

**EFFECTS OF STARCH FILLER ON THE PHYSICAL
PROPERTIES OF LYOPHILIZED CALCIUM-
ALGINATE BEADS AND THE VIABILITY OF
ENCAPSULATED CELLS**



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UNIVERSITI MALAYSIA SABAH

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TECHNOLOGY
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2011**

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**THESIS SUBMITTED IN FULFILLMENT FOR THE
DEGREE OF MASTER OF ENGINEERING**

**SCHOOL OF ENGINEERING AND INFORMATION
TECHNOLOGY
UNIVERSITI MALAYSIA SABAH
2011**

DECLARATION

I hereby declare that the material in this thesis is original except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

11 August 2010

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PS05-008-007



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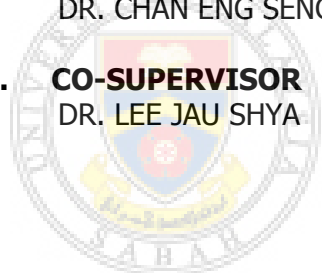
CERTIFICATION

NAME : **WONG SZE LING**
MATRIC NO. : **PS05-008-007**
TITLE : **EFFECTS OF STARCH FILLER ON THE PHYSICAL
PROPERTIES OF LYOPHILIZED CALCIUM-ALGINATE
BEADS AND THE VIABILITY OF ENCAPSULATED CELLS**
DEGREE : **MASTER OF ENGINEERING**
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ACKNOWLEDGEMENT

I would like to express my deepest gratitude and appreciation to the following individuals and organizations for their assistance and support throughout this project:

Dr. Chan Eng Seng, my main supervisor for providing the opportunity to participate in his encapsulation research project. His criticism and innovative ideas helped me throughout the development of this project. I am especially thankful for his continuous supervision and patience which led to the completion of this thesis.

Dr. Lee Jau Shya, my co-supervisor for her kind support and dedication. Her expertise in starch and inspiring guidance helped me through finding my research path since the beginning of this project.

Prof. Dr. Pogaku Ravindra for his constructive suggestions, motivations and wholehearted support along the way. His perseverance in work has made a good example for me.

Universiti Malaysia Sabah for providing scholarship under Skim Zamalah Yeoh Tiong Lay (YTL) UMS, technical support, resources and laboratory facilities for my postgraduate study.

Ministry of Science, Technology and Innovation Malaysia (MOSTI) with the grant number IRPA 03-02-10-10001-EAR for the provision of research funding.

Lecturers, laboratory assistants and staff within the university for their assistance and advice during the course of this research. I note especially for my Dean, Associate Professor Dr. Rosalam Sarbatly, Ms Sariah Abang, Dr. Rachel Mansa, Dr. Siva Kumaresan, Ms Noor Aemi Dawalih, Ms Noridah Abbas, Mr. Panjiman and Mr. Abdullah.

Professor Zhibing Zhang of University of Birmingham for assistance in providing the x-ray tomography images.

Colleagues and friends of the school and especially Encapsulation Research Group (ERG) in the past and present for their thoughtful contributions and assistance.

Finally, I would like to dedicate this project to my parents, sisters, brother and Mr. Chuah Cheng Huat for their constant support and understanding throughout my academic career, which made this project a success.

Wong Sze Ling
11 August 2010

ABSTRACT

Effects of Starch Filler on the Physical Properties of Lyophilized Calcium-Alginate Beads and the Viability of Encapsulated Cells

Calcium-alginate hydrogel is commonly used to encapsulate live cells. However, the qualities of lyophilized alginate beads could be undesirable for various reasons which may include reduction in encapsulation performance during storage. The objectives of this work were to improve the qualities of the lyophilized beads and to establish evidence if the bead qualities had significant influence on the stability of encapsulated cells. The bead qualities were manipulated by incorporating corn starch as the solid filler at various concentrations and produced using simple extrusion technique and subsequently lyophilized. The degree of shrinkage of SFAB during lyophilization was found to be dependent on the filler concentration with the control beads shrunk the most, by about 40% whereas the shrinkage reduced where beads shrunk from 18% to 9% with additional of starch filler. Sphericity of SFAB was also improved with Aspect Ratio of between 1.1 and unity. The relative porosity reduced from 71% (control sample) to 21.7% (SFAB 60%) in a near linear trend and SFAB was found to have ink bottle pore shape. With incorporation of starch fillers, SFAB with higher starch concentration had greater mechanical strength but its mechanical behaviour changed from spongy to brittle with a breakage point observed in the stress strain curve of starch concentration above 40%. Less hygroscopic behaviour was also observed from the lower EMC, range from 15% to 25% of SFAB with additional of starch compared to the control beads of EMC above 30%. Probiotic cells encapsulated within the alginate-starch beads were significantly more stable during freeze-drying process and storage, exposed to temperature, humidity and oxygen stresses in comparison to the cells encapsulated within the control beads. It is believed that with the incorporation of starch granules, stabilization on cells could occur in three possible mechanisms which are 1) shielding effect by the physical packing of starch granules 2) maintaining of particle sphericity through the presence of starch granules and 3) alteration of particle hygroscopic nature with starch granules. In conclusion, the qualities of the alginate beads were improved by incorporating starch filler and the bead qualities were found to have significant influence on the cell stability.

ABSTRAK

Hidrogel kalsium-alginat biasanya digunakan untuk mengenkapsulasikan sel hidup. Akan tetapi, kualiti manik setelah pengliofilian mungkin tidak sesuai atas beberapa sebab termasuk kejejasan prestasi enkapsulasi semasa simpanan. Objektif kajian ini adalah untuk meningkatkan kualiti manik dan membuktikan sama ada kualiti manik mempunyai kesan ketara ke atas kestabilan sel yang dienkapsulasikan. Kualiti manik dikawal dengan menambah isian kanji pada beberapa kepekatan dan dihasilkan dengan menggunakan teknik ekstrusi dan seterusnya diliofiliankan. Tahap pengecutan manik SFAB setelah diliofilian didapati bergantung kepada kandungan isian kanji dengan sampel kawalan menunjukkan pengecutan yang paling ketara, sebanyak 40% manakala manik dengan isian kanji hanya mencatatkan tahap pengecutan daripada 18% ke 9%. Kebujuran SFAB juga diperbaiki dengan catatan nisbah aspek antara 1.1 dan 1. Porositi relatif mengurang daripada 71% (sampel kawalan) kepada 21.7% (SFAB 60%) dalam tren linear dan adalah didapati bahawa SFAB mempunyai bentuk liang botol dakwat. Dengan penambahan isian kanji, SFAB yang mempunyai kandungan kanji yang lebih banyak mempunyai kekuatan mekanikal yang tinggi tetapi perangai mekanikal berubah daripada span kepada sifat rapuh dengan titik retakan yang dapat dilihat dalam graf tekanan ketegangan untuk kandungan kanji melebihi 40%. Sifat yang kurang higroskopik juga diperhatikan untuk SFAB daripada EMC rendah antara 15% kepada 25% berbanding dengan sampel kawalan yang mencatat EMC lebih daripada 30%. Sel probiotik yang dienkapsulasikan dalam manik kanji-alginat juga lebih stabil semasa proses liofilian dan penyimpanan, pendedahan kepada gangguan suhu, kelembapan dan oksigen berbanding dengan sampel kawalan. Adalah dipercayai bahawa dengan penambahan granul kanji, kestabilan pada sel mungkin terjadi dalam tiga mekanisme iaitu 1) kesan perlindungan daripada pembungkuan fizikal daripada isian kanji 2) pengekatan kebujuran manik melalui kehadiran granul kanji dan 3) perubahan sifat higroskopik semulajadi granul kanji. Secara kesimpulan, kualiti manik alginat dapat ditingkatkan dengan penambahan isian kanji dan kualiti manik didapati mempunyai kesan ketara pada kestabilan sel.

TABLE OF CONTENTS

	Page
TITLE	i
DECLARATION	ii
CERTIFICATION	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	v
<i>ABSTRAK</i>	vi
TABLE OF CONTENTS	vii
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF ABBREVIATION	xiv
LIST OF SYMBOLS	xv
CHAPTER 1: INTRODUCTION	1
1.1 Research Background	1
1.2 Research Problem	2
1.3 Research Objective	3
1.4 Thesis Organization	3
CHAPTER 2: LITERATURE REVIEW	5
2.1 Concept of Probiotics	5
2.1.1 Definition of Probiotics	5
2.1.2 Criteria for Classification as Probiotics	6
2.1.3 Probiotics from Lactic Acid Bacteria	7
2.1.4 Therapeutic and Health Benefits of Probiotics	9
2.1.5 Freeze-drying of Probiotics	11
2.1.6 Factors Affecting Stability of Probiotic Cells during Storage	12
2.2 Encapsulation	15
2.2.1 Encapsulation Functions	15
2.2.2 Encapsulation Methods	17
2.2.3 Natural Materials used for Probiotics Encapsulation	26

2.3	Selection of Calcium-alginate as Encapsulation Material for Probiotic Cells	30
2.4	Starch Polymers	31
	2.4.1 Composition of Starch Granules	33
	2.4.2 Crystallinity and Semi-Crystalline Structure of Starch Granules	36
	2.4.3 Application of Starch in Cell Encapsulation	37
2.5	Conclusions	39
 CHAPTER 3: MATERIALS AND METHODS		 40
3.1	Introduction	40
3.2	Materials	40
3.3	Preparation of Cell Culture	41
3.4	Preparation of Encapsulated Cell Beads	41
3.5	Determination of Liquid Properties	43
	3.5.1 Density Measurement	44
	3.5.2 Viscosity Measurement	44
3.6	Determination of Bead Size and Shape	45
3.7	Determination of Bulk Density, Tapped Density and Flowability	46
3.8	Imaging of Starch Filler Alginate Beads Internal Structure	46
3.9	Porosity and Pore Size Analysis	46
3.10	Determination of Mechanical Properties	47
3.11	Determination of Hygroscopicity	47
3.12	Determination of Cell Stability	48
3.13	Determination of Storage Stability	49
	3.13.1 Preparation of Different Storage Temperatures	49
	3.13.2 Preparation of Different Storage Humidity	49
	3.13.3 Preparation of Oxygen and Anaerobic Environment	50
3.14	Statistical Analysis and Control	51
3.15	Conclusion	51
 CHAPTER 4: CHARACTERIZATION OF STARCH FILLED ALGINATE BEADS		 52
4.1	Introduction	52
4.2	Size Analysis and Prediction of Starch Filled Alginate Beads	52
4.3	Shape Analysis and Surface Texture	55
4.4	Bulk, Tapped Density and Flowability of Starch Filled Alginate Beads	59
4.5	Visual Images of Freeze-dried Starch Filled Alginate Beads	61
4.6	Porosity and Pore Size Analysis of Freeze-dried Starch Filled Alginate Beads	63
4.7	Mechanical Properties of Freeze-dried Starch Filled Alginate Beads	66
4.8	Hygroscopicity of Freeze-dried Starch Filled Alginate Beads	68
4.9	Conclusions	70

CHAPTER 5:	EFFECT OF FREEZE-DRYING AND ENVIRONMENTAL FACTORS ON THE CELL SURVIVAL	72
5.1	Introduction	72
5.2	Effect of Freeze-drying Process on Cell Survival	72
5.3	Environment Factors Affecting the Stability of Freeze-dried Cells Encapsulated within Starch Filled Alginate Beads	75
	5.3.1 Temperature Effect	75
	5.3.2 Humidity Effect	78
	5.3.3 Oxygen Effect	80
5.4	Postulation of Mechanisms of Starch Filled Alginate Beads in Stabilizing the Encapsulated Cells	82
	5.4.1 Shielding Effect by the Physical Packing of Starch Granules	82
	5.4.2 Maintaining of Particle Sphericity through the Presence of Starch Granules	82
	5.4.3 Alteration of Particle Hygroscopic Nature with Starch Granules	83
5.5	Conclusions	83
CHAPTER 6:	OVERALL CONCLUSIONS AND FUTURE WORK	85
6.1	Overall Conclusions	85
6.2	Future Work	86
REFERENCES		88
APPENDIX A: LIST OF PUBLICATIONS		103

LIST OF TABLES

		Page
Table 2.1	Desirable properties of probiotic bacteria	6
Table 2.2	List of species from genera <i>Lactobacillus</i>	8
Table 2.3	Comparison among various encapsulation methods	22
Table 2.4	Characteristics of starch granules from different botanical sources	32
Table 2.5	Typical amylose and amylopectin content of starches	34
Table 3.1	The liquid properties of alginate-filler solutions and determination of κ	43
Table 3.2	Number of beads formed in 1ml of sample solution with different starch concentration	49
Table 3.3	RH generated by different saturated salt solutions	50
Table 4.1	<i>Re</i> and <i>Oh</i> of SFAB at different starch concentration	58



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LIST OF FIGURES

		Page
Figure 2.1	Effect of relative humidity on the survival of freeze-dried bacteria	14
Figure 2.2	Schematic diagram of a hard gelatin capsule	18
Figure 2.3	Schematic diagram showing compression coating of cells	18
Figure 2.4	Schematic approach of film formation	19
Figure 2.5	Schematic diagram of two types of entrapment in matrix	20
Figure 2.6	The structure of alginate chain and the egg-box model for binding of divalent cations (Ca^{2+}) to alginate	27
Figure 2.7	Chemical structure of repeating sequence of κ -carrageenan	28
Figure 2.8	Chemical structure of chitosan	29
Figure 2.9	Structure of the repeating unit of agarose	30
Figure 2.10	Light microscopy of (A) corn and (B) potato starches	33
Figure 2.11	Chemical structures of (a) amylose and (b) amylopectin	33
Figure 2.12	A and B type polymorphs of amylose	36
Figure 2.13	Diagrammatic representation of the lamellar structure of a starch granule. (A) Stacks of microcrystalline lamellae separated by amorphous growth rings, (B) Magnified view of amorphous and crystalline region, (C) Double helical structures formed by adjacent chains of amylopectin give rise to crystalline lamellae with amorphous region constitutes of branching points.	37
Figure 3.1	Process flow diagram of research methodology	42
Figure 3.2	Densitometer	44
Figure 3.3	Viscometer	45
Figure 4.1	Physical changes of calcium-alginate particle during and after freeze-drying	53

Figure 4.2	Size of SFAB before and after lyophilisation. The numbers above the line show the shrinkage percentage of the beads after freeze-dried	55
Figure 4.3	Effect of starch concentration on the aspect ratio of SFAB before and after lyophilisation. Error bars indicated the standard error of the samples at 95% confidence level.	57
Figure 4.4	Bulk and tapped density of SFAB at different starch concentration. The inset shows the Hausner ratio. The error bars indicated the standard deviation of triplicate measurements.	60
Figure 4.5	Different structural characteristics at cross-sectional area of freeze-dried SFAB. X-ray micro-computed tomography images (a-c) and SEM micrographs (d-f) of SFAB: SFAB 0% (a, d), SFAB 10% (b, e) and SFAB 60% (c, f). The bar (in a-c) corresponds to 500µm	62
Figure 4.6	Effect of starch concentration on relative porosity and pore diameter of freeze-dried SFAB	63
Figure 4.7	SEM micrograph displaying the morphology of freeze-dried SFAB with 300 times magnification at the cross-sectional area: (a) SFAB 10% and (b) SFAB 60%	64
Figure 4.8	Penetration and extraction curves of mercury during porosimetry: (a) cylindrical pore shape (Porosimeters Operating Manual, 2004); (b) conical pore shape (Porosimeters Operating Manual, 2004); (c) ink bottle shape (Porosimeters Operating Manual, 2004); (d) SFAB 10%	65
Figure 4.9	Stress-strain relationship of freeze-dried SFAB at different starch concentrations. The inset shows force at 10% deformation for different starch concentration	67
Figure 4.10	Moisture sorption of SFAB at 0%, 10%, 20%, 40% and 60% starch concentration, alginate beads at 4% (w/v), alginate and starch powders maintained at relative humidity of 80% and 30°C. The error bars indicated the standard error of the samples at 95% confidence level	69
Figure 5.1	Effect of starch concentration of SFAB on the survival of <i>L. casei</i> 01 exposed to freeze-drying process. The error bar indicated the standard error of the samples at 95% confidence level. Percentage above bars shows survival rate with standard deviation	73
Figure 5.2	Effect of temperature on the survival of <i>L. casei</i> 01 encapsulated within SFAB at different starch concentration	75

Figure 5.3	Arrhenius plot of the logarithms of k_d of <i>L. casei</i> 01 encapsulated within SFAB at different starch concentration to the reciprocals of absolute temperature. The inset shows the $E_0/2.303R$ and $\log k_0$ values at different starch concentrations	77
Figure 5.4	Effect of humidity on the survival of <i>L. casei</i> 01 encapsulated within SFAB at different starch concentration. Experiments were conducted at constant temperature of 30°C	78
Figure 5.5	Effect of oxygen on the survival of <i>L. casei</i> 01 encapsulated within SFAB at different starch concentration. Experiments were conducted at constant temperature of 30°C	81



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LIST OF ABBREVIATION

AR	Aspect ratio
AAD	Average absolute deviation
AOAC	Association of Official Analytical Chemists
cfu	Colony forming unit
Da	Dalton
d.b.	Dry basis
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
EMC	Equilibrium moisture content
ERH	Equilibrium relative humidity
G	Guluronic acid residues
L	<i>Lactobacillus</i>
M	Mannuronic acid residues
OD	Outer diameter
RH	Relative humidity
RNA	Ribonucleic acid
SEM	Scanning electron microscopy
SFAB	Starch filled alginate beads

LIST OF SYMBOLS

A	Cross-sectional area of original bead
a_w	Water activity
D	Feret diameter
D_0	Original bead diameter
d_p	Bead diameter
d_r	Dripping tip radius
d_T	Capillary tip diameter
D_{max}	Maximum feret diameter
D_{min}	Minimum feret diameter
E_a	Activation energy
F	Force exerted onto bead
g	Gravitational force
h	Collection distance
K	Correction factor
k_d	Decay constant (/day)
k_0	Experimental constant
k_{LF}	Liquid loss factor
k_{SF}	Shrinkage factor
m_p	Mass of beads
N	Cell count at a particular storage period
N_0	Cell count at the beginning of the storage period
Oh_I	Impact Ohnesorge number

P	Penetration pressure
P_r	Pore radius
R	Gas constant
Re_I	Impact Reynolds number
T	Absolute temperature
u	Impact velocity
ν	Kinematic viscosity
v_B	Bulk volume
v_T	Tap volume
γ	Surface tension
γ_m	Surface tension of mercury
ϵ	Strain
η	Viscosity
θ	Contact angle
σ	Stress
φ	Density
φ_L	Density of dripping solution
Φ_{BD}	Bulk density
Φ_{TD}	Tapped density
ΔD	Total deformation



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CHAPTER 1

INTRODUCTION

1.1 Research Background

The resident microbiota of intestine is the key to human health. By being able to deliver them to their active site, these beneficial microbial can be excellent candidates for therapeutic use. To date, a lot of researches are being developed for the use of live cells for therapeutic purposes. Among them is the probiotics. Probiotics have gained recognition to play therapeutic functions such as altering immunity, lowering cholesterol, improving lactose tolerance and avoiding cancer (Kailasapathy and Chin, 2000; Anal and Singh, 2007). Generally, the cells must remain stable in three pre-action stages: processing, storage and transit through gastrointestinal-tract in order for these therapeutic potential cells to be efficacious. With the minimum therapeutic dose suggested per day at a level of $10^8 - 10^9$ cfu/g (Kailasapathy, 2002), delivering sufficient amounts of living probiotic cells during the entire shelf-life of the product remains a great challenge.

Many attempts had been tried out to stabilize the cells and encapsulation technique had appeared to be the potential way to enhance cells viability during production and storage. With the presence of surrounding physical barrier around the cells, encapsulation material separates and protects the cells from direct exposure to the harsh environment. Numerous encapsulation methods had been developed, namely hard capsules, compression coating, film coating (spray, fluidized-bed, dry and hot-melt) and entrapment in matrix (by extrusion or emulsification). Natural or synthetic derived encapsulation materials had also been discovered to suit different purposes of encapsulation. Among the available materials, encapsulation in calcium-alginate beads, a hydrogel material, is one of the widely used biomaterials as carriers to encapsulate sensitive bioactive compounds for preservation purpose. This is because the encapsulation process could be performed in a simple, mild and safe condition.

1.2 Research Problem

In order to achieve preservation and easy handling of the encapsulated bacteria, freeze-drying process was often adopted to produce dried particulates (Zayed and Roos, 2004) with high viability. During the drying process, removal of frozen water from the alginate hydrogel by sublimation could leave void areas within the dried structure. As a result, the carriers may possess several undesirable qualities such as distorted shape, uneven size, poor mechanical strength and high porosity. These inferior characteristics may not only cause difficulty in handling and processing, it may also affect the function and performance of encapsulation.

Filler, a solid-body additive, could be incorporated to improve the characteristics of freeze-dried hydrogel particulates. Tal and co-workers (1997, 1999), Zohar-Perez *et al.* (2004) and Rassis and co-workers (2002) documented well regarding the positive effect of starch filler which improved the mechanical strength and reduced structure collapse of freeze-dried alginate particulates. Jankowski and co-workers (1997) and Sultana *et al.* (2000) were among the few reported on utilizing starch filler with calcium-alginate for cell encapsulation. However, there was still lack of literature mentioning about the carrier qualities in affecting encapsulation performance. Besides, there was no detailed study on the effect of fillers on physical characteristics of freeze-dried calcium-alginate beads. To date, only Tal *et al.* (1999), Zohar-Perez *et al.* (2004) and Rassis *et al.* (2002) had reported on the mechanical, shape and surface properties of calcium-alginate beads. However, their main concern was to produce porous particle for cell immobilization in water treatment and for food application.

Most of the literatures had shown that calcium-alginate encapsulated cells had better survival compared to the non-encapsulated state with incorporation of cryoprotectants and additional coating layers. For example, calcium-alginate beads had been proven for stabilizing cells after freeze-drying and during exposure to simulated gastric fluid and bile salt solution (Kearney *et al.*, 1990; Selmer-Olsen *et al.*, 1999; Krasaekoopt *et al.*, 2004; 2006; De Giulio *et al.*, 2005;). Yet, limited information had been found on storage stability of encapsulated cells within the freeze-dried calcium-alginate beads.

1.3 Research Objective

The main objectives of this work were two fold: 1) to improve the qualities of lyophilized calcium-alginate particles and 2) to establish evidence if the particle qualities had significant influence on the stability of encapsulated probiotic cells.

The specific objectives were:

- i. To study the effect of starch filler concentration on the characteristics of alginate particles using extrusion method. Nine characteristics studied include size, shape, bulk and tapped density, flowability, porosity, pore size, morphology, mechanical strength and hygroscopicity.
- ii. To study the effect of process variables (lyophilisation, temperature, humidity and oxygen) on stability of encapsulated cells.
- iii. To understand the interaction between particle characteristics and cell stability exposed to process variables.
- iv. To postulate the mechanisms of starch filled alginate beads in stabilization of encapsulated cells.

1.4 Thesis Organization

This thesis presents the study, firstly on the characteristics of starch filled alginate beads prior and after lyophilization process with various solid filler concentration. Lyophilization was selected as the preferred drying method based on its mild effect to probiotic cells. The second part of this thesis reports on the effects of lyophilization and other environment factors which influence the survival of probiotic cells encapsulated within the starch filled alginate beads throughout the shelf life. The outcome of this study could be used as a reference for further development in prolonging the storage of probiotic cells or any sensitive active compounds. The details of the thesis were reported in six chapters. Brief contents of each chapter were discussed as follows:

Chapter 1 presents the introduction of this thesis. Introduction chapter covers some background information on the advantages of probiotic cells to human health, current encapsulation method and materials used for storage and stabilization of this fastidious microbial. In addition, the research problems in achieving good preservation

and easy handling of the encapsulated bacteria were also highlighted. From there, an alternative solution was proposed for research. The research objectives were included into this chapter and a summary on the thesis outline was presented.

Chapter 2 focuses on the literature review where the concept of probiotics is described first. Definition, ideal properties of classification as probiotics, therapeutic potential and health benefits, factors affecting efficacy and stability of probiotics are then discussed. This chapter later provides a literature review on encapsulation functions, methods and natural materials used for cell encapsulation. By understanding of the available methods and materials, selection of method and material suited to this research is discussed. Finally, the literature regarding composition and reported application of starch in encapsulation are reviewed.

Chapter 3 is devoted to give a detailed description of the experimental materials and methodology used in this study. The experimental setups for preparation of starch filled alginate beads, methods for characterization and stability tests on model cells are also attached. Besides, the experimental designs and mathematical equations are included into this chapter.

Chapter 4 presents the results and discussions on the findings of starch filled alginate beads characteristics at different filler concentrations. A series of physical, visual and mechanical characterizations are carried out and reported. This chapter allows the understanding of the filler concentrations in affecting the starch filled alginate beads quality and handibility after lyophilization process.

Chapter 5 reveals the stability analysis of encapsulated model cells within the starch filled alginate beads after lyophilization process and subsequent storage. This knowledge on starch filled alginate beads characteristics in the previous chapter allowed further understanding on the particle qualities affecting cell stability. Meanwhile, three mechanisms of stabilizing the encapsulated cells were proposed.

Lastly, Chapter 6 concludes on the research findings of this project and some recommendations for further development in this field were addressed.

CHAPTER 2

LITERATURE REVIEW

2.1 Concept of Probiotics

For over thousands of years, people have been consuming fermented milk containing live microbe. Thus the use of live microbial food to enhance human health is not new. The concept of probiotic was scientifically introduced in 1907 by Eli Metchnikoff in his book 'Prolongation of life'. He hypothesized that the longevity of the Bulgarians was resulted from their consumption of lactobacilli which contained fermented milk. His observation had led to the growing interest on the role of probiotics in affecting human or animal health since then.

2.1.1 Definition of Probiotics

'Probiotics' originated from the Greek word 'for life'. With time, the definition evolved depending on the understanding of the mechanisms of action of their influence on the health and well-being of mankind. In 1989, Fuller gave a more precise definition of probiotics as live microbial food supplements which benefited the health of the host by maintaining or improving their intestinal microbial balance (Mattila-Sandholm *et al.*, 2002). Thereafter, Huis in't Veld expended the definition to 'a mono- or mixed-culture of live microorganisms which when applied to man or animal affected beneficially the host by improving the properties of the indigenous microflora' (Shortt, 1999). Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) included the importance of ingesting adequate amount of probiotics by defining it as 'live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers conferred one or more specified demonstrated health benefits for the host' (Anal & Singh, 2007).

2.1.2 Criteria for Classification as Probiotics

The basic requirements for the selection of probiotic micro-organisms are given in Table 2.1. It is vital that probiotic strains are originally isolated from human as most current successful strains are indicated to be of human origin. This could be due to better function of the strain in a similar environment (e.g. human gastrointestinal tract). Other required properties include resistance to acid and bile during passage through the stomach and at least temporarily to be able to colonize the intestinal tract. The use of probiotic bacterial cultures stimulates the growth of preferred microorganisms, crowds out potentially harmful bacteria and reinforces the body's natural defense mechanisms (Saarela *et al.*, 2000). To impose greater probiotic effects, adherent strains of probiotic bacteria are favoured because they are more prone to become established in the intestinal tract. The mechanism of anti-pathogenic effect may be through decreasing the luminal pH by the production of short chain fatty acids such as acetic acid, lactic acid or propionic acid rendering vital nutrients unavailable to pathogens, altering the redox potential of the environment, producing hydrogen peroxide or producing bacteriocins or other inhibitory substances (Kailasapathy & Chin, 2000). Above all, the strains should be safe and tested for human use. Safety assessments include studies on basic toxicology, risk of microbial invasion, degradation of intestinal mucosa and epidemiological data and evidence of safety (Lee & Salminen, 1995).

Table 2.1: Desirable properties of probiotic bacteria

-
- Human origin.
 - Resistance to acid and bile.
 - Adherence to human intestinal cells.
 - Colonization of the human gut.
 - Production of antimicrobial substances.
 - Antagonism against carcinogenic and pathogenic bacteria.
 - Safety in food and clinical use.
 - Clinically validated and documented health effects.
-

Source: Lee & Salminen (1995), Saarela *et al.*, (2000).

2.1.3 Probiotics from Lactic Acid Bacteria

Most studies on probiotics had focused on the lactic acid bacteria, particularly the genera that were of human intestinal origin, *Lactobacillus*, *Bifidobacterium* and *Streptococcus* or *Enterococcus*, either singly or in mixed culture (Scheinbach, 1998). Each genus and species had different characteristics but they were generally chained cocci or rod shaped gram-positive, nonmotile, nonsporulating bacteria that produced lactic acid as a major or sole product of fermentative metabolism (Salminen *et al.*, 1993).

a. Genus *Lactobacillus*

Lactobacilli are among the bacteria mostly commonly used as probiotics in human foods (Coélet *et al.*, 2004). *Lactobacilli* are distributed in various ecological niches throughout the gastrointestinal and genital tracts and constitute an important part of the indigenous microflora of man and also animal. Their distribution is affected by several environmental factors, which include pH, oxygen availability, level of specific substrates, presence of secretions and bacterial interactions. At present, 56 species of the genus *Lactobacillus* have been recognized (Gomes & Malcata, 1999.) and listed in Table 2.2. However, not all of them can be categorized as probiotics because some are non-human origin and there is lack of studies and documented clinical efficacy on each of the species.