# BIOACTIVITY OF SOYMILK FERMENTED BY LACTIC ACID BACTERIA IMMOBILIZED ON AGROWASTES

by

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## Acknowledgment

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**Nomenclature**

| | |
|---|---|
| ACE | Angiotensin-converting Enzyme |
| ATCC | American Type Culture Collection |
| MRS | de Mann, Rogosa, Sharpe broth |
| CFU/ml | Colony forming units per milliliter |
| $p$NPG | $p$-nitrophenyl-$\alpha$-D-galactopyranoside |
| $Na_2CO_3$ | Sodium (II) carbonates |
| U | units |
| w : v | ratio of weight to volume |
| w/v | ratio of weight per volume |
| SEM | Scanning Electron Microscopy |
| $\log_{10}CFU/g$ | log of colony forming units per gram |
| OPA | $o$-phthaldialdehyde |
| HCl | Hydrochloric acid |

# BIOAKTIVITI SUSU SOYA DIFERMEN OLEH BAKTERIA ASID LAKTIK TERSEKAT-GERAK PADA SISA PERTANIAN

## ABSTRAK

Susu soya adalah substrat cecair yang terbaik untuk pertumbuhan probiotik selain susu kerana susu soya kaya dengan oligosakarida seperti stakiosa dan rafinosa. Namun, kedayahidupan probiotik di dalam makanan cecair biasanya tidak melepasi tahap keperluan sesuatu makanan probiotik. Tambahan, medium cecair tidak dapat menyokong kemandiran bakteria probiotik untuk jangka masa yang panjang. Oleh itu, banyak cara termasuk penyekat-gerakan sel, telah dikaji untuk mengekalkan kedayahidupan sebanyak $10^7$ CFU/ml bagi membekalkan kesan terapeutis kepada kesihatan manusia. Objektif kajian ini adalah untuk mengkaji kesan sisa pertanian daripada durian (*Durio zibethinus*), cempedak (*Artocarpus champeden)* dan manggis (*Garcinia mangostana*) sebagai agen penyekat-gerak untuk laktobasilus yang ditumbuhkan di dalam susu soya. Lima belas probiotik yang berlainan strain telah disaring untuk toleransi terhadap asid dan hempedu bagi menilai kemandirian mereka di bawah tekanan gastrousus dan kebolehan untuk menghasilkan $\alpha$-galaktosidase. Lima strain probiotik yang terbaik (*Lactobacillus acidophilus* FTDC 1331, *L. acidophilus* FTDC 2631, *L. acidophilus* FTDC 2333, *L. acidophilus* FTDC 1733 and *L. bulgaricus* FTCC 0411) dipindahkan ke susu soya untuk kajian yang seterusnya. Kulit dalaman daripada sisa pertanian telah diasingkan daripada kulit luar, kemudian dikeringkan dan dihancurkan untuk menjadi tepung (bersaiz 150 µm) yang digunakan sebagai agen penyekat-gerak. Mikroskop penskanan elektron menunjukkan sel laktobasilus adalah melekat dan terikat pada permukaan agen pengimobil. Sel-sel laktobasilus tersekat-gerak diinokulat ke dalam susu soya dan

disimpan pada tiga suhu yang berbeza (37, 25, 4°C), dan ciri-ciri pertumbuhannya telah dikaji selama 168 jam. Susu soya yang ditambahkan sel bebas digunakan sebagai kawalan. Sel terimobil menunjukkan pertumbuhan yang lebih tinggi, pengurangan gula seperti stakiosa, rafinosa, sukrosa, frutosa and glukosa yang lebih tinggi, pengeluaran asid laktik dan asid asetik yang lebih tinggi dan pH yang lebih rendah dalam susu soya jika dibandingkan dengan kawalan bagi semua suhu yang dikaji. Keputusan kajian ini menunjukkan sisa pertanian boleh digunakan sebagai penyokong pepejal untuk pengimobilan laktobasilus di dalam medium cecair. Oleh itu, aktiviti proteolitis dan aktiviti penghambatan ACE in-vitro pada probiotik tersekat-gerak di dalam susu soya terfermentasi dinilai dengan lebih lanjut. Susu soya yang ditambahkan dengan sel probiotik bebas digunakan sebagai kawalan. Susu soya yang ditambahkan dengan sel tersekat-gerak pada tepung kulit cempedak memberi aktiviti proteolitis dan aktiviti penghambatan ACE yang paling tinggi. Aktiviti proteolitis dan aktiviti penghambatan ACE adalah lebih tinggi pada suhu 25 dan 37°C dibandingkan dengan aktiviti pada 4°C. Hal ini adalah seiring dengan pertumbuhan yang lebih tinggi pada 25 dan 37°C daripada pertumbuhan pada 4°C.

higher reduction of sugars such as stachyose, raffinose, sucrose, fructose and glucose, higher production of lactic and acetic acids, and a lower pH in soymilk compared to the control for all temperatures studied. Our results revealed that agrowastes could be used as solid supports for the immobilization of lactobacilli in a liquid medium. Hence, proteolytic activity and the in-vitro ACE inhibitory activity of the immobilized probiotics in the fermented soymilk were further evaluated. Soymilk supplemented with cells immobilized on cempedak rind powder exerted the highest proteolytic and the ACE inhibitory activities. The proteolytic and the ACE inhibitory activities were higher at 25 and $37^{\circ}C$ compared to $4^{\circ}C$. This was in tandem with higher growth at 25 and $37^{\circ}C$ compared to $4^{\circ}C$.

# CHAPTER 1

## INTRODUCTION

### 1.1 Background

Probiotics are live microorganisms that benefit the host via upon consumption in adequate amounts. Other definition of probiotics includes single or mixed culture of live microorganisms that beneficially modulates indigenous microflora when orally given to human and animal (Shortt, 1998). Many strains of lactic acid bacteria (LAB) have been found to possess probiotic properties. Previous studies have found that LAB could provide health promoting effects to host such as antimutagenic properties (Hsieh and Chou, 2006), alleviation of lactose intolerance, reduction of cancer risks, decreasing of serum cholesterol levela (Gomes et al., 1999), and antihypertension (Donkor et al., 2007). Examples of LAB and bifidobacteria include *L. acidophilus*, *L. casei.*, *L. fermentum, L. bulgaricus, B. bifidum, B. lactis* and *B. breve.*

Previous studies documented that the total viable count of LAB in some commercial yoghurts did not exceed the desired level required for the exhibition of therapeutic effects (Kailaspathy and Chin, 2000). The International Dairy Federation has stated that growth should be present at least $10^7$ CFU/g in food products to the minimum durability date in order to provide therapeutic effects to the hosts (Ouwehand and Salminen, 1998). LAB and bifidobacteria have to overcome physical and chemical barriers such as acid and bile in the gastrointestinal tract in order to exert health benefits to hosts (Liong and Shah, 2005; Gibson *et al.*, 2000). Hence, it is vital in selecting strains of LAB and bifidobacteria that possess excellent acid and bile tolerance.

with antihypertensive effects but most of the peptides are inactive in the forms of their parent protein structure. Lactic acid bacteria have the capability to degrade protein, releasing bioactive peptides via proteolysis (Fung *et al.*, 2008). These bioactive peptides have been found to exhibite ACE inhibitory activity, which inhibit angiotensin-I converting enzyme (ACE), the key enzyme responsible for hypertension. ACE (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) is a carboxypeptidase that converts angiotensin-I to angiotensin-II, a potent vasoconstrictor and by degrading bradykinin, a vasodilatory peptides, thus giving rise to a net hypertensive effect (Sentandreu and Toldra, 2007). The use of lactic acid bacteria to yield bioactive peptides is economic and convenient, where bioactive peptides are released directly into soymilk without purification or processing compared to conventional chemical synthesis (Florence *et al.*, 2003). However, there has been no documented evidence on the bioactivity of such a fermented soymilk upon cell immobilization on agrowastes.

## 1.2 Research objectives

In this study, agrowastes were used as immobilizers for lactobacilli in a food product, namely soymilk. The general objective of this project was to evaluate the growth and bioactivity patterns of the immobilized cells in soymilk using agrowastes such as durian, cempedak and mangosteen rind powder over prolonged storage.

The specific objectives were:

i)      To evaluate the acid and bile tolerance, and $\alpha$-galactosidase producing ability of lactobacilli and bifidobacteria

ii)     To develop a method for cell immobilization on agrowastes.

CHAPTER 2

LITERATURE REVIEW

## 2.1 Probiotic

Probiotics are "live microorganisms that are used as dietary supplements with the purpose of benefiting the health of hosts by positively influencing the intestinal microbial balance" (Crittenden *et al.*, 2001). In fact, the word 'probiotic' came from Greek which means 'for life'. Probiotics can be defined as "live organisms, which when consumed in adequate amounts, confer a health effect on the host" (Liong, 2007). Early of this century, Nobel Laureate Elie Metchnikoff from Pasteur Institute found that consumption of bacteria present in yoghurt increased health and longevity of human (Metchnikoff, 2004). This bacteria was believed to control infections caused by enteric pathogens and regulate toxaemia.

Human began to consume probiotics in cultured dairy products such as yoghurt for centuries. People thought the bacteria possess beneficial effects to host until scientists began to investigate deeply behind those benefits provided by the bacteria in the 1900s. Lactic acid bacteria (LAB) such as those from the genus of *Lactobacillus* have been widely documented for probiotic properties. In addition, other genera such as *Leuconostoc, Pediococcus, Bifidobacterium* and *Enterococcus* have now been identified to possess probiotic properties. *L. acidophilus*, *Bifidobacterium spp.* and *L. casei* are common strains of probiotics that are also natural inhibitants in the human intestines and have been widely applied in the manufacturing of dairy products such as yoghurt and cheese (Shah, 2007). Additionally, *Lactobacillus* and *Bifidobacterium* are non-pathogenic and non-toxigenic, retain desirable viability during storage and survive passage through the

*al.*, 2005). Hence, *Lactobacilli sp.* was used in food bioprocessing of fermented dairy and plant products. Examples of the spesies that used in food bioprocessing are *L. delbrueckii subsp. bulgaricus*, *L. reuteri*, *L. casei subsp. casei*. Some species can even grow at low temperatures in food products that stored at 4°C, such as *L. sake* and *L. curvatus*. Some of the strains can produce bacteriocins which used as biopreservatives in food (Ray, 1996).

They are approximately 56 species of the *Lactobacillus* were found recently (Playne *et al.*, 2003). Among all species, *L. acidophilus* was widely used because it can survive and grow at 45°C while the optimum temperature for growth is at the range of 35 – 40°C (Curry and Crow, 2003). The authors reviewed that *L. acidophilus* has higher acid tolerance activity, which is around 0.3 – 1.9 % from titratable acidity and it can grow well at around pH of 6.4 – 4.5.

Previous studies documented that *Lactobacillus spp.* are able to utilize oligosaccharides, monosaccharides, aldehyde hexoses, carbohydrates and polyhydroxy alcohols by homofermentation to lactic acid or by heterofermentation to lactic acid and acetic acid, alcohol and carbon dioxide, where lactic acid and acetic acid function as preservative in food products (Salle, 1967). Additionally, high concentration of small peptides and free amino acids in soymilk functions as substrates for the growth of lactobacilli. Acid resistance property of lactobacilli enables them to grow when the pH value decreased too low from the production of natural organic acids.

## 2.1.2 *Bifidobacterium sp.*

Bifidobacteria are non-motile, mesophilic Gram positive bacteria with anaerobic pleomorphic rods of various shapes, appear as single cell or in chains,

arranged in "v" or star-like shape (Ray, 1996). Bifidobacteria are natural inhibitant in human intestine (Hou *et al.*, 2000). Hence, they function as regulation of microbial ecology of the human and animal gut (Scalabrini *et al.*, 1998). Bifidobacteria also found largely in human milk where they predominatetly account for up to 95 % of all cultivable bacteria and protect against infection in infants but do not occur highly in adults (Brock, 1970). Bifidobacteria can grow optimally at 37 – 41°C with the minimum growth is at 25 – 28°C while the maximum growth is at 43 – 45°C and pH for growth is ranged between 8.0 – 4.5 (Ray, 1996).

Most of Bifidobacteria are found from human and animal sources such as faeces, vagina, dental caries, rumen and intestinal tracts. For examples, *B. infantis, B. longum, B. dentium, B. pseudocatenulatum, B. bifidum* are found in infants while B. adolescentis and B. longum are found in adults (Shah and Lankaputhra, 2002).

## 2.1.3 Health Benefits of Probiotics

A lot of past studies had clinically proven the health effects from the consumption of probiotic. Each probiotic strain exerts specific health benefit and there is no even single strain that can exert all benefits purpose. Many studies have documented that strains of probiotic possess antimicrobial properties which they produce bacteriocins and reuterine that can inhibit pathogenic bacteria (Ray, 1996). Another study revealed that *Bifidobacterium breve* strain Yakult and *Bifidobacterium pseudocatenulatum* DSM 20439 possess anti-infectious activity by inhibiting shiga toxin-producing Escherichia coli cells (Asahara *et al.*, 2004). Probiotics produced organic acids such as lactic and acetic acids which can provide bacteriocidal or bacteriostatic effect by lowering the pH of intestine.

## 2.2 Soymilk

Soybean (*Glycine max*) is one of the most economic and nutritious legume crops with production reaching 130 million tons world wide (Thippeswamy and Mulimani, 2002). The consumption of soy is higher in Asia especially Japan compared to other countries in the world. Soy is often processed in various ways to produce varying products, such as via fermentation with *Rhizopus spp.* to produce 'tempeh' (McCue and Shetty, 2004), tofu, soy sauce and bean sprouts. The Japanese also blanched soybeans and eat them as snack.

Soymilk is an aqueous extract from soybeans. The consumption and manufacturing of soymilk is increasing throughout the world due to its benefits to human health. Soymilk does not contain cholesterol and lactose, which can replace milk for those with lactose intolerance. It could be a good source of protein for vegans. Soymilk also contains soy proteins that consist of higher quality of amino acid balance compared to animal proteins (Connes *et al.*, 2003).

### 2.2.1 Nutrient Content and Suitability for Probiotic Growth

Generally, soymilk consists of approximately 40% of protein, 20% of carbohydrates, 20% of oil, 5% of ash and 5% of fibers. Additionally, soymilk contains some vitamin B 12, vitamin D and calcium. The fiber in soymilk consists of cell wall components from soybeans such as hemicelluloses, cellulose and pectic substances. The oil content in soymilk basically consists of unsaturated and polyunsaturated fatty acid.

Soymilk consists of large concentration of soy protein compared to other nutrient composition. It contains high concentrations of essential amino acids and is claimed as the only plant source of complete protein (Hendry, 2006). Hence, the high

concentration of protein in soymilk could be a good substrate for probiotic growth. Probiotics have been found to possess proteolytic activity which could liberate amino acid and peptides (Ng et al., 2008).

The carbohydrate of soymilk comprises of oligosaccharides such as stachyose and raffinose, simple sugars such as sucrose and glucose. These carbohydrates are the main source for the growth of probiotics. Oligosaccharides such as stachyose and raffinose cannot be digested by human due to the lack of enzyme α-galactosidase and this may cause gastrointestinal discomfort to humans. The beany and green off-flavour of soymilk has also been attributed to the oligosaccharides of soymilk (Donkor et al., 2006). Past studies reported that soybeans contain 1.6 % raffinose and 3.3 % stachyose (w/w) while soymilk contains 3 g/L of raffinose and 8 g/L of stachyose (Connes et al., 2004). Many strains of probiotic have been found to possess α-galactosidase which enable them to utilize oligosaccharides in soymilk as carbon sources (LeBlanc et al., 2004).


## 2.2.2 Benefits of Soymilk to Human Health

Soymilk has been suggested as an alternative to replace cow's milk which contain lactose. Lactose is a disaccharide comprising of glucose and galactose (Heyman, 2006). The lactose intolerance population cannot digest lactose due to lack of β-galactosidase (lactase) activity as this would cause bloating, diarrhea and flatus (Suarez et. al., 1997). Soymilk is an alternative for this population as soymilk is rich in protein and does not contain lactose. Additionally, soymilk contains calcium, zinc, magnesium, iron and essential amino acids which is an excellent nutritional value for human diet (Sacks et al., 2006).

The cholesterol-lowering effect of soymilk has been recognized by most studies. Past studies reported that amino acid of proteins in soymilk contributes to hypocholesterolaemic effects (Potter *et al.*, 1996). In 1999, the United States Food and Drug Administration (FDA) documented that the minimum 6.25 g of soy protein per serving can reduce the amount of saturated fat and cholesterol and it also claimed to reduce the risk of heart disease (Food and Drug Administration, 1999). Previous studies also showed that hamsters fed with probiotic-fermented soymilk either in cholesterol free diet or cholesterol-enriched diet successfully decreased the level of total cholesterol and low-density lipid (LDL) cholesterol (Kikuchi-hayakawa *et al.*, 1998). Probiotic-fermented soymilk has also been found to enhance the excretion of total bile acid and the proportion of cholesterol entering the cholic acid biosynthesis pathway in hamsters.

Soymilk contains isoflavanoids structurally similar to the hormone estrogen which can interact with the estrogen receptors (Wuttke *et al.*, 2003). Past studies have shown that these isoflavonoids in soymilk reduced the risks of cardiovascular diseases due to their ability to reduce cholesterol (Ridges *et al.*, 2001), prevent in vitro aggregation of platelet and in vivo thrombogenesis in mouse arteries (Cheng *et al.*, 2003). Due to their estrogen mimetic properties, soybean phytoestrogens have been found able to eliminate symptoms of menopause (Murkies *et al.*, 1995), prevent osteoporosis during menopause (Chiechi *et al.*, 2002) and are more effective than hormone replacement therapy (HRT) because HRT possess side effect such as estrogen linked cancers (Goodman *et al.*, 1997).

## 2.2.3 Bioactive properties of probiotic-fermented soymilk

Soymilk contains peptides with antihypertensive effects but most of the peptides are inactive in the forms of their parent protein structure. Lactobacilli have been found able to degrade protein, releasing bioactive peptides via proteolysis (Fung *et al.*, 2008). Probiotic possess a very complex proteolytic system that comprises extracellularly located serine proteinase, which act as a transport system specific for di-, tri-, and oligopeptides, and a multitude of intracellular peptides (Liong, 2007). These proteinase have been found able to hydrolyze more than 40% of the peptide bonds of $\alpha_{S1}$-casein ($\alpha_{S1}$-CN) and $\beta$-casein ($\beta$-CNs), and some of the resultant peptides possess ACE inhibitory activity with antihypertension effects. Recent studies found that peptides such as valine-proline and isoleucine-proline-proline possess ACE inhibitory activity that can lead to antihypertensive effect (Yamamoto *et al.*, 1999). The bioactive peptides that exhibited ACE inhibitory activity are able to inhibit angiotensin-I converting enzyme (ACE), the key enzyme responsible for hypertension. ACE (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) is a carboxypeptidase that converts angiotensin-I to angiotensin-II, a potent vasoconstrictor and by degrading bradykinin, a vasodilatory peptides, thus giving rise to a net hypertensive effect (Sentandreu and Toldra, 2007). The use of probiotic to yield bioactive peptides is economic and convenient, where bioactive peptides are released directly into soymilk without purification or processing compared to conventional chemical synthesis (Florence *et al.*, 2003).

It has been previously suggested that probiotic exhibited relatively poorer growth in cows' milk than soymilk due to insufficient free amino acids and peptides in cows' milk (Abu-Tarboush, 1996). This is because probiotic possess proteinases and peptidases activity which are not specific on milk casein. Soymilk is more

tofu in Japan (Arvanitoyannis and Tserkezou, 2007). There are approximately 17,000 hectare of land in Malaysia that are utilized for the cultivation of fruit, producing approximately 0.25 million tonnes of fruits (Liong *et al.*, 2008). However, only 20% of the whole fruit is edible, while the skin, core, base and rind are normally discarded as wastes.

Although agrowastes are not classified as hazardous wastes, they are produced abundantly from crops each year, leading to environmental and economical issues. Due to increased economical and environmental concerns, agrowastes are used as bedding for animals and livestock feeding or added into soil as green fertilizer. Additionally, agrowastes are used as soil conditioners or fertilizers, biofuels, thermoplastics, activated charcoal and components of other composite materials (Panthapulakkal and Sain, 2002).

It has been found that agrowastes are a good source of dietary fiber. Residues from cumin, legumes, beans, potato peel and coconut have been re-processed as sources of dietary fiber. Corn cob has been reported to contain a high amount of soluble dietary fiber while okara has a high amount of total dietary fiber (Kuan and Liong, 2008). High dietary fiber intake has been shown to exert beneficial hypocholesterolemic effect, reduce the risks of cardiovascular diseases, improve hypertension, obesity, diabetes, bowel cancers, and alleviate gastrointestinal disorders (Schneeman, 2002). Additionally, fruit agrowastes also contain an abundant amount of sugars and could be used as substrates for the growth of microorganisms. Wastes from the pineapple fruit have been used as fermentation broth for the cultivation of *Saccharomyces cerevisiae* and *Candida utilis* (Liong *et al.*, 2008), while pineapple cannery wastes have been used as substrate for the fermentation of *Saccharomyces cerevisiae* in order to produce ethanol (Nigam, 1999).

Sago waste and palm oil sludge have been used for the cultivation of *Myceliophthora thermophila* and *Trichoderma harzianum* respectively, in submerge fermentations (Sabaratnam, 2009).

### 2.3.3 Mangosteen, Durian and Cempedak

Durian (*Durio zibethinus*), from the family Bombacaceae, is one of the highly valued and desired seasonal fruit among Southeast Asia, particularly Malaysia Indonesia, Thailand and Philippines. They grow very well in this region, attributed to the warm and wet conditions of the equatorial tropics (Tongdee *et al.*, 1990). Durian is ovoid or ovoid-oblong to nearly round shaped and it weights around 2 – 4.5 kg depending on their variety (Hokputsa *et al.*, 2004). Durian has five locular units and each locular unit in turn contains 1 – 5 pulp umits (Siriphanic, 1994). The pulp unit contains seed, which is covered by a creamy, white, yellow or golden yellow aril, the edible portion of the fruit (Nanthachai, 1994). Durian rind normally weights more than half of the total fruit weight, covered by green to yellowish brown, thick and semi-woody with sharply pointed pyramidal thorns (Hokputsa *et al.*, 2004). The rind portion is usually not consumed and discarded as waste.

Cempedak (*Artocarpus champeden*) grows in many parts of Asia, which are more prevalent in India and Bangladesh (Reddy *et al.*, 2004). The origin of cempedak is from India and later spread out to other tropic regions, including Indonesia (Prahas *et al.*, 2008). Cempedak can be eaten unripe or ripe, and cooked or uncooked and used in South and Southeast Asia cuisines. Cempedak weights around 10 – 25 kg at maturity with approximately 59 % comprising of rind (Bhatia *et al.*, 1955). Cempedak rind is generated abundantly each year and is considered of no economic value, thus discarded as waste and leads to environmental issues.

millions/mg) in a short time (300 h) (Ignacio *et al.*, 2003), attributed to the large pore size of the polyurethane foam (400 μm) that entrapped more cells.

## 2.4 Protection of Probiotic Cells

The viability of probiotics should exceed $10^7$ CFU/g of product in order to exhibit therapeutic effects in the host, and maintain at a minimum level of $10^6$ CFU/g to be recognized as a probiotic food. Moreover, The International Dairy Federation has stated that probiotics should be present at least $10^7$ CFU/g in food products to the minimum durability date in order to provide therapeutic effect to host (Ouwehand and Salminen, 1998). However, previous studies documented that total viable count of probiotic in some commercial yoghurts does not exceed the desired level that is required to exhibit therapeutic effects (Kailaspathy and Chin, 2000), mostly attributed to the low survivability during processing, transport and storage.

Additionally, stresses to probiotic organisms begin in the stomach, in the presence of acids and with pH between 1.5 and 3.0, and in the upper intestines that contain bile (Liong and Shah, 2005). The time from entrance to release from the stomach has been estimated to be approximately 90 min, with further digestive processes requiring longer residence time (Berada *et al.*, 1991). Hence, protections of probiotic cells need to be done in order to improve the growth of probiotic in medium.

## 2.4.1 Protection Methods

Various methods have been used and evaluated to increase the viability of probiotic cells. Some of the methods include buffering of yoghurt with whey proteins (Sultana *et al.*, 2000), addition of prebiotics (Van Loo *et al.*, 1999) and selection of

## 2.4.2 Cell Immobilization

Cell immobilization has been found to protect and increase the growth of probiotic cells. Cell immobilization can be defined as the entrapment of biomass within various gel matrices such as alginate, agar, gelatin and polyacrylates (Walsh and Malone, 1995). Various materials have been used as carriers for cell immobilization from previous studies, such as cryo-polyvinyl-alcohol gels (cryoPVAGs) for the immobilization of *Saccharomyces cerevisiae* (Lozinsky, 1994), natural fibers and artificial fibers for the immobilization of bioluminescent *Escherichia coli* (Chu *et al.*, 2009) and polyethyleneimine (PEI) had been successfully used in the immobilization of baker's yeasts (D'Souza and Melo, 2001). Tuli *et al.* (1985) has previously evaluated several solid phases for cell immobilization and found that agar was effective in entrapping cells of *L. casei* for the production of lactic acid. Kosseva *et al.*, (1998) used calcium pectate gel and chemically modified chitosan beads as supports for the immobilization of *L. casei* in the fermentation of chardonnay wine. Alginate was also evaluated as a matrix for *L. casei* in the production of lactic acid (Yoo *et al.*, 1996).

Additionally, various food grade supports have also been evaluated, such as cereals which have been found to provide protection to *L. plantarum* and increased viability of cells under acidic and bile conditions (Haruhito *et al.*, 2006). Barley, malt and wheat have also been reported as good carriers for probiotic, leading to enhanced growth (Charalampopoulos *et al.*, 2002). Apple pieces have been used as immobilization supports for *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* in wine making, while *L. casei* was immobilized on apple pieces to ferment milk (Kourkoutas *et al.*, 2005). These studies showed increased viability during storage and improved sensory quality of the fermented products upon

cell immobilization. Apple and pear pieces were also found to be suitable immobilization carriers for *L. casei* in cheese production, with improved viability, taste and aroma of the final product (Kourkoutas *et al.*, 2006).

Agrowastes contain sugars, fibers and minerals which could be utilized by microorganisms (Rodriguez-Couto, 2008). Hence, agrowastes could be used as cells immobilizers in fermentation and this could also serve as an alternative to overcome environmental issues due to the utilization of waste generated. Also, the low cost of agrowastes is an added advantage to be considered as immobilizers. However, less information is available on the potential of agrowastes as immobilizers for probiotics. Additionally, the growth properties of immobilized probiotic on agrowastes in a liquid medium remain unknown.

## 2.4.3 Mechanisms of Cell Immobilization

Most microbial immobilization methods that are currently available can be applied to lactic acid bacteria. In general, immobilization methods are divided into two categories, namely the 'passive' and 'active' methods.

Passive immobilization can be defined as natural attachment of microorganism on surfaces of carriers and tendency to grow on carriers (Robinson *et al.*, 1986). Various types of carriers had been used to immobilize cells. Most carriers used are either natural or synthetic. It has to be noted that passive immobilization does not require any interaction of chemical bonding, charges between surfaces and cross linking. In general, food ingredients, food grade supports, plants, fruits and organic compounds are used as natural carriers for passive immobilization. Apple pieces have been used as support material for *Saccharomyces cerevisiae* immobilization in wine production (Kourkoutas *et al.*, 2006). Wine produced by

Active immobilization can be defined as attachment of cells to support material via charges between cells and support, chemical bonding and cross-linking surface. Most supports of active immobilization required chemical treatments which are not coat effective. Basically, active immobilization involves flocculant agents, chemical attachment and gel entrapment (Codd, 1987). Flocculant function as binding of cells to surface through adsorbing different charges between cells and surfaces. Chitosan is the most widely used flocculant agent and could be obtained from chitin alkaline deacetylation. The chemical structure of chitosan is a linear amino polysaccharide of $\beta$-D-glucosamine (2-amino-2-deoxy-$\beta$-D-glucan) units joined by 1-4 linkages (Oungbho and Müller, 1997). The charge of chitosan is positive which can bind cells by absorbing negative-charged cells.

Chemical attachment involves the attachment cells to support materials through chemical interactions such as covalent bonding, ionic strength and cross-linking. Chemical attachment is less used in cell immobilization because chemical interaction such as covalent bonding and cross-linking will damage cellular surface of cell and reduce viability of cells (Codd, 1987).

Gel entrapment is widely applied in cell immobilization for food industries and water treatment facilities. Basically, gel entrapment involves the use of synthetic polymers, natural polysaccharides and proteins (Codd, 1987). Synthetic polymers for gel entrapment include the use of acrylamide, photocrosslinkable resins, polyurethanes and silica gel. Polycarbamoylsulphonate (PCS) was used to immobilize *Paracoccus denitrificans* and it improved survival of cells to greater than 99 % (Willke *et al.*, 1994). Polyvinyl and polyurethane foams were used to immobilize cells in order to remove nitrate from water (Garbisu *et al.*, 1991). Poly(vinyl alcohol) cryogels were used as support material to immobilize

galactopyranosyl units and (1-4)-linkages to 3,6-anhydro-α-D-galactopyranosyl units (Burdin and Bird, 1994). Agar was used in the immobilization of *Chlorella vulgaris* in biosorption of Cu(II) (Aksu *et al.*, 1998). Cell entrapment into polysaccharides gels such as alginates, carrageenan and agar requires penetration of cells into porous matrix of carrier. Growth of immobilized cells in the porous matrix is affected by various factors including diffusion limitations imposed by the porosity of the material and accumulation of biomass (Park and Chang, 2000).

Cell immobilization has been proven to enhance the growth of cells. Different types of cell immobilization possess varying effects on the growth of cells. It has been found that the use of wheat starch granule as support material in immobilizing *Saccharomyces cerevisiae* successfully increased the growth rate of *S. cerevisiae* (Farmakis *et al.*, 2007). This was attributed to the stability of solid support materials with better mechanical properties such as strength, rigidity and porosity. The surfaces of *S. cerevisiae* and wheat starch granules possessed hydrophilic characteristic, leading to a hydration force between cells. This led to a high stability of cell suspension over a wide range of pH. The immobilization of *S. cerevisiae* onto wheat starch granules also involved physical adsorption without electrostatic or covalent binding between cells and support materials. Wheat starch granules also protected cells without direct interaction between cells and support material (Kourkoutas *et al.*, 2004). Cell immobilization was also found to produce high levels of biomass and prolonged the shelf life of yeast biomass. This was due to the decomposition of the starch granules by yeasts in the absence of sufficient growth nutrients in the medium (Farmakis *et al.*, 2007). Also, the new cells produced were immediately immobilized onto the support material, leading to enhanced grow and longer survival (Farmakis *et al.*, 2007). The immobilization of *L. plantarum* onto

| (A1) | (A2) | (A3) |
|------|------|------|
| Adsorption on a surface | Electrostatic binding on a surface | Covalent binding on a surface |

| (B) | (C1) | (C2) |
|-----|------|------|
| Entrapment within a porous matrix | Natural flocculation (Aggregation) | Artificial flocculation (cross-linking) |

| (D1) | (D2) | (D3) |
|------|------|------|
| Microencapsulation | Interfacial microencapsulation | Containment between microporous membranes |

Insoluble carrier

Liquid phase

Porous matrix

Microporous membrane

Bifunctional reagent (cross-linker)

Electrostatic forces

a. IMMOBILIZATION ON THE SURFACE OF A SOLID CARRIER

b. ENTRAPMENT WITHIN A POROUS MATRIX

c. CELL FLOCCULATION (AGGREGATION)
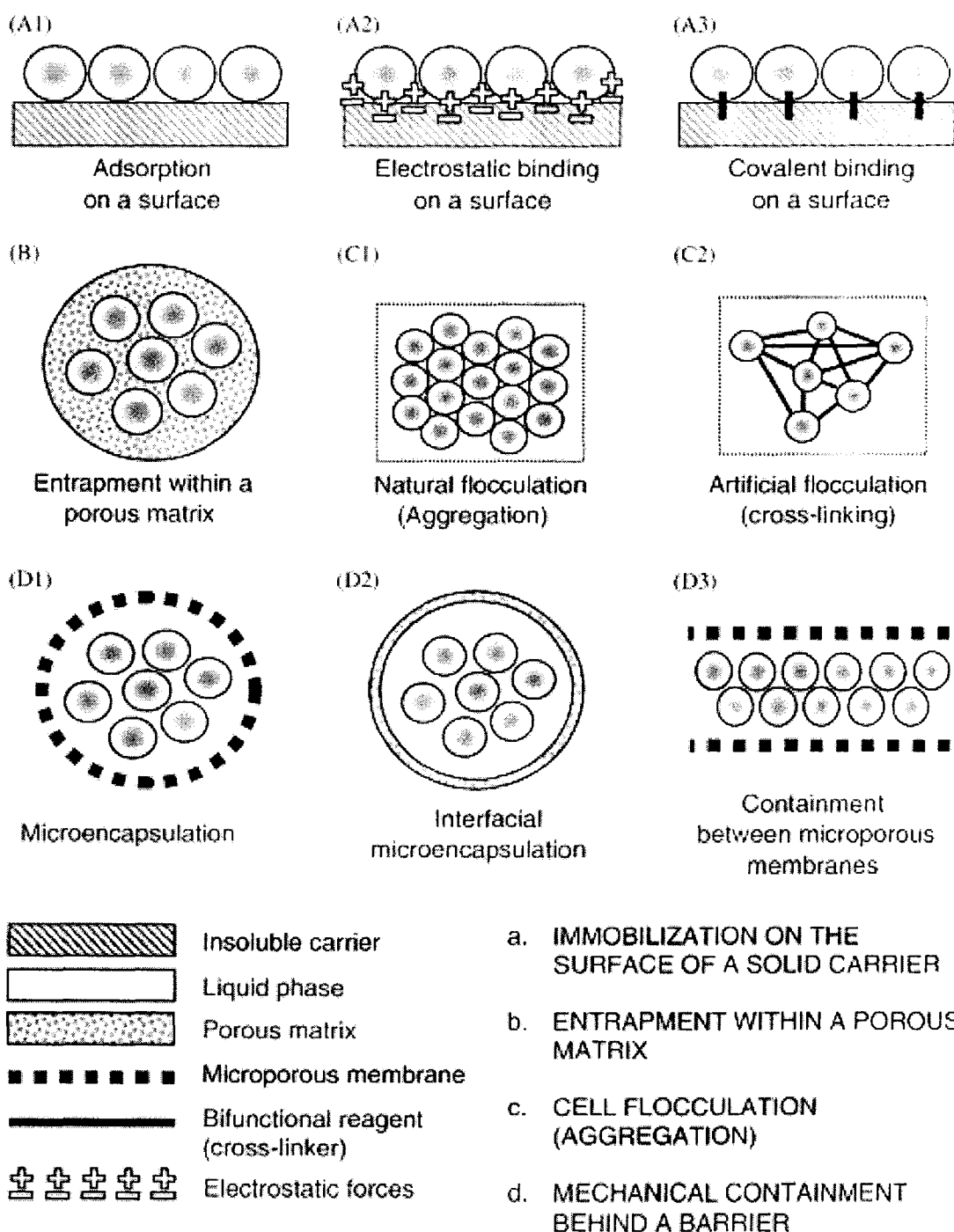
d. MECHANICAL CONTAINMENT BEHIND A BARRIER

**Figure 2.1.** Basic methods of cell immobilization: (A) attachment or adsorption on solid carrier surfaces, (B) entrapment within porous matrix, (C) self-aggregation by flocculation (natural) or with cross-linking agents (artificially induced) and (D) cell containment behind barriers (Pilkington *et al.*, 1998).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Bacterial Strains

Strains of *Lactobacillus bulgaricus* FTCC 0411, *L. casei* FTCC 0442, *L. fermentum* FTCC 0013, *L. acidophilus* ATCC 4962, *L. acidophilus* FTDC 2333, *L. bulgaricus* FTDC 1311, *Lactobacillus* spp. FTDC 1211, *L. acidophilus* FTDC 1733, *L. acidophilus* FTDC 2631, *L. acidophilus* FTDC 2133, *L. bulgaricus* FTDC 1611, *L. acidophilus* FTDC 1331, *L. bulgaricus* FTDC 1511 and *Bifidobacterium bifidium* FTDC 2142, *B. bifidium* FTCC 0012 were obtained from the Culture Collection Centre of School of Industrial Technology, Universiti Sains Malaysia, Penang, Malaysia. Each strain was propagated three times in sterile de Mann, Rogosa, Sharpe (MRS) broth (Himedia®, Mumbai, India) supplemented with 0.05 % (w/v) filter-sterilised (0.20 µm) L-cysteine · HCl (Bioshop®, Burlington, Canada) solution and incubated at 37°C for 20 hours prior to each analysis. Stock culture was stored at -20°C in 40 % (v/v) sterile glycerol.

## 3.2 Screening of Probiotic Strains

### 3.2.1 Acid Tolerance of Probiotic Strains

All probiotic strains were subcultured three times in sterile de Man, Rogosa, Sharpe (MRS) broth using 10 % (v/v) inoculum and incubated at 37°C for 20 h. Simulated gastric juices were prepared by suspending pepsin in 0.5 % (w/v) sterile NaCl to a final concentration of 3 g/L. The pH of simulated gastric juices was adjusted to 2.0, 3.0 and 4.0 with 0.1 M HCl. Probiotic culture (0.2 mL) was pippeted into sterile Eppendorf tube, followed by centrifugation at 14000 rpm and 4°C for 5

enzyme activity is defined as the amount of enzyme that release 1 μmol of *p*-nitrophenol from pNPG per milliliter per minutes under assay conditions. The specific activity was expressed as units (U) of α-galactosidase activity per milligram of protein.

Bradford assay (Bradford, 1976) was used to determine the protein concentrations of the crude enzymes extracts. The assay is based on the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm upon binding to protein.

## 3.3 Preparation of Agrowastes Immobilizers

Agricultural wastes were obtained from local orchards (Penang, Malaysia). They include durian (*Durio zibethinus*), cempedak (*Artocarpus champeden)* and mangosteen (*Garcinia mangostana*). The rind portions were cut into smaller pieces, oven-dried at 70°C for 20 h, milled with an ultracentrifugal mill (Retsch ZM 100; F-Kurt Retsch GmbH & Co., Haan, Germany) and sieved through a no. 80 test sieve (Retsch) using a vibrator sieve shaker (Retsch AS 200). The resultant powder was vacuum-packed and stored at -20°C till further use. They are used as immobilizers for the immobilization of probiotics. The average particle size was 150 μm.

## 3.4 Cell Immobilization

Agrowastes powder 2 % (w/v) was added into universal bottles containing 10 ml of MRS broth. The mixtures were autoclaved at 121°C for 15 minutes. All probiotic strains were subcultured 3 times in sterile de Man, Rogosa, Sharpe (MRS) broth using 10 % (v/v) inoculum and incubated at 37°C for 20 h prior to use. The final subcultures were then centrifuged at 4000 $x$ $g$ for 15 min. The pellets were

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discarded and the pellet was resuspended with McDowell Trump fixative prepared in 0.1M phosphate buffer (pH 7.2) for 2 h. The resuspended samples were centrifuged and the supernatant were discarded. Then, the pellet was resuspended in 1% (w/v) osmium tetroxide prepared in the phosphate buffer for 1 hour. The sample was washed twice with distilled water and dehydrated as follows:

Dehydration:

50% ethanol......................................10 minutes

75% ethanol......................................10 minutes

95% ethanol......................................10 minutes

100% ethanol......................................10 minutes (x2)

Hexamethyldisilazane (HMDS)....................10 minutes

The HMDS was decanted from the tube and the residue was air-dried at 25°C. The dried cells are then mounted on to a SEM specimen stub, coated with gold in a Sputter coater (Polaran Model SC515, England, United Kingdom) for 5 min and examined with a scanning electron microscope (Leo Supra 50VP Field Emission, Oberkochen, Germany).

## 3.8 Microbial Analysis

Viability test of probiotic strains in fermented soymilk was carried out at time 0, 12, 18, 24, 48, 72 and 168 h, using the pour-plate method. MRS agar was added with sterile L-cystein.HCl (0.05 % w/v) and incubated at 37°C anaerobically with gas generating kits (Merck, Darmstadt, Germany).

### 3.8.1 Preparation of MRS Agar

Fifty-two point two grams of powdered MRS broth and 20 g of agar-agar were mixed with 1 L of distilled water. The mixture was then stirred with magnetic stirrer bar while heated on hot plate until the agar was dissolved and the mixture was homogenized. Then, the mixture was sterilized in an autoclave (Hirayama Autoclave HA 240, Japan) at $121^{\circ}C$ for 15 min.

### 3.8.2 Preparation of peptone solution

Peptone solution was used as the diluents for serial dilution in the determination of cellular viability. 20 g of peptone powder (Fluka Chemie, Steinheim, Germany) was dissolved in 1 L of distilled water. The mixture was homogenized before pippetted into universal bottles. After that, the peptone solution was autoclaved at $121^{\circ}C$ for 15 min.

### 3.8.3 Dilution and Pour Plate

Fermented soymilk was vortexed with glass beads to release the immobilized cells from the carrier. Then, 1 ml of fermented soymilk was pippetted into a universal bottle containing 9 ml of peptone solution and vortexed. This homogenate represented $10^{-1}$ dilution. Then, subsequent 10-fold serial dilutions were produced. Then, 1 ml of homogenate from each dilution was pippetted into each sterilized petri dish, pour-plated by sterilized MRS agar supplemented with 0.15 % (w/v) L-cysteine·HCl. Then, the pour-plated samples were incubated at $37^{\circ}C$ anaerobically for 48 hours. After that, the colonies were counted as $\log_{10}CFU/g$.

(0.1 N) in order to precipitate residual proteins. The aliquots were filtered through a 0.20 μm filter (Sartorius, Goettingen, Germany). Organic acid contents were determined by high performance liquid chromatography (HPLC), using a Rezex ROA-organic acid H 300 x 7.80mm column (Phenomenex, California, USA), equipped with a HPLC Pump (Waters, Milford, Ireland) and a 2487 Dual $\lambda$ Absorbance Detector (Waters, Milford, Ireland). Sulfuric acid (0.001 N) was used as the mobile phase with a flow rate of 0.5 ml/min and pressure 500 psi. Organic acids such as acetic and lactic acids were detected at 254 nm. Known concentrations of acetic and lactic acids were used as standards.

### 3.11 Determination of pH

pH of the control and probiotic fermented soymilk was determined at time 0, 12, 18, 24, 48, 72 and 168 h using a pH meter with glass electrode (DELTA 320, Shanghai, China).

### 3.12 Proteolyitc Activity

Proteolytic activity was determined by measuring free $NH_3$ groups released from amino acids and peptides by using the $o$-phthaldialdehyde (OPA) method (Yeo and Liong, 2009). Fermented soymilk was centrifuged at 4000 x g for 10 min at 4°C, and 150 μl of the supernatant was added to 3 ml of OPA reagent. The mixture was then incubated at 25°C for 2 min. The absorbance of the solution was measured spectrophotometrically at 340 nm (Thermospectrosonic, Wisconsin, USA). The proteolytic activity of probiotic in the fermented soymilk was expressed as the absorbance of free amino groups measured at 340 nm compared to the unfermented soymilk as blank.

## 3.13 Angiotensin-converting Enzyme (ACE) Assay

The ACE-inhibitory activity was determined as previously described (Yeo and Liong, 2009). Briefly, fermented soymilk was centrifuged at 4000 x g for 15 min at 4°C. The supernatant was collected and the pH was subsequently adjusted to 8.3 with 2 M NaOH. The suspension was then centrifuged at 10000 x g for 10 min at 4°C, and 10 μl of the supernatant was mixed with 100 μl of hippuryl-histidyl-leucine buffer (5 mM Hip-His-Leu in 0.1 M phosphate buffer containing 0.3 M NaCl, pH 8.3) and 30 μl of phosphate buffer. The mixture was then pre-incubated for 5 min at 37°C. Subsequently, 20 μl of ACE solution (buffer containing ACE at a concentration of 0.1 unit/mL) was added into the mixture and incubated at 37°C for 30 min. The reaction was stopped by adding 125 μl of 1 M HCl and 850 μl of ethyl acetate. The mixture was centrifuged at 4000 x g for 5 min and 700 μl of the organic phase was transferred to a fresh test tube, prior to evaporation to dryness in a water bath at 100°C. The residue containing hippuric acid was dissolved in 2 ml of deionized water. The absorbance of the solution was measured spectrophotometrically at 228 nm with deionized water as a blank. The inhibition of ACE was calculated based on the equation below:

The ACE inhibitory activity (%) is expressed as:

$$ACE (\%) = [1- (b - c)/a] \times 100 \qquad (1)$$

Where   a: the absorbance of the reaction mixture without sample

b: the absorbance of the reaction mixture with sample

c: the absorbance of the blank solution (replace ACE solution with buffer)

## 3.14 Statistical Analysis

Data analysis was performed using SPSS Inc. software (version 15.0) (Chicago, Illinois, USA). A repeated measure ANOVA was used for time-based analyses. One-way ANOVA was used to evaluate the significant differences between sample means, with significance level at $\alpha = 0.05$. Mean comparisons were assessed by Tukey's-test. All data presented were mean values of duplicates, obtained from two separate runs (n=4), unless stated otherwise.