ABSTRACT

Title of dissertation: MICROENCAPSULATION OF PROBIOTIC BACTERIA IN XANTHAN-CHITOSAN POLYELECTROLYTE COMPLEX GELS

Sanem Argin, Doctor of Philosophy, 2007

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In recent years, increasing evidence indicating numerous health benefits associated with the intake of probiotic bacteria has created a big market of probiotic foods worldwide. However, maintaining high numbers of viable cells in probiotic food products during the shelf life of the product and during gastrointestinal transit is a challenge. The goal of this research is to develop a novel microencapsulation system using xanthan gum and chitosan polyelectrolyte complex gels in order to protect the probiotic cells against adverse environmental conditions, and to increase their recovery rates.

The extrusion method was used to form the xanthan-chitosan microcapsules. The effects of initial polymer concentration and chitosan solution pH on the crosslink density of the capsule network were investigated by swelling studies and modulated differential scanning calorimetry (MDSC) analysis. Once the capsule formulations resulting in a highly crosslinked network structure were determined, *P. acidilactici* cells were successfully encapsulated using these formulations. Efforts were made to

study the release kinetics of probiotic cells from these capsules in gastrointestinal conditions. Cell release was found to be negligible in simulated gastric juice. In simulated intestinal conditions, the release was relaxation controlled and complete cell release was achieved in at least 5 hours. After exposure to simulated gastric fluid (pH=2.0), encapsulation with xanthan gum and chitosan provided up to six-log and four-log preservation of the freeze-dried probiotic cells over free suspending cells for 1 and 2 hours, respectively.

These results suggest that xanthan-chitosan capsules have a good potential for delivery of probiotic cells to the intestines in high numbers where the cells can release and colonize to benefit the consumer.

MICROENCAPSULATION OF PROBIOTIC BACTERIA IN XANTHAN-CHITOSAN POLYELECTROLYTE COMPLEX GELS

by

Sanem Argın

Dissertation submitted to the Faculty of the Graduate School of the
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DEDICATION

To my daughter, Kayla Soysal, and my husband, Alkan Soysal.

Acknowledgments

First and foremost, I would like to thank my husband, Alkan Soysal, for his endless support, unconditional love, continuous encouragement, and tremendous patience. Without him, I would never have been able to come this far. He has been my coach, my best friend, and the shoulder I cried on throughout these years. Thank you for believing in me and for always being there for me. Thank you for finding a solution to every problem I had and not complaining about it even once. Many, many thanks for the millions of small things you have done to make it possible for me to write this dissertation.

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Chapter 1

Introduction

1.1 Significance

Life style and eating habits play an important role in overall health of individuals. In recent years, increasing evidence indicating numerous health benefits associated with the intake of probiotic bacteria has created a big market of probiotic foods worldwide [1]. The biggest challenge in the development of probiotic products is to maintain the adequate number of viable cells during the shelf life of the product as well as during the gastrointestinal (GI)-tract transit after consumption, so that the claimed health benefits can be delivered to the consumer [2–5]. Consequently, there has been a growing interest in developing techniques to enhance the survival of probiotic bacteria particularly during the GI-tract transit of the cells [6–14]. Microencapsulation of probiotic bacteria in hydrocolloid beads is one of the recent techniques studied to improve the viability and activity of the cells under unfavorable conditions by entrapping the bacteria within a bead matrix [15–18].

Na-Alginate and κ-carrageenan, alone or combined with other polymers and cryoprotectants, are the most commonly used hydrocolloids for the encapsulation of microorganisms [17, 19–21]. However, each system has its own limitations such as susceptibility to ions, lack of mechanical strength and scale-up difficulties [22, 23]. For this reason, there remains an important need for an effective capsule design that ensures the proper delivery of probiotics to the target destination and enables the complete release of the cells at desired conditions to benefit from the health promoting effects of probiotic bacteria.

1.2 Objectives

The ultimate goal of this work was to develop a novel microencapsulation system using polyelectrolyte complex gels formed by xanthan gum and chitosan to effectively sustain the viability of probiotics that are known to provide health benefits to human and animals. The purpose was to protect the encapsulated cells against harsh environmental conditions, particularly against the highly acidic gastric conditions, and to establish a release mechanism so that the bacteria can colonize in the gut to improve the intestinal microbiota. Specifically, emphasis was placed on the effects of complexation conditions on the capsule network structure, the release kinetics of the cells under GI-tract conditions, and the protective effects of the xanthan-chitosan capsules on probiotics during GI-tract transit.

Chapter 2

Literature Review

2.1 Microencapsulation of probiotic bacteria

Probiotics are defined as live microbial food supplements that benefit the host by improving its intestinal microbial balance [24]. Lactic acid bacteria are among the most important probiotic microorganisms (Table 2.1) associated with the human gastrointestinal (GI)-tract [25]. Known health benefits of probiotic bacteria include suppressing the growth of undesirable microorganisms in the colon and small intestine [26, 27], controlling serum cholesterol levels [28], reducing the risk of colon cancer [29], stimulating the immune system [30], improving lactose utilization [31], and controlling the allergic inflammation associated with food allergy [32].

To act as probiotics, bacteria must arrive in intestines alive and in sufficient numbers which is suggested at 10^6-10^7 cfu/g product [26, 33]. However, significant reduction of the number of viable and active cells in a food product occurs inevitably due to the environmental changes such as pH, temperature, and dis-

Table 2.1: Microorganisms considered as probiotics.

Lactobacillus species	*Bifidobacterium* species	Other lactic acid bacteria	Non-lactic acid bacteria
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis**	*Bacillus cereus* var. *toyoi**
L. amylovorus	*B. animalis/B. lactis*	*Enterococcus faecium*	*Escherichia coli* strain *nissle*
L. casei	*B. bifidum*	*Lactococcus lactis*	*Saccharomyces boulardii*
L. crispatus	*B. breve*	*Leuconostoc mesenteroids*	*Saccharomyces cerevisiae*
L. rhamnosus	*B. infantis*	*Pediococcus acidilactici**	*Propionibacterium*
*L. gallinarum**	*B. longum*	*Sporolactobacillus inulinus**	*freudenreichii**
L. gasseri		*Streptococcus thermophilus*	
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. delbrueckii subsp. *bulgaricus*			

* Main application for animals

Adopted from [25]

solved oxygen [2–5]. Freeze drying of the probiotic cells during initial manufacturing [16, 34]; the processing conditions of food carriers, especially the low pH and low temperature in fermented and frozen products; as well as the transportation and storage requirements [14, 35, 36] are some of the main reasons causing the loss of cell viability. Moreover, after their consumption, probiotic cells are subjected to various hydrolytic enzymes, acidic conditions of the stomach, and bile salts in the GI-tract [37, 38]. Various approaches such as addition of different growth promoters [6, 7, 9, 12, 13], manipulation of fermentation and storage conditions of the food carriers [8, 10, 11, 14], careful selection of the culture organisms according to their interrelationships and acid-bile resistance [14], and recently, using microencapsulation technology to reduce the cell loss [15–18] have been investigated to improve the survival rates of active cells arriving in the intestines. Microencapsulation of probiotics in hydrocolloid beads has been found to improve the viability and activity of probiotics in food products and the intestinal tract by entrapping the cells within a bead matrix, thus, segregating them from adverse environmental condi-

tions, as well as protecting them against bacteriophages [21, 33, 39]. There are two common techniques applied to the microencapsulation of probiotic bacteria: The extrusion (droplet) method [40–43] and emulsion (two-phase) system [16, 21, 42, 44]. Both systems achieve survival rates as high as 80-95%, but the extrusion method is simpler and cheaper [33]. Alginate is the most commonly used hydrocolloid for the entrapment of cells due to its simple and low cost gelling mechanism [19], excellent biocompatibility [20], and the reversibility of the immobilization [21]. However, alginate gels are unstable with phosphate and lactate ions as these ions can displace the calcium ions that stabilize the alginate gels [44]. Recent studies showed that Ca-alginate encapsulation does not significantly improve the survival of the cells in gastric conditions [15, 38, 45]. On the other hand, κ-carrageenan capsules have been reported to provide better protection against refrigeration temperatures than Ca-alginate beads [17]. However, with κ-carrageenan, flexible gels can only be formed in the presence of locust bean gum [44]. The formation of κ-carrageenan-locust bean gum capsules requires potassium ions, which can damage the probiotic cells [46]. Another approach was to use Ca-pectate gels as carriers since they are less susceptible to decalcifying and acidification than Ca-alginate gels [47]. Combining other polymers (starch, pectin, etc) and/or whey proteins with these common hydrocolloids has been shown to offer better protection of cells under low temperature and pH than when these gums are used alone for the encapsulation [41, 48, 49]. Moreover, different coating materials to coat the beads as well as some additives such as cryoprotectants were used to overcome the aforementioned disadvantages [39, 50]. However, all of these approaches have had varying degrees of success and the costs

of operation remain a concern. The main problems of bacteria encapsulation with Ca-alginate and κ-carrageenan gels can be summarized as susceptibility to ions, lack of mechanical strength and scale-up difficulties [22, 23]. Some other methods to encapsulate bacteria such as spray drying with starch reportedly did not offer any protection against acidic conditions [15]. Sun and Griffiths [51] reported that a mixture of gellan and xanthan gum, both of which are microbial exopolysaccharides, serves as a better encapsulating agent than Ca-alginate when the cells are exposed to the same environmental conditions. Produced by microorganisms and secreted to the environment to serve as a protection against desiccation, bacteriophage attacks, variations in temperature, cell wall degrading enzymes, and osmotic stress [52–56], microbial exopolysaccharides may be suggested as promising agents for encapsulation of probiotic bacteria.

2.2 Xanthan Gum

2.2.1 Source, structure and applications

Xanthan gum (Figure 2.1) is a microbial exopolysaccharide consisting of a cellulosic backbone with side chains of two mannose and one glucuronic acid on every second glucose residue [57, 58]. In nature, the plant pathogenic bacterium *Xanthomonas campestris* produce highly viscous xanthan gum which attaches to cabbage and protects itself against dehydration and access of harmful substances, or as a way of binding and neutralizing bacteriophages [52, 54]. Presently, commercial

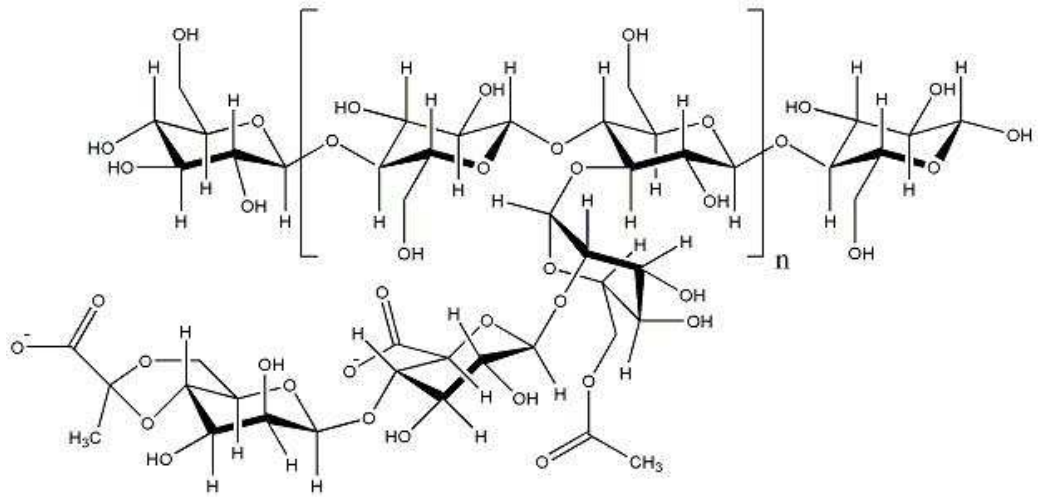


Figure 2.1: Pentasaccharide repeating unit of xanthan gum.

xanthan gum is produced from glucose by batch fermentation of *X. campestris*. The molecular weight of xanthan gum can reach up to 6 million Daltons, which makes it possible to create extremely viscous solutions at very low concentrations [59, 60].

Pyruvate and acetate substitution may vary strongly depending on the parent bacterial strain and the growth conditions of the bacteria [61]. Side chains of xanthan gum represent a very high proportion of the molecule (60%). Due to the side chains, the polymer completely hydrates in water [62]. In addition, enzymatic resistance of xanthan gum is thought to be due to the arrangement of the side chains which prevents the enzymes from attacking the β-(1-4) linkages in the backbone, thereby preventing depolymerization by enzymes, acid and alkali [63]. In aqueous solution, the stable conformation of xanthan is double helical below a transition temperature [64]. Solution studies on the conformation of xanthan gum suggest a rod-like structure with some degree of flexibility, depending on the molecular weight [65–67]. In addition to its enzymatic resistance, xanthan gum is stable

over a wide range of temperatures and pH, which finds many applications in food, pharmaceutical, cosmetic, and oil-drilling industries [68–72]. Xanthan gum was approved by the U.S. Food and Drug Administration (FDA) in 1965 and became the most widely used thickening agent in food products.

2.2.2 Rheological properties

In aqueous solution, high molecular weight xanthan molecules form a semi-flexible wormlike structure and develop a weak-gel network due to intermolecular interactions [66,67,73,74]. These intermolecular associations such as hydrogen bonding, electrostatic and hydrophobic interactions play a crucial role in the changes of rheological properties of the xanthan solutions [74, 75]. Since the junctions between the ordered chain sequences in the gel network of xanthan gum are weaker than those in the true gel networks, they can be easily broken down under stress allowing the system to flow which gives xanthan the shear-thinning behavior [76]. Due to the presence of glucuronic acid and pyruvate in its side chains, xanthan is considered to be an anionic polyelectrolyte [77]. However, the response of xanthan solutions to increasing ionic strength differs from other polyelectrolytes in that they maintain a high solution viscosity as ionic strength increases due to the increase in hydrodynamic radius [78, 79]. At low salt concentrations (up to 0.05 M), the viscosity of the xanthan gum solutions slightly decreases and beyond this, no significant effect on the steady shear and dynamic properties is observed [79, 80]. In addition, xanthan viscosity does not change significantly with temperature in the presence of salt and

the ordered conformation exists at room or elevated temperatures [77, 78, 81].

2.3 Chitosan

2.3.1 Source, structure and applications

Chitosan (Figure 2.2), poly-β-(1 4)-D-glucosamine, is the only natural cationic polysaccharide and is produced by alkaline deacetylation of chitin [82, 83]. Chitin is the second most abundant natural polysaccharide which is present in crustacea, insects and yeasts. The molecular weight of chitin and chitosan ranges between several hundred thousands to 1 million Daltons [84]. As one of the major by-products of the crabbing and shrimp canning industry, chitin is also the major source of surface pollution in coastal areas [85]. Studies on chitin and chitosan have increased since the 1990's to find value-added uses of these polysaccharides that show excellent biological properties such as biocompatibility, biodegradability, lack of toxicity, and adsorption, as well as relatively high percentage of nitrogen [82, 86–88]. The degree of acetylation (DA) is a very important property of chitosan molecules since DA affects biodegradation capability, solubility, gelling and reactivity of chitosan [89].

The unique properties of chitosan arise from its amino groups that carry positive charges at pH values below 6.5, enabling it to bind to negatively charged materials such as enzymes, cells, polysaccharides, nucleic acids, hair and skin [90]. Being a linear polysaccharide, chitosan has both reactive amino groups and hydroxyl groups that can be used to alter its physical and solution properties [90]. Chitosan

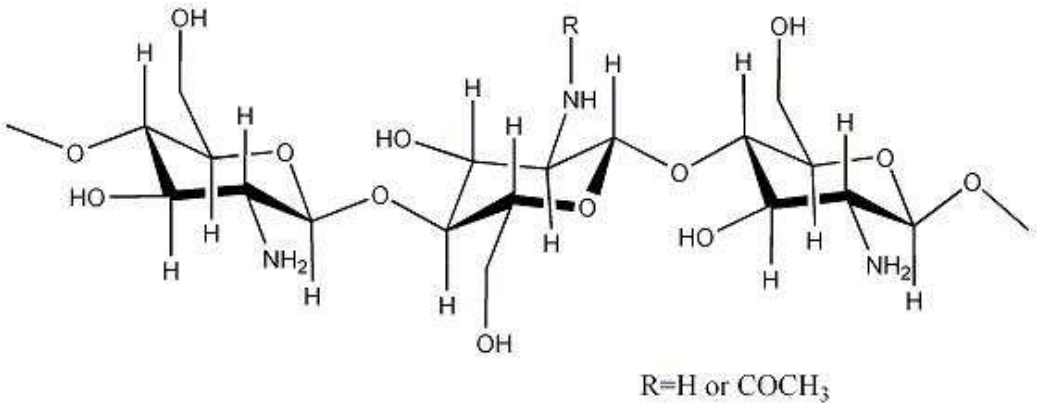


Figure 2.2: Chemical structure of chitosan.

is insoluble at alkaline and neutral pH but soluble in inorganic and organic acids such as hydrochloric acid, acetic acid, lactic acid and glutamic acid. Water soluble chitosan can be formed when hydrogen-bond formation is prevented by partial re-acetylation of chitosan molecules by several means [87, 91].

Chitin and chitosan are being extensively used in the pharmaceutical industry (cosmetics, contact lenses, artificial skin, wound dressing), paper making, photography, solid state batteries, waste water treatment, chromatography, dietary supplements and animal feed [83,92]. Considerable amount of research has been conducted to use chitosan as a drug delivery vehicle, especially for the treatment of colon diseases, such as ulcerative colitis and Crohn's disease and as a dietary supplement for lowering cholesterol and controlling overweight [84, 87, 89, 93]. Chitosan for oral administration to humans is generally recognized as safe and approved for food use in Italy, Norway, and Japan [94, 95]. In the U.S., chitosan is yet to be approved as a food additive by the U.S. Food and Drug Administration (FDA) but was approved in 1985 by Association of American Feed Control Officials (AAFCO) for its use in

animal feed, as long as the level does not exceed 0.1% of the feed [96, 97].

2.3.2 Rheological properties

Concentrated chitosan solutions exhibit shear-thinning behavior and the viscosity of dilute chitosan solutions are independent of shear rate [98]. Due to strong intermolecular hydrogen bonding even at low concentration, chitosan molecules have a tendency to entangle and form a network [99]. As a polyelectrolyte, the conformation of the chitosan molecule is affected by the electric charge density [100]. For this reason, the rheological properties of chitosan depend on factors such as pH, ionic strength, solvent selection, concentration, molecular weight, DA, and the distribution of the acetyl groups [101]. When the pH of the solution is increased, the intermolecular and intramolecular electrostatic repulsions between cationic charges are reduced and chitosan chains come closer together, lowering the hydrodynamic volume of the chitosan molecules. Such an effect may enhance the interchain and intrachain hydrogen bonding. Similarly, as the ionic strength increases, the intrinsic viscosity decreases due to shielding effect of counterions [85]. The apparent viscosities of chitosan solutions were found to be different even at the same pH when different types of acids were used to adjust the pH of the solution. The apparent viscosity of chitosan was higher when dissolved in organic acids than it was when hydrochloric acid was used. This can be attributed to the overall effect of screening and steric effects exerted by different anions on the conformation and chain stiffness of the chitosan molecules [98, 102].

2.4 Microencapsulation with xanthan gum and chitosan

2.4.1 Polyelectrolyte complex (PEC) gels by xanthan gum and chitosan

Polyelectrolytes are macromolecules having many ionizable groups such as proteins and biopolymers. They fall apart into charged polyions and many oppositely charged counterions when dissolved in polar solvents like water [103]. Their size and shape depends on the charge and interaction with counterions. With increasing charge, the flexible chain changes its shape from a contracted random coil to a fully extended one since the charges attached to the polymer repel each other [104]. When the polyelectrolyte chain stretches out, it occupies more space and the solution becomes more viscous. On the other hand, addition of low-molecular weight salt causes a decrease in the range of the intramolecular coulomb force and polymer becomes a random coil again [103]. In general, the configuration of the polyelectrolytes depends on several factors such as pH, ionic strength, type of the solvent, concentration, molecular weight and distribution of the charged group. This configuration determines the dynamic response of the polyelectrolyte solution and the resultant rheology [105].

Polyelectrolyte complex (PEC) gels formed by the electrostatic attractions between two oppositely charged polyelectrolytes mixed in an aqueous solution [106] are known to exhibit unique physical and chemical properties as the electrostatic in-

teractions are considerably stronger than most secondary binding interactions [107]. A variety of polyelectrolyte complexes can be obtained by changing the chemical structure of the components such as molecular weight, flexibility, functional group structure, charge density, hydrophilicity, hydrophobicity, and stereoregularity, as well as changing reaction conditions such as pH, ionic strength, polymer concentration, mixing ratio, and temperature [85].

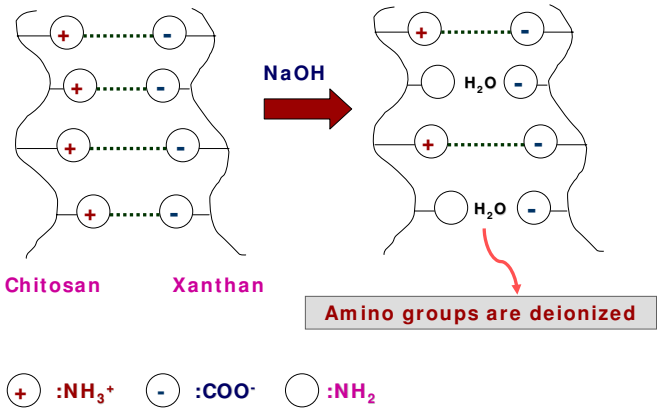
In the last decade, there has been an increasing interest in the use of PEC gels formed by chitosan and polyanions, such as alginate, carrageenan, pectin, poly(acrylic acid), carboxymethylcellulose (CMC), and xanthan gum as carriers for drug delivery and in immobilized systems [108–113]. Xanthan gum and chitosan form a three dimensional network formed by reversible ionic linkages that can absorb much more water than their own weight [113,114]. As polyelectrolyte hydrogels, xanthan-chitosan complexes were suggested as promising candidates for targeted delivery and controlled release of encapsulated products for oral administration due to the fact that only nontoxic metabolites are produced during degradation and the complex has relatively high enzymatic resistance and pH-sensitive swelling characteristics [115–117]. Xanthan-chitosan PEC gels were studied as microcarriers mostly for encapsulation of enzymes [116–119] and the studies on the applicability of the system for bacterial cells are scarce [116].

2.4.2 pH-sensitive swelling characteristics of xanthan-chitosan gels

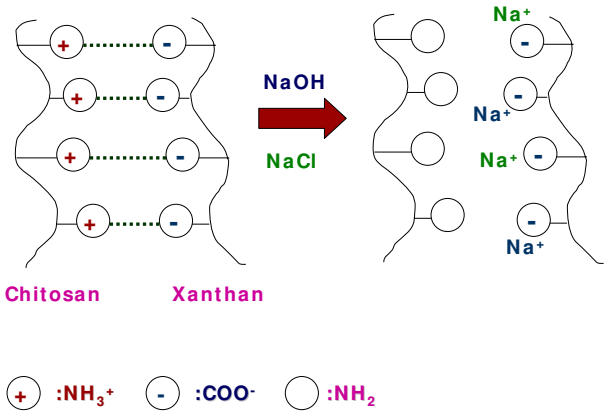
Chitosan-xanthan hydrogels are suggested as a potential candidate for targeted delivery since the pH-sensitive swelling allows the slow release of the encapsulant in the intestines while providing protection against acidic GI-tract conditions [117]. The swelling behavior of the xanthan-chitosan hydrogels is influenced by polymer properties such as molecular weight and DA of chitosan as well as the complexation conditions such as chitosan solution pH and complexation time, all of which determines the crosslink density of the complex [113, 118]. In weak acid-weak base polyelectrolyte complexes as in the case of xanthan-chitosan, the number of electrostatic bonds varies with the ambient pH because of the change in the degree of dissociation [85]. The xanthan-chitosan complex swells in the range of pH = 10-12 in NaOH solution, with a maximum equilibrium swelling ratio (defined as $[Equilibrium\ diameter/Initial\ diameter]^3$) at pH = 10, and also at pH=0 in HCl solution. In the presence of NaCl, maximum swelling can be achieved at pH = 8 since the presence of ions weakens the ionic interactions and increases the osmotic pressure difference between the ambient solution and inside of the gel [115]. The ionizable groups of the xanthan gum and chitosan, which are a carboxyl group and an amino group respectively, are oppositely charged and are electrostatically attracted to each other during the gel formation. When the pH of the solution is increased, amino groups start to deionize while carboxyl groups hold the negative charge which will cause the electrostatic linkages to disappear (Figure 2.3). Meanwhile, the car-

boxyl groups attract Na^+ ions and water diffuses into the complex. Both of these phenomena increase the osmotic pressure of the gel and consequently, the complex swells. However, equilibrium swelling ratio decreases in NaOH solution at pH values higher than 10 since too much increase in Na^+ concentration decreases the difference of osmotic pressure between the gel and ambient solution. Similarly, the swelling in acidic solutions can be explained by the neutralization of the negative charges of xanthan while the amino groups of chitosan hold their positive charge, resulting in the swelling of the gel complex [115].

Swelling capsules formed by xanthan-chitosan complexation can be described as hollow spheres that expand until equilibrium is reached in the release solution. Determination of the effective diffusion coefficient of hydrogels is very complex since their properties are affected by many parameters. Due to the swelling and destruction of the linkages between two polymers, the radius of the capsules and the amount of the impermeable segments (obstruction effect) change with time which makes the calculations more complex. However, effective diffusion coefficient is expected to increase with the increased swelling and decreased polymer fraction [120].



(a)



(b)

Figure 2.3: pH-sensitive swelling of xanthan-chitosan complex: (a) In alkaline pH (10 to 12), amino groups deionize and electrostatic linkages disappear; whereas (b) attraction of Na^+ ions increases osmotic pressure difference and causes swelling of the gel where addition of salt helps achieve the swelling at a lower pH such as pH 8.

Chapter 3

Effects of Complexation Conditions on Xanthan-Chitosan Polyelectrolyte Complex Gels

3.1 Introduction

Polyelectrolyte hydrogels formed by xanthan gum and chitosan can be used for encapsulation and controlled release of food ingredients, cells, enzymes, and therapeutic agents. A variety of xanthan-chitosan PEC gels can be obtained by changing the molecular properties of the xanthan and chitosan polymers, such as molecular weight, degree of acetylation of chitosan, and pyruvic acid content of xanthan, as well as changing the complexation conditions, such as chitosan solution pH, polymer concentration, complexation time, and mixing ratio [113,118,119,121].

This study addresses the importance of polymer concentration and chitosan

solution pH in the complexation of xanthan gum and chitosan in the form of capsules. The swelling degree of the microcapsules formed by different combinations of xanthan and chitosan was studied as an indication of the crosslink density of the hydrogel membrane. Crosslink density is an important factor determining the stability, pH-sensitive swelling behavior (thus the release properties), as well as the mechanical strength of hydrogel networks. [113, 122–124]. An increase in the crosslink density restricts the degree of swelling and reduces the pH-sensitivity by improving the stability of the network. Hence, by controlling the amount of crosslinking, xanthan-chitosan microcapsules suitable for targeted release of probiotics can be prepared.

3.2 Materials and methods

3.2.1 Preparation of chitosan and xanthan solutions

Chitosan from crab shells with a minimum deacetylation amount of 85% and a molecular weight of 370 000 (reported by the supplier) was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). A known amount of chitosan was dissolved in 1 N HCl by heating and agitating. The desired solution pH was adjusted by 1 M NaOH and DI water was added to bring it to the final volume. Xanthan gum with a molecular weight of 1.02 million (TICAXAN ®) was kindly supplied by TIC Gums (Belcamp, MD). A predetermined amount of xanthan gum was dissolved in DI water under heating and agitation. Both solutions were autoclaved before use.

3.2.2 Capsule formation and Inverted microscopy

In this study, the extrusion (complex coacervation) method was used. Capsules were formed by dropwise addition of a solution of xanthan (50 mL) into a solution of chitosan (300 mL) using a manually operated syringe with a 0.7-mm cannula (Becton-Dickinson, Franklin Lakes, NJ). The chitosan solution was agitated continuously for 40 min to allow crosslinking and avoid coalescence of capsules. The capsules were filtered through a 160 μm Millipore nylon filter, washed twice with DI water, and then freeze-dried for 24 hours.

NHS-Fluorescein labeled chitosan, prepared according to Yi et al. [125], was kindly obtained from Dr. Payne's lab in the Center of Biosystems Research at University of Maryland, College Park. Fluorescently-labeled capsules were examined under Axiovert 200 Inverted microscope (Carl Zeiss, Inc, Oberkochen, Germany).

3.2.3 Determination of the swelling degree

The effect of three complexation parameters, namely initial xanthan solution concentration (0.5, 0.7, 1.0, and 1.5% w/v), initial chitosan solution concentration (0.7 and 1.0% w/v) and chitosan solution pH (4.5, 5.5, and 6.2) on the degree of swelling of the resulting capsules were studied. Ten freeze-dried capsules were weighed and suspended in DI water overnight for each combination. The capsules were filtered, blotted to remove surface water, and weighed. The swelling degree (SD) values were calculated using the following equation:

$$\text{SD } (\%) = \frac{W_s - W_d}{W_d} \times 100 \tag{3.1}$$

where W_s and W_d are the weight of swollen capsules and that of dry capsules, respectively. The averages of four replicates for each combination were reported.

3.2.4 Differential Scanning Calorimetry (DSC) measurements

Differential scanning calorimetry (DSC) measurements were carried out on a TA Instruments Q100 DSC device (New Castle, DE). The cell resistance and capacitance calibrations were performed in two steps. The first step was heating an empty cell and the second step was heating the cell with equal weight sapphire disks on the sample and reference platforms. The cell constant and temperature calibrations were performed with an indium standard.

Standard DSC was used for the first heating and cooling runs. Approximately 5 mg of dry capsules were placed in sealed aluminum pans in small pieces. Each sample was heated up to 160℃ at a rate of 10℃/min and cooled back to 40℃ at a rate of 5℃/min to erase the thermal history of the polymers and eliminate the effect of moisture. Modulated DSC curves were obtained from the second heating run at 2℃/min. Samples were heated from 100℃ to 175℃ with a modulation period of 60 seconds and modulation amplitude of 0.318℃. A nitrogen purge was applied for all experiments. The reversing signal was used to compare the glass transitions of the samples. In addition, chitosan in flakes and xanthan in powder form were subjected to the same experimental procedure to determine their glass transition

temperatures.

3.2.5 Statistical analysis

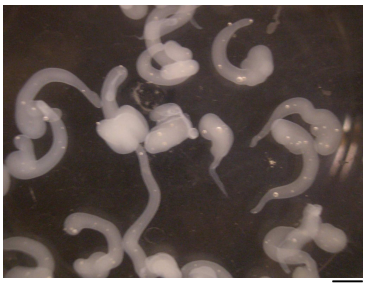
Statistical analyses were conducted using SAS 9.1.2 Software (Cary, NC). Factorial analysis of variance was used to analyze the effect of xanthan concentration, chitosan concentration, and chitosan solution pH on the swelling degree of the capsules. Differences in least square means were used for pairwise mean comparison. Analyses were performed using mixed procedure of SAS.

3.3 Results and discussion

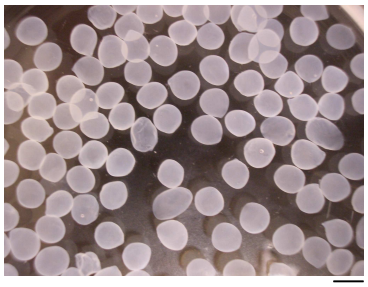
3.3.1 Capsule formation

By extruding the xanthan solution into moderate concentrations of chitosan (0.7 and 1.0% [w/v]), no stable capsules could be formed while xanthan concentrations were below 0.5% (w/v). Xanthan concentrations exceeding 1.5% (w/v) resulted in formation of amorphous capsules (Figure 3.1-a, Figure 3.1-b). The pH of chitosan solution was controlled, ranging from 4.5, where the ionization degree of chitosan is unity, to 6.2, since chitosan precipitates above its pKa value of approximately 6.3.

Capsules formed by xanthan-chitosan complexation can be described as hollow spheres. Figure 3.1-c shows a xanthan-chitosan capsule prepared by NHS-Fluorescein labeled chitosan under inverted microscope.



(a)



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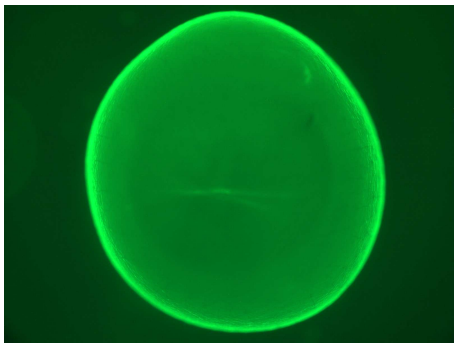


(c)

Figure 3.1: Xanthan-chitosan capsules formed when initial xanthan concentration is (a) greater than 1.5% (w/v); (b) less than 1.5% (w/v); (c) Inverted microscopy image of a 2 mm-diameter xanthan-chitosan capsule prepared with fluorescently labeled chitosan.

3.3.2 Effect of complexation conditions on the swelling degree of xanthan-chitosan capsules

The swelling degree of microcapsules formed by different combinations of xanthan and chitosan was studied as an indication of the crosslink density of the hydrogel membrane. The degree of swelling decreased by approximately 50% when using 0.7% (w/v) xanthan solution instead of 0.5% (w/v)(Figure 3.2). Therefore capsules formed from xanthan solutions at concentration of 0.5% (w/v) were not included in the statistical analysis and DSC studies.

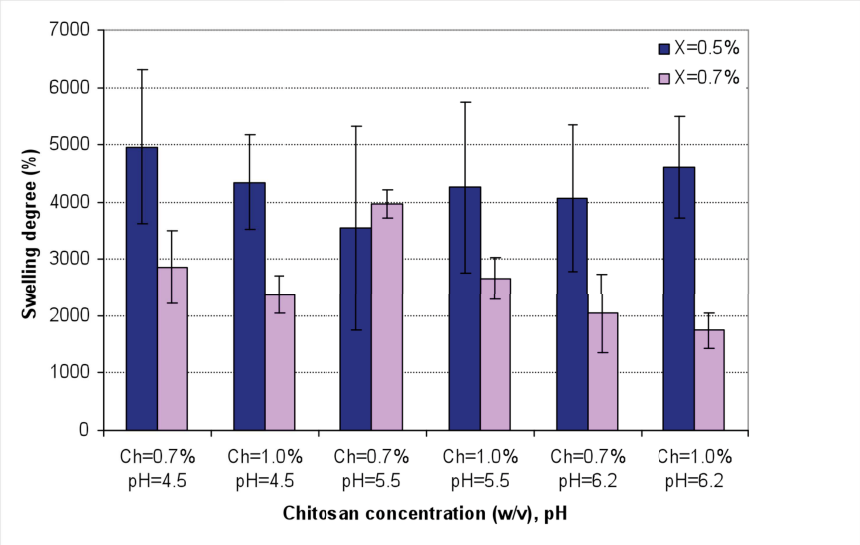


Figure 3.2: Swelling degree of xanthan-chitosan capsules formed by using 0.5% (w/v) and 0.7% (w/v) xanthan solutions.

The xanthan concentration was found to have a very significant effect on the swelling degree of xanthan-chitosan capsules at both chitosan concentrations ($P < 0.0001$) (Table 3.1). However, this effect was found to be dependent on chitosan concentration and pH. When 0.7% (w/v) chitosan was used, the high the xanthan concentration yielded a lower swelling degree under all conditions studied except when xanthan concentration was increased from 1.0% to 1.5% (w/v) at pH 6.2. On the other hand, with 1.0% (w/v) chitosan, increasing xanthan concentration resulted in significantly lower swelling degrees only when xanthan concentration was increased from 1.0% to 1.5% (w/v) at pH 4.5 and 5.5 ($P < 0.05$) and no significant difference was observed at other conditions. At both chitosan concentrations, the decrease in the swelling degrees of the capsules with the increase in xanthan

concentration was more pronounced when chitosan solution pH was 5.5 and least significant at pH 6.2.

Table 3.1: Swelling degrees of xanthan-chitosan capsules in DI water

	Chitosan (w/v)					
	0.7%			1.0%		
Xanthan (w/v)	pH 4.5	pH 5.5	pH 6.2	pH 4.5	pH 5.5	pH 6.2
0.7%	$*29^{i}$	40	21^{m}	24^{bgi}	27^{cg}	17^{dm}
1.0%	21^{j}	30	13^{an}	22^{bhj}	26^{ch}	12^{dn}
1.5%	13^{ek}	16^{el}	15^{aeo}	16^{fk}	11^{fl}	15^{dfo}

Mean values with same letter are not significantly different at P=0.05 level.

* Swelling degree/100

The effect of chitosan solution pH on the degree of swelling was more significant when the chitosan solution concentration was 0.7% (w/v) ($P < 0.0001$) than when it was 1.0% (w/v) ($P < 0.005$) (Table 3.1). With 1.5% (w/v) xanthan concentration, the increases in pH had no significant effect on the swelling degree ($P > 0.05$), whereas at other xanthan concentrations, the degree of swelling decreased significantly when chitosan pH was increased from 4.5 to 6.2. Such significant decreases in swelling degree with increasing chitosan pH could be attributed to the changes in the chain flexibility of chitosan polymer with the changes in the solution pH. The ionization degree of chitosan decreases from 1.0 to 0.5 as pH increases from 4.5 to 6.2 [126], which means that amino groups become less charged as pH increases. As a result, one may expect that fewer ionic linkages would occur between the two

polymers, resulting in higher swelling degrees as the pH approaches to its pKa value. However, since the charge density of the chitosan molecule is reduced by almost 50% as pH approaches 6.2 from a value of 4.5, the polymer chains become less extended with a smaller radius of gyration. This might result in a higher diffusion coefficient for chitosan chains at pH 6.2, consequently enhancing diffusion of chitosan into the xanthan-chitosan network and forming more linkages during the specified reaction time. This result suggests that since the formation of the xanthan-chitosan network is instantaneous upon contact, the diffusion of chitosan chains in the bulk solution through this network plays an important role to achieve a highly crosslinked structure with a small swelling degree.

Intriguingly, when 0.7 and 1.0% (w/v) xanthan solutions were extruded into 0.7% (w/v) chitosan solution, increases of chitosan pH from 4.5 to 5.5, first significantly increased the degree of swelling before it reached the lowest swelling degree at pH 6.2. This significant increase in the swelling degree when chitosan pH was raised from 4.5 to 5.5 might be associated with the slight decrease in the charge density of chitosan (ionization degree ca. 0.9) that lessens the ionic linkages between the two polymers, rendering higher swelling degrees. Magnin et al. [113] have demonstrated similar results where the swelling degree of xanthan-chitosan matrix continued to increase with increasing chitosan solution pH from 3.5 to 5.8 in bulk form by using 0.65 wt% chitosan and 0.65 wt% xanthan solutions. Moreover, our results also showed that when 1.0% (w/v) chitosan concentration was used, pH change from 4.5 to 5.5 chitosan solution had no significant effect on the swelling degree, suggesting that the swelling degree was less affected by the decrease in the charge density of the

chitosan chains as pH approaches to 5.5 when chitosan concentration is increased.

The effect of chitosan solution concentration on the swelling degree of capsules was less pronounced than the effect of xanthan solution concentration and chitosan solution pH. Increasing chitosan concentration from 0.7 to 1.0% (w/v) significantly decreased the degree of swelling only when chitosan pH was 5.5 and at xanthan concentrations of 0.7 or 1.0% (w/v). No significant difference was observed at other conditions. These findings indicate that the parameters studied cannot be viewed as independent parameters, as the effect of one parameter on the degree of swelling depends on the other two parameters. While swelling studies were capable of identifying the combined effect of polymer concentration and chitosan solution pH on the crosslink densities of xanthan-chitosan hydrogel capsules, further investigation is needed to understand the differences in the membrane structure as influenced by the xanthan-chitosan hydrogel preparation conditions. Therefore, DSC analysis was performed to compare the changes in thermal transitions in order to elucidate the changes in the crosslinking density of the capsule network.

3.3.3 DSC analysis of xanthan-chitosan capsules

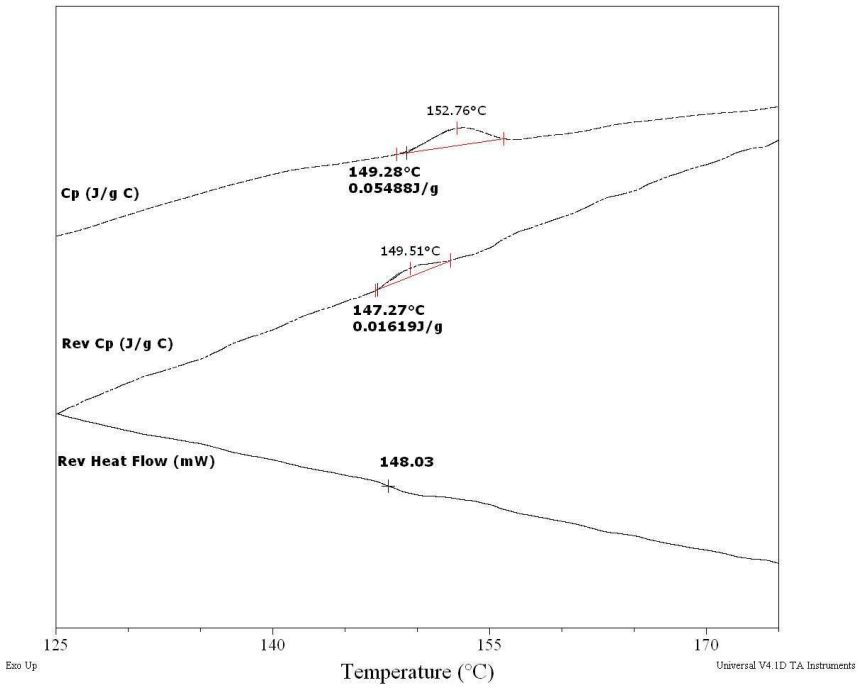
Conventional DSC was used for the first heating and cooling runs. The first heating run of each sample gave single endothermic peak at about 100℃, which was attributed to the absorbed water. Samples were cooled back to 40℃ before the second heating. The modulated differential scanning calorimetry (MDSC) technique was used for the second heating. MDSC applies two simultaneous heating rates to

the sample. The linear heating rate provides total heat flow as conventional DSC and sinusoidal (modulated) heating rate provides the heat capacity-related (reversing) component of the total heat flow. The reversing signal was used to quantify the glass transition since it separates the glass transition completely from other non-reversing processes [127, 128]. The transition enthalpies were calculated by integration of the peaks on the reversing heat capacity (Rev C_p) curves as is usually done for first-order phase transitions [129].

The small glass transitions observed for both chitosan and xanthan can be explained by the fact that both polymers are partially crystalline (Figure 3.3). The inflection points of the peaks on the Rev C_p curves correspond to the glass transition temperature (T_g) of the samples. The T_g of chitosan was determined to be approximately 148℃ and the enthalpy of this transition (H) was calculated as 0.016 J/g. The T_g for xanthan gum was found to be at approximately 143℃ with a transition enthalpy of H=0.01 J/g.

Representative reversing heat flow curves from the second heating runs of the freeze-dried hydrogel capsules are shown in Figures 3.4 and 3.5. The transition enthalpies and the glass transition temperatures were determined as described above. It is evident from these data that the polymer complex shows weak transitions as expected in physically crosslinked networks. For this reason, the transition enthalpy of xanthan gum, 0.01 J/g, was selected as the threshold enthalpy to differentiate the noise from the actual transitions appearing on the MDSC curves of the hydrogel capsules.

Figure 3.4 shows the effect of initial xanthan concentration on the capsule



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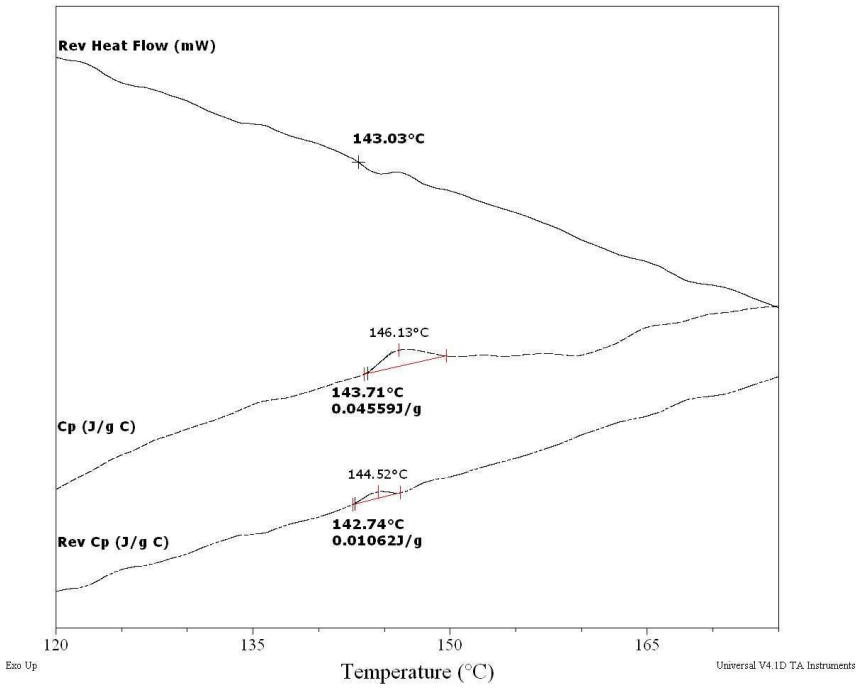
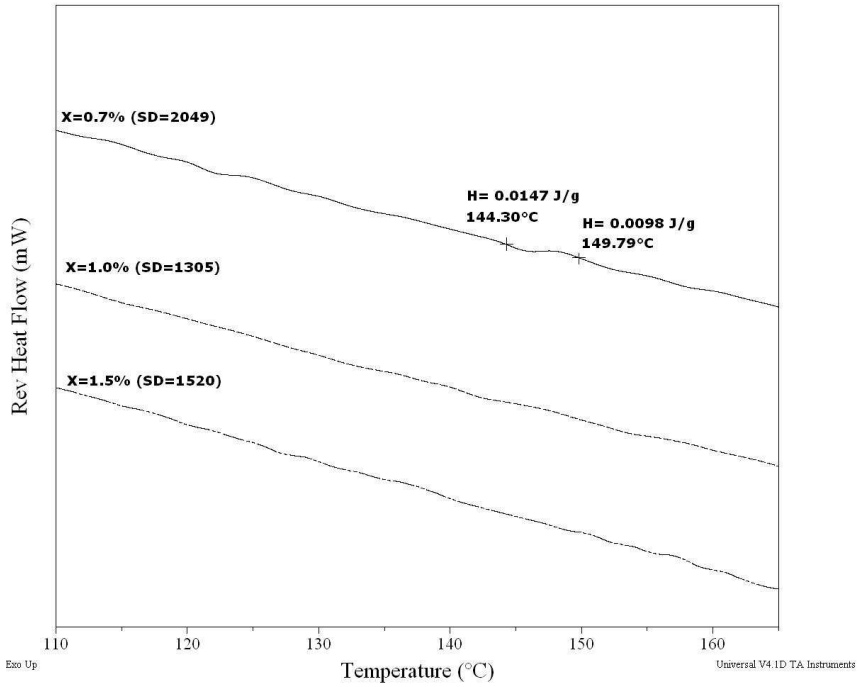
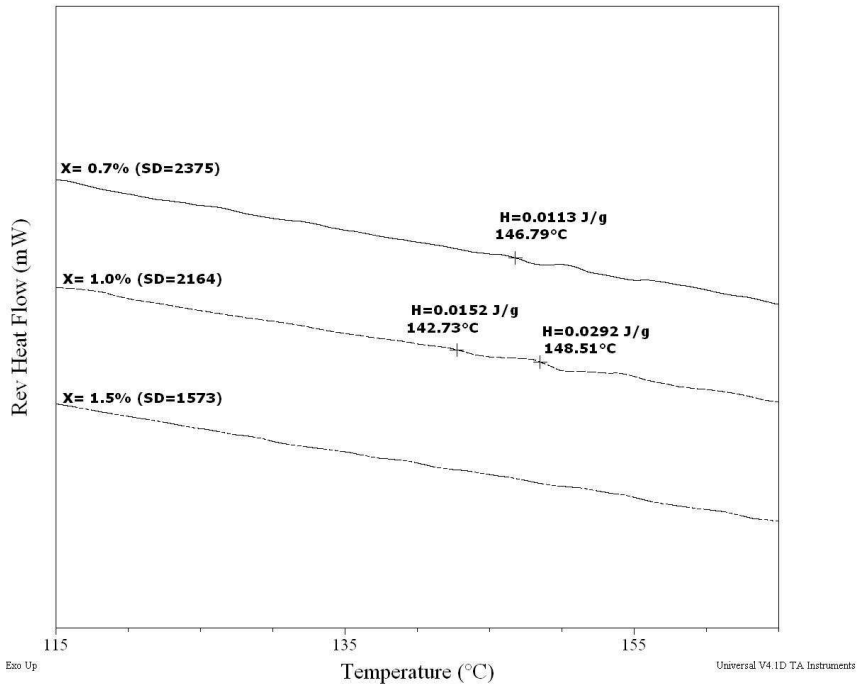


(b)

Figure 3.3: MDSC curves of (a) chitosan; (b) xanthan gum.



(a)



(b)

Figure 3.4: MDSC curves of xanthan-chitosan capsules showing the effect of initial xanthan concentration on the resulting capsule network structure. (a) Chitosan: 0.7% (w/v) pH=6.2; (b) Chitosan 1.0% (w/v) pH=4.5.

network structure. In Figure 3.4-a, the MDSC curves of capsules made from 0.7% (w/v) chitosan solution at pH 6.2 are shown. When the xanthan solution was 0.7% (w/v), two glass transition temperatures at approximately 144℃ and 149℃ were observed. This indicates the presence of uncrosslinked xanthan gum and chitosan in the network. However, these transitions disappeared in the samples formed using 1.0% (w/v) xanthan, which may indicate complete crosslinking of both polymers. This increase in crosslink density explains the significant decrease in the swelling degree of capsules when the initial xanthan concentration was increased from 0.7% (SD = 2049) to 1.0% (SD = 1305). On the other hand, increasing xanthan concentration to 1.5% (w/v) did not result in a significant difference in the degree of swelling (SD = 1520) since the network was already completely crosslinked when 1.0% (w/v) xanthan was used.

Figure 3.4-b shows the MDSC curves of capsules prepared from 1% (w/v) chitosan solution at pH 4.5. The transitions appeared approximately at 147℃ and 148.5℃ in the 0.7% and 1.0% xanthan curves, respectively, can be attributed to the glass transition of chitosan and the transition appeared in 1.0% (w/v) xanthan curve at approximately 143℃ corresponds to the glass transition of xanthan gum. This suggests the presence of uncrosslinked polymer chains in both networks. When xanthan concentration was increased to 1.5% (w/v), no transitions were observed suggesting a completely crosslinked network. These results are in good agreement with the swelling study, since there was no significant difference in the swelling degree when xanthan concentration was increased from 0.7% (w/v) (SD = 2375) to 1.0% (w/v) (SD = 2164) and with further increase in xanthan concentration to

1.5% (w/v), the degree of swelling decreased significantly (SD = 1573).

The effect of pH on the network structure of the capsules made from 1.0% (w/v) chitosan and 1.0% (w/v) xanthan solutions is shown in Figure 3.5. Both transitions from xanthan gum and chitosan observed at pH 4.5 were eliminated when the pH was increased to 6.2. This result might suggest an incomplete crosslinking between the two polymers at pH 4.5 and a complete crosslinking at pH 6.2. The swelling data also shows that there is a significant decrease in the degree of swelling when pH is increased from 4.5 (SD = 2164) to 6.2 (SD = 1187), which can be explained by the increase in the crosslink density.

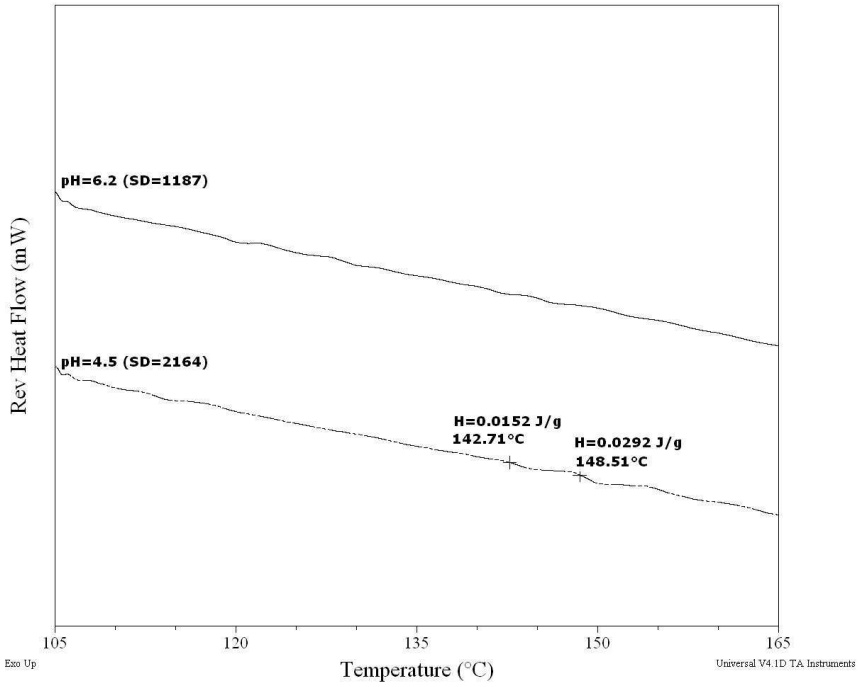


Figure 3.5: MDSC curves of xanthan-chitosan capsules showing the effect of initial chitosan solution pH on the resulting capsule network structure. Xanthan: 1% (w/v), Chitosan: 1% (w/v).

3.4 Conclusions

Characterization of factors contributing to the crosslink density of xanthan-chitosan network is important in developing hydrogels with particular mechanical and controlled release properties. Results from the swelling degree and DSC experiments showed that the crosslink density of xanthan-chitosan network was dependent on the complexation conditions employed in the present study. Xanthan concentration was found to be the most critical parameter in xanthan-chitosan network formation. The hydrogel capsules were completely crosslinked at all conditions studied when initial xanthan solution concentration was at 1.5% (w/v). The increase in xanthan concentration significantly affected the degree of swelling of the hydrogel at both chitosan concentrations. On the other hand, the effect of chitosan solution pH on the degree of swelling was more pronounced at 0.7% (w/v) than at 1.0% (w/v) chitosan concentration. The swelling degree was less dependent on chitosan concentration than xanthan concentration and chitosan solution pH. Conformational changes of chitosan polymer chains, which is dependent on the solution pH, were critical in determining the crosslinked network structure that affects the swelling degrees of resulting gels. Results from this study showed that pH and concentration effects on the xanthan-chitosan network properties are dependent on each other. It can be concluded that, the xanthan-chitosan network properties can be easily modulated by changing operationally controllable parameters, especially xanthan concentration and chitosan solution pH.

Chapter 4

The Release Kinetics and Swelling Behavior of Xanthan-Chitosan Capsules

4.1 Introduction

Xanthan-chitosan hydrogels swell in response to changes in pH, temperature, and ionic strength of the aqueous environment. In swelling controlled systems, the molecular structure as well as the nature of the polymer system control the mechanism by which a solute may be released from a polymer network [130] . At high or very low pH values, the electrostatic linkages between xanthan gum and chitosan start to disappear, allowing the network to expand and imbibe water [115]. The release of probiotic cells by such a pH-sensitive swelling-controlled mechanism is related to the macromolecular chain relaxation and to the diffusion of the cells

through the polymer membrane under countercurrent diffusion of water or biological fluids into the capsules.

It is important that the encapsulation system developed in this study keeps the cell release to a minimum until the capsules reach the small intestines where the rapid release of the cells is desirable for their colonization. For this reason, the kinetics of cell release under different conditions need to be studied in order to understand the response of the system to environmental changes, particularly variations in ambient pH. The main goal of this study was to characterize the swelling and release behavior under simulated GI-tract conditions.

4.2 Materials and Methods

4.2.1 Calibration curve

Pediococcus acidilactici (MA18/5M, National Collection of Microorganism Culture, Pasteur-France) was kindly provided by Imagilin Technology LLC (Potomac, MD). One gram of bacterial powder was hydrated in 9 mL of DI water for 30 min by shaking at 260 rpm. One mL hydrated bacteria was inoculated to 99 mL MRS broth and incubated for 24 hours at 35℃. To construct the calibration curve, a 25 mL sample was taken from MRS broth, centrifuged at 3000 rpm for 30 minutes and washed with sterile distilled water under the same conditions. After necessary dilutions, OD values at 600 nm were recorded by a Heβ IOS Spectrophotometer (ThermoSpectronic, Rochester, NY) and the corresponding samples were

oven dried overnight for mass determination.

4.2.2 Microencapsulation of *P. acidilactici*

One milliliter of hydrated *P. acidilactici* cells was inoculated into 99 mL MRS broth and incubated at 35℃ for 24 hours. Actively growing cells were recovered from MRS broth by centrifuging at 10000 rpm for 10 minutes and then were washed twice with sterile phosphate buffered saline (PBS) solution under the same centrifugation conditions. DI water was added to the cell pellet and vortexed. Xanthan and chitosan solutions were prepared as described in section 3.2.1. Encapsulation was achieved by dropwise addition of xanthan and *P. acidilactici* mixture (9:1 v/v) into chitosan solution using a manually operated syringe with 0.7-mm cannula. The chitosan solution was agitated continuously for 40 min to allow crosslinking and to avoid coalescence of capsules. The capsules were filtered through a 160μm Millipore nylon filter, washed twice with DI water, and then freeze-dried for 24 hours.

4.2.3 Kinetics of cell release

A known amount of freeze-dried capsules were suspended in release solutions, namely simulated gastric fluid (Fisher Chemicals, Suwanee, GA), simulated intestinal fluid (Fisher Chemicals, Suwanee, GA), and DI-water. The cell concentration in the solutions was monitored over time. A 1 mL sample was taken periodically in order to monitor the changes in OD values at 600 nm by using a HeβIOS Spectrophotometer (ThermoSpectronic, Rochester, NY). Consequently, 1 mL of release

solution was added after each sample is taken in order to avoid any errors in calculations. Dilutions were performed when necessary. The calibration curve of the strain was used to relate the OD values to cell concentration. Averages of two replicates for each sample were reported. Cell release kinetics were studied in deionized water for 72 hours at room temperature (under 150 rpm); in simulated intestinal fluid (composed of pancreatin, KH_2PO_4 and $NaOH$) at pH=6.8 for 24 hours at room temperature (under 150 rpm); in simulated gastric fluid (composed of pepsin, $NaCl$ and HCl) at pH=1.0 and pH=2.0 (37℃ under 150 rpm); and finally in simulated intestinal fluid after 2 hour exposure to simulated gastric fluid at pH=2.0 (37℃ under 150 rpm).

The cell release data were analyzed according to the Higuchi equation [131], eqn (4.1); Korsmeyer-Peppas equation [132], eqn (4.2); and Peppas-Sahlin equation [133], eqn (4.3) using MATLAB version 6 R13 (MathWorks Inc, Natick, MA). Nonlinear least squares fitting method was used to determine the parameters in each equation.

$$\frac{M_t}{M_f} = k' t^{1/2} \tag{4.1}$$

$$\frac{M_t}{M_f} = k t^n \tag{4.2}$$

$$\frac{M_t}{M_f} = k_d t^m + k_r t^{2m} \tag{4.3}$$

where M_t is the concentration of the cells released at time t, M_f is the concentration of the cells released at equilibrium, k and k' are constants incorporating the structural and geometric characteristics of the hydrogel, and n is the release exponent describing the mode of the transport mechanism, and m is the purely Fickian

diffusion exponent for a system of any geometrical shape.

4.2.4 Dynamic swelling behavior

Ten freeze-dried capsules were weighed and placed in simulated gastric fluid followed by simulated intestinal fluid. The capsules were carefully removed from the solutions, blotted for the removal of surface water and weighed at specified time intervals. The swelling ratio, q, was calculated as follows:

$$\mathrm{q} = \frac{W_s}{W_d} \tag{4.4}$$

where W_s is the weight of swollen capsules and W_d is the weight of the dried capsules. The averages of three replicates for each combination were reported.

The water absorption data was analyzed according to the Higuchi equation, eqn (4.1); Korsmeyer-Peppas equation, eqn (4.2); and Peppas-Sahlin equation, eqn (4.3) using MATLAB version 6 R13. Non-linear least squares fitting method was used to determine the parameters in each equation.

4.3 Results and Discussion

4.3.1 Calibration curve

The optical density of the cells in suspension was found to be related to cell concentration by a factor of 4.0773 (Figure 4.1).

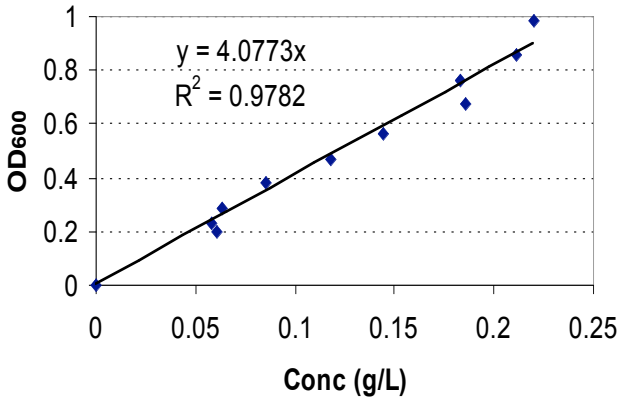


Figure 4.1: Calibration curve of *P. acidilactici*.

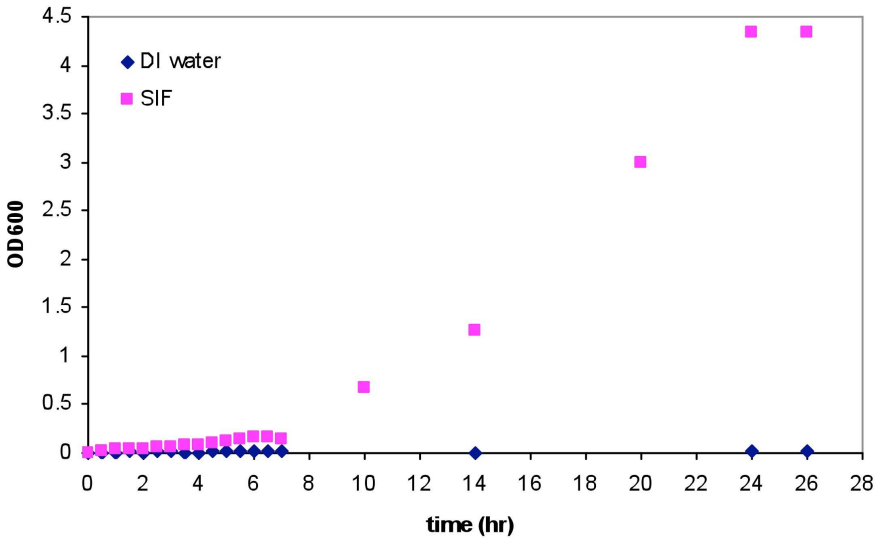
4.3.2 Kinetics of cell release

In swelling-controlled-release systems, solute transport through the polymer network is controlled by several physico-chemical phenomena such as polymer water uptake, gel layer formation, and polymeric chain relaxation [134, 135]. Delivery of the entrapped cells from hydrogel networks is a mass-transfer controlled process and depend on environmental factors such as pH, temperature, and ionic strength. The xanthan-chitosan capsules with different combinations were exposed to simulated gastric and intestinal fluid to study the cell release kinetics of the capsules under GI-tract conditions.

Figure 4.2 shows the cell release from xanthan-chitosan capsules (prepared from 0.7% [w/v] chitosan solution at pH 6.2 and 1% [w/v] xanthan solution) in DI-water, in SIF and in SGF at pH=1.0 and 2.0. The negligible release in DI-water for 72 hours (only first 26 hours is shown) indicates that capsules are stable in DI-water.

In SIF, the release from the capsules was very slow in the beginning and the release rate increased after 8 hours. Maximum release of the cells from the capsules was achieved after 24 hours in SIF. When capsules were suspended in SGF at pH=2.0, the cell release was in negligible amounts for 2 hours. However, cells were released rapidly in SGF at pH=1.0 and the OD values started to decrease after 2.5 hours, indicating cell death due to low pH. These results suggest that the pH of the SGF solution is more critical in determining the release properties of xanthan-chitosan capsules than the presence of enzyme (pepsin). The enzymatic resistance of the complex can be explained by the conformational changes that occur in chitosan after complexation with enzymatically resistant xanthan gum. These changes might make the sensitive sites of chitosan less accessible to hydrolytic action of the media [117].

Figure 4.3 shows the cell release profiles from xanthan-chitosan capsules for four different matrix formulations. Capsules were kept in SGF at pH 2.0 for 2 hours prior to be suspended in SIF at pH 6.8. For all combinations, the cell release in SGF was negligible. A time lag was observed in the early phase of the cell release from the capsules in SIF, indicating a highly-crosslinked network structure [136]. The time lag was longer for the capsules prepared from chitosan solution at pH 6.2 (60 min) than at pH 4.5 (30 min). Complete release of the encapsulated cells in SIF was achieved after 5.5 hours and 5 hours of exposure when initial chitosan solution pH was controlled at 6.2 and 4.5, respectively. Longer time lag and longer release time observed in the case of capsules prepared from chitosan solution at pH 6.2 suggests a more crosslinked hydrogel network compared to the membrane network of capsules prepared from chitosan solution at pH 4.5. These results are in agreement with the



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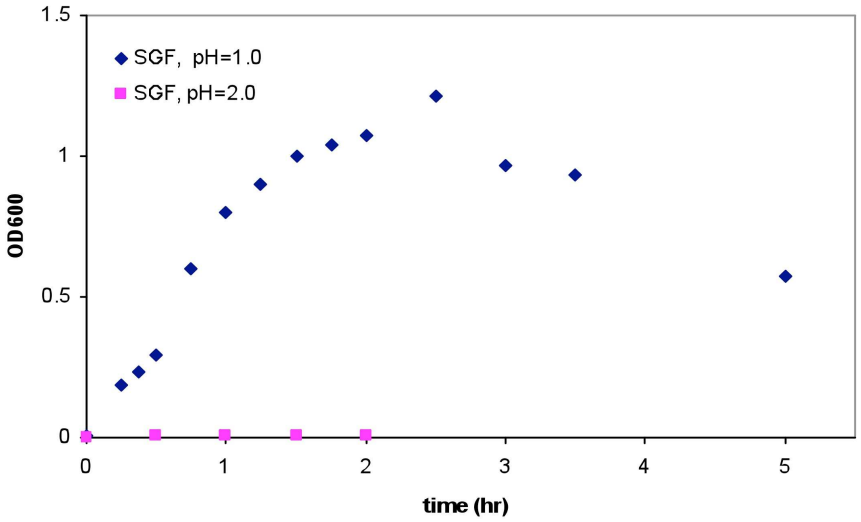


(b)

Figure 4.2: The release of *P. acidilactici* cells from xanthan- chitosan capsules (Chitosan: 0.7% (w/v), pH=6.2; Xanthan: 1.0% (w/v)) in (a) DI water and SIF; (b) in SGF at pH=1.0 and pH=2.0.

swelling degree experiments reported in Chapter 3.

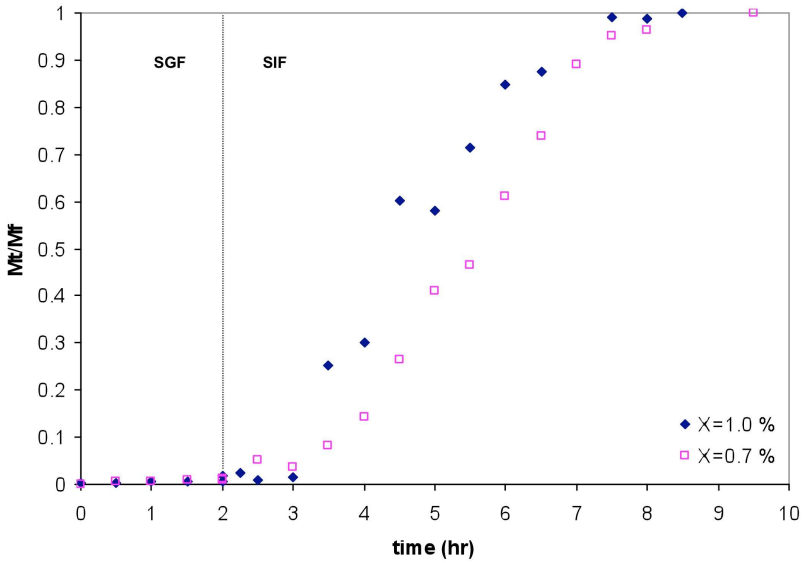
Table 4.1: Analysis of cell release data from xanthan-chitosan capsules using Higuchi eqn, Korsmeyer-Peppas eqn, and Peppas-Sahlin eqn.

Capsule combinations with 0.7% Ch	Higuchi eqn*		Korsmeyer-Peppas eqn*			Peppas-Sahlin eqn*		
	k' ($\text{min}^{-0.5}$)	r^2	n	k (min^{-n})	r^2	k_d ($\text{min}^{-0.43}$)	k_r ($\text{min}^{-0.86}$)	r^2
pH=6.2; X=0.7%	0.2374	0.7888	2.043	0.03598	0.9924	-0.5222**	0.473	0.9936
pH=6.2; X=1.0%	0.0432	0.8704	1.233	0.00098	0.9467	-0.044**	0.0116	0.94881
pH=4.5; X=0.7%	0.3304	0.9459	1.082	0.2422	0.9954	-0.1275**	0.3724	0.9946
pH=4.5; X=1.0%	0.1954	0.8878	1.284	0.1001	0.9959	-0.1646**	0.2624	0.9951

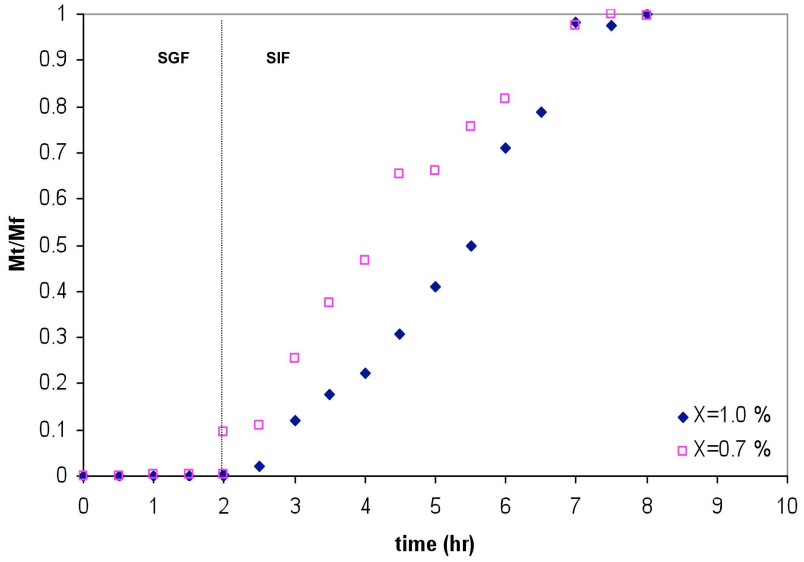
*k' ($\text{min}^{-0.5}$), Higuchi kinetic constant; k (min^{-n}), Korsmeyer-Peppas kinetic constant; k_d ($\text{min}^{-0.43}$), diffusional constant; k_r ($\text{min}^{-0.86}$), relaxational constant; r^2, correlation coefficient.

**The negative values obtained for k_d should be interpreted in terms of a diffusion process insignificant compared to the relaxation mechanism [137].

The portion of the cell release curve with a fractional release (Mt/Mf) between 0.1 and 0.6 was analyzed according to the Higuchi equation in (4.1); Korsmeyer-Peppas equation in (4.2); and Peppas-Sahlin equation in (4.3). The Higuchi model is applicable if the release is largely governed by diffusion through water-filled pores in the matrix. A good fit to Kormeyer-Peppas equation indicates the combined effect of diffusion and relaxation mechanisms for the release. For spherical systems, $n = 0.43$ indicates Fickian (diffusion controlled) transport, $0.43 < n < 0.85$ indicates non-Fickian (anomalous) transport and $n = 0.85$ implies Case II, zero-order (relaxation-controlled) transport [133,138]. Values of n higher than 0.85 for release from spheres are considered to be Super Case II kinetics [132]. The relative contribution of the diffusion and relaxation processes to the release mechanism can be analyzed by Peppas-Sahlin equation. The purely Fickian diffusion exponent, m, for xanthan-chitosan microcapsules corresponds to a value of 0.43 since spherical matrices present



(a)



(b)

Figure 4.3: Fractional release of *P. acidilactici* cells from xanthan-chitosan capsules in SGF at pH=2.0 and in SIF at pH 6.8. (a) Chitosan: 0.7 % (w/v), pH=6.2; Xanthan: 0.7% (w/v) and 1.0% (w/v); (b) Chitosan: 0.7% (w/v), pH=4.5; Xanthan: 0.7% (w/v) and 1.0% (w/v).

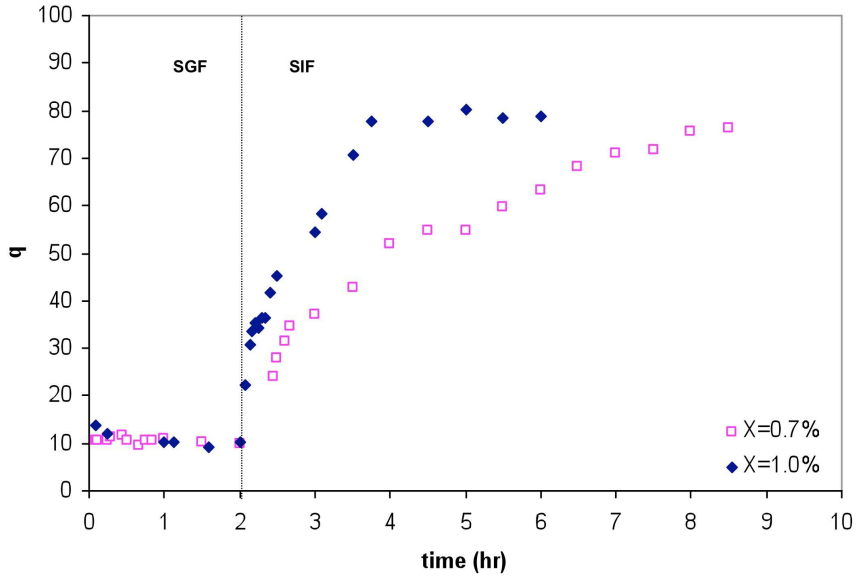
an aspect ratio of 1 [133]. The main parameter values from different equations are summarized in Table 4.1 to describe the mode of transport mechanism.

A good fit to Korsmeyer-Peppas ($n > 0.85$) and Peppas-Sahlin ($k_r \gg k_d$) equations suggests that the cell release from xanthan-chitosan capsules in SIF (after 2 hrs exposure to SGF) is controlled by polymer relaxation (Table 4.1). Since Higuchi model is only applicable to diffusion-controlled release mechanism, the release data showed a poor fit to this equation. The n values higher than 0.85 reveals a Super Case II transport mechanism for the release of cells from xanthan-chitosan capsules regardless of the combination used. Super Case II transport mechanism might result from an increased plasticization at the relaxing boundary (gel layer) due to a reduction of the attractive forces among polymeric chains [139, 140].

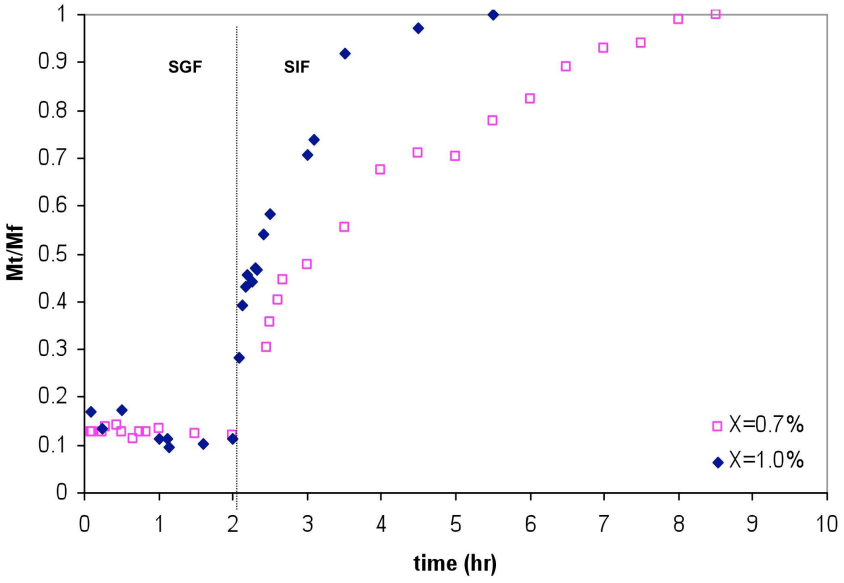
4.3.3 Dynamic swelling behavior

Xanthan-chitosan hydrogels exhibit pH-sensitive swelling characteristics due to the deionization of the functional groups in the hydrogel which significantly affects the penetrant transport mechanism of the polymer networks [115, 130]. Highly swollen hydrogels contain large amounts of unbound water which allows greater solute release [135]. The objective was to study the dynamic swelling behavior of xanthan-chitosan capsules under simulated GI-tract conditions.

Figure 4.4 shows the dynamic swelling of xanthan-chitosan capsules, prepared from 0.7% (w/v) chitosan at pH=6.2 and 0.7% (w/v) or 1.0% (w/v) xanthan in SGF at pH=2.0 and in SIF. The swelling ratio, q, was constant in SGF and increased



(a)



(b)

Figure 4.4: (a) Dynamic swelling ; (b) water absorption curve of xanthan-chitosan capsules (prepared from 0.7% [w/v] chitosan at pH=6.2 in combination with 0.7% and 1.0% [w/v] xanthan) in SGF at pH=2.0 and in SIF at pH 6.8.

rapidly for the first two hours in SIF for both combinations. This rapid swelling observed in the early stage of swelling also supports the findings in Section 4.3.2 suggesting that the rate-limiting factor for the cell release is chain relaxation. The capsules swelled to at least 80 times of their dry weights at equilibrium. While capsules with 1% (w/v) xanthan reached the equilibrium swelling ratio after two hours, at least 6 hours was needed for the capsules to reach equilibrium for capsules with 0.7% (w/v) xanthan gum.

The water absorption data was analyzed according to the Higuchi equation, eqn (4.1); Korsmeyer-Peppas equation, eqn (4.2); and Peppas-Sahlin equation, eqn (4.3). The main parameter values from different equations are summarized in Table 4.2. A good fit to Higuchi model, the low n values determined by Korsmeyer-Peppas equation and significantly higher values obtained for k_d compared to k_r by Peppas-Sahlin equation suggests that the swelling of xanthan-chitosan capsules in SIF (after 2 hrs exposure to SGF) is diffusion-controlled for both combinations.

Table 4.2: Analysis of dynamic swelling data for xanthan-chitosan capsules using Higuchi eqn, Korsmeyer-Peppas eqn, and Peppas-Sahlin eqn.

Capsule combinations with 0.7% Ch	Higuchi eqn*		Korsmeyer-Peppas eqn*			Peppas-Sahlin eqn*		
	k' ($\text{min}^{-0.5}$)	r^2	n	k (min^{-n})	r^2	k_d ($\text{min}^{-0.43}$)	k_r ($\text{min}^{-0.86}$)	r^2
pH=6.2; X=0.7%	0.0536	0.9792	0.3966	0.0094	0.9926	0.0863	-0.0007**	0.9971
pH=6.2; X=1.0%	0.0911	0.9537	0.326	0.1889	0.9826	0.158	-0.0057**	0.9859

*k' ($\text{min}^{-0.5}$), Higuchi kinetic constant; k (min^{-n}), Korsmeyer-Peppas kinetic constant; k_d ($\text{min}^{-0.43}$), diffusional constant; k_r ($\text{min}^{-0.86}$), relaxational conctant; r^2, correlation coefficient.

**The negative values obtained for k_r should be interpreted in terms of a relaxation mechanism insignificant compared to the diffusion process [137].

4.4 Conclusions

In swelling-controlled systems, the coupling of diffusion and macromolecular relaxation control the release mechanism. The xanthan-chitosan capsules were found to be stable in DI water. The pH of SGF was critical in determining the release properties of the capsules. The cell release in SGF at pH 2.0 was negligible suggesting that xanthan-chitosan capsules have a good potential for delivery of probiotic cells to intestines. The cell release from xanthan-chitosan capsules in SIF after 2 hr exposure to SGF at pH 2.0, exhibited a Super Case II transport mechanism ($n > 0.85$) regardless of the formulation used. The complete release of the cells from the capsules was achieved in at least 5 hours. Xanthan-chitosan capsules were found to swell by a diffusion-controlled mechanism. The rapid swelling observed also supported the findings that the cell release is relaxation-controlled ($k_r \gg k_d$).

Chapter 5

Protective effects of Xanthan-Chitosan Encapsulation on *P. acidilactici* cells

5.1 Introduction

For probiotic bacteria to be beneficial to the host, they should be able to survive gastric transit and reach the small intestine in sufficient numbers [26, 33]. The harsh environment of the GI-tract (the low pH conditions of the stomach and the presence of bile in the intestines) adversely affect the viability of probiotic cultures [37, 38]. In this study, *Pediococcus acidilactici* cells were encapsulated in xanthan-chitosan PEC gels to increase the survival rates during GI-tract transit.

Among the available techniques for encapsulating bacterial cells, entrapment in xanthan-chitosan capsules has not been used for the encapsulation of probiotic

bacteria. The only application of xanthan-chitosan PEC gels as microcarriers for bacterial cells was reported by Chu et al. [116] for the encapsulation of *Corynebacterium glutamicum*, a Gram (+) soil bacterium. The immobilization was carried out by mixing xanthan-chitosan PEC solution with the cell suspension and adding this mixture to distilled water through a syringe.

In this work, a simple and cost-effective extrusion method was used to encapsulate the probiotic cells. The goal was to determine the protective effects of encapsulation with xanthan gum and chitosan on *P. acidilactici* cells against freeze-drying and simulated gastric fluid at pH 2.0.

5.2 Materials and Methods

P. acidilactici cells were encapsulated as described in Section 4.2.2. Fifty milliliters of cell-xanthan mixture were dropped into 200 mL of chitosan solution by using a manually operated syringe to form the capsules. Freeze-dried capsules were subjected to simulated gastric fluid (SGF) at pH 2.0 for 1 hour and 2 hours at 37℃ under 150 rpm. Two different combinations (chitosan: 0.7% [w/v], pH 6.2; xanthan: 1.0% [w/v] and chitosan: 1.0% [w/v], pH 6.2; xanthan: 0.7% [w/v]) were used to encapsulate the bacteria. Capsules were filtered from SGF solution and suspended in SIF solution for 5 hours at 37℃ under 150 rpm to release the cells from the capsules. One milliliter aliquots from the release solutions were serially diluted and 3 replicates for each dilution were plated on MRS agar and incubated for 48 hours at 35℃. The experiments were repeated twice for each combination.

Smaller capsules were prepared by spraying the xanthan gum solution at 0.7% (w/v) through a 0.7 mm nozzle (Büchi B-290 Spray Dryer, Flawil, Switzerland) into chitosan solutions at 3 different concentrations (0.4, 0.7, and 1.0 % [w/v]) and 2 different pH values (4.5 and 6.2). Moreover, the effect of increasing xanthan concentration from 0.7 to 1.5% (w/v) on the protective properties of the capsules was studied. Capsules were subjected to SGF at pH 2.0 for 1 hour and cells were released from capsules in SIF solution. One milliliter aliquots from the release solutions were serially diluted and 3 replicates for each dilution were plated on MRS agar and incubated for 48 hours at 35℃. The experiments were repeated three times for each combination.

Free cells were mixed with 10% (w/v) skim milk (9:1 v/v), transferred to freeze-drying flask and frozen in dry ice before connected to the freeze dryer (ThermoSavant, Holbrook, NY). Freeze-dried cells were suspended in SGF for 1 hour and 2 hours under 150 rpm. One milliliter samples from free-cell solution were serially diluted and 3 replicates for each dilution were plated on MRS agar and incubated for 48 hours at 35℃.

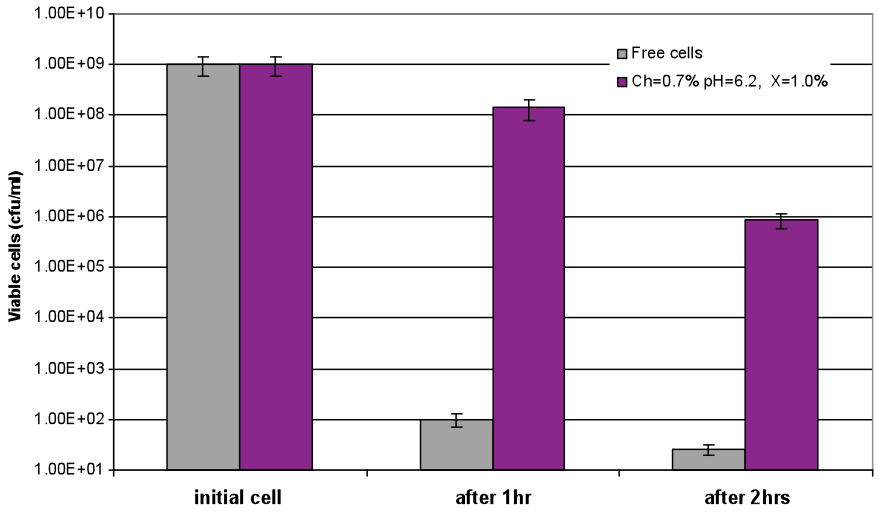
5.3 Results and Discussion

P. acidilactici are used in dietary supplements and in animal feed for their probiotic effects. They can produce large amounts of lactic acid that helps keep a proper balance of microflora in the digestive system [141]. Nevertheless, to act as a probiotic, the bacteria must survive in acidic stomach conditions and arrive in

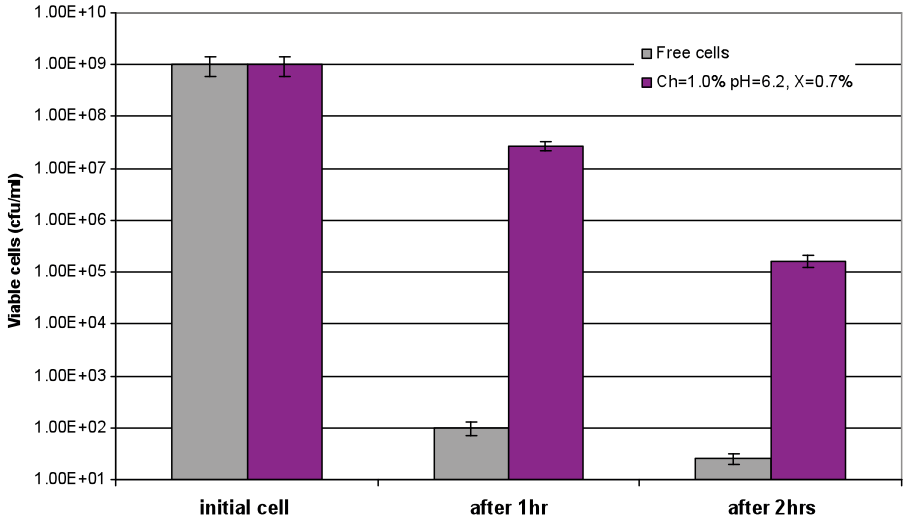
the intestines in high numbers. Gastric juice has a very low pH of 0.9 in healthy adults. However, the presence of food may raise the pH value to 3.0 and it takes 2 to 4 hours for stomach to empty after ingestion of food [142]. In this work, free and encapsulated *P. acidilactici* cells were subjected to SGF at pH=2.0 for 1 hr or 2 hours at 37℃ with moderate shaking to evaluate the effect of encapsulation on the survival of *P. acidilactici* cells in stomach conditions.

Two different combinations based on the swelling degree and DSC studies (Chapter 3) were chosen to encapsulate the bacteria in the capsules prepared by using a syringe. The capsules formed with these combinations were highly crosslinked with relatively small swelling degrees (Table 3.1). Xanthan-chitosan capsules prepared from 0.7% (w/v) chitosan at pH 6.2 and 1.0% (w/v) xanthan showed six-log preservation of *P. acidilactici* cells over free suspending cells after exposed to SGF (pH=2.0) for 1 hr and over four-log retention when exposure time was 2 hours (Figure 5.1-a). The protection effect of the capsules prepared from 1.0 (w/v) chitosan at pH 6.2 and 0.7% (w/v) xanthan was more than five logs and three logs after 1hr and 2 hours in SGF, respectively (Figure 5.1-b).

The resistance of lactic acid bacteria to freeze-drying and low pH is strain specific. It has been reported that survival rates of *P. acidilactici* in simulated gastric juice (SGF) is similar to the survival rates of *Lactococcus lactis* subsp. *lactis* and slightly lower than *Lactobacillus casei* subsp. *casei* [143]. Xanthan-chitosan microcapsules were found to provide better protection to *P. acidilactici* cells against freeze-drying and SGF than the protection provided by alginate capsules to *L. casei* cells [144, 145].



(a)



(b)

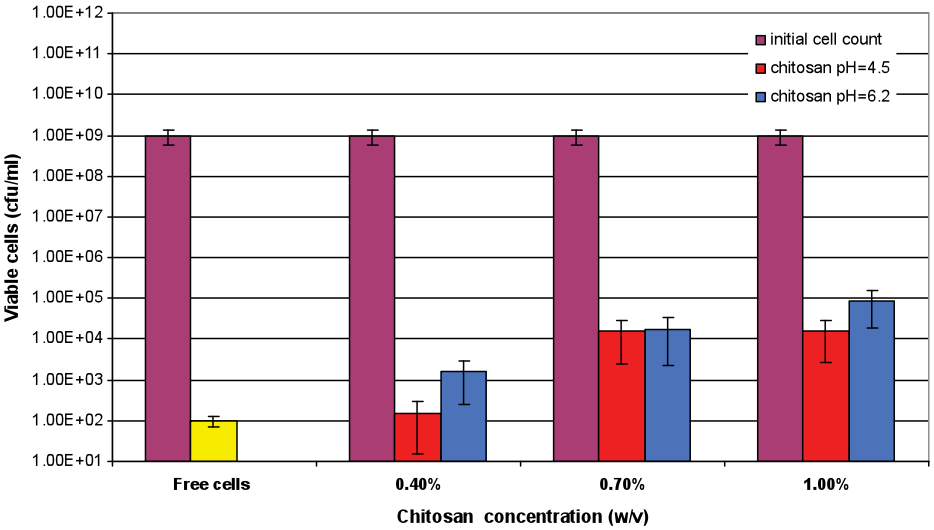
Figure 5.1: Protective effects of encapsulation on the viability of *P.acidilactici* cells against freeze-drying and SGF (pH=2.0) exposure (n=2) using (a) 0.7% and 1.0% chitosan.

The higher protection observed with the capsules prepared from 0.7% (w/v) chitosan at pH 6.2 and 1.0% (w/v) xanthan might be attributed to higher concentration of xanthan gum used compared to the other combination. Xanthan gum is known to be enzymatically resistant and is stable at low pH in contrast to other gums possessing carboxylic acid function, which are greatly influenced by pH [70, 71, 117, 146]. Sun and Griffiths [51] also reported that encapsulation of bifidobacteria in xanthan-gellan capsules increased their tolerance of high acid environments compared to alginate encapsulation.

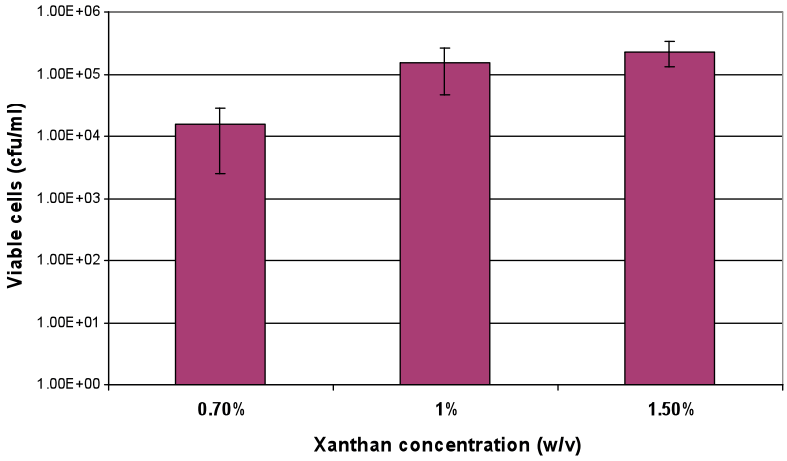
Smaller size capsules were prepared by using a 0.7-mm nozzle to see the effect of capsule size on the protective properties of xanthan-chitosan capsules (Figure 5.2).

Figure 5.2: Relative size of capsules prepared by using a syringe and a nozzle.

Although encapsulation in smaller capsules provided some protection against freeze-drying and low pH, protective effects observed in the case of smaller capsules (Figure 5.3-a) were significantly lower than that of provided by larger capsules (Figure 5.1). For instance, when capsules were prepared from 1.0% (w/v) chitosan at pH 6.2 and 0.7% (w/v) xanthan gum, encapsulation in larger capsules improved the cell



(a)



(b)

Figure 5.3: Protective effect of xanthan-chitosan capsules prepared by using a 0.7-mm nozzle on the viability of *P. acidilactici* cells against freeze-drying and SGF exposure for 1 hr. (a) Effect of initial chitosan solution concentration and pH (Xanthan= 0.7 % (w/v), n=3), (b)Effect of initial xanthan gum concentration (Chitosan=0.7 % (w/v) & pH 4.5, n=3).

viability at least three logs compared to the encapsulation in small capsules. Similar results have been reported by other researchers. Lee and Heo [45] reported that survival of bacterial cells under in vitro gastric conditions decreased with decreasing capsule size (diameter 1-2.6 mm). Sheu and Marshall [21] indicated that larger bead diameters provided more protection for *Lactobacillus bulgaricus* in frozen desserts. Moreover, it has been reported that small beads of size less than 100 μm did not significantly protect the probiotic bacteria in simulated gastric juice compared to free cells [147]. Finally, the effect of increasing initial xanthan concentration on the protective properties of xanthan-chitosan capsules was studied for the capsules prepared by nozzle. It was found that increasing initial xanthan gum concentration from 0.7 to 1.5% (w/v) only slightly improved the cell viability when chitosan solution concentration and pH were 0.7% (w/v) and 4.5, respectively (Figure 5.3-b).

These results suggest that xanthan-chitosan hydrogels have a promising protective mechanism for probiotic preservation.

5.4 Conclusions

Encapsulation of probiotic bacteria in capsules composed of xanthan gum and chitosan increased the survival rate of the cells during freeze-drying and in simulated gastric fluid compared to non-encapsulated cells. The viability of freeze-dried *P. acidilactici* cells in simulated gastric conditions was found to increase with increased capsule size. This approach might be useful in delivery of probiotic cultures to the GI-tract in high numbers to benefit from the health promoting effects of probiotic

cells.

Chapter 6

Conclusions

In this dissertation, a microencapsulation system using polyelectrolyte complex gels formed by xanthan gum and chitosan was developed to sustain the viability of probiotic bacteria in simulated gastrointestinal tract conditions. First, the effects of initial polymer concentration and chitosan solution pH on the crosslinking density of the capsule network were investigated by swelling studies and modulated differential scanning calorimetry analysis. Xanthan concentration was found to be the most critical parameter in xanthan-chitosan network formation. Conformational changes of chitosan polymer chains as pH approaches to 6.2 were critical in determining the crosslinked network structure. Swelling degree was less dependent on chitosan concentration than xanthan concentration and chitosan solution pH. Results from this study showed that when initial xanthan concentration was 1.5% (w/v) complete crosslinking can be achieved at all conditions studied. However, it was also possible to obtain a completely crosslinked network at 1% (w/v) xanthan when chitosan solution pH was 6.2.

In the second part of this dissertation, kinetics of cell release from highly crosslinked xanthan-chitosan capsules was studied under different conditions. It was found that xanthan-chitosan capsules were stable in DI water and cell release was negligible in simulated gastric fluid (SGF) at pH 2.0 for 2 hrs. These findings suggest that xanthan-chitosan capsules have a good potential for the delivery of probiotic cells to intestines with minimum damage to the cells in stomach conditions. The cell release from xanthan-chitosan capsules in simulated intestinal fluid (after 2 hrs exposure to SGF at pH 2.0) was found to be relaxation-controlled and exhibited Super Case II transport mechanism. The maximum cell release was achieved at least in 5 hrs. This rapid release in intestinal conditions was desirable for the colonization of the cells in the intestines.

Finally, the protection effects of capsules on probiotic cells against freeze-drying and simulated gastric fluid at pH 2.0 were investigated. Capsules with high crosslinking densities were chosen to encapsulate *P. acidilactici* cells. Xanthan-chitosan capsules showed up to six-log and four-log preservation of probiotic cells in SGF after 1 hr and 2 hrs, respectively. The viability of the cells was found to increase with the increase in capsule size.

In conclusion, encapsulation of probiotic bacteria in xanthan-chitosan capsules might be useful in delivery of probiotic cultures to the intestines in high numbers to benefit from their health promoting effects. Based on the results reported in this study, capsules prepared from 0.7% (w/v) chitosan at pH 6.2 and 1.0% (w/v) xanthan gum might be suggested as the optimum combinations to encapsulate the probiotic cells.

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