DEVELOPMENT OF WHEY PROTEIN-PULLULAN MICROCAPSULES FOR THE ENCAPSULATION OF *Lactobacillus acidophilus* NRRL-B 4495 AS A FUNCTIONAL FOOD INGREDIENT

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ABSTRACT

DEVELOPMENT OF WHEY PROTEIN-PULLULAN MICROCAPSULES FOR THE ENCAPSULATION OF *Lactobacillus acidophilus* NRRL-B 4495 AS A FUNCTIONAL FOOD INGREDIENT

The aim of this study was to develop whey protein-pullulan (WPI-pullulan) microcapsules in order to assess its protective effect on the viability probiotic bacteria under *in vitro* gastrointestinal conditions. The probiotic bacteria used in this study was of *Lactobacillus acidophilus* NRRL-B 4495. This study demonstrated that probiotic bacterial cells were successfully encapsulated into WPI-pullulan gel matrix. The appropriate microcapsule formation parameters include formation of primary emulsion having 50% inner polymer aqueous phase having whey protein in the presence of 10.8% pullulan with sunflower oil. Optimal concentration of bacteria for microencapsulation was decided to be kept at 10.00 log CFU/g since increasing initial load resulted in decreasing microencapsulation efficiency. The incorporation of pullulan into whey protein wall matrix created spherical microcapsules with denser and smoother surface structure. Microcapsules with varying pullulan concentrations in the wall matrix obtained the mean diameters between 68 μm to 80μm. The survival rates of *Lactobacillus acidophilus* NRRL-B 4495 under *in vitro* gastrointestinal conditions were significantly ($p<0.05$) improved by the presence of pullulan. WPI-pullulan wall matrix resulted in a 87.18% survival rate as compared to 73.19 % for free cells after 3h incubation in simulated gastric solution. Protein-polysaccharide gel network formed in the microcapsules restricted diffusion of bile salts, as well. During spray drying experiments with high concentration of pullulan, fibrous structure formation was observed. Optimal conditions for spray drying of probiotic cells were decided to have 145 ºC as an outlet temperature in the presence of WPI-pullulan wall matrix with 4.5:1 protein:pullulan ratio. Moreover pullulan incorporation in contributed to release properties of microcapsules by altering the dissolution and porosity properties. On the other hand, encapsulation into developed polymer wall matrix in wet and spray dried forms did not provide any positive advantage for the responses of probiotic microorganism against heat stress.

ÖZET

FONKSİYONEL GIDA KATKI MADDESİ OLARAK *Lactobacillus acidophilus* NRRL-B 4495'İN ENKAPSÜLASYONU İÇİN PEYNİR ALTI SUYU PROTEİNİ-PULLULAN MİKROKAPSÜLLERİNİN GELİŞTİRİLMESİ

Bu çalışmanın amacı *in vitro* gastrointestinal koşullar altında probiyotik bakterilerin canlılığı üzerine etkisini gözlemlemek amacıyla, peynir altı suyu proteini-pullulan (PAS-pullulan) mikrokapsüllerinin geliştirilmesidir. Bu çalışmada probiyotik bakterileri olarak *Lactobacillus acidophilus* NRRL-B 4495'in kullanımına karar verilmiştir. Bu çalışma, probiyotik bakteri hücrelerinin PAS-pullulan jel matris içine başarılı bir şekilde kapsüllenmiş olduğunu göstermektedir. Uygun mikrokapsül oluşumu parametreleri şu şekilde belirlenmiştir; ayçicek yağı ile %10.8 oranında pullulan içeren protein jel matrise sahip, %50 iç polimer sulu fazdan oluşan birincil emülsiyon oluşumu ve 10.00 log KOB/g optimum bakteri oranı olarak kararlaştırılmıştır. Peynir altı suyu proteininden oluşan duvar matrisi içerisine pullulan eklenmesi ile daha yoğun ve pürüzsüz yüzey yapısına sahip küresel mikrokapsüllerin oluştuğu gözlemlenmiştir. Duvar matris içinde bulunan farklı pullulan konsantrasyonları ile 80µm-68µm arasında ortalama çapa sahip mikrokapsüller elde edilmiştir. *Lactobacillus acidophilus* NRRL-B 4495'in *in vitro* gastrointestinal koşullar altında canlı kalma oranları pullulan varlığıyla önemli ölçüde ($p < 0.05$) artmıştır. Simüle edilen mide çözeltisi içerisinde 3saat inkübasyon sonucunda PAS-pullulan duvar matrisi %87.18 oranında bakteri canlılığı gösterir iken serbest hücreler için bu oran %73.19 olarak hesaplanmıştır. Protein-polisakkarit jel ağı ile mide çözeltisinin yanında safra tuzunun da difüzyonu sınırlandırılmıştır. Püskürterek kurutma işlemi esnasında yüksek pullulan varlığı sonucu fibril yapıların oluşumu gözlemlenmiştir. Probiyotik hücrelerin protein:pullulan oranı 4.5:1 olan Pas-pullulan duvar matrisi varlığında 145 ºC dış sıcaklıkla püskürtülerek kurutulması en uygun koşul olarak belirlenmiştir. Ayrıca pullulan varlığı ile mikrokapsüllerin salınım özellikleri de değişim göstermiştir. pH tolerans testi ile PAS-pullulan matrisine kapsüllenen bakterilerin uygun ortam koşulları altında çoğalmasının mümkün olduğu sonucuna varılmıştır. Öte yandan, geliştirilmiş polimer duvar matrisine kapsülleme işleminin bakterilerin ısı stresine karşı dayanıklılıklarında olumlu bir sonuç elde edilememiştir.

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CHAPTER 1

INTRODUCTION

The intestinal microbial community has an essential role in maintaining human health since microbial balance provides beneficial fermentation products, helps the production of vitamins and increases the bioavailability of essential minerals. The relationship between food consumption and health is becoming more and more important with each passing day. With the consumers' interest to natural products and functional foods, demands for functional food products show significant increase. Functional food market is growing rapidly worldwide. Compared with Europe and the America, the functional food market in Turkey, even a long way behind, has been developing quite rapidly. Probiotic products occupy one of the hugest areas within the functional food group. Probiotic bacteria represent a group of live microorganisms that exert beneficial effects for the host animal when consumed in adequate amounts. Probiotics have reported to exert several health benefits such as lowering serum cholesterol level and stimulating immune system. However, to exert these beneficial health effects, probiotics should maintain the viability throughout storage and consumption. After many studies conducted on probiotics, International Dairy Federation (IDF) stated that in colon, concentration of 10^7 live cells per gram or ml of the product should exist in order to exert expected benefits (Bouhnik, 1993).

Many nutraceutical and functional food components benefit from being encapsulated in appropriate edible delivery systems, including vitamins, probiotics, prebiotics, omega-3 fatty acids, plant extracts, antimicrobials, antioxidants, flavors, colors, and minerals. As well as probiotics, these active ingredients in foods must remain fully functional and be transported and discharged appropriately to have the desired nutritional effect since bioactive ingredients are prone to degradation and can interact with other food components resulting in loss of bioavailability and thus there is a need to protect them throughout their shelf-life as both an ingredient and in fortified food products, with the specific food systems without affecting the sensory properties, color or flavor of food products. Therefore, microencapsulation of probiotics is considered as an effective approach for their efficient survival under gastrointestinal

conditions and to improve the viability during shelf life to maintain their health promoting effects. Many studies have been conducted to find an effective microencapsulation matrix and method until today. Despite wide applications of several matrix and methods for probiotics, it is still a challenge to produce microcapsules for preservation of desired bacterial culture with high viability which does not cause non desirable texture in the final product.

Proteins and polysaccharides have been widely used for the aim or microencapsulation of probiotics. Between these polymers whey protein and alginate are two major substances used as wall matrix. Benefits of whey proteins in food applications include its high protein and amino acid content; low calorie, fat, and sodium content; lack of pathogens, toxic compounds, and antinutritional factors; good emulsification capacity; compatibility with other ingredients; ready availability; and the perception that it is a "natural" product (Renner et al., 1991). It is an effective encapsulating agent in microencapsulation of bioactive ingredients because of their ability to form microcapsules easily under mild conditions using different techniques.

However, these studies showed that whey protein does not significantly enhance the survival of probiotics during gastrointestinal transit. Therefore, administration of polysaccharides was needed to increase the viability and stability of probiotics in microencapsulation system. Filmogenic properties of protein-polysaccharide polymer microencapsulation permit barrier creation for the protection of sensitive ingredients against moisture, diffusion of acid and their transport in aqueous media (Ducel et al., 2004; Mendanha et al., 2009; Pereira et al., 2009; A. P. Pierucci et al., 2007; A. P. T. R. Pierucci et al., 2006; C. Yu et al., 2007). Based on these improved functional properties of protein-polysaccharide complexes, we decided to investigate the potential of whey protein-pullulan complex to encapsulate *L. acidophilus* NRRL B-4495.For this objective, combination of whey protein with different sorts of polysaccharides has been studied for microencapsulation of bioactive substances. Among these polysaccharides, alginate, xhanthan, starch and maltose have been widely studied. Pullulan is not extensively studied for the microencapsulation of probiotics. It has been most widely searched to form films with proteins including whey protein and soy protein due to its non-toxic, water-soluble, colorless, tasteless, odorless and heat stable characteristics.

The overall aim of this thesis was to microencapsulate the probiotic, *L. acidophilus*, into protein and polysaccharide mixture that are composed of whey protein

isolate and pullulan to provide sufficient protection against simulated gastrointestinal conditions.

Several objectives were established to meet this goal. The first objective was to investigate the effect of selected encapsulation process parameters including oil phase type, inner phase volume ratio, pullulan to whey protein ratio and initial bacterial population to reach the maximum encapsulation efficiency without forming non desirable large microcapsules. The second objective was to investigate the characteristics of developed microcapsules to observe the resistance to the physical conditions including high salt and sucrose concentration and varying heat and pH changes. Moreover, the resistance and release properties of microencapsulated bacteria in simulated gastrointestinal conditions and the surface structures of the designed capsule were investigated. The final objective was to obtain dry microcapsules containing probiotic bacteria for further use.

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CHAPTER 2

PROBIOTICS

2.1. Human Gastrointestinal Tract

The human gastrointestinal tract is colonized by different species of microorganisms. The prevalence of these microorganisms depend several factors including age, pH of the medium, diet, oxidation-reduction potential of the tissues and bacterial adhesion. Colonization starting with the birth changes over time. Adult human gastrointestinal microbiota consists of nearly 10^{14} microbial cells. Infants born with sterile colon and after birth the infant gastrointestinal tract is colonized by bacteria from environment and feces. *Bifidobacterium* predominates firstly in gastrointestinal tract of breast-fed infants, but later facultative anaerobes *Enterobacteria* and *Enterococcus* start to colonize and enable anaerobe microorganisms to dominate. Starting to take solid foods, adult type bacterial communities develop and *Bacterioides, Clostridium, Eubacterium, Enterococcus, Enterobacteria, Escheichia, Parabacteroides, Ruminococcus, Lactobacillus and Bifidobacterium* are the main genera present in the intestine (Figure 2.1). The intestinal microbial community plays a major role in health and diseases of its host in positive ways through fermentation products, increasing resistance to pathogen colonization, immunomudulation, producing vitamins or in negative ways through producing infections and increasing cancer risk. (Gibson and Roberfroid, 1999; Ouwehand and Vaughan, 2006).

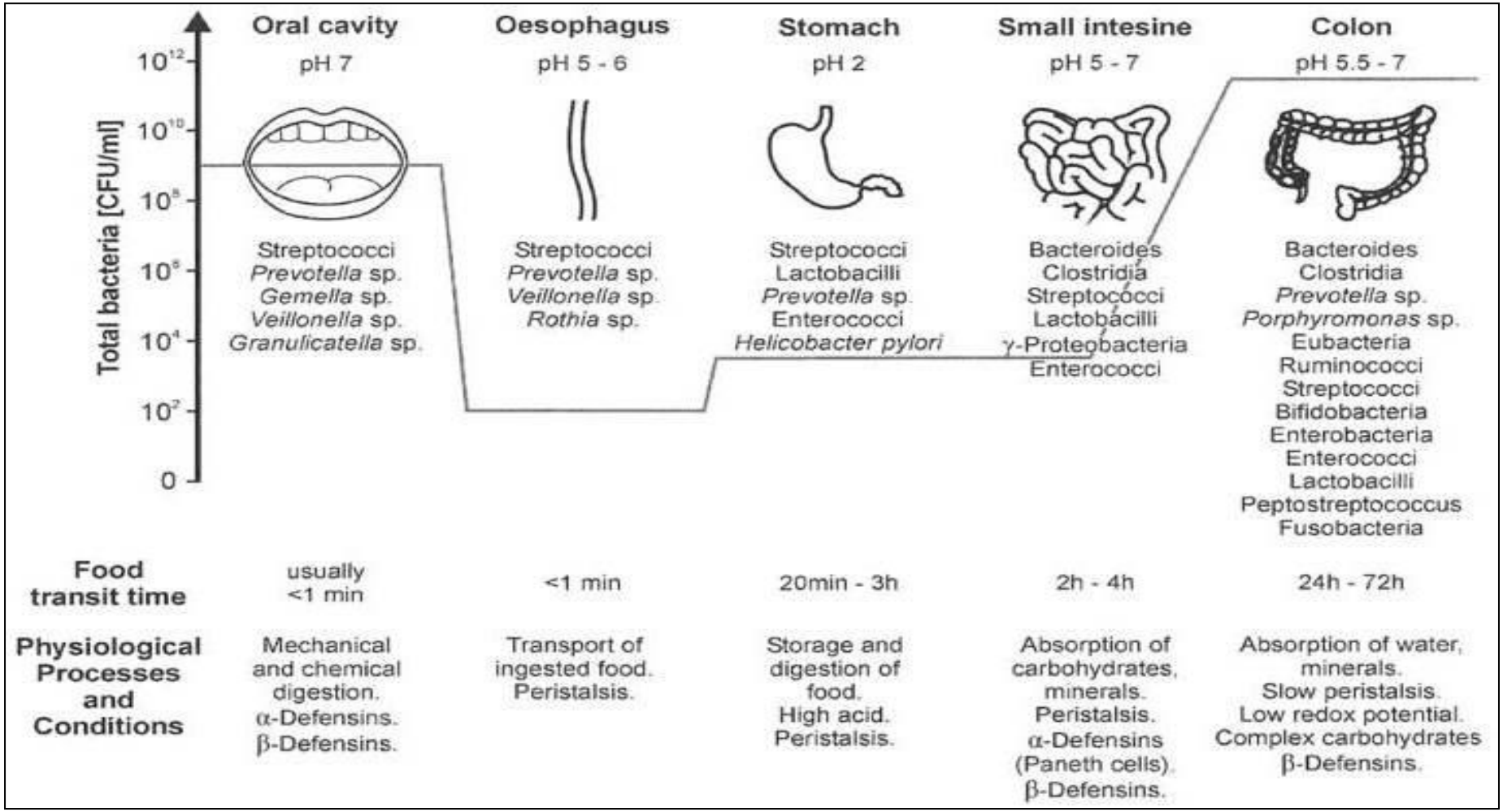

Figure 2.1. The distribution of the microbial communities inhabiting on the gastrointestinal tract of humans (Source: Huffnagle and Noverr, 2008)

2.2. History of Probiotics

Elie Metchnikoff, Nobel Prize winner scientist, demonstrated the health promoting benefits of consumption of fermented milk products containing harmless lactic acid bacteria at the turn of 20th century. However, the "probiotic" term was first proposed by Lilly and Stillwell as microbially derived factors that stimulate the growth of other organism in 1965.

Several varying definitions of probiotic microorganisms, including "organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance" (Parker, 1974) and improved as "probiotics are the live microbial feed supplements that exert beneficial effects for the host animal by improving its intestinal microbial balance" by Fuller in 1989. Several approaches to define probiotics has adopted and today probiotics are defined as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" by (Lorenzo Morelli and Capurso, 2012) are generally used.

2.3. Role of Probiotics on Human Health

Several benefits of probiotic organisms on human health have been suggested including lowering of blood cholesterol level, improvement of immunity, alleviation of symptoms of lactose intolerance, treatment of diarrhea, suppression of pathogens, anticarcinogenic and antihypertensive properties (Lourens-Hattingh and Viljoen, 2001; Shida et al., 2011). Some major probiotic microorganisms available for human use are listed in Table 2.1.

Table 2.1. Probiotic microorganism used in human health
(Source: Leroy et al., 2008; Pyar et al., 2013)

Genus	**Species**
Lactobacillus	*acidophilus*
	brevis
	casei
	rhamnosus
	helveticus
	johnsonii
	lactis
	paracasei
	fermentum
	reuteri
	salivarius
	plantarum
Bifidobacterium	*breve*
	longum
	lactis
	infantis
Lactococcus	*lactis subsp lactis*
	lactis subsp. cremoris
Enterococcus	*faecium*
Bacillus	*subtilis*
	cereus
Saccharomyces	*boulardii*
	cerevisiae
Pediococcus	*acidilactici*

2.3.1. Lowering Serum Cholesterol Level

High cholesterol levels are considered risk factors for developing of human coronary heart diseases (Lim et al., 2004). Although cholesterol-lowering medications for the treatment of high cholesterol are the major treatment strategy, they are mostly expensive and often have unwanted side effects (Z. Guo et al., 2009; M. Kumar et al., 2012; Tomlinson and Mangione, 2005). Therefore, there has been considerable interest in use of probiotic dietary supplementation for the reduction of blood cholesterol level. Possible mechanisms are assimilation of cholesterol during growth of probiotic cells, binding of cholesterol to the cellular membrane of cells, enzymatic deconjugation of bile acids by bile salt hydrolase BSH) activity of probiotics, production of short-chain fatty acids by oligosaccharides and the conversion of cholesterol to coprostanol by cholesterol reductase (Anderson and Gilliland, 1999; Pavlović et al., 2012) There are several successful *in vivo* and *in vitro* studies showing the effectiveness of use of probiotics for the reduction of serum cholesterol levels *in vitro* and *in vivo*.

Ziarno (2009) using *in vitro* experiments, reported significant cholesterol removal from MRS medium by all tested strains of *Lactobacillus acidophilus* during 5 h of growth period. Fukushima and Nakano (1996) showed *Lactobacillus reuteri* as a potential cholesterol lowering effect as it caused 38% decrease in total cholesterol in hypercholesterolemic mice. Similarly, in a study conducted by Cani et al. (2007) use of some *Bifidobacterium* species resulted in a reduction in serum cholesterol levels in rats. Anderson and Gilliland (1999) studied the effect of probiotic yoghurt on serum cholesterol levels in hypercholesterolemic humans. Results showed that human originated *L. acidophilus* L1 supplemented yoghurt samples reduced the serum cholesterol levels by 2.4% and 3.2% in two studies of 4-week treatment. In another research, Xiao et al. (2003) studied the effects of fermented milk products by *Bifidobacterium longum* BL1 on humans and concluded significant decreases in total serum cholesterol levels having hypercholesterolemia.

2.3.2. Immune System Stimulation

The human intestine contains large numbers of complex microbial community that exert both beneficial and harmful effects on host health. Recent researches have indicated relationships between intestinal microbioata and host immune defense mechanism (Macpherson et al., 2014; Maloy and Kullberg, 2008; Witte et al., 2010).

Epithelial cells in the intestinal tract (IECs) interact with immune cells of lymphoid tissue, lamina propria lymphocytes (LPL), and intraepithelial lymphocytes (IEL) to contribute the regulation of the mucosal immune response (Kagnoff and Eckmann, 1997; Medici, et al. 2004).

Medici Medici et al. (2004) studied the effect of feeding with probiotic fresh cheese on phagocytic activity of peritoneal macrophages, number of IgA+ producing cells in both small and large intestine, the ratio of CD4+ and CD8+ T lymphocytes in the small intestine of mice.

2.3.3. Lactose Intolerance

Lactose intolerance is caused by the deficiency of the β-galactosidase enzyme (Panesar et al., 2006). Unfortunately significant fraction of global population is lactose intolerant (Figure 2.2), limiting the consumption of milk and dairy products. Such persons not only suffer from malabsorption but also suffer from general impairment of the normal digestion process.

Lactose is hydrolyzed by an enzyme β-galactosidase known as lactase in the intestinal mucosa. As a result the lack of lactase, the hydrolysis of sugar lactose is incomplete. The undigested sugar pulls fluid into the intestine, moreover when the colonic bacteria acts on the undigested sugar lactic acid, hydrogen and other organic acids are produced. At the end the combined osmotic effect results in the passage of acidic diarrheal stools. Lactose intolerance describes the presence of gastrointestinal symptoms such as abdominal pain, bloating, flatulence, nausea or diarrhea. In its most severe form, lactose intolerance leads to dehydration and failure to thrive (Panesar et al., 2006; Roy and Gupta, 2003)

Probiotics have shown to have a significant role in the alleviation of lactose intolerance by the action of their extracellular β-galactosidase activity during the

ingestion of non-fermented foods. In fermented foods, lactose is used as an energy source during growth, thus reducing its content in the final products (Vasiljevic and Shah, 2008). However this activity is based on the protection of the probiotic bacteria. During gastrointestinal transit, the gastric enzymes and bile salts cause the degredation of lactase enzyme (Montalto, et al. 2006). Another important factor is the probiotic strain and the other carbohydrates existing in the medium (de Vrese et al., 2001). The utilization of lactose in presence of 2 strains of *B. longum* in a glucose containing medium were studied (Jiang, et al. 1996). It was shown that lactase activity was increased in the glucose free medium, improving lactose digestion and decreasing the hydrogen exhalation.

In another study by Montes et al. (1995), the consumption of acidophilus milk with or without inoculated yogurt bacteria have been investigated in children. Probiotic inoculated milk samples with or without a yoghurt culture were associated with decreased symptoms for lactose intolerant trials.

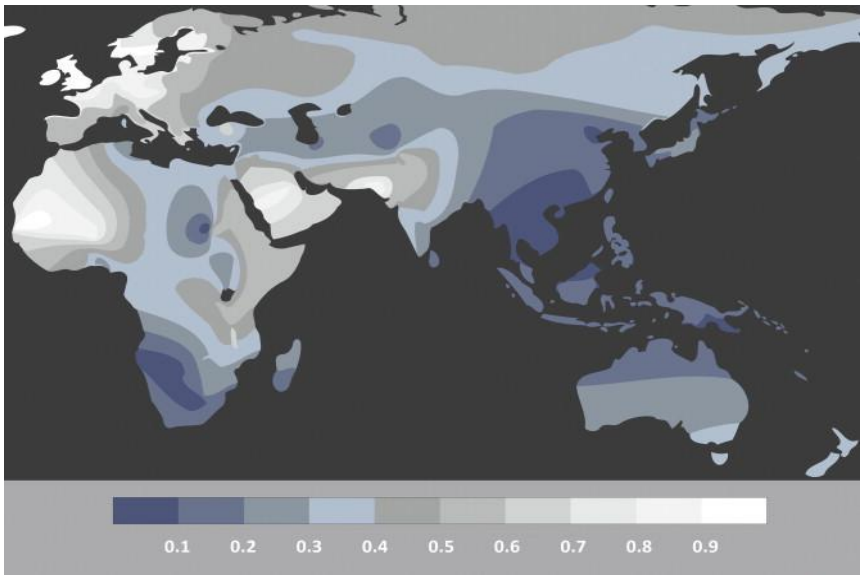

Figure 2.2. Lactase tolerance frequencies worldwide
(Source: Leonardi et al., 2012)

2.3.4. Diarrhoea

Diarrhea is a common disease causing some serious problems such as mortality and morbidity in case of lasting a long time. Antibiotic associated diarrhea (AAD) is a common complication exposed by the use of broad-spectrum antibiotics. *Clostridium*

difficile diarrhea is one of the most common type of infectious diarrhea caused by *Clostridium difficile*. It is caused when the competing bacteria in the gut is reduced as a result of antibiotic intake. The effectiveness of probiotic usage for treatment of infectious and AAD has been published in many clinical studies.Hickson et al. (2007) studied the efficiency of a probiotic drink on antibiotic associated diarrhea. The study was conducted on 135 antibiotic taking patients supplementing with a probiotic drink containing *Lactobacillus casei*, *Lactobacillus bulgaricus*, and *Streptococcus thermophiles*. Results showed that a significant (p= 0.007) reduction in the antibiotic associated diarrhea can be obtained by the use of probiotic drink compared to placebo group.

Additionally the effect of probiotic use in healthy infants for the prevention of infections was observed (Weizman et al., 2005). Infants were fed with two types of probiotics; *Bifidobacterium lactis* (BB-12) and *Lactobacillus reuteri*. As a result it was concluded that infants fed with both probiotics showed a significant reduction in incidence of diarrhea during experimental period and *L. reuteri* was more effective.

The other most commonly seen type is travelers' diarrhea and it is recorded to expose between the ranges of 30% to 70% of traveling population. In a study, experiments on an animal model to observe the effects of probiotic formulation on travelers' diarrhea were conducted (Bisson et al., 2010).

2.3.5. Suppression of Pathogens

The action mechanism of probiotics on pathogens can be explained by different ways. Secretions of antimicrobial substances, production of lactic acid thus lowering the illuminal pH and inhibiting the adhesion of pathogens to mucosal cells are involved in main action mechanisms. de LeBlanc Ade et al. (2010) demonstrated that continuous administration of *L. casei* CRL 431 has significant inhibitory effect on *Salmonella typhimuriumin* infection on an animal model. When various commercial probiotic strains (*L. rhamnosus* GG (ATCC 53103), *L. rhamnosus* LC705 (DSM 7061), *B. breve* 99 (DSM 13692), *P. freudenreichii* spp. *shermanii* JS (DSM 7067) were tested for their ability to inhibit the adhesion or displace the adhered pathogens.

In another study conducted by Luoto et al. (2014) it was found that oral intake of probiotic *Lactobacillus rhamnosus* GG supplemented with prebiotics

(galactooligosaccharide and polydextrose mixture) achieved protection against viral respiratory tract infections in infants and lowered the incidence of rhinovirus-induced episodes. Collado et al. (2007) found that the combination *L. rhamnosus* LGG with *L. rhamnosus* LC705 was significantly effective in inhibiting *Staphyloccoccus aureus*, *Escherichia coli* and *Salmonella enterica* serovar *typhimurium* over 50% rate. Additionally, (Cano and Perdigon, 2003) reported in their study that *Lactobacillus casei* CRL 431 prevented *Salmonella* and *E. coli* infections in an animal model.

2.3.6. Anticarcinogenic Properties

The incidence rate of cancer has been steadily increasing with time. An important association of diet and intestinal microbial population with cancer risk, especially for colon and rectal cancer types, has been observed by many researchers (Fraser, 1999; Giovannucci and Willett, 1994; Verberne et al., 2010). Thus, the potential protective effect of probiotic bacteria has gained a considerable interest against cancer risk.

In vitro and *in vivo* studies revealed the importance of inhibitory effects of probiotics on carcinogenesis. The anti-carcinogenic effects of probiotics does not depend on a single mechanism, in fact complex system is involved in such as production of antitumourigenic or antimutagenic metabolites, enhancing the hosts' immune response and acidification of the colon or suppression of pathogens thus modulation of bacterial enzymes promoting carcinogenic substances (M.-T. Liong, 2008)

In an early study, Campbell and Hayes (1976) found that colon cancer risk could be reduced by growth of *Bifidobacterium* in colon stimulated the production of metabolites that effect the conversion of azoxymethane from proximate to ultimate carcinogen. Goldin et al. (1996) reported the anticarcinogenic effect of *L. casei* subsp. *rhamnosus* GG on DMH-induced intestinal tumorigenesis in rats. In another study, the anticarcinogenic activity of probiotic *Bacillus polyfermenticus* on various cancer cells was investigated (Ma et al., 2010). Results revealed that *B. polyfermanticus* exerts anticarcinogenic activity by suppressing the growth of skin, breast and lung adenocarcinoma cells. Moreover human colon cancer cells including HT-29, DLD-1 and Caco-2 cells were inhibited successfully by *B. polyfermanticus.*

The positive effects of probiotics in cancer prevention have also been investigated on human volunteers. For instance, intake of probiotic *L. acidophilus* showed a significant decrease in urinary and fecal mutagenic activities which were increased with the ingestion of fried meat in diet during the study (Lidbeck et al., 2011).

2.4. Selection Criteria for Probiotics

To exert the beneficial effects on the host, probiotic bacteria should stay metabolically active in the final product throughout the storage. Additionally, the ability of probiotic bacteria to survive through the gastrointestinal tract, reach viable and colonize in the intestine of the host is another important factor. The origin of the probiotic bacteria if used in human health benefit is suggested to be human origin. This is due to the consideration that the beneficial effect of probiotics is host specific. However this criteria is changing by the increase in studies showing that the beneficial effects of the probiotic are strain dependent (R. G. Crittenden et al., 2003; L. Morelli, 2007; Sanders, 2003). Additionally ability to adhere to and colonize in the intestinal mucosa is the other essential criteria for probiotics. As the time increases the probiotic microorganism stays in the intestine, the health promoting effects will last longer (Kailasapathy and Chin, 2000; Parvez et al., 2006). Among numerous selection criteria for probiotics stated above, main ones are summarized in Figure 2.3.

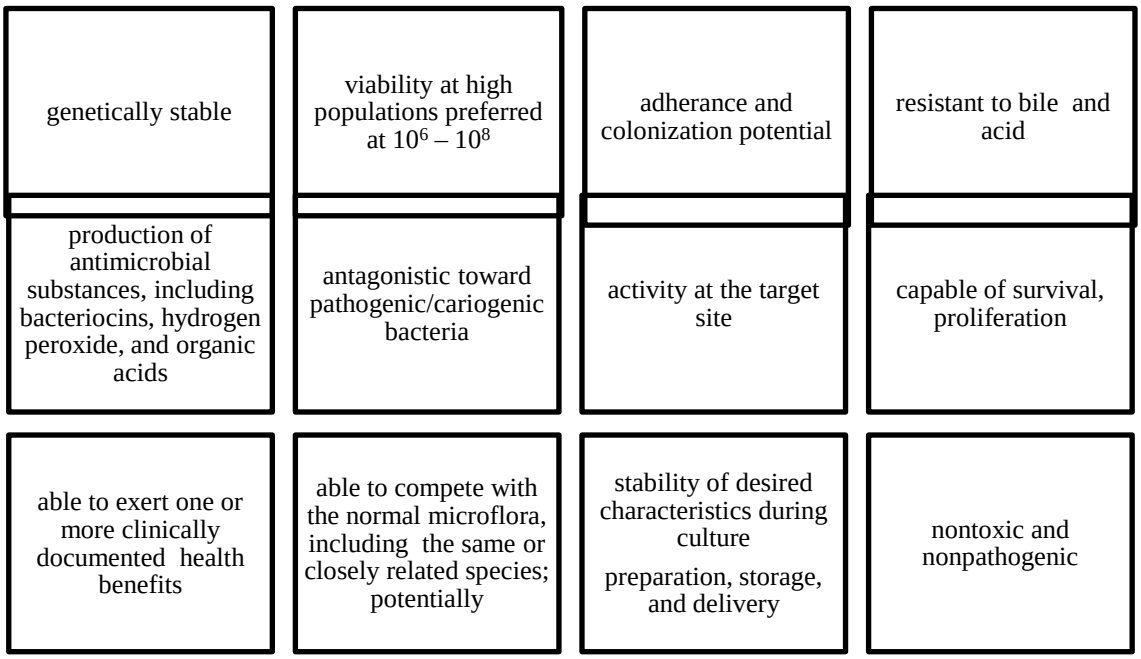

Figure 2.3. Selection criteria for probiotics

2.5. Lactic Acid Bacteria and Probiotics

After the introduction of "*Bacterium lactis*", first pure culture by by Joseph Lister in 1873, Lactic acid bacteria (LAB) have a long history of use. In many kinds of industrial applications that include fermentation technology, development of functional foods and production of enzymes/metabolites, LAB are widely used.

LAB are Gram (+), non-spore forming, catalase-negative and non-motile rods or cocci producing lactic acid as a major metabolite. They are generally recognised as safe (GRAS) and *Streptococcus, Leuconstoc, Pediococcus*, *Bifidobacterium* and *Lactobacillus* are the five main genera of LAB. Besides industrial uses, revealed health and nutritional benefits provided increased importance and moved LAB to form the base of probiotic bacteria. Within this group, most commonly used microorganisms as probiotic belong to the members of the genus *Lactobacillus* and *Bifidobacterium.* (Ghoddusi, 2011; Sanders and Klaenhammer, 2001).

2.5.1. *Lactobacillus acidophilus*

L. acidophilus exists in the normal physiologic microflora of the mammalian gastrointestinal tract. It is rod shaped homofermentative (ferments only lactose to lactic acid) bacteria (Figure 2.4). It grows optimum between pH 5.5-6.0 and 35-38 °C. It produces beta-galactosidase enzyme that ferments lactose to simple sugars glucose and galactose.

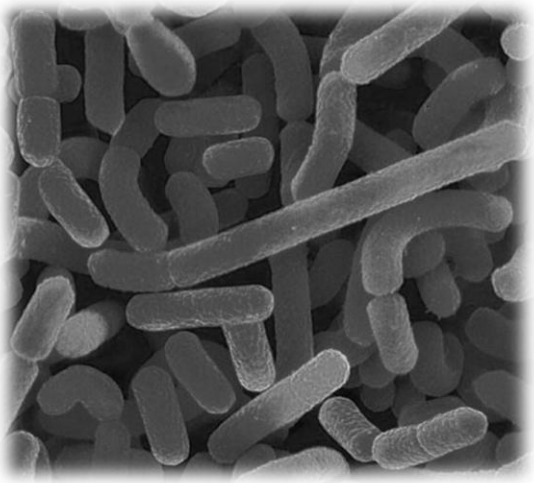

Figure 2.4. Microscopic image of *L. acidophilus*
(Source: Phillips, 2009)

It is one of the most well-known bacteria known as probiotic among LAB. In addition, its use as a probiotic, its activity against foodborne pathogens in foods has recently been investigated (Hatakka et al., 2007; Madureira et al., 2011; Shu and Gill, 2002). Many *in vivo* and *in vitro* reports and studies have been reported for *L. acidophilus* as a probiotic.

Recently Pyar et al. (2013) studied the effect of metabolites of *L. acidophilus* FTC-4462on selected pathogens. The results showed a significant bactericidal activity against pathogen *E. coli* and *S. aureus.* In another study testing the effectiveness of *L. acidophilus* and *B. lactis* on type 2 diabetic subjects (Ejtahed et al., 2012) were conducted. 4.5% and 7.45% decrease observed in total and LDL cholesterol levels, respectively. Michetti et al. (1999) demonstrated the antimicrobial activity of *L. acidophilus* LA1 on growth of *Helicobacter pylori.*

Large number of probiotic studies was mostly conducted on milk and dairy products aimed to develop functional probiotic foods. For instance in one study probiotic cheese was produced by using *L. acidophilus* LA-5 (Madureira et al., 2011). High viable bacterial numbers were obtained during the shelf life of samples and according to sensory tests, cheese samples produced by addition of 0.4% (w/v) *L. acidophilus* LA-5 accepted by consumers.

Yoghurt with *Bifidobacterium animalis* subsp. *lactis* (BB-12) and *L. acidophilus* (LA-5) was consumed with healthy adults daily for 4 weeks (Savard et al., 2011). Results proved that daily consumption of probiotics helped to maintain the desired therapeutic viable numbers in intestinal microflora. In another study, pomegranate juice was fermented with of *L. acidophilus* and *L. plantarum* separately (Mousavi et al., 2011). As a result, DPPH radical-scavenging effects of fermented fruit juice increased about 20% compared to unfermented control. This showed that addition of *L. acidophilus* and *L. plantarum* improved the beneficial effects of pomegranate juice.

2.6. Probiotic Products in Industry

The term “functional food” was used in a project searching for the effects of food components on human body systems at first time in Japan. The relationship between food consumption and health is becoming more and more important with each passing day. With the consumers’ interest to natural products and functional foods,

demands for functional food products show significant increase. Functional food market is growing rapidly worldwide. Compared with Europe and the America, the functional food market in Turkey, even a long way behind, has been developing quite rapidly. The positive effects on human health and the importance of probiotics increasingly emphasized in many studies and an increase in the consumption of functional foods has been observed. Table 2.2 showed the commonly used probiotic microorganism and the probiotic products in the industry worldwide.

Table 2.2. Commonly used probiotic lactic acid bacteria in food industry (Source: Heathcote et al., 2008)

Strain	**Brand name**	**Company name**
Bifidobacterium animalis DN 173 010	Activia	Danone
Bifidobacterium breve Yakult	Bifiene	Yakult
Bifidobacterium lactis HN019 (DR10)	Howaru Bifido	Danisco
Enterococcus LAB SF 68	Bioflorin	Cerbios-Pharma
Lactobacillus casei Shirota	Yakult	Yakult
Lactobacillus johnsonii La1 (Lj1)	LC1	Nestlé
Lactobacillus reuteri ATTC 55730	Reuteri	BioGaia Biologics
Lactobacillus rhamnosus ATCC 53013 (LGG)	Vifit and others	Valio
Lactobacillus acidophilus CL1285 and *Lactobacillus casei* Lbc80r mixture	Bio K+	Bio K+ International
Lactobacillus acidophilus LA-5	-	Chr. Hansen
Lactobacillus acidophilus NCFM	-	Danisco
Lactobacillus rhamnosus LB21	Verum	Norrmejerier
Saccharomyces cerevisiae (boulardii) lyo	DiarSafe	Wren Laboratories

CHAPTER 3

MICROENCAPSULATION

3.1. Microencapsulation Techniques

Microencapsulation refers to the physical confinement of bioactive materials in an environment where bioactive materials cannot escape. This technique is extremely popular for the immobilization of whole cells, because the transport of the nutrients and metabolites to and from the cell is possible through the gel.

Viability losses during storage and gastrointestinal transit due to detrimental conditions such as harsh acidic environment, oxygen stress and enzymatic reactions reduce the functionality of probiotics to exert health benefits. Thus, microencapsulation of probiotics is considered as an effective approach for their efficient survival under gastrointestinal conditions and to improve the viability during shelf life to maintain their health promoting effects. Successful applications of microencapsulation techniques using different materials including proteins, polysaccharides and other polymer mixtures were shown in literature (Goosen et al., 1987; Hébrard et al., 2010; Nag et al., 2011). Additionally, mechanical protection of probiotic cells during incorporation process into food product is another advantage of microencapsulation (Doleyres and Lacroix, 2005; Shahidi and Han, 1993).

Despite wide applications of several microencapsulation materials for probiotics, it is still a challenge to produce microcapsules for preservation of desired bacterial culture with high viability causing no undesirable texture characteristics in the final product (Hansen et al., 2002). Besides the undesirable textural and sensorial properties, the release of used probiotic cells at the target area is also another important consideration for the development of functional food products. Therefore, in addition to laboratory scale researches, clinical studies should also be conducted to prove the efficiency of developed microcapsules and probiotic food products (Anal and Singh, 2007). Among varying types of microencapsulation techniques (Figure 3.1) extrusion, emulsion, spray drying and freeze drying techniques are the most commonly used ones for microencapsulation for bacterial cells.

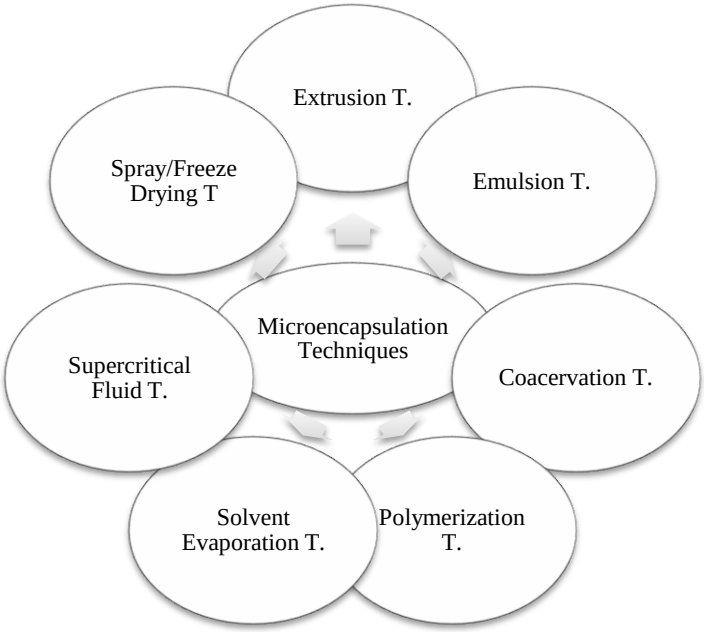

Figure 3.1. Microencapsulation techniques
(Source: Gutcho, 1976; Jyothi et al., 2010)

3.1.1. Extrusion Technique

This method is the oldest and most widely used microencapsulation technique. The advantage of this method is that the wall material surrounds the core ingredients providing a full protection. Also it provides simple, easy and low cost applications. In this method cell damage is low due to mild microencapsulation conditions (no heat treatment, no harmful solvents). However besides all the advantages mentioned above, large particle size (above 100μm) is generally obtained that limits the use (K. Y. Lee and Heo, 2000). The size of the formed microcapsules depends on the wall material concentration, viscosity of the wall material, concentration of the hardening solution, the distance between syringe and hardening solution, the gauge of the syringe and hardening time. This technique is based on mixing the probiotic microorganism with a polymer solution. This probiotic-polymer mixture is forced through a nozzle or a syringe needle at high pressure in to a hardening solution (Figure 3.2). This method has been widely studied for the microencapsulation of probiotics. For instance, Amine et al. (2014) prepared native alginate (NA), palmitoylated alginate (PA) and a blend of native-pamitoylated alginate (NA-PA) microcapsules using droplet extrusion technique using 3% $CaCl_2$ and subjected freeze drying to observe the viability of *B. longum* with changing alginate concentrations.

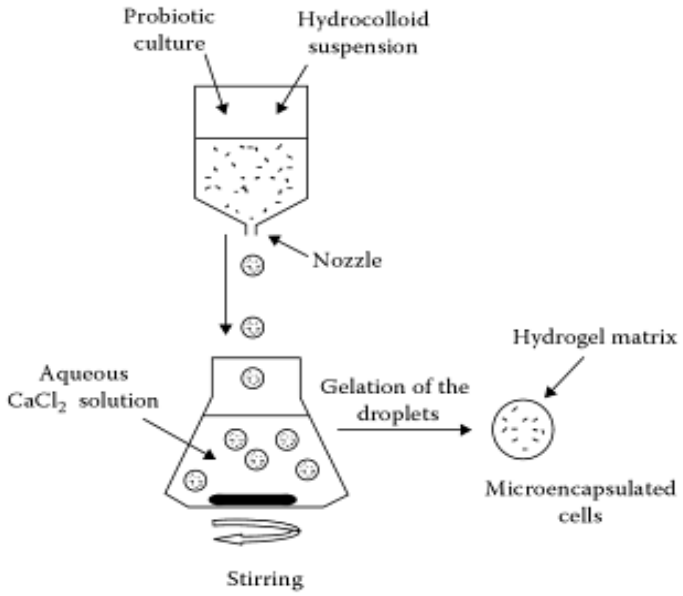

Figure 3.2. Schematic representation of extrusion technique ($CaCl_2$ represents a sample of hardening solution) (Source: Fanun, 2010)

The authors reported 65% microencapsulation yield with this technique. However compared this technique with spray drying and observed that spray drying technique were more effective with a loss of 2 log CFU/g compared to extrusion technique where 2.9 log CFU/g loss in cell counts were calculated. Khan et al. (2013) also reported that microencapsulation of *Bifidobacterium adolescentis* within legume protein isolate–alginate microcapsules with different needle gauges (16, 18, 19, 23 and 27G) yielded in a decreasing mean diameters ranging from 2.79 (16G) to 1.23 mm (27G). Moreover, it was reported that encapsulated cells improved the survival rates in simulated gastric juice where 2.25 log CFU/ml cell reduction were observed as opposed to free cells having no viable numbers.

3.1.2. Emulsification Technique

This technique has 3 different types; internal gelation, ionic gelation and enzymatic gelation. 1) In ionic gelation, process is based on the formation of a single emulsion (W/O) with the aqueous polymer-probiotic mixture and large volume of continuous oil phase. After the formation of an emulsion, hardening solution is added to form microcapsules containing probiotic bacteria. 2) In internal gelation technique, microcapsules are formed after the addition of an oil soluble acid such as glacial acetic

acid to continuous phase under stirring. The addition of acid initiates gelation by release of calcium ions from hydrocolloid probiotic mixture containing calcium complex. 3) In enzymatic gelation method, probiotic hydrocolloid mixture contains enzyme that initiates gelation by thermal induction of enzymatic coagulation after the formation of W/O emulsion. This is mostly used when milk proteins is used as a carrier (Krasaekoopt et al., 2003; Nedovic and Willaert, 2004).

When compared to extrusion technique, smaller but variable microcapsules size ranges are obtained in this method. Moreover, this method provides higher microencapsulation efficiencies and easy solutions to scale up (Winder et al., 2003). Besides these advantages, large size distribution, poor survival through gastrointestinal transit due to decreased microcapsule size and low microencapsulation yields are reported to as the disadvantages. *L. acidophilus* and *Bifidobacterium* species were encapsulated within a polymer composed of alginate and hi-maze resistant starch using emulsification technique. In this study $CaCl_2$ was used as a hardening solution and microcapsules with the size range of 500 to 3000 μm were obtained. Moreover, encapsulated cells showed better survival (2 log CFU/ml difference) compared to free one (K. Sultana et al., 2000). In another study, gelatin-maltodextrin polymer complex microcapsules were obtained to entrap *B. adolescentis* (Borza et al., 2010)

3.1.3. Spray Drying Technique

Spray drying is one of the oldest methods adapted to many industrial areas to make powders and capturing bioactive components. Microencapsulation with spray drying technique has been widely applied for microencapsulation of flavor, fish oil, probiotics and vitamins (Desai and Jin Park, 2005). This technique is based on mixing the probiotic microorganism with a polymer solution. This probiotic-polymer solution is either homogenized with oil phase in order to form emulsions and then used or used directly to form spray dried microcapsules. Formed polymer mixture/emulsion is sprayed through a nozzle into a drying chamber circulating hot air. Microcapsule size and functionality of encapsulated bioactive materials are mainly based on inlet and outlet temperatures, noozle dispersion size, viscosity, solid concentration and flow rate of stock polymer-bioactive solution (Figure 3.3). Despite high production rates in a short time, water soluble shell materials avoids controlled release of the bioactive core

material in aqueous medium (Chen and Mujumdar, 2009; Lakkis, 2008). Protective effects of microencapsulation using spray drying methods were extensively investigated by researchers. *L. acidophilus* was microencapsulated using spray drying within alginate (Lorenz 2009). Calculated cell loss of microencapsulated cells was 1.05 log CFU/g in the first hour of incubation in simulated gastric juice (pH 3.0). Castro-Cislaghi et al. (2012) encapsulated *Bifidobacterium* Bb-12 within whey protein matrix using spray drying technique and survival during gastrointestinal transit as well as tolerance to NaCl were investigated. Tolerance to acidic conditions for microencapsulated bacteria was higher when compared to free cells; there was a decrease of 0.73 log CFU/g in microencapsulated cells, while a decrease of 1.52 log CFU/g at pH 2.0.

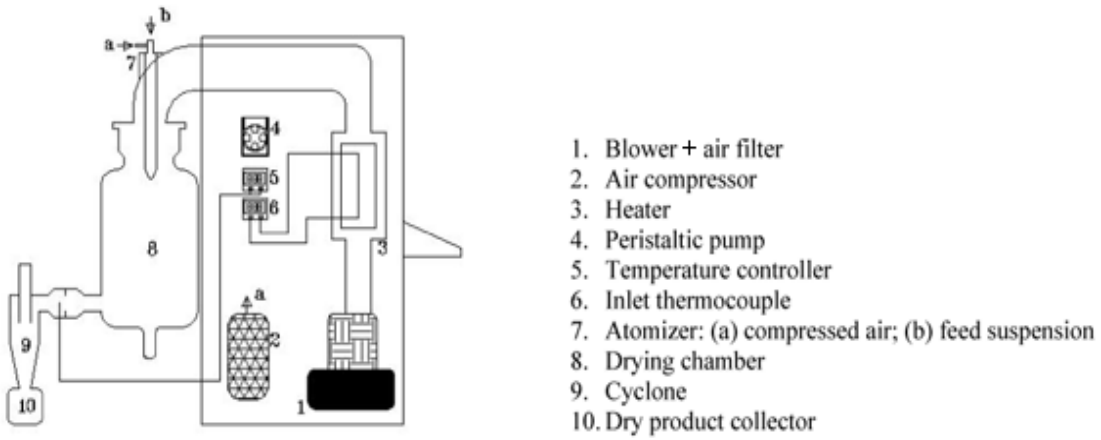

Figure 1: Schematic diagram of the spray dryer used.

Figure 3.3. Schematic representation of spray drying process
(Source: Sanders, 2003)

3.2. Selecting Wall Materials for Microencapsulation

Selection, development and characterization of biodegradable, biocompatible, safe and environmental friendly materials suitable for utilization as encapsulating agent is an important aspect of microencapsulation. Commonly used wall materials are proteins and polysaccharides including whey protein isolates and concentrates, soy protein isolates, caseinates, sodium alginate, maltodextins, arabic gum, gelatin and cellulose based polymers. Due to amphiphilic nature and good emulsifying characteristics, proteins offer suitable properties required for microencapsulation.

Moreover, salt induced gelation of proteins makes them ideal wall matrix for heat sensitive bioactive materials. On the other hand, polysaccharides show good solubility in water and posses low viscosity at high concentrations, compared to protein. Excellent oxygen and moisture barrier properties of some polysaccharides also afford good protection for the encapsulated bioactive materials. Besides all the advantages, each wall materials cannot meet all the listed properties (Kagami et al., 2003; Murano, 1998; SHEU and Rosenberg, 1995). The important point is selecting the most suitable one to meet the specific requirements for the objective of the current microencapsulation process.

3.2.1. Whey Protein

Milk proteins have been widely studied in microencapsulation of bioactive materials due to their gelation properties and ability to form interaction with other polymers to form complexes. Milk proteins are divided into 2 major categories; casein proteins and whey proteins. Whey as a waste product of cheese manufacturing process is considered to be a cheap protein source and whey proteins are functionally and nutritionally valuable. Whey proteins include β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins and other minor proteins (Gounga et al., 2007; Gunasekaran et al., 2007; Reid et al., 2005). Whey proteins are used in food industry in various areas; to improve the emulsifying and whipping properties of ice creams, to improve the flavor, aroma and texture of the bakery products, to improve the textural and nutritional quality of yoghurts, to improve the palatability of confectionary products and to prevent the shrinkage and syneresis of meat products during cooking and syneresis (R. G. Crittenden et al., 2003).

Whey protein is the most widely studied protein for the microencapsulation of probiotics since it is proved to be an effective encapsulating agent because of their ability to form microcapsules easily under mild conditions using different techniques (Table 3.1) (Heelan and Corrigan, 1998; S. Lee and Rosenberg, 1999; Rosenberg and Lee, 2004). Heat or acid induced gelation of whey proteins are widely used for the formation of microcapsules. However heat or acid sensitive bioactive materials such as probiotics make these processes unpractical for microencapsulation. Therefore, cold-induced gelation (ionotropic or enzymeatic gelation) method is preferred for the

microencapsulation of sensitive bioactive materials (Heidebach et al., 2009; Hongsprabhas and Barbut, 1997). In enzymatic gelation, addition of proteolytic enzymes such as transglutaminase and protease in protein solution result in aggregation and gelation. In ionotropic gelation direct addition of salt in the form of calcium or sodium chloride shields electrostatic interactions between proteins and due to lack of electrostatic repulsion, molecules aggregate and gel formation occurs (Nag and Das, 2013; Narkar et al., 2010; C.-Y. Yu et al., 2009). In cold gelation process, before addition of salt/acid, whey protein proceeds by an activation step followed by rapid cooling, in which an initial thermal treatment is required to denature protein molecules (Madene et al., 2006; Maskan and Altan, 2011).

Another alternative approach to form microcapsule wall material was to use proteins in presence of polysaccharides. Several approaches have shown that combination of proteins with polysaccharides improve the emulsifying properties and strengthen barrier properties of protein-based microcapsules. Filmogenic properties of protein-polysachharide polymer microencapsulation permit barrier creation for the protection of sensitive ingredients against moisture, diffusion of acid and their transport in aqueous media (Ducel et al., 2004; Mendanha et al., 2009; Pereira et al., 2009; A. P. Pierucci et al., 2007; A. P. T. R. Pierucci et al., 2006; C. Yu et al., 2007). Based on these improved functional properties of protein-polysaccharide complexes, we decided to investigate the potential of whey protein-pullulan complex to encapsulate *L. acidophilus* NRRL B-4495.

Table 3.1. Microencapsulation studies with whey protein based wall materials.

Microencapsulation process	**Wall material**	**Core material**	**Reference**
Emulsification method	Native and/or denatured whey proteins	*Lactobacillus rhamnosus* GG	(J Burgain et al., 2013)
Emulsification and/or spray drying method	Denatured whey proteins	*Bifidobacterium breve* R070 and *Bifidobacterium longum* R023	(Picot and Lacroix, 2004)
Spray drying method	Whey protein isolate (WPI), WPI-modified resistant starch complex	*L. rhamnosus* GG	(Ying et al., 2013)
Emulsification method	Pectin–whey protein complex	*L. acidophilus* La5	(Gebara et al., 2013)
Emulsification method	Native, denatured and hydrolysed (WPI)	*L. rhamnosus* GG	(Doherty et al., 2010)
Electrospinning method	Whey protein concentrate (WPC), pullulan	*Bifidobacterium animalis* subsp. *lactis* Bb12	(López-Rubio et al., 2012)
Spray drying	WPI	*Saccharomyces boulardii*	(Duongthingoc et al., 2013)
Complex coacervation	WPI- kappa carrageenan complex	*L. plantarum*	(Hernández-Rodríguez et al., 2014),
Freeze drying/ spray drying	WPI-alginate complex	*L. plantarum*	(Rajam et al., 2012)

3.2.2. Pullulan

Pullulan is an extracellular polysaccharide produced by *Aureobasidium pullulans*. It is a glucan composed mainly of maltotriose units linked by a.-I ,6-glycosidic linkages (Figure 3.4) (Barnett et al., 1999). Many different industrial wastes such as potato peel, grape skin and olive oil wastes found to be used as a carbon source for the synthesis of pullulan (Barnett et al., 1999; Singh et al., 2008). Pullulan is especially used as a coating material in the food industry, paper industry and

pharmaceutical area because of its non-toxic, water-soluble, colorless, tasteless, odorless and heat stable characteristics (Wu and Imai, 2011). It can easily dissolve in water and form viscous solutions. In food industry it is used to replace starch ingredients as a low calorie viscosity enhancer. Moreover, pullulan is used for packaging of meat and vegetables, pullulan films is involved in packaging applications for tea and coffee bags as a water soluble sugar packet (Barnett et al., 1999).

In microencapsulation technology, pullulan was used as a microencapsulation matrix for the delivery of drugs in pharmaceutical industry. For instance, Ravi et al. (2014) developed pullulan nanoparticles for the delivery of human immunodeficiency virus (HIV) protease inhibitor Laponivar. According to results, high microencapsulation efficiency and higher metabolic protection and bioavailability of Laponivar than free drug were achieved. In another study, adriamycin was encapsulated in pullulan nanocapsules to overcome drug-resistance of cancer cells and obtained significant release results with (H. Guo et al., 2014). Pullulan was also used to encapsulate *B. animalis* Bb12 using electrospinning technique (López-Rubio et al., 2012). Milk and phosphate-buffered saline (PBS) as a dissolving media instead of water were used. Pullulan capsules were compared with capsules made of whey protein concentrate and it was found that pullulan was not as effective as whey protein concentrate in stabilization of *B. animalis* Bb12 cells.

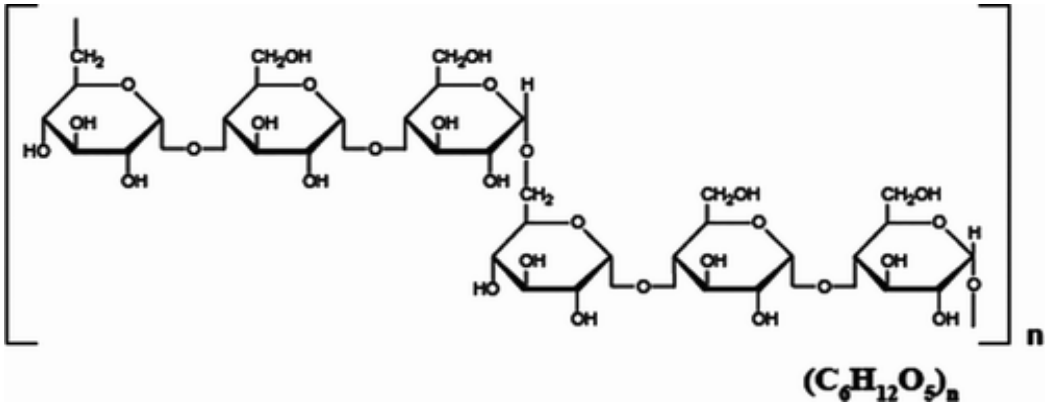

Figure 3.4. Chemical structure of pullulan
(Source: Barnett et al., 1999)

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials

Commercial strain of *L. acidophilus* NRRL-B 4495 was obtained from the ARS Culture Collection (NRRL, USA). Ox-bile was obtained from Fluka Biochemica (Switzerland). Trypsin (from bovine) was purchased from Merck (Darmstadt, Germany) and pepsin (from porcine stomach mucosa) from Sigma (St. Louis, MO, USA). Sunflower oil was obtained from a local store.Pullulan was a gift from Hyarashiba Co. (Japanese). Whey protein isolate (WPI), was obtained from BiPro, Danisco. All other chemicals were obtained from Sigma.

4.2.Methods

4.2.1. Bacterial Strain and Culture Preparations

L. acidophilus NRRL-B 4495 cells were inoculated into 5 ml of MRS broth in 0.1 % ratio and incubated at 37 ºC for 24 h under anaerobic conditions. The cultures were then subcultured into 20 ml of MRS broth and incubated under same conditions for 12 h. The cells were harvested by centrifugation at 15000g for 10 min at 4 ºC from 20 ml of a 12 h culture at the initial stationary phase. The supernatant was decanted, and the cells were re-suspended in 100 ml of pullulan-WPI solution obtaining a cell load of about 8.5 log CFU ml^{-1}.

4.2.2. Emulsion Encapsulation/Cold Gelation Method

4.2.2.1. Formation of WPI-Pullulan Wall Matrix

Whey protein isolate-pullulan microcapsules were prepared according to the method of Wood (2010) with some modifications. Briefly, whey protein isolate (WPI) was dispersed by mixing the protein powder in sterile distilled water at ambient temperature. Protein solution was stirred for approximately 3 h using a magnetic stirrer to ensure proper dissolution under 4 ºC and after hydration protein solution was denatured at 80 ºC for 30 min. Denatured protein solution was cooled to room temperature in ice bath. Pullulan in varying concentrations were dissolved in distilled water at ambient temperature and polysaccharide solution was stirred for approximately 3 h using a magnetic stirrer to ensure proper dissolution. Pullulan solution was then sterilized using by 0.45 μm filter and mixed with the denatured WPI solution at various final concentrations. Final concentrations of protein solution were 9% (w/v).

4.2.2.2. Preparation of Microcapsules

In the first step, primary water-in-oil emulsions (W_1/O) were formed by emulsifying an inner aqueous phase (W_1) made up by WPI-pullulan polymer complex containing bacteria into an oil phase (O) containing 1% PGPR as an emulsifier. W_1/O emulsion was prepared by an Ultra Turrax homogenizer (Ultra Turrax, model T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) at 3000 rpm for 5 min. This emulsion was then homogenized in $CaCl_2$ solution (100 mM). For 2 minutes at 3.400 rpm. After formation of microcapsules, this slurry was orbitally shaked at 160 rpm for 30 minutes to harden the microcapsules. The hardened microcapsules were separated from the solution and oil phase by two sets of homogenization at 1000 rpm for 1 h. It is important to state that in order to avoid the destructive heat generation possibly formed during homogenization; microencapsulation process was conducted inside the ice bath.

4.2.2.3. Bacterial Enumeration

Viable counts of non-encapsulated *L. acidophilus* NRRL-B 4495 were determined by a pour plate method using MRS agar after serial dilutions in peptone water. The plates were incubated anaerobically at 37 ºC for 72 h and colony forming units were counted.

For enumeration of microencapsulated bacteria in microcapsules, 10 g of microcapsules were suspended in peptone water. This peptone water containing microcapsules was homogenized at 11000 rpm for 5 min. Under these conditions, the microcapsules were broken and samples of 1 ml of the peptone water were diluted to an appropriate dilution and plated by the pour plate technique using MRS agar. Colonies were counted after 72 h of incubation at 37 ºC. Viable cell number was express as CFU per gram of microcapsule (CFU/g) and the efficiency of was determined as following equation (4.1);

$$\text{Microencapsulation yield (\%)} = 100 \times \frac{\text{cfu/ml after encapsulation}}{\text{cfu/ml before encapsulation}} \qquad (4.1)$$

4.2.2.4. Heat Tolerance Test

For the heat tolerance test, 1.0 ml of free or 1.0 g of microencapsulated cells with similar initial counts were resuspended in 10 ml sterile distilled water as suspending medium then heated to 45, 50, 55, 60, 65 and 70 ºC for 60 minutes in water bath. After heat treatment, samples were cooled to room temperature (25 ºC) then viable cells were enumerated (Ding and Shah, 2007).

4.2.2.5. Salt Tolerance Test

1.0 ml of free or 1.0 g of microencapsulated bacteria were resuspended in 10 ml of distilled water supplemented with different concentrations of NaCl (0, 3, 6 and 9% (w/v)). The free and encapsulated cell suspensions were stored at 4 ºC for 7 day. After incubation samples were removed and viable cells were enumerated periodically.

4.2.2.6. pH Tolerance Test

pH tolerance of free or microencapsulated cells were measured by resuspending 1.0 ml of free or 1.0 g of encapsulated bacteria in 10 ml of MRS broth having different pH values ranging from 2.0 to 8.0 (adjuested with NaOH or HCl) for 120 minutes at 37 ºC. After pH treatment, samples were removed and then viable cells were enumerated(Ding and Shah, 2007).

4.2.2.7. Survival of Free and Microencapsulated Bacteria in Simulated Gastric Juice

Simulated gastric juice was prepared according to the method described by (Z. Guo et al., 2009) with some modifications. Saline solution was prepared and the pH was adjusted to the 3.0 with 0.1N HCl and sterilize at 121°C for 15 min. Pepsin solution was prepared, sterilized by 0.22 μm filter and mixed with the saline at a final concentration of 3 g/L.

1.0 ml of free or 1.0 g of microencapsulated bacteria were transferred into 9.0 ml of simulated gastric juice and incubated at 37 °C under orbital shaking at 160 rpm for 3 h. After the incubation, samples were removed and viable bacteria were enumerated by using procedure in Section 4.3.3.

Survival rate (%) was calculated according to the following equation (4.2);

$$\text{Survival rate (\%)} = 100 \times (N_1/N_0) \quad (4.2)$$

N_1= the total viable count of strains after treatment by gastric juice (log CFU/g)
N_0= the total viable count of strains before treatment (log CFU/g)

4.2.2.8. Survival of Free and Microencapsulated Bacteria in Bile Salt

The bile tolerance was examined according to the method described by Bao et al. (2010). MRS media was supplemented with 0.6% ox-bile, 1.0 ml of free or 1.0 g of

microencapsulated *L. acidophilus* NRRL-B 4495 were inoculated into 9.0 ml of prepared bile salt solution and incubated at 37°C under orbital shaking at 160 rpm for 24 h. After the incubation, samples were removed and viable bacteria were enumerated and survival rate (%) was calculated.

4.2.2.9. Release of Microencapsulated Bacteria into Simulated Intestinal Juice

Simulated intestinal juice (SIJ) was prepared by supplementing saline with trypsin. pH of saline adjust to the 8.0 with 0.5M NaOH and sterilize at 121°C for 15 min. The trypsin solution was prepared, sterilized by sterilized by 0.45 μm filter and added at a final concentration 1g/L (Z. Guo et al., 2009). 1.0 ml of free or 1.0 g of encapsulated cells were transferred into the 9.0 ml of simulated intestinal juice and incubated at 37°C under orbital shaking at 160 rpm for 24h. After the incubation, samples were taken from supernatant and viable bacteria released in SIJ were enumerated. Survival rate (%) was calculated according to the equation 2.

4.2.2.10. Diameter Distribution

1.0 g of microcapsules was diluted with 9.0 ml of deionized water. A drop of the resulting emulsion was placed on a glass microscope slide with a cover slide. Microscopic pictures were taken using an Olympus CX31 Microscope, fitted with an Olympus DP25 Camera and diameter analysis was done with software (Olympus DP2-BSW).

4.2.2.11. Morphology of Microcapsules

The morphology of microcapsules were examined by scanning electron microscopy at 10.0 kv (Hitachi S- 3400, Germany) and environmental scanning electron microscopy (ESEM).

4.2.2.12. Moisture Content and Water Activity Measurements

Water activity of the microcapsules was determined using a Hygrolab C1 water activity meter (Hygrolab C1, Rotronic , Bassersdorf, Switzerland) (D. Dianawati et al., 2012). The moisture content of the microcapsules was determined gravimetrically by oven-drying at 105 ºC for 24 h to reach weight equilibrium (Rajam et al., 2012). The mean MC was estimated by the following equation (4.3):

$$MC\ (\%) = [(W_{wet} - W_{dry})/W_{wet}]*100 \quad (4.3)$$

Where, W_{wet} is the weight of the wet microcapsules and W_{dry} is the weight of fully dry microcapsules.

4.2.2.13. Color Measurement

Konica Minolta colorimeter (Model CR 410, Tokyo, Japan) was used for this experiment. The CIE Lab system, defined in rectangular coordinates (L*, a*, b*), where L* represents lightness, a* represents red-green and b* represents yellow-blue.

4.2.2.14. Swelling Properties

The swelling degree of the probiotic bacteria loaded WPI-pullulan microcapsules were characterized by suspending 10 freeze dried microcapsules in denionized water/SGJ/SIJ overnight at 37 ºC. After incubation, deionized water was removed from the medium carefully and then the microcapsules were removed. Filter papers were used to absorb excessive water on the surface of the microcapsules which were then weighed (Klemmer et al., 2011).The swelling degree (SD) values of freeze dried WPI-pullulan microcapsules were calculated using the following equation (4.4):

$$SD\ (\%) = [(W_s - W_d)/W_d]*100 \quad (4.4)$$

where, Ws is the weight of the swollen microcapsules and W_d is the weight of freeze dried microcapsules.

4.2.2.15. Measurement of Bacterial Growth with Pullulan

A 10.8% suspension of pullulan was prepared in sterile water (w/v), and the suspension was sterile-filtered (0.2 μm Minisart, Sartorius AG, Göttingen, Germany), and stored at 4 °C until use. Probiotic bacteria was inoculated in this pullulan solution and incubated for 24 h at 37 ºC. After incubation, viable bacterial number was counted.

4.2.2.16. Freeze Drying

Microcapsules were frozen at -20 ºC. Thereafter, the microcapsules were freeze-dried in a Lablanco freeze-dryer (Freezone 18, Kansas, USA) for 24 h at -55 ºC and at 0.050 mBar vacuum. After freeze drying, dried microcapsules were stored at 4 ºC for further experiments.

4.2.3. Spray Drying Method

4.2.3.1. Formation of WPI-Pullulan Wall Matrix

Whey protein isolate-pullulan microcapsules were prepared according to the method of Wood (2010) with some modifications. Briefly, whey protein isolate (9% w/v) was dispersed by mixing the protein powder in sterile distilled water at ambient temperature. Protein solution was stirred for approximately 3 h using a magnetic stirrer to ensure proper dissolution under 4 ºC and after hydration protein solution was denatured at 80 ºC for 30 min. Denatured protein solution was cooled to room temperature in ice bath. Pullulan in varying concentrations were dissolved in distilled water at ambient temperature and polysaccharide solution was stirred for approximately 3 h using a magnetic stirrer to ensure proper dissolution. Pullulan solution was then sterilized using by 0.45 μm filter and mixed with the denatured WPI solution at various final concentrations.

4.2.3.2. Preparation of Microcapsules

WPI-pullulan polymer blend containing *L. acidophilus* NRRL B-4495 with the initial cell load of 9.7 log CFU/g to be microencapsulated by spray drying was carried out using laboratory spray dryer (BÜCHI Mini Spray dryer B-290, BÜCHI Labortechnik AG, UK). In this study, all conditions were fixed (aspiration rate of 70%, flow rate of the peristaltic pump 15 ml/min) and the outlet air temperatures were 35, 45, 50 and 55 °C and. The powders were collected in a single-cyclone separator. The microencapsulation of bacteria under different conditions of encapsulation was performed in triplicate. The resultant spray-dried bacteria were stored separately in 5 g quantities in sealed sterile glass bottles at 4 °C.

4.2.3.3. Bacterial Enumeration

Viable counts of non-encapsulated *L. acidophilus* NRRL-B 4495 were determined by a pour plate method using MRS agar after serial dilutions in peptone water. The plates were incubated anaerobically at 37 ºC for 72 h and colony forming units were estimated.

For enumeration of microencapsulated bacteria in microcapsules, 10 g of spray dried microcapsules were suspended in peptone water. This peptone water containing microcapsules was stirred at 890 rpm for 5 min for complete dissolution. Samples of 1 ml of the peptone water were diluted to an appropriate dilution and plated by the pour plate technique using MRS agar. Colonies were counted after 72 h of incubation at 37 ºC. Viable cell number was express as CFU per gram of microcapsule (CFU/g) and the yield of was determined as following equation (4.5);

$$\text{Survival rate (\%)} = 100 \times (N/N_0) \qquad (4.5)$$

Where N_O the number of bacteria before drying, and N was the number of bacteria in the spray-dried powder.

4.2.3.4. Heat Tolerance Test

For the heat tolerance test, 1.0 ml of free or 1.0 g of microencapsulated cells were resuspended in 10 ml sterile distilled water as suspending medium then heated to 45, 50, 55, 60, 65 and 70 ºC for 60 minutes in water bath. After heat treatment, samples were cooled to room temperature (25 ºC) then viable cells were enumerated (Ding and Shah, 2007).

4.2.3.5. pH Tolerance Test

pH tolerance of free or microencapsulated cells were measured by resuspending 1.0 ml of free or 1.0 g of microencapsulated bacteria in 10 ml of MRS broth having different pH values ranging from 2.0 to 8.0 (adjuested with NaOH or HCl) for 120 minutes at 37 ºC. After pH treatment, samples were removed and then viable cells were enumerated(Ding and Shah, 2007).

4.2.3.6. Survival of Free and Microencapsulated Bacteria in Simulated Gastric Juice

Simulated gastric juice (SGJ) was prepared according to the method described by Z. Guo et al. (2009) with some modifications. Saline solution was prepared and the pH was adjusted to the 3.0 with 0.1N HCl and sterilize at 121°C for 15 min. Pepsin solution was prepared, sterilized by 0.22 μm filter and mixed with the saline at a final concentration of 3.0 g/L.

1.0 ml of free or 1.0 g of microencapsulated bacteria were transferred into 9.0 ml of simulated gastric juice and incubated at 37 °C under orbital shaking at 160 rpm for 3 h. After the incubation, samples were removed and viable bacteria were enumerated by using procedure in Section 4.2.3.3. Survival rate (%) was calculated according to the equation 4.2.

4.2.3.7. Survival of Free and Microencapsulated Bacteria in Bile Salt

The bile tolerance was examined according to the method described by Bao et al. (2010). MRS media was supplemented with 0.6% ox-bile, 1.0 ml of free or 1.0 g of microencapsulated *L. acidophilus* NRRL-B 4495 were inoculated into 9.0 ml of prepared bile salt solution and incubated at 37°C under orbital shaking at 160 rpm for 24 h. After the incubation, samples were removed and viable bacteria were enumerated and survival rate (%) was calculated.

4.2.3.8. Release of Microencapsulated Bacteria in Simulated Intestinal Juice

Simulated intestinal juice (SIJ) was prepared by supplementing saline with trypsin. pH of saline adjust to the 8.0 with 0.5M NaOH and sterilize at 121°C for 15 min. The trypsin solution was prepared, sterilized by sterilized by 0.45 μm filter and added at a final concentration 1g/L (Z. Guo et al., 2009). 1.0 ml of free or 1.0 g of microencapsulated bacteria were transferred into the 9.0 ml of simulated intestinal juice and incubated at 37°C under orbital shaking at 160 rpm for 24 h. After the incubation, samples were taken from supernatant and viable bacteria released in SIJ were enumerated.

4.2.3.9. Morphology of Microcapsules

The morphology of microcapsules were examined by scanning electron microscopy at 10.0 kv (Hitachi S- 3400, Germany) and environmental scanning electron microscopy (ESEM).

4.2.3.10. Diameter Distribution

0.1 g of microcapsules was placed on a glass microscope slide with a cover slide. Microscopic pictures were taken using an Olympus CX31 Microscope, fitted with

an Olympus DP25 Camera and diameter analysis was done with software (Olympus DP2-BSW).

4.2.3.11. Moisture Content and Water Activity Measurements

Water activity of the microcapsules was determined using a Hygrolab C1 water activity meter (Hygrolab C1, Rotronic , Bassersdorf, Switzerland) (D. Dianawati et al., 2012). The moisture content of the microcapsules was determined gravimetrically by oven-drying at 105 ºC for 24 h to reach weight equilibrium (Rajam et al., 2012). The mean MC was estimated by the following equation (4.6):

$$MC\ (\%) = [(W_{wet} - W_{dry})/W_{wet}]*100 \qquad (4.6)$$

where, W_{wet} is the weight of the wet microcapsules and W_{dry} is the weight of fully dry microcapsules.

4.2.3.12. Color Measurement

Konica Minolta colorimeter (Model CR 410, Tokyo, Japan) was used for this experiment. The CIE Lab system, defined in rectangular coordinates (L*, a*, b*), where L* represents lightness, a* represents red-green and b* represents yellow-blue.

4.2.3.13. Dissolution Time

The dissolution time of microencapsulated bacteria were characterized by suspending 5 g of spray dried microcapsules in 50 ml of denionized water/SGJ/SIJ and follewd by stirring at 880 rpm. The time as seconds required for spray dried microcapsules was given in terms of dissolution time.

4.2.4. Statistical Analysis

Results were expressed as means ± standard deviation. Mean values and standard deviation were calculated from data obtained with triplicate trials. Data

analysis was carried out using Minitab 14.0 software (Minitab Inc., State College, PA). Significance of differences between formulations were performed by analysis of variance (ANOVA) test followed by Tukey's test.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Effect of Pullulan on The Growth of Bacterial Cells

The effect of varying levels of pullulan on growth of probiotic *L. acidophilus* NRRL B-4495 cells after incubation at 37 °C for 24 h was shown in Table 5.1. This study was conducted in order to observe the promoting effect of pullulan for growth of bacterial cells. Results illustrated that increase in bacterial number increased regardless of increased pullulan amount ($p>0.05$). The increase in cell numbers was calculated at average 2 log units. This increase was supposed to favor the survival of encapsulated cells during storage and gastrointestinal transit. Additionally, as seen clearly, pullulan did not improve the bacterial population in high numbers. This can be an advantage for our study since the increase in cell numbers at higher rates is not a desired condition; destruction of formed microcapsules followed by leakage of encapsulated bacterial cells could occur. Moreover in this study pullulan is selected due to its film forming characteristics. Therefore the use of pullulan by bacterial cells in excessive amount could create the destruction of microcapsules that can further lead to diffusion of gastric acid and bile salts resulting in viability loss in addition to undesired leakage.

Table 5.1. Effect of pullulan concentration in dH_2O on bacterial cells after anaerobic incubation for 24 h at 37°C

Pullulan concentration (w/v,%)	**Initial cell count (log CFU/ml)**	**Cell count after incubation (log CFU/ml)**
4.0	8.16±0.99	8.86±0.87
9.0	8.26±1.00	10.29±0.67
10.8	8.52±0.24	10.74±.010
11.7	8.31±0.68	10.45±.059
12.6	8.74±0.82	10.89±0.98

5.2. Emulsion Encapsulation/Cold Gelation

5.2.1. Development of Microcapsules

In this study, microencapsulation process depended on emulsion microencapsulation technique. In this technique as mentioned before the first step is the formation of a primary water-in-oil emulsion and second step is hardening of the microcapsules formed in emulsion. In this study, parameters including homogenization speed, whey protein and hardening solution ($CaCl_2$) concentrations were not changed. Varying protein concentrations have been used for microencapsulation of probiotic microorganism and other bioactive compounds (Fukushima and Nakano, 1996; Montes et al., 1995). Generally, protein concentrations between 7-12% (w/v) are widely used for probiotic encapsulation studies (J. Burgain et al., 2013; Doherty et al., 2011; Duongthingoc et al., 2013). Therefore in order to select the protein concentrations, WPI-pullulan microcapsules consisting of 7%, 8%, 9%, 10% , 11% and 12% (w/v) WPI in presence of fixed amount of pullulan (9% , w/v) was formed and microcapsule formation was observed under light microscopy. After this experimental trial, wall materials having WPI at 9% and above concentrations were observed to form more compact and smother spherical shaped microcapsules. On the other hand other microcapsules containing 7% and 8% (w/v) protein easily disturbed during the removal of microcapsules from oil phase. Therefore 9% (w/v) protein concentration was selected to be used. Moreover, since the process formed microcapsules with mean diameters lower than the selected critical limit (100 μm) (Annan et al., 2008; Vivek, 2013) and microcapsules were easily formed with selected concentration of $CaCl_2$, there was also no need to optimize the homogenization speed and hardening solution concentration.

5.2.1.1. Effect of Oil Phase Type

When the microencapsulation yield of microcapsules was considered, in presence of sunflower oil, the highest microencapsulation yield (81.0%) was obtained. With respect to sunflower oil, almond oil and sesame oil showed lower microencapsulation yields; about 76.39% and 64.02%, respectively.

Table 5.2 showed the change in microencapsulation yield and mean diameter sizes of microcapsules by the effect of the oil types. It is clear that both mean droplet sizes of microcapsules and microencapsulation yields, were affected significantly ($p<0.05$) with the change in oil phase. Among three different oils, sunflower oil showed the largest of microcapsule sizes of all the microcapsule samples.

Chain length of fatty acids and fatty acid composition in oils were reported to have an effect on the stability and particle size of emulsions; the oils with long chain fatty acid will form larger diameter droplet size and more unstable the emulsion will be. (Knoth et al., 2005; Rao and McClements, 2012). Sunflower oil mainly contains high amount of long chain fatty acids (linoleic and oleic acid) (Akhtar et al. 2009). Therefore the difference in mean droplet sizes of microcapsules can be explained by the difference in fatty acid compositions. As a result, due to ability of sunflower oil to form microcapsules with desirable size and encapsulate probiotic bacteria with high efficiency, following experiments were conducted with sunflower oil as a continuous phase.

Table 5.2. Effect of oil types on mean diameters and encapsulation efficiency of WPI-pullulan microcapsules.

Oil types	Encapsulation efficiency (%)	Mean particle size (µm)
Sesame oil	64.02±2.18[a]	34.7±3.53[a]
Sunflower oil	81.00±3.11[b]	51.5±2.41[b]
Almond oil	76.39±1.21[b]	48.1±4.03[b]

[a-b] Means ± standard deviation with different superscript letters in the same line indicate significant differences ($P < 0.05$) among the studied samples

5.2.1.2. Effect of Internal Phase Volume Ratio

In the second stage, the internal phase volume ratio, the ratio of the volume of the internal phase (WPI-pullulan polymer blend) to that of the external continuous phase (sunflower oil), was examined. This ratio was determined by the addition of internal phase in different volume quantities (10-50%, v/v) to form 100 ml of primary emulsion (W_1/O). It has been reported that the dispersed phase ratio persuaded the droplet size distribution; as the ratio changes, the viscosity of the primary emulsion

changes thus changes the size of the microcapsule. The change in mean particle size results in the changes of the microencapsulation yield (Anisa and Nour, 2010). It is seen in Table 5.3, volume of the internal phase had significant effects ($p<0.05$) on both parameters. Microencapsulation yield values are directly proportional with the dispersed phase volume fraction of primary emulsions. To obtain a microcapsule with microencapsulation yield over 80%, internal phase volume ratio of 40% or 50% was necessary in the primary emulsion. Additionally by the increase in dispersed phase volume ratio, there was a significant increase ($p<0.05$) in size of microcapsules. The average droplet size for 10 % (v/v) and 50 % (v/v) were 18 μm and 58 μm, respectively. Especially, a very sharp increase in mean diameters of microcapsules was observed when the dispersed phase volume increased from 10% to 30%. Results also demonstrated that even microcapsules having the highest dispersed phase still have the mean droplet size below critical limit. As a result, following experiments were conducted with internal phase volume ratio of 50% (v/v).

Table 5.3. Effect of dispersed phase volume ratio on mean diameters and encapsulation efficiency of WPI-pullulan microcapsule

Volume ratio (%)	**Encapsulation efficiency (%)**	**Mean particle size (μm)**
10	35.00 ± 4.00^{a}	17.9 ± 3.65^{a}
20	54.97 ± 5.75^{b}	29.1 ± 5.37^{b}
30	80.63 ± 3.65^{c}	48.9 ± 3.00^{c}
40	84.61 ± 4.07^{d}	52.9 ± 4.87^{d}
50	88.16 ± 4.82^{e}	57.8 ± 5.00^{e}

[a-e] Means ± standard deviation with different superscript letters in the same line indicate significant differences ($P < 0.05$) among the studied samples

5.2.1.3. Effect of Pullulan Concentration in Wall Matrix

At first experimental procedure, protein to polysaccharide ratios had been selected as 1:1, 1:2 and 2:1. However, during experiments, formation of microcapsules with pullulan to protein concentrations at and above 13.5% could not be achieved.

Higher concentration of pullulan led to increase in viscosity and formation of emulsion could not be possible at desired structure; due to sticky nature of the formed emulsion, microcapsule formation could not be achieved and handling was very difficult.

It was interesting that in microcapsules having 4.5% w/v polymer blend was observed to form fibrous particles in addition to non-uniform and non-spherical microcapsules when discovered under the microscope (Figure 5.1). It seems that probiotic bacteria could not to get encapsulated within this small sized fibrous particle. By discarding these concentrations it can be seen that vary narrow ranges of effect of concentration of pullulan was studied for the effect on probiotic encapsulation. However in literature, studies conducted for the effect of pullulan on probiotics is very restricted and therefore, the negative or positive effects of pullulan even in very law amounts on microencapsulation process are not clear and well known. Therefore, these concentrations were discarded and pullulan concentrations were arranged as shown in Table 5.4. WPI-pullulan polymer blends (WPI-pullulan$_y$) where y stands for the weight ratios 1:0, 2:1, 1:1, 1:1.2, 1:1.3 and 1:1.4 between WPI:pullulan.

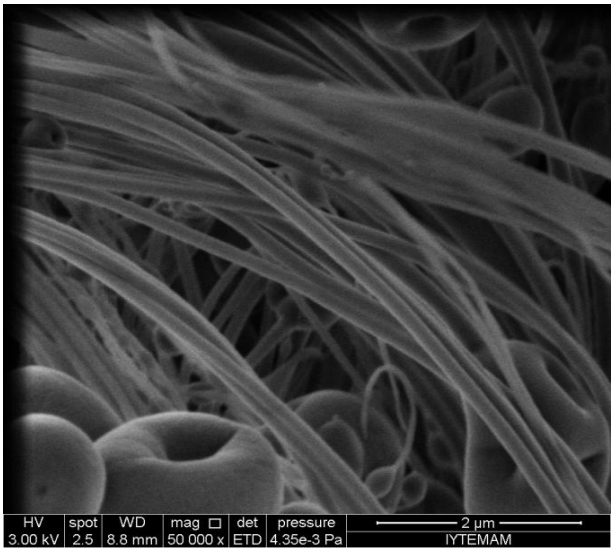

Figure 5.1. SEM of formed WPI-pullulan fibers with 4.5% pullulan concentration at 50000X magnification.

Table 5.4. Effect of pullulan concentration on mean diameters and encapsulation efficiency of WPI-pullulan microcapsules

Pullulan concentrations (%)	**WPI-pullulan$_y$**	**Encapsulation efficiency (%)**	**Mean particle size (µm)**	**Microbial load (log CFU/g)**
0	WPI-pullulan$_{1:0}$ (control)	93.55±0.03^{cb}	60.01±0.45^{a}	7.91±0.10
9.0	WPI-pullulan$_{1:1}$	92.06±0.81^{b}	68.8±1.21^{b}	7.75±0.72
10.8	WPI-pullulan$_{1:1.2}$	93.05±0.55^{cb}	71.0±0.44^{c}	7.90±0.46
11.7	WPI-pullulan$_{1:1.3}$	94.12±0.98^{c}	77.2±1.10^{d}	8.27±0.86
12.6	WPI-pullulan$_{1:1.4}$	94.09±0.66^{c}	80.7±2.46^{d}	8.42±0.54

$^{a-d}$ Means ± standard deviation with different superscript letters in the same row indicate significant differences ($P < 0.05$) among the studied samples.

Cell loading and microencapsulation efficiency values increased with increasing pullulan content, however when the microencapsulation yields were compared with control microcapsules, it seems that addition of pullulan significantly ($p<0.05$) changed microencapsulation yields (Table 5.4). Maximum cell loading was achieved with the highest pullulan content representing 94.09% microencapsulation efficiency. Addition of pullulan resulted in a bigger mean diameter sizes of microcapsules (Figure 5.3). Formulation of WPI-pullulan$_{1:1}$ led to a microcapsule size of 68.8 µm whereas, Formulation of WPI-pullulan$_{1:1.4}$ led to a microcapsule size of 80.7 µm and the mean diameters of microcapsules containing pullulan were significantly ($p<0.05$) higher than WPI microcapsules. It was observed in Table 5.4 that by the increase in polymer concentration, the viscosity of in the inner phase of the primary emulsion was increased, causing resistance to break into smaller droplets and resulting in larger microcapsule sizes (Biswal et al., 2011; Lokhande et al., 2013). Similar to our study, some researchers also pointed out that as the concentration of biopolymer mixtures increased, size of capsules became bigger (K. Y. Lee and Heo, 2000; Sandoval-Castilla et al., 2010).

5.2.1.3.1. Survival in Simulated Gastric Juice (SGJ)

In order to understand the effect of pullulan incorporation into wall material on survival of probiotic cells, *in vitro* gastrointestinal tests were conducted The changes in

viable counts of free and encapsulated probiotic *L. acidophilus* NRRL-B 4495 cells in simulated gastric juice (SGJ) were illustrated in Figure 5.2. Viable cell numbers of free cells dropped from 8.48 log CFU/g to 6.24 log CFU/g of microcapsule indicating a survival rate of 73.55% whereas WPI-pullulan microcapsules obtained increased cell survival rate up to 89.13% after 3 h incubation in SGJ. With increasing pullulan content, WPI-pullulan microcapsule samples exhibited decreasing cell loss of 1.28 log CFU/g, 1.20 log CFU/g, 1.03 log CFU/g and 1.00 log CFU/g, respectively. In contrast, viable counts of WPI-microencapsulated cells decreased about 2.32 log CFU/g with a survival rate of 74.25%. According to results, presence of pullulan created this significance but increase in pullulan concentration did not show any significant difference ($p>0.05$) in cell survival in varying pullulan concentrations. Results indicated that bacterial cells were better protected in the presence of pullulan in wall material than the WPI sample after 3 h exposure to low pH. This can be related to the formation of smoother and denser surface in microcapsules with the presence of pullulan which limits the diffusion of SGJ into microcapsules core, leading to protection of encapsulated cells. In literature there exist various studies conducted on both protein-polysaccharide and polysaccharide-polysaccharide capsules that show increasing survival rates of probiotic bacteria when exposed to lethal conditions by the addition of polysaccharides. For instance, Lotfipour Lotfipour et al. (2012) incorporated polysaccharide psyllium in alginate to encapsulate probiotic bacteria of *L. acidophilus* DMSZ20079 and concluded that increasing psyllium concentration resulted in an increase in viable cell numbers when microcapsules exposed to *in vitro* gastric acid. Nag, Dianawati Dianawati and Shah (2011) combined sodium caseinate with gellan gum for the protection of *Lactobacillus casei.* Formed microcapsules exhibited 3.1 log reduction when compared to control having 4.6 log reduction in viable cells. Moreover, Albertini et al. (2010) observed increased survival of probiotics (*L.acidophilus* LA14 and *Bifidobacterium lactis* BI07) when xanthan gum was incorporated in wall matrix. Guerin Guerin et al. (2003) explained the effect of porous membrane on the survival of probiotic microorganisms using SEM images in details and concluded that having more porous structure of the microcapsule, easier the penetration of acid and bile causing reduction in the entrapped cell number. Therefore, to understand the possible protective effect of pullulan, surface microstructure was investigated under ESEM in further sections. Moreover in the present study, differences in capsule size were thought to be reflecting

the survival during an acid challenge. By increasing the size of the capsules, the ratio of surface area to volume decreases which decreases the exposure of the capsules to the environment. Therefore, as capsule size increases, the ability of the SGJ to diffuse into the capsules decreases, thus increasing probiotic survival.

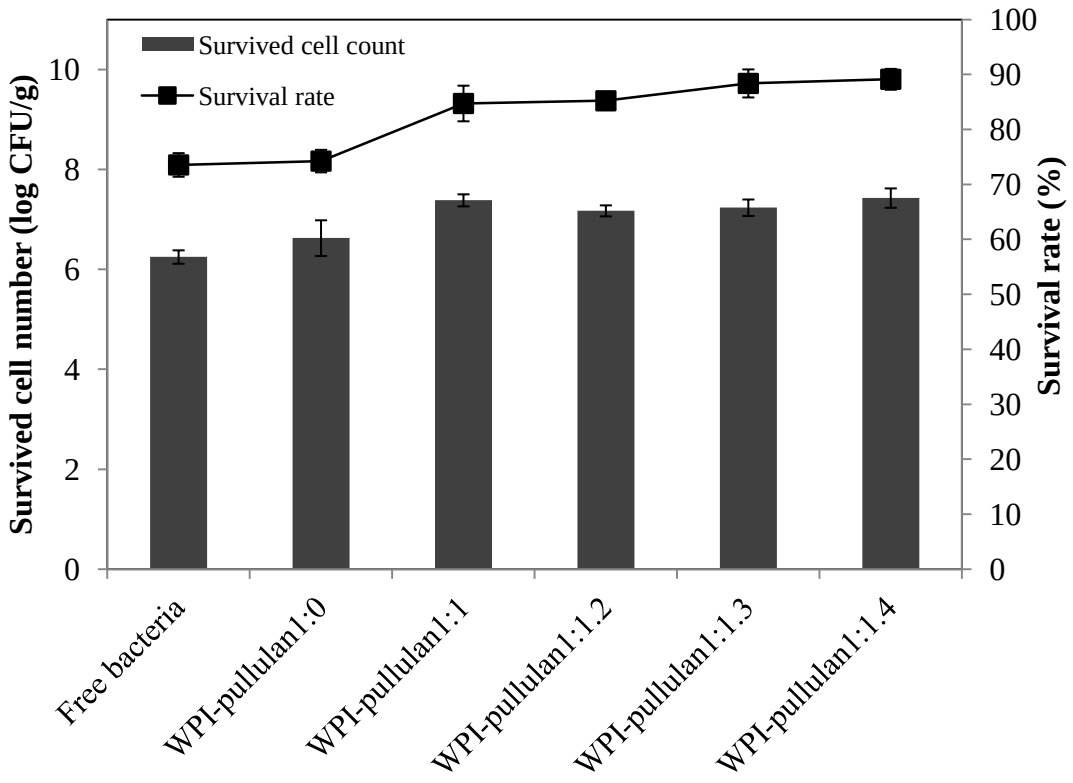

Figure 5.2. Survived cell counts and survival rate of free and encapsulated *L. acidophilus* NRRL-B 4495 with different pullulan concentrations simulated gastric juice at 37 ºC at pH 3.0

5.2.1.3.2. Survival in Bile Salt

After survival through acidic and enzymatic condition of stomach, microorganisms arrive the duodenum and action of bile salts takes place here. Therefore, bile resistance is an important criteria for the further establishment and growth of probiotics in the intestinal tract (Hill and Drasar, 1968); Floch et al., 1972).

Survivals of free and microencapsulated cells after 24 h exposure to *in vitro* bile salt solution were illustrated in Figure 5.3. Irrespective to varying pullulan concentrations, incorporation of pullulan had a positive effect ($p<0.05$) on the survival cell numbers in bile salt solution. On the other hand, statistically no significance

($p>0.05$) between WPI-pullulan$_{1:1}$ and WPI-pullulan$_{1:1.4}$ in survival rate was noted in microencapsulation wall material.

Thus, it is concluded that use of WPI-pullulan as a microencapsulation matrix was effective in protecting the microencapsulated cells with levels of at least 7.56 log CFU/g surviving cells as opposed to levels of 6.53 log CFU/g surviving cells for free bacteria after 24 h exposure to bile salt solution. Moreover, results clearly indicated that WPI-pullulan microcapsules could provide a good protection against the damage of the bile salt solution compared to WPI capsules; viability of WPI encapsulated cells reduced to 7.11 log CFU/g (84.07% survival rate), whereas WPI-pullulan microcapsules provided survival rate op to 90.02% survival rate. As various concentrations and sources of bile salt solutions and different probiotics were used by researchers, it is difficult to make an exact comparison with other studies. As an instance, in a study conducted by Lotfipour,et al. (2012) probiotic *L. acidophilus* DMSZ20079 cells were encapsulated into beads of alginate (ALG) and aliginate-psyllium (ALG-PSL). It was found that probiotic bacteria encapsulated in either ALG or ALG-PSL beads showed better survivability (less than 2.50 log reduction) after 2.0 h bile exposure compared to the free cells of which the viability dropped by around 4.50 log CFU/ml after 2.0 h bile exposure.

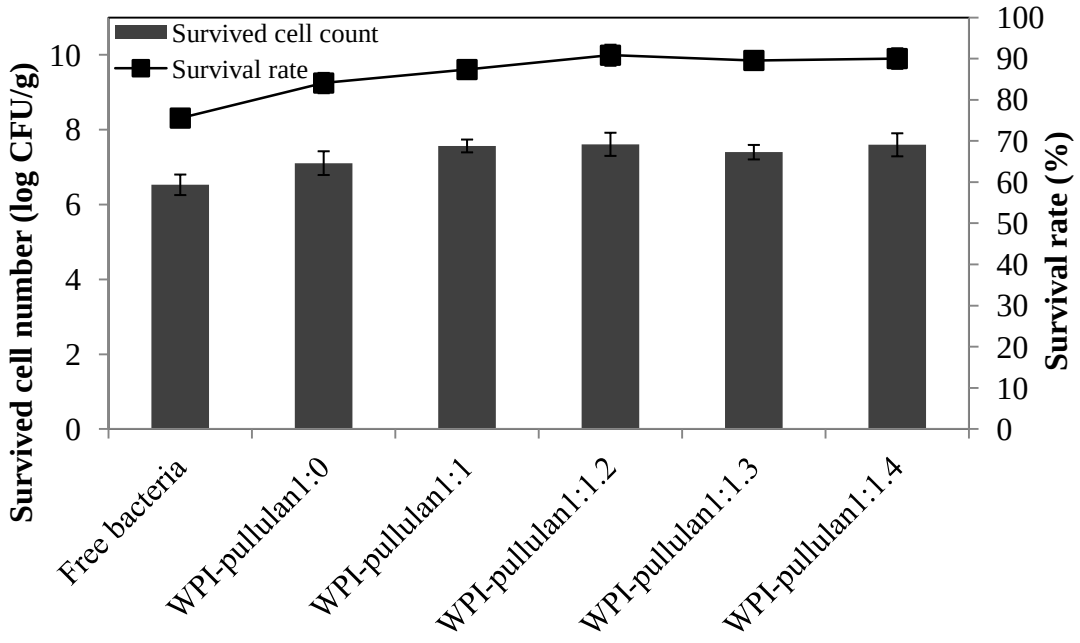

Figure 5.3. Survived cell counts and survival rate of *L. acidophilus* NRRL-B 4495 free and microencapsulated with different pullulan concentrations in bile salt solution at 37ºC

5.2.1.3.3. Release in Simulated Intestinal Juice

Release characteristics of microencapsulated *L. acidophilus* NRRL-B 4495 in WPI and WPI-pullulan microcapsules were studied in simulated intestinal juice (SIJ) and revealed in Figure 5.4. Concerning the results, all microcapsule formulations reached cell number above 7.00 log CFU/g into SIJ with the greatest cell release observed in microcapsules prepared with the addition of 10.8 g/100 ml pullulan (WPI-pullulan$_{1:2}$) after 24 h of incubation. Efficient release properties of WPI-pullulan polymer blend indicated that this polymer blends had desired properties; opening up to liberate viable cells in intestine. The decrease in the release rate from 94.38 % to 89.63 % with increase of pullulan concentration could be related to the fact that higher pullulan concentration affects the microcapsule diameter sizes (Table 5.5). In literature, different release results were stated. J.-S. Lee et al. (2011) reported increased release rates of *Xanthophyllomyces dendrorhous* as the mean diameter of alginate microcapsules decreased. Shi et al. (2013) encapsulated *L. bulgaricus* in alginate-milk microcapsules and observed that release rates of microencapsulated bacteria decreases by the increasing alginate concentrations. Gebara et al. (2013) used pectin-whey protein microcapsules and observed a reduction of 354 units for free cells. On the other hand native or denatured whey protein-pectin encapsulated cell decreased about 1.59 and 1.67 log units, respectively. However, on the contrary to our research, Mandal et al. (2006) reported the release characteristic of encapsulated probiotic did not significantly change with the increase of polymer concentration in the wall matrix.

Interestingly in this research, it was observed that release rate of microcapsules containing pullulan is higher than the non pullulan containing control (WPI-pullulan$_{1:0}$) microcapsules. However, pullulan is a slowly digested carbohydrate by human gastrointestinal enzymes (Peters, et al. 2011, Wolf, et al. 2013). This behavior of pullulan to intestinal enzymes should have stabilized the gel characteristics of the microcapsules in the intestine after exposure to SGJ and bile. Moreover, larger pore sized microcapsules were obtained by pullulan that was expected to limit the diffusion of SIJ solution. Since, large diameter size capsules have a lower surface area-to-volume ratio, thus these capsules usually would not undergo the same rate of capsule matrix degradation as the smaller-sized ones causing more prolonged release profile (Wang et al. 2014, Cui et al., 2000; Klemmer et al., 2011). Besides this expectation ,as it is seen

in Figure 5.4, by the addition of pullulan, increased release rates in WPI-pullulan$_{1:1}$ and WPI-pullulan$_{1:1.2}$ microcapsule formulations were calculated and this increase decreased by further increase of pullulan concentration in the wall material. Therefore, microscopic analysis was further conducted in order to explain these unexpected results.

Microscopic images obtained by SEM (Appendix E) showed that with WPI-pullulan$_{1:1}$ formulation, microcapsules were covered with fibrous particles which were not dissolved in SIJ solution easily. Therefore, it was concluded that the low release rate of microcapsules with WPI-pullulan$_{1:1}$ formulation was due to these fibrous particles. And higher release rate of pullulan containing microcapsules compared to non pullulan containing control (WPI-pullulan$_{1:0}$) can be explained by the swelling of WPI-pullulan microcapsules depended upon the pH sensitive factor. Because the microcapsule swelled higher than WPI microcapsules due to ionization of carboxylic groups (–COOH) of pullulan at pH 8.0 and therefore the diffusion of SIJ increased and the amount of released bacteria enhanced (Table 5.5).

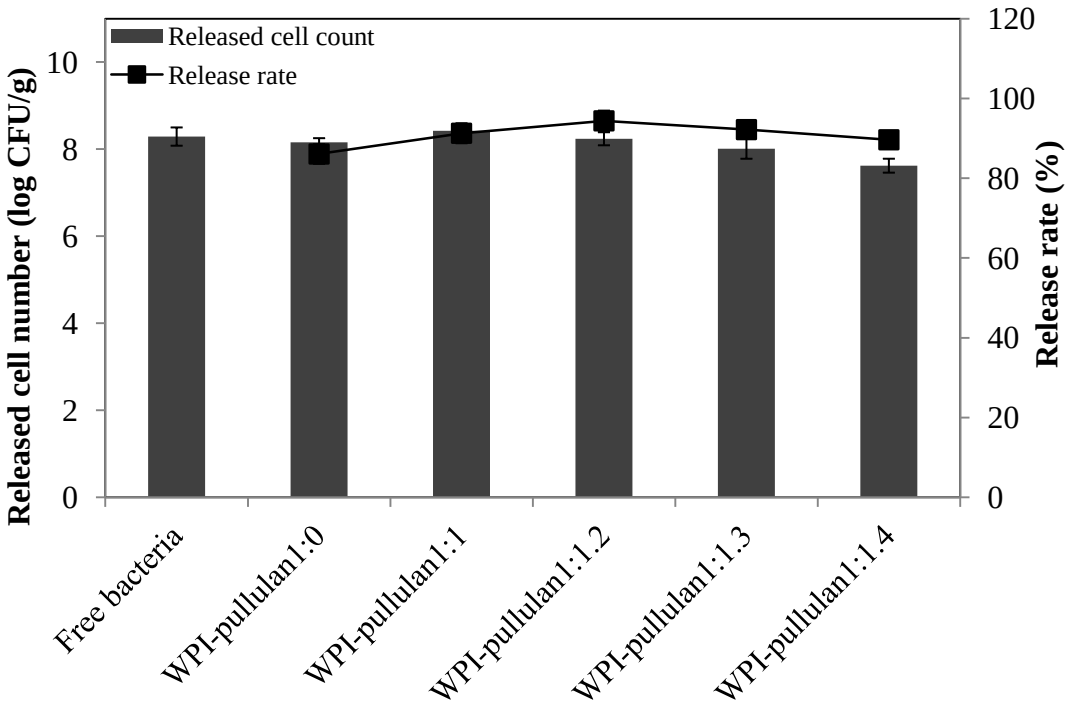

Figure 5.4. Released cell counts of free and encapsulated *L. acidophilus* NRRL-B 4495 with different pullulan concentrations in simulated intestinal juice at 37 ºC at pH 8.0

Table 5.5. Swelling degree of WPI-pullulan microcapsules with different pullulan concentrations under simulated intestinal juice exposure after 24h at 37 ºC

WPI-pullulan$_y$	**Swelling degree (%)**
WPI-pullulan$_{1:0}$	113.00±1.35^{a}
WPI-pullulan$_{1:1}$	121.98±0.78^{b}
WPI-pullulan$_{1:1.2}$	129.69±1.32^{c}
WPI-pullulan$_{1:1.3}$	139.06±1.20^{d}
WPI-pullulan$_{1:1.4}$	146.89±2.93^{e}

$^{a-e}$ Means ± standard deviation with different superscript letters in the same line indicate significant differences ($P < 0.05$) among the studied samples.

5.2.1.3.4. Storage Stability

To evaluate the efficiency of addition of pullulan into microencapsulation wall material under refrigerated storage, free cells, WPI microcapsules and WPI-pullulan microcapsules were stored for one month at 4 ºC. Results showed that after storage, a significant reduction ($p<0.05$) in viability of free cells was observed (Table 5.6). Moreover, microencapsulation into WPI microcapsules afforded a decrease of viable cell numbers from 8.44 log CFU/g to 6.91 log CFU/g after 30 days of storage at 4 ºC. Of the four pullulan concentrations investigated, WPI-pullulan$_{1:1.4}$ was found to retain the highest viability of probiotic bacteria (8.43 log CFU/g) showing the lowest viability loss (0.51 log CFU/g). As a result, comparing viabilities of free, and encapsulated cells, the protective effect of microencapsulation into WPI-pullulan polymer complexes on the stability of probiotic bacteria during refrigerated storage is obviously evident.

Microencapsulation should not only ensure the survival through gastrointestinal transit, but also should ensure the survival during the storage. Various reports exposing positive effects of microencapsulation exist in literature. Oliveira et al. (2014) used complex coacervation followed by spouted-bed- drying method for microencapsulation of *L. acidophilus* (LAC 4) in casein/pectin polymer complex. After storage for 30 days, no significant decrease in cell numbers was calculated; viable cells decreased from 9.16 log CFU/g to 8.92 log CFU/g. In another study, it was aimed to co-encapsulate probiotic microorganism *L. acidophilus* (LAC-04) in solid lipid microparticles with inulin and

polydextrose. Storage stability tests were conducted throughout 120 days at -18, 7 and -22 °C under vacuum or with controlled relative humidity. At the end of 30 and 120 days at 7 °C, encapsulated bacteria presented a logarithmic reduction of 1.83 and 4.59 log CFU/g for inulin and 1.47 log CFU/g and 2.15 log CFU/g for polydextrose under controlled relative humidity of 11%, respectively. On the other hand no surviving numbers were detected under vacuum for both encapsulated cells. Although, that study stored microcapsules with a controlled relative humidity in the presence of known prebiotics, the percentage surviving cell numbers (in other words survival rates) of WPI-pullulan encapsulated bacteria was higher. López-Rubio et al. (2012) used electrospinning technique to encapsulate *Bifidobacterium* strains in a protein (whey protein concentrate) and a carbohydrate (pullulan). After 120 days of storage, pullulan microcapsules exposed a significant drop of 4.0 log units ($p<0.001$) in viable cells of *B. animalis* Bb12 when compared to WPI encapsulated cells having decrease of 2 log units.

Table 5.6. Viable cell counts of *L. acidophilus* NRRL-B 4495 microencapsulated with different pullulan concentrations under storage at 4 ºC for 4 weeks

WPI-pullulan$_y$	**Initial cell number (log CFU/g)**	**Survived cell number (log CFU/g)**	**Survivial rate (%)**
Free bacteria	8.67±0.21	4.93±0.39	56.85±0.17^{a}
WPI-pullulan$_{1:0}$	8.44±0.12	6.91±0.21	81.89±0.53^{b}
WPI-pullulan$_{1:1}$	8.66±0.11	8.04±0.19	92.91±0.88^{c}
WPI-pullulan$_{1:1.2}$	8.37±0.44	7.79±0.34	93.10±1.48^{c}
WPI-pullulan$_{1:1.3}$	8.26±0.18	7.76±0.26	93.91±0.78^{c}
WPI-pullulan$_{1:1.4}$	8.43±0.39	7.92±0.15	93.85±2.86^{c}

$^{a-c}$ Means ± standard deviation with different superscript letters in the same line indicate significant differences ($P < 0.05$) among the studied samples.

5.2.1.3.5. Morphological Analysis

The environmental scanning electron microscopy (ESEM) uses low pressures (100–1000 Pa) without any conductive coating of the samples unlike SEM that prevents soft and sensitive samples from being destroyed (Fratesi et al., 2004, Schwartz et al. 2009). To visualize the morphology changes of microcapsules due to incorporation of pullulan, the samples were observed directly by ESEM in the hydrated state (T = 20 ºC). Figure 5.5 showed the cross sectional and surface views of the microcapsules with different concentrations of pullulan. As it can be seen from ESEM images, incorporation of pullulan in protein wall material created smoother texture that can provide better barrier properties against diffusion of gastric acid. This result could support our observations about the increased survival in *in vitro* gastrointestinal conditions of WPI-pullulan microcapsules compared to WPI microcapsules. As a result of all experiments conducted to optimize pullulan concentration in wall material, following experiments were conducted with pullulan concentration of 10.8% (w/v, formulation:WPI-pullulan$_{1:1.2}$).

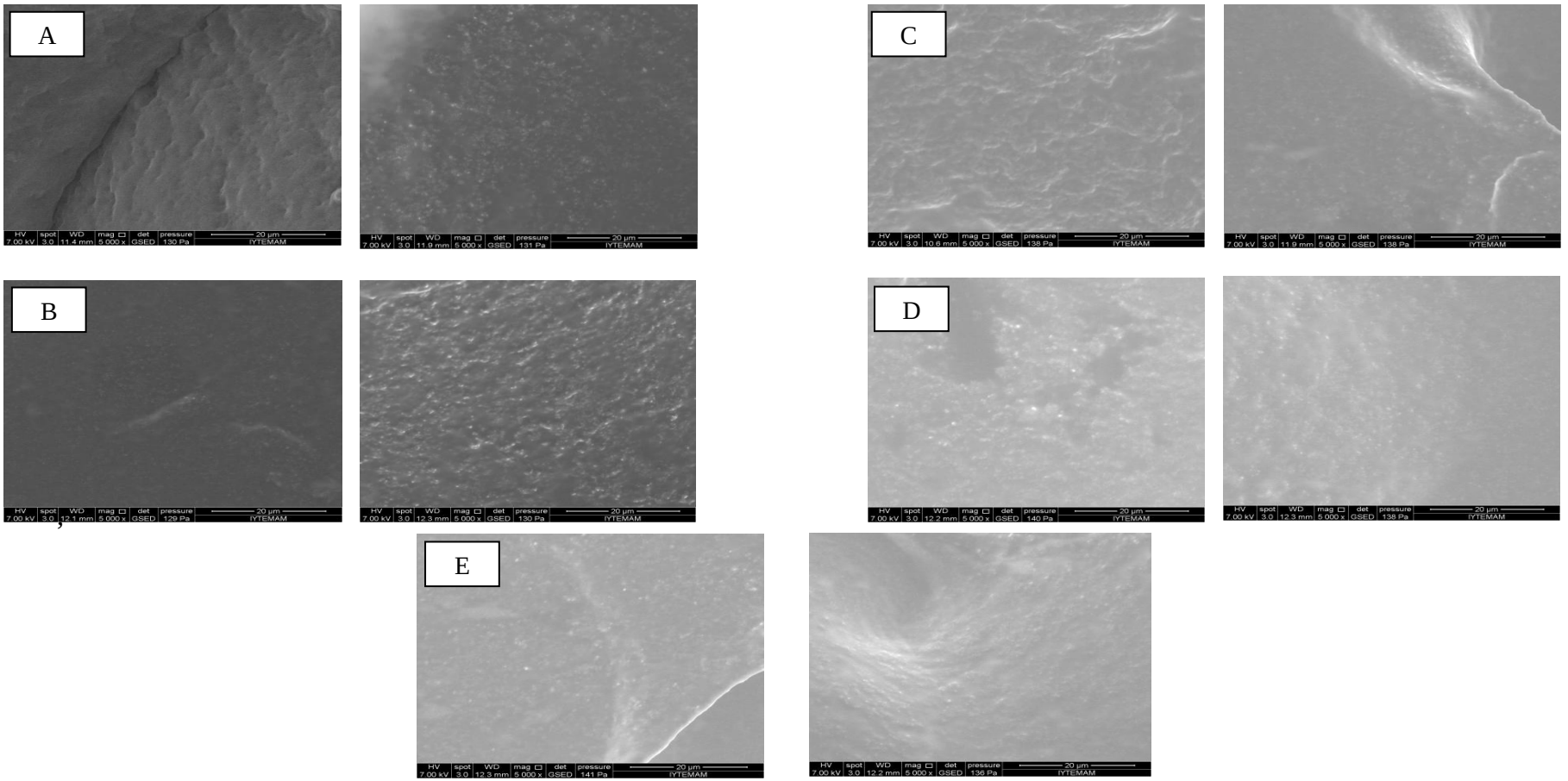

Figure 5.5. Environmental scanning electron microscopy image of a crosssection and surface of A) WPI-pullulan$_{1:0}$, B) WPI-pullulan$_{1:1}$, C) WPI-pullulan$_{1:1.2}$, D) WPI-pullulan$_{1:1.3}$, E) WPI-pullulan$_{1:1.4}$ microsphere at 5000X magnification

5.2.1.4. Effect of Initial Cell Concentration

The last parameter investigated for the development of WPI-pullulan microcapsules were initial cell load. Table 5.7 showed the change of encapsulated cell numbers by increasing initial cell concentration introduced to wall matrix. Microencapsulation yields of formed microcapsules containing different amount of cells vary considerably. It seemed that small amount of bacteria were lost during microencapsulation process. Since effect of homogenization speeds and $CaCl_2$ concentration used in microencapsulation process were observed to have no negative effect on viability (Appendix B and Appendix C). Cells might be lost into the oil phase during emulsion formation and lost during the centrifugation steps after hardening. Moreover, it was observed that increasing initial load resulted in decreasing microencapsulation efficiency and when cells were encapsulated over 10.00 log CFU/g, encapsulated cell number did not show any change. Therefore, the optimal concentration of bacteria for microencapsulation was should be kept at 10.00 log CFU/g. Increased initial bacterial load could decrease the strength of the microcapsules, thus decrease the stability of encapsulated cell (M. Y. Wang et al., 2005)

Table 5.7. Effect of initial bacterial concentration on encapsulation yield of WPI-pullulan microcapsules.

Initial cell concentration (log CFU/g)	**Cell count after microencapsulation (log CFU/g)**	**Microencapsulation yield (%)**
4.00	3.85±0.13	96.25±3.31^{c}
6.00	5.64±0.12	94.00±2.05^{c}
8.00	7.37±0.17	92.13±2.14^{c}
10.00	9.35±0.40	93.50±3.98^{c}
12.00	9.40±0.27	78.33±2.21^{b}
14.00	9.38±0.34	66.43±2.44^{a}

$^{a-c}$ Means ± standard deviation with different superscript letters in the same line indicate significant differences ($P < 0.05$) among the studied samples.

5.2.2. Characterization of Developed WPI-Pullulan Microcapsules

There have been extensive studies conducted to evaluate the viability changes of probiotics in *in vitro* gastrointestinal conditions. However, before consumption, probiotic bacteria should first resist the physical conditions of the incorporated food products. According to International Dairy Federation (IDF), in order to exert beneficial health effects, a minimum of 10^7 CFU probiotic microorganisms are expected to be per g or ml of product at the time of consumption. Thus, the tolerance to different stress conditions provides significant information about the probiotic microorganism for the selection of ideal matrix and successful applications into it. Moreover, this information may provide estimation for further behavior in gastrointestinal conditions (Ross et al., 2005).

Different responses of probiotic microorganism against heat stress have been reported earlier. The mechanism of inactivation depends on irreversible denaturation of cellular components such as ribosomes and nucleic acids (Jaesung Lee and Kaletunç, 2002). P. Teixeira et al. (1997) reported that heat induced inactivation mechanism of *L. bulgaricus* depended on the damage of cell membrane structures below 65 °C and ribosome and⁄or protein denaturation above 65 °C.

As it is well known, type of the food matrix should be significantly considered for the preservation of the functionality of the probiotic microorganism. Mostly, dairy products such as yoghurt, kefir and cheeses have been widely preferred as a probiotic carrier matrix. However, attention to non-dairy functional foods containing high salt (fermented sausages, salad dressings); high sugar content (chocolate bars and jams) has been increasing and having low pH (fruit juices, fermented products). Therefore, it is necessary to understand the response of the probiotic bacteria to under these stress conditions as long as these challenges are present during the shelf-life of functional foods, which are stored under refrigerated conditions. This chapter characterizes the developed microcapsules and analyses the resistance of encapsulated and free probiotic bacteria to stress conditions induced by heat, salt, sucrose and pH pretreatments.

5.2.2.1. Physicochemical Characterization

Color values of the formed microcapsules, shown in Table 5.8, revealed that these microcapsules have white color and can be applied dairy based food such as yoghurt, cheese and whey based drinks. However, color of the formed microcapsules may undergo some color changes when added to different food groups and this may be another topic of further research.

The moisture content and water activity (A_w) of the microcapsules was found to be 82.36% and 0.95, respectively. Moisture content and water activity affect the stabilities of the microcapsules throughout storage; higher moisture contents and water activities have been noted to increase oxygen permeability of wall materials and cause higher decline in surviving viable numbers of microorganisms during storage (Clementi and Rossi, 1984). Furthermore, low moisture content and water activity values in microcapsules contribute to improved physical and bulk properties (Onwulata et al., 1995). In this work, all formulations exhibited high water activity and moisture content values that can create some problems for use in some food products for long time storage. For this reason, for the possible prolonged storage of encapsulated bacteria in functional foods, it was necessary to study the effect of drying to reduce the moisture content for practical uses.

Table 5.8. Physicochemical characterization of encapsulated *L. acidophilus* NRRL B-4495

Physicochemical characteristics	**WPI-pullulan microcapsules**
Moisture Content (%)	82.36±0.023
Water activity (A_w)	0.95±0.04
Color	
L^*	80.29±0.04
a^*	-1.46±0.01
b^*	1.35±0.04

5.2.2.2. Diameter Distribution

Microcapsule size is an important parameter that both reflect the quality of added food product and protection of cells during gastrointestinal transit. In industrial production, low capsule sizes are required since high capsule sizes above 100 μm create sandy and gritty undesirable textural property in the food product in which they are used (Annan et al., 2008; Vivek, 2013). On the other hand, with low capsule sizes surface area to volume increases which can further lead to reduced survival during gastrointestinal transit (K. Sultana et al., 2000).

Figure 5.6 showed the frequency of diameter distribution of developed WPI-pullulan microcapsules. The microcapsules obtained in this work had an average mean diameter of 76.40 μm and diameter distributions were between 30 and 172 μm. Large sized microcapsules (>100 μm) also existed however, the highest frequencies for the WPI-pullulan microcapsules were obtained between 55 and 95 μm. Moreover when the figure was investigated the frequency distribution of large sized microcapsules was lower than 10 and the frequency distribution also showed that it is skewed more towards the smaller diameter values side relative to the most frequently occurring value. Also small sized diameter distribution did not create disadvantage as smaller diameter in microcapsules generally results in decreased microencapsulation efficiency and protection against harsh conditions. As a result frequency distribution of microcapsules represents that by emulsification/cold gelation method, with WPI-pullulan polymer blend formed microcapsules showed ideal size for food applications.

Different attempts were made in order to encapsulate probiotic microorganism to provide survival during gastrointestinal storage in effective ways. In these studies, both techniques were used and microcapsules with mean diameters higher or lower than the critical limit were obtained. For instance, (J. Wang et al., 2014) used chickpea protein incorporated with κ-carrageenan/alginate to encapsulate *Bifidobacterium adolescentis* (ATCC 15703) cells using the emulsion technique. In the study, the presence of κ-carrageenan created larger particle sizes of about 838.5 μm when compared to alginate having 22 μm mean diameters. Another study conducted by Heidebach and Kulozik (2009) again emuslion technique was used to encapsulate probiotic *Lactobacillus paracasei* ssp. *paracasei* F19 and *B. lactis* Bb12. In that study, microcapsules were formed by transglutaminase-catalysed gelation of casein and microcapsules having

median diameter of 165 µm were obtained. Compared to our results, microcapsule size were higher however in the case of incubation in SGJ at pH 3.6, both survival rates and survived viable cell numbers were lower. It was observed that encapsulated cells were reduced by about 1.8 log CFU/g and 2.75 log CFU/g for *L. paracasei* F19 and *B. lactis* Bb12, respectively. Khan et al. (2013) used extrusion technique to form legume protein isolate–alginate microencapsulation system. Mean diameters ranged from 2.79 (16G) to 1.23 mm (27G) as a function of needle gauge. Very high survival cell numbers of probiotic *B. adolescentis* were calculated after exposure to SGJ with pH 1.8 after 120 minutes. As a result, microcapsules formed with extrusion technique generally provide better protection. On the other hand, large sizes of these microcapsules create undesirable texture. Thus, studies on microencapsulation of probiotics to form small microcapsules providing higher protection still continues. In our study, we obtained small microcapsules having good protection results and this increased cell survival in our study can be attributed to the presence of polysaccharide having good film forming properties and prebiotic potential.

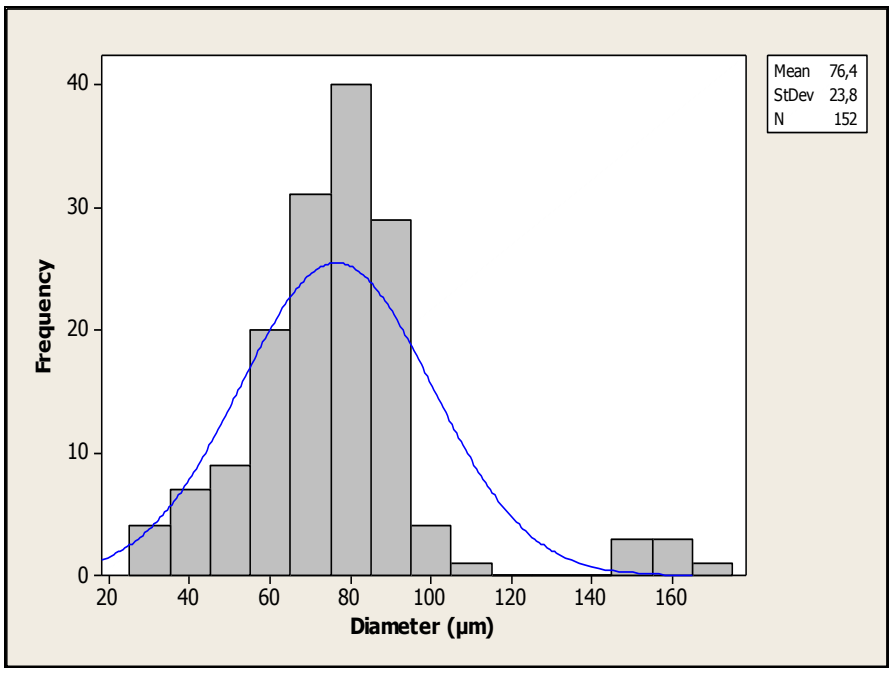

Figure 5.6. Frequency distribution of diameters of the WPI-pullulan microcapsules

5.2.2.3. Survival in Simulated Gastric Juice and Bile Salt

One of the main objectives of microencapsulation is providing protection of probiotic cells during exposure to low pH gastric environment. Figure 5.7 showed the number of survived cells of free and microencapsulated *L. acidophilus* NRRL B-4495 under SGJ. After 1 hour of incubation, *L. acidophilus* NRRL B-4495 was reduced by 1.0 log units when added as free cells. Survived cell numbers of encapsulated cells were 0.6 log units higher than free cells after 1 hour of exposure compared to initial numbers. As can be seen, free cells followed nearly a linear reduction in cell numbers. At the end of incubation, viable cell numbers of free cells decreased to 5.67 log CFU/ml possessing the survival rate of 73.19%. However, microencapsulation into WPI-pullulan microcapsules offered significant ($p<0.05$) protection compared to free cells. Survived cell number were more than 7.0 log CFU/g for WPI-pullulan encapsulated cells throughout the incubation time.

Our results suggested that free cells of *L. acidophilus* NRRL B-4495 were sensitive to low pH gastric environment. It is possible that microencapsulation into WPI-pullulan microcapsules can restrict the diffusion of SGJ. By restricting the diffusion of gastric juice, the time needed to destroy viable cells under 7.0 log CFU/g level can get longer. Also protein structure of wall matrix can provide higher pH (buffering effect) within the microcapsule that protects the cells under low pH environment (Krasaekoopt, 2013). Moreover it can be hypothesized that pullulan gel network formed in the microcapsules reduced the degradation of whey protein by pepsin due to restricted diffusion of gastric juice.

The effect of the bile salt on the viability of free and microencapsulated *L. acidophilus* NRRL B-4485 is presented in Figure 5.8. For free cells, initial viable count of 9.31 log CFU/ml was reduced to 8.64 log CFU/ml after 1 h and were further reduced to 7.75 log CFU/ml after 10 h for free cells and then reduced to 7.1 log CFU/ml at the end of incubation. In contrast to free cells, the encapsulated cell count decreased by only 0.75 log units after 10 h of incubation. Beyond 10 h of incubation, viable cells of encapsulated bacteria showed a slight decrease. Survival of encapsulated cells after exposure to bile was 8.39 log CFU/g after 24 h.

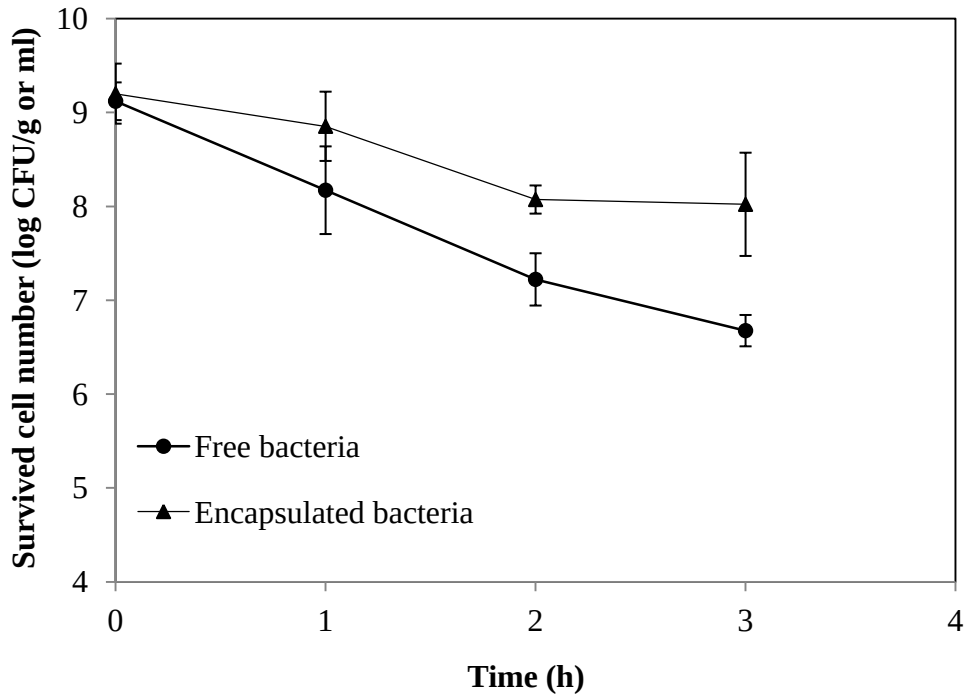

Figure 5.7. Survived cell counts of free and encapsulated *Lactobacillus acidophilus* NRRL B-4495 in simulated gastric juice at 37 ºC at pH 3.0

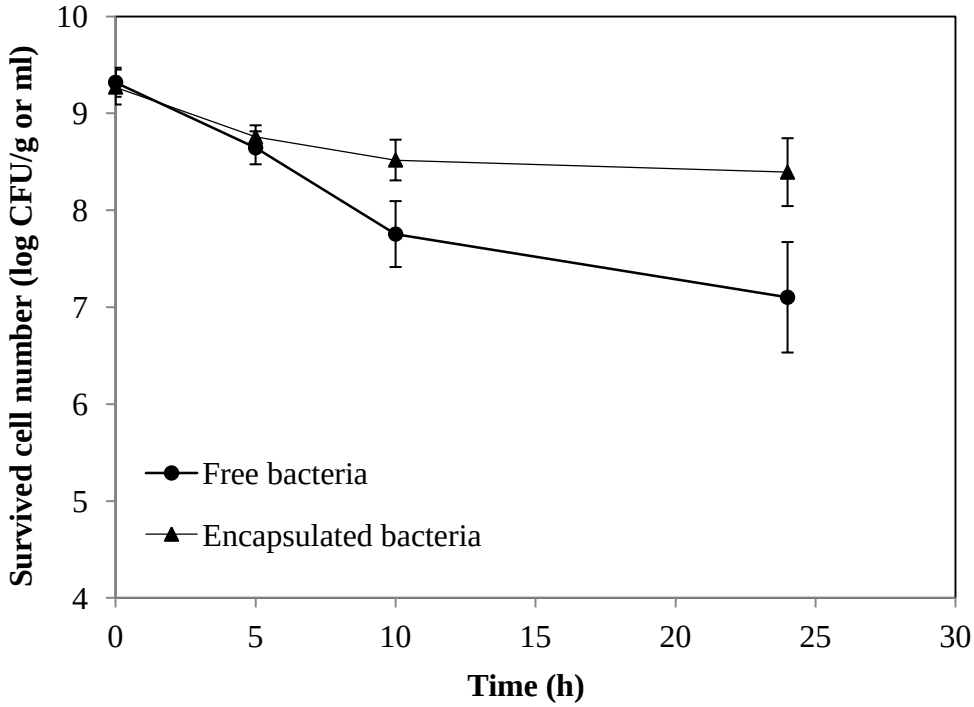

Figure 5.8. Viability of free and encapsulated *Lactobacillus acidophilus* NRRL B-4495 in bile salt solution at 37 ºC

5.2.2.4. Release in Simulated Intestinal Juice

The release characteristic of the developed WPI-pullulan microcapsules was investigated since release of viable cells in the intestine is one of the main objectives of microencapsulation. The investigated intestinal release characteristic of the encapsulated cells from the WPI-pullulan microcapsules was revealed in Figure 5.9. For this assay, microcapsules were first exposed to SGJ at pH 3.0 for 3 h after which the same microcapsules were transferred into SIJ at pH 8.0 and incubated for further 24 h. Results indicated that in 1 h, more than 70% of encapsulated bacteria was liberated from microcapsules, and within 7 h release of encapsulated cells nearly completed. The initial high release rate of WPI-pullulan microcapsule can be attributed to the swelling capacity. Moreover, during SGJ exposure microcapsules had undergone some extent of degradation and reduce the density of outer membrane permeability thus the penetration of SIJ got easier. At the same time microcapsule wall material at pH 8.0 can also be degraded by probiotic cells.

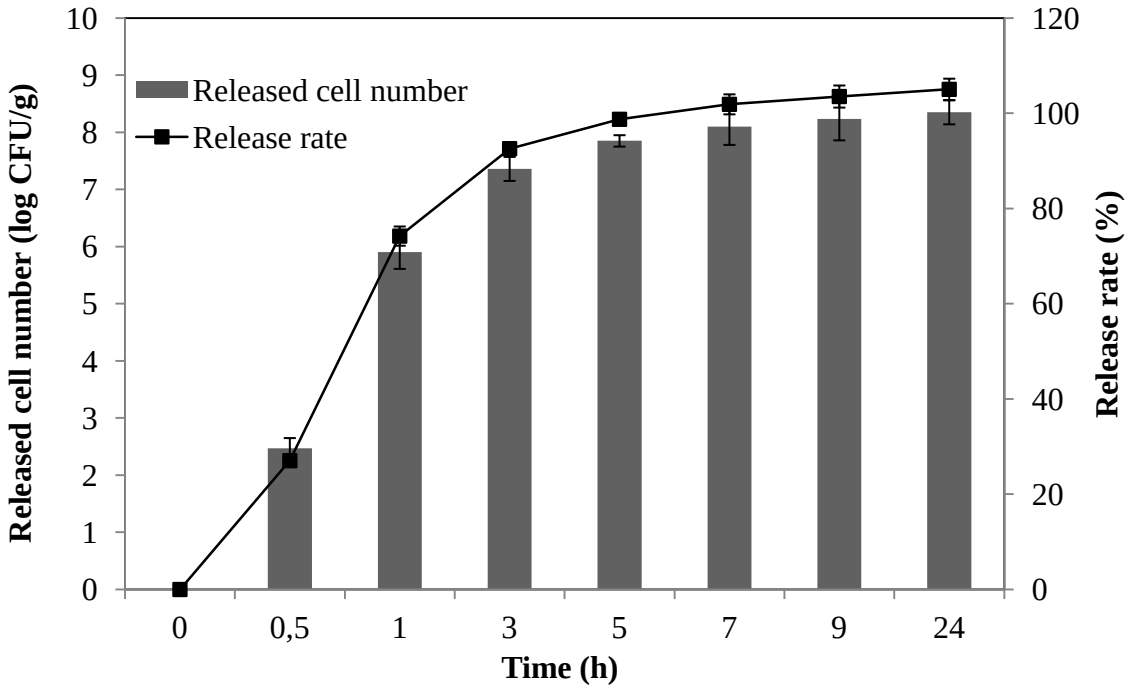

Figure 5.9. Released cell counts and release rate of encapsulated *L. acidophilus* after 3 h of incubation (37 °C) in SGJ (pH 3.0) to SIJ (pH 8.0)

5.2.2.5. Morphological Analysis

Figure 5.10 and Figure 5.11 showed the morphological structure and shape of WPI-pullulan microcapsules obtained by SEM and light microscopy, respectively. It was observed that the polymers complexes formed microcapsules spherical in shape with a smooth and homogeneous surface with small pores. As could be seen, on the external surface of the microcapsules, also probiotic *L. acidophilus* NRRL B-4495 cells were visible. From light microscopy, it is evident that microcapsules were spherical in shape. A compact and smooth surface of microcapsules due to film forming characteristics of pullulan provided better barrier properties to stop diffusion of acid and bile and release of encapsulated bacteria when compared to whey protein microcapsules. Also in Figure 5.11, the changes in structure of microcapsules after 4 weeks of storage were revealed. Size and spherical shape of microcapsules were not altered but surface of microcapsules obtained more porous structure. This porous structure of stored microcapsules might result in increased diffusion of oxygen during storage and gastric and intestinal solutions during gastrointestinal transit. Further gastrointestinal survival tests during longer storage periods should be conducted.

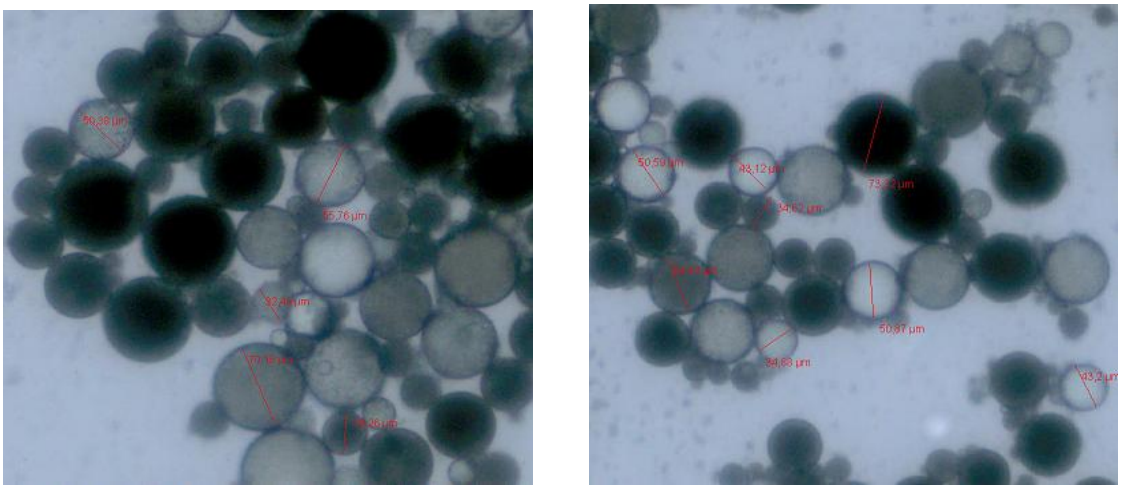

Figure 5.10. Optical micrographs of WPI-pullulan microcapsules at 100X

Moreover as seen in Figure 5.12, WPI-polymer wall matrix conserved its spherical shape and integrity against bile salts and simulated gastric acid. Also this figure showed that in simulated intestinal juice, formed polymer blend wall matrix is destructed into small particles completely and providing higher release rates. But incorporation of any polysaccharide into protein wall matrix not always provides barrier functions For instance; Guerin et al. (2003) incorporated pectin into whey protein wall matrix and used this polymer complex to encapsulate probiotic *B. bifidum*. After exposure of formed microcapsules to simulated gastrointestinal conditions, they observed significant reduction in viable cell numbers.

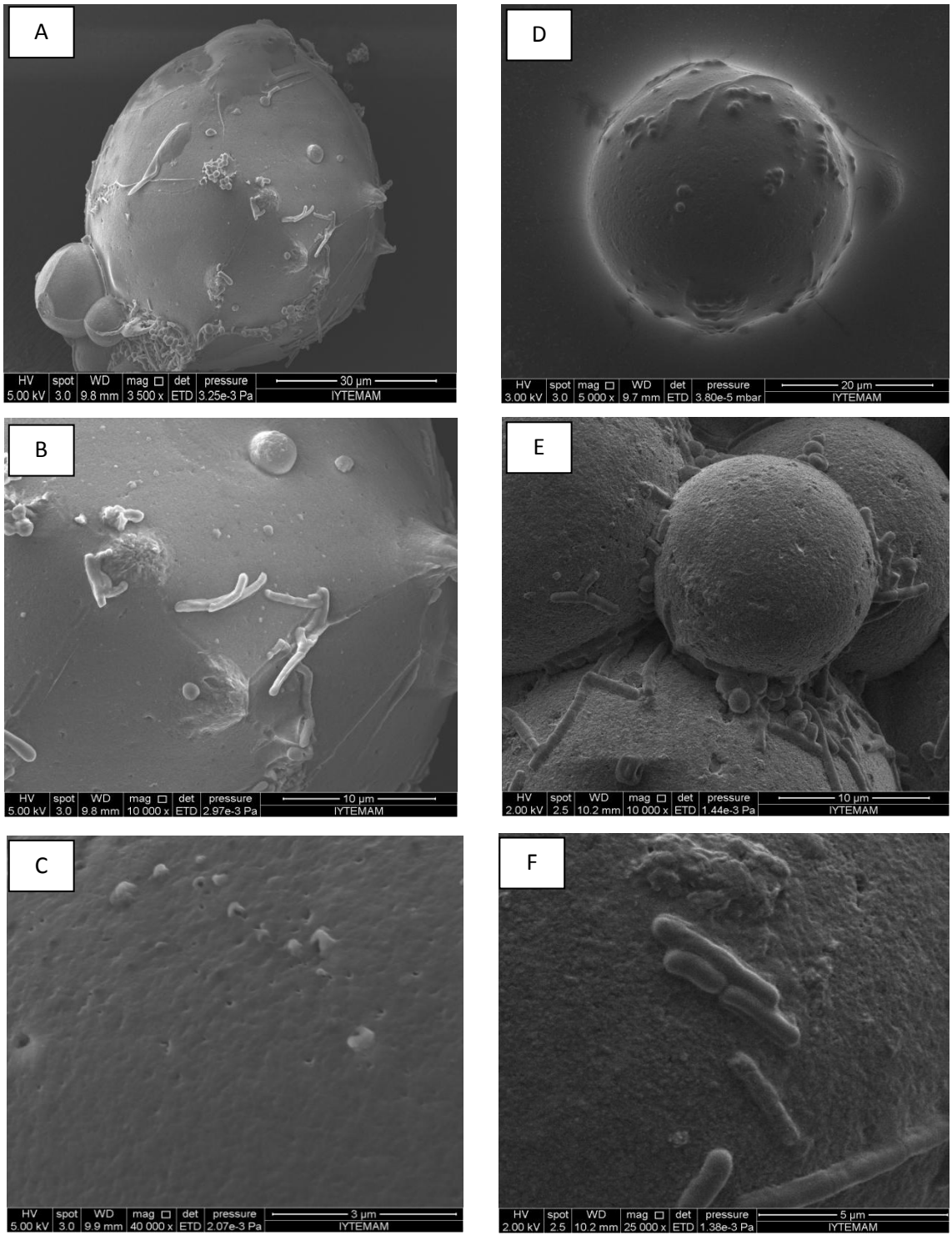

Figure 5.11. SEM images of WPI-pullulan microcapsules after production under A) 3500X, B) 10000X and C) 40000X magnifications and after storage for 4 weeks under A) 5000X, B) 10000X and C) 25000X magnifications

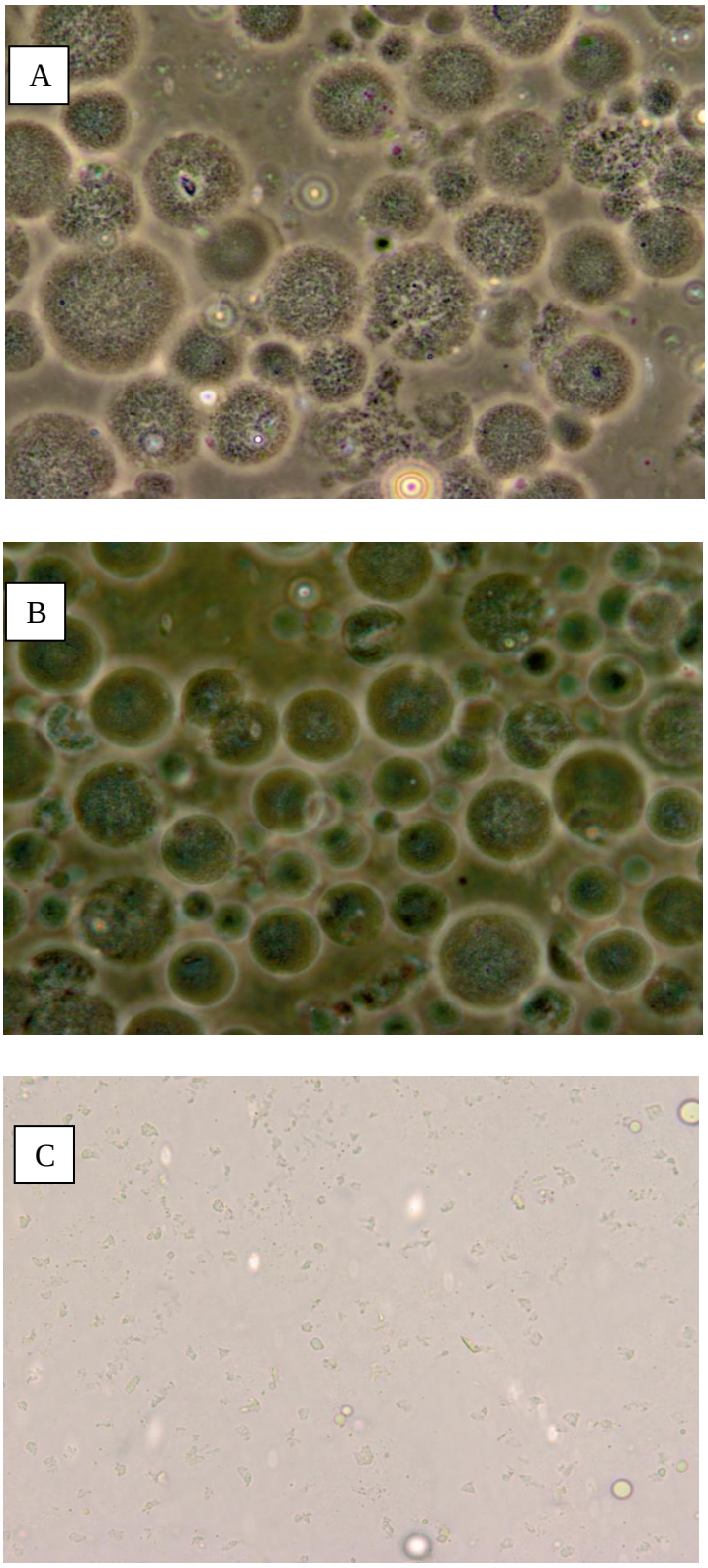

Figure 5.12. Optical micrographs of WPI-pullulan microcapsulesAa) after SGJ, B) after bile salt and C) after SIJ exposure at 100X

5.2.2.6. pH Tolerance Test

Effect of pH on the viability of free and encapsulated cells was investigated. Data obtained and presented in Table 5.9 showed that at pH 2.0, free bacterial cells could not survive. Meanwhile, WPI-pullulan encapsulated bacteria showed significant difference ($p<0.05$) in survival numbers having 7.10 log CFU/g viable cells at pH 2. At pH 3.0, while WPI-pullulan encapsulated cells obtained the viable cell number of 7.74 log CFU/g cells, free cells again failed to survive at desired theurophatic level and obtained viable cell numbers of 5.88 log CFU/ml. This higher viability of WPI-pullulan microencapsulated bacteria compared to free ones at low pH is probably because denser microcapsules having pullulan led to maintain higher local pH value compared to the surrounding medium (Annan, Borza, and Hansen (2008). Above pH 5.0, both free and WPI-pullulan encapsulated cells showed an increase in number and viable cell number reached above 9.00 log CFU/g

Jiménez-Pranteda et al. (2012) attributed to study the stability of *Lactobacillus plantarum* CRL 1815 and *L. rhamnosus* ATCC 53103 encapsulated in different sorts of polymers including xanthan gum, gellan gum, pullulan gum and jamilan. At pH values at 5.0 and 6.5, free and microencapsulated forms showed an increased cell numbers. But microencapsulation into polymers failed to survive cells at low pH values and decreased viable numbers at the level of 5.0 log CFU/g were observed. Moreover, in this study, it was observed that pullulan was unable to form microcapsules independent of its concentration.

In literature the increase in cell number in the microcapsules was explained by the use of nutrients due to the diffusion into pores of microcapsules (Lisboa et al., 2007; Peirone et al., 1998). Moreover, increase in number in pullulan containing capsules can be the result of the use of pullulan by probiotic bacteria in microcapsules. The growth experiment conducted in earlier chapter proves that this approach could be suitable for WPI-pullulan microcapsules. Consequently, results showed WPI-pullulan microcapsules could be used in food with the pH range of 3.0 to 8.0 effectively as the addition of pullulan enhances the survival of microencapsulated cells.

In literature there are several researches conducted on microencapsulated bacteria to observe the viability and survival under various pH values. Satyabama et al

(2014), showed significant improvement ($p<0.05$) in surviving cell numbers at low pH values when prebiotics (sugarbeet and chicory) were incorporated into alginate matrice.

Table 5.9. Viability of free and microencapsulated *L. acidophilus* NRRL-B 4495 at different pH values

pH values	Initial cell number (Log CFU/g or ml)		Survived cell number (Log CFU/g or ml)		Survival rate (%)	
	Free bacteria	WPI-pullulan microcapsule	Free bacteria	WPI-pullulan microcapsule	Free bacteria	WPI-pullulan microcapsule
2	9.43±0.06	9.04±0.03	0.0±0	7.10±0.06	0[a]	79.92±0.71[a]
3	9.43±0.06	9.04±0.03	5.88±0.05	7.74±0.01	69.41±0.61[b]	87.09±0.05[b]
4	9.43±0.06	9.04±0.03	7.79±0.02	8.34±0.02	91.98±0.26[c]	92.28±0.21[c]
5	9.43±0.06	9.04±0.03	9.81±0.03	8.94±0.05	104.05±0.30[d]	98.93±0.50[d]
6	9.43±0.06	9.04±0.03	10.38±0.01	9.71±0.01	110.10±0.06[e]	107.71±0.03[e]
7	9.43±0.06	9.04±0.03	10.34±0.08	9.55±0.03	109.62±0.89[e]	106.06±0.30[e]
8	9.43±0.06	9.04±0.03	10.32±0.01	9.68±0.02	109.42±0.06[e]	107.49±0.23[e]

[a-e]Means ± standard deviation with different superscript letters in the same column indicate significant differences ($P < 0.05$) among the studied samples.

5.2.2.7. Heat Tolerance Test

For practical use of probiotic bacteria delivery system to the consumers, the effect of different temperatures on the viability of bacteria had to be investigated. This study attempted to understand the protective effect of WPI-pullulan microencapsulation under heat exposure. Table 5.10 showed the viability of free and microencapsulated *L. acidophilus* at different temperatures (45, 50, 55, 60, 65 and 70 ºC) for 2 h.

WPI-pullulan complex showed significant ($p<0.05$) difference in protective effect to heat exposure when compared to free cells; free cell numbers decreased to 2.09 log CFU/ml, meanwhile both encapsulated cells preserved their viability at the level of minimum 4.75 log CFU/g. Experimental results suggest that the increase in heat tolerance is more pronounced under lower heat treatments between free cells and encapsulated bacteria. At 45 ºC, 0.2 log CFU/ml and 1.0 log CFU/ml decrease observed in viable cell counts of free and WPI-pullulan encapsulated bacteria, respectively. When free cells were exposed to 50 ºC, cell counts decreased from 9.76 log CFU/ml to 3.79

log CFU/ml. At 55 ºC, WPI-pullulan microencapsulation resulted in a cell loss of 2.8 log CFU/g, while 6.21 log CFU/g cell loss in free cells were calculated.

Even tough, addition of pullulan improved the survival rate of encapsulated cells significantly ($p<0.05$) when compared to free cells, the heat tolerance of the WPI-pullulan microcapsules is not evident. In fact none of the encapsulated cells were successfully survived at desired viable numbers (≥7 log CFU/g) at high temperatures (≥55 ºC) and. At 70 ºC, encapsulated cells in WPI-pullulan microcapsules showed viable numbers of 4.76 log CFU/g where free cells had 2.09 log CFU/ml after one hour.

These results suggest that WPI-pullulan microencapsulation did not provide heat tolerance at 55 ºC and above temperatures. Therefore, both free and microencapsulated cells of *L. acidophilus* NRRL B-4495 could not be used in foods which high heat treatment are applied during production. In literature, there are limited numbers of studies conducted to observe the effect of microencapsulation against heat treatments. For instance, Abbaszadeh et al. (2013) reported that chitosan coated alginate beads for the microencapsulation of *Lactobacillus rhamnosus* GG significantly protected the viable cells during heat exposure at 55 ◦C, 60◦C and 65 ◦C. Free cells experienced about 5 log cycles reduction after heat treatment at 65◦ C for 30 min, whereas encapsulated *L. rhamnosus* GG was reduced by only 2.55 log cycles. In another study, free and encapsulated probiotic bacteria were exposed to 65 °C for up to 1 h (Ding and Shah, 2007). After 30 min of incubation, free bacteria revealed cell loss of 6.74 log CFU/ml while, encapsulated bacteria showed a reduction of 4.17 log CFU/ml. However at the end of incubation period, microencapsulation into alginate did not show any significant ($p>0.05$) improvement in survival. Effect of microencapsulation into whey protein gels on the survival of probiotic *L. rhamnosus* GG after exposure to heat treatment was also studied (Doherty et al. 2010). Free and encapsulated cells were incubated at 52, 55, 56 and 57 °C for 5 min. Encapsulated bacteria in hydrolyzed and denatured whey protein gels exhibited the highest survival at 57 °C with 7.3 log CFU/ml and 6.5 CFU/ml viable cells, while free bacteria showed no survivals.

Table 5.10. Viability of free and microencapsulated *L. acidophilus* NRRL-B 4495 at different temperatures

Temperature (ºC)	Initial cell number (Log CFU/g or ml)		Survived cell number (Log CFU/g or ml)		Survival rate (%)	
	Free bacteria	WPI-pullulan microcapsule	Free bacteria	WPI-pullulan microcapsule	Free bacteria	WPI-pullulan microcapsule
45	9.76±0.07	9.28±0.10	9.56±0.08	8.28±0.17	97.91±1.02^{e}	89.22±1.94^{c}
50	9.76±0.07	9.28±0.10	3.79±0.03	7.74±0.23	38.83±0.32^{d}	83.37±2.60^{c}
55	9.76±0.07	9.28±0.10	3.55±0.01	6.37±0.19	36.30±0.04^{c}	68.68±2.17^{b}
60	9.76±0.07	9.28±0.10	2.77±0.02	6.19±0.04	28.44±0.24^{b}	66.75±0.41^{b}
65	9.76±0.07	9.28±0.10	2.20±0.01	4.84±0.27	22.57±0.05^{a}	52.24±3.00^{a}
70	9.76±0.07	9.28±0.10	2.09±0.01	4.75±0.25	21.50±0.07^{a}	51.29±2.78^{a}

$^{a-e}$Means ± standard deviation with different superscript letters in the same column indicate significant differences ($P < 0.05$) among the studied samples.

5.2.2.8. Salt Tolerance Test

Most foods contain salt as a taste enhancer and also as a preservative to restrict microbial growth when a certain concentration is used. The effects of varying salt concentrations on the survival of free and encapsulated cells of *L. acidophilus* during a week of storage are demonstrated in Figure 5.13 and Figure 5.14, respectively.

The viable cells of free bacteria decreased during the storage in 3%, 6% and 9% NaCl concentrations after one week of storage and reached 8.81 log CFU/g, 9.21 log CFU/g and 8.51 log CFU/g., respectively. At day 3, viable bacteria decreased from 9.78 log CFU/g to 9.46 log CFU/g by increasing salt concentration. On the other hand, no significant change in survival rates was observed between treatments for free cells at the end of storage. Moreover free cells survived above theurophatic level during exposure. Concerning the WPI-pullulan encapsulated bacteria, at the end of 7 days, slight increase in viable cell counts were observed. Storage of encapsulated cells in WPI-pullulan microcapsules in different NaCl solutions, showed 9.00 log CFU/g , 9.07 log CFU/g and 9.14 log CFU/g viable cell numbers for 3%, 6% and 9% salt solutions at end of storage time.

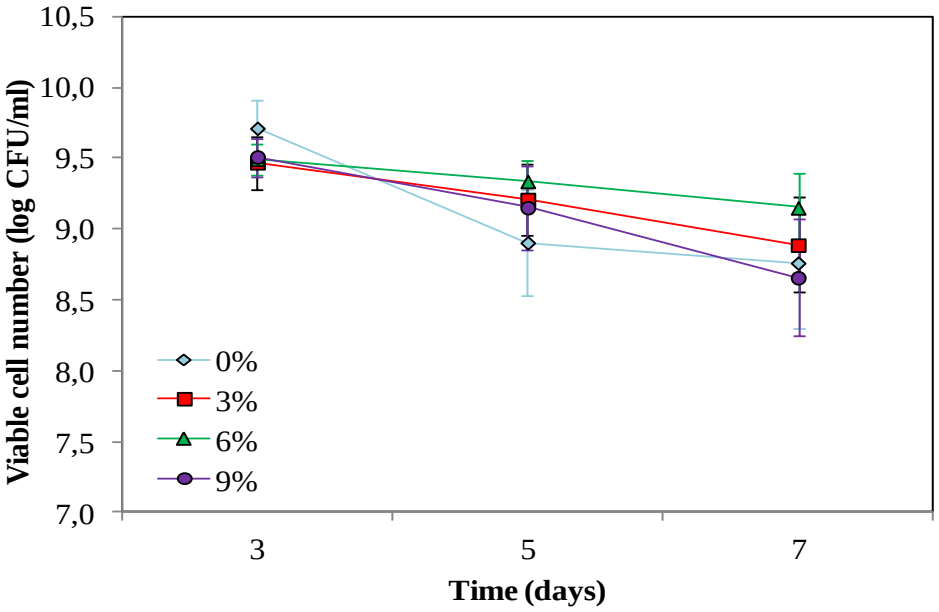

Figure 5.13. Viability of free *L. acidophilus* NRRL-B 4495 at different NaCl concentrations during 7 days of storage at 4 ºC

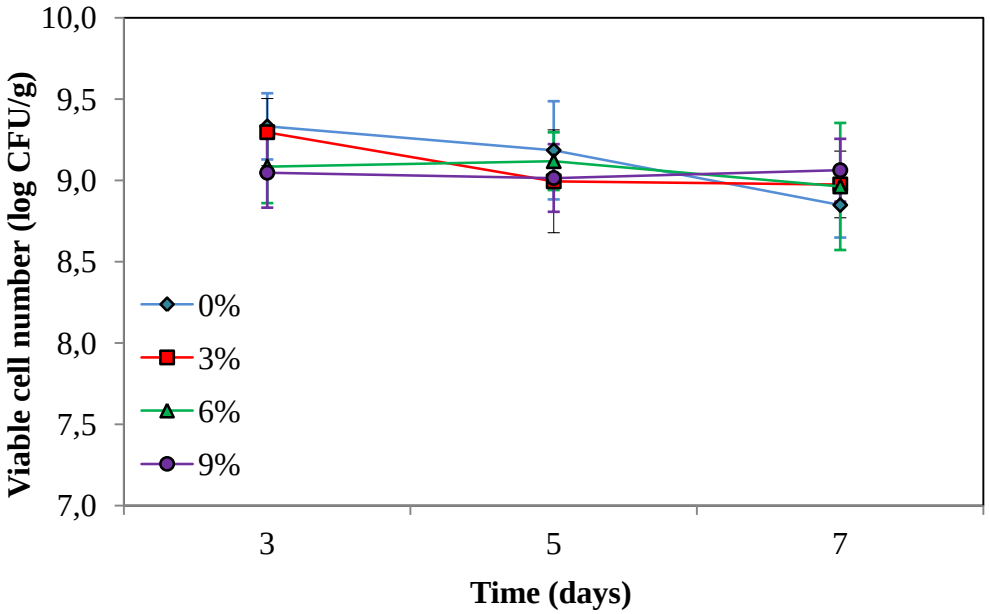

Figure 5.14. Viability of encapsulated *L. acidophilus* NRRL-B 4495 at different NaCl concentrations during 7 days of storage at 4 ºC

5.3. Effect of Freeze Drying on Characteristics of Developed WPI-Pullulan Microcapsules

Due to long storage periods or difficulties in handling in large volumes, bioactive compounds including probiotics are generally preferred to be used in dehydrated forms (Carvalho et al., 2004b; De Giulio et al., 2005; Su et al., 2007). Freeze drying also known as lyophilization is the most commonly used drying method for preserving sensitive bioactive compounds by preparation solid particles. This technique based on removing the moisture from the medium by sublimation at ambient temperature and thereby exerting a very minimum heat stress to the living cells. However, in the literature there are many studies conducted on viability loss of probiotic bacteria after freeze drying. These studies indicated that additional action had to be taken to overcome the negative sides of freeze drying. Therefore, further studies focused on the use of sugars as cryopreservatives and microencapsulation methods to avoid these effects (P. Kumar and Mishra, 2004; JS Lee et al., 2004; Peighambardoust et al., 2011; Khalida Sultana et al., 2000). For instance; Capela et al. (2006) studied the effect of microencapsulation and cryoprotectants on survival of four probiotic species in freeze dried yoghurt and obtained an improved viability when microencapsulated in alginate beads. In another study, casein based microcapsules were reported to improve the viability of *L. paracasei* ssp. *paracasei* F19 cells significantly (lost about 10% in survival rate) whereas free cells was lost the viability about 30% after freeze drying. (Heidebach et al., 2010). These studies also showed that survival rates of probiotic bacteria changes depending on the added cryoprotectants and microencapsulation techniques/materials. For this reason in this section, the aim was to study the effect of microencapsulation into developed WPI-pullulan microcapsules.

5.3.1. Effect of Freeze Drying on Cell Viability

The effect of freeze drying on both free and encapsulated cells is shown in Table 5.11. Combination of whey protein with pullulan resulted to a protective effect on survival of probiotic cells after freeze drying. Results from experiments showed that initial count of 9.41 log CFU/g has been reduced to 8.51 log CFU/g after freeze drying process for encapsulated bacteria. Microencapsulation resulted in survival of probiotic

bacteria with 90.42% survival rate. On the other hand, the log reductions of 1.48 which was equal to 84.39% survival rate were observed here in free cells. It seems that free cells of *L. acidophilus* used in this study are also resistant to freeze drying process. But further studies showed that free bacteria were more labile to simulated gastrointestinal conditions than encapsulated ones.

Table 5.11. Effect of freeze drying on survival of encapsulated *L. acidophilus* NRRL-B 4495

	Initial cell number (log CFU/g or ml)	**Survived cell number (log CFU/g or ml)**	**Survival rate (%)**
Free bacteria	9.24±0.18	7.76±0.54	84.39±1.63[a]
WPI-Pullulan microcaspsules	9.41±0.88	8.51±0.65	90.42±2.81[b]

[a-e]Means ± standard deviation with different superscript letters in the same column indicate significant differences ($P < 0.05$) among the studied samples.

There are many studies in literature showing the positive effects of microencapsulation for freeze drying. Priya et al. (2011), enhanced survival of probiotic *L. acidophilus* by microencapsulation with nanostructured polyelectrolyte layers. The number of viable cells was stated to decrease from 9.0 log CFU/ml to 6.49 log CFU/ml (73%) after freeze-drying for uncoated cells. However, the encapsulated cells showed lesser viability losses, and the viable cell count decreased from an initial count of 9.25 log CFU/ml to 8.48 log CFU/ml (91.6%) after freeze drying. In another study conducted by Ainsley-Reid, et al. (2005), whey protein based microcapsules were used to protect probiotic bacteria.

Polysaccharides and disaccharides including lactose, mannitol, maltodextrin, maltose, lactulose and inulin were reported to play an important role in protecting the viability of cells in freeze drying process. The cryoprotective effect of sugars during freeze drying is attributed to their capacity to form glassy structure providing protection of sensitive protein functionality in the cell and their ability to replace water associated with polar head groups of membrane lipids (Wang et al. 2006, Carvalho et al 2004, Leslie et al. 1995, Fatemeh et al., 2011). Ainsley-Reid, et al. (2005) reported an increase in survival rate of probiotic in whey protein microcapsules by supplemented sucrose

and lactose during FD. In another study using alginate as an microencapsulation material (Martin, et al. 2013), unmodified starch was added to wall matrix and significant protective effect on survival of live bacteria was observed. Dianawati et al. (2013) used various protein-sugar combinations in order to observe the protective effect against freezing and freeze drying. Varying survival rates up to 97.4% was obtained and as a result, the importance of combination of proteins with sugars in microencapsulation of probiotic bacteria was revealed.

5.3.2. Survival in Simulated Gastric Juice and Bile Salt

Figure 5.15 showed the viable counts of free and encapsulated *L. acidophilus* NRRL-B 4495 cells in FD, wet and free forms in simulated gastric juice for 3 h. The results indicated that in SGJ, free bacteria showed a sharp decrease from 7.91 log CFU/ml to 6.97 log CFU/ml after 1 h, whereas in presence of pullulan, detected viable cell numbers were 8.32 log CFU/g after 1 h declining from the initial count of 8.35 log CFU/g. Additionally in every sampling time, WPI-pullulan microcapsules obtained significantly higher survived cell numbers when compared to free cells. After 3 h exposure in gastric pH, free cells declined to 5.87 log CFU/ml which is equal to 2.04 log cycles reduction in viability. This result suggested that free *L. acidophilus* NRRL-B 4495 was unable to show desired health effect that ingestion of free cells would result in reduced viability as previously stated. On the other hand, in WPI-pullulan microcapsule samples, about 1.12 log reduction in viable cell numbers was observed at the end of exposure and microcapsules obtained a viable number 7.23 log CFU/g. Table 5.12 also compares the survival rates of encapsulated *L. acidophilus* NRRL-B 4495 in free, wet and FD forms in simulated gastric juice. Microencapsulated FD cells showed slight increase in survival when compared to wet ones as wet ones showed reduction of viable cell numbers from 8.76 log CFU/g to 7.36 log CFU/g which is equal to the net viable cell loss of 1.40 log CFU/g. Higher viability reduction in wet microcapsules was an expected results, because easier diffusion of acid through the pores of wet surface compared to dried ones has been previously reported by some authors (Fang et al., 2011; Nag, 2011).

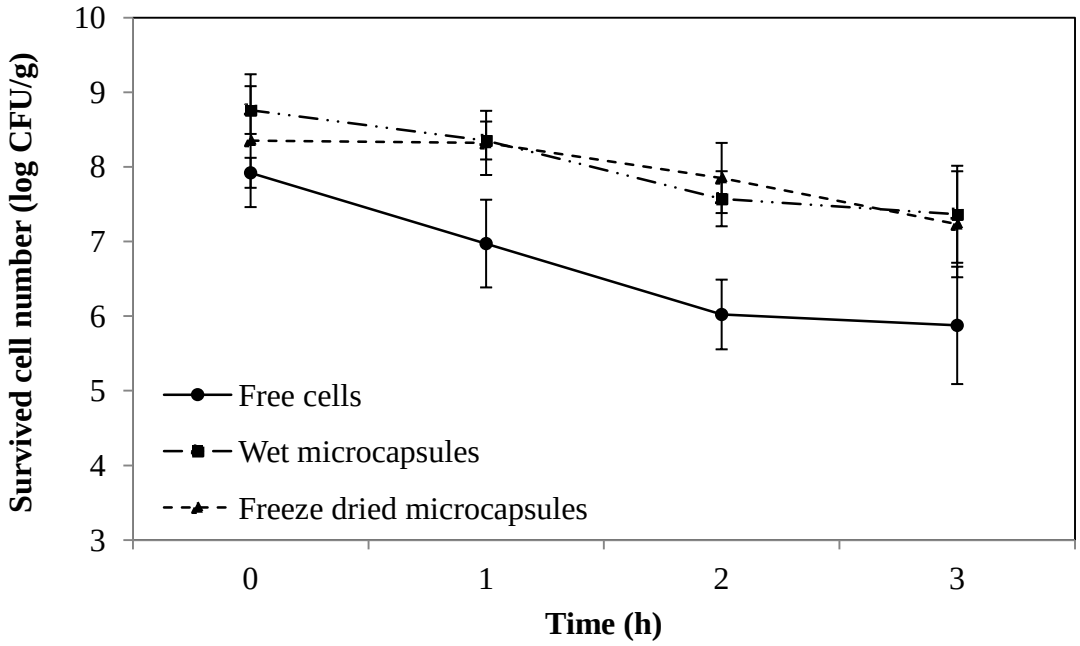

Figure 5.15. Survived cell counts of free and encapsulated *L. acidophilus* NRRL-B 4495 in wet and freeze dried forms in simulated gastric juice at 37 ºC at pH 3.0

Table 5.12. Survival rate of wet and freeze dried encapsulated *L. acidophilus* NRRL-B 4495 in simulated gastric juice at 37 ºC at pH 3.0

	Initial cell number (log CFU/g)	**Survived cell number (log CFU/g)**	**Survivial rate (%)**
Free bacteria	7.91±0.20	5.87±0.17	74.19±2.12[a]
FD WPI-Pullulan microcaspsules	8.35±0.89	7.23±0.29	86.58±2.01[b]
Wet WPI-pullulan microcapsules	8.76±0.32	7.36±0.55	84.06±1.78[b]

[a-b] Means ± standard deviation with different superscript letters in the same line indicate significant differences ($P < 0.05$) among the studied samples.

Bile in small intestinal tract of the hosts has an important role as a surfactant and helps emulsify fats (Nyberg et al., 1998). The actual physiological concentration of human bile in duodenum is in the range of 0.3% to 0.5% (Vinderola and Reinheimer, 2003) and bile salts will be released once fatty meal is present. This natural phenomenon could be harmful for probiotic bacteria as their membrane composition comprises lipids and fatty acids (M. Liong and Shah, 2005). After incubation in bile salt solution, significant difference ($p<0.05$) in survival rates and numbers between free and encapsulated cells was observed. As seen in Figure 5.16, the reduction in cell viability

for free cells was 0.68 log CFU/ml with a viable count of 7.24 log CFU/ml after 5 h of incubation in bile salt. In presence of pullulan, encapsulated cells showed 3% increase in survival rates after 5 h of incubation. After incubation in bile solution for 10 h, the final viable cell numbers were 6.35 log CFU/ml and 7.65 log CFU/g having the survival rates of 84.02% and 95.63% for free and WPI-pullulan encapsulated cells, respectively. It was calculated that free cells of *L. acidophilus* NRRL-B 4495 showed a net reduction of 1.84 log CFU/g with a survival rate of 76.76% after 24 h. In contrast, microencapsulation in presence of pullulan, probiotic cells obtained significantly higher ($p<0.05$) survival rate of 93.87%. In comparison between the encapsulated cells, the viability of probiotic cells in wet microcapsules declined to 7.89 log CFU/g from initial count of 8.77 log CFU/g. Freeze drying of microcapsules resulted in a decrease in viable cell counts from 8.0 log CFU/g to 7.51 log CFU/g after 24 h of incubation in bile, in which survival rate was about 4% higher than that of wet microcapsules (Table 5.13). It seems that the action of bile salts on encapsulated bacteria was less pronounced for FD ones. Our results suggest that the shrinkage of microcapsules as a result of freeze drying causes reduction in the pore sizes and provides more difficult diffusion of both bile salts and simulated gastric juice into microcapsule core.

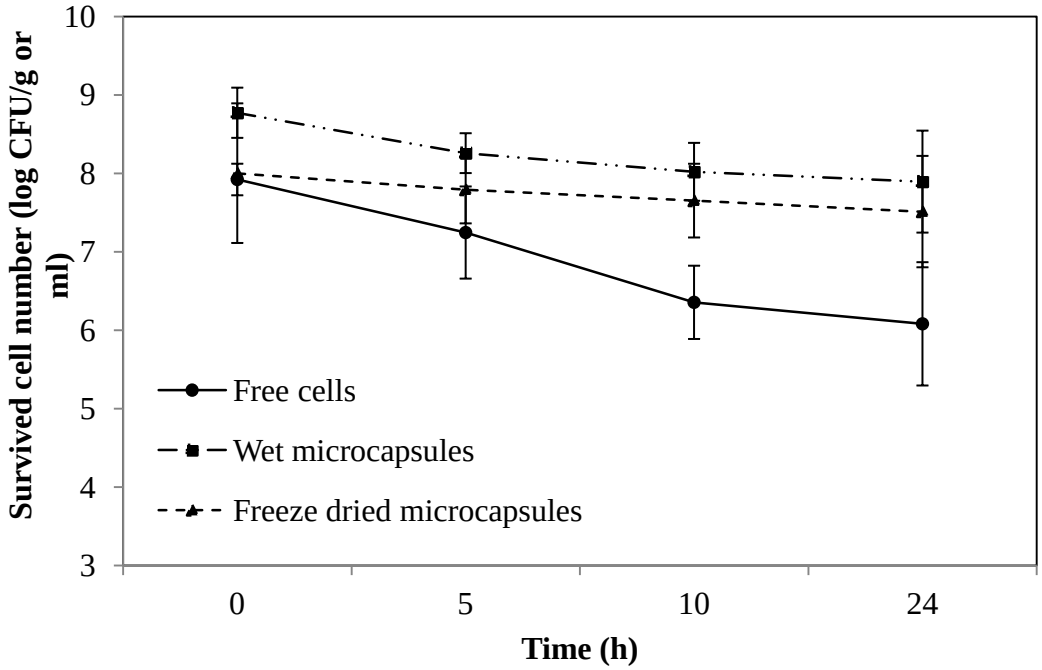

Figure 5.16. Survived cell counts of free and encapsulated *L. acidophilus* NRRL-B 4495 in wet and freeze dried form in bile salt solution at 37 ºC

Table 5.13. Survival rate of wet and freeze dried encapsulated *L. acidophilus* NRRL-B 4495 in bile salt solution at 37 ºC

	Initial cell number (log CFU/g)	**Survived cell number (log CFU/g)**	**Survival rate (%)**
Free bacteria	7.92±0.15	6.07±0.57	76.76±1.65[a]
FD WPI-Pullulan microcaspsules	8.00±0.18	7.51±0.35	93.87±0.51[c]
Wet WPI-pullulan microcapsules	8.77±0.21	7.89±0.22	89.98±1.21[b]

[a-c] Means ± standard deviation with different superscript letters in the same line indicate significant differences ($P < 0.05$) among the studied samples.

Microencapsulation into WPI-pullulan capsules could ensure greater survival in the gastric environment that cell number was high enough to meet the minimum probiotic theurophatic level required for beneficial effects. Albertini et al. (2010) studied incorporation of xanthan gum (XG) and cellulose acetate phthalate (CAP) into alginate for microencapsulation of *L. acidophilus* LA14 and *B. lactis* BI07 and reported 7% increase in survival rate in SGJ by incorporating 0.5% XG to matrix. In another study, Dianawati Dianawati and Shah (2011) incorporated mannitol in microencapsulation system. After incubation in SGJ solution, encapsulated bacteria exhibited 0.106 log decrease in the presence of mannitol, whereas 1.47 log decrease was obtained in the absence of mannitol. However after 8 h incubation in bile salt supplemented MRS broth, mannitol incorporation did not show any difference in viable cell numbers. Additionally, Lee at al. (2004) showed that microencapsulation with chitosan-Ca alginate improved the survival of probiotic *L. bulgaricus* cells in simulated gastric juice in freeze dried forms. On the other hand, no change in viable numbers was calculated when cells were encapsulated in denatured WPI-sodium alginate. Oliveira et al. (2007) also encapsulated *B. lactis* (BI-01) and *L. acidophilus* (LAC 4) using the combination of casein and pectin and further dried by the use of use of spouted-bed technology. In this study capsulated cells interestingly showed more sensitivity to low pH values. Rajam et al. (2012) used native/denatured whey protein isolate with sodium alginate for microencapsulation of *Lactobacillus plantarum* MTCC 5422. After 4h

incubation in MRS broth supplemented 2% bile salt, no significant ($p>0.05$) improvement in survival numbers were calculated in case of microencapsulation in native WPI-sodium alginate matrix. Hernández-Rodríguez et al. (2014) entrapped probiotic cells in whey protein isolate: k-carrageenan complex coacervates. As the ratio of protein to polysaccharide increased, nearly 3.5 log CFU/g increase in survived cell numbers was calculated after exposure to gastric acid and bile salts.

5.3.3. Release in Simulated Intestinal Juice

The release rates of WPI, WPI-pullulan encapsulated bacteria and survival of free bacteria in simulated intestinal juice were revealed in Table 5.14. For all of the capsule designs, release of bacterial cells was shown to have high release rates. Additionally, survival of free cells with 97% survival rate here suggested that once released, probiotic bacteria will be able to survive the simulated intestinal juice conditions. These results demonstrated that in freeze dried forms, survival rates of WPI-pullulan microcapsules were lower than wet ones for intestinal delivery of probiotic *L. acidophilus*, but about 90% of WPI-pullulan encapsulated bacteria were released into the environment from FD microcapsules. The release rates of wet and freeze dried encapsulated bacteria in simulated intestinal juice were also compared. For all of the capsules, release of bacterial cells was shown to have high release rates. Lower release rates of freeze dried WPI-pullulan microcapsules can be explained by diffusion limitation due to denser capsule structure and formation of smaller pore sizes after freeze drying. Wet and freeze dried capsules obtained release rates of 94.98% and 90.19%, respectively. Additionally, survival of free cells (97.40% survival rate) here suggests that once released, probiotic bacteria will be able to survive the simulated intestinal juice conditions. These results demonstrated that in both freeze dried and wet forms, WPI-pullulan microcapsules were successful for intestinal delivery of probiotic *L. acidophilus* and the presence of pullulan in the microencapsulation wall material seemed to have protective effect against simulated gastrointestinal conditions including gastric acid and bile salt solution even in the FD forms.

Previous studies showed that, release of encapsulated bacteria depends on the type of the polysaccharide incorporated into wall matrix. Cheow et al. (2014) incorporated locust bean gum (LBG) and xanthan gum (XG) in wall matrix for

microencapsulation of *L. rhamnosus.* The release of encapsulated bacteria was evaluated by subsequent exposure microcapsules from SGJ to SIJ for 4 h. Final released bacteria were calculated as 5.5 log CFU/mg and 4.7 log CFU/mg for LBG and XG, respectively. In another study, alginate–pectinate complex was selected for the microencapsulation and it is observed that after 2.25 h, all the entrapped cells were released from the freeze dried capsules.

Table 5.14. Release/Survival rate of wet and freeze dried encapsulated *L. acidophilus* NRRL-B 4495 in simulated intestinal juice at 37 ºC at pH 8.0

	Initial cell number (log CFU/g)	**Released cell number (log CFU/g)**	**Release/Survival rate (%)**
Free bacteria	8.47±0.29	8.25±0.10	97.40±2.14[c]
FD WPI-Pullulan microcapsules	8.21±0.16	7.41±0.13	90.19±1.58[a]
Wet WPI-pullulan microcapsules	8.66±0.05	8.22±0.52	94.98±0.67[b]

[a-c] Means ± standard deviation with different superscript letters in the same line indicate significant differences ($P < 0.05$) among the studied samples.

5.3.4. Storage Stability

In addition to survival through gastrointestinal conditions, microencapsulation should also ensure the viability during the storage. In this study, freeze dried microcapsules and freeze dried free bacterial cells were stored at refrigeration temperature for 4 weeks. It was clear that microencapsulation of probiotic bacteria cells leaded to a higher survival compared to free bacteria cells under same conditions. For free bacteria, gradual decrease in viable cell numbers thus in survival rate was observed. Initial cell counts of 7.43 log CFU/ml decreased to 5.68 log CFU/ml after 4 weeks of storage (Figure 5.17). However, this protective effect is more pronounced in FD microcapsules; WPI-pullulan microencapsulated bacteria obtained 92% survival rate with the viable cell counts of 7.21 log CFU/g bacteria. The difference in survival rate between wet and FD microcapsules can be due to the porous structure of microcapsules after 4 weeks of storage as previously observed in Figure 5.11.

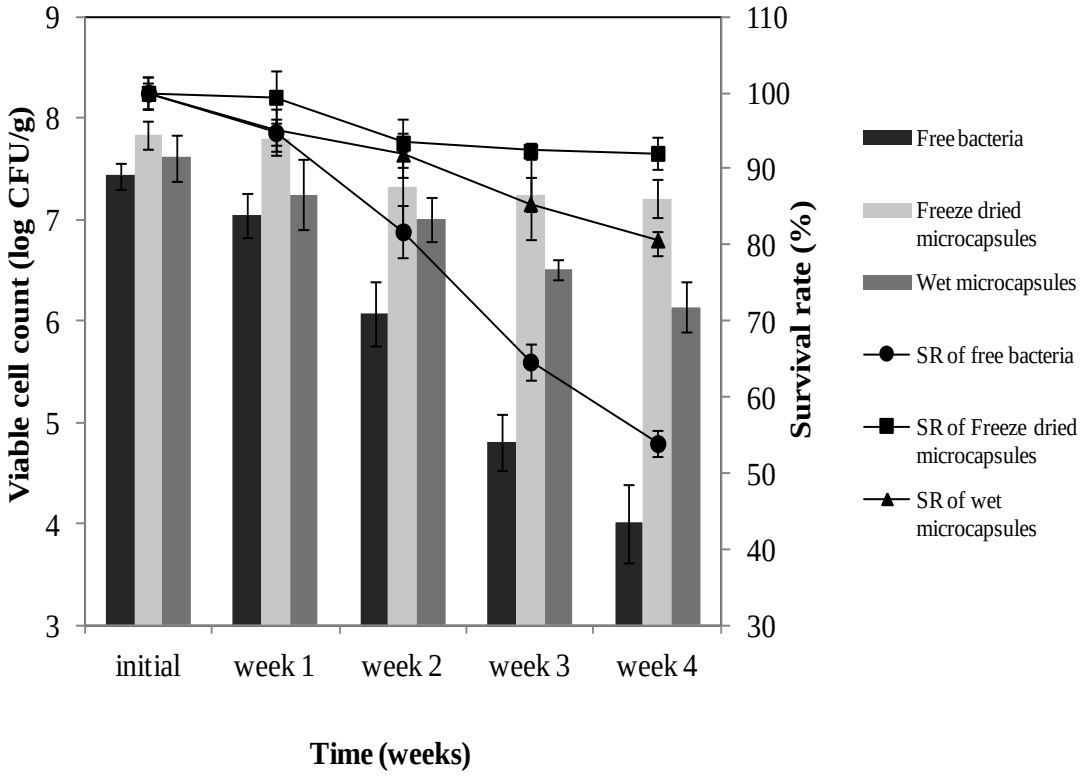

Figure 5.17. Viable cell counts of free and encapsulated *L. acidophilus* NRRL-B 4495 in wet and freeze dried forms under storage at 4 ºC for 4weeks

5.3.5. Morphological Analysis

The wall material surface structure and porosity of both FD microcapsules were observed by scanning electron microscopy (SEM) and shown in Figure 5.18. The agglomerated freeze dried microcapsules was observed during examination with SEM. It can be explained by the possible existence of residual oil droplets on the formed microcapsules. In microencapsulation technology, porosity of wall material is an important factor for the preservation and survival of encapsulated bioactive materials (Allan-Wojtas et al., 2008). Microscopic images clearly provide information for the formation of smoother surface in the presence of pullulan as well as ESEM images. It was also shown that due to freeze drying, pores of the microcapsules got shrunk, thus provided restriction against diffusion of *in vitro* gastrointestinal conditions and better survival compared to wet microcapsules was obtained. Another Figure 5.19 revealed the destructed freeze dried microcapsules under simulated gastrointestinal conditions. In both simulated gastric juice and bile salt solution, freeze dried microcapsules did not destructed at higher rates and conserved their integrity. On the other hand, undestructed

microcapsules existed after exposure to simulated intestinal juice. The decrease in release rate could be explained by these non destructed microcapsules existed after exposure in simulated intestinal juice.

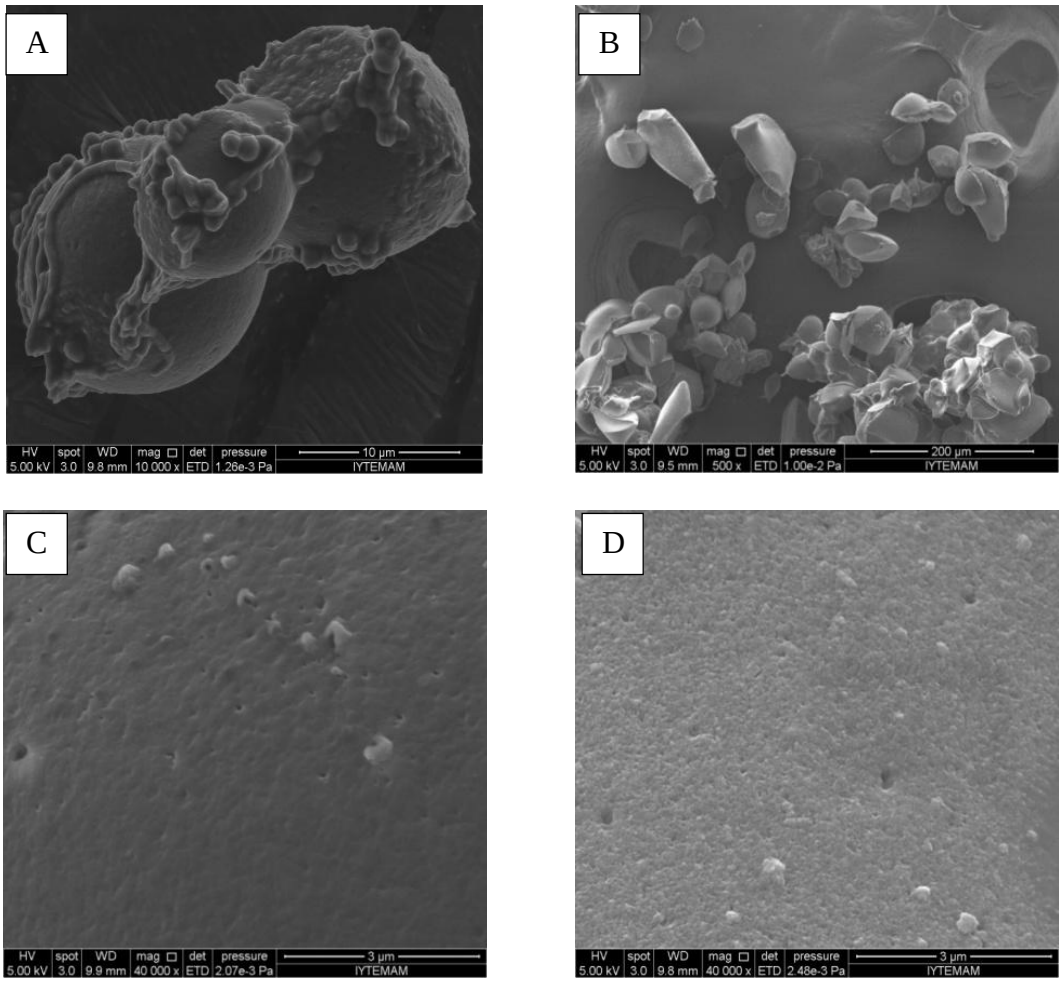

Figure 5.18. Scanning electron microscopic images of freeze dried microcapsules. A) WPI-pullulan microcapsules with bacteria B) WPI-pullulan microcapsules without bacteria C) surface of WPI-pullulan microcapsules at high magnification D) surface of WPI microcapsules at high magnification

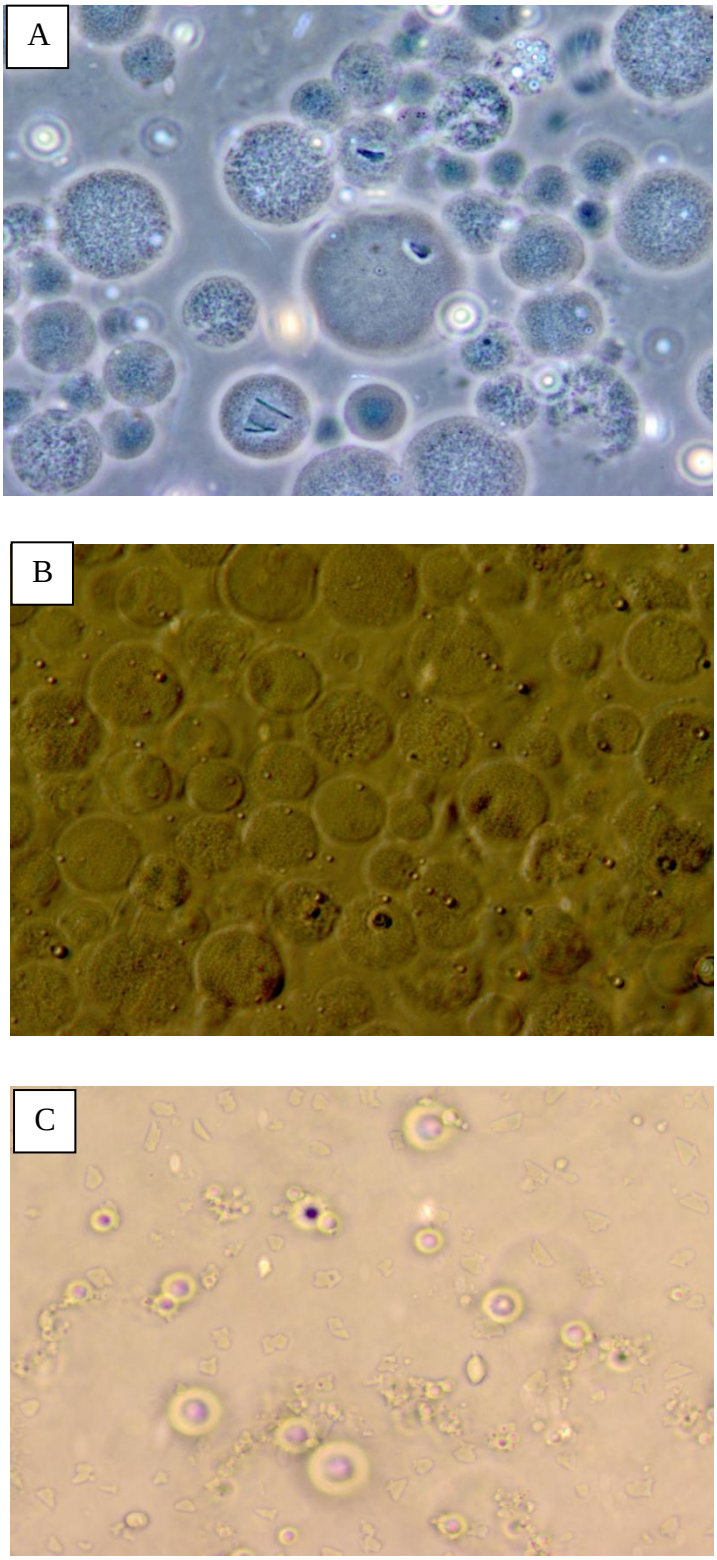

Figure 5.19. Optical micrographs of freeze dried WPI-pullulan microcapsules A) after SGJ, B) after bile salt and C) after SIJ exposure at 100X

5.3.6. Physicochemical Characterization

The average moisture content, water activity, swelling degree and color values of freeze dried probiotic microcapsules are given in Table 5.15. A_w value of freeze dried microcapsules was also found to be within desirable limits (0.28–0.65) for the survival of probiotic bacteria (Kosanke et al. 1992). On the other hand maximum level of moisture content of dry products required for prolonged storage was stated to be at or below 4%. Higher moisture content above 10% is not desired as caking usually occurs in powders and creates problems during handling (Desmond et al., 2002; Masters, 1979). In this research freeze drying of microcapsules resulted in formation of powders with moisture content and water activity of 6.14% and 0.06, respectively. It can be seen that moisture content of freeze dried samples are higher than the recommended levels but a direct conclusion based on moisture content is not possible, since the moisture distribution in the microcapsules can be in homogeneous with respect to the different constituents of the samples (Linders et al., 1997). Moreover higher moisture content levels of freeze dried probiotic microcapsules with high survival rates during storage were reported by other authors (Chávez and Ledeboer, 2007; Gbassi et al., 2009; Kim et al., 2008; Reid et al., 2005). Additionally, during storage stability tests, no adverse effects such as cake formation and microbial growth such as molds were not visually observed.

The swelling degree is the rehydration capacity of the dried microcapsules when exposed to water. WPI-pullulan microcapsules retained a significant fraction of water within its structure having 198.2% swelling degree which indicates that few or no ionic linkages between whey protein and pullulan since lower swelling degree is generally attributed to the higher degree of cross-linking within polymer matrix.

Table 5.15. Physicochemical characterization of freeze dried encapsulated *L. acidophilus* NRRL B-4495

Physicochemical characteristics	**WPI-pullulan microcapsules**
Moisture Content (%)	6.14±1.16
Swelling Degree in dH_2O (%)	198.20
Swelling Degree in SGJ (%)	69.11
Swelling Degree in SIJ (%)	103.23
Water activity (A_w)	0.062±0.04
Color	
L^*	90.88±0.34
a^*	-1.52±0.01
b^*	3.51±0.02

5.4. Spray Drying

5.4.1. Development of Microcapsules

5.4.1.1. Effect of Pullulan Concentration in Wall Matrix

The incorporation of some simple sugars and polysaccharides has found to influence the viable cell numbers of dried probiotic bacteria (Carvalho et al., 2004a). In this study, five different concentrations of pullulan were used for formation of spray dried microcapsules with WPI-pullulan polymer blend for the encapsulation of *L. acidophilus* NRRL B-4495 (Table 5.16). WPI-pullulan polymer blends (WPI-$pullulan_y$) where y stands for the weight ratios 1:0, 4.5:1, 2:1, 1:1 and 1:2 between WPI:pullulan.

Table 5.16. Pullulan concentrations used in spray drying studies

Pullulan concentration (%)	**WPI-pullulan$_y$**
0 (control)	WPI-pullulan$_{1:0}$
2.0	WPI-pullulan$_{4.5:1}$
4.5	WPI-pullulan$_{2:1}$
9.0	WPI-pullulan$_{1:1}$
18	WPI-pullulan$_{1:2}$

While conducting spray drying experiments, WPI-pullulan polymer blend with selected concentrations of pullulan mostly created huge content of fibers and proper microcapsule formation was not achieved except WPI-pullulan$_{4.5:1}$ formulation (Figure 5.20). Moreover these formed fibers showed very high resistance against dissolution in simulated intestinal juice. Formation of fibers with pullulan has also been reported by Koç et al. (2011) while studying the microencapsulation of whole egg by spray drying. Moreover, increasing pullulan content decreased the recovery of final product due to high adhesion of powder on the walls of drying cyclone unit. This can be explained by the high hydrophilic nature of pullulan as increasing pullulan content caused higher moisture level in the final spray dried microcapsules (Leonardi et al., 2012).

After spray drying procedure, produced WPI-pullulan$_{4.5:1}$ microcapsules were further compared with non pullulan containing control microcapsules in order to observe the protective effect of pullulan on *L. acidophilus* NRRL B-4495 survival. For this aim survival after spray drying procedure, resistance against simulated intestinal juice and bile salt solution, release rates in simulated intestinal juice, moisture contents and mean particle sizes were compared. Results given in Table 5.17 revealed that presence of pullulan provided enhanced survival of probiotic bacteria during spray drying. Moreover, in our study, pullulan containing wall material improved survival of encapsulated *L. acidophilus* NRRL B-4495 under detrimental simulated gastric conditions. Ying et al. (2012) and Behboudi-Jobbehdar et al. (2013) observed that increase in survival rate depended on the type of the carbohydrate used in the formulation.

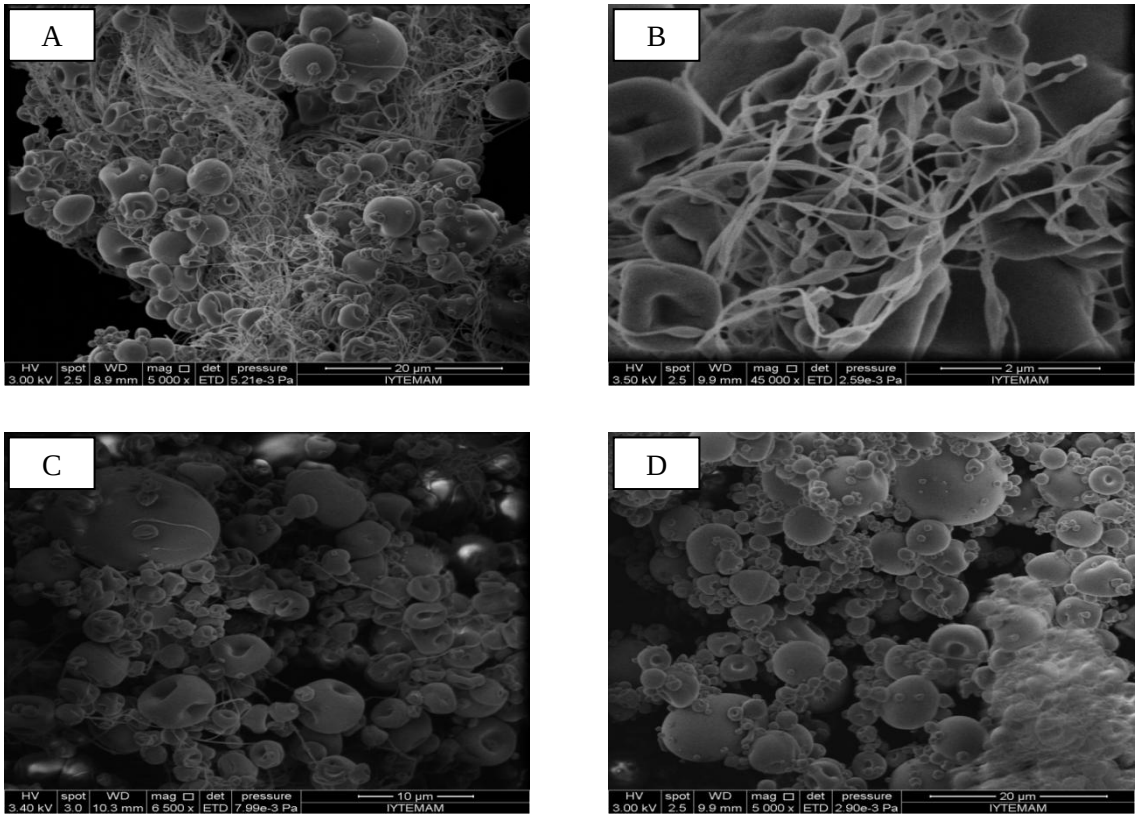

Figure 5.20. Scanning electron microscopic images of spray dried microcapsules A) WPI-pullulan$_{1:2}$, B) WPI-pullulan$_{1:1}$, C) WPI-pullulan$_{2:1}$ and D) WPI-pullulan$_{4.5:1}$

For prolonged storage and ease of handling, it is generally stated that moisture content and water activity values should be around 4-5% and 0.4 for dried food ingredients and products, respectively (Beuchat, 1981). However, incorporation of pullulan to spray drying matrix markedly influenced the moisture content and A_w values of spray dried microcapsules. Spray dried WPI-pullulan$_{4.5:1}$ microcapsules obtained higher water activity value compared to WPI-pullulan$_{1:0}$ microcapsules which can be due to the increase of the number of polar sites with the presence of pullulan. The higher relative humidity level of spray dried microcapsules revealed the need for optimization of spray drying temperature.

Table 5.17. Comparison of physicochemical properties and gastrointestinal survival of spray dried WPI-pullulan microcapsules loaded with *L. acidophilus* NRRL B-4495

Microcapsule properties	Microcapsule formulation	
	WPI-pullulan1:0 (control)	**WPI-pullulan$_{4.5:1}$**
Survival rate (%)	86.43^{A}	94.21^{B}
Microbial load (Log CFU/g)	7.99^{A}	8.72^{B}
SGJ survival (%)	71.11^{A}	81.03^{B}
Bile survival (%)	81.36^{A}	86.54^{B}
SIJ release (%)	100^{A}	107.5^{B}
Mean diameter (μm)	69.9^{A}	73.7^{B}
Moisture content (%)	9.61^{A}	13.82^{B}
Aw	0.57^{A}	0.71^{B}

$^{A-B}$ Means ± standard deviation with different superscript letters in the same column indicate significant differences ($P < 0.05$) among the studied samples.

5.4.1.2. Effect of Outlet Temperature

As shown in Table 5.18 air outlet temperature of the process had significant effects ($p<0.05$) on both survival level and physicochemical properties of *L. acidophilus* NRRL B-4495. Increase in the air outlet temperature was linked to an enhancement in recovery of spray dried microcapsules, but also linked to a decrease in survival rate during storage. All samples represented decreased viable cell counts to 9.07, 8.82, 8.71 and 7.58 log CFU/g from an initial cell load of 9.86 log CFU/g. This result is not surprising given that free bacteria were thermally sensitive and rapidly killed at temperatures above 50°C. Low A_w values and moisture contents (4–5%) are significant parameters for the commercial production of spray-dried powders for good handling characteristics such as high flow ability, low stickiness and agglomeration as well as maximum probiotic viability (Beuchat, 1981). At temperatures below 50°C, as the evaporation rate was slower, spray dried microcapsules with high moisture content were obtained causing high stickiness of the spray dried powders on the walls of drying chamber and reducing the recovery of the final product. However, high recovery values were obtained at 50 °C and 55 °C. It seems that at an outlet temperature of 50°C *L. acidophilus* NRRL B-4495 cells were encapsulated satisfactorily to produce spray dried

microcapsules in which surviving cell numbers of 8.70 log CFU/g with low moisture content were obtained. Decreasing survival rate by increasing outlet temperature has been stated by various researchers. For instance, Behboudi-Jobbehdar et al. (2013) studied the optimization of spray drying conditions for microencapsulation of *L. acidophilus* NCIMB 701748 and increase in survival rate to 84% was calculated due to reduction in outlet temperature from 91.5 °C to 60°C. In another study, similar to our observations, it was observed that increasing the outlet temperature resulted in a decreased survival rate for *L. paracasei* NFBC 338 (Gardiner et al., 2000).

Table 5.18. Effect of outlet temperature on physicochemical properties and gastrointestinal survival of spray dried WPI-pullulan microcapsules loaded with *L. acidophilus* NRRL B-4495.

Physicochemical characteristics	**Temperature (°C)**				
	Initial	**35**	**45**	**50**	**55**
Survival (%)	-	95.43[a]	90.99[b]	87.03[b]	75.84[c]
Recovery (%)	-	60[a]	69[b]	84[c]	86[c]
Microbial load (CFU/g)	9,862472	9.14[a]	8.82[b]	8.70[b]	7.59[c]
Moisture Content (%)	-	13[a]	9.80[b]	4.09[c]	3.12[d]
Aw		0.68[a]	0.53[b]	0.43[c]	0.34[d]
Diameter (μm)	-	71.4[a]	66.8[b]	46.9[c]	31.8[d]

[a-d]Means ± standard deviation with different superscript letters in the same row indicate significant differences ($P < 0.05$) among the studied samples.

5.4.1.3. Effect of Initial Cell Concentration

The effect of initial cell load on the viability of *L. acidophilus* NRRL B-4495 is displayed in Table 5.19. It was found that the yield of microencapsulation decreased significantly ($p < 0.05$) with an increase in the cell concentration. This is probably due to the fact that as the lower the ratio of the wall material to the concentration of cells, the lower the protective effect of wall material on the cells. Because formed microcapsules have a fix volume and by increasing initial cell load, part of the cells was located at the microcapsule surface (Lidbeck et al., 2011). Moreover, in microencapsulation of probiotic microorganisms, viable cell load should be obtained at and over 8.0 log CFU/

g of microcapsule to achieve successful survival during storage and gastrointestinal passage. As can be seen from the results, 80% encapsulation yield was achieved at 11.00 log CFU/g initial cell concentration. Therefore, probiotic inoculum load of 11.00 log CFU/g prior to encapsulation was selected as an optimum level since this concentration ensured viability at a satisfactory level.

Table 5.19. Effect of initial probiotic cell concentration on diameters and encapsulation efficiency of spray dried WPI-pullulan microcapsules.

Initial cell concentration (log CFU/g)	**Cell count after microencapsulation (log CFU/g)**	**Microencapsulation yield (%)**
4.00	3.49±	87.18±0.51[c]
6.00	5.20±	86.65±1.64[c]
8.00	6.79±	84.98±1.99[b]
10.00	8.11±	81.23±1.00[b]
11.00	8.81±	80.65±1.69[b]
12.00	10.55±	75.35±1.53[a]

[A-B] Means ± standard deviation with different superscript letters in the same column indicate significant differences ($P < 0.05$) among the studied samples.

5.4.2. Characterization of Developed WPI-Pullulan Microcapsules

Spray drying is a simple method, but high heat treatment in this method generally causes cellular injuries and death for temperature sensitive probiotic microorganisms. However, recent studies have shown that inclusion of carbohydrates improves the survival of spray dried probiotics (Carvalho et al., 2004a; Chávez and Ledeboer, 2007; Ying et al., 2012). In encapsulation with spray drying technique, quite smaller particle sizes can be obtained compared to emulsification/cold gelation technique. However, control of particle size is not easy with this technique. Since as stated throughout this thesis, another important prerequisite of probiotic microencapsulation is that the formed of microcapsules should not change the sensory profile of incorporated food product. Besides *in vitro* gastrointestinal resistance tests,

microscopic observations showing the changes in physical integrity of microcapsules during exposure to detrimental gastrointestinal conditions would reveal significant additional information for the stability and effectiveness of formed microcapsules to protect encapsulated cells.

Therefore the present study under this title aimed to investigate the physicochemical properties and behavior of spray dried WPI-pullulan microcapsules loaded with *L. acidophilus* NRRL B-4495 to understand the response of the encapsulated cells under *in vitro* gastrointestinal conditions.

5.4.2.1. Physicochemical Characterization

Moisture content and water activity affect the stabilities of the microcapsules throughout storage; higher moisture contents and water activities have been noted to increase oxygen permeability of wall materials and cause higher decline in surviving viable numbers of microorganisms during storage (Champagne et al., 1996; Onwulata et al., 1995). Moisture content and water activity (A_w) of the obtained microcapsules was calculated as 4.21 and 0.41, respectively (Table 5.20). It has been reported by various researchers that about 4% moisture content is an ideal level for prolonged survival of probiotic bacteria during storage (Simpson et al., 2005). In this study, formed WPI-pullulan microcapsules obtained acceptable moisture and water activity levels for spray-dried powders. Moisture content values for spray dried probiotic microcapsules varies depending on the used polymer type.

Generally during spray drying of proteins in presence of reducing sugars Maillard reaction takes place causing shades in color tending to red and yellow (De Castro-Cislaghi et al., 2012). Color vales of the formed microcapsules showed that, these microcapsules had white color and no significant change ($p<0.05$) occurred due to high heat treatment in color. This feature might allow the application of these spray dried microcapsules in a dairy based products without any visual disadvantage. While providing protection against stomach acid and bile salts, formed microcapsules must be capable of releasing their entrapped cells into intestine. Dissolution times of spray dried microcapsules showed that the time needed to get dissolved in SGJ is higher than in SIJ and water (Table 5.20) under high stirring speed of 880 rpm. Additionally while *in vitro*

intestinal survival test were conducting, at 160 rpm, spray dried microcapsules showed very low dissolution in SGJ unlike in SIJ (Figure 5.21).

Table 5.20. Physicochemical characterization of spray dried WPI-pullulan microcapsules loaded with *L. acidophilus* NRRL B-4495

Physicochemical characteristics	**WPI-pullulan microcapsules**
Moisture Content (%)	4.21±0.06
Water activity (A_w)	0,41±0.13
Dissolution in SGJ (sec)	420
Dissolution in Water (sec)	300
Dissolution in SIJ (sec)	180
Color	
L*	91.21±1.96
a*	-1.12±0.08
b*	4.89±0.29

Figure 5.21. Images of dissolved and non-dissolved microcapsules loaded with *L. acidophilus* NRRL under *in vitro* gastrointestinal conditions A) in SIJ and B) in SGJ

5.4.2.2. Diameter Distribution

The particle size distribution of WPI-pullulan spray dried microcapsules loaded with *L. acidophilus* NRRL B-4495 ranged from approximately 5 to 160 µm, with an average size of around 50 µm (Figure 5.22). As stated in earlier sections, microcapsules with high frequency of large sized particles have been reported to affect the textural and sensory acceptability of added food products Mean diameters smaller than 100 µm are generally preferred to avoid the formation of undesirable sensory and textural problems (Annan et al., 2008; Vivek, 2013). Frequency distributive curve of microcapsule sizes revealed that within 3434 samples, only 350 samples exhibited mean diameters above 100 µm. Several researches have reported particle size distributions over a very wide range for different types of microcapsules and encapsulation techniques. De Castro-Cislaghi et al. (2012) reported mean microcapsule size around 11 µm with whey. Soukoulis et al. (2014) used whey protein concentrate with maltodextrin to encapsulate *L. acidophilus* NCIMB 701748 and reported microcapsules having mean diameter of 10.96 µm. R. Crittenden et al. (2006) microencapsulated *B.infantis* Bb-02 and obtained small microcapsules having particle sizes below 20 µm.

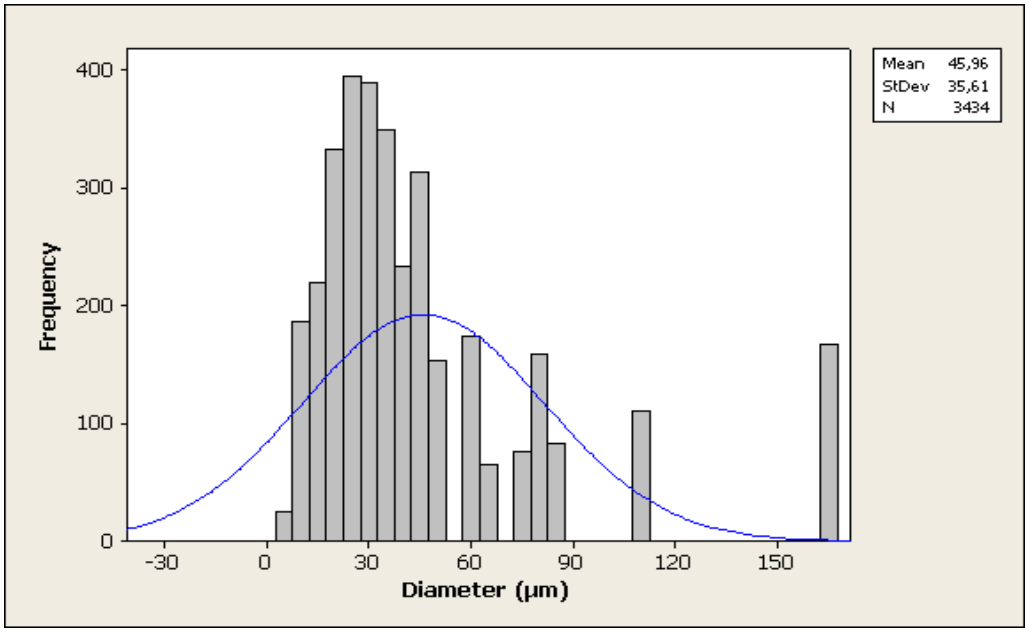

Figure 5.22. Frequency distribution of diameters of the spray dried WPI-pullulan microcapsules

5.4.2.3. Survival in Simulated Gastric Juice and Bile Salt

Figure 5.23 showed the time course of the survival of free cells and microencapsulated cells of *L. acidophilus* NRRL B-4495after 3 h exposure to SGJ. It was found that, the survived cell numbers of free and microencapsulated cells showed significant ($p<0.05$) difference. After 60 min of incubation in SGJ, the viability of free bacteria declined to 6.24 log CFU/g and finally declined to 6.12 log CFU/g after 180 min of incubation. In contrast, survival of the encapsulated bacteria was higher than that of the free cells; survival rate of probiotic bacteria increased approximately 26% by encapsulation in WPI-pullulan polymer blend. When compared with initial cell number, spray drying in the presence of WPI-pullulan polymer blend decreased only 4% in viable cell numbers and reached to 8.18 log CFU/g after 180 min. The better survival rate in encapsulated bacteria can be attributed to buffering effect of whey protein (Guerin et al., 2003; Kos et al., 2000) and pH influenced dissolution behavior of the spray dried WPI-pullulan microcapsules. Similarly, Picot and Lacroix (2004) concluded that presence of whey protein in wall matrix during spray drying provided better protection against exposure to SGJ for *Bifidobacteria.* Additionally when the dissolution data was analyzed, presence of pullulan prolonged the release of encapsulated bacteria in SGJ due to formation of compact structure by the interaction between pullulan and whey protein. Similar protective effect of pullulan-Eudragit® S100 microparticles was observed on release of risedronate in SGJ (Leonardi et al., 2012). Improved survival against SGJ with the incorporation of carbohydrates has been reported earlier. For instance, Lapsiri et al. (2013) obtained improved surviving cell numbers of spray dried *L. plantarum* when maltodextrin or trehalose was added to wall material. *L. paracasei* NFBC 338 was spray dried in the presence of gum acacia and encapsulated bacteria were more resistant to simulated gastric stress than control cells; 6 log reduction in the viability of control while, encapsulated cells showed 4 log reduction after 120 min exposure at 37 ºC (Desmond et al., 2002).

Survived cell numbers of microencapsulated cells in bile salt solution was significantly ($p<0.05$) higher than free cells throughout the exposure. Between 2 and 3 h of incubation, viable cell numbers of encapsulated bacteria remained unchanged while free bacteria decreased as the exposure time increased (Figure 5.24). After 24 h, only 11% decrease representing 1.0 log cycles was calculated for encapsulated bacteria and

finally decreased to 7.86 log CFU/g. On the other hand, free *L. acidophilus* NRRL B-4495 cells decreased about 1.27 and 1.86 log CFU/g at 5 h and 10 h of incubation, respectively and then decreased to the cell count of 6.46 log CFU/g after 24 h. This was probably due to the incomplete dissolution of spray dried microcapsules containing bacteria in bile salt solution as stated in previous section. These results were in agreement with the improved dissolution time. The slower rehydration of WPI-pullulan microcapsules avoided release of encapsulated cell into bile salt solution thus increased the viable cell numbers when compared to free ones. These results are in accordance with literature. Fritzen-Freire et al. 2013 evaluated the effect of inulin and oligofructose on the survival of *B. Bifidum* BB-12 under stres conditions. It was found that spray drying in the presence of inulin and oligofructose-enriched inulin promoted survival of encapsulated bacteria when exposed to bile salt solution (5.0 g/L). Significant ($p<0.05$) improvement in resistance to simulated gastrointestinal conditions has also been observed by Favaro-Trindade and Grosso (2002), O'riordan et al. (2001), Páez et al. (2013) depending on the spray drying matrix. Improved bile resistance of spray dried *L. acidophilus* A9 was reported by Páez et al. (2013), but viable cell number was not high enough (3.99 log CFU/ml) for further exert beneficial effects. Tehrefore, the results obtained in this present study showed that spray drying in presence of pullulan could provide good protection for *L. acidophilus* NRRL B-4495 against gastric juice and bile salts.Moreover the viable cell number fater exposure to SGJ and bile salt achieved the required minimum level (7 log CFU/g).

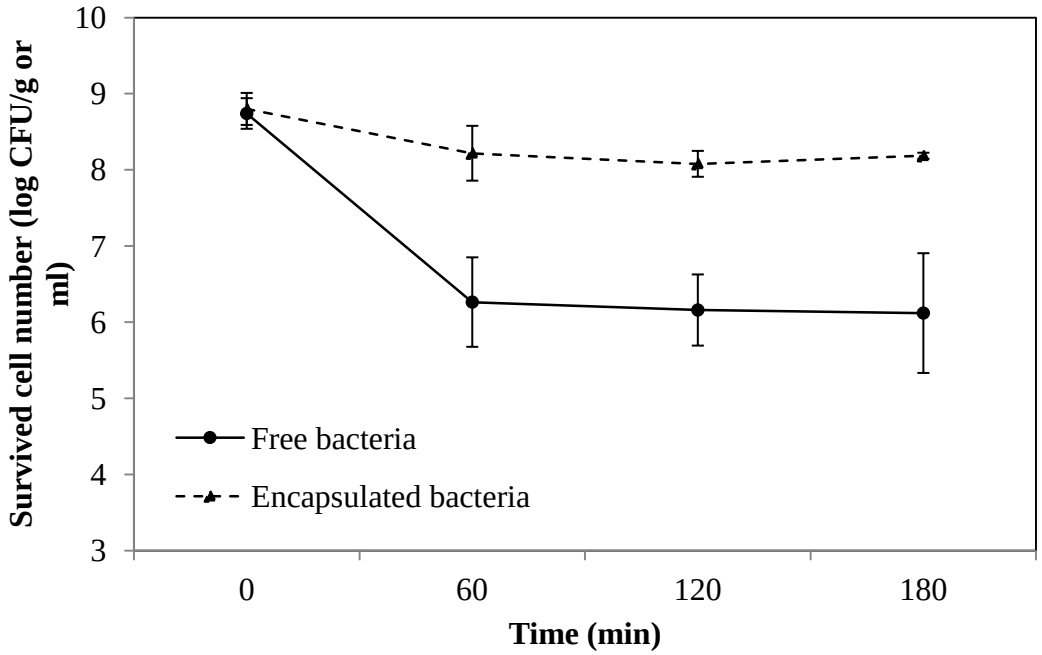

Figure 5.23. Survived cell counts of free and spray dried *L. acidophilus* NRRL B-4495 in simulated gastric juice at pH 3.0 at 37 ºC

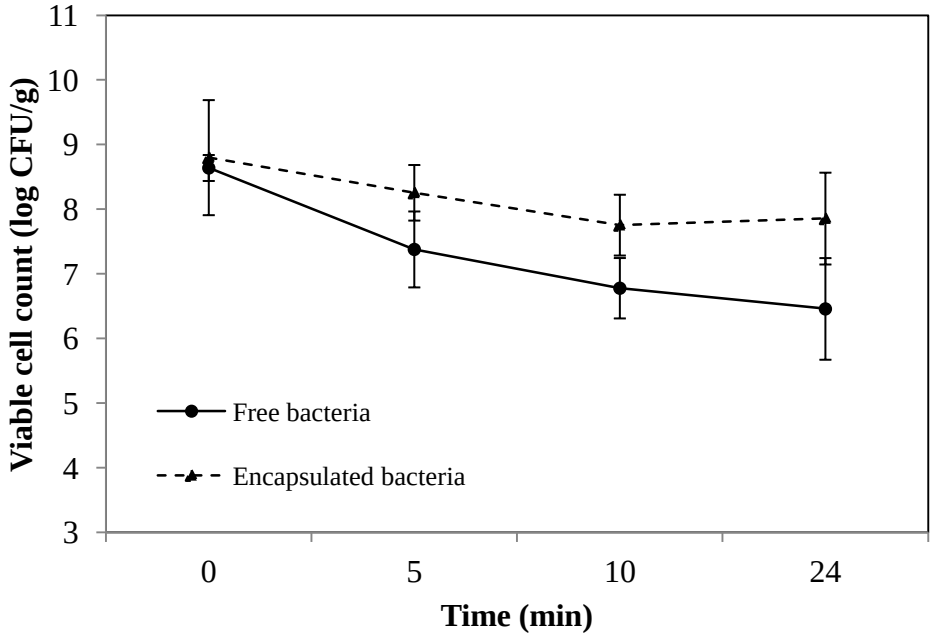

Figure 5.24. Survived cell counts of free and spray dried *L. acidophilus* NRRL B-4495 in bile salt solution at 37 ºC

5.4.2.4. Release in Simulated Intestinal Juice

In emulsion encapsulation and extrusion technique, probiotic bacteria are entrapped within hydrogels which generally remain water insoluble. In spray drying technique, microcapsules prepared from polysaccharides and proteins are water soluble (Carvalho et al., 2007). Therefore, during incubation under *in vitro* gastrointestinal conditions, encapsulated cells are released. In this study, it was observed that the dissolution time so the release rate of powdered microcapsules was higher than dissolution time in SGJ. After transferring the microcapsules from SGJ to SIJ, released cell number increased very fast in first 30 min of incubation (Figure 5.25). Figure 5.25 also showed that cell counts of released bacteria increased with exposure time. 7.93 log CFU/g viable cells liberated after 3 h, while complete cell liberation was performed following 5 h of incubation.

Release profile of encapsulated cells was in accordance with dissolution results. Within 30 min dissolution of the bulk of the microcapsules in SIJ enabled the release of most of the encapsulated cells. In conclusion, results support our hypothesis that the incorporation of pullulan to the whey protein improved the cell release characteristics, where limiting the release in SGJ and majority of the encapsulated cells are released in SIJ. Similar pH dependent release behavior of dried probiotic has been reported by other researchers. L. Morelli (2007) observed that encapsulated bacteria were not released into SGJ in significant numbers. On the other hand high numbers of viable cells were enumerated after passing to intestinal region with pH > 6. Similarly, Lee et al. maintained complete release of encapsulated *L. bulgaricus* KFRI 673 in SIJ at pH 7.4 after 3 h.

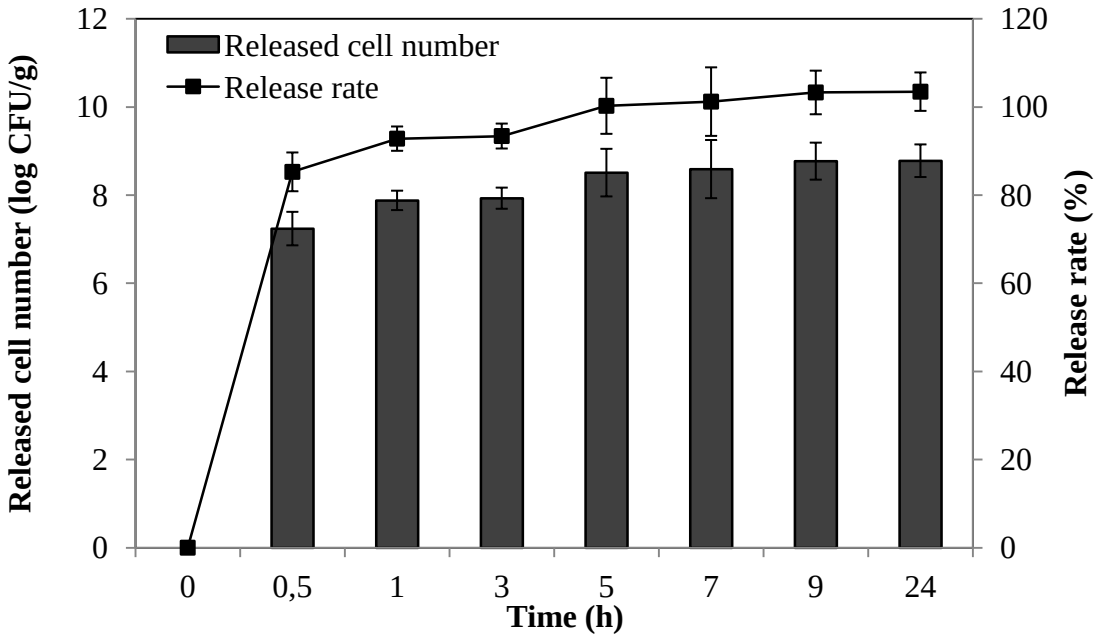

Figure 5.25. Released cell counts and release rate of spray dried *L. acidophilus* NRRL B-4495 in simulated intestinal juice at at 37 ºC at pH 8.0

5.4.2.5. Morphological Analysis

Morphological characterization of spray dried WPI-pullulan microcapsules containing *L. acidophilus* NRRL B-4495 was carried out by optical and scanning electron microscopy. Optical microscopy and SEM images showed that relatively spherical microcapsules with homogenous surface were obtained by polymer blend (Figure 5.26 and Figure 5.27). The probiotic cells did not appear on the surface of microcapsules, indicating that they were trapped within the microcapsule. Additionally, microcapsules varied in size and some shape irregularities like concavities could be observed in microcapsules. Similar shape irregularities especially in presence of polysaccharides called as "flat ball effect" in spray dried microcapsules have been reported by some researchers (Campbell and Hayes, 1976; De Castro-Cislaghi et al., 2012; Rodríguez-Huezo et al., 2007). Compared to the control samples, presence of pullulan led to the formation of shriveled spherical particles. Similar findings were also seen by Campbell and Hayes (1976).

After 4 weeks of storage, spray dried microcapsules were investigated under SEM in order to observe the morphological changes during storage. It was observed that during storage, no significant changes in surface composition of microcapsules occured but microcapsules increased in size due to hydration. This increase was

insignificant. Additionally no crack formation on the surface or distinct changes were observed showing that encapsulated cells did not proliferate and increase in number

The morphological changes of spray dried WPI-pullulan microcapsules after exposure to *in vitro* gastrointestinal conditions were observed by SEM. As seen in Figure 5.28 microcapsules partially dissolved in bile salt solution and simulated gastric juice; spherical shape of some microcapsules could clearly be observed visually. On the other hand, WPI-pullulan microcapsules lost their structure integrity and dissolved completely to liberate encapsulated cells in SIJ solution. In Figure 5.28(c), encapsulated bacteria within the dissolved WPI-pullulan polymer blend were seen. All SEM images revealed that WPI-pullulan microcapsules exhibited smooth and dense surface structure which provided protection for encapsulated cells by limiting the diffusion of acidic gastrointestinal solutions and thus restricting the release of encapsulated bacteria.

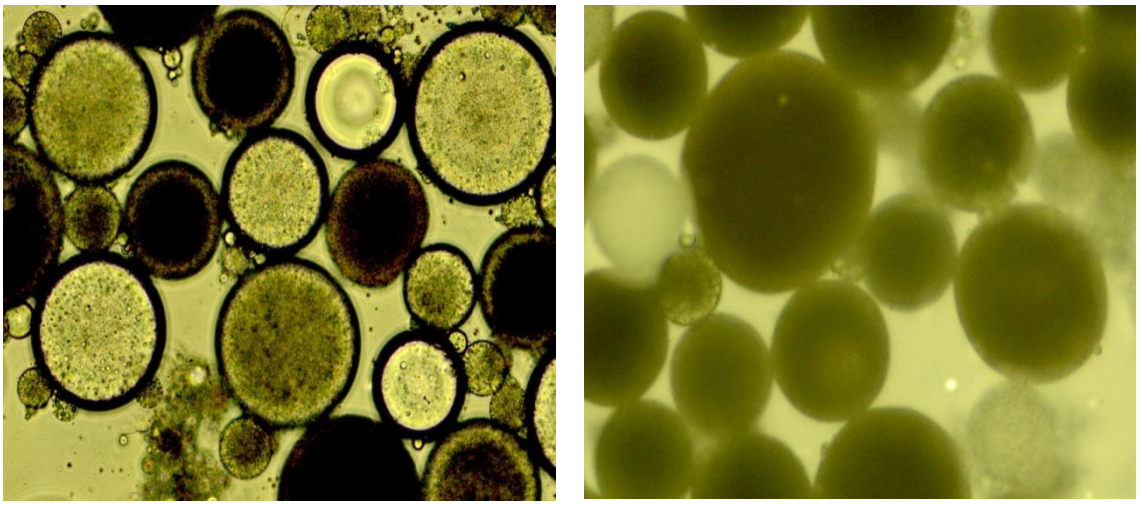

Figure 5.26. Optical micrographs of spray dried WPI-pullulan microcapsules at 100X

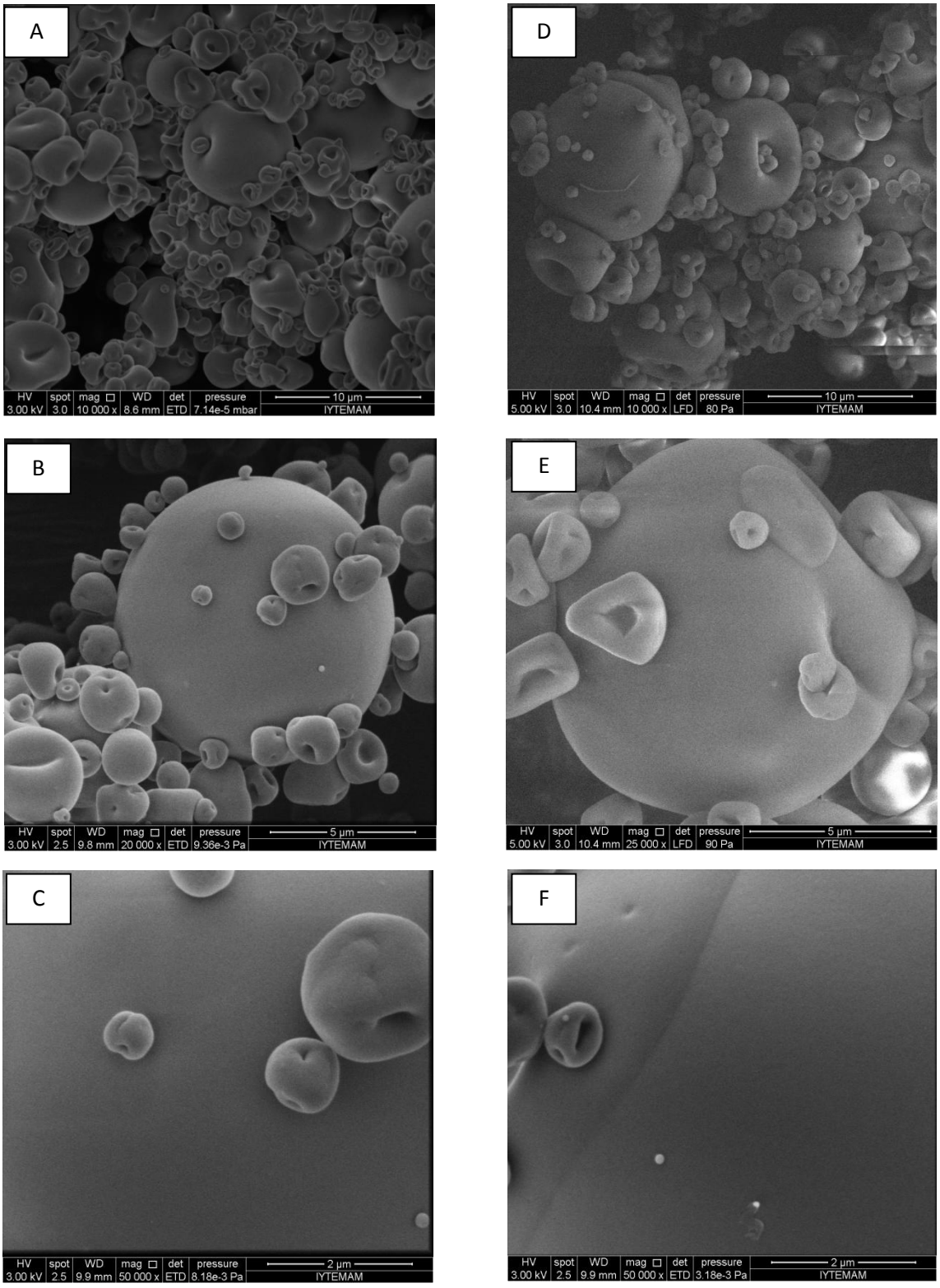

Figure 5.27. SEM images of spray dried WPI-pullulan microcapsules after production under A) 10000X, B) 20000X and C) 50000X magnifications and after storage for 4 weeks under D) 10000X, E) 20000X and F) 50000X magnifications.

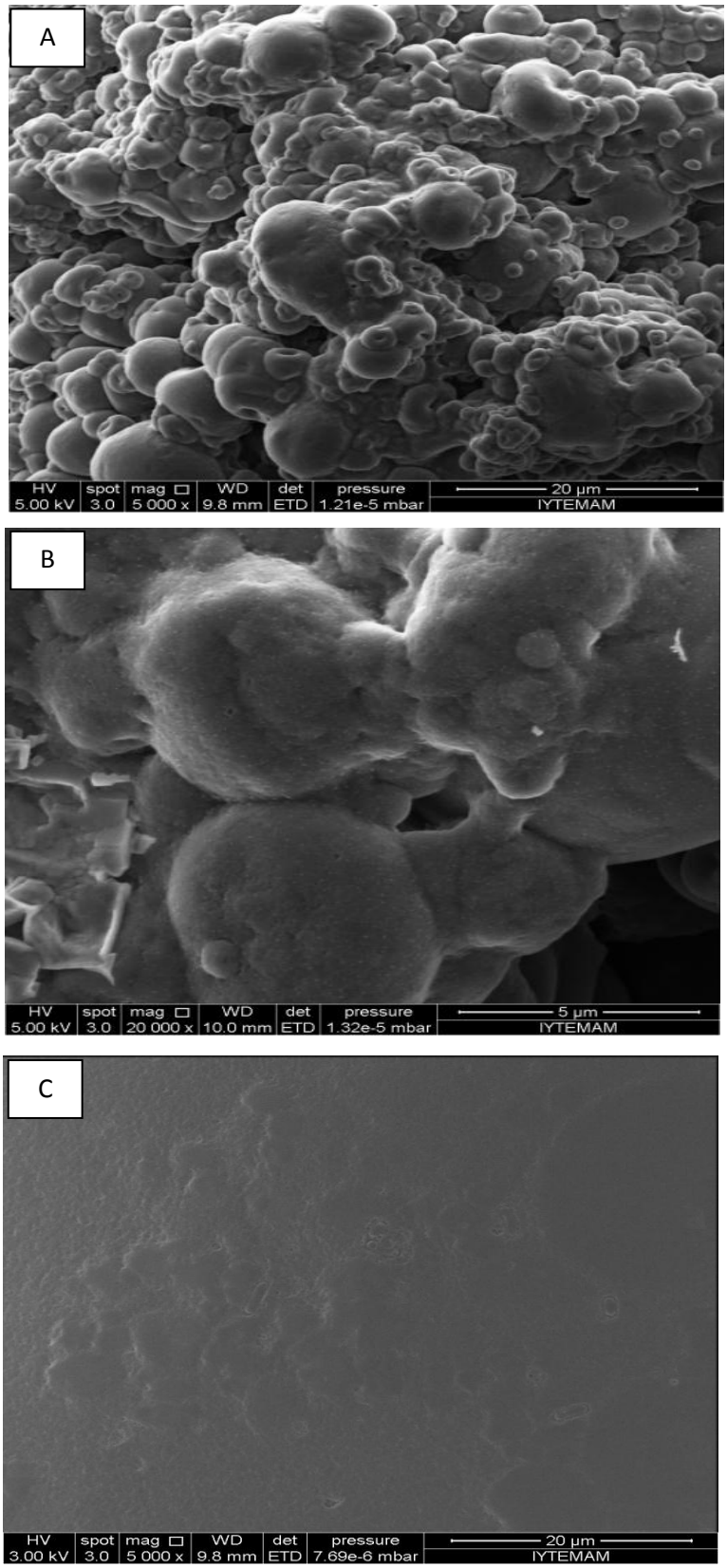

Figure 5.28. SEM images of WPI-pullulan microcapsules A) after SGJ, B) after bile salt and C) after SIJ exposure

5.4.2.6. pH Tolerance Test

Effect of pH on the viability of free and microencapsulated cells was investigated and illustrated in Table 5.21. As shown, at pH 2 and 3, spray dried bacteria in WPI-pullulan polymer blend obtained survival rates of 80.47% and 93.79%, respectively. In contrast, free cells could not survive at minimum desired level (7 log CFU/g) at low pH values. It was also seen that increasing pH value of the medium resulted in an increasing viable cell count. But when compared with free bacteria, encapsulated cells obtained it initial cell load only at pH 6.

A potential drawback of spray-drying is the high proportion of injured cells resulting from the high heat treatment during the process. Therefore generally a significant lag time is required to spend rehydrating and adapting the environment. Moreover time is needed for injured cells to repair and further grow. Lower cell proliferation of encapsulated cells during incubation might be due to existence of injured cells within microcapsule after spray drying. As a result, encapsulated bacteria could not able to grow at the same rate of free ones during incubation (Bustos and Bórquez, 2013).

Microencapsulation in the presence of carbohydrates not always improves the survival of probiotic bacteria under different stress conditions. O'riordan et al. (2001) investigated the effect of starch on the survival of *Bifidobacterium* PL1 at different pH values and observed no positive impact of starch on survival rate of bacterial cells; after 3 incubation, free cells declined 0.97 log CFU/g while encapsulated cells declined 0.95 and 1.10 log CFU/g with the increasing starch concentration at pH 7.0. Additionally at pH 2.8 no viable cells were detected even in encapsulated forms

.

Table 5.21. Viability of free and spray dried *L. acidophilus* NRRL-B 4495 at different pH values

pH values	Initial cell number (Log CFU/g or ml)		Survived cell number (Log CFU/g or ml)		Survival rate (%)	
	Free bacteria	WPI-pullulan microcapsule	Free bacteria	WPI-pullulan microcapsule	Free bacteria	WPI-pullulan microcapsule
2	9.43±0.06	8.86±0.25	0.0±0	7.13±0.05	0[a]	80.47±0.67[a]
3	9.43±0.06	8.86±0.25	5.88±0.05	8.07±0.12	69.41±0.61[b]	93.79±1.35[b]
4	9.43±0.06	8.86±0.25	7.79±0.02	8.21±0.11	91.98±0.26[c]	95.14±1.24[bc]
5	9.43±0.06	8.86±0.25	9.81±0.03	8.23±0.23	104.05±0.30[d]	98.53±2.60[cd]
6	9.43±0.06	8.86±0.25	10.38±0.01	8.37±0.18	110.10±0.06[e]	100.11±2.03[d]
7	9.43±0.06	8.86±0.25	10.34±0.08	8.13±0.05	109.62±0.89[e]	95.14±0.43[bc]
8	9.43±0.06	8.86±0.25	10.32±0.01	8.05±0.20	109.42±0.06[e]	94.24±2.26[bc]

[a-e]Means ± standard deviation with different superscript letters in the same column indicate significant differences ($P < 0.05$) among the studied samples.

5.4.2.7. Heat Tolerance Test

Efficacies of microencapsulation into WPI-pullulan polymer blend in protecting probiotic bacteria against varying heat treatments was shown in Table 5.22. Cell viability in free cells was affected by temperature increase from 45 ºC to 55 ºC; showing 5.77 log CFU/g cell loss and resulting in a final cell count of 3.79 log CFU/g.

Microencapsulation in WPI-pullulan blend by spray drying provided the highest survival against heat stress at 45 ºC. On the other hand, microencapsulation failed to prevent dramatic cell loss by increasing temperatures; significant decrease in viable cell numbers was calculated due to temperature rise to 55 ºC with cell loss of 2.61 log CFU/g. During spray drying, due to dehydration and high heat, cytoplasmic membrane of bacterial cells undergo damage (Gong, pimin et al. 2014). Thus, the probable reason for decreasing cell viability by increasing temperature might be that the cell membrane damage increased sensitivity of injured cells to heat.

Table 5.22. Viability of free and spray dried *L. acidophilus* NRRL-B 4495 at different temperatures

Temperature (ºC)	Initial cell number (Log CFU/g or ml)		Survived cell number (Log CFU/g or ml)		Survival rate (%)	
	Free bacteria	WPI-pullulan microcapsule	Free bacteria	WPI-pullulan microcapsule	Free bacteria	WPI-pullulan microcapsule
45	9.76±0.07	8.59±0.38	9.56±0.08	8.02±0.01	97.91±1.02^{e}	93.23±0.08^{f}
50	9.76±0.07	8.59±0.38	3.79±0.03	7.03±0.07	38.83±0.32^{d}	81.83±0.90^{e}
55	9.76±0.07	8.59±0.38	3.55±0.01	5.41±0.12	36.30±0.04^{c}	62.87±1.39^{d}
60	9.76±0.07	8.59±0.38	2.77±0.02	4.86±0.18	28.44±0.24^{b}	56.47±2.06^{c}
65	9.76±0.07	8.59±0.38	2.20±0.01	4.16±0.14	22.57±0.05^{a}	48.41±1.67^{b}
70	9.76±0.07	8.59±0.38	2.09±0.01	3.72±0.22	21.50±0.07^{a}	43.30±2.52^{a}

$^{a-f}$Means ± standard deviation with different superscript letters in the same column indicate significant differences ($P < 0.05$) among the studied samples.

5.4.2.8. Storage Stability

Figure 5.29 described the changes in viable cell numbers of microencapsulated cells during refrigerated storage for 4 weeks. The differences in survived cell numbers observed in this study indicated that WPI-pullulan polymer blend was more effective than free one in protecting *L. acidophilus* NRRL B-4495 throughout storage. Cell numbers using WPI-pullulan polymer blend as a microcapsule wall material decreased from 8.60 log CFU/g to 8.48 log CFU/g after 2 weeks and further decreased to 7.95 log CFU/g which represents 92.55% survival rate at the end of storage. However, the numbers decreased to 6.48 log CFU/g representing 76.14% survival rate at the end of storage.

Additionally when a short comparison between spray dried microcapsules and microcapsules produced with emulsion encapsulation/cold gelation method, over 10% difference in survival rates can be calculated. This difference could be attributed to the changes of microcapsule morphology during storage. While spray dried microcapsules conserved its dense matrix during storage, gel microcapsules obtained more porous structure. Thus spray dried microcapsules showed more resistance to oxygen diffusion and obtained higher survival rate.

The stability of dried probiotic is mainly related to the water activity and moisture content of final product. For example, P. C. Teixeira et al. (1994) reported that high A_w value accelerated mortality during storage of *L. delbruekii.*

Also, presence of some simple sugars and polysaccharides has been reported to increase storage stability of dried probiotics by replacing water molecules in cell membranes due to hydroxyl groups of carbohydrates, and thus stabilizing the structure of proteins by hydrogen bonding with polar groups of proteins (Strasser et al., 2009). In another study, *B. bifidum* BB-12 spray dried in the presence of gum arabic, gelatin and pectin (Salar-Behzadi et al., 2013). After storage at 5 ºC for 1 month, nearly 2 log CFU/g, 2 log CFU/g and 1.5 log CFU/g decrease was calculated for spray dried cells in the presence of gum arabic, gelatin and pectin, respectively. Goderska and Czarnecki (2008) used different types of starch (N-tack Hylon VII, N-lok) to encapsulate *L. acidophilus* DSM 20079. Initial couns of $1.24x10^{10}$ CFU/g decreased to $4.42x10^{7}$ CFU/g after 10 days and further storage did not decrease the viable cell numbers below 10^{7} CFU/g in the presence of N-tack starch. On the other hand, Rodríguez-Huezo et al. (2007) found that none of the polymer blend combinations (whey protein-maltodextrin-mesquite gum, gum arabic- mesquite gum, gum arabic-mesquite gum-maltodextrin) succeeded to reach the desired level of viable cell numbers (7 log CFU/g) after 5 weeks of storage.

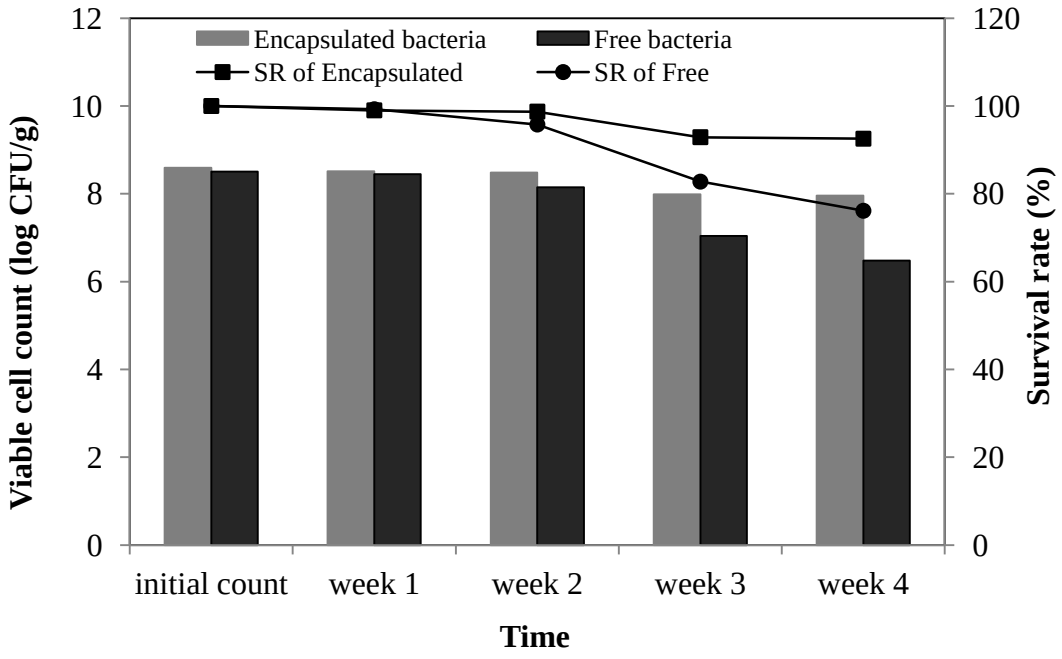

Figure 5.29. Viable cell counts of free and encapsulated *L. acidophilus* NRRL-B 4495 under storage at 4 ºC for 4 weeks.

CHAPTER 6

CONCLUSION

Presence of pullulan had a positive impact on the viability of *L. acidophilus* NRRL B-4495 at *in vitro* gastric acid and bile salt solution since it helps in maintaining the network integrity of the whey protein based microcapsules. Besides increasing the protection against adverse gastrointestinal conditions, pullulan can be used as a carbohydrate source available during gastrointestinal transit. It is likely that survived cell numbers were high enough to meet the minimum probiotic theurophatic level required for beneficial effects. SEM analysis of microcapsules indicated that the presence of pullulan in wall matrix provided formation of smoother surfaced microcapsules that could limit diffusion of harsh acidic conditions and cell leakage of bacteria. However that obtained polymer matrix could release enough viable cells recommended by the International Dairy Federation (10^7 CFU/g) for potential health benefits. Besides the protective effect of WPI-pullulan microcapsules, calculated mean diameters did not exceed 100 μm that for the further studies inclusion of microencapsulated bacteria in a food product is not expected to form sandy and gritty texture which is non-desirable for consumers. Formed microcapsules were effective in protecting the viable cells of *L. acidophilus* NRRL B-4495 at low pH values. On the other hand, no protective effect against high heat treatment was observed. And in the presence of 3% to 9% salt concentrations, probiotic cells was observed to increase in number compared to free cells.

Due to ability to form glass structures and ability to interact with cell membrane components, sugars are generally preferred as cryoprotective agents during freeze drying. In the present study, survival rate of *L. acidophilus* NRRL-B 4495 was enhanced by employing pullulan in protein encapsulation matrix. WPI-pullulan microencapsulation retained more than 90 % of viable cells after freeze drying. Although WPI-pullulan microcapsules need to be further investigated for characterization of the encapsulation system and need to be tested in *in vivo* conditions, obtained microcapsules efficiently improved the resistance to *in vitro* gastrointestinal conditions in freeze dried forms. Moreover analysis demonstrated that obtained polymer

matrix could release enough viable cells recommended by the International Dairy Federation (7 log CFU/g/10^7 CFU/g), viable cells for potential health benefits.

Spray drying technique was also applied in this thesis since it is one of the oldest methods adapted to many industrial areas to make powders and capturing bioactive components Process conditions including outlet temperature, pullulan content and initial cell concentration were investigated. Outlet temperature of 50°C *L. acidophilus* NRRL B-4495 cells with 11.00 log CFU/g initial cell load into WP-pullulan polymer blend containing 2% (w/v) pullulan were encapsulated satisfactorily to produce spray dried microcapsules. As a consequence of low dissolution of spray dried probiotic microcapsules in gastric acid and bile salt, encapsulated cells showed higher survival rates when compared to free ones. In vitro release test was carried out and rease data clearly showed that WPI-pullulan$_{4.5:1}$ formulation released most of the *L. acidophilus* NRRL-B 4495 cells in first 30 min. Moreover results also showed that encapsulated cells survived at minimum desired level (7 log CFU/g) at low pH values in contrast to free cells.

In conclusion, use of pullulan offers an effective cryoprotective behavior when combined with whey protein in microencapsulation process. In conclusion, these results demonstrated that WP-pullulan microcapsules could be a promising matrix for microencapsulation of *L. acidophilus* NRRL-B 4495.

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APPENDIX A

GROWTH CURVE

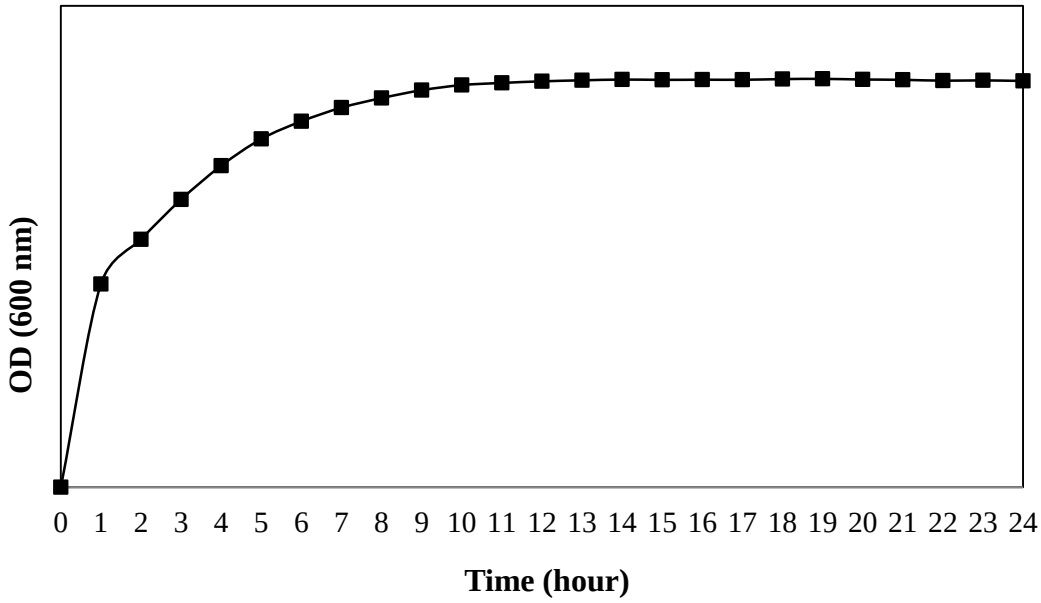

Figure A.1. Growth curve of *L. acidophilus* NRRL B-4495 at 37 ºC for 24 h

APPENDIX B

HOMOGENIZATION SPEED

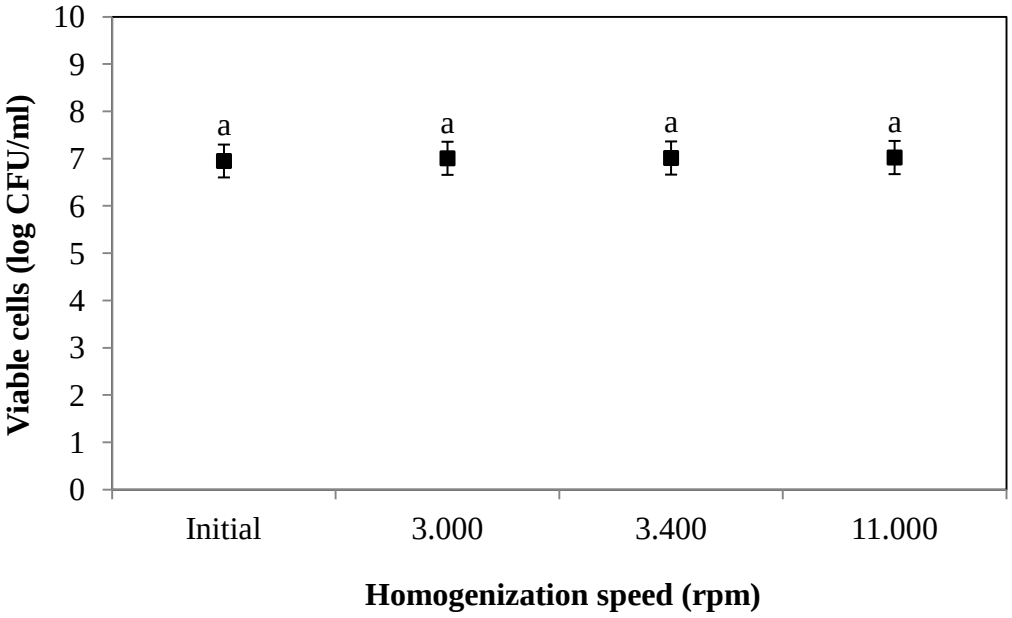

Figure B.1. Effect of homogenization speeds on viable cell count of *L. acidophilus* NRRL B-4495 used in WPI-pullulan microcapsule production

APPENDIX C

HARDENING SOLUTION

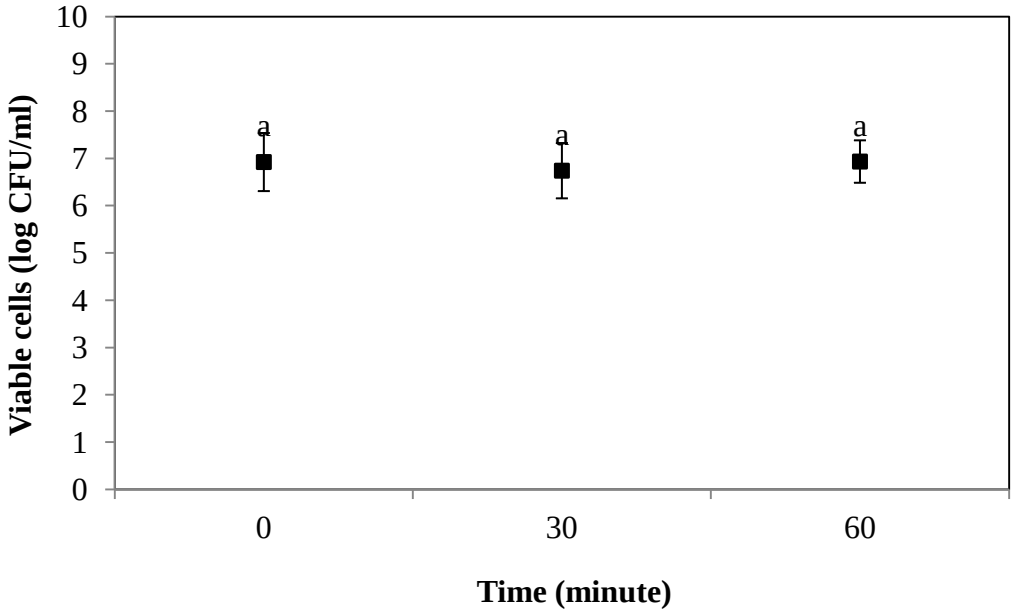

Figure C.1. Effect of hardening solution on viable cell count of *L. acidophilus* NRRL B-4495 for 60 min of incubation at ambient temperature (0.2M $CaCl_2$)

APPENDIX D

PULLULAN CONCENTRATION

Table D.1. Effect of pullulan concentration in dH_2O on viable cell numbers of bacterial cells after anaerobic incubation for 24 h at 37°C.

Pullulan concentration (w/v,%)	**Initial cell count (log CFU/ml)**	**Count after incubation (log CFU/ml)**
4.0	8.16±0.99	8.86±0.87
9.0	8.26±1.00	10.29±0.67
10.8	8.52±0.24	10.74±.010
11.7	8.31±0.68	10.45±.059
12.6	8.74±0.82	10.89±0.98
13.5	8.39±0.13	10.51±1.01

APPENDIX E

WPI-PULLULAN$_{1:1}$

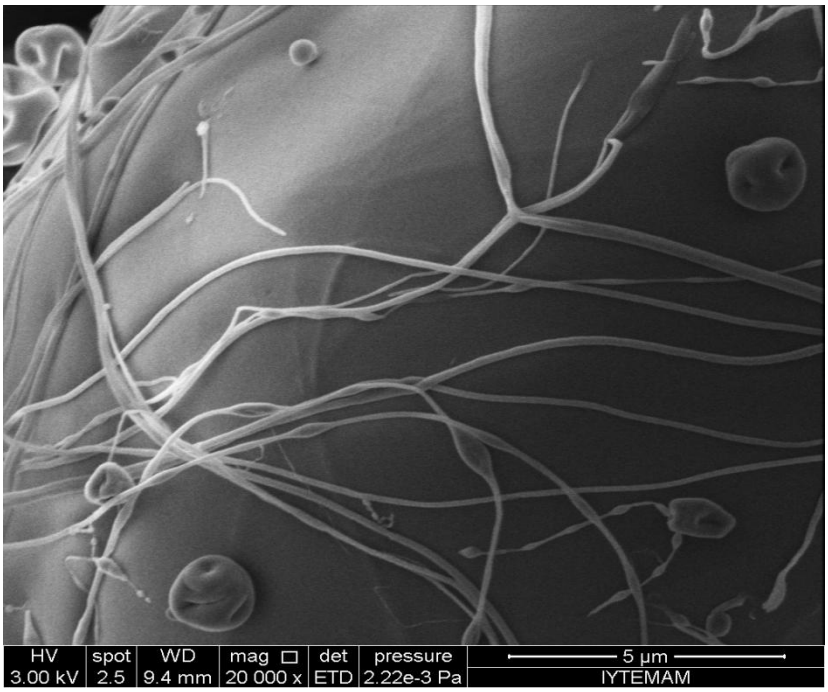

Figure E.1. SEM image of WP-pullulan$_{1:1}$ microcapsules at 20000X magnification

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PUBLICATIONS

- Çabuk, B. Tarı, C. and Harsa S. (2014). β-Galactosidase Immobilization on Chitosan-Hydroxyapatite Complex: Effects of Immobilization Conditions. Journal of Nutritional Health and Food Engineering. 1(1): 00004.
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