active site interaction (Feng et al., 2013). Many authors have reported the activity loss of immobilized enzymes upon increasing GA concentration, including trypsin (Chui and Wan, 1997), lipase (Wu et al., 2006), levansucrase (Ortiz-Soto et al., 2009) and glucose isomerase (Stanley et al., 1976).

Immobilization temperature

Temperature also had significant effect on enzyme immobilization yield ($p = 0.0220$). Enzyme immobilization has been known to be conducted at cool temperature to avoid loss of enzyme activity. However, in this experiment, it was observed that immobilized enzyme activity is higher when immobilization is done at room temperature (20 °C). Rate of molecular vibration and overall enzyme–support reaction rate is enhanced at moderately high temperatures. Immobilization at higher temperature was only better at 5% GA and not at 10% concentration and this could be due to GA inhibition of the enzyme at higher concentrations or the

possibility of excessive cross-linking which results in enzyme rigidification and inaccessibility of substrate to the catalytic active sites. These results are reliable because in a parallel control experiment where soluble trypsin was incubated at the two temperatures, it was observed that the loss of enzyme activity for soluble trypsin at 20 °C was statistically same as activity loss for trypsin stored at 4 °C. Statistical analysis showed that there was correlation between glutaraldehyde concentration and immobilization temperature (results not shown). This interaction can be attributed to changes in the cross-linking reaction mechanism of GA at higher temperatures and higher concentration (Migneault et al., 2004). Optimum values of GA concentration and immobilization reaction temperature were 5% v/v and 20 °C respectively (Figure 2) and these were chosen for subsequent immobilization procedures.

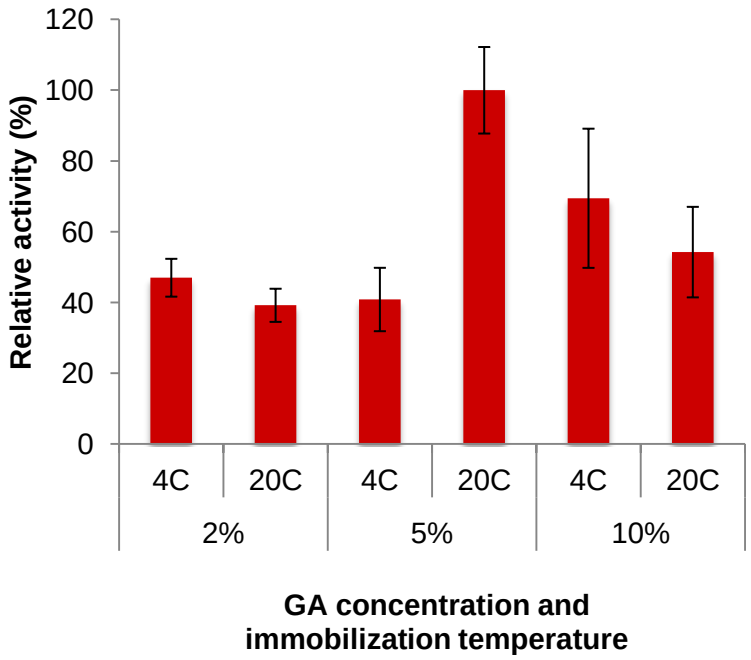


Figure 2: Effect of glutaraldehyde concentration (% v/v) and immobilization temperature (°C) on trypsin immobilization yield. Mean of n = 4 experiments. Amount of soluble trypsin loaded = 0.1 mg.

Immobilization pH

Enzyme immobilization is usually conducted at neutral pH. However, it has been suggested that enzyme incubation at alkaline pH improves enzyme-support reactions because higher pH improves the reactivity of the nucleophilic groups such as lysine in the protein molecule (Mateo et al., 2007). However, in this study, the optimum immobilization pH was observed to be pH 7.0 and immobilization efficiency decline at pH values higher than 7.0 (Figure 3). Experiments aimed at estimating the effect of pH on immobilization efficiency did not cover pH range lower than 6.0. This is because this immobilization method relies on the formation of Schiff bases and Schiff bases have been known to be sensitive to acidic pH below 5 (Migneault et al., 2004).

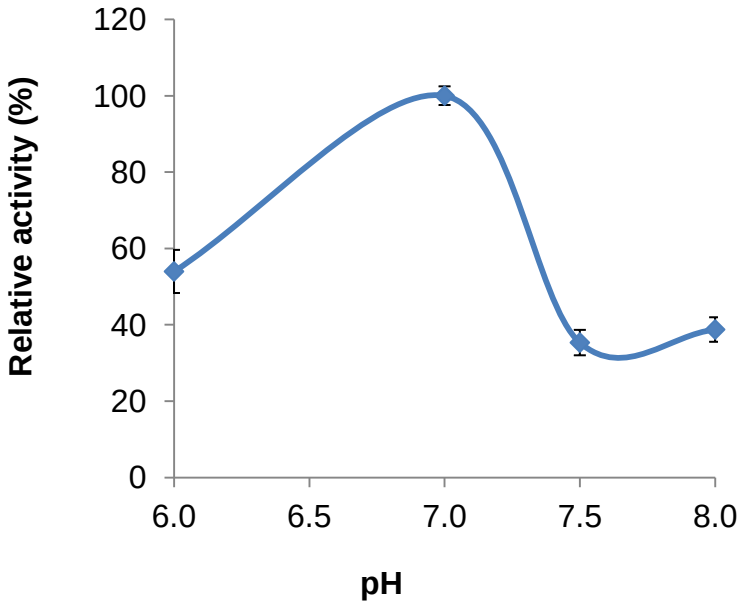


Figure 3: Effect of pH on trypsin immobilization yield. Mean of n = 4 experiments. Amount of soluble trypsin loaded = 1 mg.

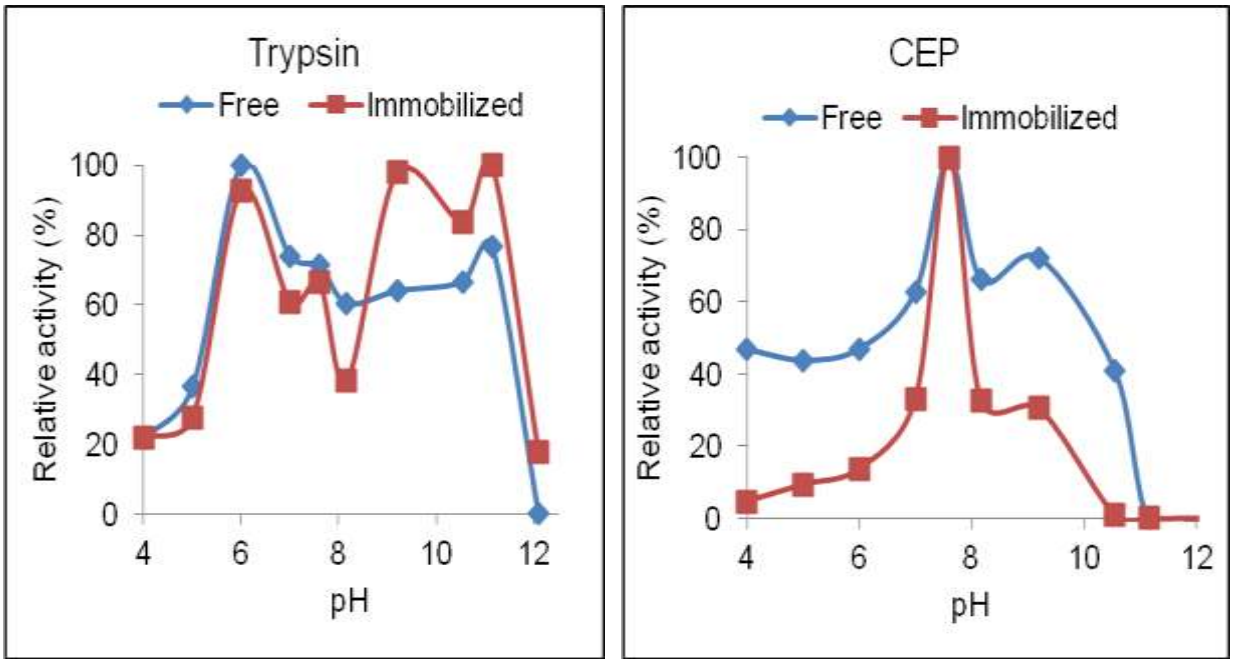
Use of spacer molecules

Literature has shown that direct fixation of trypsin onto polyester fabric without the use of spacer molecules could give rise to relatively low immobilization yields and poor storage stability (Nouaimi et al., 2001; Wang and Hsieh, 2004). This phenomenon has been attributed to steric hindrance and environmental effects encountered during direct contact of enzymes with the rather less polar polyester surface and longer or large globular spacer molecules have been shown to efficiently improve and maintain immobilized enzyme activity (Purcena et al., 2009; Wang and Hsieh, 2004). However, in this study, the use of BSA as a bulky spacer did not have any positive effect on immobilization yield (results not shown). Polyester activation in this study was achieved by the use of ethylenediamine whereas in most other studies (Nouaimi et al., (2001), for example) N,N-dicyclohexylcarbodiimide and N-hydroxysuccinimide (NHS) were used to activate the polyester fleece. The different effects of BSA linker observed in this study and that of Nouaimi et al., (2001) could be attributed to the differences in activation conditions of polyester fabric. In this experiment, the reaction of polymer with EDA created a short spacer which may have been important for crosslinking efficiency.

Optimum pH for proteinase activity

The stability of most enzymes has been known to be affected by changes in pH. The pH-dependent activity profiles of both free and immobilized enzymes are shown in Figure 4. Overall, both the immobilized and free enzyme forms of CEP exhibited a very similar pH profile with an optimum activity at pH 7.6. This implies that immobilization of CEP on polyester did not significantly alter the enzyme's pH profile. On the other hand, although free and immobilized trypsin showed a maximum activity at pH 6.0, the immobilized trypsin

showed higher stability at basic pH retaining > 86% of activity between pH 9 – 11. The change in pH profile of free and immobilized trypsin could be attributed to the effect of surface properties and residual charges on the solid polymer and the local pH value in the immediate vicinity of the enzyme active site (David et al., 2006; Purcena et al., 2009; Yiğitoğlu and Temoçin, 2010).



Figures 4: pH profiles of free and immobilized enzymes; trypsin and CEP

The treatment of most synthetic polymers (such as polyester and polyaniline) with GA leaves the polymers in the reduced state. In this state, as pH increases in the bulk solution, groups on the polymer surface become ionized and maintain an unchanged pH in the vicinity of the enzyme (Purcena et al., 2009). This could explain why immobilized trypsin was active even at

higher pH. The gradual decrease in trypsin activity between pH 6 and 8 is a phenomenon that has been observed by other authors and could be a property characteristic of trypsin within that pH region (Purcena et al., 2009). Additionally, the type of buffer used for the experiment may have had an effect on the observed activity since each reaction medium pH was attained by employing a different buffer solution: 50 mM Na-acetate buffer for pH 4-5; 50 mM Na-phosphate buffer for pH 6-7.6; 50 mM Tris-HCl for pH 8.2-12.1. For CEP on the other hand, the free enzyme observed higher pH activity than immobilized forms over the entire pH rang tested (Figure 4). Immobilization may have caused a conformational change of the enzyme making it sensitive to pH alterations in the bulk liquid environment.

Optimum temperature

The effect of temperature on both free and immobilized enzyme activity was determined by measuring the hydrolysis of s-ala at temperatures ranging from 20 to 70 °C. As shown in Figure 5, the temperature profiles of immobilized trypsin or immobilized CEP differed from the free enzymes; thus immobilization on polyester significantly altered the enzymes' temperature dependent activity. Free trypsin observed an optimum temperature of 50 °C beyond which enzyme activity decreased. On the other, immobilized trypsin was still active at temperatures beyond 50 °C and remained active even at 70 °C. CEP on the other hand did not observe any characteristic shift in the optimum temperature of 30 °C after immobilization of the enzyme. However, immobilized CEP was stable at high temperatures between 30 – 70 °C. This demonstrates high immobilized enzyme stability at elevated temperatures, for both immobilized trypsin and immobilized CEP.

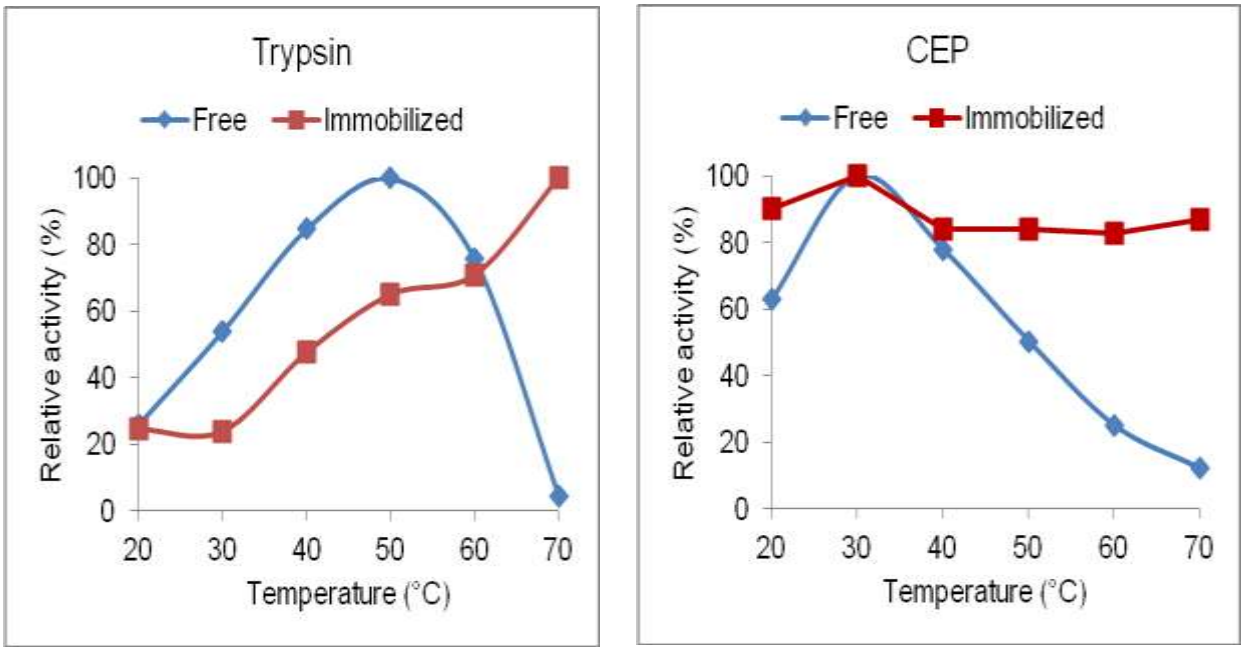


Figure 5: Effect of temperature on free and immobilized enzymes; trypsin and CEP

Changes in temperature dependent activity profile of immobilized enzymes have been well reported in literature and the observed thermal stability of both immobilized enzymes can be attributed to several factors. First, immobilization onto polyester may have segregated the individual enzyme molecules thus minimizing enzyme aggregation and its effects (Zhao et al., 2013a). Any form of aggregation in the soluble forms of both trypsin and CEP can render both enzymes susceptible to auto-degradation, especially at higher temperatures (Agyei et al., 2013; Purcena et al., 2009). Secondly, there are multiple lysine residues in each enzyme molecule; trypsin has 14 lysine groups, and this favours the formation of multiple covalent bonds which achieves some form of enzyme rigidification and activity retention, even at elevated temperatures (Mateo et al., 2007). Additionally, the random nature of enzyme conjugation unto the polyester may have resulted in some positive conformational changes

which makes the enzymes able to resist distorting conditions making them less affected by heat (Zhao et al., 2013b).

Comparison of enzyme kinetics properties

Immobilization resulted in an increase in Km values for both CEP and trypsin, indicating that substrate affinity differed for the free and immobilized forms of both enzymes. This difference can be due to several effects such as conformational changes, steric effects and increased diffusion limitation. During immobilization, multipoint covalent attachment can alter the three-dimensional active conformation of the enzyme's active site thereby affecting substrate affinity. In most cases, enzymes immobilized by covalent bonding would show a decrease in substrate affinity due to conformation changes (Migneault et al., 2004; David et al., 2006; Yiğitoğlu and Temoçin, 2010). A second reason that can be proposed for the reduced substrate affinity is steric effects. Steric effects are caused by molecular crowding and protein-protein interaction that inhibit the flexible stretching of enzyme as enzymes get loaded on the support (Zhang et al., 2013). The use of casein, which is itself is a relative large substrate (MW between 19-25 kDa), can also further increase the levels of intermolecular steric effects. Immobilization could also result in increased diffusion limitation which causes lower accessibility of substrate to enzyme (Yiğitoğlu and Temoçin, 2010). These may explain why the Km of immobilized trypsin is increased about 3 times that of the free enzyme and Km of CEP increased by about 18 times that of free enzyme (Table 1).

Table 1: Comparison of the kinetic properties of free and immobilized enzymes using the Michaelis–Menten equation

	Biocatalyst	V_{max}(mM)	K_m(mM)
Trypsin	Soluble	296.8 ± 6.1	1.8 ± 0.0
	Immobilized	1.0 ± 0.0	5.0 ± 0.1
CEP	Soluble	0.4 ± 0.0	2.5 ± 0.3
	Immobilized	0.7 ± 0.1	46.0 ± 7.2

Values are the mean ± standard deviation of three independent experiments; [#] Fit of mean values; CEP, cell-envelope proteinases

Thermodynamic parameters of enzymes

Arrhenius plots of data obtained from Figure 5 showed a linear relationship within the range of 20 – 50 °C and 20 – 30 °C for free trypsin and free CEP respectively; and 30 – 70 °C and 20 – 30 °C for immobilized trypsin and immobilized CEP respectively. These indicate an increase in enzyme activity with increasing temperature up to an optimal maximum. Further increase in temperature however results in enzyme denaturation and consequent drop in activity. Thus, the slopes of the linear graphs within the temperature activation region where temperature dependent denaturation did not occur were used to determine the activation energies of the reactions using Equation [3]. According to some authors, when enzymes are immobilized on matrixes the activation energies of immobilized forms are similar or greater than that of free enzymes (David et al., 2006; Freitas et al., 2011). The same phenomenon can account for the higher *Ea* and ΔH^* values of immobilized trypsin compared to the free form (Table 2).

Table 2 Comparison of thermodynamic parameters of free and immobilized enzymes

	Biocatalyst	***Ea* (kJ/mol)**	**ΔH^***
Trypsin	Soluble	17.31	14.63
	Immobilized	28.43	25.67
CEP	Soluble	34.24	31.69
	Immobilized	7.68	4.91

Values estimated from the mean of experimental data (n = 3)

The *Ea* of immobilized CEP on the other hand showed that immobilization of CEP lowered the *Ea* from 34.24 to 7.68 kJ/mol (a 78% decrease). The lowered *Ea* signifies decreased energy barrier, such that immobilized CEP will not require elevated temperatures to reach its highest activity. Further, The ΔH^* values of immobilized CEP was 4.91 kJ/mol; this value is about 27 kJ/mol lower than that of soluble CEP (a 85% decrease). This implies that the immobilization procedure rendered CEP thermodynamically more efficient for catalysis since immobilized CEP will require less energy to form the enzyme–substrate activated complex compared to free CEP.

Immobilized enzyme activity on macromolecular substrate

Enzymatic hydrolysis is a widely used approach for improving the functional, nutritional and physiological properties of many natural food proteins (Rodríguez Patino et al., 2007). To such end therefore, an immobilized proteolytic enzyme system should be able to release peptides from macromolecular proteins.

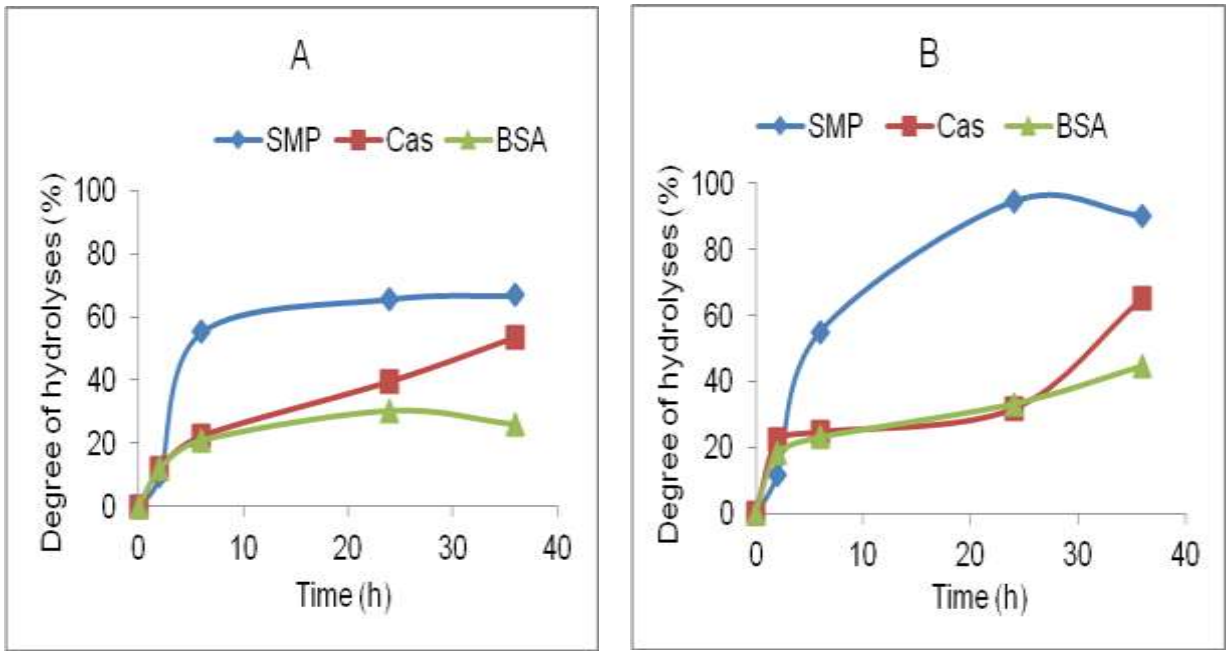


Figure 6: Degree of hydrolysis of various proteins by immobilized trypsin (A); and immobilized CEP (B); SMP -skimmed milk proteins, Cas - Casein, BSA-bovine serum albumin; (Average of duplicate experiments)

In this study, the immobilized enzymes were able to hydrolyze three different macromolecular proteins; namely, commercial skimmed milk proteins (SMP), casein from bovine milk, and bovine serum albumin (BSA), with concomitant release of peptides. Peptides released were followed by the use of the *o*-phthaldialdehyde (OPA) method which is a rapid assay that also allows real-time monitoring of protein hydrolysis (Rutherfurd, 2010). Another parameter which was used to describe protein hydrolysis by the immobilized enzymes is the degree of hydrolysis (DH). It is a practical and convenient way of estimating the proportion of cleaved peptide bonds in a protein hydrolysate (Adler-Nissen, 1982; Rutherfurd, 2010).

Results of protein hydrolysis differed for both enzymes (Figure 6). This could be due to the differences in catalytic specificities as well as differences in enzyme activities between trypsin and CEP. Peptide release was observed for all three proteins in the first few hours so that after 6 h incubation all hydrolysates showed over 20% DH (taking sample reading at 48 h as 100% DH). The DH results show that immobilized CEP was more effective in hydrolyzing the milk proteins (casein and SMP), especially after long incubation times. At 36 h, higher DH values were recorded for immobilized CEP (casein - 65%; SMP - 90%) than for immobilized trypsin (casein - 53%; SMP - 67%). This could partly be due to the fact that CEPs are biospecific for dairy proteins since; in lactobacilli, they are useful for the early stages of casein degradation into peptides and amino acids. By recording higher DH values, SMP proved to be more digestible than the other protein tested in this study. Hydrolysis of BSA was low and could be accounted for by the fact that the ordered, globular, ellipsoidal and 'heart-shaped' structure of BSA (Carter et al., 1989; Wright and Thompson, 1975) hinders access of internal peptide bonds by the immobilized enzymes.

Enzymatic hydrolysis is a widely used approach for improving the functional, nutritional and physiological properties of natural food proteins (Rodríguez Patino et al., 2007). Higher level of proteolysis (higher DH values) is a desirable property for immobilized enzyme for use in industrial applications such as waste treatments. However, for the purposes of producing functional/physiologically active peptides, a higher DH is not necessarily better. This is because; peptide functionality and bioactivity are affected by DH since these properties are dependent on the size, type and amino acids sequence of the hydrolysates. Over-hydrolysis of the protein molecule may therefore result in peptides with no functionality or bioactive

properties, or may convert peptides to amino acids! This phenomenon is confirmed in several studies where activity and functionality of protein hydrolysates has been shown to be lost when DH of protein is outside a critical range (Klompong et al., 2007; Rodríguez Patino et al., 2007; Wu et al., 2008).

SDS PAGE profile of milk proteins hydrolyzed by immobilized enzyme

A representative SDS–PAGE electrophoregram for casein degradation is shown in Figure 7A. Casein hydrolysis by immobilized trypsin was very effective such that after 6 h, all casein had been hydrolyzed to peptides. Several of these peptides were ≤ 30 kDa. After 24 h, no peptides were visible, due either to the fact that 4-15% acrylamide gel used was not able to show the small molecular peptides or the Coomassie blue staining was unable to visualize them (results not shown). Casein hydrolysis on the other hand was rather slow for immobilized CEP as it took about 48 h for the casein peak to be degraded to peptides.

The efficiency of both immobilized trypsin and immobilized CEP to degrade skimmed milk protein (SMP) was also evaluated. Results from the SDS-PAGE gels (Figure 7B) illustrate that both immobilized enzymes efficiently degraded skimmed milk protein. SMP contains a mixture of proteins such as caseins, and whey proteins (β-lactoglobulin, α-lactalbumin, immunoglobulin, serum albumin).

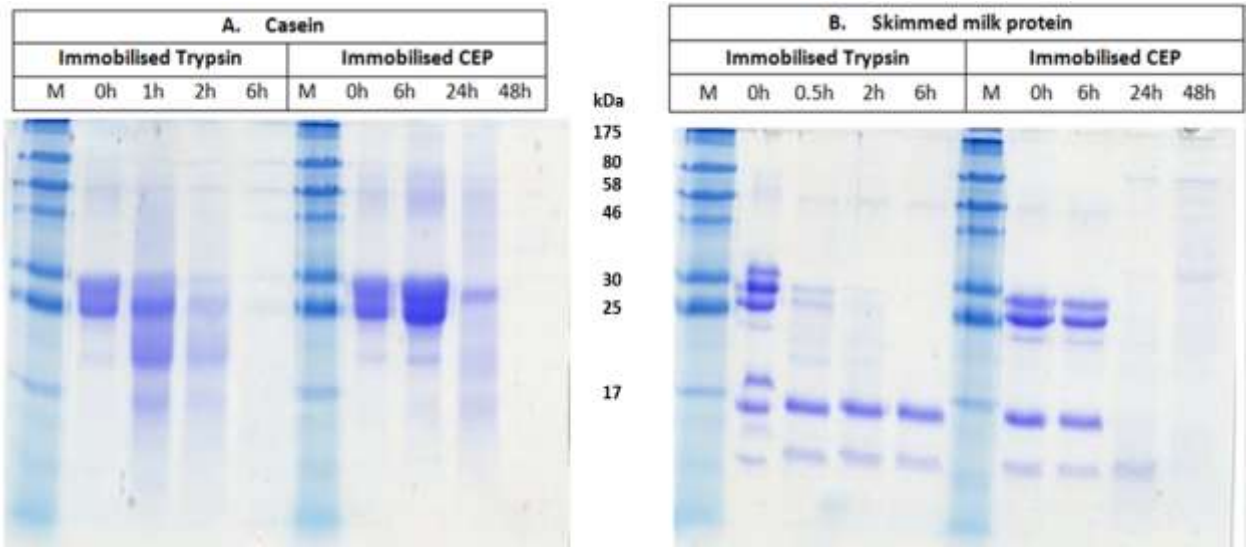


Figure 7: Action of immobilized trypsin and immobilized CEP on (A) Casein and (B) Skimmed milk protein; M- molecular weight marker

For the 0^{th} hour sample the bands corresponding to ~ 17 kDa represent β-lactoglobulin, the most abundant whey protein present in SMP (Kilara and Panyam, 2003). It is observed that trypsin degrades the casein component in SMP within 6 h, but no further significant changes are observed for the whey proteins. In contrast, CEP was shown to completely degrade most of the components present in SMP within 48 h. This unique property of the immobilized CEP to degrade other components of SMP is of significant importance in valorization, thereby possibly providing a solution to the long term problems of whey protein disposal faced by the dairy industry (Smithers, 2008). Additionally, that immobilized CEP was able to hydrolyze purified caseins as well as casein in its native state (i.e. in SMP) shows its high potential for the production of protein/peptide-rich product mixture from bovine milk, for food applications.

Interestingly, both immobilized enzymes demonstrated stability against self-digestion of already attached enzyme molecules. This is shown by the absence of auto-proteolytic soluble enzyme products in an SDS–PAGE electrophoregram (results not shown). This is a useful characteristic of this immobilization protocol considering that trypsin and CEPs from LDL 313, in the absence of stabilizers, are both highly susceptible to self digestion (Agyei et al., 2013; Purcena et al., 2009). The demonstrated proteolytic properties and stability of both immobilized enzymes demonstrate that the immobilization strategy presented in this study has the potential for the preparation of stable protease biocatalysts for several industrial applications.

Reusability of immobilized enzymes

An important parameter of immobilized enzyme systems is the reusability and stability. The loss of enzymatic activity during repeated use is important in determining how industrially feasible the immobilized enzyme system is (Feng et al., 2013). Covalent immobilization of enzymes is known to result in loss of mobility and subsequent decrease of activity. Thus, a successful enzyme immobilization system must give a minimum activity loss and a maximum number of possible cyclic uses. In this study, the immobilized enzymes retained activity in repeated assays and the activity retention shows the potential of immobilized trypsin and CEP for recovery and reusability. Activities reduced to about 38% (immobilized trypsin) and 41% (immobilized CEP) of the original activity after five cyclic uses for both enzymes (Figure 8).

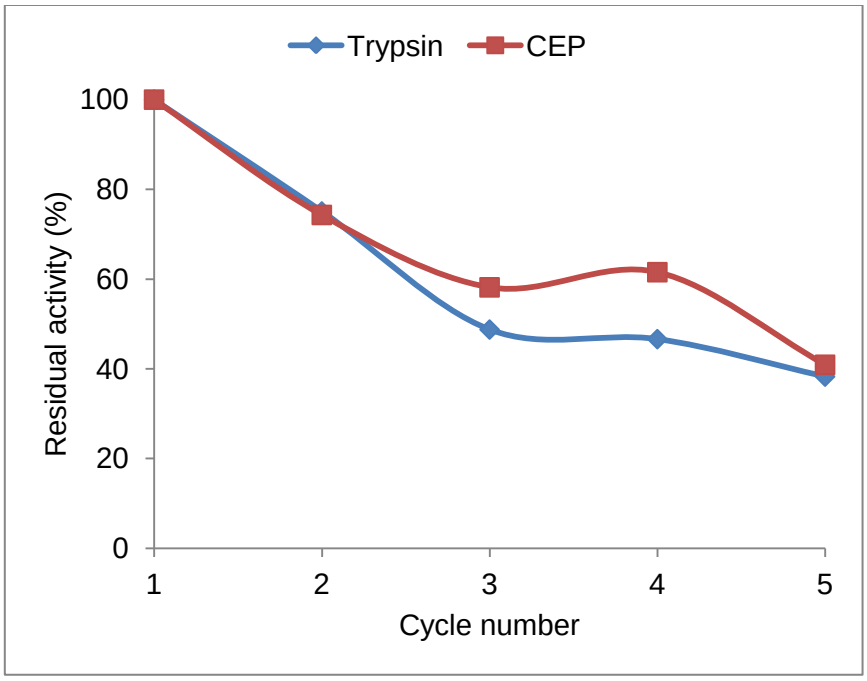


Figure 8: Reusability of immobilized trypsin (♦) and immobilized CEP (■) on polyester fabric.

The integral activity of immobilized enzymes is an industrially relevant parameter with huge economic significance. It is computed as the product of relative activity and the number of reuses, assuming the relative activity remains almost constant (Opwis, 2010). In this study, immobilized trypsin and immobilized CEP lost almost 59% and 62% of its activity respectively after recycling five times. However, the integral activity after these five reuses is much higher than the activity of free enzyme forms which could only be used once (i.e. 100%) in catalytic processes (Table 3). Also, protein loading yields on polyester fabric was high for trypsin (~ 68%) than for CEP (~ 22%) but residual activity yields of 4.1% (trypsin) and 85.6% (CEP) were observed. Thus immobilized CEP recorded a higher residual activity although a crude preparation of the enzyme was used for immobilization. The high loading but low residual activity values for trypsin could be due to the fact that soluble trypsin was used at a

higher concentration (1 mg ml^{-1}) than crude CEP (25 μg ml^{-1}) and some degree of autoproteolysis might have occurred before enzymes could be finally grafted unto functionalized polyester surface. Also, direct fixation of trypsin onto polyester fabric has been shown to give relatively low immobilization yields (Nouaimi et al., 2001; Wang and Hsieh, 2004).

The activity loss of CEPs after five cycles was quite significant. Possible reasons for this observation include leaching of weakly bound enzymes, loss of support material and enzyme inactivation during repeated use. During successive usage as the setup is subjected to several wash steps involving the use of relatively high ionic strength buffers, weakly bound enzymes are liable to 'wear-off' from the polyester support fabric. Moreover, during shaking of the reaction setup, some polyester fibres were observed to break off into the reaction mixture. Such loss in support material along with enzymes immobilised on them results in decreasing retained enzyme activity after multiple reuses. Other well noted reasons such as denaturation or inactivation of the enzyme due to continuous use could also account for the low reusability (Nakane et al., 2001). To improve CEP reusability, the immobilization conditions should be optimized specifically for CEPs and this may require the use of pure enzymes at a relatively higher concentration.

Table 3: The enzyme loading capacities and activities

Parameter	Immobilized Trypsin	Immobilized CEP
Retained activity (%)	4.07 ± 0.01	85.57 ± 5.75
Protein loading yield (% w/w)	67.71 ± 1.70	21.93 ± 4.64
Relative activity after 5 cyclic uses (%)	38	41
Integral activity after 5 cyclic uses (%)*	190	205

Values are mean ± standard error of mean of duplicate experiments; or mean of duplicate experiments; *Integral activity = relative activity of immobilized enzyme after several reuse × number of times reused; Relative activity of free enzyme forms = 100%.

Storage stability of immobilized enzymes

Storage stability is an industrially relevant process parameter that affects the transport and long-term storage of immobilized enzymes. In this study, both immobilized trypsin and CEP displayed good storage stability under the different long term storage conditions (Table 4). After 10 weeks (70 days) of storage, immobilized trypsin stored in buffer (4 °C) lost about 83% of activity and ~ 80% of activity loss was observed with samples stored under freeze-dried or air-dried conditions. Immobilized CEP on the other hand retained about 96% and 62% of initial activities respectively for samples stored in buffer at 4 °C, and lyophilized samples stored at -22 °C. Lyophilization did not have any significant effect on crude soluble enzyme activity ($p = 0.102$) when assayed immediately after freeze-drying. Thus, the observed storage stability behavior can be attributed to a combined effect of immobilization and storage conditions. Although immobilized CEP possessed good storage stability under wet-cold conditions, storage by air drying at room temperature (22 °C) resulted in about 92% loss of activity (Table 4).

Table 4: Storage stability of immobilized enzymes under different conditions after 70 days

Immobilized Biocatalyst	Condition (Storage temperature)	Enzyme activity (U ml^{-1} support)	Residual activity (% of initial activity)
Trypsin	Sodium phosphate buffer* (4 °C)	0.045 ± 0.001	16.63
	Air dried (22 °C)	0.056 ± 0.000	20.83
	Freeze-dried (-20 °C)	0.053 ± 0.001	19.72
CEP	Sodium phosphate buffer* (4 °C)	0.007 ± 0.000	96.40
	Air dried (22 °C)	0.001 ± 0.000	8.63
	Freeze-dried (-20 °C)	0.004 ± 0.000	61.87

*Buffers were supplemented with 0.02% sodium azide to control bacterial growth

Both immobilized CEP and immobilized trypsin displayed low activity retention when stored air-dried and this could be accounted for by structural changes in protein molecule at dried conditions. Immobilization can restrain the free movement of the protein side chains that are necessary for the protein molecules to adapt to dehydrated environments, and this can lead to a considerable loss of activity (Purcena et al., 2009; Soares et al., 2002). Also, for various immobilized enzymes, the recovery of activity after any method of drying (including lyophilization) is reportedly very low (Purcena et al., 2009; Soares et al., 2002; Wu et al., 2007). Thus, for immobilized CEP, the high recovery of activity (> 61%) from lyophilized preparations bears significance in the long term transport and storage.

Morphological examination of immobilized enzyme on polyester fabric

The success of immobilization in this study was confirmed by FTIR spectra (results not shown) and scanning electron micrograph (SEM). Figure 9 shows a scanning electron micrograph of polyester material before and after enzyme immobilization. SEM images show that the enzymes are well distributed over the PET fiber surfaces. Overall, the structure of polyester remained unchanged with the various treatments employed in this immobilization protocol (Figure 9, when Image A is compared with C and D). In comparison with the untreated fiber material (Figure 9B), the modified PET fibers can be seen covered with aggregates of the fixed enzyme (Figure 9C and Figure 9D). Maintenance of the physical and structural integrity of the polyester fibers (after immobilization) is an important and desirable property especially when the fibres are intended for the preparation of an immobilized enzyme packed-bed reactor.

Also, protein loading yields on polyester fabric was high for trypsin (~ 68%) than for CEP (~ 22%) but residual activity yields of 4.1% (trypsin) and 85.6%(CEP) were observed (Table 3). The high protein loading levels observed for trypsin could be attributed to the fact that a pure trypsin sample was used. This means there is no competitive binding (as it might have happened for CEP) and therefore more enzyme molecules are able to be attached unto the polyester fabric. The use of relatively higher concentration of trypsin (1 mg ml^{-1}) also means a higher degree of cross-linking is achieved with glutaraldehyde since the amount of reactive amino groups will be higher.

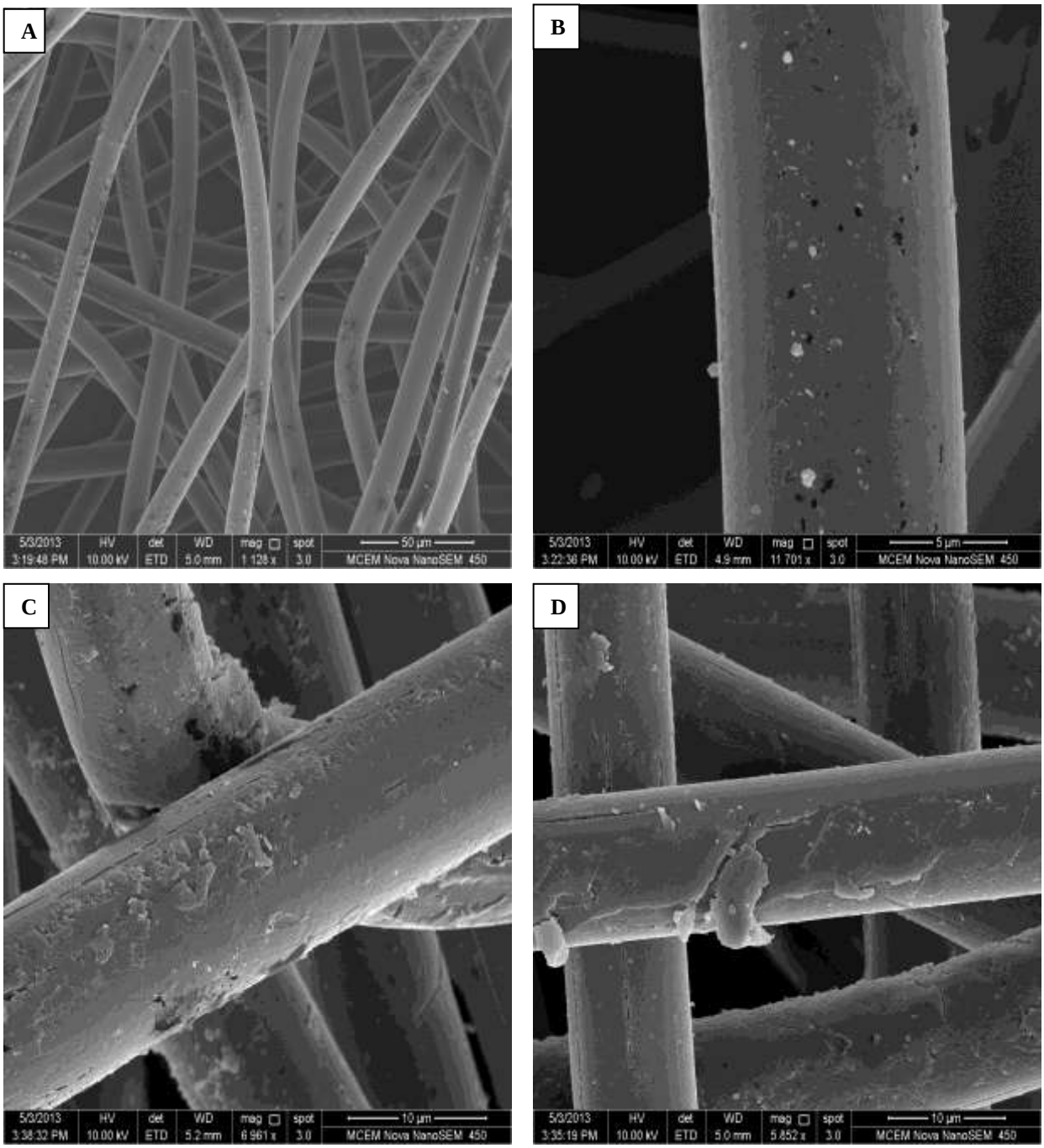


Figure 9: Scanning electron microscopy images of polyester material before treatment (A); untreated polyester fibre (B); and fibre after trypsin (C) or CEP (D) immobilization

5.6. Conclusion

In this study, low-cost polyester was used as carrier materials for the immobilization of cell-envelope proteinases (CEP) from *Lactobacillus delbrueckii* subsp. *lactis* 313 313 and a model of conventional proteinases, trypsin. The method involves amine functionalization of polyester followed by immobilization of enzymes via glutaraldehyde cross-linking. The immobilization procedure is simple and quick, and requires low preparative and economic expense for fabrics (polyester), amine linkers (ethylenediamine) and cross-linkers (glutaraldehyde). Immobilization resulted in increased operational stability, recyclability and enhanced storage stability, demonstrating their potential for the preparation of stable protease biocatalysts. Additionally, the polyester support has high operational stability and excellent resistance to compression and these properties make them suitable in the development of automated flow column bioreactors in future. The immobilized enzymes particularly CEP, were capable of hydrolyzing native proteins such as casein and SMP. This highlights their potential use of immobilized CEP for protein degradation to yield products and the process can be applied in food, pharmaceuticals, diagnostics, waste treatments, and the textile industries.

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6. CHAPTER 6: Cross-Linked Cell-Envelope Proteinase Aggregates

Section 6.1

6.1. Evaluation of cross-linked enzyme aggregates prepared from *Lactobacillus delbrueckii* subsp. *lactis* 313 cell-envelope proteinases, for protein degradation

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Monash University
Declaration for Thesis Chapter 6 Section 1

Declaration by candidate

In the case of Chapter 6 Section 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Initiation, Key ideas, Experimental Development, Results interpretations, Writing up	70

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Dr. Lizhong He	Initiation, Key ideas, Results interpretation, Writing up	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date** 12 December 2013
Main Supervisor's Signature	[redacted]	**Date** 12 December 2013

Evaluation of cross-linked enzyme aggregates prepared from *Lactobacillus delbrueckii* subsp. *lactis* 313 cell-envelope proteinases, for protein degradation

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6.2. Abstract

Enzymatic hydrolysis is a widely used approach for improving the functional, nutritional and physiological properties of food proteins; and to obtain protein hydrolysates economically the use of immobilized biocatalysts is the best choice. In this study, the design and evaluation of cross-linked enzyme aggregates (CLEAs) prepared from cell-envelope proteinases (CEPs) of *Lactobacillus delbrueckii* subsp. *lactis* 313 have, for the first time, been studied and their proteolytic properties tested using several food proteins. We have optimized cross-linking conditions including ammonium sulphate concentration, incubation temperatures, agitation speed, glutaraldehyde cross-linker concentration, reaction time and the addition of proteic feeders. Particularly, the presence of 20 mg/mg BSA improves retained activity values of cross-linked CEP aggregates (CLCEPA) from 21.5% to 40.9%. Interestingly, activity of CLCEPA was significantly improved in a number of nearly anhydrous organic solvents and this property is enhanced when CLCEPA is prepared co-aggregated with BSA. Also, quenching unreacted cross-linking groups on aggregates enhanced recyclability. CLCEPA had good thermal stability at 50°C and long-term dry storage resulted in some form of catalytic hyper-activation. CLCEPA showed proteolytic properties on several complex (chicken egg albumin, skimmed-milk protein), fractionated (bovine casein, whey protein isolate), and purified (bovine serum albumin) food proteins. Being the first report of CLEA from lactobacilli CEPs the studies demonstrates the feasibility of using LDL 313 CLCEPA for degradation of various proteins in the food industry.

Keywords: Enzyme immobilization; cross-linked enzyme aggregates (CLEA) ; *Lactobacillus delbrueckii* subsp. *lactis* 313; cell-envelope proteinases, protein degradation

6.3. Introduction

Food proteins and their hydrolysates are a useful class of biomolecules due to the many roles they play physiologically and in food processing operations. Enzymatic hydrolysis continues to be one of the most widely used approaches for improving the functional, nutritional and physiological properties of food proteins. Thus, technological and industrial applications, proteolytic enzymes must possess certain key requirements such as good stability and activity over a broad range of desired reaction conditions (e.g. extreme pH, elevated temperature, organic solvents, mechanical stress) (Brady and Jordaan 2009; Chen et al. 2006). These sets of requirement retard the application of soluble enzyme in many industrial syntheses.

Enzyme immobilization has been shown to improve enzyme stability and recyclability, thus overcoming the challenges with the use of soluble enzymes. Traditional methods of enzyme immobilization include physical adsorption, covalently binding to a carrier support, encapsulation and cross-linking (Zhao et al. 2013). Among these methods, there has been an increased interest in cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs) which are two attractive carrier-free immobilized enzymes systems (Sheldon 2011). Nevertheless, CLECs have the drawback of cost and tedious crystallization procedures, mainly because very pure enzyme preparations are required for the crystallization step (Wilson et al. 2004). CLEAs on the other hand are attractive because they can be made without extensive protein purification (Shah et al. 2006) and the final preparation has a high concentration of enzyme per unit volume (López-Serrano et al. 2002; Wang et al. 2011). Even more, cross-linking offers the advantages of improved enzyme activity, high enzyme stability,

low production costs owing to the exclusion of expensive carriers and the possibility to co-immobilize different enzymes (Cao et al. 2003; Chen et al. 2006).

Cell-envelope proteinases (CEPs) are a special class of extracellular proteolytic enzymes obtained from lactic acid bacteria. In the food industry, CEPs have been known to improve the texture and organoleptic characteristics of dairy products and also have the potential to release bioactive peptides encrypted in dairy proteins (Tsakalidou et al. 1999; Sadat-Mekmene et al. 2011). Additionally, CEPs are economical to produce and easy to extract from lactic acid bacteria (Agyei et al. 2013b). However, the use of the free CEPs in industrial processes is currently suboptimal and presents further drawbacks such as poor stability and lack of multiple utility. Being a cell membrane-associated enzyme, the most extensively used method of releasing CEPs is by washing or incubating the bacteria cells in a calcium-free buffer (Tsakalidou et al. 1999). However, this method compromises the final storage and thermal stability of CEPs so obtained (Hébert et al. 1997; Martín-Hernández et al. 1994). Additionally, Food and Agriculture Organization of United Nations (FAO) regulations suggest that enzyme preparations used in the production of consumables (such as food or pharmaceuticals) must be removed from products after processing (FAO/WHO 2006) – a difficult, if not impossible objective to achieve using soluble enzymes. The accumulative effect of all these factors makes free CEP enzyme-based process economically unfeasible.

CEPs expressed from *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) were used in this study because the production and extraction of CEPs from LDL 313 have already been optimized in previous studies (Agyei et al. 2012; D. Agyei and M. K. Danquah 2012; Agyei et

al. 2013a). The improvement of CEP stability and reusability will make it economically feasible for food processing. Screening for stable biocatalysts from LDL 313, an understudied lactobacilli species (Agyei et al. 2012) will enhance the application prospects of this species, especially in the food industry. Further, to the best of our knowledge, no study has reported the preparation of CLEA from lactobacilli CEPs. In this study therefore, cross-linked enzyme aggregate technology was used to develop stable forms of CEPs expressed by LDL 313. Conditions for quick and efficient CLEA preparation were studied and the catalytic properties of aggregates were tested on several food proteins.

6.4. Experimental

Strain and growth condition

Lactobacillus delbrueckii subsp. *lactis* 313 (ATCC® 7830™) was obtained from ATCC (Manassas, USA) and propagated twice in deMan, Rogosa and Sharpe (MRS) Broth (Acumedia, Michigan, USA) at 37 °C, 5% CO_2 and stored at -70 °C. The revived culture was grown to early-stationary phase (optical density at 560 nm (OD_{560}) of ~ 1.0) in MRS broth. Culture was centrifuged and the cell pellets suspended in an equal volume of 50 mM phosphate buffer (pH 7). This was used to inoculate fresh MRS broth to an initial OD_{560} of 0.1. Anaerobic fermentation was carried out in 500 mL Erlenmeyer flasks, in a gyratoy shaker (100 r.p.m).

Preparation of crude cell-envelope proteinase

Culture was grown at 45 °C to stationary phase (optical density at 560 nm (OD_{560}) of ~ 2.8 ± 0.4, about 0.58 g/L dry cell weight), harvested by centrifugation (4,000×g, 10 min, 4°C), and resuspended to an OD of 5 in the extraction solution containing 50 mM sodium phosphate buffer supplemented with 5 mM EDTA (pH 7) for cell-envelope proteinase (CEP) extraction via incubation (30 °C, 2 h, 50 rpm).The suspension was centrifuged (4,000×g, 10 min, 4°C) and the supernatant was retained, filtered through 0.45 μm membrane filters (Acrodics, Pall Life Sciences) and designated as crude cell-envelope proteinase (CEP). Protein concentration was determined with a Protein Assay Kit according to manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA).

Preparation of cross-linked enzyme aggregates

The crude proteinase, CEP in Na-phosphate buffer (50 mM, pH 7) was placed in a micro centrifuge tube to which solid $(NH_4)_2SO_4$ was added to obtain an appropriate level of w/v saturation. After brief vigorous mixing (~ 1 min), glutaraldehyde (25% w/v in water) was added to give a desired final concentration. The mixture was incubated with shaking and CLEA formed washed via centrifugation (10,000g, 5 min, room temperature) until supernatant was colorless signifying removal of free glutaraldehyde. This required washing twice with buffer (50 mM Na-phosphate buffer, 1 M NaCl, pH 7) and a final rinse with fresh Na-phosphate buffer (50 mM, pH 7). The final enzyme preparation was kept in Na-phosphate buffer (50 mM, pH 7). If necessary, the solid was dispersed with the aid of a pipette tip. In some instances, a blocking step was done where free carbonyl groups were quenched by reacting washed CLEA with 1 M Tris-HCl pH 7.6 (250 rpm, 1 h, 20°C) and washing twice with Na-phosphate buffer via centrifugation (10,000g, 5 min).

For experiments on the effect of proteic feeders on CLEA activity recovery, appropriate amounts (mg) of BSA from a 0.25 g/mL stock solution was added to crude CEP before the addition of precipitation and cross-linking agents. Optimization of the cross-linking conditions (i.e. ammonium sulphate concentration, incubation temperatures, agitation speed, glutaraldehyde cross-linker concentration, $CaCl_2$ concentration, reaction time and the addition of proteic feeders) were done in a sequential manner and in the order presented by fixing values for all other factors and varying one. The results of the optimum value selected for one conditions is then used for the optimisation of subsequent ones. A full description of the actual values which were fixed or varied is given for each condition in the figure caption at the Results and Discussion section.

The efficiency of immobilized enzymes was evaluated in terms of retained activity (%) as follows:

$$\text{Retained activity (\%)} = \frac{\text{Activity of immobilized enzyme aggregates}}{\text{Activity of free enzyme introduced}} \times 100\% \quad [1]$$

Proteinase Activity Assay

Proteinase assay was determined with the Protease Colorimetric Detection Kit (Sigma) with casein as substrate by measuring tricarboxylic acid (TCA) soluble peptides that produce colour equivalent to 1.0 μmol (181 μg) of tyrosine per minute at pH 7.0, 35 °C. Aliquots of 500 μL CEP or aggregates prepared from the same was added to 500 μL of substrate solution (2 mg/mL casein in 50 mM sodium phosphate buffer; pH 7.0) and incubated (35 °C, 2 h). The reaction was stopped by adding 500 μL of 2% TCA, holding for 20 min at 35 °C followed by centrifugation (15,000 rpm, 5 min). A 250 μL aliquot of supernatant was taken carefully, to which was added 625 μL of Na_2CO_3 followed by 125 μL of 4-times diluted 2.0 N Folin & Ciocalteu's Reagent. After incubating (35 °C, 30 min) and allowing cooling to room temperature, the absorbance at 660 nm was measured. One enzyme unit is defined as hydrolyzing casein to produce color equivalent to 1.0 μmol (181 μg) of tyrosine per hour at pH 7.0 at 35 °C (color by Folin & Ciocalteu's Reagent). The amount of tyrosine was determined from the tyrosine standard curve.

Hydrolytic action of CLEA on macromolecular protein substrate

CLEA prepared from 500 μL of crude CEP (with about 1.33×10^{-3} U/mL original activity) was added to 1 mL of protein substrates in 50 mM Na-phosphate buffer pH 7.0. Five different protein substrates were used, including 2% v/v chicken egg albumin, 2 mg/mL whey protein isolate, 2 mg/mL bovine serum albumin, 2 mg/mL skimmed-milk protein and 2 mg/mL total casein from bovine milk, each supplemented with 0.5% benzyl alcohol and incubated (35 °C, 250 rpm). Peptides generated were monitored with the o-phthaldialdehyde (OPA) assay as described below.

Peptide assay via o-phthaldialdehyde (OPA) reaction

The OPA (o-phtaldialdehyde) reaction was used to estimate proteolysis of macromolecular protein substrates by CLEA via the measurement of free amino acids and peptides containing primary amino acids. OPA reagent preparation and methodology were as described by (Nielsen et al. 2001) and (Alhaj et al. 2010) respectively, with slight modification as follows. About 5 μL of unhydrolysed protein or protein hydrolysed by immobilized enzyme were added to 50 μL of OPA reagent containing dithiothreitol (DTT), mixed briefly, followed by the addition of 150 μL of Mill-Q water and left to incubate at room temperature for 10 min. The absorbance of the solution was measured at 340 nm. The absorbance readings were discounted from the initial reading of unhydrolysed proteins. The degree of hydrolysis for each sample was determined according to the following equation:

$$\%\ \text{degree of hydrolysis} = \frac{(S-C)}{D} \times 100 \qquad [2]$$

where C is the reading for unhydrolysed protein (control), S is the sample reading of the protein hydrolysate formed by CLEA (samples), and D the difference between the sample

reading after 24 h (taken as 100% degree of hydrolysis) and the unhydrolysed protein reading taken as 0%.

Study of the Protein Release from the Cross-linked Aggregates

To determine the strength of covalent intermolecular attachment, crude CEP and CLEA (with and without BSA as proteic feeders) were boiled (5 min) in equal volume of sample solubilisation buffer (Laemmli 1970) which contains 2% sodium dodecyl sulfate (SDS) denaturant. This released noncovalently attached protein molecule in the aggregate into the medium. The supernatants were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS−PAGE), and stained with Coomassie blue as described (Laemmli 1970).

Thermal stability assay

Thermal stability of the soluble enzyme (about 1.33×10^{-3} U/mL) and aggregated enzyme (CLEA-0.29×10^{-3} U/mL and 0.54×10^{-3} U/mL-CLEA-BSA) was determined by incubating them in 50 mM sodium phosphate buffer, pH 7.0 and 50 °C. Samples of the suspension were withdrawn at intervals and enzyme activity was analyzed as described in previous sections. To describe the deactivation kinetics, each experimental result was converted to residual activity (i.e. the fraction of the original activity defined as the ratio between the activity after each run and the activity of the first run).

CLEA Stability in Organic Solvent

Stability of CEP and CLEA in ethanol/buffer mixtures at different concentrations (0 - 100%) was done by incubating biocatalyst in the solvent at 20 °C for 30 min, followed by activity test with 2 mg/mL casein as substrate. Crude CEP was freeze dried before the addition of the ethanol/buffer mixtures. After incubation, the solvent-catalyst mixtures were transferred to preheated incubator (35 °C) to which the casein substrate was added assayed for residual enzyme activity as described above.

Storage stability of enzyme aggregates

The storage stabilities of the enzyme aggregates were assayed by washing, drying and storing at different conditions for 2 weeks after which residual enzyme activity was assayed. Five different conditions were studied, namely, washing with buffer and storing in buffer at 4°C; washing with buffer and storing in buffer at 20 °C; washing in buffer, air-drying and storing at 20 °C; washing in ethanol, air-drying and storing at 20 °C; and, washing in buffer, freeze-drying and storing at 20 °C.

Morphological studies by SEM

Scanning electron microscopy (SEM) images of enzyme aggregates were made using a Nova™ NanoSEM 430, FEI after being coated with Au.

6.5. Results and Discussion

Selection of precipitation agent

Ionic precipitants (inorganic salts such as ammonium sulphate) are among the most widely used agents in protein precipitation and purification. Ammonium sulphate is low in cost compared to non-ionic polymers like polyethylene glycol or organic solvents such as *tert*-butyl alcohol, making $NH_4 (SO_4)_2$ a good precipitant of choice for a cheap and sustainable preparation of enzyme aggregates. In this study, the concentration of ammonium sulphate used had significant effect on CLEA activity and high ammonium sulphate concentrations (≥ 90 % w/v concentration) yielded higher activities (results not shown). Similar observation has been made by Schoevaart et al., (2004) who recorded that the use of high precipitant concentration in CLEA preparation actually gives higher recovery rates. The high retention of activity was attributed to differences in outcomes of competition between protein aggregation and denaturation (Schoevaart et al. 2004). Thus, 100% w/v ammonium sulphate concentration was chosen for subsequent experiment in this study.

Optimization of cross-linking conditions

Cross-linking temperature

Cross-linking temperature also had significant effect on CLEA activity. It is generally known that enzyme immobilization is best conducted at cool temperature to avoid loss of activity. However, in this study, CLEA activity is higher when immobilization is done at 45 °C (Fig. 1). This is because molecular vibrations and overall enzyme aggregates–cross-linking reaction rates are enhanced at high temperatures. Also, this observation can be attributed to changes in the cross-linking reaction mechanism of glutaraldehyde at higher temperatures (Migneault et

al. 2004). The optimum cross-linking temperature of 45 °C was therefore chosen for subsequent immobilization procedures.

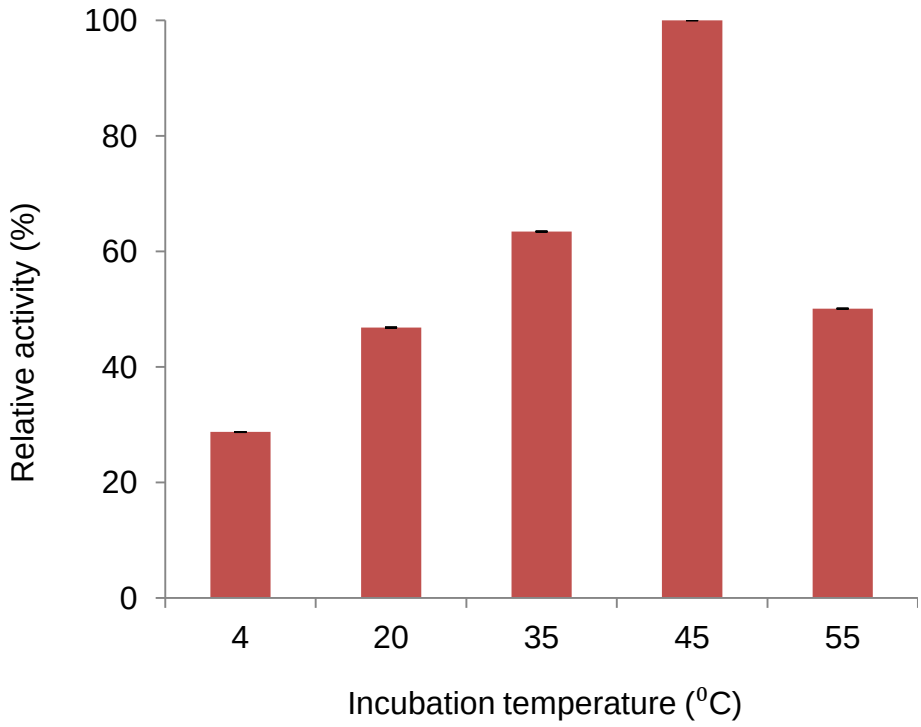

Fig. 1 Effect of cross-linking temperature on CLEA activity; (100% w/v ammonium sulphate concentration; cross-linking conditions: 0.08% v/v glutaraldehyde; 30 min incubation time; 250 rpm agitation speed); Values were normalised to highest recorded activity, standard deviation was within ± 1%; n = 4

Cross-linking agitation

Agitation speed also significantly affected CLEA activity and the optimum speed was 50 rpm (results not shown). During experimentation, CLEA prepared at slower agitation speeds had coarse appearance compared to those prepared at higher agitation speeds (> 100 rpm) which had had a finer appearance. Unquestionably, higher agitation speeds enhances cross-linking. However, beyond a critical value the aggregates could become over cross-linked which results in enzyme rigidification and inaccessibility of substrate to the catalytic active sites. This can compromise recovered enzyme activity, explaining why higher agitation speeds gave lower recovered activities.

For this study, agitation was attained by the use of an orbital shaker (Thermoline Scientific, Australia, Model TL-M-530) and speeds were given in revolutions per minute (rpm). As the current work is carried out at a small scale using microcentrifuge tubes, the parameter was not converted to shear stress for practical reasons. However, future scale-up studies could be conducted in bioreactors such as continuous stirred-tank reactor (CSTR) where the sheer rates could be estimated from the rotational speeds. Such information will also give some insight into the mechanical strength of enzyme aggregates during operation conditions.

Cross-linker (glutaraldehyde) concentration

Glutaraldehyde (GA) is a cheap and very versatile *bis*-aldehyde homobifunctional cross-linker. Although the main reactive species that participates in the cross-linking process is unknown, it has been shown that GA mainly reacts with ε-amino groups, followed by α-amino, guanidinyl, secondary amino, and hydroxyl groups at near neutral pH (Ortiz-Soto et al.

2009; Migneault et al. 2004). GA concentration had significant effect on CLEA activity and the optimum was ca. 0.8% vol/vol (Fig 2).

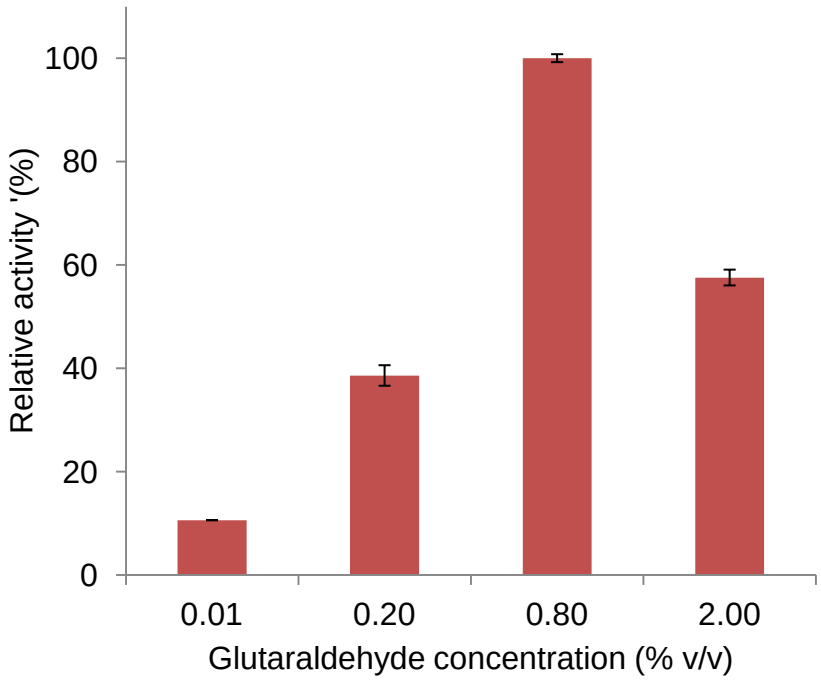

Fig. 2 Effect of cross-linker concentration on CLEA activity; (100% ammonium sulphate concentration; cross-linking conditions: 30 min incubation time; 45°C incubation temperature, 50 rpm agitation); Relative activity values (mean ± standard deviation) were normalised to highest recorded activity; n = 4

The usage of GA at low concentration resulted in insufficient cross-linking giving low levels of recovered activity. However, at high concentrations, GA may deactivate the immobilized enzyme due to excessive and unselective cross-linking which leads to lower activities (Feng et

al. 2013; Kim et al. 2013). The detrimental effect of GA at high concentrations has been observed with many other enzymes (Chui and Wan 1997; J. C. Wu et al. 2006; Ortiz-Soto et al. 2009; Stanley et al. 1976). It is worth noting that, for this part of the study, the aim was to identify different factors that affect aggregate enzyme activity. As the data shows GA concentration is one critical factor, further optimization needs to be carried out in future scale up studies intended for industrial applications.

Effect of incubation time

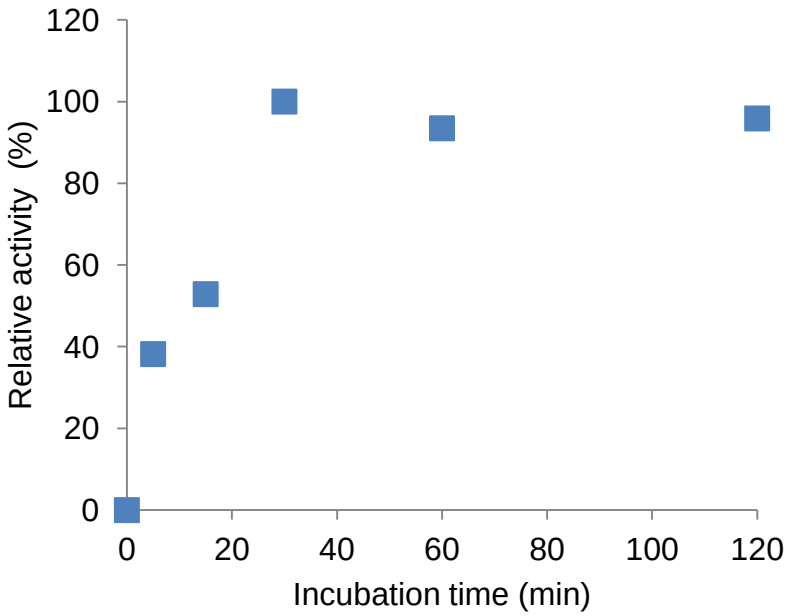

Fig. 3 Effect of incubation time on CLEA activity (100% w/v ammonium sulphate concentration; cross-linking conditions: 45°C incubation temperature, 50 rpm agitation, 0.4% v/v glutaraldehyde, 0 mM $CaCl_2$); Values were normalised to highest recorded activity, standard deviation was within ± 1%; n = 4

Cross-linking incubation time also significantly affected CLEA activity and in a manner as other conditions such as cross-linking temperature and GA concentration. Shorter incubation times are insufficient for maximum recovery whereas longer incubation times do not necessarily improve CLEA activity recovery. In this study, CLEA activity did not improve after 30 min of cross-lining (Fig. 3). The optimum incubation time was therefore chosen to be 30 min.

Effect of $CaCl_2$ additives

Calcium (II) ions are important for proteinase synthesis in lactobacilli. The role of Ca^{2+} ions in inducing and stabilizing active conformations of the bound proteinases on cell envelope surfaces has been reported (Coolbear et al. 1992; Siezen 1999). The functional involvement of calcium ions in the active conformation of the proteinase has been demonstrated and calcium ions have been shown to protect the enzyme against autoproteolytic release (Coolbear et al. 1992; Exterkate and Alting 1999; Siezen 1999). It has also been observed that activity loss of crude CEPs extracted with Ca-free buffer is restored by the addition of Ca^{2+} ions. Further, the stability of enzyme activity is maintained when crude CEPs are supplemented with Ca^{2+}. This relation of enzyme activity/stability is proportional to the concentration of added Ca^{2+} to about 10 mM (Exterkate and Alting 1999). However, it is observed that Ca^{2+} does not have any positive effect on CLEA activity recovery (results not shown). This may be due to the fact that Ca^{2+} ions exert their influence on CEP only when in aqueous form in soluble CEPs and not when CEP exists in precipitated or aggregated forms.

Effect of proteic feeders (BSA)

In this study, BSA as a proteic feeder was added to CEP in CLEA preparation (CLEA-BSA) and it was observed that BSA significantly improved the expressed CLEA activity. The addition of various amounts of BSA always resulted in higher activities than CLEA samples prepared without BSA. The optimum mg ratio of BSA: CEP was 20: 0.08, giving relative activity that was ~ 3.7 times higher than CLEA samples without BSA (Fig. 4).

In their pioneering work, Shah et al., (2006) demonstrated that the inclusion of proteic feeders (such as BSA) in CLEA preparation improves enzyme aggregate activity yield. This is especially so, in cases where the protein concentration in the enzyme solution is low, or the enzyme has low levels of lysine groups, and/or when the enzyme activity is compromised due to high glutaraldehyde concentrations needed to obtain aggregates (Shah et al. 2006; Tükel et al. 2013; Guauque Torres et al. 2013; Galvis et al. 2012). Further, enzyme aggregates have been prepared from glutaryl acylase with poly(ethylene glycol) and glutaraldehyde by co-aggregation of the enzyme with the aminated polymer polyethyleneimine (López-Gallego et al. 2005). The addition of polyethyleneimine supplied the necessary reactive and abundant primary amino groups which could be co-aggregated with the primary amino groups on the enzyme surface resulting in a stable, more efficient biocatalyst (López-Gallego et al. 2005).

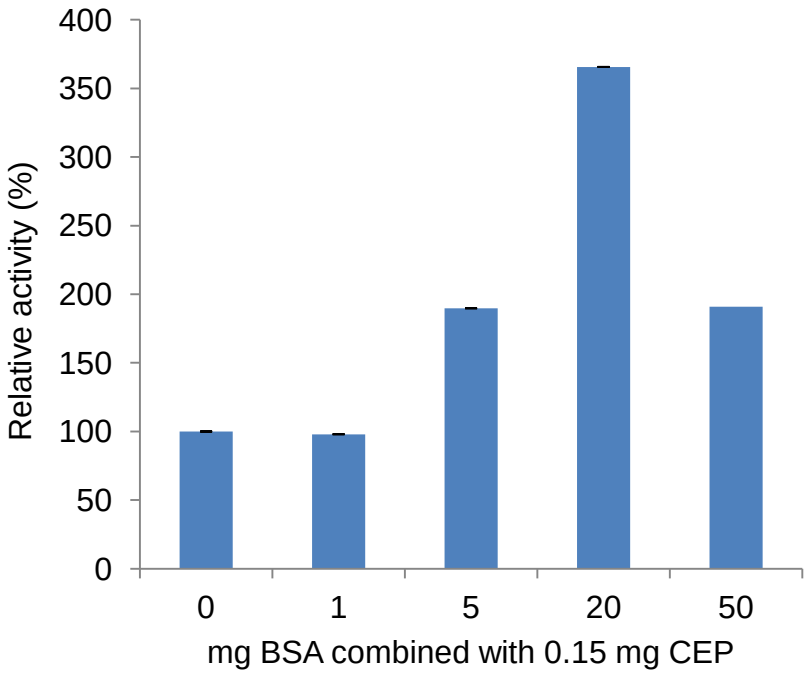

Fig. 4 Effect of BSA on CLEA activity; (100% w/v ammonium sulphate concentration; cross-linking conditions: 30 min incubation time; 45°C incubation temperature, 50 rpm agitation); Values were normalised to recorded activity for 0 mg BSA, standard deviation was within ± 2%; n = 4

In the lactobacilli, cell envelope-associated proteins comprises, among others, of the ribosomal proteins; cell-surface permeases; surface layers (S-layer) proteins; and cell-membrane-associated proteinases responsible for extracellular hydrolysis of proteins to peptides (D. Agyei and M. Danquah 2012). Thus, crude CEP used in this study putatively had contaminating proteins which could also be regarded as endogenous proteic feeder. However, these extra proteins (total protein of 0.15 mg) did not exist in quantities that could give observable improvement in CLEA activity, necessitating the addition of BSA.

There existed an optimum range of BSA concentration below which there are not enough free amino groups to prevent excessive cross-linking (Fig 4). Beyond this optimum range, the free amino groups of BSA compete with free amino groups in CEP and prevent the necessary cross-linking of the enzyme molecules. Thus, no improvement in activity was observed at BSA concentrations of 50 mg. At such high concentrations, the total protein in the mixture was stoichiometrically in excess over the amount of solid $(NH)_2SO_4$ saturation needed to give 100% saturation. It was observed, during experimentation that a precipitated protein 'gel' resulted which could not be effectively mixed at 50 rpm. Concomitantly, not all the proteins were effectively precipitated and cross-linked, giving rise to the low observed CLEA activity.

Table 1: Activity recovery of CEP, and aggregates prepared in the presence (CLEA- BSA) and absence of BSA (CLEA)

Enzyme aggregates*	**U/mL (µmol/mL/h) $\times 10^{-3}$**	**Residual activity (%)**	**U/g dry aggregate**	**Wet weight/dry weight ratio**
CEP	1.33 ± 0.02	100	-	-
CLEA	0.29 ± 0.00	21.5	2.44	36.59
CLEA-BSA	0.54 ± 0.02	40.9	1.44	12.23

*CLEAs were prepared according to the procedure described under Materials and methods using optimum conditions (100% w/v ammonium sulphate concentration; cross-linking conditions: 30 min incubation time; 45 °C incubation temperature, 50 rpm agitation, ± 20 mg BSA).

A combination of the optimum cross-linking conditions described above (section 3.2.1 to 3.2.6) gave enzyme recovery values of ca. 41% and 22% for CLEA prepared with (CLEA-BSA) and without BSA (CLEA), respectively (Table 1). Further, CLEA had higher hydration as observed from high wet weight/dry weight ratio compared to CLEA-BSA and the activity of CLEA-BSA per dried weight was lower than that of CLEA (Table 1). These observations could be explained by the presence and effect of BSA in CLEA-BSA which favors a more intense cross-linking of the aggregates thus 'pushing' out as much of the free water in the aggregate composite and leaving the aggregates drier.

Reusability of CLEA

Reusability and stability is an important parameter of immobilized enzyme systems. The loss of enzymatic activity during repeated use is therefore important in determining the industrial feasibility of the immobilized enzyme system (Feng et al. 2013; Opwis 2010). In this study, recyclability of CLEA prepared with and without a blocking step was tested. Blocking was done by reacting CLEA with 1 M Tris-HCl pH 7.6, to quench unreacted carbonyl groups on aggregate surface. The enzyme aggregates were separated from the reaction mixture by centrifugation (10,000 g, 10 min, room temperature).

It was observed that blocking significantly improves the stability and activity retention of CLEA during recycle. The operational stability of CLEA in recycle mode was tested by performing five successive runs of casein hydrolysis. Unblocked CLEA lost about 79% of activity in the second recycle alone, whereas blocked CLEA lost only 9% and still had retained activity of ca. 21% after five successive runs (Fig. 5). The activity retention of blocked CLEA shows the potential of CLEA for recovery and reusability.

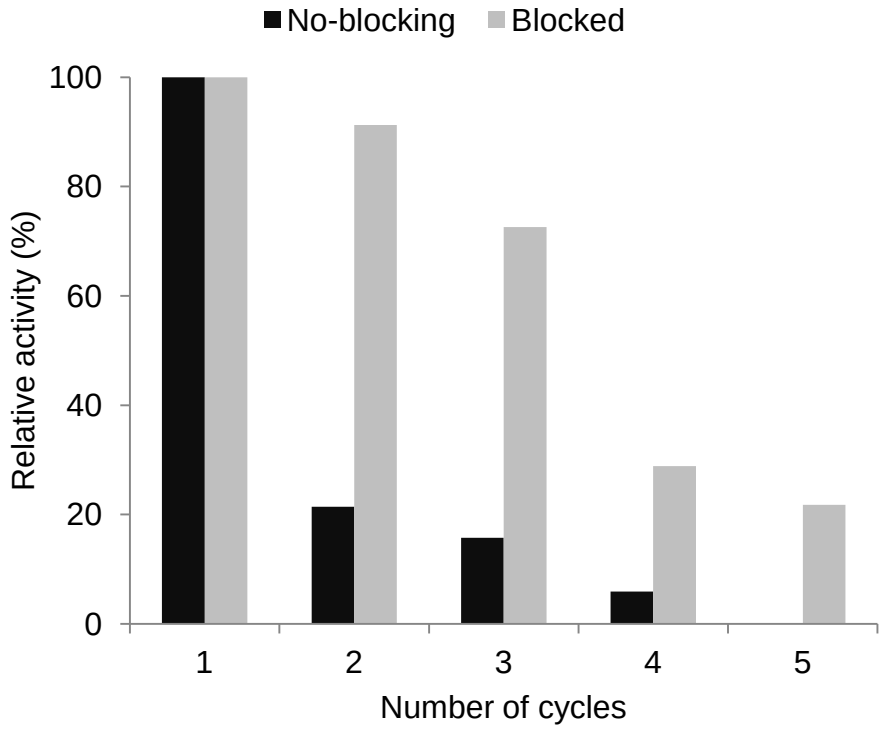

Fig. 5 Reusability of CLEA, blocked and unblocked with Tris; (100% w/v ammonium sulphate concentration; cross-linking conditions: 45°C incubation temperature, 50 rpm agitation, 0.4% v/v glutaraldehyde, 0 mM $CaCl_2$); standard deviation was within ± 2%; n = 4

Effect of blocking

CLEA prepared with no blocking step were unstable and lost activity in a rapid manner (Fig. 5). This is attributable to the fact that, without blocking there exist reactive carbonyl groups on aggregate surface. The introduction of a protein substrate (casein) therefore creates a competition and the substrate is left to interact with aggregate either by proteolysis or in a carbonyl-amine reaction. However, the carbonyl groups on the surface are able to react easily and quickly with proteinaceous substrates. Arguably, some molecules of casein used in the first run of activity assay must have reacted with the free active carbonyl groups on the

aggregate surface. However, this kind of 'blocking' significantly reduced the residual activity of CLCEPA in the second run, due to the sheer bulky nature of casein which could form a sheath around aggregates and hinder the diffusion and access of free substrates groups to the catalytic aggregates.

When aggregates are blocked however, there are little or no free carbonyl groups available for chemical attachment to casein molecules. Thus, more substrates molecules are free to access the catalytic aggregates (having nowhere else to bind unto aggregates). This results in a higher observed catalytic activity. The type of blocking agent has also been shown to affect the activity and chemical properties of immobilized enzymes. Yang and Chase, (1998) have reported that the use of 2-mercaptoethanol instead of ethanolamine as a better blocking agent which avoids the formation of charged secondary amino groups on enzyme-support conjugates.

Study of the Enzyme Release from the Aggregates

An SDS−PAGE analysis of enzyme released from aggregates and CEP was conducted. It was observed that enzyme molecules in crude CEP are clearly seen whereas all enzyme molecules remained aggregated in the case of aggregates prepared with or without BSA (results not shown). This shows the presence of strong inter- and intra-molecular cross-linking of the aggregates for enhanced stability. These results also show that improvement in activity observed with CLEA-BSA is not due to enhanced inter-and intramolecular stability but could be due to changes in kinetic and/or thermodynamic properties of aggregates as a results on BSA.

Caseinolytic potential of CLEA and hydrolysis of other macromolecular substrates

CLEA from LDL 313 CEPs were able to hydrolyze several macromolecular proteins including total casein, commercial skimmed milk proteins (SMP), whey protein isolate (WPI), chicken egg albumin (CEA) and bovine serum albumin (BSA). The *o*-phthaldialdehyde (OPA) assay was used to monitor peptide evolution in a real-time manner. The release of peptides from total casein by CLEA is shown in Fig. 6a. Another useful parameter for describing protein hydrolysis is the degree of hydrolysis (DH). DH provides a practical and convenient way of estimating the proportion of cleaved peptide bonds in a protein hydrolysate (Rutherfurd 2010). It is also a useful measurement to estimate the effect of the tested proteases (J.-H. Wu et al. 2008).

Results of peptides released from the other protein substrates (SMP, WPI, CEA, SMP) were converted to degree of hydrolysis and shown in Fig. 6b. The OPA assay showed that peptides were generated from all the proteins within the first 12 h of incubation and showed over 50% DH (taking sample reading at 24 h as 100% DH) (Fig. 6b). CEPs from lactobacilli are very effective in hydrolyzing the milk proteins (casein, SMP and WPI). This is because, like other dairy lactobacilli, LDL 313 is auxotrophic for several amino acids and therefore equipped with a plethora of proteolytic enzymes responsible for casein degradation into peptides and amino acids (Espeche Turbay et al. 2009).

Enzymatic hydrolysis is a widely used approach for improving the functional, nutritional and physiological properties of natural food proteins (Rodríguez Patino et al. 2007); and it has been reported that the final application of protein hydrolysates is determined by the degree of

hydrolysis (DH) (Vioque et al. 2001). In this study, cross-linked CEP aggregates (CLCEPA) were able to hydrolyze purified caseins as well as casein in its native state (i.e. in SMP). Whey proteins were also degraded and this shows the potential of CLCEPA for the commercial production of peptide mixtures from milk proteins. The problem of whey protein disposal is an environmental challenge faced by the dairy industry (Smithers, 2008). The ability of CLCEPA to degrade whey indicates its potential for the valorization or treatment of whey waste. Additionally, these peptide-mixtures, based on their DH, could be used in various capacities as foaming agents and emulsifiers, flavourings and food texture enhancers, and also as health- enhancing ingredients in 'physiologically active' functional foods.

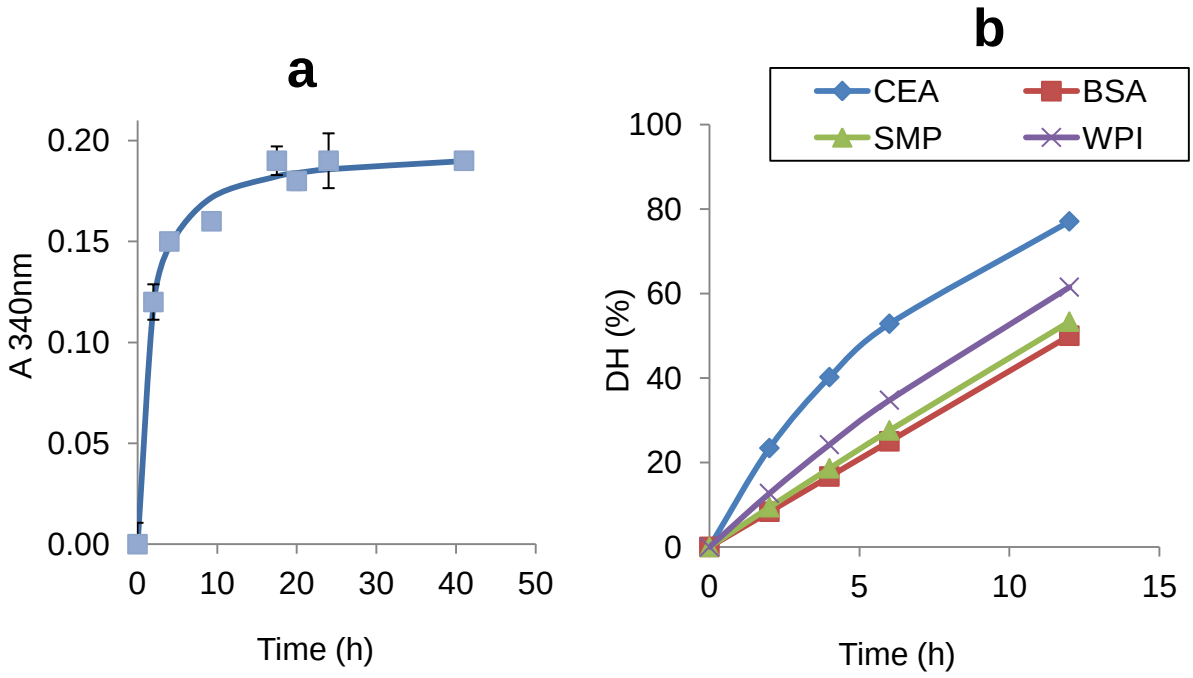

Fig. 6 Evolution of peptides released from 2 mg/mL casein by CLEA (a); and degree of hydrolysis of various macromolecular protein substrates using CLEA as biocatalyst (b); CEA- 2% v/v Chicken egg albumin; BSA – 2 mg/mL Bovine serum albumin; SMP – 2 mg/mL Skimmed milk protein; WPI – 2 mg/mL Whey protein isolate. Values are calculated from model estimates of experimental mean values (n = 4)

Thermal stability of aggregates

The thermal stability profile and kinetics of immobilized enzymes is an important parameter of much industrial relevance, especially if the biocatalysts are intended for large scale applications (Ribeiro and Rabaca 2011). In this study, although the enzyme aggregates did not have improved thermal stability over free enzyme, it is observed that all three biocatalyst forms had good thermal stability, recording residual activities of ca. 50%, 72% and 75% respectively for CLEA-BSA, CLEA and crude CEP after 49 h of incubation at 50 °C (Fig. 7). Degradation of most proteins in aqueous solutions as well as in solid state has been described by first order expressions kinetics (Yoshioka et al. 1991; Ribeiro and Rabaca 2011). In this study, crude CEP and its aggregates (with and without BSA) showed different patterns of inactivation (Fig. 7). The solid lines in the figure represent calculated values from nonlinear regression analyses of experimental data (n = 4). The experimental data were fit to the exponential decay model (Equation 3) by using 1st Optimization Statistical software (Levenberg-Marquardt method).

$$A = A_1 * exp^{(-k_1 * t_1)} + A_2 * exp^{(-k_2 * t_2)} + A_0 \quad [3]$$

k_1 and k_2 are first-order deactivation rate coefficients, A_0, A_1, and A_2 are relative activities of the initial active enzyme, enzyme intermediate, and final enzyme state, respectively. Curve fitting suggested that an exponential decay formula provided a good fit for thermal deactivation (Table 2).

CEP in this study was obtained from cultures grown at 45 °C, which is the optimum temperature for CEP yield (Agyei et al. 2012). CEP from cultures grown at 45 °C was active

even at high temperatures (i.e. 60 °C, results not shown) probably because cell cultures produce heat-shock proteins necessary for cell growth and survival at high growth temperatures (D. Agyei and M. K. Danquah 2012). Additionally, the crude CEP used in this study obviously contained several other biomolecules which might have induced some thermo-protectant properties on CEP, accounting for the high thermal stability of the crude CEP.

Table 2: Thermal decay kinetic constants for CEP, and aggregates prepared in the presence (CLEA- BSA) and absence of BSA (CLEA), at 50°C

Biocatalyst form	**Parametric decay constants**						**Pearson's coefficient, *R;* and (*F*-statistic)**
	A_0	A_1	A_2	k_1 (h^{-1})	k_2 (h^{-1})	$t_{1/2}$ (h)	
CEP	57.27	39.77	2.96	0.02	88.98	12.97	0.97; (128.85)
CLEA	71.40	31.07	-2.47	0.06	34.45	11.98	0.97; (100.94)
CLEA-BSA	52.53	15.43	32.10	1.67	0.15	3.92	0.99; (239.41)

The decay model was applied to the mean residual activity, i.e. ratio of observed activity at a specific time and the activity of catalyst at t = 0 h, (n=4) of experimental data; k_1 and k_2 are first-order deactivation rate coefficients; A_0, A_1, and A_2 are relative activities (%) of the initial active enzyme, enzyme intermediate, and final enzyme state, respectively (see Eq. [3]); $t_{1/2}$, half-life of the biocatalyst.

The three biocatalyst forms observed differences in thermal deactivation profiles. The enzyme aggregate preparation therefore influenced the thermal stability profile of CEP. A close-up view of the model predicted data for CLEA showed an initial rise (from t = 0 to t = 1 h), followed by an irreversible first-order inactivation. The initial rise in relative activity is known to occur when the specific activity of the intermediate is higher than the specific activity of the

initial enzyme state. Once a peak is reached it goes down in a first-order activity-time manner (Henley and Sadana 1985). Deactivation data for CLEA-BSA on the other hand followed a biphasic deactivation pattern involving a reversible first-order deactivation sequence and an active intermediate. There appeared to be stabilization, or near stabilization of activity for CLEA-BSA after 24 h and this is assumed to be due to a mechanism involving an irreversible deactivation as well as a reversible pathway (Fig. 7).

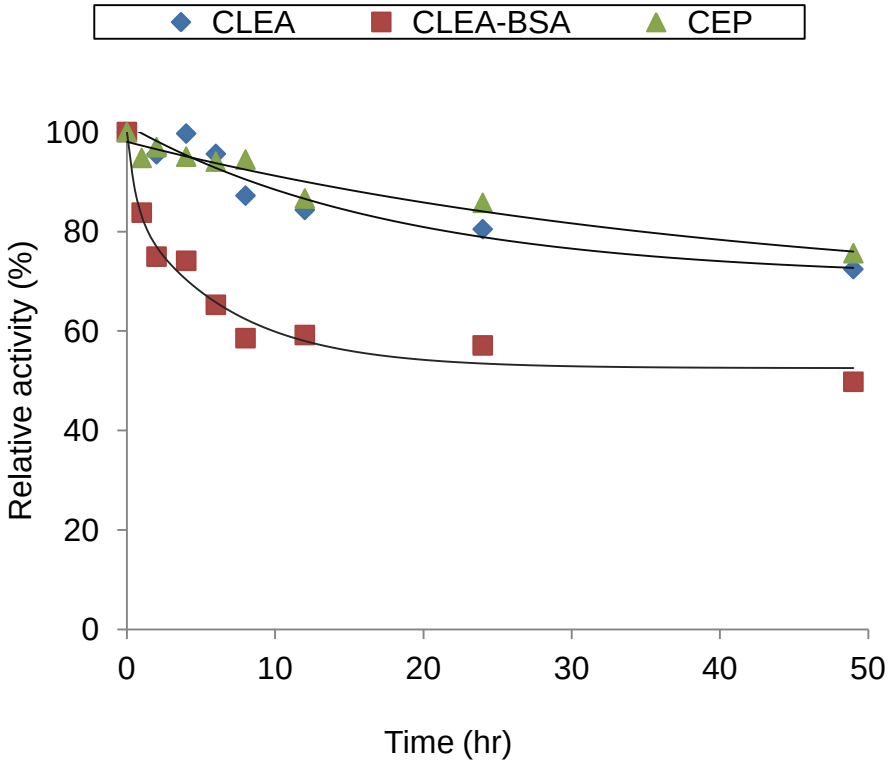

Fig. 7 Thermal degradation of CEP, CLEA and CLEA-BSA at 50°C

The time required for half the enzyme activity to be lost as a result of deactivation, the half-life of the biocatalyst, $t_{1/2}$, was estimated to be 12.97, 11.98 and 3.92 h respectively for CEP, CLEA and CELA-BSA. The difference in decay kinetics and decay mechanism is reflected in the half-life values observed by the different biocatalyst forms. Also, unlike CLEA-BSA, it is observed that CEP and CLEA recorded lower A_2 values as well as low deactivation rate constant, k_1 (Table 2), showing that only a smaller proportion by mass of these two catalysts gets deactivated from the initial state, A_0 to denatured state A_2.

CLEA stability in organic solvent

It is conventionally known that enzymes require water for catalytic activity. However, most enzyme molecules only require just a few monolayers of localised water around them; as such, the bulk of water can be replaced with an organic solvent without adversely affecting enzyme activity (Zaks and Klibanov 1985; Shinyashiki et al. 2009). Alcohol solvents have been known to cause structural changes in peptides and proteins and could also act as molecular lubricant which enhances the conformational flexibility enzyme to ease catalytic activity (Wiggers et al. 2007). An important property of immobilized enzymes which improves their technical application is their ability to tolerate harsh environmental conditions such is organic solvents (Nouaimi et al. 2001). Thus, in this study, the stability of CEP biocatalysts in cosolvent/buffer mixtures of decreasing polarity was studied.

CEP and its aggregates retained activity even after 30 min of incubation in organic solvents. Fig. 8 shows the stability of free and aggregated CEP in ethanol/water mixtures with different concentrations. The different concentrations of ethanol in the mixture did not have a

detrimental effect of the CEP activity. A maximum increase to 107% (relative to activity in 0:100% v/v ethanol:buffer mixer) was observed. However, the activities of the aggregates were significantly affected by the presence of ethanol. Activity of CLEA seems to have been stimulated then inhibited at low (20%) and high (above 40%) concentrations of ethanol respectively. Strikingly, the activity of CLEA-BSA on the other hand was significantly improved by incubation in ethanol and activity increased with increasing solvent concentration and decrease in solvent polarity. CLEA-BSA recorded an improved relative activity of ca. 237% after incubation in 100% ethanol whereas CLEA and CEP recorded ca. 140% and 107% respectively (Fig. 8) signifying that the improvement in activity of the enzyme aggregates is attributable to their nature as cross-linked enzyme aggregates.

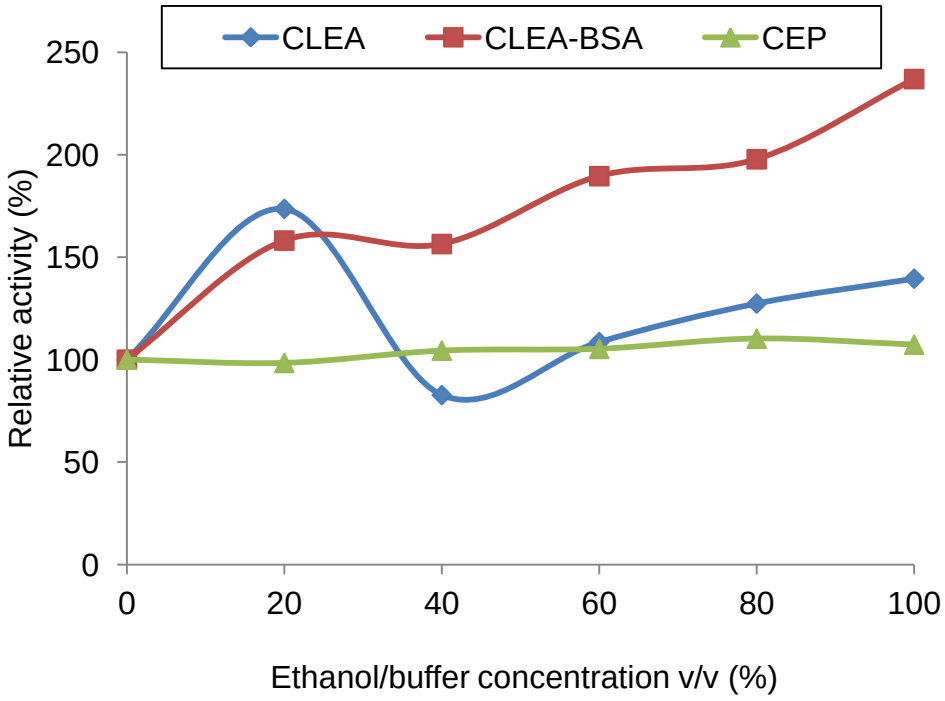

Fig. 8 Stability of free CEP and aggregated CEP (with BSA, CLEA-BSA; without BSA, CLEA) in ethanol/water mixtures with different concentrations (n = 4)

Additionally, the presence of BSA must have contributed to the stabilization and modification of catalytic activity via conformational changes which perturb the structure of enzyme active site (Wiggers et al. 2007) or the formation of stronger tertiary linkages (such as disulphide bonds) with CEP during the aggregation/precipitation step. The high stability of biocatalysts against denaturation or unfolding in the presence of solvents has been attributed to the presence of catalytically significant disulfide bonds (Ogino et al. 2001). CLEAs have also been shown to be tolerant to organic solvents (Chen et al. 2006).

Among the six major classes of enzymes, the hydrolases are known to catalyze the hydrolysis of a chemical bond in aqueous environment but this study shows that CLEA is able to hydrolyze protein even in the presence of organic solvent such as ethanol. CLEA of CEP theretofore could act as catalyst in an anhydrous organic solvents and this property is enhanced when CLEA is prepared co-aggregated with BSA. This property is also important for the storage of CLEA in organic solvent or inclusion of organic solvents to control microbial contamination during degradation reactions.

CLEA cluster size, shape and morphology

Aggregate cluster characteristics (shape and size) are an important property of CLEA and have significance in industrial application of CLEA for biotransformation. This is because the number of enzyme molecules and the way they are packed together in an aggregate affects the overall activity of the aggregate. Studies on factors for the optimal control of particle shape and size can therefore enhance the application of CLEA as well-defined catalytic entities (Schoevaart et al. 2004). In this study, CLEA-BSA aggregates were smaller in shape and finer

in consistency, but also formed large clusters (50 – 90 μm diameter) with less defined shapes. CLEA preparations were more uniform, small clusters with denser and coarser aggregates (40 – 50 μm diameter) than CLEA-BSA (Fig. 9). Aggregate formation is influenced by several factors. During aggregation, solubility of enzyme in surrounding medium decreases and increasing the speed of aggregation always gives a completely active aggregate (Schoevaart et al. 2004). Another condition that affects aggregate formation is agitation and surface charge properties (hydrophilicity or hydrophobicity) of aggregates.

CLEAs have been known to have good mechanical properties (Schoevaart et al., 2004; Ribeiro and Rabaca, 2011). However, although the addition of 'impurities' (as in the case of CLEA-BSA) improves recovered activity, it could compromise the mechanical integrity of the product resulting in aggregates with loose structures that could shear easily during the hydrolysis operation. The use of BSA putatively might have improved the level of cross-linking between enzyme and protein molecules. This is expected to help improve the mechanical strength of the enzyme aggregates but it remains to be determined to what extent this is so. It is therefore important to estimate the mechanical strength of the aggregates and correlate that with their ability to withstand shear stress.

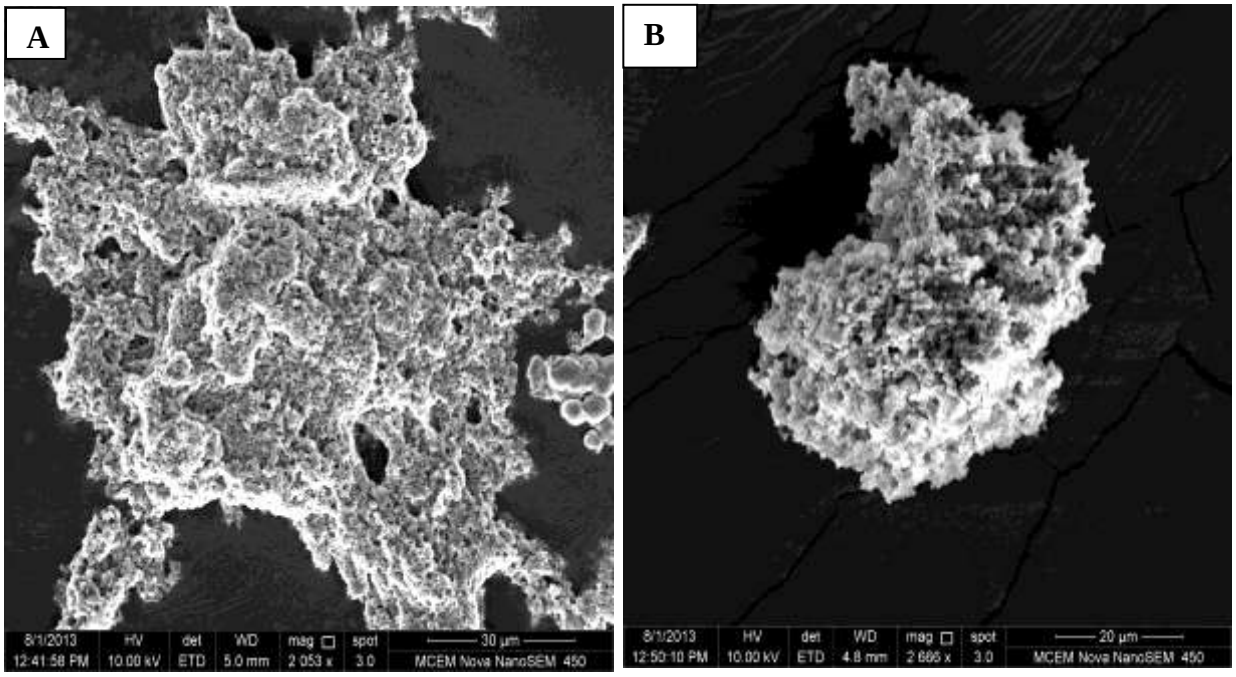

Fig. 9 Enzyme aggregates cluster size, shape and morphology; A-CLEA-BSA; B-CLEA

Cluster formation has an effect on aggregate enzyme activity since cluster size affects mass-transfer limitations. Small uniform-sized enzyme aggregates lend themselves to encapsulation, when size is controlled during preparation (Wilson et al. 2004). Big aggregates on the other hand can be due to preparatory operations such as centrifugation which lead to increased cluster formation by squeezing aggregates together (Schoevaart et al. 2004). It was observed, in this study that higher agitation speeds produced finer aggregates. The hydrophilic/ lipophilic ratio of CEPs remains unknown, but being a crude enzyme containing varying amounts of several other biomolecules such as peptidoglycans, lipopolysacharides and proteins, it can be safely assumed that CEP is hydrophilic. Aggregates built from enzymes with more hydrophilic surface residues are usually smaller in diameter (Schoevaart et al. 2004). Theoretically, the presence of BSA makes CLEA-BSA more hydrophilic than CLEA, and this could account for the observed small, fine consistency of CLEA-BSA.

Shelf stability of CLEA

Understanding the changes in properties of the hydrated protein is of much technical and industrial importance in protein science. This is because, hydrated protein at room temperature are known to undergo conformational changes which in turn affect the biological activity of the proteins (Frauenfelder et al. 1991; Desimone et al. 2008).

In this study, it was observed the different pre-treatment and storage conditions had an effect on enzyme activity of aggregates (Fig. 10). Some of the treatment conditions resulted in a hyperactivation of enzyme aggregates as reflected by activities several orders higher that of the initial value. When stored in buffer (conditions 1 and 2), CLEA-BSA retained more activity than CLEA. The high proteic mass of CLEA-BSA, due to the presence of BSA, increases the hydration level of aggregates which affects the enzyme activity.

The dynamics or behaviour of proteins has been shown to be physically related to the presence of solvent in the hydration shell (Shinyashiki et al. 2009). On the contrary, CLEA aggregates exhibited hyperactivity under dry storage conditions (conditions 3 – 5). Research has shown that protein dehydration is usually accompanied by structural and dynamical changes (Smith et al. 2004) which can affect enzyme activity. This phenomenon has been observed in other enzymes systems. For example, the stability and storage of enzymes (such as lipase) are enhanced at reduced water activity levels (Darvishi et al. 2012). The wet weight/dry weight ratio of CLEA is higher than that of CLEA-BSA (Table 1) indicating that CLEA is more hydrated than CLEA-BSA.

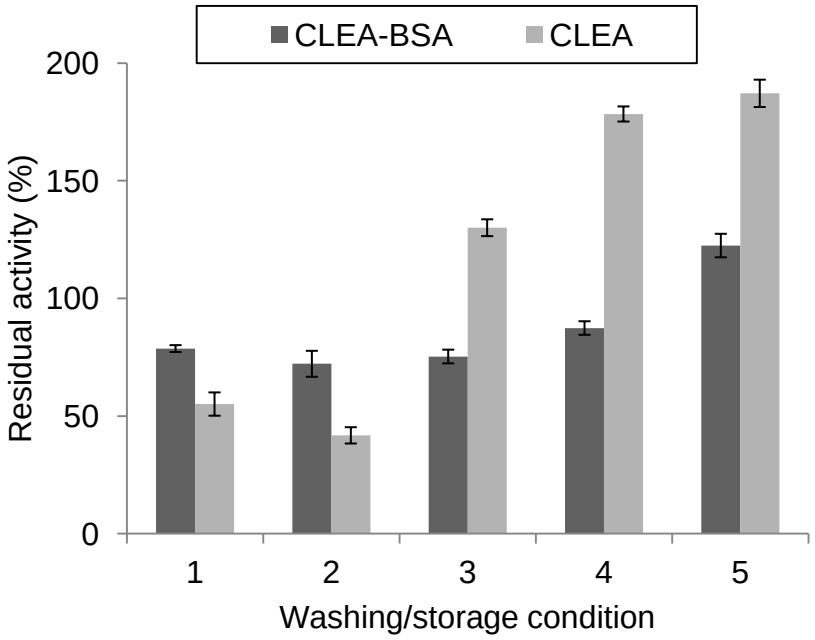

Fig. 10 Residual activity (relative to initial value) of enzyme aggregates after storage at different conditions for 2 weeks; Washing/storage conditions: 1-washed with buffer and stored in buffer at 4°C; 2-washed with buffer and stored in buffer at 20°C; 3-washed with buffer, dried in air and stored at 20°C; 4-washed with ethanol air dried and stored at 20°C; 5-washed with buffer, freeze-dried and stored at 20°C

However, upon drying, CLEA is able to lose more free water than CLEA-BSA. This is because, theoretically, BSA proteins in CLEA-BSA "hold on" to localized water molecules making it more hydrophilic and high in water activity. CLEA on the other hand is able to respond well to the drying conditions, resulting in activity hyper-activation. For CLEA samples, treatment condition 4 (when aggregates were washed in absolute ethanol and dried at room temperature) gave activity values that were statistically same as condition 5 (when aggregates were washed with buffer, freeze-dried and stored at room temperature, 20°C). Treatment condition 5 gave the highest levels of aggregate enzyme activity. However, lyophilization did not have any significant effect on activity of aggregates enzymes when

assayed immediately after freeze-drying. Thus, the observed storage stability behavior can be attributed to the combined effect of pretreatment and the long-term storage conditions. Additionally, CLCEPA activity is maintained at dried conditions and this suggests that some hydrophobic groups may be involved in its proteolytic action. The actual molecular mechanism responsible for this behavior is the subject of further investigation in our labs.

6.6. Conclusion

The design and evaluation of stable and robust cross-linked CEP aggregates (CLCEPA) from *Lactobacillus delbrueckii* subsp. *lactic* 313 is a major research endeavour, having several application prospects. We have demonstrated the plausibility of preparing aggregates from CEPs and optimized preparation conditions and parameters to improve activity recovery. The presence of proteic feeders and quenching of unreacted groups on aggregates significantly improved recovered activity and recyclability respectively. Whereas the incorporation of BSA into CLEA improved aggregates activity; it conduced to a compromise in drying stability, as well as the thermal stability at 50°C. Interestingly, treatment of enzyme aggregates in polar organic solvents and long-term storage under dried conditions resulted in hyper-activation of the enzyme activity. Molecular interpretation and mechanisms behind this behaviour requires further investigation. The proteolytic properties of CLCEPA together with their long-term storage and operational stability make them promising candidates for the production of protein-peptide mix products for health and functional food applications.

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7. CHAPTER 7: Concluding Remarks and Future Work

7.1. Conclusion

Proteolytic enzymes have a wide range of industrial applications in sectors such as medical, diagnostics, food, cosmetic, pharmaceuticals, leather, detergent, textile, photographic, bioremediation, and waste management. Most of these areas of applications have not been exploited for cell envelope proteinases (CEPs) of lactobacilli. Advancements in the industrial applications of lactobacilli CEPs will hinge on studies in the growth characterization and proteinase production by lactobacilli, as well as the downstream extraction and immobilization of CEPs to yield cheap and stable biocatalyst that can be used for various protein degradation processes.

In this study, the bioprocessing and immobilization of CEPs were explored for proteinases obtained from *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313). The key aim for the study was two-fold: (a) to produce CEPs cheaply by optimizing cell growth and fermentation parameters and conditions; and (b) to make CEPs produced recyclable and stable by deploying immobilization technologies. The objectives of this PhD research as outlined in Chapter 1 have been achieved and the core findings are reiterated in this section.

The growth conditions and metabolic processes responsible for profuse cell growth and enhanced proteinase production by LDL 313 have been identified. Being a thermophilic bacteria species, LDL 313 grows at optimum rate between temperatures from 37 to 45 ºC and the growth levels and cell densities increases with increase in temperature. Profuse cell growth and high production yields of products (organic acids) was observed when LDL 313 was cultured under anaerobic conditions, as opposed to the microaerophilic conditions thought to be the best gaseous

regime for lactobacilli. Additionally, significant quantities of cell-envelope proteinases are produced in the mid-exponential phase of fermentation cycle and anaerobic cultures produce more proteinases than microaerophilic cultures. LDL 313 was able to utilize several carbon sources (glucose, maltose, lactose) and maltose proved to be the best carbon source for cell growth and CEP production. Also, partial characterization of LDL 313 CEPs showed that since this bacterium is proteolytic for β-casein and κ-casein, CEPs from LDL 313 are to be classified in the class I (CEP $_{I}$) of the lactococcal proteinase classification system.

The culture conditions necessary to improve the expression of CEPs from LDL 313 grown in MRS medium have also been studied and optimised. It was found that the batch culture conditions studied (and optimum values) that enhance CEPs expression by LDL 313 are inoculum concentration (2% v/v), culture agitation speed (150 rpm), incubation temperature (45 °C), culture initial pH (6.0), and carbon/nitrogen ratio of production medium (1.0). This is the first reported study that deals with optimization of culture conditions for enhanced proteinase production in lactobacilli.

After addressing the issue of CEP expression, work was also done to identify the most effective extraction agent for LDL 313 cell-envelope proteinases. It was shown that CEPs are subject to autoproteolytic release and ionic misfolding in calcium-free buffer, and this forms the basis for their extraction. Incubation of cells in LiCl buffer released significant amounts of cell-envelope proteinases. The study also identified the sub-cellular localisation of CEPs in LDL 313 and found it to be located in the peptidoglycan cell-wall and not in the cell-membrane. In all, the problem of CEP production was addressed by the outcomes of the first two objectives of this

project. A cheap fermentation medium was utilized for the study and very simple strategies were used in the optimization of LDL 313 growth parameters to improve CEP yield. CEP extraction was achieved by the use of simple, inexpensive and non-toxic buffers and salts. These results therefore demonstrate that CEPs can be produced cheaply and economically. The production of CEPs constituted Part I of the entire Thesis.

Although the production and extraction of CEPs were studied and optimized in Part I, another challenge – that of poor storage and operational stability of soluble CEP – still remained. Therefore, two immobilization techniques were used in the preparation of immobilized CEPs that were stable, recyclable and also had improved 'hardiness' and operational stability. Two strategies for immobilization (covalent binding onto polyester fabric and cross-linked CEP aggregates (CLCEPA)) were developed where immobilization conditions were optimized for each method. These two methods were chosen for their ease of preparation, low cost of materials and reagents, food-compatibility and potential for scalability.

Covalently bound CEPs on polyester provided good recovered activity i.e 85% and also retained activity at other than usual conditions such as high temperatures (40 - 70 °C). Further, the recyclability was demonstrated by good retaining ~ 41% of initial activity after 5 times reuse. The storage stability of CEPs on polyester was also significantly improved with about ~ 96% of initial activity retained when stored in buffer at 4 °C.

Regarding cross-linked CEP aggregates (CLCEPA)) the presence of BSA improved retained activity almost twofold. CLCEPA retained activity at high temperatures (50 °C) as well as in

organic solvent conditions (i.e. 20 – 100 % ethanol/buffer mixtures). The recyclability of CLCEPA was reasonably good (~ 22% initial activity left after recycled 5 times). CLCEPA had good storage stability and was hyper-activated when dried in air during a-two week storage.

On the application side, both CLCEPA and CEPs on polyester were able to degrade several proteins including complex ones (chicken egg albumin, skimmed-milk protein), fractionated proteins (bovine casein, whey protein isolate), and purified proteins (bovine serum albumin, β-lactoglobulin). The application of immobilized CEPs in protein degradation to obtain industrially relevant products namely protein/peptide-rich product mixture from casein; and whey protein/peptide-based surface active foams was also explored. These are presented in Appendices A and B. The immobilization studies formed Part II of the Thesis.

Thus, by the application of simple optimization experiments, storage-stable and recyclable immobilized CEPs have been prepared. CEP production and extraction utilized cheap growth media as well as simple growth and extraction processes. Also, both immobilization methods employed were simple and quick, and also required cheap and food-compatible materials and reagents. These make them scalable with an industrial potential.

In summary, the potential of *Lactobacillus delbrueckii* subsp. *lactis 313* to produce cell-envelope proteinases has been explored and the stable forms of these enzymes have also been developed, optimized and tested with several food proteins. The outcome is a bioprocess for the production of cheap and cost-effective biocatalysts that can be used for various industrial applications in protein degradation and/or peptide production.

7.2. Future work

Enzymatic hydrolysis has been used in many industrial processes to improve the functional, nutritional and physiological properties of food proteins. Since the production of several useful products relies on the action of enzymes on proteins, it follows that the development of simple, cost-effective and scalable bioprocess is a worthwhile research endeavour.

In this study, the production and immobilization of cell-envelope proteinases (CEPs) have been investigated and optimized using *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) as the model organism. This PhD project represents an integrated bioprocess involving the development and establishment of an enzyme framework for protein degradation. It is a systematic engineering approach involving the production of cell-envelope proteinase raw materials in a cheap manner, and the preparation of stable biocatalysts that are also recyclable – making the bioprocess scalable with an industrial potential for protein hydrolysis. However, to achieve the full potential of CEPs in protein degradation, further related research work needs to be done.

First, future work elucidating the structure, composition and biochemical properties of CEPs needs to be carried out. For this project, crude enzymes were used in the immobilization studies. This has an economic advantage by eluding time consuming and relatively expensive purification steps. However, CEP purification and characterisation can assist in unravelling the biochemical properties of CEPs which, in turn, can guide the specific and rational design of the immobilization methods for an improvement in activity retention and immobilized enzyme performance.

Secondly, the molecular interpretation and mechanisms behind the behaviour of the immobilized CEPs requires further investigation. This is the first reported study of cross-linked enzyme aggregates for lactobacilli and it opens some interesting scientific questions. The cross-linked CEP aggregates which exhibited some interesting results such as high stability in organic solvents, but its reusability is not very high. The scientific understanding behind the remarkable improvement in activity (merely by changing certain physical factors such as drying conditions, aggregate size and presence of organic solvents) needs to be unravelled. The mechanical strength of the enzyme aggregates and their ability to withstand shearing at various agitation speeds must also be studied. This is particularly important for bioreactor scale-up protein hydrolysis operations. The discovered knowledge can be used to guide design of the second generation of cross-linked CEP aggregates with better reusability.

Lastly, future work resulting from this thesis should focus on the techno-economic aspects of the bioprocess. To develop an efficient and scalable enzyme-based bioprocess, prior cost budgeting and profitability analysis needs to be carried out to examine the commercial viability of using this bioprocess at industrial scale. The cost of bacteria cultivation, harvesting, proteinase extraction and immobilization, as well as utilities and energy involved also needs to be evaluated for the system using key economic indicators such as payback period, rate of return and net present value. The scale-up of cultures from flask to lab scale bioreactor and from pilot plant to industrial level will be important steps in the establishment of an industrial scale production platform for food peptides by the use of immobilized proteinases. The major steps involved in such a bioprocess (i.e. fermentation, enzyme extraction and enzyme immobilization) should

therefore be studied and assessed in order to estimate the economic feasibility as well as impacts on the environment, ideally by performing a life-cycle assessment (LCA).

8. APPENDICES

8.1. APPENDIX A - Immobilized proteinase packed-column reactor

APPENDIX A

Packed-bed enzyme bioreactor using immobilised cell-envelope proteinase: low-cost process for degradation of milk proteins

Abstract

A method for *in situ* immobilization of cell-envelope proteinases from *Lactobacillus delbrueckii* subsp. *lactis* 313 has been described to prepare a packed-bed enzyme reactor for degradation of dairy proteins. Polyester fabrics were packed in a column and enzymes were attached to polyester matrix via amine functionalization and glutaraldehyde cross-linking. The packed-bed enzyme reactor was evaluated for the degradation of casein protein and skimmed milk proteins (SMP) and showed, via a time course monitoring of the degree of hydrolysis (DH), that degradation and subsequent peptide formation trends were comparable for both proteins. RP-HPLC and SDS PAGE profiles of hydrolysates showed an assortment of peptide fragments having properties different from the parent proteins, demonstrating that the packed-bed reactor is effective for degradation of dairy proteins. The process for the development of this bioreactor is simple, cheap and scalable, making the bioreactor industrially feasible for the production of peptides from milk proteins.

Keywords: immobilized enzyme, cell-envelope proteinase, polyester fabric, peptide production, dairy proteins

INTRODUCTION

Proteolytic enzymes play a unique role in research and in many industries ranging from food, pharmaceuticals and diagnostics to waste treatments and the textile industry. In the dairy industry, enzymatic hydrolysis of proteins is a mild, non-toxic useful process that can be used to improve the physical, chemical, and organoleptic properties of the original food without compromising the nutritive value. Partial pre-digestion of food also improves intestinal absorption and bioavailability (Gonzàlez-Tello, Camacho, Jurado, Páez, & Guadix, 1994).

Cell-envelope proteinases (CEPs) are a special class of proteolytic enzymes produced by lactic acid bacteria to aid cell growth in protein-rich media (Tsakalidou, Anastasiou, Vandenberghe, van Beeumen, & Kalantzopoulos, 1999). In the food and pharmaceutical industry, CEPs are used in the production of bioactive peptides and also to improve texture and organoleptic characteristics of dairy products. However, the use of free proteases (CEPs especially) is currently suboptimal due to drawbacks such as high cost, poor stability, and lack of multiple utility. These drawbacks make soluble enzyme-based processes economically unfeasible, hindering their potential broad application.

Enzyme immobilization has been used to improve enzyme stability and biocatalysis performance for various technological and industrial applications (Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007). Immobilization also minimizes unacceptable enzyme-enzyme interactions that compromise enzyme activity or causes auto-degradation (Goradia, Cooney, Hodnett, & Magner, 2006; Purcena, Caramori, Mitidieri, & Fernandes, 2009). Despite the relatively large body of research on immobilized enzymes, some of them are still

economically unfeasible. This is attributable to difficulty of scaling up, high cost of the carrier and the immobilization techniques employed, and/or limitations on the types of reactor that can be selected (Yang & Chase, 1998).

In the food industry, conventional fixed-bed reactors with immobilized enzyme have been used in commercial-scale operations for the production of several products (Nakajima, Nishizawa, & Nabetani, 1993). For the purposes of bioreactor applications, the use of immobilized enzymes on synthetic polymer is particularly practical and advantageous since it offers certain advantages over the use of porous beads. For example, the use of porous beads in fixed bed reactors is plagued with low mass-transfer efficiencies and thus less efficient utilization of the immobilized biocatalyst, especially for bulky macromolecular substrates with low diffusion coefficients (Yang & Chase, 1998). The use of synthetic polymers ensures direct surface-to-surface contact of substrate and catalyst while offering high flexibility and resistance against mechanical stress (Nouaimi, Möschel, & Bisswanger, 2001; Purcena, et al., 2009).

In a previous study (See Chapter 5) we examined the use of poly(ethylene terephthalate) (PET) as a support for immobilization of enzymes via amine functionalization and cross-linking with glutaraldehyde. The immobilization procedure is simple and quick; and requires low preparative and economic expense for fabrics (polyester), amine linkers (ethylene diamine) and cross-linkers (glutaraldehyde). Enzyme immobilization by this method has wide industrial and economic significance in bioactive peptide production since it offers the advantages of enhanced stability, protection against autolysis, repeated use of the catalytic material and ability to release peptides from macromolecular protein substrates. Additionally, the polyester support has high operational

stability and excellent resistance to compression and these properties make them suitable in the development of automated flow bioreactors.

In this study, we have developed a low-cost fixed-bed bioreactor for peptide production using cell-envelope proteinases (CEP) from *Lactobacillus delbrueckii* subsp. *lactis* 313. Operational conditions were optimized for peptide production in the bioreactor and the peptides generated were analyzed by high performance liquid chromatography. We have demonstrated that immobilized CEP column bioreactor has an attractive efficiency in the digestion of a variety of dairy proteins. This simple and low-cost system can widely be used in the dairy industry.

MATERIALS AND METHODS

Materials

Non-woven polyester material [poly(ethylene terephthalate)] (PET), was provided by Dr Yuan Gao at CSIRO Materials Science and Engineering Division. Ethylene diamine (EDA, 99%), grade II glutaraldehyde solution (25%), and casein from bovine milk (technical grade) were all obtained from Sigma-Aldrich (Castle Hill, Australia); buffer substances (Tris hydrochloride, sodium mono phosphate and disodium phosphate); Ethylenediaminetetraacetic acid (EDTA), glycine and sodium chloride were obtained from Merck (Darmstadt, Germany). Skimmed milk proteins were obtained from Murray Goulburn (Melbourne, Australia). Protein assay kit was obtained from Biorad Laboratories (Richmond, CA).

Cell biomass and preparation of crude cell envelope extract

Cell biomass and preparation of crude cell envelope extract is described in (Agyei & Danquah, 2012a). *Lactobacillus delbrueckii* subsp. *lactis* 313 (ATCC® 7830™; LDL 313) was obtained from American Type Culture Collection, propagated twice in deMan-Rogosa-Sharpe (MRS) Broth (Oxoid Pty Ltd, Australia) at 37 °C and stored at -70 °C. Frozen cells were thawed, plated, and cultured in fresh MRS Broth (Oxoid Pty Ltd, Australia) at 37 °C for 12 h, and aliquot taken to inoculate fresh MRS medium (Acumedia, Michigan, USA) to an initial optical density at 560 nm (OD_{560}) of 0.1 ± 0.005. Cells were allowed to grow (shake flask, 45 °C, 100 rpm), to late exponential phase (OD_{560} = 1.117 ± 0.070). This OD corresponds to about 229 μg ml^{-1} dry cell weight, from a previously prepared calibration curve for converting OD 560 nm units to dry cell weight units. Cells were harvested by centrifugation (4,000×g, 4°C, 10 min) and concentrated to 5 times the initial OD_{560} in the extraction solution containing 50 mM sodium phosphate buffer,

10 mM EDTA (pH 7). This system was incubated (28 °C, 2 h, 50 rpm) after which the setup was centrifuged (4,000×g, 4 °C, 10 min). The supernatant was designated crude cell-envelope proteinase (CEP). Protein concentration was determined with a Protein Assay Kit (Bio-Rad Laboratories).

Immobilization of enzymes on polyester

PET fabric was cut into circular pieces (1.5 cm diameter and ~ 44 mg each) with a puncher. The polyester fabrics were packed in a high-density polyethylene (HDPE) column to a compressed height of 6 cm (total mass of fabric was 3.22 g), and connected in a setup as shown (Figure A1). Internal diameter of column was 1.5 cm, the length of column being 6 cm. Packed-bed volume was 10.6 ml and void volume of system (column and tubings) is ~ 3 ml

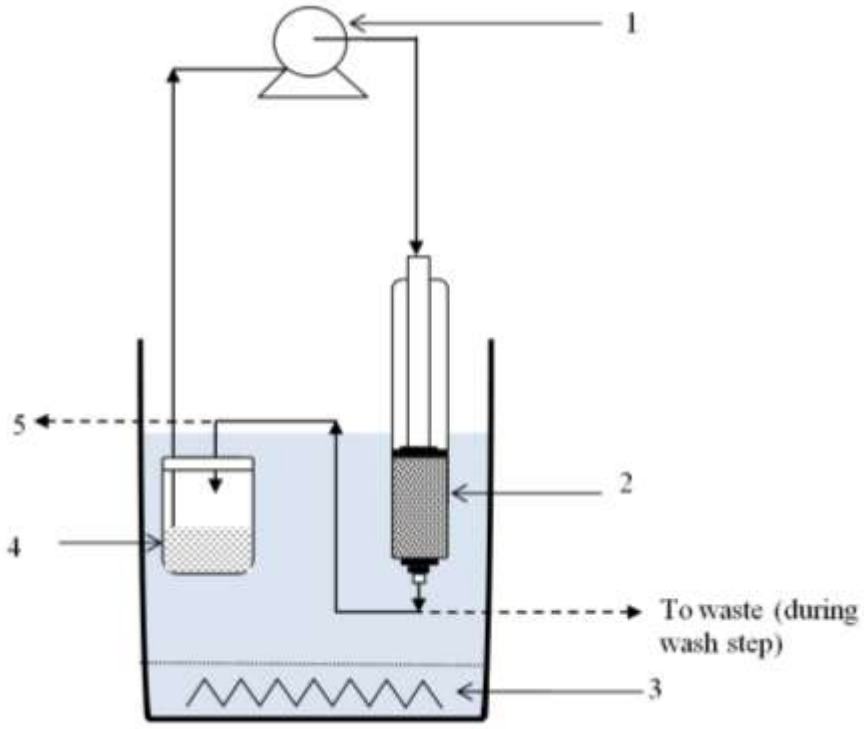


Figure A1: Setup for the preparation of immobilized enzyme bioreactor. 1- Peristaltic pump; 2- packed-bed column; 3- preset water bath incubator; 4- reservoir for activation reagents or substrate; 5- sampling point

The packed fabric was reacted with 99% EDA in a recycle mode at a flow rate of 0.5 ml min^{-1} (with incubation at 60 °C in a water bath for 90 min). This ensured the fabric was treated with a minimum of four column volumes of EDA. EDA activated fabrics were washed intensely with deionised water by connecting and fresh feed of water and discarding the waste until no EDA was detected (EDA removal was tracked by measuring pH of waste stream).

The EDA-activated fabrics were reacted with 5% v/v glutaraldehyde (GA) in phosphate buffer by placing GA in the reservoir and allowed to run at 0.5 ml min^{-1} and 90 min at room

temperature. GA-treated fabric in column was washed intensely with deionized water. GA removal was tracked by reacting waste stream with 1.25 M glycine and measuring absorbance at 405 nm. A wavelength scan was first conducted which revealed a concentration-dependent peak at 405 nm. The GA activated fabrics were then treated with enzyme (13 ml of 0.251 mg ml^{-1} crude CEP) by running the setup at 0.5 ml min^{-1} in a cycle mode for 18.5 h. Washing of free and adsorbed CEP was done with buffer (50 mM Na-phosphate buffer, 1 M NaCl, pH 7.0) and run until no free enzymes were detected in washing buffer (as measured by absorbance at 280 nm). Unreacted functional groups on fabric were quenched by treating with 1 M Tris.HCl pH 7.6 (0.1 ml min^{-1}, 2 h, room temperature) and washed again with 1 L of buffer (50 mM Na-phosphate buffer, 1 M NaCl, pH 7.0). When in storage, the immobilized CEP packed-bed column was saturated with 50 mM Na-phosphate buffer, supplemented with 0.02% NaN_3 to discourage microbial attack.

System configuration

The system configuration for operation of immobilized CEP bioreactor in recycle mode is shown in Figure A1. Reagents, buffers and substrates used in this immobilized CEP bioreactor system could easily be pumped into the process equipment. This is a desired feature under industrial settings and lends itself for the development of a continuous and *in situ* process for bioreactors. It ensures containment of chemicals during operation addressing the safety concern for the operators and production personnel.

Optimization of protein hydrolysis conditions (effect of flow rate and substrate concentration)

Flow rate and substrate concentration were optimized for the bioreactor by the use of casein supplemented with 0.5% v/v benzyl alcohol to control microbial growth that may arise due to prolonged incubation. Casein samples were reacted in bioreactor at 35 °C in a recycle mode for 24 h at varying flow rates and substrate concentrations. Samples of volume in the range of 0.05 – 0.1 column volume were collected at intermittent time interval for further analysis. Hydrolysis of milk proteins (casein and skimmed-milk proteins) were conducted on the column using the optimum conditions of flow rate and substrate concentration.

Peptide assay via o-phthaldialdehyde (OPA) reaction

The OPA (*o*-phtaldialdehyde) reaction was used to estimate peptides released from macromolecular protein substrates by immobilized enzymes bioreactor via the measurement of free amino acids and peptides containing primary amino acids. OPA reagent was prepared according to (Nielsen, Petersen, & Dambmann, 2001) and the OPA method used was according to (Alhaj, Kanekanian, Peters, & Tatham, 2010) with slight modification. About 20 μL of unhydrolysed protein or protein hydrolysed by immobilized enzyme were added to 50 μL of OPA reagent containing dithiothreitol (DTT), then 130 μL of deionised water was added. This was mixed briefly and left to stand at room temperature (21°C) for 10 min. The absorbance of the solution was measured at 340 nm. The absorbance readings were discounted from the initial reading of unhydrolysed proteins. The degree of hydrolysis for each sample was determined according to the following equation:

$$\% \text{ Degree of Hydrolysis} = \frac{(S-C)}{D} \times 100 \quad [5]$$

where C is the reading for unhydrolysed protein (control), S is the sample reading of the protein hydrolysate formed by immobilized enzyme (samples), and D the difference between the sample reading after 56 h (taken as 100% degree of hydrolysis) and the unhydrolysed protein reading taken as 0%.

Reversed –phase HPLC analytical method for detection of peptides

Reversed-phase HPLC (RP-HPLC) was carried out using an analytical C18 column (Jupiter, 250mm×4.6mm, 10µ,300 Å (Phenomenex, Torrance, CA, USA) connected to a Bio-Rad Biologic DuoFlow FPLC system (Hercules, CA, USA) with a mobile phase A (0.1% (v/v) trifluoroacetic acid (TFA) in water) and B (0.1% (v/v) TFA in acetonitrile). The concentration of protein and formation of peptides were monitored at 214 nm wavelength. Separation was achieved by using a gradient from 10% (v/v) B to 65% (v/v) B in 15 min at a flow rate of 1 mL min^{-1}. The samples were collected at regular time intervals from the packed column outlet and a volume of 0.1mL was injected for analysis.

Protein hydrolysis estimation by SDS-polyacrylamide gel electrophoresis (SDS PAGE)

Aliquots of protein samples taken intermittently as described above were mixed in a 1:1 ratio with solubilisation buffer (Kojic, Fira, Banina, & Topisirovic, 1991), heated for 5 min at 100 °C, and analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (16 % separating, 8% stacking) according to the standard method of Laemmli (1970).

RESULTS AND DISCUSSION

The immobilization protocol presented here is cheap, simple and scalable. Glutaraldehyde activation of immobilization supports is simple and its *Generally Recognized As Safe (GRAS)* status allows for its utilization in food and pharmaceutical industries (Guisan, Bentacor, & Fernandez-Lorente, 2013). Nonwoven polyester ([poly(ethylene terephthalate)], PET) was used as support material in this study. The synthetic polymer is partially intertwined mass of fibres adhering together and forming a fluffy porous fabric sheet. The structure remains unchanged after the immobilization protocol. The rigid structure and mechanic tightness make it an excellent support for enzyme immobilization (Nouaimi-Bachmann, Skilewitsch, Senhaji-Dachtler, & Bisswanger, 2007; Nouaimi, et al., 2001). Like other polymer fibres, the absence of pores is an important morphological characteristic of PET that allows direct and quick access of enzyme by substrate thereby eluding time consuming substrate diffusion steps (Goradia, et al., 2006; Purcena, et al., 2009). The chemical inertness of PET enables it to be used in procedures involving chemicals and higher-than-normal temperatures. Moreover, when used in reactors, fabrics and products from the reactor are automatically separated without any filtration step; unlike granule– or aggregate–based immobilization products which must be filtered after the enzymatic reactions and often results in contamination of final product with catalyst residues (Opwis, 2010).

Cell-envelope proteinases of lactobacilli, like most microbial enzymes, are relatively inexpensive since expression and extraction are cheap (Agyei & Danquah, 2011). In previous studies by Agyei et al, the batch conditions necessary for the production of high yields of CEPs from

Lactobacillus delbrueckii subsp. *lactis* 313 have been optimized (Agyei & Danquah, 2012b; Agyei, Potumarthi, & Danquah, 2012). In this study, crude CEP from LDL 313 was used and the immobilization yields were high (55% total protein immobilized). Additionally, the immobilized CEP bioreactor will provide economic or and technical advantages making it industrially relevant. Crude CEP used in this work did not require prior purification, thus overall production cost of this immobilization-bioreactor system. The operation configuration of the immobilized CEP bioreactor system is given in experimental section.

Selection of operation modes for peptide hydrolysis

Proteins hydrolysis by the immobilized CEP packed-bed bioreactor was tested by the use of total casein, in three modes, namely, 'stagnant', recycle and continuous modes. With the 'stagnant' mode, about 3 ml of 2 mg ml^{-1} was pumped into the column and stopped at the exit. This was left to incubate (35°C) for 11.5 h after which the protein-peptide mixture was pumped out. Casein hydrolysis by recycle mode was done by recycling 6 ml of 2 mg ml^{-1} casein at a flow rate of 0.5 ml min^{-1} for 26 h. The continuous mode was operated by running 10 ml of 2 mg ml^{-1} casein through the column at a flow rate of 0.1 ml min^{-1} without recycling. Peptides generated from all hydrolysis modes were monitored by OPA reaction assayed. It was observed that peptide production by recycled mode was higher than other modes of operation (results not shown). Thus, a recycle mode has been chosen for the remaining part of this work.

Optimization of operational conditions

Flow rates

In bioreactor studies, the flow rate of operation is directly related with residence time for reaction of substrate in column bioreactor in a recycle mode. Figure A2 shows the effect of flow rates on the release of peptides from casein. It was observed that small flow rate of 0.1 ml min^{-1} gave the highest yield of peptides especially after over 4 h of hydrolysis. At this flow rate the residence time of substrates was 100 min and increasing flow rate further resulted in a decrease in peptide yields (Figure A2). The flow rate of 0.1 ml min^{-1} was enough to allow maximum contact of substrates with immobilized enzymes thus giving high peptide yields and was therefore chosen for subsequent experiments.

The reaction rates and conversion and/or selectivity of a reactor is substantially influenced by transport limitations either inside a biocatalyst (for bead-based immobilized systems) or through the film between the biocatalyst surface and the flowing substrate (Eigenberger, 1984). Several studied on bioconversion with the used of packed-bed reactors indicate that for continuous processes, the use of higher feed flow rates results in smaller conversion percentage, due to lower residence times (Al-Mayah, 2012; Fischer, et al., 2013; Lee, Ahn, & Ryu, 1983). In a recycle mode, it is expected that increasing flow rate should increase yield due to enhanced mass transfer at a constant average reaction time. However, the contrary was observed in this study where peptide yields decreased with increase in substrate feed flow rate.

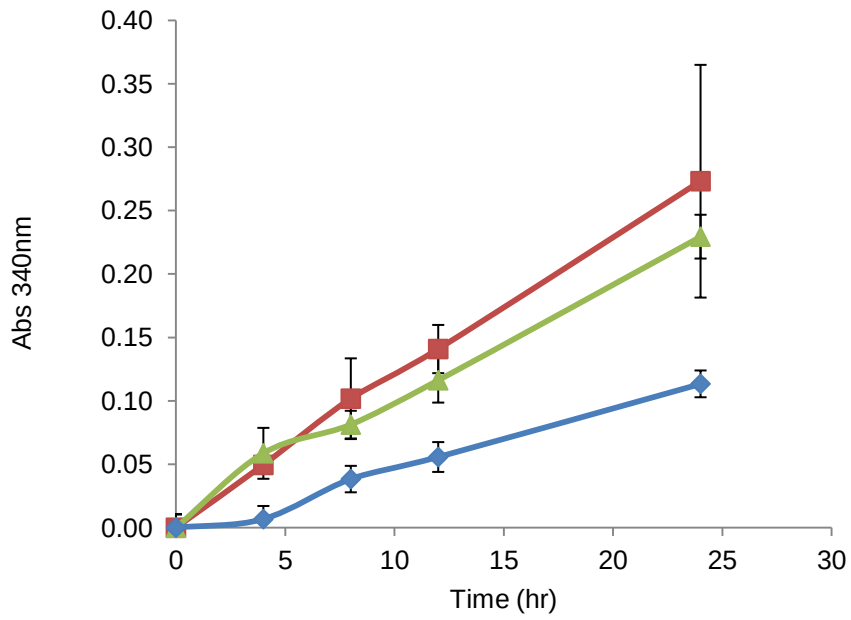
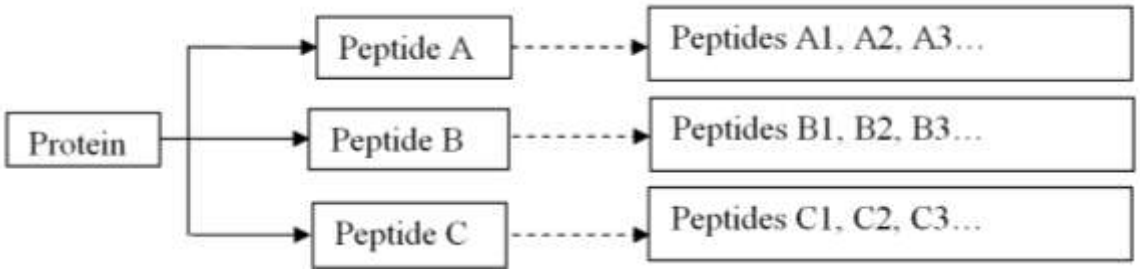


Figure A2: Time course of casein hydrolysis at different flow rates. (Experiment conditions: recycle mode, 2 mg ml^{-1} casein, 35°C; 0.1 ml min^{-1} (■); 0.25 ml min^{-1} (▲); 0.5 ml min^{-1} (♦)).

In a recycle mode, increase of flow rate will decrease the residence time per cycle while increasing the number of cycles if total reaction time is fixed. The observation in Figure A2 is probably linked to unique reaction mechanism associated with protein hydrolysis because macromolecular proteins are susceptible to sequential hydrolysis where the product of one step serves the reactant of another thus giving a large combination of plausible peptide products (See Scheme A1). Compared with processes such as the conversion of glucose to ethanol, enzymatic hydrolysis of proteins is not a single step reaction and the susceptibility of proteins/peptides to further degradation is a phenomenon that is enhanced when the reaction occurs at lower feed flow rates (i.e. higher residence times in one cycle). Thus, at lower flow rates more peptides are hypothetically released from the proteins, with peptide yield being a function of enzyme specificity and mean residence time.



Scheme A1: Schematic of sequential hydrolysis of proteins and peptides

Additionally, the OPA assay used in estimating peptide yield is a non-specific assay for peptides and quantifies all species with primary amino groups. Thus, more products (peptides) are obtained at feed flow rates with longer residence time and higher susceptibility to sequential hydrolysis (i.e. 0.1 ml min^{-1}, residence time 100 min), compared with higher flow rate of 0.5 ml min^{-1} with the residence time of only 20 min. This could also account for why the typical asymptotic curve observed with other single transformation enzyme reaction (such as conversion of glucose to ethanol) is not seen in this study, even after prolonged incubation time.

Substrate concentration

In order to investigate the effect of substrate concentration on casein hydrolysis, different casein substrate concentrations (0.5 mg ml^{-1}; 1.0 mg ml^{-1}; 2 mg ml^{-1}; and 5 mg ml^{-1}) were used. Sample flow rate of 0.1 ml min^{-1} and incubation temperature of 35 °C were used in the experiment. The results are shown in Figure A3. It can be seen that increasing the substrate concentration increases the casein hydrolysis or peptide yield since rate of enzyme reaction is proportional to substrate concentration.

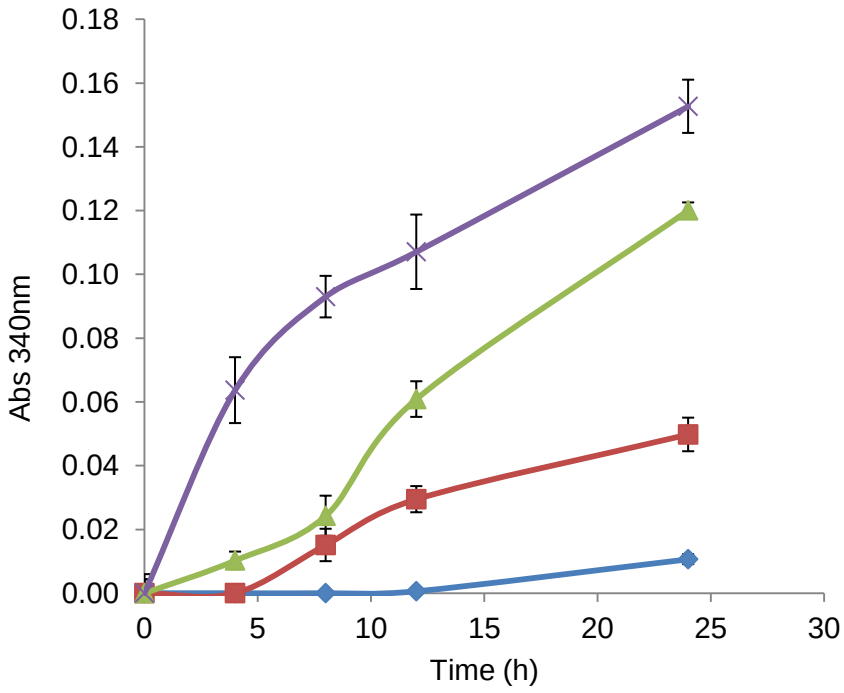


Figure A3: Time course of casein hydrolysis at different substrate concentrations (Experiment conditions: recycle mode; flow rate 0.1 ml min^{-1}; incubation temperature 35°C; Legend: 0.5 mg ml^{-1} (♦); 1.0 mg ml^{-1} (■); 2 mg ml^{-1} (▲); 5 mg ml^{-1} (×))

The catalytic efficiency of enzymes is a well known phenomenon that increases with substrate concentration until an optimal substrate concentration is attained where enzyme active sites become saturated and can no more bind to new substrates. Enzymatic protein hydrolysis is usually studied up to maximum substrate concentrations of 10% (w/v). However, this concentration dependence is protein- and enzyme- specific and higher concentrations have been shown to affect the protein hydrolysis (Butré, Wierenga, & Gruppen, 2012; Chabanon, Chevalot, Framboisier, Chenu, & Marc, 2007; Gonzàlez-Tello, et al., 1994; Márquez & Vázquez, 1999). Within the range of casein concentration tested in this study, it was observed that 5 mg ml^{-1}

casein gave the highest yields of peptides at all times during incubation. The fact that increasing the casein concentration causes an increase in reaction rate (Fig. 3), suggests that new molecules are attacked several times. The increase in the peptide yield may be the result of a higher reaction rate, indicating that the system was under-saturated at the lower casein concentration.

For a typical enzymatic reaction conducted at varying substrate concentrations, the observed activities can be transformed to calculate Michaelis-Menten constants for the reaction. However, for protein hydrolysis, a kinetic study of the process is complex. This is because different types of peptide bonds are involved and each differs in vulnerability to attack by enzymes during the hydrolytic process (Gonzàlez-Tello, et al., 1994; Márquez & Vázquez, 1999). The phenomenon is represented above in Scheme 1A and the multiple reactions make it difficult to interpret the experimental results via simple kinetic equations. As such characterization of protein hydrolysis is usually studied in terms of the degree of hydrolysis achieved in the course of reaction (Gonzàlez-Tello, et al., 1994). In this study, the degree of hydrolysis of casein was therefore used to study the catalytic performance of the immobilized CEP bioreactor. Results are presented in subsequent sections.

Degree of hydrolysis (DH)

The degree of hydrolysis (DH) of total casein and skimmed-milk protein (SMP) hydrolysed by immobilized CEP bioreactor is shown in Figure A4. DH of both proteins was similar at initial reaction times (< 12 h). Casein hydrolysis however was marginally higher than that of SMP, after 12 h of hydrolysis. After 48 h of reaction DH values of 64.3% and 73.0% were achieved for casein and SMP hydrolysis respectively.

SMP comprises of the caseins (including α-, β-, and κ-caseins) and whey proteins (α-lactalbumin, β-lactoglobulin, serum albumin, and immunoglobulins). Because CEP- immobilized bioreactor was able to hydrolyze purified caseins as well as casein in its native state (i.e. in SMP) and whey proteins, it has a high potential for the production of peptides from bovine milk.

The disposal of whey by-product has remained a major challenge for the dairy industry and the conversion of whey to bioactive peptides has been argued as an industrially feasible and lucrative option (Koutinas, et al., 2009; Morais, et al., 2013). The bioreactor system developed here can therefore be used for the conversion of whey proteins to useful bioproducts. The ability of CEPs in the conversion of whey can be exploited in overcoming the challenge of whey disposal and to provide additional benefits to the functional food and pharmaceutical industries.

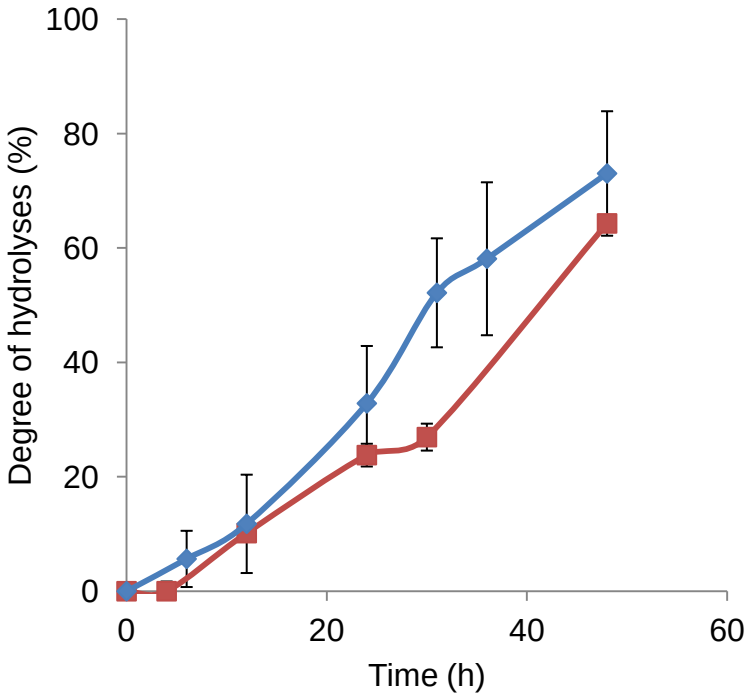


Figure A4: Comparison of the degree of hydrolysis of casein (♦) and skimmed-milk protein (■) by immobilized CEP bioreactor. (Mean of n = 4 experiments)

In the food industry, the functional and bioactive properties of protein hydrolysates have been shown to be affected by DH (Chen, Chi, Zhao, & Xu, 2012; Morifuji, et al., 2010). For example, protein hydrolysates intended for use in infant formulas must be hypoallergenic and have acceptable flavor. These properties are achieved when the hydrolysates are composed of peptides with two to six amino acids since humans absorb more nitrogen when it is delivered as di- or tripeptides rather than as whole protein or free amino acids (Gonzàlez-Tello, et al., 1994; Morifuji, et al., 2010). However, whereas higher levels of proteolyses (higher DH values) is a desirable property for immobilized enzyme for use in industrial applications such as waste treatments, a higher DH is not necessarily better for the purposes of peptides production intended for functional food applications. This is because, peptide bioactivity is determined by the size, type and amino acids sequence of the peptide. Over-hydrolysis of the protein molecule may cause the peptide to lose its bioactive properties, or may convert peptides to amino acids. This phenomenon is confirmed in several studies where the biological activity of protein hydrolysates has been shown to be lost when DH of protein is higher than a critical value (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Wu, Wang, & Xu, 2008).

Analysis of peptides by reversed-phase liquid chromatography

Reversed-phase HPLC analytical method using C18 column was used to monitor the progress of the protein hydrolysis by immobilised CEP. Casein and SMP contain a mixture of different protein species which were eluted as multiple peaks (a-e) (Figure A5). These being major components of milk are very hydrophobic proteins and therefore eluted towards the end of the gradient.

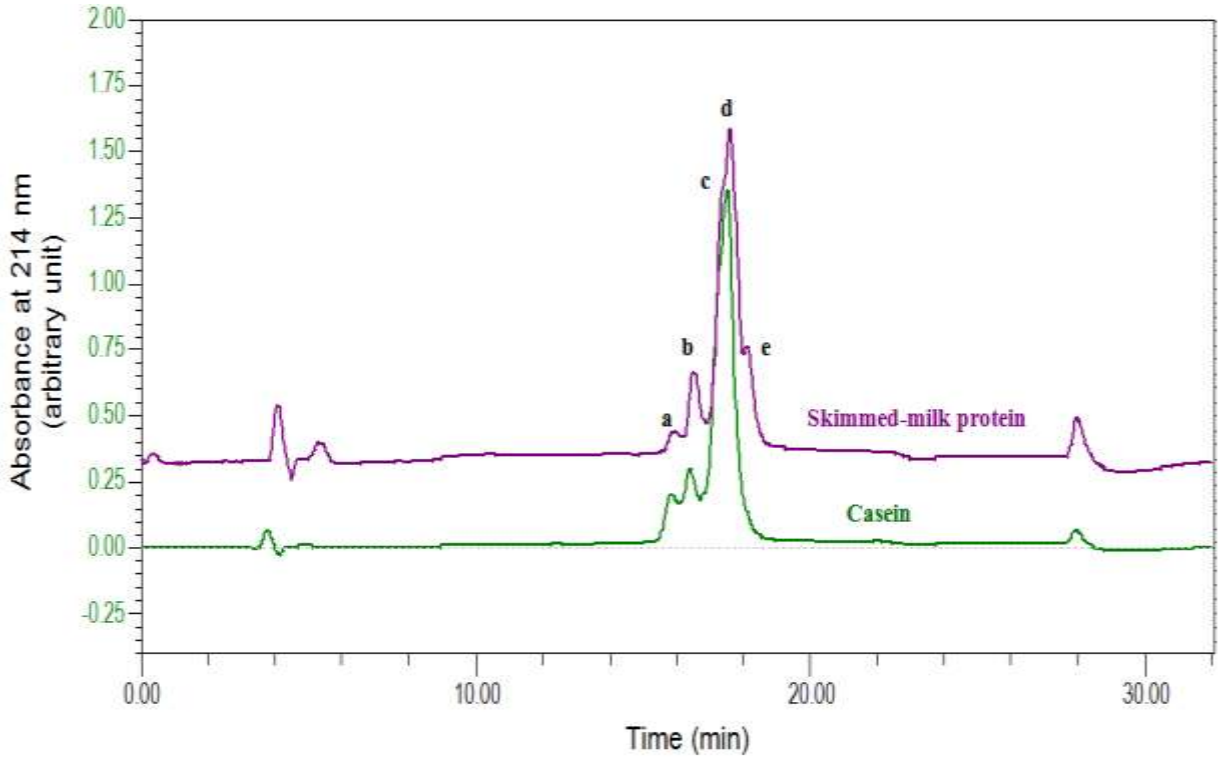


Figure A5: RP-HPLC profiles of casein and SMP before under linear gradient condition from 10% - 65% (v/v) buffer B in 15 min at 1 ml min^{-1}. For clarity, profile of SMP was shifted with constant baseline unit of 0.30 AU

Immobilised CEP in a packed column when operated in the optimum conditions showed degradation and generation of peptides over time (Figure A6A). The first eluted fragment (peak 1) was observed in 6 h and showed a steady increase with time. The lower retention time of peak 1 indicates that the specie (peptide or mixture thereof) is hydrophilic compared to casein. The difference of retention time between peak 1 and casein suggests that peak 1 contains peptide species that may have properties very different from native casein. Other protein hydrolysates were generated after 36 h of reaction and observed as significant peaks at 48 h with peak 3 being the most hydrophilic peptide generated. The RP-HPLC results also illustrate that the immobilised CEP efficiently hydrolysed SMP (Figure A6B).

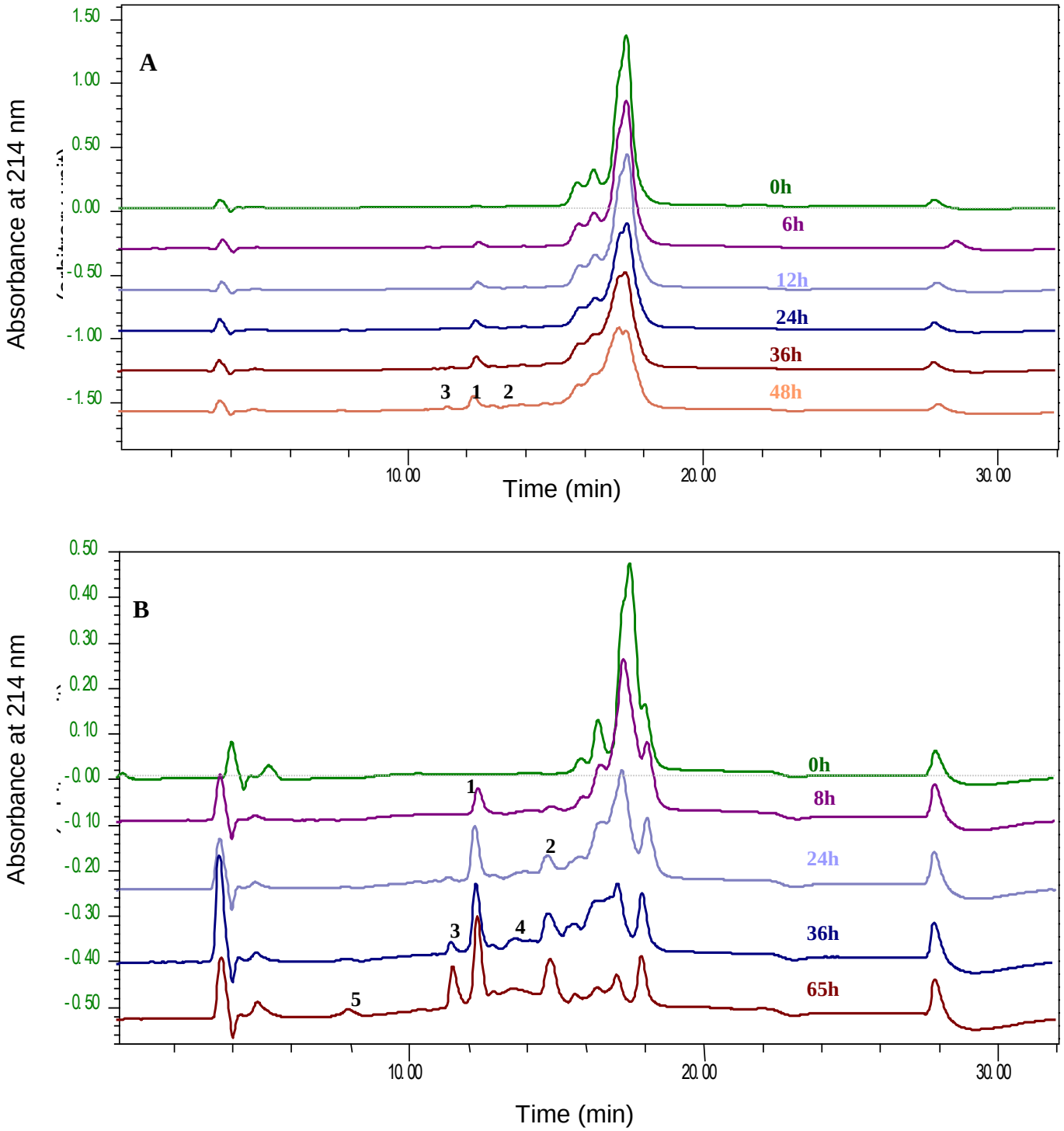


Figure A6: RP-HPLC profiles of casein (A) and SMP proteolysis (B) at different degradation times. Samples were eluted on HPLC column under linear gradient condition from 10% - 65% (v/v) buffer B in 15 min at 1 ml min^{-1}. For clarity, HPLC profiles have been sequentially shifted with constant baseline unit of 0.31AU and 0.1AU for (A) and (B), respectively.

The first hydrolysate fragment (peak 1) was observed at 8 h and continued to increase with time. The second product of proteolysis is peak 2 which appears as a distinct peak at 24 h and onwards. The retention time of peak 2 is very close to intact SMP and can be deduced to be partially cleaved SMP rather than peptide fragments. Peaks 3 and 4 appear simultaneously after 36 h of proteolytic reaction with significant degradation of SMP. At this stage the partial cleavage of SMP have exposed new cleavage sites within SMP and resulted in peptides with different hydrophobic property. Peak 5 appears almost during the end of the process and is the most hydrophilic peptide fragment cleaved during the reaction. The late appearance of peak 5 can be attributed to the process being performed in recycle mode. As a result of recycling intact SMP, partially cleaved SMP and hydrolysates thereof are being introduced repeatedly to the proteolytic action of CEP. Consequently, there is a possibility that peptides already formed may undergo further cleavage (as shown in Scheme 1) to form smaller peptide mixtures that are relatively less hydrophobic than the parent peptide.

Comparing degradation patterns based on RP-HPLC data it is realised that peaks 1 and 3 of casein hydrolysates and peaks 1 and 3 of SMP hydrolysates are alike. This is evident from the similarity of the retention times of these peaks. Since casein is also one main component in SMP it is apparent that some extent of similarity in the peptide fragments is expected. On the whole, it can be inferred from RP-HPLC profile data that specific mixed–peptide species are formed at specific stages of the reaction. This provides scope for the process to be tuned for the generation of specific peptides of interest. Also the operational mode of this bioreactor allows the intermittent collection of eluate for specific selection and simultaneous isolation of unique

hydrolysates or peptide species. By fine–tuning the process parameters it is anticipated that this process can be exploited for the production of different peptides from a single system.

SDS PAGE of skimmed milk proteins

Figure A7 shows *SDS PAGE analysis of* the hydrolysis products of SMP. The typical profile of skimmed-milk proteins shows the following bands at ~ 70-80 kDa, 25-30 kDa, and ~17 kDa which corresponds to serum albumin, and immonoglobulins; casein; and lactalbumin and β-lactoglobulin respectively (Nikkhah, 2011). In this study, hydrolytic degradation products are observed as bands at between 17-25 kDa for aliquots sampled at 4, 12 and 24 h hydrolysis time. After 12 h reaction time, two new bands are observed at 7-17 kDa. Amongst the various protein species present in SMP, maximum degradation was observed for casein, which is evident from the diminishing bands corresponding to the molecular weight of casein. Although macromolecular proteins are degraded with time, a corresponding increase in peptide bands intensity is not observed. This could be due to, either the sequential hydrolysis of the peptide product, or the inability of Coomassie blue stains to visualize low molecular weight peptides at lower concentration (Jang & Lee, 2005). However, RP-HPLC chromatogram confirms the presence of peptides during the later stages of the reaction (Figure A6).

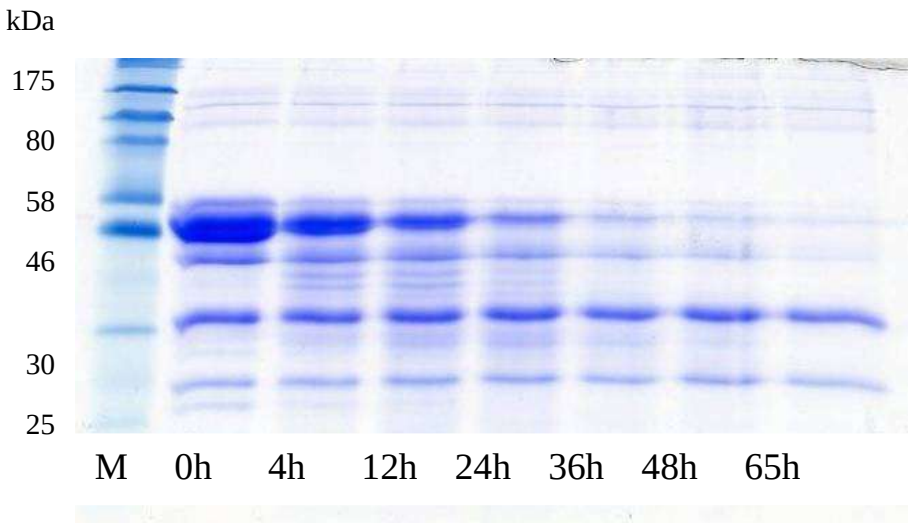


Figure A7: SDS PAGE profile for the hydrolysis of skimmed-milk protein; 16% separating gel, 8% stacking. Samples were taken at the hour times indicated. M, molecular weight ladder.

CONCLUSION

In a quest to develop an industrially feasible and low-cost process for peptide production, this work demonstrates the potential of a packed-bed immobilized enzyme reactor for peptide production from milk proteins using cell enveloped proteinase. Low-cost polyester fabric was used as carrier material for the immobilization of cell-envelope proteinases (CEP) from *Lactobacillus delbrueckii* subsp. *lactis* 313. An *in situ* immobilization approach provides a simple and controllable process that can be scaled-up for industrial operations. The *in situ* method also addresses the safety aspect by containment of the chemical immobilization process within the column reactor. The choice of raw materials ranging from the support matrix to chemicals and substrates in this work are inexpensive, and also have "generally regarded as safe" (GRAS) status that are essential for industrial production of peptides for food and pharmaceutical applications. The option of recyclability of the chemicals like EDA and GA

provide additional benefit of cost reduction. A detailed knowledge combining the properties of produced peptides and degradation kinetics could guide fine-tuning the process parameters such as reaction time to generate specific peptide species of interests. Such optimization of degradation process can help reduce purification costs in the subsequent steps. Moreover, by controlling the reaction time, different peptides can be produced at specific stages of the reaction. Therefore this technology provides the possibility of producing different peptides from a single bioreactor system.

In conclusion, the study demonstrates the utilization of immobilized CEP packed-bed column for the degradation of milk proteins and the generation of specific and unique peptides for food and pharmaceutical applications. However, more experiments are needed to provide data that gives a full description of the biocatalytic properties of the column against the background of bioprocess economics. Some of the experiments to be done are enumerated below:

1. What is its operational stability?
2. What is the activity of the immobilised enzyme in the column?
3. What is the immobilisation yield?
4. Recyclability: are there any possibilities for reuse of the column reactor?
5. Are there any possibilities for continuous use of column reactor? If so, how long?
6. Are there any economic advantages of this column? Provide quantitative data
7. Estimate the effect of operation mode (recycle, continuous and stagnate modes) – include results for each mode.
8. Effect of flow rate on product yield (wider range)
9. Effect of substrate concentration (wider range), i.e. 0 - 10% (w/v) [0 – 100 mg/ml]
10. Effect of incubation temperature
11. Chemical stability (resistance to inactivation by urea or Gn-HCl)
12. Degree of skimmed-milk hydrolysis by selecting optimum conditions from above

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8.2. APPENDIX B - Foaming food protein hydrolysates

APPENDIX B

Interfacial and foaming properties of protein hydrolysates prepared using immobilized proteinases from *Lactobacillus delbrueckii* subsp. *lactis* 313

ABSTRACT

Protein hydrolysates are amphiphilic substances that are used as emulsifiers in food dispersion products as a result of their surface active properties and interfacial characteristics. It has been shown that these interfacial properties are influenced by processing conditions such as such as hydrolysis. Thus, foaming behaviour can be obtained from some food protein hydrolysates via controlled enzymatic hydrolysis. In this study, immobilized cell-envelope proteinases were used to degrade several food proteins to produce hydrolysates and the foaming behaviour of these hydrolysates were studied. It was observed that casein and chicken egg albumin lost their foaming properties when hydrolysed. However, hydrolysates of β-lactoglobulin and whey protein isolates had good foaming behaviours to different degrees, and these foaming properties were affected by the extent of hydrolysis. X-ray reflectometry analysis showed that film thickness and the presence of film multi-layers affects foaming strength and that thin films were more stable. This study demonstrates the plausibility of using food protein hydrolysates helps provide material with complex properties which can be exploited in industrial applications such as food industry, cosmetics, pharmaceutical.

INTRODUCTION

Foams are pockets of gases in a continuous medium of liquid, semi-solid or solid phase dispersant (Ashoori et al., 2011). They are thermodynamically unstable due to three physical processes namely the drainage of liquid in films, coalescence and disproportionate of bubbles (Eisner et al., 2007). The viscosity and elasticity of interfacial layers on the air/liquid interface play a key role in foam stabilisation (Langevin, 2000).

Soft matter foams have a large market and the practical interest of foams are realised in many industrial settings such as mineral processing, oil recovery, waste-water treatment, paper production, chemical industries, fermentation, pharmaceutical formulation and food processing (Debrégeas et al., 1998; Dexter et al., 2006; Eisner et al., 2007; Middelberg et al., 2008). In the food industry, foam stability in desirable and thus the interfacial layers in a foam are therefore often stabilized by chemical or polymer surfactants which induce stability to coalescence of bubbles (Middelberg et al., 2008).

Surface active agents are either obtained from biological sources or chemical source. However, those from biological sources (bio-surfactants) have a number of advantages over chemical surfactants. These include environmentally friendly nature, ability to be used under extreme conditions, low toxicity, vast diversity and possibility of large-scale production (Banat et al., 2000; Rahman et al., 2002). A number of peptide surfactants (e.g. AM1 (Dexter et al., 2006) and DAMP4 (Middelberg and Dimitrijev-Dwyer, 2011)) have been designed and expressed in *E. coil* host systems. Although these designed peptide surfactants allows a better control of structure-function studies, the cost of their production is high. Research has also identified several naturally occurring bio-surfactants including ranaspumins from frog nests (Andrade and

Abe, 1997; Hostache and Mol, 1998), latherin from horse sweat (McDonald et al., 2009), and hydrophobins from filamentous fungi (Linder, 2009). These often contain a mixture of proteins and other surface active biomolecules such as glycolipids, lipopeptides and a range of higher molecular weight polymers (Rosenberg and Ron, 1999; Banat et al., 2000; Youssef et al., 2007). It follows from the complexities of these mixtures that there will be a high processing cost in the extraction and purification to isolate the active group.

Interestingly, many food proteins and their hydrolysates have been shown to possess interfacial properties and physical, enzymatic and chemical modifications have been applied to various proteins to change the conformation and physicochemical properties and thus enhance foaming behaviour (Corzo-Martínez et al., 2012). Chemical modifications alter the chemical composition of the proteins molecules and are also under strict legislative control. Physical modification on the other hand is easy to apply and is not constrained by legislative guidelines (Pittia et al., 1996) but could be relatively expensive and difficult to scale up. Enzymatic modification of proteins is however, attractive, giving 'tailored' products of high quality. Additionally, the use of immobilized enzyme can help cut down cost and overcome the challenge of using highly expensive soluble enzymes.

In this study the Interfacial and foaming properties of several food protein hydrolysates prepared from immobilized proteinases of *Lactobacillus delbrueckii* subsp. *lactis* 313 were explored. Experimental work on characterizing foam stability and its link to interfacial architecture and properties was also carried out by applying advanced interface-sensitive techniques (x-ray reflectometry). The work is ongoing and preliminary results are shown below. Further work will

look at exploring the foaming potential of other food protein and their hydrolysates. The foams and interfaces formed by peptide mixtures will also be characterised.

METHODOLOGY

Cell growth, cell-envelope proteinase extraction and cross-linked enzyme aggregates preparation

Lactobacillus delbrueckii subsp. *lactis* 313 (ATCC® 7830™) culture was grown at 45 °C to stationary phase (optical density at 560 nm (OD_{560}) of ~ 2.8 ± 0.4, about 0.58 mg/mL dry cell weight), harvested by centrifugation (4,000×g, 10 min, 4°C), and resuspended to an OD of 5 in the extraction solution containing 50 mM sodium phosphate buffer supplemented with 5 mM EDTA (pH 7) for cell-envelope proteinase (CEP) extraction via incubation (30 °C, 2 h, 50 rpm).The suspension was centrifuged (4,000×g, 10 min, 4 °C) and the supernatant was retained, filtered through 0.45 μm membrane filters (Acrodics, Pall Life Sciences) and designated as crude cell-envelope proteinase (CEP). Protein concentration was determined with a Protein Assay Kit according to manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA).

The crude proteinase, CEP in Na-phosphate buffer (50 mM, pH 7) was placed in a micro centrifuge tube to which solid $(NH_4)_2SO_4$ was added to obtain 90% w/v saturation. Glutaraldehyde (25% w/v in water) was also added to give a desired final concentration of 1% v/v. The mixture was incubated with shaking (45 °C, 250 rpm) and CLEA formed washed until supernatant was colorless signifying removal of free glutaraldehyde. This required washing twice with buffer (50 mM Na-phosphate buffer, 1 M NaCl, pH 7) and a final rinse with fresh Na-phosphate buffer (50 mM, pH 7). The final enzyme preparation was kept in Na-phosphate buffer (50 mM, pH 7). Unreacted carbonyl groups were quenched by reacting washed cross-linked enzyme aggregates (CLEA) with 1 M Tris.HCl pH 7.6 (250 rpm, 1 h, 20°C) and washed twice with Na-phosphate buffer via centrifugation.

Hydrolysates preparation

CLEA prepared from 10 mL of crude CEP was added to 50 mL of each protein substrates in 50 mM Na-phosphate buffer pH 7.0, namely, 2 mg/ml total casein from bovine milk, 5% v/v chicken egg albumin, 5 mg/mL whey protein isolate, 5 mg/mL β-lactoglobulin, each supplemented with sodium azide at a final concentration of 0.02%. This gives a v/v enzyme: substrate ratio of 1:5 and was incubated (35 °C, 200 rpm, 25 h). Aliquots (2 ml) were taken intermittently, centrifuged (10,000g, 10 min, 4°C) and the supernatant taken for foaming studies.

Foaming behaviour studies

Foam characterisation involved elucidating the stability of bulk foam and the interaction between individual films of foams. Foam stability was measured in cylindrical containers. A dynamic equilibrium between formation and decay of foams was first established by a constant input of a gas then foams will be allowed to collapse without gas input, giving kinetic foam stability. Foaming assay was performed with a custom-made foam preparation apparatus where air is pumped into 1 mL of sample solution through a porous glass frit at the base of the column using syringe pump (NE-1600 6-channel Syringe Pump,) operating at 10 mL/min.

X-ray reflectometry

X-ray reflectometry studies were done using facilities (i.e. Panalytical X'Pert Pro X-ray spectrophotometer) at the Australian Nuclear Science and Technology Organisation (ANSTO). Only the protein hydrolysates that had good foaming behaviour from previous experiment were selected for X-ray reflectometry studies.

RESULTS (PRELIMINARY) AND DISCUSSION

Protein Hydrolysates as foams -examples

Foaming is a very important functional effect of emulsifiers which, when present in food assist mixing, impart structure and provide desirable sensorial properties to many aerated foods, including bread, ice cream, cakes, whipped toppings and beers (Rodríguez Patino et al., 2007b; Martinez et al., 2009). The foaming characteristics (foaming power and foam stability) of many food proteins and their hydrolysates have been shown to be dependent on factors such as extent of hydrolysis, concentration, pI, etc. Due to these factors, the hydrolysates or peptides obtained from many food proteins have better foaming properties than the native protein. For example, in contrast to native proteins Some protein hydrolysates display high solubility across a wide range of pH and temperatures, (Rodríguez Patino et al., 2007b). The pI of casein is 4.6 and as such cannot be used as foaming agents in acidic food products that have pH close to its isoelectric point since the protein capacity to be adsorbed at the air/water interface can be decreased due to the loss of protein solubility. However, with peptides mixtures, foaming properties can be observed even in a range of pH values since the pI or peptide mixtures often differs from that of the native protein. For example, the pI of casein macropeptide species differ from that of native casein (Kreuß et al., 2009b).

Foaming capacity and stability studies

Results of the foaming studies for the different proteins are shown below. The protein hydrolysates were sampled from the reaction mixture at the different times (i.e. 0 h, 6.5 h, 12 h,

and 25 h). Photographs of the foams in the glass column were taken at irregular times; depending on how stable the foams are (these times are shown above the figures).

Bovine Casein

Putatively, bovine casein has been known to foam. However, hydrolysed casein samples proteins did not form stable foams as shown (Figure B1).

Sodium caseinate (SC) is the most abundant milk protein having wide industrial application due to its high nutritional value and versatile functional properties Additionally, SC is widely used in food formulations due to its good rheological properties as well as foaming and emulsifying capacities (Corzo-Martínez et al., 2012).

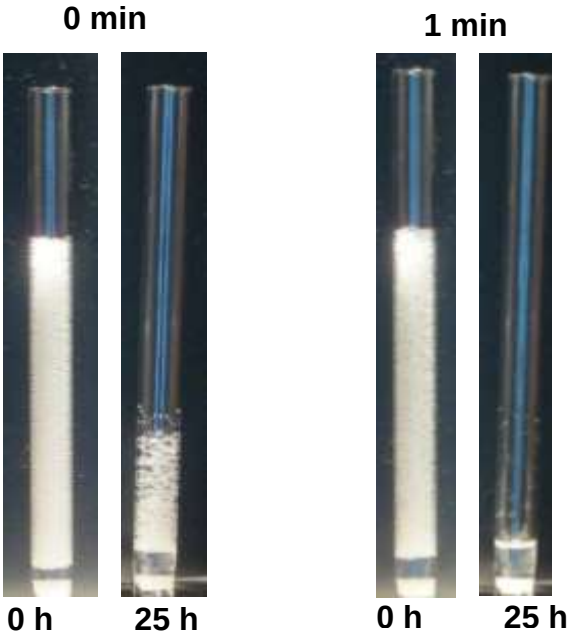

Figure B1: Foaming behaviour of casein and its hydrolysates

The combine effect of highly flexible amphiphilic structure and relatively small molecular size gives caseins the capacity to adsorb at thin air/water interface, lower interfacial tension and produce foams (Pittia et al., 1996; Rodríguez Patino et al., 2007a). Casein predominantly shows foaming behaviour in the intact form and hydrolysis has been implicated in a negative impact on long-term emulsion/foam stability (Rodríguez Patino et al., 2007b). This was the observation in this study. Whereas some level of foaming was observed for casein samples hydrolysed for 25h, the stability of the foam was poor leading to foam collapse within 1 min (Figure B1).

It must be mentioned, however, that some casein hydrolysates have also been known to exhibit a great foaming capacity. An example of this is casein glycomacropeptide (CMP). CMP is a multifunctional casein-derived peptide which is a bioactive peptide and also exhibit high surface activity (Martinez et al., 2013). CMP is released during the renneting of milk and is a hydrophilic product of the endopeptidase action of chymosin on of κ-casein (Martinez et al., 2013). A high level of structure-function relationship exist in CMP owing to a high degree of posttranslational phosphorylation and glycosylation (Kreuß et al., 2009b). Such chemical modification gives CMP some time- and pH-dependent properties. For example, at pH below 4.5 and at room temperature, CMP has been reported to undergo self-assembly which eventually leads to the formation of gels (Farías et al., 2010). However, although CMP has high surface activity it is also known to form foams of low stability (Kreuß et al., 2009a; Kreuß et al., 2009b). β-casein has also been reported to foam due to a high molecular flexibility and a high tendancy to adsorb at air/water interface. High pressure treatment has been used to modify the structure and improve the foaming properties of β-casein (Pittia et al., 1996).

In this study, no foaming behaviour was observed for casein proteins tested and this could be attributable to several factors; namely: (a) low concentration of casein (2 mg/ml) was used since foaming behaviour of proteins is dependent on concentration, (b) catalytic specificity of enzymes used in this study did not result in the release of surface active peptides.

Egg albumin

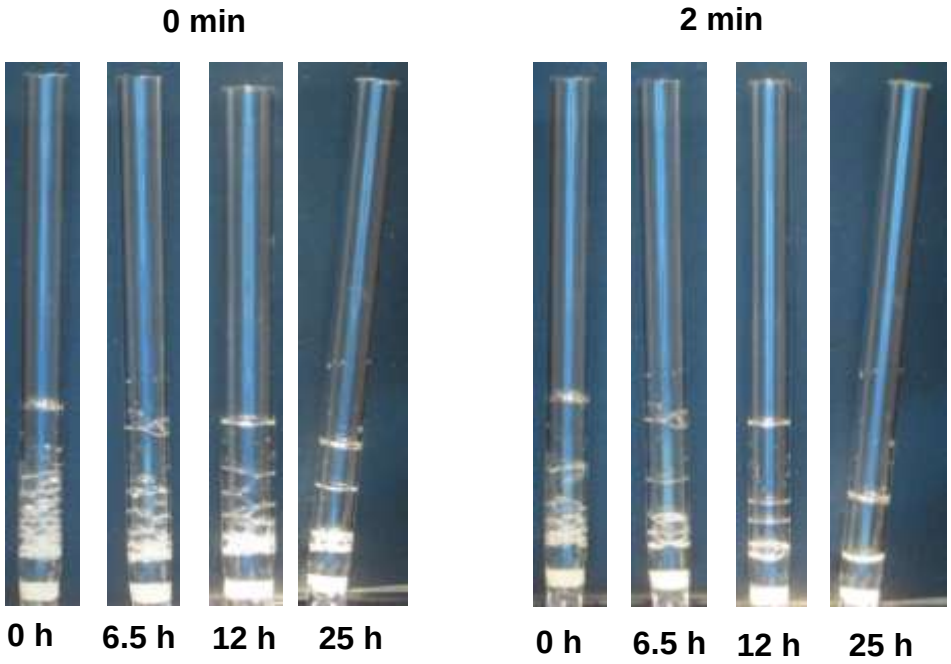

Figure B2: Foaming behaviour of egg albumin and its hydrolysates

Chicken egg albumin proteins and hydrolysates thereof did not form stable foams. The results are shown in Figure B2. This could also be due to a number of reasons. Either chicken egg albumin does not have foaming peptides encrypted in its structure, or if it did, the enzymes used in this study dones not have the neccesary chatalytic specificty to release those peptides.

β-lactoglobulin

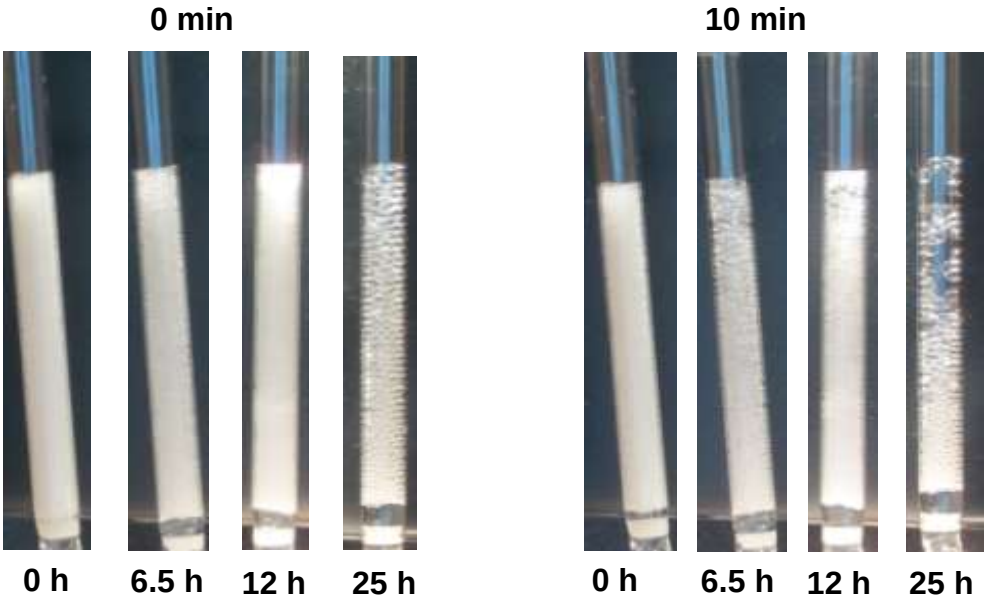

Figure B3: Foaming behaviour of β-lactoglubulin and its hydrolysates

Among the proteins studied for foaming properties, β-lactoglobulin (β-LG) was the one that formed the most stable foams. Both intact protein and hydrolysates exhibited foaming behaviour that was stable even after about 10 min (Figure B3). It can be observed that very stable foam was formed for β-LG, prior to hydrolysis. Additionally, subsequent hydrolysed samples also showed significant foaming with the 12 h samples having higher foaming stability and overall structural integrity compared to 6.5 h and 25 h hydrolysed samples each. This suggests the possibility of some foam stabilizing peptide being generated at 12 h.

It was also observed that extensive hydrolysis of the samples compromised he foaming stability. This is why the 25 h samples had poor foaming stability. It has been reported that the final application of protein hydrolysates is determined by the degree of hydrolysis (DH) and that

extensive hydrolysis of proteins typically has a negative impact on long-term emulsion/foam stability (Vioque et al., 2001). Limited protein hydrolysis (DH of 1-10%) has been shown to give hydrolysates which results in functionality changes, affecting properties such as fat- and water-holding capacity, emulsion capacity and stability, and foaming capacity and stability (Pokora et al., 2013). Additionally, the effect of DH as been shown to affect, not only the equilibrium surface tension being reached by peptides but also affect the time required to achieve a critical reduction in the surface tension and to other interfacial characteristics of the adsorbed film (structure, thickness, surface mechanical properties under shear or dilatation, etc.) (Rodríguez Patino et al., 2007b). These phenomena may account for the observation in Figure 3B.

β-LG is the most abundant whey protein present in bovine milk. The unique foaming properties of this protein can be traced back to its peculiar molecular structure. β-LG has a dimer structure and the presence of a free thiol in each monomeric subunit is capable of facilitating thiol—disulfides interchange reactions (Suttiprasit et al., 1992). Thus, β-LG is able to interact at the interface through disulfide bridges (Corzo-Martínez et al., 2012). β-LG is used as a model whey protein for many studies because it is susceptible to limited enzymatic hydrolysis which normally results in enhancing its interfacial properties by exposing more hydrophobic areas on the protein structure (Perez et al., 2012). Certain oligopeptides have been implicated in the improved surface activite properties of β-LG hydrolysates (Kilara and Panyam, 2003).

Whey protein isolates

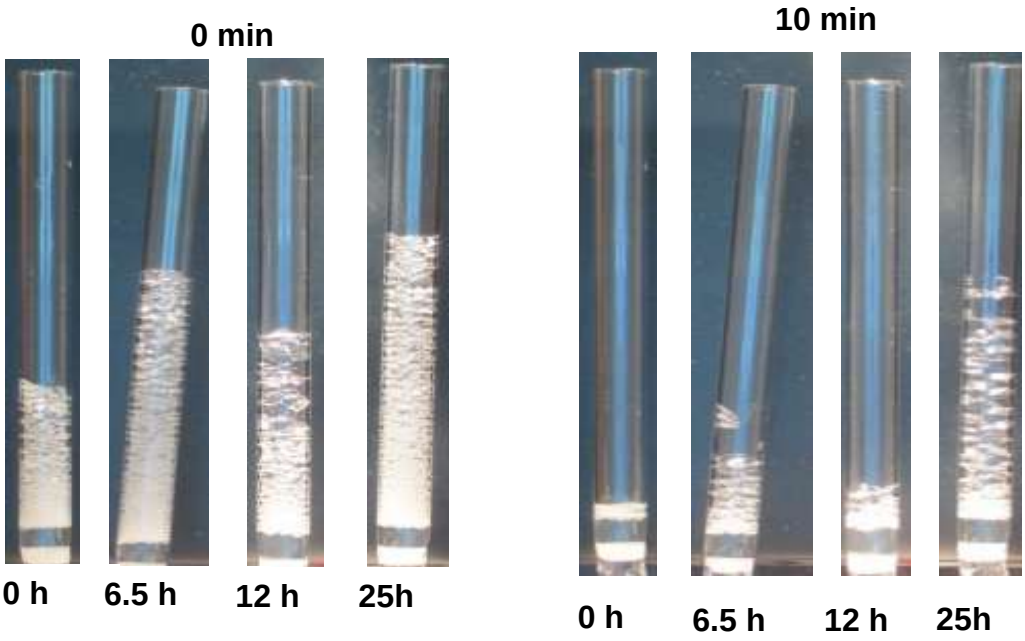

Figure B4: Foaming behaviour of bovine whey protein isolate and its hydrolysates

Whey protein isolates are a commercial by product of cheese manufacturing. In this study, the intact proteins as well as the hydrolysates had observed some foaming behavior which was stable within 10 min, although the foam strength was weak when compared to that of β-LG (Figure B4). It is observed too that 25 h hydrolyzed samples actually had better foaming properties than all other samples, including the intact protein. Whey proteins are a mixture of various proteins namely: β-lactoglobulin, α-lactalbumin, immunoglobulin, serum albumin and the ability of WPI to foam can be attributed to one or more of the following components. That WPI can form stable foams is an important technological advancement which can be exploited to overcome the problem of whey protein waste disposal faced by the dairy industry (Smithers, 2008). Other means of whey valorization include its hydrolysis to give physiologically active peptides for use in functional foods.

X-ray reflectometry analysis

X-ray reflectometry (XRR) **is** a surface-sensitive analytical technique used to characterize and study the morphological parameters of thin films surfaces and multilayers (Zhou and Chen, 1995; Kozhevnikov, 2003; Schieda et al., 2013). This technique relies on an analysis of the incident angle dependence of scattered rays from a material (Fujii, 2013). The results of the x-ray analysis of the various protein hydrolysate samples are shown in Table B1 and Figure B5.

Results from the X-ray reflectometry data show some interesting results which are corroborated by the foaming test. First, it can be observed (Table 1B) that film thickness affects foaming stability and that the thin films are more stable. This is particularly true for β-LG hydrolysates where the 12 h samples which had the thinnest films (13.98 Å) also gave foams with high stability (see Figure B3). The same is observed for WPI where the 25 h samples had thin film thickness of 14.30 Å and also gave stable foams (Figure 5B).

Table B1: X-ray reflectivity description for various hydrolysates

Sample	**Film-thickness (A)**	**Error (χ^2)**	Number of layers
β-LG_0 h	16.27	9.99e-19	1
β-LG_6.5 h	47.50; and 20.55	3.05e-19	2
β-LG_12 h	13.98	4.88e-18	1
WPI_0 h	17.54	2.81e-18	1
WPI_25 h	14.30	9.17e-19	1

β-LG, β lactoglobulin; WPI, whey protein isolates;

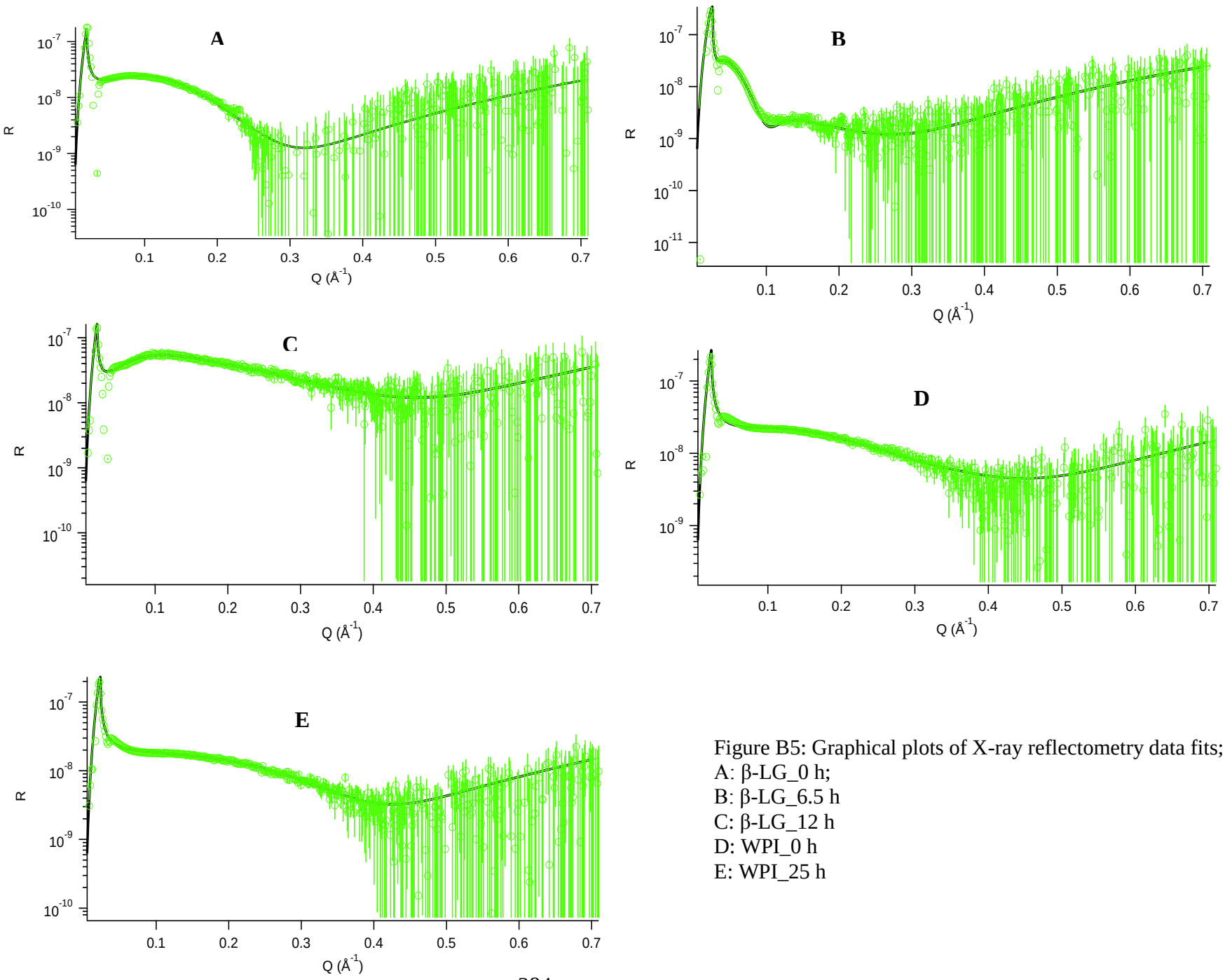

Figure B5: Graphical plots of X-ray reflectometry data fits;
A: β-LG_0 h;
B: β-LG_6.5 h
C: β-LG_12 h
D: WPI_0 h
E: WPI_25 h

The mechanism and role of thickness on the stability of complex protein/peptide foams (such as in this study) remain to be deciphered. However, thick films are more susceptible to forming aggregates at interface which may lead to instability and collapse of foams. Additionally, drainage is of the principal mechanisms of foam destruction and it is due to a gravity-driven flow of liquid along the liquid channels between intersecting foam films (Varade et al., 2011). Thus, putatively, the influence of gravity on thick films will be more pronounced than that on thin films, explaining why the thick films were relatively unstable.

Another observation is that multi-layered films had reduced foam stability. This was observed for the β-LG sample 6.5 h which showed a double layer, each with relatively high film thickness (47.5 Å and 20.6 Å). The combination of the multi-layer and the high film thickness might have contributed to the instability of the foam compared to samples at 12 h.

Further work

Further studies to be done will consist of doing more work with other food proteins and testing the following properties:

A. Quantitative estimation of foaming capacity and foam stability
B. Effect of protein concentration on foaming behaviour
C. Effect of degree of hydrolysis on foaming behaviour
D. SDS PAGE profile of the protein hydrolysates

This is important since molecular size seemed to be an important factor governing incorporation of protein into an interfacial layer. This is closely linked with the degree of hydrolysis and results

from SDS PAGE profile will also provide insight into how the peptide distribution affects foaming performance.

E. Advanced analysis of surface active food-derived peptides

Surface activity of a protein molecule is a cumulative property that is contributed to by factors such as size, shape, charge, and thermal stability (Suttiprasit et al., 1992). Such interfacial characteristics can be measured by estimating surface tension reduction as a function of concentration and DH. (Rodríguez Patino et al., 2007b). Thus, to measure structural changes at the film interface, more analysis are needed with the aid of advanced equipment that can measure structural properties such as surface tension, interfacial elasticity, and protein film structure.

F. Purification of surface active peptide species

With the aid of reversed-phase HPLC techniques, the active peptide species in the hydrolysates that exhibits foaming behaviour will be purified and studied in-depth to help reveal the foam stabilization mechanisms and thereby establish the link between interfacial structure, properties and application.

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8.3. APPENDIX C - Other Related Publications

Carbohydrate utilization affects *Lactobacillus delbrueckii* subsp. *lactis* 313 cell-envelope-associated proteinase production

Published in Biotechnology and Bioprocess Engineering 2012;17(4):787-94

Monash University
Declaration for Thesis Section Appendix C

Declaration by candidate

In the case of Appendix C the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Key ideas, Experimental, Development, Results interpretations, writing up	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**
Dr. Michael K Danquah	Initiation, key ideas and writing up

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 12 December 2013
Main Supervisor's Signature	[redacted]	**Date** 12 December 2013

Biotechnology and Bioprocess Engineering 17: 787-794 (2012)
DOI 10.1007/s12257-012-0106-2

RESEARCH PAPER

Carbohydrate Utilization Affects *Lactobacillus delbrueckii* subsp. *lactis* 313 Cell-enveloped-associated Proteinase Production

Dominic Agyei and Michael K. Danquah

Received: 16 February 2012 / Revised: 29 March 2012 / Accepted: 2 April 2012

Abstract The effect of different sugars (glucose, glycerol, maltose, galactose and lactose) on cell-membrane-associated proteinase production by *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313) was investigated. The experimental results showed that aside glycerol and galactose, all the other sugars supported high growth levels of LDL 313, with glucose displaying the maximum biomass concentration of 0.85 mg/mL dry cell weight for cells harvested at the mid-exponential phase of ~12 h after inoculation. The specific proteinase yield, a measure of the rate of proteinase production relative to cell wall biosynthesis, was used to evaluate the preferential degree of proteinase metabolism as induced by the consumption of different sugar substrates by LDL 313. It was found that maltose displayed the highest specific proteinase yield of 12.59 U/mg sugar consumed. Further, molecular differences were observed in the SDS electrophoretic profile of cell surface proteins generated for the different carbon substrates. This is a preliminary study which supports the inference that different sugars stimulate the production of different cell-surface proteins with a significant effect on cell proteinase activity.

Keywords: cell-envelope-associated proteinases, lactobacilli, sugar metabolism, proteolysis, bioactive peptides

1. Introduction

Cell-envelope-associated proteinases (CEPs) are important bio-molecules obtainable from several species of lactic acid bacteria. As bio-products they are useful in the development of sensory characteristics of dairy products, being responsible for the first stages of casein and peptide degradation during nitrogen metabolism [1]. The CEPs may also cause the release, from proteins, of bioactive peptides which have been shown to contribute to health improvement beyond basic nutrition [1-3]. The usefulness and application of CEPs, in coming years, is likely to expand beyond the manufacture of products with nutritional, organoleptic and therapeutic properties. Such advances will hinge on detailed optimisation of fermentation parameters essential for the generation of proteolytic enzymes at optimum process and economic conditions. The search for novel methods to improve CEP production and yields is therefore a major research endeavor.

Among the lactic acid bacteria, the genera lactobacilli are widely known for their industrial and technological significance [3]. Their wonted metabolism ensures the production of many useful bio-products; for example, CEP production in the lactobacilli is motivated by the limited capacity with which lactobacilli synthesize amino acids using inorganic nitrogen sources. Being dependent on the presence of pre-formed small peptides and amino acids in the growth medium as a nitrogen source [3,4] lactobacilli are equipped with a complex proteolytic system comprising of CEPs, transport proteins, and intracellular peptidases [5] for protein degradation and subsequent utilization of the degradation products. It is also well known that production of extracellular proteases by microorganisms is greatly influenced by fermentation process variables. These variables can therefore be controlled or 'finetuned' to positively influence the production and over-expression of proteases from lactobacilli [6,7]. Amongst the important variables is the carbohydrate content of the growth media which has been reported to influence the CEP activity of some

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thermophilic lactobacilli species [8,9].

When bacteria are exposed to a myriad of carbon sources they undergo a metabolic adaptation and selection program to favour the pathway of maximum survival [8]. Repression of secondary carbohydrate utilization is achieved through several mechanisms that are collectively termed as carbon catabolite repression (CCR), and such control is promoted by two independent mechanisms: inducer exclusion and genetic repression by the catabolite control protein (CcpA) and repressor proteins [10]. Genes encoding CcpA and CcpA-like proteins have been described from a number of lactic acid bacteria, namely *Lactobacillus pentosus*, *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Streptococcus mutans*, and *Lactococcus lactis* [8,11-13]. The genetic organization of *ccpA* genes is in the order *pepQ-ccpA*, meaning *pepQ*, which encodes a prolidase, and *ccpA* are always divergently transcribed [12]. The fact that *ccpA* in lactobacilli is always linked to a divergently transcribed *pepQ* gene encoding a proline-specic peptidase suggests that *pep* gene expression may be regulated by CcpA and may therefore be coordinated with carbon regulation, thereby linking carbon utilization to proteolysis [8].

Several authors have studied the utilization of sugars by lactobacilli for numerous applications and simultaneous carbohydrate utilization has been demonstrated in few different species [10,14-16]. However, little is known on the effect of carbon source on the proteinase activity of lactobacilli, and the few studies that have been reported largely focus on genetic control mechanisms. Biochemical studies relevant to validate and confirm the link between carbohydrate metabolism and proteinase synthesis are scarce. This study was aimed at probing the impact of carbon on microbial cell proteolytic activity by studying growth and CEP production by *Lactobacillus delbrueckii* subsp. *lactis* (LDL 313) in a modified MRS medium supplemented with different carbon sources.

2. Materials and Methods

2.1. Strain and growth conditions

Lactobacillus delbrueckii subsp. *lactis* 313 (ATCC® 7830™) was obtained from ATCC and propagated twice in deMan-Rogosa-Sharpe (MRS) Broth (Oxoid Pty Ltd, Australia) at 37°C, and stored at -70°C. Frozen cells were thawed, plated, and cultured in fresh MRS Broth (Oxoid Pty Ltd, Australia) at 37°C for 12 h, and 200 μL aliquot was used to inoculate each semi-defined media to an initial optical density at 560 nm (OD_{560}) of 0.1 ± 0.005. 30 mL of semi-defined media containing different carbon sources (glucose, glycerol, maltose, galactose, and lactose) at a concentration of 2% w/v were used to culture cells at 37°C and 100 rpm.

2.2. Biomass concentration

The growth of bacteria cells was estimated by determining the cell suspension turbidity measured as optical density at 560 nm (OD_{560nm}) with a spectrophotometer (Shimazu UV-visible spectrophotometer, UV-2450). During cell growth, samples were withdrawn at the said times and immediately cooled on ice. The samples were then washed with via centrifugation with 0.15 M sterile saline water, and the cell pellets resuspended in distilled water for cell growth determination. OD_{560nm} readings were converted to cell densities with a previously prepared calibration curve from which 1.0 AU corresponds to 200 μg/mL of dry cell concentration. The specific growth rate during the exponential phase (μ_{max}) was calculated from the slope of a semi-logarithmic plot of OD_{560} versus time (h).

2.3. Culture pH and total sugar analysis

The pH values of the cultures were recorded with a pH meter (TPS Digital pH-mV-temperature meter, model 1,852 mV). Total sugars were analysed by the phenol-sulphuric acid method, according to Fournier [17]. In this method, aliquots of fermentation culture were put in a glass test tube to which 500 μL of 4% phenol was added followed by the addition of 2.5 mL 96% concentrated sulphuric acid. The absorbance of setup was at 490 nm with a spectrophotometer (Shimazu UV-visible spectrophotometer, UV-2450). The amount of sugar present was then determined by comparison from calibration curves. Commercially available solutions of each sugar were used as standards.

2.4. Media composition

Glucose, maltose, glycerol, galactose and lactose (as lactose monohydrate) were each added to the growth medium at a concentration of 2% w/v. The base medium, with composition given in Table 1, was adjusted to pH 6.5 with 1 M NaOH and sterilized by autoclaving at 121°C for 15 min. The sugar solutions were sterilized by membrane filtration

Table 1. Composition of the semi-defined base medium

Composition	Concentration (g/L)
Yeast extract	10
Peptone	15
Tween 80	1
$MgSO_4$	0.2
$MnSO_4$	0.05
Na acetate	5
NH_4 citrate	2

(0.22 μm) (Acrodisc syringe filters, Pall Life Sciences).

2.5. Preparation of the cell envelope extract

Cultured cells were harvested by centrifugation at 4,000×*g* and 4ºC for 10 min, washed with an equal volume of 0.15 M sterile saline water supplemented with 10 mM $CaCl_2$, and concentrated to 3 times the initial OD_{560} in the extraction solution containing 5 M LiCl and 50 mM sodium phosphate buffer (pH 7) in a 1:1 vol/vol ratio. This system was incubated at 30ºC for 1 h with slight shaking, and then centrifuged at 4,000×*g* and 4ºC for 10 min. The supernatant was decanted and filtered through a 0.22 μm membrane (Acrodics, Pall Life Sciences). The filtrate was subjected to buffer-exchange and concentration using Ultracel 3K (3000MWCO, Amicon Ultra, Millipore) according to the manufacturer's instruction. Protein concentration was determined with a Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA).

2.6. Proteinase activity assay of LiCl extracts

Proteinase activity was assayed according to method described by Exterkate with slight modifications [18]. About 284 μL of reaction mixture containing 50 μL of enzyme solution buffered with 25 mM NaH_2PO_4, pH 7.0, 37.5 mM imidazole, and 1 mM of the peptide succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide (Sigma) was incubated at 40ºC for 10 min after which the absorbance was read immediately. The released nitroaniline was measured at 410 nm by using a SpectraMax M2e Multi-Mode Microplate Reader (Molecular devices, Sunnyvale, USA) with a thermostatically controlled cell compartment used for the sample incubation. The release of *p*-nitroaniline was monitored at 410 nm ([E 410] = 8,800/M/cm). One unit of proteinase activity is defined as the amount required to liberate 1 μmol of nitroaniline per minute.

2.7. Electrophoretic analysis

Extract electrophoresis was carried out with the Experion™ system and Pro260 Assay Kit (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. This assay kit integrates protein separation, detection, and data analyses within a single platform and uses smaller sample and reagent quantities that standard analysis method. Briefly, the LiCl extracts were mixed with sample buffer and heated at 95ºC for 5 min to denature the proteins, and 4 μL aliquot of each concentrated extract (Fig. 3) was loaded into each well.

2.8. Statistical analysis

Data were analyzed by Least Significant Difference (Statsgraphics centurion version XV). Signicant differences were defined at $P \leq 0.05$.

3. Results and Discussion

3.1. Cell growth and culture pH

Although differences in their growth profiles were observed, all the different carbon sources supported the growth of *Lactobacillus delbrueckii* subsp. *lactis* 313 (LDL 313). A similar lag phase of ~4 h was observed for the three most effective sugars for biomass production namely glucose, maltose, and lactose (Fig. 1). Glucose, lactose and maltose supported profuse growth of LDL 313 showing maximum specific growth rates of 0.49, 0.42, and 0.32/h respectively. The growth levels observed for the different carbon sources corroborated the changes in pH observed. Decrease in culture pH recorded over the entire fermentation period for glucose, lactose and maltose were 2.83, 2.72, and 2.70 respectively (from Fig. 1). This demonstrates a high level of acid tolerance by LDL 313, an important trait in the selection of probiotic strains [19,20]. Thus LDL 313 has potential applications in fermented acid-based food products.

Galactose weakly supported the growth of LDL 313 (specific growth rates of 0.14/h), and this has been previously observed [3,21,22]. In lactobacilli, galactose is catabolised through the tagatose-6-phosphate or the Leloir pathway; both of which require the action of permeases, transferases and epimerases [3,23,24]. Homofermenters like LDL 313 largely are unable to ferment galactose as the sole carbon substrate in a growth medium, however some level of galactose utilization is observed by the addition of a suitable 'enhancer' *i.e.* metabolizable energy source [25]. In this study, a low maximum specific growth rate and dry cell concentration of 0.14/h and 0.27 mg/mL respectively were obtained implying a low level of utilization of galactose. Galactose metabolism in lactobacilli is an important factor considered when choosing starters for dairy products since the fermentation of galactose is more heterolactic than that for glucose and lactose [25].

Further, cell growth was poorly supported by glycerol as the sole carbon source. A low specific growth rate of 0.09/h was observed. Glycerol acts as an organic electron acceptor in some heterofermenters and this results in the production of lactate, acetate, CO_2 and 1, 3-propanediol [3]. On the other hand, homofermenters are largely unable to catabolise glycerol due to the absence of effective glycerol transporter genes, especially at high glycerol concentrations of above 5% (v/v) [26].

The base medium with no added sugar, used as a negative control, showed limited growth with maximum growth rate and dry cell concentration of 0.09/h and 0.2 mg/mL respectively. Some components of the base medium, such as yeast extract and peptone, which contain carbon, partially supported the carbon requirement of the cells. However, the levels of these carbon sources are low, resulting in

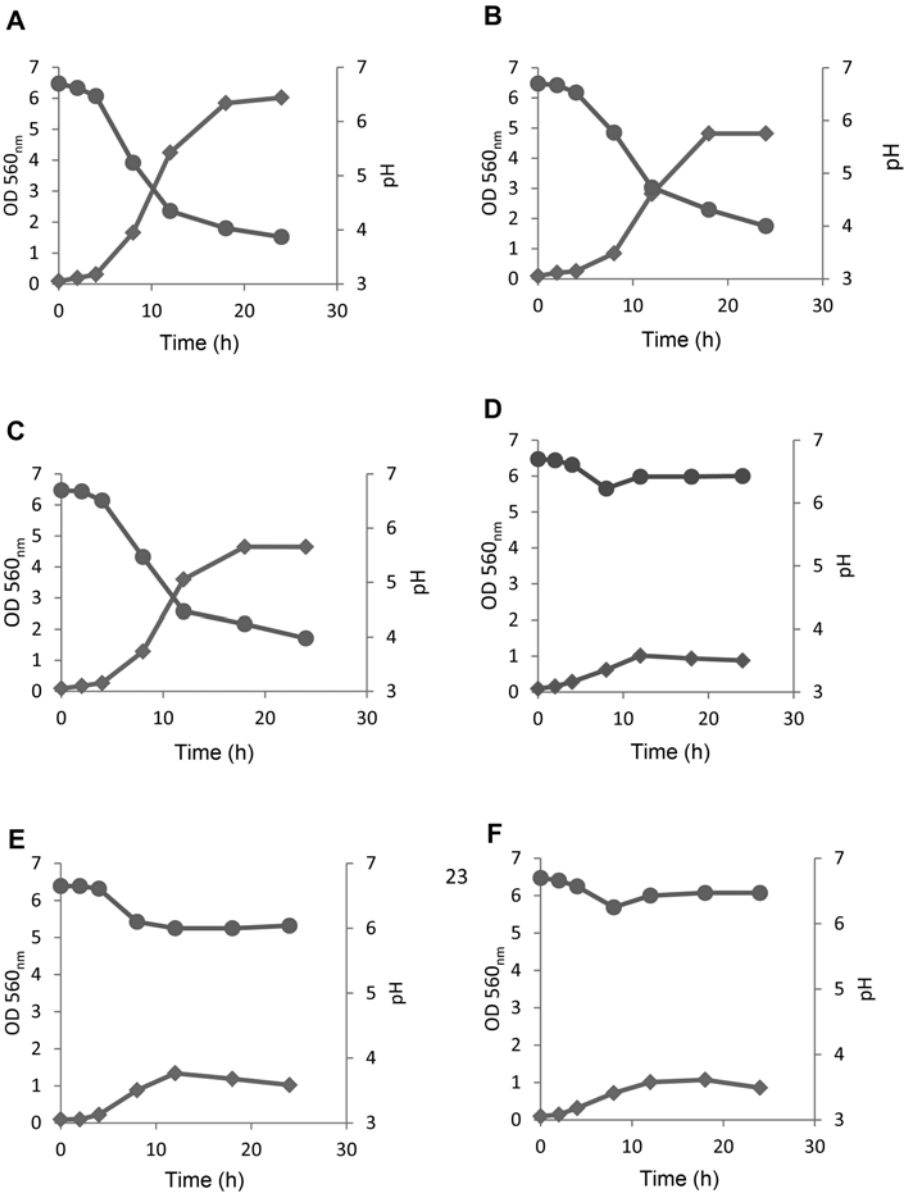

Fig. 1. Fermentation profiles of glucose (A), maltose (B), lactose (C), glycerol (D), galactose (E), and BM (base medium) (F); OD_{560nm} (◆) and pH profile (●). Fermentations were done in duplicate and a representative dataset is shown.

limited cell growth.

3.2. Sugar consumption and utilization

The consumption of sugar by LDL 313 is shown in Fig. 2. Analysis of the fermentation broth revealed that by mid-exponential phase (after 12 h of incubation) when cell-surface proteinases were harvested, ~17, 21, and 29% of glucose, maltose and lactose respectively, had been consumed. Altogether, about 43, 58, and 55% of glucose, maltose and lactose respectively were consumed after 24 h of cell cultivation. Glucose resulted in the highest biomass concentration of 0.85 mg/mL although the amount of total sugar consumed was lowest for glucose. Glucose is putatively the most preferred sugar for metabolism leading to biomass production by many lactic acid bacteria [3,10].

LDL 313 is a homofermentative lactobacillus which utilizes the Emden-Meyerhof-Parnas catabolic pathway. By this pathway, carbohydrates are converted to pyruvate which is in turn converted to lactate by lactate dehydrogenase, under conditions of reduced oxygen and excess carbon [3]. The disaccharides, maltose and lactose, supported the growth of LDL 313 yielding a maximum dry cell concentration of 0.56 and 0.72 mg/mL, and the uptake of both disaccharides were demonstrated by pH and sugar consumption profiles (Figs. 1 and 2). Lactose is the primary carbon and energy source in milk and thus lactobacilli with diary significance

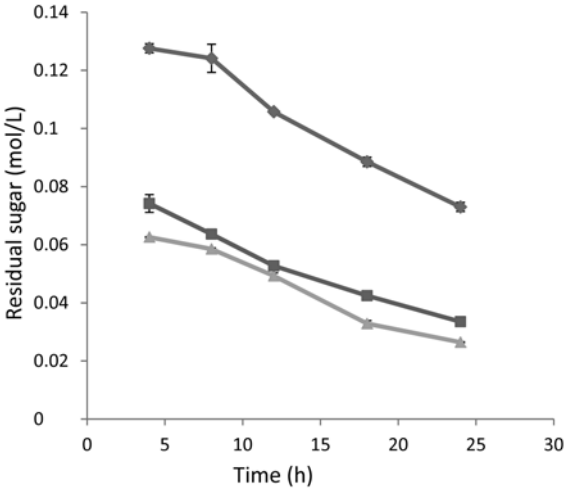

Fig. 2. Sugar consumption profile for glucose (◆), maltose (▲), and lactose (■). (Mean ± standard error of duplicate experiments, error margins smaller than marker sizes for maltose and lactose).

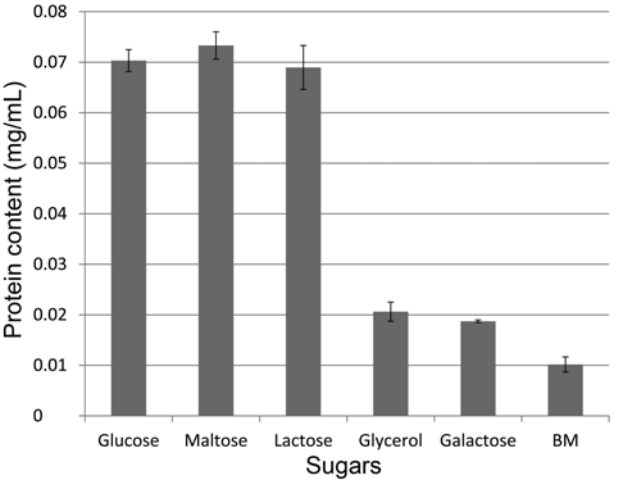

Fig. 3. Protein contents of LiCl soluble extracts from cells grown in media with different carbon sources. Values are the mean ± standard error of two independent experiments.

are highly adapted to growth on this carbon source. However, for LDL 313, biomass yield with respect to substrate consumption indicated that maltose is a more efficient sugar for biomass production than lactose. Biomass yield coefficients (Yx/s) of 2.46 mg DCW/mg substrate and 1.98 mg DCW/mg substrate for maltose and lactose respectively were recorded. A common trait shared in the metabolism of maltose and lactose is that once transported into the cell cytoplasm they are both cleaved into their respective monosaccharide units. However, for some lactobacillus strains, the presence of high concentrations of the monosaccharide units represses cell growth. For example, in *L. sanfrancisco*, uptake of maltose at a high rate, followed by hydrolysis to glucose and glucose-1-phosphate, could lead to the production of glucose at a rate that exceeds the rate of conversion of glucose to glucose-6-phosphate. Thus, part of the glucose is released via the uniport mechanism to avoid the generation of excessive concentrations of free glucose inside the cell [27]. Further, although lactose is efficiently transported and hydrolyzed intracellularly, some lactobacilli species, including *Lactobacillus delbrueckii* subsp. *lactis*, do not grow on galactose and only metabolize the glucose moiety of lactose, while the galactose is excreted into the medium in amounts stoichiometric with the uptake of lactose [21,22,25]. For this study, carbohydrate metabolism is also shown indirectly through the decrease in pH of the medium, as this parameter is directly related to the ability of the cells to produce organic acids from the carbon substrates [20].

3.3. Effect carbon source on proteinase production

The amount of proteins in the LiCl extract from the cells is presented in Fig. 3. The extraction agent LiCl works as a mild chaotropic agent. The lithium ions attack the S-layer protein sub-units which are non-covalently linked to each other and disintegrates it into monomers *via* cation substitution [28]. Thus, LiCl is able to remove both cell wall-bound proteinases and other cell surface localized

Table 2. Cell growth and proteinase production by LDL 313; analysis was performed after 12 h of fermentation

Sugar	Cell density[a] (mg/mL DCW)	Specific growth rate (μ_{max}/ h)	*Y*p/s (U/mg)[b]	*Y*p/x (U/mg DCW)[c]	*Y*x/s (mg/mg)[d]	Proteinase assay (U/mL)
Glucose	0.85±0.005	0.49	6.53±0.410	1.51±0.092	4.34±0.269	1.28±0.078
Maltose	0.56±0.002	0.32	12.59±0.827	5.11±0.113	2.46±0.163	2.86±0.064
Lactose	0.72±0.002	0.42	3.82±0.382	1.93±0.042	1.98±0.198	1.39±0.028
Glycerol	0.20±0.002	0.09	ND	ND	ND	0.35±0.092
Galactose	0.27±0.296	0.14	ND	ND	ND	0.59±0.016
BM	0.20±0.002	0.09	ND	ND	ND	0.45±0.014

Values are the mean of duplicate measurements ± standard error. ND: not determined.
[a]DCW = dry cell weight.
[b]$Y_{p/s}$ = yield of proteinase with respect to substrate (U/mg).
[c]$Y_{p/x}$ = specific product formation (SPF) of proteinase with respect to biomass (U/mg DCW).
[d]$Y_{x/s}$ = yield of biomass with respect to substrate (mg/mg).

proteins [29,30].

The cell-envelope-associated proteinase (CEP) activities of LiCl extract from cells grown in different sugars are captured in Table 2. One unit of proteinase activity is defined as the amount required to liberate 1 μmol of nitroaniline per minute. Glucose and lactose displayed proteinase activities of approximately 1.28 and 1.39 U/mL respectively. Maltose recorded the highest proteinase activity of about 2.86 U/mL with the highest Yp/x of 5.11 U/mg DCW (Table 2). The yield of proteinase with respect to substrate consumed (Yp/s) was also highest for maltose (about 12.59 U/mg). These shows that maltose preferentially promotes a higher rate of proteinase production relative to biomass synthesis in comparison to the other carbon substrates.

The high proteinase activity recorded for maltose may be accounted for by the effect of maltose on microbial cell membrane morphology. During maltose metabolism, the β-glucose-1-phosphate moiety released from maltose may be used as a precursor for cell wall synthesis [3]. Further, maltose is used as an effective protecting agent for cell membranes and proteins during microbial cell dehydration processes [31]. Thus, the high proteinase activity observed for maltose could be accounted for by the action of maltose in maintaining the morphological integrity of the bacterial cell envelope. This can ensure a tight association of the proteinase with the cell envelope during growth in maltose, thus reducing possible autoproteolytic release of cell-envelope-associated proteinases into the culture medium. Further, since LDL 313 displayed a much lower growth rate on maltose than lactose or glucose, the levels of glycolytic intermediates from maltose might have regulated enzyme (CEP) synthesis positively.

The results of this study intimate that CEP activity of LDL 313 is dependent on carbon source; however, other studies have shown the contrary. In their study, Hebert *et al.* [26] demonstrated that proteinase activity of *Lactobacillus delbrueckii* subsp. *lactis* CRL 581 is independent of carbon source. This apparent contradiction may be attributed to growth media condition, *i.e*. the type and concentration of nitrogen in the growth medium. Hebert *et al*., in their study used a chemically defined medium containing 20 amino acids in their free form, as compared to the semi-defined medium used in this study. Growth media rich in peptides have been shown to repress the expression of several genes encoding peptidases in lactobacilli [32] and this may account for the disparity in results. Moreover, the mechanism by which proteolytic genes are repressed is mediated by the regulator CodY which, among other functions, is also involved in the carbon overflow metabolism as well as sugar catabolism [26,33]. While a peptide rich medium (such as the one used in this study) is known to repress proteolytic genes [26], regulation of sugars can also be equally affected since both activities are mediated by CodY.

Another reason that may account for the disparity is that in lactobacilli, the various inducers and inhibitors involved in genetic control of nutrient metabolism are largely specie specific. In a study on *Lactococcus lactis*, the response of sixteen proteolytic system genes to carbon control showed that only *pepP* encoding an aminopeptidase confirmed sugar-dependent expression while *pepQ*, for example, was not subject to carbon regulation [34]. On the contrary, catabolite control of PepQ and corresponding differences in transcription of *pepQ* have been demonstrated in *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290 [9]. These seem to suggest that *pepQ* is sugar controlled in *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290. However, there is no sugar-dependent expression of *pepQ* in *Lactococcus lactis.* Thus, genetic control of nutrient metabolism in the lactic acid bacteria is specie-specific which justifies the disparity between our findings and that observed in literature for other lactobacilli species.

3.4. PAGE profile for LiCl soluble extracts

Overall, there was a clear visual difference between the electrophoretic profiles of cell extracts for the different sugar substrates (Fig. 4). There existed some molecular size similarities between the three effective sugars, glucose (lane 1), maltose (lane 2), and lactose (lane 3) around the 20, 35, and 260 kDa range. Several higher size bands above 100 kDa were observed for glucose, maltose and lactose compared to the other carbon sources. However, the profile for maltose (lane 2), unlike the other sugars, shows several bands. The less favorable growth carbons, glycerol (lane 4) and galactose (lane 5), each showed a unique PAGE profile with a common band at 78 kDa.

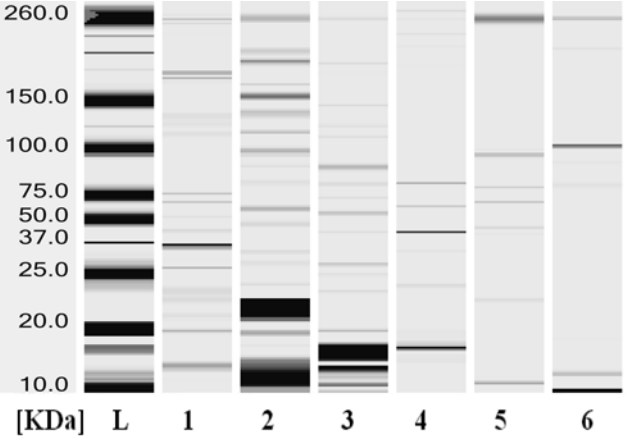

Fig. 4. SDS-PAGE profile for LiCl soluble extracts of cells grown with media containing different carbon substrates. Ladder (L); Glucose (1); Maltose (2); Lactose (3); Glycerol (4); Galactose (5); BM (6).

From the electrophoretic profiles of cell extract for each sugar, the type of cell surface proteins produced by LDL 313 is dependent on the type of sugar present in the growth medium. This pattern is indicative of the role of the different sugars inducing the synthesis of different cell-surface proteins (including CEPs). It is therefore not surprising that a different proteolytic activity was observed for each carbon substrate in this study.

A positive correlation ($r = 0.93$, $P \leq 0.05$) existed between protein content of cell extract and the cell biomass *i.e.* sugar substrates that observed high cell biomass also had high protein content of cell extract. This intimates the role of cell surface proteins in the growth of LDL 313 cells since the proteinases are the key enzymes responsible for nitrogen metabolism and growth [1]. However, although CEPs are protein-based enzymes, a high proteinase activity does not necessarily indicate high total cell protein concentration. This is because; during bacterial growth, cell proteins synthesized may not be proteolytic, implying a high total cell protein level but low cell proteinase activity. Thus, the specific product formation (SPF) of proteinase with respect to biomass is a better basis of comparing the performances of the sugars (See Table 2).

Generally, in the lactobacilli the total cell envelope-associated proteins comprises, among others, of the ribosomal proteins; permeases of the glycolytic pathway [35]; the crystalline surface layers (S-layer) proteins which are responsible for cell protection, adhesion and cell surface recognition [30,36,37]; and cell-membrane-associated proteinases responsible for hydrolyses of proteins to peptides extracellular [1,2]. The identification of the types of different proteins represented by the various bands in each lane (Fig. 4) is the subject of further study.

4. Conclusion

In summary the effect of different sugars on cell proliferation and proteinase production from LDL 313 was studied. Profuse cell growth was observed only for glucose, lactose and maltose (μ_{max} = 0.49/h, 0.42 and 0.32/h respectively). Further, the amount of cell surface proteins produced was dependent on the carbon substrate and proteinase activity was found to be dependent on the carbon source, with maltose recording the highest proteinase activity (2.86 U/mL). SDS PAGE of LiCl soluble extract indicated that the cell surface protein profiles differ with the growth medium carbon source. The results of this study show that greater amounts of proteinase could be obtained from lactobacilli with minimal effort simply by optimising medium carbon compositions. A further study to identify and characterize the specific proteins types produced with each sugar will increase understanding of the metabolic activities of the different carbon substrates in relation to CEP synthesis.

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"*Magna opera Domini exquisita in omnes voluntates ejus*"

"The works of the Lord are great, sought out of all them that have pleasure therein"

[Psalm CXI: 2].