CHAPTER 2
LITERATURE REVIEW

2.1 Bifidobacteria in starter culture

Bifidobacteria were first isolated in 1899 by Frenchman Tissier from the faeces of healthy infants fed on breast milk. For initial isolation, general anaerobic condition is essential. However, the bacteria could slightly grow on aerobic plates in the presence of CO_2 gas (Ballongue, 1998). Currently, 30 different species of bifidobacteria have been identified which have been isolated from different sources such as the faeces of humans, animals, birds and sewage, human vagina, bees, and dental caries (Scardovi, 1986; Tamime, 2002). Nine species of bifidobacteria, including *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium catenulatum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium dentium* have been isolated essentially from humans (Ballongue, 1998). Among which only six species have attracted attention in the dairy industry for the manufacture of "bio" fermented dairy products. These organisms are *B. adolescentis*, *B. breve*, *B. bifidum*, *B. infantis*, *B. lactis*, and *B. longum*. Bifidobacteria which are isolated from human origin should adhere to cells in the walls of the colon of another human. However, *Bifidobacterium thermophilum* and *B. pseudolongum*, which were isolated from animals, also adhere to both human and animals. It was reported that non-human strains of *Bifidobacterium animalis*, which has been reclassified as *B. lactis*, can adhere to human cells in tissue culture, so that the question of which species should be permitted in bio-yogurts is a matter of some discussions (Tamime, 2002). The important criteria for selection of probiotic organisms are acid and bile tolerance and adherence properties (Shah, 2000). Acid and bile tolerance present the probiotic ability to survive in the acidic environment of the

fermented milk products and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive in the bile concentrations usually encountered in the intestine. Not all probiotic strains are acid and bile tolerant (Lankaputhra and Shah, 1995). Clark *et al.* (1993) and Lankaputhra and Shah (1995) showed that *B. longum* survives in acidic conditions and is able to tolerate a bile concentration as high as 4%. Dave and Shah (1997) reported that acid and bile tolerance is strain dependent. Adherence property is one of the important selection criteria for probiotic bacteria. The desired effects of probiotic microorganisms are produced only if probiotic are able to adhere, colonize, and multiply in the intestine. The ability of probiotic bacteria to adhere to the intestine will improve their chances of winning the competition against "unfriendly bacteria" to occupy the intestinal "niches". Adherence to the intestinal cell wall should be taken to select strains on the basis of these attributes (Shah, 2001).

2.1.1 Morphology of bifidobacteria

Bifidobacteria are Gram-positive, non-motile, obligately anaerobes, non spore forming rods with dimension of 0.5–1.3 x1.5–8 μm. The rods of bifidobacteria often have an irregular shape, with a slightly curved central region, and swollen ends. Bifidobacteria do not form long chains, but it is not unusual to encounter cells that are coccoid. The cells may be V-shaped, Y-shaped, X-shaped, bent or club-shaped rods depending on the constituents of the medium on which the colony is growing (Ballongue, 1998; Tamime, 2002). The cell morphology could change to produce more branched cells, when the cell grow under adverse conditions. For example, in a medium deficient in N-acetylglucosamine and amino acids, the cells become more branched. The addition of certain amino acids such as serine, alanine or aspartic acid can transform X-shaped or Y-shaped cells into curved rods. Similarly, Samona and Robinson (1991) transformed coccoid cells of *B. bifidum* into the Y-shaped cells through the

addition of sodium chloride to a medium. However, it was noted that neither *B. longum* nor *B. adolescentis* reacted in the same way. Changing of the morphology results in altering pattern of carbohydrate fermentation. This phenomena suggests that the permeability of cell membrane to certain sugars was being modified in parallel with the structural changes taking place in the cell wall. Nevertheless, this tendency of some species to alter in shape, the cell morphology of bifidobacteria grown anaerobically on the Scardovi's tryptone phytone yeast medium (TPY) showed a tendency to adopt distinctive cellular shapes. For example, *B. bifidum* forms groups of amphora like cells, cells of *B. breve* are the thinnest and shortest among bifidobacteria. *B. longum* appears as very elongated, relatively thin cells with slightly irregular shapes (cited in Ballongue, 1998).

2.1.2 Respiratory of bifidobacteria

Bifidobacteria are catalase-negative, obligate anaerobic microorganisms. The degree of oxygen tolerance depends on the individual species and culture of the medium. Three types of response are observed during the switch from anaerobic to aerobic condition:

- Aerobic growth without the accumulation of hydrogen peroxide (H_2O_2): A strain of *B. bifidum*, which is aerotolerant, forms small quantities of H_2O_2 by reduced NAD or NADH oxidation. The absence of H_2O_2 shown in liquid aerobic culture devoid of catalase of NADH peroxidase activity can be explained by an unknown peroxidase system which can destroy H_2O_2.
- Limited growth with the accumulation of H_2O_2. The accumulation of H_2O_2 is considered to be toxic for fructose-6-phosphate phosphoketolase (F6PPK, EC.4.1.2.22), the key enzyme in the sugar metabolism of *Bifidobacterium.*
- No growth without the accumulation of H_2O_2.

In the presence of CO_2, the sensitivity of bifidobacteria to oxygen varies considerably on the strain. The strains most sensitive to oxygen had low NADH

peroxidase activity, resulting in an accumulation of toxic H_2O_2. Another possibility would be the prevention of multiplication by the presence of active oxygen such as superoxide (cited in Ballongue, 1998).

2.1.3 Optimum temperature and pH of bifidobacteria

The optimum growth temperature of the human species bifidobacteria is 36-38°C. The animal species bifidobacteria have a slightly higher optimum temperature of 41-43°C and it can reach to 46.5°C. Bifidobacleria can not grow at temperature below 20°C or above 46°C. *B. bifidum* dies at 60°C (Ballongue, 1998).

The initial optimum growth pH for bifidobacteria is between 6.5 and 7.0. Bifidobacteria cannot grow below pH of 5.0 or above 8.0 (Ballongue, 1998). Chang, *et al.* (2000) reported that bifidobacteria is sensitive to low pH and it can not survive in the human stomach which has a pH of 1.5-2.0.

2.1.4 Composition of cell wall of bifidobacteria

The principal component of the cell wall of bifidobacteria is peptidoglycan, also known as murein. Peptidoglycan is a macromolecule that consists of linear polysaccharide chains (glucose, galactose, and rhamnose), which are linked to each other by tetrapeptide bridges. The cell wall peptidoglycan consists of linear chains of N-acetyl-muramic acid and N-acetylglucosamine molecules alternating along the length of the chain (Ballongue, 1998).

2.1.5 Starch adhesion, carbohydrate utilization, metabolites, and enzymatic system of bifidobacteria

2.1.5.1 Starch adhesion of bifidobacteria

Some intestinal bacteria can adhere to starch *in vitro* and that adhesion is sometime required for efficient utilization of the substrate. Granular starches synthesized by a number of food plants, for example, native maize, potato, oat,

barley, and tapioca starch. Some starches could escape from the digestion during passage through the human small intestine and arrive in the colon as fermentable carbohydrate sources for intestinal bacteria. These starches are called "resistant starches". In animal models, inclusion of resistant starches in the diet has been shown to increase the population of bifidobacteria in the intestinal tract. Then resistant starches have also been proposed as potential prebiotics. However, the role of adhesion in starch metabolism by bifidobacteria is currently not clear (cited in Crittenden *et al.*, 2001).

Crittenden *et al.* (2001) studied the adhesion of 19 amylolytic and non amylolytic *Bifidobacterium* strains to native maize, potato, oat, and barley starch granules and investigated the links between adhesion and starch utilization. The result showed that starch adhesion was not characteristic of all the bifidobacteria tested. Adherent bacteria bound similarly to the different types of starch and was mediated by a cell surface protein(s). The adhesion model of organisms tested, appeared to be specific for alpha-1,4-linked glucose sugars. The binding capacity of the starch (number of bacteria per gram) correlated to the surface area of the granules. *B. bifidum* VTT E-001559 showed the strong adhesion to starch while *B. longum* CSCC 5532 showed poor adherent. *B. pseudolongum* (ATCC 25526) was the highly adherent strain and has the extracellular amylase activity. Highly adherent strains were able to hydrolyze the granular starches, but not all amylolytic strains were adherent, indicating that starch adhesion is not a prerequisite for efficient substrate utilization for all bifidobacteria. The growth phase during batch fermentation did not influence the degree of adhesion to the starch granules.

2.1.5.2 Carbohydrate utilization of bifidobacteria

In the genus *Bifidobacterium*, hexose is degraded individually and specifically by the fructose-6-phosphate pathway. Different species of bifidobacteria utilise different types of carbohydrates and such different

fermentations are used for identification purposes. One key enzyme involved is F6PPK known as "bifidus shunt". F6PPK cleaves fructose-6-phosphate into acetyl phosphate and erythrose-4-phosphate, and F6PPK can be used to identify the genus of *Bifidobacterium*. However, not all strains produce enough F6PPK that could be detectable (Ballongue, 1998).

2.1.5.3 Metabolites of bifidobacteria

Bifidobacterium spp., carry out a variant form of heterofermentation, in which 1M glucose produces 1M lactic acid and 1.5M acetic acid. However, the proportions of the final fermentation products vary considerably from one strain to another and even within the same species. For example, small quantities of succinic acid are produced by some strains and a small amount of CO_2 may be produced during the degradation of gluconate. The final fermentation products are formed by the sequential action of transaldolase, transketolase, xylulose-5-phosphate phosphoketolase and the enzymes of the Embden Meyerhoff-Parnas pathway, which act on glyceraldehyde-3-phosphate (Ballongue, 1998).

2.1.5.4 Enzymatic system of bifidobacteria

The essential enzyme in sugar metabolism of *Bifidobacterium* is F6PPK. The F6PPK is absent in the anaerobic bacteria, including, *Lactobacillus*, *Acthrobacter*, *Propionibacterium*, *Corynebacterium*, and *Actinomycetaceae*, which could be morphologically confused with the bifidobacteria (Ballongue, 1998).

Transaldolase is apparently an essential enzyme and characteristic of the fructose-6-phosphate shunt. In contrast, 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) is apparently nonfunctional in the bifidobacteria, at least in cells cultured on glucose which are generally deficient in detectable glucose 6-phosphate dehydrogenase (Ballongue, 1998).

2.1.6 Detection method, culture media, and culture parameters of bifidobacteria

2.1.6.1 Detection methods

Several methods could be applied for the detection of bifidobacteria in dairy products, such as plate count method, DNA-based method or enzymatic method (Roy, 2001). The availability of simple and inexpensive methods for detection, identification, and enumeration of *Bifidobacterium* is consequently important in food microbiology (Nebra and Blanch, 1999). According to Hartemink *et al.* (1996), plate count method is still preferable for routinely determination of the initial inoculum and estimation the storage time period that bifidobacteria remain viable, and for quality control measurement in dairy products. The incubation condition is generally anaerobic at 37°C.

2.1.6.2 Culture media

Culture media for bifidobacteria may be divided into basal, elective, differential, and selective culture medium. Non-selective media are useful for routine enumeration of bifidobacteria in fermented or non-fermented milks for determining the initial inoculum and survival during storage (Rasic and Kurmann, 1983; Samona and Robinson, 1991; Arroyo *et al.*, 1994). Reinforced Clostridial Agar and De Man Rogosa Sharpe (MRS) supplemented with cysteine and agar available commercially are the media of choice for industrial quality control laboratories (Roy, 2001).

L-cysteine is regarded as an essential nitrogen source for bifidobacteria (Shah, 1997) and has the additional function of reducing the redox potential which improves the anaerobic condition required by bifidobacteria. Modified MRS agar supplemented with 0.5% L-cysteine.HCl provides an excellent recovery of bifidobacteria. The mMRS is recommended for bifidobacteria enumeration from pure cultures (Payne *et al.*, 1999; Roy, 2001). The percentage recovery obtained by Payne *et al.* (1999) on mMRS for *B. longum* was 59% by spread plate and

45% by pour plate. For *B. adolescentis*, it was 92% by spread plate and 45% by pour plate and for *B. bifidum*, it was 117% by spread plate and 107% by pour plate (cited in Roy, 2001).

Several media used for selective or differential isolation such as Arroyo, Martin and Cotton Agar (AMC), Modified Rogosa's agar (RMS), NPNL and Blood-glucose-liver agar with oxgall and gentamycin (BL-OG) have been described for enumeration of bifidobacteria from other lactic acid bacteria. Columbia agar base media supplemented with lithium chloride and sodium propionate and MRS medium supplemented with neomycin, paromomycin, nalidixic acid, and lithium chloride can be also recommended for selective enumeration of bifidobacteria in dairy products. The selective media supported the growth of *Bifidobacterium* while inhibiting the growth of *Lactococcus lactis* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *Bulgaricus*, and *Streptococcus salivarius* subsp. *thermophilus*. *Lactobacillus* sp. might grow with bifidobacteria in mixed culture or in commercial fermented dairy products (Payne *et al.*, 1999). From the large number of selective media available, it can be concluded that there is no standard medium for the detection of bifidobacteria (Roy, 2001). However, AMC is based on a commercially available medium to which the specific supplements and antibiotics are added and is relatively quick to prepare, then AMC is a good choice for the routine enumeration of bifidobacteria from mixed cultures in dairy products. AMC gives higher percentage recoveries for *B. longum*. It is 59.3% by spread and 118.9% by pour plate (Payne *et al.*, 1999).

2.1.6.3 Sample plating

Preparation of samples for dilution plates may consist of manually shaking with a known volume of diluent or macerated with a diluent in a stomacher. Zero point one percent of peptone water and peptone water with 0.85% saline are among the most commonly used diluents for enumeration of bifidobacteria in dairy products (Roy, 2001).

Collins and Hall (1984) tested the following diluents for use in enumerating cultures of bifidobacteria: 0.9% saline, 0.1% peptone, 0.9% saline plus 0.1% peptone, and saline plus peptone buffered with 0.03% KH_2PO_4 and 0.06% Na_2HPO_4. No significant difference was obtained except with the peptone alone. The counts obtained from peptone were about 70% of those with the other diluents.

Hartemink and Rombouts (1999) tested the effects of diluting solution under aerobic and anaerobic conditions on plate counts and no significant differences were observed. The viable counts decrease rapidly, however, when the plates are kept aerobically for some time before incubation.

The plating technique can make a significant difference to the results of enumeration of bifidobacteria. The pour-plate technique is preferred to the spread-plate technique for enumeration of bifidobacteria in fermented dairy products. However, a study carried out by Payne *et al.* (1999) to compare spread-plate and pour-plate techniques for the enumeration of three species of bifidobacteria: *B. longum*, *B. adolescentis*, and *B. bifidum* indicated that spread-plates gave higher recoveries than the pour-plates. The differences in percentage observed using the spread-plate and pour-plate techniques are not certain, depend on species and sensitivity to oxygen of bifidobacteria (Scardovi, 1986). It is thus important to compare spread-plate and pour-plate techniques to select the combination of medium and plating technique that gives the most accurate representation of the bifidobacteria viable count (Payne *et al.*, 1999).

2.1.6.4 Culture parameters

The appearance of the colonies of *Bifidobacterium* cultured on agar medium under anaerobic conditions may vary in function of the medium and the species used, but also within a given species. Scardovi (1986) distinguished two differing types of colony for *B. bifidum*. Some colonies were smooth, convex, white, and shiny, whereas other colonies were rough with uneven edges and

map. However, the colonies formed in general one round, dull or glossy and of variable diameters (Ballongue, 1998).

2.2 Use of bifidobacteria in fermented milk products

Traditionally, yogurt is manufactured using *L. bulgaricus* and *S. thermophilus.* These yogurt bacteria are claimed to provide some health benefits. However, *L. bulgaricus* and *S. thermophilus* are not natural inhabitants of the intestine and do not survive under the acidic conditions and bile concentrations usually encountered in the gastro-intestinal tract. Therefore, for yogurt to be considered as a probiotic product, bifidobacteria are incorporated as a dietary adjunct. The application of bifidobacteria to yogurt regularly is referred to as "bio-yogurt" (Roy, 2001).

Bifidobacteria are commonly used for the production of yogurt or fermented milks, alone or in combination with other lactic acid bacteria (Roy, 2001). Fermented milk with only bifidobacteria could be manufactured, but the incubation period is long and the product quality is affected, since bifidobacteria produce appreciable amounts of acetic acid. Thus the normal practice is to make product with both yogurt and probiotic bacteria. The strains that are widely used are *B. bifidum* and *B. longum*, in combination with other lactic acid bacteria. High viable counts and survival rates during stomach passage are necessary to allow live bifidobacteria from the fermented milk products to play a biological role in the human intestine (Shah, 2001).

To provide functional properties, the minimum level of viable bacteria is approximately 10^6 bifidobacteria per mL of product at the time of consumption. The suggested therapeutic dose is 10^8–10^9 viable cells per day (Gardiner *et al.*, 2002). Research was carried out in Japan, and milk products using bifidobacteria began to appear on the market since 1971. Yogurts with bifidobacteria have been launched for decades in Europe and Japan and their consumption is increasing (Mitsuoka, 2000).

When bifidobacteria are used in fermented milk, the complex techniques are needed for product storage. This is because bifidobacteria are rapidly killed when pH goes below 5 due to lack of acid tolerance, and bifidobacteria are obligately anaerobic. They are two types of product those in which milk is fermented directly with bifidobacteria and those in which a concentrated culture of bifidobacteria is added to a fermented milk base. Bifidobacteria are heterofermentative lactic acid bacteria and produce acetic acid in addition to lactic acid. Thus, it is necessary to add some extracts or flavorings to the direct-fermented milk with bifidobacteria to mask unpleasant flavors. For adding concentrated bifidobacteria, bulk starters can be supplied frozen, and the starters are stable with quite high bacterial counts (10^{10}-10^{11} bifidobacteria per g). Among fermented milks on the other hand, there are products with: (i) bifidobacteria combined with the normal yogurt starters, *L. bulgaricus* and *S. thermophilus*; (ii) *L. bulgaricus* replaced by *Lactobacillus acidophilus*; and (iii) *L. acidophilus*, *L. bulgaricus*, and *S. thermophilus* all being used (Mitsuoka, 2000).

The main processing features of the fermented milk products containing bifidobacteria are as follows:

- **Bifidus milk**

Cow's milk is inoculated with a pure culture of *B. bifidum* or *B. longum* at the concentration of 10% w/v followed by incubation at 37°C until the pH reaches 4.5. The product characteristics are mild, acid, and slightly spicy taste. The viable count of bifidobacteria is 10^{8}-10^{9}CFU/mL with a decline of 2 log cycles during storage (Ballongue, 1998).

- **Acidophilus bifidus yogurt.**

Acidophilus bifidus yogurt is similar to the bifidus yogurt. Pasteurized milk is inoculated with separate cultures of yogurt bacteria, *L. acidophilus*, *B. bifidum* or *B. longum* at 40-42°C. The final product contained *L. acidophilus* 1-3 x 10^{7} CFU/mL, and *B. bifidum* 1-3 x 10^{7} CFU/mL (Ballongue, 1998).

2.3 Factors affecting viability of bifidobacteria in bio-products

The bio-products market offers great potential for manufacturers and has continued to gain momentum, despite the complex processing challenges of formulating products with the beneficial microorganisms. *Bifidobacterium* often die during the food manufacturing process or during the passage to the intestine. Shelf life is unpredictable for bifidobacteria, and the industry has had difficulty backing up label claims (Siuta-Cruce and Goulet, 2001).

The stability of bifidobacteria has been a major issue with bio-yogurt manufacturers and consumers alike for quite same time. In fact, many active bifidobacteria die even before the consumer receives any of the health benefits, i.e., during manufacturing, storage, or transportation of finished product. Carr *et al.* (1999) studied the viability of bifidobacteria in bio-yogurt produced in North Carolina, USA. The results showed that 44 out of 58 or 75.9% commercial bio-yogurt contained viable cultures of bifidobacteria. The counts for bifidobacteria were varied among the tested samples ranging from 0 to 5.0 log CFU/mL. None of the tested products contained the number of bifidobacteria exceeded 5 log. However, if bifidobacteria stay alive during food processing conditions, the bacteria must also survive the passage from the mouth to the intestines. Specifically, bifidobacteria are extremely sensitive to their environment. Such environmental factors as water, oxygen, temperature, and acidity affect the overall viability of bifidobacteria (Siuta-Cruce and Goulet, 2001). The survival of bifidobacteria in bio-yogurt depends on various factors as follows:

2.3.1 Yogurt acidity

One of the most pressing disadvantage associated with the use of bifidobacteria in fermented milk products is the lack of acid tolerance of some species and strains. When the lactic acid content increases during fermentation, pH levels correspondingly decrease. Over-acidification or post production acidification after fermentation occurs and during storage at refrigerated

temperature. Excessive acidification is mainly due to the uncontrollable growth of *L. bulgaricus* at low pH and refrigerated temperatures. The pH of the fermented milk may decline to a level as low as 3.6 (Lankaputhra *et al.*, 1996). The low pH may result in the inhibition of the growth of bifidobacteria since their growth is retarded below pH 5.0 (Gilliland, 1979). Martin and Chou (1992) reported that a pH of 5.5–5.6 was determined as being the minimum pH for survival of some species/strains of bifidobacteria. However, acid tolerance of *Bifidobacterium* is strain specific. Lankaputhra and Shah (1995) studied the survival of nine strains of *Bifidobacterium* spp. in acidic conditions (pH 1.5–3.0) and concluded that *B. longum* and *B. pseudolongum* survived in acidic conditions more than *B. bifidum*. The growth of *B. bifidum* was retarded below pH 5.0.

More recently, Reilly and Gilliland (1999) evaluated four strains of *B. longum* survival as related to pH during growth and found that one of the strains, *B. longum* S9, was more stable than the others regardless of pH during growth. Overall, most strains of bifidobacteria are sensitive to pH values below 4.6. Therefore, for practical application, a pH value of the final product must be maintained above 4.6 to prevent the decline of bifidobacteria populations (Modler *et al.*, 1990; Laroia and Martin, 1991).

Over-acidification can be prevented by controlling pH more than 5 (Varnam and Sutherland, 1994) by applying heat shock (58°C for 5 min) to yogurt (Marshall, 1992) before the addition of the probiotic cultures, lowering storage temperature to less than 3–4°C and improving the buffering capacity of yogurt by the addition of whey protein concentrate (Kailasapathy and Rybka, 1997).

2.3.2 Co-culture and species interaction

The composition of the species participating in the fermentation has been found to affect the survival of Bifidobacterium species. A potential growth medium, such as bio-yogurt, contains metabolic products secreted by other

microorganisms, which influence the viability of bifidobacteria. Klaver *et al.* (1993) reported that some strains of *B. bifidum* could not survive in pure milk, due to the bacteria lack of proteolytic activity. Using co-culture with proteolytic species such as *L. acidophilus* could promote the growth of *B. bifidum* (Ishibashi and Shimamura, 1993).

2.3.3 Dissolved oxygen

Since bifidobacteria are strictly anaerobic, oxygen toxicity is an important and critical problem. Milk with a low initial oxygen content should be used to obtain the low redox potential required in the early phase of incubation to guarantee the growth of bifidobacteria (Klaver *et al.*, 1993). During yogurt production, oxygen easily penetrates and dissolves in milk. Oxygen also permeates through packages during storage. To avoid the oxygen problem, it has been suggested to inoculate *S. thermophilus* and *Bifidobacterium* simultaneously during fermentation. *S. thermophilus* has a high oxygen utilization ability, which results in the depletion of dissolved oxygen in yogurt and an enhancement in the viability of bifidobacteria (Ishibashi and Shimamura, 1993).

2.4 Technology to improve viability of bifidobacteria in food products

Currently, ingredient suppliers offer consumers and food manufacturers probiotic cultures that are stable at ambient temperatures by packaging the probiotic in gel capsules, similar to vitamin capsules. In order to prevent the probiotic bacteria from reacting unfavorably with oxygen, moisture, light, and heat. However, the application of such large capsules is impractical in food systems and is costly. Therefore, it is obvious that the ability of probiotic to reproduce in the gastrointestinal tract is an important factor that is beneficial for human body. Such technologies as coating and immobilization have been suggested and investigated as promising methods for maximizing the protection of the biological integrity of probiotic products. One important aspect of the

immobilization technology is the ability to deliver a large concentration of viable cells of *Lactobacillus* spp. to the jejunum and the ileum and of *Bifidobacterium* spp. to the upper of colon. This is a significant improvement over the unprotected probiotic products that cannot escape the harsh acidity of the stomach and the bile (Siuta-Cruce and Goulet, 2001).

2.4.1 Cell immobilization technology

An immobilized cell is defined as a cell that is prevented from moving independently or the cell that the freedom of movement is restricted in supporting material. The development of techniques for cell immobilization has followed that of enzyme immobilization. The knowledge of enzyme immobilization can be used also for cell immobilization (Tampion and Tampion, 1987). There are two common method of cells immobilization that has been reported: adsorption, and encapsulation.

2.4.1.1 Adsorption

Adsorption is based on the physical adsorption of cellular protein on a surface of the carriers. Adsorption of cells onto the solid surface is probably the mildest and cheapest of cell immobilization techniques. The success of the technique depends, in the first instance, upon the properties of the cells themselves. The natural evolution of species has produced many organisms that are capable of adhering to surface. It has the disadvantage that the adsorbed cells may be leaked from the carrier during usage due to a weak binding-force between the cells and the carrier. The binding force is mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces. However, the problem could be solved by the development of stronger binding or multiple attachment (Tampion and Tampion,1987). Adsorption plays a major role in biofilm accumulation in the initial stages of colonization. Adsorption occurs by the interphase accumulation cells from the bulk liquid directly on the

substratum. Subsequently, growth of cells is most often the dominant process (Peyton and Characklist, 1994).

2.4.1.2 Encapsulation

Encapsulation has been one of the most studied of all immobilization techniques because of its mildness, ease of operation and wide applicability. Encapsulation is the method to incorporate cell into the lattices of a semi-permeable gel or enclosing the cell in a semi-permeable polymer membrane. The cells that are coated or entrapped, are referred to by various names such as core material, payload actives, fill or internal phase. The material that forms the coating is referred to as the wall material, carrier, membrane, shell or coating. The protection provided by encapsulation can prevent cell degradation due to exposure to light or oxygen (Risch and Reineccius, 1995).

The distinction of encapsulation and microencapsulation depends the particle size of the final products. Microencapsulation as a strict term apply to particles size of 0.2-5,000 μm while those larger than 5,000 μm are classified as macro- and those smaller than 0.2 μm are classified as nano-microcapsules. If the core material is very large then the process is referred to as coating. Ideally, the encapsulated particle is spherical. However, the shape is influenced by the structure of the core material (cited in Godward, 2000).

The variety of encapsulation techniques have been used in both food and pharmaceutical industries. For example, spray drying, spray cooling, coacervation, fluidized bed coating, liposome entrapment, rotational suspension separation, extrusion and inclusion complexation (Risch and Reineccius, 1995). Some studies have been used encapsulation in order to protect bifidobacteria from high acid condition of foods and intestinal gut.

Hansen *et al.* (2002) reported that 20, 70 micron microencapsulated bifidobacteria with alginate did not significantly improve survival over free cells when exposed to simulated gastric juice (USP without pepsin, pH 2.0 at

37°C for 120 min). The enumeration of bifidobacteria was determined by using MRS supplemented with 0.5 g/L cysteine at 37°C for 72 h anaerobically using Gas Pak Plus System. The enumeration of *B. bifidum* Bb-11, *B. infantis* Bb-02 and *B. longum* Bb-46 after exposed to simulated gastric juice were less than 1.0±0, less than 1.0±0, and 3.2±0.57 log CFU/mL, respectively.

Sultana *et al.* (2000) reported that 0.5-1 mm microencapsulated *Bifidobacterium* spp. with Ca-alginate could not demonstrate a significant increase in the survival when exposed to in vitro high acid and bile salt conditions. *In vitro* gastrointestinal conditions, the milk-based medium NGYC (12% non-fat skim milk, 2% glucose, 1% yeast extract, and 0.05% cysteine) at pH 2.0, 3.0, and 4.0 without pepsin was used. The enumeration of *B. infantis* CSCC 1912 after exposed to the low pH media (pH 2.0) at 37°C for 2 h was $7.1\pm1.0\times10^8$ CFU/mL.

Sun and Griffiths (2000) used 3 mm acid-stable bead made of 0.75% gellan and 1% xanthan gums to immobilize bifidobacteria. The acid-tolerant immobilized *B. infantis* ATCC 15697 was studied for its survival in peptone water pH 4, pasteurized yogurt, and simutated gastric juice. In peptone water, the cell count reduction of the immobilized *B. infantis* was not significantly different from that obtained from the free cells. The free cells were inoculated and kept for 6 weeks at 4°C. Cell counts of the immobilized *B. infantis* remained significantly higher than free cells ($P<0.0001$) when exposed to the simulated gastric juices at pH 2.5, 2.0, and 1.5. At pH 2.5, the viable count of free cells dropped from 1.2×10^9 CFU/mL to an undetectable level (less than 10 CFU/mL) in 30 min, while the viable count of immobilized cells decreased by 0.67 log cycle at the same length of time. The immobilized cells also survived significantly higher than free cells ($P<0.05$) in pasteurized yogurt stored at refrigeration temperature for 5 weeks. However, immobilized cells could not survive in simulated gastric juice when stored at pH 2.0 for 30 min or at pH 1.5 for 15 min.

2.4.2 Techniques for examination or determination of immobilized cells

The visual examination is considered to be an important source of information on the physical relationship of the cells to the support or gel matrix material. The use of the light microscope for this purpose is infrequent due to the limitations of magnification and particularly its generally inadequate depth. The most commonly used instrument is the Scanning Electron Microscope (SEM). SEM offers the possibility of variable magnification up to an amount that is more than sufficient even for bacterial cells. The great depth of focus allows porous gels and support materials to be easily examined and photographed. Being essentially a surface examination technique, it is applicable to all types of support material, even those which are completely opaque to both light and electrons. Although observation of fresh immobilized cells, in the frozen state on cryogenic stages, is possible, this simple technique is limited by lack of availability of the facility to many researchers. Generally, semi-permanent preparations are prepared by critical point drying of the specimen on a standard SEM specimen stub and evaporative coating with an appropriate electron conducing layer. The transmission electron microscope (TEM) is less frequently used than the SEM. The major role of TEM is in the examination of sections of cells immobilized in or on organic supports, which can be sectioned in the same way as the cells themselves. Careful fixation, staining, embedding, and sectioning are necessary. TEM demonstrates the intact or degraded state of cell membranes, cell structure after immobilization or deliberate permeabilization (Tampion and Tampion, 1987).

Determination at the time of immobilization is in general relatively straightforward. The quantity of cells before immobilization can be readily determined by sampling. In the case of cells embedded in gel matrices, virtually all the cells can be washed after immobilization, recovered and the mass measured. In cases where cell number rather than mass is required, samples can be analyzed by simple light microscopy or by automated cell counting

system, such as a Coulter Counter. The simplest technique involves a calculation of differences in number of cells before and after immobilization. Direct measurement of cells in an entrapped preparation is possible if the gel matrix, as in the case of alginate, can be reconverted to a completely soluble form to liberate the cells again. This may only be possible immediately after adsorption because cell growth or metabolism may result in much stronger adhesion to the surface support. The amount of leakage of cells from a support is an important characteristic of any immobilization system, and methods capable of detecting small numbers of cells, such as direct cell counting, are then useful. Cell leakage may arise from loss of the same cells as were originally immobilized or from subsequent cell division and loss of the unattached daughter cells (Tampion and Tampion,1987).

2.5 Edible films and coatings

Edible film is defined as an edible coating material on food components. The film is used to inhibit migration of moisture, oxygen, and carbon dioxide between the commodity and the external atmosphere. Edible film has been used to promote the anaerobic condition within the coated material. The edible films should be effectively coated on foods by dipping, spraying, or panning (Krochta and Mulder-Johnston, 1997).

2.5.1 Film components

Components of edible films have been divided into four categories: polysaccharides, proteins, lipids, and composites.

2.5.1.1 Polysaccharides-based edible films

In general, polysaccharides such as cellulose, pectin, starch, carrageenan, and chitosan, can adhere to surfaces of food products and effectively allow gas transfer. However, polysaccharide films are not effective moisture barriers.

Polysaccharide films have been used to provide the desirable modified atmospheres due to the CO_2 and O_2 permeability. A number of cellulose derived coatings such as methyl cellulose (MC), hydroxypropylmethyl cellulose (HPMC), hydroxypropyl cellulose (HPC), and carboxymethyl cellulose (CMC), are available commercially, most taking advantage of the modified atmosphere condition (Krochta and Mulder-Johnston, 1997; Nisperos-Carriedo, 1994).

2.5.1.2 Proteins-based edible films

Proteins such as casein, soy, and zein, can adhere to hydrophilic surface of food products and are easily modified to form films. However, protein films allow water diffusion. Unlike lipid-based barriers, protein-based barriers do not require the addition of a support matrix. Since the protein acts as both the water vapor barrier and structural component of the film (Krochta and Mulder-Johnston, 1997). Protein films are excellent gas barriers and poor moisture barriers (Rayas *et al.*, 1997; as cited by Chick and Hernandez, 2002). Plasticized protein films also exhibit good mechanical properties. Sorbitol is more effective than glycerol as a plasticizer in both sodium caseinate and whey protein concentrate film (McHugh and Krochta, 1994). Increased plasticizer concentrations resulted in increased oxygen permeability (Perez *et al.,* 2001).

2.5.1.3 Lipids-based edible films

Lipids including waxes, oils, resins, acetylated monoglycerides, natural waxes, and surfactants are commonly utilized in edible coatings. Lipid films have been widely used for intact food products in two distinct forms, laminates and emulsions. Lipid-based edible films show good water barriers but show high oxygen permeability (Avena Bustillos and Krochta, 1993). Miller and Krochta (1997) reported that as the percent crystallinity of a polymer increases, the oxygen permeability decreases. Fatty acids as film formers lack structural integrity and durability in their free form. Then fatty acid-based edible film has

more oxygen permeability than beeswax-based edible films. Lipid crystals in wax coatings are excellent barriers to moisture and gas but the permeability properties are dependent on the packing of the lipid crystals and the orientation to the direction of permeate flow. Beeswax are very good moisture barriers because of the tight orthorhombic arrangement of the crystals (Gontard *et al.*, 1994; Baldwin *et al.*, 1997). However, lipid films require a support matrix to reduce brittleness, and have difficulty adhering to the hydrophilic surfaces of food products. The common compounds used for supporting the matrices are polysaccharide and protein-based edible films (Krochta and Mulder-Johnston, 1997; Baldwin *et al.*, 1997).

2.5.1.4 Composite films

The composite edible films contain both lipid and polysaccharide or protein components in the form of bilayer or emulsion of the two components. The combination of composite films can be formulated to gain the advantages of the two components and to reduce the limitation of each individual component (Donhowe and Fennema, 1994). Edible films produced from natural polysaccharides or proteins provide generally good oxygen barrier but show high permeability to water (Rayas *et al.*, 1997; as cited by Chick and Hernandez, 2002). Whereas lipid films show good water barriers but show high oxygen permeability (Avena Bustillos and Krochta, 1993). By combining polysacchardie or protein and lipid films to produce the composite bilayer film, it is possible to decrease the water vapor permeability and at the same time keep the high oxygen barrier values. The bilayer films have the barrier for moisture and gas migration more than that of the emulsion films (Wong *et al.*, 1992; as cited by Wong *et al.*, 1994). However, the gas barrier properties of composite films depend on the relative humidity (RH) value of environment. At high RH values, composite edible films were found to have higher oxygen and carbon dioxide permeabilities than at low RH value (Guilbert *et al.*, 1996).

The use of wheat gluten and beeswax bilayer films was investigated by Gontrad *et al.* (1994). Wheat gluten provided a structural layer, whereas the beeswax layer functioned to prevent moisture loss. Results showed that beeswax placed on the wheat gluten, provided the best at moisture retention.

Greener and Fennema (1989) examined barrier properties of films based on methylcellulose (MC), fatty acid, and beeswax. MC lends strength to the film, while the beeswax prevents moisture loss. Results indicated the films with the least water vapor permeability (WVP) (at 97% relative humidity) were composed of a double beeswax coating applied to a molten fatty acid-MC base.

2.5.2 Film formation

Many techniques have been developed for films forming and described by Krochta and Mulder-Johnston (1997).

2.5.2.1 Coacervation

Coacervation involves separation of a polymeric coating material from a solution by heating, altering pH, adding solvents, or altering the charge on the polymer involved. Coacervation has been divided into two categories: simple or complex coacervation.

- **Simple coacervation**

Simple coacervation occurs after the biopolymer from polysaccharide or protein was dispersed in water and precipitated or undergoes a phase change after solvent evaporation by drying or after the addition of a hydro-soluble or non-electrolyte in which the biopolymer is insoluble, or after pH adjustment by adding an electrolyte which induced salting out or cross-linking.

- **Complex coacervation**

Complex coacervation occurs when two biopolymer solutions from polysaccharide and/or protein with opposite electron charges are mixed, thus causing interaction and precipitation of the polymer complex.

2.5.2.2 Solvent removal

For film-forming materials dispersed in aqueous solutions, solvent removal is a necessity for solid film formation. Rate and temperature of drying has been found to influence the resulting crystallinity and mechanical properties of bilayer films (Greener and Fennema, 1989).

2.5.2.3 Solidification of melt by cooling

Solidification of the melt by cooling is a common technique for preparing lipid films. Similar to the rate of solvent removal, the rate of cooling plays an important role in the overall physical properties of the resulting film. The rate of cooling influences the predominant polymorphic state, as well as degree of recrystallization in the solidified film. Kester and Fennema (1989) reported that after forming lipid film by solidification, the water vapor and oxygen resistances of lipid films were dependent on the polymorphic state and altered by tempering (cited in Donhowe and Fennema, 1994).

2.5.3 Application of film to food products

Any of film-forming techniques can be utilized with any of the following application techniques.

2.5.3.1 Dipping

This method lends itself to food products. It might require several applications of coating materials or require a uniform coating on an irregular surface. After dipping, excess coating material is allowed to drain from the product, and it is then dried or allowed to solidify. This method has been used to apply films of acetylated monoglycerides to meats, fish, and poultry or apply coatings of wax to fruits and vegetables (Krochta and Mulder-Johnston, 1997). Citrus fruits were the first types of fruit to be coated by this method. However, other

types of fruits, including tomatoes, rutabagas, and peppers have also been coated by dipping (Grant and Burns, 1994).

2.5.3.2 Spraying

Films applied by spraying can be formed in a thinner, more uniform manner than those that applied by dipping. Spraying, unlike dipping, is more suitable for applying a film to cover only one side of a food. Spraying is desirable when protection is needed on only one surface. For example, when a pizza crust is exposed to a moist sauce. Spraying can also be used to apply a thin second coating, such as the cation solution needed to cross-link alginate or pectin coatings (Krochta and Mulder-Johnston, 1997).

2.5.4 Selected coating materials

2.5.4.1 Caseinates

Caseinate is the water-soluble form of casein. The most commonly used caseinate is spray-dried sodium caseinate. It is manufactured by mixing wet acid casein curd with water at 40°C to make a solid content of about 25% and milled in a colloid mill. The slurry is then mixed with 2.5 M NaOH to give a final pH of 6.6–6.8. The viscous slurry is then vigorously mixed and heated to 75°C in a series of vats to complete solubilisation and then further heated to 95°C. The pH of the solution is adjusted with NaOH if necessary, to give a caseinate required pH. The sodium caseinate solution is then spray dried. Holding time at high temperature is minimized to limit Maillard browning of the casein. The exposure time to high pH during dissolving is minimized to prevent lysinoalanine formation and the production of off-flavors. Roller-dried sodium caseinate can be prepared by mixing moist curd with an alkaline sodium salt and feeding the slurry onto the drum of the drier. Granular sodium caseinate may be produced by reacting acid casein curd (40% moisture content) with sodium carbonate. The curd is then agitated and dried in pneumatic ring

driers. Dried sodium caseinate from pneumatic ring drier has high bulk density and dispersability when compared to spray- or roller-dried sodium caseinate. (McHugh and Krochta, 1994).

Caseinates easily form films from aqueous solutions due to their random-coil nature and ability to form extensive intermolecular hydrogen, electrostatic, and hydrophobic bonds. Caseinate-based films have limited moisture barrier ability similar to most protein-based films, due to their hydrophilic nature compared to the commonly used synthetic plastic films. Caseinate films are transparent, colorless, tasteless, odorless, and smooth (McHugh and Krochta, 1994). Ho (1992) reported that the water vapor permeability (WVP) of casein materials increased in the following order: magnesium ceseinate, calcium caseinate, micellar caseinate, sodium caseinate, potassium caseinate, and rennet caseinate. Emulsion film from beeswax & sodium caseinate had lower WVP than emulsion film from paraffin or carnuba waxes & sodium caseinate. Increased lipid concentrations decreased WVP (Ho, 1992).

2.5.4.2 Beeswax

Beeswax is also known as white wax. Beeswax is secreted by honey bees for comb building. The wax is harvested by centrifuging the honey from the wax combs, and then melted with hot water, steam, or solar heating. The wax is subsequently refined with diatomaceous earth or activated carbon, and finally bleached with permanganates or bichromates. Beeswax consists mostly of monofunctional alcohols C24-C33, hydrocarbons C25-C33, and long-chain acids C24-C34. Beeswax is very plastic at room temperature, but becomes brittle at colder temperatures. It is soluble in most other waxes and oils. Beeswax are allowed as coating agents or components by the Food and Drug Administration (FDA) of the United State of America. Beeswax are considered generally recognized as safe (GRAS) in the Code of Federal Regulations (CFR), 21 CFR 184.1973 (Baldwin *et al.*, 1997). Beeswax is allowed for direct using in food

with some limitations. Maximum allowed levels are 0.065 % in chewing gum, 0.005% in confections and frostings, 0.04% in hard candy, 0.1% in soft candy, and 0.002% in all other categories (McHugh and Krochta, 1994).

2.5.4.3 Fatty acids and monoglycerides

Fatty acids, polyglycerides, and their derivatives are used primarily as emulsifiers and dispersing agents. Most fatty acids derived from vegetable oils are considered GRAS and are commonly used in combination with glycerides as emulsifiers. Mono- and diglycerides are the most commonly used as food emulsifiers and are usually prepared by transesterification of glycerol and triglycerol. Common sources of triglycerides and their derivatives are vegetable oils such as soybean oil (Hernandez, 1994). Long-chain alcohols such as stearyl alcohol ($C_{18}H_{32}O$) are commonly used as additives in edible coatings due to their high melting point and hydrophobic characteristics. Long-chain alcohols are usually extracted from sperm whale oil and are allowed for use as emulsifiers in foods, pharmaceuticals, and cosmetics. Long-chain fatty acids such as stearic and palmitic acids are also commonly used in edible coatings for their higher melting points and hydrophobicity (Hernandez, 1994). Palmitic acid and mono- and diglyceride are allowed as coating agents or components by FDA of USA. Fatty acids have been used as a coating component for fresh citrus, lubricant, and defoamer. Mono- and diglycerides is considered GRAS in the Code of Federal Regulations (CFR), 21 CFR 184.1505 (Balwin *et al.*, 1997). Avena-Bustillos *et al.* (1994) added acetylated monoglyceride in calcium caseinate films solution in order to reduce water loss from zucchini and decrease internal oxygen concentrations during storage. A 0.8% calcium caseinate and 0.7% acetylated monoglyceride film was the most effective at increasing zucchini shelf life by increasing water-vapor resistance by 90%.

2.6 Freezing technology

Freezing is a unit operation in which the temperature of a food is reduced below its freezing point and a proportion of the water undergoes a change in state to form ice crystals. The immobilization of water to ice and the resulting concentration of dissolved solutes in unfrozen water lower the water activity (a_w) of the food. Preservation is achieved by a combination of low temperatures and reduced water activity. There are only small changes to nutritional or sensory qualities of foods when appropriate freezing and storage procedures are followed (Fellow, 2000; Clark, 2002).

2.6.1 Effect of freezing on food structure

The main effect of freezing on food structure is cell damage by growing of ice crystal. The extent of damage depends on the size of the crystals and hence on the rate of heat transfer. Figure 1 showed the influence of freezing rate on plant tissues.

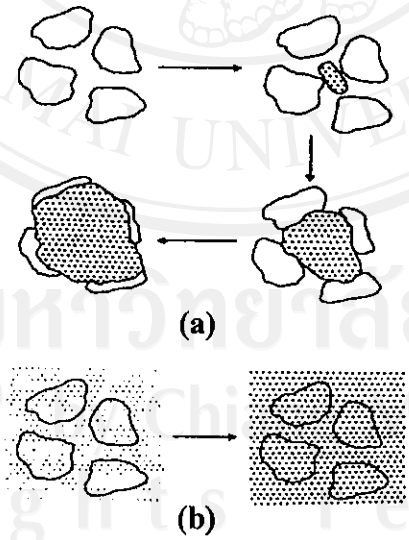

Figure 1 Effect of freezing on plant tissues: (a) slow freezing; (b) fast freezing. (Cited in Fellow, 2000).

During slow freezing, ice crystals grow in the intercellular spaces that cause deforming and rupturing of the adjacent cell walls. Ice crystals have a lower water vapor pressure than the regions within the cells. Water, therefore, moves from the cells to the growing crystals. Cells become dehydrated and permanently damaged by the increased solute concentration resulting in collapse and deformation of cell structure. On thawing, cells do not regain their original shape and turgidity. The food is softened and cellular materials leak out from the ruptured cells. The drip loss is occurred. In fast freezing, smaller ice crystals form within both cells and intercellular spaces. There is little physical damage to cells. The water vapor pressure gradients are not formed, hence there is minimal dehydration of the cells. The texture of the food is thus retained to a greater extent. However, rapid freezing causes the food surface to form a crust and prevents further expansion. This causes internal stresses to build up in the food and makes pieces of food product more susceptible to cracking or shattering, especially when the food suffer impacts during freezing method (Fellow, 2000; Clark, 2002).

2.6.2 Selected freezing methods

2.6.2.1 Cooled-air freezing or chamber freezing

In chamber freezing, food is frozen in a stationary that the natural-circulation of air at temperature between -20°C and -30°C is operated. Chamber freezing is not suitable for commercial freezing due to the low freezing rates of 3–72 h. Cooled air freezing results in poor process economics and loss of product quality. Air is usually circulated by fans to improve the uniformity of temperature distribution, but heat transfer coefficients are low (Fellow, 2000).

2.6.2.2 Cryogenic freezing

Cryogenic freezing uses solid or liquid carbon dioxide, liquid nitrogen directly in contact with the food. Both liquid-nitrogen and carbon dioxide

refrigerants are colorless, odorless, and inert. Cryogenic freezing are characterized by a change of state in the refrigerant or cryogen as heat is absorbed from the food. The heat from the food therefore provides the latent heat of vaporization or sublimation of the cryogen. The cryogen is in intimate contact with the food and rapidly removes heat from all surfaces of the food to produce high heat transfer coefficients and rapid freezing (Fellow, 2000). Two advantages of cryogenic freezing when compared to mechanical systems are lower capital cost and flexibility to process a number of different products without major changes to the system (Miller, 1998). Cryogenic freezing equipment is normally less expensive than mechanical refrigeration equipment because it does not require the compressors, evaporators, and condensers of a mechanical system. However, the operating cost of cryogenics is usually higher because the cost of electricity. Since operating the liquefaction compressors is the major cost (Clark, 2002).

2.7 Freeze drying

Freeze drying is an important operation for drying expensive foods, which have delicate aromas or textures. For example coffee, mushrooms, herbs & spices, fruit juices, meat, seafood, vegetables, and complete meals for military rations. Also for the expeditions of which consumers are willing to pay higher prices for superior quality. The first stage of freeze-drying is to freeze the food in conventional freezing equipment. Small pieces of food are frozen rapidly to produce small ice crystals and to reduce damage to the cell structure of the food. In liquid foods, slow freezing is used to form an ice crystal lattice, which provides channels for the movement of water vapor. The next stage is to remove water during subsequent drying and hence dry the food. If the water vapor pressure of a food is held below 4.58 Torr (610.5 Pa) and the water is frozen, the solid ice in heated food sublimes directly to vapor without melting. The water vapor is continuously removed from the food by keeping the

pressure in the freeze drier cabinet below the vapor pressure at the surface of the ice. Vapor is removed with a vacuum pump and condensed on refrigeration coils. As drying proceeds, a sublimation front moves into the frozen food, leaving partly dried porous food behind it. Foods are dried in two stages. First by sublimation to approximately 15% moisture content. Second, by evaporative drying or desorption of unfrozen water to 2% moisture content (Fellow, 2000). Freeze-drying yields a high-quality, lightweight, and easily rehydrated product that retains the original shape of the starting material, unlike conventional drying, in which shrinking and surface hardening can occur (Clark, 2003).

Freeze-drying has been used for microbial cultures in food processing to prolong shelf life prior to inoculum generation (Fellow, 2000). Saxelin *et al.* (1999) reported that *B. lactis* BB12 could survive excellent during the freezing in liquid nitrogen (-176°C) or in a freezer at -80°C of the concentrated cultures in liquid nitrogen and during freeze-drying with the cell counts higher than 10^{11} CFU/g. *B. lactis* BB12 tolerated well the two-week period at 25°C that might possibly be needed for transportation. Freeze-dried *B. lactis* BB12 could be stored for one year at -18°C without any significant loss in viability. The results demonstrate that freeze-drying is a very effective way of storing probiotic cultures.

Maitrot *et al.* (1997) studied the immobilization of *B. longum* in k-carrageenan and locust bean gum gel beads, and cultured in a medium containing MRS broth and whey-permeate. The same beads were incubated for 5 successive batch fermentations and freeze-dried following mixing with a protective solution. Viable population in the beads increased from 8×10^7 to 4.7×10^{10} CFU/g or 10.67 log CFU/g after three batch fermentations, but no further increase in viable cell population could be achieved in the last two fermentations. However, increasing bifidobacterial cells inside the beads by using 3-5 successive batch fermentation, consumed a lot of time.

Freeze-drying has been used to modify the structure of the hydrocolloid beads for using in many biotechnological purposes, such as water denitrification, matrices for the immobilization of denitrifying isolates, carriers of bacteria or spores for biological control of soil-borne root diseases, and carriers of Gram positive lactic acid bacteria starter cultures involved in dairy and food fermentation. The dried hydrocolloid beads are also used as a vehicle to delivery drug into the human gasrointestinal tract and control release drug the target organ (Zohar-Perez *et al.,* 2004).

Shan-Yang *et al.* (1999) studied the effect of quick and slow freezing on the porosity of freeze-dried poly (*N*-isopropylacrylamide) beads and reported that QF-beads had more porosity than SF-beads after drying. However, poly (*N*-isopropylacrylamide) microgel beads had a diameter ca 50 μm then the large ice crystal of slow freezing caused the structure of dried beads collapsed.

Fwu-Long *et al.* (2002) produced the freeze-dried chitosan beads for immobilizing an anti-inflammatory drug. The morphology of the freeze-dried chitosan bead had an interconnected porous structure comprising particulates around the pores.

Tal *et al.* (1997) modified the structure of alginate beads by freezing the gel beads at -80°C for 24 h before freeze-drying and studied the effect of potato starch as a filler and carbon source at concentrations of 10, 20, 30, and 40% (w/w) to improve the mechanical and biological properties of freeze-dried denitrifying alginate beads. The denitrifying bacterium was *Pseudomonas* sp. Freeze-dried beads containing high concentrations of starch were found to have higher ($p<0.05$) mechanical and denitrifying properties than beads containing low concentrations of the starch filler.

2.8 Tapioca starch and tapioca starch beads

2.8.1 Tapioca starch

Tapioca starch is obtained from cassava root. The cassava plant has been classified as *Manihot esculenta* of the family Euphorbiaceae. Cassava is the term usually applied in Europe and the United States to the roots of the plant. Whereas tapioca is referred to the processed products of cassava. The cassava plant have been cultivated in the tropical regions of North and South America, Brazil, Madagascar, Africa, India, Malaysia, Indonesia, and Thailand. The roots from the plant are an important carbohydrate food source in many regions of the tropics (Whistler *et al.*, 1984).

Tapioca starch has been applied over a wide range of products, either as a raw material or as an additive. Tapioca starch plays an important role in many industries, for example, foods, pharmaceuticals, and textiles. In food industry, tapioca starch has been used as thickener, gelling agent, bulking agent, anti-stick agent, and raw material for fermentation. Tapioca starch may also be enzymatically converted into dextrose, maltose, fructose, and maltodextrin (Whistler *et al.*, 1984; Swinkels, 1985).

2.8.1.1 Chemical composition of tapioca starch

Tapioca starch is a polysaccharide, consisting of anhydrous glucose units ($C_6H_{10}O_5$). The glucose units are linked through glucosidic bonds. This bond is hydrolysable in the presence of enzyme or acid, but the bond is stable under alkaline conditions (Swinkels, 1985). Essentially, the starch is a mixture of amylose and amylopectin. The ratio of amylose and amylopectin is specific for each type of starch. Swinkels (1985) reported that tapioca starch contains about 17% linear polysaccharides of amylose and 83% branched polysaccharides of amylopectin. The approximate composition of tapioca flour/starch is 97.50% starch, 0.30% protein, 0.40% fiber, and 0.20% ash by dry basis (Ministry of Industry, Thailand, 1978)

2.8.1.2 Physicochemical properties of tapioca starch

Physicochemical properties include the dispersion, gelatinization, and pasting characteristics, as well as chemical composition of the starch (Whistler *et al.*, 1984; Swinkels, 1985). The physical structure of the starch granules such as the alignment and association of the polymers chains within the granules would govern the water absorption, swelling power, and solubility of the starch components. Minor chemical components, such as fat, protein, and fiber, would also influence the properties, the processing and product quality (cited in Whistler *et al.*, 1984). Size and shape of the starch granules vary widely, depending upon the botanical sources of the starch. Size of the tapioca starch granules varies between 4-35 micron. Shape of the granule is oral and some lop (Swinkels, 1985).

2.8.2 Tapioca starch beads

Tapioca starch beads (TSB) or tapioca starch pearls are recognized as food ingredient in Thailand including many countries in Asia and South-East Asia. Tapioca starch beads are made from the tapioca starch (*Manihot esculenta* Crantz). The commercial production of TSB involved: adjusting the moisture content of tapioca starch powder to the level that the particle of starch could be attached to each other, forming the bead in the rolling bowl, drying, and size screening (Thaiwa Co. Ltd., Bangkok, Thailand, unpublished data). The diameter of the beads is between 1.4 and 2.8 mm for the small size and between 4.75 and 6.7 mm for the large size (Thai Standard for Industrial Products 1011-2533, 1991). The advantages of TSB are non-toxic in nature, very low cost (ca 12 baht or 0.30 US$ per kg), easy to handling and commercial available as food ingredient.