CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

The lactic acid bacteria are a group of Gram positive bacteria, non-respiring, non-spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. They are the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread, sorghum beer, all fermented milks, cassava (to produce *gari* and *fufu*) and most "pickled" (fermented) vegetables. Historically, bacteria from the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* are the main species involved. Several more have been identified, but play a minor role in lactic fermentations (Axelsson, 1998).

These organisms are heterotrophic and generally have complex nutritional requirements because they lack many biosynthetic capabilities. Most species have multiple requirements for amino acids and vitamins. Because of this, lactic acid bacteria are generally abundant only in communities where these requirements can be provided. They are often associated with animal oral cavities and intestines (e.g. *Enterococcus faecalis*), plant leaves (*Lactobacillus, Leuconostoc*) as well as decaying plant or animal matter such as rotting vegetables, fecal matter, compost, etc. (Dugas, 2008).

The metabolism of LAB are two main hexose fermentation pathways that are used to classify LAB genera. Under conditions of excess glucose and limited oxygen, homolactic LAB catabolize one mole of glucose in the Embden-Meyerhof-Parnas (EMP) pathway to yield two moles of pyruvate. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to

lactic acid. This process yields two moles ATP per glucose consumed. Representative homolactic LAB genera include *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus* and group I lactobacilli. Heterofermentative LAB use the pentose phosphate pathway, alternatively referred to as the pentose phosphoketolase pathway. One mole Glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO_2. The resulting pentose-5-phosphate is cleaved into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP is further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. Theoretically, end products (including ATP) are produced in equimolar quantities from the catabolism of one mole glucose. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, *Weissella*, and group III lactobacilli (Wikipedia, 2008)

2.2 Lactic acid bacteria in health and disease

There has been much recent interest in the use of various strains of lactic acid bacteria as probiotic, *L. acidophilus* and *L. casei*, as viable preparations in food or dietary supplements to improve the health of humans and animals. Lactic acid bacteria were originally defined as certain species of *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Pediococcus* (Hammes and Tichaczek, 1994). However, following recent taxonomic studies and the creation of new genera, the lactic acid bacteria now include *Aerococcus, Alloiococcus*, *Carnobacterium, Enterococcus*, *Lactobacillus, Lactococcus*, *Leuconostoc, Pediococcus, Streptococcus*, *Tetragennococcus* and *Vagococcus* (Axelsons, 1990). These genera consist of Gram-positive bacilli and cocci that metabolize carbohydrates fermentatively, producing lactic acid as the major end-product (homo-fermentative strain) or as a significant component in a mixture of end-product (hetero fermentative strain) (Triamchanchoochai, 2002).

Lactic acid bacteria have been used as probiotic to manage intestinal disorders such as lactose intolerance, acute gastroenteritis due to rotavirus and other enteric pathogens, adverse effects of pelvic radiotherapy, constipation, inflammatory bowel disease and food allergy. These disease states are associated with disturbances of the intestinal microflora and various degrees of inflammation of the intestinal mucosa leading to increased gut permeability. To be successful in the treatment of these condition a probiotic strain should be able to survive gastric acidity, adhere to the intestinal epithelial cells and colonize the intestine at least temporarity. Strains with these characteristics are promising candidates for use in functional foods for the management of specific diseases of the gastrointestinal tract (Triamchanchoochai, 2002).

2.3 Application of Lactic acid bacteria

2.3.1 Chemical product

2.3.1.1 Pure lactic acid production

Lactic acid bacteria can produce lactic acid which used in many industries such as foods, beverages, pharmaceutics and cosmetics (Sakai and Yamanami, 2006).

2.3.1.2 Food preserved substance

Lactic acid bacteria can produce antimicrobial substances such as bacteriocins. These antibacterial peptides are of great interest in food industry as natural preservatives and possible substitutes for chemical preservation (Abee *et al.,* 1995; Cleveland *et al.,* 2001). Bacteriocins produced by lactic acid bacteria have been classified on the basis of their biochemical characteristics in three groups (Klaenhammer, 1993; Nes *et al*., 1996).

Class I bacteriocins, termed lantibiotics, are small and heat-stable peptides that contain thioether amino acid, like lanthionine. The model of this group, nisin, is probably the most well-known and studied bacteriocins produce by lactic acid bacteria (Cleveland *et al*., 2001).

Class II contain small heat-stable, nonmodified peptide. Which are subdivided into Class IIa, or antilisterial peptide, that contain the recently described lactococcin MMFII (Ferchichi *et al.,* 2001) and sakacin G (Simon *et al*., 2002) and Class IIb, consisting in the association of two different peptides for full activity, such as lactococcin G or lacticin F (Nissen-Meyer *et al*., 1992).

Class III contains large heat-labile proteins,this group is not well documented (Muriana and Klaenhammer, 1991).

Nisin is produced by fermentation using the bacterium *Lactococcus lactis*. Commercially, it is obtained from natural substrates including milk and is not chemically synthesized. It is used in processed cheese production to extend shelf life by suppressing gram-positive spoilage and pathogenic bacteria. There are many other applications of this preservative in food and beverage production. Due to its highly selective spectrum of activity it is also employed as a selective agent in microbiological media for the isolation of gram-negative bacteria, yeast and moulds (Wikipedia, 2007).

2.3.1.3 Exopolysaccharides

Lactic acid bacteria producing exopolysaccharides (EPS), also play major industrial role in the production of ferment dairy products, in particular, production of yogurt, ayran (drinking yogurt), cheese, fermented cream and milk-based desserts (De Vuyst and Degeest, 1999; Jolly *et al.,* 2002). Their production significantly contributes to texture, rheology, mouth feel, taste perception and stability of the final products (Pszczola, 2001; Welman and Maddox, 2003). Other physiological benefits include enhancement of gastrointestinal colonization of the EPSs in the

gastrointestinal tract (Welman and Maddox, 2003). In addition, EPSs produced by lactic acid bacteria have been claimed to have antitumor, antiulcer, immunostimulatory effects (Gill, 1998; De Vuyst and Degeest, 1999) and are claimed to lower blood cholesterol levels (Ruas-Madiedo *et al.,* 2001).

2.3.1.4 Biodegradable polymer

Biopolymers are polymers which are present in, or created by, living organisms such as wood, cotton, corn, wheat and bacteria or these polymers from renewable resources such as beets, corns, potatoes and vegetable oils that can be polymerized to create bioplastics. For bacterial cell, there are two ways fermentation can be used to create biopolymers and bioplastics.

- **Bacterial Polyester Fermentation** – Bacteria are one group of microorganisms that can be used in the fermentation process. Fermentation, in fact, is the process by which bacteria can be used to create polyesters. Bacteria called *Ralstonia eutropha* are used to do this. The bacteria use the sugar of harvested plants, such as corn, to fuel their cellular processes. The by-product of these cellular processes is the polymer. The polymers are then separated from the bacterial cells.

- Lactic Acid Fermentation – Lactic acid is fermented from sugar, much like the process used to directly manufacture polymers by bacteria. However, in this fermentation process, the final product of fermentation is lactic acid, rather than a polymer. After the lactic acid is produced, it is converted to polylactic acid using traditional polymerization processes (Greenplastics.com, 2002).

2.3.2 Food fermentation

Food fermentation has been used for centuries as a method to preserve perishable food products. The raw materials traditionally used for fermentation are as diverse as: fruits, cereal, honey, vegetables, milk, meat, fish. Lactic acid bacteria are widely used in the production of fermented food, and they constitute the majority of the volume and the value of the commercial starter cultures. The primary activity of the culture in a food fermentation is to convert carbohydrates to desired metabolites as alcohol, acetic acid, lactic acid or CO_2. In the table 1 shown main food products produced by fermentation with the raw materials used and the type of culture employed (Hansen, 2002).

Table 1 Type of fermented food with a long history of use in large geographical area of the world.

Product	Raw material	Starter culture
Wine	Grape juice	Yeast, Lactic acid bacteria
Bread	Grains	Yeast, Lactic acid bacteria
Soy sauce	Soy beans	Mould (Aspergillus), Lactic acid bacteria
Sauerkraut, Kimchi	Cabbage	Lactic acid bacteria
Fermented Sausages	Meat	Lactic acid bacteria
Fermented milks	Milk	Lactic acid bacteria
Cheese	Milk	Lactic acid bacteria

Source: Hansen, (2002)

2.3.3 Functional foods

Functional food is a dietary ingredient that has cellular or physiological effect above the normal nutritional value such as prebiotics, probiotics, cereal, fibre, etc. (Gibson, 1998).

2.3.3.1 Prebiotic

Prebiotic is a nondigestible food ingredient that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number, of bacteria in the colon that can improve host health (Gibson and Roberfroid, 1995). Prebiotic carbohydrates are found naturally in such fruit and vegetables as bananas, berries, asparagus, garlic, wheat, oatmeal, barley (and other whole grains), flaxseed, tomatoes, Jerusalem artichoke, onions and chicory, greens (especially dandelion greens but also spinach, collard greens, chard, kale, mustard greens, and others), andlegumes (lentils, kidney beans, chickpeas, navy beans, white beans, black beans). And the various oligosaccharides classified as prebiotics and added to processed foods and supplements include Fiber gums, Fructo-oligosaccharides (FOS), Inulins, Isomalto-oligosaccharides, Lactilol, Lactosucrose, Lactulose, Oligofructose, Pyrodextrins, Soy oligosaccharides, Transgalacto-oligosaccharides (TOS) and Xylo-oligosaccharides. Prebiotics have many benefits for health such as anticarcinogenic activity, antimicrobial activity, lower triglyceride levels, stabilize blood glucose levels, boost the immune system, improve mineral absorption and balance, rid the gut of harmful microorganisms and prevent constipation and diarrhea (Innvista.com, 2008).

2.3.3.2 Probiotic

Probiotics are living microorganisms which when ingested have beneficial effects on the equilibrium and the physiological functions of the human intestinal microflora (Fuller, 1992). Probiotic bacteria, which are commensals of the human gut, have been reported to inhibit the growth of undesirable microorganisms and food poisoning bacteria, such as *Salmonella*, that can be encountered in the gastrointestinal tract (Huges and Hoover, 1991; Lim *et al.,* 1993). The characteristics of a successful probiotic are acid and bile tolerance, antimicrobial activity against intestinal pathogens, and ability to adhere and colonize the intestinal tract. Beneficial effects of probiotic include alleviation of lactose intolerance, control of diarrhea, inhibition of intestinal pathogens, enhanced immune response and anticarcinogenic activity (Mishra and Prasad, 2005).

2.4 Beneficial effects of probiotics bacteria

2.4.1 Managing Lactose Intolerance

Because of lactic acid bacteria convert lactose into lactic acid, their ingestion may help lactose intolerant individuals tolerate more lactose than what they would have otherwise (Sanders, 2000).

2.4.2 Prevention of Colon Cancer

In laboratory investigations, lactic acid bacteria have demonstrated anti-mutagenic effects thought to be due to their ability to bind with (and therefore detoxify) hetrocylic amines; carcinogenic substances formed in cooked meat (Wollowski *et al.,* 2001). Animal studies have demonstrated that lactic acid bacteria can protect against colon cancer in rodents, though human data is limited and conflicting. Most human trials have found that lactic acid bacteria may exert anti-carcinogenic effects by decreasing the activity of an enzyme called

β-glucuronidase (which can regenerate carcinogens in the digestive system) (Brady *et al*., 2000).

2.4.3 Cholesterol Lowering

Animal studies have demonstrated the efficacy of a range of lactic acid bacteria to be able to lower serum cholesterol levels in animals, presumably by breaking down bile in the gut, thus inhibiting its reabsorption (which enters the blood as cholesterol). Some, but not all human trials have shown that dairy foods fermented with lactic acid bacteria can produce modest reductions in total and LDL cholesterol levels in those with normal levels to begin with, however trials in hyperlipidemic subjects are needed (Sanders, 2000).

2.4.4 Lowering Blood Pressure

Several small clinical trials have shown that consumption of milk fermented with various strains of lactic acid bacteria can result in modest reductions in blood pressure. It is thought that this is due to the ACE (Angiotensin Converting Enzyme) inhibitor like peptides produced during fermentation (Sanders, 2000).

2.4.5 Improving Immune Function and Preventing Infections

Lactic acid bacteria are thought to have several presumably beneficial effects on immune function. They may protect against pathogens by means of competitive inhibition (i.e., by competing for growth) and there is evidence to suggest that they may improve immune function by increasing the number of IgA-producing plasma cells, increasing or improving phagocytosis as well as increasing the proportion of T-lymphocytes and Natural Killer cells (Reid *et al*., 2003; Ouwehand *et al*., 2002). Clinical trials have demonstrated that probiotics may decrease the incidence of respiratory tract infections and dental caries in children (Nase *et al*., 2001) as well as aid in the treatment of *Helicobacter pylori* infections (which cause peptic ulcers) in adults when used in combination with standard medical treatments (Hamilton-Miller,

2003). Lactic acid bacteria foods and supplements have been shown to be effective in the treatment and prevention of acute diarrhea; decreasing the severity and duration of rotavirus infections in children as well as antibiotic-associated and travelers diarrhea in adults (Reid *et al*., 2003; Ouwehand *et al*., 2002).

2.4.6 Reducing Inflammation

Lactic acid bacteria foods and supplements have been found to modulate inflammatory and hypersensitivity responses, an observation thought to be at least in part due to the regulation of cytokine function. Clinical studies suggest that they can prevent reoccurrences of Inflammatory Bowel Disease in adults (Reid *et al*., 2003) as well as improve milk allergies (Kirjavainen *et al*., 2003) and decrease the risk of atopic eczema in children (Kalliomaki *et al*., 2003).

2.5 Charecteristics of a good probiotic (Triamchanchoochai, 2002)

2.5.1 Should be a strain which is capable of exerting a beneficial effect on the host e.g. increased growth or resistance to disease.

2.5.2 Should be non-pathogenic and non-toxic.

2.5.3 Should be present as viable cells, preferably in large number, although we do not know the minimum effective dose.

2.5.4 Should be capable of surviving and metabolizing in the gut environment, e.g. resistant to low pH and organic acids.

2.5.5 Should be stable and capable of remaining viable for long periods under storage and field conditions.

However, the microorganism can not survive during the processing and shelf life of food and supplements, transit through high acidic conditions of the stomach and bile salts in the small intestine (Kailasapathy, 2002; Mandal, 2006).

Microencapsulation has been considered have good potential for protecting probiotic bacteria against adverse conditions in food and during passage through the GI tract. Microencapsulation has been shown to be capable of protecting bacterial cells by retaining them within a polymer membrane or matrix and yielding increased survival (Audet *et al.,* 1988; Sheu and Marshall, 1993).

2.6 Microencapsulation

Microencapsulation is a process in which the cells are retained within an encapsulating matrix or membrane (Krasaekoopt *et al.,* 2004). Microcapsules are small particles that contain an active agent or core material surrounded by a coating or shell, wall material, carrier or encapsulant (Vilstrup, 2001 and Madene *et al.,* 2006).

Microencapsulation are several techniques such as spray drying, coacervation, cocrystrallization and freeze-drying, have been widely applied in food industry for encapsulating vitamins, minerals and other sensitive ingredients (Yoo *et al.,* 2006). Microencapsulation of probiotic in hydrocolloid beads has been tested for improving their viability in food products and GIT transit (Kebary *et al.,* 1998).

Hydrocolloid beads can be classified into 2 groups, depending on the method used to form the beads: extrusion (droplet method) and emulsion or two-phase system. Both extrusion and emulsion technique increase the survival of probiotic bacteria by up to 85-90% (Audet *et al.,* 1988; Rao *et al.,* 1989).

2.6.1 Extrusion technique

Extrusion method is the oldest and most common procedure of producing hydrocolloid capsules (King, 1995). In general, it is a simple and cheap method with gentle operations which makes cell injuries minimal and causes relatively high viability of probiotic cells. Biocompatibility and flexibility are some of the other specifications of this method (Klien *et al*., 1983; Tanaka *et al*., 1984; Martinsen *et al*., 1989). However, the most important disadvantage of this method is that it can not be feasibly used for large-scale production due to slow formation of the microbeads. In other words, it is difficult to be scaled up. Generally, the diameter of beads formed in this method (2-5 mm) is larger than those formed in the emulsion method. Extrusion method in the case of alginate capsules consists of the following stages: preparation of hydrocolloid solution, addition of probiotic cells into the mentioned solution in order to form cell suspension and extrusion of the cell suspension through syringe needle in a way that the resulting droplets directly drip into the hardening solution (setting batch). Hardening solution consists of multivalent cations (usually calcium in the form of calcium chloride). After dripping, alginate polymers immediately surround the added cells and form three-dimensional lattices by cross linkages of calcium ions (Krasaekoopt *et al*., 2003) (Figure 1). It is common to apply concentration ranges of 1-2% and 0.05-1.5 M for alginate and calcium chloride, respectively. Most of the generated beads have a range of 2-3 mm in diameter (Krasaekoopt *et al*., 2003). This parameter is strongly influenced by the factors such as type of alginate, viscosity of alginate solution, distance between the syringe and setting batch and particularly diameter of the extruder orifice (needle) (Smidsrod and Skjak-Braek, 1990)

2.6.2 Emulsion technique

Emulsion technique has been successfully applied for the microencapsulation of lactic acid bacteria (Audet *et al*., 1988; Lacroix *et al*., 1990). In contrary with the extrusion technique, it can be easily scaled up and the diameter of produced beads is considerably smaller (25 μm-2 mm). However, this method requires more cost for performance compared with the extrusion method due to need of using vegetable oil for emulsion formation (Krasaekoopt *et al*., 2003). In this technique, a small volume of cell/polymer slurry (as a dispersed phase) is added to the large volume of vegetable oil (as a continuous phase) such as soy-, sun flower-, corn-, millet or light paraffin oil (Groboillot *et al*., 1993). Resulting solution becomes well homogeneous by proper stirring/ agitating, till Water-in-oil emulsion forms. Emulsifiers can be used for better emulsion formation. Tween 80 at the concentration of 0.2% has been recommended as the best choice (Sheu and Marshall, 1993; Sheu *et al*., 1993). Once W/O emulsion forms, the water soluble polymer becomes insoluble after addition of calcium chloride, by means of cross linking and thus makes gel particles in the oil phase. Smaller particles of the water phase in W/O emulsion will lead to the formation of beads with smaller diameters. Agitation rate of the mixture and type of emulsifier used are also determinable factors from the beads diameter point of view (Krasaekoopt *et al*., 2003) (Fig. 1). Using emulsifiers causes formation of beads with smaller diameters, because these components decrease interfacial tension of the water and oil phases. It has been claimed that by applying emulsifiers of tween 80 and lauryl sulphate together, beads with a range of 25-35 μm in diameter can be produced (Sheu and Marshall, 1993). It has been reported that concentration and viscosity of the encapsulation mix before gelation and its agitation rate are the main parameters that control the diameter of the final formed microbeads (Truelstrup-Hansen *et al*., 2002). It should be reminded that the beads diameter, apart from having a crucial effect on the viability of probiotic cells, their metabolic rate and sensory properties of the final product, also affects distribution and dispersion quality of the microbeads within the product (Picot and Lacroix, 2003).

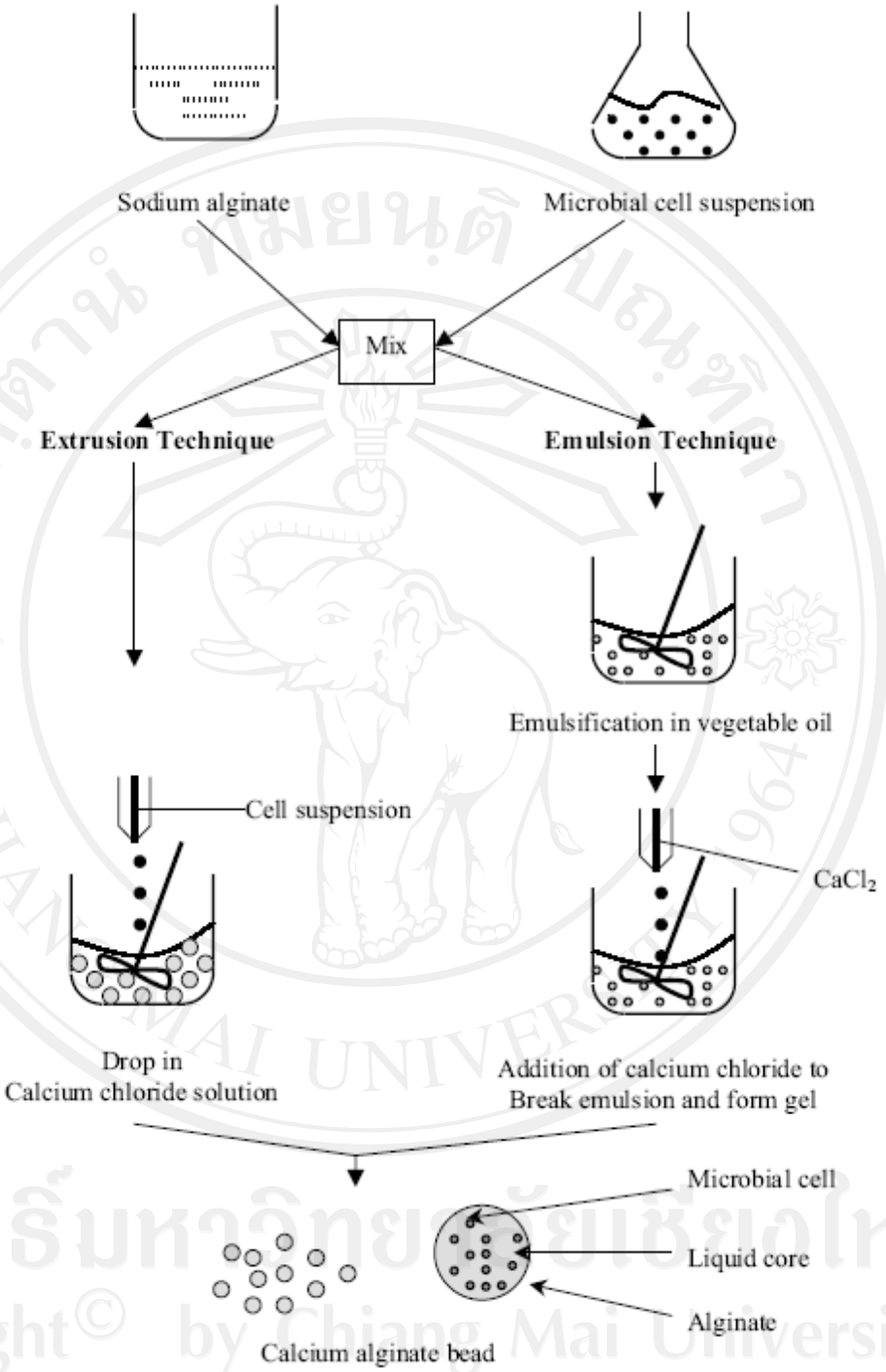


Figure 1 Flow diagram of encapsulation of bacteria by the extrusion and emulsion techniques (Krasaekoopt *et al.*, 2003).

2.7 Groups of encapsulation materials

The natural polymers used as carrier materials in the encapsulation technology have the great advantage of being nontoxic, biocompatible and biodegradable. The most used natural polymers are algal polysaccharides such as alginate carrageenan and agarose. Chitosan and aminopolysaccharide derived from chitin Gellan gum is an extracellular anionic polysaccharide secrete from microorganisms, have also been proposed for some applications (Murano, 1998).

2.7.1 Alginate

Alginates constitute a family of linear binary copolymers of I,4-linked β-D-mannuronic acid and α-L-guluronic acid of varyingcomposition and sequence (Fig.2). These two sugars are arranged in the alginate molecules in homopolymeric regions, blocks of guluronic (GGGGG . . .) and mannuronic residues (MMMMM . . .) and regions of alternating guluronic and mannuronic residues (GMGMGM . . .). The composition and block structure of the alginate molecules are very dependent upon algal source and tissue, and are important in controlling the gelling characteristics of the alginate and consequently its functional properties as immobilization matrix (Haug *et al.,* 1974; Smidsrod and Skjak-Braek, 1990). In particular, the length of the G blocks is the principal structural feature contributing to gel formation (Smidsrod, 1974). Since its introduction as a suitable entrapment matrix in 1977 (Kierstan and Bucke, 1997) alginate remains the preferred material for entrapment of cells, enzymes and drugs (Caunt and Chase 1987; Skjak-Braek and Martinsen, 1991; Tzeng *et al.,* 1991; Declerck *et al.,* 1996; De Riso *et al.,* 1996; Walsh *et al.,* 1996).

Alginates are produced by marine brown algae of the genera *Macrocystis, Laminaria, Eklonia, Ascophvllum, Fucus and Pelvetia* and by bacteria such as *Azotobacter vinelandii* (Gorin and Spencer, 1966) and *Pseudomonas* (Govan *et al.,* 1981).

These polyuronates found many applications in food and other industrial uses as thickening, stabilizing, gelling and film forming agents (Indergaard and Ostgaard, 1991). They give thermally irreversible ionotropic gels in the presence of cations such as lead, copper, cadmium. barium, strontium, calcium and zinc, with the regions of guluronic blocks mainly responsible for the gelation. Mechanical and swelling characteristics of alginate gels, and consequently release properties, are strongly affected by the monomer composition, block structure and molecular weight of alginate molecules (Martinsen *et al.,* 1989; Smidsrod and Skjak-Braek, 1990; Aslani and Kennedy, 1996; Draget *et al.,* 1996). In the case of calcium-alginate the stability of the gel is reduced by the presence of chelating agents such as lactate, phosphate and citrate. Selection of alginates with definite chemical structure and relationship between structure and functional properties depends on the cell or substance to been capsulated and the final goal to be achieved.

2.7.2 Agar/ Agarose

Agarocolloids are a family of gel-forming galactans derived mainly from the red algae *Grlidiunz, Gracilaria and Pterocladia* (Craigie, 1990). Historically, the "agar-type" galactans were separated into two components: agarose, a nonsulphated fraction, and agaropectin, a mixture of various sulphated molecules (Araki, 1966). However, the study of Duckworth and co-workers demonstrated that agar may be regarded as a complex family of polysaccharides containing several extremes in structure (Duckworth and Yaphe, 1971). The basic structure of this binary linear copolymer consists of strictly alternating 3-O-linked β-D-galactopyranose and 4-O-linked α-L-galactopyranose with a large part of the 4-O-linked galactose units existing as the 3.6- anhydro form (Fig. 2) (Araki, 1956; Rees, 1969). Such structural regularity may be masked or modified in a number of ways by the substitution of hydroxyl groups with sulphate hemiesters and methyl ethers in various combinations and more rarely with a cyclic pyruvate ketal as 4,6-O-(1 -carboxyethylidene) group (Duckworth and Yaphe, 1971; Craigie, 1990).

Agar and agarose aqueous solutions are clear and form, between 32 and 40 ^{o}C, firm gels which do not melt below 85 ^{o}C. Gels exhibit a mechanical strength weaker than those of carrageenan and alginate, however, the procedure for preparing the gel is simple and does not require ions or gel stabilization. The type and extent of chemical substitution and molecular weight and molecular weight distribution widely affect the physical properties of agar polymers. The gelation behaviour of agar is determined by the regularity of alternation of D-galactose and 3,6-anhydro-L-galactosere residues, the content and position of charged groups like sulphate and pyruvate, the content of methyl ethers and the molecular weight and molecular weight distribution of the native polymers (Guiseley, 1970; Watase and Nishinari, 1983; Armisen and Galatas, 1987; Murano, 1995). In particular, two kinds of agar can be chemically and physically distinguished by their methoxyl content and gelling temperatures: agars obtained from *Gelidium* and *Pterocladia* having gelling temperatures between 30 ^{o}C and 36 ^{o}C, and agars produced by *Gracilaria* with gelling temperatures around 40 ^{o}C. The gel structure, which is based on cross-links involving noncovalent co-operative binding of chains in ordered conformations, is greatly affected by these chemical substituents. In fact. aggregation of helices, and consequently the typical hysteresis of the thermoreversible order-disorder transition of agar, can be highly perturbed by the presence of charged groups which can interfere with intermolecular hydrogen bonding, stabilizing the ordered regions (Norton *et al.,* 1986; Tako and Nakamura, 1988).

Agar and agarose have been extensively used and are continuing to be used as stabilizing and gelling additives in food preparations. However, agar became best known as a thermoreversible ion-independent solidifier in bacteriology. Recently, agar and agarose have found application also in pharmaceutical products as a thickener and suspending and gelling agent, in formulations for controlled-release of drugs and in biotechnology for immobilization of bacteria, yeasts and animal cells (Gin *et al.,* 1987; Guiseley, 1987; Renn, 1990; Neufeld *et al.,* 1991; Tun *et al.,* 1996).

2.7.3 κ-carrageenan

Among algal polysaccharides, carrageenans exhibit the greatest diversity with respect to molecular structure and range of properties. There is a continuous spectrum of carrageenan molecules and several types have been described as differing in their sulphate content. The major algal sources of carrageenan are the genera *Chondrus, Eucheuma, Ahnfeltia* and *Gigartina* (Craigie, 1990). All carrageenans have a common backbone of alternating β (1,3)-D-galactose and α (I,4)-D-galactose with the " κ " family composed of linear chains in which the β (1,3)-unit is a 4-sulphate-D-galactose (Figure 2) (Bodeau-Bellion, 1983). Highly viscous κ-carrageenan water solutions produce gels in the presence of potassium, sodium and calcium ions (Rochas and Rinaudo, 1984). Gelation conditions strongly affect the properties of the final gel matrix.The mechanical strength of the gel increases with increasing carrageenan concentration whereas monovalent cations such as potassium caesium and ammonium give stronger gels compared with mono- and bivalent metal ions (Stanley, 1987). Recently, κ-carrageenan has found application in the immobilization field for the encapsulation of bacterial, fungal, algal and plant cells (Tosa *et al.,* 1979; Chevalier and de la Noue, 1985; Willetts, 1988; Lacroix *et al.,* 1990; De Riso *et al.,* 1996).

2.7.4 Chitosan

Chitosan is a linear polyglucosainine obtained by deacetylation of chitin (Figure 2). Like alginate, chitosan forms ionotropic gels at pH below 6.0 by cross-linking with cations and polyanions such as polyphosphate and polyaldehydrocarbonic acid (Vorlop and Klein, 1981). Chitosan has successfully been used to encapsulate bacterial cells, mycelia, drugs (Berthold *et al.,* 1996; Chu *et al.,* 1996; De Riso *et al*., 1996; Shinonaga *et al.,* 1996; Yoshino *et al.,* 1996). Several parameters have shown to affect the preparation, and physical and release characteristics of chitosan-based materials. In particular, the degree of de-acetylation, the pH and NaCI concentration during the gel formation strongly influence the release

percentage of molecules from the chitosan gel (Berthold *et al.,* 1996; Chen *et al.,* 1996).

2.7.5 Gellan

Gellan gum is a natural anionic microbial polysaccharide obtained from *Pseudomonas elodea*. The primary structure is based on a tetrasaccharidic repeating unit consisting of two β-D-glucose, one β-D-glucuronic acid and one α-L-rhamnose residues (Figure 2) (O'Neill *et al.,* 1983). The two acyl groups present in the same glucose residue in the native gellan are removed in the commercial preparations. Gellan gum is capable of gelation upon heating and cooling in the presence of a large variety of ions, among which the divalent calcium and magnesium exhibit the greatest efficiency (Grasdalen and Smidsrod, 1987; Camelin *et al.,* 1993). Gellan gum can be used as a structuring and gelling agent in a wide range of applications to mimic the texture of existing gelling agents or to create new textures (Sanderson, 1990). Recently, it has been proposed for several applications in the field of cell immobilization and controlled release of bioactive materials (Rozier *et al.,* 1989; Norton and Lacroix, 1990; Deasy and Quigley, 1991; Sanzgiri *et al.,* 1993; Alhaique *et al.,* 1996).

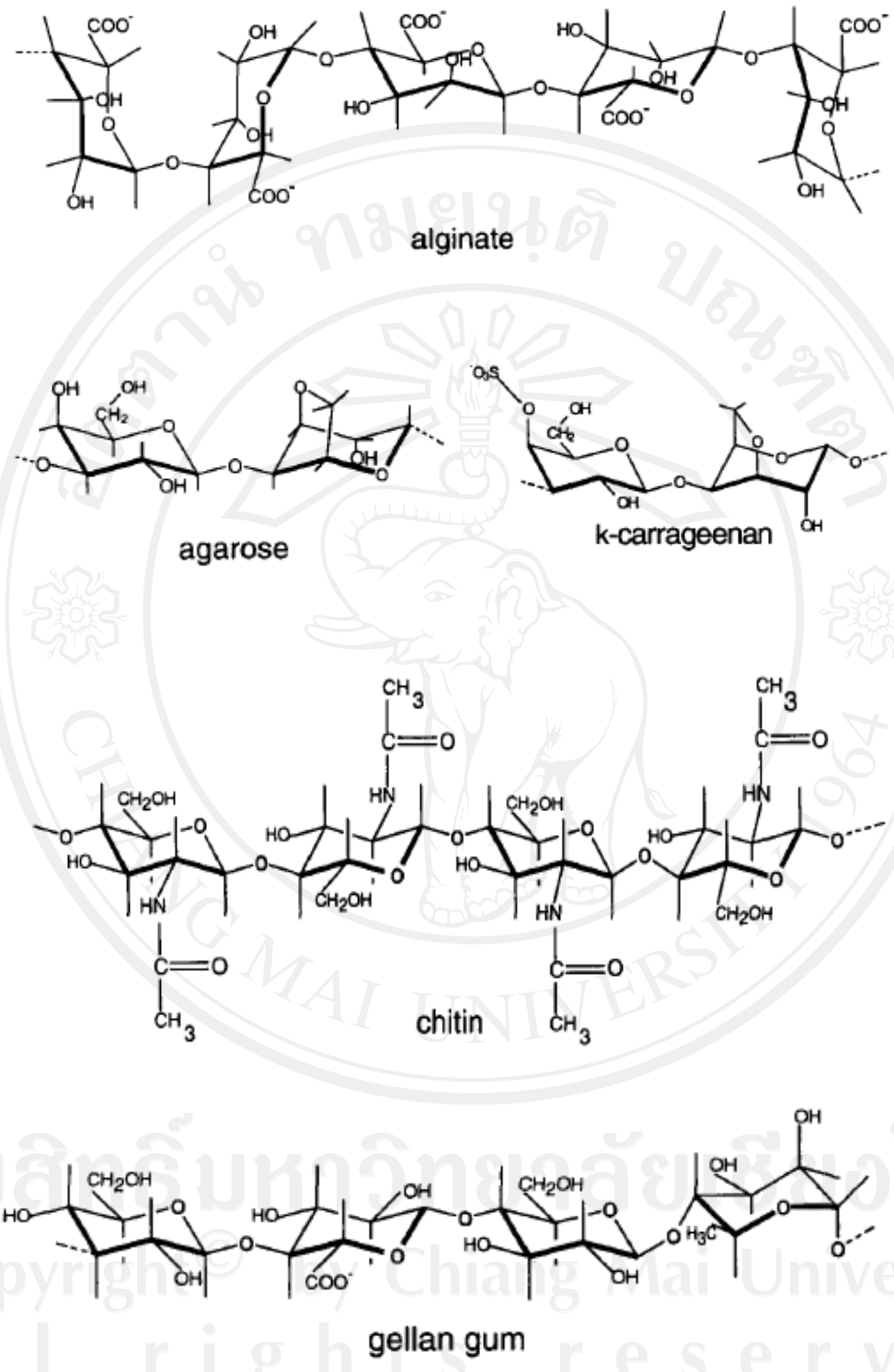


Figure 2 Chemical structure of the repeating sequences of the most used natural polysaccharides in encapsulation techniques (Murano, 1998).

The encapsulation of *B. bifidum* in κ-carrageenan beads maintained cell viability for as long as 24-weeks of cheddar cheese ripening, with no negative effects on texture, appearance and flavor (Dinakar and Mistry, 1994).

Rao *et al*. (1998) found that encapsulation of *Bifidobacterium pseudolongum* with cellulose acetate phthalate (CAP), increased the survival of bacteria under simulated gastric acid conditions as compared with the non-encapsulated bacteria.

Encapsulation of *Bifidobacterium* spp. with calcium alginate significantly improved their viability in mayonnaise with pH 4.4 (Khalil and Mansour, 1998).

Encapsulation of *Lactobacillus rhamnosus* in alginate improved survival at pH 2.0 up to 48 h, while the free cells were destroyed completely (Goderska *et al.,* 2003). Similarly, survivability of *B. longum* were encapsulated with calcium alginate in the simulated conditions of gastric juice (pH 1.5) could be considerably increased (Lee and Heo, 2000).

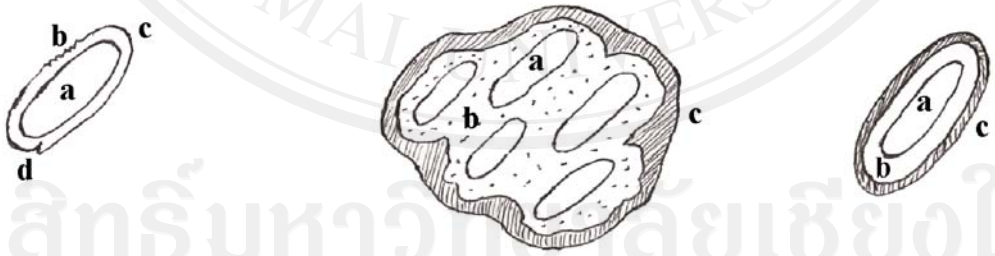
Immobilization of *B. longum* in κ-carrageenan (Adhikari *et al*., 2000) and *B. infantis* in gellan-xanthan beads (Sun and Griffiths, 2000) allowed to maintain high-cell concentrations during 5-week storage of yoghurt, with no change in sensorial properties (Adhikari *et al*., 2000).

The improvement of *B. bifidum* viability in yogurt after encapsulation with calcium alginate was in a way similar that throughout the 3 weeks refrigerated storage at 4 ^{o}C, its viable counts did not fall below 10^7 cfu/ml. Also, no undesirable sensory properties were observed in the final product. The result were also obtained after frozen storage of the product (Sultana *et al*., 2000).

From these studied also confirms that the microencapsulation techniques will be useful for ensuring high number of cell survive in harsh conditions such as during processing in dairy products and during the passage through the stomach and intestinal tract (Kailasapathy, 2002; Krasaekoopt *et al*., 2003).

2.8 Structural details of microbeads

In Fig. 3 presents structural characteristics of microbeads. Each microbead consists of hydrocolloids (also called capsule) coated around the bacterial cell(s). If the capsule has a gel-like structure, the microbead is named gel-bead. Because the geometrical shape of a microbead is usually spherical to elliptical, it is also called a "microsphere". Beads might have even/smooth or rough surfaces (Fig. 3, part 3.1). Each bead might consist of one or several cells. When several cells are enclosed by the capsule, the interstitial liquid from solution fills the free spaces of the microbead. Superficial and / or deep cracks might appear in the beads (Fig. 3, part 3.1). Extension of these cracks leads to pore formation, which considerably reduces the encapsulation efficiency. Microbeads can be coated with a second layer of chemical compounds in order to increase microencapsulation efficiency. The second layer is a so-called coat or support or shell. Microbeads with (Fig. 3, part 3.3) or without the coat are named coated- and uncoated beads, respectively. The constituents entrapped within the coat are known as the "core" (Sultana *et al.*, 2000; Truelstrup-Hansen *et al.*, 2002; Dimantov *et al.*, 2003; Krasaekoopt *et al.*, 2003; Chandramouli *et al.*, 2004).



3.1 3.2 3.3

Figure 3 Structural details of microbeads. 3.1 Single cell bead: Bacterial cell (a), uneven surface (b), even surface (c), crackled surface (d); 3.2 Multicell bead: Bacterial cells (a), interstitial liquid (b), capsule (c); 3.3 Coated bead: Bacterial cell (a), capsule (b), coat/shell (c) (Mortazavain *et al.*, 2007).

2.9 Factors affecting microencapsulation effectiveness of probiotics

2.9.1 Coating of the capsules

For coating of capsules is an efficient way to improve their physicochemical characteristics. For example, shell coating on the alginate capsules makes them resistant to the chelating agents of calcium ions. Also, increases their mechanical strength (Smidsrod and Skjak-Braek, 1990). Coating calcium chloride on the alginate capsules, especially at high concentrations of alginate, makes strong beads with good stratification (Chandramouli *et al*., 2004).

2.9.2 Bead diameter

Final beads diameter are important factors in the encapsulation effectiveness. In parallel with increasing beads diameter, their protective effects against the violent environmental factors increase (Truelstrup-Hansen *et al*., 2002).

2.9.3 Effect of bacteria on the capsule materials

There is a report regarding digestion of starch capsules by encapsulated bacteria (Takata *et al*., 1977). Therefore, prior to selection of a capsule material for encapsulation, ability of the enclosed bacteria digest starch should be considered (Mortazavain *et al*., 2007).

2.9.4 Modification of capsule materials

Chemical modification of capsule materials is a common practice to improve encapsulation effectiveness. Structural modification of the capsule materials might be done by direct structural changes and/or addition of special additives. For example cross-linked alginate matrix (produced at low pHs) is obtained from modified alginate structures applied to probiotics encapsulation (Mortazavain *et al*., 2007).

2.9.5 Initial concentration of microbial cells

As concentration of microbial cells in the encapsulation solution increases, the number of entrapped cells in each bead (cell load) and as a result, quantitative efficiency of encapsulation increases (Mortazavain *et al.,* 2007).

2.9.6 Conditions of processing factors

Special attention should be made on the processing factors during microencapsulation process such as freezing (cryogenic freezing or freeze drying), spray drying and storage conditions in order to avoid injuries to the beads and contained cells (Sultana *et al*., 2000). Also, process factors can influence some important parameters related to bead effectiveness such as beads diameter (Truelstrup-Hansen *et al*., 2002).

2.10 Application of Microencapsulation (Sriamornsak, 1998)

Microencapsulation have found application in many fields such as in pharmaceutical, agriculture, food, cosmetics, photography and printing etc.

2.10.1 Agriculture and veterinary applications

- Sustained release of pesticides.
- Slow release of fertilizers.
- Isolation of animal feed additions.
- Delayed release of biocontrol agents.
- Food and dietary supplements of animals.

2.10.2 Food applications

- Extended shelf life stability of ingredients, flavors and aroma.
- Increased oxidative stability of nutrient oils and additives.
- Improved protection of vitamins, nutrients and minerals.
- Odor, taste and color masking.

2.10.3 Commercial/consumer applications

- Triggered release of fragrance.
- Release-on-demand in deodorants and antiperspirants.
- Isolation of detergent additives.

2.10.4 Industrial applications

- Isolation of reactive adhesive components.
- Controlled release of oil well additives.
- Thermal release of catalysts for polymer curing.

2.10.5 Pharmaceutical applications

- Controlled release of orally ingested drugs.
- Prevention of protein denaturation.
- Taste masking.
- Rapid dissolution of drugs.
- Targeted delivery of drugs.