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Development of a microencapsulation technique for probiotic bacteria *Lactobacillus casei* 431 using a protein-polysaccharide complex

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Abstract

According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are defined as ‘‘live microorganisms which when administered in adequate amounts confer a health benefit for the host’’ (FAO/WHO, 2001). *Lactobacilli* and *Bifidobacteria* are two major group of organisms considered to have probiotic properties. Probiotic bacteria are accepted universally for conferring beneficial effects to human gut health. However, the successful delivery of these bacteria to the human intestine via a proper food matrix is challenging because the stresses encountered by the probiotics during processing, storage and gastric transition cause major loss of viability. The primary objective of this study was to develop a novel protection system using a complexation product of dairy protein and a bacterial exo-polysaccharide which should be able to protect the probiotic bacteria during their gastric transit and also release them under suitable conditions in the intestine. *Lactobacillus casei* 431, a commercial strain from Chr Hansen, Denmark, was chosen as the experimental strain and the protein-polysaccharide complex was made up of sodium caseinate and gellan gum. Gelation of the sodium caseinate and gellan gum mixture was achieved by a gradual decrease of pH with slow hydrolysis of glucono-delta-lactone (GDL) and *Lactobacillus casei* 431 cells were successfully entrapped into this gel matrix. An intermediate step of forming a water-in-oil emulsion was involved in this process for producing micron level gel particles. The appropriate combination of ingredients, based on final elastic modulus to attain adequate gel strength, was finally decided as 10% (w/w) sodium caseinate, 0.25% (w/w) gellan gum and 2.5% (w/w) GDL. This combination resulted in a very fine and uniform capsule size distribution and up to 89% encapsulation efficiency was achieved.

The gelled microcapsules were freeze dried to obtain better shelf stability and easy handling properties. The particles obtained had diameters ranging from 40 to 1100 µm for wet and 46 to 631 µm for freeze dried microcapsules. The mean diameters (D_{32}) of wet and freeze dried microcapsules were found as 287 and 152 µm, respectively.

Scanning electron microscopic examination of the freeze dried particles showed irregular surfaces and the presence of numorous pores.

Tolerance of free and encapsulated bacterial cells in simulated gastric juice at pH 2.0 was tested in an *in vitro* model and the results showed better survivability of encapsulated cells in both wet and dry microcapsules as compared to the free cells. The log CFU reduction figures after a 2 hour incubation period, were 4.56 for free cells, 3.03 for cells inside wet capsules and 2.28 for cells protected inside freeze dried particles. Incubation of free and encapsulated cells in the presence of 1% (w/v) bile extract for 8 hours showed 2.51 log CFU/gm reductions for free cells with almost no detrimental effect on wet microencapsulated cells and 2.44 log CFU reductions for freeze dried cells.

Further research work was undertaken to improve the post freeze drying survivability of the *L. casei* 431 cells by including cryoprotective solutes in both the culture growth and the drying media. Trehalose and lactose were chosen as cryoprotecting agents. Compared to an average 1.70 log CFU reduction in case of control (no cryoprotectant) samples, trehalose and lactose containing samples both showed a much better survival rate; only 0.84 and 0.37 log CFU/gm reduction respectively, in cell population, were recorded. A membrane coating over the produced microcapsules was applied and the properties of such coated samples were checked separately. The coating process aided in the post drying survivability and only 0.53 and 0.13 log CFU/gm reductions were recorded for trehalose and lactose supplemented samples, respectively. The presence of cryoprotecting compounds proved to be beneficial against the simulated gastric environment and the membrane coating gave additional improvement in this regard. During the gastric fluid incubation tests, cryoprotected samples (freeze dried) containing trehalose and lactose shown a higher survival of 3.13 log CFU/gm and 2.04 log CFU/gm respectively, compared to cells in free form. Improvements offered by the membrane coating were recorded as an additional 0.23 log CFU/gm and 0.66 log CFU/gm higher survival for trehalose and lactose respectively. The same trend was observed for bile salt tolerance also. Cryoprotected samples (freeze dried) containing trehalose and lactose showed a higher survival of 0.41 log CFU/gm and 0.84 log CFU/gm respectively, compared to cells in free form. Additionally, the membrane coating process contributed

towards further improvement in viability of 0.25 log CFU/gm and 0.26 log CFU/gm for trehalose and lactose respectively. Overall, lactose has been found to be a marginally better protectant of cells than trehalose against freeze drying, acid and bile salt stresses.

The membrane coating process helped in forming a very smooth surface morphology devoid of any visible pores. Perhaps the presence of a membrane coating was responsible for this better protective nature of coated microcapsules. But as a drawback, this coating process resulted into higher particle mean diameters, both for wet and freeze dried beads.

Storage of freeze dried samples at 37°C proved to be more detrimental to the entrapped cells than at 4°C. But the results obtained were better compared to the situation where no protective compounds were used. It was found that lactose and trehalose helped in maintaining high levels of viable cell populations during the storage period but the cell degradation rate was positively correlated with the storage temperature.

Therefore, it can be concluded that a low pH sodium caseinate-gellan gum gel matrix can offer adaptation and protection to the probiotic cells before encountering a high acid stomach environment and therefore can be utilized as an effective microencapsulation technique. The survibility of the *L. casei* 431 cells could be further improved during freeze drying as well as gastrointestinal transit by incorporation of protectants, viz., lactose or trehalose and applying a membrane coating of gellan gum. High acid food preparations such as, yogurt and fruit juice could be the probable applications for the current findings.

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Contents

Abstract……………………………………………………………………………………I
Acknowledgements…………………………………………………………………..V
Contents……………………………………………………………………………VII
List of tables………………………………………………………………………XVI
List of figures…………………………………………………………………….XVII

Chapter 1. Introduction…………………………………………………….......1

Chapter 2. Literature review……..……………………………………………..……5
2.1 Introduction……………………………………………………………………5
2.2 What are probiotic bacteria?…..……………………………………………….5
2.2.1 Definitions…………………………………………………………….…..5
2.2.2 Characteristics of probiotic bacteria ………………………………………6
2.2.2.1 General microbiological criteria …………………………………7
2.2.2.2 Technological aspects ……………………………………………7
2.2.2.3 Functional benefits of probiotic bacteria……..…………………....7
2.2.3 Probiotics in human gastro-intestinal tract ………………………………..8
2.2.4 Some common probiotic species and strains ……………………………10
2.2.4.1 *Lactobacillus* species……………………………………………..12
2.2.4.2 *Bifidobacterium* species…………………………………………..13
2.2.5 Health benefits and human clinical trials with probiotic bacteria..……....13
2.2.6 Regulations governing foods containing probiotics ……………………..16
2.2.7 Mechanism of probiotic bacteria functionalities ………………………...17
2.2.7.1 Reduction of luminal pH………………………………………...18
2.2.7.2 Production of hydrogen peroxide………………………………...19
2.2.7.3 Production of bacteriocins...…....19
2.2.7.4 Fermentative production of carbon dioxide……………..………19
2.2.7.5 Diacetyl production by fermentation…………………….……..20
2.2.7.6 Deconjugation of bile acids……………………………...……..20

2.2.8 Challenges involved in incorporating probiotics into food………………...21

2.2.8.1 Food processing factors…………………………………..………21

2.2.8.2 Exposure to gastric acid present in stomach …………..………21

2.2.8.3 Exposure to bile salts present in intestinal fluid……………….…22

2.2.8.4 Oxygen intolerance of probiotic strains……………………………22

2.2.9 Selection chriteria of a probiotic strain for microencapsulation and subsequent food applications…………………………………………………………23

2.3 Microencapsulation overview……………………………………………………24

2.3.1 Definition……………………………………………………..…………24

2.3.2 Purpose of microencapsulation…………………………..……………25

2.3.3 Structure of microcapsules…………………………………………...25

2.3.4 Microencapsulation techniques………………………………………28

2.3.4.1 Extrusion technique for microencapsulation……………………28

2.3.4.2 Emulsion technique for microencapsulation……………………31

2.3.4.3 Use of drying technology for microencapsulation………………32

2.3.4.3.1 Spray drying as a technique of microencapsulation..…32

2.3.4.3.2 Microencapsulation of probiotics with freeze drying.....35

2.3.4.3.3 Other drying methods used for microencapsulation…...36

2.3.4.4 Microencapsulation with spray chilling…………………………36

2.3.4.5 Coacervation technique for microencapsulation………………..37

2.3.4.6 Co-crystalization method……………………………………..38

2.3.4.7 Molecular inclusion…………………………………………..…39

2.3.4.8 Centrifugal extrusion technique………………………………...40

2.3.5 Selection criteria of choosing encapsulating matrix…………………41

2.3.6 Gelation mechanism of dairy proteins…………………………..…..42

2.3.6.1 Acid gelation of casein…………………………………..…...42

2.3.6.2 Enzymatic gelation……………………………………………..43

2.3.7 Use of milk proteins in microencapsulation…………………..……..44

2.3.7.1 Introduction……………………………………………..……44

2.3.7.2 Whey protein as wall material for microencapsulation………...44

2.3.7.3 Use of sodium caseinate as microencapsulating agent…………46

2.3.8 Use of glucono-delta-lactone in gelation of milk proteins…………..….47
2.3.9 Use of gellan gum as microencapsulation wall material……………..……48
2.4 Drying of probiotic culture and related physiological stresses……………...….50
2.4.1 Use of cryoprotective agents in the freeze drying media…………...…….50
2.4.2 Trehalose and lactose as cryoprotective compounds…………………..…..52
2.4.3 Improving freeze drying viability by proper cell harvesting……………..53
2.4.4 Use of additives in the growth media for enhanced protection during drying……………………………………………………………...………53
2.5 Summary and conclusion……………………………………………….…………54

Chapter 3.0 Development of the microencapsulation process and studying the properties of produced microcapsules……………………………………………..56

3.1 Introduction……………………………………………………………………..56
3.2 Materials and methods……………………………………..…………………56
3.2.1 Ingredients and chemicals…………………...……………………………57
3.2.2 Studying the gel formation and gel behavior of the encapsulating media..57
3.2.3 Preparation of cell suspension…………………………………………….59
3.2.4 Microencapsulation process of *L. casei* cells……………………….....….59
3.2.5 Optimization of the Core -Wall ratio……………………..………………60
3.2.6 Particle size distribution analysis…………………………….………...61
3.2.7 Evaluation protocol for protection of bacterial cells in simulated gastric juice………………………………………………………………...…..62
3.2.8 Survival of encapsulated and free cells in presence of bile salts….………63
3.2.9 Statistical analysis……………………………………………….…….64
3.3 Results and discussion…………………………………………………………..65
3.3.1 Gelation of sodium caseinate and gellan gum mixture……………......…65
3.3.2 Encapsulation efficiency……………………………………………..……..68
3.3.3 Particle size distribution……………………………………………….……69
3.3.4 Survival of encapsulated and free cells in SGF and under simulated bile conditions……………………………………………………………...……70

3.4 Conclusion……………………………………………………………..……..74

Chapter 4.0 Effect of freeze drying on the physical properties and viability of probiotic cells entrapped in the microcapsules …………………………………...76

4.1 Introduction……………………..………………………………………..76
4.2 Materials and methods…………………………………………………..77
4.2.1 Ingredients and chemicals……………………………………….…...77
4.2.2 Preparing *L. casei* cell suspension…………….…………………………...77
4.2.3 Microencapsulation technique……………………………………......77
4.2.4 Freeze drying of wet gelled microcapsules………………………………..77
4.2.5 Measuring size distribution of freeze dried microcapsules………………..78
4.2.6 Scanning Electron Microscopic (SEM) examination……………………...78
4.2.7 *L. casei* cell viability during *in vitro* gastric transit and in presence of bile salts…………………………………………………………………...78
4.2.8 Storage stability test and moisture content determination ………….……..79
4.2.9 Statistical analysis…………………………………………….………...79
4.3 Results and discussion……………………………………………...……80
4.3.1 Particle size distribution of freeze dried microcapsules…………………...80
4.3.2 Scanning electron microscopic examination………………………………82
4.3.3 Effect of freeze drying on microencapsulated *L. casei* cell viability……..84
4.3.4 Protection of microencapsulated and freeze dried *L. casei* cells in simulated gastric juice……………………………………………………..87
4.3.5 Effect of bile salts on encapsulated cells in freeze dried microcapsules…..89
4.3.6 Enumeration of viable cells…………………………………………......90
4.3.7 Storage stability of the freeze dried microcapsules……………..………...91
4.4 Conclusion……………………………………………………………...…..94

Chapter 5.0 Improvements in post drying cell survival and gastric tolerance by incorporating cryoprotectants and by applying membrane coating around the microcapsules……………………………………………………………..……96

5.1 Introduction……………………………………………………………………...96
5.2 Materials and methods………………………………………………………….98
5.2.1 Ingredients and chemicals…………………………………………………98
5.2.2 Growth media formulation and culturing of *L. casei* cells………………...98
5.2.3 Microencapsulation process……………………………………………....98
5.2.4 Membrane coating process around the microcapsules…………………...100
5.2.5 Particle size distribution…….……………………………………………...100
5.2.6 Electron microscopic examination of the microcapsules………………...101
5.2.7 Survivability of *L. casei* cells in simulated gastric and bile salt environments……………………………………………………………..102
5.2.8 Storage stability test……………………………………………………...102
5.2.9 Statistical analysis………………………………………………………..102
5.3 Results and discussion…………………………………………………………103
5.3.1 Particle size distributions of membrane coated microspheres……………103
5.3.2 Microscopic examination of the membrane coated freeze dried particles.105
5.3.2.1 Light microscopic imaging of the freeze dried TMB and LMB microcapsules…………………………………………………105
5.3.2.2 Transmission electron microscopic view of dry TMB and LMB microcapsules…………………………………………………107
5.3.2.3 Scanning electron microscopic images of the dried TMB and LMB capsule surfaces………………………………………………..109
5.3.3 Analysis of cell survivability out of freeze drying……………………….111
5.3.4 Role of compatible solutes added in cell growth media………………….114
5.3.5 Effect of cryoprotective compounds and membrane coating process on acid tolerance (simulated gastric conditions) of *L. casei* cells………………...115
5.3.5.1 Behavior of free *L. casei* cells in simulated gastric fluid………...115
5.3.5.2 Effect of trehalose and membrane coating on acid tolerance of *L.*

casei cells……………………………………………………….116

5.3.5.3 Effect of lactose and membrane coating on acid tolerance of *L. casei* cells…………………………………………………………….117

5.3.6 Effect of bile salts on free and encapsulated *L. casei* cells……………….120

5.3.6.1 Behavior of free cells in presence of bile salts……………………..120

5.3.6.2 Effect of trehalose and membrane coating on bile salt tolerance of *L. casei* cells………………………………………………………121

5.3.6.3 Effect of lactose and membrane coating on bile salt tolerance of *L. casei* cells……………………………………………………….122

5.3.7 Storage stability of the *L. casei* cells in freeze dried microcapsules……127

5.3.7.1 Survival of encapsulated freeze dried cells stored at 4°C………..127

5.3.7.2 Survival of encapsulated freeze dried cells stored at 37°C……...128

5.3.8 Conclusion…………………………………………………………..130

Chapter 6.0 Overall conclusion and future recommendations……..……………132

References……………………………………………………………………….135

Appendices

Appendix 3.1 Measuring growth curve and identifying the stationary phase of *L. casei* 431 bacteria grown in MRS broth with 1% (v/v) inoculation…………………………170

Appendix 3.2 Particle size distribution data for wet microcapsule samples prepared in Chapter 3.0 experiments…………………………………………………………….171

Appendix 3.3 Survival of L. casei cells in free form and inside wet microcapsules when subjected to simulated gastric juice at pH 2.0 without pepsin………………………...172

Appendix 3.4 Action of simulated gastric juice at pH 2.0 with 0.32% pepsin on *L. casei* cells in free form and inside wet microcapsules………………………………………173

Appendix 3.5 Survival of *L. casei* cells in free form and microencapsulated in wet capsules in a simulated bile solution with 1.0% (w/w) bile extract…………………...174

Appendix 4.1 Volume distribution data for freeze dried particles as prepared in Chapter 4.0……………………………………………………………………………………..175

Appendix 4.2 Survival of *L. casei* cells microencapsulated in wet and freeze dried form during incubation in simulated gastric fluid……………………………….…………..176

Appendix 4.3 Action of bile salts on *L. casei* cells encapsulated in wet and freeze dried microcapsules…………………………………………………………….………177

Appendix 4.4 Moisture content analysis of wet and freeze dried microcapsules……..178

Appendix 4.5 Changes in cell viability of freeze dried microencapsulated beads containing *L. casei* cells, during storage (Chapter 4.0)……………………………..179

Appendix 5.1 Comparative particle size distribution data for both wet and dried uncoated, TMB and LMB samples…………………………...…………………180

Appendix 5.2 Loss of viability due to freeze drying (Chapter 5.0 experimental data)..181

Appendix 5.3 Survival of *Lactobacillus casei*, in free form and microencapsulated in wet and dried TB samples form when treated in simulated gastric fluid…………………..182

Appendix 5.4 Survival of *Lactobacillus casei*, in free form and microencapsulated in in wet and dried TMB samples form when treated in simulated gastric fluid……………183

Appendix 5.5 Survival of *Lactobacillus casei*, in free form and microencapsulated in in wet and dried LB samples form when treated in simulated gastric fluid………..……..184

Appendix 5.6 Survival of *Lactobacillus casei*, in free form and microencapsulated in in wet and dried LMB samples form when treated in simulated gastric fluid……………185

Appendix 5.7 Response of free, wet and dry encapsulated (TB) *L. casei* cells in presence of 1% porcine bile extract……………………..…………………………………….186

Appendix 5.8 Survivability of free and membrane coated (TMB) *L. casei* cells in simulated bile environment………………..…………………………………………187

Appendix 5.9 Response of free, wet and dry encapsulated (LB) *L. casei* cells in presence of 1% porcine bile extract…………………………………………………………...188

Appendix 5.10 Survivability of free and membrane coated (LMB) *L. casei* cells in simulated bile environment……………………………………………………..….189

Appendix 5.11 Storage stability of control sample (freeze dried) with no cryoprotectant or membrane coating (Chapter 5.0)…………………………………………………190

Appendix 5.12 Storage stability of freeze dried TB samples prepared as per chapter 5.0……………………………………………………………………………...…191

Appendix 5.13 Storage stability of freeze dried TMB samples prepared as per chapter 5.0………………………………………………………………………………...192

Appendix 5.14 Storage stability of freeze dried LB samples prepared as per chapter 5.0………………………………………………………………………………...193

Appendix 5.15 Storage stability of freeze dried LMB samples prepared as per chapter 5.0………………………………………………………………………………...194

List of tables

Table 2.1 Microorganisms used or considered for use as probiotics in human…..……11

Table 2.2 Different probiotic bacteria strains and their health benefits……….……….15

Table 3.1 Optimization of the core-wall proportions by adding various concentrations of free cell suspensions……………………………………………………………….…68

Table 4.1 Sieve analysis of freeze dried microcapsules done in Malvern Mastersizer Scirocco-2000 unit………………………………………...……………..………..81

List of figures

Figure 2.1 Microbial colonization of the human gastro-intestinal tract……………..…….....9

Figure 2.2 Schematic diagram of two representative types of microcapsules, A: Matrix type and B: Aggregate type capsule…………………………………………………….27

Figure 2.3 Various forms of microcapsules used in the food industry………………….27

Figure 2.4 2-D Skeletal image of Glucono-delta-lactone molecule…………………….47

Figure 2.5 Structural unit of gellan gum molecules…………………………………….48

Figure 3.1 Optimized process flow diagram of the cell culturing and microencapsulation of *Lactobacillus casei* bacteria……………………………………………………….58

Figure 3.2 Changes in elastic modulus G' of 10% sodium caseinate solution mixed with 1.0% (♦), 1.5% (●), 2.0% (■) and 2.5% (▼) glucono-δ-lactone………………………………………………………………………………..65

Figure 3.3 Changes in elastic modulas (G') after the addition of 0.1% (■), 0.25% (♦) and 0.50% (▼) gellan gum into a control sample (●) containing 10% sodium caseinate and 2.5% glucono-δ-lactone ………………………..…………………………………...66

Figure 3.4 Changes in the pH by the action of 2.5% glucono-δ-lactone on 10% sodium caseinate (■) and 10% sodium caseinate mixed with 0.25% gellan gum (▲) during incubation for 240 min at 20 ^{o}C ……………………………………………………..67

Figure 3.5 Particle size distribution curve of the microcapsules, as obtained using a Malvern Mastersizer………………………………………………………………….69

Figure 3.6 Survival of encapsulated cells (∆, with pepsin; ○, without pepsin) and free cells (▲, with pepsin; ●, without pepsin) of *Lactobacillus casei* in simulated gastric fluid incubated at pH 2.0 for 120 min. The error bars indicate standard deviations from the mean values of three replicated experiments………………………………………………71

Figure 3.7 Survival of encapsulated cells (■) and free cells (♦) of *Lactobacillus casei* under simulated bile conditions during 8 h of incubation. The error bars indicate standard deviations from the mean values of three replicated experiments……..……..73

Figure 4.1 Volume distribution curve of wet (▲) and freeze dried (x) particles plotted against surface based diameters measured in Malvern Mastersizer, U.K……………...80

Figure 4.2.1 Surface of a particle viewed at 5000x magnification……………………..82

Figure 4.2.2 Agglomerated microcapsules viewed at 250x magnification……………..82

Figure 4.2.3 Cross sectional view at 2500x magnification showing presence of a few *L. casei* cells (black arrows)……………………………………………………….....82

Figure 4.2.4 Uneven surface properties viewed at 1000x magnification……………....82

Figure 4.2.5 Surface morphology at 2500x magnification showing presence of numerous pores………………………………………………………………………......83

Figure 4.2.6 Rod shaped *L. casei* cell visible on a cross sectional view at 20000x magnification…………………………………………………………………………83

Figure 4.3 Effect of freeze drying on the survival of microencapsulated *L. casei* cells..85

Figure 4.4 Survival of *L. casei* cells microencapsulated in wet (♦) and freeze dried (■) form during incubation in simulated gastric fluid. The values shown are the mean values of 3 replicates……………………………...……………………………………87

Figure 4.5 Action of bile salts on *L. casei* cells encapsulated in wet (♦) and freeze dried (■) microcapsules……………………………………………………………………......89

Figure 4.6 *L. casei* colonies plated on MRS agar…………………………………….....91

Figure 4.7 Changes in cell viability of freeze dried microencapsules containing *L. casei* cells, during storage at -20°C (■), 4°C (♦) and 37°C (▲). Error bars representing standard deviations from the mean value of 3 replicated counts………………….……....92

Figure 5.1 Process flow diagram of the microencapsulation technique………………...99

Figure 5.2 Particle size distributions of wet (♦) and freeze dried (■) TMB microcapsules, compared with no-membrane coated wet (▲) and dried (x) samples…………………103

Figure 5.3 Particle size distributions of wet (♦) and freeze dried (■) LMB microcapsules, compared with without-membrane wet (▲) and dried (x) samples…………………...104

Cross sectional images of TMB microcapsules at 200x (Fig. 5.4.1), at 400x (Fig. 5.4.2) magnifications and the same for LMB microcapsules at 200x (Fig. 5.4.3) and at 400x (Fig. 5.4.4) viewed by light microscopy with toluidine blue staining……………..…..106

Figure 5.4.5 TEM image of TMB capsule cross section showing caseinate, gellan & trehalose complexation……………………………………………...……………107

Figure 5.4.6 Cross sectional view of LMB capsule by TEM image…………………..107

Figure 5.4.7 TEM image showing presence of *L. casei* cells inside TMB microcapsule…………………………………………………………………………...108

Figure 5.4.8 *L. casei* cells entrapped in LMB capsule, viewed via TEM imaging…....108

Figure 5.4.9 TEM image view of membrane coating formation around TMB capsule.108

Figure 5.4.10 TEM image showing presence of membrane coating on LMB capsule..108

Figure 5.4.11 SEM image of TMB capsule surface viewed at 5000x magnification…109

Figure 5.4.12 Surface of LMB capsules, viewed at 5000x magnification on SEM…..115

Figure 5.4.13 Surface morphology of a TMB microcapsule at 10000x magnification..110

Figure 5.4.14 Surface morphology of a LMB microcapsule at 10000x magnification..110

Figure 5.4.15 Particle shape of TMB microbeads as viewed via SEM image………...110

Figure 5.4.16 Particle shape of LMB microbeads as viewed via SEM image………...110

Figure 5.5 Comparative cell viability before (Wet ■) and after freeze drying (Dry ■) of uncoated capsules containing trehalose (TB) & lactose (LB) and membrane coated capsules containing trehalose (TMB) and lactose (LMB). Error bars representing standard deviations from the mean value of 3 replicated counts………………………111

Figure 5.6 Survival of *Lactobacillus casei,* in free form (♦) and microencapsulated in uncoated, trehalose containing beads (TB), in wet (■) and dried (▲) form when treated in simulated gastric fluid (SGF). Error bars representing standard deviations from the mean value of 3 replicated counts……………………….…………………………116

Figure 5.7 Survival of *L. casei* cells in free form (♦) and protected in membrane coated trehalose containing capsules (TMB), in both wet (■) and dried (▲) forms, when incubated in simulated gastric fluid. Error bars representing standard deviations from the mean value of 3 replicated counts..………………………………...………………….117

Figure 5.8 Survival of *Lactobacillus casei,* in free form (♦) and microencapsulated in uncoated, lactose containing beads (LB), in wet (■) and dried (▲) form when treated in simulated gastric fluid (SGF). Error bars representing standard deviations from the mean value of 3 replicated counts..118

Figure 5.9 Survival of *L. casei* cells in free form (♦) and protected in membrane coated lactose containing capsules (LMB), in both wet (■) and dried (▲) forms, when incubated in simulated gastric fluid. Error bars representing standard deviations from the mean value of 3 replicated counts..119

Figure 5.10 Response of free (♦), wet (■) and dry (▲) encapsulated (TB) *L. casei* cells in presence of 1% porcine bile extract. Error bars representing standard deviations from the mean value of 3 replicated counts..121

Figure 5.11 Survivability of free (♦) and membrane coated (TMB) *L. casei* cells in simulated bile environment. Membrane coated capsules were tested for wet (■) and dried (▲) forms. Error bars representing standard deviations from the mean value of 3 replicated counts..122

Figure 5.12 Response of free (♦), wet (■) and dry (▲) encapsulated (LB) *L. casei* cells in presence of 1% porcine bile extract. Error bars representing standard deviations from the mean value of 3 replicated counts 123

Figure 5.13 Survivability of free (♦) and membrane coated (LMB) *L. casei* cells in simulated bile environment. Membrane coated capsules were tested for wet (■) and dried (▲) forms. Error bars representing standard deviations from the mean value of 3 replicated counts..124

Figure 5.14 Result summary for comparing the effect of cryoprotecting compounds in wet microcapsules (TB x, TMB ■, LB ▲, LMB *) on bile tolerance, against the wet control sample (●) with no protectant. Error bars representing standard deviations from the mean value of 3 replicated counts……………...…………………………………125

Figure 5.15 Result summary for comparing the effect of cryoprotecting compounds in dry microcapsules (TB x, TMB ■, LB ▲, LMB *) on bile tolerance, against the dry control sample (●) with no protectant. Error bars representing standard deviations from the mean value of 3 replicated counts………………...………………………………126

Figure 5.16 Stability of microencapsulated *L. casei* cells in freeze dried microparticles when stored at 4°C. Graphs showing TB (●), LB (■), TMB (■) and LMB (▲) samples in comparison with the control sample (♦). Error bars representing standard deviations from the mean value of 3 replicated counts..…………..……………………………..128

Figure 5.17 Stability of microencapsulated *L. casei* cells in freeze dried microparticles when stored at 37°C. Graphs showing TB (●), LB (■), TMB (■) and LMB (▲) samples in comparison with the control sample (♦). Error bars representing standard deviations from the mean value of 3 replicated counts..…...……………………………………..129

Chapter 1.0. Introduction

At the beginning of the 20th century, the importance of *Lactobacilli* for human health was first hypothesized by the famous Russian biologist Ilya Metchnikoff but he considered the total community of gut microbes as detrimental and suggested that the desirable effects can be obtained if they are substituted by the yoghurt bacteria (Holzapfel *et. al*., 1998). Since then a lot of research works and commercial practices have been guided towards improving the human and animal health status by modulating the gut microbiota with live microbial adjuncts, also known as probiotics.

Probiotic bacteria are natural inhabitants of the human gastrointestinal tract but due to the action of antibiotic compounds (e.g. treatment of diseases with antibiotics) the presence of the microflora is badly affected. Therefore, it is important to restore their balance in the gut, by supplying from an outside source in the form of dietary supplements or functional foods containing live probiotic cultures. In doing so, the cultures need to be grown, stored and applied to food products. During the manufacturing process used in food products, the probiotic bacteria are subjected to various kinds of stresses such as, heat, cold, osmotic pressure and oxygen. The most detrimental among them is the heat stress (e.g. during the spray drying process). A huge loss of cell viability takes place during storage as an ingredient or as a part of any food product.

Following ingestion with a food or in the form of capsules (dietary supplement), the bacteria are exposed to the very harsh acidic environment in the stomach where the pH ranges from 1.0 to 2.0 and thereafter again to the detrimental bile salts present in the intestinal pathway. Probiotics are also exposed to milder acidic environments when added to fermented food, juice or beverages. The exact mechanism of bile salt toxicity is yet to be completely understood but it has been reported that bile salts being surface active amphipatic molecules, possess some antimicrobial properties and their detergent like nature aids in disassembling bacterial cell membranes (De Angelis and Gobbetti, 2004). Osmotic stress is induced to probiotic bacteria when the solute concentrations of

the surrounding media changes suddenly. It is important to maintain the cytoplasmic solute concentration at a constant level for the bacteria to survive. An increase in media osmolarity results in diffusion of water from cell inside to outside, thereby causing changes in cell volume and subsequent cell deaths (Poolman and Glaasker, 1998). *Lactobacillus* and *Bifidobacterium*, the major two probiotic species are anaerobic in nature and therefore exposure to oxygen during processing and storage creates another stressful environment to them (Guerzoni *et. al.*, 2001). Overall, it can be seen that a number of challenges are involved in the process of successful delivery of viable probiotic cultures right from the collection and growth stage up to the human intestinal walls where they are supposed to colonize in order to impart the expected health benefits.

The choice of ingredients for the protective matrix is an important factor in increasing the stability of the probiotic bacteria. A variety of food materials including carbohydrates, polysaccharides, plant-hydrocolloids, bacterial exopolysaccharides, plant and milk proteins and cellulose have been used in the encapsulation matrix by researchers, the most common among them being sodium alginate gels in the presence of divalent cataions (Anal and Singh, 2007). The use of dairy proteins in microencapsulation (also referred as encapsulation or entrapment throughout this thesis) technology has been studied with a great interest due to its well-known functional properties (Rosenberg and Sheu, 1996; Chen and Subirade, 2007). Some of the functional properties of dairy proteins, for example, bland flavor, high solubility, emulsifying and film forming nature and low viscosity in solutions have made them very attractive for use as one of the shell materials in the microencapsulation process (Madene *et. al.*, 2006; Heidebach *et. al.*, 2009). Food proteins easily meet GRAS standards, have high nutritional value and excellent gelation, foaming and water binding capacity, thus making them highly suitable for probiotics or any kind of neutraceutical carrier material, which can be orally administered (Chen *et. al.*, 2005).

After the microencapsulation process is completed it is important to convert the microparticles into a dried form, to ensure prolonged storage and future use in functional

food or neutraceutical applications. This dehydration also aids in easy handling and transport of the probiotic cultures. The most common drying methods applied in such circumstances are freeze drying and spray drying, but both of them impart high physiological stresses to the live bacteria during the processing steps. Spray drying generally involves exposure of the microencapsulated particles to a high inlet air temperature (varying from 160 to 220°C) for a small duration. This causes extensive heat stress to the bacteria and consequent loss of integrity of the live cells. The cytoplasmic membranes, cell wall, DNA and RNA of the cells are affected due to heat exposure which leads to loss of metabolic activity (Teixeira *et. al.*, 1995; 1997). However, compared to spray drying, freeze drying is much less stressful to the live bacterial cells. Freeze drying involves a freezing step at sub zero temperature followed by sublimation of the formed ice crystals under vacuum. The sublimation cum drying step is carried out at a moderate temperature of around 20°C and hence is not stressful to the live cells (Tsvetkov and Brankova, 1983). Therefore, freeze drying is preferred most of the time to stabilize heat sensitive bioactive materials such as probiotic bacteria.

The major objectives of the current study were:

1) To develop a delivery system of probiotics to the human gastro-intestinal (GI) tract by developing a protective matrix surrounding the bacterial cells. The protection should be against the external processing stresses and also internal harsh GI tract environment as mentioned above. Finally the cells need to be successfully released from the matrix in the intestine. Therefore, the encapsulating matrix should be stable at low pH but should go into solution and release the content at neutral pH.

2) To develop the encapsulated probiotic ingredient in a freeze dried form to ensure longer storage life and easy handling properties.

3) To overcome the stresses associated with the freeze drying process, by incorporating suitable cryoprotective compounds in the encapsulating matrix. This will ensure a higher cell population in the developed ingredient, which is important from legal and economic points of view.

4) As mentioned earlier, a major challenge in probiotic food development is to confer adequate protection to the live cells against the harsh acidic and bile salts environment in the gastro-intestinal tract. Therefore, another objective of this project was to study whether the microencapsulated matrices are able to offer any such benefit to the encapsulated cells against the prevailing GI conditions, in an *in vitro* model.

Chapter 2.0. Literature review

2.1 Introduction

The literature review is divided into 3 parts. Part 1 provides an introduction to probiotic bacteria, their selection criterion and the challenges associated with handling them. This part also discusses the health benefits of the probiotic strains and associated regulatory aspects in short.

The second part discusses the various microencapsulation technologies for the probiotic bacteria for successful delivery and controlled release. The advantages and disadvantages of various encapsulation techniques are discussed. In particular, microencapsulation technology using dairy proteins has been discussed in detail and the potential of developing newer methods has been identified.

Part 3 of this review discusses the challenges associated with freeze drying of encapsulated probiotic cells and various approaches for addressing the challenges. The focus is primarily on application of a range of cryoprotectants to improve cell survival after freeze drying.

2.2 What are probiotic bacteria?

2.2.1 Definitions

Probiotic bacteria have been defined in many ways by different people but perhaps the most widely used and accepted definition is proposed by Fuller (1992) according to which probiotics are living microorganisms which when ingested have beneficial effects on the host by improving the physiological functions of the intestinal microflora. Another definition had been provided by Havenaar and Huis In't Veld (1992), which explains probiotics as "a preparation of or a product containing viable, defined micro-

organisms in sufficient numbers, which alter the microbiota (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host". Probiotics have also been defined as "live microbes which transit the gastro-intestinal tract and in doing so benefit the health of the consumer" (Tannock *et. al.*, 2000), which contradicts the earlier definitions focusing only on the interactions between the probiotic organisms and the original intestinal microflora (Fuller, 1989). A recent definition can be found in The Food and Agricultural Organization of the United Nations and the World Health Organization's documents which defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). But all of these definitions agree in one point that probiotics must deliver some kind of health benefits and should be living in nature, and able to form colonies on the intestinal wall.

2.2.2 Characteristics of probiotic bacteria

A particular bacterial strain is considered as probiotic in nature only when it fulfils certain criteria. These include the physiological and manufacturing demands through which the bacterial strain must survive and also they should have all the capabilities to exert the beneficial effects on the host as per their basic definition (B. O'Grady, 2005). One of the conditions namely colonization was however challenged by Tannock (2003), according to whom the transit time of the intestinal tract allows smooth multiplication of bacteria even if there is cell loss with faecal material. This becomes important while considering the nature of allochthonous microorganisms which exerts effect from their luminal habitat and this proves colonization is not an essential condition for a probiotic strain (B. O'Grady, 2005).

Probiotic dairy products are categorized as 'novel type' and the European market study shows a steadily increasing range of yoghurt like products claiming to contain probiotic strains (Reuter, 1997). A probiotic microorganism is supposed to promote health of the host in general but to critically identify a particular strain as probiotic, following three

categories of key criteria have been identified, which are desirable in any probiotic strain (Havenaar and Huis In't Veld, 1992; Holzapfel *et. al*., 1998).

2.2.2.1 General microbiological criteria

The factors to be considered in this case are safety or non-pathogenicity, survival strength through the human defense system in the upper part of GIT (containing saliva, gastric juice and bile juice), should be of human origin and must have genetic stability.

2.2.2.2 Technological aspects

The strain should be adaptive to the most common manufacturing stresses and should impart an acceptable shelf life and sensory qualities in terms of color, aroma, taste and texture. Viability throughout the period of claimed shelf life is also an important factor.

2.2.2.3 Functional benefits of probiotic bacteria

This is the most important and a particular strain is considered as probiotic if it is able to confer certain beneficial traits to the human body.

The important functional benefits obtained in general from a single or group of probiotic strains include immune modulation and improving the strength of gut mucosal barrier. These are achieved by modification of gut microflora, capability to adhere with intestinal mucosa which in turn helps to prevent pathogen adherence or activation, by modifying the dietary proteins and bacterial enzyme capacity and by influencing the gut mucosal permeability (Holzapfel *et. al*., 1998).

According to Kailasapathy and Chin (2000), "probiotics have been reported to play a therapeutic role by modulating immunity, lowering cholesterol, improving lactose tolerance and preventing some cancers." The probiotic health benefits are also

associated with digestive and respiratory functions, prevention and restriction of infectious disease in children and other high risk populations (FAO/WHO, 2001).

2.2.3 Probiotics in human gastro-intestinal tract

The human gastrointestinal tract (GI) is a very complex and sophisticated ecosystem comprising of more than 400 different species and out of them about 40 species predominate (Freter, 1992; B. O'Grady, 2005). The GI tract starts from the oral cavity, which is made up of mouth, nose and throat and a complex mibrobiota exists here (Marsh, 1980; B. O'Grady, 2005). It has been reported that bacteria in plenty of numbers can be found on the posterior and anterior tongue, sub and supragingival plaque, buccal mucosa and vestibular mucosa (Willis *et. al.*, 1999; B. O'Grady, 2005). The acidic environment inside the stomach provides a highly effective barrier against pathogenic and benign invading microorganisms and here the cell count falls below 10^3 colony forming units (CFU) ml^{-1} of the gastric contents. The small intestine does not provide a healthy environment that promotes the growth of bacteria due to rapid transit time, low pH and presence of bile. The same holds true for duodenum which also has low microbial population and secrets intestinal fluid creating a hostile environment (Sanford, 1992; B. O'Grady, 2005). However, along the jejunum and ileum the numbers as well as the variety of probiotic species increase progressively. The large intestine is favorable for the growth of these bacteria due to factors like slow transit time, a favorable pH environment and availability of nutrients (Suau *et. al.*, 1999; B. O'Grady, 2005). The small intestine contains species such as, *Enterococci, Enterobacteria, Lactobacilli, Bacteroides* and *Clostridia* in the range of 10^4 – 10^6 cfu/ml, and this population goes up to 10^{11} – 10^{12} cfu/ml in the large intestine (Salminen *et. al.*, 1998; B. O'Grady, 2005).

The human gastrointestinal tract remains sterile until the birth of a newborn and then the microbial colonization process begins during the delivery process. The inoculum is mainly obtained from the mother’s vaginal or fecal flora in case of conventional birth or from the environment when there is a caesarean delivery. This probiotic population

gradually evolves in a fashion which can best cope with the physiological and microbiological pressure encountered within the ecosystem. This resistance, also known as 'barrier effect', helps the indigenous microbiota to fight against invading pathogens and to compete for nutrients (Alderbeth *et. al.*, 2000; B. O'Grady, 2005). In the very beginning, *E. coli* and *Streptococcus* dominate but gradually along with breast feeding, *Bifidobacterium* population starts increasing with a decrease of the other two mentioned. There is strong indication that the type of diet greatly influences the diversity of the intestinal microflora of a newborn (Bullen *et. al.*, 1977; Mevissenverhage *et. al.*, 1985; Holzapfel *et. al.*, 1998).

The healthy intestinal epithelium having a good intestinal flora can create a barrier against the uptake of pathogens, antigens and other harmful compounds from the gut lumen. The intestinal mucosa also is very efficient in assimilating antigens and evoking specific immune responses (Heyman *et. al.*, 1982; Holzapfel *et. al.*, 1998). Baba *et. al.* (1991) showed that intestinal probiotics challenge the invading microorganisms and prevent diseases through competitive exclusion and this was best demonstrated by showing that germ-free animals were more susceptible to infection and diseases. It is therefore understood that composition of intestinal microflora greatly influences the gastrointestinal health and affects the host health (B. O'Grady, 2005).

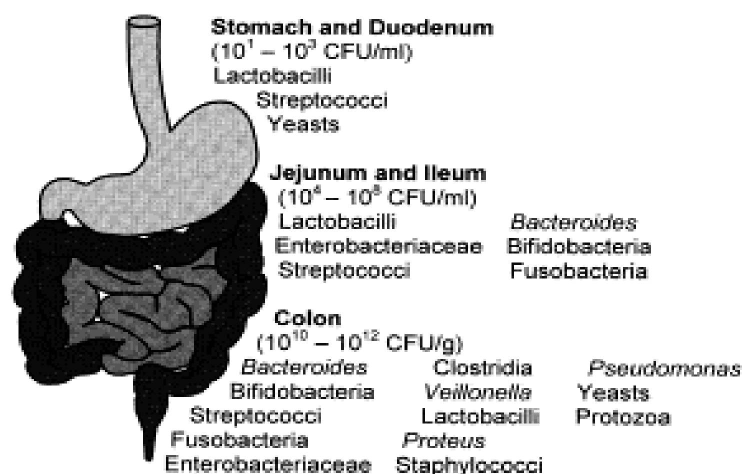


Figure 2.1 Microbial colonization of the human gastro-intestinal tract (Simon and Gorbach, 1982; Holzapfel *et. al.*, 1998)

The intestinal flora has a wide range of beneficial activities such as degrading certain food components, producing certain B vitamins, stimulating the immune system and producing digestive and protective enzymes. It also helps in metabolizing some carcinogenic substances. The colonic flora produces short chain fatty acids (SCFA) which are passively absorbed by the enterocytes (Hoverstad, 1989; Holzapfel *et. al*., 1998).

2.2.4 Some common probiotic species and strains

Nowadays a large number of species have been recognized as having probiotic characteristics. Major probiotic bacteria, mainly the *Lactobacilli* and *Bifidobacterium* are of high importance to the researchers and majority of researches have been done on them. Consequently, they have been used widely in the food industry as probiotic organisms. Probiotics are commercially marketed either in a lyophilized form or as fermented food products. *L. acidophilus*, *L. casei* strain *Shirota*, *L. rhamnosus* and *L. reuteri* are the most popular choices and have a long application history followed by some *Bifidobacterium* spp. and also a few non-lactics which are mainly used in pharmaceutical applications (Holzapfel *et. al*., 1998). Table 2.1 below shows a list of popular probiotic strains.

Table 2.1 Microorganisms used or considered for use as probiotics in human (Vuyst *et. al.*, 2004)

Lactobacillus species	*Bifidobacterium* species	Other lactic acid bacteria	Other organisms
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis**	*Bacillus cereus (e.g toyoi)**$
L. amylovorus	*B. animalis*	*Enterococcus faecum**	*Escherchia coli (e.g Nissle*, 1917)$
L. casei	*B. bifidum*	*Lactococcus lactis*	*Propionibacterium freudenreichii**$
L. crispatus	*B. breve*	*Leuconostoc mesenteroides*	*Saccharomyses cerevisiae*$
L. gallinarum	*B. infantis*	*Pediococcus acidilactici*	*Saccharomyses boulardii*$
L. gasseri	*B. lactis*	*Sporolactobacillus Inulinus**	
L. jhonsonii	*B. longum*	*Streptococcus thermophilus*	
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			
L. salivarius			

* Mainly applied in animals.

$ Mainly applied in pharmaceutical preparations.

2.2.4.1 *Lactobacillus* species

Lactobacilli are one of the most abundant probiotic organisms and are characterized by these most common properties like gram-positive, non-sporulating, catalase-negative and oxidase-negative, devoid of cytochromes, non-aerobic in nature but can tolerate oxygen to some extent, are fastidious and strictly fermentative. The end product of their sugar fermentation is lactic acid (Axelsson, 1993; Anal and Singh, 2007;). They have beneficial effects to the gut health in the form of easy digestion, immune stimulation and inhibition of pathogens (Stanton *et. al.*, 2003). *Lactobacilli* are not the major species present in the gastrointestinal tract but normally they are very easily cultivated there and they have been used safely for a long time in various food applications (Stanton *et. al.*, 2003). The most common among the all species found in the human gut is *Lactobacillus acidophilus* complex, *Lactobacillus salivarius* and *Lactobacillus casei* complex (Dunne *et. al.*, 1999). *Lactobacillus acidophilus* complex group comprises of 6 similar phenotype traits but *L. acidophilus* is most commonly used in probiotic preparations due to its acid and bile resistant qualities, antimicrobial activity and survivability during the gastro-intestinal transit (Dunne *et. al.*, 1999). *Lactobacillus salivarius* is known as beneficial for its resistant property to acid and bile, good adherence quality to gastric epithelial cells and smooth transportability through the intestinal tract. *Lactobacillus casei* complex includes *Lactobacillus casei/paracasei*, *Lactobacillus rhamnosus GG* and *Lactobacillus casei shirota* (Vuyst *et. al.*, 2004).

2.2.4.2 *Bifidobacterium* species

Bifidobacteria are classified as Gram-positive, non-sporulating, catalase-negative, non-acid fast bacilli. They are strictly anaerobes and are difficult to cultivate in milk and other food preparations (Vuyst *et. al*., 2004). They have a low tolerance to acid, only tolerant to pH 4.5 and above, and are unable to grow in a medium with high oxidative potential (Stanton *et. al*., 2003). The carbohydrate fermentation end product of *Bifidobacteria* are mainly acetic acid, lactic acid but not carbon dioxide, butyric acid or propionic acid (Anal and Singh, 2007). *Bifidobacteria* are the predominant variety in infant colon, with the *Bifidobacterium longum*, *B. infantis* and *B. breve* dominating and they are the third most populous in the adult colon with the maximum presence of *Bifidobacterium longum* and *B. adolescentis* and therefore are frequently used as probiotic culture (Vuyst *et. al*., 2004).

2.2.5 Health benefits and human clinical trials with probiotic bacteria

There is a huge body of research publications regarding the wide range of probiotic applications on human clinical trials. This subject is not in the scope of current review, but a short glimpse of the important and common studies are mentioned below.

Idiopathic inflammatory bowel disease is the most common disease associated with disturbed gut microflora and two major forms of this are Crohn's disease and ulcerative colitis. Several attempts have been made to treat these two diseases (Guslandi *et. al*., 2000; 2003; Ishikawa *et. al*., 2003; O'May and Macfarlane, 2005) with the application of probiotics.

Pouchitis is a type of chronic inflammation of the ileal pouch which follows the colectomy in ulcerative colitis patients. The effectiveness of probiotics in treating this disease has been investigated in a number of studies, the most interesting one being that of Gionchetti *et. al*. (2000), who used a randomized controlled trial among 40 patients and found that a significant percentage of people receiving probiotics showed

improvement in pouchitis condition in comparison to those taking only maize starch placebo (O'May and Macfarlane, 2005).

The other important gastro-intestinal diseases on which clinical trials have been conducted with probiotics and found successful are irritable bowel syndrome (O'Sullivan and O'Morain, 2000; Sen *et. al*., 2002; Kim *et. al*., 2003), antibiotic associated diarrhoea and colstridium difficile associated diarrhoea (Thomas *et. al*., 2001; Seki *et. al*., 2003; Plummer *et. al*., 2004), traveller's diarrhoea (Oksanen *et. al*., 1990; Katelaris *et. al*., 1995), infant diarrhoea (Rosenfeldt *et. al*., 2002; Gaon *et. al*., 2003). The results have been summarized by O'May and Macfarlane (2005).

Among the extra-gastrointestinal conditions in which probiotics application have been widely tested are atopic dermatitis and bacterial vaginosis (Kalliomaki *et. al*., 2003; Reid *et. al*., 2003; O'May and Macfarlane, 2005). In this context, another summarized and published table from Salminen *et. al*. (1996) is thought to be relevant and therefore reproduced in Table 2.2.

Table 2.2 Different probiotic bacteria strains and their health benefits (Salminen *et. al.*, 1996; Holzapfel *et. al.*, 1998)

Strain	**Reported effects in clinical studies**
Lactobacillus acidophilus LA1	Immune enhancer, adjuvant, adherent to human intestinal cells, balances intestinal microflora.
Lactobacillus acidophilus NCFB 1748	Lowering feacal enzyme activity, decreased feacal mutagenicity, prevention of radiotherapy related diarrhea, treatment of constipation.
Lactobacillus GG (ATCC 53013)	Prevention of antibiotic associated diarrhea, treatment and prevention of rotavirus diarrhea, treatment of relapsing Clostridium difficile diarrhea, prevention of acute diarrhea, Crohn's disease, antagonistic against cariogenic bacteria, vaccine adjuvant.
Lactobacillus casei Shirota	Prevention of intestinal disturbances, treatment of rotavirus diarrhea, balancing intestinal bacteria, lowering feacal enzyme activities, positive effects in the treatment of superficial bladder cancer, immune enhancer in early colon cancer, immune enhancement.
Streptococcus thermophilus & *Lactobacillus bulgaricus*	No effect on rotavirus diarrhea, no immune enhancing effect during rotavirus diarrhea, no effect on feacal enzymes.
Bifidobacterium bifidum	Treatment of rotavirus diarrhea, balancing intestinal microflora, treatment of viral diarrhea.
Lactobacillus gasseri (ADH)	Feacal enzyme reduction, survival in the intestinal tract.
Lactobacillus reuteri	Colonising the intestinal tract, mainly animal studies so far, possibly an emerging human probiotic.

2.2.6 Regulations governing foods containing probiotics

Though the concept of using beneficial bacteria in food products originated at the very beginning of the 20th century, the term ‘probiotic’ appeared in the documents only in the 1960s. From a legal perspective, a number of definitions have appeared in the scientific literatures but no internationally recognized legal definition has been published so far. However, since the products containing probiotic cultures are becoming more and more popular, a risk is always there that consumers will be misled in terms of health claims or number of viable probiotic cells present in the product (Hicky, 2005). In the European Union (EU), the proposed regulation defines probiotic bacteria as “live food supplements which benefit the health of consumers if consumed regularly in sufficient quantities”. It also proposes that any kind of health claim should be supported by sufficient scientific data and a product label should contain the quantity of food to be consumed necessarily to obtain the claimed health benefit, the persons who should avoid the product and if applicable, a warning showing the possible bad effects of consuming the product in excess of the recommended quantity (Hicky, 2005). According to an experts’ report from United Nations Food and Agriculture Organization (FAO) and World Health Organization (WHO), a product claiming to have probiotic culture in it should contain at least 10^6 – 10^7 cfu live viable bacteria per gram of the product (FAO/WHO, 2001).

United Kingdom, being a member state of the EU, is expected to implement the same community legislation. Even before the EU came into existence, in order to protect consumers and to promote fair trade, a Functional Food Initiative was taken up by the Ministry of Agriculture, Food and Fisheries (MAFF) in 1994 to get an overview of the marketplace and thereafter a Joint Health Claim Initiative (JHCI) was established in 1997 which involved consumer groups, enforcement authorities and industry representatives. The JHCI came up with some regulations which clearly mentioned the health claim requirements (Hicky, 2005).

In the USA however, there is no specific legislation on probiotic or functional foods. The Food and Drug Administration (FDA) has classified all the possible foods into four categories, viz. conventional foods, dietary supplements, foods for special dietary use and medicinal foods. In theory, probiotic products can be placed into any of the above categories but mostly the commercial products have been marketed as a dietary supplement only (Hicky, 2005).

The Codex Alimentary Commission (CAC) has adopted a new standard after its 26th session in July 2003 which is expanded to a broad range of fermented products including yogurt, sweetened/flavoured yogurt, kefir, acidophilus milk and koumiss. Though this standard does not mention probiotics but includes a minimum level of starter culture organisms of 1×10^7 cfu g^{-1} and if a specific microorganism other than the normal starter culture is claimed to be present then the minimum level should be 1×10^6 cfu g^{-1} (FAO/WHO, 2003; Hicky, 2005).

2.2.7 Mechanism of probiotic bacteria functionalities

The microbiota of the intestine is mainly involved in the fermentation process of various endogenous and exogenous growth substrates, the metabolic end products of which are beneficial to human health (Macfarlane and McBain, 1999; B. O'Grady, 2005). It is believed that one of the mechanisms utilized by the beneficial probiotics present in the gut is by colonic fermentation of starches, which are resistant to the action of pancreatic amylases and also by saccharolysis of few oligosaccharides and a variety of sugars, which results in the production of various short-chain fatty acids (SCFA) such as, butyrate, acetate, propionate and lactate. These SCFA's finally result in a lower pH of the lumen which protects against invading pathogenic microorganisms and also reduces the transformation of primary bile acids into secondary pro-carcinogenic bile acids (Cummings and Macfarlane, 1997; B. O'Grady, 2005).

The mechanism of the beneficial traits offered by probiotic microorganisms to human host has been well described (Dunne *et. al.*, 1999; Kailasapathy and Chin, 2000; Naidu

and Clemens, 2000; Andersson *et. al.*, 2001; Isolauri and Salminen, 2005). By reinforcing body's natural defenses and keeping out harmful pathogens by competitive exclusion, probiotics make their way to grow and colonize in the intestinal wall (Anal and Singh, 2007). This anti-pathogenic mechanism consists of several antagonist properties including, (a) reduction of luminal pH by producing a series of volatile short-chain fatty acids (SCFA); (b) making specific nutrients unavailable to the pathogenic organisms; (c) decreasing the redox potential thus suitable for only anaerobic probiotic growth; (d) production of hydrogen peroxide in anaerobic condition; and/or (e) production of pathogen inhibitory compounds known as bacteriocins (Naidu and Clemens, 2000; Hoover and Chen, 2005).

2.2.7.1 Reduction of luminal pH

The fermentation process of carbohydrates in the lumen of the colon uses lactate dehydrogenase to convert pyruvate and thereby produces lactic acid and other short chain fatty acids such as acetic, propionic and butyric acid (Kailasapathy and Chin, 2000). These acids reduce the intestinal lumen pH which is unfavorable for both Gram-positive and Gram-negative bacteria.

Lipophillic acids such as, lactic and acetic acid are present in undissociated forms in the colon. These acids possibly penetrate through the pathogenic cell membranes and inhibit their growth (Naidu and Clemens, 2000). Acetic and lactic acids produced by *Bifidobacteria* have been shown to have inhibitory effects against *Salmonella* spp., *E. coli* and *Staphylococcus aureus* (Naidu and Clemens, 2000).

Apart from pH reduction, probiotics have been reported to produce phenols, ammonia, steroid metabolites and bacterial toxins which are also helpful in pathogenic bacterial growth restriction (Hidaka and Eida, 1988; Naidu and Clemens, 2000).

2.2.7.2 Production of hydrogen peroxide

Lactic acid bacteria (LAB) such as *Bifidobacterium* and *Lactobacilli* are able to produce hydrogen peroxide (H_2O_2) by using the electron transport chain. The strong oxidizing property of H_2O_2 affects the permeability of the pathogenic cell membranes. Specifically, the actions on cell components such as nucleic acids are responsible for the antimicrobial activity of LAB (Naidu and Clemens, 2000).

2.2.7.3 Production of bacteriocins

Bacteriocins are defined as a variety of antimicrobial proteins including metabolic products, antibiotic-like substances and bactericidal proteins produced by probiotic bacteria (Naidu and Clemens, 2000). Bacteriocins act through adsorption to receptors on the surface of target bacteria and they are species specific. The destruction of the target bacteria is the result of biological, morphological and metabolic changes caused by the bacteriocins.

Bacteriocins are mainly of 4 major types – antibiotics, heat-stable peptides, large heat-labile proteins and complex proteins whose activity require the association of carbohydrate or lipid moieties (Naidu and Clemens, 2000; Hoover and Chen, 2005).

The major bacteriocins documented are nisin, pediocin, sakacin and reuterin, all produced by LAB. Nisin, produced by *L. lactis* has a specific antagonistic activity to *L. monocytogenes* through the formation of pores in cell membranes (Hoover and Chen, 2005). Similarly, reuterin produced by *L. reuteri* is a broad spectrum bacteriocin capable of inhibiting Gram-negative species such as, *Salmonella* and *Sigella* and Gram-positive species such as, *Clostridia* and *Listeria* (Hoover and Chen, 2005).

2.2.7.4 Fermentative production of carbon dioxide

The production of carbon dioxide by *Lactobacilli* is well described by Naidu and Clemens (2000). Hexose fermentation by heterofermentative *Lactobacilli* results in the

production of carbon dioxide. The production of carbon dioxide displaces oxygen in the colon and also decreases the pH. Thereby, an anaerobic environment is created where strict aerobic organisms find difficulty in proliferating.

2.2.7.5 Diacetyl production by fermentation

By the fermentation of pyruvates, *Lactobacilli* are able to produce diacetyl and acetaldehyde. Diacetyl interferes with arginine utilization in arginine-binding protein of Gram-negative species, thereby making them very susceptible (Jay, 1991; Naidu and Clemens, 2000). *Escherichia coli,* for example is one of the most susceptible species to diacetyl but some other species also reported to be susceptible are *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Salmonella anatum* (Motlagh *et. al*., 1991; Naidu and Clemens, 2000).

2.2.7.6 Deconjugation of bile acids

Bile acids in the form of conjugates with glycine or taurine are secreted in the duodenum. *Bifidobacterium* can reduce the cholesterol reabsorption by deconjugating bile acids and by hydrolysis of sodium taurocholate and glycocholate in the colon (Ballongue, 2004).

It is proven that certain lactic acid bacteria (LAB) have specific immune regulating properties and as a result they interact with mononuclear phagocytes and endothelial cells of the host (Brassart and Schiffrin, 1997; Holzapfel *et. al*., 1998). Some particular strains of LAB showed adjuvant properties after infection with pathogenic microorganisms and this is achieved by stimulation of a specific antibody response (Pouwels *et. al*., 1996; Holzapfel *et. al*., 1998).

2.2.8 Challenges involved in incorporating probiotics into foods

2.2.8.1 Food processing factors

Common food processing parameters are designed to prevent pathogenic contaminations as well as to kill the existing harmful microbial population to make the food safe for consumption. Therefore, incorporating probiotic bacteria into the same foods become difficult if the established processing conditions are followed. For example, heat treatment and drying are the two common techniques for enhancing the shelf life of a food but both of them are detrimental to some extent for the probiotic bacteria. Another big challenge is to restrict the multiplication of probiotic bacterial cells once they are added into a food, otherwise instead of adding value to it, such bacterial growth would lead to spoilage of the product itself.

Other detrimental conditions for the survival of any probiotic culture in fermented dairy products reported are titratable acidity, pH, hydrogen peroxide, storage temperature, presence of other species and strains, lactic and acetic acid concentration and presence of whey protein concentration (Kailasapathy and Supriadi, 1996; Lankaputhra *et. al*., 1996; Dave and Shah, 1997; Anal and Singh, 2007).

Also, some physiological traits such as the susceptibility of probiotic *Lactobacilli* and *Bifidobacteria* towards acid, bile and oxygen stresses have made incorporation of them difficult in dairy foods (Stanton *et. al*., 2003). Each of these factors is described below in detail.

2.2.8.2 Exposure to gastric acid present in stomach

Acid tolerance is another important quality a probiotic strain should possess because the gastric pH frequently falls below 2.0 (McLauchlan *et. al*., 1998; Stanton *et. al*., 2003). *L. acidophilus* have been able to show a good resistance around pH 2.0 and maintain near neutral cytoplasmic pH (Kashket, 1987; Stanton *et. al*., 2003). Acid tolerance can be

improved in a number of ways such as, up-regulation of the gene responsible for stress protection and by acidic environment adaptation (Kullen and Klaenhammer, 1999; Shah, 2000; Stanton *et. al*., 2003). Similarly, the acid and bile tolerance in case of *Bifidobacteria* also vary a lot among different strains. Species such as, *B. longum*, *B. pseudolongum*, and *B. animalis* are highly aciduric in nature with *B. longum* has been reported to survive up to 4.0% bile concentration (Clark and Martin, 1994; Lankaputhra and Shah, 1996; Stanton *et. al*., 2003).

2.2.8.3 Exposure to bile salts present in intestinal fluid

An essential condition to select a particular probiotic strain is their ability to survive the transit through small intestine and the tolerance towards bile salts present there (Charteris *et. al*., 1998; Stanton *et. al*., 2003). Haller *et. al.* (2001) reported that *Lactobacilli* of intestinal origin are more bile resistant than those obtained from fermented foods. It was estimated that only 1.3 to 1.5% of the orally ingested *L. acidophilus* cells via fermented foods ultimately survived through the intestinal transit. Some strains of *Lactobacilli* have been reported as bile stress tolerant and hence they are a good choice of selecting as a probiotic culture. There are some strains capable of deconjugating bile acids using the bile salt hydrolase enzyme, though the importance of this activity has not been completely understood (Marteau *et. al*., 1993; Stanton *et. al*., 2003).

2.2.8.4 Oxygen intolerance of probiotic strains

The oxygen content and the redox potential of the environment containing probiotics play very important roles in deciding their viability. The anaerobic probiotic strains are directly affected by the presence of oxygen in both intestinal microbial ecosystem and exogenous oxidative stress conditions (Stanton *et. al*., 2003). The survival of *L. acidophilus* in yogurt was shown directly related to the oxygen permeability of the packaging materials (Dave and Shah, 1997). An anti-oxidative strain of *Lacobacilli*, identified as *L. fermentum*, have been found more viable in presence of oxygen

compared to other non-oxidative strains (Kullisaar *et. al*., 2002; Stanton *et. al*., 2003). The future research towards understanding the molecular mechanisms responsible for this particular anti-oxidative stress behaviour could lead to the development of more aero-tolerant *Lactobacilli* strains (Stanton *et. al*., 2003). Since *Bifidobacterium* is highly anaerobic, the oxygen sensitivity is very important in selecting a strain. They are more or less affected in several ways due to presence of oxygen and it was reported that *B. longum* in an oxygenated environment experienced an extended lag phase and growth was limited, the cell morphology altered, with the cells becoming longer in size and also the cellular fatty acid profiles changed (Ahn *et. al*., 2001; Stanton *et. al*., 2003).

Therefore, it is apparent that the probiotic strains when administered along with a food preparation have to pass through many adverse conditions as discussed above. To increase their resistance against these adverse environments, several approaches have been proposed such as, selection of acid and bile resistant strains, packaging in oxygen protected materials, double-step fermentations, pre-adaptation to various stress conditions, adding micronutrients in the form of amino-acids and peptides and most importantly, microencapsulation (Gismondo *et. al*., 1999; Anal and Singh, 2007).

2.2.9 Selection chriteria of a probiotic strain for microencapsulation and subsequent food applications

Several probiotic strains available commercially may have been proven clinically beneficial to human host but all of them may not be suitable for food applications because of the challenges as discussed in section 2.8. Though the purpose of microencapsulation is to provide adequate protection to the strains against such adverse environment, it is always preferable to select strains which are already resistant (Ref). In a recent study, Kotikalapudi et.al. (2010) tested 4 commercial probiotic strains against in vitro acid tolerance, bile salt exposure and registance against anti-microbial compounds. It was reported that each strain acted differently against such challenges and finally *L. acidophilus* ATCC 11975 was found to be most suitable for encapsulation purpose. The importance of selecting acid and bile tolerant strains has also been stressed upon in a

review by Sarkar (2010) where many strains of *Lactobacillus* and *Bifidobacterium* spp. have been found to be vulnerable to acid and bile stress. It was reported that *B. longum* survived better than *B. infantis*, *B. adolescentis* and *B. bifidum* in an acidic incubation test. Among the *Lactobacilli* strains, *L. acidophilus* ATCC 4962, *L. casei* ASCC 290 and *L. casei* ASCC 292 were found to be very robust against acidic condition.

2.3 Microencapsulation overview

2.3.1 Definition

A general definition of the microencapsulation technique is given by Champagne and Fustier (2007), according to which, it is a method of packaging a desired food material in miniature sealed capsules, made up of a suitable food grade matrix, which are able to release their contents at controlled rates and under controlled environmental triggers, such as, shear, temperature, enzymes, pH etc. The size range of the microcapsules generally varies from sub micrometer to a few millimeters depending upon the materials and technology adapted to produce them. Microencapsulation of probiotic cultures basically involves applying a physical barrier around the living cells which should ideally be capable of providing shelter against all the adverse external conditions, which it might face during processing, storage and gastro-intestinal passage. Earlier, immobilized or entrapped cells were mainly used in bio-technological applications, the basic purpose being to keep the cells separate from metabolites. But in relatively recent times, the refined processes of microencapsulation have been applied to stabilize the cells, enhance the viability and stability during the production, storage and handling of lactic acid cultures etc. (Kailasapathy, 2002).

Immobilization and encapsulation are two terms that are often used interchangeably but in fact immobilization refers to entrapping the core material within or throughout a whole matrix whereas encapsulation is the process of making a continuous coating around the core particle so that it is completely surrounded by the wall material, thus leaving a little chance of surfacing out (King, 1995; Kailasapathy, 2002).

Microencapsulation has many advantages over the immobilization technique. They are semi permeable, thus allowing the metabolites to pass through but made of strong shell materials retaining the bacterial cells inside. The encapsulated core material is control released either by mechanical breaking of matrix, dissolution of the wall material or melting of the wall, by diffusion or by pressure (Brannon-Peppas, 1997; Kailasapathy, 2002).

2.3.2 Purpose of microencapsulation

Microencapsulation process was developed approximately 30 years ago to protect certain compounds or biological cells from the surrounding environment which could be destructive to the core. The process generally allows smaller molecules to enter or exit the capsules. Examples of encapsulation seen in nature are bird's egg, plant seed, bacterial spore-wall, skin and seashells (Gibbs *et. al*., 1999). Lactic acid bacteria (LAB) were first immobilized in 1975 on Berl saddles and later in alginate beads for the purpose of continuous yogurt fermentation (Linko, 1985; Gibbs *et. al*., 1999). Microencapsulation improves the flow properties of core material and protects them from moisture, oxygen and heat. The process can also improve the nutritional quality of certain oxygen sensitive ingredients such as, vitamins and lipids. In food as well as in pharmaceutical industry, controlled release of flavors, aroma, perfumes, drugs, detoxicants etc. plays a very important role and this is achieved by microencapsulation techniques. In case of effective drug delivery, microencapsulation helps in proper time release, improved stability of medical formulations and flavor masking of obnoxious compounds (Gibbs *et. al*., 1999).

2.3.3 Structure of microcapsules

A microbead or microcapsule is made up of a single or combination of natural food ingredients such as, sugars, gums, proteins, polysaccharides, lipids and synthetic or modified polymers coated around the core component. They are produced either in soft gel form (sometimes referred as "gel beads") or in dried powder form. The surface

morphology of the beads can be smooth or irregular, with or without the presence of pores. Presence of pores is responsible for reduced encapsulation efficiency (Mortazavian *et. al*., 2007). The formed microparticles can take different shapes as illustrated in Figure 2.2 and Figure 2.3 and their encapsulating properties also vary accordingly. The most common classifications are based on matrix materials such as, with single wall material (sodium alginate) or a mix of materials (xanthan and gellan gums, alginate and chitosan, alginate and whey protein etc.) and microcapsule shape formation. The capsule shapes can be of regular and spherical with smooth surfaces or irregular shapes with uneven surface morphology. Sodium alginate microcapsules are generally regular in shape with smooth surface properties (Muthukumaraswamy *et. al*., 2006). On the other hand, presence of milk proteins in the wall material composition and their slow gelling properties generally lead to irregular shaped capsules (Ainsley-Reid *et. al*., 2005). It was also reported that same wall materials produce different shaped and sized capsules depending upon the microencapsulation techniques adapted to produce them. In a study, almost perfect spherical particles with very smooth surfaces were produced with sodium alginate in extrusion process but emulsion technique resulted into irregular shaped, rough surfaced particles (Muthukumaraswamy *et. al*., 2006). Another way to classify microencapsulation is by the manufacturing techniques such as, extrusion, emulsion, drying etc., which are discussed in more details in the following section. Any relationship between the microcapsule structures and the functionality of the probiotic bacteria entrapped therein could not be found in published literatures.

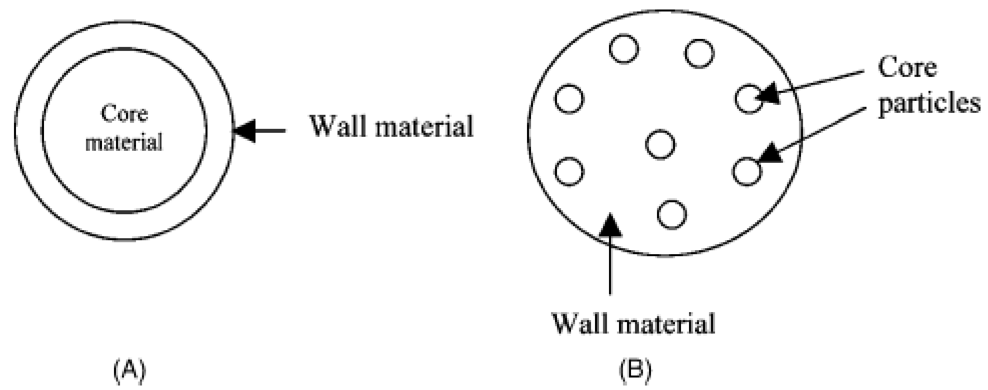


Figure 2.2 Schematic diagram of two representative types of microcapsules, A: Matrix type and B: Aggregate type capsule (Desai and Park, 2005)

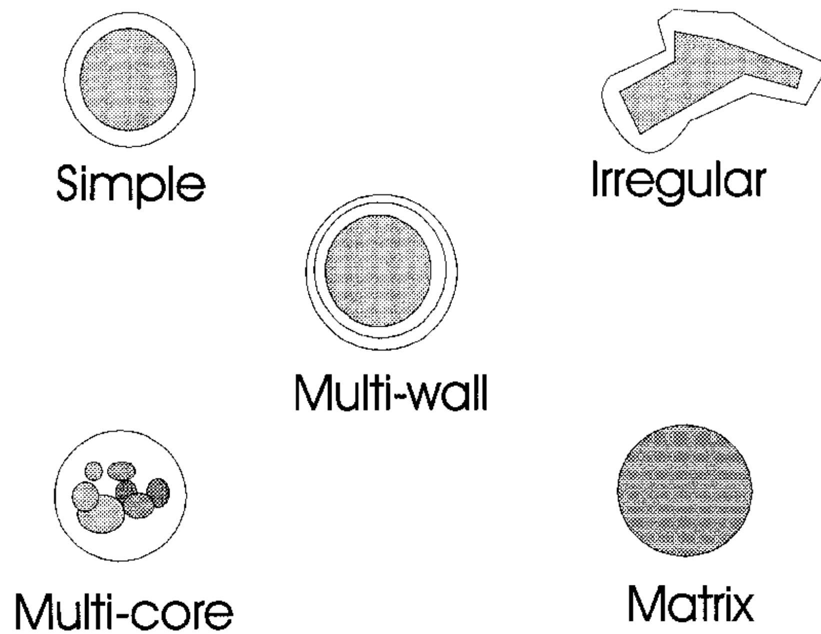


Figure 2.3 Various forms of microcapsules used in the food industry (Gibbs *et. al*., 1999)

2.3.4 Microencapsulation techniques

The most common methods of microencapsulation applied to probiotic bacteria are entrapping into a gel matrix either by extrusion or emulsion techniques (using ionotropic gel forming mechanism), spray drying, spray chilling or coating and freeze drying. The less popular methods in probiotic technology but quite common in other fields of food and neutraceutical world are coacervation, liposomes, molecular inclusion in β-cyclodextrin molecules, fluidized bed coating, centrifugal suspension separation etc. (Champagne and Fustier, 2007). The common techniques of microencapsulation are reviewed below in more details.

2.3.4.1 Extrusion technique for microencapsulation

Desai and Park (2005) described the extrusion process steps in a review. The core material is completely mixed with a molten carbohydrate mass. The carbohydrate mass may comprise of more than one compound. The adhered carbohydrate is then solidified by immersing into a dehydrating liquid and thereby entrapping the core material. The encapsulated matrix is then separated from the liquid bath, and dried using a suitable technology.

Extrusion is the oldest and most common technique for converting hydrocolloids into microcapsules (King, 1995). The property of certain biopolymers such as alginate, carrageenan and pectin to form gels in presence of minerals such as, calcium and potassium has been successfully applied to entrap probiotic bacteria using extrusion method. The chemical explanation behind such gel formation is the bonding of the multiple free carboxylic radicals by gelling ions and thereby formation of the gels (Champagne and Fustier, 2007). Various concentrations of sodium alginate solutions have been successfully used by several researchers to form tiny gel particles by spraying over a calcium chloride solution followed by filtering them out (Eikmeier and Rehm, 1987; Champagne *et. al.*, 1992; Desai and Park, 2005). Sodium alginate is a heteropolysaccharide obtained from different species of sea algae. It is a polymer of L-

guluronic acid which gets bound with divalent cations and thereby bacterial cells suspended in alginate solutions get encapsulated into the resultant gel (Smidsrod and Skjakbraek, 1990).

Extrusion is a relatively new technique compared to some other popular forms such as spray drying and involves entrapping of core materials within a glassy carbohydrates mix (Madene *et. al.*, 2006). The extrusion technique is highly preferred to coat volatile and hence unstable flavours and oils. This method has been reported to increase the shelf life of such compounds dramatically by prohibiting oxygen diffusion through the matrix (Gouin, 2004; Desai and Park, 2005; Madene *et. al.*, 2006).

In a recent work, Li *et. al*. (2009) microencapsulated *L. casei* cells in a mix of sodium alginate and gelatin using extrusion process. This combination was reported to successfully protect the cells during gastro-intestinal transit but the beads were relatively larger in size with a mean diameter of 1.1 ± 0.2 mm. Starch was used in combination with alginate to encapsulate *L. paracasei* cells in another study by Babu *et. al*. (2009) and very good thermotolerance as well as salt, acid and bile tolerances have been reported. Another study using the extrusion technique was carried out where bacterial cell suspension was added into a mix of 20% non fat milk and 1.8% sodium alginate solution at a ratio of 1:1 (v/v) and found to be effective in protecting lactic acid bacteria against gastric environment (Ross *et. al*., 2008). Though not exactly for probiotics, chitosan in combination with alginate and extrusion technique was used to microencapsulate a bacteriophage (Felix O1), and very high acid resistance was reported (Ma *et. al*., 2008). Free bacteriophages were completely destroyed at pH 3.7 within 5 min of exposure whereas only 0.67 log CFU reduction was observed for microencapsulated cells even when the medium pH was 2.4. The protective effect of microencapsulation against oxygen was studied by Talwalkar *et. al*. (2003). *B. lactis* and *L. acidophilus* cells entrapped in calcium alginate-starch beads were grown aerobically and then incubated into yogurt in presence of oxygen. All the 6 strains of tested *L. acidophilus* were found nicely protected due to microencapsulation and 1.0 log higher viability was recorded over the free cells.

Some of the studies reported about microencapsulation of probiotic culture using extrusion technique followed by observing their behavior in different food products. Very recent ones worth mentioning are addition of *L. acidophilus* and *B. bifidum* into Kasar cheese by Ozer *et. al*. (2008) and evaluation of storage survibability of *L. acidophilus* and *B. lactis* into Iranian yogurt drink by Mortazavian *et. al*. (2008). The former one used both extrusion and emulsion techniques and found no significant difference between two in the result. The microencapsulated cells performed equally well with regard to count, proteolysis and sensory qualities than their counterparts in the free cell form. In the later case, the calcium alginate entrapped cells were extracted from the stored product and subjected to artificial gastric environment. The encapsulated cells showed 26.3 to 34.0% better survival rate in alleviated harsh conditions than the free ones. In another study, storage viability of probiotics microencapsulated into calcium alginate beads in presence of resistant starch was observed by incorporating into ice-cream (Homayouni *et. al*., 2008). After a period of 180 days at -20°C, encapsulated cells showed 30% better viability than the free cells. Shah and Ravula (2000) freeze dried calcium alginate beads prior to their incorporation into a frozen dessert. They concluded an improved viability of cells due to the encapsulation. In another study, alginate and Hi-Maize starch combination was used for microencapsulation and glycerol was added as a cryoprotectant before freeze drying by Sultana *et. al*. (2000). Eight weeks of storage study was performed after adding them in yogurt and only 0.5 log reduction in 8 weeks were reported. However, the control over bead size distribution was poor. *Bifidobacterium bifidum* and *B. lactis* in encapsulated form were added in mayonnaise having pH 4.42 and it was reported that the free cells died completely by 2 weeks but the encapsulated cells survived well up to 12 weeks and 8 weeks respectively (Khalil and Mansour, 1998).

A major advantage of the extrusion process is its shell-core character, where the encapsulated material is completely covered and protected by the wall material and residual or surface core material is removed by a dehydrating liquid, generally isopropyl alcohol (Gibbs *et. al*., 1999; Desai and Park, 2005). This is important when the presence

of residual core material may impart undesirable sensory properties to the product, (e.g. fish oils). The carbohydrate coating provides an excellent barrier property against oxygen and this fact is validated by some studies where flavor oils have been found to retain up to 5 years of shelf life (Gouin, 2004).

However, the main disadvantage has been reported as very poor payload of typically 8%. High payload i.e., higher ratio of core to shell material is important in economic as well as sensory perspectives. Attempts to increase the pay load had resulted into unstable microcapsules and leakage of core materials (Gouin, 2004). Other disadvantages worth mentioning are susceptibility of carbohydrates towards damage and structural defects, a larger particle size distribution, limited choice of wall materials etc. (Gouin, 2004; Madene *et. al*., 2006).

2.3.4.2 Emulsion technique for microencapsulation

This method uses a continuous and a dispersed phase to form either a permanent or temporary emulsion, followed by a separation step where the phases are separated and the dispersed phase encapsulates the probiotic bacteria as core material (Krasaekoopt *et. al*., 2003). Similar to the extrusion process, sodium alginate has been a favorite material to the researchers for encapsulating probiotic bacteria using emulsion method. Instead of spraying or dropping from a needle, the sodium alginate as dispersed phase is emulsified into any vegetable oil and thereafter insolubilized by slowly adding a solution of calcium chloride. Finally the beads are collected using filtration or mild centrifugation (Sheu and Marshall, 1993). This process forms much smaller beads compared to extrusion, which is a desired property and the emulsion process is easy to scale up to an industrial level. The particle size distribution is controlled by the speed of agitation and homogenization parameters. An emulsion of sodium alginate in corn salad oil was made by passing through a microporous glass membrane (MPG) and this was used to encapsulate *L. casei* YIT 9018 cells; a good stability with narrow particle size distribution was achieved (Song *et. al*., 2003). The encapsulated cells in that study performed consistently better than the free ones, in artificial gastric and bile salt solutions as well as during storage at

different temperatures. Materials other than alginate commonly used in emulsion technique are a mix of κ-carageenan and locust bean gum (Audet *et. al.*, 1988), chitosan (Groboillot *et. al.*, 1993), gelatin (Hyndman *et. al.*, 1993) and cellulose acetate phthalate (Rao *et. al.*, 1989).

The emulsion technique for microencapsulation has been successfully used with many materials other than alginate. Two such examples are mentioned here. Adhikari *et. al.* (2000) used 2% κ-carrageenan and 0.9% NaCl dispersed into vegetable oil and emulsified the mix with Tween 80, followed by immobilization with potassium chloride. Free and encapsulated *B. longum* cells were tested for their resistance in the acidic medium of yogurt during 30 days of refrigerated storage. Encapsulated cells showed significantly better survival rate (> 70.5 to 78%) than the free ones. In the other study, gelipin-gelatin cross linked microcapsules were produced to entrap *B. adolescentis* cells and harvested from the emulsion phase using NaCl and Tween 80 solution as washing liquid (Annan *et. al.*, 2008). This flexibility of the emulsion technique has encouraged researchers to experiment with various other hydrocolloids such as casein which does not have an instant gelation property and hence not suitable to be used in extrusion method. A detailed discussion on the use of proteins as encapsulating matrix has been presented later in this chapter (section 2.3.7).

2.3.4.3 Use of drying technology for microencapsulation

Drying of the encapsulated mixture using different techniques such as spray drying, freeze drying and fluidized bed drying are common in the industry. The added advantage of drying as a means of microencapsulation is the stability of the encapsulated culture during prolonged storage.

2.3.4.3.1 Spray drying as a technique of microencapsulation

Microencapsulation via the spray drying process is used since the 1950s in a variety of applications such as flavor oil, vitamins, minerals, fish oils and probiotic cultures

(Gouin, 2004; Desai and Park, 2005). The drying process in case of probiotic cultures always causes some level of viability losses, mainly due to physical injury to microcapsules, release of bacterial cells and the heat generation essential during drying process (Mortazavian *et. al*., 2007). Some of the efforts to reduce the cell death during drying were using a mix of proper cryoprotectants during freeze drying, optimizing the inlet and outlet temperatures for spray drying, using a combination of spray and fluidized bed drying to minimize the heat shock and pre-adaptation to heat sustenance prior to spray drying (Champagne and Fustier, 2007). A major limitation of spray drying is the limitation in choice of shell materials. The shell material has to be water soluble for spray drying to take place, causing immediate release of the core material in aqueous medium and thus controlled release can not be achieved.

Some hydrophobically-modified polysachharides such as, octyle-substituted starches have been successfully used as shell material in spray drying, to encapsulate up to 50% flavor oils (Gouin, 2004). In the current decade, Mesquite gums for cardamom oil (Beristain *et. al*., 2001) and Polyvinyl pyrrolidone (PVP) in combination with dextran for microorganisms (Millqvist-Fureby *et. al*., 2000) have been used as shell materials for spray drying microencapsulation process. Mesquite gum was able to encapsulate upto 83.5% of cardamom oil with very good flavor masking property. In the later case, a coacervation process was adapted using the polymers PVP and dextran, and *Enterococcus faecium* cells were successfully encapsulated followed by spray drying. The control release mechanisms of both mesquite gum and PVP-dextran systems were dissolution into aqueous solutions.

O'Riordan *et. al.* (2001) used spray drying technology to coat *Bifidobacterium* cells with starch. No significant advantage of microencapsulation was noticed during acid exposure and storage assay but milder spray drying parameters resulted into less than 1.0 log reduction in cell decay. Preparing a double layered emulsion comprising of an aqueous inner phase surrounded by an intermediate oil phase and finally an outer aqueous phase (w/o/w type) and then finally spray drying the emulsion have been successful to entrap the core materials in the innermost water phase. This process gave a

better stability to the entrapped material but resulted in a much lower payload of the encapsulated material (Gouin, 2004). *B. lactis* were found highly resistive against spray drying stress conditions in a study by Favaro-Trindale and Grosso (2002) who observed negligible reduction at 130ºC inlet and 75ºC outlet temperatures. On the contrary, *L. acidophilus* cells in the same study showed 2.0 log reduction under similar drying parameters. The encapsulating media used there was cellulose acetate phthalate and very good particle morphology with no pores on the surface was achieved. In another study to protect *L. paracasei* during spray drying, the encapsulating effect of gum acacia was investigated. Cells were grown in 10% gum acacia and also in 10% RSM media as a control (Desmond *et. al.*, 2002). Even at a high outlet temperature of 95ºC the cells encapsulated in gum acacia survived 10 times better than the control sample. This process also improved viability by 20 times during storage at 4ºC. In the same study, a 100 fold higher survival was recorded for gum acacia treated cells during gastric juice incubation. The major advantages of the spray drying process are ease of scaling up, low operational cost, continuous operation and adaptability to most common industrial equipment. However, spray drying may not be suitable particularly for probiotic bacteria due to the requirement of high temperature drying (Gibbs *et. al.*, 1999; Kailasapathy, 2002; Madene *et. al.*, 2006). The loss of viability also depends upon the type of carriers used, for example, log reduction in soluble starch was found to be higher compared to other carriers such as gelatine, gum arabic and skim milk (Lian *et. al.*, 2002; Santivarangkna *et. al.*, 2007). In an attempt to reduce the viability loss during drying, a modified spray drying process involving coating of milk fat and denatured whey protein isolate was tried. The said coating was applied around the micronized freeze dried cultures during spray drying. It was concluded that this is a suitable method for scaling up industrially and also is economic in nature (Picot and Lacroix, 2003; 2004). Another aim of the experiment was to optimize the mixing parameters for large scale production of micronized encapsulated particles with greater control over particle sizing.

2.3.4.3.2 Microencapsulation of probiotics with freeze drying

Freeze drying is a better alternative which does not require low temperature transportation and consequently no risk of thawing, but involves high cost of operation, is difficult to scale up and the process is not continuous (Santivarangkna *et. al*., 2007). Moreover, freezing causes damage to the cell membranes due to ice crystal formation and it also imparts stress conditions by high osmolarity resulting from the high concentration of media solutes. Significant mortality of bacterial cells has been reported after freeze drying due to the loss of membrane integrity and denaturation of macromolucles (Franks, 1995; Thammavongs *et. al.*, 1996; De Angelis and Gobbetti, 2004). The freeze drying process has been traditionally used to stabilize probiotic bacteria but the combination of freeze drying and encapsulation is relatively newer concept. In a very recent work by Heidebach *et. al*. (2010), *Lactobacillus* F19 and *Bifidobacterium* Bb12 cells were first encapsulated into enzymatically gelled sodium caseinate and the gel particles were freeze dried to study the storage stability. They reported significantly better post drying survival as well as storage viability for encapsulated cells compared to the free cells (cell protein mixture). In another recent work, gelatinized starch and lecithin were incorporated into the alginate microcapsules which contained probiotic organisms in encapsulated form and the beads were freeze dried to evaluate the storage stability at different temperatures (Donthidi *et. al*., 2010). It was shown that encapsulated bacteria had much better stability at 23ºC for 12 weeks and lecithin helped in obtaining higher encapsulation efficiency and more stability to the *L. casei* cells when added into yogurt. A comparison of spray, freeze and fluid bed drying of the microencapsulated *L. acidophilus* and *B. bifidum* cells was reported in another work (Goderska and Czarnecki, 2008). It was found that highest post drying survival was achieved in case of freeze drying when skimmed milk was added into the encapsulation mix as cryoprotectant.

Therefore, it can be seen that the detrimental action of freeze drying process on bacterial cells can be offset to some extent by proper microencapsulation.

2.3.4.3.3 Other drying methods used for microencapsulation

The other less popular forms of drying are fluidized bed drying, vacuum drying and a system of mixed drying processes. The major advantages of a fluid bed drying process are total control over the drying temperature, low or comparable cost of operation and the disadvantage being relatively longer duration (up to 2 hours) involved in drying (Santivarangkna *et. al.*, 2007). In a fluid bed, only granular materials can be dried hence the probiotic cultures are encapsulated first in supporting materials such as skim milk (Roelans and Taeymans, 1990), potato starch (Linders *et. al.*, 1997), calcium alginate (Selmer-Olsen *et. al.*, 1999) or casein (Mille *et. al.*, 2004). Vacuum drying is very suitable for heat sensitive products such as encapsulated probiotic cultures since the drying takes place at a much lower temperature under vacuum. The oxidation reactions during the drying can also be reduced in this process. The disadvantages of the process are batch operations and relatively very long drying time (Santivarangkna *et. al.*, 2007). However, the drying time can be minimized by using a continuous vacuum dryer where cost is only one third of a freeze dryer and the materials can be dried at 1-4% moisture level at 40.C and within 5-10 min (Hayashi *et. al.*, 1983).

2.3.4.4 Microencapsulation with spray chilling

Though not much scientific data are available on the use of spray chilling technology for encapsulating probiotic bacteria, but this method is used extensively in other food ingredients applications (Champagne and Fustier, 2007). The basic operations involve a dry core material on which a lipid based coating material is sprayed. The core material is kept under motion and the lipid in liquid form is sprayed as a mist, ensuring proper mixing and coating. The temperature of the coating material is maintained above the melting point of the lipid but a strong increase in core material temperature is preferably avoided. Cooling air with temperatures between 10 to 50°C is used to solidify the lipid and strengthen the coating. This process can also be used for double coating a particle with lipid as the first layer, followed by a protein or gum for stabilization or to adjust the specific gravity of a formulation (Shin *et. al.*, 2002). A reverse process is also described

in the patent by Smith and Lambrou (1974) where the first coating on flavor oil was formed with gum acacia followed by an outer layer of melted fat. Spray chilling or cooling has been successfully used to encapsulate vitamins, minerals, acidulents, other frozen liquids and heat sensitive materials (Gibbs *et. al.*, 1999).

2.3.4.5 Coacervation technique for microencapsulation

In a coacervation process, colloidal particles are separated from a solution and deposited around a targeted core material. It's a popular process in encapsulating flavor oil but has also been used in fish oils, vitamins, enzymes, nutrients and preservatives. The process can be termed as simple where only one hydrocolloid is involved whereas complex coacervations involve two or more polymers.

The coacervation process has been described as a three step method comprising of phase separation, deposition and solidification (Desai and Park, 2005). In the first step, the coating material consisting of usually one or more polymers goes through a phase separation process and forms a coacervate. Core materials remain in the suspended or emulsified form and as soon as the wall material particles coalesce, it causes a decrease in surface area and total free interfacial energy of the system. This process favors the coacervate nuclei adsorption to the surface of the core material and a uniform layer or coating forms around the core particles. The final step causes the solidification of coating material, generally by a cross linking reaction using chemical, thermal or enzymatic methods. The formed microparticles are then collected by filtration or mild centrifugation followed by drying (Desai and Park, 2005; Madene *et. al.*, 2006).

Microencapsulation of probiotic bacteria *Lactobacillus* E1 using the coacervation technique was investigated by Sun *et. al*. (2009). A double emulsion (w/o/w type) was made using diatomite, sodium alginate, dextrin and gelatin. Good storage stability at 10ºC over a period of 37 days was reported. A coacervation process followed by spray drying to encapsulate *B. lactis* and *L. acidophilus* was tried by Oliveira *et. al*. (2007).

Casein and pectin complex was used as the wall material. A higher shelf life of spray dried culture mass and very good *in vitro* acid tolerance was reported in this study.

The coacervation technique is advantageous for microencapsulation purpose because of its very high pay load of up to 99% and total control over the release of core materials (Gouin, 2004). The process can be carried out at room temperature making it particularly suitable for heat sensitive objects such as probiotic bacteria (Desai and Park, 2005).

A major disadvantage of using the coacervation technique is its high costing particle isolation procedure in the last step and the complexity of the technique. But it was suggested that the optimization of the last step and use of a spray dryer instead of fluidized or freeze dryer can reduce the overall cost dramatically (Gouin, 2004). Glutareldehyde is used often as the cross linking agent but it is not applicable in the food industry due to toxicity issues (Desai and Park, 2005). Instead of glutareldehyde, cross linking enzyme transglutaminase is used to address this problem (Truong *et. al*., 2004).

2.3.4.6 Co-crystalization method

Co-crystalization is one of the simplest forms of encapsulation used mainly for fruit juices, essential oils, flavors and brown sugar (Madane *et. al*., 2006). The process is carried out by crystallization of a super saturated sucrose solution with the desired core materials dispersed into it.

The supersaturated sucrose solution is maintained at a high temperature to prevent crystallization. The heat is gradually released allowing the solution to crystallize with the core material already added into it. Following encapsulation into the crystal matrix, the product is dried and sieved as per the particle size requirements (Bhandari *et. al*., 1998).

No scientific publication related to microencapsulation of probiotic bacteria using co-crystalization technique was found, probably due to the high temperature required to maintain the supersaturated sucrose solution.

The process has advantages of being economic, with high pay load of up to 90%. This process has found maximum utility in the confectionary and pharmaceutical industries as because liquid products can be readily formed into tablets with simple processing. However, the co-crystallization process demands a tighter control rate of nucleation and crystallization and also a strict thermal balance during various stages of operations (Desai and Park, 2005).

2.3.4.7 Molecular inclusion

This method is also known as inclusion complexation, which involves entrapment of smaller molecules inside the hollow cavity of a larger molecule (Hedges and McBride, 1999; Madane *et. al*., 2006). A very few such molecules exist which are appropriate for food applications. Cyclodextrins are commonly used for this type of encapsulation process. However, use of cyclodextrins in food formulations is restricted to certain countries only. Therefore this is not a popular method to encapsulate probiotic bacteria.

Cyclodextrins are formed from the enzymatic hydrolysis of starch molecules and during hydrolysis; linear fragments are created, which are finally joined to form circular structures with hollow cavities inside. Therefore, cyclodextrins can form inclusion complexes with small enough compounds which can fit inside these hollow cavities (Gouin, 2004; Madane *et. al*., 2006).

Molecular inclusion in β-cyclodextrin has found its popularity in encapsulating flavors, aromas, vitamins and minerals. Hydrophobic vitamins (A, E and K) are good substrates for this type of encapsulation. The control release mechanism is also very interesting where the core materials are released when displaced by more favorable substrates. An example is the flavor burst in the mouth from cyclodextrin complex because compounds

found in the mouth are more favorable substrates for cyclodextrin (Gouin, 2004). It has been reported that β-cyclodextrin molecules containing core compounds are highly heat stable, can tolerate up to 200ºC temperature and highly resistant to chemical degradation (Hedges and McBride, 1999; Gouin, 2004).

No research publication was found which involved the molecular inclusion property of cyclodextrin to encapsulate probiotic bacteria.

Some of the major limitations of this molecular inclusion technology are very low payload (Gouin, 2004) and very high cost of raw materials (Madane *et. al*., 2006).

2.3.4.8 Centrifugal extrusion technique

The centrifugal extrusion technique involves the pumping of the core and coating materials through separate tubes to the surface of a rotating cylinder. With the rotational motion of the cylinder, both materials are mixed and extruded as a fluid rod which is broken by the centrifugal force. The coating over the core material to form capsules is caused by the difference in surface tensions. Finally, the formed capsules are placed on a moving bed of starch, which absorbs excess moisture and cushions the impact (Desai and Park, 2005).

This is a popular method used in the food industry to encapsulate a wide range of ingredients such as flavors and seasonings (Desai and Park, 2005), aspartame, vitamins, methionine etc. (Gibbs *et. al*., 1999). Compared to extrusion, smaller particles are produced in this technique and a wide range of coating materials such as gelatin, alginate, carrageenan, starches, fatty acids, waxes etc. can be used in centrifugal extrusion (Gibbs *et. al*., 1999; Desai and Park, 2005).

Major advantages of this system as mentioned by Gouin (2004) are slower release properties of the capsules and higher throughput rate in comparison to the spray drying process.

2.3.5 Selection criteria of choosing encapsulating matrix

The release of any entrapped core material is controlled by the capsule wall properties (matrix control) or the coating material over the wall (membrane control). Releasing of encapsulated core materials is dependent upon the wall materials response towards the environmental triggers such as pH, temperature, sheer stress etc. The permeability through the matrix, the solubility of the core component and the vapor pressure (in case of a volatile compound) are also important factors in choosing the right ingredient combination of a microencapsulation process (Gibbs *et. al*., 1999). For example, it was reported that calcium alginate beads produced by ionotropic gelation are found to be porous, facilitating easy transport of the core materials out of the encapsulating matrix (Anal and Singh, 2007). A range of various biodegradable polymers have been used by several researchers to microencapsulate probiotic bactera. A detailed list of such materials can be found in a review by Anal and Singh (2007). Each of these materials or combinations thereof has some unique advantages and disadvantages. κ-Carrageenan alone or in combination with locust bean gum was tried in many studies (Audet *et. al*., 1988; 1990; 1991; Doleyres *et. al*., 2002; 2004) to encapsulate probiotic bacteria but the gelling agent involved in the process was potassium chloride, which was found to have an inhibitory effect on *S. thermophilus* and *L. bulgaricus* (Audet *et. al*., 1988, Anal and Singh 2007). The choice of wall ingredients also depends upon the objective of encapsulation. A certain material mix may be good in providing good protection against gastric passage but the idea of incorporating the same materials in food products may not be very attractive. For example, enteric polymer coatings in the form of mainly cellulose derivatives are used extensively in the pharmaceutical industry for tablet coating purpose. One such polymer, cellulose acetate phthalate, was used to encapsulate *L. acidophilus* cells and excellent protection in gastric environment was noted (Rao *et. al*., 1989).

The goodness of milk components as food ingredients is accepted universally and therefore may be considered as a good choice for encapsulating probiotic bacteria. Microparticles made of milk proteins are finding rapidly increasing applications in the

food industry because of their flexibility to encapsulate any type of hydrophilic, hydrophobic or biological substances such as, probiotic bacteria. The property of milk proteins to form gels in the presence of certain compounds is utilized for the encapsulation purpose and this is being discussed in detail below.

2.3.6 Gelation mechanism of dairy proteins

As mentioned in the previous section, the property of milk proteins to form gels is attracting interests in the microencapsulation industry. In order to successfully apply this property for microencapsulation of food ingredients, particularly probiotic bacteria, it is very important to understand the mechanism of such gel formations.

The gelation properties of dairy proteins have been studied widely for the purpose of microencapsulating various nutrients and probiotic bacteria (Rosenberg *et. al*., 1993; Rosenberg and Sheu, 1996; Chen and Subirade, 2007). Dairy proteins are broadly categorized as casein and whey protein, the later is made u p of β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins and minor proteins. The largest fraction in whey protein is β-lactoglobulin (50% by weight) which is the main factor for whey protein gel formation at temperatures above 75°C (Kilara and Vaghela, 2004). The whey proteins can be gelled chemically, thermally or enzymatically. Gelation is the result of unfolding of the protein and subsequent aggregation. Mostly, the non-covalent bonds stabilize the gel network but covalent bonds, such as disulphide bridges may also contribute to such stabilization (Eleya and Gunasekaran, 2006).

Casein is the principal part of the milk protein complex and the main factors controlling the gelation of casein are the presence of acid and enzymes.

2.3.6.1 Acid gelation of casein

Gelation of casein is caused by its aggregation in the acidic condition. Near its isoelectric point, the net charge of a protein is zero and consequently the repulsive forces

are minimum among the molecules. This aids in easy movement and aggregation of molecules. Therefore, by adjusting the environmental conditions such as pH, it is possible to induce gelation of proteins.

Lucey *et. al*. (1998) described the mechanism of acid gelation. The fractal aggregation theory suggested by them assumes spherical particles under Brownian motion can aggregate when they encounter with each other. When the repulsive forces reach near zero, there is virtually no resistance to form a cluster, which acts as a building block to develop a solid gel structure.

2.3.6.2 Enzymatic gelation

The enzymatic gelation of protein was described by Aguilera and Rademacher (2004), as the introduction of artificial covalent bonds into food proteins. Common food-grade enzymes used in cross-linking food proteins are transglutaminase, peroxidase and polyphenol oxidase.

The use of transglutaminase in the food and pharmaceutical industries has been studied with great interest because it can act on a range of substrates and is able to cross-link a variety of proteins (Aguilera and Rademacher, 2004; Bonisch *et. al*., 2007). Microbial transglutaminase (MTG) is a metabolic product from the bacterium *Streptoverticillium mobaraense*. This enzyme particularly catalyses the acyl-transfer reactions, resulting in the formation of molecular cross-links in proteins (Cho *et. al*., 2003).

The functional and physicochemical properties of the transglutaminase cross-linked proteins depend upon factors like enzyme concentration, incubation time and the type and concentration of proteins. Some of the properties that can be mentioned as examples are gelation properties, gel strength, gel elasticity and water holding capacity of the end product.

Microencapsulation of fish oils using MTG cross-linked proteins was investigated by Cho *et. al*. (2003). The performances of whey protein isolate, soluble wheat protein, isolated soy protein and sodium caseinate were observed. Increasing protein concentration resulted in increase in emulsion stability for isolated soy protein and MTG. However, in the case of whey protein isolate and soluble wheat protein, the stability increased with MTG treatment but no effect of protein concentration was observed. The success of isolated soy protein presented a promising alternative to use whey protein as an encapsulating agent.

2.3.7 Use of milk proteins in microencapsulation

2.3.7.1 Introduction

Protein hydrogels are very convenient for this purpose because in liquid and semi-solid food applications, the matrix size can be decreased to a level acceptable for sensory evaluations (Augustin, 2003; Chen *et. al*., 2005). Another big advantage of using protein hydrogels as neutraceutical carriers is their inherent and desirable property of stabilizing the food texture (Chen *et. al*., 2005).

2.3.7.2 Whey protein as wall material for microencapsulation

The gelation of whey protein has been traditionally achieved by heat treatment, by which the polypeptides chains unfold and subsequently self-aggregate to form a three dimensional network, capable of entrapping water. But the requirement of heat treatment makes this process unsuitable for encapsulating many heat sensitive materials such as probiotic bacteria (Chen *et. al*., 2005). The cold-induced gelation methods of whey proteins have been suggested as a potential solution to this (Barbut and Foegeding, 1993; Maltais *et. al*., 2005; Heidebach *et. al*., 2009; 2009a).

The application of cold-set gel has been seen in encapsulating ferrous salt in a denatured β-lactoglobulin gel matrix (Remondetto *et. al*., 2002). Whey protein in denatured form

has been extensively used so far as shell material (Rosenberg *et. al*., 1993; Hogan *et. al*., 2001a; Picot and Lacroix, 2003; 2004). Whey protein isolate has been used to encapsulate probiotics using the ionotropic gelation technique (Kailasapathy and Sureeta, 2004; Ainsley-Reid *et. al*., 2005). The protein gelaton mechanism is explained by Aguilera and Rademacher (2004). Addition of cations in the form of calcium or sodium chloride shields electrostatic charges on the surface of the proteins and allows the molecules to move close together. Consequently, molecules have a tendency to aggregate due to the absence of electrostatic repulsion and gelation can occur. This method is carried out at room temperature making it suitable for encapsulating heat sensitive probiotic microorganisms. A combination of alginate, pectin and whey protein was used to encapsulate *Bifidobacterium* cells and a membrane formation surrounding the beads was achieved by the conjugation of whey protein and pectin. It was found that membrane coated capsules offered much better protection to the cells against low pH of simulated gastric juice and bile salts present in simulated intestinal fluid (Guerin *et. al*., 2003). Beaulieu *et. al.* (2002) reported a very slight degradation of whey protein microcapsules when subjected to acidic gastric juice. Whey protein when used in combination with carbohydrates, acts as an emulsifying and film-forming agent while the carbohydrates form the encapsulating network (Sheu and Rosenberg, 1998). Another form of encapsulating materials recently attracting attention is emulsion gel. The oil-in-water emulsions of whey protein isolates (WPI) easily form gels by heat treatment and impart very good encapsulating, organoleptic and amphiphilic properties (Chen and Dickinson, 1998; Chen *et. al*., 1999; Chen, *et. al*., 2005). These gels were tested for the degradation possibilities in the gastro-intestinal environment and were found to be very resistant (only 20% degradation reported), giving them good potential to be as encapsulating agents (Leung *et. al*., 2005; 2005a). Milk proteins are gradually becoming popular as encapsulating material for probiotic bacteria. Specially the whey proteins have unique dissociating and aggregating properties under different physical conditions and this can be used to form particles with a wide range of diameter ranging from 40 nm to 2 mm (Chen *et. al*., 2005). In their study, a cold gelation cum emulsification process to prepare microbeads was developed by emulsification of denatured whey protein followed by calcium induced gelation. The enzymatic actions of pepsin and pancreatin

were studied and it was found that the microbeads were resistant against the action of pepsin at low pH medium but completely dissolved in presence of pancreatin and released its bioactive contents in the simulated intestinal environment (Chen *et. al.*, 2005).

2.3.7.3 Use of sodium caseinate as microencapsulating agent

Sodium caseinate appears to offer ideal physical and functional properties required for microencapsulation because of its amphiphilic and emulsifying characteristics (Hogan *et. al.*, 2001; Madene *et. al.*, 2006). Though whey protein has been reported to possess very good oxygen barrier properties (Kim *et. al.*, 1996), it was shown that the encapsulating capability of sodium caseinate is much higher than whey protein (Faldt and Bergenstahl, 1996). The previous use of sodium caseinate as encapsulating material was however limited to flavor oil and fish oil only (Kim *et. al.*, 1996; Millqvist-Fureby *et. al.*, 1999; Madene *et. al.*, 2006). In this regard, a patented technology recently developed by Singh *et. al.* (2009) is worth mentioning which used an emulsion of casein and whey protein to encapsulate fish oil. The invention is currently going through a promising commercialization process. Hogan *et. al.* (2001) mentioned about the high potential of sodium caseinate in combination with polysaccharides as cost effective encapsulating material. Heidebach *et. al.* (2009) has recently developed a method in which *L. paracasei* and *B. lactis* strains were encapsulated by enzymatic gelation of sodium caseinate, by crosslinking with transglutaminase enzyme. The gel structure was broken and converted into microcapsules by suspending the particles in an oil base under continuous stirring, during the gel formation from soluble state. In another attempt they have taken a similar approach for casein gelation to encapsulate probiotic bacteria but used rennet as a coagulating agent in place of transglutaminase enzyme (Heidebach *et. al.*, 2009a). In a report, Charteris *et. al.* (1998) showed that when sodium caseinate and whey protein isolate were added as buffering media with two strains, *L. casei* 212.3 and *B. infantis* 25962 during their *in vitro* study of gastro-intestinal transit, the tolerance improved drastically. They have also found further improvement in viability of *L. casei* F19 when milk proteins were added to the simulated pancreatic juice.

The protein medium surrounding the bacterial cells provides a very good buffer compared to plant hydrocolloid materials. The milk proteins help to increase the pH of gastric juice significantly and thus increase the survivability of the encapsulated bacteria (Charteris *et. al.*, 1998; Ross *et. al.*, 2005). As discussed earlier, casein in the form of sodium caseinate aqueous solution can be coagulated and gelled either by actions of enzymes such as rennet and transglutaminase (Heidebach *et. al.*, 2009; 2009a), by cross-linking with glutaraldehyde (Latha *et. al.*, 2000) or by slow acidification with glucono-delta-lactone (GDL) (Lucey *et. al.*, 1997).

2.3.8 Use of glucono-delta-lactone in gelation of milk proteins

Glucono-delta-lactone or GDL is commonly used as an acidulant in the food and meat industry and is a cyclic ester with molecular formula $C_6H_{10}O_6$ and molar mass of 178.14 g/mol. It is a harmless food additive used in various products such as sausages, frankfurters, yogurt and other dessert dishes (Trop and Kushelevsky, 1985).

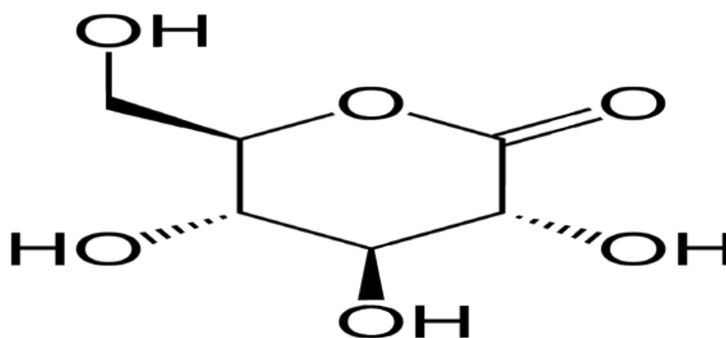


Figure 2.4 2-D Skeletal image of Glucono-delta-lactone molecule (Wikipedia 1)

GDL in the presence of water hydrolyses partially into gluconic acid and maintains a balance between the lactone form and acid form until a chemical equilibrium is reached. This results into a slow pH drop of the surrounding medium. GDL has been extensively used for making acid milk gels and studying their rheological properties (Cobos *et. al.*, 1995; van Vliet and Keetels, 1995; Lucey *et. al.*, 1997a). Lucey *et. al.* (1998a) found

that GDL induced skim milk gels attain a much higher storage modulus (G') value in a given time compared to bacterial culture fermented gels. GDL has been reported to be non-reactive to the amino groups present in milk proteins (Deane and Hammond, 1960). But it was also found that the interaction between GDL and milk proteins takes place through the L-lysine residues (Trop, 1984).

The gelation of milk protein with glucono-delta-lactone and subsequent use of it to microencapsulate probiotic bacteria was not found in a thorough scientific literature search. This was thought to be a good opportunity and the potential has been investigated in this current project, as detailed in the following chapters.

2.3.9 Use of gellan gum as microencapsulation wall material

Gellan gum is a bacterial exopolysaccharide obtained by the aerobic submerged fermentation of *Sphingomonus elodea* (ATCC 31461). This is a linear tetrasaccharide, high in molecular weight consisting of about 50000 residues. The functionality of gellan gum depends mostly upon the degree of acylation and the ions present in it. The most unique functionality of this is the ability to hold small particles in a suspension but without increasing its viscosity significantly (Baird and Pettitt, 1991).

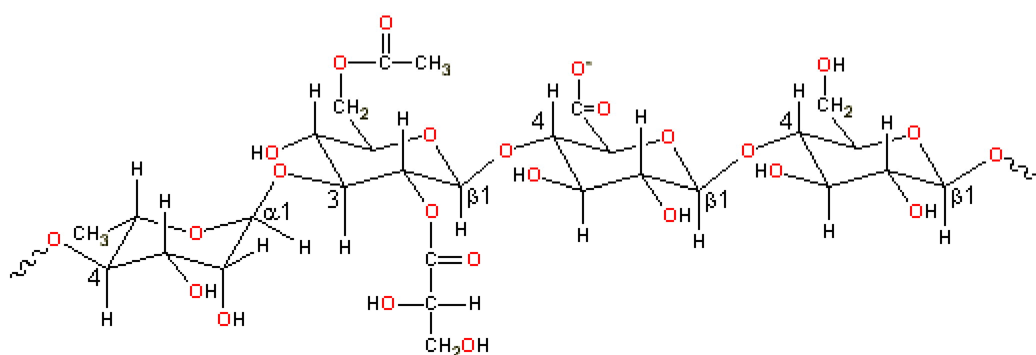


Figure 2.5 Structural unit of gellan gum molecules (London South Bank University Website)

Gellan gum forms very firm and brittle gels but melts easily in the mouth, releasing the water and any flavor from its gel network (Sun and Griffiths, 2000). Though gellan gum itself is able to form good microcapsules when gelled in presence of calcium ions but this process needs a pre-heating of up to 80ºC for 1 hour (Sanderson, 1990) which is not suitable for heat sensitive core materials like probiotics. Therefore, for encapsulation purpose, it has always been used in combination with another gum such as, xanthan (Sun and Griffiths, 2000; McMaster *et. al*., 2005; Muthukumarasamy *et. al*., 2006) or a few sequestrants such as sodium citrate, sodium metaphosphate and EDTA (Camelin *et. al*., 1993). Gellan gum in combination with sodium alginate and pectin has found its use as wall material in encapsulating casein and hydrogenated vegetable fat (Mukai-Correa *et. al*., 2005). In a study, *Bifidobacterium longum* cells were immobilized for continuous fermentation purpose in gellan gum beads in the presence of 0.2% sodium citrate at 47ºC and 0.1M calcium chloride solution was used to harden the microcapsules. It was concluded that this immobilized cell technology using gellan can be used very effectively for high volumetric productivity and high concentration biomass production in a continuous fermentation process (Doleyres *et. al*., 2002). The other prominent application of gellan gum as encapsulating agent is found in cheddar cheese ripening where protease enzymes (flavorzyme) were entrapped and added to the milk during cheese manufacture and the enzyme activities were studied (Kailasapathy and Lam, 2004). To our knowledge, use of gellan gum in combination with sodium caseinate, as probiotic encapsulating medium has not been tried so far but the physicochemical and rheological characteristics of an oil-in-water emulsion made of this particular combination and 30% sunflower oil was studied by Sosa-Herrera *et. al*. (2008). They found some intermediate complex formation in a caseinate-gellan mix at pH 5.4 where both polymers were negatively charged, so the possibility of a coacervation was ruled out. It was concluded by them that the observed phenomenon could be due to electrostatic interaction as suggested by De Kruif and Tuinier (2001) and more recently by Ye *et. al*. (2006) for sodium caseinate/gum arabic mix or intermolecular hydrogen bonding by Dickinson (2003).

2.4 Drying of probiotic cultures and related physiological stresses

Among several techniques available, freeze drying is the most popular and widespread technology for drying probiotics. Though milder processing conditions in freeze drying have been proved to be less detrimental than spray drying, the freezing step involved here causes maximum injury to the cells. It was found that 60 to 70% of the cells which survived the freezing step could easily live through the desiccation step (To and Etzel, 1997). Freezing leads to extra-cellular ice crystal formation and consequent increase in extra-cellular osmolality, which in turn causes dehydration of bacterial cells (Meng *et. al.*, 2008). Disintegration of cell membranes and macromolecular denaturation are other detrimental effects of freezing and responsible for viability loss (De Angelis and Gobbetti, 2004). Bacterial cell size also plays an important role here. Fonseca *et. al.* (2000) demonstrated that higher surface area of the cell leads to higher damage of the cell membranes during the freezing process and shown that spherical shaped *Enterococci* were more resistant than rod shaped *Lactobacilli* during the freeze drying process. Other factors responsible for the degree of cryopreservation, as stated in a review by Hubalek (2003) are temperature, pH and composition of the growth medium, osmolarity and aeration, cell water and lipid composition, cooling rate during freezing etc. Different researchers have taken different approaches to control cell damage during the freeze drying process. Some of them are discussed in the following sections.

2.4.1 Use of cryoprotective agents in the freeze drying media

The technology to protect the bacterial cells during freeze drying is known as cryopreservation and the compounds used to achieve this protection are called cryoprotectants. Addition of cryoprotecting compounds prior to fermentation or drying helps in adaptation of probiotic cells in the changed environment. The added compounds slowly start accumulating inside the bacterial cells, which helps in reducing the osmotic difference between inside and outside of cells (Kets *et. al.*, 1996; Meng *et. al.*, 2008). The cryoprotecting agent or CPA can either be added to the growth or the drying media and their action varies with different strains. But certain general compounds such as

non-fat milk solids, lactose, trehalose, glycerol, betaine, adonitol, sucrose, glucose, dextran etc. have been regarded as suitable protectants for many species (Hubalek, 2003; Morgan *et. al*., 2006).

The protection mechanism is better understood by classifying the CPA's into two broad groups namely, (i) amorphous glass forming and (ii) eutectic crystallizing salts. The first group comprises of carbohydrates, proteins and polymers and they act by imparting very high viscosity at the glass transition phase and thereby restricting the molecular mobility of the cells. Most successful cryoprotentants for probiotic bacteria fall into this group. The other group contains certain eutectic salts which tend to crystallize as the temperature approaches near the freezing point but instead of providing protection, they have been sometimes reported as detrimental to the cell membranes (Orndorff and Mackenzi, 1973; Morgan *et. al*., 2006).

The CPA's are sometimes classified in a slightly different manner into three groups namely, (i) highly permeable compounds such as, monovalent alcohols, amides and sulfoxides, (ii) slowly permeable compounds such as glycerol and (iii) non-permeable compounds such as mono-oligo-polysaccharides, sugar alcohols, proteins, polyalcohols etc. Permeable compounds bind the intracellular water and prevent dehydration. Non-permeable protectants form a layer on the cell surface, allow partial outflow of water from cell body, reduce toxic effect of salts, stop excessive growth of ice crystals and maintain their structures (Hubalek, 2003; Saarela *et. al*., 2005).

Apart from offering protection during the drying process, compatible solutes have been shown to improve the acid tolerance property for probiotic cultures. The presence of glucose as a compatible solute was reported to increase the viability of cells by 6.0 log during simulated gastric juice trial (Corcoran *et. al*., 2005).

2.4.2 Trehalose and lactose as cryoprotective compounds

Trehalose has been found to be an excellent protectants against dehydration stress by acting as a stabilizer of membranes and proteins and replacer of water from the macromolecular structures (Rudolph and Crowe, 1985; Morgan *et. al*., 2006). Trehalose is a disaccharide containing two water molecules and found naturally as internal pool in many types of yeasts. It has a molecular weight of 378.33 with the chemical formula $C_{12}H_{22}O_{11}.2H_2O$. Conrad *et. al.* (2000) demonstrated the high cryoprotective efficiency of trehalose in a study and achieved high survival of *L. acidophilus* cells during freeze drying. The high glass transition temperature (T_g) of trehalose has been suspected to be responsible for such property. Compounds with high T_g can remain relatively immobile at higher temperatures and help to produce more stable freeze dried matrix (Crowe *et. al*., 1996; Sun and Davidson, 1998; Morgan *et. al*., 2006). Trehalose is also regarded as a good cryoprotectant due to its property to form dihydrate crystals, which leaves the remaining matrix as glass and do not reduce the T_g or stability of the matrix (Crowe *et. al*., 1998). Trehalose and sucrose were proved to be very effective protectants when *Escherichia coli* DH5 alpha and *Bacillus thuringiensis* I-ID-I were subjected to freeze drying (Leslie *et. al*., 1995). In another recent study, trehalose, sucrose and sorbitol were used as cryoprotectants for *lactobacilli* cells and sucrose was found the best agent among the group (Siaterlis *et. al*., 2009). However, in one experiment, trehalose was found to offer poorer result than control sample when used in media prior to air drying (Linders *et. al*., 1997).

Lactose is another popular cryoprotective compound with molecular weight of 360.31 and chemical formula of $C_{12}H_{22}O_{11}.H_2O$. Its effectiveness over glycerol has been shown by Chavarri *et. al.* (1988), when *Lactobacilli* cells were frozen and stored at -20 to -70°C. Lactose has been found to be an effective cryoprotectant for certain species such as, *L. lactis*, *E. coli*, *L. delbrueckii* and *S. cerevisiae* but moderately effective for *Streptomyces tenebrarius* and completely ineffective for *S. platensis* (Hubalek, 2003). Lactose along with a few other sugar substrates have been added to the growth media for

L. delbrueckii and a marked improvement in post drying survival, storage stability and thermotolerance has been recorded (Carvalho *et. al*., 2004).

2.4.3 Improving freeze drying viability by proper cell harvesting

All the probiotic species do not show equal vulnerability towards freeze drying. The technological robustness of a species or strain depends upon several factors such as age of the culture, cell size and shape, concentration of cells in the drying media, stress preventive mechanism of the cell and chemical structure of the cell membranes. For example, gram-positive cells have been found to be more robust than the gram-negative ones (Heckly, 1985; Carvalho *et. al*., 2004a). The survivability during freeze drying can also be improved by proper and timely harvesting of the cells. Generally, cells at their stationary growth phase have been found to be most robust towards drying stresses (Mary *et. al*., 1986; Rees *et. al*., 1995; Corcoran *et. al*., 2004). The explanation for such behavior has been cited as the physiological state during the stationary phase where starvation of carbon and other nutrients induce stress response to the cells and make them more adaptive towards subsequent drying stresses (Morgan *et. al*., 2006).

2.4.4 Use of additives in the growth media for enhanced protection during drying

Manipulating the culture growth media with various additives has been proved to be beneficial for the cells. The factors responsible for this can be identified as compatible solutes accumulation inside the cells, exopolysaccharides production and change in the cell membrane fatty acid profile (Carvalho *et. al*., 2004a). Various sugars in the form of compatible solutes help to regain osmotic balance of the bacterial cells during the low water activity stage of freezing and subsequent drying. This osmoregulation is performed by movement of the solutes through the cell membranes, which helps in adaptation of the cells towards osmotic stress (Bayles and Wilkinson, 2000; Carvalho *et. al*., 2004a). Compatible solutes are able to accumulate at high levels into the cell cytoplasmic fluid. This process needs adequate time and adding them in the growth media instead of drying media helps in obtaining better results (Carvalho *et. al*., 2004a).

Few examples of such compatible solutes are peptone, tryptone, yeast and meat extracts and various sugar substrates. Though the exact mode of action of these solutes against drying stress is quite complex but Ko *et. al*. (1994) suggested that it is very similar to that by cryoprotectants, i.e. prevention of aggregation of cell proteins and changing the cell membrane physical properties. A wide range of sugar substrates have been used by various researchers as supplements in the growth media, which has been summarized in a review paper by Carvalho *et. al*. (2004a). This includes glycine betaine, proline betaine, acetylcarnitine, carnitine, gamma-butyrobetaine and 3-dimethylsulphoniopropionate (Bayles and Wilkinson, 2000), non-fat skim milk and sucrose (Costa *et. al*., 2000), adonitol (Devaldez *et. al*., 1983), manitol (Kets *et. al*., 1996), etc.

2.5 Summary and conclusions

This chapter covered the basic aspects of probiotics, identified the issues related to probiotic stability and reviewed in fair detail two most common preservation techniques, encapsulation and freeze drying. This review helped to find out the research gaps and to develop hypothesis on potential courses of work to close the research gap. A completely successful stabilization technique should be able to immobilize the growth of cells to prevent product spoilage, should retain their viability during storage as an ingredient and also during the product shelf life. When the product containing these microencapsulated cells is consumed, the protective barrier must ensure their survival during gastric transit and at the same time it must ensure their proper release in the intestine in order to ultimately achieve the intended benefits. Several techniques for microencapsulation of probiotic bacteria were found to be partially successful in achieving the targeted and controlled delivery. Each combination of encapsulating material and encapsulation technology has some specific advantages and disadvantages. Producing bigger size microcapsules and thereby ensuring better protection is easier but bigger microcapsules reduce sensory quality in finished products. So all the criteria must be met to deliver something which is a real need for the industry. From this literature search, no published work has been found which dealt with all of these together and completely achieved the

targeted milestones. The current research was planned to explore some combination of encapsulation techniques as well as freeze drying media with a target of stabilizing probiotic bacteria. Further details of the research objectives and methodologies are described in the following chapters.

Chapter 3.0. Development of the microencapsulation process and studying the properties of produced microcapsules

3.1 Introduction

Lactobacillus casei 431 cells have been used throughout this study as the encapsulated core material. *Lactobacillus* is a part of the group known as lactic acid bacteria or LAB and being the largest genus among this group, it contains a very large number of species. Several health benefits have been reported to be associated with *lactobacilli* strains. Among many probiotic strains it was important to choose one for therapeutic dosing which has favorable properties against all the possible manufacturing (chemical and physical) and physiological stresses. *L. casei* has been reported to retain higher survivability, when exposed to heat stress conditions. It also shows better adaptability in a low temperature environment and continues to grow at 20°C, although at a reduced rate (De Angelis and Gobbetti 2004). *Lactobacillus* strains in general show a cryotolerance tendency towards freeze drying when pre-adapted in a similar environment (De Angelis and Gobbetti 2004).

Choosing a low pH medium for entrapping the *L. casei* cells (as discussed in this chapter) can be justified with the proven fact that acid adaptation to *Lactobacilli* species improves their acid tolerance capability. Survival in extreme acidic conditions, as in the human stomach, is very much affected positively by the acid tolerance response (ATR) (Foster and Hall, 1991). De Angelis *et. al*. (2001) showed that *L. sanfranciscensis* CB1 when acid adapted at low pH of 5.0, showed much higher viability in stressed environment compared to the counterparts grown at pH 6.5. The present experiment was designed in a similar fashion where *L. casei* cells were first exposed to a gradual decrease in pH for 2 hours during the microcapsule formation until pH reaches ≈5.2 and thereafter the capsules, entrapping the cells, were stored overnight at 4°C for further reduction in pH up to 4.6 - 4.7, followed by freeze drying.

3.2 Materials and Methods

3.2.1 Ingredients and chemicals

Sodium caseinate containing 90% (w/w) protein was obtained from Fonterra Co-Operative Ltd. (Palmerston North, New Zealand). High acyl type gellan gum and glucono-delta-lactone (GDL) were purchased (supplier code 418 and 575 respectively) from Hawkins Watts (Auckland, NZ). Canola oil was purchased from the local market. Commercial strain of *Lactobacillus casei* 431 in freeze dried form was obtained from Chr Hansen, Denmark. This strain is being marketed as a probiotic by one of the biggest culture manufacturing company in the world, hence this one was selected to further improve on its delivery mechanisms. MRS broth and peptone water were supplied by Difco Laboratories, U.S.A. MRS agar was purchased from Merck KGaA, Germany. Pepsin in powder form (P7000) and porcine bile extract powder (B8631) were supplied by Sigma Aldrich, USA. NaCl and NaOH were obtained from VWR International Ltd., England.

3.2.2 Studying the gel formation and gel behavior of encapsulation media

Sodium caseinate and gellan gum solutions of various concentrations were mixed with GDL and the rheological properties were studied. The purpose for this was to identify the most appropriate gel strength which will have sufficient rigidity to form individual microparticles. A time sweep test for a duration of 180 minutes was chosen to monitor the elastic (G') and loss (G") modulus development during the slow gel formation period. The rheometer used was from TA Instruments, U.K (Model AR-G2) and the procedure selected was comprised of oscillatory mode with flat plate geometry at a fixed strain 0.02 and a frequency of 1 Hz. The temperature of the samples was maintained at 30°C. Firstly, 10% sodium caseinate solution (w/w) was acidified with various concentrations of GDL (1.0, 1.5, 2.0 and 2.5%, w/w) with no gellan gum addition. In the next set of experiments, the caseinate solutions (10% w/w) containing different concentrations of gellan gum (0.1, 0.25 and 0.5%, w/w) were studied, maintaining the GDL concentrations at a fixed 2.5% (w/w) level.

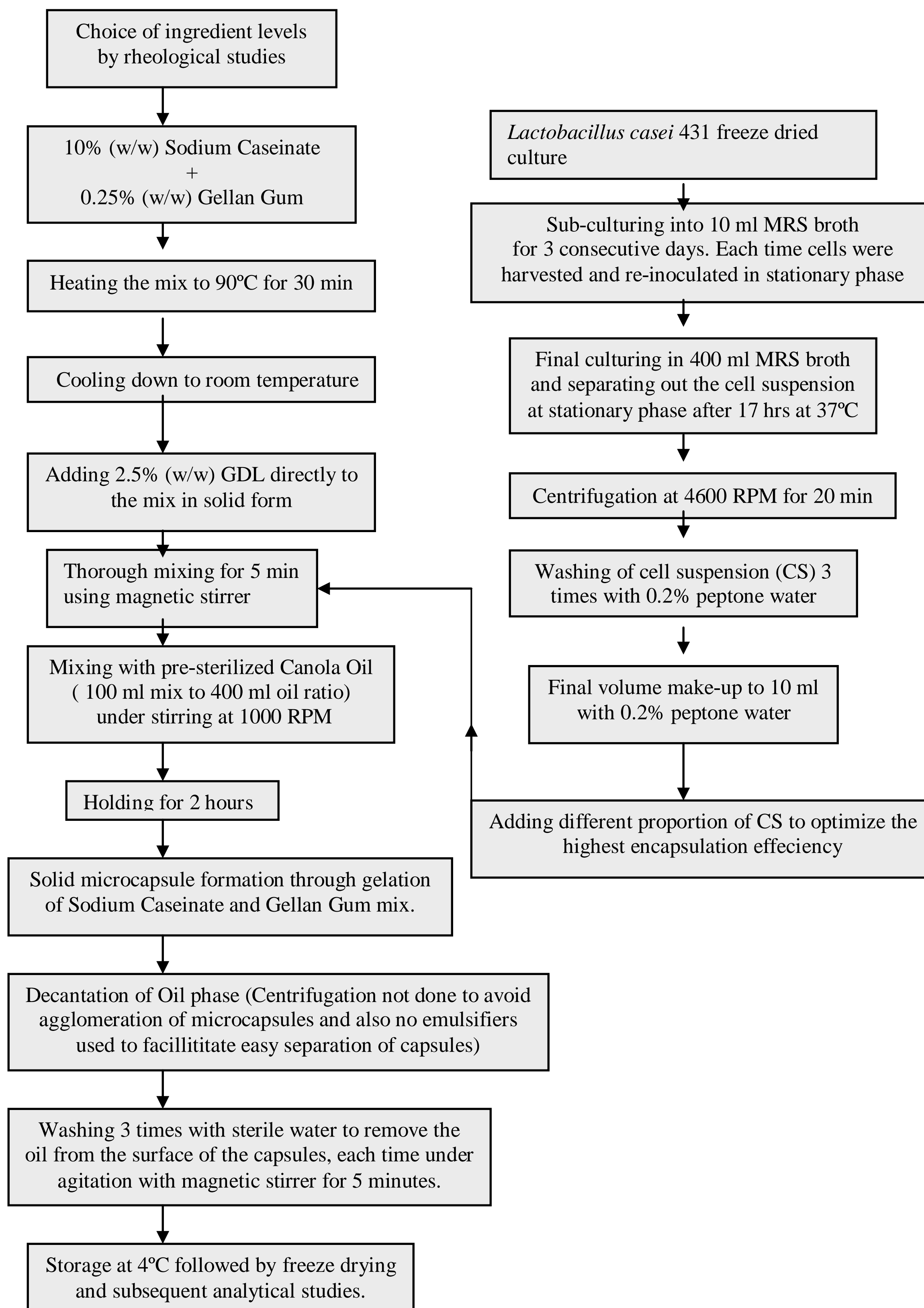


Figure 3.1 Optimized process flow diagram of the cell culturing and microencapsulation of *Lactobacillus casei* bacteria

3.2.3 Preparation of cell suspension

Lactobacillus casei 431 cells in freeze dried form were grown in MRS broth media (Man *et. al.*, 1960) with 1.0% (w/v) inoculation. The standard growth curve of *L. casei* cells was determined by checking the optical density (OD) at 610 nm using proper dilution factors in a spectrophotometer (Hitachi International, Japan). Growth phases were identified as per the graph shown in Appendix 3.1. Stationary phase cells were cultivated after 17 hours of incubation at 37ºC and re-inoculated in MRS broth for consecutively 3 times (Capela *et. al.*, 2006; Ding and Shah, 2009). All the inoculation works were done under sterile biological hood with laminar air flow. Only the incubation part was performed under anaerobic conditions using Gaspak™ EZ anaerobic container system from Becton, Dickinson and Company, Maryland, USA and all other activities up to the microencapsulation and storage were done under normal ambient atmosphere. Finally, 400 ml of the media was used for bulk culturing and cells were harvested at stationary growth phase by centrifuging at 4600 rpm for 20 min using a laboratory centrifuge (Thermo Fisher Technology, Germany). It was earlier reported that centrifugation does not have any detrimental effect on cell viability (Ainsley-Reid *et. al.*, 2005). The supernatant was discarded and the cell precipitate was washed thoroughly with sterilized 0.2% (w/v) peptone water. This washing process was repeated 3 times and after discarding the final wash water, the cell slurry was made up to 10 ml by adding required quantity of sterile peptone water and mixed thoroughly using a vortex shaker.

3.2.4 Microencapsulation process of *L. casei* 431 cells

The process flow diagram in Fig. 3.1 shows the microencapsulation technique used. The sodium caseinate and gellan gum were first rehydrated into required quantity of R.O water (a mix of 10% Na-caseinate and 0.25% gellan gum w/w) for 16 hours at 4°C under slight agitation with a magnetic stirrer (Biolab, NZ) and thereafter completely dissolved at 60°C for 5 min, followed by heating the mix at 90°C for 30 min for sterilization. All the glassware was autoclaved at 121°C for 15 min before use but autoclaving of the protein-polysaccharide mix was avoided because it resulted in the development of an

undesirable pink color. The mix was then cooled to room temperature and GDL (at the rate of 2.5% w/w) was added in solid form directly into the mix with continuous stirring at 300 RPM using a magnetic stirrer. The action of GDL on sodium caseinate solution alone and caseinate-gellan gum mix was studied by observing the gradual drop in pH. The pH meter used for this purpose and in all further occasions was from Eutech Instruments, Malaysia (Model pH 501). After stirring for 5 minutes and complete mixing of GDL into the solution, *L. casei* 431 cell suspension in various proportions (as outlined in section 3.2.5) was added to the caseinate-gellan gum mix under continuous stirring for another 5 minutes. One hundred gram of this mix was then added slowly into an Erlenmeyer flask containing 400 ml pre-sterilized canola oil under constant stirring at 1000 rpm with a magnetic stirrer. This resulted in a very fine and uniform distribution of the dispersed phase of caseinate-gellan mix into the continuous phase of canola oil. A stable emulsion was not desired as that would make the separation of microcapsules difficult at the later stage; hence no emulsifier was used in the process. The stirring process helped the discrete phase particles to remain suspended in the oil media. After holding the system for 120 minutes, each liquid droplet (dispersed phase) was converted into semi-solid gelled particles due to the action of GDL hydrolysis and consequent slow gelation of sodium caseinate. The particles were allowed to settle down and the oil was separated out by decantation. Centrifugal separation from the oil phase was avoided to prevent agglomeration of the gel particles. The microcapsules were washed 3 times with sterilized R.O water to remove the residual oil adhered to the particle surface. The sample was then stored overnight at 4°C for further analysis.

3.2.5 Optimization of the core -wall ratio

To determine the highest encapsulation efficiency, 4 different samples were prepared. Keeping the wall material mix constant in all cases, various proportions of cell suspensions were added into them as described below and the bacterial counts were measured before and after the microencapsulation process. Ten ml of the bacterial cell suspension as obtained previously (section 3.2.3) was divided into 4 parts of 1, 2, 3 and 4 ml and made up to the final volume of 5 ml by adding the balance quantity of

sterilized 0.2% peptone water. Each of these portions was then added to 100 gm of the wall material mix. The encapsulation efficiency (EE) was calculated using a modified equation of Heidebach *et. al.* (2009) as mentioned below.

$$EE\ (\%) = \frac{\text{Total Solids}_{wcm}}{\text{Total Solids}_{capsule}} \times \frac{\text{CFU. (g capsule)}^{-1}}{\text{CFU. (g WCM}^{*})^{-1}} \times 100\%$$

*WCM: Wall material and cell suspension mix.

Dry matter content of WCM and capsules was determined by measuring a known quantity and drying the same in an oven maintained at 105°C for 24 hours, and thereafter measuring the difference in weight due to moisture evaporation. The plate count of capsule slurry was done by breaking the capsules with a Colworth 400 laboratory stomacher (Model BA6021, A.J. Seward, London) and thereby releasing the cells, followed by serial dilution in peptone water, pour plating and incubating in MRS agar media at 37°C for 48 hour duration. The calculated encapsulation efficiencies for 4 samples are listed in Table 3.1 of results section 3.3.3.

3.2.6 Particle size distribution analysis

The wet and freeze dried microcapsules were analyzed for their mean diameter (D_{32}) and the cumulative size distribution in a Malvern Mastersizer 2000 Ver. 5.54 (Malvern Instruments Ltd., Malvern, U.K) using laser diffraction technology. The principle behind this technology is based on the diffraction of a laser beam on a diluted aqueous medium containing suspended particles. The diffracted laser beam is collected at different scattering angles by numerous semi-circular photoelectric diodes. The surface based particle diameter distribution is calculated by measuring the diffracted intensities for each angle using the Lorenz-Mie theory. The equation used by the instrument for calculating the D_{32} values is as follows: (McKay, 2002)

$$D[3,2] = (\sum n_i d_i^3 \ / \ \sum n_i d_i^2)$$

Where n_i is the number of microcapsules suspended with diameter d_i.

The samples were added gradually until the obscuration reached more than 15%. For wet microcapsules, a standard operating procedure (SOP) designed for casein micelle was used with particle refractive index (RI) of 1.370 and absorption of 0.001. The dispersant used was RO water with RI 1.330 and sample concentration 0.66% v/v. Samples were analyzed in duplicate and the average result is reproduced in the results section 3.3.4.

3.2.7 Evaluation protocol for protection of bacterial cells in simulated gastric juice

As one of the basic purposes of microencapsulation is to offer adequate protection to probiotic microorganisms against harsh acidic condition during stomach transit, a simulated stomach environment was prepared *in vitro* and the free and encapsulated *Lactobacillus casei* 431 cells were incubated in it. Simulated gastric fluid (SGF) was prepared as per US pharmacopeia (USP31-NF26, 2008) with 0.2% (w/v) NaCl and the pH was adjusted to 2.0 with hydrochloric acid. Pepsin (800-2500 units/mg of protein) in powdered form, obtained from porcine gastric mucosa was added into this preparation at 0.32% (w/v). The purpose was to observe any enzymatic degradation effect of pepsin onto the protein-polysaccharide matrix because such degradation would result into easy release of encapsulated bacterial cells and expose them to the artificial gastric fluid. One gm of wet and freeze dried microcapsules were added to test tubes containing 9 ml of pre-warmed (37°C) SGF preparations and incubated in a water bath maintained at 37°C under orbital agitation at 100 rpm (Guerin *et. al*., 2003). The pH measurements after the final incubation of 120 min did not show any significant change in value. After every 30 min, one sample aliquot was taken out and immediately the pH of the medium was raised to 7.0 with 0.1N NaOH to stop the enzymatic reaction as well as to destabilize the gelled protein matrix for easy breakdown of capsules. The pH meter electrode was

sterilized prior to the experiment with chlorine solutions and rinsed with sterile distilled water. The capsules were then smashed with the stomacher to release the entrapped live cells followed by serial dilution and pour plate counting on MRS agar (37°C for 48 hours). For the free cells a similar approach was taken and 1 ml of cell suspension was added for every 9 ml of SGF. Neutralization followed the incubation. In each experiment a negative control in peptone water (pH 7.0) was analyzed to measure the initial population. The cell counts were adjusted to a common denomination of per gram solid for the ease of comparison.

3.2.8 Survival of encapsulated and free cells in the presence of bile salts

A simulated environment containing bile salts was prepared as per the method described by Muthukumarasamy *et. al*. (2006). Monobasic potassium phosphate (KH_2PO_4) at 0.68% (w/v) and 1.0% (w/v) porcine bile extract were dissolved into deionized milliQ water (Millipore, France) and the pH adjusted to 6.8 with 0.2N sodium hydroxide solution. This solution was then autoclaved at 121°C for 15 min duration and used to evaluate the resistance of free and microencapsulated cells against the action of bile salt. Wet microcapsules (1 gm) were added in 4 pairs of kimax tubes containing 9 ml of prepared bile solutions each, pre-warmed at 37°C and incubated in a water bath maintained at 37°C with orbital agitation at 100 rpm. The same process was followed for free cell count where rehydrated cultures were grown in MRS broth with 1.0% inoculation for 17 hours and 1 ml of the cell suspension was added to 9 ml of prepared bile solutions in four kimax tubes. Samples from both groups were tested for viable counts in every 2, 4, 6 and 8 hours interval by serial dilution in 0.2% peptone water and pour plating on MRS agar (37°C for 48 hours). The encapsulation matrix was found to be weak at this almost neutral pH and moderate temperature combination. The beads went into solution after 8 hours, releasing the cells free. Therefore, any mechanical action to break them was not needed in this case.

For all the acid and bile tolerance experiments, samples were plated in duplicate for each dilution level and the whole experiment was replicated three times to obtain the mean values plotted and the standard deviations from the mean were represented as error bars.

3.2.9 Statistical analysis

Statistical analysis of the obtained results was done using Minitab 15.1.0 software from Minitab Inc., State College, PA, USA. Analysis of Variance (ANOVA) between two series of data was performed and a difference is considered to be of significant importance only at more than 95% confidence level ($p \leq 0.05$).

3.3 Results and discussion

3.3.1 Gelation of sodium caseinate and gellan gum mixture

An increase in the concentration of GDL resulted in more rapid formation of the sodium caseinate gel and higher G' values (Fig. 3.2). Gel formation was initiated after about 30 min of incubation with 2.5% GDL; lower concentrations of GDL led to slower gel formation and the addition of 1.0 and 1.5% GDL was not sufficient to produce a gel with adequate strength, even after 180 min of incubation. Three distinct phases of gelation were visible with 2.5% GDL (Fig. 3.2): the initiation of gel formation; a rapid increase in gel strength; a trend to a plateau when the GDL hydrolysis was possibly complete and no more gluconic acid was available for a further reduction in pH. Based on the elastic modulus and the relative gelation time, a GDL concentration of 2.5% was chosen for subsequent analysis.

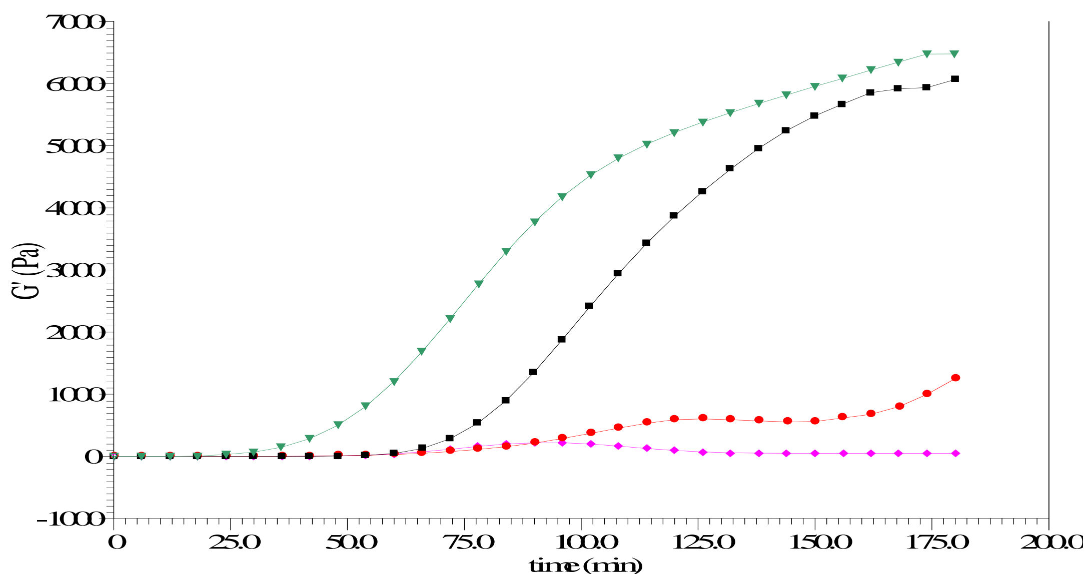


Figure 3.2 Changes in the elastic modulus (G') of 10% sodium caseinate solution mixed with 1.0% (♦), 1.5% (●), 2.0% (■) and 2.5% (▼) glucono-δ-lactone.

Gellan gum at different concentrations (0.10, 0.25 and 0.50%) was added to the combination of 10% sodium caseinate and 2.5% GDL. The interaction between sodium caseinate and gellan gum was evident by a marked increase in the *G'* of the mixture at the end of 180 min of incubation (Fig. 3.3). Because a very high *G'* of the wall material might create difficulty in releasing the core material from the gel network, a gellan gum concentration of 0.25% was chosen.

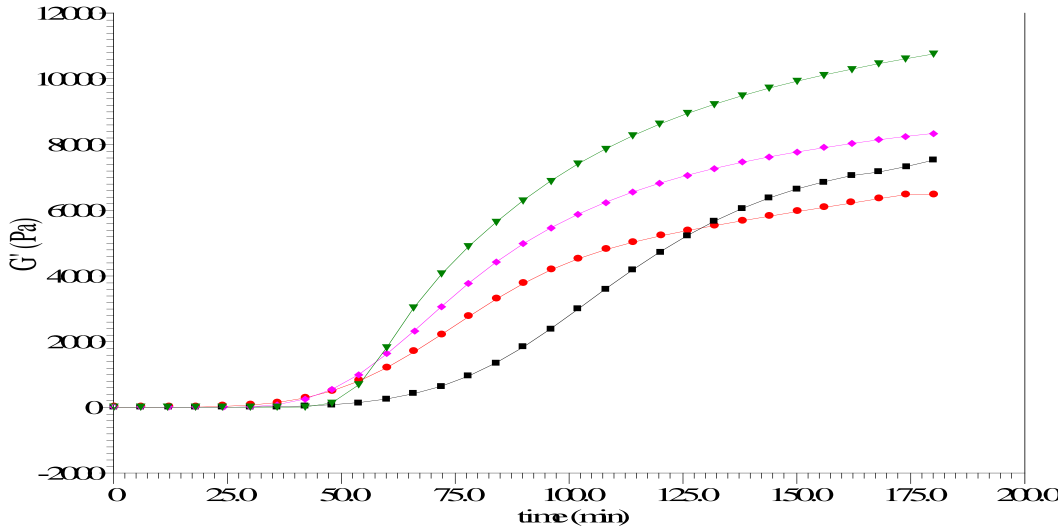


Figure 3.3 Changes in the elastic modulus (*G'*) after the addition of 0.1% (■), 0.25% (♦) and 0.50% (▼) gellan gum into a control sample (●) containing 10% sodium caseinate and 2.5% glucono-δ-lactone.

The rate of decrease in pH by the addition of 2.5% GDL was similar in both sodium caseinate solution and the sodium caseinate and gellan mixture solution (Fig. 3.4).

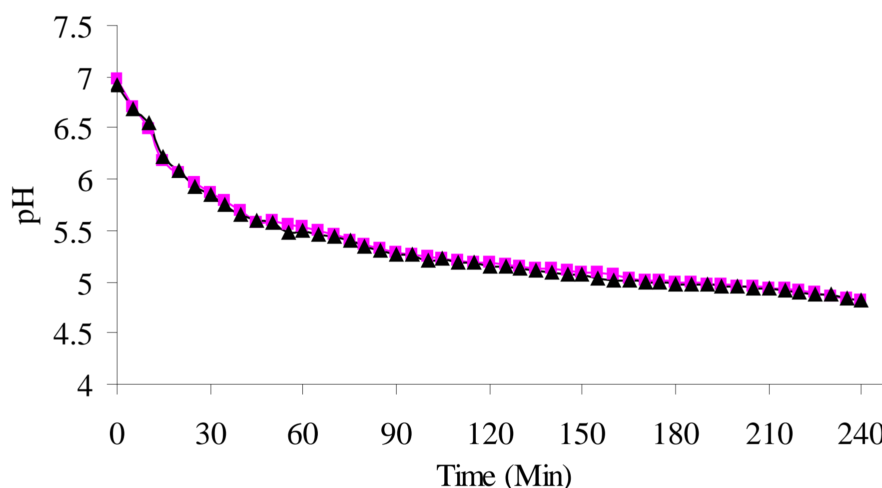


Figure 3.4 Changes in the pH by the action of 2.5% glucono-δ-lactone on 10% sodium caseinate (■) and 10% sodium caseinate mixed with 0.25% gellan gum (▲) during incubation for 240 min at 20 °C.

The rheological properties of sodium caseinate gels induced by cold acidification with GDL have been studied extensively (Lucey *et. al.*, 1997). In the present study, increasing the concentration of GDL resulted in more rapid formation of the sodium caseinate gel and higher *G'* values, which is in agreement with the findings of Lucey *et. al.* (1997) and Menendez *et. al*. (2004). However, the strength of the sodium caseinate gel appeared to be inadequate for forming microcapsules with sufficient rigidity. It was important that the soft gel particles retained their shape and that their size was intact without any coalescence. Therefore, we designed a protein–polysaccharide complex using sodium caseinate and gellan gum. Another reason for including a polysaccharide was the instability of sodium caseinate gels at higher pH. Gellan gum in combination with sodium caseinate was found to provide a synergistic effect in terms of the gel strength and the stability at higher pH values.

In the dynamic microencapsulation system used in our study, the droplets move around continuously and tend to coalesce, form larger particles and flocculate to form aggregates in the absence of any emulsifier. McClements (1999) explained this phenomenon as electrostatic attractions between the casein molecules near the

isoelectric point. In order to avoid this situation, the entire microencapsulation process was completed within approximately 2 h, when the pH of the mixture had decreased to about 5.2. Although a final pH of around 4.6 was needed to complete the coagulation and to attain a firmer gel structure, the final pH decrease may have continued after the microcapsules were stored at 4°C in a distilled water suspension. Our process was capable of preventing further agglomeration of the casein particles.

3.3.2 Encapsulation efficiency

The EE of various concentrations of free cell suspensions was calculated (Table 3.1). The EE varied from 41.9 to 89.5% with an increase in the cell loading but showed a slight decrease on further increase in the cell concentration above a certain level (3 mL of cell suspension).

Table 3.1 Optimization of the core-wall proportions by adding various concentrations of free cell suspensions

Sample	Composition	Log CFU/Gm Solid	Encapsulation Efficiency (%)
1	1.0% free cell suspension	10.96	41.91
2	2.0% free cell suspension	11.17	67.27
3	3.0% free cell suspension	11.29	89.54
4	4.0% free cell suspension	11.28	86.62

The high EE can be attributed to the entire process not including any detrimental steps such as heat treatment or high shear force. In addition, gellan gum in combination with sodium caseinate may improve the EE. The EE appeared to be in agreement with the results of Heidebach *et. al*. (2009), who used a similar process with a sodium caseinate emulsion. As shown in Table 3.1, the EE improved steadily with an increase in the cell

loading but there was a slight decrease after a certain optimum loading, because the cells present on the droplet surface were possibly lost into the oil phase or drained with the washing water.

3.3.3 Particle size distribution

The size distribution for the capsules was found to be uniform and exhibited a diameter range from about 40 to 1100 µm (Appendix 3.2). The surface-weighted and volume-weighted mean diameters of the capsules were found to be about 287 and 399 µm respectively (Fig. 3.5).

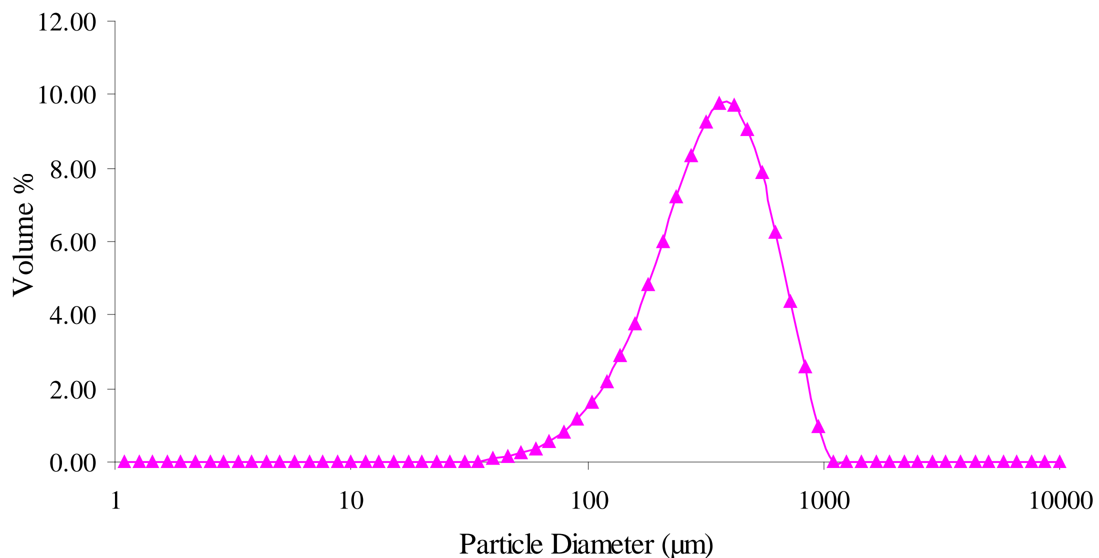


Figure 3.5 Particle size distribution curve of the microcapsules, as obtained using a Malvern Mastersizer.

Microparticle size distributions over a very wide range have been reported by several researchers. Muthukumarasamy *et. al*. (2006) encapsulated *Lactobacillus reuteri* using a variety of gel matrices and concluded that an extrusion process generally produces beads of much larger diameter (average 2–4 mm) than an emulsification process (from 20 µm to 1 mm). Adhikari *et. al*. (2003) used a combination of emulsification and coacervation

to encapsulate *Bifidobacterium longum* and reported microcapsules in the range 22−350 μm. The optimum microcapsule size is a compromise between the effectiveness of encapsulation and the sensory properties. In general, coarseness in the mouth occurs for a particle size above 1000 μm but is not detectable below 3 μm (Singer and Dunn, 1990). A minimum diameter of 100 μm has been suggested to offer better protection for *Bifidobacterium* in gastric juice (Hansen *et. al*., 2002) and an optimum range of 100−200 μm has been proposed (McMaster *et. al*., 2005). The sizes of the capsules produced in this study were slightly above this suggested range. However, the actual impact of this size distribution can be measured only by proper sensory analysis after incorporating the microcapsules in a suitable food formulation.

3.3.4 Survival of encapsulated and free cells in SGF and under simulated bile conditions

The incubation time tested for *in vitro* gastric juice tests varied among different researchers. As inspired by the *in vivo* trials carried out by Berrada *et. al*. (1991), some researchers have tried a 90 min duration (Ainsley-Reid *et. al*., 2005; Heidebach *et. al*., 2009; 2009a), whereas testing up to 2 hours have been also reported by other researchers (Sun and Griffiths, 2000; Guerin *et. al*., 2003; Ding and Shah, 2009). The optimum pH level in gastric environment is difficult to define because of its dynamic nature, but it is considered to be varying between 1.5 and 3.5. Martoni *et. al*. (2007) investigated the behavior of *L. plantarum* 80 cells in a computer controlled dynamic gastro-intestinal model and reported that an average human secretes about 2.5 L of gastric fluid in a day with an average pH of 2.0-2.5. Based on these findings, a combination of pH 2.0 and 120 min incubation time have been chosen in this study for the simulated gastric tests.

The initial count of free cells was adjusted with suitable dilution to about 10.7 log cfu, to match with the initial cell population of the capsules. After 30 min of incubation in SGF without pepsin addition, the viability of free cells declined to 9.4 log cfu and finally reached 4.9 log cfu after 120 min of incubation. In contrast, addition of pepsin to the SGF provided protection to the free cells (Fig. 3.6) because the viable count in SGF with

pepsin decreased only to 5.9 log cfu after 120 min of incubation, which was significantly higher ($p \le 0.05$) than for the free cells in SGF without pepsin. However, pepsin had almost no effect on the viability of encapsulated cells in SGF. With or without the presence of pepsin in SGF, the viable count of the encapsulated cells was significantly higher ($p \le 0.05$) than that of the free cells.

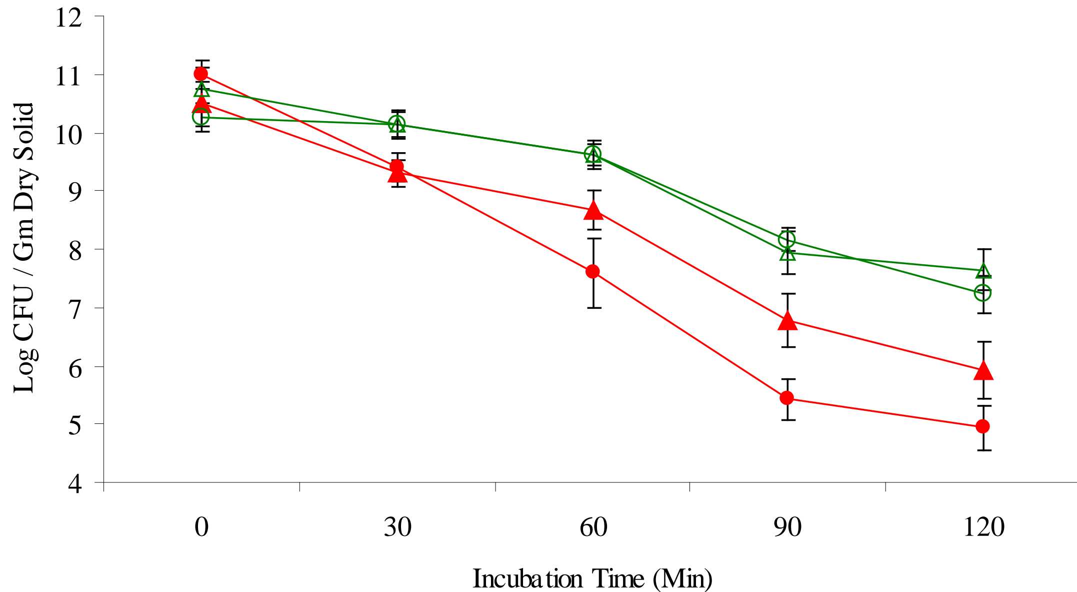


Figure 3.6 Survival of encapsulated cells (Δ, with pepsin; ○, without pepsin) and free cells (▲, with pepsin; ●, without pepsin) of *Lactobacillus casei* 431 in simulated gastric fluid incubated at pH 2.0 for 120 min. The error bars indicate standard deviations from the mean values of three replicated experiments.

The reduction in viability of free cells in SGF without pepsin of about 6.1 log cfu after 120 min of incubation can be compared with the 5 log cfu reduction of *L. paracasei* observed after 90 min at pH 2.5 by Heidebach et al. (2009). However, Song *et. al.* (2003) observed better resistance of *L. casei* YIT 9018 and only 4 log cfu reduction was recorded after 3 h of incubation at pH 1.2. A more pronounced lethal effect was reported by Ding and Shah (2009); when nine strains of *lactobacilli* and *bifidobacteria* were tested for acid resistance at pH 2.0; all strains were found to be badly affected, with an average log cfu reduction of 6.5−7.0 after 120 min of incubation. The addition of pepsin,

a proteolytic enzyme that is secreted in the stomach, to SGF appeared to have a protective effect on free cells (Fig. 3.5). In this study, after 120 min of incubation, the reduction in free cells was only about 4.6 log cfu in SGF with pepsin compared with 6.1 log cfu in SGF without pepsin. This observation is similar to the findings of Saarela *et. al.* (2005), who explained that it could be due to the presence of other unknown compounds in commercial pepsin extracts obtained from porcine gastric mucosa and also due to the strain-specific action of pepsin.

The viability of encapsulated cells in SGF (both with and without pepsin) was reduced by only about 3.1 log cfu after 120 min of incubation. When compared with the reduction for free cells, this finding is important for the current microencapsulation technique. Heidebach *et. al.* (2009) used sodium caseinate gelled with transglutaminase enzyme for *L. paracasei* encapsulation and similarly found about 3.0 log cfu reduction after 90 min of incubation at pH 2.5. They also investigated a different gelling technique using rennet but the differences in survival rate for encapsulated cells compared with free cells of *L. paracasei* and *B. lactis* were only 0.8 and 2.8 log cfu higher respectively (Heidebach *et. al.*, 2009a).

The better survival rate in an encapsulated environment can be attributed to the absence any direct contact of the cells with the acidic medium, which is common for any kind of encapsulation technique; additionally, the buffering nature of milk protein might provide some enhanced protection (Kos *et. al.*, 2000; Guerin *et. al.*, 2003; Ainsley-Reid *et. al.*, 2005). In this study, the better survivability of encapsulated cells in SGF might also be explained by the synergistic effect of gellan gum as well as by the pre-adaptability of bacterial cells in low pH caseinate gels.

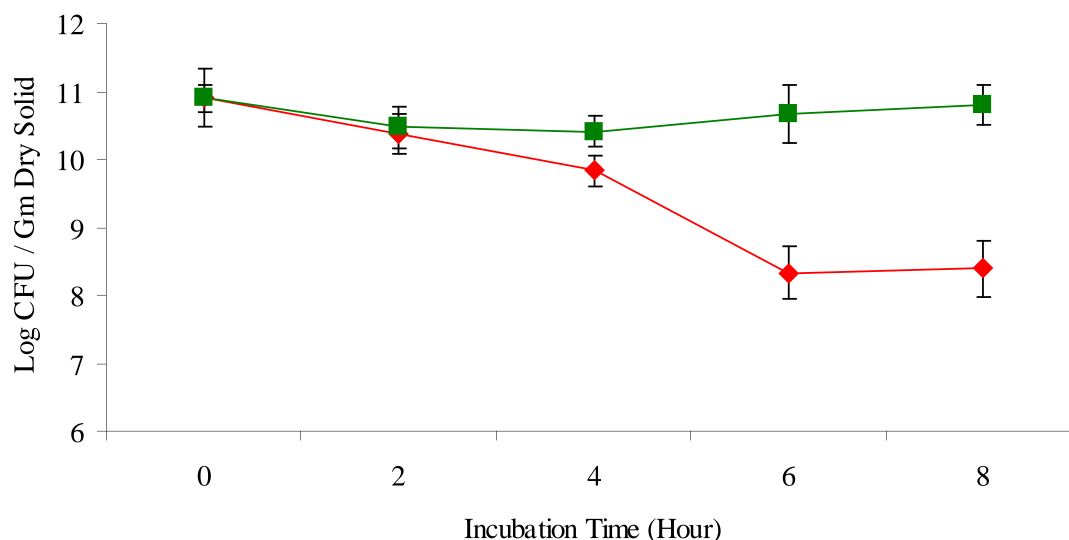


Figure 3.7 Survival of encapsulated cells (■) and free cells (♦) of *Lactobacillus casei* 431 under simulated bile conditions during 8 h of incubation. The error bars indicate standard deviations from the mean values of three replicated experiments.

The viable count of encapsulated cells in bile salt solution was also significantly higher ($p \leq 0.05$) than that of free cells after 4 h of incubation (Fig. 3.7); beyond 4 h, the detrimental action of bile salt on the free cells was accelerated. The free cell count had decreased by about 2.6 log cfu at 6 h but then remained almost constant between 6 and 8 h of incubation. For the encapsulated cells, the viable count decreased by only about 0.5 log cfu during the first 4 h of incubation, increased slightly at 6 h and then almost reached the initial cell count level after 8 h of incubation.

The neutral pH of bile extract solution may cause destabilization of the gel network and the properties of the bile salts could possibly cause emulsification of the entrapped or surface oil to some extent, thereby releasing the *L. casei* cells (Ding and Shah, 2009). In the present study, a very high bile tolerance was observed for encapsulated cells when compared with free cells. As different researchers have used various concentrations and sources of bile salts, it is difficult to make any comparison with the current study results. However, a lethal action of bile salts on probiotic bacteria has generally been observed

(Guerin *et. al*., 2003; Song *et. al*., 2003; Ding and Shah, 2009). Favaro-Trindale and Grosso (2000) and Guerin *et. al*. (2003) observed opposite results; free cells and encapsulated *bifidobacteria* cells showed higher viability after 3 h of incubation in the presence of bile salts.

3.4 Conclusion

The novel encapsulation technique developed in present study offers a gel network which is easy to handle with a good control over particle sizing and provides adequate protection for probiotic bacteria against a harsh acidic environment and the detrimental action of bile salts. It can be concluded that this system may be effectively utilized to deliver probiotic bacteria in a protected matrix and via a low pH carrier food to human intestine for further colonization there and conferring health benefits. The obtained data suggests that if the initial population can be maintained at a level of 10^{10} to 10^{11} cfu/gm then even after considering the cell mortality during gastric transition, the capsules should be able to deliver viable cells to the level of 10^{7} cfu/gm in the intestine. It is generally agreed that foods containing probiotic bacteria should have at least 10^{6} to 10^{7} cfu/gm viable cells per gram of it (Anal and Singh, 2007). Therefore, the high initial density of cells is very important from a commercial angle. It is preferable if one can restrict the addition of microencapsulated probiotics to below 1% level in any food formulation in order to avoid any unfavorable sensory responses (Gobbetti *et. al*., 1998; Muthukumarasamy *et. al*., 2006; Reid *et. al*., 2007). Therefore, an initial load of 10^{9} to 10^{10} cfu/gm of capsules is essential for this, which was achieved in the current research.

The microcapsules obtained here were made of soft gels of coagulated sodium caseinate and gellan gum complex, which needed to be stored in refrigerated conditions only, otherwise growth of the entrapped bacteria would destabilize the gel network. However, it is always preferable to develop a food ingredient which is stable at ambient storage conditions. Therefore, drying of the wet microcapsules is necessary to obtain a prolonged shelf life of the encapsulated bacteria in viable form. Developing the drying process and studying the effect of drying on the microcapsules' physical properties as

well as the physiological effects on cell viability became the major objective of the following chapters' work.

Chapter 4.0. Effect of freeze drying on the physical properties and viability of probiotic cells entrapped in the microcapsules

4.1 Introduction

The developed microcapsules in the form of soft gel beads (Chapter 3.0) contained on average 87.5 $^{+}/_{-}$ 1.5% moisture and had water activity (a_w) in the range of 0.93-0.95, which are considered to be very high and favorable for the multiplication of the entrapped viable cells. This could not be allowed because growth of cell population in the immobilized matrix would lead to leakage of bacteria from the capsules, which is not desired. For prolonged storage of the microcapsules containing probiotic bacteria, it was necessary to reduce this moisture content by choosing a suitable drying method. A dried form of storage is also important as it provides a sufficiently high dose of probiotic cells into any food product by reducing the bulk. Food manufacturers prefer dry ingredients because it provides economic benefits in the form of low rate of addition and the impact on sensorial qualities is minimum. Dried probiotic culture has substantial advantages over the liquid or frozen forms because refrigerated storage and distribution are expensive. Among the probable and popular drying methods, freeze drying was considered to be the most suitable for this particular project because freeze drying parameters impart low heat stress to the entrapped cells. Also, the handling properties of the semi-solid gelled particles were not suitable for other popular drying methods such as, spray drying or fluidized bed drying.

4.2 Materials and methods

4.2.1 Ingredients and chemicals

The wet microcapsules were produced following the procedure described in Chapter 3.0; hence all the ingredients and chemicals used in these experiments are the same as mentioned under section 3.2.1.

4.2.2 Preparing *L. casei* 421 cell suspension

As mentioned in Chapter 3.0, the method remains the same for growing and concentrating the bacterial cells. After 3 consecutive sub-culturing, stationary phase cells grown in MRS broth were harvested after 17 hours of incubation by centrifugation and washed thoroughly 3 times with peptone water. The obtained cell suspension was then mixed with the encapsulating materials as described in Chapter 3.0.

4.2.3 Microencapsulation technique

Wet microcapsules made of optimized proportions of sodium caseinate and gellan gum were prepared by cold gelation with GDL as described in sections 3.2.4 and 3.2.5 of Chapter 3.0. The bacterial cell suspension obtained above was entrapped into this matrix by the emulsion technique (Fig. 3.1) and the gelled particles were stored overnight at 4°C.

4.2.4 Freeze drying of wet gelled microcapsules

Freeze drying of soft gel particles were carried out in a freeze drier (W.G.G. Cuddon Ltd., Blenheim, N.Z, Model 0610). The steps involved in the process were freezing at -18°C, followed by drying with sublimation at 20°C under vacuum of 0.4 Torr, for a duration of 48 hours. The dried particles obtained were granular in texture with very good flowability.

4.2.5 Measuring size distribution of freeze dried microcapsules

The Scirocco-2000 unit of the Malvern Mastersizer 2000 Ver. 5.54 (Malvern Instruments Ltd., Malvern, U.K) was used to determine the mean particle size and surface based diameter range. The procedure and principle behind the laser diffraction technology are the same as described in the case of wet particles in section 3.2.6 (Chapter 3.0). The refractive index of the casein micelle, i.e; 1.370 was used as reference because the exact value of the same for this complex encapsulating marix and bacterial cells was unknown. Duplicate samples were analyzed and the average result is presented in the results section.

4.2.6 Scanning electron microscopic (SEM) examination

The morphological properties of the surface as well as the cross sectional areas of freeze dried capsules were observed under a FEI Quanta 200 scanning electron microscope (Eindhoven, The Netherlands). The powdered particles were sliced with a razor blade, mounted on a standard SEM sample stud made of aluminum, with the help of double sided sticky tape. The sample was then sputter coated with gold with the help of a BAL-TEC SCD 050 sputter coater under vacuum of 5 x 10^{-2} milli bar. The images were recorded in TIF format using 20 kV accelerated voltage and 250 to 20,000 times magnification.

4.2.7 *L. casei* 431 cell viability during *in vitro* gastric transit and in presence of bile salts

The analytical methods adopted were exactly the same as described in section 3.2.6 and 3.2.7 for wet microcapsules.

4.2.8 Storage stability test and moisture content determination

The freeze dried microparticles were divided into three parts for the shelf stability test and stored at -20°C, 4°C and 37°C (accelerated stability test). The samples were packed in low density poly-ethylene (LDPE) sachets under normal atmospheric conditions, as described by Saarela *et. al*. (2005). Each sample was tested for viable cell counts every 7 days and allowed to be rehydrated for 120 min at 4°C followed by homogenizing in a stomacher, before pour plating on MRS agar. The moisture content at each point was determined by measuring a known quantity of the particles and drying it in a hot air oven maintained at 105°C for 24 hours duration and thereafter by calculating the difference in weight (Appendix 4.4). The viable cell counts were converted into a common denominator of per gram solid for ease of comparison.

4.2.9 Statistical analysis

Statistical analysis of the obtained results was done as per the methods described in chapter 3.0 under section 3.2.9.

4.3 Results and discussion

4.3.1 Particle size distribution of freeze dried microcapsules

The freeze dried particles were analyzed for their size distribution and found to have a lower range and lower mean diameter compared to the wet ones. This was expected because drying causes shrinkage of the particles, thereby producing smaller microcapsules (Appendix 4.1). The particles diameter ranged from 45 to 630 µm with a surface weighted mean (D_{32}) of 151.65 µm. Fifty four percent of the sample population was below 182 µm (Fig 4.1).

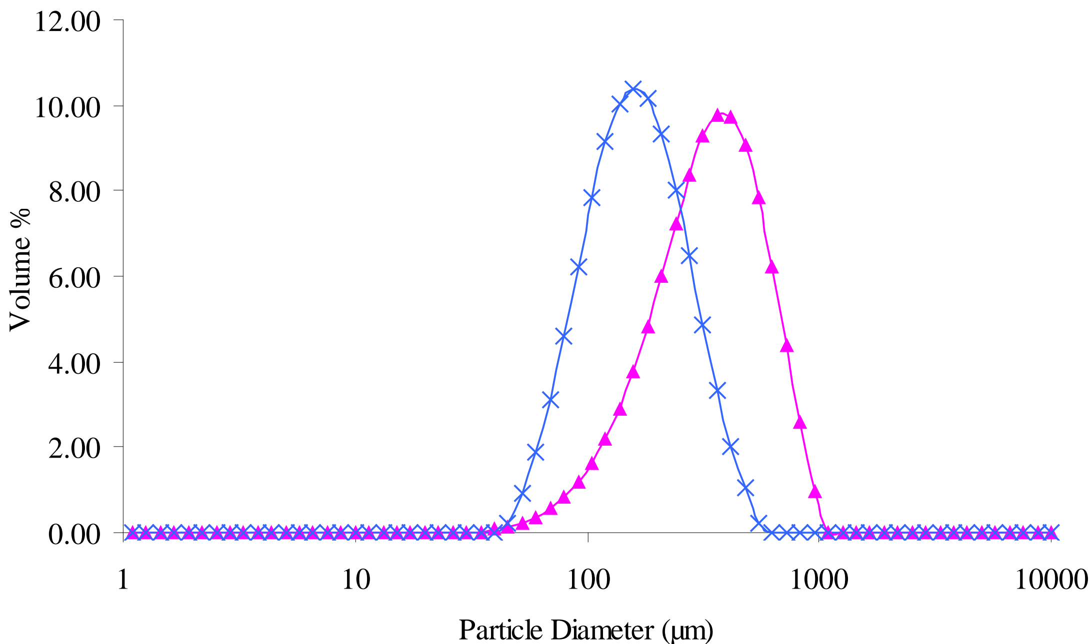


Figure 4.1 Volume distribution curve of wet (▲) and freeze dried (x) particles plotted against surface based diameters measured using Malvern Mastersizer, U.K.

The comparative size distribution between the wet capsules obtained in Chapter 3.0 and the dried particles in this experiment can be observed from Fig. 4.1. The higher mean diameter in wet particles is visible from the right hand shift of the curve whereas the

smaller particle size range in case of dried samples is represented by the narrower bell - shaped curve.

Table 4.1 Sieve analysis of freeze dried microcapsules done in Malvern Mastersizer Scirocco-2000 unit.

Mesh No	Aperture μm	Volume In %	Vol Below %
8	2000	0.00	100.00
10	1700	0.00	100.00
12	1400	0.00	100.00
14	1180	0.00	100.00
16	1000	0.00	100.00
18	850	0.00	100.00
22	710	0.00	100.00
25	600	0.86	100.00
30	500		99.14

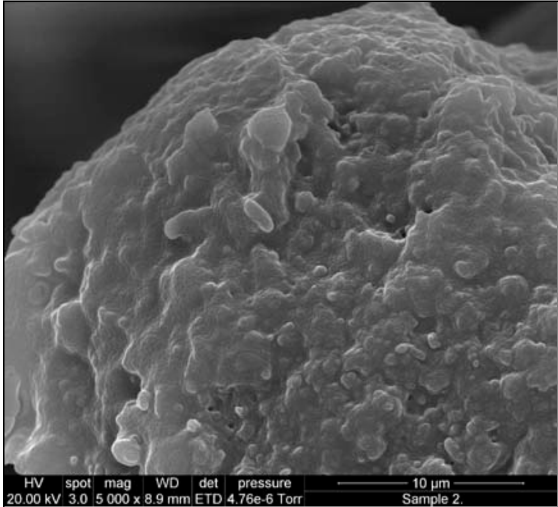
Mesh No	Aperture μm	Volume In %	Vol Below %
30	500	2.10	99.14
36	425	4.38	97.04
44	355	6.45	92.66
52	300	9.68	86.21
60	250	10.82	76.53
72	212	11.99	65.71
85	180	13.66	53.72
100	150	12.66	40.06
120	125		27.40

Mesh No	Aperture μm	Volume In %	Vol Below %
120	125	9.64	27.40
150	106	7.38	17.76
170	90	5.57	10.37
200	75	3.04	4.81
240	63	1.38	1.77
300	53	0.39	0.39
350	45	0.00	0.00
400	38		0.00

The mesh analysis of the sample population (Table.4.1) shows 100% of the particles passed through 25 mesh, 40% passed through 100 mesh sieve and the particle with lowest diameter could pass through a 300 mesh sieve.

In Chapter 3.0 it was discussed that some previous researchers proposed an optimum range of 100 to 200 μm in particle diameter distribution to maintain a good balance between protection ability and sensorial qualities. In the present study, freeze drying helped to reduce the overall size range and mean particle diameter. The maximum size was also reduced to 630 μm from the maximum value of 1096 μm in wet sample population. The average mean size of the microcapsules was reduced from 287 μm to 152 μm after freeze drying. This value is well within the proposed range and is therefore considered to be an improvement step in process optimization.

4.3.2 Scanning electron microscopic examination



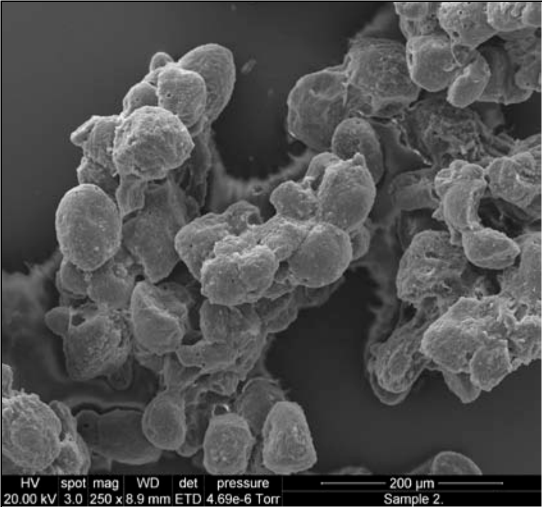
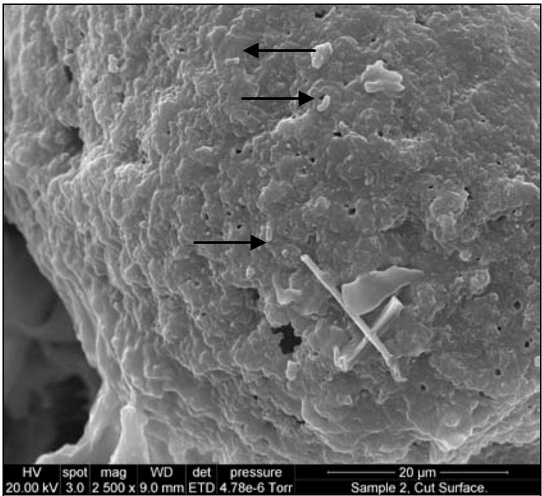


Fig. 4.2.1 Surface of a particle viewed at 5000x magnification.

Fig. 4.2.2 Agglomerated microcapsules viewed at 250x magnification.



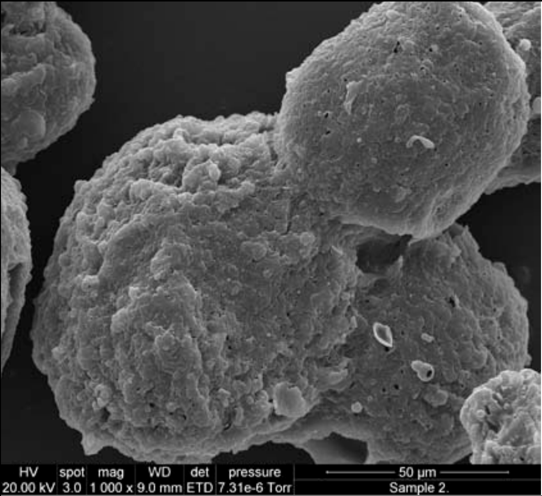


Fig. 4.2.3 Cross sectional view at 2500x magnification showing presence of a few *L. casei* cells (pointed with black arrows).

Fig. 4.2.4 Uneven surface properties viewed at 1000x magnification.

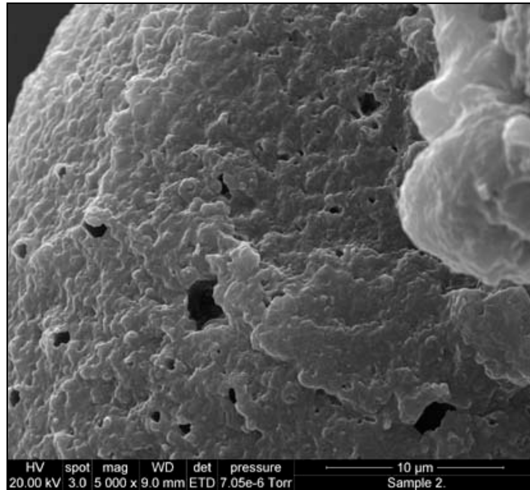


Fig. 4.2.5 Surface morphology at 2500x magnification showing presence of numerous pores.

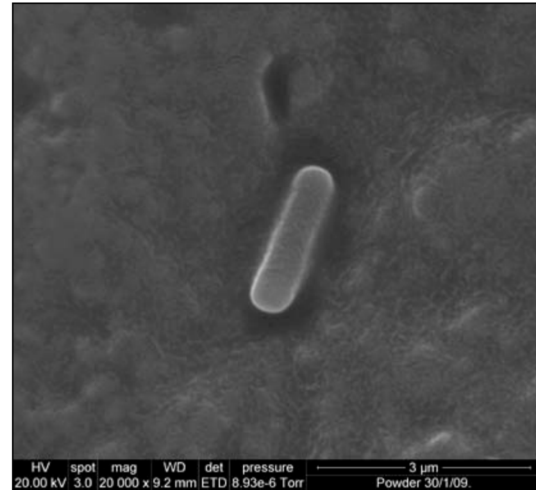


Fig. 4.2.6 Rod shaped *L. casei* cell visible on a cross sectional view at 20000x magnification.

Fig. 4.2.1 - Fig. 4.2.6 show scanning electron micrographs of the freeze dried particles at different magnification levels. It is evident from Figs. 4.2.2 and 4.2.4 that the microcapsules produced were not perfectly spherical in shape, as opposed to alginate capsules generally produced by the extrusion method. A possible explanation is available from Ainsley-Reid *et. al*. (2005) who used a Ca^{2+} induced whey protein gel for encapsulation and concluded that the slower gelling property of milk protein was the reason behind irregular shape of capsules. This was confirmed by Muthukumarasamy *et. al*. (2006) who found almost perfect spherical particles with smooth surface in the extrusion process but the same wall material (sodium alginate) in the emulsion process produced irregular shaped and rough surfaced particles, very similar to what was obtained in the present study (Fig. 4.2.1). Most of the individual particles were actually agglomerated, made up of several smaller, near spherical particles. After agglomeration they had taken up the irregular shapes. But the whey protein gel network mentioned above (Ainsley-Reid *et. al*. 2005) produced a much smoother and compact surface compared to these results. A compact surface without pores can provide much better barrier properties to stop acid or bile diffusion and escape of bacterial cells from the encapsulated matrix. This remains a challenge because a regular smooth surface and

uniform size distribution are important for industrial processing and the future objectives of the current project will be focused on this aspect. The *L. casei* cells were not visible on the surface of the particles but the cross- sectional view showed a few rod shaped cells, as shown in Figs. 4.2.3 and 4.2.6 and also their presence is confirmed by plate counts during subsequent tests. This is in agreement with the findings of Muthukumarasamy *et. al*. (2006).

The stickiness or agglomerating character of the freeze dried microparticles can be explained with the degree of free oils present on the particle surface and the binding property of oil. Hogan *et. al*. (2001) also found similar agglomerated spray dried particles made from Na-caseinate and carbohydrate blends and reported the presence of surface oil as the probable reason.

Guerin *et. al*. (2003) prepared a protein-polysaccharide gel matrix to entrap *B. bifidum* cells and further coated the beads with a membrane by a transacylation reaction between pectin and whey protein at high pH. In their study, the control capsules without membrane coating had numerous pores present on the surface which facilitated acid and bile juice penetration inside them and consequent reduction in entrapped cell viability. Similarly, in the present study the presence of clear pores on the capsule surface was found via SEM pictures (Fig. 4.2.4, Fig. 4.2.5). To apply an effective barrier coating around the dried particles, various possibilities are being tried out but a similar transacylation reaction might not be suitable for this method because it needs the reaction to occur at pH 8.0-9.0 and at that level the gelled sodium caseinate matrix would become unstable and solubilize, thereby releasing the entrapped bacterial cells.

4.3.3 Effect of freeze drying on microencapsulated *L. casei* 431 cell viability

It can be seen from Fig. 4.3 that freeze drying affected the viability of the encapsulated cells and a considerable loss was observed. Results from 3 independent experiments showed that the initial count of 11.04 Log CFU per gm dry solid has been reduced to 9.34 Log CFU/gm after the drying process.

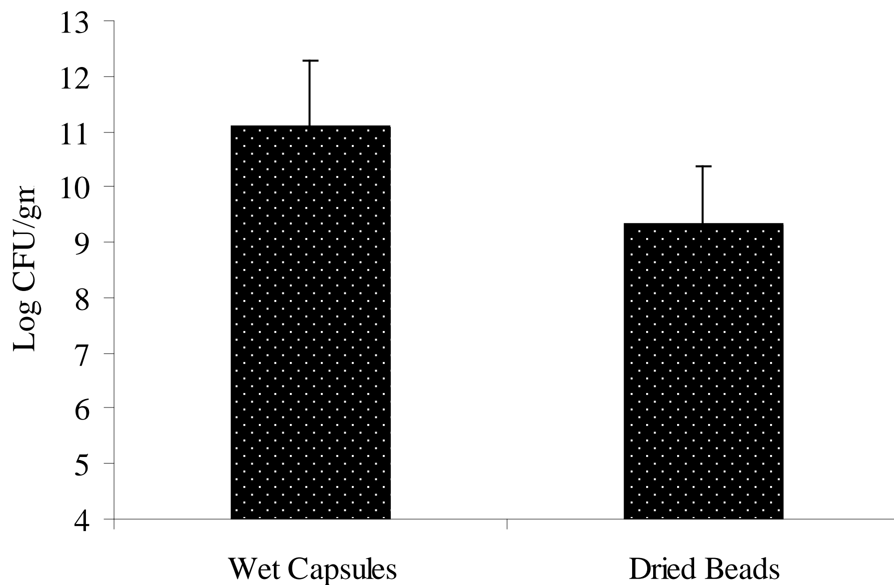


Figure 4.3 Effect of freeze drying on the survival of microencapsulated *L. casei* 431 cells

Freeze drying is a popular method for preserving sensitive bioactive compounds such as, microorganisms because it removes the moisture from the medium by sublimation at ambient temperature and thereby exerting the very minimum heat stress to the living cells. But it has been reported that all the organisms do not survive equally well during freeze drying and certain strains do better in this regard (Rybka and Kailasapathy, 1995). *Lactobacillus* spp. and especially *L. casei* 1520 has been found to be more adversely affected by freeze drying than the *Bifidobacterium* spp. because of their cell wall membrane compositional differences (Carvalho *et. al.*, 2004; Capela *et. al.*, 2006). This difference in survival rate remained pronounced even when cryoprotectants were used. With the same cryoprotectant material Unipectine™ RS 150, *B. longum* cells showed 80% improvement in viability whereas *Lactobacillus* spp. showed only 30% increment (Capela *et. al.*, 2006).

The post drying cell population obtained in the result reported above was not very encouraging. The log reduction of 1.70 observed here (Fig. 4.3) is very high even

compared to the free cells freeze dried by Capela *et. al*. (2006). Four strains of *Lactobacillus casei* 279, 292, 1520 and 2607 were freeze dried in that experiment and recorded a post drying log reduction of 0.12, 0.16, 0.39 and 0.12, respectively. These strains were not microencapsulated but they were dried in a neutral pH media containing 14% w/v reconstituted skim milk. Ainsley-Reid *et. al*. (2005) freeze dried the free and whey protein based microencapsulated cells in media supplemented with sucrose, lactose, milk and they found satisfactory survival rate (ranging from 34-52%) after drying. Based on these results it can be concluded that the lethal action of freezing in the present study has been prompted by the absence of milk sugar or any similar cryoprotectant in the encapsulated matrix and also the low pH of the encapsulating wall materials. Freeze drying at low pH is considered to be a major cause of cell mortality because as the solute becomes evaporated gradually, the concentration of hydrogen ions around the cell membranes becomes high (Saarela *et. al*., 2005). A similar conclusion was drawn by Baati *et. al*. (2000), when exposure of *L. acidophilus* cells to pH 5.4 prior to freezing led to a considerable loss in post drying viability. The improvement in post drying survivability using a suitable cryoprotectant and thereby offsetting the lethal effect of low pH remains an important objective for the next phase of work of this project.

4.3.4 Protection of microencapsulated and freeze dried *L. casei* cells in simulated gastric juice

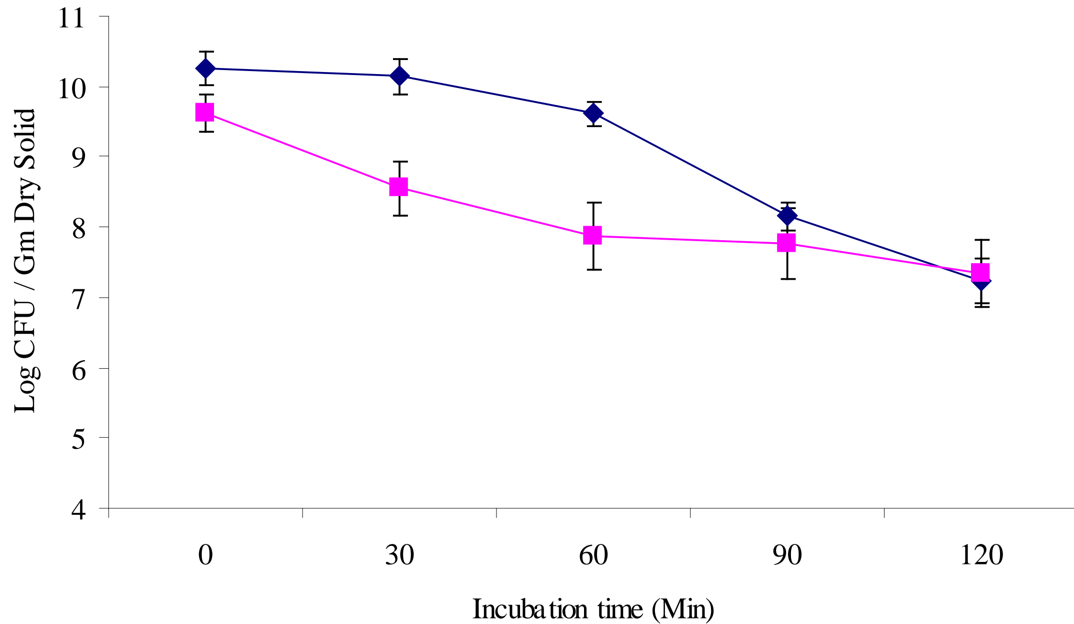


Figure 4.4 Survival of *L. casei* cells microencapsulated in wet (♦) and freeze dried (■) form during incubation in simulated gastric fluid. The values shown are the mean values of three replicates.

Fig. 4.4 shows the comparative protection levels offered by the wet and freeze dried particles to the *L. casei* 431 cells entrapped therein, against the acidic medium (SGF) at pH 2.0. For the wet microcapsules, the viability reduced from 10.27 log CFU/gm at the beginning to 7.23 log CFU/gm at the end of 120 min (as reported in section 3.3.5 of Chapter 3.0). That equals to a net loss of 3.04 log CFU in cell viability. The antimicrobial action of gastric acid was comparatively less pronounced on the freeze dried microcapsules described in this chapter. During the experiment, the initial load could not be increased above 9.62 log CFU/gm, on average, due to some viability loss during drying, but after the 120 min incubation period at pH 2.0, the final average colony count per gm solids was recorded as 7.34 log CFU. That equals to a 2.28 log reduction on net basis. Comparison of the net log reduction figures for wet and dry

samples showed that dried capsules were able to offer 36 times better protection than the wet ones. Also, statistical analysis supported this finding and showed significant difference ($p \leq 0.05$) between the rates of cell decay in two samples.

In case of freeze dried samples, the rate of decline in viable cells number was slower compared to the wet particles, as evident from the slope of the curve (Fig. 4.4). Drying causes shrinkage of particle volume and reduction in surface pore diameters. Therefore, a possible explanation could be the easier penetration of acid through the wet surface pores of the microcapsules, compared to the dried ones and thereby imparting lethal effect on the bacterial cell membranes.

It was earlier stressed in Chapter 3.0 that the better protective nature of the produced microcapsules against simulated gastric fluid is probably contributed by the presence of milk protein in the wall material mix. Though Ainsley-Reid *et. al*. (2005)'s work involving whey protein gelation with $CaCl_2$ did not yield very good acid tolerance result but they suspected that sub lethal damage of cells during Ca^{2+} ion exposure was a probable reason for such poor viability. Picot and Lacroix (2004) believed the presence of protein in their spray dried encapsulating matrix was one of the reason to offer better protection against gastric acid and the same matrix was easily able to degrade in intestinal fluid, thereby releasing the *Bifidobacteria* cells completely. In the present study, the presence of gellan gum is also a probable factor for better protectability of the microcapsules. Gellan gum is known to be resistant to acidic environment and might have had some synergistic effect in combination with casein, which has contributed to the better survival rate in simulated gastro-intestinal conditions. After the complete 120 min of incubation, the microcapsules were found intact and stable with no adverse effect of low pH gastric juice, in contrast to the report from Heidebach *et. al*. (2009) who found about 40% protein release into the gastric juice from sodium caseinate based microcapsules. Microencapsulation has been reported by many researchers as a very effective way to protect the cells from gastric juice. Almost all of these studies have been concentrated on providing a neutral pH environment around the bacterial cells. Any

literature discussing about encapsulating the cells in a low pH matrix (pH 4.7) was not found. Therefore it is difficult to compare these results with similar work done before.

4.3.5 Effect of bile salts on encapsulated cells in freeze dried microcapsules

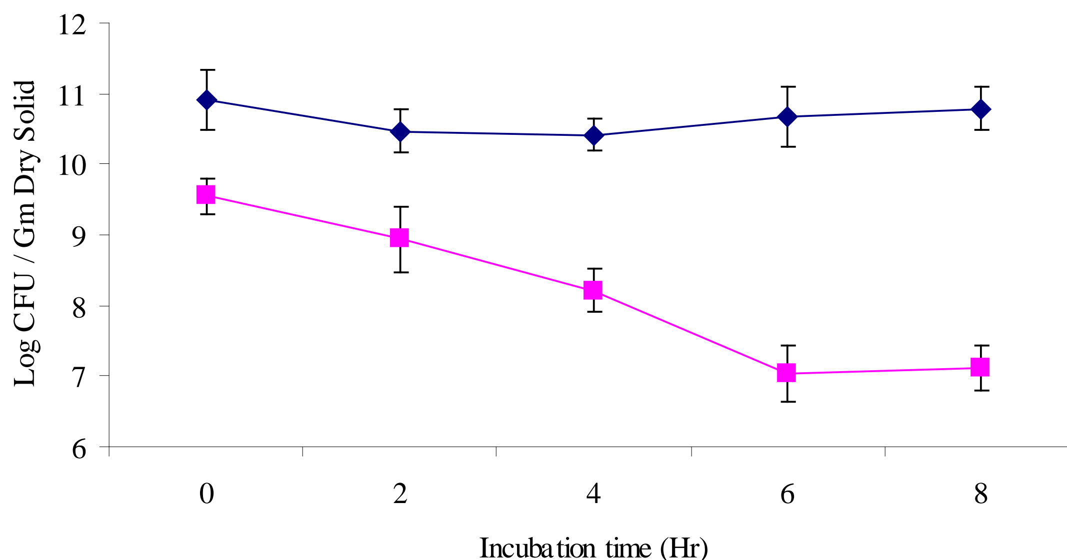


Figure 4.5 Action of bile salts on *L. casei* cells encapsulated in wet (♦) and freeze dried (■) microcapsules

In this section, a comparative analysis is shown between the protective actions of wet and dried microcapsules against the bile salts encountered by the probiotic bacteria during simulated intestinal transit. Fig. 4.5 shows the results for wet capsules already reported previously in Chapter 3.0 and reproduced here for comparison purposes. Excellent tolerance against bile salts was observed when cells were entrapped into wet microparticles. A slight decline of 0.50 log CFU in viable cells was recorded in first 4 hours but thereafter the number became steady and slightly increased at the end of 8 hours. The initial and final readings after 8 hours were 10.9 and 10.8 respectively. However, the freeze dried particles seemed to offer poor protection to the cells and the viability was badly affected in the first 6 hours which came down from 9.6 to 7.0 log CFU. Thereafter a slight improvement was observed and at the end of the 8 hour period the final count was 7.1 log CFU per gram solid.

This higher mortality for freeze dried cells can be compared with the works of Saarela *et. al*. (2005) who used the same 1% bile extract, neutral pH 7.0 and observed 2.5 to 3.0 log CFU reduction of freeze dried *B. animalis* cells. In the present study, *L. casei* cells were significantly better ($p \leq 0.05$) protected in wet microcapsules by approximately 200 times compared to the freeze dried microcapsules. This higher protection obtained in wet capsules could not be supported by searching the published studies. Physical observation showed that the neutral pH medium of the simulated intestinal fluid caused destabilization of the gel network and the gelled particles were completely dispersed into the bile solution at the end of the experiments. The detergent like property of bile salt might have caused emulsification of the entrapped and surface oil and thereby releasing the *L. casei* cells to bile salt solution (Ding and Shah, 2009). The lipophilic nature of bile salts is thought to be the primary reason for bacterial cell mortality. The actual detrimental action as suggested by Kurdi *et. al*. (2006) is by accumulation on the cell membrane, resulting into structural and functional alteration of the membrane. Bile salts make the bacterial cell membrane permeable to protons, causing cell death (Kurdi *et. al*., 2006; Ruiz *et. al*., 2009). From the transmission electronic microscopic images, the above authors found severe damage to *B. animalis* cell surface due to action of bile salts and suggested pre-adaptability in gradually increasing concentration of bile solution as a measure to protect the cells during intestinal transit.

4.3.6 Enumeration of viable cells

The use of stomacher a to break the capsules during enumeration did not seem to have any detrimental effect on cell viability. Other methods reported for capsule breaking are immersion in sodium phosphate/citrate buffer (in case of alginate beads) followed by sonication (McMaster *et. al*., 2005), pasting with a mortice (Sun and Griffiths, 2000; Muthukumarasamy *et. al*., 2006), homogenization (Guerin *et. al*., 2003; Ainsley-Reid *et. al*., 2005), or gentle shaking (Sheu and Marshall, 1993; Song *et. al*., 2003). Use of a combination of stomacher, homogenizer and colloid mill was reported by Heidebach *et. al*. (2009) for protein-based microcapsules. Hansen *et. al*. (2002) preferred mechanical

disruption for releasing the cells from alginate capsules, over the phosphate buffer immersion process and found equal release of cells in a less time consuming process. In the present study, because of very good stability of the encapsulating matrix at low pH, it was difficult to smash the particles completely with only a stomacher when they were suspended in either simulated gastric juice or peptone water. Therefore, the encapsulating network had to be weakened by neutralizing the medium to pH 7.0 with NaOH, without imparting any stress to the cells. Neutralization did not seem to have any adverse effect on cell viability and this was supported by the findings of Saarela *et. al.* (2005). This was not needed during the enumeration of bile solution experimental samples because they were already suspended into a neutral pH media.

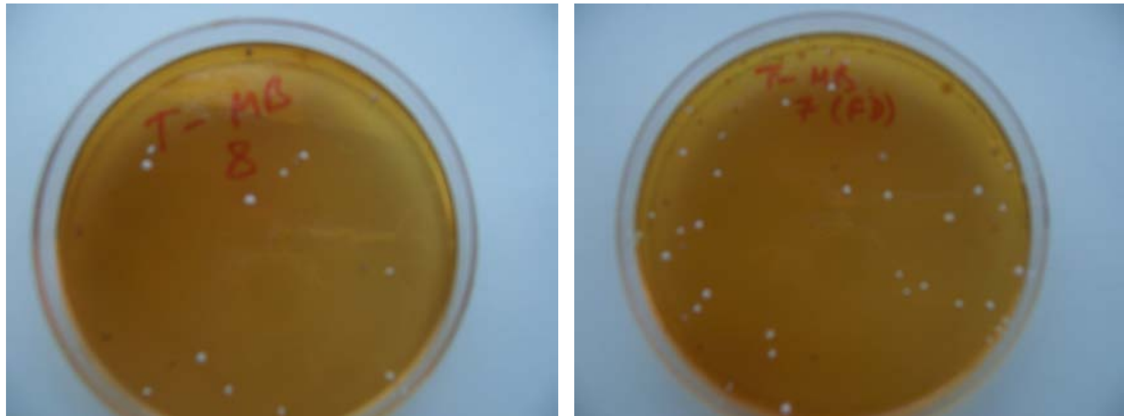


Figure 4.6 *L. casei* colonies plated on MRS agar

4.3.7 Storage stability of the freeze dried microcapsules

Fig. 4.7 represents the viable cell counts for freeze dried particles stored at different temperatures under normal atmospheric conditions. Each sample was tested weekly during a period of one month. Storage at -20°C proved to be most successful. The cell count starting from 9.47 Log CFU was slightly increased to 9.61 at the end of 1st week and thereafter reduced to 8.41 Log CFU after 4 weeks. This was a satisfactory result with only 1.06 log CFU loss in viability during the entire period. A non-consistent trend was observed during 4°C storage. The viable count declined considerably after the first

week from 9.47 log to 8.08 log CFU but then gradually went up to 9.50 log at the end of 3rd week and finally reduced to 8.02 log CFU after 4th week. The accelerated storage test at 37°C showed a steady decline in viable cell count. The rate of decline was fastest in the first week from 9.47 log to 5.74 log CFU per gm solid, followed by a steady decline till 3.35 log CFU at the end of 4 weeks. The higher temperature storage had a fatal impact on the bacterial cells.

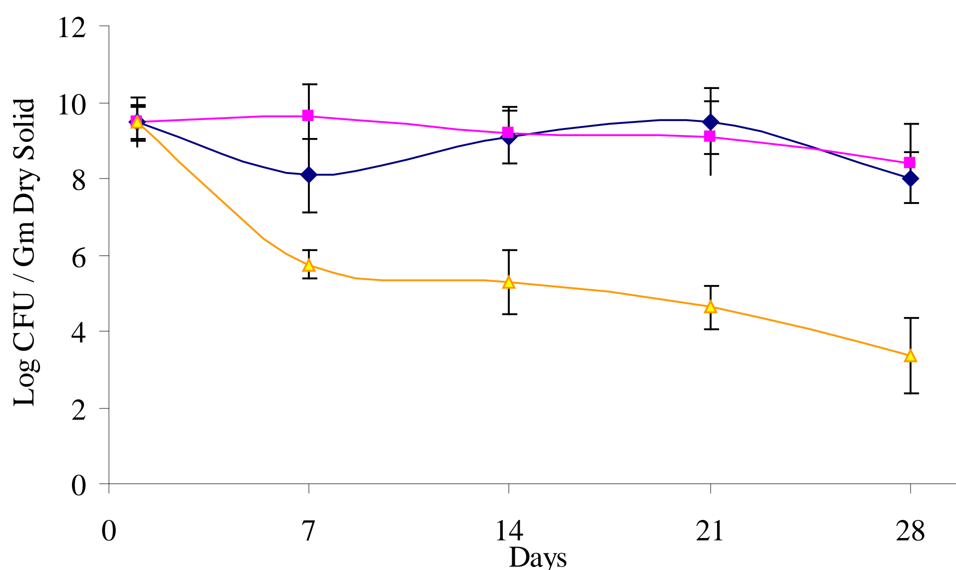


Figure 4.7 Changes in cell viability of freeze dried microencapsules containing *L. casei* cells, during storage at -20°C (■), 4°C (♦) and 37°C (▲). Error bars representing standard deviations from the mean value of 3 replicated counts.

In order to facilitate the ease of transportation and storage, the encapsulated probiotics must be produced in a dried form. In this project, the best result was obtained in -20°C storage with only 1.06 log CFU reduction in cell count, followed by storage at 4°C where a net reduction of 1.45 log CFU/gm was recorded (Fig. 4.7). Dry storage of both encapsulated and free cells at 4°C have been previously reported to show good retention of viability, up to 4 weeks for free and up to 6 weeks for encapsulated cells (Kim *et. al.*, 1988; Koo *et. al.*, 2001; Song *et. al.*, 2003). However, at 23°C storage the above researchers found higher reduction in log cycles for free cells compared to the encapsulated ones. As shown in Fig. 4.7, high temperature storage at 37°C had definitely affected the encapsulated cell viability. According to Baati *et. al.* (2000), a sudden

change in environment causes physiological stresses to probiotic cells and it was suggested that a pre-adaptation to cryogenic conditions is preferable before freeze drying of microorganisms. The cell population stored at 37°C had gone through a lot of temperature fluctuations such as freezing from ambient conditions to -18°C, drying at 20°C and then the extreme storage conditions. The loss of moisture from the microparticles during storage at 37°C is also a probable cause for poor survival of the probiotic cells. Another hypothesis for explaining the gradual loss in viability during elevated temperature storage was postulated by Bozoglu *et. al*. (1987). According to that, the presence of oxygen in the storage environment causes poor cell recovery. Oxygen gradually starts diffusing inside the dry cells because cells remain permeable during the storage period. This in turn causes accumulation of free radicals inside the cells. In a non-metabolic state, bacteria are unable to transport these compounds out of the cell body, which triggers an irreversible damaging process within the cell (Bozoglu *et. al*., 1987; Morgan *et. al*., 2006). The freeze dried samples in this study were stored without any special protection against oxygen such as any oxygen absorber, which could possibly explain the poor survival rate at elevated temperature.

Capela *et. al*. (2006) observed a similar decline in viability when freeze dried *Lactobacilli* strains were stored at 37°C. After a period of 4 weeks the decline was from average 8.95 log CFU to 5.50 log CFU and from 8 weeks onwards the cell count in most of the samples showed negligible viability. The same study showed a moderate level of decline when the samples were stored at 21°C. Therefore it can be concluded that storage of microcapsules containing probiotic bacteria at 37°C becomes too stressful and should be avoided. Proper oxygen protected packaging and a moisture proof storage environment should be maintained.

The moisture content of the wet microcapsules was in the range of 86 to 89% and for freeze dried particles it varied from 3 to 4 % (Appendix 4.4). This range is in conformation with the recommendations for good storage stability for dried cultures (Champagne *et. al*., 1996; Picot and Lacroix, 2003) and better physical and bulk properties (Onwulata *et. al*., 1995; Picot and Lacroix, 2003). A much higher range in

moisture content (8.30% to 17.00%) in freeze dried whey protein microcapsules was reported in the work of Ainsley-Reid *et. al*. (2005). They found that the highest residual moisture level yielded the best post drying survival for probiotics but at a compromise of handling properties, such as flowability. The storage stability data has not been provided in that study but Clementi and Rossi (1984) reported that a higher moisture content in freeze dried powders generally ends up with a maximum decline in viability during storage. In this present study, a steady count of viable bacteria in the microcapsules (when stored at 4°C and -20°C) over a period of 4 weeks proved that the presence of aerobic environment during the manufacturing or storage did not adversely affect its viability. The substantial loss in viability was observed in the sample stored at 37°C which is a shortcoming of this endeavor and needs to be improved during further research.

The emulsification process during the encapsulation process generally entraps some oil inside the microcapsules and it has been reported as unfavorable for bacterial viability (Krasaekoopt *et. al*., 2003; McMaster *et. al*., 2005). But in this storage stability results, no such adverse effect of oil entrapment was noticed. Instead, it can be hypothesized that the cells surrounded by an oil film might have some protection against oxygenated environment. During commercial production and storage, maintaining anaerobic conditions are not always feasible. Hence it is technologically essential to develop a microencapsulation process which is not adversely affected by the presence of oxygen, especially during prolonged storage.

4.4 Conclusion

The main objective of the current chapter works was to convert the soft gelled microcapsules (obtained in Chapter 3.0) into a dried form and freeze drying was chosen as the most appropriate method for doing so. The wet gel particles were successfully dried and a free flowing granular mass of microencapsulated *L. casei* 431 cells was obtained. The average particle diameter and size distribution were within the limit which is considered to be generally acceptable for sensory evaluation purpose.

Microscopic examinations of the dried particles revealed almost spherical shapes but a slightly agglomerated texture. However, the particle surface was porous and this is not a favorable property to entrap and protect the live bacterial cells.

A high mortality of cells occurred after freeze drying. Minimizing the cell mortality is of commercial interest because a higher payload of bacterial cells into the microcapsules is an important economic criterion which reduces ingredient cost to a food formulation, as well as helping to meet the legal requirements. Therefore, it is the main objective of next chapter to improve on post-drying viability.

Apart from handling properties and ease of storage, another advantage of the freeze dried sample was its better protection ability during simulated gastric transit. The net log reduction and rate of decline in cell viability was less compared to the wet particles. Still there is a scope for improvement in this aspect and will be tried by modifying the encapsulating matrix in following chapter.

A different result was obtained when both the wet the dried capsules were subjected to bile salt solution. Wet capsules performed considerably better than the dried ones in terms of cell protection ability. It was suspected that bile salts aided emulsification of entrapped oil inside the microcapsules which consequently weakened the gel matrix. Therefore, a firmer gel network needs to be formed, possibly by applying a membrane coating and this remains an objective for the next level of studies.

Chapter 5.0. Improvements in post drying cell survival and gastric tolerance by incorporating cryoprotectants and by applying membrane coating around the microcapsules

5.1 Introduction

Probiotic cultures in a suitable microencapsulated format must be dried to complete the process and to obtain the maximum benefit from the entire effort. Therefore, the retention of high viability during drying and also during prolonged storage at moderate conditions is very important for the successful delivery of probiotic cells.

Drying of microencapsulated probiotic cultures involves many challenges. As discussed previously in Chapter 3.0 and 4.0, the microcapsules produced from the gelation of sodium-caseinate-gellan gum complex were freezed dried as per a standard procedure but a very high log reduction in viability was noticed immediately after drying. The reduction of 1.70 log CFU per gm solid was high compared to the other reported work and it has therefore been an objective of the present work to improve the viability after drying. After going through the relevant published literature, it was decided to use a suitable cryoprotecting material in the encapsulation matrix as well as in the growth media, as compatible solute, and to examine any improvement in post drying viability. In this chapter, almost the same process flow line has been followed as Fig. 3.1 (Chapter 3.0) but with the incorporation of compatible solutes and cryoprotectants. Trehalose or lactose have been incorporated into the wall material mix and growth media. Samples were analyzed to observe any improvement in cell viability during freeze drying, simulated gastric transit and storage.

The microcapsules produced in this study (Chapter 3.0 and 4.0) had uneven surface morphology and show the presence of many pores on the surface, which may cause acid and bile salt penetration inside the capsules leading to high mortality of probiotic bacteria, *L. casei*. This needed to be addressed and it was hypothesized that coating the

individual microcapsules with a membrane could be useful. From the literature search it was found that only a few attempts have been made previously to coat the microbeads, containing probiotic bacteria, with an additional membrane. Guerin *et. al*. (2003) used a transacylation reaction between whey protein and pectin in the presence of NaOH to form the membrane around the particles, encapsulating *Bifidobacterium bifidum* cells. A very good acid and bile resistance for the membrane coated beads was achieved compared to the free cells and uncoated capsules. The capsules used in that study were made of whey protein gels coagulated with calcium ions and hence the transacylation reaction at alkaline pH did not cause any destabilization of the gel network. But this was not possible in the current project since the sodium caseinate gel was not stable at high pH. Another attempt was taken by Ding and Shah (2009), who coated the calcium alginate beads first with palm oil and then a second layer was applied by ionic bonding with poly-L-leysine. The underlying mechanism was based on electrostatic interaction between alginate and poly-L-leysine because both are oppositely charged molecules. Since gelled sodium caseinate at its isoelectric point bears neutral charge, this technique could not be adapted in the present study. In a related study by Klinkenberg *et. al*. (2001), chitosan, a cationic polysaccharide had been used to form a coating over alginate particles by electrostatic complexation and improved cell release properties during fermentation were reported.

Some biopolymers like alginate and gellan gum have been popularly used as edible coatings for fruits and vegetables to enhance their shelf life, by reducing the respiration rate and gas exchange (Baldwin and Nisperos-Carriedo, 1994; Nussinovitch, 2000). The unique colloidal properties and gel forming ability of gellan gum and sodium alginate in the presence of divalent cataions have been previously used to provide an edible coating on fresh-cut apples (Rojas-Grau *et. al*., 2008), fresh-cut papaya (Tapia *et. al*., 2008) and melon (Oms-Oliu *et. al*., 2008). This principle has been modified by using gellan gum alone, adapted in this project to form a membrane coating on the sodium caseinate-gellan gum complex microcapsules. The objective for this was to obtain smooth surface and also to reduce the pores found in the previous results (Chapter 4.0).

5.2 Materials and methods

5.2.1 Ingredients and chemicals

Sodium caseinate containing about 90% (w/w) protein was obtained from Fonterra Co-Operative Ltd. (Palmerston North, New Zealand). High acyl type gellan gum and Glucono-δ-lactone (GDL) were purchased from Hawkins Watts (Auckland, NZ). α-Lactose monohydrate and trehalose dihydrate were supplied by Sigma Aldrich, Steinheim, Germany. Canola oil was purchased from a local market. All chemicals used in this study were of analytical grade and obtained from Sigma Aldrich, USA.

5.2.2 Growth media formulation and culturing of *L. casei* cells

The basic procedure used is described in Chapter 3.0. MRS broth was prepared as per standard procedure by dissolving 52 gm per 1000 ml of distilled water. This was then divided into two equal portions for further modification with disaccharides (as compatible solutes). The portions were supplemented with trehalose or lactose powders at 2% (w/v), followed by autoclaving at 121°C for 15 min as suggested by Carvalho *et.al.*, (2004). *Lactobacillus casei* 431 cells in lyophilized form were inoculated into these two media @ 0.1% (w/v). After anaerobic incubation at 37°C for 17 hours, stationary phase cells were harvested (Appendix 3.1) and re-inoculated in the respective supplemented media. This process of sub-culturing was repeated 3 times to increase the viability of the cells and also to allow the compatible solutes (trehalose and lactose) to accumulate inside the cells. On the 4th day, bulk culturing of the cells were done in two parts of 400 ml MRS broth, supplemented with trehalose or lactose. The rest of the process was similar to the method described in Chapter 3.0.

5.2.3 Microencapsulation process

The microencapsulation process carried out in this part of the project is summarized in the process flow diagram (Fig. 5.1). All the process parameters were followed as per

Figure 5.1 Process flow diagram of the microencapsulation technique

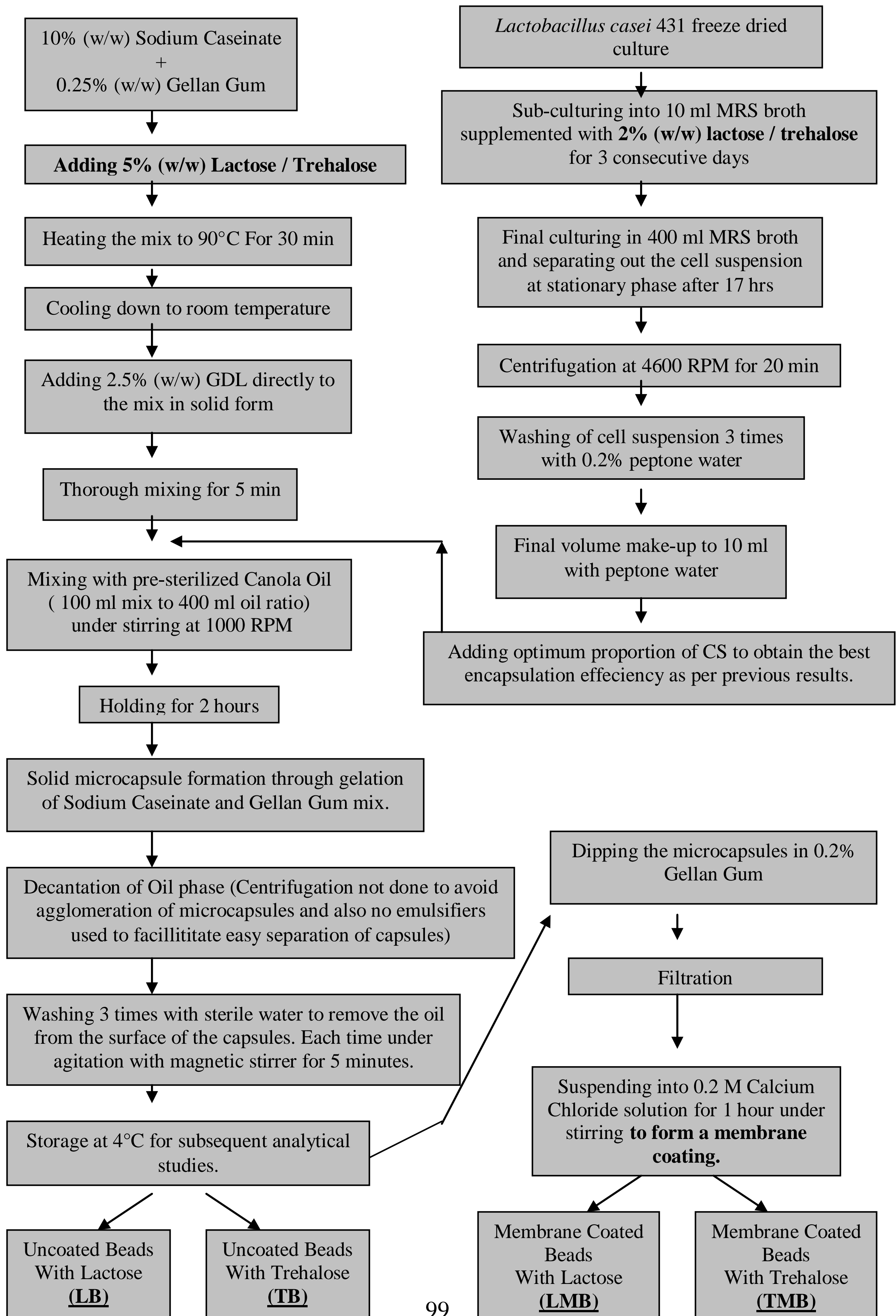


Fig. 3.1 (Chapter 3.0) except the encapsulating material mix composition. Cells grown in the presence of trehalose were encapsulated in a mix comprising of 10% (w/w) sodium caseinate and 0.25% gellan gum, supplemented with 5% (w/w) trehalose. This mix was then gelled with 2.5% (w/w) glucono-delta-lactone (GDL) and emulsified in canola oil exactly as described in section 3.2.4 of Chapter 3.0. For the *L. casei* 431 cells grown in the presence of lactose, a similar procedure was followed and the encapsulating mix was added with 5% (w/w) lactose in this case. The derived microcapsules in wet as well as in final freeze dried forms were named as **TB** and **LB,** respectively (Fig. 5.1).

5.2.4 Membrane coating process around the microcapsules

Prior to freeze drying, a portion of the trehalose (TB) and lactose (LB) treated microcapsules were subjected to a membrane coating process as described below. A solution of 0.2% (w/v) gellan gum was prepared in distilled water and autoclaved at 121°C for 15 min. After cooling down to room temperature, the wet microcapsules from both the batches were dipped into this solution under stirring and held for 5 min. The microcapsules from each batch were then filtered out from the gellan gum solution by a Whatman 50 paper and dipped into the previously autoclaved 0.2M $CaCl_2$ solution. Slow stirring at 300 rpm with a magnetic stirrer was continued for 60 min for proper gellan gum gel formation in presence of $CaCl_2$ and subsequent coating around the wet capsules. The coated capsules were then partially stored at 4°C for further analysis and the remaining material were freeze dried as per the normal procedure detailed in section 4.2.4 (Chapter 4.0). The membrane coated beads (both wet and dried) were named as **TMB** (trehalose) and **LMB** (lactose) for future references (Fig. 5.1).

5.2.5 Particle size distribution

Membrane coated particles (TMB and LMB) in both wet and dry forms were analyzed for their surface based mean diameters, ranges and size frequency distributions. The

equipment and procedure used for wet and dry samples were exactly the same as described in Chapter 3.0 and Chapter 4.0 respectively.

5.2.6 Electron microscopic examination of the microcapsules

Membrane coated LMB and TMB samples (freeze dried) were examined under scanning electron microscope (SEM) to observe the effect of coating process on the surface morphology. The procedure and equipment used are described in Chapter 4.0.

The presence and position of encapsulated *L. casei* cells inside the wet TMB and LMB microcapsules were examined with the help of transmission electron microscopy (TEM). The microscope used for this purpose was from Philips Export Dvn., Eindhoven, The Netherlands (Model CM10). The samples were first observed under light microscopy, which is a preliminary step towards TEM observation. The sample preparation steps included primary fixation with 3% glutaraldehyde in 0.1M phosphate buffer, holding for two hours at room temperature followed by centrifugation at 4000 rpm. The pellet was then mixed with 20% bovine serum albumin (BSA), coagulated the BSA with 25% glutaraldehyde and the resultant pellet was sliced into thin pieces. After 3 washes with buffer, the thin pellets were dehydrated with a gradually increasing concentration of acetone. One micron cross sections were cut from the thin blocks and placed on microscopic slides after staining with Toluidine Blue. The samples were then ready for light microscopic observations.

Further processing of these resin blocks was done to prepare for TEM observation. The blocks were trimmed to approximately half by half a millimeter square sizes which included the area of interests and then made into small 100 nm sections using a diamond knife and Ultra-microtome. The cross-sections were then stretched using chloroform vapor and double stained first with saturated Uranyl Acetate in 50% ethanol for 4 min and again with Lead Citrate for next 4 min. The equipment used for cutting the resin blocks was Leica Ultracut R from Microsystem GmBH, Austria.

5.2.7 Survivability of *L. casei* cells in simulated gastric and bile salt environments

The same procedure and methods were used as mentioned in Chapter 3.0 and Chapter 4.0, to perform the simulated gastric fluid (SGF) and bile tolerance tests for wet and freeze dried microcapsules containing *L. casei* cells. In simulated gastric fluid preparations, pepsin at the rate of 0.32% (w/v) was added in all the samples.

5.2.8 Storage stability test

Freeze dried samples obtained from the four categories listed above were placed at 4°C and 37°C rooms after packing in LDPE sachets. Storage conditions were normal atmospheric with no humidity and oxygen control. Samples were tested each week for its viable count for a total duration of four weeks. Sample rehydration and moisture content analysis were performed as per the method described in Chapter 4.0.

5.2.9 Statistical analysis

Statistical analysis of the results was done as described in Chapter 3.0 under section 3.2.9.

5.3 Results and discussion

5.3.1 Particle size distributions of membrane coated microcapsules

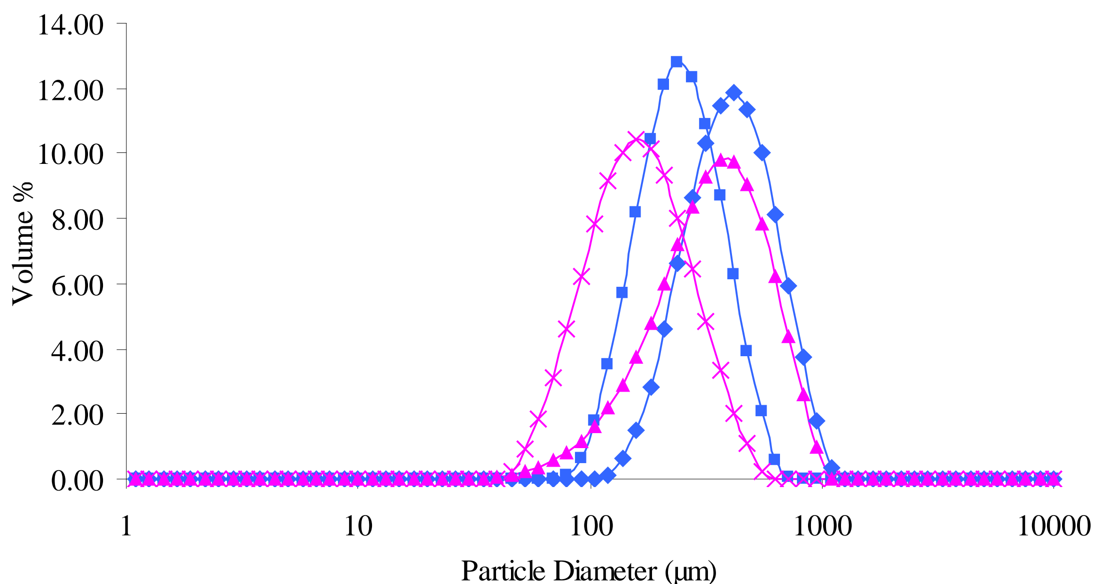


Figure 5.2 Particle size distributions of wet (♦) and freeze dried (■) TMB microcapsules, compared with no-membrane coated wet (▲) and dried (x) samples.

In order to characterize and compare the microcapsules for their mean diameters and range, only membrane coated samples TMB and LMB were chosen. The other two samples without any membrane coating (TB and LB) were not tested because they were made using the same procedure as in Chapter 3.0.

Trehalose containing capsules (TMB) showed a very uniform, unimodal volume distribution curve (Fig. 5.2). The surface weighted diameters of the wet particles ranged from 105 µm to 1096 µm with a mean (D_{32}) value of 340 µm. The diameter range for wet LMB capsules was same as TMB samples but the mean D_{32} was slightly reduced to 315 in this case (Fig.5.3). Nearly 80% of the TMB and 84% of the LMB particles were under 550 µm. Similar distribution curve for both TMB and LMB indicated similar impact of trehalose and lactose on the particle size distribution of the coated

microcapsules. Also, Fig. 5.2 and 5.3 show the size distribution graphs obtained in Chapter 3.0 for particles without any membrane coating. The diameter range of the sample population was almost same for all the three samples which are represented by the overlapping bell shaped curves.

Freeze drying of the particles caused a reduction in overall range of particle diameters. The minimum and maximum values for TMB particle diameters were 79 μm and 724 μm and the corresponding figures for LMB particles were 35 μm and 631 μm.

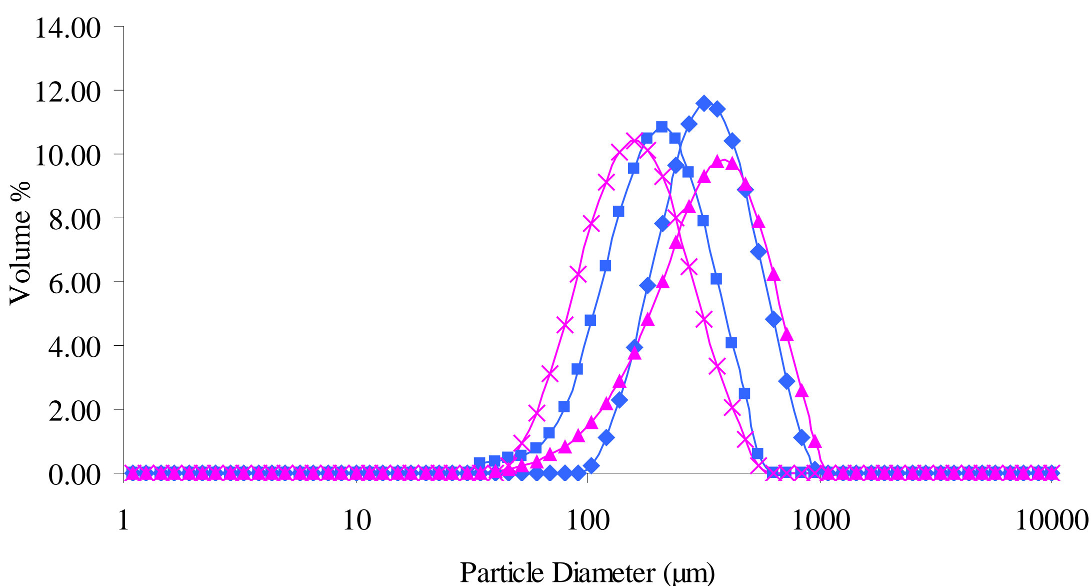


Figure 5.3 Particle size distributions of wet (♦) and freeze dried (■) LMB microcapsules, compared with without-membrane wet (▲) and dried (x) samples.

D_{32} values of freeze dried TMB and LMB particles were measured as 209 μm and 181 μm respectively. According to the volume distribution data obtained (shown in Appendix 5.1), approximately 55% of the particles were below 240 μm in size for TMB samples and for the LMB samples 59% of the sample mass were under 240 μm in diameter. By comparing the size distribution trend of dried and membrane coated capsules with those obtained in chapter 4.0 results (uncoated), it can be seen that the volume distribution of the dry TMB as well as LMB samples were shifted on the right,

indicating a larger average particle diameters throughout the population mass (Fig. 5.2 and 5.3).

The membrane coating formation was evident from analyzing the particle diameters as well as from observing the surface morphology under the microscope. The D_{32} values of wet TMB and LMB microcapsules were increased by 53 µm and 28 µm compared to the uncoated wet samples analyzed and reported in Section 3.3.3. A similar trend was observed in case of freeze dried samples. The mean diameters of dried TMB and LMB capsules were increased by 57 µm and 29 µm respectively, compared to the uncoated dried particles reported in Section 4.2.1. The presence of a smooth membrane was evident from the scanning electron microscopic pictures, reported in Section 5.3.2 of this chapter.

5.3.2 Microscopic examination of the membrane-coated freeze dried particles

In Chapter 4.0, it was shown that microscopic examination of the uncoated and dried microcapsules revealed the presence of numerous pores on the surface and very uneven surface morphology. This can possibly be explained by the shrinkage of gel network due to moisture evaporation during freeze drying. To improve the surface properties, a membrane coating around the particles was applied. Therefore, further microscopic examinations were necessary to observe the effects of such membrane coating. Also, the images presented in Chapter 4.0 did not show any significant number of bacterial cells whereas the microbial plate count of the capsules supported their presence in very high numbers. Therefore, in this chapter, a cross sectional view was observed under light and transmission electron microscopy to locate the position of the *L. casei* cells.

5.3.2.1 Light microscopic imaging of the freeze dried TMB and LMB microcapsules

As discussed in Section 5.2.6 of this chapter, sample slides prepared for transmission electron microscopy (TEM) were first observed under light microscope at 200 and 400x magnification levels. Cross sectional views of spherical TMB and LMB particles are presented in Figs. 5.4.1 - 5.4.4.

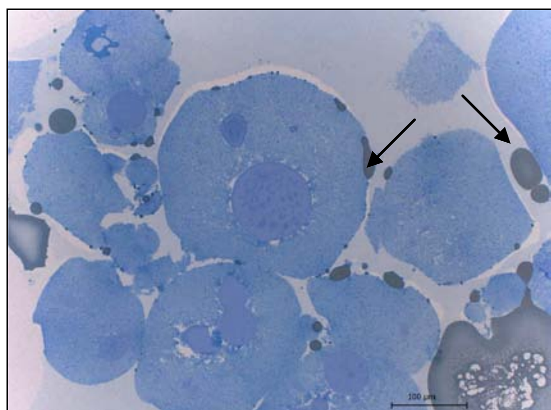


Fig. 5.4.1

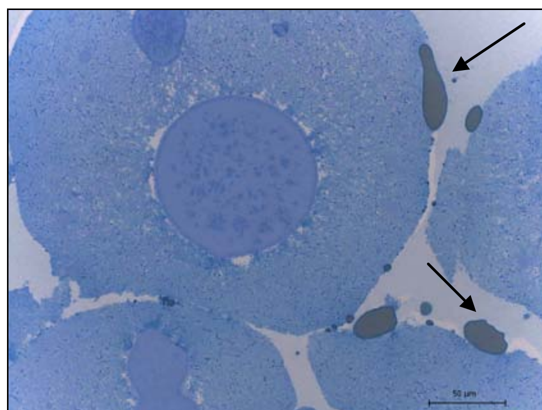


Fig. 5.4.2

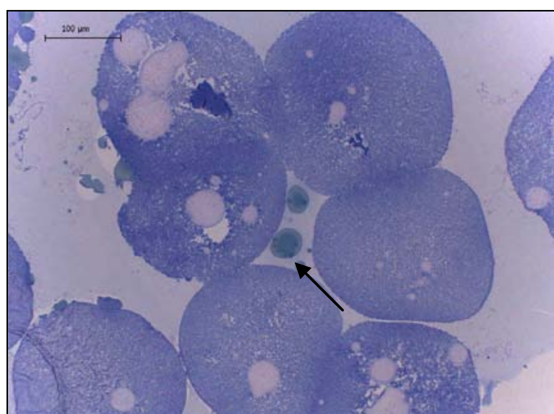


Fig. 5.4.3

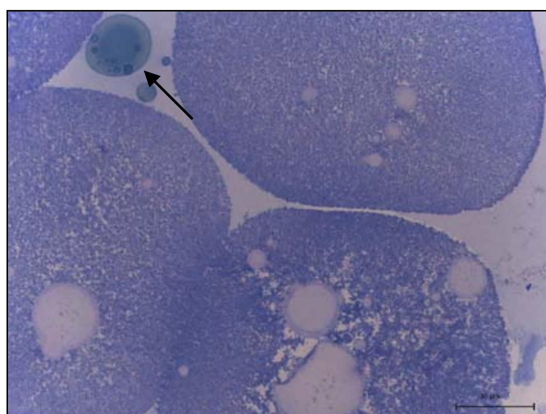


Fig. 5.4.4

Cross sectional images of TMB microcapsules at 200x (Fig. 5.4.1), at 400x (Fig. 5.4.2) magnifications and the same for LMB microcapsules at 200x (Fig. 5.4.3) and at 400x (Fig. 5.4.4) viewed by light microscopy with toluidine blue staining

The presence of rod shaped *L. casei* cells can be seen inside both of the sample particles and the distribution is very uniform. A higher cell density and more compact gel network can be observed in case of LMB samples (Fig. 5.4.3 and 5.4.4).

Another interesting observation here is the presence of very small residual oil droplets around the particle surfaces but not in an encapsulated form inside them (see black arrows on figures 5.4.1 to 5.4.4). The presence of these oil droplets is the possible reason of particle agglomeration, as shown later via scanning electron microscopic images in

Fig. 5.4.15 and 5.4.16. The cross sectional images viewed under light microscopy (Fig. 5.4.1 to 5.4.4) show a dense gel network with the bacterial cells entrapped inside. It was also visible that the individual particles were near spherical shaped, which was apparently in contradiction with SEM images in Fig. 5.4.15 and 5.4.16, which shows irregular shaped, agglomerated particles. This can be explained possibly by the agglomeration of very fine round, spherical microcapsules due to the gelling action during the membrane coating process and also due to the presence of oil droplets acting as a binding agent.

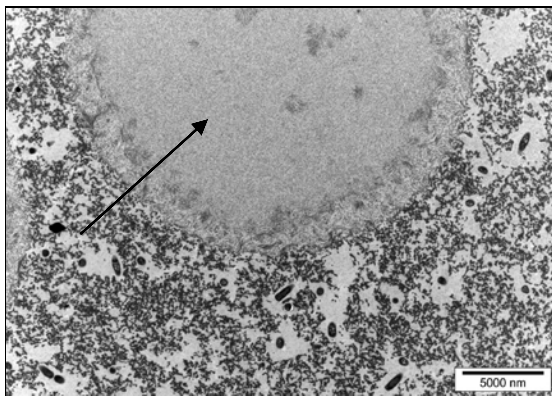


Fig. 5.4.5 TEM image of TMB capsule cross section showing caseinate, gellan and trehalose complexation

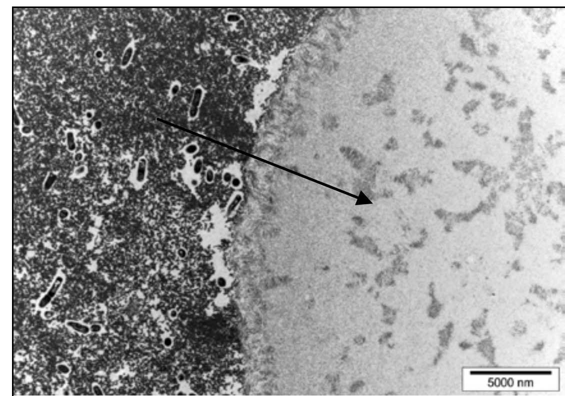
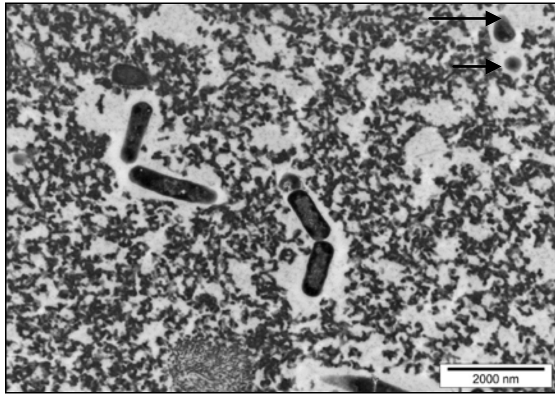


Fig. 5.4.6 Cross sectional view of LMB capsule by TEM imaging

5.3.2.2 Transmission electron microscopic view of dry TMB and LMB microcapsules

TEM images shown in Figs. 5.4.5 - 5.4.10 showed the complex structures formed by the encapsulating ingredients (marked with arrows). It can be seen that minor ingredients such as, lactose/trehalose and gellan gum, surrounded by the main encapsulating component, i.e, gelled sodium caseinate. Another view of this complexation can be seen on the light microscopic images.



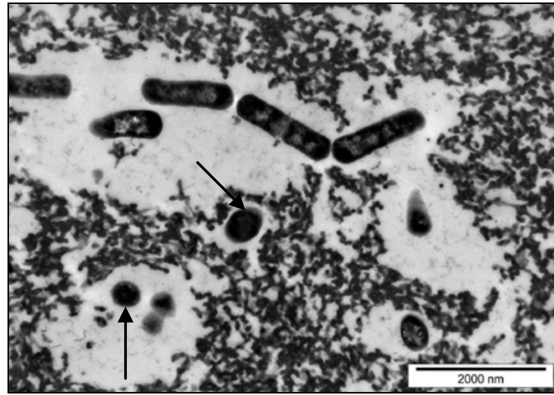
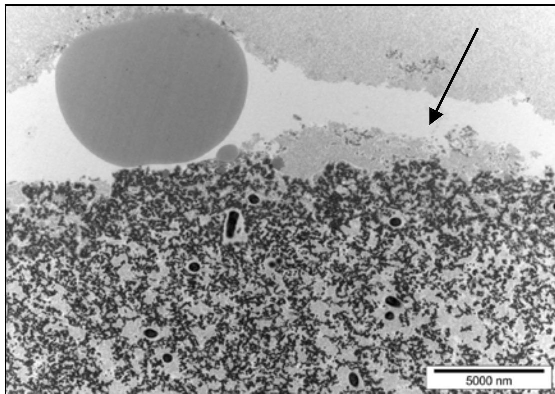


Fig. 5.4.7 TEM image showing presence of *L. casei* cells inside TMB microcapsule

Fig. 5.4.8 *L. casei* cells entrapped in LMB capsule, viewed via TEM imaging

Figs. 5.4.7 and 5.4.8 showed the presence and position of rod shaped *Lactobacillus casei* 431 cells encapsulated inside the microcapsules. The small round or dot shaped figures are cross sectional view of those cells (indicated with black arrows).



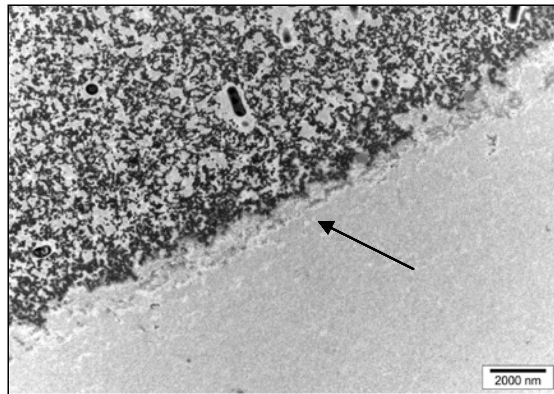


Fig. 5.4.9 TEM image view of membrane coating formation around TMB capsule

Fig. 5.4.10 TEM image showing presence of membrane coating on LMB capsule

The membrane coating process was performed by the ionic gelation of gellan gum and its presence was observed by the TEM and SEM images. The cross sectional views of the particles indicated its presence (Fig. 5.4.9 and 5.4.10), although not in a very distinct manner.

5.3.2.3 Scanning electron microscopic images of the dried TMB and LMB capsule surfaces

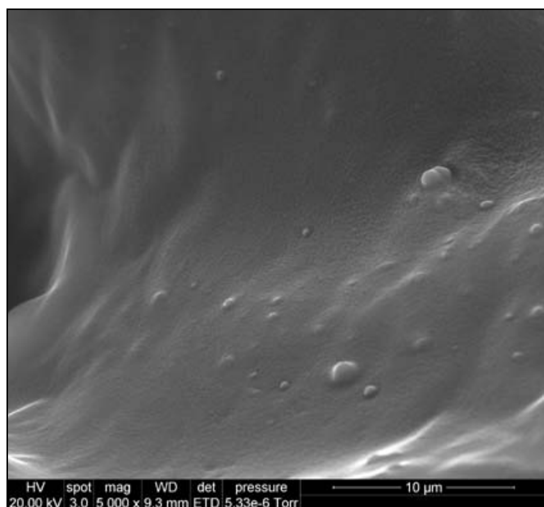


Fig. 5.4.11 SEM image of TMB capsule surface viewed at 5000x magnification

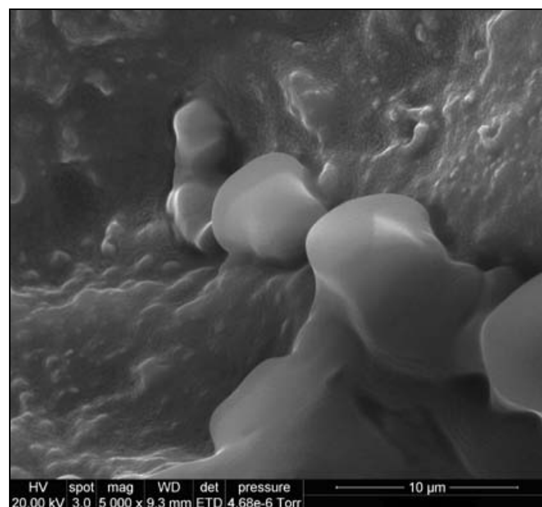
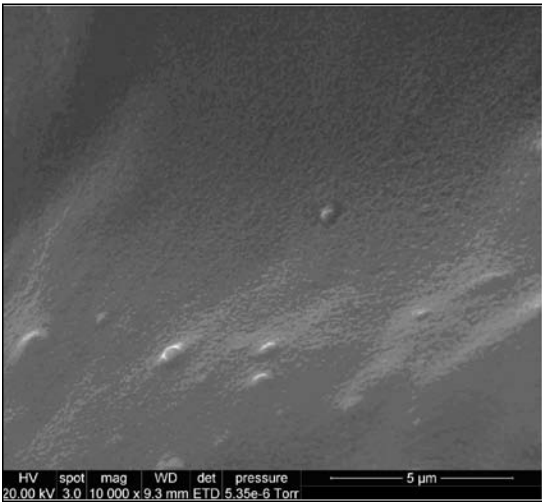


Fig. 5.4.12 Surface of LMB capsules, viewed at 5000x magnification on SEM

SEM imaging of the TMB and LMB capsules was helpful in understanding the presence of membrane coating and its effect on surface morphological properties (Fig. 5.4.11 and 5.4.12). A further closer look (1000x magnification) (Fig. 5.4.13 and 5.4.14) revealed very smooth and compact surfaces which are completely different than what were observed in Chapter 4.0. This confirmed the formation of a membrane around each particle.



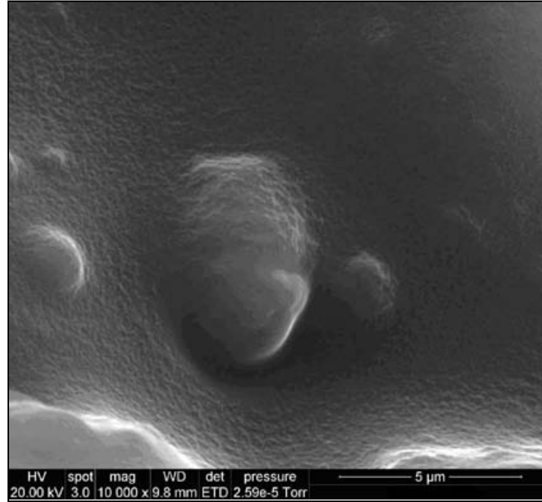
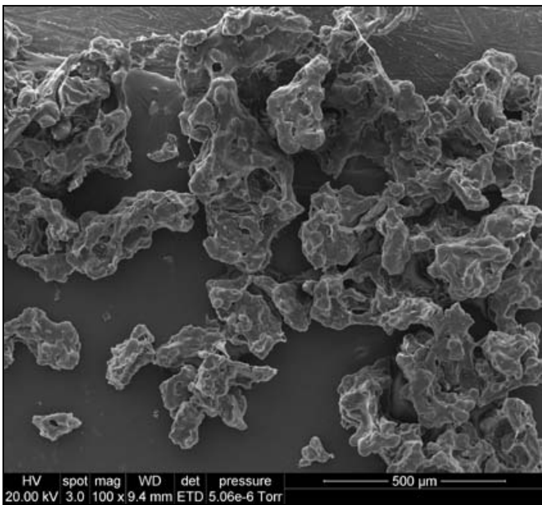


Fig. 5.4.13 Surface morphology of a TMB microcapsule at 10000x magnification

Fig. 5.4.14 Surface morphology of a LMB microcapsule at 10000x magnification

Agglomerated and irregular particle shapes with sharp edges can be noticed in Figs. 5.4.15 and 5.4.16 for TMB as well as LMB samples.



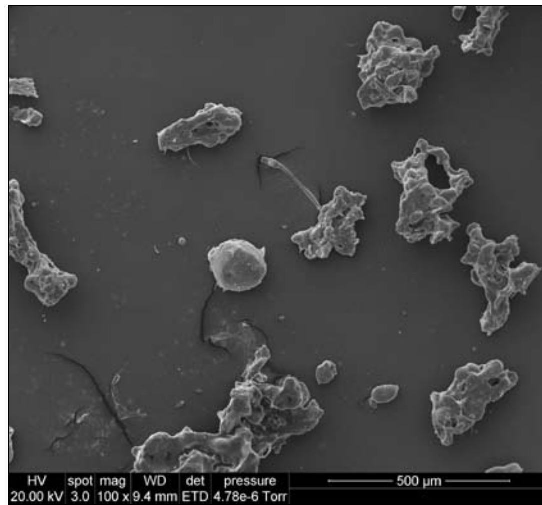


Fig. 5.4.15 Particle shape of TMB microcapsules as viewed via SEM image

Fig. 5.4.16 Particle shape of LMB microcapsules as viewed via SEM image

The smooth surface obtained in the process and absence of any visible pores (Figs. 5.4.13 and 5.4.14) are deemed to be a confirmation of the membrane formation process

discussed above. The oil droplets were found to be present only in the outer space and not in an entrapped form. Therefore, any complexation of oil and other ingredients was not evident. A complex formation via sodium caseinate, gellan gum and disaccharides interactions can be clearly viewed on the TEM images reported in Fig. 5.4.5 and 5.4.6 (marked with arrows). When these images were visually compared with the reported work of Guerin *et. al*. (2003), who used a transacylation reaction between protein, pectine and alginate to form membrane coatings, high similarities were found in surface structures. The presence and absence of pores were demonstrated in coated and uncoated beads, very similar to the SEM images obtained in this current study.

5.3.3 Analysis of cell survivability out of freeze drying

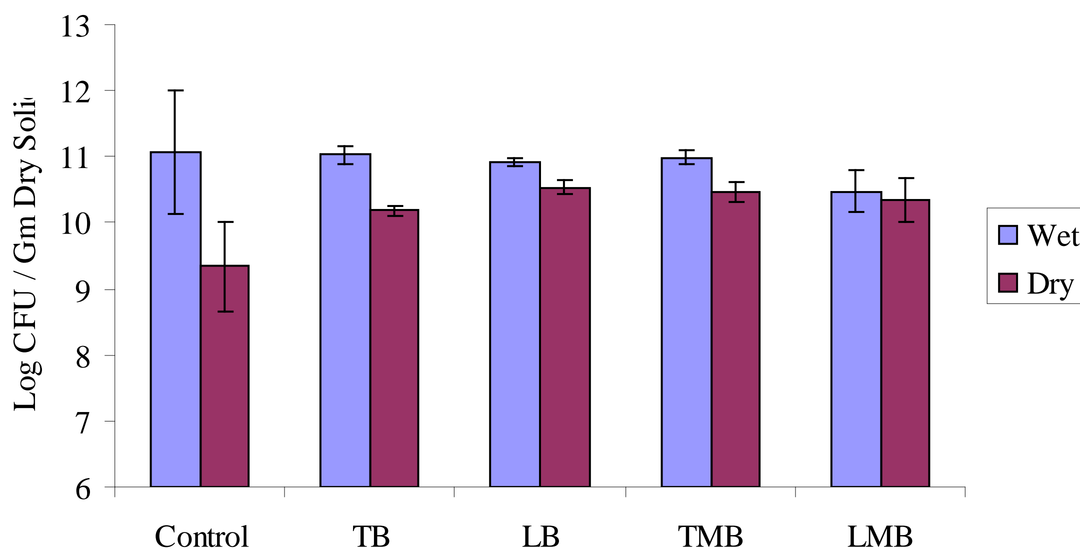


Figure 5.5 Comparative cell viability before (Wet ■) and after freeze drying (Dry ■) of uncoated capsules containing trehalose (TB) and lactose (LB) and membrane coated capsules containing trehalose (TMB) and lactose (LMB). Error bars representing standard deviations from the mean value of 3 replicated experiments.

Fig. 5.5 shows the cell counts (CFU) per gm of dry solids of wet and freeze dried microcapsules. The mean values from three independent trials are shown on the logarithmic scale. Samples without the additional membrane coating, i.e, TB and LB

showed good improvements compared to the control sample without any compatible solute or cryoprotective compounds. The initial average populations per gm solid in wet particles were 11.04 (for control), 11.03 (for TB) and 10.91 (for LB) log CFU which were reduced to 9.34 (control), 10.19 (TB) and 10.54 (LB) log CFU respectively in the dried form. Thus, the control sample showed 1.70 log CFU/gm reduction in cell viability, whereas the TB and LB samples showed only 0.84 and 0.37 log CFU/gm reduction. The only changes made in these two samples were addition of lactose and trehalose in the culture growth media as well as in the encapsulating/drying media. Therefore, the improvement in post drying viability can possibly be attributed to these two protectants.

The LMB and TMB samples were produced with an additional membrane coating step with gellan gum as detailed in section 5.2.4. From Fig. 5.5 it is evident that this step helped in obtaining comparatively better results than the uncoated beads. Viable cell count for TMB sample reduced to 10.46 log from the initial count of 10.99 log, while the values for LMB sample were 10.35 and 10.48 log. Thus, the average log CFU reduction for TMB sample was 0.53 and the same for sample LMB was only 0.13. Statistical analysis of the individual series of data showed that the results for TMB and LMB samples were significantly ($p \leq 0.05$) better than the control sample with no protectants and also better than the uncoated samples with cryoprotective agents.

Bacteria and microbial spores have sometimes been observed to maintain a good survival level when freeze dried without any protecting agent, but the presence of a suitable agent has proved to be beneficial most of the times (Hubalek, 2003). As discussed in the previous paragraph, the rate of survival improved after freeze drying when cryoprotective compounds were included in the microencapsulation matrix. Trehalose containing microcapsules have shown better protective ability than the control sample which was as per expectation and the cryoprotective properties of trehalose may be responsible for this. The cryoprotective nature of trehalose has been well studied and established (Coutinho *et. al.*, 1988; Leslie *et. al.*, 1995; Diniz-Mendes *et. al.*, 1999). Various researchers have shown this property of trehalose tested on viruses,

psychrophilic yeasts, mycorrhizal fungus and *Lactobacilli* bacteria (Hubalek, 2003). In a review, Carvalho *et. al*. (2004a) mentioned that trehalose was more effective protectant than most others when applied on dry biomaterials, liposomes, isolated biological membranes and intact cells. The mechanism for such protection as pointed out by them is protection of protein functionality by preventing unfolding and aggregation, an increase in glass transition temperature, thereby restricting the molecular mobility, and binding of trehalose as a compatible solute. Trehalose was used as a cryoprotectant, probably for the first time, on *Lactobacillus bulgaricas* by Antoni *et. al*. (1989). They found that the action of trehalose is strain specific and highly dependent upon the media composition. These factors make it difficult for any direct comparison of the current results with other researchers' findings. Moreover, it must be kept in mind that the microencapsulation material mix in the present work was subjected to a gelation step which reduced the pH of the medium, which is not favorable for probiotic bacteria during freeze drying (Saarela *et. al*. 2005). However, using the process flow described in section 5.2, a very good protective effect of trehalose has been obtained. The *L. casei* cells encapsulated in TB and TMB samples showed only 0.84 and 0.53 log CFU/gm loss of viability respectively, compared to 1.70 log in untreated control samples (Fig. 5.8). This result is in aggrement with many other studies where trehalose has shown similar cryopreservation properties (Leslie *et. al*., 1995; De Giulio *et. al*., 2005; Zayed and Roos, 2004).

Lactose is not only considered to be a good cryoprotectant but also acts as the starting material of compounds with prebiotic properties. For example, lactulose, lactitol and lactobionic acid are unabsorbable lactose derivatives and have been shown to impart prebiotic benefits on various *Lactobacillus* and *Bifidobacterium* species. They reported higher acid and bile tolerance of *Lactobacillus* strains when grown in presence of above mentioned prebiotic lactose derivatives (Saarela *et. al*., 2003). In the current study, lactose was found to be the best protectant against the freeze drying stress (Fig. 5.5). The net log reduction obtained for LB and LMB samples were 0.37 and 0.13 respectively. These values are quite low compared to the trehalose samples mentioned above. The

prebiotic attribute of lactose is possibly one of the reasons which contributed towards this better protection ability.

5.3.4 The role of compatible solutes added in cell growth media

It was earlier hypothesized that the likely action of compatible solutes in growth media is by environmental adaptation (Carvalho *et. al*., 2004a; Meng *et. al*., 2008). They postulated that the growing of cells in the presence of these sugar substrates imparts a distinct physiological and morphological trait to them. Bacterial action on these solutes produces some metabolites such as manitol, sorbitol and glutamate, which slowly start accumulating inside the cells and change their response towards drying. The sugar substrates used as cryoprotectants, in this case trehalose and lactose, have to enter the cell membranes and also be present in the drying medium to effectively protect the cells. If they are metabolized into monosaccharides by the bacteria prior to drying, the effectiveness may be reduced. However, two recent studies concluded that even if the fermentation activity continues, the ultimate protection ability does not change significantly (Zayed and Roos, 2004; Pehkonen *et. al*., 2008). In the current project, *L casei* cells were grown in media supplemented with trehalose and lactose, hence the cells had enough time to accumulate these solutes inside the membrane. Since the microencapsulation process was completed in about 2.5 hours and thereafter the cells were stored under chilled conditions, it can be assumed that it was not possible for the bacterial cells to metabolize a significant quantity of trehalose or lactose present in the encapsulating mix.

Stabilization of the probiotic cells is important to maintain a high viability rate during drying. It was suggested that a glass type amorphous state of protectant matrices helps in overcoming freezing and desiccation stresses. The water plasticization of the drying media and interactions of residual water with dehydrated cells are also deemed to be important factors (Pehkonen *et. al.*, 2008). Both trehalose and lactose are known to possess a high glass transition temperature and cushion the osmotic shock by leveling the intra- and extra-cellular concentrations.

5.3.5 Effect of cryoprotective compounds and membrane coating process on acid tolerance (simulated gastric conditions) of *L. casei* cells

Trehalose or lactose was incorporated into the encapsulation mix with the main purpose of improving the post drying survival of *L. casei* 431 cells. However, it was also important to observe their effects on the acid tolerance property of the encapsulated matrix. Hence a similar experimental protocol for simulated gastric fluid incubation as per Chapters 3.0 and 4.0 was followed and the results are reported here.

5.3.5.1 Behavior of free *L. casei* cells in simulated gastric fluid

To compare the results, *L. casei* concentration in free cell form was adjusted to the level of the encapsulated population by proper dilution of growth media (MRS broth) and the initial count of 11.03 log CFU gradually was found to decrease in value during the 120 min. The viable colony counts after 30, 60, 90 and 120 min were 10.27, 7.96, 6.82 and 6.10 log CFU, respectively (Fig. 5.6), with a final net reduction of 4.93 log CFU.

5.3.5.2 Effect of trehalose and membrane coating process on acid tolerance of *L. casei* cells

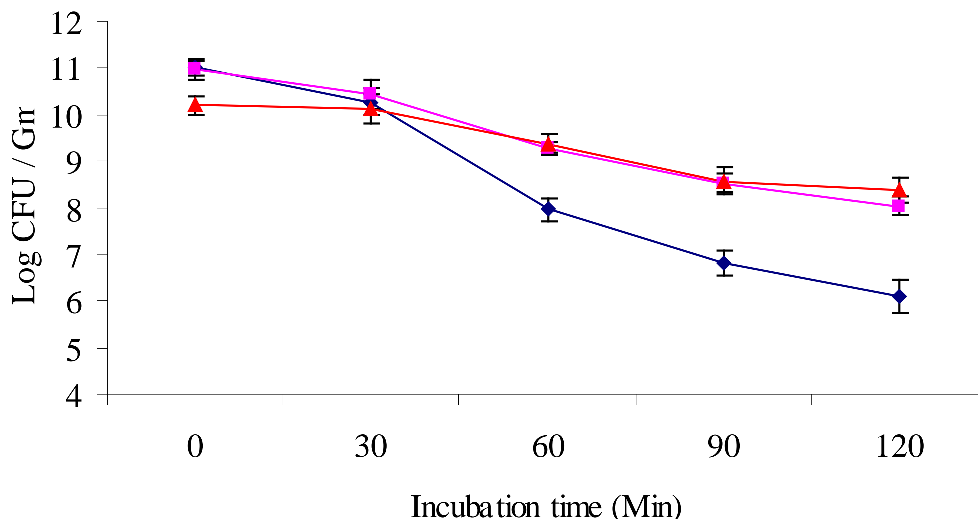


Figure 5.6 Survival of *Lactobacillus casei,* in free form (♦) and microencapsulated in uncoated, trehalose containing beads (TB), in wet (■) and dried (▲) form, when treated in simulated gastric fluid (SGF). Error bars representing standard deviations from the mean of 3 replicates.

Uncoated capsules treated with trehalose (TB) were tested in both wet and dried forms. Bacterial cells contained in the wet sample suffered a moderate loss in count and the population decreased from an initial level of 10.96 to 8.03 at the end of the test. This is equivalent to a net log reduction of 2.93. The corresponding figures for dry capsules were 10.19 and 8.39, which is only 1.80 log CFU reduction during the same period (Fig. 5.6).

Membrane coated samples with trehalose added as a protectant (TMB) seemed to be marginally more robust than the uncoated ones (TB) (Fig. 5.7). Though the cells inside wet particles suffered a viability loss of 2.83 log CFU/gm (from 10.87 to 8.04), freeze dried capsules were able to provide better protection and only log CFU reduction of 1.57 was recorded (from 10.18 to 8.61). Statistical analysis showed no significant ($p \geq 0.05$)

difference in the case of wet TB and TMB samples but freeze dried samples were significantly ($p \leq 0.05$) different.

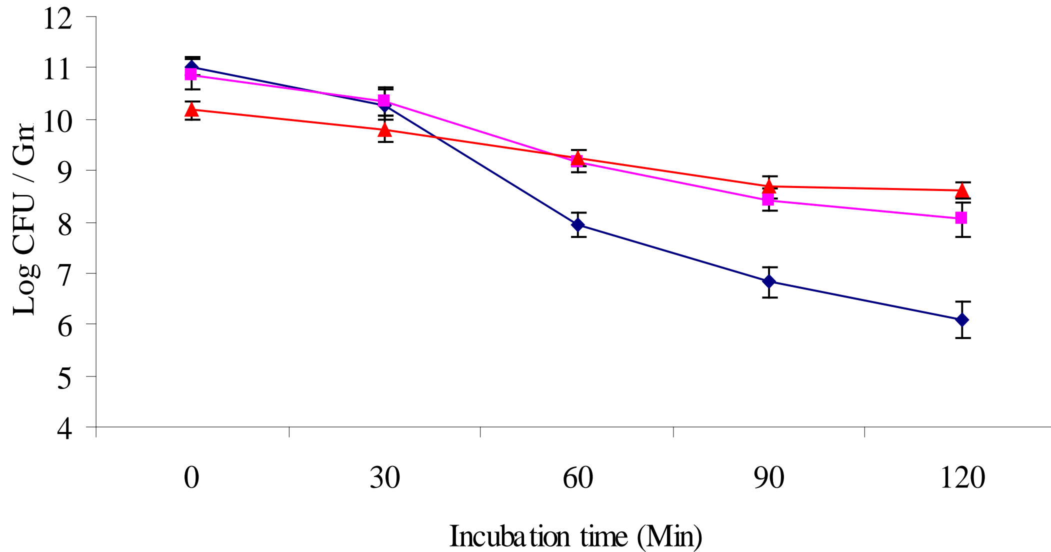


Figure 5.7 Survival of *L. casei* cells in free form (♦) and protected in membrane coated trehalose containing capsules (TMB), in both wet (■) and dried (▲) forms, when incubated in simulated gastric fluid. Error bars representing standard deviations from the mean of 3 replicates.

5.3.5.3 Effect of lactose and membrane coating process on acid tolerance of *L. casei* cells

Lactose supplemented uncoated capsules (LB) showed very similar performance to the TB samples. Wet capsules subjected to SGF recorded a decline in cell count as follows. The starting population of 10.88 log CFU/gm was gradually reduced to 10.73, 9.15, 8.49 and 7.79 log after 30, 60, 90 and 120 min respectively (Fig. 5.8).

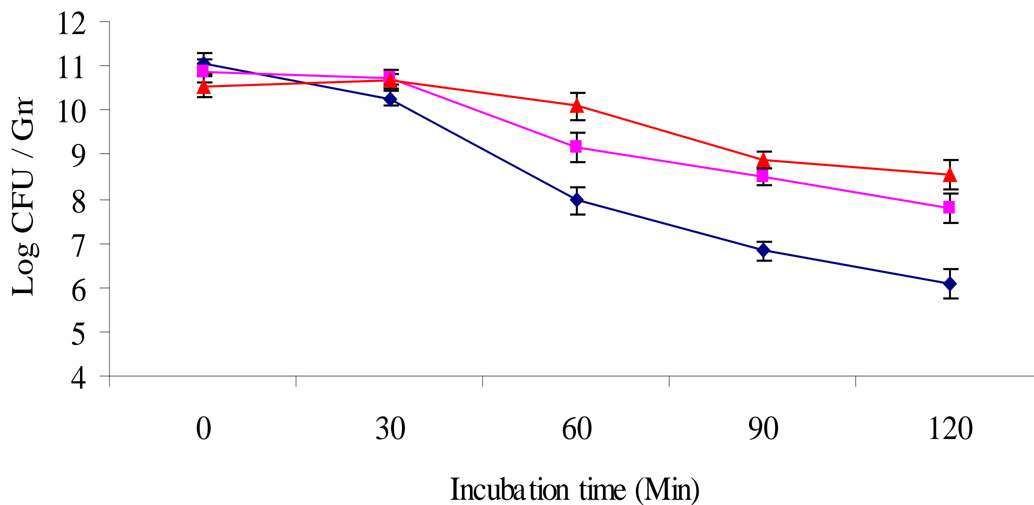


Figure 5.8 Survival of *Lactobacillus casei,* in free form (♦) and microencapsulated in uncoated, lactose containing beads (LB), in wet (■) and dried (▲) form, when treated in simulated gastric fluid (SGF). Error bars representing standard deviations from the mean of 3 replicates.

Thus, the net reductions in viability count were 3.09 log CFU/gm solids of wet capsules. Bacteria encapsulated in freeze dried particles were stable up to initial 30 min and thereafter declined at a faster rate (Fig. 5.8). Finally, the viable count reached 8.55 log CFU/gm at the end of 120 min test period, which represents a net reduction of 1.99 log CFU. Statistical comparison of the data series obtained for TB and LB samples showed no significant difference ($p \geq 0.05$) between the effects of trehalose and lactose. However, they improved the acid tolerant property (when used in growth and drying media) of the encapsulating matrix significantly ($p \leq 0.05$) in comparison to the cells in free form.

Coated samples containing lactose (LMB) apparently best protected the cells against the harsh acidic environment. For wet microbeads, initial viable count of 10.92 log CFU showed gradual decrease at a much slower rate during the 120 min test. The viable colony counts after 30, 60, 90 and 120 min were 10.64, 9.53, 9.07 and 8.51 log CFU, respectively (Fig. 5.9), showing only a net reduction of 2.41 log CFU. Freeze dried LMB samples also seemed to offer good protection to the cells. The cell population was only reduced to 9.38 from an initial level of 10.76 log CFU/gm, thus showing only 1.38 log

CFU/gm net reduction. Test of significance showed that the bacterial cells were significantly better ($p \leq 0.05$) protected in freeze dried LMB microcapsules than all the other samples.

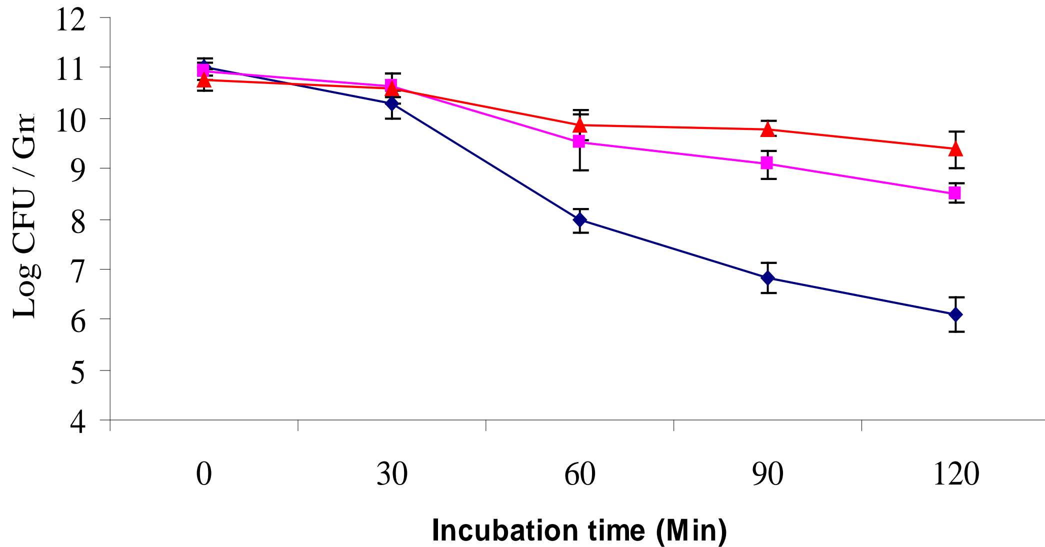


Figure 5.9 Survival of *L. casei* cells in free form (♦) and protected in membrane coated lactose containing capsules (LMB), in both wet (■) and dried (▲) forms, when incubated in simulated gastric fluid. Error bars representing standard deviations from the mean of 3 replicates.

The combined results reported above clearly indicate that the cryoprotectants lactose and trehalose improved the acid tolerance properties of microencapsulated cells, when compared to both the free and encapsulated cells without any cryoprotectant (Chapter 3.0 and 4.0). It can also be seen from the results that an additional coating of gellan gum over the TB and LB microcapsules helped in achieving better protection to the cells entrapped inside. Reddy *et. al.* (2009) studied three freeze dried strains of probiotic LAB (*P. acidilactici*, *L. plantarum* and *L. salivarius* CFR -2158) in presence of trehalose, lactose and some other cryoprotectants, followed by testing of their acid resistance capabilities (at pH 2.5). Very high survival rates in the range of 95% for *L. plantarum* and *P. acidilactici* and 80-85% for *L. salivarius* were reported, when trehalose and lactose were used in the drying media. Lactose was found to be more protective than

trehalose in their study for all the three strains. The values reported by them can not be compared directly with the current results because the drying media composition and cell growth conditions were totally different, but a general trend in improvement observed in both cases can be justified easily. The better performance of lactose over trehalose obtained throughout Chapter 5.0 results is also in line with the findings of Reddy *et. al*. (2009). In another study, skim milk, added as a cryoprotectant has been shown to impart enhanced gastro-intestinal resistance to *L. plantarum* cells (Leite *et. al*., 2009). Thus, the beneficial effect of cryoprotecting compounds on acid tolerance as obtained in the current study is in accordance with other studies.

5.3.6 Effect of bile salts on free and encapsulated *L. casei* cells

The concept of microencapsulating probiotic bacteria is based on providing sufficient protection during high acidic gastric passage and releasing them at the neutral pH environment. After passing through the stomach and before reaching the colon, the probiotic cells also need to pass through the duodenum and the small intestine comprising of jejunum and ileum. The duodenum contains intestinal fluid made up of bile salts, pancreatic juices and these are known to create a hostile environment for bacteria (Holzapfel *et. al*., 1998). The environment in the small intestine is not very bacteria friendly due to rapid transit time, high pH and presence of bile. Therefore, it was important to test the efficiency of the developed microencapsulation technique for their ability to protect against bile salts and compare the results with the behavior of free cells in the same simulated environment.

5.3.6.1 Behavior of free cells in presence of bile salts

Free *L. casei* cells grown in MRS broth were incubated in a simulated bile environment for 8 hours and aliquots were tested for viable counts in every 2 hours interval. The initial cell population of 11.03 log CFU/ml gradually diminished to 10.56, 9.72, 8.93 and 8.86 after 2, 4, 6 and 8 hours, respectively (Fig. 5.10). This 2.17 log reduction in viable

count indicated the detrimental action of bile salts and used as a control to judge the performance of other samples.

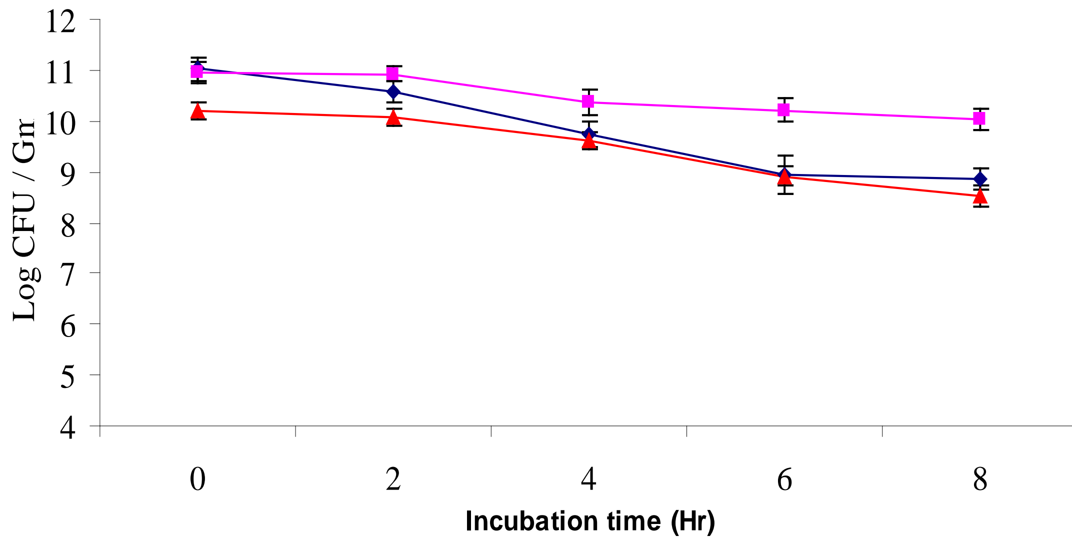


Figure 5.10 Response of free (♦), wet (■) and dry (▲) encapsulated (TB) *L. casei* cells in presence of 1% porcine bile extract. Error bars representing standard deviations from the mean of 3 replicates.

5.3.6.2 Effect of trehalose and membrane coating on bile salt tolerance of *L. casei* cells

Similar to the protocol followed in Chapter 3.0 and Chapter 4.0, wet and freeze dried microcapsules were tested for their protection ability against the detrimental action of bile salts. Uncoated trehalose containing beads (TB) showed very good protection and the cell population was reduced only by 0.93 log CFU/gm for wet and 1.66 log CFU/gm for the dried particles (Fig. 5.10).

Applying a membrane coating of gellan gum on the trehalose treated microparticles (TMB) seemed to offer further protection to the *L. casei* cells encapsulated in them (Fig.

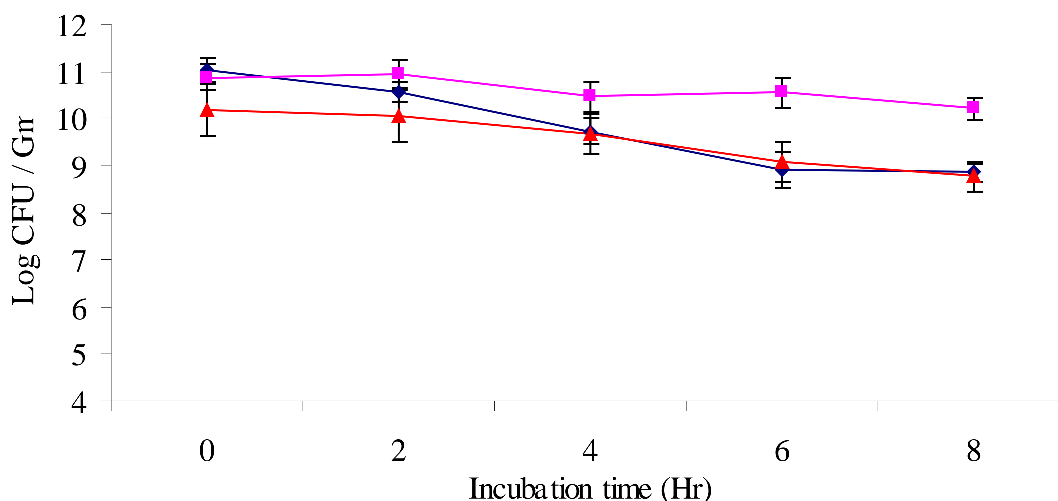


Figure 5.11 Survivability of free (♦) and membrane coated (TMB) *L. casei* cells in simulated bile environment. Membrane coated capsules were tested for wet (■) and dried (▲) forms. Error bars representing standard deviations from the mean of 3 replicates.

5.11). The wet particles contained an average initial cell population of 10.87 log CFU/gm which showed a slight decrease to 10.21 log CFU/gm after 8 hours. In case of dried capsules, the initial cell population decreased from 10.18 to 8.77 during the same period. The net log reduction values for wet and dried particles were 0.66 and 1.41 log CFU/gm respectively. Statistical analysis of each series comprising of 6 data points showed that both wet and dry TMB capsules were significantly better ($p \leq 0.05$) than the corresponding TB as well as free cell samples. Also, the TB samples offered better protection ($p \leq 0.05$) to the cells in encapsulated form compared to the free cells.

5.3.6.3 Effect of lactose and membrane coating on bile salt tolerance of *L. casei* cells

The lactose containing (LB) samples performed slightly better than the trehalose containing samples, although no statistical significance ($p \geq 0.05$) against the TB samples could be established. The cell deaths were recorded as a 0.78 log CFU/gm reduction (from 10.88 to 10.10) for wet capsules and 1.33 log CFU/gm (from 10.54 to 9.21) for dried ones (Fig. 5.12). Wet particles offered good protection until four hours (only 0.18

log reduction), but thereafter the viability reduced at a higher rate. For freeze dried capsules, cell mortality was low during the first 2 hours and increased at a sharper rate for next 4 hours.

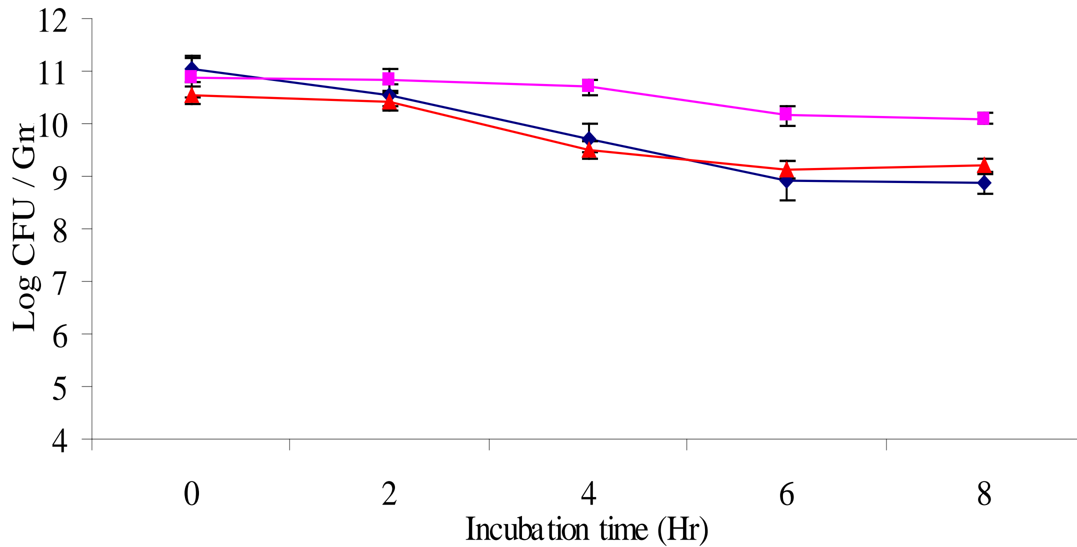


Figure 5.12 Response of free (♦), wet (■) and dry (▲) encapsulated (LB) *L. casei* cells in presence of 1% porcine bile extract. Error bars representing standard deviations from the mean of 3 replicates.

Fig. 5.13 shows the action of bile salts on the *L. casei* cells, in free form and inside LMB microcapsules. The best results from all these samples were obtained in case of LMB capsules. Only 0.50 log CFU population reduction was recorded in the wet samples and 1.07 log in the case of freeze dried samples. The rates of decline were very similar between the two samples and the major viability loss was recorded from 4 hour onwards. Both wet and freeze dried LMB samples were significantly better ($p \leq 0.05$) than free cell, TB, LB as well as TMB samples (sections 5.3.6.1 and 5.3.6.2).

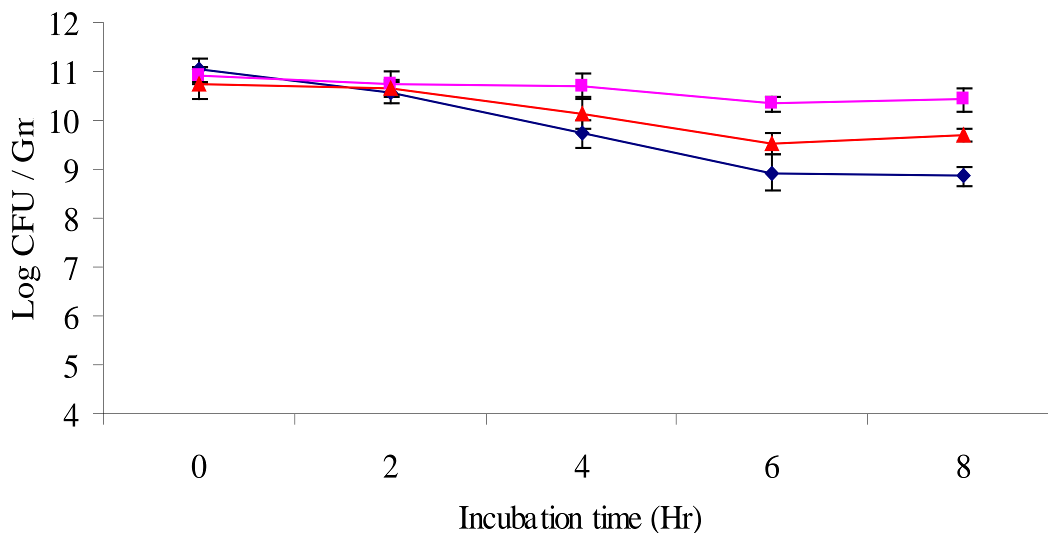


Figure 5.13 Survivability of free (♦) and membrane coated (LMB) *L. casei* cells in simulated bile environment. Membrane coated capsules were tested for wet (■) and dried (▲) forms. Error bars representing standard deviations from the mean of 3 replicates.

Analyzing the overall results of bile tolerance experiments conducted throughout Chapter 3.0, 4.0 and 5.0, it can be concluded that the bacterial cells inside the wet microcapsules were better protected than the cells in the freeze dried ones. Wet samples of TB, LB, TMB and LMB offered much better protection than the freeze dried samples of corresponding treatments. The net log reduction figures for wet TB, LB, TMB and LMB were 0.93, 0.78, 0.66 and 0.50 respectively and the corresponding values for freeze dried samples were 1.66, 1.33, 1.41 and 1.07 (Fig. 5.14 and 5.15). It can be seen that the membrane coating helped in achieving better protection and lactose was more effective than trehalose in this aspect, but the freeze drying process offset this beneficial effect to some extent. A probable explanation for poor survival in freeze dried capsules could be due to the physiological stresses experienced by the cells during freezing and subsequent drying process. Also, freeze drying causes shrinkage of the particles thereby exposing the bacterial cells more towards the surface, which might have resulted into easier contact with the bile salt solution.

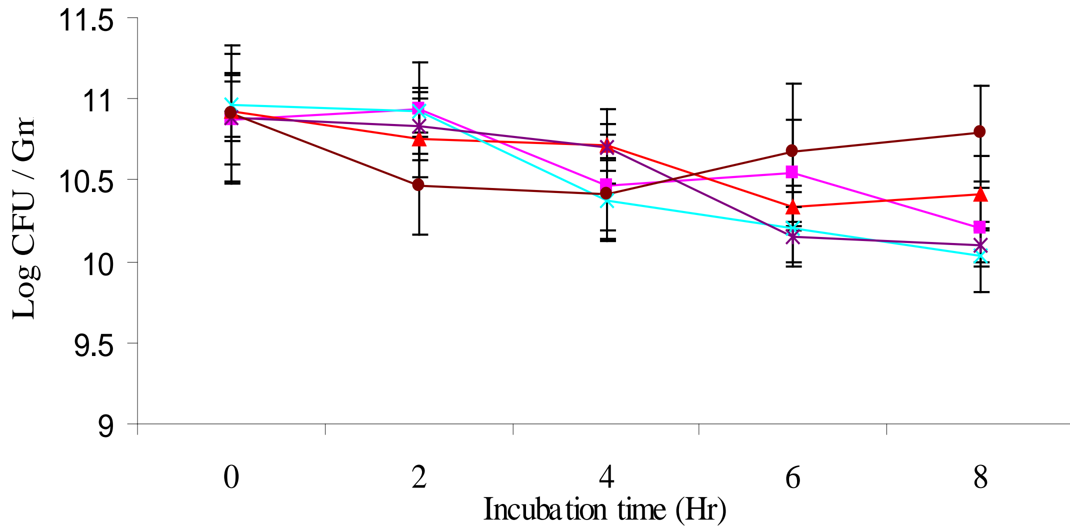


Figure 5.14 Comparison of the effect of cryoprotecting compounds in wet microcapsules (TB x, TMB ■, LB ▲, LMB *) on bile tolerance, against the wet control sample (●) with no protectant. Error bars representing standard deviations from the mean of 3 replicates.

The resistance of *L. casei* cells against bile salts had been improved due to the encapsulation and membrane coating process but any distinct benefit coming from the cryoprotectants could not be established. When looked at the wet capsules with and without cryoprotectants (Fig. 5.14), it can be seen that control samples (Chapter 3.0) performed better than all the other four samples with cryoprotectants added. The range of log reductions for cryoprotected samples varied from 0.50 to 0.93 compared to the average value of 0.12 obtained in control sample with protectant. However, the result summary in Fig. 5.15 shows that without-protectant freeze dried sample (chapter 4.0) was a poorer protector than 3 other samples except for TB, where equal log reductions (2.44) was recorded. Therefore, a clear benefit of cryoprotecting solutes in growth and drying media against the detrimental action of bile salts could not be claimed with confidence.

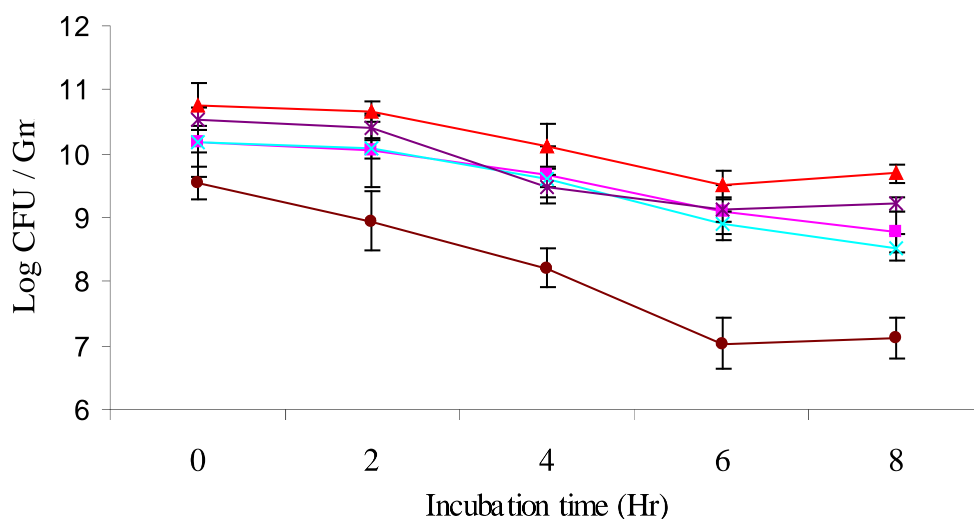


Figure 5.15 Comparison of the effect of cryoprotecting compounds in dry microcapsules (TB x, TMB ■, LB ▲, LMB *) on bile tolerance, against the dry control sample (●) with no protectant. Error bars representing standard deviations from the mean of 3 replicates.

In a similar previous work, trehalose, lactose and a few other cryoprotectants were shown to impart very good resistance towards bile but no control sample without these protectants was reported and only 0.3% concentration of bile salt was used in that case (Reddy *et. al.*, 2009). Hence, it was difficult to directly compare the results. Bile extracts (as used in this project) have been reported to be more destructive than bile acids and up to 3.5 and 3.0 log reduction of fresh and freeze dried cell population respectively were observed (Saarela *et. al.*, 2005). The freeze dried cell mass in that study contained sucrose as a cryoprotectant. One percent bile extract was used in the test fluid, similar to the conditions maintained in this research. The degree of destruction in the free as well as freeze dried cells was higher because more sensitive *B. animalis* cells were tested, compared to the *L. casei* cells in the current research.

5.3.7 Storage stability of the *L. casei* cells in freeze dried microcapsules

Maintaining a high level of cell viability throughout the storage period is very important for the end use of either as direct-to-vat starter or as adjunct inoculation in the form of probiotics. The degree of survival during storage and maintaining the functional properties depend upon the type of drying technology and the drying matrix used to stabilize the bacteria (Carvalho *et. al.*, 2004a). In the present study, combined effect of microencapsulation and incorporation of compatible solutes as well as cryoprotectants, on survival of bacteria during storage at both refrigerated and high temperatures were investigated.

5.3.7.1 Survival of encapsulated freeze dried cells stored at 4°C

The control samples did not have any protectant either in the growth or drying media and resulted in high mortality after freeze drying. Therefore, the initial cell density could not be kept to the same levels as of the experimental samples which were treated with lactose or trehalose. Control samples stored at 4°C showed a reduction in cell viability from 9.47 to 7.87 log CFU/gm at the end of four weeks, causing a net 1.60 log reduction (Fig. 5.16). Among the experimental samples, TB showed maximum susceptibility with a net 1.2 log CFU/gm reduction in count, followed by LB which was moderately affected with 0.71 log CFU reduction. The rates of cell death were quite similar up to the 3rd week but cells in the TB samples decayed at a much faster rate in the last week, compared to the cells within LB samples (Fig. 5.16). The membrane coating process seemed to offer better protection. Cells inside TMB samples, on average, showed 0.33 log reduction in 4 weeks (from 10.18 to 9.85) and the best result was obtained in LMB samples which came down to 10.50 from an initial figure of 10.76 log CFU, thus showing only 0.26 log reduction in the viability. The degree of viable cell decay between TMB and LMB was not statistically significant ($p \geq 0.05$) but both of them were significantly better ($p \leq 0.05$) than TB, LB and contro l samples.

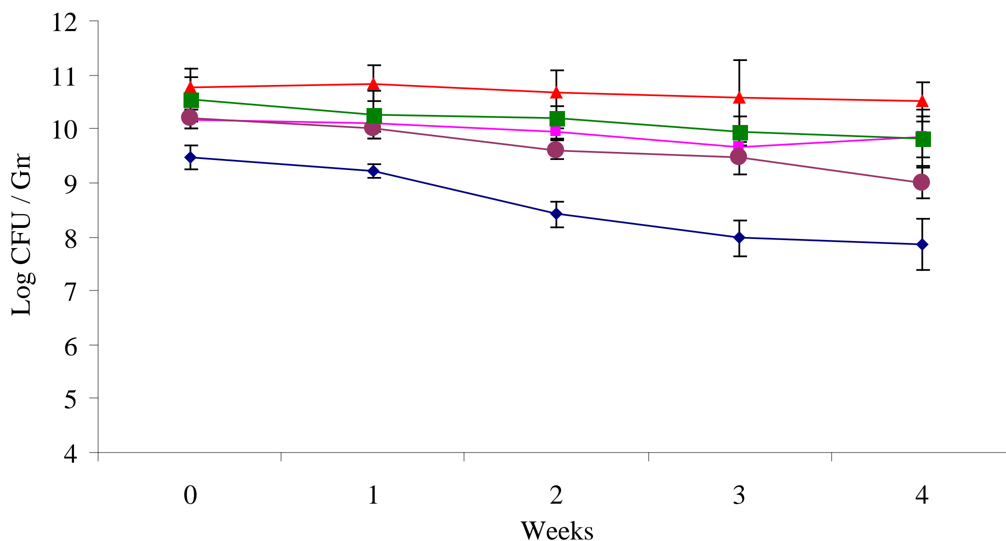


Figure 5.16 Stability of microencapsulated *L. casei* cells in freeze dried microparticles when stored at 4°C. Graphs showing TB (●), LB (■), TMB (■) and LMB (▲) samples in comparison with the control sample (♦). Error bars representing standard deviations from the mean of 3 replicates.

5.3.7.2 Survival of encapsulated freeze dried cells stored at 37°C

At 37°C storage, the control sample showed 4.79 log reduction in viability per gm solid of the capsules (Fig. 5.17). All the four cryoprotected and membrane coated samples showed much better retention of viable cells. LB, LMB and TMB samples had loss of viability with 2.18, 2.11 and 2.16 log CFU reduction, respectively, during the entire 4 weeks test. TB samples looked slightly adversely affected with 2.45 log reduction, but significantly better ($p \leq 0.05$) compared to the control samples.

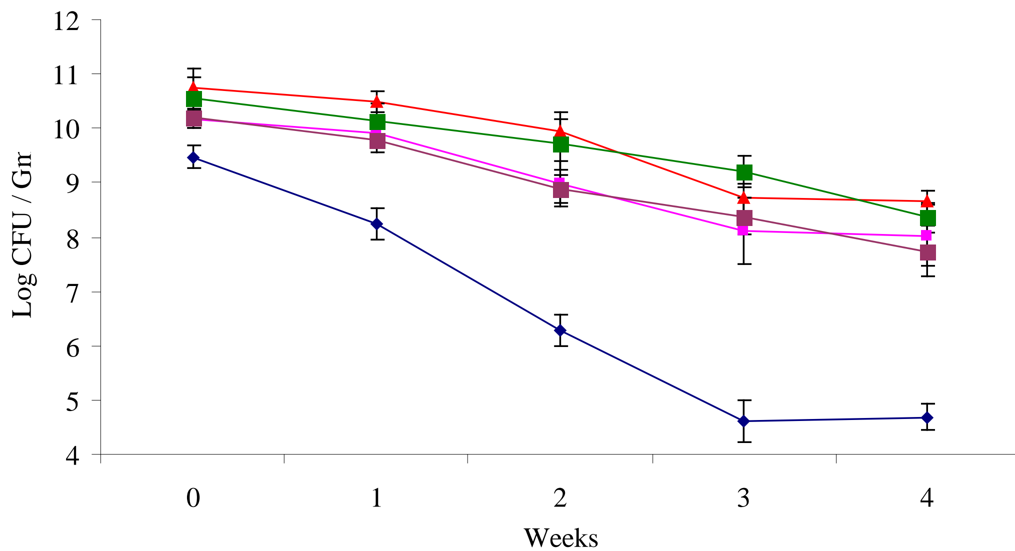


Figure 5.17 Stability of microencapsulated *L. casei* cells in freeze dried microparticles when stored at 37°C. Graphs showing TB (■), LB (■), TMB (■) and LMB (▲) samples in comparison with the control sample (♦). Error bars representing standard deviations from the mean of 3 replicates.

The use of cryoprotective compounds during freeze drying has been found helpful in maintaining better viability of cells during prolonged storage (Carvalho *et al*., 2002) but actually many other internal as well as external factors such as, moisture content, relative humidity, presence of oxygen, light exposure etc. are equally important (Meng *et. al*., 2008). It was also reported that the storage viability is highly related to the temperature and it decreases at elevated storage temperatures (Champagne *et. al*., 1996; Abadias *et. al*., 2001). Results shown in Fig. 5.16 and 5.17 very clearly support this and it can be observed that the storage of microencapsulated and freeze dried *L. casei* cells at 4°C was much more effective than at 37°C. The same trend also held well for free cells too. Between the two additives used, lactose was found to be more effective at 4°C storage. However, at 37°C, all the encapsulated samples suffered from high mortality and the net reduction figures were very close for all four samples. The protective mechanism of sugars has been explained by their high viscous glass forming character at room temperature when they are subjected to dehydration. High storage viability of *L. rhamnosus* GG and a direct relationship with the presence of trehalose, lactose/trehalose

and lactose/maltose have been reported (Meng *et. al.*, 2008). A distinctive advantage of using protective additives can be seen when the control sample results at both 4°C and 37°C are compared. Moreover, it was also observed that membrane coated microcapsules were able to retain more live cells during storage at low temperature, with the LMB samples showing slightly better results (Fig. 5.16). The contribution of the microencapsulation process towards enhanced viability has been confirmed by Koo *et. al.* (2001), who found encapsulated cells had similar viability level to the non-encapsulated ones at 4°C, but at elevated temperatures encapsulated cells performed much better. But at the same time, study by O'Riordan *et. al.* (2001) did not report any enhanced protection for starch encapsulated *bifidobacterium* cells during storage at room temperatures. Hence, it can be seen that the role of microencapsulation in enhanced storage stability can not be concluded with confidence but a general consensus about the effect of common cryoprotective compounds is evident. The results obtained throughout this project also support this fact where microencapsulation alone was not very effective in enhanced stability test (Chapter 4.0) but cryoprotection helped in obtaining comparatively better results at both 4°C and 37°C storage (Chapter 5.0).

5.3.8 Conclusions

The main objectives of this chapter were to minimize the cell death occurring due to freeze drying and also to improve the survivability of cells during simulated gastric transit. Two modifications in the process flow diagram were planned. It was hypothesized that addition of compatible solutes in the growth media as well as in the drying matrix would help in better survival of bacterial cells. The results obtained were encouraging for both lactose and trehalose. Lactose was proved to be a very good protectant for cells not only during freeze drying but also during simulated gastric fluid incubation. The other protectant used, trehalose, was also helpful in these two aspects as compared to control samples. A similar trend was noted in the case of bile salt tolerance tests, where lactose containing samples showed better viability than the control and trehalose containing samples. The second modification planned was application of an additional membrane coating over the produced wet microcapsules. The gellan gum

coating was helpful in obtaining better results for both the objectives mentioned above. Membrane coated samples (TMB and LMB) proved to be more robust against the stresses of freeze drying and the challenging gastric environment, in comparison to the non coated samples (control, LB and TB). A smoother surface area with no visible pores is thought to be the primary reason for this effect. Lactose containing membrane coated samples gave the best results in all aspects and further developments on the manufacturing process flow are suggested for this study.

Chapter 6.0. Overall conclusions and future recommendations

The first part of this project focused on developing a novel microencapsulation technology targeting probiotic bacteria. After thorough searching of previous literature (Chapter 2.0) on this subject area it was found that dairy proteins in a combination with polysaccharides could be a good potential carrier material for encapsulating a lactobacillus strain, namely *L. casei* 431. The high density gel network produced from sodium caseinate and gellan gum nicely entrapped the probiotic cells and its low viscosity helped to have a control over particle size distribution. High encapsulation efficiency (up to 89%) was achieved and the mean diameter of the produced microcapsules in soft gel form was slightly higher than the generally acceptable range. The acid tolerance of the encapsulated cells was compared with the free cells in an *in vitro* gastric model and a significantly higher survival was recorded for encapsulated cells. A similar trend was observed in the presence of bile salts.

In the second part of the project (Chapter 4.0), the wet gel microcapsules were subjected to freeze drying and its effects were studied on capsule physical properties, cell survival after drying, surface morphology and changes in protection ability against simulated gastric and intestinal environments. The freeze dried microcapsules were found to have certain shortcomings such as poor cell survival following freeze drying and only moderate protection ability against gastric environment due to the presence of a porous surface structure.

The third part of the research (Chapter 5.0) addressed the shortfalls and modified the encapsulation matrix with the incorporation of the disaccharides trehalose and lactose. The surface properties were improved by developing a membrane coating process. Overall improvements in post drying survival of *L. casei* 431 cells, their acid and bile salt tolerance and storage stability were recorded. Lactose was found to be better protectant than trehalose during freeze drying, as weel as in simulated gastric and bile salt environments.

The developed technology is therefore considered to carry considerable potential towards application in the food industry, where the successful targeted delivery of probiotic bacteria is still a challenge.

A basic shortcoming of this technology which could not be improved by any modification is that the whole concept was based on acidic gelation of sodium caseinate and hence the capsule matrix is highly unstable in a neutral to high pH zone. Therefore, this process is only appropriate in a low pH food carrier such as yoghurt, fruit juice, mayonnaise, fruit smoothies etc.

However, certain areas on which more focus can be directed and further development can be carried out are being listed below.

Further recommendations

1) The microencapsulation process and the benefits obtained need to be validated using other probiotic strains including *bifidobacterium* varieties, to verify whether similar protections are achievable.
2) After observing satisfactory results in the *in vitro* model, the developed system can be further validated using *in vivo* model via a series of suitable animal trials.
3) The ultimate application of this technology is targeted for a suitable food product. Hence a thorough product stability study and sensory evaluation would be important as a preliminary step towards successful commercialization of this technology.
4) It is also important to study the behavior of the produced microcapsules during the most common processing steps applicable in the food industry. For example, the viability of the entrapped bacteria needs to be enumerated when the food products containing the microcapsules are subjected to normal pre-heating or pasteurization processes. The effects of some other unit operations like homogenization, centrifugation and extrusion on the physical properties of the

microcapsules should be studied. Any adverse effect on cell viability also needs to be given importance in this regard.

5) The capsule integrity during the acid and bile tolerance examinations can be examined at some intervals during the incubation, with the help of SEM/TEM imaging. This will probably help in understanding the gradual capsule breakdown process, acid and bile penetration behavior and release of probiotic bacteria in real time.
6) The storage stability test for the dry capsules should be continued for at least one year because DVS cultures and probiotic ingredients supplied by the manufacturers normally claims one year of storage stability at refrigerated conditions.

Overall, the current research introduces a promising technology for microencapsulating not only probiotic bacteria but also other bioactive materials such as, minerals, vitamins, oxidation sensitive lipids etc. Further development and experimental designs can be conceived based on the suggestions listed above. The health benefits of probiotic rich foods are well accepted and clinically proven. But the regulatory authorities throughout the world are becoming stricter and the food manufacturers in today's world will have to ensure that the minimum stipulated quantity of viable bacteria are delivered properly. Therefore, the successful targeted delivery of probiotic bacteria to the human intestine has tremendous market potential and it will be highly welcomed by the industry if a complete solution can be offered at an affordable cost.

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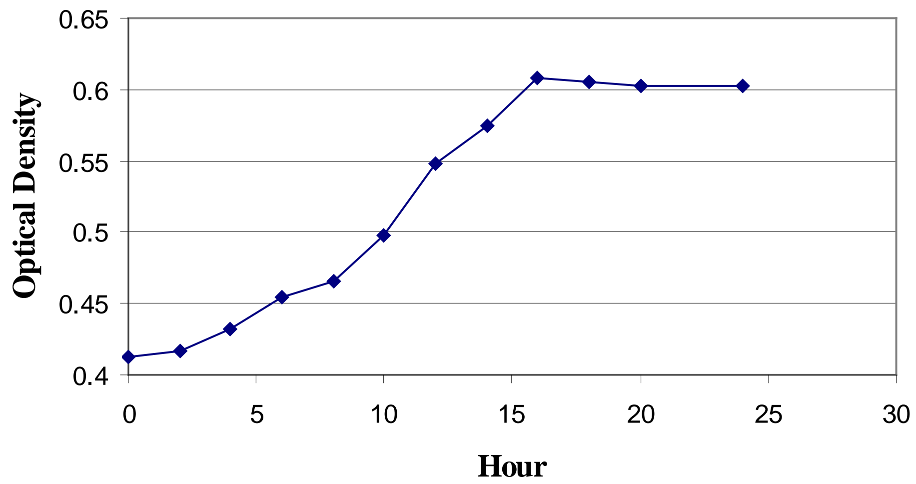

Appendix 3.1 Measuring growth curve and identifying the stationary phase of *L. casei* 431 bacteria grown in MRS broth with 1% (v/v) inoculation.

Appendix 3.2: Particle size distribution data for wet microcapsule samples prepared in Chapter 3.0 experiments

Size µm	Volume in %	Size µm	Volume in %	Size µm	Volume in %	Size µm	Volume in %	Size µm	Volume in %	Size µm	Volume in %
0.010		0.105		1.096		11.482		120.226		1258.925	
	0.00		0.00		0.00		0.00		2.17		0.00
0.011		0.120		1.259		13.183		138.038		1445.440	
	0.00		0.00		0.00		0.00		2.88		0.00
0.013		0.138		1.445		15.136		158.489		1659.587	
	0.00		0.00		0.00		0.00		3.75		0.00
0.015		0.158		1.660		17.378		181.970		1905.461	
	0.00		0.00		0.00		0.00		4.81		0.00
0.017		0.182		1.905		19.953		208.93		2187.762	
	0.00		0.00		0.00		0.00		5.98		0.00
0.020		0.209		2.188		22.909		239.883		2511.886	
	0.00		0.00		0.00		0.00		7.21		0.00
0.023		0.240		2.512		26.303		275.423		2884.032	
	0.00		0.00		0.00		0.00		8.36		0.00
0.026		0.275		2.884		30.200		316.228		3311.311	
	0.00		0.00		0.00		0.00		9.27		0.00
0.030		0.316		3.311		34.674		363.078		3801.894	
	0.00		0.00		0.00		0.01		9.77		0.00
0.035		0.363		3.802		39.811		416.869		4365.158	
	0.00		0.00		0.00		0.08		9.72		0.00
0.040		0.417		4.365		45.709		478.630		5011.872	
	0.00		0.00		0.00		0.14		9.07		0.00
0.046		0.479		5.012		52.481		549.541		5754.399	
	0.00		0.00		0.00		0.23		7.86		0.00
0.052		0.550		5.754		60.256		630.957		6606.934	
	0.00		0.00		0.00		0.37		6.23		0.00
0.060		0.631		6.607		69.183		724.436		7585.776	
	0.00		0.00		0.00		0.57		4.37		0.00
0.069		0.724		7.586		79.433		831.764		8709.636	
	0.00		0.00		0.00		0.83		2.58		0.00
0.079		0.832		8.71		91.201		954.993		10000.000	
	0.00		0.00		0.00		1.17		0.98		0.00
0.091		0.955		10.000		104.713		1096.478			
	0.00		0.00		0.00		1.61		0.00		0.00
0.105		1.096		11.482		120.226		1258.925			

Appendix 3.3: Survival of *L. casei* cells in free form and inside wet microcapsules when subjected to simulated gastric juice at pH 2.0 without pepsin.

Free Cells	Control		30 Min		60 Min		90 Min		120 Min	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	5.37 x 10^10	10.73	2.51 x 10^9	9.4	4.36 x 10^7	7.64	7.24 x 10^5	5.86	7.41 x 10^4	4.87
Expt 1.2	6.61 x 10^10	10.82	4.17 x 10^9	9.62	1.86 x 10^7	7.27	2.88 x 10^5	5.46	9.77 x 10^4	4.99
Expt 2.1	1.95 x 10^11	11.29	2.09 x 10^9	9.32	4.90 x 10^8	8.69	1.55 x 10^5	5.19	4.47 x 10^4	4.65
Expt 2.2	7.24 x 10^10	10.86	1.17 x 10^9	9.07	9.55 x 10^6	6.98	7.24 x 10^5	5.86	2.57 x 10^4	4.41
Expt 3.1	8.31 x 10^10	10.92	2.95 x 10^9	9.47	2.24 x 10^7	7.35	1.35 x 10^5	5.13	1.82 x 10^5	5.26
Expt 3.2	2.19 x 10^11	11.34	3.89 x 10^9	9.59	4.07 x 10^7	7.61	1.23 x 10^5	5.09	2.69 x 10^5	5.43
Average		10.993		9.412		7.590		5.432		4.935
Std. Dev		0.257		0.202		0.591		0.356		0.378

Wet Capsules	Control		30 Min		60 Min		90 Min		120 Min	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	7.76 x 10^10	10.89	2.09 x 10^10	10.32	7.26 x 10^9	9.86	2.45 x 10^8	8.39	1.62 x 10^8	8.21
Expt 1.2	2.57 x 10^10	10.41	9.12 x 10^9	9.96	2.14 x 10^9	9.33	3.09 x 10^7	7.49	2.45 x 10^7	7.39
Expt 2.1	8.51 x 10^10	10.93	1.20 x 10^10	10.08	5.13 x 10^9	9.71	9.55 x 10^7	7.98	3.39 x 10^7	7.53
Expt 2.2	5.89 x 10^10	10.77	7.24 x 10^9	9.86	2.04 x 10^9	9.31	8.13 x 10^7	7.91	4.57 x 10^7	7.66
Expt 3.1	1.82 x 10^11	11.26	1.74 x 10^10	10.24	5.89 x 10^9	9.77	3.31 x 10^7	7.52	1.62 x 10^7	7.21
Expt 3.2	1.55 x 10^10	10.19	2.34 x 10^10	10.37	5.37 x 10^9	9.73	1.86 x 10^8	8.27	6.76 x 10^7	7.83
Average		10.742		10.138		9.618		7.927		7.638
Std. Dev		0.385		0.205		0.237		0.372		0.352

Appendix 3.4: Action of simulated gastric juice at pH 2.0 with 0.32% pepsin, on *L. casei* cells in free form and inside wet microcapsules.

Free Cells	**Control**		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	6.02 x 10^10	10.78	4.57 x 10^9	9.66	1.23 x 10^9	9.09	2.45 x 10^7	7.39	2.57 x 10^6	6.41
Expt 1.2	1.70 x 10^10	10.23	2.09 x 10^9	9.32	2.88 x 10^8	8.46	6.17 x 10^6	6.79	9.33 x 10^5	5.97
Expt 2.1	8.32 x 10^9	9.92	1.02 x 10^9	9.01	1.66 x 10^8	8.22	1.86 x 10^6	6.27	3.80 x 10^5	5.58
Expt 2.2	3.09 x 10^10	10.49	1.70 x 10^9	9.23	3.80 x 10^8	8.58	3.39 x 10^6	6.53	1.55 x 10^5	5.19
Expt 3.1	3.72 x 10^10	10.57	2.95 x 10^9	9.47	9.55 x 10^8	8.97	1.78 x 10^7	7.25	2.88 x 10^6	6.46
Expt 3.2	8.91 x 10^10	10.95	1.51 x 10^9	9.18	4.90 x 10^8	8.69	2.88 x 10^6	6.46	9.55 x 10^5	5.98
Average		10.490		9.312		8.668		6.782		5.932
Std. Dev		0.373		0.229		0.323		0.451		0.487

Wet Capsules	**Control**		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	2.95 x 10^10	10.47	1.86 x 10^10	10.27	6.61 x 10^9	9.82	2.14 x 10^8	8.33	5.62 x 10^7	7.75
Expt 1.2	2.04 x 10^10	10.31	1.34 x 10^10	10.13	3.80 x 10^9	9.58	1.02 x 10^8	8.01	1.62 x 10^7	7.21
Expt 2.1	6.76 x 10^9	9.83	4.68 x 10^9	9.67	2.95 x 10^9	9.47	1.86 x 10^8	8.27	1.20 x 10^7	7.08
Expt 2.2	2.14 x 10^10	10.33	1.90 x 10^10	10.28	5.75 x 10^9	9.76	1.17 x 10^8	8.07	2.40 x 10^7	7.38
Expt 3.1	1.66 x 10^10	10.22	1.33 x 10^10	10.13	4.79 x 10^9	9.68	2.45 x 10^8	8.39	1.44 x 10^7	7.16
Expt 3.2	2.69 x 10^10	10.43	2.34 x 10^10	10.37	2.34 x 10^9	9.37	7.59 x 10^7	7.88	6.31 x 10^6	6.8
Average		10.265		10.142		9.613		8.158		7.230
Std. Dev		0.231		0.249		0.173		0.201		0.318

Appendix 3.5: Survival of *L. casei* cells in free form and microencapsulated in wet capsules in a simulated bile solution with 1.0% (w/w) bile extract.

Free Cells	Control		30 Min		60 Min		90 Min		120 Min	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	1.51 x 10^11	11.18	6.76 x 10^10	10.83	5.13 x 10^9	9.71	1.02 x 10^9	9.01	1.45 x 10^9	9.16
Expt 1.2	3.80 x 10^10	10.58	1.38 x 10^10	10.14	6.46 x 10^9	9.81	1.66 x 10^8	8.22	1.86 x 10^8	8.27
Expt 2.1	8.13 x 10^11	10.91	2.29 x 10^10	10.36	3.89 x 10^9	9.59	1.10 x 10^8	8.04	1.23 x 10^8	8.09
Expt 2.2	6.76 x 10^10	10.83	1.17 x 10^10	10.07	5.75 x 10^9	9.76	2.82 x 10^8	8.45	1.86 x 10^8	8.27
Expt 3.1	8.32 x 10^10	10.92	4.07 x 10^10	10.61	1.78 x 10^10	10.25	2.24 x 10^8	8.35	2.95 x 10^8	8.47
Expt 3.2	1.02 x 10^11	11.01	1.74 x 10^10	10.24	6.92 x 10^9	9.84	8.13 x 10^7	7.91	1.17 x 10^8	8.07
Average		10.905		10.375		9.827		8.330		8.388
Std. Dev		0.199		0.293		0.225		0.387		0.405

Wet Capsules	Control		30 Min		60 Min		90 Min		120 Min	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	7.76 x 10^10	10.89	6.02 x 10^10	10.78	4.07 x 10^10	10.61	1.07 x 10^11	11.03	1.32 x 10^11	11.12
Expt 1.2	3.02 x 10^11	11.48	1.62 x 10^10	10.21	2.45 x 10^10	10.39	1.10 x 10^10	10.04	1.95 x 10^10	10.29
Expt 2.1	4.07 x 10^10	10.61	1.15 x 10^10	10.06	1.32 x 10^10	10.12	2.14 x 10^10	10.33	5.13 x 10^10	10.71
Expt 2.2	2.24 x 10^11	11.35	5.37 x 10^10	10.73	1.99 x 10^10	10.3	4.37 x 10^10	10.64	4.90 x 10^10	10.69
Expt 3.1	2.69 x 10^10	10.43	2.45 x 10^10	10.39	2.04 x 10^10	10.31	1.45 x 10^11	11.16	8.13 x 10^10	10.91
Expt 3.2	4.90 x 10^10	10.69	4.68 x 10^10	10.67	5.37 x 10^10	10.73	6.46 x 10^10	10.81	1.02 x 10^11	11.01
Average		10.908		10.473		10.410		10.668		10.788
Std. Dev		0.421		0.299		0.223		0.425		0.296

Appendix 4.1: Volume distribution data for freeze dried particles as prepared in Chapter 4.0

Size µm	Volume in %	Size µm	Volume in %	Size µm	Volume in %	Size µm	Volume in %	Size µm	Volume in %	Size µm	Volume in %
0.010		0.105		1.096		11.482		120.226		1258.925	
	0.00		0.00		0.00		0.00		9.14		0.00
0.011		0.120		1.259		13.183		138.038		1445.440	
	0.00		0.00		0.00		0.00		10.05		0.00
0.013		0.138		1.445		15.136		158.489		1659.587	
	0.00		0.00		0.00		0.00		10.40		0.00
0.015		0.158		1.660		17.378		181.970		1905.461	
	0.00		0.00		0.00		0.00		10.14		0.00
0.017		0.182		1.905		19.953		208.93		2187.762	
	0.00		0.00		0.00		0.00		9.31		0.00
0.020		0.209		2.188		22.909		239.883		2511.886	
	0.00		0.00		0.00		0.00		8.02		0.00
0.023		0.240		2.512		26.303		275.423		2884.032	
	0.00		0.00		0.00		0.00		6.48		0.00
0.026		0.275		2.884		30.200		316.228		3311.311	
	0.00		0.00		0.00		0.00		4.85		0.00
0.030		0.316		3.311		34.674		363.078		3801.894	
	0.00		0.00		0.00		0.00		3.34		0.00
0.035		0.363		3.802		39.811		416.869		4365.158	
	0.00		0.00		0.00		0.00		2.03		0.00
0.040		0.417		4.365		45.709		478.630		5011.872	
	0.00		0.00		0.00		0.24		1.07		0.00
0.046		0.479		5.012		52.481		549.541		5754.399	
	0.00		0.00		0.00		0.94		0.22		0.00
0.052		0.550		5.754		60.256		630.957		6606.934	
	0.00		0.00		0.00		1.87		0.00		0.00
0.060		0.631		6.607		69.183		724.436		7585.776	
	0.00		0.00		0.00		3.11		0.00		0.00
0.069		0.724		7.586		79.433		831.764		8709.636	
	0.00		0.00		0.00		4.62		0.00		0.00
0.079		0.832		8.71		91.201		954.993		10000.000	
	0.00		0.00		0.00		6.23		0.00		0.00
0.091		0.955		10.000		104.713		1096.478			
	0.00		0.00		0.00		7.82		0.00		0.00
0.105		1.096		11.482		120.226		1258.925			

Appendix 4.2 : Survival of *L. casei* cells microencapsulated in wet and freeze dried form during incubation in simulated gastric fluid

Wet Capsules	**Control**		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	2.95 x 10^10	10.47	1.86 x 10^10	10.27	6.61 x 10^9	9.82	2.14 x 10^8	8.33	5.62 x 10^7	7.75
Expt 1.2	2.04 x 10^10	10.31	1.34 x 10^10	10.13	3.80 x 10^9	9.58	1.02 x 10^8	8.01	1.62 x 10^7	7.21
Expt 2.1	6.76 x 10^9	9.83	4.68 x 10^9	9.67	2.95 x 10^9	9.47	1.86 x 10^8	8.27	1.20 x 10^7	7.08
Expt 2.2	2.14 x 10^10	10.33	1.90 x 10^10	10.28	5.75 x 10^9	9.76	1.17 x 10^8	8.07	2.40 x 10^7	7.38
Expt 3.1	1.66 x 10^10	10.22	1.33 x 10^10	10.13	4.79 x 10^9	9.68	2.45 x 10^8	8.39	1.44 x 10^7	7.16
Expt 3.2	2.69 x 10^10	10.43	2.34 x 10^10	10.37	2.34 x 10^9	9.37	7.59 x 10^7	7.88	6.31 x 10^6	6.8
Average		10.265		10.142		9.613		8.158		7.230
Std. Dev		0.231		0.249		0.173		0.201		0.318

Dried Capsules	**Control**		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	6.46 x 10^9	9.81	8.51 x 10^8	8.93	1.95 x 10^8	8.29	2.34 x 10^8	8.37	1.07 x 10^8	8.03
Expt 1.2	3.24 x 10^9	9.51	2.69 x 10^8	8.43	3.55 x 10^7	7.55	3.09 x 10^7	7.49	1.44 x 10^7	7.16
Expt 2.1	8.91 x 10^9	9.95	1.20 x 10^9	9.08	2.34 x 10^8	8.37	1.82 x 10^8	8.26	4.68 x 10^7	7.67
Expt 2.2	4.37 x 10^9	9.64	3.72 x 10^8	8.57	1.15 x 10^8	8.06	8.32 x 10^7	7.92	1.51 x 10^7	7.18
Expt 3.1	1.62 x 10^9	9.21	1.62 x 10^8	8.21	1.32 x 10^7	7.12	1.20 x 10^7	7.08	4.68 x 10^6	6.67
Expt 3.2	3.72 x 10^9	9.57	1.32 x 10^9	8.12	5.75 x 10^7	7.76	3.39 x 10^7	7.53	2.09 x 10^7	7.32
Average		9.615		8.557		7.858		7.775		7.338
Std. Dev		0.256		0.385		0.477		0.497		0.467

Appendix 4.3 : Action of bile salts on *L. casei* cells encapsulated in wet and freeze dried microcapsules

Wet Capsules	**Control**		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	7.76 x 10^10	10.89	6.02 x 10^10	10.78	4.07 x 10^10	10.61	1.07 x 10^11	11.03	1.32 x 10^11	11.12
Expt 1.2	3.02 x 10^11	11.48	1.62 x 10^10	10.21	2.45 x 10^10	10.39	1.10 x 10^10	10.04	1.95 x 10^10	10.29
Expt 2.1	4.07 x 10^10	10.61	1.15 x 10^10	10.06	1.32 x 10^10	10.12	2.14 x 10^10	10.33	5.13 x 10^10	10.71
Expt 2.2	2.24 x 10^11	11.35	5.37 x 10^10	10.73	1.99 x 10^10	10.3	4.37 x 10^10	10.64	4.90 x 10^10	10.69
Expt 3.1	2.69 x 10^10	10.43	2.45 x 10^10	10.39	2.04 x 10^10	10.31	1.45 x 10^11	11.16	8.13 x 10^10	10.91
Expt 3.2	4.90 x 10^10	10.69	4.68 x 10^10	10.67	5.37 x 10^10	10.73	6.46 x 10^10	10.81	1.02 x 10^11	11.01
Average		10.908		10.473		10.410		10.668		10.788
Std. Dev		0.421		0.299		0.223		0.425		0.296

Dried Capsules	**Control**		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	5.13 x 10^9	9.71	4.68 x 10^7	7.67	7.76 x 10^6	6.89	3.47 x 10^7	7.54	1.70 x 10^7	7.23
Expt 1.2	2.82 x 10^9	9.45	2.45 x 10^7	7.39	1.55 x 10^6	6.19	4.57 x 10^6	6.66	8.71 x 10^6	6.94
Expt 2.1	7.76 x 10^9	9.89	7.41 x 10^6	6.87	1.23 x 10^6	6.09	2.69 x 10^6	6.43	1.44 x 10^7	7.16
Expt 2.2	3.80 x 10^9	9.58	2.69 x 10^6	6.43	3.39 x 10^6	6.53	8.32 x 10^6	6.92	2.95 x 10^7	7.47
Expt 3.1	1.41 x 10^9	9.15	1.29 x 10^7	7.11	1.32 x 10^6	6.12	4.68 x 10^6	6.67	3.63 x 10^6	6.56
Expt 3.2	3.24 x 10^9	9.51	4.37 x 10^6	6.64	1.82 x 10^6	6.26	3.24 x 10^6	6.51	1.91 x 10^7	7.28
Average		9.548		7.018		6.347		6.788		7.107
Std. Dev		0.250		0.465		0.309		0.405		0.318

Appendix 4.4 Moisture content analysis of wet and freeze dried microcapsules

Wet microcapsules as per Chapter 3.0

		Moisture Content (%)
Experiment No. 1	Sample No. 1	86.32
Experiment No. 1	Sample No. 2	86.78
Experiment No. 2	Sample No. 1	87.66
Experiment No. 2	Sample No. 2	88.34
Experiment No. 2	Sample No. 1	87.51
Experiment No. 2	Sample No. 2	88.83

Freeze dried microcapsules as per Chapter 4.0

		Moisture Content (%)
Experiment No. 1	Sample No. 1	3.23
Experiment No. 1	Sample No. 2	3.78
Experiment No. 2	Sample No. 1	3.66
Experiment No. 2	Sample No. 2	4.03
Experiment No. 2	Sample No. 1	4.22
Experiment No. 2	Sample No. 2	3.89

Appendix 4.5 Changes in cell viability of freeze dried microencapsulated beads containing *L. casei* cells, during storage (Chapter 4.0)

At 4.C	**Day 0**		**Day 7**		**Day 14**		**Day 21**		**Day 28**	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	4.68 x 10^9	9.67	2.69 x 10^8	8.43	1.86 x 10^9	9.27	2.04 x 10^9	9.31	5.13 x 10^8	8.71
Expt 1.2	1.70 x 10^9	9.23	1.15 x 10^8	8.06	2.29 x 10^9	9.36	4.79 x 10^9	9.68	2.29 x 10^8	8.36
Expt 2.1	3.23 x 10^9	9.51	1.35 x 10^8	8.13	6.92 x 10^8	8.84	3.39 x 10^9	9.53	4.37 x 10^7	7.64
Expt 2.2	1.35 x 10^9	9.13	6.46 x 10^7	7.81	2.34 x 10^8	8.37	3.24 x 10^9	9.51	2.40 x 10^7	7.38
Expt 3.1	5.25 x 10^9	9.72	2.34 x 10^8	8.37	1.17 x 10^9	9.07	6.76 x 10^9	9.83	3.55 x 10^8	8.55
Expt 3.2	3.39 x 10^9	9.53	4.68 x 10^7	7.67	3.39 x 10^9	9.53	2.34 x 10^9	9.37	3.02 x 10^7	7.48
Average		9.465		8.078		9.073		9.538		8.020
Std. Dev		0.237		0.300		0.419		0.193		0.586

At -20.C	**Day 0**		**Day 7**		**Day 14**		**Day 21**		**Day 28**	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	4.68 x 10^9	9.67	2.57 x 10^9	9.41	1.86 x 10^9	9.27	2.45 x 10^9	9.39	6.17 x 10^8	8.79
Expt 1.2	1.70 x 10^9	9.23	5.37 x 10^9	9.73	1.35 x 10^9	9.13	8.51 x 10^8	8.93	2.04 x 10^8	8.31
Expt 2.1	3.23 x 10^9	9.51	1.86 x 10^9	9.27	5.75 x 10^8	8.76	4.37 x 10^8	8.64	1.07 x 10^8	8.03
Expt 2.2	1.35 x 10^9	9.13	5.62 x 10^9	9.75	3.16 x 10^9	9.5	2.14 x 10^9	9.33	1.29x 10^9	9.11
Expt 3.1	5.25 x 10^9	9.72	4.90 x 10^9	9.69	9.33 x 10^8	8.97	3.72 x 10^8	8.57	1.86 x 10^8	8.27
Expt 3.2	3.39 x 10^9	9.53	6.46 x 10^9	9.81	3.98 x 10^9	9.6	3.72 x 10^9	9.57	9.12 x 10^7	7.96
Average		9.465		9.610		9.205		9.072		8.412
Std. Dev		0.237		0.217		0.318		0.418		0.450

At 37.C	**Day 0**		**Day 7**		**Day 14**		**Day 21**		**Day 28**	
	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm	CFU/Gm	Log CFU/Gm
Expt 1.1	4.68 x 10^9	9.67	2.69 x 10^5	5.43	1.95 x 10^5	5.29	4.79 x 10^4	4.68	6.17 x 10^8	3.27
Expt 1.2	1.70 x 10^9	9.23	1.90 x 10^6	6.28	5.25 x 10^5	5.72	1.15 x 10^5	5.06	2.04 x 10^8	3.55
Expt 2.1	3.23 x 10^9	9.51	8.13 x 10^5	5.91	4.57 x 10^5	5.66	1.29 x 10^5	5.11	1.07 x 10^8	3.07
Expt 2.2	1.35 x 10^9	9.13	1.86 x 10^5	5.27	6.60 x 10^4	4.82	2.34 x 10^4	4.37	1.29x 10^9	3.39
Expt 3.1	5.25 x 10^9	9.72	3.09 x 10^6	6.49	1.74 x 10^5	5.24	1.86 x 10^4	4.27	1.86 x 10^8	3.64
Expt 3.2	3.39 x 10^9	9.53	1.17 x 10^5	5.07	8.51 x 10^4	4.93	1.58 x 10^4	4.2	9.12 x 10^7	3.17
Average		9.465		5.742		5.277		4.615		3.348
Std. Dev		0.237		0.574		0.367		0.400		0.220

Appendix 5.1: Comparative particle size distribution data for both wet and dried uncoated, TMB & LMB samples

Size µm	Uncoated Wet Volume in %	Uncoated Dry Volume in %	TMB Wet Volume in %	TMB Dry Volume in %	LMB Wet Volume in %	LMB Dry Volume in %
1.096	0.00	0.00	0.00	0.00	0.00	0.00
1.259	0.00	0.00	0.00	0.00	0.00	0.00
1.445	0.00	0.00	0.00	0.00	0.00	0.00
1.660	0.00	0.00	0.00	0.00	0.00	0.00
1.905	0.00	0.00	0.00	0.00	0.00	0.00
2.188	0.00	0.00	0.00	0.00	0.00	0.00
2.512	0.00	0.00	0.00	0.00	0.00	0.00
2.884	0.00	0.00	0.00	0.00	0.00	0.00
3.311	0.00	0.00	0.00	0.00	0.00	0.00
3.802	0.00	0.00	0.00	0.00	0.00	0.00
4.365	0.00	0.00	0.00	0.00	0.00	0.00
5.012	0.00	0.00	0.00	0.00	0.00	0.00
5.754	0.00	0.00	0.00	0.00	0.00	0.00
6.607	0.00	0.00	0.00	0.00	0.00	0.00
7.586	0.00	0.00	0.00	0.00	0.00	0.00
8.71	0.00	0.00	0.00	0.00	0.00	0.00
10.000	0.00	0.00	0.00	0.00	0.00	0.00
11.482	0.00	0.00	0.00	0.00	0.00	0.00
13.183	0.00	0.00	0.00	0.00	0.00	0.00
15.136	0.00	0.00	0.00	0.00	0.00	0.00
17.378	0.00	0.00	0.00	0.00	0.00	0.00
19.953	0.00	0.00	0.00	0.00	0.00	0.00
22.909	0.00	0.00	0.00	0.00	0.00	0.00
26.303	0.00	0.00	0.00	0.00	0.00	0.00
30.200	0.00	0.00	0.00	0.00	0.00	0.02
34.674	0.01	0.00	0.00	0.00	0.00	0.29
39.811	0.08	0.00	0.00	0.00	0.00	0.38
45.709	0.14	0.24	0.00	0.00	0.00	0.45
52.481	0.23	0.94	0.00	0.00	0.00	0.53
60.256	0.37	1.87	0.00	0.00	0.00	0.74
69.183	0.57	3.11	0.00	0.00	0.00	1.21
79.433	0.83	4.62	0.00	0.10	0.00	2.03
91.201	1.17	6.23	0.00	0.66	0.01	3.23
104.713	1.61	7.82	0.02	1.76	0.22	4.77
120.226	2.17	9.14	0.14	3.49	1.12	6.46
138.038	2.88	10.05	0.64	5.69	2.3	8.15
158.489	3.75	10.4	1.52	8.16	3.93	9.54
181.970	4.81	10.14	2.85	10.42	5.86	10.48
208.93	5.98	9.31	4.61	12.10	7.84	10.81
239.883	7.21	8.02	6.60	12.78	9.65	10.45
275.423	8.36	6.48	8.62	12.34	10.96	9.43
316.228	9.27	4.85	10.33	10.89	11.58	7.87
363.078	9.77	3.34	11.48	8.71	11.39	6.03
416.869	9.72	2.03	11.84	6.28	10.44	4.07
478.630	9.07	1.07	11.34	3.93	8.87	2.5
549.541	7.86	0.22	10.04	2.09	6.92	0.57
630.957	6.23	0.00	8.14	0.56	4.82	0.00
724.436	4.37	0.00	5.94	0.05	2.86	0.00
831.764	2.58	0.00	3.75	0.00	1.1	0.00
954.993	0.98	0.00	1.80	0.00	0.14	0.00
1096.478	0.00	0.00	0.35	0.00	0.00	0.00
1258.925	0.00	0.00	0.00	0.00	0.00	0.00
1445.440	0.00	0.00	0.00	0.00	0.00	0.00
1659.587	0.00	0.00	0.00	0.00	0.00	0.00
1905.461	0.00	0.00	0.00	0.00	0.00	0.00
2187.762	0.00	0.00	0.00	0.00	0.00	0.00
2511.886	0.00	0.00	0.00	0.00	0.00	0.00
2884.032	0.00	0.00	0.00	0.00	0.00	0.00
3311.311	0.00	0.00	0.00	0.00	0.00	0.00
3801.894	0.00	0.00	0.00	0.00	0.00	0.00
4365.158	0.00	0.00	0.00	0.00	0.00	0.00
5011.872	0.00	0.00	0.00	0.00	0.00	0.00
5754.399	0.00	0.00	0.00	0.00	0.00	0.00
6606.934	0.00	0.00	0.00	0.00	0.00	0.00
7585.776	0.00	0.00	0.00	0.00	0.00	0.00
8709.636	0.00	0.00	0.00	0.00	0.00	0.00
10000.000	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 5.2: Loss of viability due to freeze drying (Chapter 5.0 experimental data)

	Control sample (Wet)			Control sample (Dry)	
	CFU/Gm	Log CFU/Gm		CFU/Gm	Log CFU/Gm
Expt 1.1	5.89 x 10^9	9.77		1.78 x 10^8	8.25
Expt 1.2	1.74 x 10^10	10.24		1.51 x 10^9	9.18
Expt 2.1	1.48 x 10^12	12.17		2.04 x 10^10	10.31
Expt 2.2	1.15 x 10^11	11.06		4.37 x 10^9	9.64
Expt 3.1	1.95 x 10^11	11.29		2.95 x 10^9	9.47
Expt 3.2	8.32 x 10^11	11.92		1.62 x 10^9	9.21
Average		11.08			9.34
Std. Dev		0.934			0.675

	Sample TB (Wet)			Sample TB (Dry)	
	CFU/Gm	Log CFU/Gm		CFU/Gm	Log CFU/Gm
Expt 1.1	1.06 x 10^11	11.02		1.55 x 10^10	10.19
Expt 1.2	7.76 x 10^10	10.89		1.91 x 10^10	10.28
Expt 2.1	1.20 x 10^11	11.08		1.17 x 10^10	10.07
Expt 2.2	1.91 x 10^11	11.28		1.70 x 10^10	10.23
Expt 3.1	9.12 x 10^10	10.96		1.38 x 10^10	10.14
Expt 3.2	9.13 x 10^10	10.96		1.62 x 10^10	10.21
Average		11.03			10.19
Std. Dev		0.137			0.073

	Sample LB (Wet)			Sample LB (Dry)	
	CFU/Gm	Log CFU/Gm		CFU/Gm	Log CFU/Gm
Expt 1.1	9.77 x 10^10	10.99		4.17 x 10^10	10.62
Expt 1.2	8.13 x 10^10	10.91		3.63 x 10^10	10.56
Expt 2.1	7.41 x 10^10	10.87		3.09 x 10^10	10.49
Expt 2.2	9.12 x 10^10	10.96		4.68 x 10^10	10.67
Expt 3.1	8.32 x 10^10	10.92		2.34 x 10^10	10.37
Expt 3.2	6.76 x 10^10	10.83		3.39 x 10^10	10.53
Average		10.91			10.54
Std. Dev		0.058			0.105

	Sample TMB (Wet)			Sample TMB (Dry)	
	CFU/Gm	Log CFU/Gm		CFU/Gm	Log CFU/Gm
Expt 1.1	9.59 x 10^10	10.98		3.72 x 10^10	10.57
Expt 1.2	1.15 x 10^11	11.06		5.25 x 10^10	10.72
Expt 2.1	6.76 x 10^10	10.83		2.04 x 10^10	10.31
Expt 2.2	8.51 x 10^10	10.93		2.75 x 10^10	10.44
Expt 3.1	1.26 x 10^11	11.1		2.04 x 10^10	10.31
Expt 3.2	1.05 x 10^11	11.02		2.57 x 10^10	10.41
Average		10.99			10.46
Std. Dev		0.097			0.160

	Sample LMB (Wet)			Sample LMB (Dry)	
	CFU/Gm	Log CFU/Gm		CFU/Gm	Log CFU/Gm
Expt 1.1	1.62 x 10^10	10.21		1.29 x 10^10	10.11
Expt 1.2	1.20 x 10^11	11.08		1.02 x 10^11	11
Expt 2.1	2.45 x 10^10	10.39		1.86 x 10^10	10.27
Expt 2.2	2.19 x 10^10	10.34		1.55 x 10^10	10.19
Expt 3.1	3.80 x 10^10	10.58		2.24 x 10^10	10.35
Expt 3.2	1.79 x 10^10	10.25		1.51 x 10^10	10.18
Average		10.48			10.35
Std. Dev		0.324			0.329

Appendix 5.3: Survival of *Lactobacillus casei* , in free form and microencapsulated in in wet and dried **TB** samples form when treated in simulated gastric fluid.

	0 Min		30 Min		60 Min		90 Min		120 Min	
Free Cells	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.38 x 10^11	11.14	3.39 x 10^10	10.53	1.15 x 10^8	8.06	2.34 x 10^6	6.37	1.95 x 10^6	6.29
Expt 1.2	6.76 x 10^10	10.83	3.89 x 10^10	10.59	1.95 x 10^8	8.29	8.32 x 10^6	6.92	7.24 x 10^5	5.86
Expt 2.1	1.17 x 10^11	11.07	2.63 x 10^10	10.42	8.13 x 10^7	7.91	1.07 x 10^7	7.03	8.13 x 10^5	5.91
Expt 2.2	1.74 x 10^11	11.24	1.70 x 10^10	10.23	6.17 x 10^7	7.79	1.41 x 10^7	7.15	4.27 x 10^5	5.63
Expt 3.1	6.61 x 10^10	10.82	9.55 x 10^9	9.98	1.17 x 10^8	8.07	4.07 x 10^6	6.61	1.86 x 10^6	6.27
Expt 3.2	1.20 x 10^11	11.08	7.41 x 10^9	9.87	4.17 x 10^7	7.62	6.92 x 10^6	6.84	3.89 x 10^6	6.59
Average		11.03		10.27		7.96		6.82		6.09
Std. Dev		0.170		0.296		0.236		0.286		0.352

	0 Min		30 Min		60 Min		90 Min		120 Min	
Wet Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.74 x 10^11	11.24	6.46 x 10^10	10.81	3.24 x 10^9	9.51	7.24 x 10^8	8.86	1.95 x 10^8	8.29
Expt 1.2	1.44 x 10^11	11.16	4.90 x 10^10	10.69	2.40 x 10^9	9.38	2.95 x 10^8	8.47	1.35 x 10^8	8.13
Expt 2.1	9.55 x 10^10	10.98	2.24 x 10^10	10.35	1.48 x 10^9	9.17	2.04 x 10^8	8.31	7.08 x 10^7	7.85
Expt 2.2	6.76 x 10^10	10.83	4.17 x 10^10	10.62	1.66 x 10^9	9.22	3.89 x 10^8	8.59	1.17 x 10^8	8.07
Expt 3.1	5.50 x 10^10	10.74	1.38 x 10^10	10.14	1.62 x 10^9	9.21	2.09 x 10^8	8.32	1.20 x 10^8	8.08
Expt 3.2	6.17 x 10^10	10.79	1.05 x 10^10	10.02	1.35 x 10^9	9.13	4.07 x 10^8	8.61	5.50 x 10^7	7.74
Average		10.96		10.44		9.27		8.53		8.03
Std. Dev		0.206		0.318		0.145		0.207		0.199

	0 Min		30 Min		60 Min		90 Min		120 Min	
Dried Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	2.24 x 10^10	10.35	1.48 x 10^10	10.17	2.34 x 10^9	9.37	6.76 x 10^8	8.83	4.37 x 10^8	8.64
Expt 1.2	9.55 x 10^9	9.98	6.76 x 10^9	9.83	5.25 x 10^9	9.72	8.71 x 10^8	8.94	2.69 x 10^8	8.43
Expt 2.1	1.86 x 10^10	10.27	6.17 x 10^9	9.79	3.24 x 10^9	9.51	2.04 x 10^8	8.31	1.86 x 10^8	8.27
Expt 2.2	2.57 x 10^10	10.41	8.51 x 10^9	9.93	1.29 x 10^9	9.11	1.66 x 10^8	8.22	1.62 x 10^8	8.21
Expt 3.1	8.13 x 10^9	9.91	2.95 x 10^10	10.47	2.14 x 10^9	9.33	2.45 x 10^8	8.39	1.17 x 10^8	8.07
Expt 3.2	1.55 x 10^10	10.19	3.39 x 10^10	10.53	1.70 x 10^9	9.23	5.25 x 10^8	8.72	5.50 x 10^8	8.74
Average		10.19		10.12		9.38		8.57		8.39
Std. Dev		0.201		0.323		0.215		0.300		0.259

Appendix 5.4: Survival of *Lactobacillus casei* , in free form and microencapsulated in in wet and dried **TMB** samples form when treated in simulated gastric fluid.

	0 Min		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
Free Cells	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.38 x 10^11	11.14	3.39 x 10^10	10.53	1.15 x 10^8	8.06	2.34 x 10^6	6.37	1.95 x 10^6	6.29
Expt 1.2	6.76 x 10^10	10.83	3.89 x 10^10	10.59	1.95 x 10^8	8.29	8.32 x 10^6	6.92	7.24 x 10^5	5.86
Expt 2.1	1.17 x 10^11	11.07	2.63 x 10^10	10.42	8.13 x 10^7	7.91	1.07 x 10^7	7.03	8.13 x 10^5	5.91
Expt 2.2	1.74 x 10^11	11.24	1.70 x 10^10	10.23	6.17 x 10^7	7.79	1.41 x 10^7	7.15	4.27 x 10^5	5.63
Expt 3.1	6.61 x 10^10	10.82	9.55 x 10^9	9.98	1.17 x 10^8	8.07	4.07 x 10^6	6.61	1.86 x 10^6	6.27
Expt 3.2	1.20 x 10^11	11.08	7.41 x 10^9	9.87	4.17 x 10^7	7.62	6.92 x 10^6	6.84	3.89 x 10^6	6.59
Average		11.03		10.27		7.96		6.82		6.09
Std. Dev		0.170		0.296		0.236		0.286		0.352

	0 Min		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
Wet Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	6.61 x 10^10	10.82	2.63 x 10^10	10.42	1.07 x 10^9	9.03	3.71 x 10^8	8.57	1.95 x 10^8	8.29
Expt 1.2	2.45 x 10^10	10.39	2.45 x 10^10	10.39	7.41 x 10^8	8.87	2.04 x 10^8	8.31	1.05 x 10^8	8.02
Expt 2.1	9.55 x 10^10	10.98	1.17 x 10^10	10.07	1.82 x 10^9	9.26	1.74 x 10^8	8.24	1.86 x 10^8	8.27
Expt 2.2	5.62 x 10^10	10.75	1.32 x 10^10	10.12	2.34 x 10^9	9.37	1.38 x 10^8	8.14	2.04 x 10^8	8.31
Expt 3.1	1.32 x 10^11	11.12	1.86 x 10^10	10.27	1.29 x 10^9	9.11	5.25 x 10^8	8.72	7.41 x 10^7	7.87
Expt 3.2	1.51 x 10^11	11.18	6.76 x 10^10	10.83	2.57 x 10^10	9.41	3.98 x 10^8	8.6	2.95 x 10^7	7.47
Average		10.87		10.35		9.18		8.43		8.04
Std. Dev		0.289		0.274		0.209		0.231		0.329

	0 Min		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
Dried Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	2.57 x 10^10	10.41	1.35 x 10^10	10.13	3.02 x 10^9	9.48	4.27 x 10^8	8.63	4.57 x 10^8	8.66
Expt 1.2	1.95 x 10^10	10.29	6.92 x 10^9	9.84	1.86 x 10^9	9.27	2.34 x 10^8	8.37	2.00 x 10^8	8.3
Expt 2.1	1.32 x 10^10	10.12	5.75 x 10^9	9.76	1.44 x 10^9	9.16	7.59 x 10^8	8.88	4.17 x 10^8	8.62
Expt 2.2	8.32 x 10^9	9.92	1.20 x 10^10	10.08	1.62 x 10^9	9.21	3.09 x 10^8	8.49	6.03x 10^8	8.78
Expt 3.1	1.74 x 10^10	10.24	4.07 x 10^9	9.61	1.02 x 10^9	9.01	6.76 x 10^8	8.83	3.55 x 10^8	8.55
Expt 3.2	1.17 x 10^10	10.07	2.69 x 10^9	9.43	1.70 x 10^9	9.23	6.17 x 10^8	8.79	5.62 x 10^8	8.75
Average		10.175		9.81		9.23		8.67		8.61
Std. Dev		0.174		0.269		0.153		0.204		0.174

Appendix 5.5: Survival of *Lactobacillus casei* , in free form and microencapsulated in in wet and dried **LB** samples form when treated in simulated gastric fluid.

	0 Min		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
Free Cells	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.38 x 10^11	11.14	3.39 x 10^10	10.53	1.15 x 10^8	8.06	2.34 x 10^6	6.37	1.95 x 10^6	6.29
Expt 1.2	6.76 x 10^10	10.83	3.89 x 10^10	10.59	1.95 x 10^8	8.29	8.32 x 10^6	6.92	7.24 x 10^5	5.86
Expt 2.1	1.17 x 10^11	11.07	2.63 x 10^10	10.42	8.13 x 10^7	7.91	1.07 x 10^7	7.03	8.13 x 10^5	5.91
Expt 2.2	1.74 x 10^11	11.24	1.70 x 10^10	10.23	6.17 x 10^7	7.79	1.41 x 10^7	7.15	4.27 x 10^5	5.63
Expt 3.1	6.61 x 10^10	10.82	9.55 x 10^9	9.98	1.17 x 10^8	8.07	4.07 x 10^6	6.61	1.86 x 10^6	6.27
Expt 3.2	1.20 x 10^11	11.08	7.41 x 10^9	9.87	4.17 x 10^7	7.62	6.92 x 10^6	6.84	3.89 x 10^6	6.59
Average		11.03		10.27		7.96		6.82		6.09
Std. Dev		0.170		0.296		0.236		0.286		0.352

	0 Min		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
Wet Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	4.68 x 10^10	10.67	3.23 x 10^10	10.51	7.59 x 10^8	8.88	1.62 x 10^8	8.21	1.12 x 10^8	8.05
Expt 1.2	2.69 x 10^10	10.43	3.09 x 10^10	10.49	1.35 x 10^9	9.13	4.90 x 10^8	8.69	4.90 x 10^7	7.69
Expt 2.1	8.13 x 10^10	10.91	5.37 x 10^10	10.73	1.51 x 10^9	9.18	1.55 x 10^8	8.19	8.71 x 10^7	7.94
Expt 2.2	1.05 x 10^11	11.02	4.37 x 10^10	10.64	2.69 x 10^9	9.43	2.75 x 10^8	8.44	1.29 x 10^8	8.11
Expt 3.1	9.77 x 10^10	10.99	8.13 x 10^10	10.91	2.19 x 10^9	9.34	5.75 x 10^8	8.76	2.34 x 10^7	7.37
Expt 3.2	1.74 x 10^11	11.24	1.32 x 10^11	11.12	8.71 x 10^8	8.94	4.37 x 10^8	8.64	3.63 x 10^7	7.56
Average		10.88		10.73		9.15		8.49		7.79
Std. Dev		0.286		0.244		0.216		0.248		0.294

	0 Min		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
Dried Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	3.31 x 10^10	10.52	7.41 x 10^10	10.87	2.40 x 10^10	10.38	1.20 x 10^9	9.08	4.37 x 10^8	8.64
Expt 1.2	2.04 x 10^10	10.31	3.47 x 10^10	10.54	1.20 x 10^10	10.08	1.86 x 10^9	9.27	4.90 x 10^8	8.69
Expt 2.1	4.27 x 10^10	10.63	1.74 x 10^10	10.24	1.48 x 10^10	10.17	6.16 x 10^8	8.79	6.46 x 10^8	8.81
Expt 2.2	5.50 x 10^10	10.74	6.17 x 10^10	10.79	8.71 x 10^9	9.94	3.55 x 10^8	8.55	1.82 x 10^8	8.26
Expt 3.1	4.90 x 10^10	10.69	5.37 x 10^10	10.73	1.32 x 10^10	10.12	3.09 x 10^8	8.49	1.74 x 10^8	8.24
Expt 3.2	2.19 x 10^10	10.34	6.46 x 10^10	10.81	6.92 x 10^9	9.84	9.33 x 10^8	8.97	4.37 x 10^8	8.64
Average		10.54		10.66		10.09		8.86		8.55
Std. Dev		0.181		0.236		0.188		0.305		0.238

Appendix 5.6: Survival of *Lactobacillus casei* , in free form and microencapsulated in in wet and dried **LMB** samples form when treated in simulated gastric fluid.

	0 Min		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
Free Cells	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.38 x 10^11	11.14	3.39 x 10^10	10.53	1.15 x 10^8	8.06	2.34 x 10^6	6.37	1.95 x 10^6	6.29
Expt 1.2	6.76 x 10^10	10.83	3.89 x 10^10	10.59	1.95 x 10^8	8.29	8.32 x 10^6	6.92	7.24 x 10^5	5.86
Expt 2.1	1.17 x 10^11	11.07	2.63 x 10^10	10.42	8.13 x 10^7	7.91	1.07 x 10^7	7.03	8.13 x 10^5	5.91
Expt 2.2	1.74 x 10^11	11.24	1.70 x 10^10	10.23	6.17 x 10^7	7.79	1.41 x 10^7	7.15	4.27 x 10^5	5.63
Expt 3.1	6.61 x 10^10	10.82	9.55 x 10^9	9.98	1.17 x 10^8	8.07	4.07 x 10^6	6.61	1.86 x 10^6	6.27
Expt 3.2	1.20 x 10^11	11.08	7.41 x 10^9	9.87	4.17 x 10^7	7.62	6.92 x 10^6	6.84	3.89 x 10^6	6.59
Average		11.03		10.27		7.96		6.82		6.09
Std. Dev		0.170		0.296		0.236		0.286		0.352

	0 Min		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
Wet Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	8.13 x 10^10	10.91	6.76 x 10^10	10.83	1.91 x 10^10	10.28	3.09 x 10^9	9.49	5.37 x 10^8	8.73
Expt 1.2	1.32 x 10^11	11.12	8.71 x 10^10	10.94	1.45 x 10^10	10.16	1.74 x 10^9	9.24	2.45 x 10^8	8.39
Expt 2.1	4.07 x 10^10	10.61	2.04 x 10^10	10.31	2.88 x 10^9	9.46	8.71 x 10^8	8.94	3.72 x 10^8	8.57
Expt 2.2	7.41 x 10^10	10.87	4.68 x 10^10	10.67	1.51 x 10^9	9.18	5.25 x 10^8	8.72	2.88 x 10^8	8.46
Expt 3.1	9.12 x 10^10	10.96	4.17 x 10^10	10.62	1.10 x 10^9	9.04	1.07 x 10^9	9.03	4.57 x 10^8	8.66
Expt 3.2	1.17 x 10^11	11.07	2.88 x 10^10	10.46	1.17 x 10^9	9.07	9.55 x 10^8	8.98	1.70 x 10^8	8.23
Average		10.92		10.64		9.53		9.07		8.51
Std. Dev		0.180		0.232		0.555		0.266		0.184

	0 Min		**30 Min**		**60 Min**		**90 Min**		**120 Min**	
Dried Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	2.34 x 10^10	10.37	5.23 x 10^10	10.72	1.82 x 10^10	10.26	1.05 x 10^10	10.02	6.92 x 10^9	9.84
Expt 1.2	6.61 x 10^10	10.82	4.90 x 10^9	10.69	1.29 x 10^10	10.11	7.59 x 10^9	9.88	6.17 x 10^9	9.79
Expt 2.1	4.90 x 10^10	10.69	1.95 x 10^10	10.29	4.37 x 10^9	9.64	3.55 x 10^9	9.55	2.09 x 10^9	9.32
Expt 2.2	1.02 x 10^11	11.01	1.55 x 10^10	10.19	9.33 x 10^9	9.97	5.50 x 10^9	9.74	1.48 x 10^9	9.17
Expt 3.1	7.41 x 10^10	10.87	5.75 x 10^10	10.76	5.25 x 10^9	9.72	4.90 x 10^9	9.69	1.51 x 10^9	9.18
Expt 3.2	6.03 x 10^10	10.78	7.76 x 10^10	10.89	2.95 x 10^9	9.47	6.92 x 10^9	9.84	9.12 x 10^8	8.96
Average		10.76		10.59		9.86		9.79		9.38
Std. Dev		0.217		0.281		0.302		0.163		0.359

Appendix 5.7: Response of free, wet and dry encapsulated **(TB)** *L. casei* cells in presence of 1% porcine bile extract

Free Cells	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.70 x 10^11	11.23	2.82 x 10^10	10.45	4.17 x 10^9	9.62	9.12 x 10^8	8.96	1.17 x 10^9	9.07
Expt 1.2	5.37 x 10^10	10.73	4.07 x 10^10	10.61	2.14 x 10^9	9.33	5.01 x 10^8	8.7	4.17 x 10^8	8.62
Expt 2.1	6.17 x 10^10	10.79	5.25 x 10^10	10.72	6.92 x 10^9	9.84	1.62 x 10^9	9.21	1.20 x 10^9	9.08
Expt 2.2	1.48 x 10^11	11.17	7.24 x 10^10	10.86	1.17 x 10^10	10.07	2.95 x 10^9	9.47	7.76 x 10^8	8.89
Expt 3.1	1.95 x 10^11	11.29	2.57 x 10^10	10.41	8.13 x 10^9	9.91	7.24 x 10^8	8.86	6.46 x 10^8	8.81
Expt 3.2	9.12 x 10^10	10.96	2.04 x 10^10	10.31	3.47 x 10^9	9.54	2.45 x 10^8	8.39	4.68 x 10^8	8.67
Average		11.028		10.560		9.718		8.932		8.857
Std. Dev		0.237		0.207		0.271		0.380		0.195

Wet Capsules	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.44 x 10^11	11.16	8.13 x 10^10	10.91	1.74 x 10^10	10.24	1.32 x 10^10	10.12	1.44 x 10^10	10.16
Expt 1.2	1.23 x 10^11	11.09	1.32 x 10^11	11.12	3.09 x 10^10	10.49	1.86 x 10^10	10.27	4.36 x 10^9	9.64
Expt 2.1	8.71 x 10^10	10.94	7.59 x 10^10	10.88	2.14 x 10^10	10.33	7.76 x 10^9	9.89	1.35 x 10^10	10.13
Expt 2.2	1.17 x 10^11	11.07	8.71 x 10^10	10.94	6.46 x 10^10	10.81	1.23 x 10^10	10.09	8.71 x 10^9	9.94
Expt 3.1	7.76 x 10^10	10.89	1.05 x 10^11	11.02	1.62 x 10^10	10.21	2.57 x 10^10	10.41	1.17 x 10^10	10.07
Expt 3.2	4.07 x 10^10	10.61	5.25 x 10^10	10.72	1.38 x 10^10	10.14	3.02 x 10^10	10.48	1.79 x 10^10	10.25
Average		10.960		10.932		10.370		10.210		10.032
Std. Dev		0.198		0.135		0.247		0.220		0.218

Dried Capsules	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	8.32 x 10^9	9.92	1.82 x 10^10	10.26	6.61 x 10^9	9.82	1.38 x 10^9	9.14	2.04 x 10^8	8.31
Expt 1.2	2.34 x 10^10	10.37	1.55 x 10^10	10.19	4.79 x 10^9	9.68	1.05 x 10^9	9.02	3.89 x 10^8	8.59
Expt 2.1	2.04 x 10^10	10.31	1.10 x 10^10	10.04	2.75 x 10^9	9.44	5.37 x 10^8	8.73	5.13 x 10^8	8.71
Expt 2.2	1.74 x 10^10	10.24	1.29 x 10^10	10.11	4.37 x 10^9	9.64	7.76 x 10^8	8.89	2.19 x 10^8	8.34
Expt 3.1	1.10 x 10^10	10.04	9.33 x 10^9	9.97	4.90 x 10^9	9.69	1.10 x 10^9	9.04	2.45 x 10^8	8.39
Expt 3.2	1.90 x 10^10	10.28	6.76 x 10^9	9.83	2.95 x 10^9	9.47	5.13 x 10^8	8.71	6.76 x 10^8	8.83
Average		10.193		10.067		9.623		8.922		8.528
Std. Dev		0.175		0.155		0.144		0.175		0.215

Appendix 5.8: Survivability of free and membrane coated **(TMB)** *L. casei* cells in simulated bile environment

	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
Free Cells	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.70 x 10^11	11.23	2.82 x 10^10	10.45	4.17 x 10^9	9.62	9.12 x 10^8	8.96	1.17 x 10^9	9.07
Expt 1.2	5.37 x 10^10	10.73	4.07 x 10^10	10.61	2.14 x 10^9	9.33	5.01 x 10^8	8.7	4.17 x 10^8	8.62
Expt 2.1	6.17 x 10^10	10.79	5.25 x 10^10	10.72	6.92 x 10^9	9.84	1.62 x 10^9	9.21	1.20 x 10^9	9.08
Expt 2.2	1.48 x 10^11	11.17	7.24 x 10^10	10.86	1.17 x 10^10	10.07	2.95 x 10^9	9.47	7.76 x 10^8	8.89
Expt 3.1	1.95 x 10^11	11.29	2.57 x 10^10	10.41	8.13 x 10^9	9.91	7.24 x 10^8	8.86	6.46 x 10^8	8.81
Expt 3.2	9.12 x 10^10	10.96	2.04 x 10^10	10.31	3.47 x 10^9	9.54	2.45 x 10^8	8.39	4.68 x 10^8	8.67
Average		11.028		10.560		9.718		8.932		8.857
Std. Dev		0.237		0.207		0.271		0.380		0.195

	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
Wet Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	6.17 x 10^10	10.79	7.41 x 10^10	10.87	3.55 x 10^10	10.55	5.50 x 10^10	10.74	2.45 x 10^10	10.39
Expt 1.2	1.62 x 10^11	11.21	9.12 x 10^10	10.96	6.46 x 10^10	10.81	4.79 x 10^10	10.68	1.48 x 10^10	10.17
Expt 2.1	3.72 x 10^10	10.57	4.68 x 10^10	10.67	7.24 x 10^9	9.86	9.33 x 10^9	9.97	3.55 x 10^10	10.55
Expt 2.2	1.38 x 10^11	11.14	1.95 x 10^11	11.29	4.07 x 10^10	10.61	2.19 x 10^10	10.34	1.48 x 10^10	10.17
Expt 3.1	8.91 x 10^10	10.95	1.74 x 10^11	11.24	2.75 x 10^10	10.44	5.89 x 10^10	10.77	6.92 x 10^9	9.84
Expt 3.2	3.71 x 10^10	10.57	4.17 x 10^10	10.62	3.24 x 10^10	10.51	6.16 x 10^10	10.79	1.38 x 10^10	10.14
Average		10.872		10.942		10.463		10.548		10.210
Std. Dev		0.276		0.280		0.321		0.328		0.242

	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
Dried Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.90 x 10^10	10.28	1.62 x 10^10	10.21	6.61 x 10^9	9.82	5.37 x 10^8	8.73	3.09 x 10^8	8.49
Expt 1.2	3.09 x 10^10	10.49	4.37 x 10^10	10.64	3.39 x 10^9	9.53	3.72 x 10^8	8.57	5.37 x 10^8	8.73
Expt 2.1	6.46 x 10^10	10.81	2.75 x 10^10	10.44	1.62 x 10^10	10.21	5.50 x 10^9	9.74	1.66 x 10^9	9.22
Expt 2.2	2.19 x 10^10	10.34	1.70 x 10^10	10.23	9.12 x 10^9	9.96	2.34 x 10^9	9.37	1.10 x 10^9	9.04
Expt 3.1	7.24 x 10^9	9.86	4.68 x 10^9	9.67	3.72 x 10^9	9.57	7.41 x 10^8	8.87	2.45 x 10^8	8.39
Expt 3.2	1.95 x 10^9	9.29	1.29 x 10^9	9.11	8.81 x 10^8	8.94	1.48 x 10^9	9.17	5.50 x 10^8	8.74
Average		10.178		10.050		9.672		9.075		8.768
Std. Dev		0.533		0.563		0.438		0.437		0.316

Appendix 5.9: Response of free, wet and dry encapsulated **(LB)** *L. casei* cells in presence of 1% porcine bile extract

	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
Free Cells	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.70 x 10^11	11.23	2.82 x 10^10	10.45	4.17 x 10^9	9.62	9.12 x 10^8	8.96	1.17 x 10^9	9.07
Expt 1.2	5.37 x 10^10	10.73	4.07 x 10^10	10.61	2.14 x 10^9	9.33	5.01 x 10^8	8.7	4.17 x 10^8	8.62
Expt 2.1	6.17 x 10^10	10.79	5.25 x 10^10	10.72	6.92 x 10^9	9.84	1.62 x 10^9	9.21	1.20 x 10^9	9.08
Expt 2.2	1.48 x 10^11	11.17	7.24 x 10^10	10.86	1.17 x 10^10	10.07	2.95 x 10^9	9.47	7.76 x 10^8	8.89
Expt 3.1	1.95 x 10^11	11.29	2.57 x 10^10	10.41	8.13 x 10^9	9.91	7.24 x 10^8	8.86	6.46 x 10^8	8.81
Expt 3.2	9.12 x 10^10	10.96	2.04 x 10^10	10.31	3.47 x 10^9	9.54	2.45 x 10^8	8.39	4.68 x 10^8	8.67
Average		11.028		10.560		9.718		8.932		8.857
Std. Dev		0.237		0.207		0.271		0.380		0.195

	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
Wet Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	3.47 x 10^10	10.54	1.10 x 10^11	11.04	4.37 x 10^10	10.64	1.78 x 10^10	10.25	1.44 x 10^10	10.16
Expt 1.2	2.24 x 10^10	10.35	4.57 x 10^10	10.66	5.25 x 10^10	10.72	1.86 x 10^10	10.27	9.55 x 10^9	9.98
Expt 2.1	6.92 x 10^10	10.84	3.09 x 10^10	10.49	5.75 x 10^10	10.76	1.29 x 10^10	10.11	1.20 x 10^10	10.08
Expt 2.2	8.13 x 10^10	10.91	8.51 x 10^10	10.93	6.76 x 10^10	10.83	2.34 x 10^10	10.37	1.38 x 10^10	10.14
Expt 3.1	2.51 x 10^11	11.4	9.55 x 10^10	10.98	2.75 x 10^10	10.44	1.05 x 10^10	10.02	7.76 x 10^9	9.89
Expt 3.2	1.74 x 10^11	11.24	7.76 x 10^10	10.89	6.31 x 10^10	10.8	7.41 x 10^9	9.87	1.29 x 10^10	10.11
Average		10.880		10.832		10.698		10.148		10.060
Std. Dev		0.400		0.212		0.143		0.184		0.104

	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
Dried Capsules	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.70 x 10^10	10.23	1.86 x 10^10	10.27	4.68 x 10^9	9.67	1.44 x 10^9	9.16	1.55 x 10^9	9.19
Expt 1.2	4.37 x 10^10	10.64	2.34 x 10^10	10.37	3.55 x 10^9	9.55	1.86 x 10^9	9.27	1.62 x 10^9	9.21
Expt 2.1	3.39 x 10^10	10.53	2.24 x 10^10	10.35	2.57 x 10^9	9.41	6.92 x 10^8	8.84	1.17 x 10^9	9.07
Expt 2.2	4.68 x 10^10	10.67	4.79 x 10^10	10.68	1.82 x 10^9	9.26	1.10 x 10^9	9.04	1.86 x 10^9	9.27
Expt 3.1	2.75 x 10^10	10.44	1.86 x 10^10	10.27	4.68 x 10^9	9.67	2.24 x 10^9	9.35	1.32 x 10^9	9.12
Expt 3.2	5.13 x 10^10	10.71	3.72 x 10^10	10.57	2.34 x 10^9	9.37	1.35 x 10^9	9.13	2.34 x 10^9	9.37
Average		10.537		10.418		9.488		9.132		9.205
Std. Dev		0.180		0.169		0.169		0.179		0.107

Appendix 5.10: Survivability of free and membrane coated **(LMB)** *L. casei* cells in simulated bile environment

Free Cells	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.70 x 10^11	11.23	2.82 x 10^10	10.45	4.17 x 10^9	9.62	9.12 x 10^8	8.96	1.17 x 10^9	9.07
Expt 1.2	5.37 x 10^10	10.73	4.07 x 10^10	10.61	2.14 x 10^9	9.33	5.01 x 10^8	8.7	4.17 x 10^8	8.62
Expt 2.1	6.17 x 10^10	10.79	5.25 x 10^10	10.72	6.92 x 10^9	9.84	1.62 x 10^9	9.21	1.20 x 10^9	9.08
Expt 2.2	1.48 x 10^11	11.17	7.24 x 10^10	10.86	1.17 x 10^10	10.07	2.95 x 10^9	9.47	7.76 x 10^8	8.89
Expt 3.1	1.95 x 10^11	11.29	2.57 x 10^10	10.41	8.13 x 10^9	9.91	7.24 x 10^8	8.86	6.46 x 10^8	8.81
Expt 3.2	9.12 x 10^10	10.96	2.04 x 10^10	10.31	3.47 x 10^9	9.54	2.45 x 10^8	8.39	4.68 x 10^8	8.67
Average		11.028		10.560		9.718		8.932		8.857
Std. Dev		0.237		0.207		0.271		0.380		0.195

Wet Capsules	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	7.41 x 10^10	10.87	6.46 x 10^10	10.81	5.89 x 10^10	10.77	2.63 x 10^10	10.42	4.68 x 10^10	10.67
Expt 1.2	8.51 x 10^10	10.93	4.57 x 10^10	10.66	2.57 x 10^10	10.41	2.19 x 10^10	10.34	2.69 x 10^10	10.43
Expt 2.1	1.51 x 10^11	11.18	9.33 x 10^10	10.97	7.76 x 10^10	10.89	1.86 x 10^9	10.27	1.86 x 10^10	10.27
Expt 2.2	6.17 x 10^10	10.79	2.04 x 10^10	10.31	2.75 x 10^10	10.44	2.14 x 10^10	10.33	5.25 x 10^10	10.72
Expt 3.1	1.15 x 10^11	11.06	8.13 x 10^10	10.91	7.75 x 10^10	10.89	3.24 x 10^10	10.51	1.62 x 10^10	10.21
Expt 3.2	4.68 x 10^10	10.67	7.59 x 10^10	10.88	7.24 x 10^10	10.86	1.29 x 10^10	10.11	1.55 x 10^10	10.19
Average		10.917		10.757		10.710		10.330		10.415
Std. Dev		0.184		0.243		0.225		0.136		0.233

Dried Capsules	0 Hr		2 Hr		4 Hr		6 Hr		8 Hr	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	4.17 x 10^10	10.62	2.34 x 10^10	10.37	1.51 x 10^10	10.18	4.37 x 10^9	9.64	5.50 x 10^9	9.74
Expt 1.2	8.13 x 10^10	10.91	5.50 x 10^10	10.74	5.37 x 10^9	9.73	3.63 x 10^9	9.56	3.80 x 10^9	9.58
Expt 2.1	1.74 x 10^11	11.24	5.75 x 10^10	10.76	2.04 x 10^10	10.31	7.41 x 10^9	9.87	5.49 x 10^9	9.74
Expt 2.2	9.12 x 10^10	10.96	3.80 x 10^10	10.58	5.62 x 10^9	9.75	2.45 x 10^9	9.39	7.76 x 10^9	9.89
Expt 3.1	3.09 x 10^10	10.49	6.46 x 10^10	10.81	1.82 x 10^10	10.26	1.91 x 10^9	9.28	4.79 x 10^9	9.68
Expt 3.2	2.14 x 10^10	10.33	4.68 x 10^10	10.67	3.47 x 10^10	10.54	2.19 x 10^9	9.34	3.09 x 10^9	9.49
Average		10.758		10.655		10.128		9.513		9.687
Std. Dev		0.338		0.161		0.324		0.221		0.139

Appendix 5.11: Storage stability of **control** sample (freeze dried) with no cryoprotectant or membrane coating (Chapter 5.0)

At 4.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	4.17 x 10^9	9.62	1.86 x 10^9	9.27	1.74 x 10^8	8.24	1.07 x 10^8	8.03	1.55 x 10^8	8.19
Expt 1.2	2.69 x 10^9	9.43	2.75 x 10^9	9.44	5.13 x 10^8	8.71	7.59 x 10^7	7.88	8.51 x 10^7	7.93
Expt 2.1	1.48 x 10^9	9.17	1.48 x 10^9	9.17	3.09 x 10^8	8.49	8.51 x 10^7	7.93	2.88 x 10^7	7.46
Expt 2.2	4.57 x 10^9	9.66	1.17 x 10^9	9.07	1.48 x 10^8	8.17	1.86 x 10^8	8.27	1.35 x 10^7	7.13
Expt 3.1	1.86 x 10^9	9.27	1.51 x 10^9	9.18	1.66 x 10^8	8.22	2.34 x 10^8	8.37	1.35 x 10^8	8.13
Expt 3.2	4.79 x 10^9	9.68	1.29 x 10^9	9.11	4.90 x 10^8	8.69	2.63 x 10^7	7.42	2.34 x 10^8	8.37
Average		9.472		9.207		8.420		7.983		7.868
Std. Dev		0.216		0.133		0.244		0.336		0.477

At 37.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	4.17 x 10^9	9.62	2.34 x 10^8	8.37	1.07 x 10^6	6.03	1.17 x 10^5	5.07	7.41 x 10^4	4.87
Expt 1.2	2.69 x 10^9	9.43	1.17 x 10^8	8.07	2.19 x 10^6	6.34	3.55 x 10^4	4.55	2.14 x 10^4	4.33
Expt 2.1	1.48 x 10^9	9.17	4.9 x 10^8	8.69	6.46 x 10^6	6.81	1.95 x 10^4	4.29	3.24 x 10^4	4.51
Expt 2.2	4.57 x 10^9	9.66	1.29 x 10^8	8.11	1.66 x 10^6	6.22	1.23 x 10^5	5.09	7.59 x 10^4	4.88
Expt 3.1	1.86 x 10^9	9.27	7.08 x 10^7	7.85	1.48 x 10^6	6.17	3.55 x 10^4	4.55	8.13 x 10^4	4.91
Expt 3.2	4.79 x 10^9	9.68	2.45 x 10^8	8.39	1.23 x 10^6	6.09	1.48 x 10^4	4.17	4.07 x 10^7	4.61
Average		9.472		8.247		6.277		4.620		4.685
Std. Dev		0.216		0.296		0.282		0.386		0.239

Appendix 5.12: Storage stability of freeze dried **TB** samples prepared as per chapter 5.0

At 4.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.91 x 10^10	10.28	1.29 x 10^10	10.11	5.50 x 10^9	9.74	3.39 x 10^9	9.53	1.35 x 10^9	9.13
Expt 1.2	2.19 x 10^10	10.34	1.62 x 10^10	10.21	4.47 x 10^9	9.65	4.07 x 10^9	9.61	1.74 x 10^9	9.24
Expt 2.1	1.20 x 10^10	10.08	6.61 x 10^9	9.82	2.19 x 10^9	9.34	2.19 x 10^9	9.34	1.07 x 10^9	9.03
Expt 2.2	1.74 x 10^10	10.24	1.05 x 10^10	10.02	3.89 x 10^9	9.59	2.45 x 10^9	9.39	7.24 x 10^8	8.86
Expt 3.1	1.48 x 10^10	10.17	9.55 x 10^9	9.98	4.07 x 10^9	9.61	3.09 x 10^9	9.49	9.12 x 10^8	8.96
Expt 3.2	1.05 x 10^10	10.02	7.24 x 10^9	9.86	5.13 x 10^9	9.71	2.57 x 10^9	9.41	5.50 x 10^8	8.74
Average		10.188		10.000		9.607		9.462		8.993
Std. Dev		0.122		0.148		0.143		0.100		0.181

At 37.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	1.91 x 10^10	10.28	8.51 x 10^9	9.93	3.72 x 10^8	8.57	2.75 x 10^8	8.44	1.07 x 10^8	8.03
Expt 1.2	2.19 x 10^10	10.34	1.20 x 10^10	10.08	1.74 x 10^9	9.24	1.62 x 10^8	8.21	8.71 x 10^7	7.94
Expt 2.1	1.20 x 10^10	10.08	3.63 x 10^9	9.56	4.07 x 10^8	8.61	1.95 x 10^8	8.29	3.72 x 10^7	7.57
Expt 2.2	1.74 x 10^10	10.24	6.31 x 10^9	9.8	5.75 x 10^8	8.76	2.34 x 10^8	8.37	2.40 x 10^7	7.38
Expt 3.1	1.48 x 10^10	10.17	5.13 x 10^9	9.71	1.48 x 10^9	9.17	2.88 x 10^8	8.46	4.07 x 10^7	7.61
Expt 3.2	1.05 x 10^10	10.02	4.57 x 10^9	9.66	8.91 x 10^8	8.95	3.24 x 10^8	8.51	7.59 x 10^7	7.88
Average		10.188		9.790		8.883		8.380		7.735
Std. Dev		0.122		0.190		0.284		0.113		0.253

Appendix 5.13: Storage stability of freeze dried **TMB** samples prepared as per chapter 5.0

At 4.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	2.34 x 10^10	10.37	1.95 x 10^10	10.29	8.51 x 10^9	9.93	2.40 x 10^9	9.38	5.13 x 10^9	9.71
Expt 1.2	1.29 x 10^10	10.11	1.35 x 10^10	10.13	1.78 x 10^10	10.25	3.89 x 10^9	9.59	5.50 x 10^9	9.74
Expt 2.1	2.09 x 10^10	10.32	1.74 x 10^10	10.24	1.05 x 10^10	10.02	4.68 x 10^9	9.67	7.76 x 10^9	9.89
Expt 2.2	9.33 x 10^9	9.97	8.71 x 10^9	9.94	6.61 x 10^9	9.82	4.07 x 10^9	9.61	8.13 x 10^9	9.91
Expt 3.1	2.57 x 10^10	10.41	1.32 x 10^10	10.12	4.90 x 10^9	9.69	7.41 x 10^9	9.87	7.76 x 10^9	9.89
Expt 3.2	7.76 x 10^9	9.89	8.51 x 10^9	9.93	8.13 x 10^9	9.91	6.76 x 10^9	9.83	8.91 x 10^9	9.95
Average		10.178		10.108		9.937		9.658		9.848
Std. Dev		0.220		0.149		0.190		0.178		0.098

At 37.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	2.34 x 10^10	10.37	1.38 x 10^10	10.14	7.59 x 10^8	8.88	2.88 x 10^8	8.46	3.72 x 10^8	8.57
Expt 1.2	1.29 x 10^10	10.11	8.71 x 10^9	9.94	1.02 x 10^9	9.01	1.86 x 10^8	8.27	1.49 x 10^8	8.17
Expt 2.1	2.09 x 10^10	10.32	1.62 x 10^10	10.21	5.50 x 10^9	9.74	6.76 x 10^7	7.83	3.63 x 10^7	7.56
Expt 2.2	9.33 x 10^9	9.97	5.62 x 10^9	9.75	8.32 x 10^8	8.92	1.51 x 10^7	7.18	1.91 x 10^7	7.28
Expt 3.1	2.57 x 10^10	10.41	4.90 x 10^9	9.69	1.48 x 10^8	8.17	1.15 x 10^8	8.06	1.38 x 10^8	8.14
Expt 3.2	7.76 x 10^9	9.89	6.46 x 10^9	9.81	1.35 x 10^9	9.13	7.24 x 10^8	8.86	2.34 x 10^8	8.37
Average		10.178		9.923		8.975		8.110		8.015
Std. Dev		0.220		0.213		0.504		0.576		0.494

Appendix 5.14: Storage stability of freeze dried LB samples prepared as per chapter 5.0

At 4.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	4.68 x 10^10	10.67	2.82 x 10^10	10.45	2.57 x 10^10	10.41	1.38 x 10^10	10.14	1.02 x 10^10	10.01
Expt 1.2	3.24 x 10^10	10.51	1.55 x 10^10	10.19	2.40 x 10^10	10.38	7.76 x 10^9	9.89	3.72 x 10^9	9.57
Expt 2.1	3.09 x 10^10	10.49	3.72 x 10^10	10.57	1.74 x 10^10	10.24	1.51 x 10^10	10.18	5.13 x 10^9	9.71
Expt 2.2	4.17 x 10^10	10.62	1.20 x 10^10	10.08	1.55 x 10^10	10.19	5.62 x 10^9	9.75	5.75 x 10^9	9.76
Expt 3.1	5.13 x 10^10	10.71	1.66 x 10^10	10.22	8.71 x 10^9	9.94	1.32 x 10^10	10.12	1.10 x 10^10	10.04
Expt 3.2	1.66 x 10^10	10.22	1.17 x 10^10	10.07	1.23 x 10^10	10.09	4.07 x 10^9	9.61	8.13 x 10^9	9.91
Average		10.537		10.263		10.208		9.948		9.833
Std. Dev		0.178		0.204		0.177		0.235		0.184

At 37.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	4.68 x 10^10	10.67	2.75 x 10^10	10.44	1.41 x 10^10	10.15	2.34 x 10^9	9.37	1.17 x 10^8	8.07
Expt 1.2	3.24 x 10^10	10.51	1.78 x 10^10	10.25	6.46 x 10^9	9.81	2.24 x 10^9	9.35	6.92 x 10^8	8.84
Expt 2.1	3.09 x 10^10	10.49	5.62 x 10^9	9.75	3.80 x 10^9	9.58	1.38 x 10^9	9.14	1.78 x 10^8	8.25
Expt 2.2	4.17 x 10^10	10.62	2.04 x 10^10	10.31	9.12 x 10^9	9.96	1.91 x 10^9	9.28	4.07 x 10^8	8.61
Expt 3.1	5.13 x 10^10	10.71	1.48 x 10^10	10.17	3.24 x 10^9	9.51	1.17 x 10^9	9.07	1.10 x 10^8	8.04
Expt 3.2	1.66 x 10^10	10.22	9.33 x 10^9	9.97	1.74 x 10^9	9.24	1.07 x 10^9	9.03	1.44 x 10^8	8.16
Average		10.537		10.148		9.708		9.207		8.328
Std. Dev		0.178		0.250		0.330		0.146		0.324

Appendix 5.15: Storage stability of freeze dried **LMB** samples prepared as per chapter 5.0

At 4.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	6.46 x 10^10	10.81	8.32 x 10^10	10.92	6.92 x 10^10	10.84	4.27 x 10^10	10.63	3.98 x 10^10	10.6
Expt 1.2	4.90 x 10^11	10.69	6.46 x 10^10	10.81	3.80 x 10^10	10.58	3.55 x 10^10	10.55	2.95 x 10^10	10.47
Expt 2.1	5.62 x 10^10	10.75	5.75 x 10^10	10.76	4.47 x 10^10	10.65	2.34 x 10^10	10.37	2.63 x 10^10	10.42
Expt 2.2	5.13 x 10^10	10.71	7.76 x 10^10	10.89	6.46 x 10^10	10.81	5.13 x 10^10	10.71	5.13 x 10^10	10.71
Expt 3.1	5.25 x 10^10	10.72	5.89 x 10^10	10.77	3.02 x 10^10	10.48	3.31 x 10^10	10.52	2.34 x 10^10	10.37
Expt 3.2	7.08 x 10^10	10.85	7.24 x 10^10	10.86	5.13 x 10^10	10.71	5.37 x 10^10	10.73	2.51 x 10^10	10.4
Average		10.755		10.835		10.678		10.585		10.495
Std. Dev		0.063		0.065		0.137		0.134		0.133

At 37.C	Day 0		Day 7		Day 14		Day 21		Day 28	
	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm	Log CFU/Gm
Expt 1.1	6.46 x 10^10	10.81	4.37 x 10^10	10.64	1.74 x 10^10	10.24	1.41 x 10^9	9.15	7.41 x 10^8	8.87
Expt 1.2	4.90 x 10^11	10.69	5.13 x 10^10	10.71	7.76 x 10^9	9.89	6.92 x 10^8	8.84	2.95 x 10^8	8.47
Expt 2.1	5.62 x 10^10	10.75	3.24 x 10^10	10.51	6.76 x 10^9	9.83	4.07 x 10^8	8.61	4.37 x 10^8	8.64
Expt 2.2	5.13 x 10^10	10.71	5.62 x 10^10	10.75	1.10 x 10^10	10.04	1.05 x 10^8	9.02	6.61 x 10^8	8.82
Expt 3.1	5.25 x 10^10	10.72	8.71 x 10^9	9.94	7.76 x 10^9	9.89	3.47 x 10^8	8.54	2.45 x 10^8	8.39
Expt 3.2	7.08 x 10^10	10.85	2.40 x 10^10	10.38	5.89 x 10^9	9.77	1.66 x 10^8	8.22	5.13 x 10^8	8.71
Average		10.755		10.488		9.943		8.730		8.650
Std. Dev		0.063		0.301		0.171		0.342		0.190