PROTEOLYSIS ENHANCEMENT OF CHEDDAR CHEESE AND ENZYME-MODIFIED CHEESE BY FREE OR ENCAPSULATED FORM OF NATURAL AND RECOMBINANT ENZYMES OF *LACTOBACILLUS RHAMNOSUS* S93

By

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ABSTRACT

A recombinant aminopeptidase (pepN) of *Lactobacillus rhamnosus* S93 was encapsulated in chitosan-coated alginate beads using an extrusion method. The effects of alginate, $CaCl_2$, chitosan concentrations, hardening time, pH, and alginate/enzyme ratios on the encapsulation efficiency (*EE*) and the enzyme release (*ER*) were then investigated. Chitosan in the gelling solution significantly increased the encapsulation efficiency (*EE*) and also retarded the enzyme release (*ER*) from the beads. Th e greatest *EE* was observed in a pH 5.4 solution (chitosan-$CaCl_2$) during bead formation. Increasing the $CaCl_2$ concentration over 0.1 M neither affected the *EE* nor the *ER*. Increasing the hardening time beyond 10 min led to a decrease in *EE* and alginate: enzyme ratio (3:1) was optimal to prevent the *ER*.

Using the free or encapsulated form of the recombinant pepN at three different concentrations (50, 500, 2000 units 18 L^{-1}milk), control and experimental cheeses were prepared in vats containing 18 L milk. The effects of two enzyme forms at the similar concentration were also investigated in larger scale of cheesemaking trial (200 L milk). The amounts of nitrogen soluble in phosphotungstic acid (PTA-N) and free amino acids (FAA) were enhanced by increasing the enzyme concentration. While proline disappeared at 4 months of ripening period, leucine was the most dominant amino acid in the experimental cheeses. Cheese with the encapsulated enzyme at the highest concentration received significantly higher mean levels of PTA-N and total FAAs than the other cheeses. The use of encapsulated enzyme resulted in an acceleration of 70% in proteolysis and superior sensory properties than the control cheese. Almost all encapsulated enzymes were entrapped into the cheese matrix, revealing the stability of the capsules during Cheddar cheese manufacturing.

The application of natural and recombinant enzymes of *Lactobacillus rhamnosus* S93 was also investigated to develop a process of Cheddar-EMC flavorings. Water soluble nitrogen, PTA-N and FAA levels were significantly higher in the proteinase (Neutrase) added EMCs. The highest levels of PTA-N and FAA were evident in samples containing the highest levels of combined natural and recombinant enzymes. Proline disappeared in the experimental EMCs compared to the control over maturation times.

RÉSUMÉ

Une aminopeptidase recombinée (pepN) provenant de *Lactobacillus rhamnosus* S93 fut encapsulée par un procédé d'extrusion en billes d'alginate recouvertes de chitosane. Les effets des concentrations en alginate, $CaCl_2$, et chitosane, de la durée de durcissement, du pH, et du rapport alginate: enzyme sur l'efficacité d'encapsulation (*EE*) et la perte d'enzyme (*PE*) furent alors étudiés. L'ajout de chitosane dans la solution de gélification augmenta *l'EE* de façon significative, et retarda aussi la *PE* à partir des billes. L'*EE* la plus élevée fut notée lorsqu'une solution avec chitosan-$CaCl_2$, à un pH de 5.4, servit à la formation des billes. Une augmentation de la concentration en $CaCl_2$ au delà de 0.1 M n'influença ni la *EE,* ni la *PE*. Étendre la durée du durcissement des billes au delà de 10 min eut pour résultat de diminuer la *EE*. Un rapport alginate : enzyme ratio (3:1) a diminué la *PE*.

Utilisant la forme libre ou encapsulée de PepN recombiné à trois différentes concentrations (50, 500, 2000 unités 18 L^{-1} lait) des fromages expérimentaux et témoins furent produits dans des cuves contenant 18 L de lait. L'effet des deux formes de l'enzyme, ajoutées à la même concentration, fut étudié dans une épreuve de fabrication de fromage à plus grande échelle (200 L de lait). Une augmentation de la concentration de l'enzyme a amélioré la teneur d'azote soluble en acide phosphotungstique (PTA-N) et d'acides aminés libres (AAL) des fromages. Si la proline disparut après 4 mois d'affinage, la leucine demeura l'acide aminé dominant des fromages expérimentaux. Par rapport aux autres fromages, ceux ayant reçu l'apport maximum d'enzyme encapsulé montrèrent des niveaux significativement plus élevés de PTA-N et d'AALs totaux. L'utilisation d'enzyme encapsulé accéléra la protéolyse de 70% et permis le développement de propriété organoleptique supérieure par rapport aux fromages témoins. Les enzymes encapsulés furent presque entièrement retenus dans la matrice du fromage, mettant en relief la stabilité des capsules durant la manufacture de fromage Cheddar.

De plus, l'utilisation d'un extrait brut de cellules bactériennes et/ou dénzyme recombinante provenant de *L. rhamnosus* S93 fut étudié en vue de développer un procédé de production de substances aromatisantes de fromage Cheddar modifié par enzymes (SAFME). Les niveau d'azote soluble dans l'eau, de PTA-N et d'AALs

furent significativement plus élevés pour les SAFME avec ajout de protéinase (Neutrase). Les niveaux les plus élevés de PTA-N and AALs furent décelés dans les échantillons ayant reçu les niveaux les plus élevés d'extrait brut de concert avec la peptidase recombinée. Les SAFME générés avec la peptidase recombiné seule, et ceux ayant été traités avec des niveaux élevés d'extrait brut montrèrent une perte totale de proline.

TABLE OF CONTENTS

	PAGE
ABSTRACT	ii
RÉSUMÉ	iii
TABLE OF CONTENTS	v
LIST OF TABLES	ix
LIST OF FIGURES	xi
ACKNOWLEDGEMENTS	xiv
FOREWARD	xv
CONTRIBUTION OF AUTHORS TO MANUSCRIPTS FOR PUBLICATION	xvii
PREFACE	xix
GENERAL INTRODUCTION	1
CHAPTER 1	4
LITERATURE REVIEW	
1.1 INTRODUCTION	5
1.2 CHEESE	6
1.2.1 Milk and Cheese Production	6
1.2.2 Cheese Proteins	7
1.2.3 Cheesemaking Process	7
1.3 RIPENING OF CHEDDAR CHEESE	8
1.3.1 Ripening Agents	8
1.3.2 Reactions Involved in Ripening	9
1.3.2.1 Proteolysis	9
1.3.2.2 Lipolysis	10
1.3.2.3 Glycolysis	11
1.4 ACCELERATION OF CHEESE RIPENING	12
1.4.1 Methods for Accelerating Cheddar Cheese Ripening	12
1.4.1.1 Elevated Temperature	12
1.4.1.2 Addition of Enzymes	13
1.4.1.2.1 Lipases	14
1.4.1.2.2 Plasmin	15
1.4.1.2.3 Other Proteinases and Peptidases	16
1.4.1.2.4 β-Galactosidase	18
1.4.1.3 Enzyme Modified Cheese	19
1.4.1.4 Attenuated Starters	19
1.4.1.4.1 Cell Lysis Techniques	20
1.4.1.5 Adjunct Cultures	22
1.4.1.6 Genetically Engineered Starters and/or Recombinant Enzymes	27
1.4.1.6.1 Over-Expression of LAB Enzymes	28
1.4.1.6.2 Starter Lysis and Food Grade Systems	30
1.4.1.7 Microencapsulation of Ripening Enzymes	30
1.5 CONCLUSIONS	36

CHAPTER 2 44
Microencapsulation of a Recombinant Aminopeptidase (pepN) from *Lactobacillus rhamnosus* S93 in Chitosan-Coated Alginate Beads
2.1 ABSTRACT 45
2.2 INTRODUCTION 45
2.3 MATERIALS AND METHODS 47
2.3.1 Materials, Strain and Growth of *E.coli* clones 47
2.3.2 Purification of the pepN and Electrophoresis 47
2.3.3 Enzyme and Protein Assays 48
2.3.4 Preparation of Alginate and Chitosan-Coated Alginate Beads 49
2.3.5 Determination of the *ER* and Polymer/Enzyme Ratio 50
2.3.6 Freeze Drying of Beads 51
2.3.7 Scanning Electron Microscopy (SEM) 51
2.3.8 Statistical Analyses 51
2.4 RESULTS AND DISCUSSION 51
2.4.1 Enzyme Preparation and Encapsulation 51
2.4.2 Effects of Different Parameters on the *ER* of Beads 54
2.4.3 Scanning Electron Microscopy (SEM) 56
2.5 CONCLUSIONS 57

CHAPTER 3 72
Effect of Recombinant Aminopeptidase of *Lactobacillus rhamnosus* on Cheddar Cheese Ripening
3.1 ABSTRACT 73
3.2 INTRODUCTION 73
3.3 MATERIALS AND METHODS 74
3.3.1 Materials, Strain and Growth of *E.coli* clones 74
3.3.2 Enzyme Preparation and Purification 75
3.3.3 Enzyme and Protein Assays 75
3.3.4 Preparation of Alginate and Chitosan-Coated Alginate Beads 75
3.3.5 Cheddar Cheese Manufacture 76
3.3.6 Compositional Analysis 76
3.3.7 Analysis of Proteolysis Indices 77
3.3.8 Sensory Evaluation 77
3.3.9 Microbiological Analysis 77
3.3.10 Statistical Analysis 78
3.4 RESULTS AND DISCUSSION 79
3.4.1 Enzyme Preparation and Purification 79
3.4.2 Enzyme Encapsulation 79
3.4.3 Compositional Analysis 79
3.4.4 Microbiological Analysis 80
3.4.5 Proteolysis Analysis 81
3.4.6 Sensory Evaluation 83
3.5 CONCLUSIONS 83

Chapter 4 96
Application of Free or Encapsulated Recombinant Aminopeptidase of *Lactobacillus rhamnosus* S93 in Acceleration of Cheddar Cheese Ripening
4.1 ABSTRACT 97
4.2 INTRODUCTION 97
4.3 MATERIALS AND METHODS 99
4.3.1 Materials, Strains and Growth of *E.coli* clones 99
4.3.2 Purification of Recombinant Enzymes and SDS-PAGE 99
4.3.3 Enzyme and Protein Assays 100
4.3.4 Preparation of Chitosan-Coated Alginate Beads 100
4.3.5 Cheddar Cheese Manufacture 100
4.3.6 Estimation of the Encapsulated Enzyme Entrapment in the Curd 100
4.3.7 Compositional Analysis 101
4.3.8 Analysis of Proteolytic Indices 101
4.3.9 Sensory Evaluation 101
4.3.10 Microbiological Analysis 101
4.3.11 Statistical Analysis 101
4.4 RESULTS AND DISCUSSION 101
4.4.1 Enzyme Preparation and Purification 101
4.4.2 Enzyme Encapsulation 102
4.4.3 Compositional Analysis 102
4.4.4 Microbiological Analysis 102
4.4.5 Proteolysis Analysis 103
4.4.6 Sensory Evaluation 105
4.5 CONCLUSIONS 106

CHAPTER 5 117
Enhancement of Cheddar-EMC Proteolysis Using Natural and Recombinant Enzymes of *Lactobacillus rhamnosus*

5.1 ABSTRACT 118
5.2 INTRODUCTION 118
5.3 MATERIALS AND METHODS 119
5.3.1 Chemicals and Preparation of Crude Enzyme of *Lactobacillus rhamnosus* S93 119
5.3.2 Preparation of Recombinant Aminopeptidase from *Lactobacillus rhamnosus* S93 119
5.3.3 Purification of Recombinant Enzyme and SDS-PAGE 120
5.3.4 Enzyme and Protein Assays 120
5.3.5 Preparation of Enzyme-Modified Cheese 120
5.3.6 Physicochemical Analyses 120
5.3.7 Statistical Analysis 121
5.4 RESULTS AND DISCUSSION 121
5.4.1 Enzyme Preparation and Purification 121
5.4.2 Compositional Analysis 121
5.4.3 Proteolysis Analysis 121

5.5 Conclusions 124

GENERAL CONCLUSIONS 140
FUTURE STUDIES 142
REFERENCES 143

LIST OF TABLES

PAGE

Table 1.1. Enzymes used in acceleration of Cheddar cheese maturation (Modified from M. El Soda, and S. Pandian: Recent developments in accelerated cheese ripening. *J. Dairy Sci.* 74: 2317–2335, 1991). 37

Table 1.2. Commercial enzymes to accelerate ripening of cheese (Modified from B. A. Law: Controlled and accelerated cheese ripening: the research base for new technologies. *Int. Dairy J.* 11: 383– 398, 2001). 38

Table 1.3. Genetic engineering approaches applied to LAB or to the overproduction of enzymes for flavor enhancement (Modified from B. A. Law: Controlled and accelerated cheese ripening: the research base for new technologies. *Int. Dairy J.* 11: 383– 398, 2001). 39

Table 1.4. Recombinant proteolytic and lipolytic enzymes for cheese and other industrial applications (Adapted from B. H. Lee: Methods of enzyme incorporation into cheese. *IDF Monograph* 2007, In press). 40

Table 1.5. Peptidases and esterases cloned into *Lactococcus lactis* and food grade systems (Adapted from B. H. Lee: Methods of enzyme incorporation into cheese. *IDF Monograph* 2007, In press). 41

Table 2.1. Summary of the purification steps of recombinant aminopeptidase from *Lactobacillus rhamnosus* S93. 58

Table 3.1. Protocol for Cheddar cheese manufacturing. 84

Table 3.2. Compositions of milk used for making of cheeses. 85

Table 3.3. Composition of control and experimental chesses at 15 days of ripening. 86

Table 3.4. Eigenvalues of the correlation matrix calculated from PRINCOMP procedure. 87

Table 3.5. Duncan's multiple range test for responses. 88

Table 4.1. Protocol for Cheddar cheese manufacturing in three trials. 107

Table 4.2. Compositions of milk used for making of experimental Cheeses. 107

Table 4.3. Composition of control and experimental chesses (at 15 days of ripening). 108

Table 4.4. Duncan's multiple range test for response. 109

Table 4.5. Eigenvalues of the correlation matrix calculated from PRINCOMP Procedure. 110

Table 5.1. Protocol for Cheddar-EMC production. 125

Table 5.2. Composition of grain fresh Cheddar cheese used for making of Cheddar EMCs. 126

Table 5.3. Compositions of the slurries (at manufacturing day) used for preparation of EMCs. 126

Table 5.4. Duncan's multiple range test for responses. 127

Table 5.5. Eigenvalues of the correlation matrix calculated from PRINCOMP procedure. 128

LIST OF FIGURES

PAGE

Figure 1.1. Different proteolytic enzymes in cheese during ripening (Adapted from M. J. Sousa, Y. Ardö, and P. L. H. McSweeney: Advances in the study of proteolysis during cheese ripening. *Int. Dairy J.* 11: 327– 345, 2001). 42

Figure 1.2. Basic cheese ripening biochemistry (Adapted from B. A. Law: Controlled and accelerated cheese ripening: the research base for new technologies. *Int. Dairy J.* 11: 383– 398, 2001). 43

Figure 2.1. Effect of chitosan concentration on the encapsulation efficiency. Sodium alginate concentration (2% w/v); alginate/enzyme ratio (3:1); hardening time (10 min); $CaCl_2$ concentration (0.1 M); agitation speed (70 rpm); chitosan-$CaCl_2$ pH (5.4). Results are presented as mean ± standard deviation. 59

Figure 2.2. Effect of alginate concentration on the encapsulation efficiency. Chitosan concentration (0.1% w/v); hardening time (10 min); $CaCl_2$ concentration (0.1 M); agitation speed (70 rpm); chitosan-$CaCl_2$ pH (5.4). Results are presented as mean ± standard deviation. 60

Figure 2.3. Effect of $CaCl_2$ concentration on the encapsulation efficiency. Sodium alginate concentration (2 % w/v); chitosan concentration (0.1% w/v); alginate/enzyme ratio (3:1); hardening time (10 min); agitation speed (70 rpm); chitosan-$CaCl_2$ pH (5.4). Results are presented as mean ± standard deviation. 61

Figure 2.4. Effect of hardening time on the encapsulation efficiency. Sodium alginate concentration (2% w/v); chitosan concentration (0.1% w/v); $CaCl_2$ concentration (0.1 M); alginate/enzyme ratio (3:1); agitation speed (70 rpm); chitosan-$CaCl_2$ pH (5.4). Results are presented as mean ± standard deviation. 62

Figure 2.5. Effect of chitosan-$CaCl_2$ solution-pH on the encapsulation efficiency. Sodium alginate concentration (2% w/v); chitosan concentration (0.1% w/v); $CaCl_2$ concentration (0.1 M); hardening time (10 min); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation. 63

Figure 2.6. Effect of alginate concentration on the enzyme release from the chitosan treated beads in release buffer. Chitosan concentration (0.1% w/v); $CaCl_2$ concentration (0.1 M); hardening time (10 min); chitosan-$CaCl_2$ pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation. 64

Figure 2.7. Effect of chitosan concentration on the enzyme release from chitosan treated beads in citrate buffer. Na-alginate concentration (2% w/v); $CaCl_2$ concentration (0.1 M); hardening time (10 min); chitosan-$CaCl_2$ pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation. 65

Figure 2.8. Effect of $CaCl_2$ concentration on the enzyme release from 66

chitosan treated beads in citrate buffer. Na-alginate concentration (2% w/v); chitosan concentration (0.1% w/v); hardening time (10 min); chitosan-$CaCl_2$ solution-pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation.

Figure 2.9. Effect of chitosan-$CaCl_2$ solution-pH on the enzyme release from chitosan treated beads in citrate buffer. Na-alginate concentration (2% w/v); chitosan concentration (0.1% w/v); hardening time (10 min); $CaCl_2$ concentration (0.1 M); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation. 67

Figure 2.10. Effect of Na-alginate/enzyme ratio on the enzyme release from the Ca-alginate beads. Na-alginate concentration (2% w/v); chitosan concentration (0.1% w/v); hardening time (10 min); $CaCl_2$ concentration (0.1 M); chitosan-$CaCl_2$ solution-pH (5.4); agitation speed (70 rpm). Results are presented as mean ± standard deviation. 68

Figure 2.11. The SEM images of wet beads. The surface view of chitosan-free (a, c, e) and chitosan-coated beads (b, d, f, g, h): Original magnification (a, b, g, × 1.0k; c, d, ×10k; e, f, h, ×80); the inside view of Ca-alginate (i, k) and Ca-alginate coated with chitosan (j, l) (original magnification: i, j, ×1.0k; k, l, ×10k); voltage (5.00 kV); encapsulation conditions: alginate concentration (2%); $CaCl_2$ concentration (0.1M); chitosan concentration (0.1%); hardening time (10 min); chitosan-$CaCl_2$ pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm). 69

Figure 2.12. The scanning electron microscopy images of the freeze-dried Ca-alginate coated with chitosan beads. The surface view of freeze dried chitosan-coated alginate beads (original magnification: m, × 50; n, q, ×250; o, × 80; p, r, × 1.0k); Voltage: 5.00 kV. Encapsulation conditions: Na-alginate concentration (2 %); $CaCl_2$ concentration (0.1 M); chitosan concentration (0.1%); hardening time (10 min); chitosan-$CaCl_2$ pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm). 71

Figure 3.1. Changes in lactococci populations (a) cheeses with the free enzyme, (b) cheeses with the encapsulated enzyme. Results are presented as mean ± standard deviation. Probability level: $P < 0.05$. See Table 1 for identification of cheeses codes. 89

Figure 3.2. Changes in lactobcilli populations (a) cheeses with the free enzyme, (b) cheeses with the encapsulated enzyme. Results are presented as mean ± standard deviation. Probability level: $P < 0.05$. See Table 1 for identification of cheeses codes. 90

Figure 3.3. Changes in (a) WSN (expressed as a percentage of total nitrogen), (b) PTA-N (expressed as a percentage of total nitrogen) and (c) total FAA contents. Results are expressed as mean ± standard deviation. Probability level: $P < 0.05$ (WSN); $P < 0.01$ (PTA-N, total FAA). See Table 1 for identification of cheeses codes. 91

Figure 3.4. Concentration of individual free amino acids at (a) 60 days, (b) 120 days, (c) 180 days of ripening in control and experimental cheeses. Results are expressed as mean ± standard deviation. See Table 1 for identification of cheeses codes. 93

Figure 3.5. PCA of the individual FAA in the control and experimental cheeses. a, b, c; d, e, f; g, h, i; j, k, l; m, n, o stand for the control cheese, F500U, F2000U, E500U, and E2000U ripened at 4°C after 2, 4, and 6 months, respectively. 95

Figure 4.1. Changes in microbial counts (a) lactococci, (b) lactobacilli during ripening of the cheeses. Results are presented as mean ± standard deviation. Probability level: $P < 0.05$. 111

Figure 4.2. Changes in (a) WSN (expressed as a percentage of total nitrogen), (b) PTA-N (expressed as a percentage of total nitrogen) and (c) total FAA contents. Results are expressed as mean ± standard deviation. Probability level: $P < 0.05$ (WSN); $P < 0.01$ (PTA-N, total FAA). 112

Figure 4.3. Concentration of individual FAA at (a) 1d, (b) 2 months, (c) 4 months and (d) 6 months of ripening in control and experimental cheeses. Results are expressed as mean ± standard deviation. 114

Figure 4.4. PCA of the individual FAA in the control and experimental cheeses. a, b, c, d; e, f, g, h; I, j, k, l stand for the control cheese, cheese with the free enzyme and cheese with the immobilized enzyme ripened at 4°C after 1 d, 2, 4, and 6 months, respectively. 116

Figure 5.1. Changes in WSN (expressed as a percentage of total nitrogen) during maturation times, (a) Neutrase-added EMCs, and (b) non-Neutrase-added EMCs. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes. 129

Figure 5.2. Changes in PTN (expressed as a percentage of total nitrogen) during maturation times, (a) Neutrase-added EMCs, and (b) non-Neutrase-added EMCs. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes. 130

Figure 5.3. Changes in Total FAA contents. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes. 131

Figure 5.4. Concentration of individual free amino acids in control and Neutrase-added EMCs. (a) 1 day, (b) 3 days, (c) 6 days of ripening. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes. 132

Figure 5.5. Changes in Total FAA contents of the two groups of EMCs at 1 day. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes. 135

Figure 5.6. Concentration of individual free amino acids of the two groups of EMCs at 1 day of ripening. (a) 10 units, (b) 25 units, (c) 50 units. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes. 136

Figure 5.7. PCA of the individual FAA in the control and experimental EMCs. 1 Day: EMC1 (a), EMC5 (b), EMC2 (c), EMC8 (d), EMC 6(e), EMC3 (f), EMC9 (g), EMC7 (h), EMC4 (i), EMC10 (A); 3 Days: EMC1 (B), EMC5 (C), EMC2 (D), EMC8 (E), EMC 6(F), EMC3 (G), EMC9 (H), EMC7 (I), EMC4 (J), EMC10 (K); 6 Days: EMC1 (L), EMC5 (M), EMC2 (N), EMC8 (O), EMC 6(P), EMC3 (Q), EMC9 (R), EMC7 (S), EMC4 (T), EMC10 (U). 139

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FOREWARD

This thesis is submitted in the format of papers suitable for journal publication. The chapters of this thesis are written in a way to link among the topics to elaborate the contents of the thesis using a series of manuscript. The general introduction presents the rational and objectives of the research. Chapter 1 (literature review) reviews and details the relevant methods and approaches to accelerate cheese ripening. Chapter 2 describes a method for encapsulation of recombinant aminopeptidase of *Lactobacillus rhamnosus* S93. Chapters 3 and 4 focus on the applications of the encapsulated or free form of this enzyme in accelerating Cheddar cheese ripening. Chapter 5 covers the use of the natural or recombinant pepN of *Lactobacillus rhamnosus* to develop a process for Cheddar-EMC flavorings. The summary of the major conclusions and recommendations for future studies are included at the end of the main text body. A thorough bibliography is the final part of this thesis.

This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines Concerning Thesis Preparation, which are as follows:

"Candidates have the option, subject to the approval of the Department, including as part of the thesis the text of an original paper, or papers suitable for submission to learn journals for publication. In this case, the thesis must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional materials (experimental and design data as well as descriptions of equipment) must be provided sufficient detail to allow a clear and precise judgment to be made of the importance and originally of the research reported. The abstract, full introduction and conclusion must be included, and where more that one manuscript appears, connecting texts and common abstracts, introduction and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted. While the inclusion of manuscripts co-authored by the candidate and others is not prohibited by McGill, the candidate is warned to make an explicit

statement on who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the External Examiner is made more difficult in such cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis."

Although all the work reported in this thesis is the responsibility of the candidate, Dr. Byong H. Lee from Department of Food Science and Agricultural Chemistry, Macdonald Campus, had direct advisory input as the work as it progressed.

CONTRIBUTION OF AUTHORS TO MANUSCRIPTS FOR PUBLICATION

The chapters of this thesis have been presented at scientific conferences and published or are in process of publication in peer reviewed journals. The author of this thesis was responsible for the plan and conduct of experiments, collection and analysis of the data, and preparation of manuscripts for publication. Dr. Byong H. Lee is the thesis supervisor, and had direct supervision on the research. He was also involved in editing and reviewing the manuscripts. Dr. Varoujan Yaylayan provided scientific advice. Dr. Daniel St-Gelais provided advice, financial support and technical assistance to carry out cheesemaking trials at Agriculture & Agri-Food Canada, Food R & D Centre (St-Hyacinthe, QC, Canada). Dr. Claude Champagne (Food R & D Centre, St-Hyacinthe, QC, Canada) provided advice for enzyme encapsulation, financial support, and editorial assistance in the final stage of paper submission. Dr. Kieran Kilcawley (Department of Food Safety and Cultures, Teagasc, Moorepark, Cork, Ireland) provided technical assistance for analysis of free amino acids and editorial assistance in the final stage of paper submission. Mr. Normand Robert provided technical support for enzyme preparation and purification. Dr. Hassan Sabik provided advice and technical support.

List of publications and scientific presentations related to the thesis

A. Part of this thesis has been published or submitted as follows;

Azarnia, S., Robert, N. and Lee, B. H. (2006). Biotechnological methods to accelerate Cheddar cheese ripening. *Crit. Rev. Biotechnol.* 26: 121-143.

Azarnia, S., Lee, B. H., Robert, N. and Champagne, C. P. (2008). Microencapsulation of a recombinant aminopeptidase (pepN) from *Lactobacillus rhamnosus* S93 in chitosan-coated alginate beads. *J. Microencapsulation* 25: 46-58.

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Azarnia, S. and Lee, B. H. (2006). EMC-Flavors and Cheddar Cheese Ripening by Native and Recombinant *Lactobacilli* Enzymes. (Oral presentation). Food R & D Centre, Agriculture and Agrifood Canada, St-Hyacinthe, QC, Canada.

Azarnia, S., Lee, B. H., St-Gelais, D. and Champagne, C. P. (2007). Acceleration of Cheddar cheese ripening by free and encapsulated recombinant aminopeptidase of *Lactobacillus rhamnosus.* (Oral presentation). *IFT 2007 Chicago, IL, USA.*

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Azarnia, S., Lee, B. H., St-Gelais, D. and Champagne, C. P. (2007). Enhancement of proteolysis and flavors in Cheddar cheese using encapsulated recombinant aminopeptidase of *Lactobacillus rhamnosus* S93. (Oral presentation). (2008). *IDF. Cheese Symposium. Bern, Switzerland.* (Accepted).

PREFACE

CLAIMS OF ORIGINAL RESEARCH

1. **Developed a method to encapsulate a recombinant aminopeptidase (90 kDa) of *Lactobacillus rhamnosus* S93 in chitosan- coated alginate beads.**

   This enzyme was successfully encapsulated in chitosan-coated alginate beads prepared by an extrusion method. The optimal conditions (alginate, $CaCl_2$, chitosan concentrations, hardening time, pH, and alginate/enzyme ratios) were evaluated and resulted in the high encapsulation efficiency and the low enzyme release from the capsules during beads formation.

2. **Developed a method to accelerate Cheddar cheese ripening using the encapsulated recombinant aminopeptidase of *Lactobacillus rhamnosus* S93.**

   This is the first report of the application of chitosan-alginate particles to improve enzyme delivery and activity into the cheese matrix. Adding the encapsulated form of recombinant aminopeptidase of *Lactobacillus rhamnosus* S93 at renneting step resulted in acceleration of ripening (70%) with superior sensory characteristics in experimental Cheddar cheeses compared to the control. This encapsulation technique led to the high rate of incorporation of the enzyme into the cheese matrix, indicating the stability of the capsules during Cheddar cheese manufacturing stages.

3. **Developed a method to accelerate Cheddar cheese ripening using the free form of recombinant aminopeptidase of *Lactobacillus rhamnosus* S93.**

   Adding the free form of this enzyme at the salting step into the cheese matrix led to higher proteolysis and superior sensory properties in experimental Cheddar cheeses than the control cheese.

4. **Developed a process of Cheddar-Enzyme Modified Cheese (EMC) flavorings using natural or recombinant enzymes of *Lactobacillus rhamnosus* S93.**

   The proteolysis of experimental EMCs by these enzymes was significantly increased compared to the control. The highest level of the secondary

proteolysis was achieved using both the recombinant and natural enzymes at the highest concentration in the presence of a proteinase (Neutrase). Proline disappeared in EMCs treated with the recombinant aminopeptidase or natural enzymes alone or in combination over maturation times.

GENERAL INTRODUCTION

Cheese ripening is a microbiological and biochemical process involving in the enzymatic digestion of curd components driven by three basic reactions of proteolysis, lipolysis and glycolysis. The purpose of this process is to change a raw curd into a cheese with a distinct flavor, aroma and texture (Spreer, 1998).

In hard cheeses such as Cheddar, proteolysis is the most important reaction for flavor formation. The primary proteolysis results from the actions of chymosin and plasmin on the caseins leading to the formation of large and intermediate-sized peptides which in turn are degraded to smaller peptides by residual coagulants and enzymes derived from starter and non-starter bacteria. Intracellular bacterial peptidases are responsible for degradation of small peptides to free amino acids. These free amino acids are degraded into flavor and aroma compounds (Law, 2001; Sousa *et al.*, 2001).

Maturation of Cheddar cheese normally takes place at 5°C to eliminate defects causing by the growth of spoilage microorganisms. Thus, ripening is slow and an expensive process for the cheese industry and reduction of this period without destroying the quality of matured cheese has economic and technological advantages for the cheese industry (Fox *et al.*, 1998).

Elevated ripening temperature, addition of enzymes and cheese slurry, attenuated and adjunct cultures, genetically engineered starters, and encapsulation of ripening enzymes are important methods to accelerate cheese ripening.

Elevated temperature is the simplest method for accelerating the ripening of Cheddar cheese. However, this method could cause equal acceleration of all biochemical reactions involved in cheese ripening that result in unbalanced or off-flavors (Hannon *et al.*, 2005). Furthermore, it may lead to the growth of unwanted or survival of pathogenic and food-poisoning microorganisms (Fox *et al.*, 1996).

Addition of exogenous enzymes is another way to reduce the maturation time. The poor availability of approved commercial enzymes for cheese ripening, non-uniform distribution of enzymes in the curd and their losses in the whey are the main drawbacks to this method (Wilkinson, 2004; Kailasapathy and Lam, 2005).

Enzyme modified cheeses (EMC) are typically produced by enzyme addition to the cheese after manufacture or maturation and made into a paste by the inclusion of water and emulsifier under shearing (Moskowitz and Noelck, 1987). The industrial demand for EMCs has increased due to its application as a cheese flavor ingredient in processed foods and in low-fat and non-fat products. Production of EMC is the most economic way to produce an intense natural Cheddar cheese flavor (Lee *et al.*, 2007).

Starter bacteria have also an important role in the ripening and development of flavor in cheese. Using an attenuated starter culture reduces the acid-producing ability of the cells without the destruction of their intracellular enzymes (Law, 2001). Lysis of lactic acid bacteria (LAB) causes the involvement of the intracellular starter enzymes in the gradual process of cheese flavor formation (Lortal and Chapot-Chartier, 2005).

Non-starter lactic acid bacteria (NSLAB) have a significant role in proteolysis and flavor development in cheese during ripening (di Cagno *et al.,* 2006). They always predominate in producing Cheddar cheese with good quality. However, using an adjunct starter has disadvantages of rapid and excess acidification, resulting in atypical flavor in cheese (Lee *et al*, 2007).

The LAB system has the advantage of being an *in situ* microflora containing the enzymes that are desirable for cheese flavors and ripening. However, LAB enzymes are mostly intracellular and extraction from a low yield of LAB biomass is expensive. Therefore, the use of over-expressed enzymes of *Lactobacillus casei* species, i.e. *Lactobacillus rhamnosus* S93 which naturally contains high levels of intracellular proteinase, peptidase and esterase could be an alternative application in cheese ripening (Lee *et al.*, 2004; Azarnia *et al.*, 2006b).

A balanced breakdown of caseins into small peptides and amino acids is important in development of acceptable cheese flavor and texture (Visser, 1993). For this purpose, encapsulation of ripening enzymes could be a cost effective way for uniform delivery of enzymes into the cheese matrix (Azarnia *et al.*, 2006a; Kailasapathy and Lam, 2005).

Liposomes or artificial lipid membrane vesicles could be used to encapsulate the enzymes and added cheese milk to accelerate Cheddar cheese ripening. However,

high costs, limitation for large-scale production and low encapsulation efficiency are the main disadvantages of this method (Kailasapathy and Lam, 2005).

Another alternative is the use of natural polysaccharide or food gums to encapsulate enzymes used in accelerating cheese ripening. Alginate has been used for living cells and enzymes due to its cost- effective, biocompatible and non-toxic matrix as a food additive (Sankalia *et al.*, 2005). It is a linear polysaccharide of guluronic and mannuronic acid residues extracted from brown algae (Draget *et al.*, 2006). This polyanionic polymer forms a three-dimensional gel network in the presence of divalent cations, such as calcium, resulting from ionic crosslinking via calcium bridges between guluronic acid residues (Draget *et al.*, 2006). Alginate has been used to immobilize enzymes such as lipase (Betigeri and Neau, 2002), dextransucrase (Kubik *et al.*, 2004), inulinase (Catana *et al.*, 2005), α-galactosidase (Prashanth and Mulimani, 2005), glucose oxidase (Blandino *et al.*, 2000; Khani *et al.*, 2006), and Flavorzyme (Kailasapathy *et al.*, 2006). Alginate-pectin capsules have also been used for the fortification of Cheddar cheese with folic acid (Madziva *et al.*, 2006).

However, alginate gels have large pores that could result in enzyme release during the operation (Tanaka *et al.*, 1984). This problem could be reduced by formation of a polyelectrolyte complex between alginate as an anionic polymer and chitosan as a cationic polymer (Draget *et al.*, 2006).

Although alginate has been used as an immobilization matrix for biomolecules and microorganisms (Vårum and Smidsrød, 2006), no data are found on the encapsulation of lactobacilli peptidases in alginate-chitosan particles and their applications to accelerate cheese ripening.

The objectives of this research, therefore, were (1) to encapsulate the recombinant aminopeptidase (pepN) of *Lactobacillus rhamnosus* S93 in chitosan-coated alginate beads, (2) to evaluate the effects of either free or encapsulated form of this enzyme on proteolysis and sensory properties of Cheddar cheese in two different scales of cheesemaking trials, and (3) to investigate the application of natural or recombinant enzymes derived from *Lactobacillus rhamnosus* S93 for developing a process of Cheddar-EMC flavorings.

CHAPTER 1

LITERATURE REVIEW

Biotechnological Methods to Accelerate Cheddar Cheese Ripening

Cheese is one of the dairy products that can result from the enzymatic coagulation of milk. The basic steps of the transformation of milk into cheese are coagulation, draining, and ripening. Ripening is the complex process required for the development of a cheese's flavor, texture and aroma. Proteolysis, lipolysis and glycolysis are the three main biochemical reactions that are responsible for the basic changes during the maturation period. As ripening is a relatively expensive process for the cheese industry, reducing maturation time without destroying the quality of the ripened cheese has economic and technological benefits. Elevated ripening temperatures, addition of enzymes, addition of cheese slurry, attenuated starters, adjunct cultures, genetically engineered starters and recombinant enzymes and microencapsulation of ripening enzymes are traditional and modern methods used to accelerate cheese ripening. In this context, an up to date review of Cheddar cheese ripening is presented.

The paper entitled "Biotechnological methods to accelerate Cheddar cheese ripening" has been published in *Critic. Rev. Biotechnol.* 26: 121-143, 2006.

1.1 INTRODUCTION

Cheesemaking serves to preserve bovine milk or its nutritional components for later human consumption (Spreer, 1998; Eck, 2000). About 95% of cow's milk nitrogen occurs in the form of proteins, amongst which casein, which mainly exists in the form of calcium phospho-caseinate, is the most prominent (Fox, 1989a; Spreer, 1998). During cheesemaking, about 95% of the casein is transformed from milk into curd (Renner, 1993). This transformation occurs in three basic steps: coagulation, draining, and ripening. Classified as a hard cheese, Cheddar has an intermediate texture and is white or orange in color (Fox, 1993; O'Riordan and Delahunty, 2003a,b). American Cheddar cheese is more cohesive, with a waxy texture and a bland flavor (Guinee, 2003), whereas English Cheddar cheese is crumbly with a sharp and acid flavor (Kosikowski and Mistry, 1997). The textural changes and flavor enhancement that occur during the ripening of Cheddar cheese are part of a complex process driven by three basic reactions: proteolysis, lipolysis and glycolysis (Sousa *et al.*, 2001; Collins *et al.*, 2003a). These reactions, responsible for the changes that occur in the curd during ripening, are carried out by chymosin (rennet) or rennet substitutes, indigenous milk enzymes, adventitious starter and non-starter bacteria. Factors such as maturation time, pH and salt concentration also affect the rate of ripening (Fox *et al.*, 1996).

In the flavor formation of matured hard cheeses such as Cheddar, proteolysis, in generating small peptides and amino acids, is the main biochemical flavor-generating process (Fox *et al.*, 1996; Forde and Fitz-Gerald, 2000; Law, 2001). Amines, aldehydes, alcohols and ammonia, derived from amino acid degradation, make up a significant portion of cheese flavor and aroma compounds. Furthermore, the breakdown of a cheese's proteinaceous network causes textural changes in the cheese matrix (Sousa *et al.*, 2001).

Also important in the development of cheese flavor, lipolysis (Forde and Fitz-Gerald, 2000; Collins *et al.*, 2003a,b) results in the formation of free fatty acids (FFAs) from the hydrolysis of triglycerides. Volatile compounds, such as methyl ketones, thioesters and lactones, result from the catabolism of FFAs (Sousa *et al.*,

2001; Collins *et al.*, 2003a,b). These FFA(s) have a major role in Cheddar cheese flavor, short chain ones causing rancid off-flavors (Deeth and Touch, 2000). However, the contribution of individual components to Cheddar cheese flavor is not well understood (Wijesundera and Drury, 1999). Decomposition of the lactose by starter bacteria affects both the texture and flavor of Cheddar cheese. Compounds, such as diacetyl, acetic and propionic acid, are derived from glycolysis (Forde and Fitz-Gerald, 2000). Since the metabolism of lactose is restricted by salting, most of the lactic acid is produced before Cheddar cheese salting (Choisy *et al.*, 2000). Citrate metabolism leads to an undesirable openness and floating curd in Cheddar cheese. Since Cheddar cheese ripening is a long and expensive process, shortening the duration of ripening reduces manufacturing expenses. The most common methods to accelerate maturation are to increase the ripening temperature, use exogenous enzymes, use attenuated and adjunct starter cultures, add cheese slurry, employ genetically modified starters or recombinant enzymes, and microencapsulate ripening enzymes. Changes in ripening conditions significantly affect Cheddar cheese characteristics, and the advantages and disadvantages of traditional and new methods used to accelerate Cheddar cheese ripening are discussed below.

1.2 CHEESE

1.2.1 Milk and Cheese Production

Rising 14% from 1995, 2004 total world milk production reached 613 million tons, and is expected to reach 747 million tons in 2014 (Bulletin of the International Dairy Federation, 2005). Of different milk sources, cow's milk accounted for the largest share of total milk production, its 2004 production totaling 516 million tons (Bulletin of the International Dairy Federation, 2005). In 2004, the United States was the top cow's milk producing country with 77.6 million tons. In 2004, global cheese production was 17.8 million tons, accounting for about 40% of all milk processed worldwide, and of which 80% was made from cow's milk. Hard/semi-hard cheeses account for the largest portion (50.9%) of total cheese production (Bulletin of the International Dairy Federation, 2005). Internationally, the Finns are the largest

consumers of milk at 180.9 kg/capita, while the Greeks are the largest consumers of cheese at 28.7 kg/capita (Bulletin of the International Dairy Federation, 2005).

1.2.2 Cheese Proteins

Different varieties of cheese contain from 20 to 35% protein and are thus good sources of essential amino acids (Renner, 1993; Dillon and Berthier, 2000). Casein and whey proteins (serum proteins) are the two main protein fractions in bovine milk (Spreer, 1998; Walstra *et al.*, 1999). Casein exists in the form of calcium phospho-caseinate and accounts for about 80% of the total protein of bovine milk (Spreer, 1998; Lucey and Singh, 2003). The four main types of casein in the casein micelle are α_{s1}-, α_{s2} -, β- and κ- caseins and their relative proportions are about 45, 12, 35 and 8%, respectively (St-Gelais and Haché, 2005). There are two main regions in the primary structure of the peptide chain. While the amino terminal (1-105) of κ- casein is hydrophobic, the carboxyl terminal (106–169) is acidic and hydrophilic (Brule *et al.*, 2000). Casein micelles occur in combination with appropriate quantities of colloidal calcium phosphate (CCP). The isoelectric point of casein is at pH 4.6 (de Kruif and Holt, 2003; Lucey and Singh, 2003; Swaisgood, 2003). K-Casein accounts for 8–15% of total casein and its isoelectric point is at pH 4.1– 4.5. The phenylalanine105-methionine106 peptide bond is sensitive to enzymatic hydrolysis, which generates the hydrophobic para-κ-casein (residues 1–105) and a hydrophilic macropeptide or glycomacropeptide (CMP or GMP, residues 106–169) (Spreer, 1998; Walstra *et al.*, 1999; Hyslop, 2003). The α- and β-caseins are sensitive to calcium ions, while κ-casein plays a role in the stabilization of casein micelles in the presence of calcium salts in milk (de Kruif and Holt, 2003; Hyslop, 2003).

1.2.3 Cheesemaking Process

The basic steps in the transformation of milk into cheese consist of coagulation, draining, and ripening. During the coagulation step, modification of casein micelles occurs under the action of proteolytic enzymes or lactic acid. Biochemical modifications of the curd components occur in the ripening stage (Spreer, 1998; Mulvihill and Ennis, 2003). The coagulation of milk by rennet occurs in a sequence

of three phases (Fox, 1993; Spreer, 1998): (i) destabilization of κ-casein by limited proteolysis at the 105–106 peptide linkage, leading to the formation of para-κ-casein (residues 1–105) and a caseinomacropeptide (residues 106–109) (Dalgleish, 1993; Hyslop, 2003; Mulvihill and Ennis, 2003). Para-κ-casein, being insoluble and hydrophobic, aggregates and forms the coagulum in the presence of divalent ions. The caseinomacropeptide fraction is soluble and is lost in the whey after cutting; (ii) aggregation and precipitation of the destabilized casein micelles, enhanced by the presence of calcium ions at the optimum pH and temperature; (iii) proteolysis plays a major role during the maturation period and development of a network. It has a significant effect on flavor and texture development, resulting from changes in the properties and structure of the chymosin curd.

1.3 RIPENING OF CHEDDAR CHEESE

Cheddar cheese ripening is a very complex microbiological and biochemical process which involves the enzymatic digestion of the curd components (Spreer, 1998; Choisy *et al.*, 2000). The purpose of cheese ripening is to change a raw cheese curd into a cheese with a distinct flavor, aroma, and body. Cheese flavor is a complex combination of several hundred flavor components, developed through biochemical alterations that occur during the ripening period (Forde and Fitz-Gerald, 2000; Kwak *et al.*, 2003; Lucey *et al.*, 2003). The process of Cheddar cheese ripening involves the fermentation of lactose, and the degradation of proteins and fats (Laleye *et al.*, 1987). Changes in curd composition and microflora during ripening affect the flavor development of Cheddar cheese, which is controlled by variations in temperature and duration of maturation (O'Riordan and Delahunty, 2003a,b). The microstructure of Cheddar cheese consists of an extensive network that includes αs_1-casein, β-casein and the other caseins (Lucey *et al.*, 2003).

1.3.1 Ripening Agents

Cheese ripening is catalyzed by milk enzymes, coagulant, starter lactic acid bacteria (LAB), and non-starter lactic acid bacteria (NSLAB) (Wilkinson, 2004). All milk components remaining in the curd are involved in the cheese ripening and

maturation process, which involves the enzymatic degradation of these components (Forde and Fitz-Gerald, 2000). In general, the important components involved in cheese ripening are: chymosin or rennet substitutes, natural milk enzymes, starter bacteria and their enzymes, and enzymes from secondary starter cultures and molds.

1.3.2 Reactions Involved in Ripening

Proteolysis, lipolysis, and glycolysis are the three major biochemical reactions that occur during cheese ripening (Collins *et al.*, 2003a; Lucey and Singh, 2003; Smit *et al.*, 2005). These reactions involve a variety of chemical and microbiological changes under controlled conditions. They are responsible for the basic flavor and textural changes of cheese during ripening (Collins *et al.*, 2003a; Lucey and Singh, 2003).

1.3.2.1 Proteolysis

Proteolysis is the most important biochemical reaction in cheese during ripening, and plays a vital role in the development of texture and flavor (Fox and Wallace, 1997; Spreer, 1998; Sousa *et al.*, 2001). Coagulating proteases, plasmin and microbial proteases (from starter and non-starter bacteria) influence proteolysis during ripening. These proteolytic enzymes provide a mixture of small peptides and amino acids which directly change the taste of the cheese (Law, 2001). Amino acids are also precursors of volatile aroma compounds (Smit *et al.*, 2005). Free amino acids are degraded into compounds such as amines, aldehydes, alcohols and ammonia, which are flavor and aroma compounds (Fox *et al.*, 1996; Law, 2001). The breakdown of the protein network during proteolytic activity leads to textural changes in the cheese matrix. Carboxyl and amine groups that are liberated during proteolysis cause a decrease in water activity by binding water molecules (Sousa *et al.*, 2001).

In hard cheeses such as Cheddar, proteolysis is the most important reaction in flavor formation (Forde and Fitz-Gerald, 2000; Smit *et al.*, 2005). The primary proteolysis results from the actions of chymosin and plasmin (Forde and Fitz-Gerald, 2000). Hydrolysis of caseins leads to the formation of large and intermediate-sized peptides, which in turn are degraded to smaller peptides by coagulants and enzymes

derived from starter and non-starter bacteria. Intracellular bacterial peptidases that are released after cellular lysis are responsible for degradation of small peptides to free amino acids (Sousa *et al.*, 2001). It is believed that amino acid catabolism is accomplished essentially by LAB, and is responsible for aroma formation in Cheddar cheese. Amino acids are catabolized by two different pathways: one leads to the formation of important sulfur compounds, while the other is initiated by a transamination reaction and is the main pathway for degradation of all amino acids by LAB. These reactions lead to formation of α-keto acids, which in turn are degraded to various aroma compounds (Yvon and Rijnen, 2001; Kieronczyk *et al.*, 2003). LAB and NSLAB cooperate in aroma formation in Cheddar cheese: the conversion of amino acids to keto- and hydroxyl-acids is initiated by lactobacilli, while *Lactococcus* strains further convert these products to carboxylic acids. This cooperation between LAB and NSLAB leads to an enhanced cheese flavor. The proteolytic enzymes involved in various stages of proteolysis in cheese are shown in Figure 1.1.

1.3.2.2 Lipolysis

Lipases in cheese originate from milk lipase, chymosin paste, starter, adjunct starter, non-starter bacteria, and exogenous lipases (Deeth and Fitz–Gerald, 1995; Thomson *et al.*, 1999; McSweeney and Sousa, 2000; Perotti *et al.*, 2005). Lipases catalyze the hydrolysis of triglycerides, diglycerides, monoglycerides, fatty acids and glycerol (Deeth and Fitz–Gerald, 1995; Thomson *et al.*, 1999; McSweeney and Sousa, 2000). Lipolysis has an important effect on flavor development in cheese during ripening. Fatty acid composition, lipolytic enzymes, lipolytic microorganisms, moisture, temperature, storage time, oxygen, and surface area all affect lipolysis. Lipolytic degradation of triglycerides of milkfat leads to the formation of free fatty acids, which are catabolized to volatile compounds, such as methyl ketones, thioesters and lactones (Walstra *et al.*, 1999; Forde and Fitz–Gerald, 2000; Collins *et al.*, 2003a, b). The degree of lipolysis depends on the variety of cheese. For example, in a mold-ripened cheese, extensive lipolysis is essential to flavor development, whereas it is undesirable in the case of Cheddar cheese (Forde and Fitz-Gerald, 2000; Alewijn *et al.*, 2005; Perotti *et al.*, 2005). Free fatty acids (FFAs) play a major role in the flavor

of Cheddar cheese. Large quantities of short chain fatty acids, such as butyric acid, produce rancid off-flavors (Deeth and Touch, 2000). The free caproic acid to linolenic acid ratio of Cheddar cheese is similar to that of milkfat. However, free butanoic acid occurs at a greater concentration in cheese than in milkfat (Collins *et al.*, 2003a). In Cheddar cheese LAB esterolytic or lipolytic enzymes hydrolyze esters of free fatty acids, mono-, di-, and triglycerides (Liu *et al.*, 2001). The lipolytic activity of LAB, especially of *Lactobacillus* and *Lactococcus* spp., is weaker than that of species such as *Pseudomonas*, *Acinetobacter* and *Flavobacterium*. However, because of their high numbers at the extended ripening period, they are responsible for the liberation of significant levels of FFA (Collins *et al.*, 2003a). The FFA act as precursors to produce flavor and aroma compounds in catabolic reactions leading to the formation of methyl ketones, lactones, esters, alkanes and secondary alcohols (Collins *et al.*, 2003a; Alewijn *et al.*, 2005).

1.3.2.3 Glycolysis

During cheesemaking, lactose is converted into lactic acid by the LAB and the main isomer produced during this change is L-lactate (Fox and Law, 1991). Increasing mineral solubilization and casein dissociation are the results of decreasing pH. Demineralization of the casein micelles has a major effect on cheese texture and increases cheese proteolysis (Le Graët and Gaucheron, 1999; Pastorino *et al.*, 2003). Lactose degradation influences cheese flavor and a number of flavor compounds, including diacetyl, acetic acid and propionic acid are produced from this phenomenon (Forde and Fitz-Gerald, 2000). Lactose metabolism is decreased by addition of salt during Cheddar cheese manufacture. In this case, most of the lactic acid is produced in the cheese before salting and molding. The rate of lactose fermentation depends on the salt-in-moisture percentage of the curd. At low salt-in-moisture concentrations and low populations of NSLAB, residual lactose is converted mainly to L (+) lactate by the starter bacteria (Choisy *et al.*, 2000). The level of lactate in Cheddar is about 1.5%. Matured Cheddar contains a considerable concentration of D (-) lactate, which may be formed from residual lactose by lactobacilli or by racemization of L (+) lactate. Approximately 90% of the milk's citrate is soluble and most of this is lost in

the whey. In some cheeses, citrate metabolism leads to flavor compounds, such as diacetyl, acetoin and 2, 3-butyleneglycol (Fox and Law, 1991). Citrate is metabolized rapidly in the presence of fermentable carbohydrate with the production of acetic acid, diacetyl and CO2. Citrate metabolism is responsible for undesirable openness and floating curd in Cheddar cheese (Cogan and Hill, 1993). Heterofermentative lactobacilli cause late-gassing problems in Cheddar cheese. To eliminate this problem and to shorten the Cheddar maturation time, a *Lactobacillus casei* with a high peptidolytic activity has been employed by one dairy in Quebec, Canada (Lee *et al.*, 1990a,b). Thus, in summary, proteolysis, lipolysis and glycolysis are the three main reactions involved in cheese ripening and related to formation of cheese flavor. Cheese flavor biochemistry is summarized in Figure 1.2.

1.4 ACCELERATION OF CHEESE RIPENING

Maturation of Cheddar cheese normally takes place at 5°C, thus largely eliminating defects due to the growth of spoilage microorganisms (Lee *et al.*, 1990a,b). Ripening is a relatively expensive process for the cheese industry; therefore, reduction of this period without destroying the quality of matured cheese has economic and technological advantages (Trépanier *et al.*, 1992b; Fox *et al.*, 1998): (i) savings in refrigeration, labor and inventory expenses; (ii) increased cheese production in developing countries; and (iii) rapid production of cheese flavor.

1.4.1 Methods for Accelerating Cheddar Cheese Ripening

The methods used to accelerate the ripening of cheese have traditionally included the use of enzymes, such as proteinases, peptidases, lipases and β-galactosidase. In general, both traditional and modern methods used to accelerate the maturation time can be categorized into: elevated ripening temperature, addition of enzymes, addition of cheese slurry, attenuated starters, adjunct cultures, genetically engineered starters, and the microencapsulation of ripening enzymes.

1.4.1.1 Elevated Temperature

Although cheese was traditionally ripened in caves or cellars, in modern factories cheese maturation is carried out at controlled temperatures and humidity.

Storing cheese below 10°C maintains its stability and microbiological safety. Elevated ripening temperatures pose a risk in terms of the growth of unwanted microbial contaminants and the possibility of survival of pathogenic and food-poisoning microorganisms (Wilkinson, 1993; Fox *et al.*, 1996). However, cheese made with pasteurized milk under good manufacturing condition can be ripened at temperatures up to 15°C. The most desirable acceleration of flavor and texture development in Cheddar cheese is obtained at 12°C. Under these conditions, maturation time can be decreased 60–75% without body defects (Law, 2001). Cheeses stored above 8°C showed greater total bacterial counts and a slightly elevated streptococcal population compared to control cheeses (Cromie *et al.*, 1987). The total number of NSLAB was unchanged in cheese stored at elevated temperatures. There was no relationship between the presence of bacterial groups and off-flavors developed in cheeses stored at 17.5°C for 32 weeks and 20°C for 16 weeks (Cromie *et al.*, 1987). Hannon *et al.* (2005) evaluated the effect of elevated ripening temperatures of various durations during the initial stages of ripening on the acceleration of Cheddar cheese maturation. Descriptive sensory analysis with multivariate statistical methods was used to develop a controlled-acceleration strategy. The use of elevated ripening temperatures during the early stages of maturation sped up the ripening of experimental cheeses by two months. A well balanced flavor was obtained in cheeses stored at 20°C or 12°C for 1 or 6 weeks, respectively (Hannon *et al.*, 2005). Elevated temperature is the simplest method for accelerating the ripening of Cheddar cheese. However, all biochemical reactions involved in cheese ripening may be accelerated equally at elevated temperatures and unbalanced or off-flavors may result (Law, 2001; Hannon *et al.*, 2005).

1.4.1.2 Addition of Enzymes

Increasing the amount of the key enzymes used in cheese manufacture leads to the acceleration of cheese ripening. Proteinases and peptidases in cheese include plasmin, chymosin, and cell wall and/or intracellular proteinases and peptidases of the LAB and NSLAB. Animal or fungal lipases and proteases are widely used in the manufacture of Enzyme Modified Cheese (EMC). However, the direct use of these

enzymes to develop cheese ripening and flavor is not widespread. The poor availability of approved commercial enzymes for cheese ripening and the inability to homogeneous mix the enzymes into the cheese matrix are the main drawbacks to this method (Law, 2001). Exogenous enzymes can be individual enzymes or mixtures of commercial enzymes. In the cheesemaking process, exogenous enzymes can be used with cheesemilk, with starter cultures or coagulants, by direct addition into cheese blocks, or at the dry salting stage. The latter method is used in the manufacturing of Cheddar cheese (Wilkinson and Kilcawley, 2005).

1.4.1.2.1 Lipases

Lipolysis is limited in cheese varieties such as Cheddar, Dutch and Swiss types. Although it is considerable in the extra-matured cheeses of these kinds, the products of proteolysis and glycolysis affect the formation of the well-balanced flavor (Fox and Stepaniak, 1993). Addition of pre-gastric esterase led to rancidity in Cheddar cheese. However, the use of gastric enzyme extract or a combination of gastric and pre-gastric extracts improved its flavor (Wilkinson, 1993). The role of lipolysis in the generation of flavor in Cheddar cheese is unclear and fatty acids found in the volatile fraction of Cheddar cheese may contribute to the background cheese aroma but not to taste (Wilkinson, 1993). Incubation with an *Aspergillus oryzae* culture broth produced Cheddar aroma in cream (Arbige *et al.*, 1986). Although enzyme preparations from this strain contained both protease and lipase, the lipase fraction was the major contributor to Cheddar aroma in cream (Arbige *et al.*, 1986). This enzyme was named FlavorAge (Chr. Hansen, Inc., US) and commercialized for the acceleration of cheese maturation (Arbige *et al.*, 1986; El Soda and Pandian, 1991; Wilkinson, 1993; Law, 2001). This enzyme's specificity for hydrolysis of intermediate chain length triglycerides (C_6–C_{10}) confers its importance in the Cheddar maturation process. While most lipases produce either butyric acid (C_4) or longer chain FFAs (C_{12} and above), resulting in rancid or soapy flavors, respectively (Arbige *et al.*, 1986; Wilkinson, 1993), the FFA profiles of cheeses treated with FlavorAge were similar to control cheeses, but with higher concentrations (Arbige *et al.*, 1986; Wilkinson, 1993). Matured Cheddar cheese was also obtained by using a low concentration of a

lipase from *Aspergillus oryzae* (strain ATCC 20719). Ripening duration with this lipase was two- to five-fold shorter than with conventional lipolytic enzymes and no enzyme-associated rancidity was reported. Cheddar aroma, measured by GC/MS/sniffing techniques, indicated that lipolysis products were important flavor compounds (Arora *et al.*, 1995). Piccantase (*Mucor miehei*, Gist Brocades), Palatase (*Mucor meihei*, Novo Nordisk), Capalase/Italase (Dairyland), and kid/lamb lipase/esterase (Chr. Hansen) are other microbial lipase preparations that have been used in the cheese industry. However, uniform distribution was difficult to control and defects such as bitterness, rancidity, poor texture, and non-typical flavor have been reported (Lee, 1994; Law, 2001).

1.4.1.2.2 Plasmin

The principal indigenous milk proteinase is plasmin (EC 3.4.21.7). It is a trypsin-like proteinase with a pH optimum of around 7.5, and is produced from plasminogen by the action of various plasminogen activators (Fox, 1993; Forde and Fitz-Gerald, 2000; Kelly and McSweeney, 2003; Lucey *et al.*, 2003). Most of the plasmin and plasminogen are associated with casein micelles and are incorporated into the curd during cheesemaking (Farkye and Fox, 1992; Fox, 1993). In Cheddar, the concentration of this enzyme is approximately 3 to 4.5 mg/g. An increase in plasmin activity induced by heating has been attributed to the thermal inactivation of the inhibitors of plasminogen activators. This phenomenon results in the increased conversion of plasminogen to the active enzyme. While plasmin is more important as a ripening agent in high cooked cheeses, chymosin is inactivated in this variety of cheese (Sousa *et al.*, 2001; Lucey *et al.*, 2003). Plasmin is active, especially on α_{s2}-casein and β-casein. The most susceptible casein is β-casein and its hydrolysis leads to the production of δ-caseins, whereas the κ-casein is fairly resistant to plasmin action (Forde and Fitz–Gerald, 2000; Sousa *et al.*, 2001; Lucey *et al.*, 2003). The elevated plasmin activity accelerates proteolysis of β-casein and the formation of δ-caseins. Hydrolysis of caseins by plasmin leads to the production of large and intermediate-sized peptides which are degraded by the coagulant and microbial enzymes (Sousa *et al.*, 2001). Streptokinase is an extracellular protein, produced by

the mastitis pathogen *Streptococcus uberis,* which can activate plasminogen to plasmin. Upadhyay *et al.* (2004) used a semi-purified preparation of streptokinase, or a strain of *Lactococcus* (PRA270) genetically modified to produce streptokinase. They studied effects of increased plasmin activity on proteolysis during cheese ripening. Cheddar cheese was manufactured from milk supplemented with a semi-purified preparation of streptokinase at 0.1%, 0.25%, 0.5% (v/v), and in separate trials, miniature Cheddar-type cheeses were made using *Lactococcus* strain PRA270 as starter. Both approaches resulted in the activation of plasminogen and increasing plasmin activity in the experimental cheeses. This led to the acceleration of proteolysis and hydrolysis of β-casein. Reversed-phase HPLC showed increased production of hydrophobic peptides in cheeses with higher plasmin activity; however, levels of free amino acids were unaffected (Upadhyay *et al.*, 2004). Compared with the expensive plasmin, commercial trypsin is relatively inexpensive, widely available and can be used for accelerating ripening. Genetic engineering may be the other way to reduce enzyme cost. The gene for plasmin has been cloned and expressed in a *Lactococcus* host, but the result of using this treatment in cheesemaking is not clear (Arnau *et al.*, 1997).

1.4.1.2.3 Other Proteinases and Peptidases

The effect of a neutral protease from *Aspergillus oryzae* on the development of Cheddar cheese flavor and texture was studied during 6 months of ripening at 10°C. Proteolytic enzymes at different concentrations were added to the curd at the milling stage (Fedrick *et al.*, 1986). In this study, proteolysis increased with time and with increasing enzyme concentration. The amount of free amino acids in cheese treated with 0.1 and 0.01% neutral protease after 2 and 4.5 months, respectively, was the same as in the control cheese after 6 months (Fedrick *et al.*, 1986). Addition of a metalloprotease derived from *Micrococcus caseolyticus* resulted in a modification in hard cheese texture. Bitter flavor was not reported in the treated cheese. A neutral protease from *Bacillus subtilis* has also been used to accelerate Cheddar cheese ripening; however, ripened cheeses produced with this protease had defects in body and flavor (Ridha *et al.*, 1983). Cheddar cheeses were produced with intracellular

cell-free enzyme extracts (CFE) of cheese starter bacteria and a commercial Neutrase proteinase. The typical Cheddar flavor produced with Neutrase or with CFE was more intense, after 2 months, than that of the control cheese. Cheese containing both CFE and Neutrase had a stronger flavor than those manufactured with either Neutrase alone or CFE alone (Law and Wigmore, 1983). Production of Cheddar cheese with a fungal acid proteinase led to a bitter flavor. In contrast, bacterial neutral proteinase developed the typical flavor in Cheddar cheese and increased proteolysis by about 2% over two months. Both cheeses, with either bacterial neutrase or fungal neutrase, were softer-bodied and more brittle than the control cheeses of the same age (Law and Wigmore, 1982). The survival of gram-positive bacteria was not affected by the addition of neutral proteinase and aminopeptidase to salt-added Cheddar cheese during ripening, but the death rate of gram negative bacteria was increased in the initial stages. While gram negative bacteria disappeared after 12 weeks, gram positive bacteria were detected at the end of the 16^{th} week ripening period (Bautista and Kroll, 1988). Commercial preparations of a Neutrase from *Bacillus subtilis* were used to accelerate Cheddar cheese ripening. Cheddar cheese flavor was developed without any defects at a level of 0.0125 mg/kg of cheese, but higher levels of the enzyme caused bitterness (Law and Wigmore, 1982). Acid proteinase originating from *Aspergillus oryzae* was stable at the pH of Cheddar cheese, but caused extensive proteolysis and bitterness during ripening. All the proteinase-treated cheeses were more crumbly, less elastic and less firm than the control. These defects were noticeable at low concentrations of neutral proteinase (Law and Wigmore, 1982). Accelase (LAB exopeptidase), a commercial enzyme, has been widely used in the production of different cheese varieties and their reduced-fat variants. This enzyme reduces bitterness in Cheddar cheese (Law, 2001). An enzyme extracted from kilned, germinated barley was added (500 mg/kg curd) to Cheddar curd at the salting stage. The enzyme preparation had a high dipeptidase, proteinase, and carboxypeptidase activity. Cheese produced without the enzyme extract had a higher moisture content and lower pH than cheese produced with the barley extract, Neutrase (20 mg/kg curd), or both. The best-flavored cheese was produced using barley extract plus Neutrase or Neutrase alone, although some bitterness was detectable (Law, 2001).

Commercial proteinase preparations are widely used to accelerate Cheddar cheese maturation. Although inclusion of peptidases is useful to eliminate bitterness, texture and flavor defects can be a problem (Wilkinson and Kilcawley, 2005). Application of a combination of exogenous lipases and proteinases has led to an increase in proteolysis compared to using proteinases alone (Wilkinson and Kilcawley, 2005).

1.4.1.2.4 β-Galactosidase

Using a *β*-galactosidase preparation (the Maxilact preparation) in milk prior to Cheddar cheese manufacture resulted in increased proteolysis compared to an untreated control cheese (Gooda *et al.*, 1983). According to their findings, the proteolytic activity of the *β*-galactosidase preparation was very weak, suggesting that the high levels of the proteolytic products in the experimental cheeses were due to the increased number of starter bacteria and an increase in the quantity of their peptidases. The growth of starter bacteria was stimulated by free glucose and hydrolyzed lactose in the milk. They concluded that using the Maxilact preparation could improve Cheddar cheese manufacture and accelerate its ripening period (Gooda *et al.*, 1983). Production of Cheddar cheese with hydrolyzed lactose milk (up to 60%) caused a reduction in the processing time and accelerated ripening of the cheese (Ridha *et al.*, 1983). Fermentation was much faster with a monosaccharide, glucose/galactose, than a disaccharide, lactose. Table 1.1 summarizes the enzymes from cheese-related and non-cheese related enzymes used to accelerate Cheddar cheese maturation (El Soda and Pandian, 1991).

Using mixtures of proteinase and lipase preparations is another approach to accelerate Cheddar cheese ripening. A partly purified extracellular aminopeptidase of *Brevibacterium linens* was used to accelerate Cheddar cheese ripening. The combination of this enzyme with a commercial Neutrase led to a better flavor score compared with Neutrase alone (Hayashi *et al.*, 1990). Guinee *et al.* (1992) added various levels of serine proteinases from *Brevibacterium linens* (2.9, 8.6 and 26 enzyme units/kg curd) at the salting stage to reduce Cheddar cheese maturation time, and found the greatest proteolysis to occur at the greatest enzyme concentration (26

enzyme units/kg curd). The addition of 48 mg/kg of this enzyme to Cheddar curd at the salting stage led to an increase in water soluble, ethanol soluble and 5% phosphotungstic acid-soluble nitrogen (PTA-SN).

Commercial enzymes available on the market for the reduction of ripening time are listed in Table 1.2.

1.4.1.3 Enzyme Modified Cheese

Production of enzyme-modified cheese (EMC), which is slurry of fresh cheese with different enzymes, is the most economic way to produce an intense natural Cheddar cheese flavor (Kilcawley *et al.*, 2000; Kilcawley *et al.*, 2001). The addition of specific enzymes to the cheese slurry, homogenization, incubation, and inactivation of the enzymes are the important stages in the preparation of EMCs (Wilkinson, 1993; Kilcawley *et al.*, 1998). EMCs are used as an ingredient in salad dressings, dips, sauces, snacks, fillings, cheese spreads, etc. in order to enhance the cheesy taste and to create a specific cheese character.

Lipolysis has an important role in the generation of Cheddar EMC flavor. Short chain volatile free fatty acids play an important role in enhancing the cheese flavor intensity in the final product (Kilcawley *et al.*, 1998; Wilkinson and Kilcawley, 2002). The concentration of free fatty acids is ten times higher in EMC than in young cheese. Starter bacteria, which are used for degradation of lactose to lactic acid and as a source of enzymes, affect cheese flavor development in natural cheeses. However, their role in EMCs production is as to generate flavors (Kilcawley *et al.*, 1998). There are only a few reports on the incorporation of EMC into Cheddar cheese acceleration (so-called slurry method) since most of the work in this area is unpublished and classified as confidential.

1.4.1.4 Attenuated Starters

Starter bacteria have an important role in the ripening and development of flavor in cheese. Because of their main role in the progressive acidification of cheese, increasing the number of starter bacteria can result in over-acidification of the final curd. The purpose of using an attenuated starter culture is to reduce the acid

producing ability of the cells without the destruction of their intracellular enzymes (Wilkinson, 1993; Law, 1999; Law, 2001).

The cell envelope-proteinase of lactococcal starter bacteria is referred to lactocepin. It is divided into two main specificity groups, lactocepin with type PI proteinase specificity (lactocepin I) and lactocepin with type PIII specificity (lactocepin III) (Kunji *et al.*, 1996; Reid and Coolbear, 1998). Lactocepin I breaks down β-casein and κ-casein, while lactocepin III degrades β-, κ-caseins easily and catalyses the hydrolysis of αs_1-casein. Buist *et al.* (1997) showed that lactocepin I degrades the lysozyme N-acetylmuramidase faster than lactocepin III. To control flavor development in cheeses, the autolytic properties of starter bacteria and peptidase activities of individual strains should be considered (Bruinenberg and Limsowtin, 1995). Crow *et al.* (1995a,b) showed that an elevated starter lysis in Cheddar cheese caused an increase in the formation of amino acids and ammonia without increasing lactose degradation. They concluded that the extent of autolysis of adventitious lactic acid bacteria affected the levels of hydrophobic and hydrophilic peptides in cheese extracts, indicating that the balance of the intact and autolysed bacterial cells in young curd is important in cheese ripening.

1.4.1.4.1 Cell Lysis Techniques

Lysis of lactic acid bacteria plays a crucial role in dairy ferment ations and ensures the involvement of the intracellular starter enzymes, particularly peptidases, in the gradual process of cheese flavor formation (Lortal and Chapot-Chartier, 2005). To establish the role of starter lysis in the various stages of cheese ripening, Meijer *et al.* (2004) investigated the lysis process of *Lactobacillus lactis* strains used in cheese manufacture. They also investigated the role of growth conditions on starter cell lysis and its importance for cheese manufacture. The results revealed that the cell walls of rapidly lysing strains were more sensitive to mutanolysin than those of stable strains. The sensitive strains also produced many more flavor components in experimental cheeses compared to the stable ones. A direct correlation was observed between the stability of cell walls and the accumulation of bitter flavor in cheese. The effect of growth conditions on cell lysis efficiency was studied in continuous culture. The cells

and the cell wall composition became stable at lower growth rates in the sensitive strains, while the stable strain was not affected by the changes. They concluded that the cell lysis can be controlled by growth conditions (Meijer *et al.*, 2004).

Heat shock, freeze shock, spray drying, lysozyme treatment and mutation are different approaches, which have been investigated for the production of attenuated cells (Madkor *et al.*, 2000; Law, 2001). Physical methods such as heat-shock, freeze-shock and spray drying are based on cell lysis (Khalid *et al.*, 1991). Freeze-shocked cells are normally prepared by freezing and thawing of the cells through several cycles at -20°C. Both natural and induced lysis of the cell causes an increase in enzymes released into the cheese matrix by the attenuated cells (Law, 2001). Most of the intracellular enzymes of *Lactococcus* or *Lactobacillus* are activated after the dead starter cells lyse (Chapot-Chartier *et al.*, 1994). Use of a fast-lysing strain AM2 (non-bitter) in Cheddar cheese resulted in a five-fold increase of free amino acids over the slow-lysing strain Hp (bitter) (Wilkinson *et al.*, 1994). Attenuated cultures combined with adjunct cultures can be effective in ripening, especially in reduced-fat cheeses. Trépanier *et al.* (1992a) showed that by using a mixture of attenuated and adjunct cultures, cheese flavor and texture were improved without any increase in acidity.

Homogenized heat-treated cells of *Lactobacillus casei* spp. *casei* were added to develop Cheddar cheese flavor (Trépanier *et al.*, 1992a,b). The results showed that the breakdown of peptides and the amount of amino nitrogen increased, and bitterness declined in the treated cheese. Intensity of flavor in Cheddar cheese was altered by the use of a heat-shocked culture of either *Lactobacillus casei* or *Lactobacillus helveticus*. Treating the cells at 67°C for 22 sec led to a maximum reduction of lactic acid production and minimum destruction of the proteolytic enzymes. Attenuated starters of the *Lactococcus* and *Lactobacillus* genera, along with a main starter culture, reduced maturation times and increased the degree of proteolysis and lipolysis in Cheddar cheese. An excellent quality of Cheddar cheese was obtained by using 1% heat-shocked lactobacilli. When compared to the control cheese, this treatment caused a 50% increase in flavor enhancement. Intensity of flavor was increased 60% by addition of Neutrase at a concentration of 1×10^{-5} Anson units (AU)/g of cheese (Trépanier *et al.*, 1992a,b). Flavor intensity was developed in all

reduced-fat Cheddar cheeses treated with freeze-shocked, freeze-dried and spray-dried adjunct cells of *L. helveticus* (Madkor *et al.*, 2000). In this study, *Lb. helveticus* I or *Lb. casei* T were treated with freeze-shock, heat-shock, or spray drying methods, and were then used as an attenuated culture to produce Cheddar cheese. The amounts of free amino nitrogen and free fatty acid were increased in cheeses produced with freeze-shocked or heat-shocked *Lb. helveticus* adjunct, and cheese made with the freeze-shocked *Lb. casei* adjunct, respectively. Freeze-shocked *Lb. helveticus*-treated cheeses had the highest scores for flavor and aroma (Madkor *et al.*, 2000).

Flavor Control (FC^{TM}) CR culture (Chr. Hansen A/S, Denmark), and Enzobat^{TM} (Medipharm, Sweden) are two commercial attenuated starter flavor-enhancing systems (Law, 2001). The former is a non-acidifying natural genetic variant of lactococci and the latter is a heat-shocked *Lb. helveticus*. FC^{TM} culture originally developed for full-fat hard and semi-hard cheeses. However, they were found to also improve the flavor of fat-reduced Cheddar cheese (Law, 2001).

Lactococcus lactis spp. *cremoris* AM2 (a non-bitter strain) or *Lactococcus lactis* spp. *cremoris* HP (a bitter strain) were used to produce Cheddar cheeses (Collins *et al.*, 2003b). After 238 days storage at 8°C, cell viability in cheeses of strain AM2 was lower than that of strain HP. Autolysis was much greater in the cheeses treated with AM2 than in cheeses made with HP. The amounts of caprylic, myristic, palmitic and stearic acids were significantly increased in the cheeses produced with AM2, resulting from a relationship between the amount of cell autolysis and the level of lipolysis during maturation (Collins *et al.*, 2003b).

1.4.1.5 Adjunct Cultures

Adding starter bacteria, adjunct cultures or enzymes directly to the cheese matrix can accelerate ripening. Andersen and Madsen (2004) made semi-hard cheeses from milk acidified to pH 6.4 with lactic acid. They used a needle device to inject a suspension of *Lactobacillus lactis* into the cheese matrix before final pressing. The adjunct bacteria (*Lactococcus* and *Lactobacillus* species) and the enzyme (serine protease) were injected to accelerate ripening. After 3 weeks, cheeses containing starter bacteria and adjunct had similar level of soluble nitrogen as in a 9-month-old

Cheddar cheese, but this index was greater in cheeses treated with the enzyme. In the enzyme-treated cheeses, αs_1- and β-caseins were totally degraded, whereas more than 50% of the casein was intact in the cheeses produced without the ripening enzyme. However, the amount of hydrophilic peptides increased in cheeses with adjunct bacteria, due to the release of peptidases into the cheese matrix. Hannon *et al.* (2003) investigated the use of an autolytic strain of *Lb. helveticus* DPC4571 to accelerate the ripening of Cheddar cheese. They concluded that the presence of this strain in the starter system increased the level of secondary proteolysis compared to the control cheese.

Non-starter lactic acid bacteria (NSLAB) have a significant role in proteolysis and flavor development in cheese during ripening (Peterson *et al.*, 1990; di Cagno *et al.*, 2006). NSLAB are the only bacteria that grow in Cheddar cheese during maturation, and they influence the quality of the cheese. The most important thing related to the cheese quality is the strains of NSLAB, rather than their numbers (Shakeel *et al.*, 1999). They are selected and used as commercial adjunct cultures with starter bacteria to increase proteolysis and enhance flavor intensity (Wilkinson, 1993; Law, 2001; di Cagno *et al.*, 2006). NSLAB may be derived from milk, especially raw milk, cheesemaking equipment, air, or personnel. NSLAB include *Micrococcus, Leuconostoc, Pediococcus* and *Enterococcus,* but mesophilic lactobacilli are the predominant species (Jordan and Cogan, 1993). Low cooking temperatures (38°C) and mesophilic *Lactococcus* starter strains are used in the manufacture of Cheddar cheese. However, Bley *et al.* (1985) studied the effects of higher manufacturing temperatures (42–43°C) and a starter that consisted of *St. thermophilus* and normal mesophilic starter (*Lactococcus lactis* spp. *cremoris*) to reduce maturation time. Problems included the production of CO2 and the presence of up to 33 mmol of galactose/kg curd in the cheese. Presence of galactose in the cheese is mainly responsible for the browning of heated cheeses such as pizzas or processed cheese (Mukherjee and Hutkins, 1994). Martley and Michel (2001) reported a pinkish coloration in Cheddar cheese produced by adventitious *St. thermophilus*. This color was observed in areas that contained significant amounts of galactose. They studied the behavior of *St. thermophilus* in Cheddar cheese in the

presence of the mesophilic starters *Lactococcus lactis* spp. *cremoris* or spp. *lactis*. A combination of *St. thermophilus* with normal starters at 38°C led to an increase in acidity and galactose production, with equal populations of both microorganisms, whereas at 41°C, with the high galactose content, *St. thermophilus* was more numerous than *Lc. lactis* spp. *cremoris*. *Lactobacillus rhamnosus* was intentionally added to a cheese manufactured with *St. thermophilus* starter, which contained 24 mmol galactose/kg, on the first day. The starter utilized all the galactose during the first 3 months of cheese ripening (Martley and Michel, 2001). Adventitious NSLAB also were able to utilize galactose, and there was a close relationship between their populations and the disappearance of this substrate. Since cheese has a low redox, low pH, high salt and low carbohydrate content, many microorganisms cannot grow under these circumstances (Swearingen *et al.*, 2001). When lactococci were added to cheesemilk at about 105 colony forming units (cfu)/g of cheese, after about 3 to 4 months of ripening, viable cells were not detectable (<10 cfu/g cheese) (Fox *et al.*, 1998). Unlike lactococci, NSLAB grew rapidly during the ripening period and their population increased from <10 cfu/g of cheese after manufacture to $>10^6$ cfu/g by 3 to 4 months of ripening. Lactobacilli, especially *Lb. casei*, *Lb. plantarum*, and *Lb. brevis*, were the main NSLAB in Cheddar cheese and grew as heterogeneous bacteria inside the cheese matrix. The number of NSLAB in Cheddar cheese produced from high quality pasteurized milk, in modern automated plants, increased from <100 cfu/g in 1-day-old cheese to about 10^7 cfu/g after 2 months. The flavor of the cheese made from raw milk was more intense than the cheese produced from pasteurized milk, possibly due to the presence of indigenous microflora in the raw milk (Fox *et al.*, 1998). Milk pasteurization may result in the denaturation of milk enzymes and whey proteins, and the destruction of milk microflora including NSLAB. Among these changes, it seems that the alteration of the milk's indigenous NSLAB exerts a major influence on the biochemical changes which occur during Cheddar cheese ripening, more so than heat-induced changes in indigenous enzymes (McSweeney *et al.*, 1993). To evaluate the importance of the NSLAB in biochemical changes during Cheddar cheese maturation, they compared the ripening of Cheddar cheese made from raw milk to that of Cheddar made from pasteurized and microfiltered (MF)

milk. They concluded that differences between proteolysis of the raw and pasteurized milk cheeses were attributable to the NSLAB peptidases. No difference was observed between the pasteurized and MF milk cheeses in the parameters considered (McSweeney *et al.*, 1993).

NSLAB derived from environmental contamination are important contributors to cheese ripening. Their numbers in Cheddar cheese increased up to 5×10^8 cells/g, and dying cells, by releasing enzymes, continued to ripen the cheese in the later stages. *Lactobacillus casei* types were predominant in heat-treated Cheddar (Lee, 1994). The most important factor in using NSLAB as adjuncts in Cheddar is that the NSLAB must be identified and selected correctly. In a study by Swearingen *et al.* (2001), Cheddar cheese was manufactured with selected NSLAB to evaluate their effects on proteolysis and cheese flavor. The results showed that after 6 months storage, the amount of free amino acids in all cheeses that contained adjuncts was higher than in the control cheese. In addition, these cheeses yielded higher sensory scores than the controls.

A cell-free extract of brevibacteria containing a large amount of methanethiol was added to Cheddar cheese curd. Compared to a 6-month-old Cheddar cheese, "typical flavors" were developed during the 2 month ripening period; however flavor and texture defects were observed in over-ripened cheese (Law, 1987). To improve cheese flavor, *B. linens* BL2 was added as an adjunct to the reduced-fat Cheddar (60%). *B. linens* is not a traditional culture for the manufacturing of Cheddar cheese. However, its advantage over other adjuncts is the release of methanethiol via methionine catalysis by methionine-δ-lyase (Weimer *et al.*, 1997). In addition, production of volatile sulfur compounds was increased by the addition of purified *B. linens* BL2 methionine-δ-lyase and free methionine to *Lc. lactis* spp. *cremoris* S2 (Weimer *et al.*, 1999). The effect of different levels of *Lactobacillus casei* spp. *casei* IFPL 731 or *Prop. shermanii* NCDO853 on proteolysis and flavor of Cheddar cheese were studied (Fernández-Esplá and Fox, 1998). The pH and chemical composition of the cheese were not affected using these strains. The highest scores for flavor and body were received by cheeses that were treated with low or medium levels of these adjuncts. However, sweet and nutty flavors were observed in the cheeses treated with

high level of *Prop. shermanii*. The major differences were found in the amino acid levels rather than in primary proteolysis between the control and experimental cheeses. The concentrations of amino acids increased in samples inoculated with high level of *Prop. shermanii*, while medium and high levels of this inoculum in experimental cheeses led to a decrease in content of hydrophobic peptides. The content of amino acids was not increased in samples inoculated with *L. casei* resulting from the lack of cell lysis (Fernández-Esplá and Fox, 1998). The impact of lactic acid bacteria in the maturation of Cheddar cheese was studied by adding cell homogenates and/or live cells of *Lactobacillus casei* spp. *casei* L2A at the renneting and salting stages (Trépanier *et al.*, 1991a,b). Flavor intensity increased 40% in supplemented cheeses compared to the control cheese during ripening (Trépanier *et al.*, 1991a,b). Probiotic bacteria, such as lactobacilli, bifidobacteria and enterococci, have been used as adjunct cultures in the production of different cheeses such as Gouda, Argentinean fresco cheese, white cheese, Cheddar, Cottage, and Crescenza (Bergamini *et al.*, 2006). Ong *et al.* (2006) studied the effect of probiotic adjuncts (*Lb. acidophilus, Lb. casei, Lb. paracasei* and *Bifidobacterium* spp.) on proteolytic patterns of Cheddar cheese during maturation. The results of their study showed that the presence of these bacteria led to greater secondary proteolysis in probiotic cheeses than in control cheeses, without having a significant effect on the level of the primary proteolysis. They suggested that the proteolytic pattern of Cheddar cheese may be altered using these microorganisms (Ong *et al.*, 2006).

Bacterial cells' autolysis results in the liberation of their intracellular enzymes into the cheese matrix at the initial stage of ripening, resulting in shortened maturation time and improved cheese flavor (Buist *et al.*, 1995; Lortal and Chapot-Chartier, 2005; Picon *et al.*, 2005; Kenny *et al.*, 2006). *Lb. helveticus* strains with different degrees of autolysis were used as adjuncts with the starter, to determine the link between autolysis of these bacteria and Cheddar cheese flavor development (Kenny *et al.*, 2006). The results confirmed the importance of the autolysis of *Lb. helveticus* strains for cheese flavor improvement. However, other factors also contributed to flavor, including the strain's complement of peptidases, the metabolism

of amino acids, and the stability of the released enzyme in the cheese (Kenny *et al.*, 2006).

de Wit *et al.* (2005) studied the possibility of using *Debaryomyces hansenii* and *Yarrowia lipolytica* as adjunct cultures to accelerate Cheddar cheese maturation. These yeasts are well known for their proteolytic and lipolytic activities, and their compatibility and stimulating action on LAB when they are used as co-starters. A significant difference in proteolysis and lipolysis indices was obtained by inoculating a combination of the two yeasts with LAB (de Wit *et al.*, 2005). They suggested that the increased hydrolysis rate in experimental cheeses could be due to the synergistic effect of the yeast enzymes.

1.4.1.6 Genetically Engineered Starters and/or Recombinant Enzymes

Genetic engineering techniques represent newer methods to accelerate cheese maturation time. There has been considerable progress in the genetic modification of lactic acid bacteria (LAB) used in the dairy industry. However, safety concerns and consumer and industry acceptance are major obstacles in using these methods to accelerate the cheese ripening process. Genetic engineering techniques can be divided into four categories: (i) food grade microorganisms overproducing recombinant enzymes; (ii) genetically engineered lactic starters producing enzymes from *Lactobacillus lactis* using a food grade cloning system; (iii) cloning of autolysin into lactic starters; and (iv) metabolic engineering of lactic starters (Lee, 2007).

Lactose-negative (Lac^-) and proteinase-negative (Prt^-) lactic bacteria were the first strains genetically modified, followed by peptidase negative mutants. Freeze-shocked cells of *Lactobacillus casei* or its x-prolyldiaminopeptidyl peptidases (XPDAP)-deficient- mutant strain were added to Cheddar cheese curd (El Abboudi *et al.*, 1991). Soluble nitrogen was increased 60% in both treated cheeses, compared to the control. The amounts of TCA or PTA-soluble nitrogen levels and the flavor scores for the two experimental cheeses were the same. The use of genetic engineering techniques also led to the expression of the natural proteinase from *Bacillus subtilis* in *Lactococcus lactis* spp. *lactis* (van de Guchte *et al.*, 1990). In another attempt, the gene for the Neutrase of *B. subtilis* was cloned into *Lactococcus lactis* UC 317

(McGarry *et al.*, 1994). As a result of an extensive proteolysis in the Cheddar cheese produced with this engineered culture, the texture was very soft after 2 weeks at 8°C. The cheese aroma was satisfactory but the taste was unsatisfactory. However, a mixture (80:20) of unmodified: modified cells gave the best results in regard to the control of proteolysis (McGarry *et al.*, 1994).

Chymosin extract from calf stomach has been traditionally used for cheese production. The active components of rennet are chymosin (EC 3.4.23.4) and pepsin (EC 3.4.23.1) (Green *et al.*, 1985; Bines *et al.*, 1989). Chymosin is more active than pepsin. However, both enzymes hydrolyze the same bond of κ-casein, which leads to milk coagulation (Dalgleish, 1993). Chymosin along with milk proteinase and proteinases from starter and non-starter bacteria are responsible for the proteolysis of caseins (Fox, 1989b). Due to a shortage of calf chymosin, other substitutes of chymosin have been investigated. Recombinant calf chymosin is now the major source of the cheese coagulation enzyme and has captured over 80% of the total chymosin market. Cheddar cheese produced using recombinant chymosin from *Esherichia coli* was compared to cheeses produced using standard chymosin in parallel trials with three single starter strains. There was no significant difference in proteolysis or texture development during ripening between the cheeses (Green *et al.*, 1985). Bines *et al.* (1989) used a recombinant chymosin from *Kluyveromyces lactis* in Cheddar cheese production. The composition and maturation rates of the Cheddar cheese did not differ from those made using standard chymosin at 6 weeks and taste differences between the cheeses were not detectable at 3, 6 or 12 months. Harboe (1992) used a recombinant chymosin from *Aspergillus niger* and Praaning-van Dalen (1992) used one from *Kluyveromyces lactis* . This recombinant enzyme has been shown to be structurally similar to the main component of calf chymosin (Harboe, 1992).

1.4.1.6.1 Over-Expression of LAB Enzymes

Acid production, protein utilization, bacteriophage resistance, extracellular polysaccharide synthesis, and flavor/aroma metabolism are important functions of the LAB applied in dairy technology to accelerate cheese ripening. These vital functions

form the basis of the genetic modification strategies applied to LAB for use in shortening cheese maturation times (Law, 2001). The genetic modifications of LAB, according to their proteinase type, peptidase production or lytic properties in cheese, are listed in Table 1.3.

The peptidases of LAB are probably the main enzymes involved in cheese ripening (Law, 2001). As LAB enzymes are mostly intracellular and extraction or lysis from a low yield of LAB biomass is expensive, genetic engineering has mostly been geared toward the overproduction of these enzymes from *Aspergillus*. However, the LAB system has the advantage of being an *in situ* microflora bearing the enzymes that are desirable for cheese flavors and ripening (Lee, 2007). Overproduction of recombinant enzymes obtained from lactic acid bacteria or fungi has many uses in cheese ripening. pepN and pepX have many applications in the elimination of bitter and allergenic peptides in protein hydrolysates. Peptidases are also currently used in the treatment of recombinant proteins produced by the industry to eliminate the potential allergenic reactions (Xin *et al.*, 2002). Esterases are also useful for cheese flavor generation, and inter-esterification of milkfat (Choi and Lee, 2001; Choi *et al.*, 2004).

Many native proteolytic systems have been reported from *Lb. lactis, Lb. helveticus, Lb. delbruecki* and *Lb. casei* (Arora and Lee, 1992; Habibi-Najafi and Lee, 1994; Habibi-Najafi and Lee, 1995; Shin *et al.*, 2004). They exhibit cell-wall proteinases and peptidases with different specificities (Christensen *et al.*, 1999), but the genes of these lactic proteolytic and esterolytic enzymes have not yet been over-expressed in lactic cultures or other hosts. A low expression of LAB peptidases have often been reported in *E. coli* (van Alen-Boerrigter *et al.*, 1991). *Lb. casei* pepN is an exception where a thousand-fold over-expression was obtained in *E. coli* (Lee and Robert, 1997). *Lb. casei* esterase (estl) was similarly over-expressed in *E. coli, Pichia pastoris, and Methylotroph extorquens* (Choi and Lee, 2001; Choi *et al.*, 2004). Intracellular esterase from *Lb. casei* LILA was cloned, sequenced and characterized (Fenster *et al.*, 2003a, b). Both esterases (estC and estA) were compared under cheese ripening conditions, and estC was found to be more suitable for cheese-like conditions than estA. Cloned proteolytic and lipolytic enzymes with industrial

applications are listed in Table 1.4. Addition of exogenous enzymes overproduced by gene technology could be useful in the preparation of enzyme-modified cheese to produce highly intense cheese flavors.

Because NSLAB always predominate, and the *Lb. casei* group is competent in producing good quality improved-flavor matured Cheddar cheese, few studies have been carried out to accelerate Cheddar cheese ripening with an adjunct of live or shocked cells of *Lb. casei*. However, some defects produced by such cultures led to the use of over-expressed enzymes of *Lb. casei* spp. *casei* LLG, which naturally contains a high level of intracellular proteinase, aminopeptidase and esterase. The combined activity of these enzymes results in the production of cheese flavor compounds (Lee *et al.*, 2004; Azarnia *et al.*, 2006b).

1.4.1.6.2 Starter Lysis and Food Grade Systems

Most of the genetic techniques used to accelerate cheese ripening are based on the enhancement of lactococcal proteolytic enzymes by expressing the many peptidases and esterases of *Lactobacillus* or other lactics. Because of concerns with genetically modified starter bacteria, cell lysis techniques are very important in releasing intracellular enzymes from lactococci in a food grade system. The development of cloning systems, composed solely of DNA from the homologous host or GRAS organisms without relying on antibiotic markers, is very important in food applications (Lee, 2007). Table 1.5 shows different lysis techniques and food grade systems to release LAB enzymes and to develop cheese flavors.

1.4.1.7 Microencapsulation of Ripening Enzymes

A well-balanced breakdown of the curd protein, mainly casein, into small peptides and amino acids is important in the development of acceptable cheese flavor and texture (Visser, 1993). Therefore, a great deal of attention has been given to accelerating cheese proteolysis through the addition of free proteolytic enzymes to the cheese milk or to the curd (O'Riordan and Delahunty, 2003a,b). The distribution of enzymes during ripening is affected by the degree of enzyme entrapment and distribution in the curd, as well as by enzyme activity under particular conditions.

Indigenous milk enzymes distribute well in the curd during the cheesemaking process, due to their association with the caseins or milkfat. In the case of LAB or NSLAB, the mechanisms of their entrapment during cheesemaking, result in them being localized at the interface between milkfat globules and the casein matrix. Exogenous enzymes are lost in the whey at the drainage stage. Consequently, various methods have been developed to improve the extent of their contribution (Wilkinson, 2004). Direct addition of enzymes to the milk may not result in a uniform distribution of enzymes in the curd, and may cause early proteolysis and texture defects, and a loss of enzymes in the whey resulting in cheese whey contamination (Wilkinson, 1993; Kailasapathy and Lam, 2005).

Microencapsulation technology has been used in the dairy industry to control and improve cheese flavor (El Soda, 1986). Encapsulation of cell-free extracts or viable bacterial cells are two techniques employed. Products are formed within the capsules and released into the cheese during ripening (Arnau *et al.*, 1997). Gums, milkfat and phospholipids have been used as encapsulating materials. Living cells immobilized in calcium alginate beads have been used for the fermentation of milk or whey products (Champagne *et al.*, 1989). Although the enzymes are entrapped in an alginate matrix, given its high porosity, the capsules are leaky. In an attempt to accelerate cheese ripening, Kailasapathy and Lam (2005) investigated food gums (gellan, κ-carrageenan), and a high melting point milkfat fraction as materials for enzyme encapsulation. During the ripening period, proteolysis rates were greater in all the cheeses treated with encapsulated enzyme, compared to the control cheese. Enzyme-bearing gum capsules showed a greater retention rate in the cheese matrix than did enzyme-bearing fat capsules. The nature of the gum effects on the proteolysis rate during ripening varied according to the type of gum used, the greatest rate of proteolysis occurring in cheeses treated with κ-carrageenan capsules. The cheeses' sensory or textural properties were not affected by the type of the gum used (Kailasapathy and Lam, 2005). Alginate-pectin capsules were used for the fortification of Cheddar cheese with folic acid (Madziva *et al.*, 2006). Application of the two food grade polymers (alginate, pectin) resulted in a high encapsulation

efficiency, high capsule stability in the cheesemilk, during cheese pressing and ripening, and their uniform distribution in the cheese matrix (Madziva *et al.*, 2006).

Magee and Olson (1981a) used esterate or oleate sorbitan esters to achieve high encapsulation efficiency. In their study, lipid-coated microcapsules were formed by extruding a water/oil emulsion into a cool dispersion liquid. They concluded that the encapsulation efficiency was affected by type, concentration, and proportion of emulsifiers, temperature of the dispersion liquid, ratio of carrier solution to milkfat, and the solute concentration in the encapsulated phase. The encapsulation efficiency was between 80 and 90%. The melting points of milkfat range between 30 and 40°C, so at the temperatures of cheese manufacture, lipid-coated capsules could soften and be disrupted. According to Magee and Olson (1981b), this melting point range could be increased by using emulsifiers during the encapsulation procedure. They studied heat stability of such microcapsules at 32°C. Lipid-coated capsules consisting of peptone and various proportions of Span 60 and Glycomul TS were held at 32°C. The proportions of the emulsifiers did not influence the encapsulation efficiency, but did affect capsules' temperature stability. Microcapsules coated in milkfat containing 3 to 4% of a 1:1 to 1:3 ratios of Span 60 and Glycomul TS emulsifiers showed maximum heat stability. The interaction between the emulsifier concentration and volume of the encapsulated phase influenced capsule stability (Magee and Olson, 1981b).

Maintaining enzymatic activities of the carrier mixture is a problem in forming milkfat-coated capsules due to the relatively high temperature used during the microencapsulation procedure. To solve this issue, it has been suggested that whole cells be encapsulated, since the heat stability of the essential enzymes should be greater in viable, intact cells than in cell-free extracts. As a great number of capsules would be needed to generate the cheese's appropriate flavor balance, the application of microencapsulation in milkfat appears limited, but might serve to enhance the flavor of low fat cheese and possibly in the production of a flavor-enhanced cheese (El Soda, 1986).

Given the chemical complexity of cheese flavor, the use of encapsulated multiple enzyme systems are essential in enhancing and regulating flavor development in cheese (Magee and Olson, 1981c). A cell-free extract of *St. lactis* spp.

diacetilactis, along with substrates and cofactors were encapsulated into milkfat capsules. The incorporation of these capsules into experimental cheeses caused an eight-fold increase in diacetyl and acetoin concentrations during ripening, compared to control cheeses (Magee and Olson, 1981c).

Since a complex series of reactions is responsible for producing the numerous compounds attributed to cheese flavor, it is unlikely that a single enzyme could generate all these compounds (Braun and Olson, 1986a, b). Consequently the development of adequate flavor could only be achieved using a microencapsulated multi-enzyme system. Furthermore, an acceptable flavor in low or reduced fat cheese can be developed through the addition of selected milkfat-encapsulated flavor-producing systems (Braun and Olson, 1986a). Milkfat-coated capsules were produced using a mixture of cell-free extracts from *Gluconobacter oxydans* and *Streptococcus lactis* var. *maltigenes*, with substrate and nicotinamide adenine dinucleotide (NAD). The former organism produced acetic acid from ethanol, and the latter 3-methylbutanal and 3-methylbutanol from leucine. The capsules were added to skim milk and milk with 1.1% fat to produce reduced-fat cheeses. High concentrations of 3-methylbutanal and 3-methylbutanol and a strong malty flavor were produced in cheeses treated with capsules containing a complete cell-free extract mixture (Braun and Olson, 1986a).

Kim and Olson (1989) studied aerobic and anaerobic production of methanethiol by milkfat-coated microcapsules containing *Brevibacterium linens* and methionine. The production of methanethiol was three- to four-fold greater under aerobic conditions, with most of the methanethiol being oxidized to dimethyl disulfide. Approximately 35% of the total methanethiol was absorbed by the milkfat capsules and approximately 65% was detected in the headspace. Optimum pH and temperature for methanethiol production were 8 and 26°C, respectively.

Liposome encapsulated enzymes are an alternative method for enzyme supplementation of cheeses. Liposomes are made of materials, such as phospholipids, which are normal food components. Encapsulated enzymes are released into the curd through degradation of the vesicle membrane (Wilkinson, 1993). Phospholipids form membrane structures on contact with water. Their main advantage is that they form

vesicles (liposomes) without the addition of detergents. The main types of liposomes used in cheese research include multilamellar vesicles (MLV), small unilamellar vesicles (SUV), microfluidizer liposomes (MF), reversed-phase vesicles (REV), and dehydration-rehydration vesicles (DRV) (Skeie, 1994). Alkhalaf *et al.* (1988) used REV liposomes as proteinase carriers for the accelerated ripening of Saint-Paulin type cheese. This system resulted in a uniform distribution of the enzyme in the curd since the size of the liposomes allowed their distribution in the curd in the same manner as bacterial cells (El Soda, 1986). The encapsulated enzyme was added to cheesemilk at the renneting stage (Fresta and Puglisi, 1999) and the phospholipid vesicles protected potential substrates in milk. Liposomes can be considered to perform like bacterial cells bearing a selected mixture of enzymes, but which are released at a much faster rate to the cheese. This technology also offers the possibility of preparing a wide range of vesicles with different sizes, net charge and sensitivity to pH and/or temperature (El Soda, 1986). The lipid composition of the liposome membrane affects its stability in milk and the rate of ripening (Fresta and Puglisi, 1999); however, liposome technology is currently still too expensive to be used commercially in cheese production.

Acceleration of Cheddar cheese ripening using liposome-encapsulated Neutrase was studied by Kirby *et al.* (1987). They reported high enzyme encapsulation efficiency and a high enzyme entrapment rate by the curd. Evaluation of proteolytic activity and flavor quality indices showed that cheeses produced by this method ripened twice as fast as control cheeses (Kirby *et al.*, 1987). Kheadr *et al.* (2000) studied the effect of liposome-encapsulated bacterial and fungal proteinases on properties of Cheddar cheese during ripening. While the experimental cheeses' proteolysis and texture development were faster than in control cheeses, the experimental cheeses showed greater moisture, less protein and were more brittle than control cheeses. Liposome-treated cheeses had a less compact microstructure, with liposomes being located at the fat-casein interface. Bitter and astringent peptides accumulated to a greater degree in experimental cheeses than in the control cheeses, depending on the type and concentration of enzyme, but after 3 months of ripening, the organoleptic properties of the experimental cheeses were improved and bitter off-

flavors were no longer detected. Fresta *et al.* (1995) used liposome encapsulated Neutrase to accelerate Cheddar cheese ripening. The liposomes were prepared by applying repeated freeze-thaw cycles to multilamellar vesicles. Filtration through polycarbonate filters produced evenly-sized liposomes. This study showed that encapsulation efficiency was affected by the number of freeze-thaw cycles, the highest value being achieved after seven cycles. This method led to a two-fold increase in proteolysis rate in the experimental cheese compared to the control cheese.

A commercial neutral proteinase was encapsulated in liposomes (multilamellar) and added to cheesemilk for the production of Cheddar cheese. During curd manufacture, the milk proteins were protected from proteinase attack and a significant proportion of the liposome-entrapped enzyme was retained in the curd. An increased rate of β-casein breakdown in the curd was observed (Law and King, 1985). Grosswasser *et al.* (2000) prepared capsules of small multilamellar vesicles of surfactant (spherulites) by shearing a lamellar phase. They studied encapsulation of alkaline phosphatase into spherulites. The encapsulated enzyme was shown to be unable to develop any enzymatic activity on its substrate (*p*-nitrophenylphosphate). This was due to absence of contact between the enzyme and the substrate; however, enzymatic activity was recovered after destructi on of the vesicles. In this study, encapsulation efficiency ranged from 70% to 95% and was dependent on the enzyme/phospholipid ratio.

Flavorzyme (a fungal protease and peptidase complex, from *Aspergillus oryzae)*, neutral bacterial protease (from *Bacillus subtilis*), acid fungal protease (from *Aspergillus oryzae*), and a fungal lipase preparation (Palatase M, from *Mucor miehei*) were individually encapsulated in liposomes and added cheesemilk to develop Cheddar cheese flavor (Kheadr *et al.*, 2003). In general, cheeses made with a mixture of lipase and bacterial proteases were preferred to other cheeses. The use of this enzyme cocktail led to a well-balanced equilibrium between proteolysis and lipolysis products without producing bitter or rancid flavors or poor texture defects (Kheadr *et al.*, 2003). The effect of microencapsulated iron-fortified Cheddar cheese on flavor

development during ripening was studied by Kwak *et al.* (2003) but chemical and sensory defects were not reported in this study.

1.5 CONCLUSIONS

The main characteristics of Cheddar cheese are texture, aroma and flavor development during ripening through biochemical and microbiological reactions. Maturation of Cheddar cheese is a slow and expensive process. Therefore, methods to accelerate this process have economic and technological implications for the Cheddar cheese industry. Addition of enzymes, adjuncts, attenuated cultures or genetically modified starters have been used to shorten the duration of cheese ripening. However, the poor availability of commercial enzyme preparations, difficulty in uniformly distributing them in the cheese matrix, the waste of enzymes in the whey and whey contamination are problems that arise when this approach is employed to accelerate cheddar cheese ripening. Enzyme encapsulation is one way to overcome these problems. However, the efficiency of the enzyme encapsulation process is still low (less than 50%), thus increasing cost. Liposomes have been tried with some success, but the mechanism of their release of enzymes in cheese is not well understood. The major current drawback to liposome technology is cost.

Starters with NSLAB adjuncts have been used to promote proteolysis and flavor intensity in Cheddar cheese. The mechanisms of cheese flavor production by this combination should be further investigated. Transferring genes from lactobacilli to starter lactococci could help produce desirable enzymes and enhance favored flavors. Although genetically modified starters may play a vital role, safety concerns regarding genetic modification of viable lactic cells, even with food grade vectors, is a major bottleneck. As recombinant chymosins from *E. coli*, yeast (*Kluyveromyces*) and fungus (*Aspergillus*) are successfully employed in Cheddar cheese manufacture, rather than using the GMO starters, the possibility exists to produce, purify, and encapsulate these enzymes, allowing them to be incorporated evenly into the cheese matrix without washing out during cheese manufacturing.

Table 1.1. Enzymes used in acceleration of Cheddar cheese maturation (Modified from M. El Soda, and S. Pandian: Recent developments in accelerated cheese ripening. *J. Dairy Sci.* 74: 2317–2335, 1991).

	Enzyme added	Source	Stage of addition	Parameters studied
Cheese related enzymes	Total enzymes + Neutrase	Disrupted cells of *Lactobacillus casei*, *L. lactis*, *L. plantarum*	Milk	Organoleptic
	Serine proteinases	*Brevibacterium linens*	---	Bitterness
	Cell-free extracts	*L. casei* ssp. *casei*	Curd	TCA-soluble N, bitterness
Non-cheese related enzymes	Neutral protease	*Aspergillus oryzae*	Milled Curd	Free amino acid Levels
	Total extract: Dipeptidase, Carboxypeptidase, and proteinase fractions activities with or without Neutrase	Kilned germinated Barley	Curd, at salting	Flavor
	Naturage*/Neutrase		Milk	Free fatty acid

* An enzyme mixture from starter bacteria.

Table 1.2. Commercial enzymes to accelerate ripening of cheese (Modified from B. A. Law: Controlled and accelerated cheese ripening: the research base for new technologies. *Int. Dairy J.* 11: 383– 398, 2001).

Type/Product	Sources/Availability
Animal lipases	Enzyme companies
Fungal lipases	Dairy ingredient suppliers
Fungal proteinases	Novo Nordisk (Denmark)
Fungal peptidases	Novo Nordisk (Denmark)
Rulactine (*Micrococcus* proteinase)	Rhone-Poulenc (Currently Aventis, S.A)
Flavorage (fungal lipase + proteinase)	Chr. Hansen Inc
Accelase (LAB exopeptidase)	Rhodia (UK) (Currently Danisco)
Bacterial and fungal proteinase	IBT Ltd (Currently Danisco)
Starter LAB extract	IBT Ltd (Currently Danisco)

Table 1.3. Genetic engineering approaches applied to LAB or to the overproduction of enzymes for flavor enhancement (Modified from B. A. Law: Controlled and accelerated cheese ripening: the research base for new technologies. *Int. Dairy J.* 11: 383– 398, 2001).

Genetic modification	Functional change from wild type	Ripening/flavor effect
Lactose metabolism diverted by selective elimination or enhancement of key metabolic enzymes	Increased production of diacetyl	Enhanced buttery flavor
Altered proteinase specificity and peptidase balance	Selective increases in production of aromatic amino acids (glutamate, methionine, valine, cysteine, proline, leucine, glycine)	Reduction of bitter taste, higher taste intensity, increased sweet, savory, floral, sulfurous, nutty flavor notes
Enhanced catabolic enzymes (demethiolase, deaminase, transaminase	Selective increases in production of carbonyls, esters, organic sulphur compounds, fatty acids, keto acids	Extended flavor profile through added aroma notes, intensification of aroma
Introduction of cell lysine genes under control of external promoters to trigger lysis (pH, salt temperature, Nisin)	Controlled instantaneous lysis in the cheese matrix	Accelerated ripening through faster release of "flavoring enzymes" (peptidases, esterases, amino acid catabolic enzymes)
Addition of genetically overproduced enzymes from LAB	Controlled overproduction of cheese ripening enzymes	Accelerated ripening through encapsulation

Table 1.4. Recombinant proteolytic and lipolytic enzymes for cheese and other industrial applications (Adapted from B. H. Lee: Methods of enzyme incorporation into cheese. *IDF Monograph* 2007, In press).

Enzyme	Donor	Host (Expression fold)	Promoter	Reference
Peptidases				
PepN	*Aspergillus sojae*	*A. sojae*	NT	Chien *et al.* (2002)
PepN	*Aspergillus niger*	*A. niger*	Own (24)	Basten *et al.* (2001)
PepN	*Pyrococcus horikoshi*	*E. coli*	NT	Matsui *et al.* (2000)
PepN	*Streptococcus thermophilus*	*E. coli*	T7 (452)	Chavagnat *et al.* (1999)
PepN	*Lactobacillus rhamnosus*	*E. coli* *L. lactis*	Own (1,000) Own (20)	Lee & Robert (1997)
PepN	*Lactococcus lactis*	*E. coli* *L. lactis*	T7 (NT) T7 (20)	van Alen Boerrigter *et al.* (1991)
PepC	*Lactobacillus helveticus*	*L. lactis*	pTRKH2 (250)	Tuler *et al.* (2002)
PepC	*Aspergillus niger*	*A, niger*	Own (70)	Basten *et al.* (2003)
PepI	*Lactobacillus bulgaricus*	*E. coli* *L. lactis*	Own (15,000) Own (17)	Atlan *et al.* (1994)
PepX	*Lactobacillus helveticus*	*E. coli*	Own (NT)	Kimula *et al.* (2002)
Pep X	*Lactococcus lactis*	*E. coli*	T7 (NT)	Xin *et al.* (2002)
PepX	*Lactobacillus rhamnosus*	*E. coli*	Own (10)	Varmanen *et al.* (2000)
PepX	*Streptococcus thermophilus*	*E. coli*	NT	Anastasiou *et al.* (2002)
PepX	*Lacobacillus helveticus*	*E. coli*	Own (200)	Yüksel & Steel (1996)
PepX	*Lactobacillus rhamnosus*	*E. coli*	Own (200)	Lee & Robert (1997)
Esterases				
EstI	*Lactobacillus casei*	*E. coli*, (200), *P. pastoris* *M. extorquens*	T7 (200) AOX (1000) ADH (500)	Choi *et al.* (2004)
Est	*Lactobacillus casei*	*E. coli*	NT	Fenster *et al.* (2003a, b)
Est	*Lactococcus lactis*	*L. lactis* (170)	Nisin (170)	Fernández *et al.* (2000)
Lipases				
Lipase	*R. oryzae*	*S. cerevisiae*	Own (129U/ml/h)	Minning *et al.* (2001)
Lipase	*R. niveus*	*S. cerevisiae*	Own (324 mg/ml)	Kohno *et al.* (1999)
Lipase	*G. candidium*	*P. pastoris*	AOX (200 mg/ml)	Cantoni *et al.* (1997)
Lipase	*G. candidium*	*P. pastoris*	AOX (60 mg/l)	Holmquist *et al.* (1997)

Table 1.5. Peptidases and esterases cloned into *Lactococcus lactis* and food grade systems (Adapted from B. H. Lee: Methods of enzyme incorporation into cheese. *IDF Monograph* 2007, In press).

Method	Based	Advantage	Reference
Lysis			
Autolysin	AcmA, *L.lactis*	Noninfective	Buist *et al.* (1995) Haandrikman *et al.* (1991)
Prophage	ϕUS3		De Ruyter *et al.*(1997)
Bacteriocin	Nisin	Noninfective	Cotter *et al.* (2003)
	Lacticin		
	Lactococcin		Morgan *et al.* (1997)
Food grade			
Air gene	Complementation		Bron *et al.* (2002)
Melibiose	Complementation		Boucher *et al.* (2002)
phiFC1vector	Integration		Yang *et al.* (2002)
pFG200	Amber suppressor, *supD*		Sørensen *et al.* (2000)

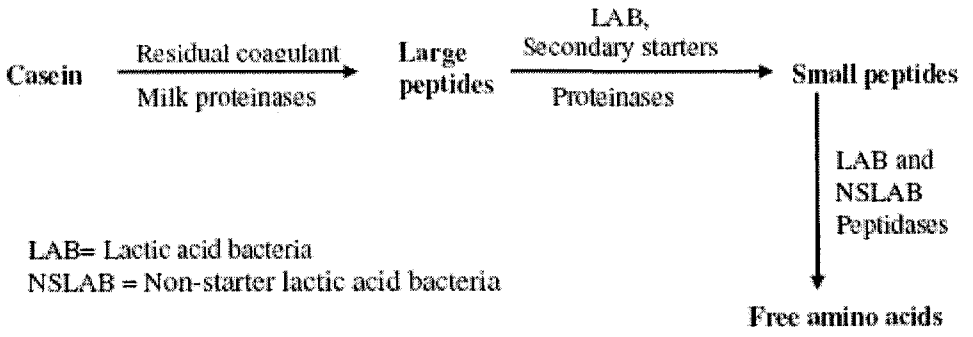


Figure 1.1. Different proteolytic enzymes in cheese during ripening (Adapted from M. J. Sousa, Y. Ardö, and P. L. H. McSweeney: Advances in the study of proteolysis during cheese ripening. *Int. Dairy J.* 11: 327– 345, 2001).

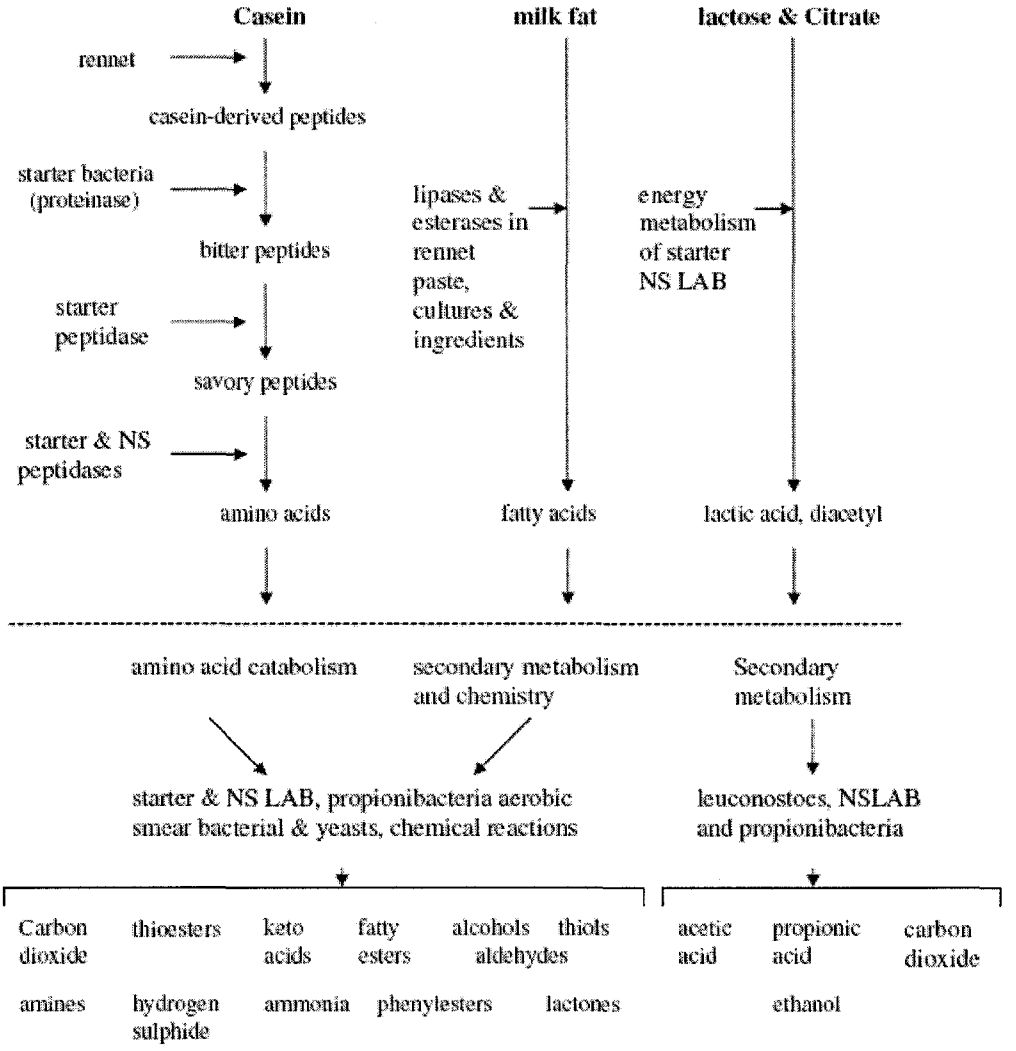


Figure 1.2. Basic cheese ripening biochemistry (Adapted from B. A. Law: Controlled and accelerated cheese ripening: the research base for new technologies. *Int. Dairy J.* 11: 383– 398, 2001).

CHAPTER 2

Microencapsulation of a Recombinant Aminopeptidase (pepN) from *Lactobacillus rhamnosus* S93 in Chitosan-Coated Alginate Beads

This chapter has focused on the encapsulation of recombinant aminopeptidase (pepN) of *Lactobacillus rhamnosus* S93 produced by *E. coli* in chitosan-coated alginate beads using an extrusion method. For this purpose, the optimal conditions (alginate, $CaCl_2$, chitosan concentrations, hardening time, pH, and alginate/enzyme ratio) were evaluated. This method has resulted in the high encapsulation efficiency and the low enzyme release from the capsules during beads formation.

Chitosan-coated alginate beads could provide a suitable matrix for encapsulation of this enzyme and its application in cheesemaking technology that were examined in Chapters 3 and 4.

The result of this research has been presented at *XVth International Workshop Bioencapsulation*, Vienna, Austria, 2007, and is in press in *Journal of Microencapsulation*. The manuscript has been co-authored by Sorayya Azarnia, Byong Lee, Normand Robert, and Claude Champagne, and written by Sorayya Azarnia and edited by Dr. Byong Lee. Claude Champagne provided advice for enzyme encapsulation, financial support, and editorial assistance in the final stage of paper submission. Normand Robert provided technical support for enzyme preparation and purification.

2.1 ABSTRACT

A recombinant aminopeptidase (90 kDa) of *Lactobacillus rhamnosus* S93 produced by *E. coli* was encapsulated in alginate or chitosan-coated alginate beads prepared by an extrusion method. We investigated the effects of alginate, $CaCl_2$, chitosan concentrations, hardening time, pH, and alginate/enzyme ratios on the encapsulation efficiency (*EE*) and the enzyme release (*ER*). Chitosan in the gelling solution significantly increased the *EE* from 30.2% (control) to 88.6% (coated). This polycationic polymer retarded the *ER* from beads during their dissolution in release buffer. An increase in alginate and chitosan concentrations led to greater *EE* and lesser *ER* from the beads. The greatest *EE* was observed in a pH 5.4 solution (chitosan-$CaCl_2$) during bead formation. Increasing the $CaCl_2$ concentration over 0.1 M neither affected the *EE* nor the *ER*. Increasing hardening time beyond 10 min led to a decrease in *EE* and the alginate: enzyme ratio (3:1) was optimal to prevent the *ER*.

2.2 INTRODUCTION

Among non-starter lactic acid bacteria (NSLAB) species, *Lactobacillus casei* strains could be used in producing a good quality of matured Cheddar cheese (Arora and Lee, 1994; Lee and Robert, 1997). The catabolism of amino acids by lactic acid bacteria (LAB) has an important role in aroma formation in Cheddar cheese. Degradation of small peptides to free amino acids is carried out by intracellular bacterial peptidases (Sousa *et al.*, 2001). Consequently, there is considerable interest in the use of these enzymes for accelerating Cheddar cheese ripening through peptide hydrolysis. However, using adjunct live or shocked cells of *L. casei* for this purpose caused defects in the final cheese product. This has led to the studies of *L.* native or recombinant enzymes such as intracellular proteinase, aminopeptidase and esterase and their applications in cheese maturation (Arora and Lee, 1994; Lee and Robert, 1997). An over-expression of pepN from *Lb. rhamnosus* S93 has been obtained in *Escherichia coli* (*E. coli*) (Lee and Robert, 1997) and this enzyme has many

applications in the elimination of bitter and allergenic peptides in protein hydrolysates.

Alginate has been used for living cells and enzymes due to its cost- effective, biocompatible non-toxic matrix as a food additive (Beshay, 2003; Sankalia *et al.*, 2005). It is a linear polysaccharide of α-L-guluronic acid (G) and ß-D-mannuronic acid (M) residues extracted from brown algae, *Phaeophyceae*, (Draget *et al.*, 2006). This polyanionic polymer forms a three-dimensional gel network in the presence of divalent cations, such as calcium, resulting from ionic cross-linking via calcium bridges between L-guluronic acid residues (Draget *et al.*, 2006).

Alginate gels have large pores that may result in enzyme release during the operation. Although this phenomenon does not affect diffusion of biomolecules within a molecular weight of 20 kDa, higher molecular weight substances such as albumin (69 kDa) are released from alginate beads (Tanaka *et al.*, 1984). The porosity of alginate gels and the subsequent release of substances could be reduced by treating them with polycationic polymers, such as chitosan. Chitosan is a hydrophilic polymer consisting of N-acetyl-D-glucosamine and D-glucosamine residues. It is produced by alkaline deacetylation of chitin, leading to the formation of the deacetylated form of the amino groups. It has the advantage of exhibiting both gel- and film-forming capacities (Vårum and Smidsrød, 2006). The electrostatic interaction between the negatively charged carboxylic acid groups of alginate and the positively charged amino groups of chitosan results in the formation of a membrane on the bead surface (Huguet *et al.*, 1996). This phenomenon reduces leakage of entrapped materials from the bead. Encapsulation of urease (Kara *et al.*, 2006), and lactase (Lu *et al.*, 2007) using chitosan-alginate polyelectrolyte complexes are good examples in using both polysaccharides for the enzyme immobilization. However, no data are found on the encapsulation of lactobacilli peptidases in alginate-chitosan particles, nor on how this affects the encapsulation efficiency and the release of the enzyme from the beads.

In the present work, attempts were made to encapsulate the recombinant pepN of *Lactobacillus rhamnosus* S93 by optimizing the encapsulation efficiency in alginate-based beads. This was assessed on the basis of the enzyme entrapment in capsules, and *ER* from the beads in a release buffer solution.

2.3 MATERIALS AND METHODS

2.3.1 Materials, Strain and Growth of *E.coli* clones

All chemicals and media used were purchased from Sigma (Sigma-Aldrich, Inc., St. Louis, MO, USA) and Difco (Difco Laboratories, Detroit, MI, USA), respectively, unless otherwise mentioned.

The *E. coli* clones containing the gene for aminopeptidase (pepN) of *Lactobacillus rhamnosus* S93 were obtained from the Agriculture & Agri-Food Canada, Food R & D Centre (St-Hyacinthe, PQ, Canada). They were plated on Luria Bertani (LB) agar supplemented with ampicillin (100μg mL^{-1}) and incubated at 37°C. After growing the cells in the antibiotic-containing LB broth at 37°C on a shaker, they were harvested by centrifugation at 4000g (30 min, 4°C) and washed twice with ice-cold phosphate buffer (0.05M, pH 7.5). The harvested cells were disintegrated by homogenization (Emulsiflex-5C, Avestine Homogenizer, Ottawa, ON, Canada). The pressure was gradually increased up to 10,000g, and the cell suspension was centrifuged (10,000g, 30 min, 4°C).

2.3.2 Purification of the pepN and Electrophoresis

The crude cell-free extracts were fractionated by salting out with solid ammonium sulphate (Bioshop, Burlington, ON, Canada) to 75% saturation. The ammonium sulphate was added gradually to the crude extracts and stored with gentle stirring overnight in cold room. The precipitate was collected by centrifugation (10,000g, 30 min), dissolved in a minimum amount of ice-cold phosphate buffer (0.05M, pH 7.5), and then dialyzed for 8 hours against ice cold water, using dialysis membranes (50,000 MWCO, Spectra/Por®, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). After the dialyzed fraction was passed through a Sephadex G-25 column (PD-10, Pharmacia Biotech LKB, Montreal, PQ, Canada), the fraction was analyzed for protein and aminopeptidase assays.

This fraction, containing recombinant pepN, was purified by one step preparative ion exchange column chromatography (Arora and Lee, 1992) using an FPLC system (Waters™ 650E Advanced Protein Purification System, Lachine, PQ,

Canada). Enzyme was loaded onto a column (BioPilot™ column Q-Sepharose® High performance 35/100, Baie d'Urfé, PQ, Canada) equilibrated by potassium phosphate buffer (0.05M, pH 7.5). Enzyme was eluted by a linear gradient of NaCl (0-0.06 M) with a flow rate of 4 mL min^{-1}. Fractions were pooled and the activity of enzyme was measured.

Aminopeptidase was analyzed by denaturing polyacrylamide gel electrophoresis (Laemmli, 1970). The bands were visualized by zinc stain solution A, imidozole, followed by destaining with zinc stain solution B, zinc sulphate (Bio-Rad Laboratories, Montreal, PQ, Canada). The molecular size of the protein was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% running and 4% stacking gels (Laemmli, 1970). The low-molecular-mass proteins (Bio-Rad Laboratories, Montreal, PQ, Canada) were used as standard and the molecular mass of the enzyme was determined from the standard curve.

2.3.3 Enzyme and Protein Assays

Aminopeptidase (pepN) activity was determined at 405 nm using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Leucine-*p*-nitroanilide (leu-*p*NA) dissolved in methanol with a final concentration of 16.4 mM was used as a substrate (Arora and Lee, 1994). The reaction mixture consisting of 10 µL of enzyme solution, 10 µL of leu-*p*NA, 10 µL of cobalt solution (20 mM) and 170 µL of potassium phosphate buffer (0.05 M, pH 7.5) was incubated at 37°C for 30 min. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitroaniline min^{-1} under the defined conditions. Specific activity was defined as enzyme units per mg protein.

Protein concentration was estimated using BCA™ (bicinchoninic acid) Protein Assay Kit (Pierce Chemical Ltd, Rockford, IL, USA) at 562 nm, using bovine serum albumin as a standard.

2.3.4 Preparation of Alginate and Chitosan-Coated Alginate Beads

A method modified of Champagne *et al.* (1992) was used to form calcium-alginate beads. The type of sodium alginate used in this study was Sobalg FD 126 (Grindsted Products, Inc., Rexdale, ON, Canada) with 40% guluronic acid content and 650 cP viscosity of 1% solution at 20°C (Champagne *et al.*, 2000).

After sodium alginates of 1.0% to 2.2 % (w/v) were dissolved in boiling water, the purified recombinant pepN was mixed with the cooled sodium alginate solution at a ratio of 1:3. The mixture was thoroughly mixed gently for 2 min. The polymer-enzyme suspension was introduced to a 10 mL syringe and added drop wise through a 20G1 needle (Becton Dickinson & Co. Rutherford, NJ, USA) to a cold (4°C) 0.1M solution of calcium chloride dihydrate (Bishop Canada Inc., Burlington, ON) at a ratio of 1:3. All capsules were allowed to harden in this gelling solution for 10 min in agitation (70 rpm) using an orbital shaker. The capsules were harvested by filtration, washed with deionized distilled water to remove the untrapped enzyme and excess calcium chloride from the surface of the beads. The encapsulation efficiency, *EE,* expressed as a percentage, was calculated as (Sankalia*., et al.*, 2005; Kailasapathy *et al.*, 2006):

$$EE = 100 \times (EA_b / EA_i) \qquad (1)$$

where EA_b ($EA_b = EA_i - EA_{rb}$), EA_i and EA_{rb} are the enzyme activity (units) in the beads, the initial enzyme activity (total units) in the polymer/enzyme mixture and the enzyme activity (units) in the release buffer (see below), respectively.

To evaluate the effect of chitosan on *EE* and the release characteristic of beads, this polymer was added to a $CaCl_2$ gelling solution. Portions (1, 2, or 3 g) of crab shell chitosan (85% deacetylated) were dissolved in 700 mL of 0.1 M glacial acetic acid and stirred overnight. After complete dissolution, the pH was adjusted to 5.4 with NaOH (1M). $CaCl_2 . H_2O$ (14.7 g) was added to the chitosan solution and the volume adjusted to 1L with deionized distilled water, yielding cationic solutions of 1, 2 or 3 g chitosan L^{-1} in 0.1M $CaCl_2$ at pH 5.4 (Zhou *et al.*, 1998). The mixture of enzyme and the cooled Na-alginate solution (2%) at a ratio of 1:3 was dropped into

the gelling solution containing $CaCl_2$ and chitosan. The capsules were allowed to harden in this gelling solution for 10 min, under agitation (70 rpm), resulting in the formation of chitosan-coated alginate beads.

The effect of $CaCl_2$ in gelling solution on *EE* and bead stability was evaluated by varying the concentration from 0.05 to 0.2 M., in the presence or absence of chitosan.

The effect of hardening duration (5 to 30 min at 5 min intervals) formed in a 0.1% chitosan-0.1M $CaCl_2$ solution at 4°C was also investigated. To evaluate the effect of gelling solution pH, the final pH of the chitosan-$CaCl_2$ gelling solution was adjusted to 3.8, 4.2, 4.6, 5.0, or 5.4 with sodium hydroxide (1 M).

2.3.5 Determination of the *ER* and Polymer/Enzyme Ratio

Various factors such as alginate, chitosan, $CaCl_2$, pH of the cationic solution, and alginate/enzyme ratios were studied on the *ER* from beads. After washing, 1 g (wet weight) of coated or uncoated beads was dissolved under gentle agitation (70 rpm) in 10 mL of trisodium citrate (20 g L^{-1}) release buffer, at room temperature (Champagne *et al.*, 1992). The enzyme activity having leaked into the release buffer was measured after 30, 60, 90, and 120 min.

The degree of *ER* during bead suspension in the release buffer was defined as:

$$ER = 100 \times [EA_{rb} / (EA_b + EA_{rb})] \qquad (2)$$

Where, EA_{rb}, EA_b ($EA_b = EA_i - EA_{rb}$) and EA_i are the enzyme activity (units) in the release buffer, the enzyme activity (units) in the beads and the initial enzyme activity (total units) in the polymer/enzyme mixture, respectively.

To determine the loading capacity of the beads, mixtures of 2% alginate and recombinant pepN in ratios of 1:1, 2:1, and 3:1 were prepared. After the mixtures were individually hardened in a 0.1% chitosan-0.1 M $CaCl_2$ solution, the effect of this factor on the *ER* characteristics of capsules was studied.

2.3.6 Freeze Drying of Beads

The encapsulated enzyme was frozen at -80°C, and was subjected to freeze-drying (FTS System, Inc. Biopharm Division, Stone Ridge, NY, USA) for 48 h at 22°C under a 90 millitor vacuum. Dried capsules (0.1 g) were dissolved in 30 mL of 20 g L^{-1} sodium citrate solution to release the entrapped enzyme and the *ER* was measured.

2.3.7 Scanning Electron Microscopy (SEM)

The alginate and chitosan-coated alginate beads were first dehydrated in a graded ethanol series (10% to 100%), and then dried using a critical point drier (Marivac, Canada). Samples were placed on an aluminum stub with double sided carbon, covered with a 10 nm layer of gold by sputtering, and observed in a scanning electron microscope (SEM) (Hitachi S-3000N, Japan) at 5 kV with a secondary electron as a detector.

2.3.8 Statistical Analyses

Single factor analysis of variance (ANOVA), using Microsoft Excel (Microsoft Office XP Professional, 2003), was used for statistical analyses. Means were compared at a significant level of 5%. All experiments were carried out in triplicate.

2.4 RESULTS AND DISCUSSION

2.4.1 Enzyme Preparation and Encapsulation

The single step of preparative ion exchange column chromatography led to a 11.6-fold purification of the recombinant pepN over the crude extract, with a recovery of 28.6 % (Table 2.1). The molecular mass of pepN was determined to be about 90 kDa by SDS-PAGE, close to that of Arora and Lee (1994).

Addition of chitosan to the gelling solution showed a significant ($P < 0.05$) effect on *EE*. The *EE* of chitosan-free alginate beads was roughly 30%, i.e. about 70% of the recombinant pepN present in the sodium alginate solution was lost during bead formation. However, for chitosan concentrations (0.1, 0.2, 0.3%), the *EEs* were

between 79 and 88% (Figure 2.1). Further addition of chitosan over 0.2% could not improve the *EE*, resulting in a viscous gelling solution and non-spherical beads.

When different alginate concentrations (1.0 to 2.2%, w/v) were adjusted in the presence or absence of chitosan at 0.1 M $CaCl_2$ and with the alginate: enzyme ratio of 3:1, the *EE* was higher (89%) than control (30%) at 2.2% alginate concentration (Figure 2.2). The *EE* remained unchanged from 1.8 to 2.2% of alginate. Moreover, alginate over 2% led to difficulties in extruding the polymer-enzyme solution as well as the formation of larger, non-spherical capsules.

The large pore size of alginate gels may result in the leakage of enzymes out of the gel matrix (Tanaka *et al.*, 1984). There are discrepancies between data on the effect of alginate concentration on diffusion of small compounds from the particles. While Tanaka *et al.*, (1984) did not find an effect, Itamunoala (1987) observed lower diffusion rates of glucose with increasing alginate concentration. Data from this study (Figure 2.2) pointed to an effect of alginate concentration are in line with the observations of Itamunoala (1987). The molecular weight of the compounds also affects diffusion rates from alginate particules. Diffusion of biomolecules with a molecular weight of less than 20 kDa was not strongly hindered from the gel beads, whereas higher molecular weight substances diffused out of the alginate beads more slowly (Tanaka *et al.*, 1984). Data show that gamma-globulins having MW of approximately 150 kDa slowly diffuse from alginate particles (Tanaka *et al.*, 1984). Thus, it was a concern that our pepN of 90 kDa would leak out of the particles during the hardening process. Encapsulation efficiency values around 30% (Figure 2.2) suggest that this was indeed the case. Data from Kailasapathy *et al.*, (2006) on flavorzyme for cheese ripening are in agreement with the results of this study.

Coating the beads with different polymers, resulting in smaller pores on the bead surface, could solve this problem. Formation of a membrane on the bead surface using polycationic polymers such as chitosan, reduced gel porosity and limited the release of immobilized materials, resulting in greater *EE* (Huguet *et al.*, 1996).

In this study, adding chitosan to the gelling solution resulted in a greater *EE*. The subsequent release of the enzyme from beads in citrate release buffer was decreased by chitosan treatment as well. A similar result was obtained in the controlled release

of hæmoglobin (Hb) (Huguet *et al.*, 1996) and Bovine Serum Albumin (Zheng *et al.*, 2004) from chitosan-treated alginate beads. Formation of the polyelectrolyte membrane between the two gums led to an increase in the retention capacity of alginate beads (Huguet *et al.*, 1996). Furthermore, the possibility of using chitosan as a polycationic polymer for interaction with alginate, a polyanionic polymer, was applied to the control of drug release from microcapsules, where the alginate-chitosan interaction could control the release rate of low and high molecular weights drugs and enhance the loading capacity of beads (Sezer and Akbuğa, 1999a, b). Besides their application to protein or enzyme delivery, alginate beads coated with chitosan have also been successfully used in the immobilization of probiotic bacteria (Krasaekoopt *et al.*, 2006).

Although an increase in chitosan led to a higher *EE* and better control of the pepN release in our study, this effect was not clearly shown over 0.2% of chitosan. This could be due to the strong interaction between chitosan and alginate in the presence of the corresponding anionic carboxyl and cationic amino groups. Zheng *et al.* (2004) concluded that when all alginate carboxyl groups were thoroughly bound up, a higher concentration of chitosan would not be necessary. Chitosan coating proved better than co-encapsulation with starch in enhancing the *EE* values. Indeed, the highest *EE* values reported with the starch-alginate matrix were approximately 70% (Kailasapathy *et al.*, 2006) while values approaching 90% were obtained with chitosan coating.

To investigate the effect of $CaCl_2$ concentration on the *EE*, the alginate-enzyme solution was hardened in 0.05, 0.1, 0.15 and 0.2 M, with or without chitosan. In the presence of chitosan, increasing $CaCl_2$ concentration from 0.05 to 0.1 M at 2% alginate significantly increased *EE* from 68.26 to 85.3%, but the efficiency remained almost unchanged over 0.1M (Figure 2.3). The enzyme entrapment was also slightly improved in uncoated capsules (Figure 2.3) with increasing $CaCl_2$ concentration from 0.05 to 0.1M, which is in agreement with data from Kailasapathy *et al.* (2006), but further increases were ineffective. Therefore, this study shows that an increase in $CaCl_2$ from 0.05 to 0.1M is more beneficial when chitosan is in the gelling solution.

When the enzyme-polymer solution at a fixed 2% alginate was hardened in a 0.1M $CaCl_2$ solution in the presence of chitosan (0.1 %) for 5 to 30 min, the *EE* peaked at 10 min and then decreased as hardening time increased from 10 to 30 min (Figure 2.4). These data differ from those of Kailasapathy *et al.* (2006) who obtained maximum enzyme recovery after 60 minutes. This suggests that chitosan shortens the time required to obtain the encapsulated enzyme. Contraction of the beads occurs during hardening (Martinsen *et al.*, 1989), thus partially expelling out its content. This could partially explain the lower values over 10 min of incubation.

When the *EE* of the alginate beads was investigated for pH values between 3.8 and 5.4, the efficiency increased continuously from pH 3.8 to 5.4 (Figure 2.5).

2.4.2 Effects of Different Parameters on the *ER* of Beads

The *ER* from chitosan treated capsules under different conditions was studied by dissolving the beads in 2% sodium citrate solution as a release buffer. The magnitude of the enzyme release was significantly decreased by increasing alginate concentration from 1.0% to 2.2% and increased with time of exposure to the release buffer (Figure 2.6). After 30 min in the release buffer, the *ERs* varied between 45 and 16% while after 60 min these values ranged between 58 and 38% (Figure 2.6).

The addition of chitosan to the cationic solution significantly ($P < 0.05$) reduced the *ER* from beads during dissolution in the citrate buffer (Figure 2.7). After one hour, almost 100% of the enzyme encapsulated in chitosan-free beads had leaked into the release buffer, whereas around 52% of the enzyme released from the beads hardened in a gelling solution containing 0.1% chitosan (Figure 2.7). By increasing $CaCl_2$ concentration from 0.05 to 0.1M during bead formation, the subsequent enzyme leakage was reduced, but no further effect was observed after at 0.1M (Figure 2.8).

The effect of chitosan-$CaCl_2$ solution-pH on the *ER* from the chitosan-coated alginate beads showed that increase of the pH value reduced the enzyme release from the capsules during the dissolution of the beads in the release buffer (Figure 2.9). The greatest reduction in enzyme release was obtained at pH 5.4.

The dissociation constants (pK_a) for chitosans range from 6.2 to 7.0, and are 3.38 and 3.65, respectively, for mannuronic and guluronic acid monomers (Draget *et al.*, 2006). Chitosan is a polycationic polymer at acidic pH-values due to the pKa of the amino group of the glucosamine residue (Draget *et al.*, 2006). By contrast, at a higher pH, the degree of amino group protonization is decreased resulting in a reduction in the interaction between alginate and chitosan. This phenomenon leads to the formation of a more diffuse polyelectrolyte membrane. In the present study, the *EE* for pepN increased with increasing pH of the chitosan-$CaCl_2$ solution, peaking at 86.85% for a pH of 5.4. This result concurs with the work of Huguet *et al.* (1996), who reported that the lowest release of bead-immobilized Hb was observed when the chitosan-$CaCl_2$ solution-pH was 5.4. Increasing the pH of the hardening solution up to pH 5.4 also caused an increase in the ionization of the alginate, resulting in a high level of ionic interactions between the two polymers, and the formation of a denser membrane at the surface of the bead than would be formed at a lower pH (Huguet *et al.*, 1996).

The alginate and $CaCl_2$ concentrations are the two other factors that may affect the *EE* and *ER* of substances from microcapsules. Given that the formation of Ca-alginate beads results from the cross-linking between COO^- groups and Ca^{2+} ions, it seems that an increase in alginate or $CaCl_2$ should delay the release of encapsultants from beads (Won *et al.*, 2005). Thus, we investigated the effect of alginate and $CaCl_2$ concentrations on *EE* and subsequent *ER* of the pepN from chitosan-treated alginate beads, showing that the *EE* increased with increasing alginate concentration up to 1.8%, while subsequently *ER* was decreased by increasing the polymer concentration (Figure 2.6). Increasing the $CaCl_2$ concentration over 0.1 M at 2.0% of alginate showed no further effect on the *EE* (Figure 2.3). These results concur with those of other investigations. The production of alkaline protease by *Teredinobacter turnirae* cells was investigated by encapsulating cells in Ca-alginate beads (Beshay, 2003). At low *vs.* high alginate, the beads were soft and showed rapid leakage of the cells from the beads. Won *et al.* (2005) studying the optimization of lipase entrapment in Ca-alginate beads, reported that the *EE* increased with increasing alginate concentration. They noted that at 1.0% alginate, the *EE* remained unchanged as $CaCl_2$ concentration

increased above 0.05M (Won *et al.*, 2005). It has also been demonstrated that diffusion of higher molecular weight substances such as albumin out of Ca-alginate beads was little affected by increasing the $CaCl_2$ from 0.05 to 0.5 M, but was considerably limited by increases in the alginate concentration (Tanaka *et al.*, 1984). Mittal *et al.* (2005) studying immobilization of dipeptidylpeptidase IV in Ca-alginate beads showed that an increase in alginate up to 3.0% prevented the leakage of the enzyme from the beads, but over 3% resulted in a high viscose alginate solution and non-uniform bead sizes. Although chitosan and alginate concentrations affect bead release behavior, greater concentrations of either polymer are not required when they exist at the same level in the solution (Zheng *et al.*, 2004).

By decreasing the enzyme proportion in the Na-alginate/enzyme ratio, the *EE* was increased by 78.49%, 83.5% and 88.02% at the ratios of 1:1, 2:1 and 3:1, respectively. The increase of enzyme portion led to a greater release of enzyme from the beads. The lowest *ER* has occurred in the capsules with a polymer: enzyme (3:1) ratio (Figure 2.10).

2.4.3 Scanning Electron Microscopy (SEM)

The surface and inner morphologies of Ca-alginate and chitosan-treated alginate beads were examined using SEM. Using chitosan as a coating material for alginate beads led to the formation of beads with a rough and compact surface (Figure 2.11(*b, d, f, g and h*)), compared to the smooth surface (Figure 2.11(*a, c and e*)) of uncoated alginate beads. The very spongy inner structure of the uncoated beads (Figure 11(*k*)) changed to one with small pores when the beads were coated (Figure 2.11(*l*)). This difference was also reported by other workers (Sezer and Akbuĝa, 1999b; Won *et al.*, 2005; Outokesh *et al.*, 2006). The porous structure of uncoated alginate beads would result in the faster release of recombinant pepN from capsules. This phenomenon was previously reported by Zheng *et al.* (2004). A collapsed center was observed on the surface of wet coated beads (Figure 2.11(*h*)). This structure was observed in dried alginate beads by Sankalia *et al.* (2005). They mentioned that this phenomenon may result from non-uniformly formed Ca-alginate beads having a compact surface and a loose core (Sankalia *et al.*, 2005).

The effect of freeze-drying on the capsule morphology was also evaluated (Figure 2.12(*m-r*)). Freeze drying resulted in irregular and non-spherical capsules (Figure 2.12(*m*)) as was reported by Sankalia *et al.* (2005).

2.5 CONCLUSIONS

The recombinant aminopeptidase (pepN) from *Lactobacillus rhamnosus* S93 was successfully encapsulated in Ca-alginate beads coated with chitosan using an extrusion method. In general, the loss of recombinant pepN from the beads during encapsulation was affected by Na-alginate, chitosan, and calcium chloride concentrations as well as the ratio of the enzyme to the alginate and the pH of chitosan-$CaCl_2$ solution. The use of chitosan as a coating material led to about 89% retention of the recombinant pepN activity in the encapsulated beads and an important reduction in enzyme release from the beads, compared to uncoated Ca-alginate beads. Thus, chitosan-treated alginate beads could provide a suitable matrix for encapsulation of this enzyme in cheesemaking technology.

Table 2.1. Summary of the purification steps of recombinant aminopeptidase from *Lactobacillus rhamnosus* S93.

Purification step	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Crude extract	9.32	34.00	3.65	1.00	100.00
Ammonium sulfate	6.71	32.40	4.83	1.32	95.30
Ion exchange	0.23	9.73	42.30	11.58	28.62

*One unit of enzyme is defined as the amount of enzyme required to release 1 μmol of *p*- nitroaniline min^{-1} under the experimental condition.

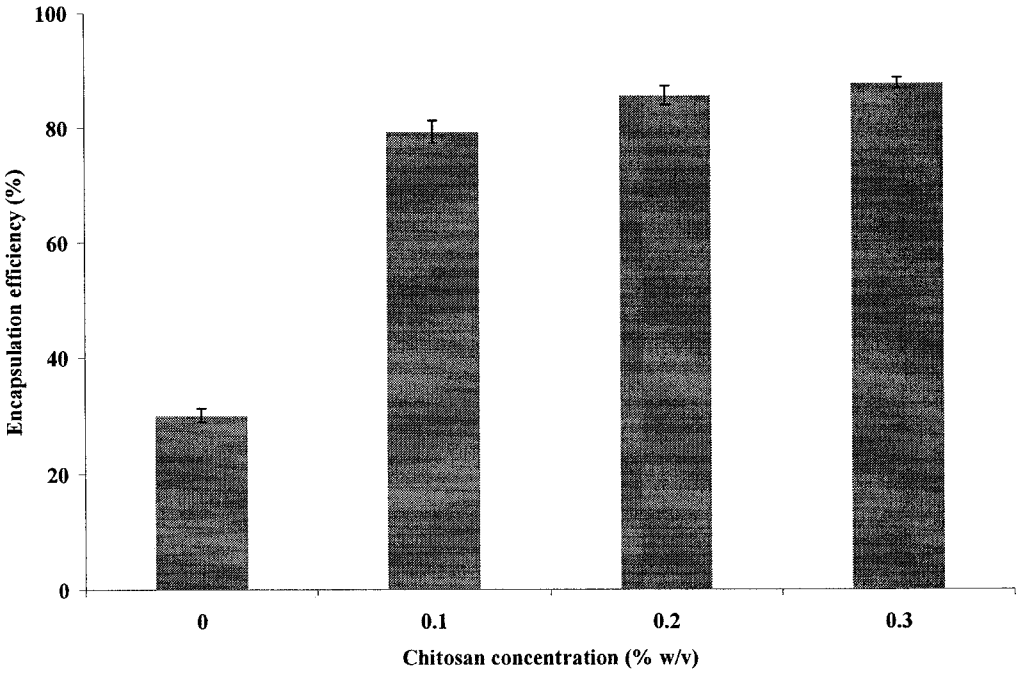


Figure 2.1. Effect of chitosan concentration on the encapsulation efficiency. Sodium alginate concentration (2% w/v); alginate/enzyme ratio (3:1); hardening time (10 min); $CaCl_2$ concentration (0.1 M); agitation speed (70 rpm); chitosan-$CaCl_2$ pH (5.4). Results are presented as mean ± standard deviation.

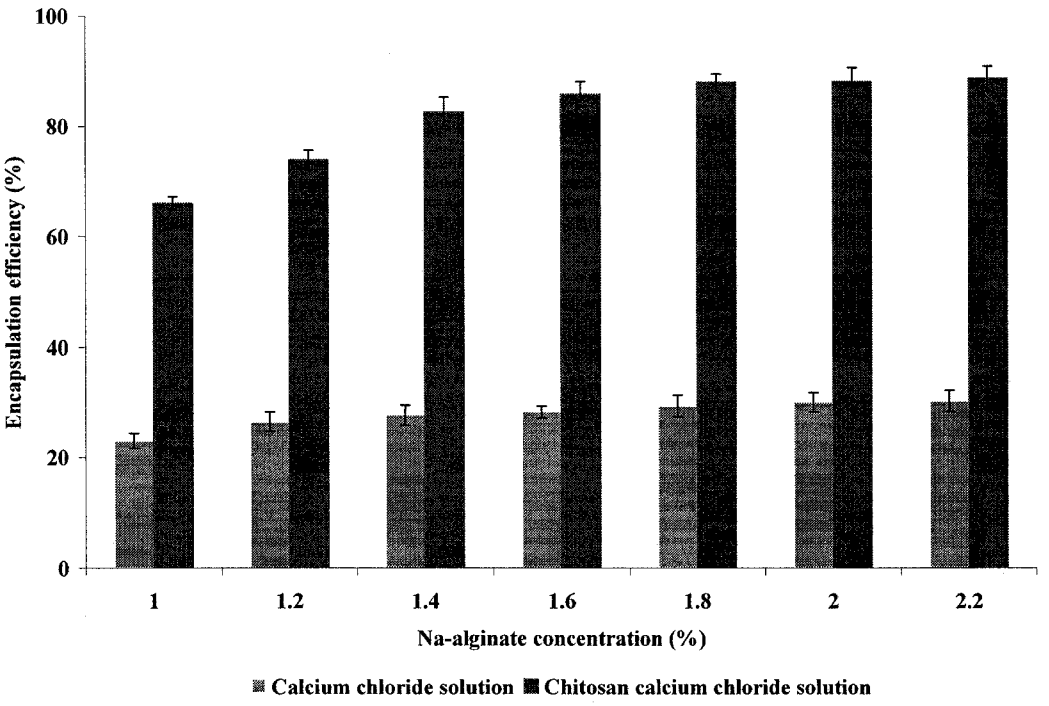


Figure 2.2. Effect of alginate concentration on the encapsulation efficiency. Chitosan concentration (0.1% w/v); hardening time (10 min); $CaCl_2$ concentration (0.1 M); agitation speed (70 rpm); chitosan-$CaCl_2$ pH (5.4). Results are presented as mean ± standard deviation.

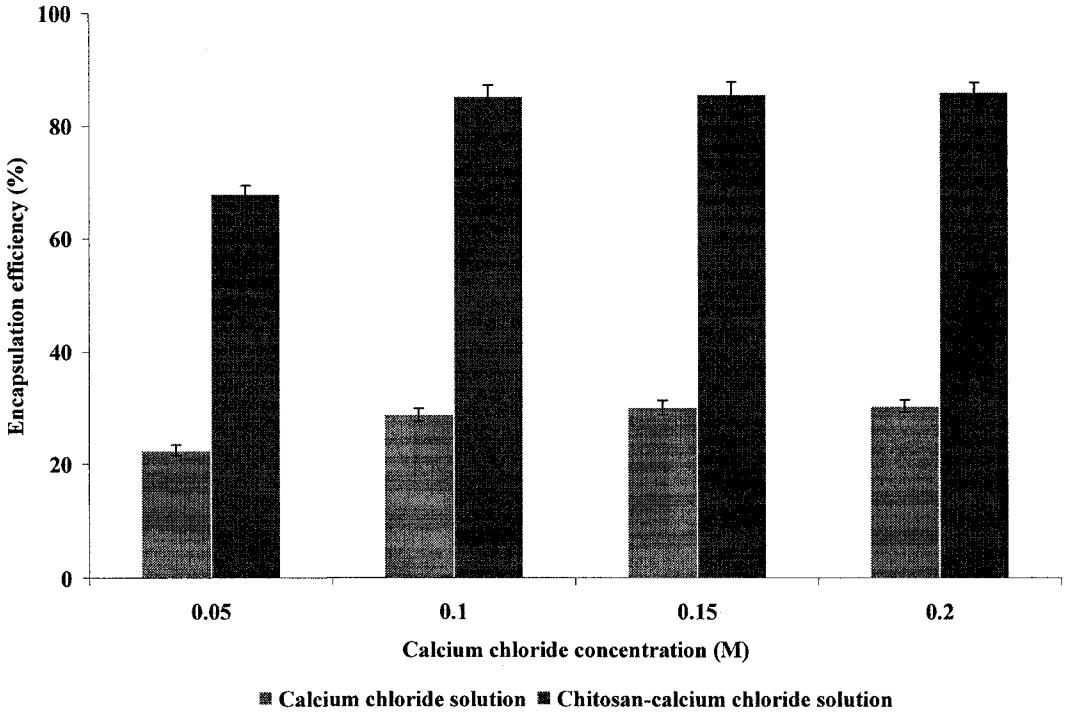


Figure 2.3. Effect of $CaCl_2$ concentration on the encapsulation efficiency. Sodium alginate concentration (2 % w/v); chitosan concentration (0.1% w/v); alginate/enzyme ratio (3:1); hardening time (10 min); agitation speed (70 rpm); chitosan-$CaCl_2$ pH (5.4). Results are presented as mean ± standard deviation.

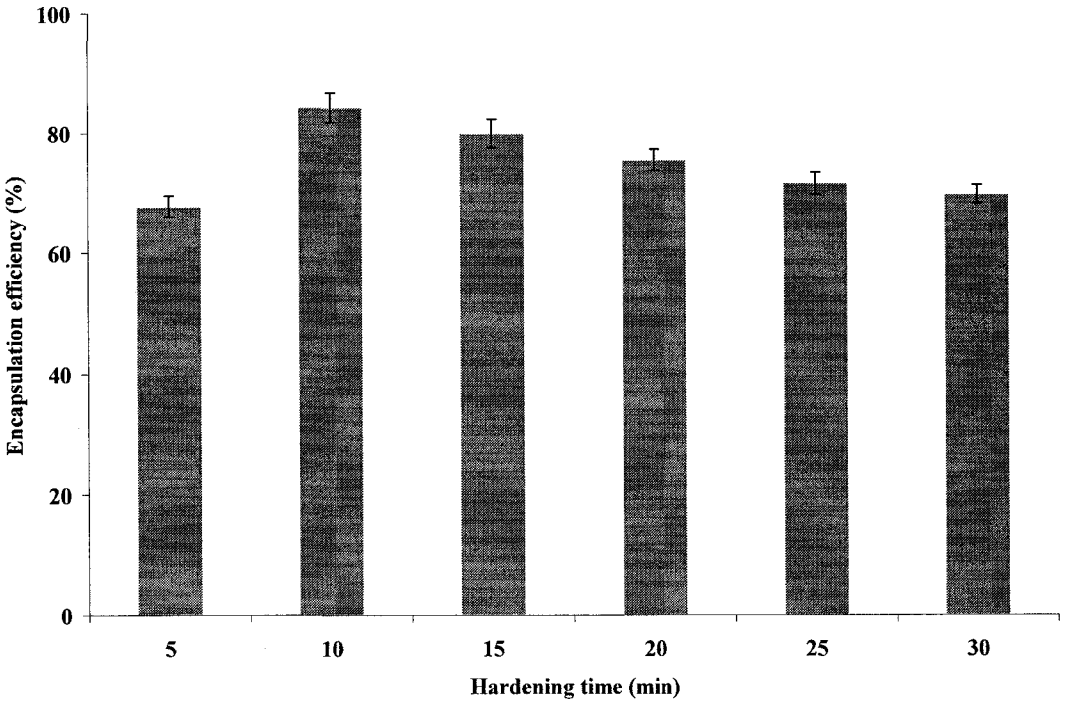


Figure 2.4. Effect of hardening time on the encapsulation efficiency. Sodium alginate concentration (2% w/v); chitosan concentration (0.1% w/v); $CaCl_2$ concentration (0.1 M); alginate/enzyme ratio (3:1); agitation speed (70 rpm); chitosan-$CaCl_2$ pH (5.4). Results are presented as mean ± standard deviation.

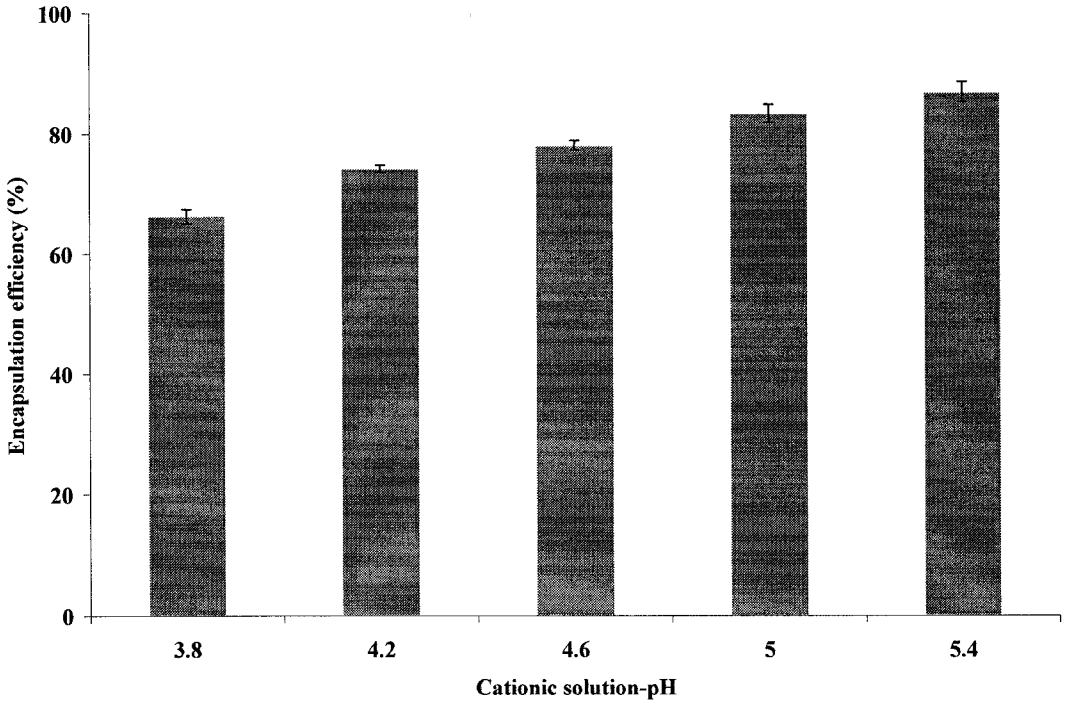


Figure 2.5. Effect of chitosan-$CaCl_2$ solution-pH on the encapsulation efficiency. Sodium alginate concentration (2% w/v); chitosan concentration (0.1% w/v); $CaCl_2$ concentration (0.1 M); hardening time (10 min); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation.

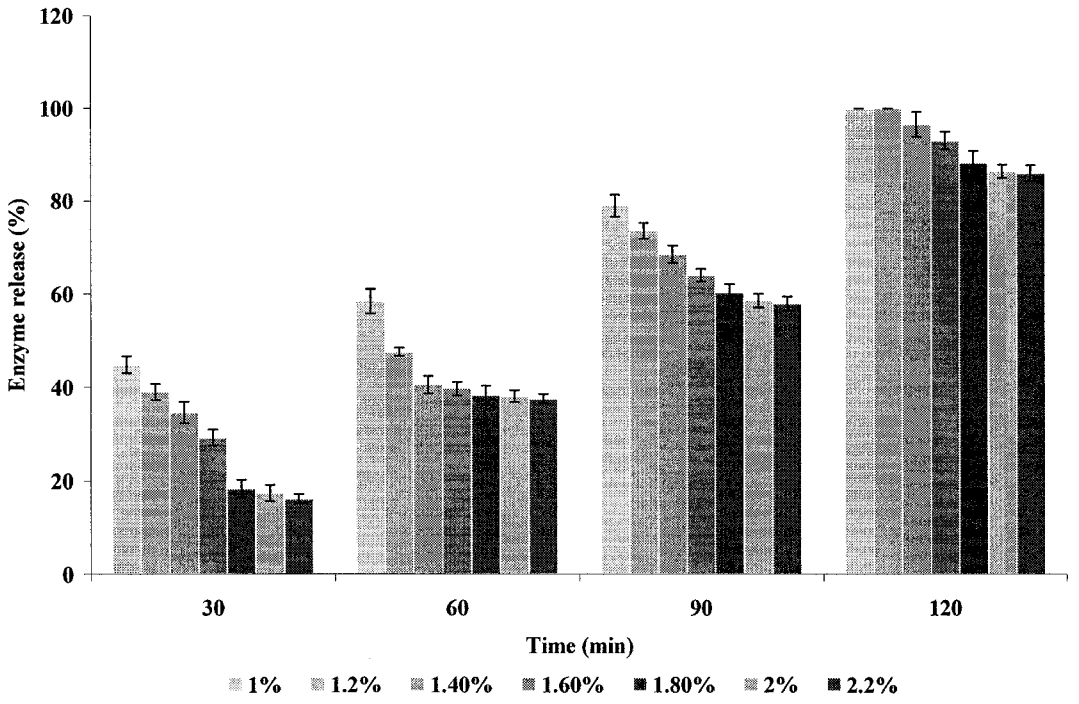


Figure 2.6. Effect of alginate concentration on the enzyme release from the chitosan treated beads in release buffer. Chitosan concentration (0.1% w/v); $CaCl_2$ concentration (0.1 M); hardening time (10 min); chitosan-$CaCl_2$ pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation.

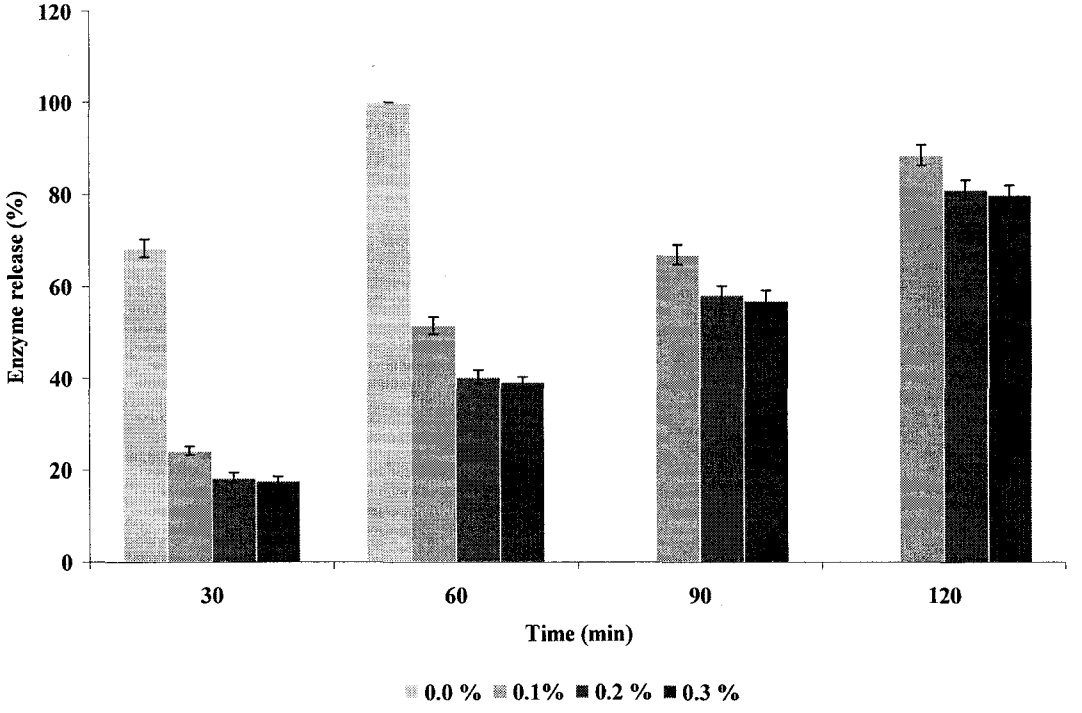


Figure 2.7. Effect of chitosan concentration on the enzyme release from chitosan treated beads in citrate buffer. Na-alginate concentration (2% w/v); $CaCl_2$ concentration (0.1 M); hardening time (10 min); chitosan-$CaCl_2$ pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation.

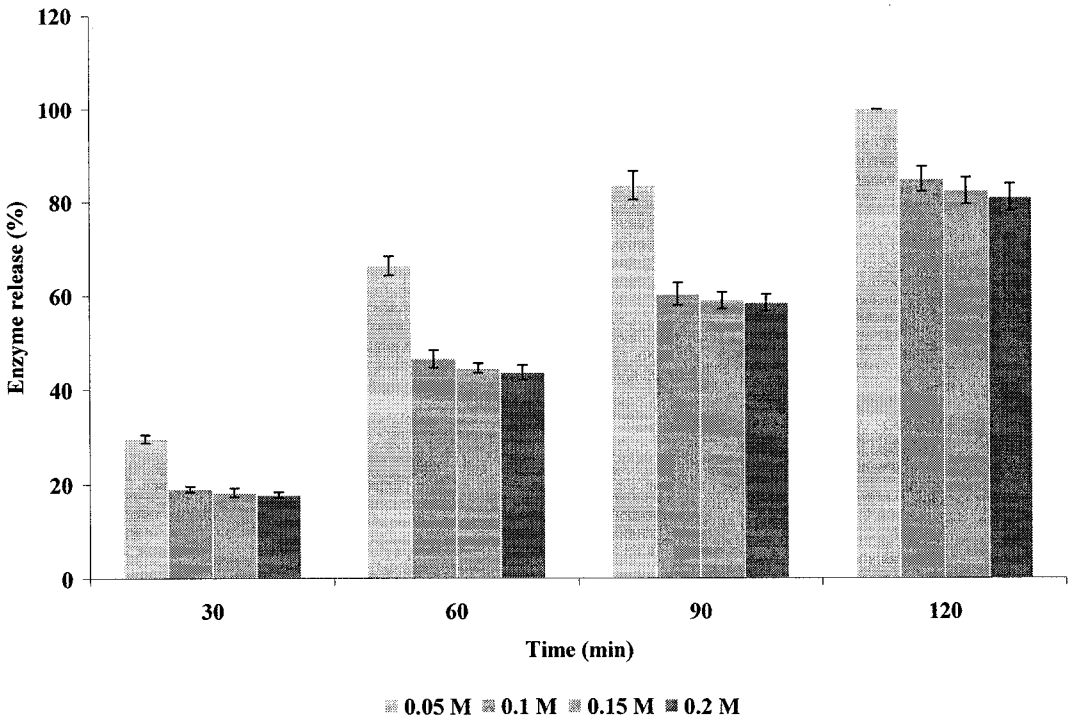


Figure 2.8. Effect of $CaCl_2$ concentration on the enzyme release from chitosan treated beads in citrate buffer. Na-alginate concentration (2% w/v); chitosan concentration (0.1% w/v); hardening time (10 min); chitosan-$CaCl_2$ solution-pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation.

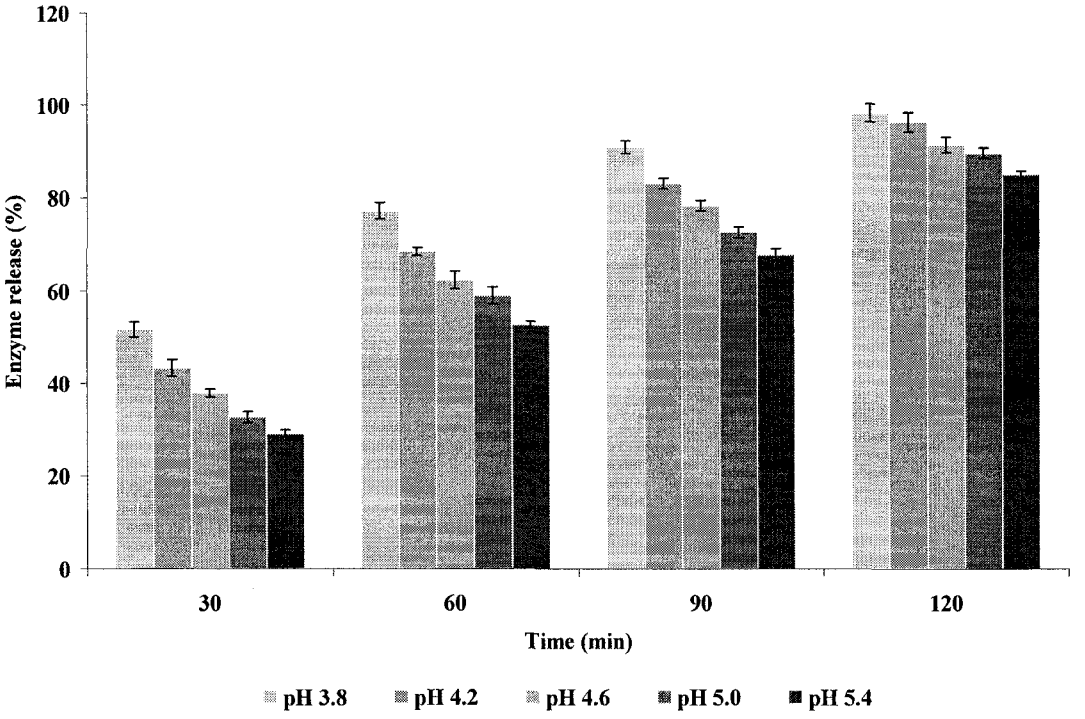


Figure 2.9. Effect of chitosan-$CaCl_2$ solution-pH on the enzyme release from chitosan treated beads in citrate buffer. Na-alginate concentration (2% w/v); chitosan concentration (0.1% w/v); hardening time (10 min); $CaCl_2$ concentration (0.1 M); alginate/enzyme ratio (3:1); agitation speed (70 rpm). Results are presented as mean ± standard deviation.

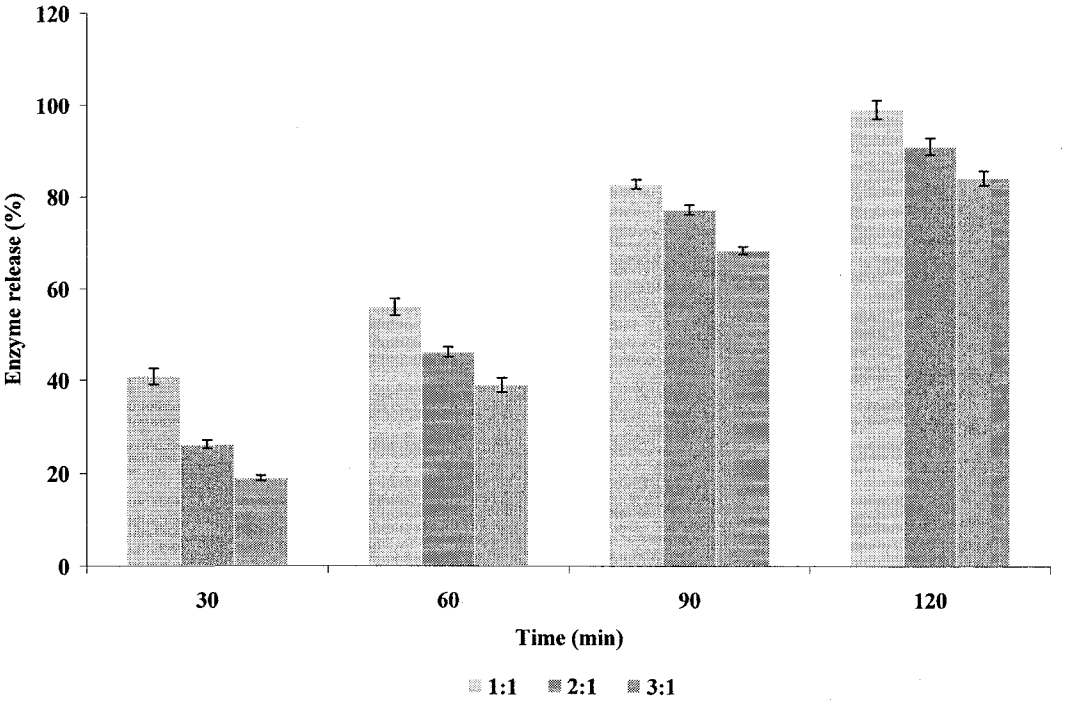


Figure 2.10. Effect of Na-alginate/enzyme ratio on the enzyme release from the Ca-alginate beads. Na-alginate concentration (2% w/v); chitosan concentration (0.1% w/v); hardening time (10 min); $CaCl_2$ concentration (0.1 M); chitosan-$CaCl_2$ solution-pH (5.4); agitation speed (70 rpm). Results are presented as mean ± standard deviation.

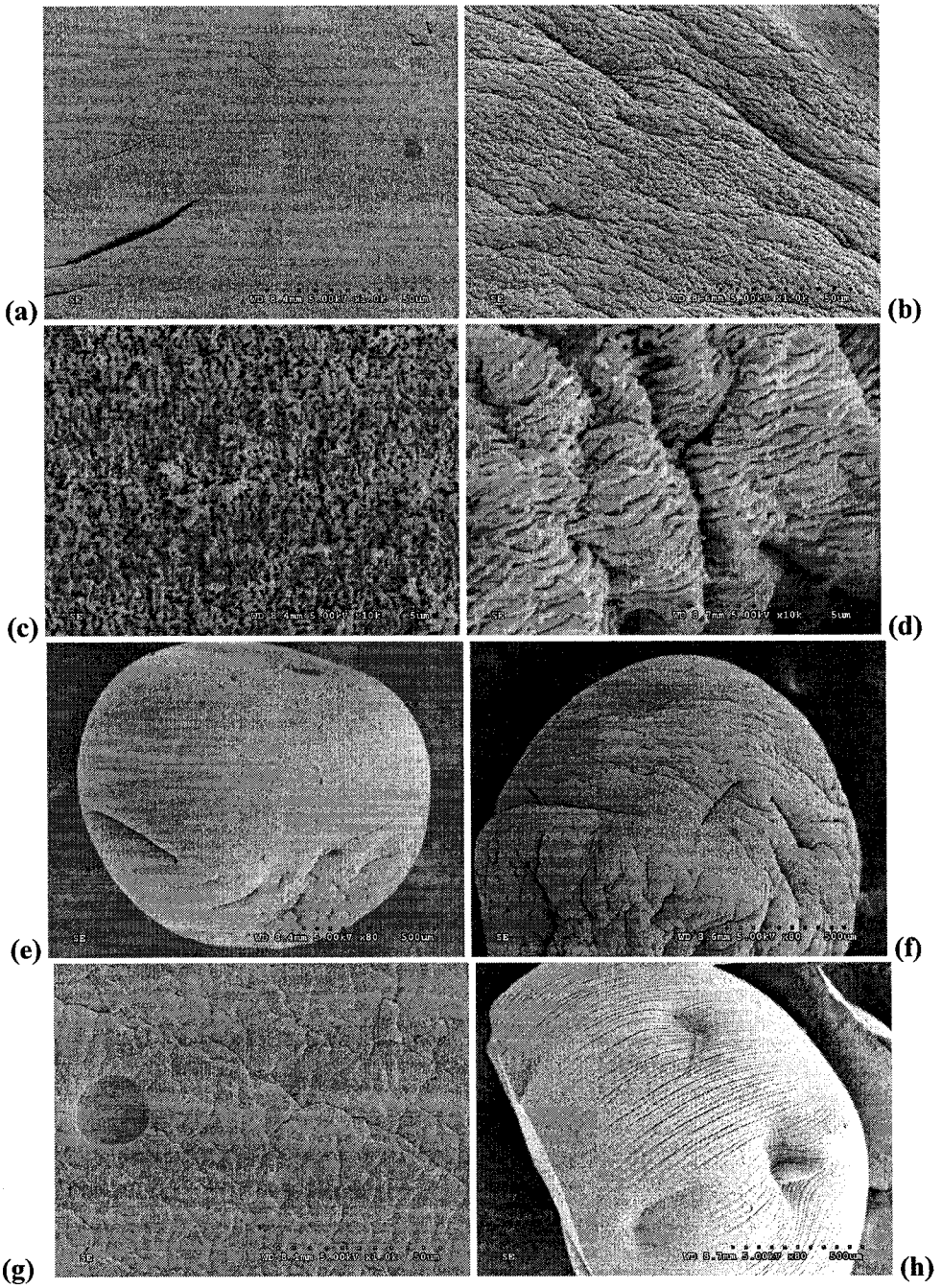


Figure 2.11. The Scanning Electron Microscopy images of wet beads. The surface view of chitosan-free (a, c, e) and chitosan-coated beads (b, d, f, g, h): Original magnification (a, b, g, × 1.0k; c, d, ×10k; e, f, h, ×80); the inside view of Ca-alginate (i, k) and Ca-alginate coated with chitosan (j, l) (original magnification: i, j, ×1.0k; k, l, ×10k); voltage (5.00 kV); encapsulation conditions: alginate concentration (2%); $CaCl_2$ concentration (0.1M); chitosan concentration (0.1%); hardening time (10 min); chitosan-$CaCl_2$ pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm).

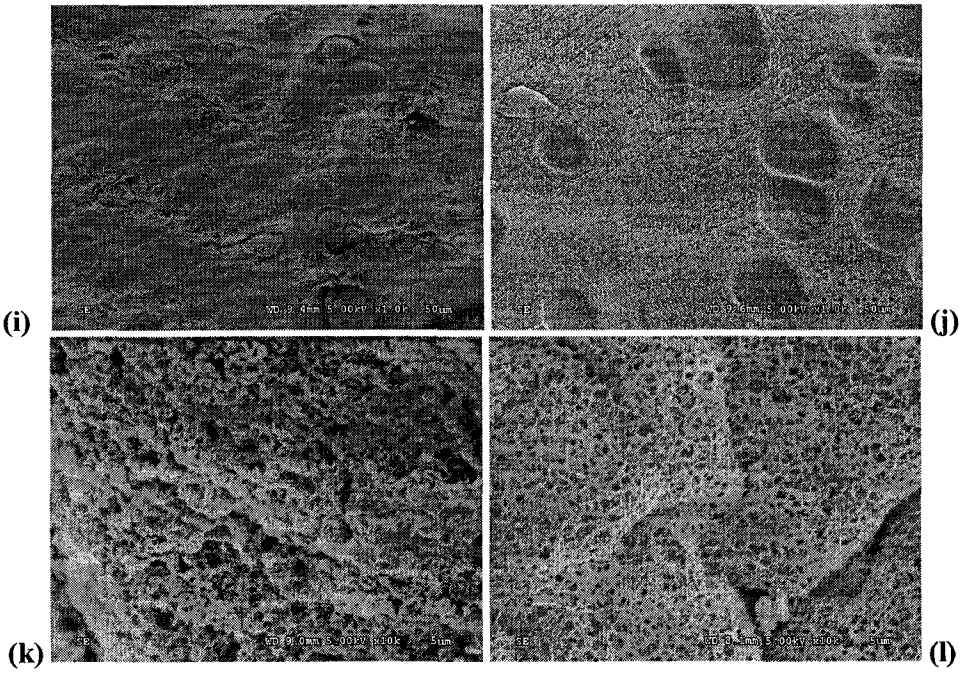


Figure 2.11. (Continued).

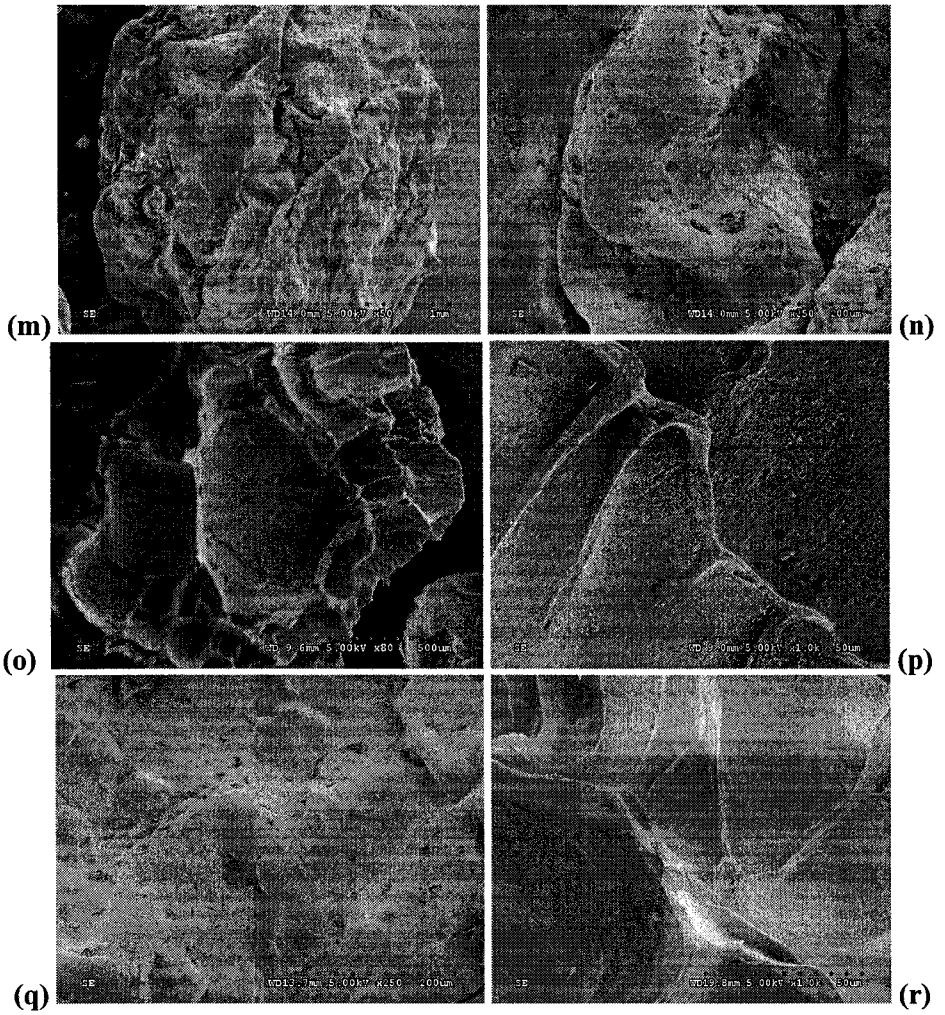


Figure 2.12. The Scanning Electron Microscopy images of the freeze-dried Ca-alginate coated with chitosan beads. The surface view of freeze dried chitosan-coated alginate beads (original magnification: m, × 50; n, q, ×250; o, × 80; p, r, × 1.0k); Voltage: 5.00 kV. Encapsulation conditions: Na-alginate concentration (2 %); $CaCl_2$ concentration (0.1 M); chitosan concentration (0.1%); hardening time (10 min); chitosan-$CaCl_2$ pH (5.4); alginate/enzyme ratio (3:1); agitation speed (70 rpm).

CHAPTER 3

Effect of Recombinant Aminopeptidase of *Lactobacillus rhamnosus* on Cheddar Cheese Ripening

As detailed in Chapters 1and 2, the LAB system has the advantage of being an *in situ* microflora containing the enzymes that are desirable for cheese ripening. However, LAB enzymes are mostly intracellular and extraction from a low yield of LAB biomass is expensive. Therefore, the use of over-expressed enzymes of *Lb. rhamnosus* S93 could be an alternative application in cheese ripening. As the major concern of addition of enzymes into milk is the enzyme loss into the whey during cheesemaking, encapsulation of ripening enzymes could be a cost effective way for uniform delivery of enzymes into the cheese matrix.

For the first time, the effect of encapsulated form of recombinant aminopeptidase (pepN) of *Lb. rhamnosus* S93 in alginate-chitosan particles on proteolysis of Cheddar cheese was investigated.

The result of this research was submitted as a manuscript to *International Dairy Journal*. The manuscript has been co-authored by Sorayya Azarnia, Byong H. Lee, Daniel St-Gelais, and Kieran Kilcawley, and written by Sorayya Azarnia and edited by Dr. Byong H. Lee. Dr. Daniel St-Gelais provided advice, financial and technical supports, and Dr. Kieran Kilcawley provided technical support and editorial assistance in the final stage of paper submission.

3.1 ABSTRACT

The recombinant aminopeptidase of *Lactobacillus rhamnosus* S93 in free or encapsulated form was investigated for its potential to accelerate Cheddar cheese ripening. An extrusion method was used to encapsulate the enzyme in alginate beads coated with chitosan. The encapsulation efficiency was increased from 30% to 87% by inclusion of chitosan during gelation. The free or encapsulated aminopeptidase were added at the renneting or salting stage at three different concentrations (50, 500, 2000 units per 18 L of milk). Indices of secondary proteolysis were enhanced by increasing the enzyme concentration. Cheeses with the highest concentration of the encapsulated enzyme had significantly higher concentrations of soluble nitrogen in phosphotungstic acid and total free amino acids and received the highest mean scores for the sensory characteristics.

3.2 INTRODUCTION

Lactic acid bacteria (LAB) play an important role in cheese ripening, namely the metabolism of lactose and the degradation of casein and lipids by its intracellular enzymes to cheese flavor compounds (Arora and Lee, 1994). The slow release of such key intracellular enzymes has long been known to be a factor involved in the long maturation times to produce Cheddar cheese. As maturation of cheese is expensive, any reduction has significant cost benefits for the cheese industry. Different methods have been employed to shorten cheese maturation times: elevated ripening temperatures, attenuation of starter bacteria, use of adjunct cultures, addition of exogenous enzymes, microencapsulation of enzymes and addition of curd slurries (Law, 2001). All of these methods have the aim of ensuring that enzyme substrate interactions are enhanced. However, as the enzymes of LAB are those responsible for cheese ripening it would appear that the solution to achieving an accelerated but balanced Cheddar cheese should involve their use and not external exogenous enzymes of other species (Lee and Robert, 1997, Lee *et al.*, 2007).

In this study, a key aminopeptidase (pepN) from *Lactobacillus rhamnosus* S93 over-expressed in *Escherichia coli* (Lee and Robert, 1997) was investigated for use in the acceleration of Cheddar cheese ripening.

However, as most enzymes are water soluble, up to 90% may be lost to the whey during cheesemaking. This may also have deleterious effects on potential uses of the whey. Microencapsulation of the enzyme can be a cost effective way for uniform delivery of enzymes into the cheese matrix, without significant losses into the whey (Kailasapathy and Lam, 2005). During ripening, encapsulated enzymes are released into the curd upon capsule breakdown (Gibbs *et al.*, 1999; Kailasapathy and Lam, 2005).

In this study, sodium alginate was investigated as a potential encapsulating agent. Sodium alginate is a natural polysaccharide which contains mannuronic and guluronic acid residues and has the capacity to bind divalent cations such as calcium, resulting in a 3-dimentional gel network (Roberts, 1992). However, alginate gels have large pores which may lead to the release of enzymes from the capsules into the whey during drainage. This problem can be reduced by formation of a polyelectrolyte complex between alginate as an anionic polymer and chitosan as a cationic polymer (Roberts, 1992). While alginate has been used as an immobilization matrix for biomolecules and microorganisms (Champagne *et al.*, 1992; Kailasapathy *et al.*, 2006; Vårum and Smidsrød, 2006), no work has been reported on the encapsulation of lactobacilli peptidases in alginate-chitosan particles or its application in accelerating cheese ripening.

In the present study, the purified recombinant aminopeptidase of *Lactobacillus rhamnosus* S93 was encapsulated in alginate beads coated with chitosan. Three different dose rates of the purified recombinant aminopeptidase in the encapsulated or free form were added at the renneting or salting stage during cheese manufacturing, and proteolysis and sensory properties of Cheddar cheeses were investigated.

3.3 MATERIALS AND METHODS

3.3.1 Materials, Strain and Growth of *E. coli* clones

Materials and media were used in this Chapter were described in Chapter 2, unless otherwise mentioned. The microbial growth condition was the same as described in Section 2.3.1.

3.3.2 Enzyme Preparation and Purification

Enzyme preparation and purification were carried out as described in Section 2.3.2.

3.3.3 Enzyme and Protein Assays

Measurements of the aminopeptidase activity and the protein concentration were carried out as described in Section 2.3.3.

3.3.4 Preparation of Alginate and Chitosan-Coated Alginate Beads

Portions (1g) of crab shell chitosan (85% deacetylated) were dissolved in 700 mL of 0.1 M glacial acetic acid and stirred overnight. After complete dissolution, the pH was then adjusted to 5.4 with NaOH (1 M). $CaCl_2$. H_2O (14.7 g) was added to the chitosan solution and the volume adjusted to 1L with deionized distilled water, yielding a cationic solution of 1g chitosan L^{-1} in (0.1 M) $CaCl_2$ (Zhou *et al.*, 1998). The purified recombinant aminopeptidase and the cooled Na-alginate (Sobalg FD 126, Grindsted Products, Inc., Rexdale, ON, Canada) solution (1.8 %) at a ratio of 1:3 were mixed gently for 2 min. The mixture was introduced to a 10 mL syringe and added drop-wise through a 20G1 needle (Becton Dickinson & Co. Rutherford, Franklin Lakes, NJ, USA) into a cold (4°C) gelling solution containing $CaCl_2$ (0.1 M) or (0.1%) chitosan- (0.1 M) $CaCl_2$ at a ratio of 1:3. The capsules were allowed to harden in this gelling solution for 10 min, under agitation (70 rpm), resulting in the formation of alginate or chitosan-coated alginate beads. After filtration, the beads were washed with deionized distilled water to remove the untrapped enzyme and excess calcium chloride from the surface of the beads. Portions (1 g; wet weight) of coated or uncoated beads were then dissolved under gentle agitation (70 rpm) in 10 mL of trisodium citrate (20 g L^{-1}) release buffer, at room temperature (Champagne et al., 1992). After complete dissolution of the beads, the enzyme activity in the release buffer was measured. All experiments were carried out in triplicate.

The encapsulation efficiency was calculated according to the equation described in Section 2.3.4. The same procedure described in Section 2.3.6 was used for preparation of freeze dried free or encapsulated enzymes.

3.3.5 Cheddar Cheese Manufacture

The starter strains used were *Lactococcus lactis* ssp. *cremoris* (LL-74, LL-275 and LL-390) at a ratio of 1: 1: 1 (Agropur, Granby, PQ, Canada). They were separately grown at 21°C for 16 hours, in heat treated (85°C for 10 min) reconstituted low heat skim milk powder (12%). Control and experimental cheeses were made from pasteurized cows' milk at the pilot plant of Agriculture & Agri-Food Canada, Food R & D Centre (St-Hyacinthe, PQ, Canada) by an experienced cheese master in vats containing 18 L milk according to the protocol listed in Table 3.1. Milk pasteurization was carried out at 73°C for 16 sec, and then $CaCl_2$ (25 g $100kg^{-1}$milk) and 1.5% (v/v) of starter culture were added to the milk. Double strength chymosin (Maxiren®, DSM Food Specialties USC Inc., Parsippany, NJ, USA) was added (0.02%, v/v) at 32°C. After about 30 min, the curd was cut and heated to 38°C. The whey was drained off, and cheddaring occurred at 38°C. After milling, the curd was salted (2.0%, w/w), placed in 2 kg hoops and pressed 1 hour under pressure (2.46 Kgf cm^{-2}). The cheese blocks were packed under vacuum condition and stored at 4°C for 6 months. The freeze dried free and encapsulated forms of the purified recombinant pepN, at concentrations of 50, 500 and 2000 units per 18 L milk were added, respectively, to the curd at the salting stage and to milk at the renneting stage. The microbial and physicochemical analyses were carried out at 1, 15, 60, 120 and 180 days of ripening. All analyses were carried out in four replicates.

3.3.6 Compositional Analysis

Contents of fat, protein, lactose and total solids of milk were obtained using a Milkoscan FT 120 (Foss North America, Eden Prairie, MN, USA).

Moisture content of cheeses was determined by drying to a constant weight at 102°C (IDF, 1982). Salt contents were measured by a Corning chloride analyzer 926 (Nelson-Jameson, Inc. Marshfield, WI, USA). The total nitrogen was determined by the macro-Kjeldahl method (IDF, 1986). Fat content was determined by the Mojonnier extraction procedure and ash by heating samples at 550°C for 16 hours in a muffle furnace. The pH of grated cheese (10 g) macerated in 10 mL distilled water

was measured (Hannon *et al.*, 2005) using a pH meter (Ross® pH electrodes, Thermo Orion, Beverly, MA, USA). All analyses were carried out in four replicates.

3.3.7 Analysis of Proteolysis Indices

The water soluble nitrogen (WSN) and the total nitrogen soluble in 5% phosphotungstic acid (PTA-N), expressed as a percentage of total nitrogen were determined by the methods described by Christensen *et al.* (1991).

Individual free amino acids (FAA) were determined on 24% trichloroacetic acid filtrates prepared from the water-soluble nitrogen fraction using a Jeol JLC-500/V amino acid Analyzer (Jeol Ltd, Garden City, Herts, UK) fitted with a Jeol sodium high performance cation exchange column (Kilcawley *et al.*, 2006) and the results expressed as $\mu g\ g^{-1}$ cheese.

3.3.8 Sensory Evaluation

The sensory properties of control and the experimental cheeses were evaluated, focusing on the Cheddar cheese characteristics, at 120 days by 8 trained panellists at the Agriculture & Agri-Food Canada, Food R & D Centre (St-Hyacinthe, PQ, Canada). The cheeses were cut into small cubes (about 5g) and tempered to room temperature for about 2 hours before tasting. Four portions (5 g) of each sample were put in an amber-glass bottle, capped and coded with a 2-digit number. Duplicate samples of each cheese were presented randomly to the testers. Deionized water was used for palate cleansing between samples. The testers were asked to evaluate, without swallowing the samples, the cheeses considering three attributes (texture, flavor or aroma) using a 5-point hedonic scale as very good (+2), good (+1), acceptable (0), bad (-1) and very bad (-2). After each tester completed the given form, a round table discussion took place at the end of the session.

3.3.9. Microbiological Analysis

All media were purchased from Difco (Difco Laboratories, Detroit, MI, USA), unless otherwise mentioned. The packaged cheese was opened and handled aseptically. Samples (10 g) were placed in 90 mL sterile 0.1% (w/w) peptone solution and blended in a Stomacher 400 circulator (Seward Lab., London, U.K.) for 3 min at

260 rpm. Bacterial numbers were determined by plating appropriate dilutions using sterile 0.1% peptone. All plates were made in four replicates according to the method described by the American Public Health Association for dairy products (APHA, 1992). M17-Agar and Rogosa Agar (adjusted pH to 5.5 with acetic acid 96%) were used for enumeration of lactococci and lactobacilli, respectively. The plates were incubated at 30°C for 4 days in an anaerobic incubator (Thermo Fisher Scientific, Fisher Canada, Nepean, ON, Canada). Yeasts and molds were counted on Rose Bengal Agar with chloramphenicol (Bishop Canada Inc., Burlington, ON, Canada) incubated at 22°C for 4 days. Total coliforms were enumerated on Violet Red Bile Agar with plate incubation at 37°C for 24 hours. Total staphylococci were counted on *Staphylococcus* Medium 110 at 37°C for 48 hours. All analyses were carried out in four replicates.

3.3.10 Statistical Analysis

A split plot design was used to evaluate the data from the WSN, PTA-N, concentration of total FAA, microbial changes, cheese and milk compositions and sensory properties The main plot factor was the treatment, control cheese, cheeses with the free or encapsulated purified recombinant aminopeptidase at three different concentrations (50, 500 and 2000 units per 18 L milk), and the sub-plot factor was ripening time. A general linear model (GLM) procedure of Statistical Analysis System (SAS, 2004) was used to perform analysis of variance (ANOVA) to evaluate the effect of treatment for all response variables. Statistically significant differences between different treatments were determined by Fisher's least significant difference (F-value). Moreover, Duncan's multiple range test was carried out to compare the means among the proteolytic indices and sensory attributes of cheeses. The level of significance was determined at $P < 0.05$ (Duncan, 1955).

Principal Component Analysis (PCA) (Johnson, 1998) was carried out on the individual FAA data to obtain the best possible analysis of the individual FAA.

3.4 RESULTS AND DISCUSSION

3.4.1 Enzyme Preparation and Purification

The purification results of the crude enzyme containing recombinant pepN after one step of preparative ion exchange column chromatography showed that the enzyme was purified 11.6-fold over the crude extract with a recovery of 28.6 percent. The molecular weight determined by the SDS-PAGE was about 90 kDa as described in Section 2.4.1.

3.4.2 Enzyme Encapsulation

The presence of chitosan in the gelling solution significantly ($P < 0.05$) increased *EE* from 30% in capsules formed in gelling solution containing $CaCl_2$ to 87% with Chitosan-$CaCl_2$ gelling solution. Chitosan, a hydrophilic polymer consisting of N-acetyl-D-glucosamine and D-glucosamine residues (Roberts, 1992; Vårum and Smidsrød, 2006; Martínez-Ruvalcaba *et al.*, 2007), is produced by alkaline deacetylation of chitin, leading to the formation of the deacetylated form of the amino groups. The amino groups are highly electronegative and can become positively charged by absorbing a proton (Vårum and Smidsrød, 2006). Therefore, electrostatic interaction between the negatively charged carboxylic acid groups of alginate and the positively charged amino groups of chitosan causes formation of a polyelectrolyte complex between the two polymers. The formation of this membrane on the bead surface reduced gel porosity and limited the release of encapsulated materials, resulting in greater *EE* (Huguet *et al.*, 1996). A similar result was obtained in the controlled release of hæmoglobin (Hb) (Huguet *et al.*, 1996) and bovine serum albumin (Polk *et al.*, 1994; Huguet and Dellacherie, 1996; Zheng *et al.*, 2004) from chitosan-treated alginate beads.

3.4.3 Compositional Analysis

There were no significant differences ($P > 0.05$) among the composition and pH of cows'milk used for preparation of the control and experimental cheeses have (Table 3.2).

No significant differences ($P > 0.05$) were observed among the cheeses for the salt-in moisture, ash, fat-in dry matter, protein, and pH values (Table 3.3) except for the moisture content. However, the moisture contents were in acceptable levels for Cheddar cheese (Food and Drugs Act and Regulations, 2006).

3.4.4 Microbiological Analysis

The Figures 3.1 and 3.2 show the changes in the populations of lactococci and lactobacilli in the cheeses during ripening time of 6 months at 4 °C. A significant ($P < 0.05$) reduction of lactococcal populations was observed during storage time in all cheeses. This progressive reduction of starter bacteria was also reported by other authors (Dabour *et al.*, 2006; Di Cagno *et al.*, 2006, Ong *et al.*, 2006).

Reduction of the starter's cell count is due to the interior cheese conditions such as low pH, low temperature, high concentration of salt and lack of lactose during the ripening period (Fox *et al.*, 1996). An increase in the intracellular enzymes, particularly peptidases of lactococci into the cheese matrix is often due to the lysis of the dead cells (Chapot-Chartier *et al.*, 1994; Law, 2001). This phenomenon is important in flavor generation in the cheese during maturation. The cells' autolyses result in the liberation of their intracellular enzymes into the cheese matrix at the initial stage of ripening (Lortal and Chapot-Chartier, 2005; Picon *et al.*, 2005; Kenny *et al.*, 2006).

On the contrary, lactobacilli grew rapidly in all cheeses during the ripening period (Figure 3.2). Non-starter lactic acid bacteria are the only bacteria which grow in Cheddar cheese during maturation (Fox *et al.*, 1998; Shakeel *et al.*, 1999). They could be originated from milk, equipment, air, or personnel. It is well known that lactobacilli are the predominant species of this group (Jordan and Cogan, 1993).

In general, the results obtained from the present study showed that using free or encapsulated enzymes did not affect the cell counts. No pathogens, yeasts and molds were found in all cheeses that it means the cheeses were prepared and stored under appropriate conditions.

3.4.5 Proteolysis Analysis

The proteolysis in control and experimental cheeses was evaluated by measuring the amount of WSN, PTA-N and total FAA (Figure 3.3). According to ANOVA, the results obtained from the assessment of WSN showed that the level of this nitrogen fraction was significantly ($P < 0.05$) increased in all cheeses with storage time. No significant ($P > 0.05$) differences in this fraction were observed among the cheeses during the ripening.

The PTA-SN and total FAA were also increased in all cheeses during the maturation time and with increasing the enzyme concentration (Figure 3.3). The ANOVA analysis showed that for the both proteolytic indices, significant differences were observed for the ripening time ($P < 0.01$), treatments ($P < 0.01$) and the interaction between the treatment and the ripening time ($P < 0.01$).

According to Duncan's test, there were significant differences at the level of 5% between the mean levels of the PTA-N among the cheeses. Cheese with the highest concentration of the encapsulated enzyme showed a significantly higher mean level of PTA-N among the cheeses over the 6 month ripening period (Table 3.5).

The results obtained from ANOVA analysis showed that the amounts of total FAA were significantly increased for all cheeses with time and with increasing the enzyme concentration. Furthermore, Duncan's test provided similar results where the cheese with the highest concentration of the encapsulated enzyme had the highest mean levels of the total FAA compared to those of the other cheeses over maturation times (Table 3.5). The amounts of PTA-N and total FAA in cheese with the highest concentration of the encapsulated enzyme after 2 months ripening were almost similar to those of the control cheese after 6 months, thus indicating the acceleration of about 70% in these proteolytic indices (Figure 3.3).

Both free and encapsulated forms of the recombinant pepN led to enhancement of proteolysis compared to that of the control. However, an important advantage of encapsulated enzyme is that it can be added directly into milk, rather than to the curd at the salting stage, so that the enzyme could be distributed in the curd uniformly. Therefore, study of the cheese microstructure could be useful to find the effects of the two methods on the uniform distribution of the enzyme in the cheese matrix.

Our results on acceleration of Cheddar cheese ripening by the recombinant aminopeptidase of *Lb. rhamnosus* could be supported by the effects of adjuncts starter cultures that can speed up the Cheddar cheese maturation. Ong *et al.* (2006) studied the effect of probiotic adjuncts on the proteolytic patterns of Cheddar cheese during maturation. Their results showed that the presence of these bacteria led to a greater secondary proteolysis in probiotic cheeses than in control cheeses, without having a significant effect on the level of the primary proteolysis (Ong *et al.*, 2006). The impact of LAB in the maturation of Cheddar cheese was also studied by adding cell homogenates or live cells of *Lactobacillus casei* subsp. *casei* L2A at the renneting and salting stages. Flavor intensity increased in these cheeses compared to the control cheese during ripening (Trépanier *et al.*, 1991a).

In general, the WSN evolution during ripening time results from hydrolysis of caseins to large and intermediate-sized peptides which in turn are degraded to smaller peptides by residual coagulants and enzymes derived from starter and non-starter bacteria (Dabour *et al.*, 2006; Sousa *et al.*, 2001).

In this study, the concentration of individual free amino acids in the control and the experimental cheeses was evaluated at 2, 4 and 6 months of the ripening time (Figure 3.4). The concentration of most individual amino acids in all cheeses increased with the ripening time and with increasing enzyme concentration, and the greatest amount was observed in cheeses with 2000 units of the recombinant pepN. However, the levels of most individual amino acids were greater in all enzyme-added *vs.* control cheeses at all sampling times, except for proline, which disappeared in the enzyme-added cheeses after 4 months of ripening (Figure 3.4). Leucine was the most dominant amino acid in the enzyme-added cheeses (Figure 3.4). It is understandable as the aminopeptidase used in this study has preference for dipeptides containing leucine as the N-terminal residue (Arora *et al.*, 1990). A large quantity of leucine has been also reported in Cheddar cheeses with added lactobacilli (Puchades *et al.*, 1989).

The PCA was carried out on the raw data of the individual FAA using the PRINCOMP procedure of the SAS software. Table 4 presents the first 10 eigenvalues of the covariance matrix, the difference between successive eigenvalues, the proportion of the covariance explained by each eigenvalue, and the cumulative

proportion of the explained variance. A PCA chart (Figure 3.5) demonstrates that PC1 and PC2 accounted for a cumulative variation of 92.16%. PC1 accounted for 86.90% of the variation and separated the samples on the basis of the enzyme concentration.

3.4.6 Sensory Evaluation

The results obtained from the sensory evaluation of the cheeses showed significant differences for the three attributes, texture ($P < 0.05$), flavor ($P < 0.05$) and aroma ($P < 0.01$) among the cheeses. Comparison of the mean scores for sensory attributes using Duncan's test showed that cheese with the highest units of the encapsulated enzyme received significantly the highest mean scores for texture, flavor and aroma compared to the other cheeses (Table 3.5).

The testers did not recognize any defect, due to the presence of alginate beads in the samples. However, the small cracks were observed on the surface of the cheeses treated with the encapsulated enzymes because of the bead size (about 2 mm). It is thought that this problem may be eliminated by the use of smaller, more uniform beads, generated with an encapsulator.

3.5 CONCLUSIONS

In this study, a recombinant aminopeptidase of *Lb. rhamnosus* S93 was successfully encapsulated into chitosan-coated alginate beads. Addition of chitosan into the gelling solution significantly increased the encapsulation efficiency.

Evaluation of the recombinant aminopeptidase in encapsulated or free form on proteolysis during the ripening of Cheddar cheese revealed that cheeses supplemented with the encapsulated enzyme had significantly higher concentrations of the PTA-N and FAA than those supplemented with the free enzyme. Cheese with 2000 units of the encapsulated enzyme had significantly the highest concentration of secondary proteolysis indices as well as the highest mean scores for the sensory characteristics.

Table 3. 1. Protocol for Cheddar cheese manufacturing.

Cheese codes	Treatments	Enzyme units 18 L^{-1}milk	Enzyme addition stage
C	Control	0	Non applicable
F50U	Cheese with free form of pepN	50	Salting
F500U	Same above	500	Salting
F2000U	Same above	2000	Salting
E50U	Cheese with encapsulated pepN	50	Renneting
E500U	Same above	500	Renneting
E2000U	Same above	2000	Renneting

Table 3.2. Compositions of milk used for the making of cheeses.

	Fat (%)	Protein (%)	Lactose (%)	Total solid (%)	pH
C[a]	3.84±0.02	3.22±0.04	4.47±0.04	12.55±0.01	6.60±0.04
F 50U	3.84±0.02	3.22±0.04	4.47±0.04	12.55±0.01	6.60±0.04
F 500U	3.84±0.02	3.22±0.04	4.47±0.04	12.55±0.01	6.60±0.04
F 2000U	3.84±0.02	3.22±0.04	4.47±0.04	12.55±0.01	6.60±0.04
E 50U	3.87±.03	3.22±.03	4.47±.02	12.56±0.03	6.59±0.03
E 500U	3.78±0.03	3.23±0.02	4.50±0.03	12.46±0.03	6.57±0.01
E 2000U	3.84±0.02	3.23±0.02	4.47±0.04	12.56±0.02	6.54±0.04

[a]See Table 1 for identification of cheese codes.

Table 3.3. Composition of control and experimental chesses at 15 days of ripening.

Cheeses	Salt (%)	Moisture (%)	S/M[a] (%)	Protein (%)	F/DM[b] (%)	Ash (%)	pH
C	1.68±0.01	36.83±0.06	4.55±0.04	24.61±0.06	51.38±0.97	3.41±0.04	5.11±0.02
F 50U	1.67±0.01	36.80±0.04	4.54±0.02	24.63±0.05	51.34±0.98	3.42±0.02	5.12±0.03
F 500U	1.67±0.01	37.14±0.06	4.52±0.04	24.50±0.10	51.27±0.89	3.46±0.01	5.11±0.03
F 2000U	1.67±0.02	37.19±0.02	4.49±0.05	24.47±0.08	51.25±0.85	3.44±0.02	5.13±0.02
E 50U	1.69±0.01	36.89±0.04	4.57±0.04	24.60±0.09	51.18±0.83	3.45±0.02	5.12±0.02
E 500U	1.68±0.02	36.98±0.02	4.54±0.04	24.54±0.09	51.23±0.84	3.42±0.01	5.11±0.03
E 2000U	1.67±0.01	37.02±0.04	4.50±0.04	24.50±0.11	51.32±0.90	3.43±0.04	5.13±0.03

[a]Salt in Moisture.
[b]Fat in Dry Matter.
See Table 1 for identification of cheese codes.

Table 3.4. Eigenvalues of the correlation matrix calculated from PRINCOMP procedure.

Principal Component	Eigenvalue	Difference	Proportion	Cumulative
1	15.6421042	14.6959339	0.8690	0.8690
2	0.9461703	0.3045333	0.0526	0.9216
3	0.6416370	0.4230001	0.0356	0.9572
4	0.2186369	0.0481779	0.0121	0.9694
5	0.1704590	0.0568071	0.0095	0.9788
6	0.1136519	0.0379950	0.0063	0.9851
7	0.0756569	0.0334696	0.0042	0.9894
8	0.0421873	0.0061636	0.0023	0.9917
9	0.0360237	0.0054133	0.0020	0.9937
10	0.0306104	0.0028546	0.0017	0.9954

Table 3.5. Duncan's multiple range test for responses.

Parameters			E2000U	F2000U	E500U	F500U	E50U	F50U	C
PTA/TN(%)		Duncan grouping	A	B	C	D	E	F	F
		Mean	2.533	2.395	2.156	2.040	1.457	1.371	1.347
TFAA (ug g^{-1} Cheese)		Duncan grouping	A	B	C	D	E		
		Mean	6819.47	6441.35	5223.42	4945.48	3038.27	-	-
Sensory attributes	Texture	Duncan grouping	A	AB	B	B	B	B	B
		Mean	1.375	1.125	0.750	0.750	0.625	0.500	0.500
	Flavor	Duncan grouping	A	AB	AB	B	B	B	B
		Mean	1.625	1.375	1.250	0.750	0.750	0.625	0.625
	Aroma	Duncan grouping	A	AB	AB	B	B	B	B
		Mean	1.500	1.000	0.750	0.625	0.250	0.250	0.250

Means within a row with the different letter are significantly different ($P < 0.05$).
See Table 1 for identification of cheeses codes. "-" Not measured.

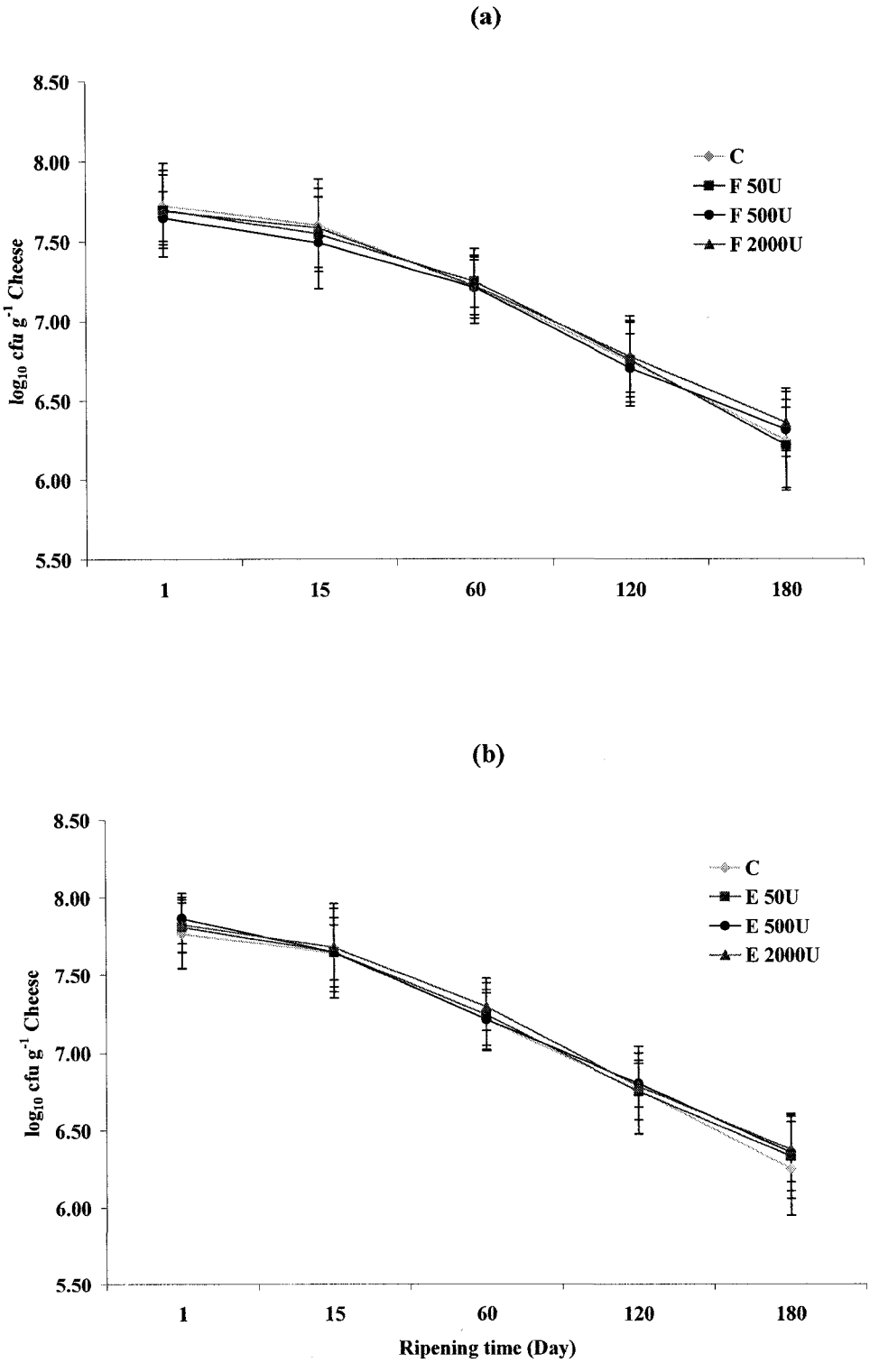


Figure 3.1. Changes in lactococci populations (a) cheeses with the free enzyme, (b) cheeses with the encapsulated enzyme. Results are presented as mean ± standard deviation. Probability level: $P < 0.05$. See Table 1 for identification of cheeses codes.

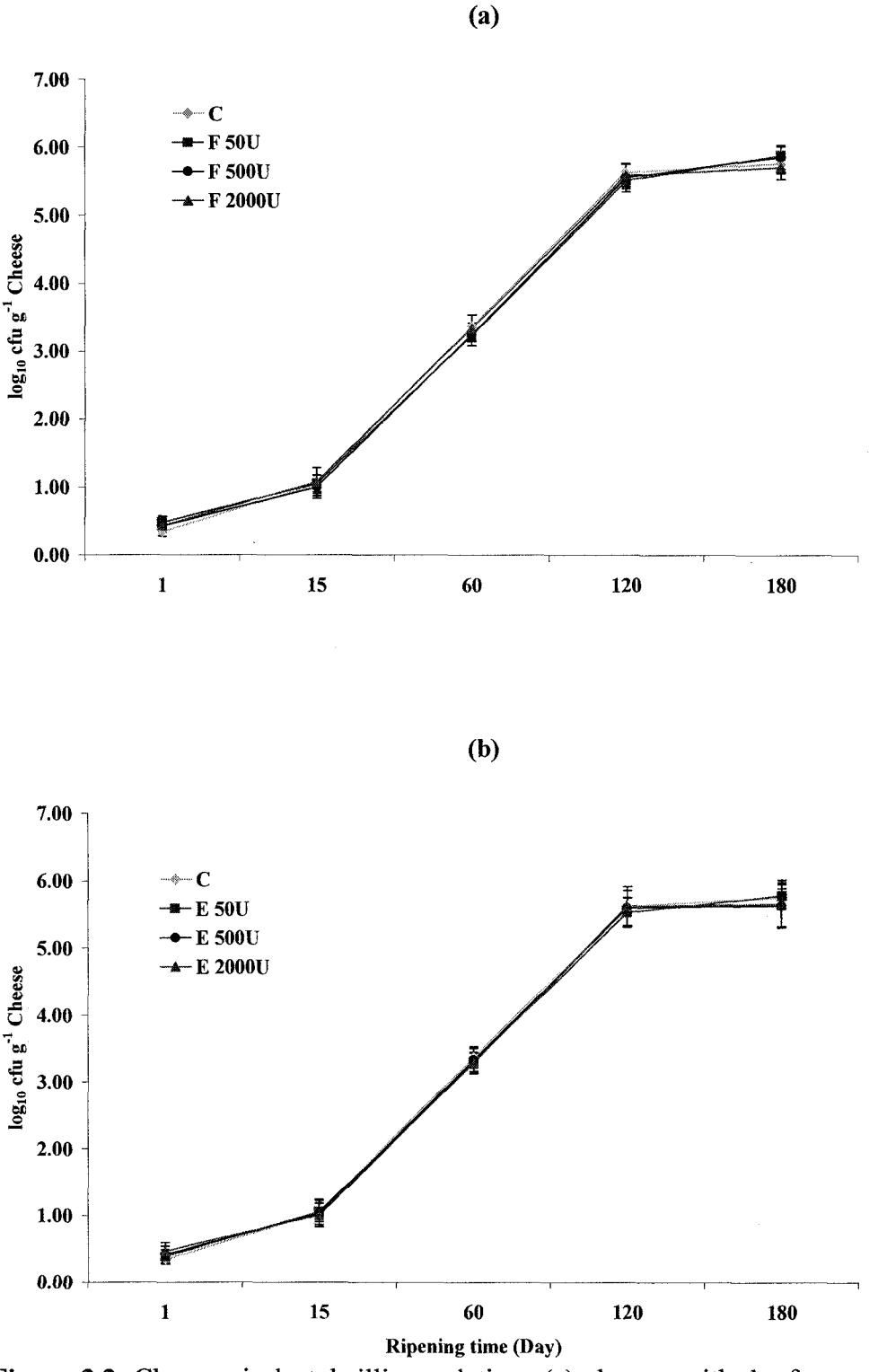


Figure 3.2. Changes in lactobcilli populations (a) cheeses with the free enzyme, (b) cheeses with the encapsulated enzyme. Results are presented as mean ± standard deviation. Probability level: $P < 0.05$. See Table 1 for identification of cheeses codes.

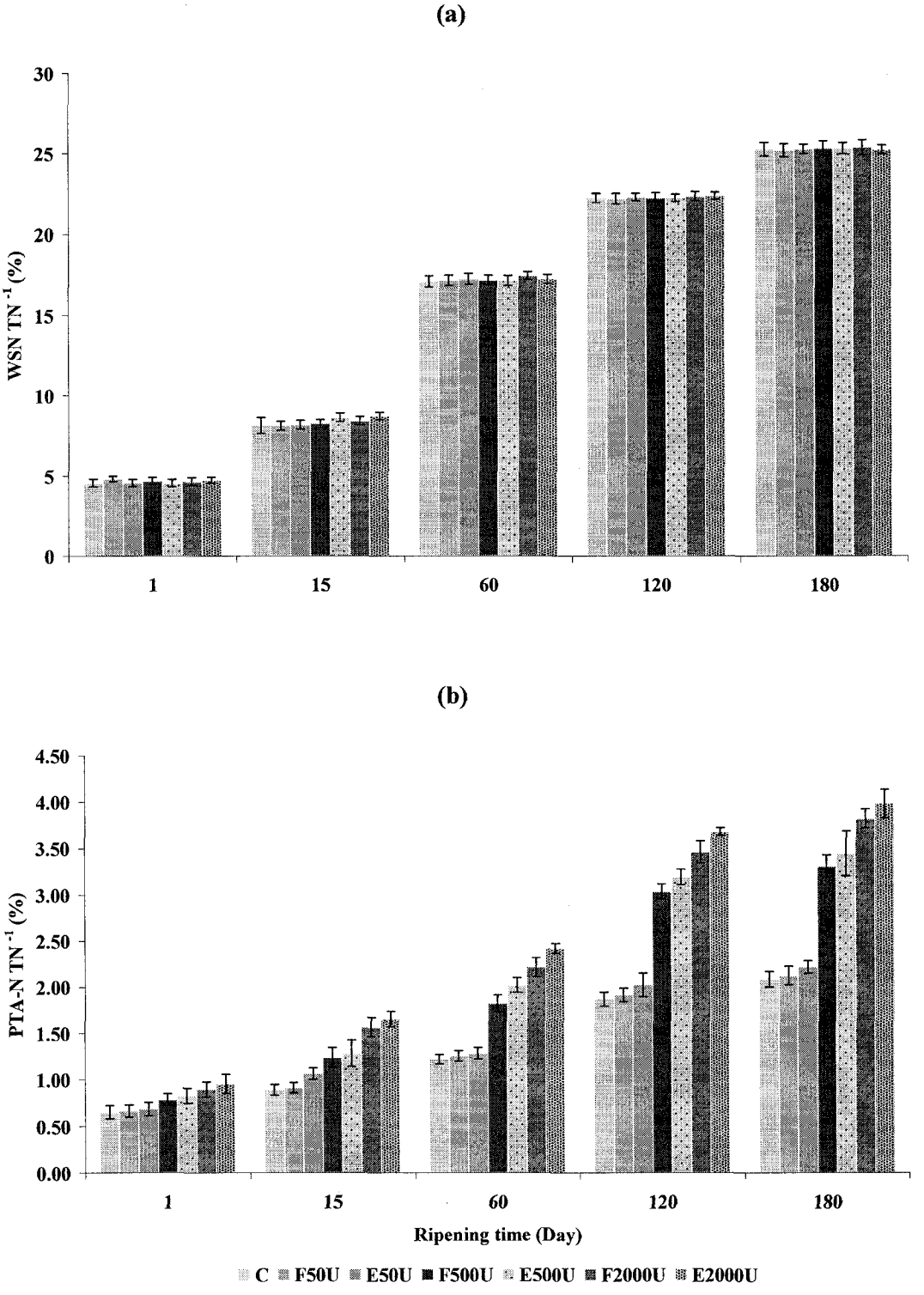


Figure 3.3. Changes in (a) WSN (expressed as a percentage of total nitrogen), (b) PTA-N (expressed as a percentage of total nitrogen) and (c) total FAA contents. Results are expressed as mean ± standard deviation. Probability level: $P < 0.05$ (WSN); $P < 0.01$ (PTA-N, total FAA). See Table 1 for identification of cheeses codes.

(c)

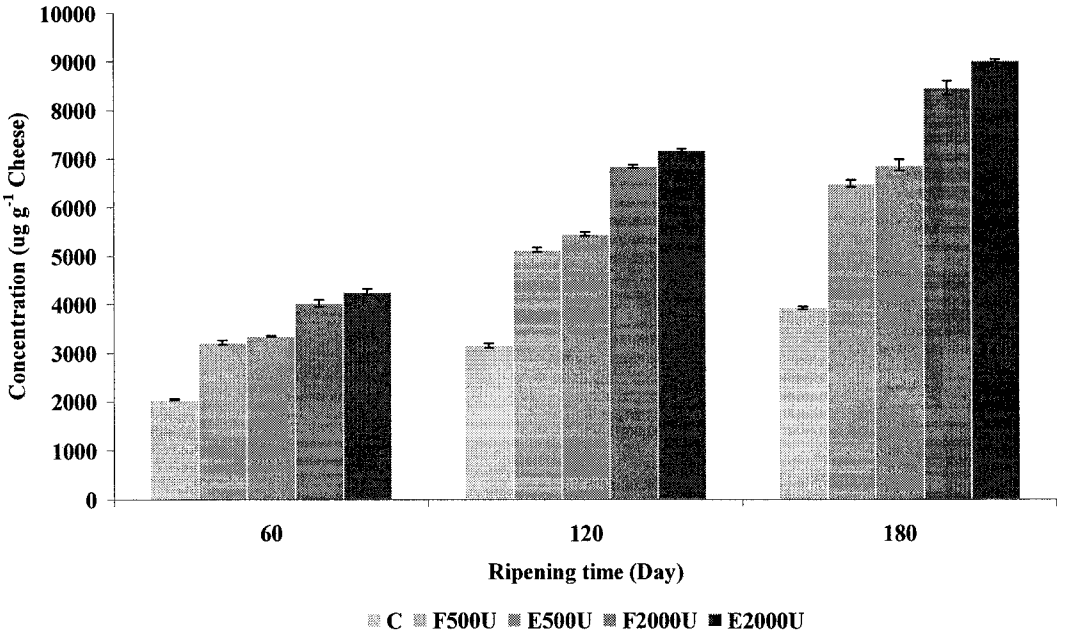
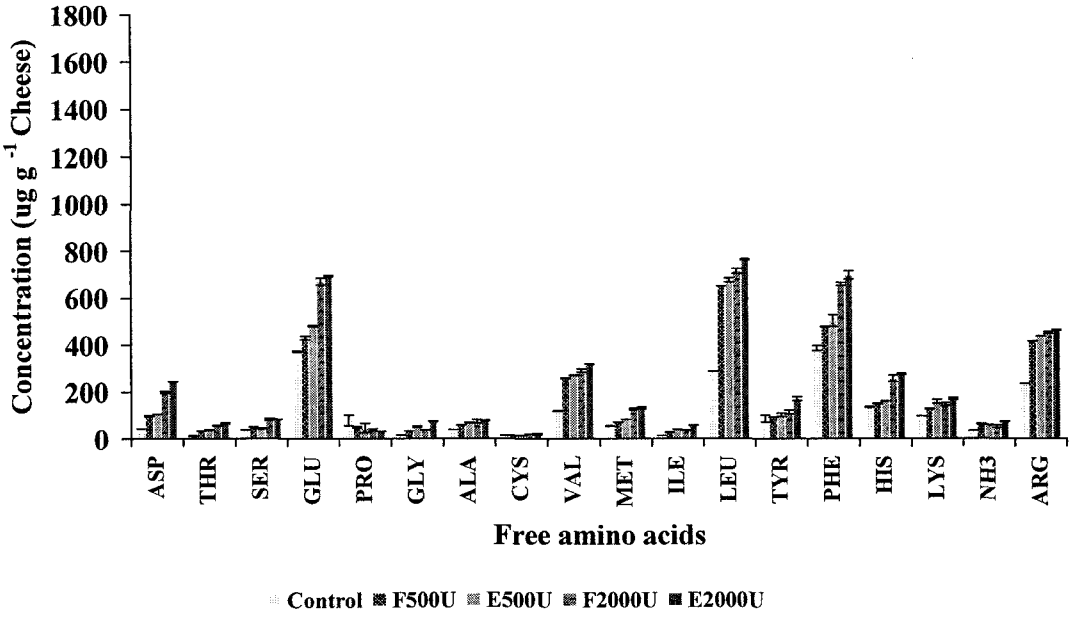


Figure 3.3. (Continued).

(a)



(b)

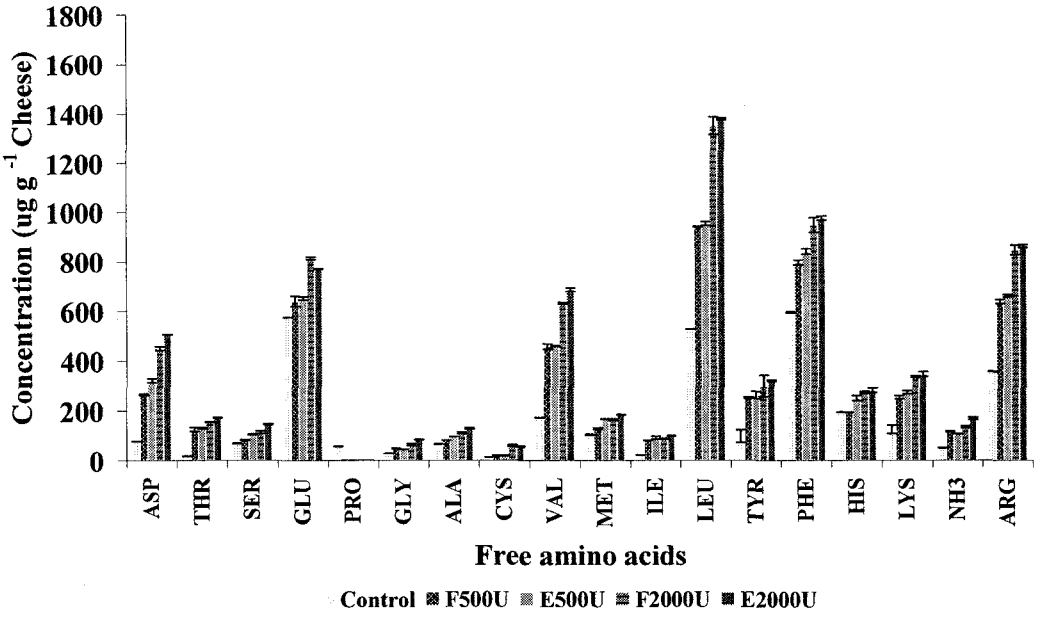


Figure 3.4. Concentration of individual free amino acids at (a) 60 days, (b) 120 days, (c) 180 days of ripening in control and experimental cheeses. Results are expressed as mean ± standard deviation. See Table 1 for identification of cheeses codes.

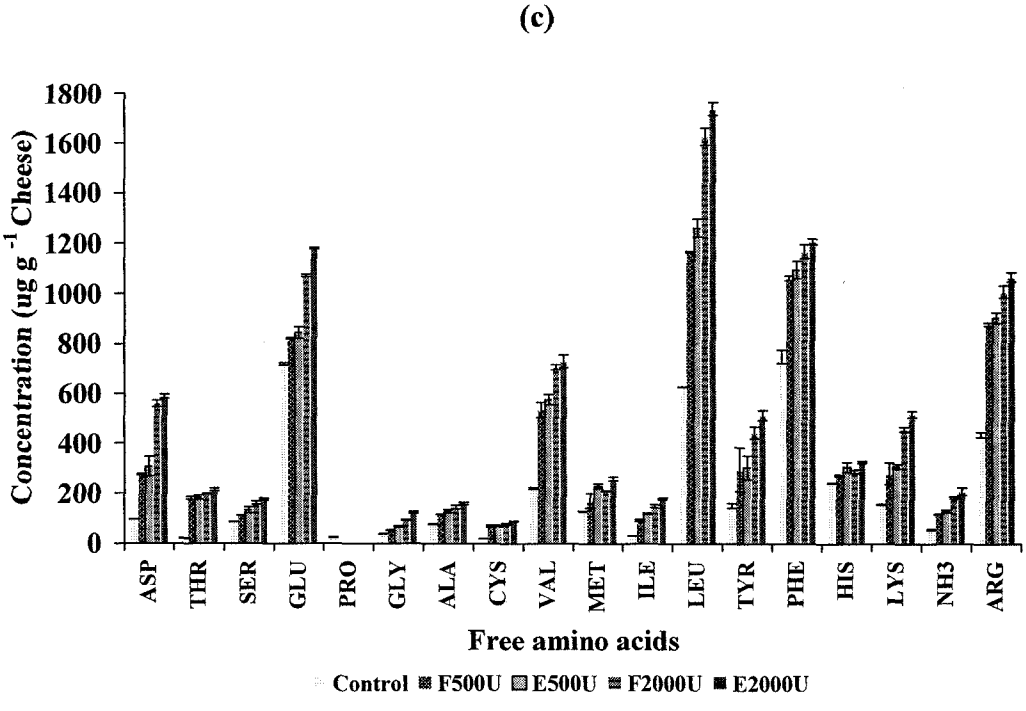
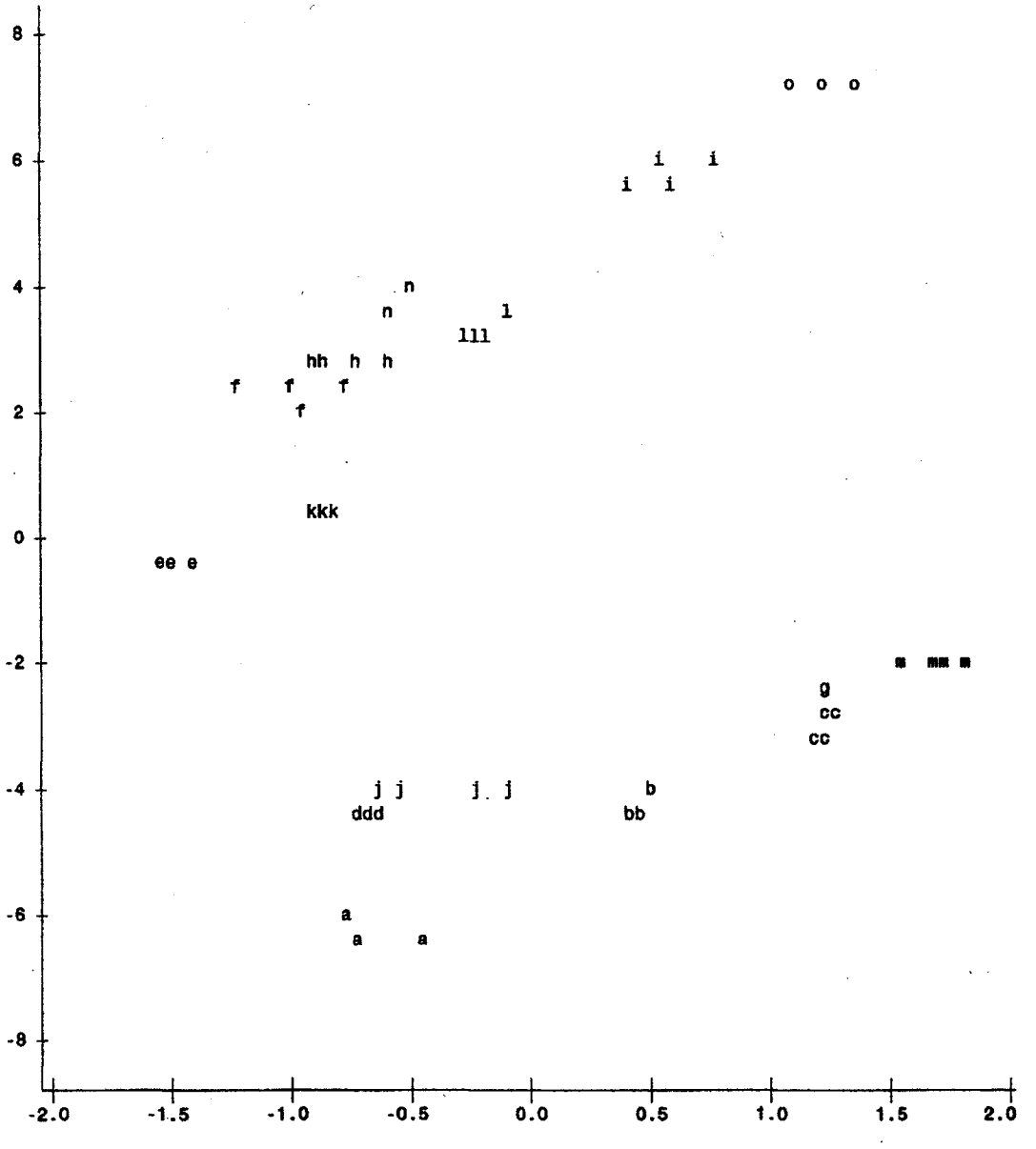


Figure 3.4. (Continued).

Principal component 1 (86.90%)



Principal component 2 (5.26%)

NOTE: 1 obs had missing values. 10 obs hidden.

Figure 3.5. PCA of the individual FAA in the control and experimental cheeses. a, b, c; d, e, f; g, h, i; j, k, l; m, n, o stand for the control cheese, F500U, F2000U, E500U, and E2000U ripened at 4°C after 2, 4, and 6 months, respectively. See Table 1 for identification of cheeses codes.

CHAPTER 4

Application of Free or Encapsulated Recombinant Aminopeptidase of *Lactobacillus rhamnosus* S93 in Acceleration of Cheddar Cheese Ripening

In this chapter, the effects of free or encapsulated form of recombinant aminopeptidase derived from *Lactobacillus rhamnosus* S93 on proteolysis of Cheddar cheese were investigated in larger scale of cheesemaking trial (200 L milk).

As reported in Chapter 3, small cracks were observed on the surface of the cheeses treated with the encapsulated enzyme. This defect was due to the handmade bead size (about 2 mm). To overcome this problem and to incorporate smaller and more uniform beads into the cheese matrix, an encapsulator equipped with a 300 μm nozzle was used for preparation of capsules.

The result of this research has been presented at *IFT 2007 Chicago*, IL, USA, and submitted as a manuscript to *Journal of Dairy Science*. The manuscript has been co-authored by Sorayya Azarnia, Byong H. Lee, Daniel St-Gelais, and Claude Champagne, and written by Sorayya Azarnia and edited by Dr. Byong H. Lee. Dr. Daniel St-Gelais and Dr. Claude Champagne provided advice, financial and technical supports.

4.1 ABSTRACT

The use of recombinant aminopeptidase (pepN) from *Lactobacillus rhamnosus* S93, in free or encapsulated form was investigated to shorten the duration of Cheddar cheese ripening. This enzyme was encapsulated in the alginate bead coated with chitosan using an extrusion method. After cheeses were made in vats containing 200 L milk, the free or encapsulated enzymes were added into the curd at the renneting or salting stage, respectively. Proteolysis was determined by measuring water soluble nitrogen, the nitrogen soluble in phosphotungstic acid (PTA-N) and free amino acids (FAA) over a 6-month period. Almost all of the encapsulated enzymes were entrapped into the cheese matrix, indicating the stability of the beads during the manufacturing stages. When the cheeses were graded by panelists, the experimental cheeses produced the superior-sensory characteristics than those of the control. The level of water soluble nitrogen was increased in all cheeses with storage time, but no significant differences in this fraction were observed between the control and experimental cheeses at different times. In contrast, the amounts of the PTA-N and the total FAA were significantly higher in the experimental cheeses than those of the control. The cheese treated with the encapsulated enzyme had significantly higher mean levels of PTA-N and total FAAs than the other cheeses. The use of encapsulated enzymes resulted in an acceleration of about 4 months in ripening compared to that of the control cheese.

4.2 INTRODUCTION

The textural changes and flavor enhancement which occur during the ripening of Cheddar cheese are part of a complex process driven by three basic reactions: proteolysis, lipolysis and glycolysis. Amongst them, proteolysis is the main biochemical flavor-generating process leading to formation of small peptides and amino acids in Cheddar cheese (Sousa *et al.*, 2001). Amino acids catabolism by intracellular lactic acid bacterial peptidases is responsible for aroma formation in Cheddar cheese (Sousa *et al.*, 2001). However, using an adjunct live or shocked cell of *Lactobacillus casei* strains for this purpose caused some defects in cheese (Lee *et*

al., 2004). Over-expressed enzymes of these strains naturally contain a high level of intracellular proteinase, aminopeptidase and esterase. Lee and Robert (1997) over-expressed an aminopeptidase (pepN) from *Lactobacillus rhamnosus* S93 in *Esherichia coli* (*E. coli*). This enzyme could have application in the acceleration of Cheddar cheese ripening (Lee *et al.*, 2007).

As cheese ripening is an expensive, slow and lengthy process, reduction of this time has economic and industrial benefits for the cheese industry. Amongst the different methods used to speed up cheese maturation, addition of enzymes into milk seems to be the simplest and cheapest one. The disadvantages of direct addition of enzymes to the milk are: non-uniform distribution of enzymes in the curd, early proteolysis and texture defects, and a loss of about 90% of enzymes in the whey during cheesemaking (Kailasapathy and Lam, 2005). Encapsulation technology could be an alternative to this method and it has been used in the dairy industry to control and improve cheese flavor (Kailasapathy and Lam, 2005).

Gums, milkfat and phospholipids have been used as encapsulating materials. Liposomes or artificial lipid membrane vesicles have been used to accelerate Cheddar cheese ripening (Kailasapathy and Lam, 2005). Enzymes such as Flavorzyme, neutral bacterial protease, acid fungal protease, and Palatase (Kheadr *et al.*, 2003) were encapsulated in liposomes and added cheese milk to develop Cheddar cheese flavor. However, this technology is expensive to be used commercially for cheese ripening purpose (Azarnia *et al.*, 2006a).

Another alternative for this purpose is using natural polysaccharides or food gums. In an attempt to accelerate cheese ripening, Kailasapathy and Lam (2005) investigated gellan, κ-carrageenan, and a high melting point milkfat fraction as materials for enzyme encapsulation. Their results showed that proteolysis rates were greater in all the cheeses treated with encapsulated enzyme, compared to the control cheese. However, enzyme-containing gum capsules showed a greater retention rate in the cheese matrix than did enzyme-containing fat capsules (Kailasapathy and Lam, 2005).

Na-alginate is an anionic polymer has the capacity to bind divalent cations such as calcium, resulting in the formation of a three dimensional gel network (Roberts,

1992). This polymer has been used to immobilize enzymes such as glucose oxidase (Khani *et al.*, 2006), and Flavorzyme (Kailasapathy *et al.*, 2006). Moreover, alginate-pectin capsules were used for the fortification of Cheddar cheese with folic acid (Madziva *et al.*, 2006).

The problem with this polysaccharide is its large pore size resulting in enzyme release during the operation (Tanaka *et al.*, 1984). Although this phenomenon does not affect diffusion of biomolecules within a molecular weight of 20 kDa, higher molecular weight substances such as albumin (69 kDa) are released from alginate beads (Tanaka *et al.*, 1984). However, this problem could be reduced by formation of a polyelectrolyte complex between alginate as an anionic polymer and chitosan as a cationic polymer (Roberts, 1992; Huguet *et al.*, 1996).

Alginate has been used as an immobilization matrix for biomolecules and microorganisms (Champagne *et al.*, 1992; Kailasapathy *et al.*, 2006; Vårum and Smidsrød, 2006) but no data are found on the encapsulation of lactobacilli peptidases in alginate-chitosan particles and its application to accelerate cheese ripening. In this study, the use of recombinant aminopeptidase (pepN) from *Lactobacillus rhamnosus* S93, in free or encapsulated form, was investigated to shorten the duration of Cheddar cheese ripening.

4.3 MATERIALS AND METHODS

4.3.1 Materials, Strain and Growth of *E. coli* clones

Chemicals and media were used in this part were described in previous chapters, Sections 2.3.1 and 3.3.1.

4.3.2 Purification of Recombinant Enzyme and SDS-PAGE

The procedures were used for the fractionation of the crude cell-free extract, and purification and determination of the molecular size of the enzyme were described in Section 2.3.2.

4.3.3 Enzyme and Protein Assays

The enzyme activity and protein concentration were determined according to the procedure described in Section 2.3.3.

4.3.4 Preparation of Chitosan-Coated Alginate Beads

The method used for preparation of alginate or chitosan-coated alginate beads in Section 3.3.4 was modified in this part of study as follows: the mixture of the purified enzyme and Na-alginate solution (1.6 %) at a ratio of 1:3 was extruded through a 300μm nozzle of an encapsulator (Inotech Labor, IE-50 R, Eulerstrasse, Switzerland) into a cold (4°C) gelling solution containing $CaCl_2$ (0.1M) or chitosan (0.1%)-$CaCl_2$ (0.1M) at a ratio of 1:3. After hardening, being filtered and washed, the encapsulation efficiency was determined as described in Section 2.3.4.

The free or encapsulated enzymes were frozen according to the procedure described in Section 2.3.6. All analyses were carried out in triplicates.

4.3.5 Cheddar Cheese Manufacture

Control and experimental cheeses were made in triplicate at the pilot plant of Agriculture and Agri-Food Canada, Food R & D Centre (St-Hyacinthe, PQ, Canada) by an experienced cheese master in vats containing 200 L milk according to the protocol listed in Table 4.1. Cheeses were made under the same conditions described in Section 3.3.5. All analyses were carried out in duplicate.

4.3.6 Estimation of the Encapsulated Enzyme Entrapment in the Curd

The rate of encapsulated enzyme inco rporation into the curd was indirectly estimated by measuring the aminopeptidase activity in the whey after drainage and pressing steps of the cheese manufacturing. Then, the aminopeptidase activity in the whey of the cheese with encapsulated enzyme was compared with that of the control cheese.

4.3.7 Compositional Analysis

The total nitrogen, moisture, fat and ash contents and pH-values of the cheeses were determined as described in Section 3.3.6. Compositions of milk were determined using a Milkoscan FT 120 (Section 3.3.6). All analyses were carried out in duplicate.

4.3.8 Analysis of Proteolytic Indices

The water soluble nitrogen (WSN), the total nitrogen soluble in 5% phosphotungstic acid (PTA-SN) and Individual free amino acids (FAA) were evaluated according to the methods used in Section 3.3.7. All determinations were made in duplicate.

4.3.9 Sensory Evaluation

The procedure was used for evaluation of the sensory properties of cheeses was described in Section 3.3.8.

4.3.10 Microbiological Analysis

The microbial changes in the cheeses during the ripening were evaluated according to the procedure described in Section 3.3.9.

4.3.11 Statistical Analysis

A split plot design with three replicates using a general linear model (GLM) procedure of Statistical Analysis System (SAS) was used to evaluate the data from the WSN, PTA-N, concentration of FAA, microbial changes; cheese and milk compositions and sensory properties as described in Section 3.3.10. PCA was carried out on the individual FAA data as described in Section 3.3.10.

4.4 RESULTS AND DISCUSSION

4.4.1 Enzyme Preparation and Purification

The recombinant pepN was purified 11.6-fold compared to the crude extract and its estimated molecular weight was 90 kDa as described in Section 2.4.1.

4.4.2 Enzyme Encapsulation

The encapsulation efficiency obtained in this part of the study was increased from 10% to 90% in capsules formed in gelling solution containing Chitosan-$CaCl_2$. Chitosan, as a hydrophilic polymer has amino groups which are highly electronegative and can become positively charged by absorbing a proton (Vårum and Smidsrød, 2006). Therefore, electrostatic interaction between the negatively charged carboxylic acid groups of alginate and the positively charged amino groups of chitosan results in formation of a polyelectrolyte complex between the two polymers. This phenomenon reduces gel porosity and limits the release of the encapsulated enzyme, therefore, resulting in greater *EE*. This result is also consistent with our results reported in Chapters 2 and 3 of this thesis.

The incorporation rate of the encapsulated enzyme into the cheese matrix was estimated by measuring enzyme activity in the whey after the drainage and the pressing steps. No aminopeptidase activity was observed in the whey obtained from the cheese with encapsulated enzyme or the control cheese. Almost all encapsulated enzymes were entrapped into the cheese matrix indicating the stability of the capsules during Cheddar cheese manufacturing.

4.4.3 Compositional Analysis

Milk compositions for production of the control and experimental cheeses are summarized in Table 4.2.

The compositions of the cheeses at 15 days of ripening time are presented in Table 4.3. There were no significant differences ($P > 0.05$) among the cheeses for the moisture, salt, salt-in moisture, fat-in dry matter, ash, protein, and pH values.

4.4.4 Microbiological Analysis

The changes in the mean populations (from the three trials) of lactococci, and lactobacilli in the cheeses during ripening time of 6 months at 4°C are demonstrated in Figure 4.1. Lactococcal populations decreased significantly ($P < 0.05$) during storage time in all cheeses. The mean population of starter lactic acid bacteria declined 1.86, 1.83, 1.85 log cycles after 6 months of ripening in control, cheeses

treated with free or encapsulated enzymes, respectively (Figure 4.1). The results showed that the free or encapsulated enzymes did not affect the cell counts. No pathogens, yeasts and molds were found in all cheeses in this study, which means that the cheeses were prepared and stored in a good condition. A progressive reduction of lactococcal populations during Cheddar cheese aging was also reported by other authors (Dabour *et al.*, 2006; di Cagno *et al.*, 2006, Ong *et al.*, 2006).

Low pH, low temperature, high concentration of salt and lack of lactose result in the reduction of the starter's cell count during the ripening period. This caused, therefore, an increase in the intracellular enzymes particularly peptidases of lactococci into the cheese matrix which is important in flavor generation in the cheese during maturation (Law, 2001; Lortal and Chapot-Chartier, 2005; Kenny *et al.*, 2006).

Conversely, lactobacilli grew rapidly in all cheeses during the ripening period and their population increased from about 0.5 $\log_{10}$ cfu/g of cheese to 6 $\log_{10}$ cfu/g of cheese after 6 months of maturation (Figure 4.1). These results are also in agreement with the results reported in Chapter 3 of this thesis.

4.4.5 Proteolysis Analysis

The extent of proteolysis in control and experimental cheeses was evaluated by measuring the amount of WSN, PTA-N and total FAA.

The results obtained from the assessment of WSN which was expressed as a percentage of total nitrogen is shown in Figure 4.2. The ANOVA analysis showed that the level of this nitrogen fraction as primary proteolysis was increased significantly ($P < 0.05$) in all cheeses with storage time. However, no differences ($P > 0.05$) were observed between the control and experimental cheeses in this fraction at each stage of ripening (Figure 4.2).

Continued increasing the WSN during ripening time can be resulted from degradation of casein to low molecular weight of water soluble peptides and amino acids by milk enzymes, residual coagulant, starter lactic acid bacteria (LAB) and NSLAB (Sousa *et al.*, 2001; Dabour *et al.*, 2006). Cheddar cheese flavor appeared to be located in this heterogeneous fraction (Christensen *et al.*, 1991).

The other proteolytic indices, i.e. PTA-N and total FAA were also increased in all cheeses during the ripening time (Figure 4.2). However, the amounts of these secondary proteolytic indices were significantly ($P < 0.01$) higher in the experimental cheeses than those of the control cheese at each sampling time (Figure 4.2), indicating the acceleration of the ripening duration. According to Duncan's test, there were significant differences at the level of 5% between the mean levels of the PTA-N and total FAA among the cheeses. As presented in Table 4.4, cheese with the encapsulated enzyme showed a significantly higher mean level of PTA-N and total FAA compared to those of the other cheeses over the 6 month ripening period. These results confirm the results obtained in our previous study reported in Chapter 3, and also are in agreement with the others in using adjuncts starter culture to speed up Cheddar cheese maturation (Swearingen *et al.*, 2001; Ong *et al.*, 2006).

Figure 4.2 compares the concentration of individual FAA in the control and the experimental cheeses at 1 day, and 2, 4 and 6 months of the ripening time. The FAA profiles for the three treatments were similar. The concentration of almost all individual amino acids in all cheeses increased with the ripening time. This is in agreement with the trends found for the PTA-N (Figure 4.2). However, the levels of all individual amino acids were higher in the experimental cheeses at all sampling times, except for proline which was higher in the control cheese than that of experimental cheeses at 2 months and disappeared in experimental cheeses at 4 months. The major FAAs in all cheeses during the ripening time were Leu, Glu, Phe, Val, Arg, Tyr, Lys, and Met (Figure 4.3). As reported in Chapter 3, leucine was the most abundant amino acid in the experimental cheeses in this part of the study as well (Figure 4.3). These are attributed to the property of the recombinant aminopeptidase used in this study which has the high activities for Leu-, Lys-, Ala-, Val-, and Met-peptides and has also preference for dipeptides containing Leu as the N-terminal residue (Habibi-Najafi and Lee, 1994). Aminopeptidase N or C (pepN or C) releases Lys, Leu, Arg, Met, or Phe from the N-terminal position and has been shown to be effective in reducing levels of bitter peptides in cheese (Habibi-Najafi and Lee, 1994; Swearingen *et al.*, 2001).

These results are also in agreement with findings of other workers such as Puchades *et al.* (1989), Wallace and Fox, (1997), and de Wit *et al.* (2005) who found that Leu, Phe, and Glu were the most abundant in Cheddar cheese. Glu, Met, Leu, Lys and Val were higher in the Cheddar cheeses with added lactobacilli as an adjunct culture than in control cheese (Gardiner *et al.*, 1998). A large quantity of Leu has also been reported in lactobacilli-added Cheddar cheeses (Puchades *et al.*, 1989).

The FAA assessment can be used as an indicator of proteolysis activity during ripening (Hannon *et al.*, 2005). Amino acid catabolism is responsible for aroma formation in Cheddar cheese. This phenomenon leads to the formation of important sulfur compounds, and also degradation of all amino acids by a transamination reaction results in formation of α-keto acids which in turn are degraded to various aroma compounds (Yvon and Rijnen, 2001). It is reported that the release of Phe, Tyr, Leu, Ileu, Val and Met was coincident with good cheese flavor (Hannon *et al.*, 2005).

The PCA was carried out on the raw data of the individual FAA to obtain the best possible analysis of these compounds. It was carried out using the PRINCOMP procedure of the SAS software. Table 4.5 presents the first 10 eigenvalues of the covariance matrix, the difference between successive eigenvalues, the proportion of the covariance explained by each eigenvalue, and the cumulative proportion of the explained variance. A PCA chart (Figure 4.4) demonstrates that PC1 and PC2 accounted for a cumulative variation of 93.39%. This result is consistent with our previous study in Chapter 3.

4.4.6 Sensory Evaluation

The sensory evaluation data were obtained at 120 days of ripening time with presenting the samples with different codes and in random order to the testers. The ANOVA analysis showed differences ($P < 0.05$) for the three attributes, texture, flavor and aroma among the cheeses. According to Duncan's test, experimental cheeses received significantly higher mean scores for the sensory properties than the control cheese (Table 4.4). The testers did not recognize any defect due to the presence of alginate beads in the samples.

4.5 CONCLUSIONS

This study presents a method to enhance the proteolysis and sensory properties of Cheddar cheese using the recombinant amino peptidase of *L. rhamnsous* S93. Cheese with the encapsulated enzyme had significantly higher mean levels of PTA-N and total FAAs than the other cheeses. The use of encapsulated enzyme resulted in an acceleration of 70% in ripening and superior sensory properties than the control cheese. Efficient incorporation of enzymes in milk before cheddaring can be achieved using this technique. Although this study was aimed to develop an encapsulation technique which can affect Cheddar cheese ripening, it would be also useful for other type of cheeses.

Table 4.1. Protocol for Cheddar cheese manufacturing in three trials.

Cheese Code	C	F	E
Treatments	Control	Cheese with free enzymes	Cheese with encapsulated enzymes
Enzyme units 18 L^{-1}milk	0.00	2000	2000
Enzyme addition stage	Non applicable	Salting	Renneting

Table 4.2. Compositions of milk used for the making of experimental cheeses.

Composition	C	F	E
Fat (%)	3.85±0.02	3.87±0.03	3.83±0.03
Protein (%)	3.31± 0.04	3.31±0.04	3.31±0.04
Lactose (%)	4.41± 0.04	4.42±0.05	4.43±0.03
Total solid (%)	12.56±0.07	12.60±0.08	12.56±0.07
pH	6.64±0.01	6.63±0.03	6.67±0.01

Results are expressed as mean ± standard deviation.
C: control cheese; F: cheese with free enzyme; E: Cheese with encapsulated enzyme.

Table 4.3. Composition of control and experimental chesses (at 15 days of ripening).

Composition	C	F	E
Salt (%)	1.51±0.03	1.50±0.03	1.50±0.02
Moisture (%)	38.76±0.27	38.73±.029	39.02±0.22
S/M[1] (%)	3.89±0.06	3.87±0.04	3.84±0.06
F/DM[2] (%)	51.26±1.27	51.28±1.27	51.39±1.15
Protein (%)	23.73±0.09	23.75±0.08	23.48±0.10
Ash (%)	3.21±0.10	3.23±0.08	3.19±0.07
pH	5.05±0.06	5.07±0.07	5.04±0.05

[1]Salt in Moisture.
[2]Fat in Dry Matter.
Results are expressed as mean ± standard deviation.
C: control cheese; F: cheese with free enzyme; E: Cheese with encapsulated enzyme.

Table 4.4. Duncan's multiple range test for responses.

Parameters		E	F	C	Parameters			E	F	C
PTA/TN (%)	Duncan grouping	A	B	C	Sensory attributes	Texture	Duncan grouping	A	A	B
	Mean	2.130	1.926	1.303			Mean	0.704	0.704	0.185
TFAA(ug g^{-1} cheese)	Duncan grouping	A	B	C		Flavor	Duncan grouping	A	A	B
	Mean	5193	4710	2635			Mean	0.704	0.593	0.074
						Aroma	Duncan grouping	A	A	B
							Mean	0.815	0.741	0.222

Means within a row with the different letter are significantly different ($P < 0.05$).
C: control cheese; F: cheese with free enzyme; E: Cheese with encapsulated enzyme.

Table 4.5. Eigenvalues of the correlation matrix calculated from PRINCOMP Procedure.

Principal Component	Eigenvalue	Difference	Proportion	Cumulative
1	15.8153346	14.8205908	0.8786	0.8786
2	0.9947437	0.5043750	0.0553	0.9339
3	0.4903687	0.1855368	0.0272	0.9611
4	0.3048319	0.2261766	0.0169	0.9781
5	0.0786553	0.0130202	0.0044	0.9824
6	0.0656351	0.0107372	0.0036	0.9861
7	0.0548980	0.0098695	0.0030	0.9891
8	0.0450284	0.0054212	0.0025	0.9916
9	0.0396073	0.0081641	0.0022	0.9938
10	0.0314432	0.0096792	0.0017	0.9956

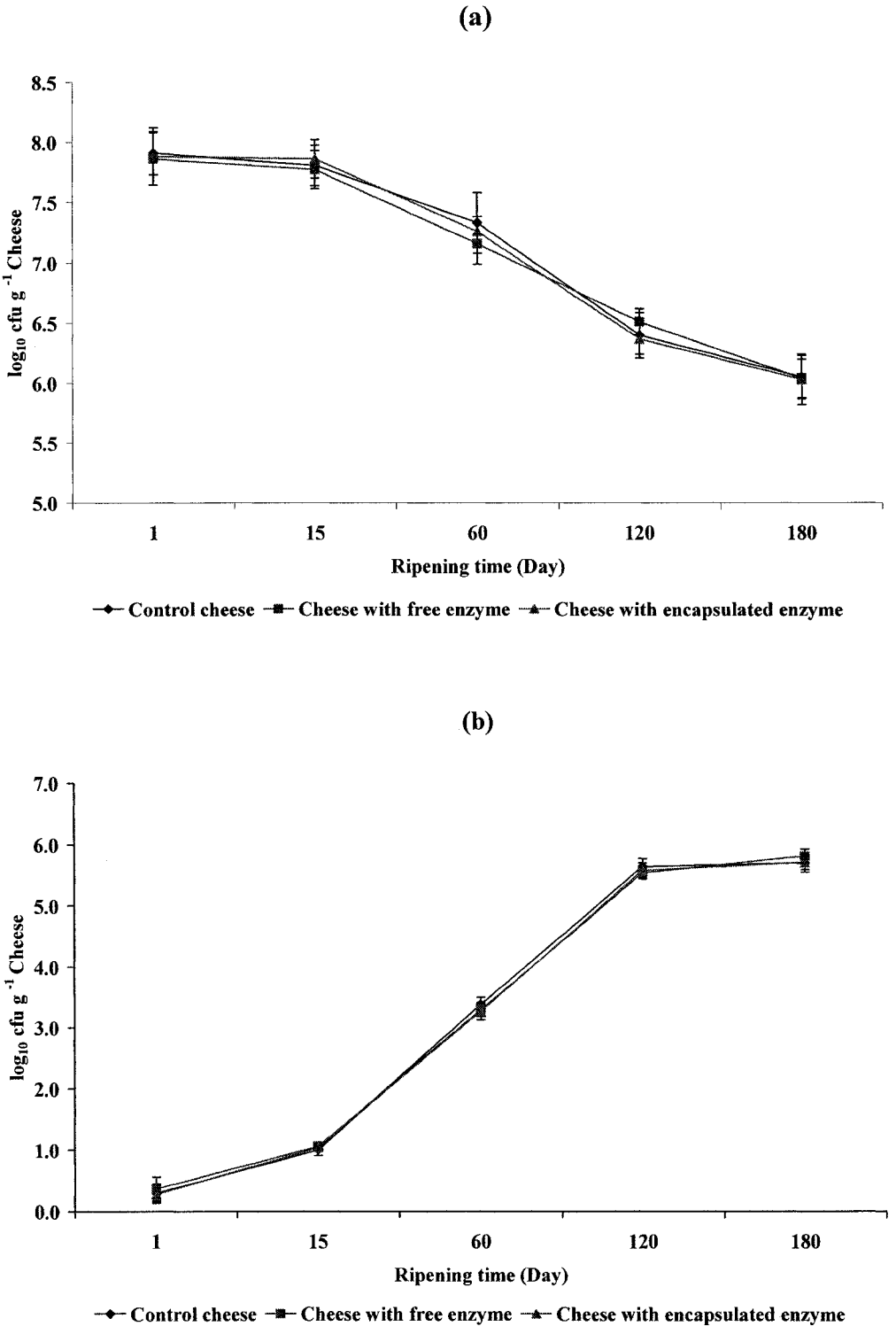


Figure 4.1. Changes in microbial counts (a) lactococci, (b) lactobacilli during ripening of the cheeses. Results are presented as mean ± standard deviation. Probability level: $P < 0.05$.

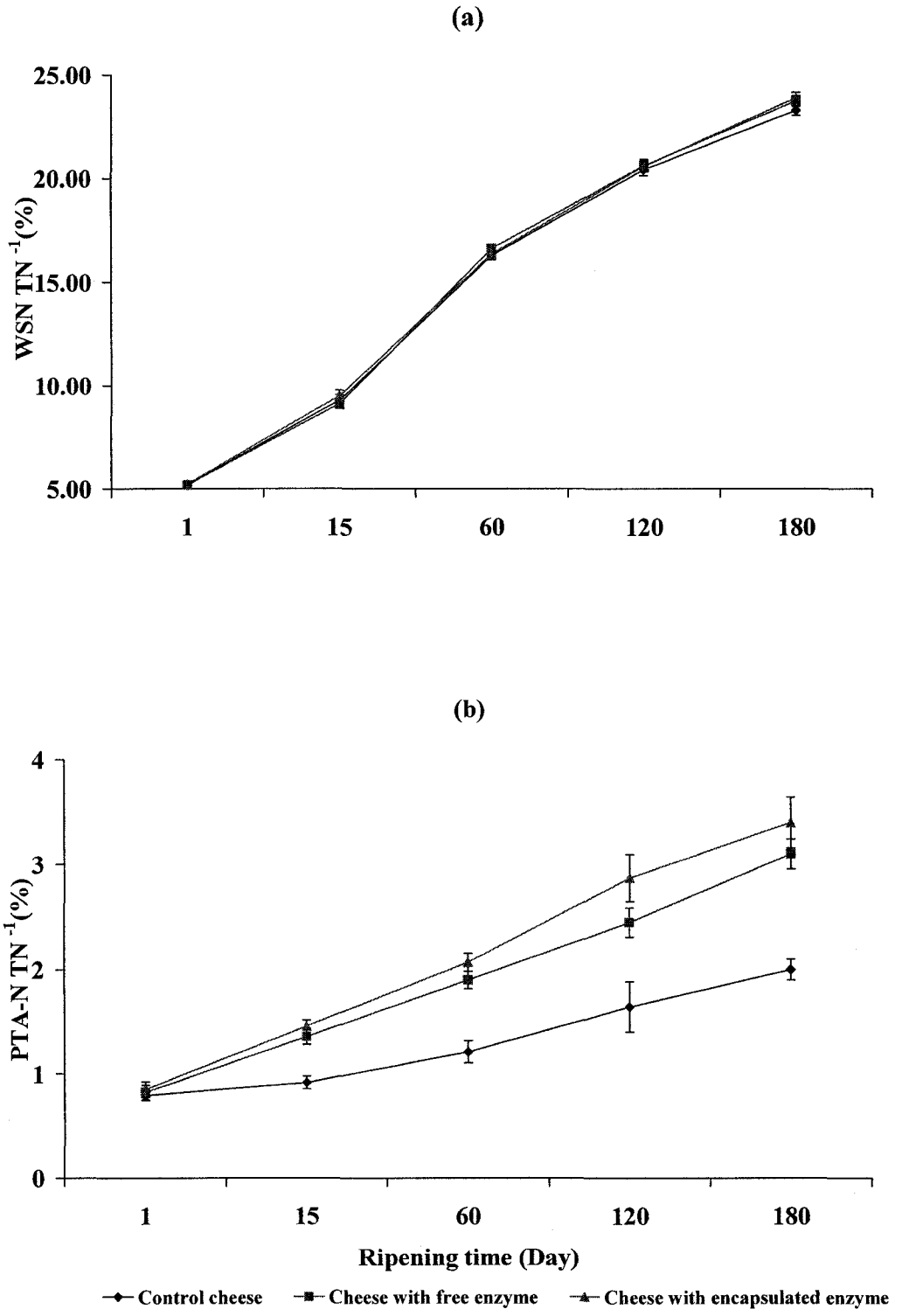


Figure 4.2. Changes in (a) WSN (expressed as a percentage of total nitrogen), (b) PTA-N (expressed as a percentage of total nitrogen) and (c) total FAA contents. Results are expressed as mean ± standard deviation. Probability level: $P < 0.05$ (WSN); $P < 0.01$ (PTA-N, total FAA).

(c)

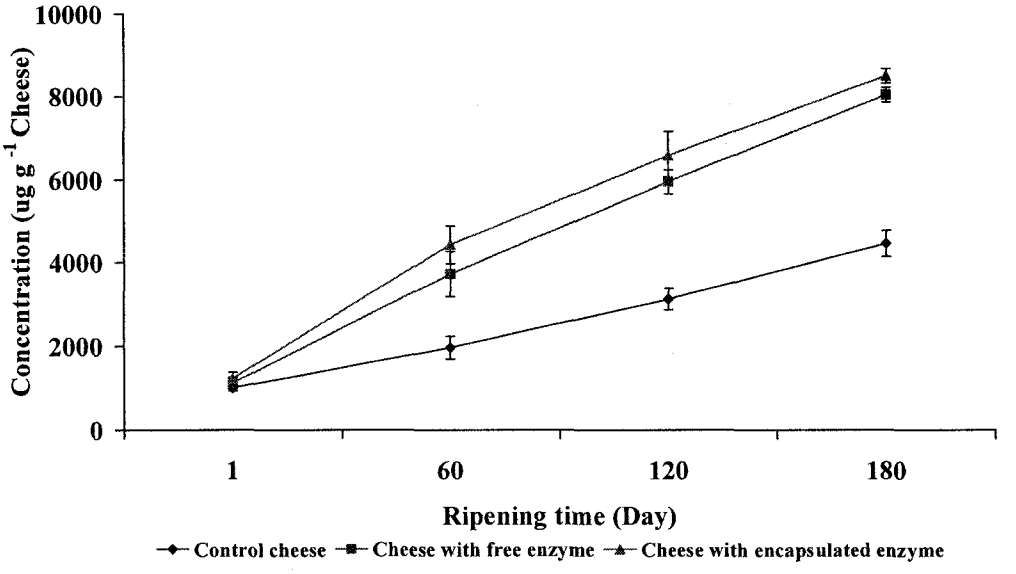


Figure 4.2. (Continued).

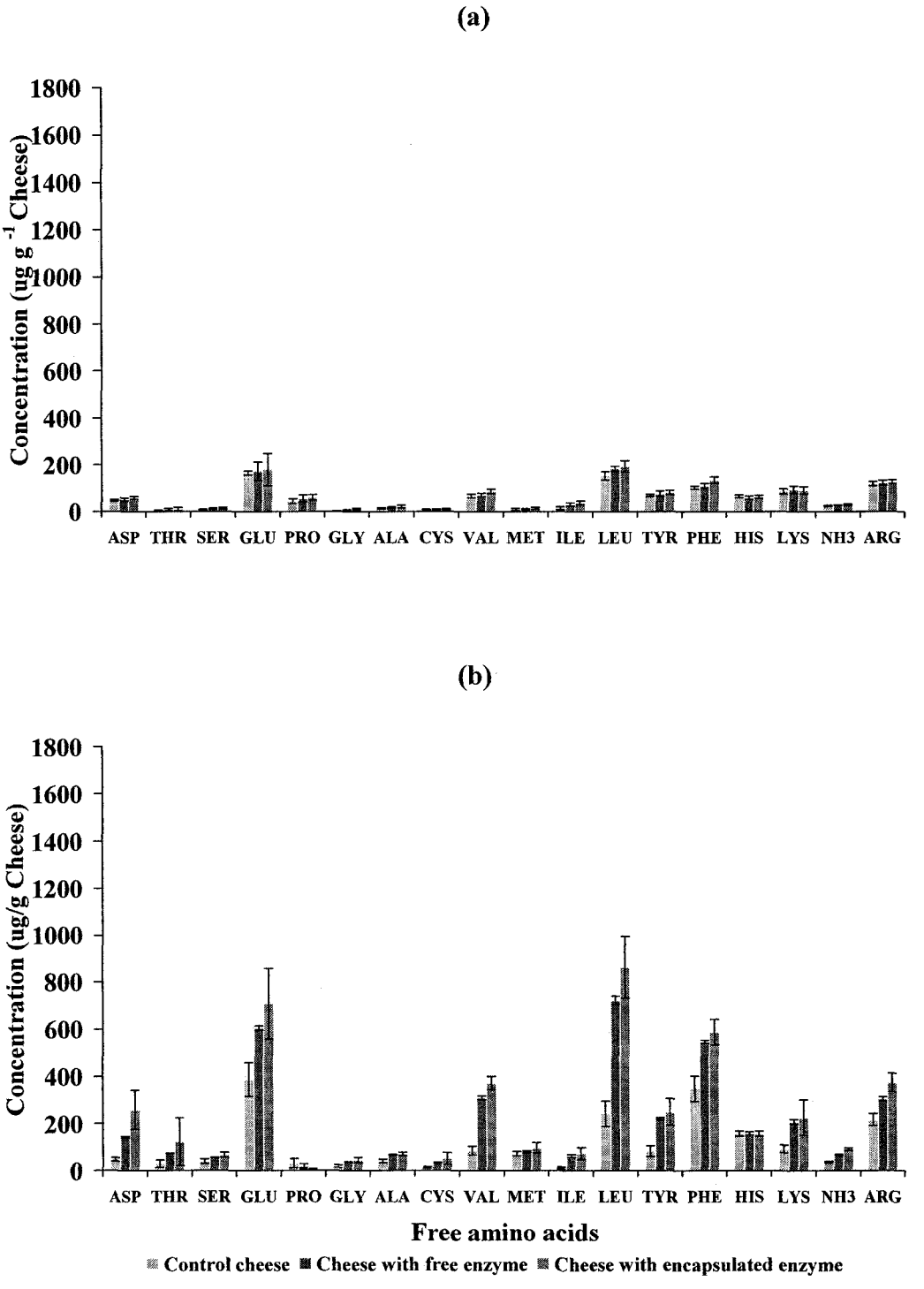
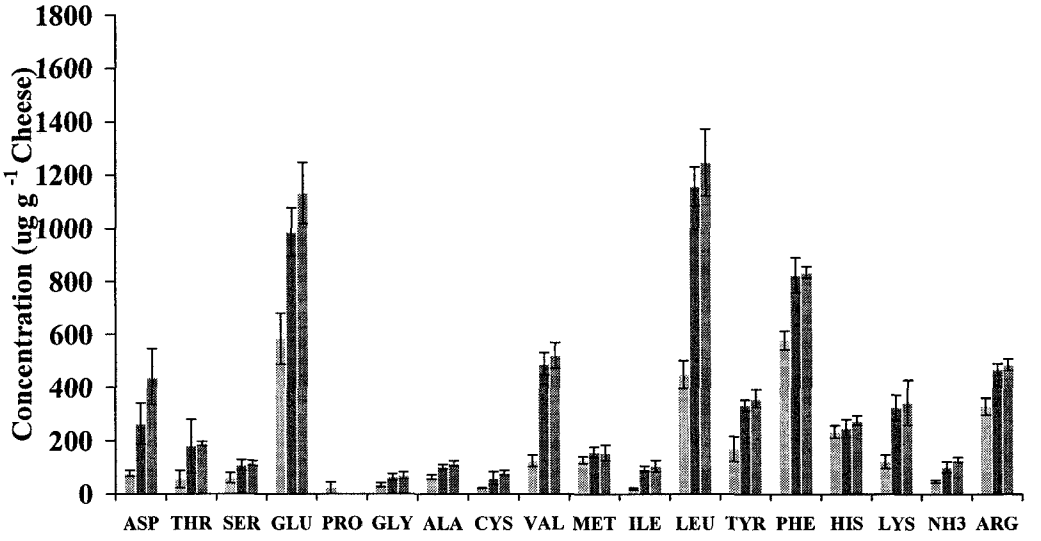


Figure 4.3. Concentration of individual FAA at (a) 1d, (b) 2 months, (c) 4 months and (d) 6 months of ripening in control and experimental cheeses. Results are expressed as mean ± standard deviation.

(c)



(d)

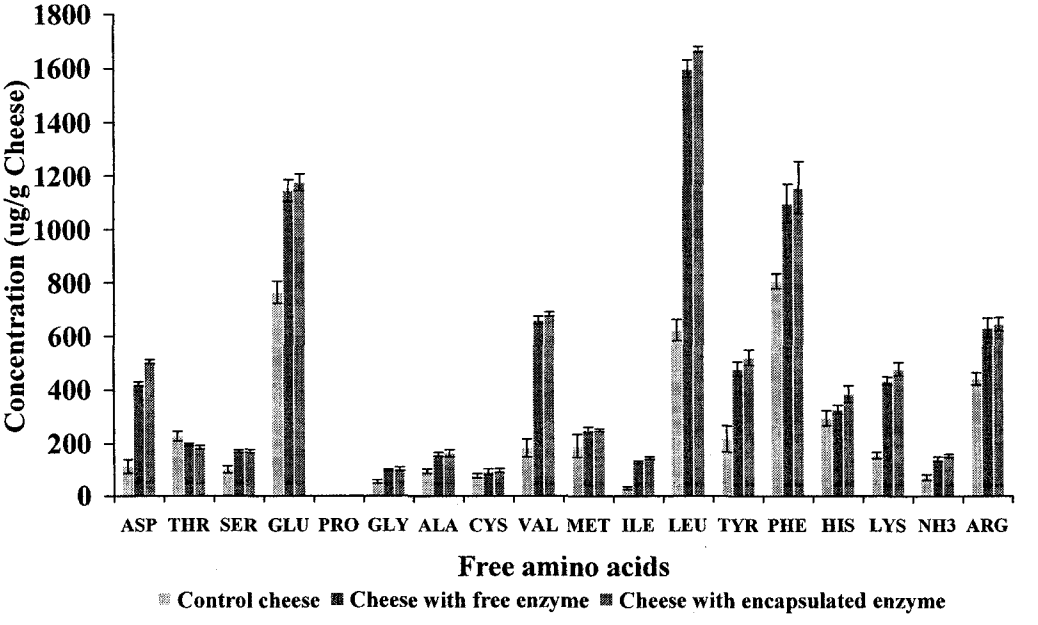
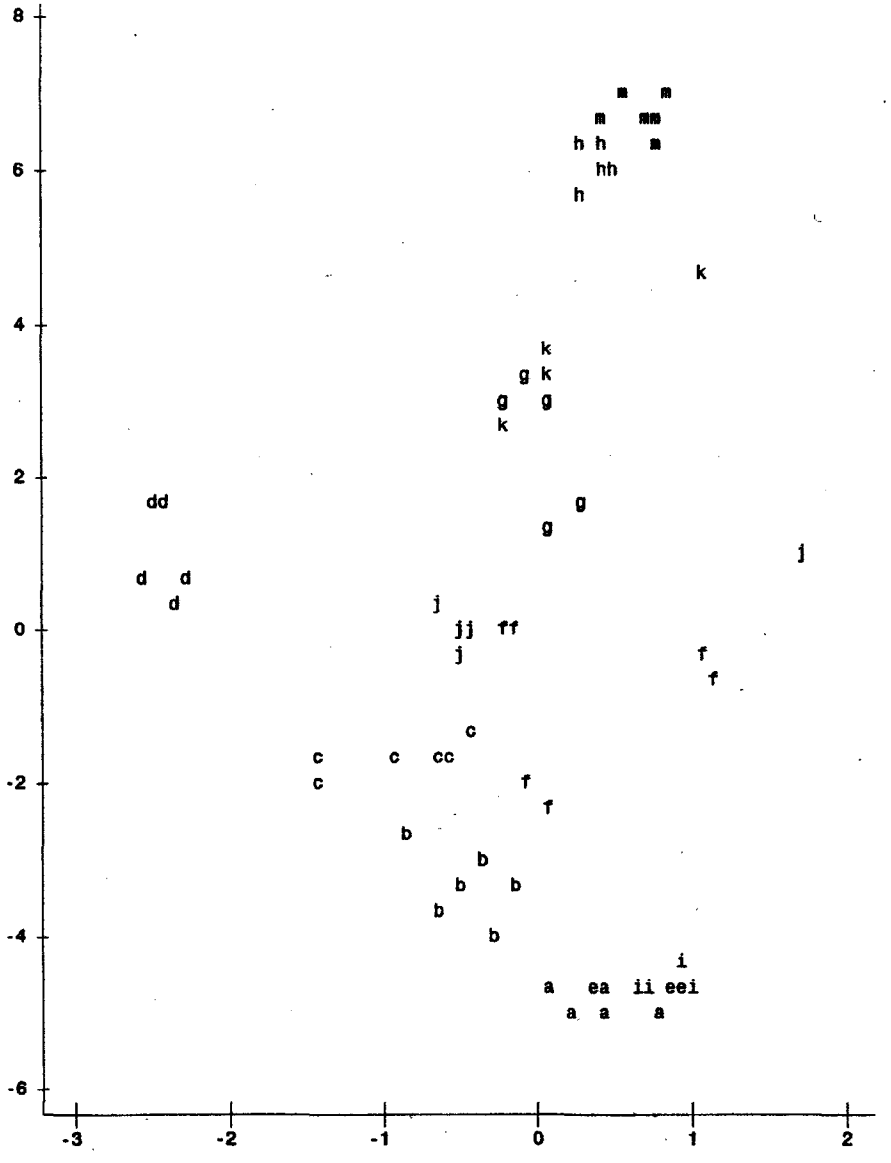


Figure 4.3. (Continued).

Principal Component 1 (87.86%)



Principal Component 2 (5.53%)

NOTE: 7 obs hidden.

Figure 4.4. PCA of the individual FAA in the control and experimental cheeses. a, b, c, d; e, f, g, h; I, j, k, m stand for the control cheese, cheese with the free enzyme and cheese with the immobilized enzyme ripened at 4°C after 1 d, 2, 4, and 6 months, respectively.

CHAPTER 5

Enhancement of Cheddar-EMC Proteolysis Using Natural and Recombinant Enzymes of *Lactobacillus rhamnosus* S93

Enzyme modified cheeses (EMC) are produced by enzyme addition to the cheese after manufacture or maturation and made into a paste by the inclusion of water and emulsifier under shearing. The industrial demand for EMCs has been increased due to its use as a cheese flavor ingredient in processed foods and in low-fat and non-fat products. Production of EMC is the most economic way to produce an intense natural Cheddar cheese flavor

In this chapter, the applications of both natural and recombinant enzymes derived from *Lb. rhamnosus* S93 individually or in combination, with or without a commercial proteinase (Neutrase, Novozymes) were investigated to produce Cheddar-EMC flavorings.

The result of this research has been presented at *2006 CIFST / AAFC Joint Conference*, Montreal, Canada, and submitted as a manuscript to *International Dairy Journal.* The manuscript has been co-authored by Sorayya Azarnia, Byong H. Lee, Varoujan Yaylayan and Kieran Kilcawley, and written by Sorayya Azarnia and edited by Dr. Byong H. Lee. Dr. Varoujan Yaylayan provided scientific advice, and Dr. Kieran Kilcawley provided technical support and editorial assistance in the final stage of paper submission.

5.1 ABSTRACT

The use of crude cellular extract (natural enzymes) and recombinant aminopeptidase derived from *Lactobacillus rhamnosus* S93 was investigated in the production of Cheddar-enzyme modified cheese flavorings. Cheese slurries were produced and pre-incubated with or without a commercial proteinase (Neutrase, Novozymes). After which different units (0-50 units per 200g curd) of the natural or recombinant enzymes were added separately or in combination, and incubated anaerobically at 37°C for 1, 3 and 6 days. Water soluble nitrogen levels, nitrogen soluble in phosphotungstic acid and free amino acids were significantly higher in the Neutrase-added enzyme modified cheeses. Highest levels of free amino acids were evident in samples containing the highest levels of natural enzymes with recombinant aminopeptidase. Proline disappeared in all enzyme modified cheeses containing the recombinant peptidase or natural enzymes alone or in combination over maturation times.

5.2 INTRODUCTION

Enzyme modified cheeses (EMC) are produced by enzyme addition to the cheese after manufacture or maturation and made into a paste by the inclusion of water and emulsifier under shearing (Moskowitz and Noelck, 1987). They are incubated for a number of days at 30-45°C. The industrial demand for EMCs has increased due to its use as a cheese flavor ingredient in processed foods and in low-fat and non-fat products. Their flavor intensity is about 15-30 times that of natural cheese (Kilcawley *et al.*, 2001).

Lactic acid bacterial enzymes are desirable for cheese flavors and ripening (Lee *et al*, 2007). However, using live or shocked cells of *Lactobacillus casei* strains for this purpose can cause some defects in cheese (Lee *et al.*, 2004), leading to the use of over-expressed enzymes of these strains. *Lactobacillus casei* species naturally express a high level of intracellular proteinase, aminopeptidase and esterase and the combined activity of these enzymes results in the production of cheese flavor compounds (Lee *et al*, 2007).

An aminopeptidase (pepN) has been over-expressed from *Lactobacillus rhamnosus* S93 in *Esherichia coli* (*E. coli*) (Lee and Robert, 1997) and may have applications in the production of enzyme modified cheeses (Lee *et al.*, 2007).

The objectives of this study were to investigate the use of both a crude cellular extract (natural enzymes) and a recombinant aminopeptidase derived from *Lactobacillus rhamnosus* S93 individually or in combination, with or without a commercial proteinase (Neutrase, Novozymes) as proteolytic agents, in the production of Cheddar-EMC flavorings.

5.3 MATERIALS AND METHODS

5.3.1 Chemicals and Preparation of Natural Enzyme of *Lactobacillus rhamnosus*

All chemicals and media were purchased from Sigma (Sigma-Aldrich, Inc., St-Louis, MO, USA) and Difco (Difco Laboratories, Detroit, MI, USA), respectively, unless otherwise mentioned. Lyophilized *Lactobacillus rhamnosus* S93 was obtained from the Agriculture and Agri-Food Canada, Food R & D Centre (St-Hyacinthe, PQ, Canada). This strain was revived by two consecutive inoculations and was grown in MRS broth for 16 hours at 37°C. The cells were harvested by centrifugation (Beckman Coulter Canada, Inc., Mississauga, ON, Canada) at 4000g (4°C, 15 min) and washed twice with ice-cold phosphate buffer (0.05 M, pH 7.5). The harvested cells were disintegrated by homogenization (Emulsiflex-5C, Avestin Homogenizer, Ottawa, ON, Canada). The pressure was increased gradually up to 10,000g. The crude enzyme extract was obtained by centrifugation (10,000g, 30 min, 4 °C) of the disintegrated cell suspension, precipitated by ammonium sulphate ,75 %, (Arora and Lee, 1994), and passed through a Sephadex G-25 column (PD-10, Pharmacia Biotech LKB, Montreal, PQ, Canada).

5.3.2 Preparation of Recombinant Aminopeptidase of *Lactobacillus rhamnosus*

The recombinant aminopeptidase of *Lactobacillus rhamnosus* S93 was prepared according to the procedure described in Section 2.3.1.

5.3.3 Purification of Recombinant Enzyme and SDS-PAGE

The procedure for purification and molecular size determination of the recombinant aminopeptidase were described in Section 2.3.2.

5.3.4 Enzyme and Protein Assays

The enzyme activity and protein concentration were determined as described in Section 2.3.3.

5.3.5 Preparation of Enzyme-Modified Cheese

The method of Park *et al.* (1995) was modified as follows: All utensils were autoclaved at 121°C for 15 min. Grain fresh Cheddar cheese was provided by Agropur (Granby, PQ, Canada). Shredded fresh Cheddar cheese (5 kg) was mixed gently with a sterile solution of Na_2HPO_4 (125 g) dissolved in distilled water (1,625 mL). The mixture was heated at 85°C for 30 min and then cooled down to 50°C. As demonstrated in Table 5.1, the cheese slurries were prepared in two groups, Neutrase-added slurries and non-Neutrase added ones. Neutrase® 0.8L (*Bacillus subtilis* proteinase, Novozymes North America Inc., Franklinton, NC, USA) was diluted (1:10) with potassium phosphate buffer (0.05M, pH 7.5), filtered with a 0.22 μm membrane filter (Millipore, Carrigtwohill, Co. Cork, Ireland). A sample (3.9 mL) of the filtrate was added to the slurry at 45°C and then mixed gently. The samples were packed under vacuum condition and kept at 45°C for 24 hours. Different units (0-50 units per 200g curd) of natural enzyme and recombinant pepN were added alone or in combination to the treated slurries, and incubated anaerobically at 37°C for 1, 3 and 6 days. The control and the experimental EMCs were prepared in duplicate according to the protocol listed in Table 5.1. Sampling was carried out at 1, 3, and 6 days of incubation at 37°C for the physicochemical analyses. All analyses were carried out in duplicate.

5.3.6 Physicochemical Analyses

Moisture, salt, ash, fat and total nitrogen contents were determined as described in Section 3.3.6. The Protein amount was expressed on the basis of dry weight. The

water soluble nitrogen (WSN), the total nitrogen soluble in 5% phosphotungstic acid (PTA-N) and free amino acids (FAA) were determined according to the procedures described in Section 3.3.7. All determinations were made in duplicate.

5.3.7 Statistical Analysis

A split plot design with two replicates using a general linear model (GLM) procedure of the SAS software was used to evaluate the data from the WSN, PTA-N, concentration of FAA, compositions of fresh Cheddar cheese and Cheddar-EMCs as described in Section 3.3.10. PCA was carried out on the individual FAA data as described in Section 3.3.10.

5.4 RESULTS AND DISCUSSION

5.4.1 Enzyme Preparation and Purification

The recombinant pepN was purified about 12-fold over the crude extract with a recovery of about 29 percent and its molecular weight estimated by SDS-PAGE was 90 kDa as described in Section 2.4.1.

5.4.2 Compositional Analysis

Table 5.2 shows the composition of grain fresh Cheddar cheese used for preparation of the control and experimental EMCs. The compositions of the slurries at manufacturing day are presented in Table 5.3. No significant differences ($P > 0.05$) were observed among the slurries in compositions. These slurries were used for production of EMCs which were prepared according to the protocol described in Table 5.1. The percentage of ash was higher in slurries compared to that of the fresh Cheddar cheese resulting from addition of the dibasic sodium phosphate solution as an emulsifying salt. The similar result has been reported in commercial Cheddar EMCs (Kilcawley *et al.*, 2000).

5.4.3 Proteolysis Analysis

In this study, the slurries were emulsified by adding the sodium phosphate solution and then heated at 85°C for 30 min to inactivate their indigenous enzymes

(Park *et al.*, 1995; Lee *et al.*, 2007). Neutrase was added to the emulsified and heated shredded fresh Cheddar cheese, followed by incubating at 45°C to partly mimic the action of rennet which is involved in the breakdown of κ-casein into peptides (Cliffe and Law, 1990; Park *et al.*, 1995). Addition of Neutrase induced the accumulation of bitter peptides, primarily through its action towards β-casein, but it is known that peptidases from the starter or non-starter lactic acid bacteria can reduce bitterness (Cliffe and Law, 1990; Park *et al.*, 1995).

The extent of proteolysis was evaluated by measuring the amount of WSN, PTA-N and FAA in the control and natural or recombinant enzymes-added EMCs. Figure 5.1 demonstrates the results obtained from the evaluation of WSN. According to ANOVA, the level of this nitrogen fraction increased significantly ($P < 0.01$) in all Neutrase-added EMCs (Group a) over maturation. However, no differences ($P > 0.05$) were observed among individual EMCs at each ripening time (Figure 1).

The level of WSN increased significantly ($P < 0.01$) in non-Neutrase added EMCs (Group b) with maturation times as well but there was no significant difference ($P > 0.05$) among group b EMCs at each maturation time (Figure 5. 1). The results obtained in this study showed that the presence of Neutrase resulted in a significant ($P < 0.01$) increase in WSN compared to the samples containing the given enzymes without Neutrase (Figure 5.1).

The increase of WSN has also been reported during ripening time of Cheddar cheese which results from degradation of casein to low molecular weight of water soluble peptides and amino acids (Sousa *et al.*, 2001; Dabour *et al.*, 2006). Intracellular bacterial peptidases are responsible for degradation of small peptides to FAA (Sousa *et al.*, 2001). Amino acid catabolism is responsible for aroma formation in Cheddar cheese (Yvon and Rijnen, 2001).

The PTA-N also increased in all EMCs during the maturation times. The levels of this nitrogen fraction were higher in natural or recombinant enzymes-added EMCs listed in Table 1 compared to that of the control during ripening (Figure 5.2). The ANOVA showed significant differences for the ripening time ($P < 0.01$), treatments ($P < 0.01$) and the interaction between the treatment and the ripening time ($P < 0.01$).

Lack of Neutrase also affected the evolution of PTA soluble nitrogen in the samples. The amounts of this secondary proteolysis index were higher in the group a EMCs than in group b EMCs (Figure 5.2). According to ANOVA, significant ($P < 0.01$) differences were found between the two groups (a and b) of EMCs for the ripening time, treatments and the interaction between the treatment and the ripening time as well.

According to the result obtained from Duncan's test, there were significant differences at the level of 5% between the mean levels for the PTA-N of the EMCs in both groups. EMC10 and EMC20 received the highest mean level within each respective group; as opposed to the EMC1 and EMC11 which received the lowest mean level. In comparison between the EMCs of the two groups, EMC10 had the highest mean level of the PTA soluble nitrogen (Table 5.4).

The concentration of total FAA significantly increased in Neutrase-added EMCs with maturation time and with increasing the enzyme concentration (Figure 5.3). The levels of this index were higher in the Neutrase-added EMCs than those of the non-Neutrase added ones (Figure 5.5). With regard to Duncan's test, EMC10 had the highest mean level of the total FAA over maturation times (Table 5.4).

The concentration of individual free amino acids was evaluated in the control and natural or recombinant enzymes-added EMCs (Figures 5.4 and 5.6). The concentration of most individual amino acids in these EMCs increased over maturation at 37°C. However, proline was not observed in EMCs 5, 6, 7, 15, 16 and 17 containing recombinant pepN during incubation times (Figures 5.4 and 5.6). It also disappeared in EMC 3, EMC4, EMC9 and EMC10 containing higher enzymes concentrations at 3 days (Figures 5.4). The concentration of proline was lower in non-Neutrase added EMCs compared to Neutrase-added ones (Figure 5.6). The highest amount of proline was observed in EMC1. Arginine disappeared as maturation progressed in all natural or recombinant enzymes-added Cheddar-EMCs (Figure 5.4). A decrease in arginine was also reported in Cheddar cheese during ripening times by other workers (Puchades, 1989; Wallace and Fox, 1997). This reduction could be resulted from its conversion into biogenic amines (Laleye *et al.*, 1987). However, the amount of arginine was higher in group b EMCs than in group a EMCs (Figure 5.6).

Leucine was the most dominant amino acid in the experimental EMCs (Figures 5.4 and 5.6). The concentrations of glutamine, lysine, valine and phenylalanine were also higher compared to other FAA during ripening times (Figure 5.4 and 5.6).

Results obtained from the evaluation of proteolysis in this part of our study are in agreement with those of Cheddar cheese which reported in Chapters 3 and 4 of this thesis.

The PCA was carried out on the raw data of the individual FAA of Neutrase-added EMCs using the PRINCOMP procedure of the SAS software. A PCA chart (Figure 5.7) demonstrated that PC1 and PC2 accounted for a cumulative variation of 85.05%. PC1 accounted for 76% of the variation and separated the samples on the basis of the enzyme concentration. A direct relationship was observed between the levels of FFA and the enzyme concentration.

5.5 CONCLUSIONS

The results of this study highlight that proteolysis of Cheddar-EMCs can be enhanced by using natural and recombinant enzymes of *Lactobacillus rhamnosus* S93, but require the addition of a broad specificity proteinase, such as Neutrase to generate more peptides from caseins and to achieve very high levels of secondary proteolysis, as evident by PTA and FAA. Levels of each FAA increased over maturation in all EMCs except for proline and arginine which disappeared over maturation in the experimental EMCs.

Addition of exogenous enzymes genetically overproduced in *E. coli* can be useful in the preparation of enzyme-modified cheese to produce highly intense cheese flavors. However, further investigation is required to establish correlations between the proteolytic parameters and the sensory characteristics of Cheddar-EMCs.

Table 5.1. Protocol for Cheddar-EMC production.

Treatments	Codes	Enzyme units $200g^{-1}$ slurry
Group a: Neutrase-added slurries		
Control	EMC1	0
Natural enzymes from *Lb. rhamnosus* S93	EMC2	10
	EMC3	25
	EMC4	50
Recombinant pepN from *Lb. rhamnosus* S93	EMC5	10
	EMC6	25
	EMC7	50
A combination of natural enzymes and recombinant pepN	EMC8	10: 10
	EMC9	25: 25
	EMC10	50: 50
Group b: Non-Neutrase added slurries		
Control	EMC11	0
Natural enzymes from *Lb. rhamnosus* S93	EMC12	10
	EMC13	25
	EMC14	50
Recombinant pepN from *Lb. rhamnosus* S93	EMC15	10
	EMC16	25
	EMC17	50
A combination of natural enzymes and recombinant pepN	EMC18	10: 10
	EMC19	25: 25
	EMC20	50: 50

Table 5.2. Composition of grain fresh Cheddar cheese used for making of Cheddar EMCs.

Moisture (%)	Salt (%)	[1]S/M (%)	Fat (%)	[2]F/DM (%)	Protein (%)	[3]P/DM (%)	Ash (%)
42.06± 0.09	1.64±0.01	3.90±0.02	29.70±0.42	51.26±0.65	22.85±0.04	39.44±0.11	3.49±0.06

[1]Salt in Moisture.
[2]Fat in Dry Matter.
[3]Protein in Dry Matter.
Results are expressed as mean ± standard deviation.

Table 5.3. Compositions of the slurries (at manufacturing day) used for preparation of EMCs.

Compositions	Neutrase-added slurries	Non-Neutrase-added slurries
Moisture (%)	55.22±0.07	55.25±0.08
Salt (%)	1.28±0.01	1.29±0.01
[1]S/M (%)	2.31±0.01	2.33±0.01
Fat (%)	23.35±0.48	23.42±0.40
[2]F/DM (%)	52.14±1.15	52.33±0.96
Protein (%)	16.15±0.09	16.19±0.07
[3]P/DM (%)	36.06±0.18	36.17±0.13
Ash (%)	4.26±0.02	4.27±0.02

[1]Salt in Moisture.
[2]Fat in Dry Matter.
[3]Protein in Dry Matter.
Results are expressed as mean ± standard deviation.

Table 5.4. Duncan's multiple range test for responses.

Parameters		EMC1	EMC2	EMC3	EMC4	EMC5	EMC6	EMC7	EMC8	EMC9	EMC10
PTA/TN(%)	Duncan grouping	J	H	E	B	I	F	D	G	C	A
	Mean	3.782	12.617	15.853	20.593	10.430	14.322	17.875	13.258	19.745	25.068
TFAA (ug g^{-1} Cheese)	Duncan grouping	J	H	E	B	I	F	D	G	C	A
	Mean	6589	9841	14655	20225	8797	12985	17396	12132	19225	26602
		EMC11	EMC12	EMC13	EMC14	EMC15	EMC16	EMC17	EMC18	EMC19	EMC20
PTA/TN(%)	Duncan grouping	J	H	E	B	I	F	D	G	C	A
	Mean	2.039	3.551	4.884	6.566	3.135	4.665	5.262	4.107	5.616	8.025
TFAA (ug g^{-1} Cheese)	Duncan grouping	J	H	E	B	I	F	D	G	C	A
	Mean	1167	1568	2490	3401	1381	2308	2812	2132	3192	3883

Means within a row with the different letter are significantly different ($P < 0.05$).
See Table 1 for identification of EMCs codes.

Table 5.5. Eigenvalues of the correlation matrix calculated from PRINCOMP procedure.

Principal Component	Eigenvalue	Difference	Proportion	Cumulative
1	13.6792411	12.0494685	0.7600	0.7600
2	1.6297725	0.7229280	0.0905	0.8505
3	0.9068446	0.1397161	0.0504	0.9009
4	0.7671284	0.4098884	0.0426	0.9435
5	0.3572400	0.1687306	0.0198	0.9633
6	0.1885095	0.0914066	0.0105	0.9738
7	0.0971029	0.0062849	0.0054	0.9792
8	0.0908180	0.0088022	0.0050	0.9843
9	0.0820158	0.0075857	0.0046	0.9888
10	0.0744301	0.0361454	0.0041	0.9930

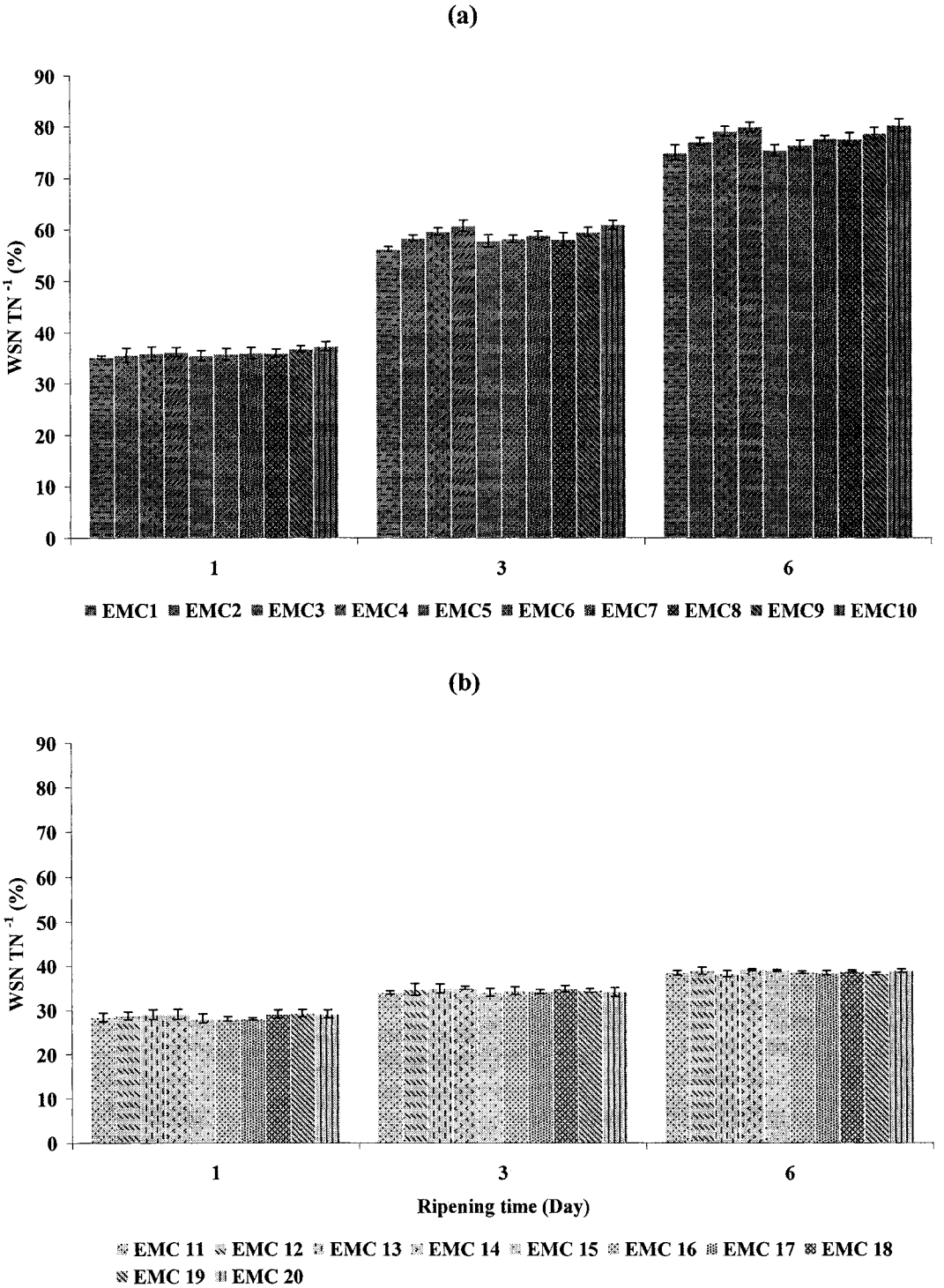
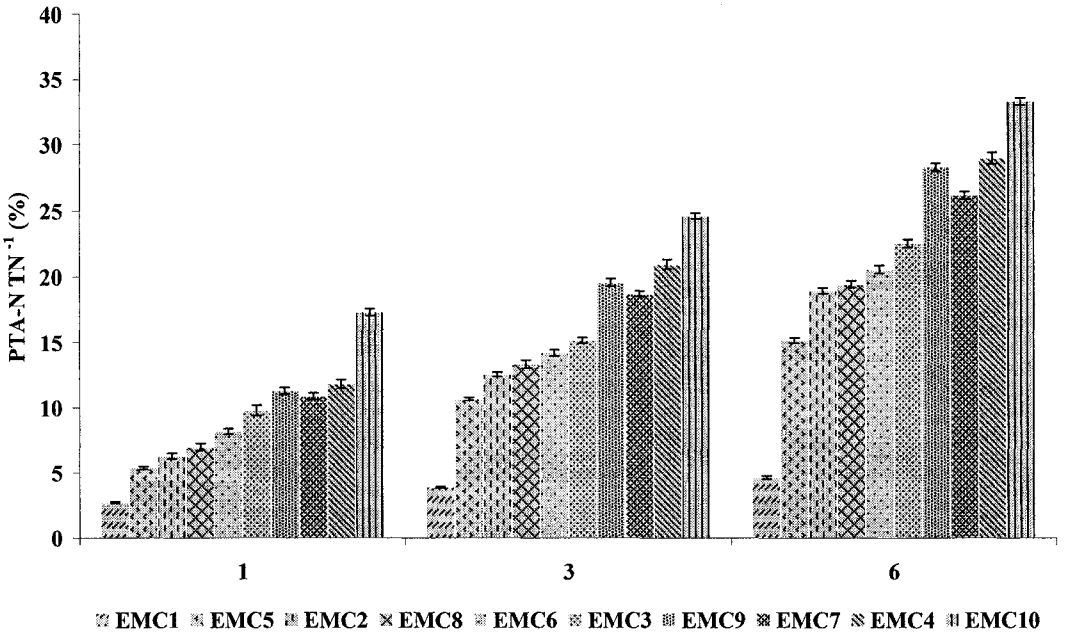


Figure 5.1. Changes in water soluble nitrogen (expressed as a percentage of total nitrogen) during maturation times, (a) Neutrase-added EMCs, and (b) non- Neutrase-added EMCs. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes.

(a)



(b)

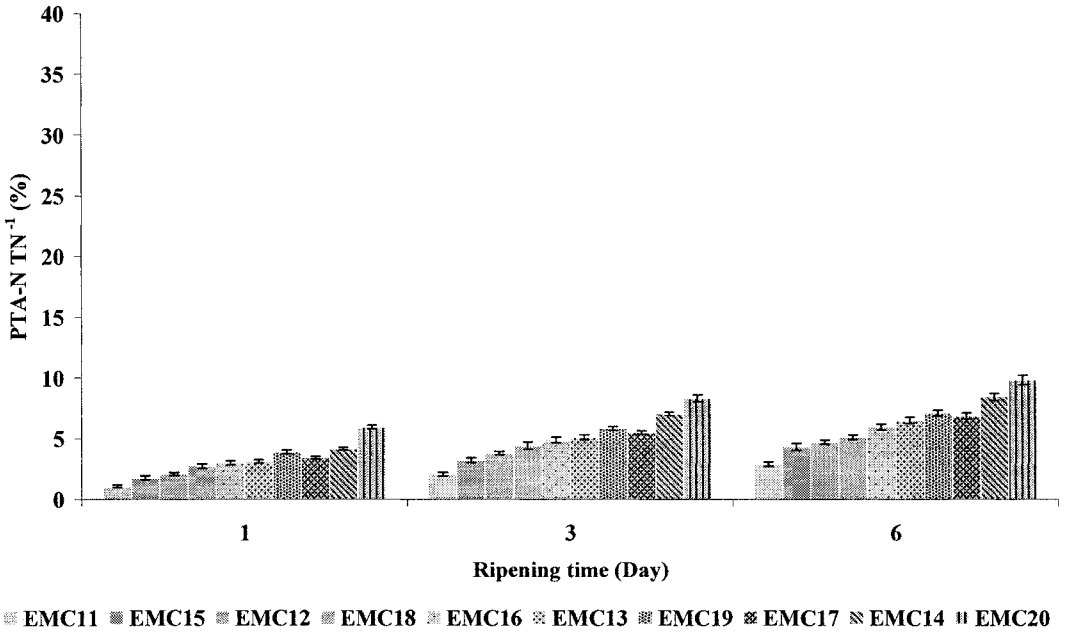


Figure 5.2. Changes in nitrogen soluble in phosphotungstic acid (expressed as a percentage of total nitrogen) during maturation times, (a) Neutrase-added EMCs, and (b) non- Neutrase-added EMCs. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes.

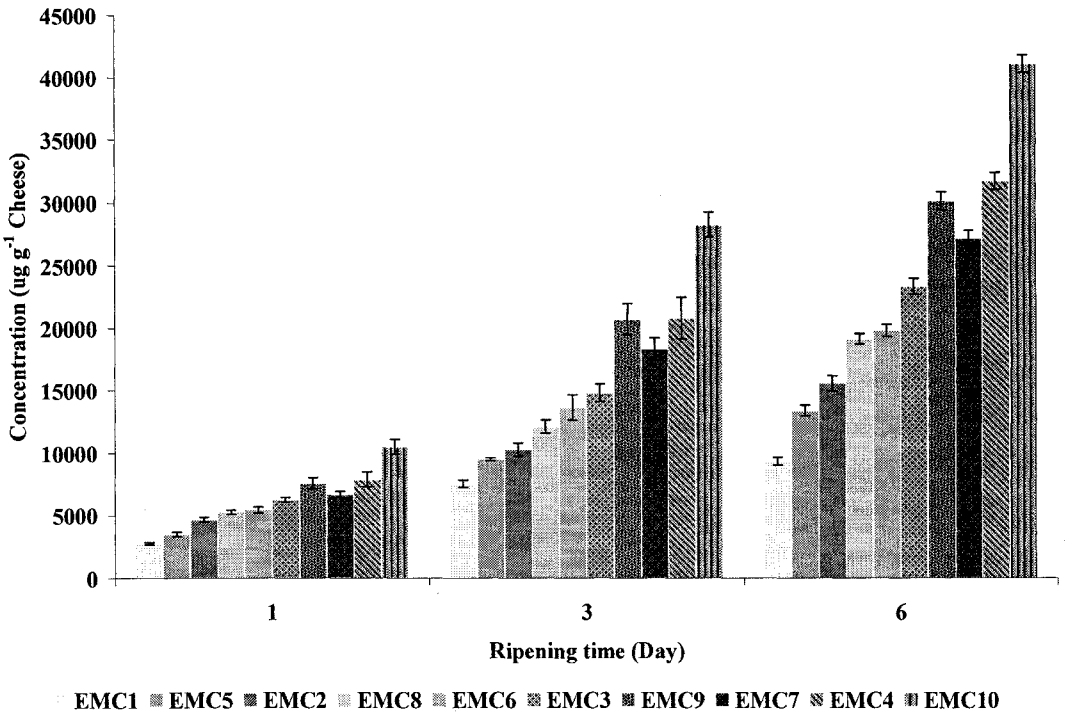


Figure 5.3. Changes in total FAA contents. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes.

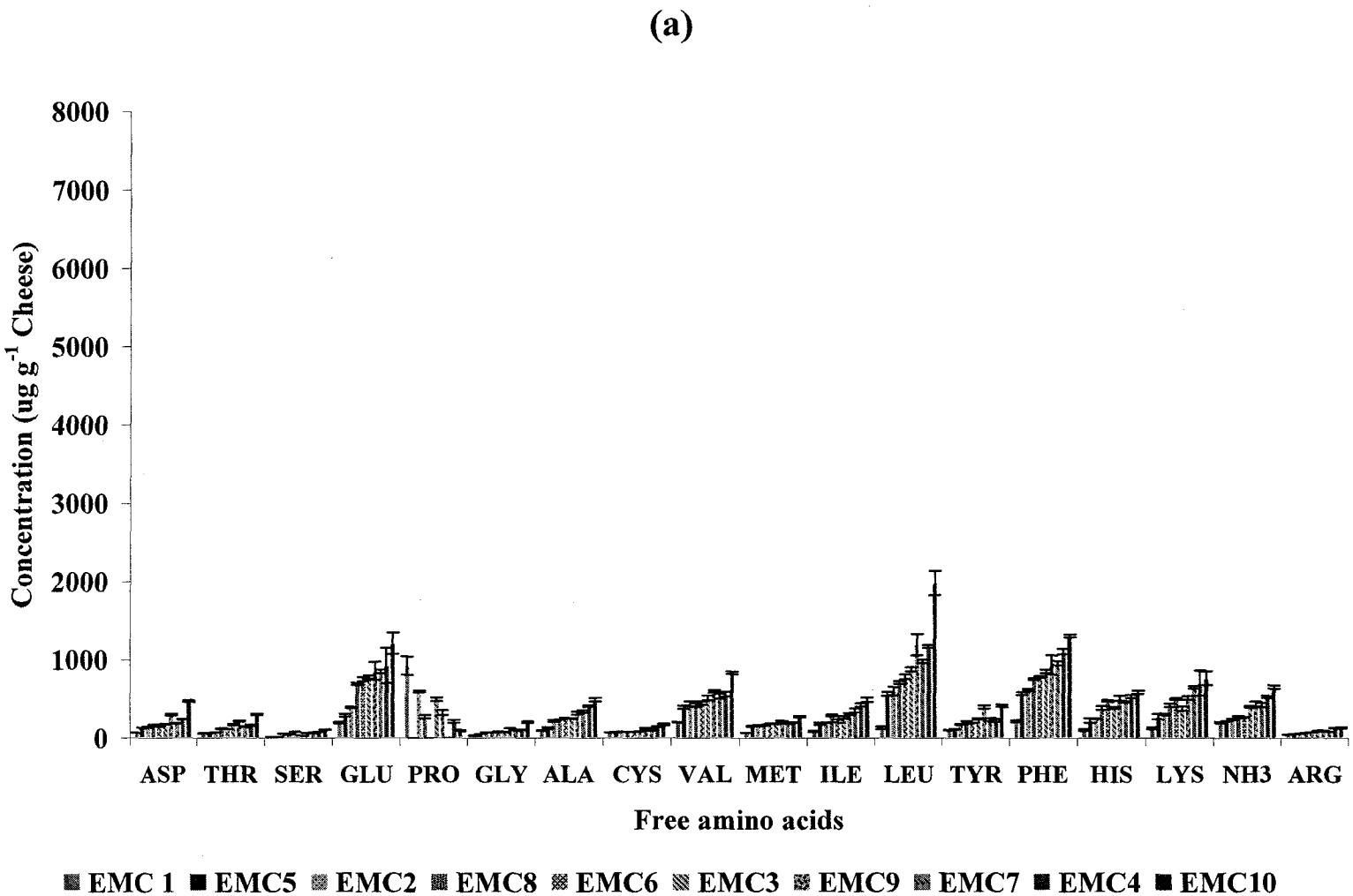


Figure 5.4. Concentration of individual free amino acids in control and Neutrase-added EMCs. (a) 1 day, (b) 3 days, (c) 6 days of ripening. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes.

(b)

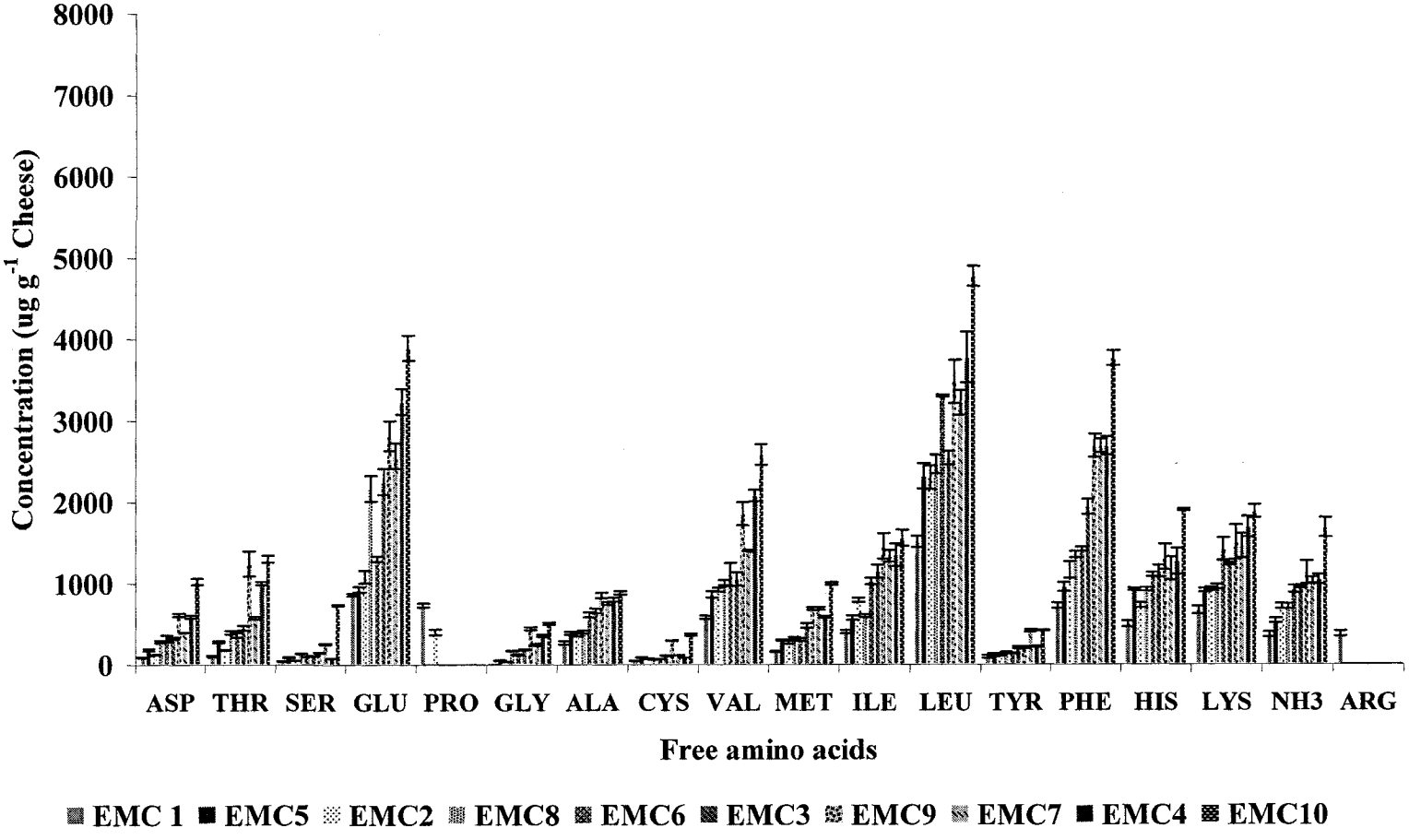


Figure 5.4. (Continued).

(c)

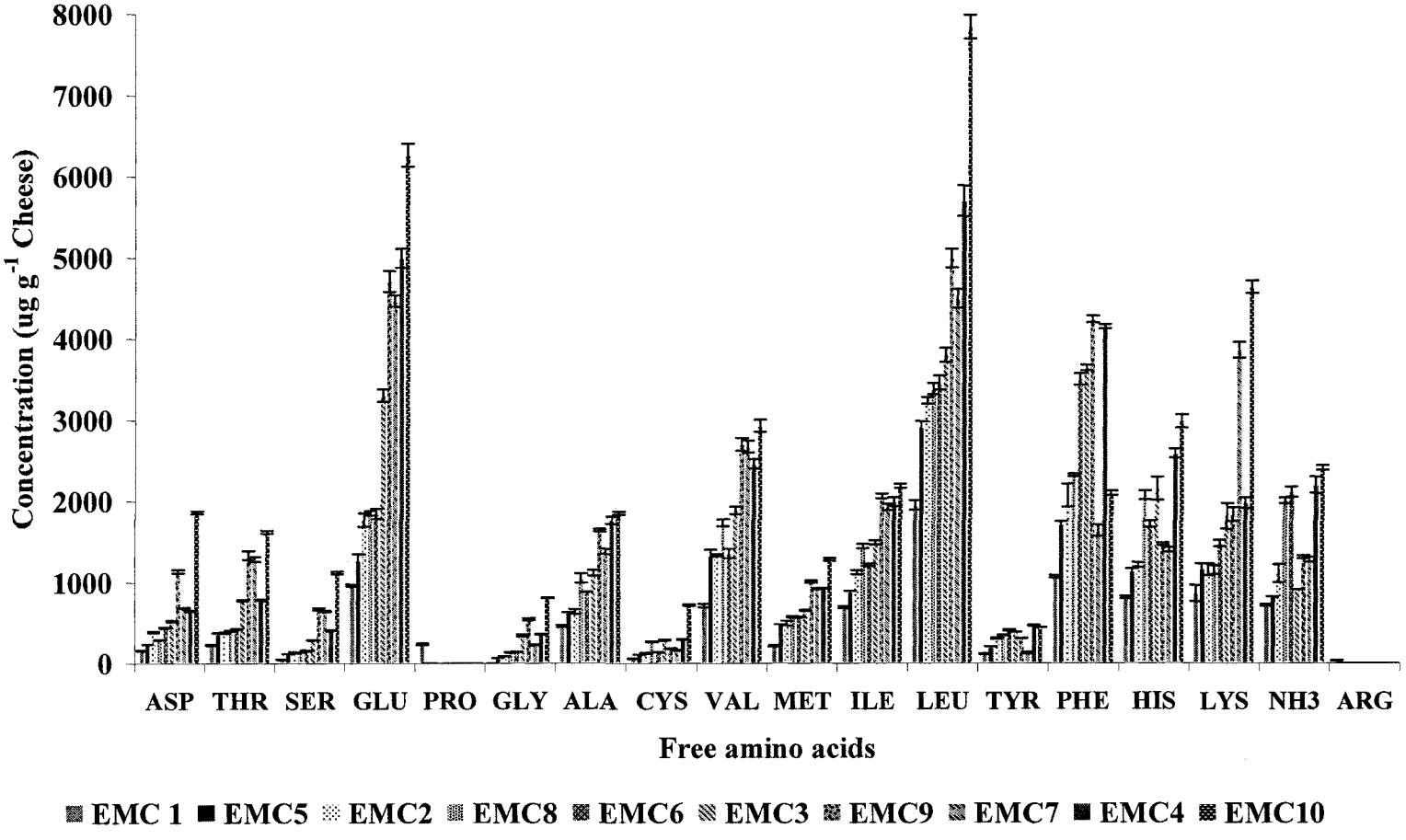


Figure 5.4. (Continued).

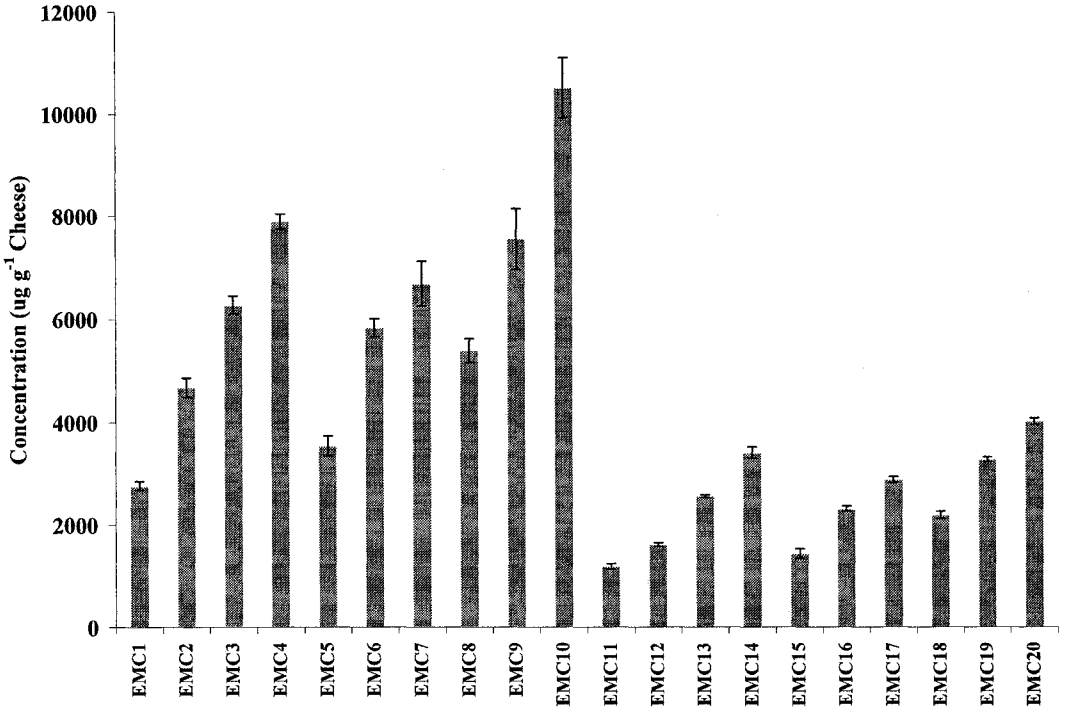


Figure 5.5. Changes in total free amino acid contents of the two groups of EMCs at 1 day. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes.

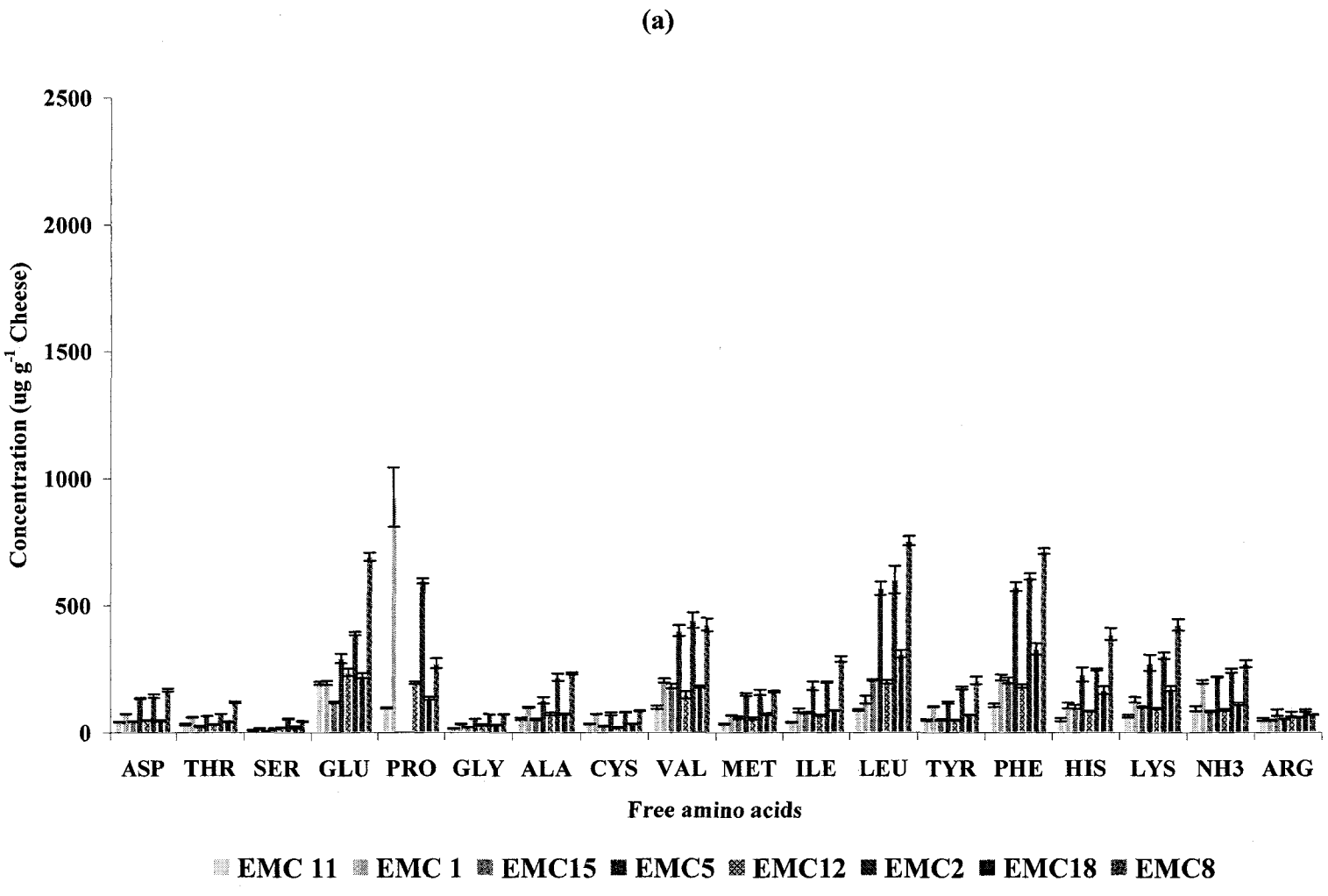


Figure 5.6. Concentration of individual free amino acids of the two groups of EMCs at 1 day of ripening. (a) 10, (b) 25, and (c) 50 units of natural or recombinant enzymes alone or combination per 200 g curd. Results are expressed as mean ± standard deviation. See Table 1 for identification of EMCs codes.

(b)

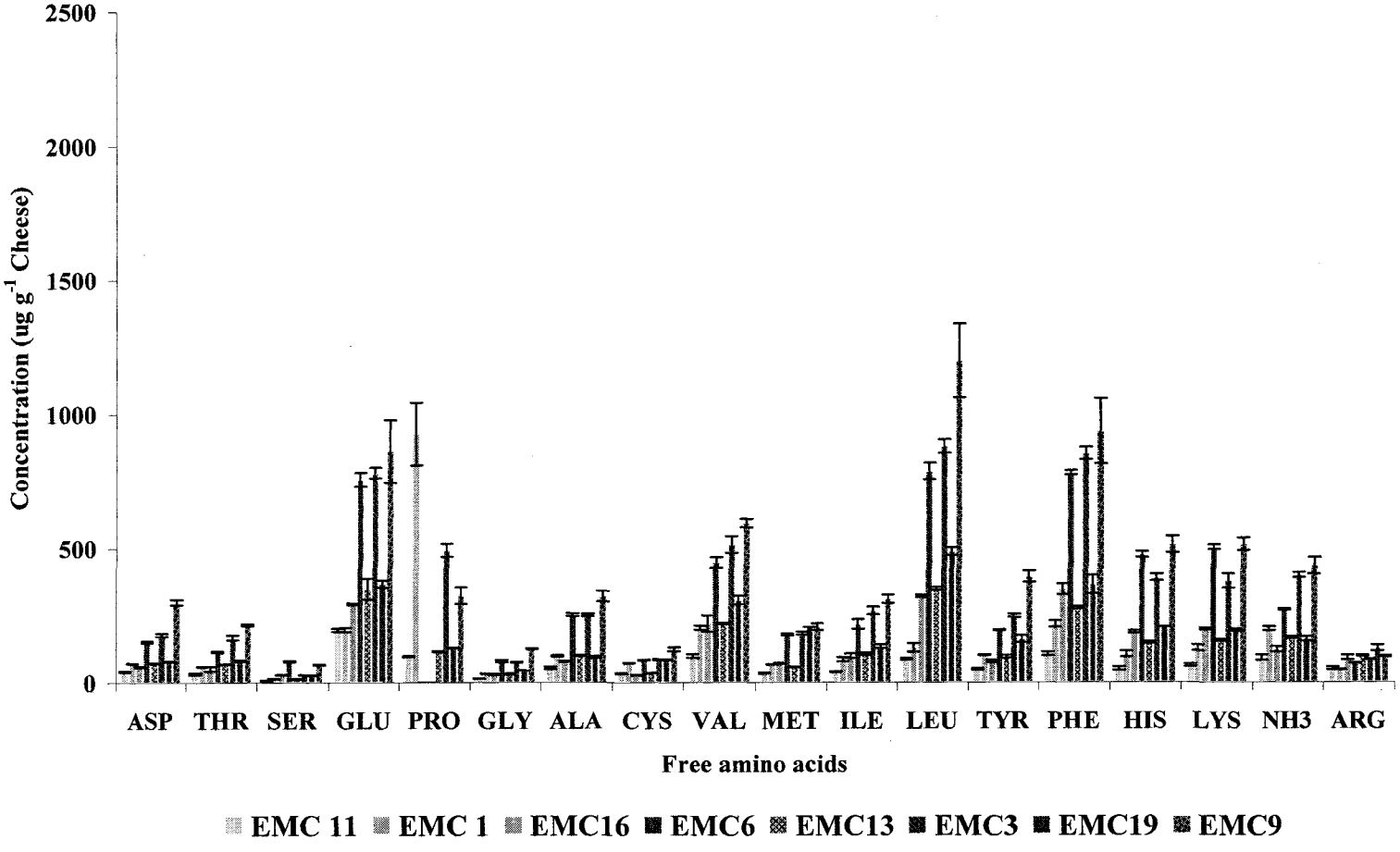


Figure 5.6. (Continued).

(c)

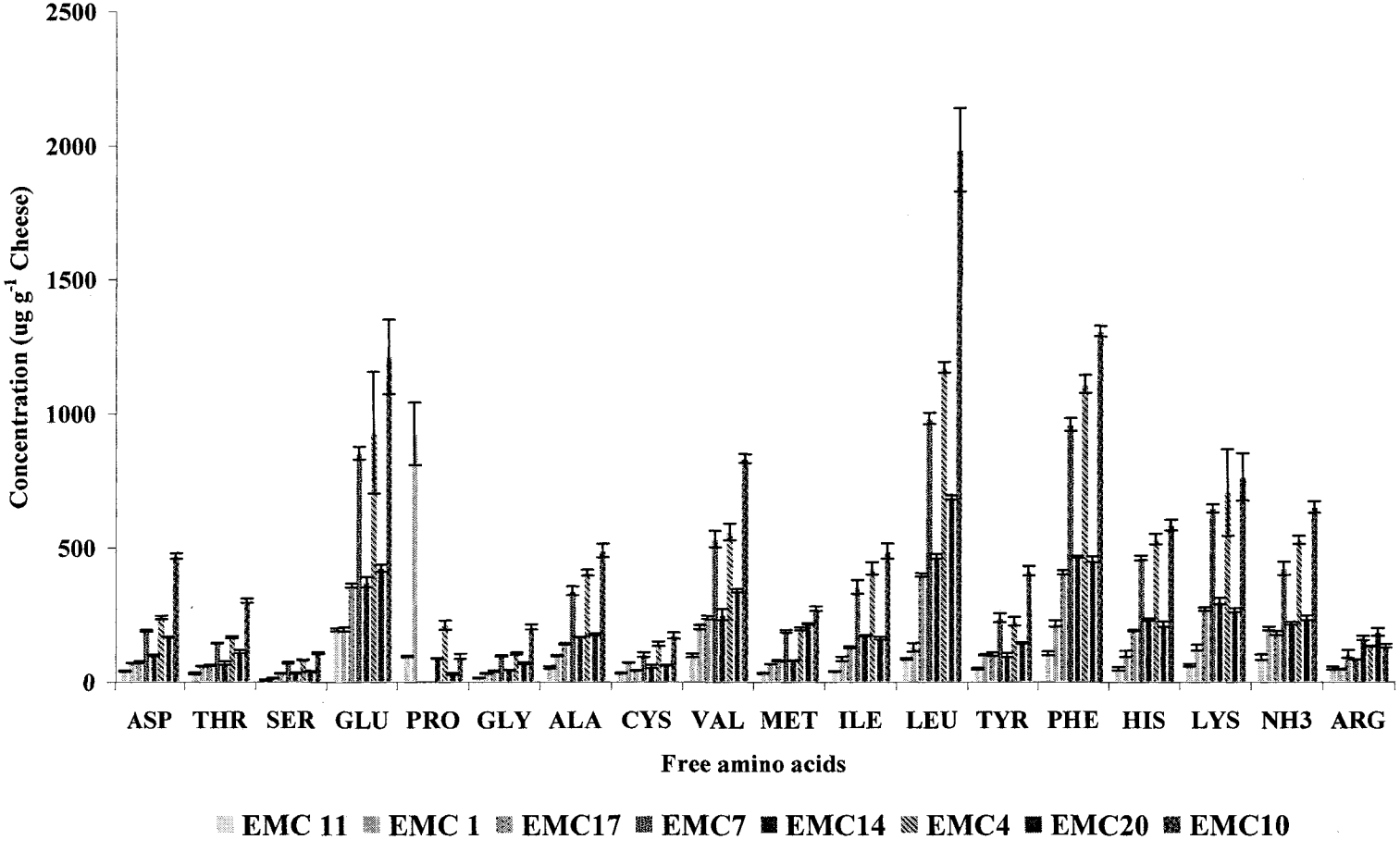
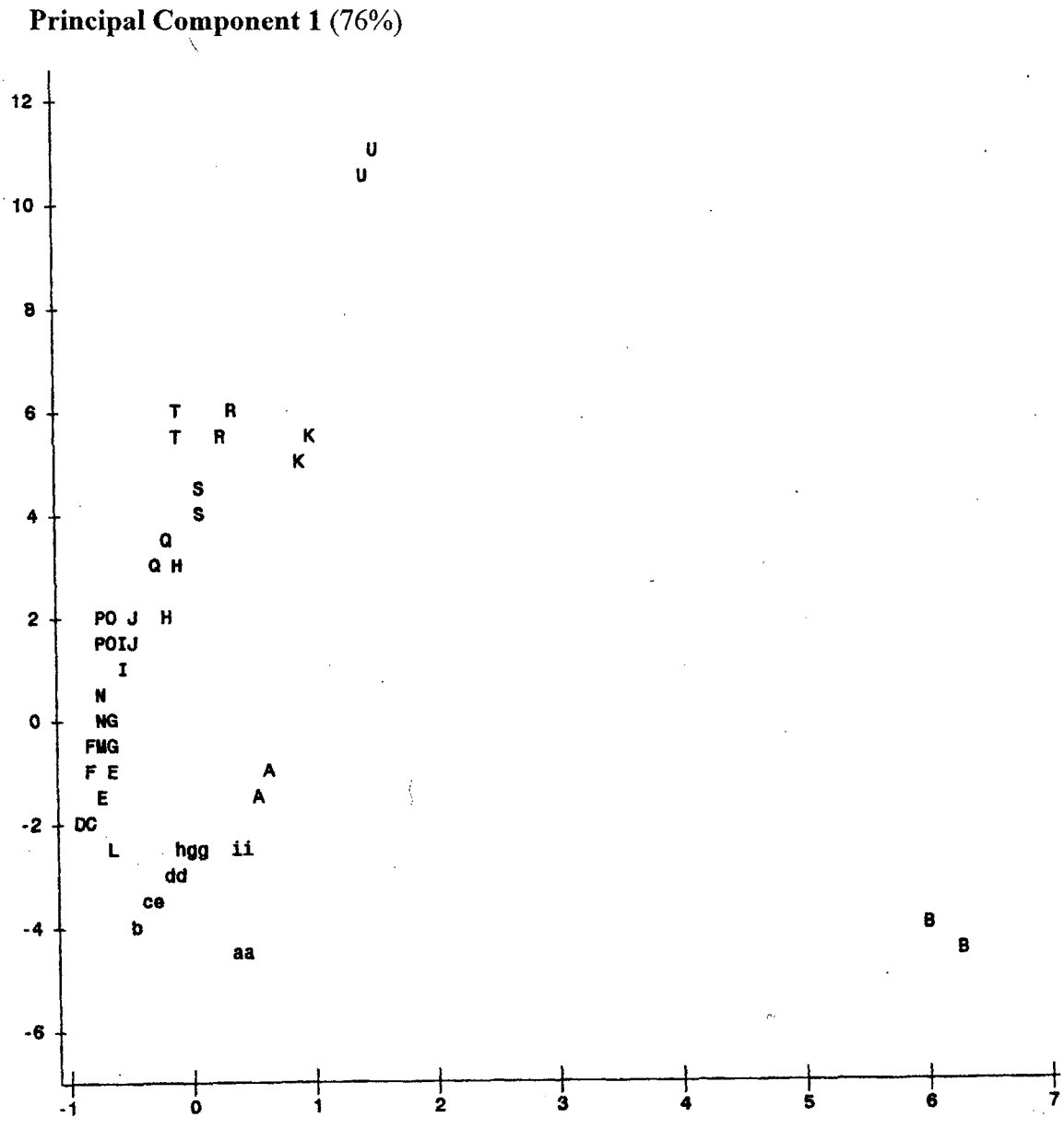


Figure 5.6. (Continued).



NOTE: 10 obs hidden.

Figure 5.7. PCA of the individual FAA in the control and experimental EMCs (with given enzymes and Neutrase). 1 Day: EMC1 (a), EMC5 (b), EMC2 (c), EMC8 (d), EMC 6(e), EMC3 (f), EMC9 (g), EMC7 (h), EMC4 (i), EMC10 (A); 3 Days: EMC1 (B), EMC5 (C), EMC2 (D), EMC8 (E), EMC 6(F), EMC3 (G), EMC9 (H), EMC7 (I), EMC4 (J), EMC10 (K); 6 Days: EMC1 (L), EMC5 (M), EMC2 (N), EMC8 (O), EMC 6(P), EMC3 (Q), EMC9 (R), EMC7 (S), EMC4 (T), EMC10 (U). See Table 1 for identification of EMCs codes.

GENERAL CONCLUSIONS

The aims of this thesis were first to encapsulate recombinant aminopeptidase (pepN) of *Lactobacillus rhamnsous* S93 in chitosan-coated alginate beads to provide a uniform delivery of this enzyme into Cheddar cheese matrix and to reduce cheese ripening period. The effects of free or encapsulated form of the enzyme on the acceleration of Cheddar cheese proteolysis were evaluated, and a process of Cheddar-enzyme modified cheese (EMC) flavorings was also developed.

The recombinant aminopeptidase (pepN) from *Lactobacillus rhamnosus* S93 was successfully encapsulated in Ca-alginate beads coated with chitosan using an extrusion method. The loss of recombinant pepN from the beads during encapsulation procedure was affected by Na-alginate, chitosan, and calcium chloride concentrations as well as the enzyme ratio to the alginate and the pH of chitosan-$CaCl_2$ solution. The use of chitosan as a coating material led to about 90% retention of the recombinant pepN activity in the encapsulated beads and an important reduction in enzyme release from the beads, compared to uncoated Ca-alginate capsules. An increase in alginate and chitosan concentrations led to greater encapsulation efficiency (*EE*) and lesser enzyme release (*ER*) from the beads. The greatest *EE* was observed in a pH 5.4 solution (chitosan-$CaCl_2$) during bead formation. Increasing the $CaCl_2$ concentration over 0.1 M affected neither the *EE* nor the *ER*. Increasing hardening time beyond 10 min led to a decrease in *EE*. The increase of enzyme portion led to a greater release of enzyme from the beads. The lowest *ER* has occurred in the capsules with a polymer: enzyme (3:1) ratio.

The effects of free or encapsulated form of the recombinant pepN on the proteolysis of Cheddar cheese were evaluated in two different scales of cheesemaking trials. Addition of free or encapsulated form of the recombinant pepN to the curd led to enhancement of proteolysis of experimental cheeses compared to that of the control. Furthermore, the results revealed that an increase in the enzyme concentration resulted in higher levels of secondary proteolytic indices during ripening times. The levels of most individual FAA were higher in all experimental cheeses at sampling times, except for proline, which disappeared in experimental cheeses at 4 months of the ripening period. In contrast, leucine was the most

dominant amino acid in the experimental cheeses during the maturation time. However, the cheese with encapsulated enzymes at the highest concentration had significantly higher mean levels of PTA-N and total FAAs than the other cheeses. The use of encapsulated enzymes resulted in an acceleration of 70% in ripening compared to that of the control cheese. This cheese also received higher mean levels of the sensory scores. The testers did not recognize any defect, due to the presence of alginate beads in the samples.

The high incorporation of the encapsulated pepN into the cheese matrix indicated the stability of the capsules during Cheddar cheese manufacturing stages. Thus, efficient incorporation of enzymes homogeneously in milk before cheddaring can be achieved using this technique.

Applications of the crude cellular extracts and recombinant aminopeptidase from *Lb. rhamnosus* S93 were investigated to develop a process of Cheddar-EMC flavorings. The results of this study highlight that proteolysis of Cheddar-EMCs can be enhanced by using crude and recombinant preparations of *Lb. rhamnosus* S93, but require the addition of a broad specificity proteinase, such as Neutrase to generate more peptides from caseins and to achieve very high levels of secondary proteolysis, as evident by PTA and FAA. The level of each FAA was increased over maturation in all EMCs, except for proline and arginine which disappeared during maturation in the experimental EMCs. Addition of exogenous enzymes that genetically overproduced in *E. coli* was useful in the preparation of enzyme-modified cheese to produce highly intense cheese flavors. However, further investigation is required to establish correlations between the proteolytic parameters and sensory characteristics of Cheddar-EMCs.

FUTURE STUDIES

1. Analysing flavor compounds of Cheddar cheese prepared with two forms of the recombinant aminopeptidase of *Lactobacillus rhamnosus* S93 by the solid-phase microextraction-gas chromatography to compare the flavor profiles.
2. Assessing the rheological properties of Cheddar cheeses made with two forms of the recombinant aminopeptidase of *Lactobacillus rhamnosus* S93.
3. Studying Cheddar cheese microstructure to find the effects of free or encapsulated form of the recombinant aminopeptidase of *Lactobacillus rhamnosus* S93 on the enzyme distribution in the cheese matrix.
4. Analysing flavor compounds of Cheddar-EMCs by the solid-phase microextraction-gas chromatography to compare the effects of both natural and recombinant enzymes of *Lactobacillus rhamnosus* S93 on the flavor profiles.
5. Evaluating the sensory properties of the Cheddar-EMC prepared with the natural and recombinant enzymes of *Lactobacillus rhamnosus* S93.

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