**Review Article**

# Principles and methods of microencapsulation of probiotic microorganisms

**Amir Mortazavian, Seyed Hadi Razavi, Mohammad Reza Ehsani, Sara Sohrabvandi**

*Department of Food Science and Engineering, Faculty of Biosystem Engineering, Campus of Agriculture, University of Tehran, P.O. Box 31587 -78659, Karaj, I.R. Iran*

***Abstract***
*Worldwide popularity of probiotic- microorganisms and their products on the one hand, and their general weak viability in food products (especially fermented types) as well as gastrointestinal conditions on the other hand, has encouraged researchers to innovate different methods of probiotics viability improvement. Microencapsulation of the probiotic cells is one of the newest and highly efficient methods, which is now under the especial attention and is being developed by various researchers. This article reviews the principles and methods of probiotic cell microencapsulation.*
***Keywords***: Microencapsulation, Probiotic, Viability.

***Table of contents:***



*Correspondence to: **Amir Mortazavian, Ph.D.**
*Telefax +98 261 2248804*
*E-mail: mortazvn@ut.ac.ir*

## INTRODUCTION

Probiotics are microorganisms which settle in the intestine medium and render healthful effects on the host (humans or animals), substantially *via* maintenance and improvement of the microbial balance (between the healthful and harmful microorganisms) of the intestine (Fuller 1989; 1991; Goldin 1998; Gismondo *et al*. 1999). Various health benefits have been attributed to probiotics such as antimutagenic and anticarcinogenic properties, antiinfection properties, immune system stimulation, serum cholesterol reduction, alleviation of lactose intolerance and nutritional enhancement (Gilliland and Speck, 1977; Kim and Gilliland, 1983; Rasic and Kurmann, 1983; Gurr, 1987; Gilliland, 1989; Surawicz *et al.,* 1989; Fuller, 1992; Buck and Gilliland, 1994; Lancaputhra and Shah, 1995; Daly and Davis, 1998; Klein *et al.,* 1998; Macfarlane and Cummings, 1999; Mombelli and Gismondo, 2000). Species of *Lactobacillus acidophilus*, *L. casei*, *Bifidobacterium bifidum*, *B. longum*, *B. breve*, *B. infantice* and *B. lactis* (*B. animalis*) are the most popular bacteria applied food probiotic products (Daly and Davis, 1998; Klein *et al*., 1998; Macfarlane and Cummings, 1999). *Saccharomyces boulardii* is the only probiotic fungus which has been successfully used for curing the intestinal infections, especially diarrhea (Surawicz *et al*., 1989; Mombelli and Gismondo, 2000). Extensive research carried out on the viability and survivability of probiotics in gastrointestinal tract and food products (especially dairy fermented products) have revealed that in general, their viability dramatically decreases

due to exposure to detrimental environmental factors such as organic acids, hydrogen ions, molecular oxygen and antibacterial components (Gilliland and Speck, 1977; Hamilton-Miller, 1999; Iwana *et al*., 1993; Lancaputhra and Shah, 1995; Shah *et al*., 1995; Dave and Shah, 1996; Dave and Shah, 1997; Kailasapathy and Rybka, 1997; Shah and Lankaputhra, 1997; Kebary *et al*., 1998; Beal *et al*., 1999; Gardini *et al*. 1999; Hamilton-Miller *et al*., 1999; Schillinger, 1999; Vinderola *et al*., 2000; Sultana *et al*., 2000; Mortazavian *et al*., 2006a,b,c,d). In addition, the beneficial effects of probiotic microorganisms appear when they arrive in the intestinal medium, viable and in high enough number, after surviving the above mentioned harsh conditions (Gilliland, 1989). The minimum number of probiotic cells (cfu/g) in the product at the moment of consumption that is necessary for the fruition of beneficial pharmaceutical (preventive or therapeutic) effects of probiotics has been suggested to be represented by the minimum of bio-value (MBV) index (Mortazavian *et al.,* 2006c). According to the International Dairy Federation (IDF) recommendation, this index should be $\geq 10^7$ cfu/g up to the date of minimum durability (Ouwehand and Salminen, 1998). In some countries such as Argentina, Prague and Brazil, the standard of $\geq 10^6$ cfu/g has been accepted in the case of bifidobacteria. This standard has been prescribed $>10^7$ cfu/g in Japan (Robinson, 1987). Also, various recommendations have been presented by different researchers such as $>10^6$ cfu/g by all probiotics in yogurt (Robinson, 1987; Kurman and Rasic, 1991) and $>10^7$ cfu/g in the case of bifidobacteria (Holcomb *et al.,* 1991). Apart from the MBV index, daily intake (DI) of each food product is also determinable for their probiotic effectiveness. The minimum amount of the latter index has been recommended as approximately $10^9$ viable cells per day (Shah *et al*., 1995; Kurman and Rasic, 1991; Mortazavian, 2006c). The type of culture media used for the enumeration of probiotic bacteria is also an important factor for determination of their viability, as the cell recovery rate of various media are different (Mortazavian, 2006c,d).

Viability loss of probiotics in food products (especially fermented types) and acidic-bile conditions of gastrointestinal tract has always encouraged researchers to find new efficient methods of viability improvement. Microencapsulation, as one of the newest and most efficient methods, has recently been under especial consideration and investigation. From a microbiological point of view, microencapsulation can be defined as the process of entrapment/enclosure of microorganisms cells by means of coating them with proper hydrocolloid(s) in order to segregate the cells from the surrounding environment; in a way that results in appropriate cell release in the intestinal medium (Sultana *et al*., 2000; Krasaekoopt *et al*., 2003; Picot and Lacroix, 2003a). Among the releasing agents (triggers), pH changes, mechanical tensions, heat, enzymatic activities, osmotic pressure, slow diffusion of the moisture through the capsule layers, presence of some chemical components and storage time can be mentioned (Gouin, 2004). Micropropagation of probiotic cells has been shown preserve them from detrimental environmental factors such as high acidity and low pH (Wenrong and Griffiths, 2000), bile salts (Lee and Heo, 2000), cold shocks induced by the process conditions such as deep freezing and freeze drying (Shah and Rarula, 2000), molecular oxygen in case of obligatory anaerobic microorganisms (Sunohara *et al*., 1995), heat shocks caused by process conditions such as spray drying, bacteriophages (Steenson *et al*., 1987) and chemical antimicrobial agents (Sultana, 2000). However, other advantages such as increase of sensory properties stability and/or its improvement (Gomes and Malcata, 1999) and immobilization of the cells for their homogeneous distribution throughout the product (Steenson *et al.*, 1987; Krasaekoopt *et al.*, 2003) can also be achieved.

Importance of the microencapsulation method, as an efficient manner for increasing probiotics viability, justifies reviewing the newest achievements in this regard. The present article reviews principles and methods of probiotics microencapsulation including discussions of microbeads structure, components used for microencapsulation, its applications and advantages regarding probiotics, factors affecting microencapsulation effectiveness, microencapsulation methods and technology and methods of microencapsulation efficiency evaluation (by the assessment of its qualitative factors).

## 2. Structural details of microbeads

Figure 1 represents 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 (Figure 1, part 1.1). Each bead might consist of one or several cells. When several cells are enclosed by the capsule, the intersti-

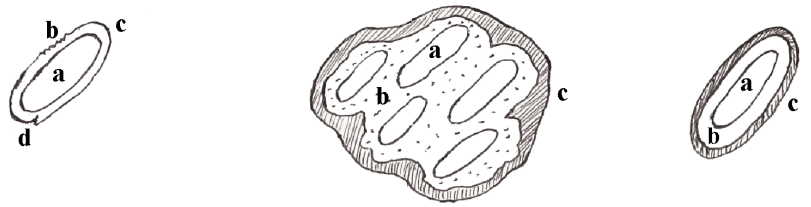


**Figure 1.** Structural details of microbeads. 1.1. Single cell bead: Bacterial cell (a), uneven surface (b), even surface (c), crackled surface (d); 1.2. Multicell bead: Bacterial cells (a), interstitial liquid (b), capsule (c); 1.3. Coated bead:Bacterial cell (a), capsule (b), coat/shell (c).

tial liquid from solution fills the free spaces of the microbead. Superficial and/or deep cracks might appear in the beads (Figure 1, part 1.1). 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 (Figure 1, part 1.3) 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. Main components used for microencapsulation of probiotics

*3.1. Alginate and its combinations:* Alginate is a linear heteropolysaccharide extracted from different types of algae, with two structural units consisting of *D*-mannuronic and *L*-guluronic acids. Calcium alginate has been widely used for the encapsulation of lactic acid- and probiotic bacteria, mainly in the concentration range of 0.5-4% (Sheu and Marshall, 1991; Sheu and Marshall, 1993; Truelstrup-Hansen *et al*., 2002; Kim *et al*., 1996; Jankowski *et al*., 1997; Khalil and Mansour, 1998; Kebary *et al*., 1998; Lee and Heo, 2000; Shah and Rarula, 2000; Sultana *et al*., 2000; Truelstrup-Hansen, 2002; Krasaekoopt *et al*., 2004). Alginate capsules have some advantages as follows (Klien *et al*., 1983; Tanaka *et al*., 1984; Martinsen *et al*., 1989; Prevost and Divies, 1992; Dimantov *et al*., 2003; Chandramouli *et al*., 2004; Gouin, 2004): Easily form gel matrices around bacterial cells, they are not poisonous to the body (is safe or biocompatible), they are cheap, mild process conditions (such as temperature) are needed for their performance, can be easily prepared and performed (simplicity and ease of handling) and properly resolve in the intestine and release entrapped cells. Alginate gel matrix appropriately surrounds the bacterial cells with a diameter of 1-3 μm and the pores sizes formed at the surface of alginate beads do not exceed 7 nm (Klien *et al*., 1983). However, some disadvantages are attributed to alginate beads. For example they are susceptible to acidic environments and their crackling and loss of mechanical stability in the lactic acid-containing environments have been verified (Eikmeier and Rehm, 1987; Roy *et al*., 1987; Audet *et al*., 1988; Ellenton, 1998). Also, because alginate gel is formed in the presence of calcium ions, its integrity is deteriorated when subjected to monovalent ions or chelating agents which absorb calcium ions such as phosphates, lactates and citrates (Roy *et al*., 1987; Smidsrod and Skjak-Braek, 1990; Ellenton, 1998). Other disadvantages include difficulties in industrial scale applications due to their high expenses and weak ability of scaling up as well as formation of crackled and porous bead surfaces (Gouin, 2004). Latter specification leads to the relatively fast diffusion of moisture and other fluids through the capsules which reduce their barrier properties against unfavorable environmental factors (Gouin, 2004). The mentioned defects can be efficiently compensated by blending of alginate with other polymer compounds, coating other compounds on its capsules and structural modification of the alginate by using various additives (Krasaekoopt *et al*., 2003). Blending alginate with starch is a common practice and it has been shown that encapsulation effectiveness of different bacterial cells especially lactic acid bacteria were improved by applying this method (Jankowski *et al*., 1997; Sultana *et al*., 2000; Sun and Griffiths, 2000; Truelstrup-Hansen *et al*., 2002; Krasaekoopt *et al*., 2003). Besides good protection from bacterial cells, alginate-starch blends render the advantage of micronutrients and metabolites diffusing through the capsules, inside and outside of the entrapped cell(s). As a result, beads would contain metabolically active cells (Jankowski *et al*., 1997). Blending calcium alginate with Hi-maite starch pro-

duces capsules with high cell viability due to formation of capsules with a good integrated structure as well as prebiotic effect of the latter compound (Sultana *et al*., 2000). Alginate-glycerol blend improved survivability of the cells deep frozen with liquid nitrogen and kept at -20ºC. This has been attributed to the cryogenic effect of glycerol (Truelstrup-Hansen *et al*., 2002). Formation of a coat/shell around the alginate capsule has been verified to considerably improve its physicochemical characteristics. It has been reported that by coating semipermeable layers of chitosan polymer (as a polycationic compound) around the alginate capsules (which have negative charges), beads with improved physical and chemical stability were produced. This structure was tolerant against the deteriorative effects of calcium chelating and antigelling agents. Also structurally, the beads were denser and much stronger, thus avoiding breaking and cell(s) release (Smidsrod and Skjak-Braek, 1990; Zhou *et al*., 1998; Krasaekoopt *et al*., 2003). In fact low-molecular-weight chitosan diffuses faster into the alginate matrix compared with the high-molecular-weight one, resulting in the formation of capsules with higher density and strength. Coating of calcium chloride on the alginate capsules has also been investigated (Chandramouli *et al*. 2004). Regarding the function of calcium ions in alginate gel formation, this coating causes generation of more stable beads with a higher protective effect on the probiotic cells, and as a result, higher viability. Poly-amino acids such as poly-*L*-lysine (PLL) are from other poly-cationic polymers coated on the alginate capsules. Similar to chitosan, these polymers make strong complexes with alginate matrix and give it the advantages as mentioned for chitosan (Smidsrod and Skjak-Braek, 1990; Champagne *et al*., 1992a; Larisch *et al*., 1994). Generation of multilayer shells of PLL on the alginate capsules has also been investigated: the first layer of PLL on the capsule surface produces positive charge, then the second alginate coat gives the beads surface negative charge. This trend can be repeated several times. As a result, layers of alginate and PLL would be formed alternatively (Champagne *et al*., 1992a; Larisch *et al*., 1994; Marx, 1989). Coatings of Polyetylenamine and glutaraldehyde (as other types of polycationic polymers) on the alginate capsules has also been reported. Cross-linked alginate matrix (produced at low pHs) is obtained from modified alginate structures applied to probiotics encapsulation. Although this kind of matrix has more density and strength compared with the alginate matrix alone, it is able to successfully release the bacterial cells into the intestine (Marx, 1989).

*3.2. Starch:* As mentioned previously (section 3.1), starch has been used as a material for coating of alginate capsules. High-amylose corn starch (HACS) can be applied for enhancing functions of capsule- or shell/coat formation (Dimantov *et al*., 2003). Lyophilized corn starch (LCS) has been reported to be used as capsule-forming material, however, its decomposes after being subjected to pancreatic enzymes (Fanta *et al*., 2001). Resistant starch (RS) is not degraded by the pancreatic amylase enters the intestine in the indigestible form. This specification apart from giving the microbeads good enteric delivery characteristic (good release of bacterial cells in the large intestine), also gives them prebiotic functionality as they can be used by the probiotic bacteria in the intestine (Kritchevsky, 1995; Muir *et al*., 1995; Phillips *et al*., 1995; Silvester *et al*., 1995; Haralampu, 2000; Thompson, 2000). HACS with 20% RS has been recognized to be suitable for the enteric delivery purpose. By applying hydro-thermal and retrogradation processes on the native high-amylose corn starch (NHACS), RS-rich fractions which are suitable for encapsulation can be prepared (Dimantov *et al*., 2004). It has been reported that fermentation of starch by microorganisms such as bifidobacteria, *Lactobacilli*, *Streptococci* and *Entrobacteriaceae* reduces the pH of the intestine *via* formation of short chain fatty acids (Macfarlane and Gummings, 1991; Kleessen *et al*., 1997; Le Blay *et al*., 1999). Also, consumption of resistant starch reduces the risk of intestinal cancer because of having dietary fiber functionality (Dimantov *et al*., 2004).

*3.3. Mixture of xanthan-gelan:* A mixture of xanthan-gelan gum has been used for the microencapsulation of probiotics (Paquin *et al*., 1990; Sanderson, 1990; Sultana *et al*., 2000; Sun and Griffiths, 2000. The optimum mixing proportion was 1:0.75 for xanthan: gelan (Sun and Griffiths, 2000). In contrary with alginate, this mixture is resistant to acidic conditions. Also, as apposed to from carrageenan which needs potassium ions for structural stabilization (it is harmful for the body in high concentrations), this gum can be stabilized with calcium ions (Klein and Vorlop, 1985; Sanderson, 1990). It should be noted that although gelan gum is able to generate gel-bead structure for microencapsulation, it is not used on its own for this purpose because of having a high gel-setting temperature (80-90°C for

about 1 h) which results in heat injuries to the probiotic cells (Sun and Griffiths, 2000).

*3.4. Carrageenan and its mixtures: K*-carrageenan is a neutral polysaccharide which requires high temperatures (60-90°C) for dissolution especially when applied at high concentrations such as 2-5% (Klein and Vorlop, 1985). When cell slurry containing probiotics is added to the sterilized and cooled (40-45ºC) solution of this polymer, subsequent cooling down to room temperature results in its gelatinization. Adding monovalent ions such as potassium in the form of KCl leads to the establishment of gel-beads (Krasaekoopt *et al*., 2003). However, KCl has been reported to have an inhibitory effect on some lactic acid bacteria such as *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (traditional yogurt bacteria) (Audet *et al*., 1988). As replacements, $Rb^+$, $Cs^+$ and $NH_4^+$ ions have been recommended. These ions, regardless of resolving the above mentioned problem, produce stronger gel beads compared with potassium ion. Mixture of *k*-carrageenan-locust bean renders good efficiency in lactic-fermented products (such as yogurt) due to its lower susceptibility to the organic acids. This mixture has been widely used for microencapsulation of probiotics in fermented products (Audet *et al*., 1988; Arnauld, 1992). However, gel formation of *k*-carrageenan-locust bean mixture is dependent on calcium ions, which have adverse effects on both viability of *Bifidobacterium* spp. and the human body. The latter property arises from its undesirable effect on the electrolyte equilibrium of liquids in the body (Paquin *et al*., 1990; Sun and Griffiths, 2000). It has been reported that the proportion of 1:2 for carrageenan-locust been gives a strong gel for microencapsulation (Miles *et al*., 1984; Takata *et al*., 1977).

*3.5. Gelatin:* Gelatin gum has been used for the microencapsulation of probiotics, alone or in mixture with other gums (Hyndman *et al*., 1993). It is a protein gum which makes a thermoreversible gel. Its amphoteric nature gives the ability of having synergistic effects with anionic polysaccharides such as gelan gum. The two mentioned polymers are miscible at pHs > 6, because of having negative charges. When pH of the solution drops below the isoelectric pH of gelatin, this gum obtaines positive charge to interact with the gelan gum (King, 1995). Mixture of gelain-toluene diisocyanate makes strong capsules which are tolerant against crackling and breaking, especially at higher concentrations. This can be attributed to the cross-link formation between these polymers. Mentioned mixture has been used for the encapsulation of *Lactobacillus lactis* ssp. *cremoris* (Hyndman *et al*., 1993). Mixture of gelatin-arabic gum has also been applied in the coating of soybean-oil capsules (Truelstrup-Hansen *et al*., 2002).

*3.6. Cellulose acetate phethalate:* This component contains negative-charge groups of phethalate. It is soluble at pHs ≥ 6, but insoluble at pHs ≤ 5 (Malm *et al*., 1951). Because of having a safe nature for purpose human ingestions, it is being widely used for drug capsulation in pharmacy (Rao *et al*., 1989; Krasaekoopt *et al*., 2003). Also, freeze dried *Bifidobacterium pseudolangum* capsulated with this compound and coated by wax has been reported to have considerably higher survivability after passing through gasteric juice (Rao *et al*., 1989).

*3.7. Chitosan:* Chitosan is a linear polysaccharide with negative charge arising from its amine groups which are obtained by deacetylation of chitin. It is soluble at pHs < 6 and like alginate, makes a gel structure by ionotropic gelation. Chitosan polymers can further polymerize by means of cross-link formation in the presence of anions and polyanions (Klien *et al*., 1983). As mentioned before (section 3.5), chitosan has been used for coating of gelatin capsules. Because its efficiency for increasing viability of probiotic cells is not satisfactory, it is most often used as a coat/shell, but not capsule. Usually, low-concentration chitosan solution (e.g. 0.4%) is applied for shell-making on capsules such as gelatin (Zhou *et al*., 1998). It has reported that mixture of chitosan and hexamethylene diisocyanate or chitosan and glutaraldehyde make stronger coats compared with chitosan alone (Groboillot *et al*., 1993). In order to coat chitosan on alginate capsules, solutions of microbeads with alginate capsules should be dripped into a chitosan-calcium chloride mixture. Presence of calcium ions is necessary for proper coating (Krasaekoopt *et al*., 2003).

*3.8. Miscellaneous compounds:* Components such as whey proteins used as capsule materials (Picot and Lacroix 2003a,b; Picot and Lacroix, 2004), soybean oil as capsule coated by a mixture of Arabic and gelatin gums (Truelstrup-Hansen *et al*., 2002), wax for coating different types of capsules (Rao *et al*., 1989) and calcium chloride for coating alginate capsules (Chandramouli *et al*., 2004) have also been used to encapsulate probiotics. Apart from the main materials

which directly form capsule and/or coat structure, additives such as SDS, tween 80 (as emulsifiers) and cryoprotectants (e.g. glycerol) are usually added to the solution for the encapsulation process (Kearney *et al*., 1990).

**4. Applications and Advantages of probiotics microencapsulation**

Applications and advantages of probiotics encapsulation can be discussed from different angles including production of starter cultures, production of food products from the aspects of probiotic cells viability in the products, their sensory properties of them and probiotic cells immobilization in the products, viability of probiotics cells in the gastrointestinal tract (GT) and usage in fermentors. These aspects are discussed below:

*4.1. Production of starter cultures:* Microencapsulation can be used efficiently for preparation of bacterial starter cultures with higher viability. It has been shown that the shelf life of encapsulated *Lactobacillus rhamnosus* VTT E-97800 which is kept under room temperature and relatively high relative humidity is at least 6 months. This shelf life was successfully increased to at least 18 months when the encapsulated cells were deep frozen in liquid nitrogen. Encapsulated cells can be directly ingested in the products and consumed. Only 10% deterioration of such beads was observed after passing through simulated gastrointestinal conditions (Mattila-Sandholm *et al*., 2002). Picot and Lacroix (2003b) encapsulated starter cells by using whey protein fragments within a milk fat medium. By applying this method, production of starter culture powder with minimum of heat damage during spray drying was achieved. It has been understood that encapsulation of starter cells with the mixture of alginate-glycerol can significantly increase their survivability after the deep freezing process (Sultana *et al*., 2000).

*4.2. Viability of probiotics in gastrointestinal tract:* Various reports confirm that microencapsulation efficiently increases the probiotics viability through the passing from acidic-enzymatic-bile conditions of the gastrointestinal tract. For instance, Rao in 1989 understood that encapsulation of *B. pseudolongum* with cellulose acetate phethalate (CAP) increased its viability in the simulated conditions of the gastrointestinal tract (Groboillot *et al*., 1993). Experiments of Lee and Heo in 2000 showed that survivability of *B. longum* encapsulated with calcium alginate in the simulated conditions of gastric juice (pH 1.5) could be considerably increased. Experiments indicated that coating of the calcium chloride on sodium alginate capsules containing *L. acidophilus* increased tolerance of the mentioned bacteria against harsh acidic (pH 2) and bile (1%) conditions (Chandramouli *et al*., 2004). Simulated conditions of the stomach (pH 1.5) led to a dramatic loss in the viable counts of *B. infantice* (from $1.23 \times 10^9$ to <10 cfu/ml after 30 min), nevertheless, its viability loss under the same conditions after microencapsulation did not exceed the 0.67% of the first viable cell amount (Sun and Griffiths, 2000). Research results have revealed that resistant starch is an efficient component for the purpose of probiotics encapsulation, because it is not dissolved or decomposed in the gastric acid, neutral pH and by the enzymatic activity of pancreas, but releases its cells when the beads enter the intestine (Englyst *et al*., 1992; Sun and Griffiths, 2002). Microencapsulation with CAP has also been claimed to have a suitable effect on the viability of *B. pseudolongum* after being exposed to the simulated gastric conditions. According to the same research, the unencapsulated cells were completely destroyed after 1 h. It should be pointed out that apart from the type of capsulation materials; diameter of capsules or coats is also a determinable factor for improving the viability of probiotics. Excessive reduction in diameter can weaken or remove the protective function of encapsulation. For example, it has been reported that survivability of encapsulated probiotics with alginate capsules under the acidic-bile conditions showed no significant difference when the diameter of gel-beads were 20 and 70 μm compared with the bigger sizes (Sultana *et al*. 2000). Also, microencapsulation of *Bifidobacterium* spp. did not significantly increase their viability when the cells encountered the simulated gastric juice (Chandramouli *et al*. 2004).

*4.3. Application in fermentors:* It has been claimed that during biomass production, microencapsulation of probiotics can include the following advantages: increasing the tolerance of microorganisms against factors such as bacteriophage infection (Steenson *et al*., 1987), chemical poisoning agents, protecting microorganism cells against unwanted changes such as genetic mutations, reaching good productivity in metabolite production especially at high agitation rates (Arnauld *et al*., 1992) and producing more dense biomass (Champagne *et al*., 1992b).

*4.4. Production of food products:* Advantages of probiotic microencapsulation in food probiotic products can be discussed from four points of view: increasing

viability of probiotics in products till the moment of consumption, achieving new methods in food manufacture, fixing and improving the sensory properties of probiotic products and immobilizing probiotic cells in the products. The above, mentioned sections are discussed separately below.

*4.4.1. Viability of probiotics:* Microencapsulation can noticeably improve the viability of probiotic microorganisms due to its protective effects against detrimental environmental factors such as high acidity, low pH, molecular oxygen (in the case of obligatory anaerobic microorganisms), poisoning agents generated during the process (especially heat treatment), digestive enzymes, bacteriophages, hydrogen peroxide, short-chain fatty acids, carbonyl-aromatic compounds (three last cases are produced by starter cultures during fermentation) and heat processing (e.g. drying) (Mortazavian *et al*., 2006a). Increasing viability of probiotics will lead to the increase of products shelf life. Undoubtably, high acidity and low pH of fermented products are the main factors that cause viability loss of probiotics, especially during refrigerated storage (Shah *et al*., 1995; Dave and Shah and Lankaputhra, 1997; Mortazavian *et al*., 2006a,b,c). Microencapsulation of *L. acidophilus* and bifidobacteria with calcium alginate did not considerably increase their viability after being subjected to the intense acid (pH 2) and bile (2%) environment, *vice versa*, however, at mild acidic conditions (natural acidity of yogurt), throughout 8 weeks of refrigerated storage improving the probiotics survivability was noticeable. Mixture of alginate-HACS or alginate-RS compared with calcium alginate alone, improves the coherency and continuity of capsule structure (alginate and starch showed synergistic effect in gel formation) and as a result, viability of probiotic cells (Sultana *et al*., 2000). Experiments made by Kebary *et al.* (1998) showed that encapsulation of bifidobacteria with alginate could significantly increase their viability in frozen ice milk, whereas, using *k*-carrageenan for this reason was not as successful as the previous one. Encapsulated *B. longum* in milk medium showed higher viability compared with free cells during storage time (Truelstrup-Hansen *et al*., 2002). According to Kalil and Mansur investigation (1998), encapsulation of *Bifidobacterium* spp. with calcium alginate significantly improved their viability in mayonnaise with pH 4.4 (Khalil and Mansour, 1998). Higher survivability of *B. infantis* in yogurt during the refrigerated storage was reported when the cells were encapsulated by mixture of gelan-xanthan. The average size of the beads was 3 mm after the encapsulation process (Sun and Griffiths, 2000). Encapsulated probiotics with an alginate-starch mixture and a bead size range of 0.5 to 1.0 mm were considerably more viable in yogurt during the storage period (Sultana *et al*., 2000). Increase in the viability probiotics Lactobacilli in frozen ice milk after encapsulation with alginate (size range from 25 to 62 μm) has been reported (Sheu and Marshall, 1993). The same results were achieved in the case of fermented frozen dairy desserts. Coating of alginate beads with PLL considerably increased probiotics viability against severs process conditions (Shah and Rarula, 2000). Other research indicated that survivability of *Bifidobacterium* spp. and *L. acidophilus* noticeably increased in fermented frozen dairy desserts when alginate with SPS and tween 80 additives were used for encapsulation (Sultana *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 4ºC, its viable counts did not fall below $10^7$ cfu/ml. Also, no undesirable sensory properties were observed in the final product. The above mentioned results were also obtained after frozen storage of the product (Sultana *et al*., 2000). Good efficiency for encapsulation process after the encapsulation of *B. infantis* with xanthan-gelan mixture in yogurt with pH 4 during the 6 wks of storage period at 4ºC has been reported. Mentioned cells showed higher survivability during the pasteurization process (Sun and Griffiths, 2000). *B. longum* ATCC 15696 cells added to cheddar cheese at the stage of curd milling, were totally viable after 24 weeks of ripening period. The cells were completely metabolically inactive during this time (Dinakar and Mistry, 1994; Sun and Griffiths, 2000). It has been verified that the viability of *Lactobacilli* encapsulated with calcium alginate could be increased up to 40% in frozen products such as ice cream and frozen ice milk (Sheu and Marshall, 1993). Because microencapsulation of probiotic starter cultures considerably decreases their metabolic activity, viability of the cells would increase due to the slower acid production rate. For instance, it has been reported that incubation time for yogurt made with *L. casei* and *L. acidophilus* up to the end point of pH 5, increased from 6 h in the case of free cells to 30 h in the case of encapsulated cells (Sultana *et al*., 2000). This fact was also evident during the refrigerated storage period. Decrease in acidification rate of starter bacteria and as a result pH drop during this period leads to the consid-

erable extension of product shelf life due to increasing probiotics viability within the storage time (Mortazavian *et al*., 2006b).

*4.4.2. Achieving new methods in food manufacture:* Nowadays, by applying encapsulated starter culture bacteria, new innovations have been achieved in the manufacture of dairy probiotic products such as yogurt. Specific encapsulation of probiotic (even traditional yogurt bacteria) cells can cause desirable rate of cellular metabolic activity. For example, new continuous method of yogurt production with encapsulated traditional yogurt bacteria (*Streptococcus salivarius* ssp. *thermophilus* and *Streptococcus delbrueckii* ssp. *bulgaricus*) has been proposed which has the following advantages compared with traditional methods: product with relatively fixed sensory properties can be produced, viability of bacteria remains very high and the proportion of *Lactobacillus delbrueckii ssp. bulgaricus*/ *Streptococcus thermophilus* bacteria from initial to final stages of fermentation process and as a result, flavor of the product can be well controllable (Krasaekoopt *et al*., 2003). In this regard, diameter of the capsules has been found to play a very important role, because it determines lactose absorption rate through the capsules and subsequently, acid production rate by the starters. Mentioned factor also has an important effect on the cell-release rate from microbeads. It has been reported that for the beads with a small diameter (0.5-1.0 mm), cell release and acid production rates are carried out with higher speed (Krasaekoopt *et al*., 2003). From the disadvantages of fermented products production by using encapsulated starter cultures to long incubation times and higher prices due to the need for larg high amounts of starter cell inocula (because there are no cells multiplication during the fermentation process) can be mentioned. Larisch *et al.* in 1994 claimed that by encapsulation of *Lactococci* with alginate (as a capsule) and PLL (as a coat/shell), the incubation time decreased by >17% compared with the conditions in which yogurt was fermented by free cells (Larisch *et al*., 1994). However, there is no other report to confirm this claim.

*4.4.3. Immobilization of probiotic cells:* Immobilization of probiotic cells has been carried out by using the encapsulation process to make homogeneous dispersion of the cells throughout the product. This specification is important especially in polyphase and viscose products such as mayonnaise (Khalil and Mansour, 1998). No excessive information is available in this regard.

*4.4.4. Fixing and improving the sensory properties of probiotic products:* Microencapsulation of probiotics helps to fix and/or improve the sensory properties of the final product. In general, sourness of fermented products (such as yogurt) produced by encapsulated starters is milder than those produced by unencapsulated ones because of the lower amount of acid production and pH drop (Adhikari *et al*., 2000). This fact could be useful especially for traditional yogurt starters rather than probiotic starters because fermented probiotic products generally have a milder acidic flavor (due to both lower acidification rate and more L+ lactic acid production instead of D- type) compared with the same traditional (non-probiotic) products, in which sharp- and over acidifications are the main reasons of their restricted shelf life (Mortazavian *et al*., 2006c). Therefore, microencapsulation of starter cultures leads to flavor fixation of fermented products because encapsulated cells are relatively or totally (regarding type and diameter of the capsulation) inactive in metabolism and do not influence flavor profile of the products, especially during the storage time. For instance, no significant change in sensory properties of yogurt containing encapsulated *B. bifidum* was observed after 3 weeks of refrigerated storage at 4ºC (Krasaekoopt *et al*., 2003). Simultaneous inoculation (coculturation) of probiotic starters and traditional yogurt bacteria (*St* and *Lb*) into the yogurt milk is a common practice in probiotic yogurt production. However, loss of probiotics viability is inevitable (Mortazavian *et al*. 2006a,b,c). One way to overcome this problem is performing stepwise fermentation. In this method, first, the pH of yogurt milk is dropped down to the required amount and then, encapsulated probiotic cells are added (Truelstrup-Hansen *et al*., 2002). By applying the mentioned procedure, besides fixing sensory properties of the product, significant increase in probiotic cell viability after fermentation can be achieved. Acetic acid produced by *Bifidobacterium* spp. gives a vinegar taint to the fermented probiotic products such as yogurt (Adhikari *et al*., 2000). This off flavor which is mainly produced during the fermentation period develops within storage time. Microencapsulation of bifidobacteria has been used to overcome this problem, because the amount of produced acetic acid in yogurt generated with encapsulated bifidobacteria was considerably lower than those produced by non-encapsulated ones thereby, improving the flavor properties of fermented probiotic products (Adhikari *et al*., 2000). Encapsulation of bifidobacteria in the fermented products not only

improves their sensory characteristics, but also improves the viability of probiotic microorganisms because acetic acid is the weakest organic acid and as a result, the strongest one with respect to the mortal effects point of view on the bacterial cells (Mortazavian *et al*., 2006c).

Although microencapsulation of probiotic cells (and even traditional starter bacteria) can be applied as an efficient method to improve the sensory attributes of the probiotic products (especially fermented types), its unsuitable usage might lead to the off flavor and/or off texture of the final product, especially defects in mouth-feeling. For instance, encapsulation of *B. longum* and *B. lactis* in milk led to a especial off flavor which was not observed in the product containing free cells of the same bacteria. This fact was attributed to changes in the metabolic pathways of the encapsulated cells which caused production of small-bitter peptides (Truelstrup-Hansen *et al*. 2002). It has been understood that microbeads with diameters more than the special limit (>100 μm, particularly more than 1 mm) can deteriorate mouth-feel properties of products such as liquid milk, yogurt and sour cream due to the appearance of the special sense of coarseness. Beads with the range of 1-3 mm in diameter can adversely affect both texture and flavor of the final product might be adversely affected (Chandramouli *et al*., 2004). It should be noted that increasing the beads diameter to more than the particular limit (regarding type of capsule and microorganism) has been proved to have no significant effect on the viability of the cells (Truelstrup-Hansen *et al*., 2002).

## 5. Factors affecting microencapsulation effectiveness of probiotics

Different parameters can be considered for evaluating the effectiveness of the probiotc encapsulation process such as viability maintenance after encountering detrimental environmental conditions, cell release/recovery ability and hardening time (time needed for capsules formation). Various factors can influence the mentioned parameters which are discussed below:

*5.1. Capsule characteristics with respect to the surrounding environment:* True selection of capsule materials regarding their purpose surrounding environment is very important. For instance, as mentioned before (section 3.1), leakage of calcium ions form alginate capsules structure leads to its decomposition. So, alginate capsules should be avoided from environments containing high acidity and chelating agents. However, in milk-based media such as liquid milk, cream and yogurt, due to availability of high levels of calcium ions, leaching of calcium ions from gel-bead structures could be considerably inhibited. Therefore, gel-beads maintain their shape and structure (Truelstrup-Hansen *et al*., 2002). Using RS as a capsule material makes beads resistant against enzymatic digestion (Dimantov *et al*., 2003). If arriving probiotic cells to the small intestine is the aim, selection of capsule material(s) should be in such a way that their decomposition occurs after subjecting them to the small intestine pH or pancreatic enzymes. Toleration of the beads against the mentioned digestive factors results in their direct excretion from the body without settling the probiotic cells in the intestine. If the beads must be settled in the large intestine, it is preferable to be tolerant against the pancreatic and small intestine (but not large intestine) conditions. However, this is not always easily achievable due to the restrictions in chemical characteristics of encapsulation materials. In this regard, when the beads open in the small intestine, released cells are expected to arrive to the large intestine. However, pancreatic shock after release of the cells in the small intestine might reduce their viability. No research which has substantially studied this phenomenon was found. Generally, all the capsules must be resistant to the acidic conditions of gastric juices (Sun and Griffiths, 2000; Mortazavian *et al*., 2006c). Some times it is necessary to use especial types of hydrophobic components for encapsulation to make the beads tolerant against the high moisture conditions of products (Truelstrup-Hansen *et al*., 2002).

*5.2. Coating of the capsules:* As mentioned in section 2, 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). This shell has also no significant effect on the diffusivity of the alginate capsules (Tanaka *et al*. 1984).

*5.3. Concentration of capsule-making solution and beads diameter:* Concentration of the capsule-making solution and 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). This has been proved in both product and body conditions (Lee and Heo, 2000; Chandramouli *et al*., 2004; Sheu and Marshall, 1993). Sultana *et al*. in 2000 perceived that alginate capsules with the range of 0.5 -1.0 mm in diameter significantly increased viability of bifidobacteria in yogurt with normal pH during refrigerated storage, but not at the simulated stomach pH. As mentioned before (section 4.4.4), increasing beads diameter more than the especial limit (regarding types of capsule and product) is inapplicable because of causing inappropriate mouth-feel and (even) flavor. Furthermore, increasing capsule diameter leads to decreasing its digestibility by pancreatic enzymes. Increasing of beads diameter especially when RS is used for capsule formation should be under attention because this component is resistant to enzymatic digestion of pancreas (Dimantov *et al*., 2003). Research relevant to the concentration of capsule-making solutions has revealed that raising concentration of alginate solution from 0.75% to 1.8% has noticeable effects on *L acidophilus* viability under the simulated gastric conditions; but at >2%, it was impossible to generate spherical and homogeneous beads due to increase in solution viscosity and decrease in its of mass diffusivity (Chandramouli *et al*., 2004). According to another observation, increasing the solution concentration containing calcium alginate and HACS (>2%, of even up to 4%) did not have any considerable effect on the protective properties of beads against intensive environmental factors (Sultana *et al*., 2000). More research is needed to achieve a comprehensive conclusion in this regard.

*5.4. Environmental conditions:* Type and severity of detrimental environmental factors are some of the most important parameters reduce encapsulation effectiveness. For instance, capsules tolerate low acidic environments such as yogurt medium much more than violent acidic conditions such as gastric juices (Sultana *et al*., 2000; Truelstrup-Hansen *et al*., 2002). It has been reported that alginate capsules with a mean diameter of 100 μm are effective enough for the most types of fermented products, but not for gastric acid (Cui *et al*., 2000). Special attention should be placed on this fact because encapsulation effectiveness only with respected to products conditions is not sufficient due to subsequent impact of the gastrointestinal tract on the capsules during delivery from the body. Considering that alginate is one of the most common encapsulating materials used for probiotic encapsulation, precautions must be taken in order to overcome its restrictions against acidic conditions (see section 3.1).

*5.5. Effect of bacteria on the capsules:* There is a report regarding digestion of starch capsules by encapsulated bacteria (Takata *et al*., 1997). Therefore, prior to selection of a capsule material for encapsulation, ability of the enclosed bacteria digest starch should be considered. More research is required in this regard.

*5.6. 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. From modified alginates produced by the first manner, the cross-linked alginate matrix can be mentioned (section 3.1). Addition of glycerol as a cryoprotectant agent gives the beads the ability of cell protection against freezing with cryogens (Kearney *et al*., 1990). By means of this method, viability of bifidobacteria and *L. acidophilus* have been shown to increase up to 88.5 (Kebary *et al*., 1998) and 90% (Sheu and Marshall, 1993), respectively. Cryoprotectants reduce the amount of ice crystallization by attaching to free water molecules. The fact that glycerol-containing beads exhibit 43% reduction in volume compared with normal types during the freezing process is an approval for the cryoprotective effect of glycerol. SDS and tween 80 are other additives which have been under investigation. These additives are capable of both increasing cells resistance against freeze drying and changing beads diameter (Thompson, 2000). The more the concentration of SDS and tween 80 in the encapsulation solution, the smaller the diameter of beads achieved. Also, it has been claimed that application of mentioned materials gives alginate capsules especial characteristics including dissolutionability of the beads in the buffer media as well as increasing their resistance against lactic acid (Krasaekoopt *et al*., 2003).

*5.7. 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. It should be underlined that the cell load more than the special limit, on the one hand, causes softening of the capsules structure and on the another hand, leads to the improper mouth-feel due

to excessive increase in diameter (section 4.4.4).

*5.8. 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, micronization (section 6.2) 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).

*5.9. Miscellaneous affecting factors:* Other remaining factors such as mixing sequence/order of the constituents during microencapsulation, their mixing proportion and mechanical tensions which might make crackling or fracturing of the beads can also affect effectiveness of microencapsulation.

**6. Methods of probiotic microencapsulation**

The technology of probiotic encapsulation can be divided in two parts: 1, microencapsulation of probiotics in the encapsulation solutions and 2, drying of encapsulation solution in order to achieve encapsulated cell powders/granules. Here, these mentioned stages are discussed separately.

*6.1. Microencapsulation stage:* Extrusion and emulsion techniques, which have also been called droplet and two-phase system methods respectively, are two basic ways for encapsulation of probiotic microorganisms (Krasaekoopt *et al*., 2003). These methods are elaborated below:

*6.1.1. Extrusion method:* 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). Howevers, 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). At low concentration levels of alginate (about 0.6%) in the encapsulation solution, gel formation of alginate polymers would be possible only if the calcium ions (concentration about 0.3 M) be present (Jankowski *et al*., 1997). 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, its concentration and as a result, viscosity of alginate solution, distance between the syringe and setting batch and particularly diameter of the extruder orifice (needle) (Smidsrod and Skjak-Braek, 1990). Beads diameter decreases along with increasing concentration and viscosity of the encapsulation solution. Using low-guluronic alginate led to the formation of beads with smaller diameter (Martinsen *et al*., 1989). For production of alginate capsules with chitosan coat, alginate solution is dripped into the hardening batch containing calcium chloride and chitosan (Overgaard *et al*. 1991; Zhou *et al*., 1998). Soaking of alginate beads in the chitosan solution (0.1%, pH 6.5) for 20 min has been observed to form beads with good properties (Krasaekoopt *et al*., 2004). Van Lengerich *et al*. in 2001 invented low-temperature extrusion for microencapsulation of microorganisms and enzymes. In this method, encapsulation is carried out in a plasticized composite matrix consisting of fat, flour and starch. After addition of mentioned mixture to the encapsulated solution, the resulting paste (20% moisture content) is chopped in a chopping system till particles with the diameter range of 0.5-1.5 mm are formed. Resulting particles are dried using air flow. As a consequence, on one hand, shelf-stable microcapsules are formed and on the other hand, heat injuries to the cells are considerably reduced due the absence of applying relatively light temperatures for the formation of capsules, in this method. Hence, this produce has been recommended for the microencapsulation of *L. acidophilus*, as a heat sensitive bacterium (Gouin, 2004).

*6.1.2. Emulsion method:* 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 emulasion 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). Using emulsifiers causes formation of beads with smaller diameters, because these components decrease interfacial tension of the water and oil phases (Adamson, 1982). 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). Microbeads produced by emulsion method are usually recovered by the membrane filtration technique (Krasaekoopt *et al*., 2003). In the emulsion technique relevant to alginate, a fat soluble acid such as acetic acid is usually added to the encapsulation mixture. Thereby, pH of alginate solution is reduced to approximately 6.5, at which gelation process of alginate with calcium ions starts (Poncelet *et al*., 1993). After gel formation, the encapsulated mixture is poured into water to separate the oil phase by decantation (Krasaekoopt *et al*., 2003). 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, 2003a).

*6.2. Drying stage:* Drying of the encapsulated mixture in order to produce cell powders/granules can be achieved by different methods. The most important of these methods are freeze drying, spray drying and fluidized bed drying (Dimantov *et al*., 2003). In general, the drying process causes some injuries to the microbeads, release of some cells and reducing viability of the cells. In the freeze drying technique, heat injuries to the cells are minimal compared with other techniques. However, this method is relatively expensive and difficult to be performed on the industrial scale. Also, cryoprotectants must be used to inhibit cold injuries to the cells. Spray drying has been recommended for this reason because it is a relatively cheap method and large volumes of solutions can be processed by this technique. However, viability loss of the cells is high due to presence of both dehydration and heating factors, simultaneously (Fu and Etzel, 1995). It seems that achieving the best method can be possible by modified techniques of spray drying, as carried out by Picot and Lacroix in 2003. Their procedure was economic with high an ability of maintaining probiotic cells viability (Picot and Lacroix, 2003a). The method consists of coating milk fat droplets containing powder particles of freeze dried cells with polymers of whey proteins, in a condition where emulsifiers and the spray drying process is used. The size of the starter culture powder particles had a determinable impact on their homogeneous distribution within the oil phase (hydrophobic phase). This size should be bigger than bacterial cells (2-4 μm) and smaller than selected fat droplets (10-50 μm) for achieving appropriate encapsulation. Mentioned size regulations were carried out by the micronization process. In previous research carried out by Picot and Lacroix in 2003a, optimization of the process parameters in the spray drying of O/W emulsions had been investigated. The results showed that optimum diameter of fat droplets for the mentioned process was 10-50 mm (Picot and Lacroix, 2003b). Micronization can be done by the size reduction system such as the impact mill, jet mill, mill with agate motor and ball mill systems. Jet mills form the best systems on both the laboratory and industrial scales (Picot and Lacroix, 2003a). This mill has been used to produce various types of wheat flour,

protein powders and pharmaceutical powders (Kurmann and Rasic, 1991, Kearney *et al*., 1990). In Picot and Lacroixs investigation (2003a), effects of process factors including grind air pressure and feeding rate on the diameter of powder particles and cells viability along with the effect of reducing powder particles size (micronization) on the heat resistance of bacterial cells during the spray drying process was studied. Micronization was found necessary to reach the homogeneous emulsion system; however, excessive reduction of particles size led to mechanical damage of the cells and considerably decreased their heat resistance during the spray drying process, especially when high temperatures were used. Therefore, micronization should be carried out with special care and in a particular limit (particularly at high temperatures of spray drying) to avoid mentioned damages. However, more researches should be carried out to find if this is possible to compensate the damaging effects of micronization with the thicker coating of fat layer and whey protein polymers. Picot and Lacroix in 2004 perceived that dispersing of *Bifidobacterium* spp. fresh cells (unfrozen dried cells) in a suspension of heat-treated whey protein base containing milk fat droplets followed by spray drying of the mixture is a suitable method on the industrial scale with respect to cells viability and economics (Picot and Lacroix, 2004). Total achievements of Picot and Lacroix are shown in Figure 2.

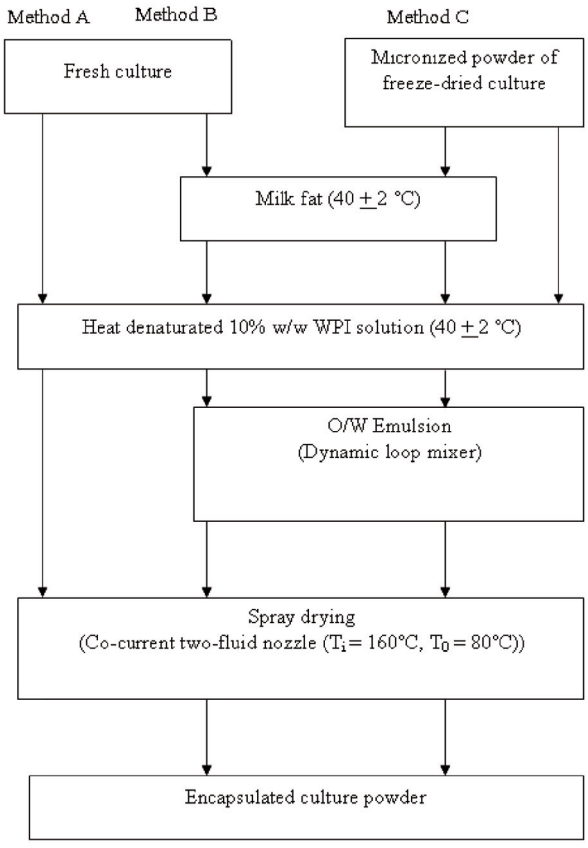


**Figure 2**. Three encapsulation techniques invented by Picot and Lacroix (2003a, b and 2004). WPI= whey protein isolate, $T_i$= temperature-in, $T_0$= temperature-out, O/W= oil in water.

**7. Methods for evaluating the microencapsulation efficiency of probiotics**

Microencapsulation efficiency of probiotics can be evaluated by the assessment of some qualitative parameters as has been discussed below:

*7.1. Viability of probiotic cells:* Efficiency of microencapsulation concerned with viability maintenance of probiotics against detrimental stress factors such as acidity can be measured by drawing and considering the kinetics of cell loss in the products and/or simulated body conditions over time. Because conditions of fermented products such as yogurt are not usually stable due to the metabolic activity of starter cultures during the storage time, the logarithmic loss of probiotic cells dose not follow linear relation, unlike the rules of thermal bacteriology. In order to define the D-value as in thermal bacteriology (here it means equal periods of time, in each of them the microbial population reduces by one logaithm, under constant conditions of pH or acidity, for example), beads encounter the artificial static conditions of the product (lactic acid with the desired pH) and/or the gastrointestinal tract (chloridric acid with pH 1.5-2 for about 2 h, and then, neutral pH of phosphate buffer, digestive enzymes and bile salts of pancreas) and the kinetics of cell loss are assessed. The temperature for all the assessment stages is recommended at 37ºC, *i.e.* normal body's temperature (Dimantov *et al*., 2003). Simulation of fermented products such as yogurt is normally carried out using lactic acid and a storage temperature of 4ºC. The concentration of lactic acid can be increased several times (e.g. 20-25%) in order to reduce the experimental period (Sun and Griffiths, 2000; Dimantov *et al*., 2003).

*7.2. Cell release ability or cell recovery rate or beads solubility/dissolution ability:* Capsules must be capable of releasing their entrapped cells into the objective place, namely intestine. Since the intestine provides the right conditions for probiotics to survive and multiply, release of cells into this site leads to probiotics

settlement and activity. By the subjecting of microbeads to the simulated gastrointestinal conditions (section 7.1), cell release percent can be measured. Released cells are enumerated and are compared with the initial cell counts before the encapsulation. Also, as produced polymers from digested capsules can be filtered by membrane processing (using suitable mesh), the weight ratio of the beads prior and subsequent to encountering with simulated conditions, or the weight ratio of retentate and filtrate particles represents the percentage of indigested beads. Beads digestibility can also be determined by measuring the ratio of the beads mean diameter before and after doing the digestivity test. This can be made possible a using light scattering technique. Direct observation with scanning electron microscopy (SEM) is also recommended in this regard (Dimantov *et al*., 2003). In order to assess the digestibility of the beads against pancreatic enzymes, they are normally incubated at 37ºC in the presence of the mentioned enzymes.

*7.3. Microgeometrical properties of the beads:* These properties include bead- size/diameter (is usually reported as an average bead size/diameter), shape, integrity and uniformity in shape and size. Beads size can be measured by applying a laser diffractometer or the light scattering technique (Picot and Lacroix, 2003a). Direct observation with a light microscope or SEM method is also suggested. Other methods such as sieving or membrane filtration of encapsulated mix can also be used. For this reason, sequential sieving (150 µm, 500 µm and 1 mm) has been recommended. Shapes of the beads are mainly spherical or elliptical. However, these can be observed directly by the two microscopic techniques mentioned above. Integrity and uniformity of the beads have an important impact on their efficiency. In this regard, studying the pores, cracks and voids of the beads surface are especially important. The best way of evaluating beads integrity and uniformity is by observation with the SEM method. Also, light scattering technique might be useful, as uneven surfaces have a higher light scattering index. Uniformity of the beads from the size point of view, apart from being considered by the SEM method, has been recommended to evaluate the use of sieving, as mentioned above (Sultana *et al*., 2000). For consideration of microgeometrical properties of the beads, self-aggregated particles that do not carry any cells, should not be mistaken for the real microbeads. This can particularly be evident for starch particles and small barely granules (Sultana *et al*., 2000; Truelstrup-Hansen *et al*., 2002).

*7.4. Capsules density:* Compression of the capsules in the beads can be evaluated by the SEM method (Sultana *et al*., 2000). Light scattering technique has also been recommended, as dense materials have more light absorption. This explains why mentioned materials seem more opaque under the light microscope or in SEM images. Surface density (mass/surface) of the capsules is possible to be assessed by breaking/dissolving the capsules, drying and weighting them and finally, dividing the result by the total surface of the beads. It is usually reported in g/cm$^2$ (Dimantov *et al*., 2003; Picot and Lacroix, 2003a). Total surface of the beads can be assessed with respect to the average bead diameter and its proportion to the surface.

*7.5. Cell load of the beads:* The best way to reach a mean of the beads cell load (number of the cells in each bead) is by direct observation of the beads from different samples by SEM method (Sultana *et al*., 2000).

*7.6. Setting/hardening trend of the capsules:* This specification can be monitored by using the SEM method, with periodic scanning.

*7.7. Dispersibility of the beads within the product:* The beads distribution homogeneity throughout the product has been recommended to be evaluated by consideration of SEM images, from different samples (Dimantov *et al*., 2003).

## CONCLUSIONS

In the present article, principles and methods of probiotics microencapsulation were reviewed. Importance and variety of probiotics and food probiotic products on the one hand, and successes achieved by applying the microencapsulation process for the purpose of maintaining of probiotic cells viability on the other hand, have necessitated extensive research in the probiotic microencapsulation field. However, general industrial application of the microencapsulation process in the case of probiotics seems still far from achieved, because many of the details are under the question. Future research could be concentrated on the aspects such as applying more efficient encapsulation materials or improving the common used ones; consideration of probiotic encapsulation in the products which have not still been investigated or a few evidences are present about them; approval or rejection of the previous research results; studying correlations

between process factors and microencapsulation effectiveness in different products; developments and new achievements in microencapsulation technology relevant to each industry and product, optimization of the process factors in order to reach the highest viability and the most satisfactory sensory properties of the products along with the lowest cost and improving or inventing new methods relevant to evaluation of microencapsulation efficiency, particularly under *in vivo* conditions. The latter issue should be given especial attention; because if the capsules do not efficiently protect probiotic cells from violent gastrointestinal tract conditions, even with a satisfactory efficiency of the products, would have no positive effect at most, due to final deterioration through delivery inside the body. However, in a few investigations this important issue has been under the attention. In conclusion, although many questions and details should be answered and revealed, in general, good hopes are visualized for the microencapsulation of probiotics in the future. This reasoning might open a new horizon of extensive investigation in this field.

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