HEAT ADAPTATION AND MICROENCAPSULATION OF *LACTOBACILLUS* SP. STRAIN 3C2-10 ISOLATED FROM CASSAVA PULPS

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การปรับตัวจากความร้อนและการห่อหุ้ม *Lactobacillus* sp. สายพันธุ์ 3C2-10 ที่แยกได้จากกากมันสำปะหลัง

นางสาวจินต์จุฑา กรรณเทพ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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จินต์จุฑา กรรณเทพ : การปรับตัวจากความร้อนและการห่อหุ้ม *Lactobacillus* sp. สายพันธุ์ 3C2-10 ที่แยกได้จากกากมันสำปะหลัง (HEAT ADAPTATION AND MICROENCAPSULATION OF *LACTOBACILLUS* SP. STRAIN 3C2-10 ISOLATED FROM CASSAVA PULPS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. รัชฎาพร อุ่นศิวิไลย์, 80 หน้า

วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาศักยภาพในการปรับตัวจากความร้อนของ *Lactobacillus* sp. สายพันธุ์ 3C2-10 ต่อการทำแห้งแบบพ่นฝอย จากนั้นศึกษาการรอดชีวิตในระบบทางเดินอาหารจำลองและศึกษาอายุการเก็บรักษาที่ระดับอุณหภูมิ 4 25 และ 40 องศาเซลเซียสของ *Lactobacillus* sp. สายพันธุ์ 3C2-10 ที่ถูกห่อหุ้มและผ่านการปรับตัวจากความร้อนโดยวิธีทำแห้งแบบพ่นฝอย การให้ความร้อนในอาหารที่แตกต่างกันและการปรับตัวจากความร้อนต่อการรอดชีวิตของเซลล์ ซึ่ง *Lactobacillus* sp. สายพันธุ์ 3C2-10 ถูกทดสอบระดับการต้านทานความร้อนที่ 45 50 52 และ 55 องศาเซลเซียส เป็นเวลา 15 นาทีในทุกอาหาร (อาหารเหลว MRS, ฟรุคโตโอลิโกแซคคาไรด์ (FOS) และ มอลโตเดกซ์ทรินชนิดทนย่อย (RMD)) ค่า D-value ($D_{60°C}$) ของ *Lactobacillus* sp. สายพันธุ์ 3C2-10 มีค่าสูงสุดเมื่อเหนี่ยวนำความร้อนที่ 47 องศาเซลเซียส เป็นระยะเวลา 15 นาที ก่อนให้ความร้อนอีกครั้งที่ 60 องศาเซลเซียส เป็นระยะเวลา 15 นาที และที่ pH 3 ตามลำดับ

ผลการทดลองพบว่าเซลล์ที่ปรับตัวจากความร้อนมีการรอดชีวิตสูงกว่าเซลล์ที่ไม่ได้ปรับตัวจากความร้อนเมื่อผ่านวิธีทำแห้งแบบพ่นฝอยที่อุณหภูมิขาออก 84-90 และ 91-97 องศาเซลเซียส ตามลำดับ อย่างไรก็ตาม การต้านทานความร้อนโดยธรรมชาติของเซลล์แบคทีเรียขึ้นกับอัตราการเจริญและปัจจัยการเจริญ ยิ่งไปกว่านั้นผลของความเครียดจากความร้อนสามารถช่วยส่งเสริมการรอดชีวิตของเซลล์แบคทีเรียได้

จากการห่อหุ้ม *Lactobacillus* sp. สายพันธุ์ 3C2-10 ที่ผ่านการปรับตัวจากความร้อนโดยวิธีทำแห้งแบบพ่นฝอย ซึ่งใช้พรีไบโอติก 2 ชนิด ได้แก่ ใช้ฟรุคโตโอลิโกแซคคาไรด์และมอลโตเดกซ์ทรินชนิดทนย่อย เป็นวัสดุห่อหุ้มและศึกษาอัตราส่วนระหว่างเชื้อกับวัสดุห่อหุ้ม พบว่าเปอร์เซ็นต์ของประสิทธิภาพการห่อหุ้มโดยใช้ฟรุคโตโอลิโกแซคคาไรด์ร่วมกับมอลโตเดกซ์ทรินชนิดทนย่อยสูงกว่า ฟรุคโตโอลิโกแซคคาไรด์และมอลโตเดกซ์ทรินต้านทานการย่อยเท่ากับ 91% ในขณะที่ผลของความชื้น, ขนาดอนุภาค, ความหนาแน่น, การละลาย และประสิทธิภาพการห่อหุ้มไม่แตกต่างกันในทุกอัตราส่วนระหว่างเชื้อกับวัสดุห่อหุ้ม (1: 1 และ1:2) อย่างไรก็ตามผลของการวิเคราะห์ธาตุและสารประกอบโดยใช้ Fourier Transform Infrared Spectrometer (FT-IR) ระหว่าง 0-60 วัน พบช่วงลายพิมพ์นิ้วมือ (fingerprint region) ของคาร์โบไฮเดรต (กลูโคสและฟรุกโตส) ลดลง และมีการเพิ่มขึ้นของกรด ในส่วนของการทดสอบความเสถียรเมื่อผ่านระบบย่อยอาหารจำลอง พบว่าชนิดของ

วัสดุห่อหุ้มที่อัตราส่วนเดียวกันแสดงผลของอัตราการรอดชีวิตไม่แตกต่างกันทางสถิติ อย่างไรก็ตาม ผลของอัตราส่วนระหว่างเชื้อกับวัสดุห่อหุ้ม 1:2 แสดงการรอดชีวิตของเชื้อมากกว่าอัตราส่วนระหว่างเชื้อกับวัสดุห่อหุ้ม 1: 1 และพบว่าการใช้ฟรุคโตโอลิโกแซคคาไรด์ร่วมกับมอลโตเดกซ์ทรินชนิดทนย่อย ที่อัตราส่วนเชื้อต่อวัสดุห่อหุ้ม 1: 2 แสดงผลการเก็บรักษาที่ 4 องศาเซลเซียส ดีกว่าการเก็บรักษาที่ 25 องศาเซลเซียส

สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2559

ลายมือชื่อนักศึกษา สินศุภา
ลายมือชื่ออาจารย์ที่ปรึกษา

JINJUTAR KUNNATHEP: HEAT ADAPTATION AND MICROENCAPSULATION OF *LACTOBACILLUS* SP. STRAIN 3C2-10 ISOLATED FROM CASSAVA PULPS. THESIS ADVISOR : ASST. PROF. RATCHADAPORN OONSIVILAI, Ph.D., 80 PP.

HEAT ADAPTATION/SPRAY DRYING/SURVIVAL RATE/ FRUCTOOLIGOSACCHARIDE/RESISTANT MALTODEXTRIN

The objectives of this study were to investigate the potential of heat adaptation as a survival to enable the *Lactobacillus* sp. strain 3C2-10 to withstand spray drying. The viability of encapsulated *Lactobacillus* sp. strain 3C2-10 after heat adaptation by the spray drying technique was investigated for stabilization during the simulated gastrointestinal tract and storage condition temperatures of 4, 25 and 40°C. The effect of heat treatment in different media and heat adaption on the survival of spray dried cells were monitored. The *Lactobacillus* sp. strain 3C2-10 exhibited heat resistance at temperatures of 45, 50, 52 and 55°C for 15 min in all media tested (MRS broth, fructooligosaccharides (FOS) and resistant maltodextrin (RMD)). The highest D-value ($D_{60°C}$) of the *Lactobacillus* sp. strain 3C2-10 was induced by pre-heating at 47°C for 15 min before being exposed to a temperature at 60°C for 15 min and pH 3, respectively. The adapted cells represented higher cell viability than non-adapted cells at both outlet temperatures of 84-90°C and 91-97°C, respectively. However, the natural heat resistance of bacterial cells depended on the growth rate conditions and growth-limiting factors. Moreover, the effect of heat stress could increase bacterial cells viability.

The encapsulation of the *Lactobacillus* sp. strain 3C2-10 cells after heat adaptation by the spray drying technique with two types of prebiotics as wall material (FOS and RMD) and ratio of core-to-wall was studied. The results showed that the efficiency percentage of encapsulation with FOS combined with RMD was at 91% and higher than encapsulation with only FOS or RMD. In addition, the effect of moisture content, particle size, bulk density, solubility and encapsulation efficiency were not significantly different in both core-to-wall ratios (1: 1 and 1: 2) tested. Moreover, the results of the elemental and compound of the encapsulated *Lactobacillus* sp. strain 3C2-10 cell after heat adaptation were analyzed using the Fourier Transform Infrared Spectrometer (FT-IR) during the storage time of 60 days. The results showed that the fingerprint region of carbohydrates (glucose and fructose) were decreased, and acids were increased. In the simulated gastrointestinal tract model, the cells survival rate were not significantly different among different wall materials at the same core-to-wall ratios. However, the core-to-wall ratio at 1:2 showed the cells survival higher than the core-to-wall ratio at 1: 1. The combination of FOS and RMD at the ratio of 1: 2 exhibited higher storage stability of the *Lactobacillus* sp. strain 3C2-10 at the temperature of 4 °C than 25°C.

School of Food Technology Student's Signature จินตนุช

Academic Year 2016 Advisor's Signature Ratchadaporn, O.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of varience
°C	Degree Celsius
CFU	Colony forming unit
et al.	et alia (and others)
(m, μ) g	(milli, micro) Gram
h	Hour
(m, μ) l	(milli, micro) Liter
(m, μ) M	(milli, micro) Molarity
min	Minute
(m, μ) mol	(milli, micro) Mole
N	Normality
%	Percentage
PCR	Polymerase chain reaction
rpm	Round per minute
s	Second
sp.	Species
supsp.	Supspecies
%v/v	Percentage volume by volume
%w/v	Percentage weight by volume

CHAPTER I

INTRODUCTION

1.1 Rational and background

Lactobacilli strains are among the lactic acid bacteria (LAB) that are the most widely used as oral probiotics to improve gut health and immunity, obesity prevention, anti-carcinogenic, and protect harmful microorganisms (Bourdichon et al., 2012). The probiotic is defined by Food and Agriculture Organization of the United Nations (FAO) as a live microorganism when administered in adequate amounts exerts host health benefit. This definition is including mono or mixed culture of live microorganisms that are applied to animals and humans. The genus *Lactobacillus* has a long history of safety use including dairy and non-dairy foods application. Delivery foods or nutritive supplements should contain probiotic populations of at least 10^6-10^7 live microorganisms per gram or milliliter at the time of consumption in order to provide health benefits to the host (Kent and Doherty, 2014). Prebiotics are defined as non-digestible foods that are necessary for stimulating the growth of probiotic bacteria in the colon and improving host health. Carbohydrate and fibers are major prebiotics such as oligosaccharides, resistance starch and inulin (Wrzosek, Moravčík, Antošová, Illeová, and Polakovič, 2013). Both prebiotics and probiotics their combination may reinforce the effect by each other. Thus, the synergistic combination of prebiotics and probiotics, termed as symbiotic enhances the viable counts of lactobacilli and bifidobacteria compared to either prebiotics or probiotics alone (Wrzosek et al., 2013).

Probiotics need to be encapsulated. Cells may be protected from adverse environment and viability of the cells throughout the storage period as well as in the human digestive tract could be improved. In manufacturing probiotics and other fermented products, freeze drying and spray drying are frequently applied techniques. Freeze drying is a technique used for preserving biological materials and starter cultures. It is an expensive process and discontinuous with low yields, and energy and time demanding. While spray drying is a promising low-cost alternative because it allowed the continuous production of high dehydration yields within short time periods. However, in the spray drying process loss of viability of the cells. When bacterial cells sense a high temperature that change membrane fluidity, alter cell protein structure or disrupt ribosomes or affect nucleic acids. Response to stress, in this, increases the organism' tolerance to the same or different type of stress. This phenomenon is occasionally described as adaptive response (Jørgensen, Hansen, and Knøchel, 1999). It is possible to induce a thermal sub-lethal effect on cells which adapts them selves to adverse conditions during drying and storage (Anekella and Orsat, 2013). Usually increase temperature of 10°C add from the optimal growth temperature leads to shock (Broadbent and Lin, 1999). Sub-lethal thermal can increase the survival of *L. acidophilus* between 12 and 14 folds depending on media (Anekella and Orsat, 2013).

Starch and maltodextrin are prebiotics that were widely used for microencapsulation by spray drying. Only a few prebiotic (as fructooligosaccharides (FOS) and galactooligosaccharide (GOS)) were reported as wall material due to stickiness behavior limits. Stickiness during spray drying can be reduced by changing the glass transition temperature (Tg) with the combination of high molecular weight

agents such as whey proteins, like polymers and gums (Rajam and Anandharamakrishnan, 2015) . Resistance maltodextrin (RMD) is an alternative prebiotic which has high glass transition temperature (Pai, Vangala, Ng, Ng, and Tan, 2015). Moreover, RMD is a randomly 1, 2-, 1, 3- and 1, 4-alpha or beta linkages that are resistant to digestion in the human digestive system and also increase probiotics populations in colon and fecal weight (Lefranc-Millot et al., 2012).

The purposes of this research were to investigate the natural heat resistance level and heat adaption of *Lactobacillus* sp. strain 3C2-10 in MRS broth, FOS and RMD and to study effect of spray drying outlet temperature on cell viability. Moreover the stability and survival of *Lactobacillus* sp. strain 3C2-10 microcapsules simulated gastrointestinal conditions were measured and monitored the storage time.

1.2 Research objectives

The objectives of this research were as follows:

1. To determine the natural heat resistance level and heat adaption of *Lactobacillus* sp. 3C2-10 in MRS broth, FOS and resistant maltodextrin.

2. To study the effect of spray drying outlet temperature on cell viability of *Lactobacillus* sp. strain 3C2-10

3. To investigate the stability of spray dried *Lactobacillus* sp. strain 3C2-10 microcapsules in simulated gastrointestinal conditions.

4. To measure the survival of spray dried *Lactobacillus* sp. strain 3C2-10 microcapsules during storage

1.3 Research hypotheses

Effect of natural heat resistant level and heat adaption of *Lactobacillus* sp. strain 3C2-10 would be increased cell viability after encapsulation by spray drying at optimal condition. The spray dried *Lactobacillus* sp. strain 3C2-10 microcapsules would increase survival after passage through simulated gastrointestinal conditions and during storage conditions.

1.4 Scope of the study

Lactobacillus sp. strain 3C2-10 was studied the natural heat resistant level in MRS broth, fructooligosaccharides (FOS) and resistant maltodextrin (RMD), respectively, and heat adaption conditions were also investigated. The heat adapted cells were encapsulated at optimum spray drying condition. FOS and RMD were used as wall materials. The microcapsules of selected strain were studied for their physical properties include moisture content, water activity, particle size, bulk density solubility, morphology and Fourier Transform Infrared Spectroscopy (FTIR). Influence of encapsulation process to survival of the selected strain was studied in which storage time of encapsulated strains would be also investigated.

CHAPTER II

LITERATURE REVIEWS

2.1 Cassava

Cassava is the common name of *Maniho esculenta Crantz*, which was originated in South America and Southern and Western Mexico (Anyanwu, Ibeto, Ezeoha, and Ogbuageu, 2015). Cassava planting areas around the world were approximately 113.8 million crops with an average yield of 1.92 tons/crop. Nigeria, Brazil and Thailand are the major locations of cassava productions in the world. However, Thailand had a higher yield per area than there of Nigeria and Brazil. Thailand is currently one of the world's biggest exporters of cassava products with the major competitors including Indonesia, Brazil and Vietnam (Kaplinsky, Terheggen, and Tijaja, 2011).

The cassava starch production is one of the most important agro-industries in Thailand. Thailand is the third largest producer of cassava starch in 2010, which are approximately 22.5 million tons per year (Veiga, Valle, Feltran, and Bizzo, 2016). The solid wastes from cassava starch production are cassava stem, soil, sand and pulp (Veiga et al., 2016). There is still lack of an interest in utilization these wastes for value adding. Nowadays, the wastes are mainly used as soil additive, animal feed and bio-alcohol production, for example. Virunanon et al. (2013) has reported that around 25-30 million tons per year of fresh cassava roots are used for cassava starch

production, which generated cassava solid waste as cassava pulp around 3-5 million tons.

Cassava pulp is a residual pulp separated during the processing of cassava starch, and the main by-product from this process. Pulp is fine and white with moisture content up to 75% and the main composition is carbohydrate (55-56%) (Freire, Ramos, and Schwan, 2015, Virunanon et al., 2013). Starch remains in the pulp approximately

50-60% of its dry weight, in which starch is trapped inside ligno-cellulose. The pulp also contains pectin, cellulose, and fiber approximately 10-15%, protein of 1.5-5%, fat of 0.1-4%, respectively, (Kurdi and Hansawasdi, 2015). Other components are minerals: Fe^{2+} of 155 ppm, Mn^{2+} of 40 ppm, Mg^{2+} of 1100 ppm, Cu^{2+} of 4 ppm and Zn^{2+} of 21 ppm per kg-dry pulp (Coulin, Farah, Assanvo, Spillann, and Puhan, 2006, Kurdi and Hansawasdi, 2015). The pulp is high in fermentable carbohydrates, rich organic nature and moisture content but low in fiber and nitrogenous compounds (Freire et al., 2015). However, it could be a good substrate for microorganisms to grow and to produce different metabolites.

2.2 Probiotics

There are many explanations about probiotics. A live microorganism when administered in adequate amounts exerts host health benefit (Morelli and Capurso, 2012, Ogueke, Owuamanam, Ihediohanma, and Iwounp, 2010). This definition is including mono or mixed culture of live microorganisms that are applied to animals and humans. Microorganisms classified as probiotics must have the characterization as suggested by FAO/WHO guidelines. In addition, this characterization must have

functional properties such as antimicrobial activity, resistance to the gastrointestinal tract, adherence to human epithelial cells, modulation immune system and activity of bile hydrolase. Most importantly, it is approved to be safe. Lactic acid bacteria (LAB) has a wide range of genera including an important number of species and generally recognized as being safe (GRAS) status for applications in food (Bourdichon et al., 2012). LAB is a Gram-positive and growing in microaerophilic to strictly anaerobic condition (Z. G. Zhang, Ye, Yu, and Shi, 2011). The most recognized genera of LAB are *Lactobacillus Lactococcus, Enterococcus, Pediococcus, Leuconostoc, Streptococcus, Tetragenococcus, Carnobacterium, Weissella* and *Bifidobacterium* (Calasso and Gobbetti, 2011, Z.G. Zhang et al., 2011). Nevertheless, LAB is focused on having similar physiological, biochemical properties and also association with the gastrointestinal tract (GIT). Species of this genera could be found in the GIT of humans and animals as well as in fermented food. The strains used as probiotics usually belong to species of the genera *Lactobacillus, Enterococcus* and *Bifidobacterium.*

Lactobacillus are in domain bacteria, phylum Firmicutes, class Bacilli, order Lactobacillales, family Lactobacillaceae and genus *Lactobacillus. Lactobacillus* is the most widely used strain which has been approved as probiotics and applied in many food products such as dairy products and non-dairy products over 40 countries for 50 years (Miao et al., 2015, Stiles and Holzapfel, 1997). For example, *Lactobacillus bulgaricus* was used for starter cultures of dairy fermentations (Teixeira, Castro, Mohácsi Farkas, and Kirby, 1997), *L. casei* 01 in lychee juice (Kingwatee et al., 2015), *L. acidophilus* NRRL B-442 and *L. rhamnnosus* NRRL B-4495 in raspberry juice (Anekella and Orsat, 2013). The taxonomy, metabolic activities, growth and

nutritional requirements of *Lactobacillus* strains are clarified to rule in the gastrointestinal tract and enhance fermentative activity (Pimentel, Madrona, Garcia, and Prudencio, 2015). The major *Lactobacillus* species of the natural normal flora in humans and animals intestine are *L. plantarum*, *L. acidophilus* and *L. fermentum*, etc (Z. Zhang, Tao, Shah, and Wei, 2016). These species are presented in term of probiotics promoting a healthy equilibrium of normal flora in the gastrointestinal system including preventing infection from pathogenic bacteria.

Lactobacillus has constructive effect in intestinal epithelial cells colonization, and activation of innate immunity response of the host and protecting the invasion of pathogenic microorganisms (DiRienzo, 2000). Moreover, *Lactobacillus* strains have been recorded to exert health promoting effects by several mechanisms. Some of these mechanisms are anti-carcinogenic properties and cancer prevention especially colon and bladder, anti-oxidative effects, improving of arthritis, reduction of dermatitis and allergic symptoms. Chingwaru and Vidmar (2017) studied *Lactobacillus* sp. from Zimbabwean dairy. The strain has antimicrobial activity which can be controlled paediatric diarrhea that is caused by *E. coli*. Moreover, the qPCR test studied by Huang et al. (2015) showed that the *Lactobacillus plantarum* ZDY 2013 enhanced the populations of *Lactobacillus* and *Bifidobacterium* in the colon or cecum and reduced the potential enteropathogenic bacteria of mice.

Probiotics application have been studied, commonly used in food fermented products such as yogurt, cheeses, natto, Kefir (Adesulu-Dahunsi, Sanni, Jeyaram, and Banwo, 2017), fufu (fermented cassava mash) and ogi (fermented maize and sorghum) (Adesulu-Dahunsi et al., 2017). Lactic acid bacteria especially *Lactobacillus* sp. were used in various materials including fruits, vegetables, meat and fish. (Nuraida, 2015).

Food matrix could be changed to be functional foods by probiotics addition. The productions of probiotics are important factor for food substrate.

Nawong, Oonsivilai, and Boonkerd (2013) have isolated probiotics from the cassava pulp. The selection procedure for probiotic strain was emphasized by properties of cholesterol lowering. They found that probiotic *Lactobacillus* sp. strain 3C2-10 profounded this activity, and its tendency to be *L. plantarum* strain WG27 (99% similarity).

2.3 Prebiotics

Prebiotics was first introduced in 1995 by Gibson and Roberfroid. It has been described as non-digestible food that usefully affects the host by stimulating the growth of normal flora in the colon and improving host health. In 2008, the definition of dietary prebiotics were updated as "a selectively fermented ingredient that results in specific changes in the activity of the gastrointestinal normal flora and improves host health" (Gibson et al., 2010). The major prebiotics used as carbohydrate and fibers includes non-digestible oligosaccharides (NDOs) include soy bean oligosaccharides lactulose, fructooligosaccharides, galactooligosaccharides, mannanooligosacharides and xylooligosaccharides. Polysaccharides such as resistant starch, and inulin and Non-starch polysaccharides (NSP) are arabinoxylan, pectin, gum. Desired characteristics of a good prebiotic are non-digestible or partially digested and non-absorbable in the small intestine, badly fermented by bacteria in the mouth but well by normal flora in the colon but badly fermented by bacterial pathogens in the colon (Lee and Salminen, 2009). Prebiotics have been suggested to give several benefit to host such as

shortening fecal transit time, immunological response promoting colonic normal flora growth and also producing short chain fatty acid (Patel and Goyal, 2011).

2.3.1 Resistant maltodextrin

Since the 1980s, it has been well known that not all starch are digested by enzymatic and absorbed in small intestine of human digestion. The part is not enzymatically digested and cannot be fermented in large intestine, and found in the feces. The starch and starch products that are not digested in the small intestine that are resistant starch (RS). The RS is classified in 4 groups including RS1 referred to the starch that is physically inaccessible to enzymatic digestion, RS2 as native starch granules or ungelatinized starches, RS3 as retrograded starch formed during the food processing, and RS4 as a starch that is chemically modified. The resistant maltodextrin are classified in the RS4.

Resistant maltodextrin (RMD) is short chain polymers of glucose that are resistant to digestion in the human digestive system. It is typically produced by hydrolysis and transglucosidation (Dextrinization process) of starch (corn, tapioca, etc.). Then dextrin solution from dextrinization process is hydrolyzed by amylases, after separation of glucose fraction results in concentrate of RMD (Figure 2.1). During dextrinization process, the 1-4 alpha and 1-6 alpha glycosidic bonds are hydrolyzed and transglucosidation creates glycosidic linkages to random such as 1,2-, 1,3- and 1,4-alpha or beta linkages (Jochym, Kapusniak, Barczynska, and Śliżewska, 2012).

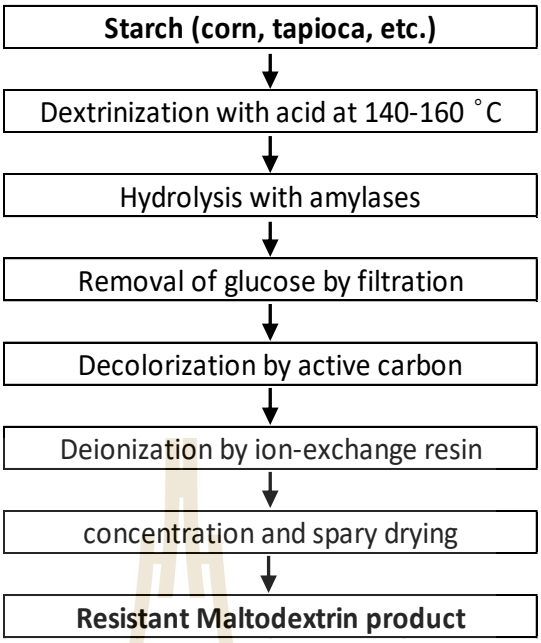
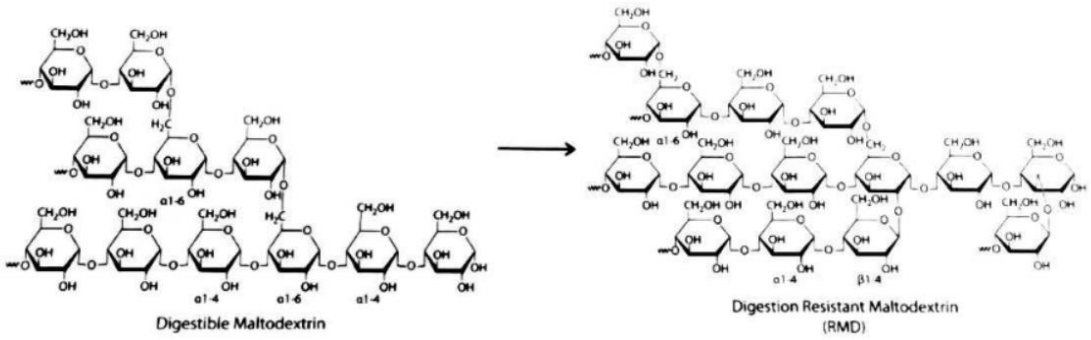


Figure 2.1 Diagram of the resistant maltodextrin manufacturing process.

From: Susan Sungsoo Cho and Samuel (2009)

The RMD consists of an average degree of polymerization (DP) of 10–15 (Jochym et al., 2012) with the average molecular weight of 2000 and low glycemic index (10% that of maltodextrin). Figure 2.2 shows the chemical structure of RMD compared to maltodextrin. The structure of maltodextrin consists of most 1, 4 glycosidic bonds but RMD not only has 1, 4 and 1, 6 glycosidic bonds but also has 1, 2 and 1, 3 glycosidic bonds. Currently, There are two type of MRD ingredients in the market, Nutriose® (Roquette's Research Centre) and Fibersol®2 (Matsutani Chemical Industry Co). Nutriose® is produced from maize or wheat starch by dextrinization process but Fibersol®2 is produced from cornstarch by pyrolysis and enzymatic conversion. Both of them have many important physiological effects and be used to for clinical nutrition.



Glycosidic bond	1 → 6	1 → 4	1 → 3	1 → 2
Maltodextrin				
- Acidic hydrolysis	5.1%	91.6%	2.2%	1.2%
- Enzymatic hydrolysis	4.5%	94.7%	0.9%	0.0%
Resistant maltodextrin	58.5%	27.0%	3.2%	11.3%

Figure 2.2 Structure and of maltodextrin prepared by acidic or enzymatic hydrolysis and resistant maltodextrin (Fibersol®-2 model)

From: Susan S Cho and Almeida, (2012); Susan Sungsoo Cho and Samuel (2009)

On 90% of the resistant maltodextrin finds in the large intestine and 50% is fermented, these findings support its physiological effects in the large intestine. After resistant maltodextrins are digested and fermented by bacteria. They produced short-chain fatty acids (SCFA) and gases. The SCFA are considered to have the following properties in the large intestine such as anti-inflammatory that a specific energy source for intestinal mucosal cell to promote cell growth, interfering with bile acid reabsorption thus lowering blood cholesterol levels and primarily butyric acid that aiding in water movement across the large intestines. RMD reported in many literature which has referred to nutritional benefits including increased bacterial populations in the colon. Ohkuma, K. et al., (1990) have found the major strains of *Bifidobacterium* that was good at RMD fermentation. Fastinger et al. (2008) examined 39 testers

healthy adult human were subjected to RMD treatment for 7 weeks. The experiment showed 15g/ day of RMD treatment resulting in the highest *Bifidobacterium* populations. In addition to this, they found the increase of the fecal SCFA and butyrate with increased RMD.

Moreover, Pai et al. (2015) studied the fundamental solid state properties of RMD. RMD thermal analysis using differential scanning Calorimeters (DSC) confirmed the glass transition temperature (T_g) of RMD at 170.7°C. This feature is close to maltodextrin, in which T_g values were based on the natural dextrose equivalent (DE) of maltodextrin, ranging from 100 to 243°C (Goula and Adamopoulos, 2008) . Among the food biopolymers of interest for spray- drying, extruded and spray coating encapsulation, maltodextrin was especially useful due to their low viscosity, high Tg and solubility.

2.3.2 Fructooligosaccharides

Oligosaccharides are carbohydrates and degree of polymerization (DP) are between 3 to 10 (Committee, 2001) or 3 to 9 (Joint and Consultation, 1998) . Fructooligosaccharides (FOS) or oligofructose or oligofructan are oligosaccharide fructans in which fructose is the principal monomer. It is extracted from plants such as chicory, jerusalem artichoke, asparagus, garlic and onion. FOS can be produced by enzymatic hydrolysis or chemical degradation of inulin. Moreover, it can be produced from sucrose by fungal enzymes (Roberfroid, Van Loo, and Gibson, 1998). FOS can be prepared in the commercial scale from sucrose through the transfructosylating action of fungal enzymes include β-fructofuranosidases and β-fructosyltransferases. Morover, it can be prepared from inulin by partial hydrolysis using endo-inulinase. They are consisted of several linear chains of fructose units, linked by 2,1 beta

glucosidic bonds. The length of fructose chain varies from 2 to 4 (DP 2-4) as a mixture of 1-kestose (1-kestotriose; GF2) , nystose (1, 1-kestotetraose; GF3) , and 1F-β-fructofuranosylnystose (1, 1, 1-kestopentaose; GF4) . Thus, FOS is a combination of GF_n unit, G is structure of Glucrose and F is Fructose (Figure 2.3)

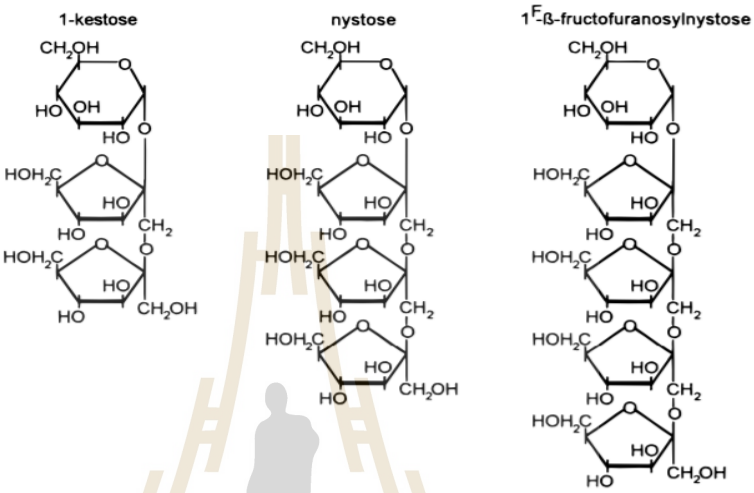


Figure 2.3 Molecular structures of the Fructooligosaccharides

From: Campbell et al., (1997)

FOS have attracted a lot of interest because of their nutritional properties. FOS is classified in non-digestible oligosaccharides (NDOs) that they are not digested in the human upper intestine but they are fermented in the colon to lactate and short chain fatty acids (propionate, butyrate and acetate) . FOS stimulate growth of probiotics (*Lactobacillus* and *Bifidobacterium*) and inhibited growth of some bacterial (*Clostridium perfringens*, *Escherichia coli* and *Bacteroides*) (Corthier and Doré, 2010). In addition, FOS have been showed to exhibit useful health effects by reduction of bacterial pathogens, reduction of serum cholesterol concentrations, improvement of the immune system as biomarkers of intestinal health support of mineral absorption and protect colon cancer. FOS are considered food ingredients due to the combination

of nutritional benefits and technological properties closely related to those of sugar and glucose syrups (Alles, Tessaro, and Noreña, 2013) . They have been used in various functional food products such as yogurt, fermented milk, cheese and cheese-based products (Akalın, Tokuşoğlu, Gönç, and Aycan, 2007; Oliveira, Sodini, Remeuf, and Corrieu, 2001). Oliveira et al. (2009) studied SCFA in yogurt containing *Lactobacillus acidophilus* and 2% FOS. The result showed increasing of SCFA than that of yogurt containing *Lactobacillus acidophilus* only. However, the use of lyophilization technique to prolonged FOS storage is limited due to the hygroscopic of FOS (Amrutha, Hebbar, Prapulla, and Raghavarao, 2014; Rajam and Anandharamakrishnan, 2015) . Crittenden and Playne (1996) reported that FOS are stable at pH 4-7 at refrigerated temperatures a year. Chiavaro, Vittadini, and Corradini (2007) studied the Raftilose95® (FOS and inulin) thermal analysis by differential scanning Calorimeters (DSC) . The result confirmed that the glass transition temperature (T_g) was lower than 80°C.

2.4 Encapsulation

Encapsulation is the first introduction in the biotechnology that make production processes. It has been defined as entrapping one substance (core or active agent) within another substance (shell or wall material) . Diameters of encapsulated particles were about nanometer to millimeter that the size and shape depend on materials and methods used to make them (Desai and Jin Park, 2005). Different types of encapsulates are produced from various wall composition and different techniques such as spray drying, freeze drying, coacervation, liposome entrapment, crystallization, extrusion, spray-freeze drying, emulsification, etc. Also depends on the

core physicochemical properties. There are two main type of encapsulates, the reservoir type and the matrix type. The reservoir type has wall material around the core that it can break by pressure to release the core. The matrix type, the core is homogenously dispersed in the wall material (Zuidam and Shimoni, 2010). The Figure 2.4 shows only spherical shaped. There are also irregular and cylindrical shaped.

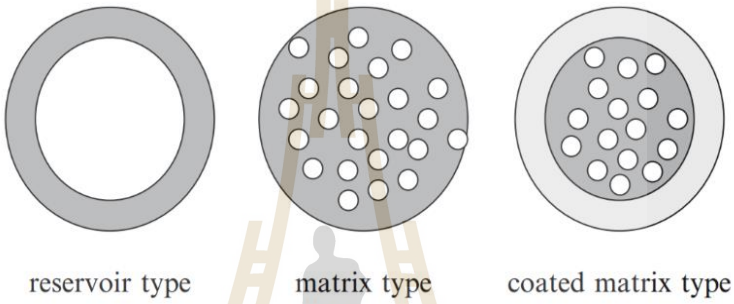


Figure 2.4 Reservoir type, matrix type, and coated matrix type encapsulates. The core is indicated in white, and the wall material in gray.

From: Zuidam and Shimoni (2010)

The benefits of microencapsulated in the food such as improved stability in product and during processing, changeable properties of active components, immobility of active agent in processing of food, controlled release (probiotics, enzymes, antioxidant and vitamin) (Gharsallaoui, Roudaut, Chambin, Voilley, and Saurel, 2007) and microencapsulation have been applied to protect probiotic bacteria from adverse environmental conditions including the low pH in stomach and bile salts, processing condition, and extending viability during storage (Chávez and Ledeboer, 2007). In manufacturing probiotics and other fermented products, freeze drying and spray drying are frequently applied techniques. Freeze drying is a technique used for preserving biological materials and starter cultures. It is an expensive process and

discontinuous with low yields and energy, and time demanding. While spray drying is a promising low-cost alternative because it allows the continuous production of high yields dehydration within short time periods (Ross Crittenden, Weerakkody, Sanguansri, and Augustin, 2006).

2.4.1 Spray drying

Spray drying has been used for periods to encapsulate food ingredients. It was the first patented design in 1872 in the United States. The history of spray drying has its beginning in World War II. It is used for the dairy industries that monopolizing the process changing the milk to milk powder. The principles of the sprayer are moisture removal by heat and controlling the humidity of the powder. The spray drying process consists of (1) atomization that is the first step to transforming liquid to powder that the spread of bulk liquid into a large number of droplets. (2) Evaporation, the droplets encounter with hot air in the chamber and rapid evaporation of moisture from droplets. (3) And the powder is collected at the bottom of the chamber (Cal and Sollohub, 2010; Chickering III, Keegan, Randall, Bernstein, and Straub, 2001).

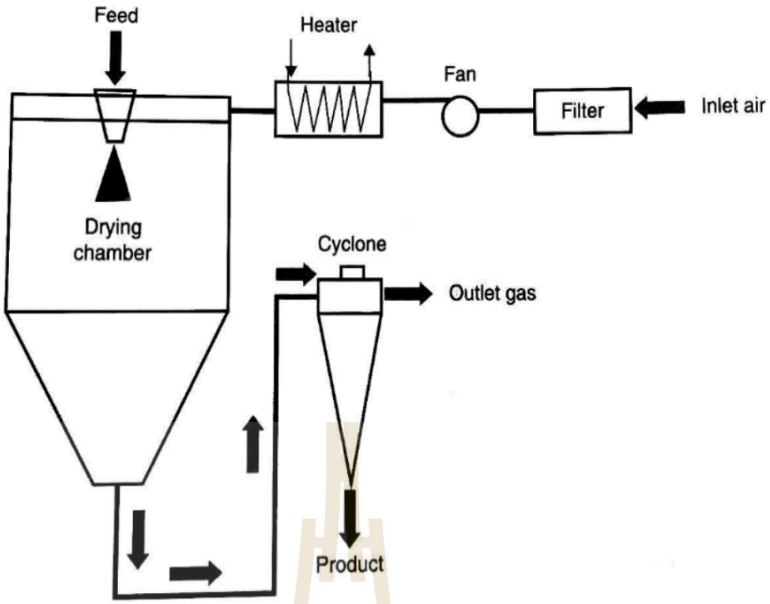


Figure 2.5 Schematic of spray dryer

From: Anandharamakrishnan (2015)

Parameters of spray drying process have affected the final product and storage. The first step is atomization, the important parameters are feed flow rate and feed viscosity. Both of these parameters have affected on the droplet size. When increasing the feed flow rate increases droplet size (Maas et al., 2011). Also when increasing the feed viscosity make increase droplet size. And next step is evaporation. The important parameters are inlet temperature and outlet temperature. Inlet temperature is the heated drying air temperature that have affected on the wet bulb temperature nearby hot air (Foster, Bronlund, and Paterson, 2005). Therefore, higher inlet temperatures increase the wet bulb temperatures nearby hot air affecting on higher throughput. On other hands lower inlet temperature have affected on the protection of functional properties. Another important parameter is outlet temperature that the temperature of air before solid particles enter the cyclone. So it have affected

on the final moisture content and morphology of encapsulation (Foster et al., 2005). Outlet temperature is related to inlet temperature, therefore it cannot be fixed.

During microencapsulation of probiotics bacteria by spray drying, the effect of heat has affected on the viability of the cell. The heat destroys or inactivates component of bacterial cells such as ribosome, protein, DNA and RNA. Base on Santivarangkna, Kulozik, and Foerst (2008) studies, they was found that the high temperature did not mainly effect on the viability but the combination of temperature and time that have the main effect. Therefore in spray drying process outlet temperature is a major impact on the viability of cell because the particle into being dry has the effect of the temperature of particle increase.

Probiotic cells are inactive by dehydration during high drying temperature. The foundation of the dehydration mode of cell inactivation is by removal of water that contributes to the stability of bacterial proteins and DNA. Dehydration also leads to changes in the membrane lipids that help in ruling membrane permeability (Carvalho et al., 2002; Meng, Stanton, Fitzgerald, Daly, and Ross, 2008) thus maintaining the structural integrity of cells. It is therefore clear that, since ribosome is critical for heat inactivation, in a similar way it is the cytoplasmic membrane for dehydration inactivation (Riveros, Ferrer, and Borquez, 2009). This is because the membrane lipid bilayer structures are thermodynamically unstable and are consequently a primary target for dehydration induced damage (Chalat Santivarangkna, Kulozik, and Foerst, 2007; Teixeira et al. , 1997) . Otherwise, dehydration decreases water availability inside encapsulated cells resulting from spray drying. So, the cells attain a state of dormancy, during which the metabolism slows down (Paul, Fages, Blanc, Goma, and Pareilleux, 1993).

Many wall materials can be used to encapsulate but they must be certified for food applications as generally recognized as safe (GRAS). Wall material selection factors are (1) solubility, the preparation process must dissolve core in wall material before spraying. (2) Viscosity, wall material at lower viscosity and high solid concentration is simple to atomization and protect the core (Sanguansri and Ann Augustin, 2010). (3) Glass transition temperature (T_g), the temperature to change physical properties from glassy state to rubbery state (Soukoulis, Lebesi, and Tzia, 2009). T_g depends on molecular weight and chain length of polymer that higher molecular weight and longer chain length have affected on higher T_g. The wall material which high T_g means good protection (Zimeri and Kokini, 2002). Normally, wall material (a polymer) exists glassy state that can limit diffusion of core. But during the thermal process or spray drying that the temperature rises over T_g of wall material it changes to rubbery state, lead to stickiness and tend to promote more diffusion of the core.

2.5 Stress response and heat adaption

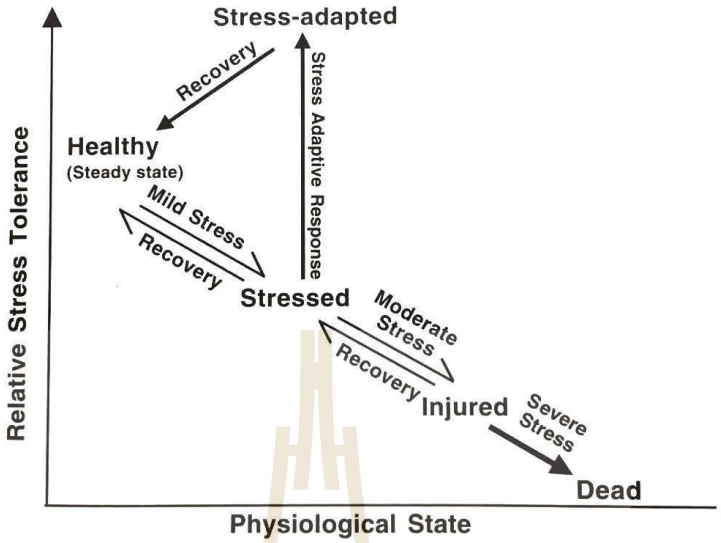


Figure 2.6 Interrelations among physiological states of microbial cell subjected to stresses.

From: Yousef and Juneja (2002)

Microorganism has a stress encountered which is vary in magnitude and outcome. Mild stress is a sublethal stress levels that reduce growth rate of microorganism. Moderate stress is a stress level that is regular lethal to the cells, cause majority reduce the cells population (Schimel, Balser, and Wallenstein, 2007). The stress response of microorganism occurs during food production and processing such as physical treatments (pressure, heat, osmotic, light and oxygen) , chemicals treatments (acids, oxidant and salts) and biological stresses (microbial metabolites, antagonism and competition) (Abee and Wouters, 1999; Yousef and Courtney, 2003). The phenomenon of bacterial cells stress response occurs to change membrane fluidity, alter cell protein structure, disrupt ribosomes, and to affect nucleic acids. (Duncan, Bott, Terlesky, and Love, 2000; Duncan and Hershey, 1989b; Yousef and

Juneja, 2002). Microorganism has an inherent tolerance level to a particular stress. LAB can tolerance to acid conditions than other bacteria (Juneja, Klein, and Marmer, 1998; Kang, Jeon, Shin, Kwon, and So, 2015). When bacteria are subject to a heat shock, cells respond by becoming resistant to lethal heat treatments. During food processing, bacterial are more likely to be killed more than injured or stressed. However, there are processing conditions that constitute a mild stress and thus induce adaptive response (Hecker, Schumann, and Völker, 1996).

Heat shock was begins described in early 1962. Started to study in *Drosophila*, he discovered that adjusted the incubator which his kept tissues to a higher temperature and was called the heat shock (HS) response (Ritossa, 1996). In bacteria, the first publications referring to the heat shock response in *E.coli*. They have studied the Transient regulation of Protein Synthesis in *E. coli* upon shift-up of growth temperature from 30 to 42°C. It was recommended that it had possible to improve the survival rate of the bacteria (Yamamori, Ito, Nakamura, and Yura, 1978). The HS response represents a distinct change in metabolism, involving a transient adjustment to new environmental conditions. One physiological consequent heat challenge which has been reported in a range of cultured mammalian cells, bacteria, yeast and fungi (Mackey and Derrick, 1986). It has been reported that protein synthesis occurs during the period of shock. These proteins have been showed to be involved in the regulation of the response (Whitaker and Batt, 1991; Yamamori et al., 1978). Due to most processing, they must have heat involved. It is reported about heat adaptation in lactic acid bacteria that increased the survival during heat stress. Kang et al. (2015) studied effect of heat adaption on *L.lactis* HE-1. They demonstrated its tolerances to various environmental stresses such as acid, ethanol, H_2O_2 and heat that. They found heat-

adapted cell showed cell survival rate higher than non-adapted cells as 5.47%, 75.00%, 37.7% when exposed to acid, ethanol and H_2O_2, respectively. While the cell viability of heat-adapted cell was 5.53 log cfu/ml but all of non-adapted cells were killed by heat treatment. This result shows that the heat shock protein is not only heat resistant, but also it tolerates something else (Kang et al., 2015). Stress proteins or heat shock proteins (HSPs) are the most conserved chaperone proteins presenting in both prokaryotes and eukaryotes. Their expression is induced in response to a wide variety of physiological and environmental insults.

The metabolism of cells respond to stress, the stress must somehow be sensed. Ribosomes were suggested as sensors for temperature shocks because of the sensitivity of these cellular components to heat (R. F. Duncan and Hershey, 1989a). In addition, changes in the membrane structure of fluidity may trigger a signal to synthesize proteins to counteract a stress (Chun, Li-bo, Di, Jing, and Ning, 2012). *Bacillus subtilis* is a wildly model of Gram-positive bacteria that were studied of response to the heat shock. When *B. subtilis* was shocked by high temperature, the expression of over 200 genes was induced (Darmon et al., 2002). These genes were classified up on transcriptional regulation as six groups. Class I and III were controlled by transcriptional repressors, Class II was controlled by σ factor that the regulates general stress response, Class IV was controlled by transcriptional activators, Class V was controlled by a two-component signal transduction system and Class VI consist operons and the other genes that regulation was unknown (Schumann, 2003). Class I heat shock genes include the *groESL* and *dnaK* operons that coding proteins chaperone complexes GroES-GroEL and DnaK-GrpE-DnaJ, respectively. Under non stressed conditions, both of operons are regulated by the HrcA protein which specific with the

inverted repeat CIRCE (controlling inverted repeat for chaperone expression) (Reischl, Wiegert, and Schumann, 2002). When the temperatures increase the GroE chaperonin is not available for binding to the HrcA protein which changes from the active (repressor) to the inactive. From a mechanism is that the HrcA protein needs the GroE chaperonin system to be able to bind the CIRCE element that has been studied in several lactobacilli such as *L. plantarum* DPC2739, *Lactobacillus rhamnosus* HN001 (De Angelis et al., 2004; Prasad, McJarrow, and Gopal, 2003). Induction of heat tolerance to pasteurization was related to the transient induction of HSPs, the major groups of the DnaK and GroEL families (De Angelis et al., 2004), classified as Class I heat shock genes in *B. subtilis* and are regulated by the HrcA/ CIRCE interaction. Castaldo, Siciliano, Muscariello, Marasco, and Sacco (2006) found that the CcpA protein in the regulation of the groESL and dnaK operons. It works as opposed to HrcA protein that is positive regulation. CcpA protein was described as the regulatory protein mediating carbon catabolite repression that is the specific sequence of DNA target in Gram-positive bacteria. It is called catabolite responsive element (CRE).

CHAPTER III

MATERIALS AND METHODS

3.1.1 Growth curve of *Lactobacillus* sp. 3C2-10

The stock cultures were maintained in deMan, Rogoda and Sharpe (MRS) broth (Himedia®, Hi media Laboratories Pvt. Ltd., Mumbai, India) containing 40% glycerol at -20°C. The cells were sub-cultured in 9 mL MRS broth at temperature 37°C for 24 h under anaerobic conditions. Then, the cultures were transferred into 40 ml MRS broth and incubated at temperature 37°C under anaerobic conditions. Five milliliters of cell suspensions were taken every 2 h. Cells were enumerated by spread plate method using MRS agar after incubation at 37°C for 48 h under anaerobic conditions.

3.1.2 Effect of resistance maltodextrin and Fructooligosaccharides on cell viability

Lactobacillus sp. strain 3C2-10 was inoculated in MRS broth containing 2% (w/ v) RMD or FOS and incubated at temperature 37°C for 48 h under anaerobic conditions. The cell suspension were monitored at time intervals of 0, 24 and 48 h for determining viable cell counts (Huebner, Wehling, Parkhurst, and Hutkins, 2008).

3.2.1 Bacterial strains and cultural conditions

Lactobacillus sp. strain 3C2-10 isolated from cassava pulp (Nawong, 2015) was used in this study. The stock cultures were stored in MRS broth (Himedia®, Hi media Laboratories Pvt. Ltd., Mumbai, India) containing 40% glycerol at temperature

-20°C. The cells were activated in 9 mL MRS broth at 37°C for 24 h under anaerobic conditions. Then, the cultures were transferred into 40 ml MRS broth and incubated at temperature 37°C under anaerobic conditions. The cells were harvested at the early stationary phase (24 h). The cells were centrifuged (5804R, eppendorf, Bangkok, Thailand) at 3,234*g* for 10 min at temperature 25°C. Cell pellets were harvested and resuspended with the sterile 0.85% sodium chloride solution, and the all suspension was centrifuged under the same conditions. Cells were freshly prepared for each experiment and enumerated by spread plate method using MRS agar after incubation at temperature 37°C for 48 h under anaerobic conditions.

3.2.2 Preparation of media

Resistant maltodextrins (Fibersol®) and fructooligosaccharides (FOS) (Sigma-Aldrich®, Sigma-Aldrich Pte. Ltd., Saint Louis, MO, USA) were dissolved in steriled water and gently stirred using a magnetic stirrer (Rajam and Anandharamakrishnan, 2015). All media solutions (20% w/v) were sterilized before use.

3.2.3 Heat resistant

Heat resistance was measured by the method described by Anekella and Orsat (2013). The early stationary phase culture was maintained in each media solution (MRS broth, FOS, and RMD) and incubated in water bath at temperatures of 45, 50, 52, and 55°C respectively. One milliliter aliquots were collected at time intervals (0, 2, 5, 7, 10 and 15 min, respectively) and permitted to standing in water bath at temperature 25°C. Viable cell counts were determined.

3.2.4 Heat adaptation

Active cells of *Lactobacillus* sp. strain 3C2-10 were incubated at temperature 37°C until the early stationary phase (24 h). All cell suspensions were induced the heat adaption at temperatures of 37, 42, 47, 50 and 52°C for 15 min in water bath. After that, the adapted and non-adapted cells were treated at temperature 60°C for 15 min (Anekella and Orsat, 2013). One milliliter aliquots was collected at time intervals (0, 5, 7, 10 and 15 min, respectively). Adapted and non-adapted cells were cooled at temperature 25°C immediately. Viable cell counts were determined on MRS agar plate.

3.2.5 Acid tolerance

Lactobacillus sp. strain 3C2-10 was incubated at temperature 37°C until the early stationary phase (24 h). The cells were incubated at temperature 47°C for 15 min to induce heat adaptation (adapted cells). The acid response was monitored at time intervals (30, 60, 90 and 120 min, respectively). The acid were adjusted using 1 M HCl at pH 2 and pH 3 (Kang, Jeon, Shin, Kwon, and So, 2015). The viable cell counts were determined on MRS agar plate.

3.2.6 The decimal reduction times (D-value) determination

The D-value was calculated following equation as

$$D = t \,/\, Log(\frac{N_o}{N_t})$$

Where N_0 is initial cells viability, N_t is the cells viability at the time t minutes, t is the heating time (minute).

3.2.7 Quantification of heat shock genes

RNA Isolation and Reverse Transcription

The heat shock genes of *Lactobacillus* sp. strain 3C2-10 were determined using quantitative real-time PCR (qRT-PCR) technique. *Lactobacillus* sp. strain 3C2-10 was harvested at 37°C until the early stationary phase (24 h). Adapted cells were induced at 47°C for 15 min. Total RNA of adapted cells ($1.05x10^{10}$ cfu/ml) was RNA kit extracted performed using NucleoSpin® (Macherey-Nagel, Dueren Germany), according to the recommendation of manufacturer. cDNA was prepared using the ReverTra Ace® qPCR RT Master Mix (Toyobo) according to the manufacturer's protocol. Briefly, total RNA template were incubated temperature at 65°C for 5 min and then permitted on ice before adding 4x DN Master mix. The reaction was incubated temperature at 37°C for 5 min. 5x RT Master mix was added to 10 µl of mixture solution. Thermal cycler conditions were set up at temperature 37°C for 15 min, 50°C for 5 min and heat up to temperature at 98°C for 5 min. Then the mixture solution was rapidly cooled down to temperature at 4°C.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

qRT-PCR was performed using QuantStudio™ 5 System (Applied Biosystems, Foster City, CA, USA) and SYBR Select Master Mix (Applied Biosystems). Briefly, the amplified products were examined using 96-well plate in a 20-µl reaction volume containing 10 µl of SYBR® Green Real time PCR Master Mix (Toyobo). Thermocycling for all targets was carried out in a solution of 20 µL under following conditions: 95°C for 2 min, 40 cycles of denaturation (15 s, 95°C), annealing (30 s, 51.5 °C), and extension (45 s, 72°C). Relative expression was determined by 2^ (-delta

delta Ct) method with the expression of *ldhD* as housekeeping reference**.** Direct sequence of 16S rRNA gene were performed using primer as follows**:** *ldhD***:** forward 5´- acgcccaagctgatgttatc and reverse 5´- agtgtcccacgagcaaagtt-3´; *ctsR***:** forward 5´-aatttggtcgatgatgctgatg-3´and reverse 5´-taagtcccggtccgttaatcc-3´; *ftsH***:** forward 5´-gcagcatccttcgaagaatcca-3´and reverse 5´-gggaaacttggttcagcaaca-3´; *dnaK***:** forward 5´-tcaaccgtgtcacccaagta-3´and reverse 5´- tccttaagttgtggcattca-3´ (Castaldo, Siciliano, Muscariello, Marasco, and Sacco, 2006, Daniela Fiocco et al**.**, 2009, Fiocco, Crisetti, Capozzi, and Spano, 2008).

3.3.1 Spray drying

Microencapsulation of *Lactobacillus* sp. strain 3C2-10 with wall materials at different ratios was performed using laboratory-scale spray dryer (BÜCHI B-290, Bangkok, Thailand). Bacterial cells were activated in 3.5 L MRS broth temperature at 37°C under anaerobic conditions until stationary phase. Heat adapted cells were prepared following 3.2.4. Thirty gram of cell pellets mixed with FOS or RMD at ratios of 1:1 and 1:2 (Table 3.1). Spray drying condition was maintained at flow rate 40 m^3/h and 100% constant air inlet temperature at 150 ± 5°C. Spray dried powder was harvested and kept in aluminium foil temperature at 4, 25 and 40°C respectively.

Table 3.1 Composition of solutions before spray drying

Wall material (core-to-wall)	Core (cell pellets, g)	Wall (wall material, g)
FOS 1:1	30	30
FOS 1:2	30	60
RMD 1:1	30	30
RMD 1:2	30	60
FOS+RMD 1:1	30	30
FOS+RMD 1:2	30	60

FOS – Fructooligosaccharides, RMD- Resistance maltodextrin

3.3.2 Enumeration of microencapsulated bacteria and encapsulation efficiency

The cells were released from microencapsules as described by Rajam et al. (2012). A half gram of spray dried powder was dispersed in 0.85% w/v NaCl in a shaking water bath (100 rpm) at temperature 37°C for 30 min to release the cells. The viable cell counts were determined on MRS agar plate after incubation at temperature 37°C for 48 h under anaerobic conditions. Encapsulation efficiency was calculated as

$$\text{Encapsulation efficiency} = \frac{N}{N_o} x\ 100$$

When N_0 is log cell number of viable cells before spray drying and N is after spray drying.

3.3.3 Moisture content and water activity

Moisture content of spray dried powder was determined according to American Association of Cereal Chemists. One gram of sample was kept in aluminum

pans at temperature 105°C. Residual moisture content was calculated using the following formula as

$$\% \text{ moisture content} = \frac{W_f - W_i}{W_i} x\ 100$$

When w_i is the weight of spray dried powder and w_f is the constant of dried powder weight after dehydration at temperature 105°C.

Water activity of spray dried powder were determined using water activity meter (OX-2, AQUA Lab, Charpa techcenter co. Ltd., Bangkok, Thailand).

3.3.4 Morphology and particle size

Morphology of spray dried powder was monitored using scanning electron microscope (SEM) (FEI, Quanta 450, Hillsboro, OR, USA). Spray dried powder adhered on sample stubs by carbon tape were sputter coated with gold for 3 nm Coater (SC7620, Quorum, East Sussex, England). Images were taken under high vacuum with voltage of 10 kV. Particle sizes were determined using ImageJ software. Approximately 150 capsules from multiple images of each treatment were randomly calculated. The report showed average diameter size (Khem, Bansal, Small, and May, 2016).

3.3.5 Bulk density

The bulk density was modified from Kingwatee et al. (2015). Five grams of spray dried powders were tapped and mixed using vortex for 2 min. The ratio of mass of the powder and the volume engaged in the cylinder determines the bulk density value (g/ml)

3.3.6 Solubility

Solubility was determined using the method modified from Cano-Chauca, Stringheta, Ramos, and Cal-Vidal (2005). Briefly, 0.5 g sample powder was added into 100 ml of distilled water and stirred for 5 min. The solutions were centrifuged at 3,234g for 10 min at temperature 25°C. The sediment was dried using vacuum oven at temperature 100 °C. The percentage solubility was calculated as

$$\%\ \text{solubility} = \frac{S_f - S_i}{S_i}\ x\ 100$$

When s_i is the weight before dehydration and s_f is the constant weight after dehydration at temperature 100°C.

3.3.7 Fourier transform infrared (FTIR) measurement

The FTIR spectra of microcapsules with probiotic cells were established with a Fourier transform infrared spectrometer (Tensor 27, Bruker, Germany) using the diamond crystal detector. The spectra of the samples were obtained in transmission mode from 600 to 4000 cm^{-1} wave number range.

3.4 Gastro-intestinal tract simulation

Simulated gastric juice (SGJ) was prepared by dissolving pepsin (P-7000, Sigma-Aldrich®, Sigma-Aldrich Pte. Ltd., Saint Louis, MO, USA) in 0.2%w/v NaCl to final concentration of 0.3 g/L and adjusting the pH to 2.0 with 0.1 M HCl. Then the mixture solution was sterilized by passing through 0.2 µm membrane filter (Corning®, Corning Incorporated, NY14831, Germany).

Simulated intestinal juice (SIJ) was prepared according to Huang and Adam (2004). Bile salts (Difco®, Becton, Dickinson and Company, Franklin Lakes, NJ,

USA) and pancreatin (P-8096, Sigma-Aldrich®, Sigma-Aldrich Pte. Ltd., Saint Louis, MO, USA) were suspended in 0.02 M phosphate buffer to final concentration of 4.5 g/L and 1g/L, respectively and adjusted to pH 7.4 with 0.1 M NaOH. Then the mixture solution was sterilized. Fresh simulated gastric and intestinal juices were prepared freshly just before test.

Washed cells of *Lactobacillus* sp. strain 3C2-10 and 0.5 g spray dried powder were add to 4.5 ml of SGJ (37°C), mixed by gentle vortexing for 10 s and incubated temperature at 37°C for 60 min. Then added 1.25 ml of SIJ (37°C) and adjusted to pH 7.4 by 0.1M NaOH. Final volume was made up to 10 ml with 0.02M phosphate buffer and incubated temperature at 37°C for 0-240 min. Surviving bacteria was enumerated as described in section 3.3.2.

3.5 Survival of microencapsulated *Lactobacillus* sp. strain 3C2-10 during storage

The cell viability of *Lactobacillus* sp. strain 3C2-10 during storage temperatures at 4, 25 and 40°C was evaluated as described in 3.3.2. Encapsulated cell viability was determined. The storage time (t, day) and the cell viability logarithmic value ($\log N_t/N_i$) were plotted as X and Y axis respectively. First order reaction kinetics model was calculated as

$$\text{Log}\,\frac{N_t}{N_i} = k_T t$$

Where N_i is number of cells viability at initial of storage (cfu/g), N_t is number of cells viability at the storage period (cfu/g), k_T is the specific rate of viability loss and t is the storage time (days).

3.6 Statistical analyses

All experiments with triplicate samples were conducted for each treatment. All statistical analysis were performed using SPSS (version 15.0, SPSS Inc., USA). Data are presented as mean ± standard deviation (SD). Means were compared by independent-samples t-test. Differences were considered significantly at $p < 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 *Lactobacillus* sp. strain 3C2-10 and wall material

4.1.1 The growth of *Lactobacillus* sp. strain 3C2-10

The growth of *Lactobacillus* sp. strain 3C2-10 was investigated with MRS agar at temperature 37°C and incubation for 48 h. The log phase was observed between 12-24 h. The stationary phase of growth after 24 h was showed in Figure 4.1. The sub-lethal treatment of *Lactobacillus* was exhibited in stationary phase which enhanced their survival during lethal treatment (Saarela et al., 2004).

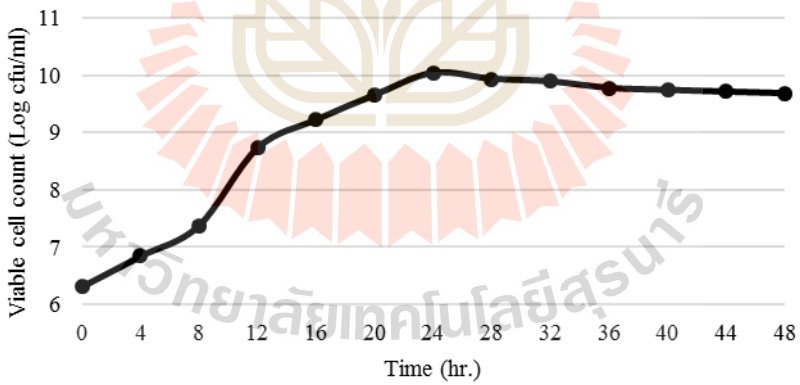


Figure 4.1 Growth of *Lactobacillus* sp. strain 3C2-10 on MRS at temperature 37°C for 48 h.

4.1.2 Effect of MRS, Fructooligosaccharides and resistance maltodextrin on cell viability of *Lactobacillus* sp. strain 3C2-10

The viability of *Lactobacillus* sp. strain 3C2-10 with MRS, FOS and RMD were investigated during incubation for 0, 24 and 48 h and the results showed in Figure 4.2. The viability of *Lactobacillus* sp. strain 3C2-10 after incubation with MRS, FOS, and RMD, respectively for 24 h showed significantly different ($p<0.05$). The highest cells viability of *Lactobacillus* sp. strain 3C2-10 incubation with MRS medium for 24 and 48 h were 10.46 ± 0.02 and 10.38 ± 0.01 log cfu/ml respectively. In addition, the cells viability of *Lactobacillus* sp. strain 3C2-10 incubation with FOS and RMD for 24 h were 9.83 ± 0.04 and 9.83 ± 0.04 log cfu/ml respectively. The cells viability of *Lactobacillus* sp. strain 3C2-10 incubation with FOS and RMD for 48 h decreased slightly at 9.75 ±0.04 and 9.30 ±0.02 log cfu/ml respectively. MRS medium is a enriched nutrients, which promotes growth of lactic acid bacteria (Jianling, Shuping, Wanling, and Yingtuan, 2007). Oku et al., (2015) reported that RMD had the effect on growth of some strains of *Bifidobacterium* and *Lactobacillus.* In addition, FOS and RMD were major prebiotics, which stimulated the growth of probiotics (Miyazato, Kishimoto, Takahashi, Kaminogawa, and Hosono, 2016).

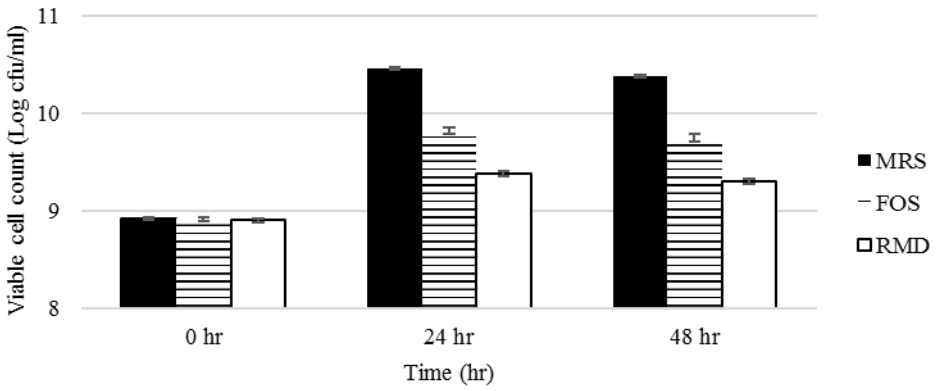


Figure 4.2 The *Lactobacillus* sp. strain 3C2-10 growth in MRS broth, Fructooligosaccharides (FOS) and resistant maltodextrin (RMD) incubated at 37°C for 48 h under anaerobic conditions.

4.2 Effect of heat resistant, heat adaptation and spray drying outlet temperature on cell viability of *Lactobacillus* sp. strain 3C2-10

4.2.1 Effect of heat resistant on cell viability of *Lactobacillus* sp. strain 3C2-10 in MRS, FOS, and RMD

The heat resistant of *Lactobacillus* sp. strain 3C2-10 was studied by incubation with MRS, FOS, and RMD and exposed to various temperature (45, 50, 52 and 55°C) for 0-15 min. The viability of Lactobacillus sp. strain 3C2-10 at the temperature of 50, 52 and 55°C is showed in Figure 4.3. The D-value of MRS medium incubation was higher than RMD and FOS incubation at all temperature tested. De Angelis et al. (2004) reported that a complex media such as MRS medium, skim milk and sterile milk had affected the survival rate of bacteria including better cells protection than a simple medium. Anekella and Orsat (2013) established that MRS medium was effective in survival of *Lactobacillus acidophilus* NRRL B-4495 and

Lactobacillus rhamnosus NRRL B-442 at temperature 50-52°C but not in raspberry juice medium.

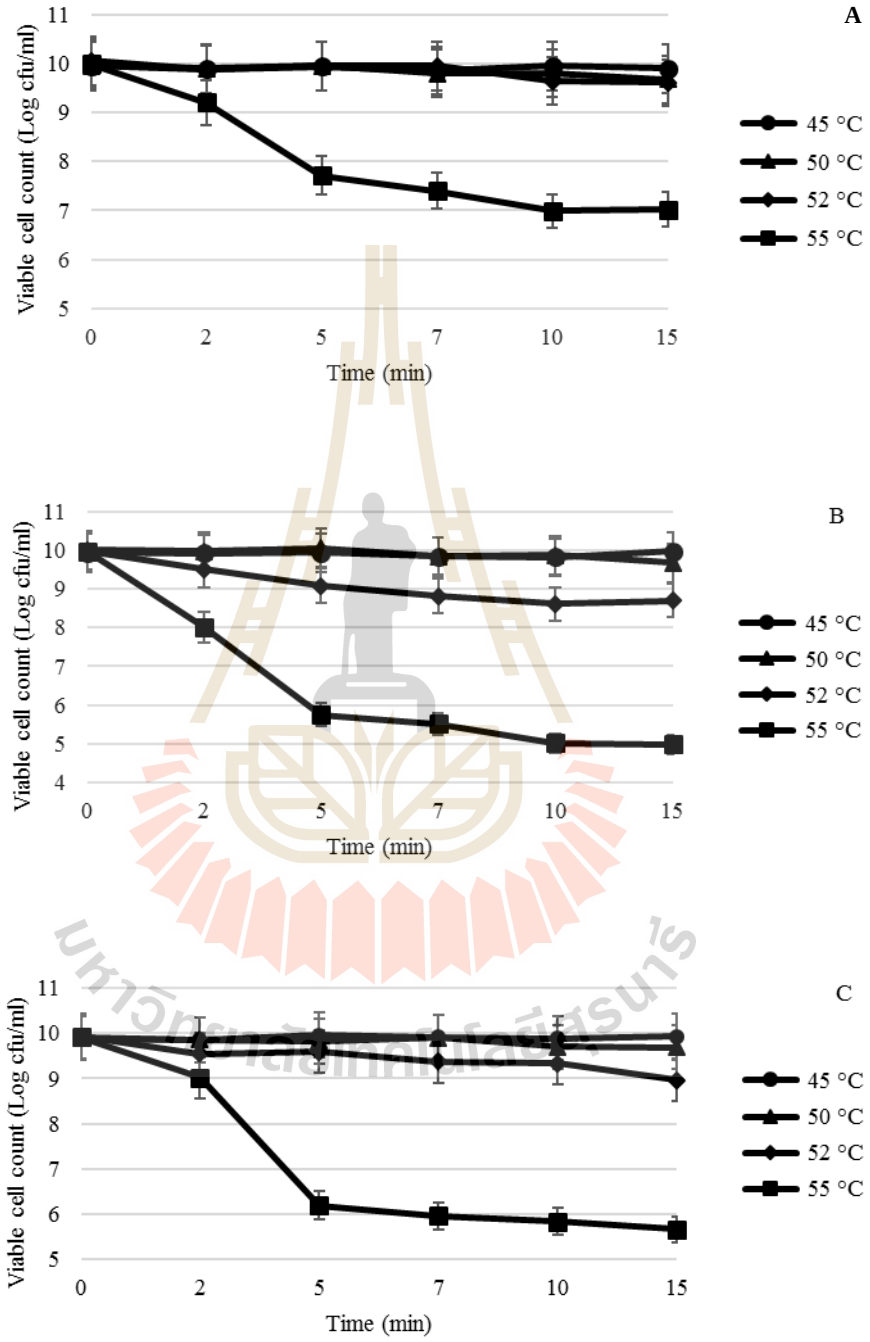


Figure 4.3 The viability of *Lactobacillus* sp. strain 3C2-10 in (A) MRS broth (B) FOS (C) RMD at the temperatures of 45, 50, 52 and 55°C

4.2.2 Effect of heat adaptation on cell viability of *Lactobacillus* sp. strain 3C2-10 in MRS broth at the various temperature

Lactobacillus sp. strain 3C2-10 in MRS broth were pre-treated with temperatures at 37, 42, 47, 50 and 52°C for 15 min. The adapted cells were exposed to temperature at 60°C for 15 min. The effect of heat adaption on cells viability of *Lactobacillus* sp. strain 3C2-10 were showed in Figure 4. 4. A D-value of *Lactobacillus* sp. strain 3C2-10 at temperature 60°C for pre-heated with temperatures at 37, 42, 47, 50 and 52°C were 3.21, 4.48, 10.14, 2.24 and 2.13 min, respectively. These result indicated the optimum temperature for heat adaptation of *Lactobacillus* sp. strain 3C2-10 was at 47°C. Previously studied found that the highest D-values of *L. lactis* HE-1 was at 7.85 min when *L. lactis* HE-1 was treated at temperature 42°C for 15 min in MRS broth before exposed to temperature at 60°C for 10 min (Kang, Jeon, Shin, Kwon, and So, 2015). Generally, the temperature increased 10°C from optimum temperature of bacterial growth leads to bacterial cells thermal shock effect (Teixeira, Castro, and Kirby, 1994). The adaptive response of cells during growth involves some genes activation from coping with adverse stress conditions and maintains cells viability. This cell adaption leads to a general stress response thus obtains more resistance cells that could survive under adverse growth conditions. De Angelis et al. (2004) found that genes in the family of DnaK and GroEL were involved in this response.

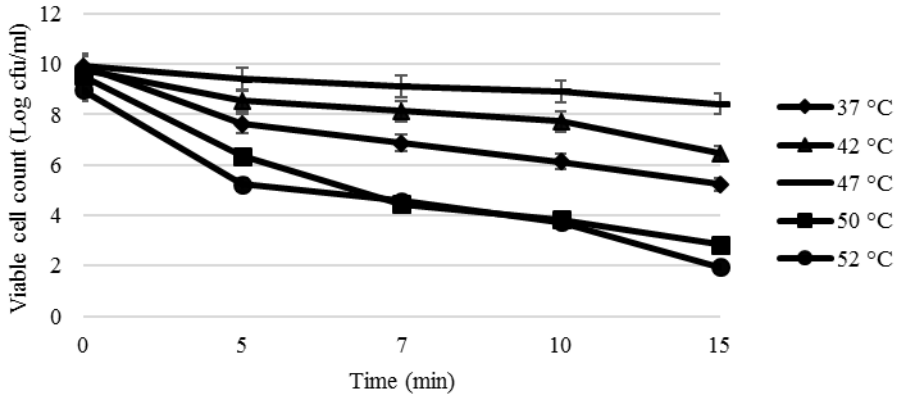


Figure 4.4 The viability of *Lactobacillus* sp. strain 3C2-10 in MRS broth were treated at the temperaturesof 37, 42, 47, 50 and 52°C before exposed 60°C 15 min.

4.2.3 Effect of heat adaptation on cells viability of *Lactobacillus* sp. strain 3C2-10 in MRS broth at various pH

The *Lactobacillus* sp. strain 3C2-10 was induced for heat adaptation in MRS broth at temperature 47°C for 15 min. Both adapted and non-adapted cells were exposed to the pH 2 and pH 3 for up to 120 min. The effect of heat adaptation on cells viability were evaluated and the results are showed in Figure 4.5. The loss of adaptive and non-adaptive cells at pH 2 condition showed no significantly different ($p>0.05$) throughout the 120 min period (approximately 4 log cfu/ ml). In the meanwhile, the viability loss of adapted cells (1.9 log cfu/ml) at pH 3 condition was lower than non-adapted cells (4 log cfu/ ml) and showed significantly different ($p<0.05$) throughout the period 120 min. Kang et al. (2015) supported that the cells viability of *L. lactis* HE-1 adapted cells were higher than non-adaptive cells before exposed to low pH condition. Lactic acid bacteria produced lactic acid during sugar fermentation, indicated imply that they were frequently exposed to acid stress (Lin et al., 1996). It is important to note that lactic acid was a weak organic acid that not

charged at low pH; therefore, it could easily passed through the cell membrane in the protonated form (Rallu, Gruss, Ehrlich, and Maguin, 2000).

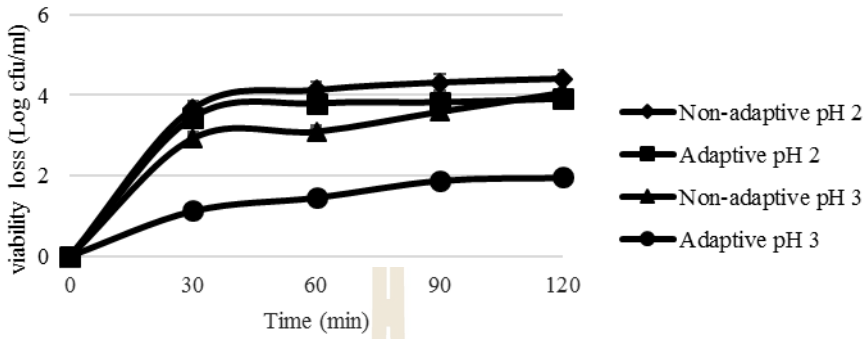


Figure 4.5 The viability of *Lactobacillus* sp. strain 3C2-10 treated at pH 2 and pH 3 after exposed to temperature at 47°C for 15 min.

4.2.4 Gene expression after heat adaptation using Quantitative Reverse Transcription Polymerase Chain Reaction

Gene expression after heat adaptation at temperature 47°C for 15 min is showed in Figure 4.6 and confirmed by using Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) technique. Gene expression data were normalized as relative fold gene expression following the method of Chiu, Guu, Liu, Pan, and Cheng, (2007) and Hernandez-Santana et al., (2016). The adapted cells showed gene expression of *ctsR*, *ftsH* and *dnaK* but not in non-adaptive cells. Gene *ctsR*, *ftsH* and *dnaK* of adaptive cells expressed as 1.5, 1.6 and 2.5 fold, respectively. Recently Fiocco et al. (2010) and Fiocco et al. (2009) studied expression of *ctsR* and *ftsH* gene in *L. plantarum* after induced by heat. The *ftsH* gene was controlled by expression of *ctsR* gene when treated with temperature at 42°C. The *ftsH* gene strongly increased about 8 folds after 10 min heat treatment. After even 1 h, the *ftsH* gene expressed 2 folds. Castaldo, Siciliano, Muscariello, Marasco, and Sacco (2006) found

that *dnaK* gene in *L. plantarum* LM3 increased 16 folds after induced with temperature at 50°C for 15 min.

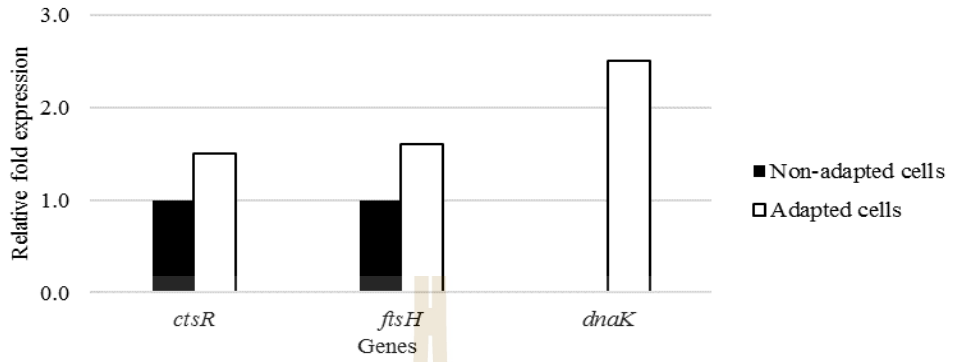
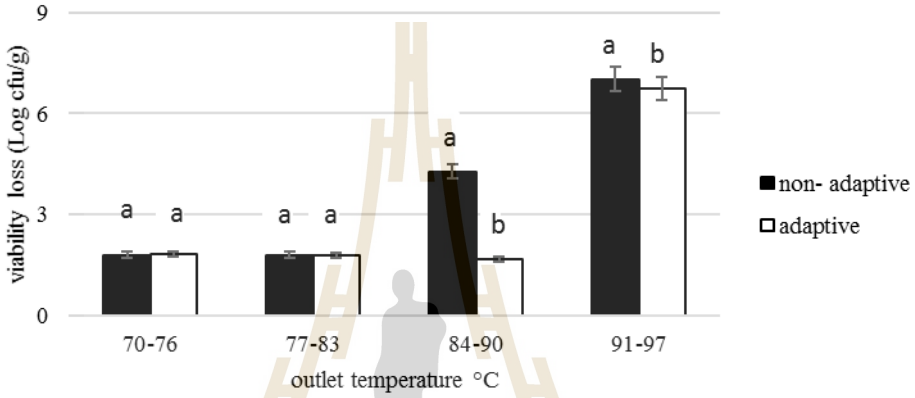


Figure 4. 6 Gene expression of adapted cells and non-adapted cells after expose at temperature 47°C for 15 min using Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

4.2.5 Effect of spray drying outlet temperature on cells viability of *Lactobacillus* sp. strain 3C2-10

The destruction of cells during spray drying process depended on the combinations of temperature and time (Santivarangkna, Kulozik, and Foerst, 2008) and affecting the heat resistance of the cells (Teixeira, Castro, Mohácsi-Farkas, and Kirby, 1997). The effect of spray drying outlet temperature on cells viability in RMD with and without heat adaptation (temperature at 47°C for 15 min) were performed and the result are showed in Figure 4. 7. The viability loss of adapted cells are significantly lower during spray drying process at the outlet temperature of 84-90°C, compared to the outlet temperatures at 70-76, 77-83, and 91-97°C respectively. However the survival rate of adapted and non-adapted cells were not significantly different ($p>0.05$) at outlet temperature 70-76 and 77-83°C, respectively (Figure 4.7). In addition, the adapted and non-adapted cells were more heat sensitive at outlet

temperature 91-97°C than lower outlet temperature tested. It had been reported that the outlet temperature at higher than 85-90°C was respected to be lethal for probiotics generally (Corcoran, Ross, Fitzgerald, and Stanton, 2004) . Heat adaptation could enable cells to survive at the outlet temperature up to 92°C (Anekella and Orsat, 2013).



[a] and [b] values in the same outlet temperature with a different letter were significant different ($p<0.05$)

Figure 4.7 The viability loss of *Lactobacillus* sp. strain 3C2-10 after spray drying in RMD with or without heat adaptation at spray drying outlet temperature between 70 and 97°C.

4.3 Encapsulation of *Lactobacillus* sp. 3C2-10 with fructooligosaccharides, resistant maltodextrin by spray drying process

4.3.1 Effect of wall material and core-to-wall ratio on viability of *Lactobacillus* sp. strain 3C2-10

The cell viability of *Lactobacillus* sp. strain 3C2-10 depended on the encapsulation efficiency (Rajam, Karthik, Parthasarathi, Joseph, and Anandharamakrishnan, 2012). *Lactobacillus* sp. strain 3C2-10 cells were encapsulated with three different

wall materials such as FOS, RMD, and FOS in combination with RMD (FOS+RMD). The percentage of encapsulation efficiency of FOS+ RMD showed higher than FOS and RMD wall materials ($p<0.05$) as showed in Table 4.1. The application of wall material combination at a core-to-wall ratio of 1: 1 and 1: 2 were not significant different ($p>0.05$) at 91.30 and 91.57% respectively. Pai, Vangala, Ng, Ng, and Tan (2015) reported that an increase in RMD loading from 20% to 40% (w/v) led to 60% to 80% the encapsulation efficiency elevation and probably, the thickness of micro particle shell walls was increased (Balasubramani et al., 2015; Lauro, De Simone, Sansone, Iannelli, and Aquino, 2007; Pai et al., 2015; Tuyen, Nguyen, and Roach, 2010). High hygroscopic and low molecular weight restricted the application of FOS as encapsulating matrix due to the difficulty in converting into dry powder form through spray drying process (Rajam and Anandharamakrishnan, 2015; Ranadheera, Baines, and Adams, 2010). The molecules of hydrophilic carbohydrate may be accumulated into cells and cause prevention of cells proteins from denaturation. The hydrogen bonds formation ensured the stable tertiary structure of proteins in the lack of water (Leslie, Israeli, Lighthart, Crowe, and Crowe, 1995).

4.3.2 Moisture content and water activity

The moisture contents of the spray dried microcapsules ars displayed at the level of 2.73 to 2.21% (Table 4.1). Moisture content depends on the inlet and outlet temperature of air drying, feed solution concentration, and composition of feed solution. The percentage of moisture content has affected to the particle size in spray drying technique. In addition, water activity for all wall materials tested were not significantly different ($p>0.05$). Darmon et al. (2002) revealed that the water activity depended on the outlet temperature including affected the microbial growth.

Generally, the water activity to limit the growth of bacteria should be maintained lower than 0.25 (Chávez and Ledeboer, 2007).

4.3.3 Particle size and bulk density

Particle size is important physical properties that directly affect the application of microcapsules into food formulation. Microcapsules exhibited a range of size varying from 8.58 to 28.42 μm diameter (Table 4.1). The microcapsules of 1:2 core-to-wall ratio showed larger particle size than 1: 1 in all wall material tested, because of wall material concentration increased. In addition, the FOS also showed a larger particle size than RMD.

The bulk density was evaluated during storage processing and packaging. The factors that affected bulk density are the particle size, morphology and moisture content. The results showed significantly different ($p<0.05$) in the bulk density of during storage time. The bulk density of microcapsules ranged from 0.41 to 0.52 g/ml (Table 4.1). The FOS encapsulates displayed higher value of bulk density due to the particle aggregation and less interspace between particles (Rajam and Anandharamakrishnan, 2015). In addition, the FOS+ RMD microcapsules had lower bulk density than FOS and RMD. Rajam and Anandharamakrishnan (2015) suggested that morphology directly influenced the bulk density, flow ability and rehydration characteristics of encapsulated powder.

4.3.4 Solubility

The solubility percentage of spray dried microcapsules are varied from 81.70 to 99.09% (Table 4.1). The RMD microcapsules of 1:1 and 1:2 core-to-wall ratio exhibited significantly higher solubility percentage than FOS and FOS+ RMD ($p<0.05$). Probably, the solubility had affected high hygroscopicity and

contradict to bulk density (Kingwatee et al., 2015). The powder with low solubility property should have high bulk density (Fazaeli, Emam-Djomeh, Ashtari, and Omid, 2012).

Table 4.1 Effect of wall material and core-to-wall ratio on encapsulation efficiency and physical properties of microcapsules.

Wall material	Core-to-wall ratio	Encapsulation efficiency (%)	Moisture content (%)	Water activity ^{ns}	Particle size (µm)	Bulk density (g/ml)	Solubility (%)
FOS	1:1	76.76 ± 0.65^{d}	2.55 ± 0.11^{abc}	0.26 ± 0.06	22.44 ± 6.26^{ab}	0.50 ± 0.01^{ab}	85.65 ± 1.33^{c}
	1:2	80.62 ± 0.16^{c}	2.73 ± 0.18^{a}	0.28 ± 0.06	28.42 ± 5.42^{a}	0.52 ± 0.04^{a}	81.70 ± 0.83^{d}
RMD	1:1	88.46 ± 0.50^{b}	2.47 ± 0.08^{bc}	0.25 ± 0.06	8.58 ± 3.37^{c}	0.44 ± 0.01^{cd}	99.09 ± 0.20^{a}
	1:2	89.37 ± 1.00^{b}	2.58 ± 0.09^{ab}	0.20 ± 0.03	12.78 ± 5.05^{bc}	0.47 ± 0.01^{bc}	97.71 ± 0.43^{a}
FOS+RMD	1:1	91.30 ± 0.27^{a}	2.21 ± 0.09^{d}	0.22 ± 0.04	16.64 ± 6.57^{bc}	0.41 ± 0.02^{d}	94.90 ± 1.12^{b}
	1:2	91.57 ± 0.92^{a}	2.33 ± 0.11^{cd}	0.26 ± 0.07	19.29 ± 6.31^{abc}	0.43 ± 0.02^{d}	93.85 ± 0.33^{b}

$^{a, b, c}$ and ^{b} values in the same column with a different letter were significant different ($p<0.05$), ^{ns} as non-significant different ($p>0.05$)

FOS = fructooligosaccharides, RMD = resistant maltodextrin, FOS+RMD = combination of FOS and RMD ratio 1:1

4.3.5 Morphology of spray dried microcapsules

Morphology of spray dried microcapsules are displayed in Figure 4.8. FOS microcapsules of 1:1 and 1:2 core-to-wall ratio are showed in Figure 4.8A and 4.8B, respectively. The RMD microcapsules of 1:1 and 1:2 core-to-wall ratio are showed in Figure 4.8C and 4.8D, respectively. All microcapsules are observed as irregular and spherical particles with wrinkled surface but shrinkage and smooth on some surface area. Nevertheless, core-to-wall ratio did not affect morphology of microcapsules (Rajam and Anandharamakrishnan, 2015).

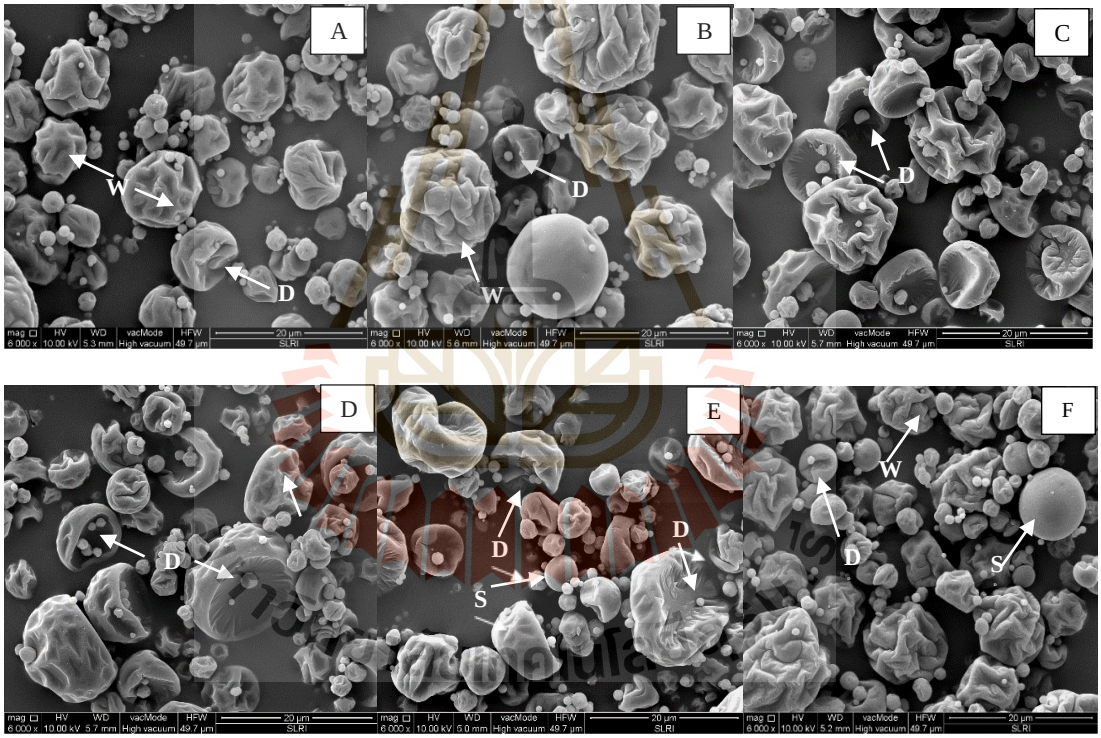


Figure 4.8 Morphology of spray dried microcapsules produced with three different of wall materials in 1:1 and 1:2 core-to-wall ratios. (A) FOS 1:1 (B) FOS 1:2 (C) RMD 1:1 (D) RMD 1:2 (E) RMD+FOS 1:1 (F) RMD+FOS 1:1. S-smooth, D-dents, and W-wrinkles.

4.3.6 Fourier transform infrared (FTIR)

The spray dried *Lactobacillus* sp. strain 3C2-10 microcapsules were analyzed by FTIR to obtain spectral information for each wall material (Figure 4.9). The spectra of all wall materials had fingerprint region of 1,200 to 800 cm^{-1} region. The C-C and C-O groups vibration modes were displayed indicating the carbohydrates showed their characteristic bands (Ibrahim, Alaam, El-Haes, Jalbout, and Leon, 2006). All wall materials tested had strong absorptions at 1,026 cm^{-1} (representing as glucose) and 990 cm^{-1} (representing as fructose). At day 0, the particles exhibited higher spectral adsorption than those at 60 days storage condition at temperature of 4, 25 and 40°C, respectively. The concentrations of glucose and fructose had decreased during storage conditions at temperature of 40°C for 60 days.

The direct measurement spectrum shows the spectral bands arising from fatty acids (1,650 to 1540 cm^{-1}) (Figure 4.10). Presumably, fatty acids found could be acetic acid, propionic acid and butyric acid (Koca, Rodriguez-Saona, Harper, and Alvarez, 2007) and produced by *Lactobacillus* sp. strain 3C2-10. FTIR spectra of fatty acids showed strong bands during storage condition at temperature of 40°C for 60 days.

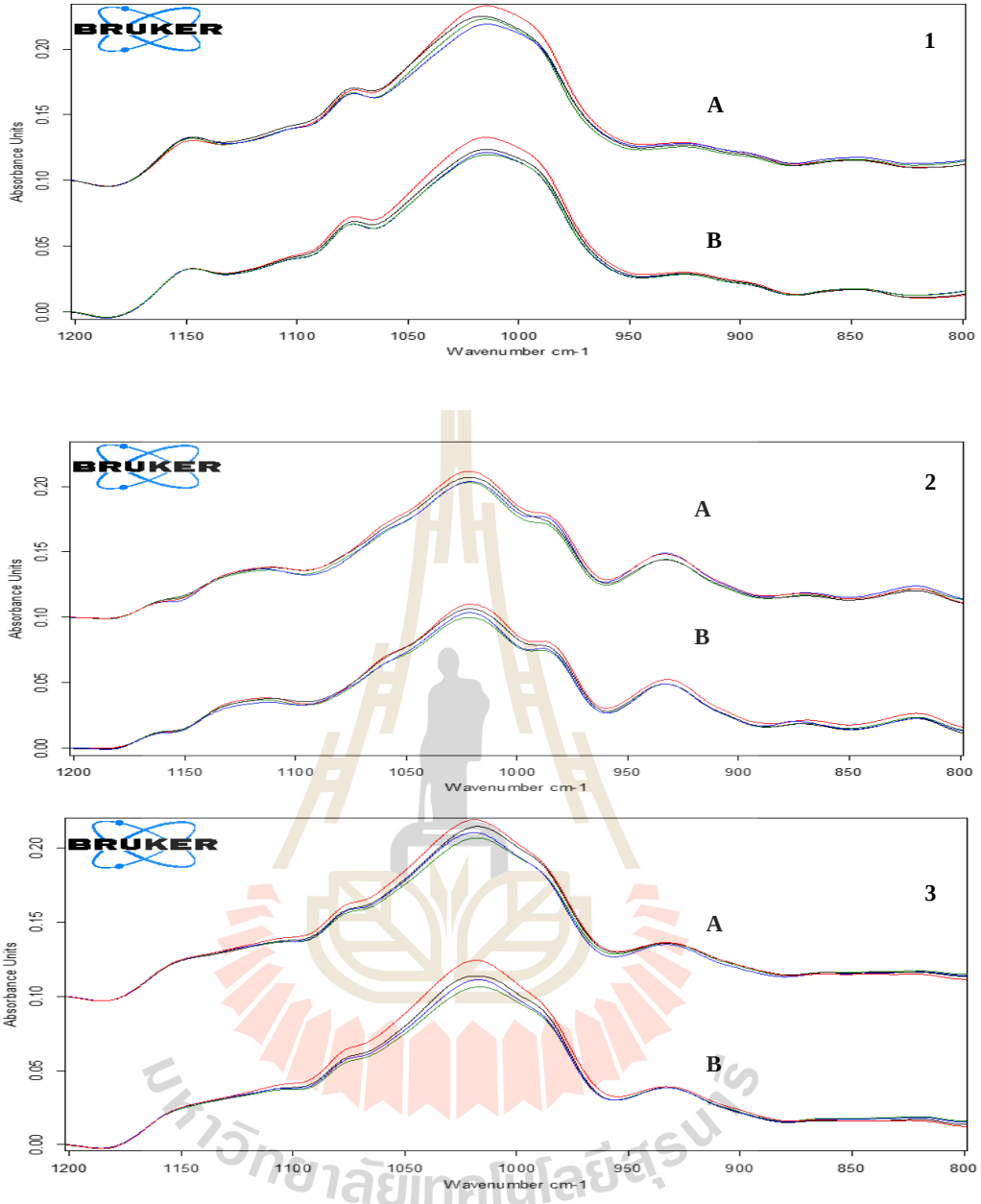


Figure 4.9 FTIR spectra of carbohydrate of spray dried *Lactobacillus* sp. strain 3C2-10 microcapsules (1) RMD, (2) FOS and (3) combination of FOS and RMD represent the storage at 0 and 60 day (—0 day, —4 °C for 60 day, — 25°C for 60 day, —40°C for 60 day) in the 1,200 to 800 cm^{-1} region. (A) core-to-wall ratio 1:1 (B) core-to-wall ratio1:2

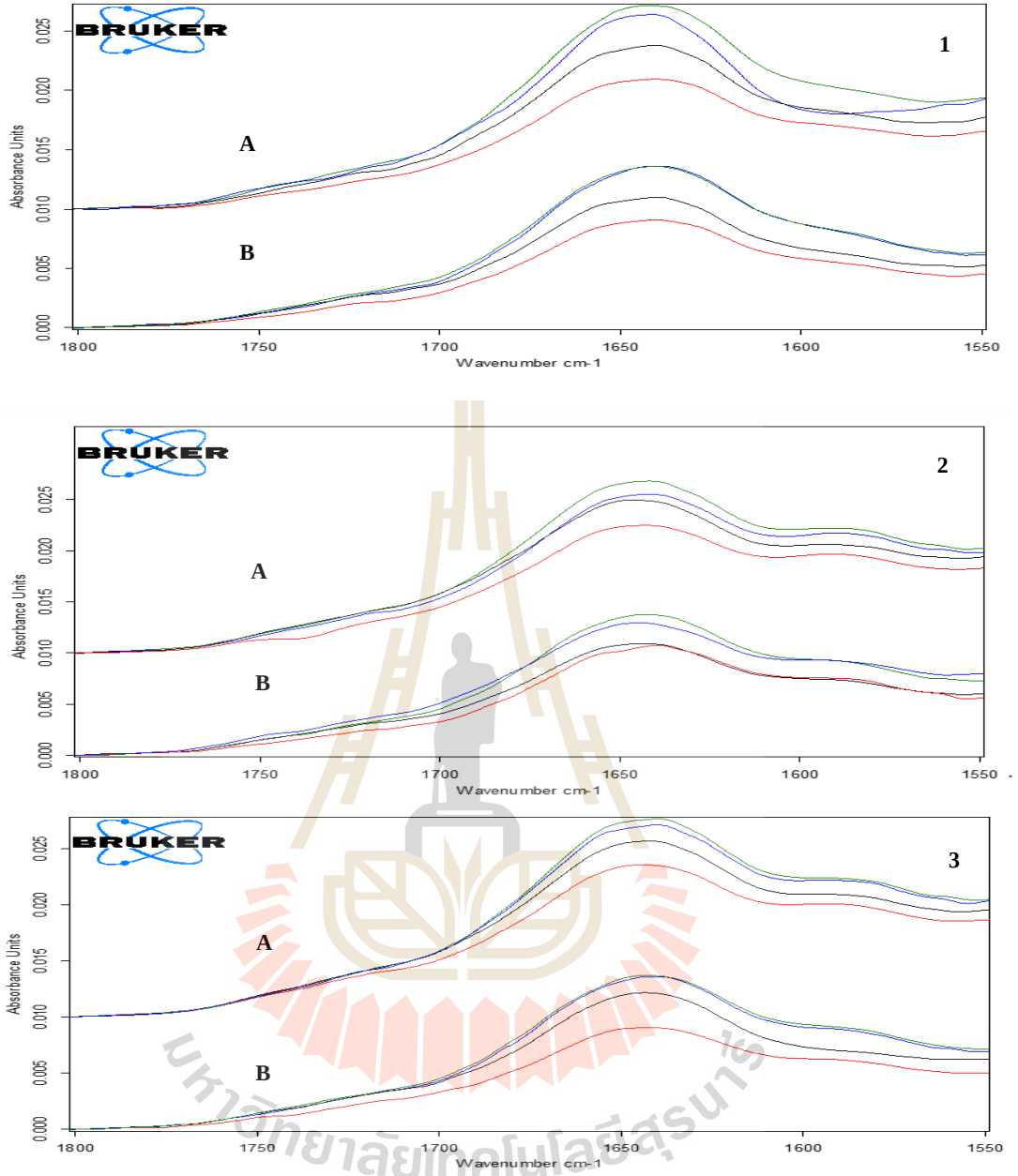


Figure 4.10 FTIR spectra of fatty acid of spray dried *Lactobacillus* sp. strain 3C2-10 microcapsules (1) RMD, (2) FOS and (3) combination of FOS and RMD represent the storage at 0 and 60 day (—0 day, —4 °C for 60 day, — 25°C for 60 day, —4°C for 60 day) in the 1,800 to 1,550 cm^{-1} region. (A) core-to-wall ratio 1:1 (B) core-to-wall ratio1:2

4.4 Stability of spray dried probiotic microcapsules in gastric condition

4.4.1 Survival of free and microencapsulated *Lactobacillus* sp. strain 3C2-10 in simulated gastric digestion.

The survival of free and microencapsulated *Lactobacillus* sp. strain 3C2-10 simulated gastric digestion was investigated. The effect of encapsulated cells on survival of *Lactobacillus* sp. strain 3C2-10 cells with the simulated gastric juice (SGJ) during 120 min is showed in Figure 4.11. The viability of free cells were decreased approximately 3.5 log cfu/g after exposured to SGJ for 30 min. A high survival rate was observed for all microcapsules of core-to-wall ratio 1:2 ($p<0.05$) in which the survival rate were decreased approximately 1.71-1.79 log cfu/g after exposured to SGJ for 120 min. In addition, the reduction in all microcapsules of core-to-wall ratio 1:1 was approximately 2.08-2.20 log cfu/g after exposured to SGJ for 120 min, free cells were not survived in the SGJ condition. However, the simulation of gastric juice *in vitro* probably overestimated viability loss relative to the scenario *in vivo* because the food components might temporarily elevate the gastric pH (Borza et al., 2010). Accordingly, a strong correlation of the final survival rate of *Lactobacillus* sp. strain 3C2-10 within FOS+RMD microcapsules of core-to-wall ratio 1:2 in SGJ for 120 min were achieved.

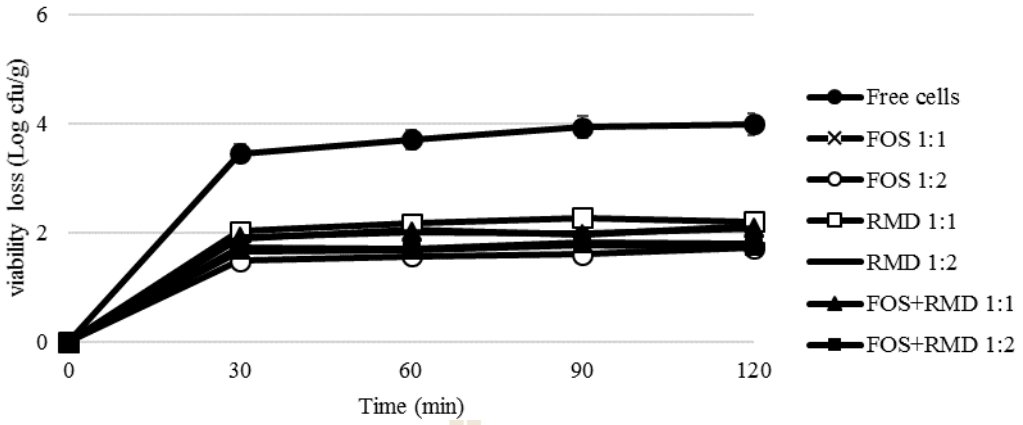


Figure 4.11 The viability loss of microencapsulated *Lactobacillus* sp. strain 3C2-10 and free cells after exposured to SGJ (pH 2) at 37°C

4.4.2 Survival rate of free and microencapsulated *Lactobacillus* sp. strain 3C2-10 in simulated gastric and intestinal juice

Lactobacillus sp. strain 3C2-10 in the microcapsules at 1:1 and 1:2 core-to-wall ratio sequential exposure to SGJ for 60 min followed by 120,180 and 240 min incubation in simulated intestinal juice (SIJ) with bile (pH7. 4) survived significantly ($p<0.05$) better than free cells (Figure 4.12). For free cells, the viability were reduced by 4. 52 log cfu/ g after the initial incubation in SIJ for 60 min and decreased up to 4. 96 log cfu/ g after incubation in SIJ for 240 min. In addition, FOS+RMD microencapsulation of 1:2 core-to-wall ratio supported the survival rate of *Lactobacillus* sp. strain 3C2-10 after sequential exposure to SGJ for 60 min followed by 240 min in SIJ. The viable numbers were reduced by approximately 2.53 log cfu/g. The FOS, RMD and FOS+RMD affected the survival rates of *Lactobacillus* sp. strain 3C2-10 in the upper gastrointestinal tract condition. The previously researcher suggested that the prebiotics such as FOS and GOS were indirect protected depending on their general abilities to stimulate and sustain the bacterial growth (Pan, Wu,

Zhang, Cai, and Song, 2009; Tymczyszyn, Gerbino, Illanes, and Gómez-Zavaglia, 2011). Schwab, Vogel, and Gänzle (2007) reported that FOS was directly interacted with membrane and maintained its integrity and fluidity of bacterial cells thus improving cellular stability to acid stress.

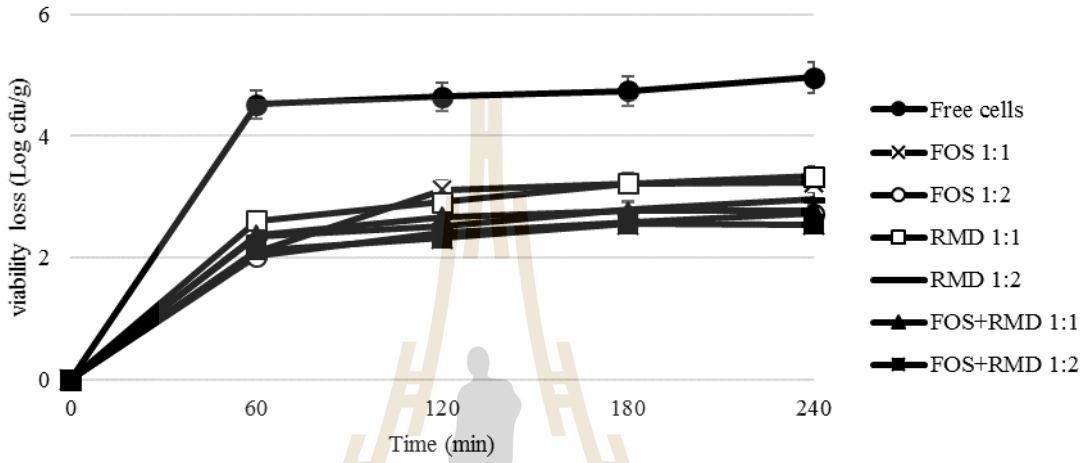


Figure 4. 12 The viability loss of *Lactobacillus* sp. strain 3C2-10 free cells and encapsulated after exposed to SGJ for 60 min following SIJ at temperature of 37°C.

4.5 Survival of microencapsulated *Lactobacillus* sp. strain 3C2-10 during storage

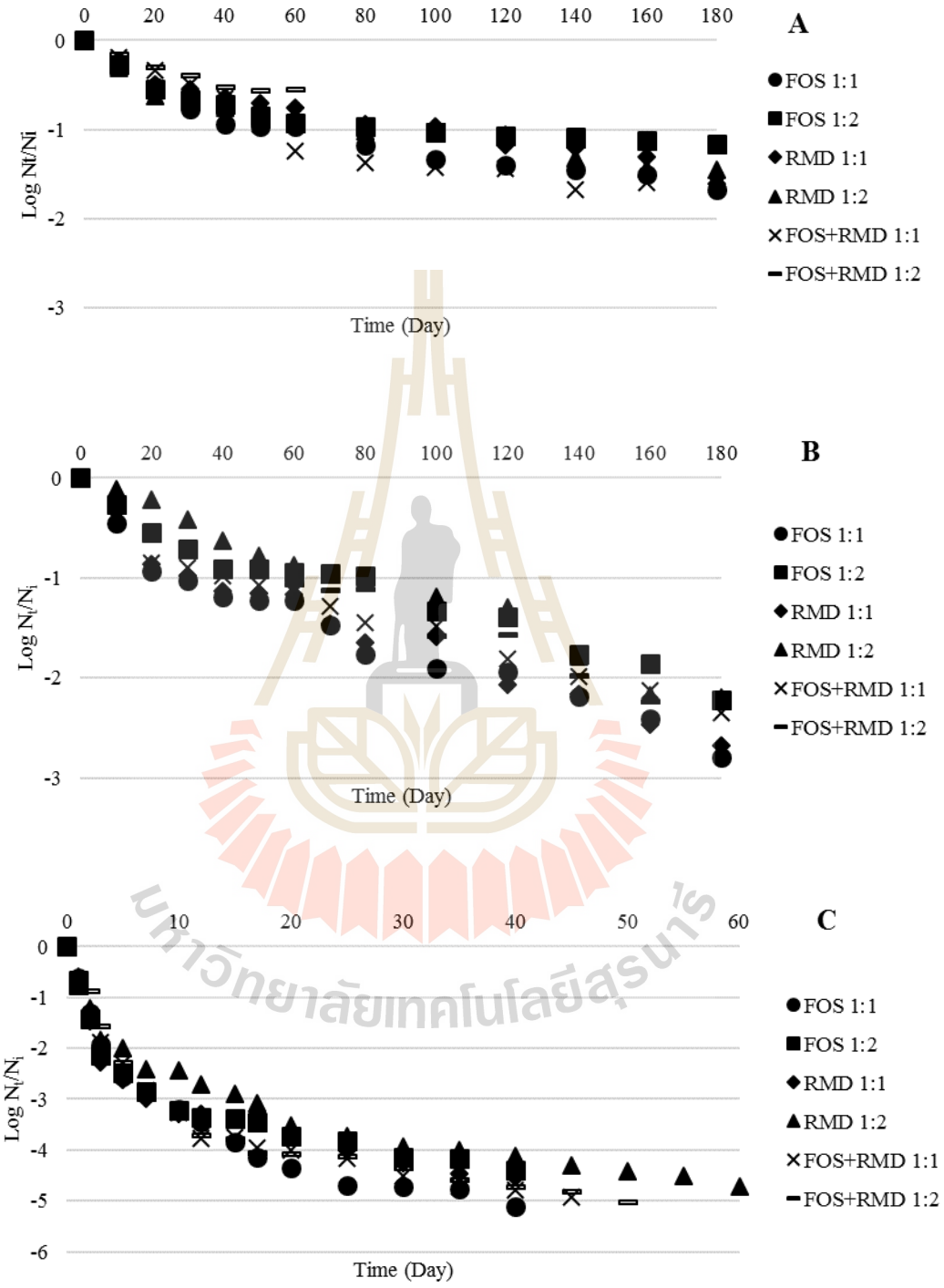


Figure 4.13 Viability loss of spray dried *Lactobacillus* sp. strain 3C2-10 at storage temperatures of (A) 4, (B) 25 and (C) 40°C

Table 4.2 The rate constant (k) and R-squared value (R^2) of linear regression at storage temperature of 4, 25 and 40°C

Wall materials	4°C		25°C		40°C	
	k (day^{-1})	R^2	k (day^{-1})	R^2	k (day^{-1})	R^2
FOS 1:1	-0.0079	0.87	-0.1776	0.96	-0.4110	0.93
FOS 1:2	-0.0052	0.75	-0.1414	0.94	-0.3968	0.90
RMD 1:1	-0.0073	0.94	-0.1795	0.96	-0.4162	0.89
RMD 1:2	-0.0066	0.88	-0.1646	0.96	-0.3312	0.89
FOS+RMD 1:1	-0.0092	0.85	-0.1537	0.96	-0.3825	0.92
FOS+RMD 1:2	-0.0062	0.89	-0.1633	0.97	-0.4226	0.98

The viability of microencapsulated *Lactobacillus* sp. strain 3C2-10 with temperature at 4°C storage condition were significantly ($p<0.05$) higher than storage condition temperature at 25 and 40°C for up to 180 days (Figure 4.13). First-order kinetics of microencapsulated cells were established in Table 4.2. The combination of FOS and RMD at ratio 1:2 exhibited a higher rate on storage stability of *Lactobacillus* sp. strain 3C2-10 temperature at 4°C than 25°C ($p<0.05$). FOS contains higher moisture and prevents excessive drying (Crittenden and Playne, 1996; Santivarangkna et al., 2008). Hoobin et al. (2013) reported that the survived cells of FOS microencapsulates deteriorated due to the higher residual moisture content. Moreover, the moisture content and molecular mobility had impacted on the survival of probiotics during storage. The RMD is an encapsulating material with spray drying process being reported on the nutritional benefits that help increasing probiotics populations in colon (Fastinger et al., 2008; Lefranc-Millot et al., 2012). Finally, the combination of FOS and RMD could enable their usage for wall materials in spray drying process application and enhance storage stability.

CHAPTER V

CONCLUSIONS

The effect of heat resistant on cell viability of *Lactobacillus* sp. strain 3C2-10 in MRS broth, fructooligosaccharides (FOS) and resistant maltodextrin (RMD) were studied to estimate the heat resistant level of *Lactobacillus* sp. strain 3C2-10. The D-value of all temperatures tested in MRS broth showed the highest viability of *Lactobacillus* sp. strain 3C2-10. MRS medium was applied for heat adaptation at temperature not exceed 52°C. The heat adaption of cell were exhibited at the optimum condition with MRS broth at temperature 47°C for 15 min. In addition, the effect of heat adaption not only increased viability of the cells during heat treatment, but increased viability of cells during exposed to pH 3 for 120 min.

The effect of wall material as the combination of FOS and RMD after passed through spray drying showed the highest encapsulation efficiency around 91%. While the effect of core-to-wall between the ratio at 1: 1 and 1: 2 was not significantly different ($p>0.05$) in encapsulation efficiency. The core-to-wall ratio affected the moisture content, particle size, bulk density and the solubility (not significant different; $p>0.05$). The FTIR spectra of spray dried microcapsules for all wall materials (FOS, RMD and FOS+RMD) established during 0 and 60 days. The result showed a reduction of carbohydrates fingerprint region especially glucose and fructose, while as the acids fingerprint increased. Encapsulation with core-to-wall ratio 1:2 revealed high survival cells than encapsulation at the ratio of 1:1. The viability of cells encapsulated (FOS, RMD and FOS+RMD) with same core-to-wall ratio showed

non-significant different ($p>0.05$) after exposed to SGJ and SIJ. The combined wall material (FOS+RMD) in core-to-wall ratio 1:2 showed the highest storage time at 4°C.

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APPENDIX

APPENDIX

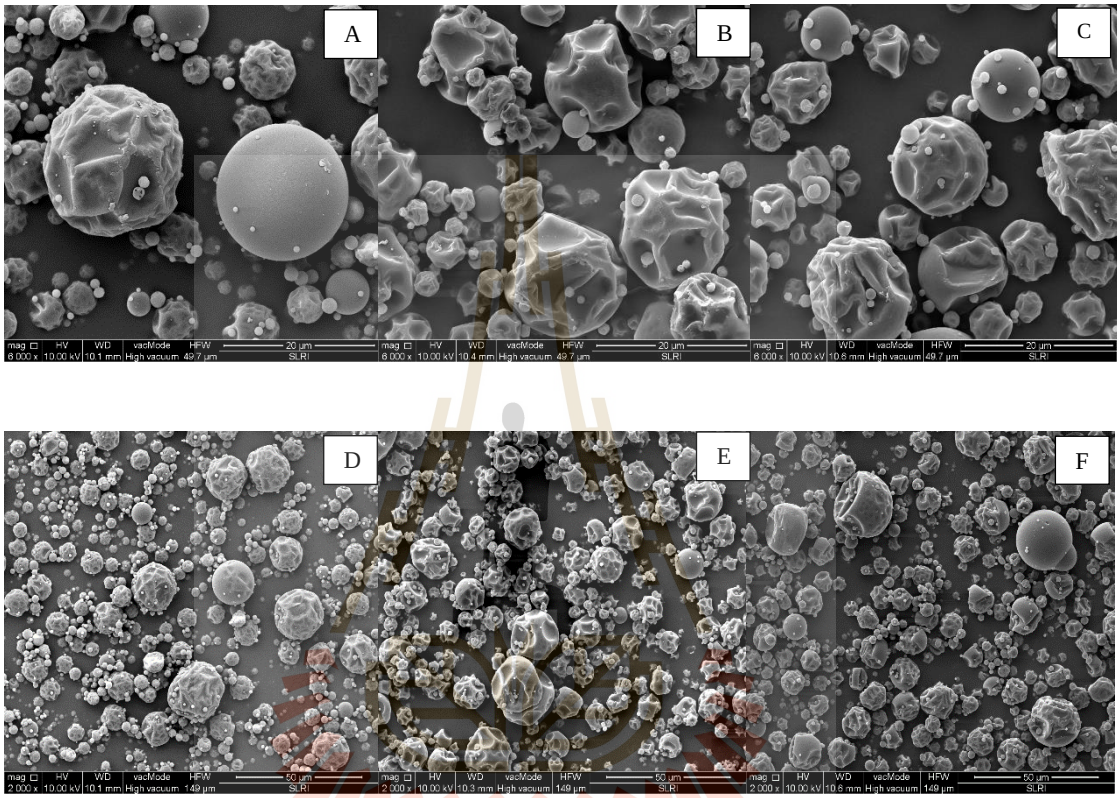

Figure 1 Morphology of spray dried microcapsules without *Lactobacillus* sp. 3C2-10 produced with three different of wall materials in 1:1 and 1:2 core-to-wall ratios. (A) FOS (B) RMD (C) RMD+FOS at 6000X magnification and (D) FOS (E) RMD (F) RMD+FOS at 2000X magnification.

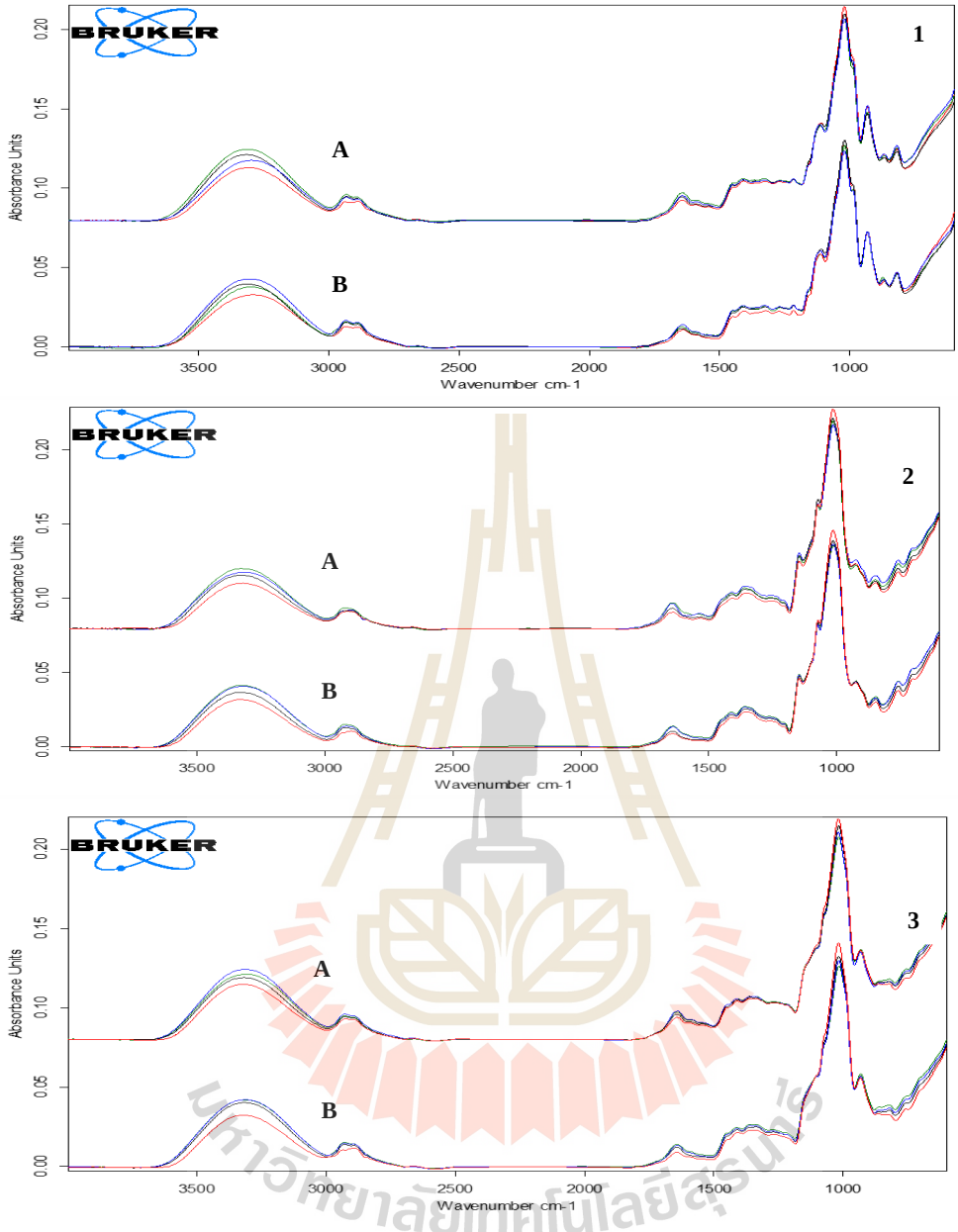

Figure 2 FTIR spectra of spray dried *Lactobacillus* sp. 3C2-10 microcapsules (1) RMD, (2) FOS and (3) combination of FOS and RMD represent the storage at 0 and 60 day (—0 day, —4°C for 60 day, —25°C for 60 day, —40°C for 60 day). (A) core-to-wall ratio 1:1 (B) core-to-wall ratio1:2

BIOGRAPHY

Ms. Jinjutar Kunnathep was born in October 28, 1991 in Bangkok and growing up in Phetchaburi Province, Thailand. She received Bachelor's Degree in B.Sc. (Food Technology) from Institute of Agricultural Technology, Suranaree University of Technology, Thailand in 2013. In 2014, she enrolled in a Food Technology Master program, Suranaree University of Technology, Thailand. Her research topic was heat adaption and microencapsulation of *Lactobacillus* sp. strain 3C2-10 isolated from cassava pulp. The results from some part of this research have been presented as a poster presentation at The 20th World congress on Clinical Nutrition (WCCN), December 14-16, 2016, Rama Gardens Hotel, Bangkok, Thailand. The results from part of this research have been presented as an oral presentation at International Food Research Conference (IFRC), July 25 – 27, 2017, Complex of the Deputy Vice Chancellor (Research & Innovation), University of Putra Malaysia. In research topic was effect of heat adaptation and spray drying outlet temperature on the survival of *Lactobacillus* sp. strain 3C2-10.