ประสิทธิภาพของแซคคาโรไมเซส ซีรีวิซิอี้ เคลือบอัลจิเนท ในการต้านเชื้อสเตรปโตคอกคัส อะกาแลคติอี้ ที่แยกได้จากปลานิลในห้องทดลอง

นางสาวคมเคียว พิณพิมาย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
ปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยา ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

IN VITRO EFFICACY OF ALGINATE-ENCAPSULATED *SACCHAROMYCES CEREVISIAE* AGAINST *STREPTOCOCCUS AGALACTIAE* ISOLATED FROM NILE TILAPIA

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Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program

in Veterinary Pathobiology

Department of Pathology

Faculty of Veterinary Science

Chulalongkorn University

Academic Year 2011

Thesis Title	*IN VITRO* EFFICACY OF ALGINATE-ENCAPSULATED *SACCHAROMYCES CEREVISIAE* AGAINST *STREPTOCOCCUS AGALACTIAE* ISOLATED FROM NILE TILAPIA
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คมเคียว พิณพิมาย: ประสิทธิภาพของแซคคาโรไมเซส ซีรีวิซิอี้ เคลือบอัลจิเนท ในการต้านเชื้อสเตรปโตคอกคัส อะกาแลคติอี้ ที่แยกได้จากปลานิลในห้องทดลอง (*In vitro* efficacy of alginate-encapsulated *Saccharomyces cerevisiae* against *Streptococcus agalactiae* isolated from Nile tilapia) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.น.สพ.ดร. นพดล พิฬารัตน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.น.สพ.ดร. ชาณรงค์ รอดคำ, รศ.สพ.ญ.ดร. อรัญญา พรพลพิสิฐ, 42 หน้า

โปรไบโอติกเป็นสารเสริมอาหารที่ได้รับความนิยมในอุตสาหกรรมการเลี้ยงปลานิล โดยในการศึกษาครั้งนี้นำการเคลือบโปรไบโอติกด้วยอัลจิเนทมาทดสอบประสิทธิภาพในการมีชีวิตรอดของโปรไบโอติกในการเก็บรักษาและการทนสภาวะต่างๆที่ไม่เหมาะสม เชื้อโปรไบโอติก แซคคาโรไมเซส ซีรีวิซิอี้ โดยกลุ่ม A นำไปผสมกับสารละลายเปปโตน กลุ่ม B นำไปผสมกับ 10% ของนมพร่องมันเนย แล้วนำไปเคลือบด้วยอัลจิเนท โดยวิธีหยดผ่านเข็มฉีดยาและทำให้แห้งในขณะเย็น ในการทำให้แห้งจำนวนที่มีชีวิตของแซคคาโรไมเซส ซีรีวิซิอี้ ในกลุ่ม B เหลือมากกว่าในกลุ่ม A อย่างมีนัยสำคัญทางสถิติ หลังจากเก็บไว้ที่อุณหภูมิห้องเป็นเวลา 7 และ 14 วัน จำนวนเชื้อที่มีชีวิตในกลุ่ม B มีมากกว่าในกลุ่ม A ตัวแคปซูลในทั้งสองกลุ่มมีรูปร่างกลมถึงรี โดยที่กลุ่ม A มีลักษณะพื้นผิวขรุขระและยุบตัวลงไปเป็นสัน ในขณะที่กลุ่ม B มีพื้นที่ผิวขรุขระและไม่ยุบตัวลงไปเป็นสัน เปอร์เซนต์การรอดชีวิตของทั้งสองกลุ่มในการทดสอบความทนทานในสภาวะกรด มีจำนวนเชื้อมีชีวิต เหลือรอดมากกว่ากลุ่มควบคุม โดยที่ในกลุ่ม B มีจำนวนเชื้อมีชีวิตเหลือรอดมากที่สุด ในการทดสอบใน 10% ของน้ำดีของปลานิล กลุ่มทดลอง A และกลุ่มทดลอง B มีจำนวนยีสต์ที่มีชีวิตมากกว่ากลุ่มควบคุม โดยที่กลุ่ม B มีจำนวนเชื้อมีชีวิตเหลือรอดมากกว่าทั้งสองกลุ่มอย่างมีนัยสำคัญทางสถิติ และเชื้อในกลุ่มทดลอง A และ B มีความสามารถในการต้านเชื้อสเตรปโตคอกคัส อะกาแลกติอี้ จำนวน 17 สายพันธุ์จากทั้งหมด 25 สายพันธุ์ จากผลการทดลองทั้งหมดบ่งชี้ว่าการเคลือบเชื้อแซคคาโรไมเซส ซีรีวิซิอี้ด้วย อัลจิเนทช่วยทำให้เชื้อมีชีวิตรอดมากขึ้นในการทำให้แห้งในขณะเย็น ในการเก็บรักษา และการอยู่ในสภาวะกรดและน้ำดี

ภาควิชาพยาธิวิทยา ลายมือชื่อนิสิต.....
สาขาวิชาพยาธิชีววิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....
ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5275555031: MAJOR VETERINARY PATHOBIOLOGY

KEYWORDS: PROBIOTIC / *S.CEREVISIAE* / ENCAPSULATION / ALGINATE

KOMKIEW PINPIMAI: *IN VITRO* EFFICACY OF ALGINATE-ENCAPSULATED *SACCHAROMYCES CEREVISIAE* AGAINST *STREPTOCOCCUS AGALACTIAE* ISOLATED FROM NILE TILAPIA, ADVISOR: ASST. PROF. NOPADON PIRARAT, DVM, Ph.D., CO-ADVISOR: ASST. PROF. CHANNARONG RODCHUM, DVM, Ph.D., ASSOC. PROF. ARANYA PONPORNPISIT, DVM, Ph.D., 42 pp.

Probiotic is a dietary supplement that has been used in tilapia culture. In this study the microencapsulation technique was applied to improve the viability of probiotic during storage and unflavored condition. The probiotic, *Saccharomyces cerevisiae* were mixed with Peptone dilution saline (group A) or 10% skim milk (group B), encapsulated with alginate via extrusion method and freeze-dried. The viability of group B after being freeze-dried was significantly higher than group A. At 7 and 14 days after being kept in room temperature, the viability of group B was higher than group A. The morphologies of the two groups of microencapsules were round to oval in shape. Group A had rough with concave polygonal ridges surface while group B had rough surface. The survival percent of both groups were higher than the control group after being exposed to NSS pH 1.5 for 1, 2, 3, and 4 h and group B was higher than group A. The survival percent of group B after being exposed to 10% tilapia bile salts was significantly higher than the control group and group A in 1, 2 and 3h. The survival percent of group A was lower than group B, but higher than the control group in every hour of exposer to 10% tilapia bile salts. Free *S. cerevisiae*, group A and group B had similar antimicrobial activity to 17 from 25 strains of *Streptococcus agalactiae*. All the results have suggested that the encapsulation of *S. cerevisiae* with alginate can improve the viability during freeze-drying and storage. Moreover, it can also confer the effect of protection in acid and bile environment.

Department : Veterinary Pathology
Field of Study : Veterinary Pathobiology
Academic Year : 2011

Student's Signature…….
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ACKNOWLEGEMENTS

I am greatly indebted to my principal adviser Asst. Prof. Dr. Nopadon Pirarat. The opportunity to study under his supervision has been most rewarding. Throughout this research, Dr. Nopadon personal guidance, understanding and encouragement have been deeply appreciated. I would also like to express my gratitude to my co-advisor Asst. Prof. Dr. Channarong Rodkhum and Assoc. Prof. Dr. Aranya Ponpornpisit for their advice and support during the course of my master degree program.

I would like to thank all of the laboratory technicians in department of Veterinary Microbiology and Pathology for their technical help and assistance with my chemical and instrumental needs. I am also thankful to my friends in Pathobiology program and friends from Chulalongkorn University music club for their support and assistance during this research project.

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LIST OF ABBREVIATIONS

cfu	colony forming unit
g	gram
h	hour
log	common logarithm
mm	millimeter
mL	milliliter
NSS	normal saline solution
PCR	polymerase chain reaction
PDS	Peptone dilution saline
S. agalactiae	*Streptococcus agalactiae*
S. cerevisiae	*Saccharomyces cerevisiae*
SDA	Sabouraud Dextrose agar
SDB	Sabouraud Dextrose broth
SEM	scanning electron microscope

CHAPTER I

INTRODUCTION

In Thailand, aquaculture continues to grow and add to the size and diversity of the country's economy. Nile tilapia is one of the most popular species for farming because Nile tilapias are the easiest and most cost-effective fish to farm (FAO Fisheries & Aquaculture *Oreochromis niloticus*, 2010). Tilapia farms are expanding in various parts of the country and intensive culture systems have been developed (FAO Fisheries & Aquaculture Thailand, 2006). In large-scale production, where aquatic animals are exposed to stressful conditions, outbreaks of infection become a significant problem resulting in serious economic losses. Streptococcosis in tilapias is a major cause of the economic losses due to high morbidity and mortality (Bromage and Owens, 2002). Antibiotics are commonly used to control this problem, but many complications often follow such as residual drugs and drug resistance, which greatly concern the consumer (Agnew and Barnes, 2007). Although vaccines are also available for Streptococcosis, vaccination of fish is limited by the number of fish, the cost involved, as well as the difficulty in administering the vaccine. Alternative methods for controlling infectious diseases are being investigated.

Various technologies, chemicals, and drugs are used to increase the production of fish to respond to the demand of the market. However, inappropriate use of these brings about environmental degradation, drug residue in produce and an increase in antimicrobial resistance in bacteria. An alternative aquaculture that is free from chemicals and drugs has been developed to replace the old ways of farming. Probiotics, beneficial microorganisms, are suitable candidates in controlling infectious diseases and in promoting growth performance in fish. At the same time they are friendlier to the environment and consumers. Different types and strains of microorganisms that are used as probiotics, naturally, give different results in hosts due to their composition, ability in producing inhibitory compounds, adhesion on gut epithelium as well as in competition for nutrients. One ambiguous issue, however, is the transfer of the antibiotic resistance gene that appears among the groups of bacteria. Some research shows that probiotic bacteria are also a reservoir of antibiotic resistance genes. Yeasts, another group of microorganism used as probiotics might be a good

solution because they are not involved in this process. Moreover, the cell wall of yeast comprises of phosphopetidomannan or phospholipomannan in the outer layer and chitin and 1,3-beta and 1,6-beta-glucan in the inner layer. They are all of pathogen-associated molecular pattern (PAMP) that stimulates the immunity via pattern-recognition receptors (PRRs).

In food animals, probiotics must be safe not only for the animals themselves but also for humans. Microorganisms that have been used in the food and drink industry e.g. baking and brewing are therefore good candidates. *Saccharomyces cerevisiae* is a species of budding yeasts that can normally be found on fruit skin such as grapes'. It is also found in the gastrointestinal tracts of humans and fish as normal flora (Gatesoupe, 2007). It has been used for food processing in the beer brewing and bread baking industries. In aquaculture, the studies of the extraction or inactive whole cells of *S. cerevisiae* give positive results in controlling infectious diseases caused by bacteria such as *Aeromonas hydrophilla* through stimulating innate immunity (Siwicki et al., 1994). *S. cerevisiae* can improve growth performance in numerous species of fish such as rainbow trout, striped bass and Galilee Tilapia (Wachè et al., 2006; Abdel-Tawwab et al., 2008; 2010). It can also reduce copper absorption and accumulation in Galilee Tilapia (Abdel-Tawwab et al., 2010).

Like other food supplements, to utilise the best effects of probiotics, an appropriate dose is necessary. However, the viability of probiotics in food products and in the action site (intestine) is still difficult to quantify because the viability of probiotics can be lost in unfavorable environments. To improve the probability of the survival of probiotics, microencapsulation technology is applied. Microencapsulation has been broadly used in many industries such as printing, chemical, textile and food industries. It is also used for pharmaceutical and medical purposes. Microencapsulation of probiotics with hydrocolloid beads has been proved to increase the survival in food products and in gastrointestinal conditions. Alginate, part of the cell wall of brown algae, is the best known material that is used for entrapping probiotics. Alginate is non-toxic to the probiotic cells and safe for the consumer. It can improve the survival of probiotics up to 80-95% (Mandal et al., 2006). Previous studies show that the concentration of alginate, concentration of calcium chloride and hardening time are the main factors that influence the characteristics of the capsule. The study of Chandramouli et al. (2004) reports a

significantly increased survival of probiotic cells under gastric conditions (*in vitro* study) by using 1.8% alginate, 30 min hardening in 0.1 M $CaCl_2$ and capsule size of 450μm with 10^9 CFU/mL initial cell load. On the other hand, several studies revealed that using alginate alone could not protect probiotic cells from high acid environments. Blending alginate with other materials such as starch and poly-*L*-lysine improved the protective ability of microencapsule (Krasaekoopt et al., 2003). Though a lot of *in vitro* studies revealed satisfactory viability of probiotics under human or land animal gastric conditions, applicable information on *in vitro* studies in aquatic animals is still limited and almost non-existent.

This study was conducted to develop and investigate an appropriate encapsulation technique for *Saccharomyces cerevisiae* to apply in tilapia culture and to determine the efficacy of encapsulated *S. cerevisiae* to fight against *S. agalactiae.*

CHAPTER II

LITERATURE REVIEW

1. Tilapia

Tilapias are tropical freshwater cichlid fish, native to Africa and the Middle East. *Oreochromis niloticus* or Nile tilapia is one of the most popular species that has been recognized as a food source for rural communities and aquaculture industry worldwide (FAO Fisheries & Aquaculture Thailand, 2006). They were introduced to Thailand in 1965 from Japan (FAO *Oreochromis niloticus*, 2010) and since then have been developed for aquaculture. Tilapias have many interesting characteristics, making them suitable for farming. They are omnivorous - eating a variety of natural food organisms (Dong et al., 2010). They can tolerate poor water quality including high water temperature, high water salinity, low dissolved oxygen and high concentration of ammonia (Prunet and Bornancin 1989; Atwood et al., 2003; Sardella et al., 2004). They can also tolerate the stress resulting from high stock density (Siddiqui et al., 1989). They grow and attain market size rapidly and finally, they are palatable (FAO Fisheries & Aquaculture Thailand, 2006). Due to the palatability, tilapia markets all over the world have been expanding in recent years at the same time as tilapia farms have. Tilapia culture has become the most important aquaculture crop in many countries including Thailand. With new research and technology, certainly, room for more development and expansion concerning tilapia aquaculture exists.

2. Streptococcosis

Streptococosis is a disease caused by *Streptococcus sp.,* which is a Gram-positive, encapsulated, facultative anaerobic cocci bacterium in pairs or chains. The bacteria in this genus cause serious diseases in a number of different hosts such as mammals, fish and also humans. In aquatic animals, the disease can occur in both wild and cultured fish. In 1957, the first identification of *Streptococcus* sp. in cultured fish (rainbow trout) in Japan was reported (Hoshina et al., 1958). The bacteria have been identified from various parts of the world particularly in intensive culture. Moreover, an outbreak of infection is always followed by a serious economic loss due to high morbidity and mortality (Bromage and Owens, 2002). *S. agalactiae* and *S. iniae* are the two species that are mostly reported. Both of them bring about similar clinical signs - spiral or erratic swimming, ocular abnormalities such as peri-orbital and intraocular haemorrhage, opacity and exophthalmia, reddening or haemorrhage in the integumental and musculoskeletal systems, ascites and ulceration (Bromage and Owens, 2002). However, in some cases the fish may not show any clinical signs before death. Traditional classification of Streptococci is based on serogrouping of carbohydrate antigens of cell wall and their hemolytic activities. *S. agalactiae* is a group B Streptococcus that can be either hemolytic or non-hemolytic on a blood agar plate. It has been recognized as the cause of mastitis in bovines, and of neonatal meningitis, sepsis, and pneumonia in humans. Although *S. agalactiae* is found in humans and animals, zoonotic transmission seems to be non-existent or insignificant (Robinson and Meyer, 1966). *S. iniae* is a non-group *Streptococcus* and is always hemolytic on a blood agar plate. *S. iniae* was first identified from multiple subcutaneous abscesses in freshwater dolphins. Like *S. agalactiae*, they can cause diseases in fish, mammals, and also in humans, but in contrast, *S. iniae* is zoonotic. *S. iniae* infections in humans can occur mainly in people with skin injuries caused by handling live or fresh fish. In humans, it brings about the development of cellulitis, which is occasionally localized in organs or joints (Facklam et al., 2002). Streptococcosis can infect fish via contaminated water. It can also infect fish via the oral route through contaminated food and cannibalism of infected fish. The conventional methods to prevent and control Streptococcosis are chemotherapy and vaccination. Regarding vaccination, there were reports of vaccination of *S. iniae* in rainbow trout farms from 1995 to 1997 with good

results in decreasing the mortality rate from 50% to 5%, but shortly after the vaccinations, new massive outbreaks occurred (Bachrachet al., 2001). Until now, there has not been any commercial vaccine that can give satisfactory results. Although using antibiotics is an effective way for treating infected fish, drug resistance usually happens amongst dense populations (Agnew and Barnes, 2007). The use of probiotics is an interesting technique that has been focused on since then. Several studies revealed the efficacy of probiotics in controlling Streptococcosis.

3. Probiotics used in aquaculture

An alternative way to control infection by using beneficial microorganism has attracted a lot of attention. Probiotics are microbial cell preparations or components of microbial cells adjunct, which beneficially affect the host animals (Fuller, 1989; Salminen et al., 1999). In aquaculture, Verschuere et al. (2000) gave a broader definition of probiotics as a live microbial adjunct, which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards diseases, or by improving the quality of its surroundings i.e. the environment of aquatic animals in which host and pathogen share the same ecosystem. The general modes of action of probiotics are modulating host immunity, enhancing digestion, providing minerals and vitamins for the host, inhibiting pathogens, producing inhibitory compounds, or competing for chemicals, energy or adhesion sites.

Since aquatic animals have different physiology from terrestrial animals and live in different environment, so the criteria in selecting probiotics for them are rather different from for terrestrial animals and humans.

3.1 Selection of probiotics

3.1.1 Safety

The safety is the first consideration in using probiotics. Among terrestrial animals, the probiotics might not be pathogens for hosts but in water, they can be harmful to other aquatic animals. This is a concern because in aquatic system, aquatic animals and pathogens share the same ecosystem (Verschuere et al., 2000). In addition, in food animals, probiotics must be safe not only for the animals, they also have to be safe for humans. For this reason, the concept of using probiotics derived from human and microorganisms in human food and drink industries are now widely accepted.

Furthermore, the mentioned safety is not only for the animals or humans but also for the environment. Probiotic strains should not contain any antibiotic resistance genes that can be transferred to other bacteria (Czerucka et al., 2007). Therefore, we see the emergence of antimicrobial drugs against pathogen bacteria in markets. In fact, a recent study reported that some strains of lactic acid bacteria, might act as reservoirs of antimicrobial resistance genes (Czerucka et al., 2007). In addition, transferring of genes

that occur only among bacteria, making yeasts a good candidate.

3.1.2 Survival in gastrointestinal tract

The important limitation of using probiotics is the viability of probiotics during their passing through gastrointestinal tract to the action site. From oral cavity to intestine, probiotics have to pass through several unfavorable environments such as low pH in fish stomach, ranging from 1 – 3.2 up depending on species. Previous studies reported that the pH of tilapia stomach is below 2 since the acid helps to break down the plant cell walls, which are a part of their feed (Nagase, 1964; Moriarty, 1973). Into proximal intestine the pH is ranged from around 6 – 7 but the bile salts from the gall bladder can also affect the viability of probiotics (Fish, 1960). Several *in vitro* studies reveal the significant loss of viability in probiotics. The microencapsulation technique that has been used in drug delivery is, therefore, applied to solve this problem.

3.1.3 Adhesion/Colonization

Adhesion is the first step that probiotics do to promote the host health: their colonization, stimulate the immunity and their antagonistic activity against pathogens via competitive exclusion (Verschuere et al., 2000). Concepts of using probiotics that are isolated from fish are widely mentioned because each probiotic stain has individual specificity for adhesion. Several studies on probiotics aquaculture showed that some strains of probiotic derived from humans or certain animals could also adhere to the fish intestinal mucus (Verschuere et al., 2000).

4. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, a microorganism in the group of budding yeasts from the brewing industry has been widely used as probiotics in farm animals and humans. It belongs to the fungal kingdom: subdivision Ascomycotina. Its size is around 5-10 mm. It can grow well on glucose, fructose, mannose, galactose, sucrose, and maltose in 20-30 °C. It can be found as microflora in fish intestine and also in humans' (Gatesoupe, 2007). In aquaculture, it has been used for growth performance and enhancing immune function. Numerous studies reveal the efficacy in growth performance via enhancing digestive enzyme and providing nutrition including fiber, vitamin B and folic acid (Abdel-Tawwab et al., 2006; Wachè et al., 2006; Gatesoupe, 2007; Abdel-Tawwab et al., 2008). It is generally believed to be a great immune stimulant, thanks to its wall structure which contain polysaccharides (85%) and protein (15%) (Lesage and Bussey, 2006). The polysaccharides comprise of 90% glucose (beta 1, 3 and beta 1, 6 glucan), 1-2% N-acetylglucosamine and 10-20% mannose (Lesage and Bussey, 2006). It was reported to be able to protect against bacterial infectious diseases such as *Aeromonas hydrophila* by through both a cellular immune response and immunoglobulin in serum (Siwicki et al., 1994). Many studies reveal that the mechanisms that *S. cerevisiaie* use to control infectious diseases might not be just only the stimulation of host immune response but also production of extracellular proteases and siderophores that help to inhibit growth of pathogens (Gatesoupe, 2007). Furthermore, studies in rainbow trout suggest that some strains of *S. cerevisiae* can adhere to and colonize the intestine. This is one of the mechanisms that help hosts to protect themselves against pathogens (Vázquez-Juárez et al., 1997; Andlid et al., 1998).

Table 1 The used of *S. cerevisiae* as probiotic in aquaculture

Fish	Pathogen	Mode of action	
rainbow trout		enhance digestive enzyme	Wachè et al., 2006
hybrid striped bass	*Streptococcus iniae*	growth performance	Li and Gatlin, 2004
hybrid tilapia		growth performance immunostimulant	He et al., 2009
Nile tilapia	*Aeromonas hydrophila*	growth performance	Abdel-Tawwab et al., 2008
hybrid striped bass	*Mycobacterium marinum*	growth performance	Li and Gatlin, 2005
Nile tilapia	*Aeromonas hydrophila*	enhance immune response	El-Boshy et al., 2010
Nile tilapia	*Aeromonas hydrophila*	enhance immune response	Reque et al., 2009
gilthead sea bream		enhance immune response	Ortuno et al., 2002
Nile tilapia		growth performance	Lara-Flores et al., 2003

5. Microencapsulation

Gut microorganism is a key role in maintaining the host health. Alterations of the gut microbial population have been associated with variation of diseases. Recent studies indicate that administration probiotics give a positive effect on host health. However, one big limitation of using probiotics is the viability during pass through gastrointestinal tract and during storage. The microencapsulation technique is applied to improve the viability of probiotics. Microencapsulation is a technique during which a single or mixed particle is coated or entrapped with other material on a small scale (Bansode et al., 2010). For probiotic encapsulation, the polymer system is one of the most widely studied. It provides advantages not only in protecting probiotic cells from unfavorable environments in food processing and under gastrointestinal conditions but also in making the particle easier to handle and therefore easier to control the dosage (Anal and Singh, 2007). The most commonly used technique is extrusion and emulsion (Anal and Singh, 2007) via temperature control or ionic cross-linking with different materials as will be described further.

5.1 Microencapsulation technique for polymer system

5.1.1 Extrusion

This technique is easy to prepare and the process of encapsulation is gentle, ensuring the viability of probiotic cells. The core materials commonly used in this technique are alginate and chitosan. The technique begins with the preparation of the hydrocolloid solution that has already been mixed with probiotic cells. The process is followed by extruding the mixed solution through a nozzle at high pressure as droplets get into a hardening solution. A capsular wall forms around the droplets by ionic cross-linking between the polymers and hardening solution. The diameter of the nozzle and the distance of free-fall control the size and shape of the capsules (Krasaekoopt et al., 2003). A syringe is a basic piece of equipment that is often used in this technique. As the manufacturing process is scaled up, it becomes difficult to control the size of the capsules, hence equipment has been developed to control variables such as the vibration of the nozzles, the oaxial flow and the eltrostatic field strength. Multi-nozzle systems, rotating disc atomizers and jet-cutting techniques are applied to encapsulate probiotics for mass.

5.1.2 Emulsion

Comparing emulsion technique with extrusion technique, it appears that emulsion technique is easier to use on a large scale and the capsule size is smaller than using extrusion without extra equipment. However, emulsion technique costs more due to extra material: vegetable oil for emulsion formation. This technique can be used in combination with spray-drying technique. Emulsion technique can be separated into two phases. First, a small volume of the cell-polymer is added to a large volume of vegetable oil. The mixture is stirred until a water-in-oil formation forms. Then, by adding cross-linked solution (if alginate is the core material, $CaCl_2$ is the cross-linked solution), the water-soluble polymer becomes insoluble to form capsules during the oil phase. The size of the capsule is controlled by the speed of stirring; the size varies from 25μm to 2 mm (Krasaekoopt et al., 2003). According to some studies, adding emulsifiers such as tween 80 and lauryl sulphate leads to smaller capsule sizes because emulsifiers decrease the surface tension of the water and oil. Although this technique has been used successfully to encapsulate lactic acid bacteria (Lacroix et al., 1990), residual oil, emulsifier and surfactant in the capsule can be toxic to probiotic cells.

5.2 Main components used for microencapsulation of probiotics

5.2.1 Alginate

Alginate is a linear heteropolysaccharide of D-mannuronic (L) and L-guluronic acid (G) extracted from brown algae. It mainly consists of L-guluronic acid (G) and D-mannuronic acid (M). The composition and sequence of L-guluronic acid (G) and D-mannuronic acid (M) varies depending on sources of the algae. The capsule is formed by L-guluronic acid (G) bound with a divalent cat ion such as Ca and the length of D-mannuronic acid (M) is the main structure of the capsule. Alginate can be used in both extrusion and emulsion techniques. The hardening solution for the alginate system is $CaCl_2$. The extrusion technique diameter of the needle, the distance of free-fall viscosity of alginate, the concentration of alginate solution and $CaCl_2$ are the factors that influence the capsule sizes and shapes. Previous studies show that the capsule sizes that are less than 100 mm do not significantly protect the bacteria in simulated gastric fluid compared with free cells (Hansen et al., 2002). There are other options in using the emulsion technique with the alginate system, besides using $CaCl_2$ in the continuous

phase such as adding oil-soluble acid, e.g. acetic acid to reduce the alginate pH from 7.5 to 6.5 and initiate gel formation with Ca^{2+}. Calcium alginate capsules increase the viability of probiotics up to 80-95% (Mandal et al., 2006). The alginate system could be studied in various ways. Various materials such as starch, pectin and whey proteins can be blended with alginate solution to improve the viability of probiotic cells. Alginate "matrices" blended with probiotic compnents such as starch and oligosaccharide are proved to increase viability of probiotic cells (Jankowski et al., 1997, Chen et al., 2005).

Although encapsulating probiotics with alginate provides many advantages, the limitation is the stability of sodium in the alginate capsule. Chelating agents such as phosphate, lactate, and citrate share affinity for calcium and destabilize the capsule. The problem might occur with probiotics in a group of lactic acid bacteria during lactic acid fermentation (Krasaekoopt at el., 2003). Coating alginate capsules with other materials is applied to improve the integrity of the capsule. Chitosan, a positively charged polyamine, is one of coating materials that has been studied. Low-molecular-weight chitosan reduces 40% of cell release due to a semipermeable film around the negative charge (alginate) (Zhou et al., 1998). It would not dissolve in Ca^{2+} chelating agents, which help to stabilize the capsule. Poly l-lysine, a poly amino acid, is an interesting one that has been used to coat alginate capsule. Double coating with poly l-lysine and alginate can reduce cell release during milk fermentation by approximately 50 % (Champagne et al., 1992).

5.2.2 Chitosan

Chitosan, the N-deacetylated product of the polysaccharide chitin, can be isolated from crustacean shells, insect cuticles and the membranes of fungi, each of which gives different properties (Anal and Singh, 2007). Similar to alginate, chitosan uses ionic cross-linked (phosphates and polyaldehydro carbonic acid) to form a capsule. It was water soluble at pHs lower than 6 (Krasaekoopt at el., 2003) and exhibited inhibitory effects on different types of lactic acid bacteria (Groboillot et al., 1993). Consequently chitosan is not well accepted for use as the main capsule but it is used as an additional coating material on alginate capsules.

5.2.3 Carrageenan

Carrageenan is polysaccharide extract from marine macroalgae. A concentration of around 2%-5% at high temperature (60-80°C) is needed to dissolve carrageenan. The formation of the capsule from carrageenan (Anal and Singh, 2007) is induced by temperature change. First, the probiotic cell suspension is blended with sterilized carrageenan solution at 40–45°C. After cooling it to room temperature, the capsule is formed. Then KCl is added, K+ helps to stabilize the capsule and prevents it from swelling. However lactic acid bacteria such as *S. thermophilus* and *L. delbrueckii* spp. *Bulgaricus* may be affected by KCl (Krasaekoopt et al., 2003). Previous studies show that a mixture of locust bean gum and carrageenan increased strength of the capsule through interaction between galactomannan chains and carrageenan. It has been used in fermentation processes since it is unaffected by organic acids.

CHAPTER III

MATERRIALS & METHODS

1. Microencapsulation

1.1 Probiotic yeast

S. cerevisiae (JCM 7255) from glycerol stock that was kept at -80 °C and cultured in Sabouraud Dextrose agar (SDA) at 30°C for 24h, before transferred to 100 mL of Sabouraud Dextrose broth (SDB).

1.2 Microencapsulation

The probiotic, *S. cerevisiae* was cultured in SDB at 30 °C for 48 h, refrigerated centrifuged at 4500 x g at 4 °C for 15 min, and washed with sterile Peptone Dilution Saline (PDS) three times. The *S. cerevisiae* was encapsulated in two groups. The first group (group A) was prepared by resuspending the *S. cerevisiae* in PDS at a final concentration of 10^9cfu/mL The second group (group B) was prepared by resuspending the *S. cerevisiae* in 10% skim milk at a final concentration of 10^9cfu/mL Under sterile conditions, *S. cerevisiae* was encapsulated according to the method described by Chandramouli et al. (2004) with certain modifications. Alginate matrix was prepared by mixing 1.8% sterile sodium alginate solution with yeast suspension. The capsules were prepared aseptically by dropping the alginate mixture 30 cm from a 10-mL syringe through a 25G needle to sterile 0.1 M calcium chloride solutions. The capsules were hardened in 0.1 M $CaCl_2$ solutions for 30 min and washed with sterile distilled water. The yeast cells in calcium alginate capsules were determined the density.

2. Viability after being freeze-dried

To determine the loss of encapsulated yeast during freeze-dried process.

The calcium alginate capsules were freeze-dried by freezing at -80°C for 2 h followed by drying at -50 °C for 48 h. The numbers of yeast were determined before and after the freeze-dry process. The yeast cell densities were determined.

3. Viability during storage

To assess the viability of encapsulated yeast during storage at room temperature (25°C)

The encapsulated *S. cerevisiae* were kept at room temperature. 0, 7, and 14 days after drying, 0.5 g of capsules were resuspended in sodium citrate and then shook at room temperature for 30 minutes. The complete release of yeast cells was determined by serializing the dilution in PDS before plating on SDA (ISO 6887-1).

4. Morphology Analysis

The size and shape of microencapsules were determined by light and scanning electron microscope. Microcapsules were examined using Scanning Electron Microscope (SEM): JSM-5410LV (JEOL, Japan). The freeze-dried microcapsules were mounted on metal grids by using double-sided tape and coated with gold under vacuum.

5. Acid tolerance

Simulated gastric solution was prepared with normal saline pH 1.5 (adjust pH by using 5M HCl). 0.5g of each type of microcapsules were placed separately in test tubes containing 4.5mL simulated gastric solution then incubated at 25°C. Triplicate samples were taken after incubation at 25°C for 0, 1, 2, 3 and 4 h. The cell counts of encapsulated yeast were enumerated on SDA to determine the survival of free and encapsulated yeast in acid environment.

6. Bile tolerance

For bile salt tolerance, 0.5 g of each type of microcapsules were placed separately in test tubes, containing 4.5 mL simulated gastric solution at 25°C. After incubation for 60 min. the simulated gastric solutions were removed and the microcapsules were placed in 10% tilapia bile salts (Nikoskelainen et al., 2001). Triplicate samples were withdrawn after incubation at 30°C for 1, 2, and 3 h. The cell counts of encapsulated yeast were enumerated on SDA agar.

7. Antimicrobial activity

The antimicrobial activity of fresh *S. cerevisiae*, encapsulated *S. cerevisiae* in PDS solution and encapsulated *S. cerevisiae* in 10% skim milk solution was determined by using agar spot test according to Pirarat et al., (2009) with modifications.

7.1 Agar spot test

S. cerevisiae from an overnight culture (24 h) in SDB and each type of encapsulated *S. cerevisiae* were spotted on the surface of SDA and incubated at 30°C for 24 h to allow the development of the colonies. After 24 h, 50 µl of each strain of *S. agalactiae* (5×10^6 cfu/mL) (25 strains) was inoculated in semi-solid TSA (TSB with yeast extract of 0.6%+ Agar 0.75%) and poured over the SDA (spotted with grown *S. cerevisiae*). The plates were incubated at 30°C for 24 h and checked for inhibition zone. The inhibition zones were classified as (-) no visible inhibition, (+) 0.5 to 6 mm inhibition, (++) 7 to 12 mm, and (+++) more than 12 mm inhibition.

The strains of *S. agalactiae* that were used in this study were isolated from the outbreak in the central area of Thailand during 2008-2011 that confirmed by biochemical method and PCR.

Table 2 Strains of *S. agalactiae* used in this study

No.	Isolation no.	Location	Molecular serotype	Isolation date
1	SA065301	Nakorn pratom	Ia	-
2	SA075302	Nakorn pratom	Ia	-
3	SA075303	Nakorn pratom	Ia	-
4	SA075304	Nakorn pratom	Ia	-
5	SA075305	Nakorn pratom	Ia	-
6	SA075306	Nakorn pratom	Ia	-
7	SA075314	Nakorn pratom	Ia	January, 2011
8	SA075315	Prajeenburi	Ia	January, 2011
9	SA075316	Prajeenburi	Ia	January, 2011
10	SA075317	Nakorn pratom	Ia	-
11	SA075318	Nakorn pratom	Ia	-
12	SA075319	Nakorn pratom	Ia	-
13	SA075320	Nakorn pratom	Ia	-
14	SA015401	Nakorn pratom	Ia	January, 2011
15	SA015403	Nakorn pratom	Ia	January, 2011
16	SA015404	Nakorn pratom	Ia	January, 2011
17	S.aga 1	Laboratory collection	Ia	2008
18	S.aga 3	Laboratory collection	Ia	2008
19	S.aga 4	Laboratory collection	Ia	2008
20	SAT 1	Laboratory collection	Ia	2008
21	SAT 2	Laboratory collection	Ia	2008
22	SAT 3	Laboratory collection	Ia	2008
23	SAT 4	Laboratory collection	Ia	2008
24	CRCU1	Laboratory collection	Ia	2008
25	SA CP	Suphan Buri	Ia	-

8. Statistical Analysis

The data of the numbers of microorganisms will be expressed as a mean ± S.D. and evaluated using one-way ANOVA followed by the Bonferroni-type multiple t-test. All tests used a significant difference level of $p < 0.05$.

CHAPTER IV

RESULTS

1. Number of cell entrapped

The microencapsules were prepared by using 1.8% alginate mixed with suspension of *S. cerevisiae* in different solutions. The cell loading was ranged from 1-2 x 10^8 cfu/g beads in both microencapsules.

2. Viability after being freeze-dried

The survival of group B was significantly higher than group A; 7.9 log cfu/mL and 6.7 log cfu/mL respectively (Fig. 1)

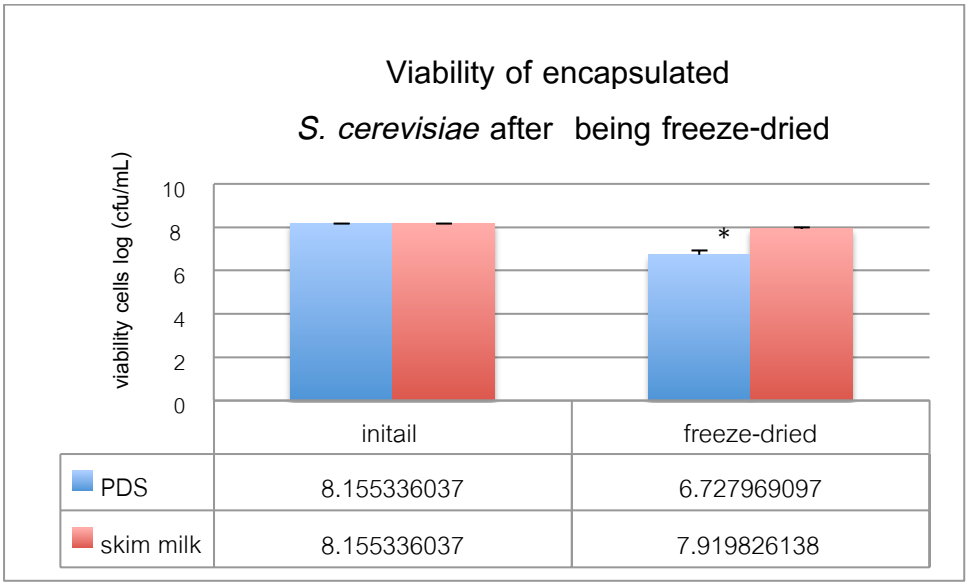


Figure 1 Viability of encapsulated *S. cerevisiae* after being freeze-dried. Significantly different between the groups at p <0.05 (*)

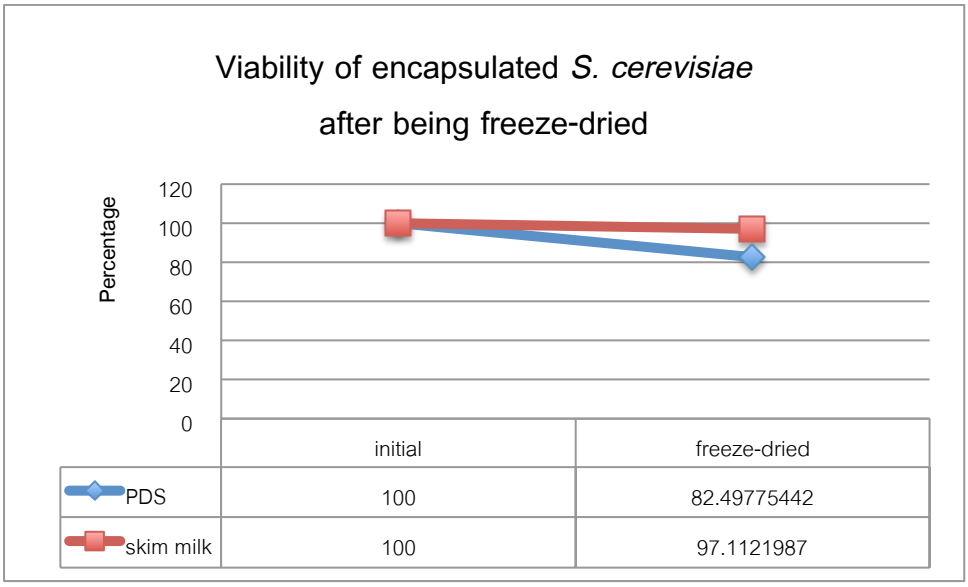


Figure 2 Percent viability of encapsulated *S. cerevisiae* after being freeze-dried

3. Viability of encapsulated *S. cerevisiae* during storage

The viability of free and encapsulated *S. cerivisiae* during storage at room temperature was shown in Fig 3. No viability of yeast cells was detected after 7 days in free cells. At 7 and 14 days of storage the viability of group B was significantly higher than group A; 91 survival percent was found in group B on 7 and 14 days and 81.8 and 79.4 survival percent was in group A on 7 and 14 days respectively.

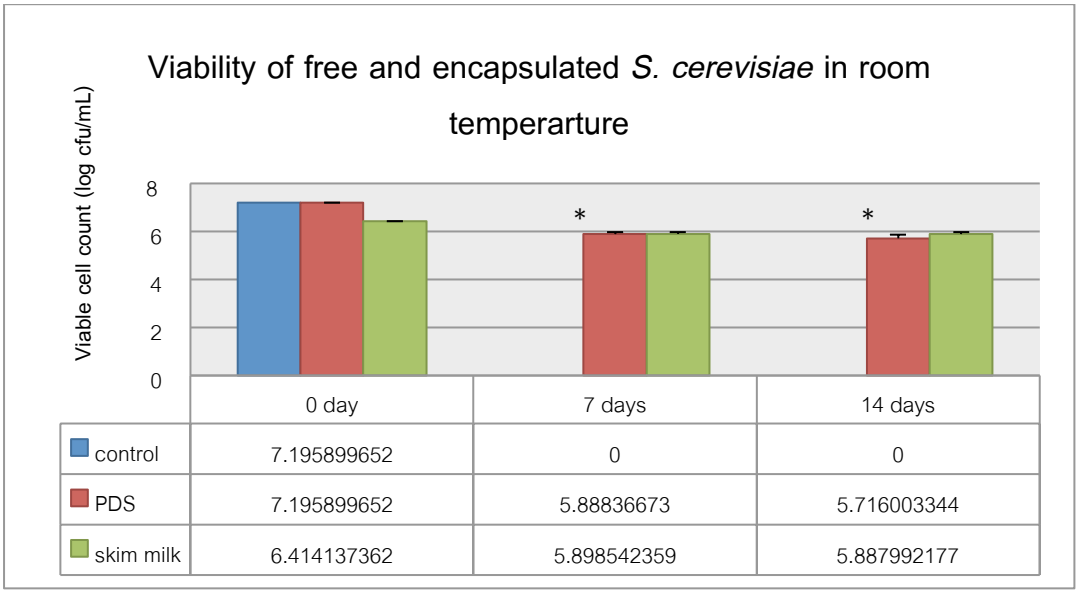


Figure 3 Viability of free and encapsulated *S.cerevisiae* during storage. Significantly different between the groups at p <0.05 (*)

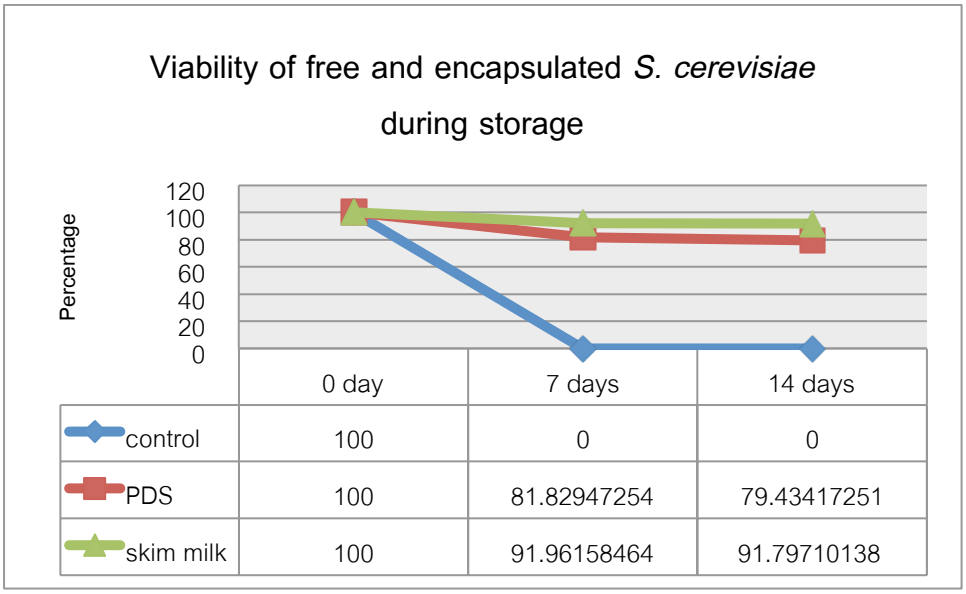
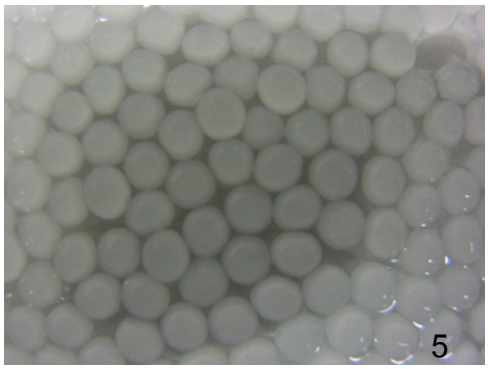
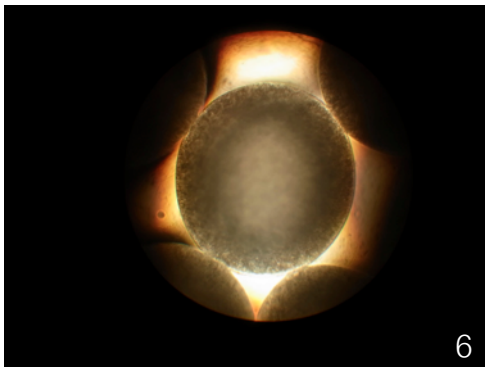


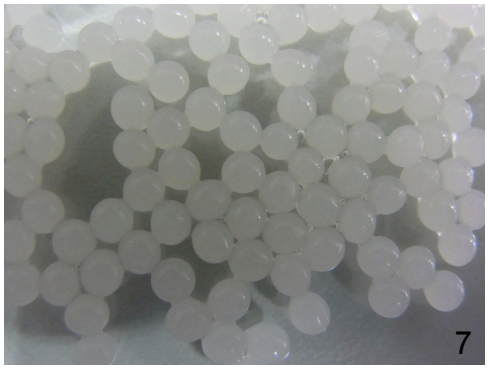
Figure 4 Percent viability of free and encapsulated *S.cerevisiae* during storage

4. Morphology Analysis

The size and shape of microencapsules were determined by light and scanning electron microscope. The extrusion technique that was used in present study resulted in microencapsule size of around 1-1.5 mm. in both groups of microecapsule. The shape of the microencapsules before being freeze-dried was generally round but a few oval shaped microencapsule were observed in both kinds of micrencapsules (Fig. 5-8). By using light microscope group A revealed yeast cells distributing though out the capsules (Fig. 6) while group B showed smooth white to light yellow surface without structure of yeast cells (Fig. 9). After being freeze-dried the morphology of microecncapsules was determined by using SEM. The microencapsules of both groups were round to oval in shape. The surface of group A was rough with concave polygonal ridges while the surface of microencapsules group B was rough. The cross section of micrencapsules of both types showed numerous of pores inside. The yeast cells linked with alginate matrix were observed in both types of microencpsules (Fig. 12)







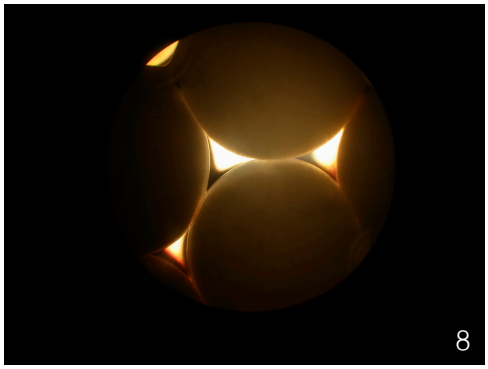
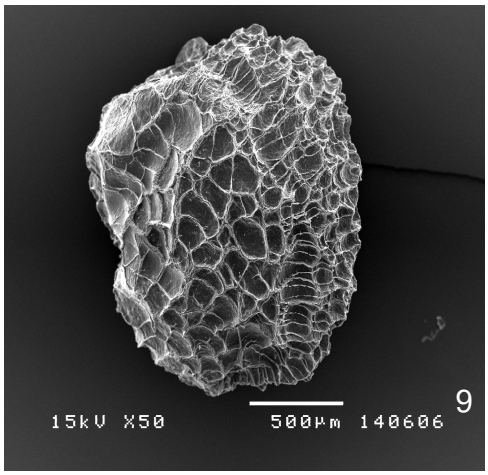


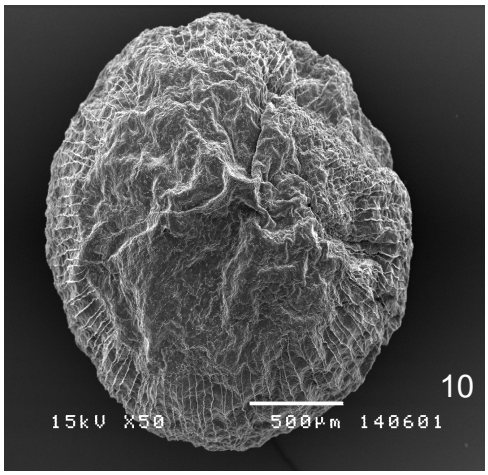
Figure 5 Microencapsules using PDS; The shape of the microencapsules was generally round but a few oval shaped microencapsule

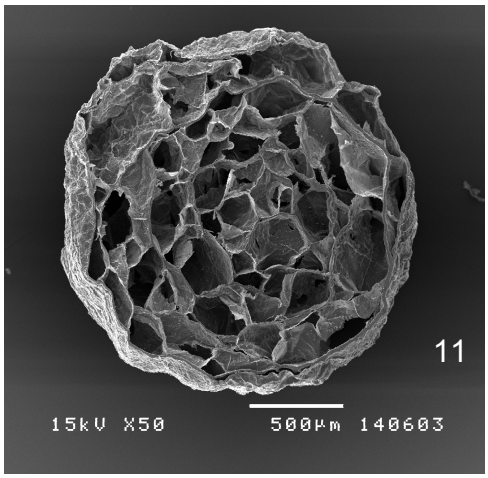
Figure 6 Microencapsules using PDS; Yeast cells distributed inside the microencapsules

Figure 7 Microencapsules using 10% skim milk; The shape of the microencapsules was round but a few oval shaped microencapsule

Figure 8 Microencapsules using 10% skim milk; Smooth white to light yellow surface







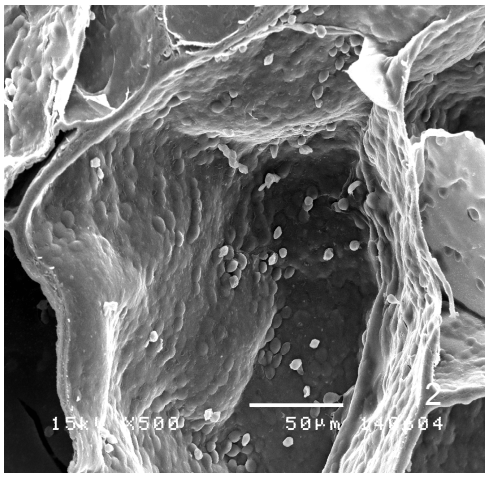


Figure 9 SEM picture of alginate-microencapsule usinPDS; The surface of microencapsules (group A) was rough with concave polygonal ridges

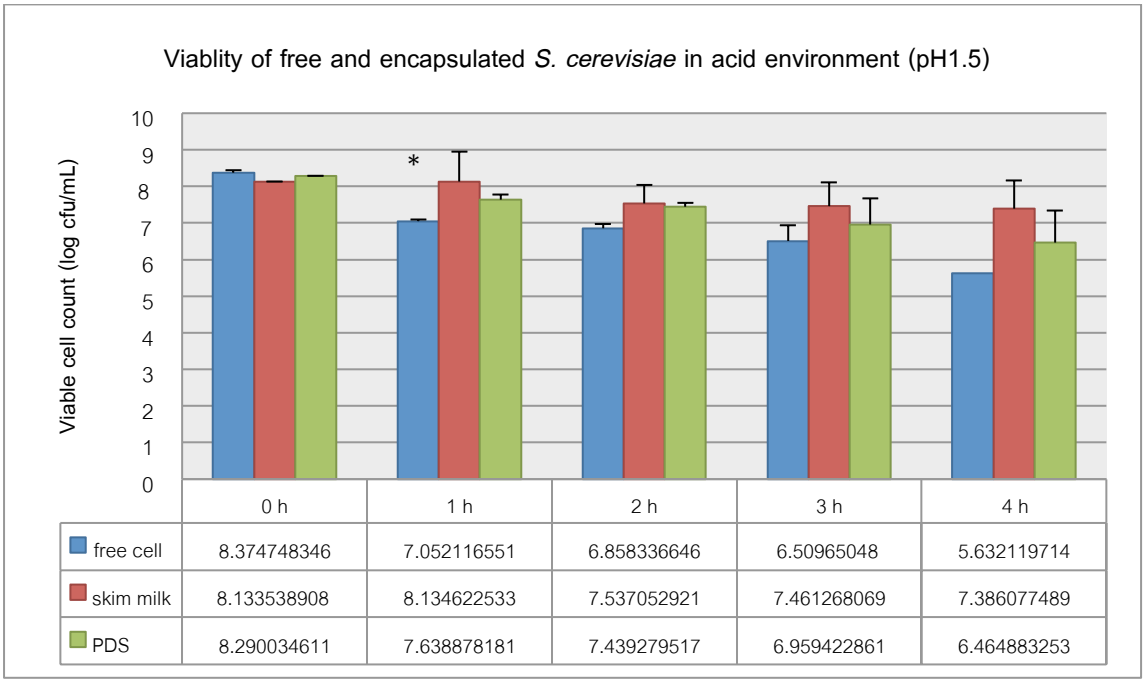
Figure 10 SEM picture of alginate-microencapsule using 10% skim milk; The surface of microencapsules (group B) was rough

Figure 11 SEM picture of cross section of alginate-microencapsule using 10% skim milk

Figure 12 SEM picture of alginate-microencapsule using 10% skim milk; Yeast cells linked with alginate matrix

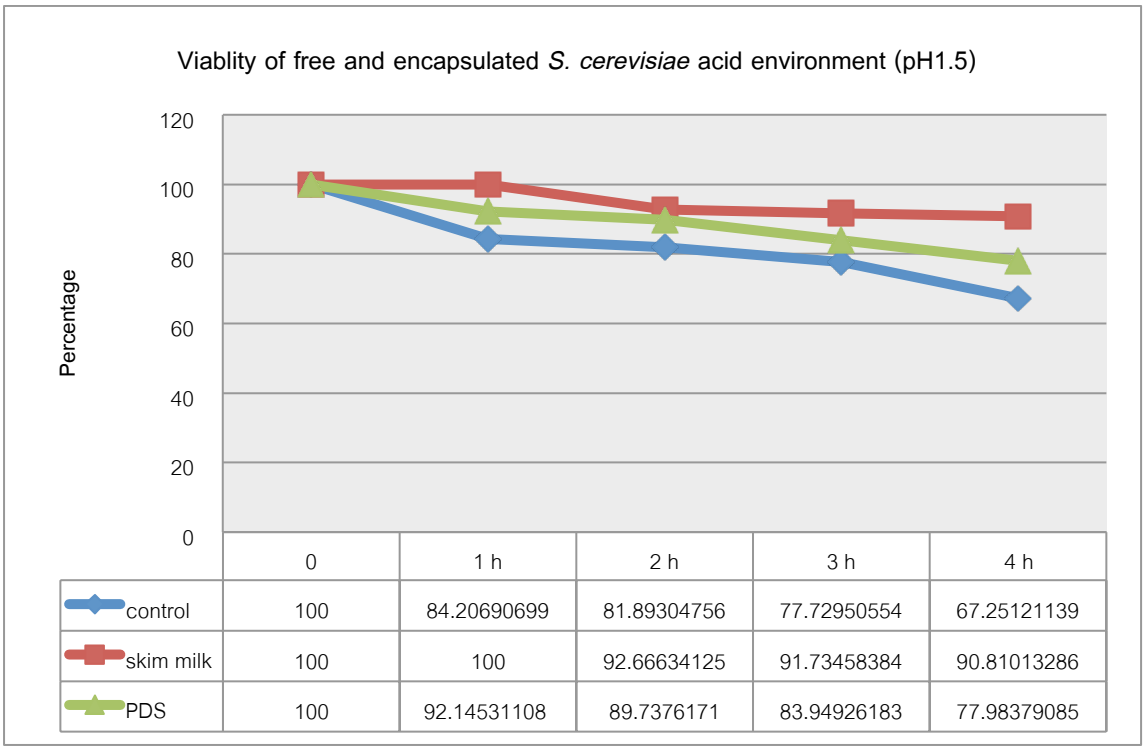
5. Acid tolerance

The survivals of free and encapsulated *S. cerevisiae* (group A and group B) in gastric condition (NSS pH 1.5) in 0, 1, 2, 3, 4 h were represented in Fig. 13. The survival of free *S. cerevisiae* continued to decreased in every passing hour but not at the same rate; at 0 h – 8.3 log cfu/mL, after 1 h – 7 log cfu/mL, 2 h – 6.8 log cfu/mL, 3 h – 6.5 log cfu/mL, 4 h – 5.6 log cfu/mL. The viability of group A also decreased which was similar to free cells but slower by 0h – 8.2 log cfu/mL, 1 h – 7.6 log cfu/mL, 2 h – 7.4 log cfu/mL, 3 h – 6.9 log cfu/mL, 4 h – 6.4 log cfu/mL. However, there was no decrease in the viability of group B after being exposed to NSS pH 1.5 for 1 h which was significantly different from that of both free cells and group A. After 2 h, 3 h, and 4 h the survivals of group B were 7.5 log cfu/mL, 7.4 log cfu/mL and 7.3 log cfu/mL, from 8.1 log cfu/mL (0 h).



	0 h	1 h	2 h	3 h	4 h
free cell	8.374748346	7.052116551	6.858336646	6.50965048	5.632119714
skim milk	8.133538908	8.134622533	7.537052921	7.461268069	7.386077489
PDS	8.290034611	7.638878181	7.439279517	6.959422861	6.464883253

Figure 13 Viability of free *S. cerevisiae*, encapsulated *S. cerevisiae* using PDS and *S. cerevisiae* using 10% skim milk in acid environment. Significantly different among the groups at $p < 0.05$ (*)



	0	1 h	2 h	3 h	4 h
control	100	84.20690699	81.89304756	77.72950554	67.25121139
skim milk	100	100	92.66634125	91.73458384	90.81013286
PDS	100	92.14531108	89.7376171	83.94926183	77.98379085

Figure 14 Percent viability of free *S. cerevisiae*, encapsulated *S. cerevisiae* using PDS and *S. cerevisiae* using 10% skim milk in acid environment

6. Survival of free and encapsulated *S. cerevisiae* in tilapia bile salts

To determine the tolerance of free and encapsulated *S. cerevisiae* in *in vitro* proximal intestine, the samples were exposed to NSS pH 1.5 for 60 min, followed by a further incubation in 10% tilapia bile salts for 1 h, 2 h and 3 h. The results were represented in Fig.15. The survivals of free *S. cerevisiae* and encapsulated *S. cerevisiae* (both groups) continued to decrease from hour to hour with significantly difference in every hour that passed. At 1 h and 2 h after being exposed to 10% tilapia bile, the highest survival was group B followed by that group A and that of free *S. cerevisiae.* At 3 h there was similar trend in the decrease among these three groups with the survivals of encapsulated *S. cerevisiae* using PDS and used 10% skim milk being significantly higher than free *S. cerevisiae* but there was no significant difference vis-à-vis encapsulated groups.

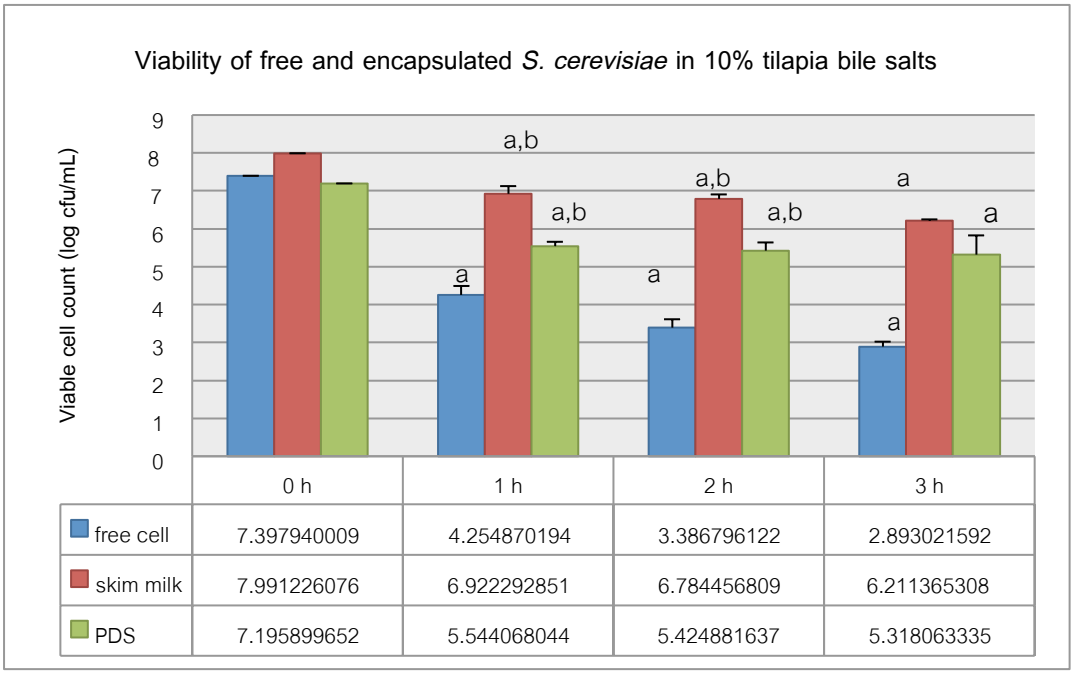


Figure 15 Viability of free *S. cerevisiae*, encapsulated *S. cerevisiae* using PDS and *S. cerevisiae* using 10% skim milk in 10% tilapia bile salts. Means having the same letter are significantly different at $P < 0.05$

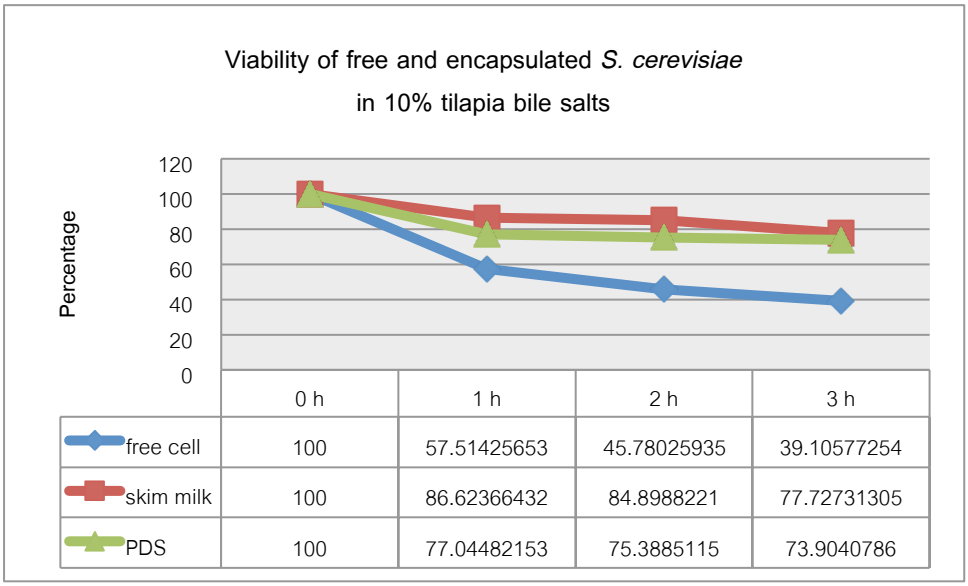


Figure 16 Percentage of survivals of free *S. cerevisiae*, encapsulated *S. cerevisiae* using PDS and *S. cerevisiae* using 10% skim milk in 10% tilapia bile salts

7. Antimicrobial activity

The agar spot test of using free *S. cerivisiae* group A and group B on SDA showed inhibition zones of 17 from 25 stains of *S. agalactiae* (Table3). There was no inhibition zone in any strains of *agalactiae* undergoing agar spot test with killed yeast.

Table 3 Inhibition zone

Isolation no.	Free *S. cerevisiae*	Group A (PDS)	Group B (skim milk)	Killed *S. cerevisiae*
SA065301	+++	++	++	-
SA075302	++	++	++	-
SA075303	-	-	-	-
SA075304	+	+	+	-
SA075305	-	-	-	-
SA075306	+	+	+	-
SA075314	+	+	+	-
SA075315	-	-	-	-
SA075316	+	++	++	-
SA075317	-	-	-	-
SA075318	-	-	-	-
SA075319	++	++	++	-
SA075320	+	+	+	-
SA015401	++	++	++	-
SA015403	-	-	-	-
SA015404	+	+	+	-
S.aga 1	++	+	++	-
S.aga 3	+	+	+	-
S.aga 4	-	-	-	-
SAT 1	++	++	++	-
SAT 2	-	-	-	-
SAT 3	+	++	+	-
SAT 4	+	+	+	-
CRCU1	+	+	+	-
SA CP	+	+	+	-

CHAPTER V
DISCUSSION

The encapsulation is a method that has been used to improve the viability of probiotics in unfavourable condition. There are a number of materials and techniques that can be used to give different advantages. In the present study the extrusion technique and alginate were used.

Sizes of microcapsules are one factor that affects to the properties of microcapsules. Larger sizes of capsules were reported to have more protection than smaller sizes (Krasaekoopt et al., 2003). The extrusion method using equipment such as syringe generally produces larger sizes which range from 2-5 mm (Mortazavian et al., 2007) than using other equipment. In this study the capsule sizes were approximately 1.5 mm. in both groups. The sizes of microcapsules depended on many factors including the viscosity and concentration of alginate, the distance between the syringe and the batch and the diameter of extruder hole (needle) (Mortazavian et al., 2007). In the present study, both groups were conducted using the same viscosity and concentration of alginate and the similar procedure but different solutions that were to resuspend yeast cells (group A: PDS, group B: 10% skim milk). The result indicated that there was no significant difference in the capsule size, suggesting that adding 10% skim milk did not affect the capsule sizes.

The loss of viability during encapsulation in the present study was extremely low in both microcapsules using PDS and 10 % skim milk. There was no significant difference of viability between the two groups of microcapsules, which also suggested that using PDS and 10% skim milk as solution had no effect on cell viability during encapsulation process. The loss of viability during encapsulation process is due to two main factors, the materials and the method. Alginate, anionic polysaccharide from algae, which was reported to be non-toxic to the probiotic cells, is the material used in this study. The alginate matrix has less than 17 nm pore size (Klein et al., 1983) that can retain *S. cerevisiae*, which has a diameter of around 5-10 μm. This result is similar to those of the studies using the extrusion method for encapsulation (Krasaekoopt et al., 2003; 2006). The extrusion technique was reported to be a gentle method to encapsulate living cells with less viability loss during the process, but it may not be practical for large-scale production because of the slow formation of the microcapsules (Mortazavian et al., 2007; Burgain et al., 2011).

In order to enhance the viability of probiotics in microcapsules, most of microcapsules

have to be stored in liquid and at low temperature, which might not be practical in aquatic farms. Drying technology has been added to improve the efficacy of encapsulated probiotics. It is useful not only for storage but it also to help maintain the viability of probiotics in dry food products. Different techniques can be used to dry encapsulated probiotics such as spray drying, freeze-drying and fluidized bed drying. The freeze-drying technique was used in this study. The freeze-drying is a technique used to immobilize living cells, which is essential to its long-term preservation. The technique, however, may damage the cells in certain ways. During the process, the cells are exposed to the risks of chemical imbalance and temperature can cause membrane damage, macromolecule denaturation and cell rupture from internal ice formation. The removal of water may also affect the properties of many hydrophilic macromolecules (Khoramnia et al., 2011). Different microorganisms have different responses to the freeze-drying procedure due to the various physiological states of the cells (Blanquet et al., 2005). The reported survival rates of *S. cerevisiae* ranged from 0.1% to 98% depending on the strains and the conducted freeze-drying conditions (Lodato et al., 1999; Blanquet et al., 2005; Cerrutti et al., 2000). In this experiment, the survival rate of group A was significantly higher than that of group B. This variance of the survival rates may have resulted from using different solutions to re-suspend the yeast cells, as skim milk has been known and used in the food industry as an effective cryo-protectant. The result of this study is similar to that of the study of Goderska, K. and Czarnecki, Z. (2008) that mentions about the higher viability of probiotics after being freeze-dried when skim milk was added into the encapsulation material. The skim milk protects the cells by stabilizing their membranes and creating a sponge-like structure that assists the rehydration process (Selmer-Olsen et al., 1999). Above all, the skim milk provides the cells protective coating during freeze-drying process. 5-20% of skim milk is suggested to be the most appropriate concentration that was used in this study. After being freeze-dried, both groups of microcapsules were stored in room temperature. On the 7^{th} and 14^{th} days after being freeze-dried the yeast cells were detected; 1-log decrease was found in group B, and 2-log decrease was found in group A. The higher viability of group B (using 10 % skim milk) might be related to the microcapsule dissolving process due to skim milk being a good media that can stimulate the growth of microorganisms.

From the light microscope the yeast cells could be seen inside the microcapsules using PDS, but the yeast cells were invisible in the microcapsules using 10% skim milk. Smooth yellow and white substance was seen instead. This may have happened because of the turbidity of

skim milk that was a suspension. After being freeze-dried the microcapsules were examined by SEM, and showed almost spherical shapes in both groups of microcapsules. However, the difference between two groups was revealed on the surface of microcapsules. The microcapsules group A presented rough with concave polygonal ridges with no-pore surface while the microcapsules group B showed a much smoother and more compact surface when compared with group A. The difference of surface appearance could result from different chemical compositions (Chen et al., 2005). This result corresponds with that of acid and bile tolerance that will be further discussed. The group B that have much more compact surface can provide better barrier to stop acid or bile diffusion and leakage of yeast cells from the encapsulated matrix.

Among the groups of microorganisms that have been used as probiotics, yeasts are generally believed to be the most tolerant species that can survive in unfavourable environment such as low pH in stomach or bile salt from proximal intestine. However, this present study showed that the survival of *S. cerevisiae* continued to decrease from hour to hour in low pH condition. Furthermore, the survival rate of *S. cerevisiae* significantly decreased after being exposed to NSS pH 1.5 and then transferred to 10% tilapia bile salts which remained around 57%. On the other hand, the survival of encapsulated *S. cerevisiae* in gastric condition and 10% bile salts was higher in both types of alginate-microcapsules when compared with free cells. The results in this study were in contrast with the previous study of Sultana et al. (2000) which reported that alginate used as the matrix for encapsulation could not protect the organisms from high acid environment. On the other hand, the results were comparable to several studies which showed that the improvement of the survival of microorganisms that have alginate as the matrix (Graff et al., 2007). The difference of the reports might result from the difference in velocity, the concentration of alginate and the size of microcapsules used in each study. Several studies revealed improvement of the survival of probiotics when a combination of alginate with other materials was used because alginate can be affected by acidic environment and chelating agent can absorb calcium iron such as phosphorus. In this study skim milk was used to mix with alginate. The microcapsules group B gave a better protection in low pH and 10% tilapia bile salts than the microcapsule group A. This may be caused by calcium ion in the skim milk that could inhibit the leaching of calcium ions from microcapsules (Hansen et al., 2002)

The present study on the agar spot test also showed the efficacy of the antimicrobial activity of *S. cerevisiae* on of 17 from 25 strains of *S. agalactiae*. The agar spot test using killed

yeasts, which showed no inhibition zones, clearly indicated that only the metabolite products, not the yeast cells, are involved in the growth inhibition of *S. agalactiae*. In this study, fresh *S. cerevisiae* and encapsulated *S. cerevisiae* gave similar results in agar spot test, which pointed out that the encapsulation did not affect the antimicrobial characteristics of *S. cerevisiae*.

The data from this experiment clearly showed that the microencapsulation of *S. cerevisiae* in alginate matrix limited the degradation of *S. cerevisiae in vitro*. In addition, the more protection can be initiated by adding 10% skim milk in alginate matrix. The microencapsulation method could be a major procedure in probiotic application in aquaculture.

CHAPTER VI

FUTURE RESEARCH DIRECTIONS

Probiotics become one of significant dietary supplement for aquaculture. The limitation of using probiotics is the viability of probiotics at the action site because probiotics risk being damaged by unfavourable environment from during the storage until the passing through the gastrointestinal tract. The microencapsulation might be the main method of keeping the viability of probiotics. Alginate is a main material that can be used in this process because it is not toxic to probiotic cells and safe for humans. However, other materials should be blended with alginate to make a better protection. The microencapsulation technique used in this study gave good results in *in vitro*, the study *in vivo* has yet to be done before the overall results can be confirmed.

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Appendix

Normal Saline Solution (NSS)

NaCl	8.5 g
DW	1000 mL

Peptone Dilution Saline (PDS)

Peptone	1 g
NaCl	8.5 g
DW	1000 mL

0.1 M Calcium Chloride solution

$CaCl_2$	11.1 g
DW	1000 mL

10% skim milk

Skim milk	10 g
DW	100 mL

BIOGRAPHY

Miss Komkiew Pinpimai was born in 1983 in Surin, Thailand. She graduated from Sansen Witayalai School with an award of the out-standing students. She finished Doctor of Veterinary Medicine from Chulalongkorn University in 2008. Her major interest is aquatic nutrition. While she was an undergrad student, she participated in many university music activities particularly those of the Chulalongkorn University Bigband (C. U. Band) of which she was elected president in 2005.