ผลของการทำแห้งแบบแช่เยือกแข็งต่อความเสถียรของกล้าเชื้อ (*Bacillus subtilis* SB-MYP-1) ที่เก็บรักษาด้วยแป้งถั่วเหลือง ซึ่งเป็นสารปกป้องจากความเย็น

นายฐิติกร มหิสนันท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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EFFECT OF FREEZE DRYING ON THE STABILITY OF STARTER CULTURE (*Bacillus subtilis* SB-MYP-1) PRESERVED WITH SOYBEAN FLOUR AS A CRYOPROTECTANT

Thitikorn Mahidsanan

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

Suranaree University of Technology

Academic Year 2016

EFFECT OF FREEZE DRYING ON THE STABILITY OF STARTER CULTURE (*Bacillus subtilis* SB-MYP-1) PRESERVED WITH SOYBEAN FLOUR AS A CRYOPROTECTANT

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ฐิติกร มหิสนันท์ : ผลของการทำแห้งแบบแช่เยือกแข็งต่อความเสถียรของกล้าเชื้อ (*Bacillus subtilis* SB-MYP-1) ที่เก็บรักษาด้วยแป้งถั่วเหลืองซึ่งเป็นสารปกป้องจากความเย็น (EFFECT OF FREEZE DRYING ON THE STABILITY OF STARTER CULTURE (*Bacillus subtilis* SB-MYP-1) PRESERVED WITH SOYBEAN FLOUR AS A CRYOPROTECTANT) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.ปิยะวรรณ กาสลัก, 126 หน้า.

วัตถุประสงค์ของงานวิทยานิพนธ์นี้คือ ศึกษาประสิทธิภาพการยึดเกาะ (surface attachment) ของแป้งถั่วเหลืองต่อการปกป้องเป้าหมายเซลล์ (stress target) เพื่อรักษาความเสถียรของกล้าเชื้อ *Bacillus subtilis* SB-MYP-1 ที่ผ่านการทำแห้งแบบแช่เยือกแข็งภายใต้สภาวะแบบ sub-lethal ขอบเขตการศึกษาคือ เตรียมเซลล์กล้าเชื้อ *B. subtilis* SB-MYP-1 ด้วยแป้งถั่วเหลืองความเข้มข้น 10% (w/v) โดยวิธีการทำแห้งแบบแช่เยือกแข็ง เพื่อให้ได้กล้าเชื้อผงที่มีคุณสมบัติสอดคล้องตามเกณฑ์มาตรฐานคุณภาพของกล้าเชื้อผง ได้แก่ การรอดชีวิต (>10^7 CFU/g powder) ความชื้น (<7 % wet basis) และปริมาณน้ำอิสระ (<0.6) รวมถึงความเสถียรภาพของเป้าหมายเซลล์กล้าเชื้อเทียบกับคุณภาพของเซลล์กล้าเชื้อสด (fresh cell starter culture) และกล้าเชื้อที่ทำแห้งแบบแช่เยือกแข็งด้วยการใช้สารปกป้องจากความเย็นทางการค้า 3 ชนิด ได้แก่ soy protein isolate (SPI) soluble starch (ST) และ maltodextrin (MD) จากนั้นติดตามศักยภาพของกิจกรรมเซลล์กล้าเชื้อผงที่มีแป้งถั่วเหลืองเป็นสารปกป้องจากความเย็น เก็บในถุงอะลูมิเนียมฟอยด์ลามิเนตสุญญากาศที่อุณหภูมิ -25 0 และ 25 องศาเซลเซียส เป็นเวลา 0 45 และ 90 วัน

ภายหลังการทำแบบแห้งแช่เยือกแข็ง พบว่า มีการยึดเกาะของแป้งถั่วเหลือง ซึ่งสามารถรักษาความเสถียรภาพของเป้าหมายเซลล์ซึ่งไม่แตกต่างกับเซลล์กล้าเชื้อสด (P>0.05) แต่ผนังเซลล์และเยื่อหุ้มเซลล์บางส่วนของกล้าเชื้อผงที่ทำแห้งแบบแช่เยือกแข็งด้วยการใช้สารปกป้องจากความเย็นทางการค้า 3 ชนิดนั้นถูกทำลาย และเมื่อติดตามประสิทธิภาพในการเก็บรักษากล้าเชื้อผง *B. subtilis* SB-MYP-1 ที่มีแป้งถั่วเหลืองเป็นสารปกป้องจากความเย็น ซึ่งเก็บรักษาในถุงอะลูมิเนียมฟอยด์ลามิเนตสุญญากาศที่อุณหภูมิ -25 องศาเซลเซียส ติดตามเป็นเวลา 90 วัน ด้วยวิธีการใช้สมการทำนายด้านจุลินทรีย์ (predictive microbiological equations) พบว่าเป้าหมายเซลล์ยังคงมีความเสถียรภาพ และมีคุณสมบัติตามมาตรฐานคุณภาพกล้าเชื้อผงที่อุณหภูมิ และระยะเวลาของการเก็บรักษาซึ่งไม่แตกต่างจากการตรวจวิเคราะห์จริง (P>0.05)

เมื่อทำการทดสอบประสิทธิภาพการทำงานของกล้าเชื้อผงในกระบวนการหมักผลิตภัณฑ์ถั่วเน่าต้นแบบ พบว่าศักยภาพการผลิตเอนไซม์อะไมเลส โปรติเอส และกรดแกมมาพอลิกลูตามิกในชั่วโมงที่ 36 ของการหมักถั่วเน่าไม่แตกต่างจากการใช้กล้าเชื้อสด (P>0.05)

ดังนั้นข้อมูลจากผลงานวิจัยแสดงให้เห็นว่า การใช้แป้งถั่วเหลืองเป็นสารปกป้องจากความเย็นเพื่อเก็บรักษากล้าเชื้อ *B. subtilis* ด้วยวิธีการทำแห้งแบบแช่เยือกแข็ง สามารถรักษาคุณสมบัติของกล้าเชื้อให้มีความเสถียรในสภาวะ sub-lethal ที่เป็นผลกระทบจากกระบวนการทำแห้งได้ รวมถึงศักยภาพการผลิตเมตาบอไลท์สำคัญเมื่อทำหน้าที่ในกระบวนการหมักผลิตภัณฑ์ถั่วเน่า ทั้งนี้สมการทำนายด้านจุลินทรีย์สามารถใช้เป็นเครื่องมือในการควบคุมหรือติดตามคุณภาพกล้าเชื้อผงในระหว่างการเก็บรักษาได้เช่นกัน นอกจากนี้ต้นแบบของการใช้แป้งถั่วเหลืองเพื่อการเก็บรักษากล้าเชื้อ *B. subtilis* ด้วยวิธีทำแห้งแบบแช่เยือกแข็ง สามารถนำไปประยุกต์ใช้ในการเก็บรักษากล้าเชื้อที่มีสปอร์ในอุตสาหกรรมถั่วเหลืองหมักได้

ปัจจุบันผู้วิจัยได้เก็บรักษากล้าเชื้อ *B. subtilis* SB-MYP-1 ไว้ที่สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย รหัสเชื้อจุลินทรีย์คือ TISTR 2397 เพื่อเป็นประโยชน์ในอุตสาหกรรมถั่วเหลืองหมักที่สนใจต่อไป

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา _____

THITIKORN MAHIDSANAN : EFFECT OF FREEZE DRYING ON THE STABILITY OF STARTER CULTURE (*Bacillus subtilis* SB-MYP-1) PRESERVED WITH SOYBEAN FLOUR AS A CRYOPROTECTANT. THESIS ADVISOR : ASST. PROF. PIYAWAN GASALUCK, Ph.D., 126 PP.

FREEZE DRYING STRESS/SOYBEAN FLOUR/CRYOPROTECTANT/ *Bacillus subtilis* SB-MYP-1 STABILITY/THUA-NAO FERMENTATION

The aim of this dissertation was to study the effective surface attachment of soybean flour to protect cell stress targets for preserving the stability of *Bacillus subtilis* SB-MYP-1 after sub-lethal stress of freeze drying. The scope of this study was to prepare the *B. subtilis* SB-MYP-1 cells with 10% (w/v) soybean flour for freeze drying, to which the gains of freeze-dried starter culture properties were accorded to the standard quality of powdered starter culture such as viability (>10^7 CFU/g powder), moisture content (<7% wet basis) and water activity (<0.6). In addition, the stability of cell stress targets was compared to the quality of fresh cell starter culture and freeze-dried starter cultures with three commercial cryoprotectant including soy protein isolate (SPI), soluble starch (ST), and maltodextrin (MD). The activities of freeze-dried cells with soybean flour cryoprotectant kept in a laminate aluminum foil vacuum bag at -25, 0, and 25°C for 0, 45, and 90 days were then monitored to determine their potentials.

After freeze drying, it was found that the surface attachment of soybean flour had occurred, which maintained the stability of cell stress targets were no different from those of the fresh cells (P>0.05), whereas, the partial cell wall and cell membrane

of freeze-dried cells with three commercial cryoprotectants were destroyed. The effective preservation of freeze-dried *B. subtilis* SB-MYP-1 with soybean flour was kept in a laminate aluminum foil vacuum bag at -25°C for 90 days, which was then monitored by predictive microbiological equations. This demonstrated that the stability of its cell stress targets was still provided and relevant to the standard quality of dried starter culture at the storage temperature and time, which were no different from the actual analysis ($P>0.05$).

The potentials of freeze-dried starter culture were then verified in a prototype of Thua-nao fermentation. It was found that the potentialities of amylase, protease and PGA production were no different from those using fresh cells at 36 h during Thua-nao fermentation ($P>0.05$).

Thus, this study revealed that using soybean flour as a cryoprotectant for preserving *B. subtilis* by freeze drying could maintain the stability of starter culture under sub-lethal effect from the drying process, as well as its potentials of significant metabolite production could function in Thua-nao fermentation. In addition, the predictive microbiological equations could be also used as a tool for controlling and monitoring the dried starter culture during storage. Furthermore, the prototype of soybean flour preserved freeze-dried *B. subtilis* could be applied to spore-forming starter cultures preservation in fermented soybean industry.

At present, the researcher has taken the *B. subtilis* SB-MYP-1 to keep at the Thailand Institute of Scientific and Technological Research with the code TISTR 2397 for future interest of the fermented soybean industry.

School of Food Technology Student's Signature Thitikorn Mahidsanan

Academic Year 2016 Advisor's Signature

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LIST OF ABBREVIATIONS

α	=	Alpha
ANOVA	=	Analysis of variance
bp	=	Base pair
β	=	Beta
CFEs	=	Cell-free extracts
CTAB	=	Cetyl trimethylammonium bromide
CFU	=	Colony forming unit
^{o}C	=	Degree celsius
DNA	=	Deoxyribonucleic acid
et al.	=	et alia (and others)
FW	=	Fresh weight
GDH	=	Glutamate dehydrogenase
GOGAT	=	2-oxoglutarate aminotransferase
GS	=	Glutamine synthetase
γ	=	Gamma
h	=	Hour
kHz	=	Kilohertz
(m, μ, p) g , L, mol, M	=	(milli, micro, pico) Gram, Liter, Mole, Molar
μm	=	Micrometre
MD	=	Maltodextrin

LIST OF ABBREVIATIONS (Continued)

min	=	Minute
nm	=	Nanometre
%	=	Percentage
cm^{-1}	=	Reciprocal centimeter
×g	=	Relative centrifugal force
rpm	=	Revolutions per minute
% (w/w)	=	Percent mass/mass
% (w/v)	=	Percent mass/volume
% (v/v)	=	Percent volume/volume
SBF	=	Soybean flour
SPI	=	Soy protein isolate
ST	=	Soluble starch

CHAPTER I

INTRODUCTION

1.1 Introduction

The significance of microbial starter cultures has been importantly used in fermented food industries. Since starter culture characteristics such as the growth at a wide range condition, enzymes and antimicrobial activities, as well as specific gene expression which contribute to the development of fermented food properties. Accordingly, its stability should be preserved for fermented food production (Holzapfel, 2002).

Bacillus subtilis SB-MYP-1 was obtained by isolation from Thai fermented soybeans (Thua-nao). It possessed amylase and protease production while these activities affected the decreasing undesirable-alcohol (octanol and butanediol), and the increasing desirable-pyrazine and nutritional values. For the nutritional metabolites which consisted of poly-γ-glutamic acid (PGA), calcium, ferric and phosphorus, affected the health-promoting compounds of human health (Gasaluck, 2010). According to *B. subtilis*-PGA production, which was operated by *pgsB* expression for controlling key intracellular enzymes pathway, including glutamate dehydrogenase (GDH), glutamine synthetase (GS), 2-oxoglutarate aminotransferase (GOGAT) to provide the slime-viscous properties of Thua-nao (Gasaluck, 2010; Mahidsanan and Gasaluck, 2011; Najar and Das, 2015 Shih and Van, 2001; Tanimoto, 2010). The enzymatic reaction of *B. subtilis* can, furthermore, convert an

iron, calcium, magnesium, potassium, selenium, copper and zinc into more soluble forms to provide the nutritional values. Meanwhile, the enzymatic degradation products of pyrazines are derived from the interaction between amino acids and carbonyl compounds in order to generate the unique volatiles in fermented soybean products (Bajaj and Singhal, 2011; Dajanta, Apichartsrangkoon, and Chukeatirote, 2011; Godwin, 2013). The novel strain of *B. subtilis* SB-MYP-1 is, then, necessary to preserve its starter culture characteristics for the functional of fermented soybean production in food industry and Small and Medium Enterprise (SMEs).

Basically, various techniques of starter culture powder preservation are used in food fermentation and SMEs. For instance, the spray-dried *Lactobacillus curvatus* in Thai Nham, freeze-dried *Bifidobacterium animalis* in enzyme production, and soybean-*Aspergillus oryzae* seed in Thai Tao-Jiaw production which were accorded toward a long term stability (Department of Agriculture, 2015; Dianawati and Shah, 2011; Valyasevi, 2005). However, the dried-culture generally gains a resistance to environmental stress via preservative techniques which those conditions may disrupt the structural cell functions, such as cell wall, cell membrane, proteins or enzymes, and genes (Wesche, Gurtler, Marks, and Ryser, 2009).

Freeze drying is used to preserve a perishable material since its process mainly operates by freezing the material, then decreasing surrounding pressure, moisture content and water activity (a_w) to provide the frozen water and sublimate from the solid phase to the gas phase. Its product properties are suitable for heat sensitive, nutritional value, and microbial control (Brennan and Grandison, 2012). Meanwhile, freeze-dried encapsulation has been interested to be the technique of starter culture preservation for fermented food industries worldwide (Morgan,

Herman, White, and Vesey, 2006), such as *B. subtilis* SB-MYP-1 (Mahidsanan and Gasaluck, 2011), *Bifidobacterium longum* 1941 (Dianawati, Mishra, and Shah, 2013), and *L. lactis*, *Streptococcus thermophilus* (Carafa, Clementi, Tuohy, and Franciosi, 2016).

Freeze drying stress describes the effects of cold temperature on cellular responses and microbial metabolism stimulation through constrains a cold shock sensor leads to reduce enzyme reaction, solute uptake, cell membrane fluidity and gene functions (Figure 1.1). At the same time, mRNA increases while cold shock protein (CSPs) is regulated at the level of transcription leads to adjust the level of unsaturated fatty acid on the cell membrane (Ulusu and Tezcan, 2001; Cavicchioli, Saunders, and Thomas, 2014; Wesche et al., 2009). Therefore, the process of microbial freeze drying has required cryoprotective substances to prevent low temperature stress on its structural cell functions owing to cryoprotectant adhesion mechanisms. Those substances are classified into the type of carbohydrate, protein, sugar alcohol, glycerine, etc (Li, Tian, Liu, Zhao, Zhang, and Chen, 2011).

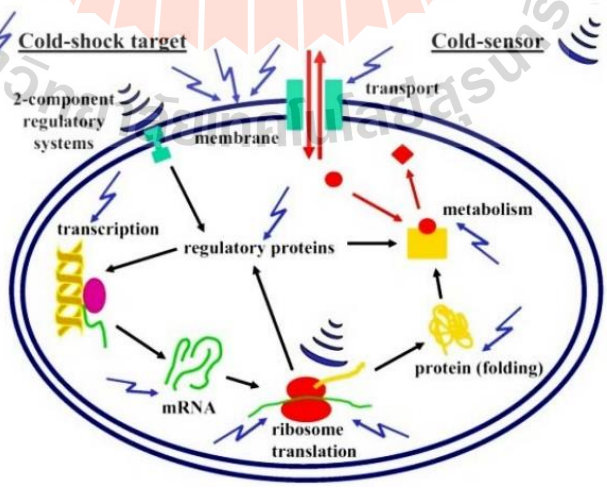

Figure 1.1 The effect of cold temperature on a generic microorganism (Cavicchioli et al., 2014).

Sugar and its derivatives are generally recognized to use in the step of dried culture preparation. It plays a role as osmotic adjustments in the protection for cell membrane and cell components due to a chemical bonding between the functional group of cells and cryoprotectants which consists of OH-group, protein amino group, amide group, phospholipid, etc (Santivarangkna**,** Higl, and Foerst, 2008; Muchova, Wilkinson, and Barak, 2011). Santivarangkna, Naumann, Kulozik, and Foerst (2010) had suggested that the sorbitol protected freeze-dried lactic acid bacterial cells by interacting with phosphate groups at cell membranes. It was observed from hydrogen-bonding sensitive of C=O and P=O stretching bands on the FT-IR spectra. At the same time, the potentiality of cryoprotectant could maintain the cell viability, membrane fluidity, metabolic rate, ATPase and other microbial enzyme activity along with its fermentation profiles. Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter, and Fisk (2014) had documentary evidence that the maltodextrin, D-glucose and trehalose maintained the stabilization of microencapsulated *L. acidophilus*. Nevertheless, the longer preservation by reducing sugars, the disadvantages of syneresis susceptibility and water activity increased. This phenomenon can induce the decreasing of starter culture stability (Abe, Uchijima, Yaeshima, and Iwatsuki, 2009; Ying, Sun, Sanguansri, Weerakkody, and Augustin, 2012).

On the other hand, there is a report that soy protein isolate was also applied in freeze-dried *Bifidobacterium lactis* BB-12. Its stability could be stored for 90 days under vacuum condition (Chavez and Ledeboer, 2007). Dianawati et al. (2013) demonstrated the soy protein isolate providing a good protection of *B. longum* 1941 under freezing condition, when it was combined with mannitol and maltodextrin.

Furthermore, in case of soybean flour was produced from 100% of whole soybean. It typically contains 30% (w/w) of carbohydrate, 18-20% (w/w) of fat and 39-41% (w/w) of protein which those substances are also the predominant microbial-carbon and nitrogen sources (Los Angeles Chinese Learning Center, 2014). All things the hypothesis of soybean flour cryoprotectant, its efficiency might be more appropriate to use than either carbohydrate or protein because it consists of mainly different polar and nonpolar substances as through lysine, arginine, histidine (Iman and Maji, 2013) as well as starch, hemicelluloses, cellulose, stachyose, raffinose, arabinose, glucose and sucrose (Esteves, Martino, Olivieira, Bressan, and Costa, 2010), which could be capable of reacting with cross linking molecules. This principle had been described by the FT-IR evidence of adhesive effect between soybean flour and negatively charged sites at the surfaces of glutaraldehyde agent model. The FT-IR intensities were found in the region of 3400-3000 cm^{-1}, 2230 and 1167 cm^{-1} which assigned to four peaks of -OH, -NH, -N-C=O and C-N stretching vibration, respectively. Those results were interpreted to the interaction between an -OH and $-NH_2$ groups present of both substance combinations (Iman and Maji, 2013). Thus, the adhesion between microbial cells and cryoprotectant depended on the mechanisms under environmental stress factors, in the meanwhile, there is no article of freeze-dried *B. subtilis* using the soybean flour protection.

Due to the revealed reports mentioned above, freeze-dried *B. subtilis* SB-MYP-1 is inoculated into the soybean fermentation avoiding cryoprotective interference. Soybean flour is used in freeze dried *B. subtilis* SB-MYP-1 preparation because its compositions are not different from soybean material in its fermentation. Therefore, the aim of this study was to use soybean flour for preserving freeze-dried

B. subtilis SB-MYP-1 cell characteristics via adherence mechanism, thereby its stabilities were maintained during storage. The application of this dried starter culture was also used in soybean fermentation.

To complete the starter culture preservation, a change of stress target function and its characteristics during storage would be considered at the response surface methodology (RSM). The predictive microbiological modelling was performed by quadratic mathematical model in order to specify a point of control measure for prolonging the shelf life based on the stability of their powder culture (Behboudi-Jobbehdar, Soukoulis, Yonekura, and Fisk, 2013; Khoramnia, Abdullah, Liew, Sieo, Ramasamy, and Ho, 2011; Kim and Rhee 2015).

In summary, the aims of this dissertation were these followings (1) to reveal the mechanisms of soybean flour on *B. subtilis* SB-MYP-1 cell protection through the function of cell wall, cell membrane, activities of three intracellular enzymes (GDH, GOGAT, and GS) and *pgsB* gene expression under freeze drying, (2) to predict the stability of freeze-dried *B. subtilis* SB-MYP-1 cell stress targets during storage by the quadratic mathematical models (RSM), and (3) to verify the starter culture potentials such as growth profiles, amylase and protease activity, and PGA production throughout solid state fermentation of soybean (Thua-nao).

1.2 Research objectives

To reveal the novel of whole soybean flour protected *B. subtilis* SB-MYP-1 cells stability under freeze drying stress, whilst the viability and functional potential of dried starter culture in Thua-nao fermentation are preserved during storage.

1.3 Research hypotheses

The cryoprotective effect of soybean flour can maintain the freeze-dried *B. subtilis* SB-MYP-1 stability, as well as the potentials of dried starter culture can perform in solid state soybean fermentation. Combination of control factors can also be used as a parameter in predictive model for the shelf life evaluation.

1.4 Scope and limitation of the study

The first part of the study, 10% (w/v) soybean flour (SBF; Doikham product) was selected to be a cryoprotectant in freeze-dried *B. subtilis* SB-MYP-1 cells (freezing stress at -60^{o}C and low vacuum stress at 0.001 mbar, 35^{o}C for 24 h). The qualities of freeze-dried starter culture namely, viability, water activity (a_w), moisture content and survival rate were considered. In addition, the cell membrane fluidity, morphological properties, matrix adhesion on cells, metabolic activity, activities of three intracellular enzymes and *pgsB* expression were also evaluated. The commercial protective agents, such as 10% (w/v) soy protein isolate (SPI; Chemipan product), 10% (w/v) soluble starch (ST; Carlo Erba product), and 10% (w/v) maltodextrin (MD; Chemipan product) were positive control treatments. Based on the results obtained, the combination criteria of freeze-dried cells were viability (>10^7 CFU/g), moisture content (<7% wet basis), and a_w (<0.6). The morphological properties, cell membrane fluidity, metabolic activity, intracellular enzyme activities and *pgsB* gene expression were maintained. Since their combination criteria were not harmful to the *B. subtilis* SB-MYP-1 characteristics.

Furthermore, freeze-dried *B. subtilis* SB-MYP-1 cells with soybean flour were then selected to preserve its stability under storage factors in the presence of oxygen

and moisture at temperature controls (25, 0 and -25^{o}C) while those samples were sealed in a laminate aluminum foil vacuum bag. The starter culture qualities based on combination criteria were evaluated at the interval times of 0, 45 and 90 days which the potentials of dried starter culture were also tested in solid state soybean fermentation within 72 h.

1.5 Expected results

1. To gain the appropriate form of freeze-dried *B. subtilis* SB-MYP-1 cells with soybean flour, the dried starter culture would be possessed at the viability (>10^7 CFU/g powder), moisture content (<7% wet basis) and water activity (<0.6) to maintain the cell membrane fluidity, morphological properties, three intracellular enzyme activities and *pgsB* expression. The potentials of amylase, protease, and PGA production through solid state fermentation of soybean could be also remained.

2. Drawn from dissertation results contributed to preserve the potentials of *B. subtilis* SB-MYP-1 strain at the Thailand Institute of Scientific and Technological Research. In addition, the technology of freeze-dried starter culture with soybean flour could be transferred to which SMEs (Small and Medium Enterprises) interested.

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CHAPTER II

LITERATURE REVIEWS

2.1 Starter culture in food fermentation

Food fermentation is the alternate preservation for perishable raw materials, generally prepared from raw or cooked raw materials prior to starter cultures for specific enzymes and metabolites producing, contributes to the characteristic properties improvement, such as taste, aroma, visual appearance, texture, shelf life and safety. Ancient fermentation, the process without the starter inoculum has been applied to food preservation through their trial and error, but the disadvantage is a competitive activity of the contamination of pathogen and spoilage microorganisms (Hansen, 2002; Holzapfel, 2002).

Recently, starter culture has been developed by the isolation of the potential function to accelerate the fermentation process under the microbial stress in a wide range condition, achieve the specific finish product quality. Their metabolites (organic acids, antimicrobial peptide, etc.) can inhibit foodborne pathogens and spoilages, may provide an additional advantage during fermentation. The highest level of safety and flexibility is achieved by using a commercial starter culture for direct inoculation. Therefore, starter culture should be preserved using an appropriate technique for further food fermentation (Holzapfel, 2002).

2.1.1 *B. subtilis* starter culture in solid state soybean fermentation

Soybean (*Glycine max L.*) is one of the nutritionally raw material, records of its food usage in worldwide. As shown in Figure 2.1, fermented soybean are produced by many types of microbial starter culture, such as lactic acid bacteria, acetic acid bacteria, yeasts, molds and other bacteria.

Figure 2.1 Fermented soybean products: Japanese natto (A), Nigerian dawadawa or iru (B), Nepalese kinema (C), Korean chungkook-jang (D), Philippines tao-si (E) and Thai thua-nao (F) (Natto, online, 2016; dawadawa, online, 2016; Kinema, online, 2016; Chung-gook-jang-jigae, online, 2011; Fermented black soybean, online, 2016).

The most of their starter cultures are involved in the alkaline fermentation as flat and thick colony of *B. subtilis* since the basic steps of fermented soybean preparation are soaking, boiling, fermentation, incubation and finally packing

(Figure 2.2). This starter culture has a high proteolytic activity that is evident by progressive increase of trichloroacetic acid-nitrogen, ammonia nitrogen and pH value during fermentation. Visessanguan, Benjakul, Potachareon, Panya, and Riebroy (2005) observed that the *B. subtilis* during thua-nao fermentation released proteinase which played important role in proteolysis of soy proteins. Its reactions were reported to be responsible on the flavor characteristic of thua-nao. In addition, three types of extracellular enzymes, namely, neutral proteases, alkaline protease and esterase were also found during fermentation (Hu et al., 2010).

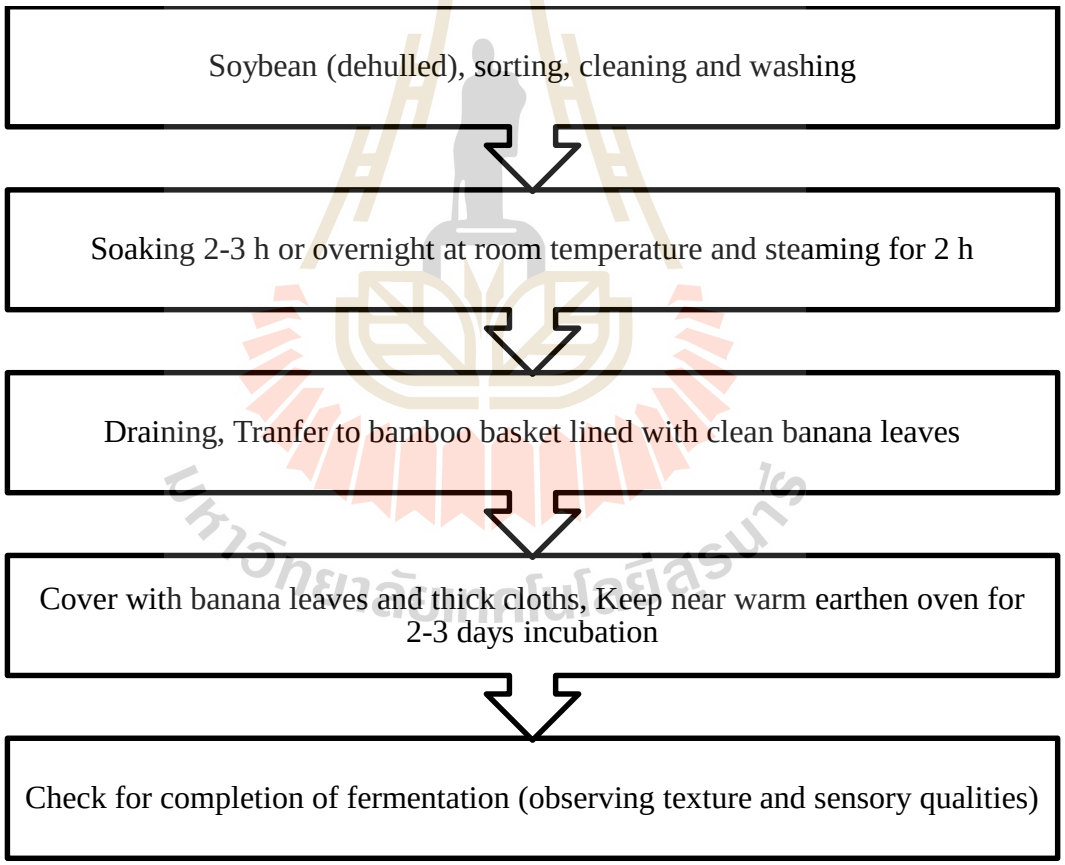

Figure 2.2 Flow chart of traditional fermentation of soybeans (Modified from Shrestha, Dahal, and Ndungutse, 2010).

In general, finished product of fermented soybean was covered by a light brown growth of bacteria, which had sticky material. Natto had a fruity or nutty aroma without strong ammonia odour, while traditional thua-nao was a dried disk with dark brown in color, strong smell and noticeable ammonia. Many researchers had studied the volatile compounds in fermented soybean, such as Gasaluck (2010) identified desirable volatiles by Gas Chromatography-Mass spectrometry in *B. subtilis* SB-MYP-1 fermented soybean product. The results showed the increasing of pyrazines volatiles, in contrast, the alcohol group was not found in the finished product. In addition, Dajanta, Apichartsrangkoon, and Chukeatirote (2011) found the predominant volatiles in pure TN51 *B. subtilis*-fermented thua-nao, namely 2,5-dimethylpyrazine, 2-methyl butanoic acid, 2,3,5-trimethylpyrazine, 2-methylpropanoic acid and acetic acid, whereas the major volatiles of naturally fermented soybean were 2,5-dimethylpyrazine, benzaldehyde, 5-methyl-3-hexanone, 2-butanone and 3-methyl-2-pentanone due to enzymatic activities from starter culture fermentation. Also *Bacillus* sp. was isolated from Thai traditional thua-nao, it could produce several extracellular enzymes with the same function, such as nattokinase, protease, amylase, phytase, lipases and glutamyl hydrolase. Enzymatic degradation products, such as dicarbonyl compounds and free amino acids, will generate further complex odorous compounds through strecker degradation and other reactions in maillard browning. The predominant volatiles from their interactions, including pyrazines, aldehydes, alcohols, acids and esters are generated. Meanwhile, precursors of major volatile-pyrazines are derived from the interaction between amino acids and carbonyl compounds.

2.1.2 Beneficial of *B. subtilis* in fermented soybean and food safety

Fermentation of soybeans with *B. subtilis*, lead to extensive hydrolysis of all the macromolecules such as proteins to smaller peptides, amino acids and simple sulfur and nitrogen compounds, starch into oligosaccharides and simple sugars and lipids into simple fatty acids. A key health benefit of *B. subtilis* fermentation almost completes removal of indigestible oligosaccharides such as trisaccharides (raffinose), tetrasaccharides (stachyose), which are responsible for indigestion and flatulence in humans (Esteves, Martino, Olivieira, Bressan, and Costa, 2010). Meanwhile, Shrestha, and Noomhorm (2001) found that the urease activity of dried-kinema led to inactivation of trypsin inhibitors in raw soybeans. A national value, enzymatic activities of starter culture can convert a mineral like iron, calcium magnesium, potassium, selenium, copper and zinc into more soluble forms, so that the body gets more of the benefit of nutrients. It increases the total vitamin and mineral content in the final product that are able to add large quantities of thiamin, nicotinic acid and biotin, making an even healthier overall product (Godwin, 2013). In addition, antimicrobial activity of starter culture is also a good property. Kalavi, Muroki, Omwega, and Mwadime (1996) claimed that *Rhizopus* fermented soybeans (Tempeh) reduced the risk of diarrhea when inoculated to the diet of malnourished children. Lee and Chang (2017) proved that bacteriocin-producing *B. subtilis* SN7 has great potential as a controlling agent of *B. cereus* and as a starter culture in Cheonggukjang. Besides, *Bacillus* sp. inhibited the growth of *Aspergillus*, and removed both aflatoxin B1 and ochratoxin A from Thai fermented soybean (Petchkongkaew, Taillandier, Gasaluck, and Lebrihi, 2008).

2.1.3 Other soy fermented products by fungal fermentation

Fungal starter culture has been normally used in soy fermentation under processing factors, such as pH value, salt concentration and raw soybean strains on each origin which its fermented products are following:

2.1.3.1 Miso

Miso is traditional Japanese seasoning that produced by rice, barley, and/or soybeans with sodium chloride salt (10-12% w/w) and *Aspergillus oryzae* (kojikin) fermentation (pH 5.0-5.4). However, the most of miso was made with soy through as the result is a thick paste used for sauces and spreads, pickling vegetables or meats, and mixing with dashi soup stock to serve as miso soup called misoshiru (Figure 2.3A) (Marui et al., 2013).

2.1.3.2 Tempeh

Tempeh (Figure 2.3B) is a traditional soy product from *Rhizopus oligosporus*, and controlled at pH 6.0-7.0 that binds soybeans into a cake form, similar to a very firm vegetarian burger patty. This fermented product is a traditional of soy food in East Asian country (Jelen, Majcher, Ginja, and Kuligowski, 2013).

2.1.3.3 Soy sauce

A condiment is produced from a fermented paste of boiled soybeans, roasted grain, brine, and fungal starter culture of *Aspergillus oryzae* or *Aspergillus sojaemolds*, and yeasts. After fermentation (pH 4.40-5.40), the paste is pressed to achieve a liquid soy sauce (Figure 2.3C), whereas a solid byproduct that is often used as animal feed (Hashimoto and Nakata, 2003).

Figure 2.3 Fermented soybean products from fungal starter culture: Miso soup (A), Tempeh (B) and Soy sauce (C) (Miso soup, online, 2016; Tempeh, online, 2016; Fermented soy sauce, online, 2016).

2.2 Poly-γ-glutamic acid

Poly-γ-glutamic acid (PGA) is an anionic biopolymer, naturally occurring homo-polyamide made of D- and L-glutamic acid units connected by amide linkages between α-amino and γ-carboxylic acid groups (Figure 2.4). It is a component of traditional Japanese natto by *B. subtilis* fermentation. Similar products from fermented soybeans have been also consumed in several areas in Asia, namely Kinema in Nepal, Thua-nao in Thailand, and Chungkookjang in Korea, etc. Moreover, PGA characteristics are water-soluble, biodegradable, edible and non-toxic toward humans. Meanwhile, the applications of PGA have been interested in industrial fields, such as food products, cosmetics, medicinal industries, and agriculture (Shih, Wu, and Shieh, 2005; Sung, Park, Kim, Poo, Soda, and Ashiuchi, 2005; Tanimoto, 2010; Wei, Wolf-Hall, and Chang, 2001).

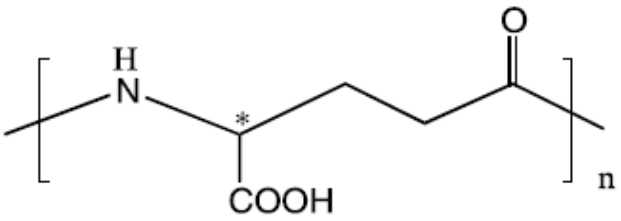

Figure 2.4 Structure of PGA (Shih and Van, 2001).

2.2.1 PGA genetic organization in *B. subtilis*

PGA was first discovered in *B. anthracis* capsules. Bovarnick revealed that the PGA was freely secreted into the growth medium of *B. subtilis* and *B. licheniformis* (Figure 2.5). Four genes-*capB*, *capC*, *capA* and *capE*, encoding a 47-amino-acid peptide are necessary for PGA synthesis in *B. anthracis*. In case of *B. subtilis*, in which three genes (*pgsB*, *pgsC* and *pgsAA*) were initially identified in polyglutamate synthesis (Figure 2.6). In fact, PGA production pathway is mainly produced from glycolysis and ammonium sulfate by intracellular enzymes whilst L-glutamic acid is produced from citric acid via isocitric acid and γ-keto glutaric acid (2-oxoglutaric acid) in the TCA cycle (Figure 2.7). The conversion of γ-keto glutaric acid to L-glutamic acid is to possess in two different ways. In absence of glutamine, the glutamate dehydrogenase (GDH) pathway is activated, in which L-glutamic acid synthesized from γ-keto glutaric acid and ammonium sulfate. In the present of L-glutamine, another pathway involved glutamine synthetase (GS) and glutamine: 2-oxoglutarate aminotranferease (GOGAT) is activated, in which L-glutamic acid is formed from γ-keto glutaric acid and L-glutamine catalyzed by GOGAT as well as L-glutamine regenerated from L-glutamic acid and ammonium sulfate catalyzed by GS (Shih and Van, 2001). The consequence of *B. subtilis* GXA-28 potential and key

enzyme activities analysis under specific temperature indicated that the flux from iso-citrate to 2-oxoglutarate and from 2-oxoglutarate to glutamate were increased with high activity of glutamate dehydrogenase, which led to enhance PGA production (Zeng, Chen, Wang, Zheng, Shu, and Liang, 2014).

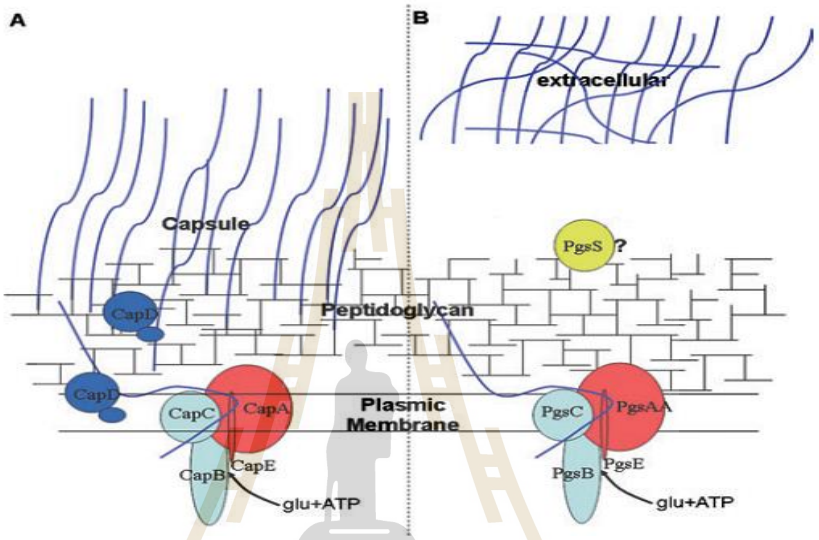

Figure 2.5 A) PGA is anchored covalently to the peptidoglycan, localized in the membrane or peptidoglycan in *B. anthracis*. B) PGA is found in the extracellular medium in *B. subtilis* and *B. licheniformis* (Candela and Fouet, 2006).

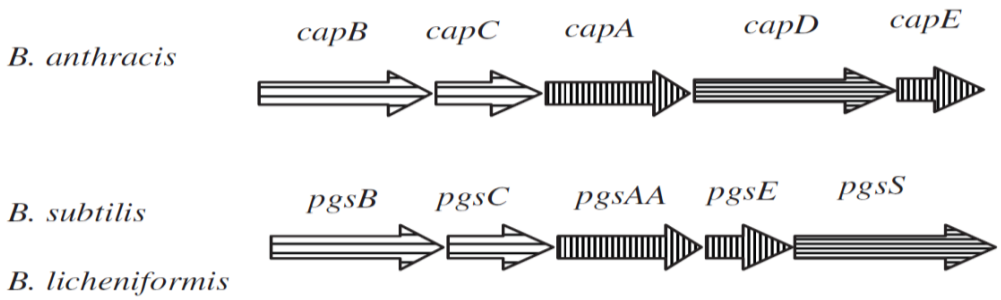

Figure 2.6 The genetic elements required for PGA synthesis in *B. anthracis*, *B. subtilis* and *B. licheniformis* (Bajaj and Singhal, 2011).

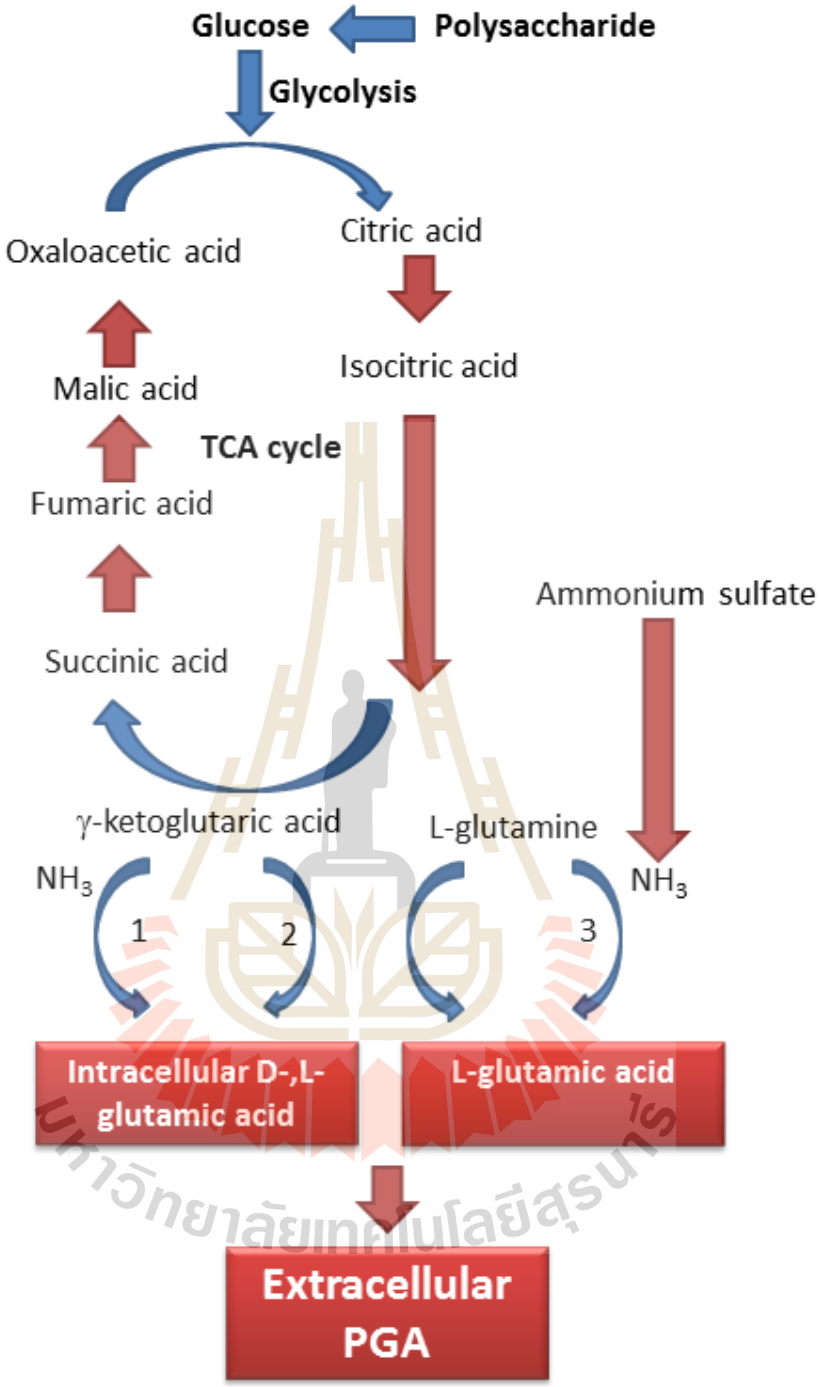

Figure 2.7 Proposed pathway of PGA synthesis in *B. subtilis* (1: Glutamate dehydrogenase (GDH), 2: 2-oxoglutarate aminotransferase (GOGAT) and 3: Glutamine synthetase (GS)) (Modified from Shih and Van, 2001).

2.2.2 PGA production by fermented *B. subtilis* starter culture

PGA production from *B. subtilis* fermentation, frozen aliquot of this starter culture is acclimatized to the submerged fermentation. Its conditions for 15-30 or 72-130 hours as long as the PGA-containing broths are then acid hydrolyzed, decolorized, and filtered multiple times. The liquid containing the purified PGA is then concentrated and heat sterilized. After further filtration, the concentrated of purified PGA is again sterilized and dried to powder by spray drying. However, PGA production depends on several factors, including strain of starter culture, nutrient source, pH, temperature, and fermentation time. Richard and Margaritis (2004) suggested that PGA was produced maximally (23 g/L) by *B. subtilis* IFO 3335 in batch fermentation at pH 7.0 along with glycerol concentration at 20 g/L in glutamic acid/citric acid medium. In the same manner, Shih and Yu (2005) recommended that *B. subtilis* (natto) maximally produced PGA at 37^{o}C for 21 hours in a basal medium (pH 7.0) containing 5% (w/w) sucrose and 1.5% (w/w) L-glutamic acid. Because *B. subtilis*-protease and amylase decompose protein and insoluble sugar in the raw food material or medium sources, thus their activities may affect the increasing of PGA during fermentation. For the previous study of *B. subtilis* SB-MYP-1 potential, it could produce PGA in nutrient broth, PGA broth as well as solid state soybean fermentation (Mahidsanan, 2012).

As noted before, the starter culture properties should be maintained its stability, namely, the viability, enzyme activity for use in further food fermentation. Hence, the moderns of microbial preservation by encapsulation become to be interested techniques which are explained in the next section.

2.3 Microbial encapsulation

Encapsulation is a method to entrap one substance within another substance, thereby producing particles with diameters of few millimeters. The substance is encapsulated may be called the core material, the active agent, fill, internal phase, or payload phase. At the same time, the substance encapsulating is often called the coating, membrane, shell, capsule, carrier material, external phase, or matrix. The carrier material of encapsulates used in food products or processes should be food grade and able to form barrier for active agent and its surroundings. As can be seen in Figure 2.8, different types of encapsulates can be found as reservoir type and the matrix type. The reservoir type has a shell around the core material this can also be called a capsule since it is made by fluidized bed coating. In the case of matrix type, the active agent is dispersed over the carrier material, and can also be found on the surface since it is made by spray drying and freeze drying. A combination of these two types gives a third type of capsule that is recovered by a coated matrix type (Burgain, Gaiani, Linder, and Scher, 2011; Nedovica, Kalusevica, Manojlovicb, Levica, and Bugarskib, 2011; Ray, Raychaudhuri, and Chakraborty, 2016).

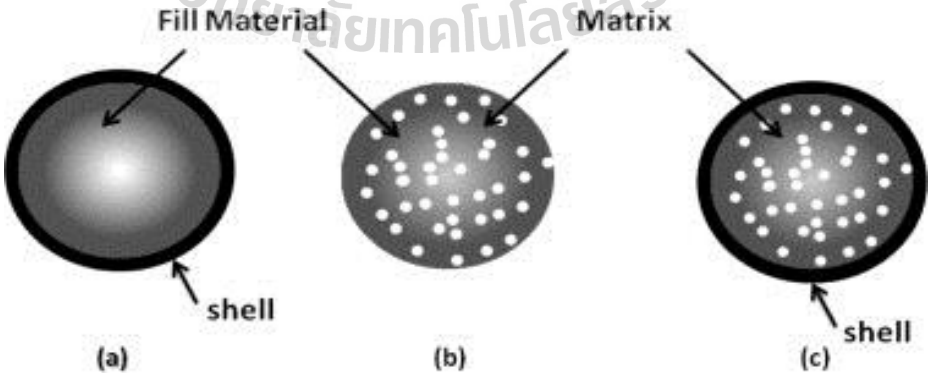

Figure 2.8 Schematic representation of encapsulation systems: (a) reservoir type, (b) matrix type, and (c) coated matrix type (Burgain et al., 2011).

At the same time, the selection of an entrapment method depends on several factors, including the potential for large scale production, cost, particle shape and resistance and, most importantly, the resulting viable cells. A comprehensive decision pathway for selecting a convenient immobilization technique is shown in Table 2.1.

Table 2.1 Cell immobilization techniques.

Encapsulation factors	**Live microorganisms**			
Type	Surface attachment	Entrapment	Containment	Aggregation
Mechanism	Adsorption on carrier	Mobility hindrance and controlled diffusion	Confinement in close membrane or interface	Natural or induced formation of self-aggregates
Agent	Natural absorption and induced bonding	Natural and synthetic polymer composites	Natural and synthetic membranes	Aggregation agents or polyelectrolyte cross linkers
Media	Carriers (Natural, synthetic, inorganic)	Gels, porous materials, films, fibers	Capsules or liposomes	Colloidal particles biofilms

From: Corona-Hernandez, Alvarez-Parrilla, Lizardi-Mendoza, Islas-Rubio, Ia Rosa, and Wall-Medrano, 2013.

A long time ago, the microbial encapsulation has been preferred for long term storage of cultures for worldwide. There are extensive culture collections that depend on these drying methods to preserve a huge diversity of cells for future propagation. In addition to culture collections and food industries have found drying technologies to be the preferred methods for preserving a multitude of different food in bulk quantities.

It would appear that drying technologies are still many varied methods of desiccating microorganisms, and no generic drying method for all applications. Quality control testing used positive control sample of reference microbial strain, and is distributed by using drying techniques to make cultures stable and transportable at ambient temperatures. In general, the objective of drying process is to enable long term storage of microorganisms to cell viability preservation. Due to the length of time required to get the actual data of dried samples over extended periods of time, a majority of the data discussed in this review is recovery of cells directly after drying. The limited data was found that the indication of the stability of the dried microorganisms over extended periods of time, especially when precise numbers of organisms had been preserved. A Figure 2.9 shows a general process step of starter culture encapsulation by drying methods (Morgan, Herman, White, and Vesey, 2006). However, the main of methodologies for microbial encapsulation based on drying principle is explained in the next section.

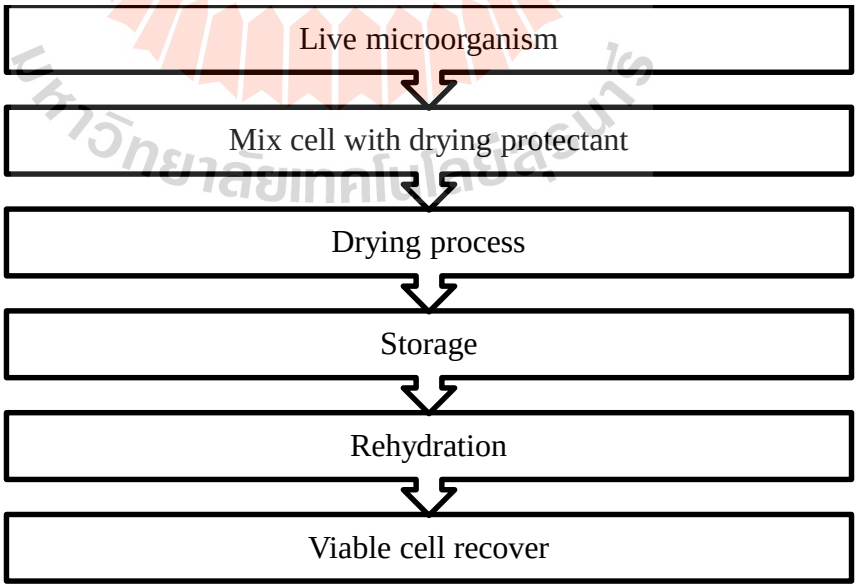

Figure 2.9 Starter culture drying process (Morgan et al., 2006).

2.3.1 Freeze drying

Freeze drying has been performed to preserve microorganisms in worldwide culture collections, such as the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC) since its material allows easy and inexpensive shipping and handling. This process is generally recommended to freeze dry concentrated cultures of $>10^7$ CFU/g in ensuring sufficient cells remaining after the freeze drying, long term storage and reconstitution for further propagation of the strain. During the freeze drying process involves the removal of water from frozen cell suspension by sublimation under reduced pressure. Sublimation is the process whereby water is removed as water vapor directly from ice, without passing through the liquid state. Cell concentration ($>1\times10^8$ CFU/ml) has been decided on the basis that the higher the initial cell concentration, the longer viable cells will survive within the freeze-dried sample (Morgan et al., 2006). In 2013, Sohail et al. showed a gel encapsulated *Lactobacillus rhamnosus* GG probiotic, which was freeze-dried exhibited less death after 6 months at 4°C, and the research of Dianawati, Mishra, and Shah (2013) had shown of freeze-dried *L. acidophilus* and *L. lactis* sp. *cremoris* bacteria, which condition was set to achieve -100 Torr of internal pressure before freeze drying at temperature of -88°C, 44 h of primary freeze drying, and 4 h of secondary freeze drying, and then dried culture kept in foil pouch containing sodium hydroxide (a_w 0.07) or lithium chloride (a_w 0.11) desiccant conditions. The result showed that the survival rate of freeze-dried cells was higher than that of spray-dried cells kept under the same conditions after 10 weeks of storage.

2.3.2 Foam formation

Foam formation is a new drying technique which uses sugar solution to transform biological suspensions into mechanically stable dry foam. These foams are made by boiling under vacuum at ambient temperatures to induce a process, which produces immobile amorphous, non-crystalline glass foams directly from a liquid. The foams are then subjected to further drying at elevated temperatures to increase their stability at ambient temperatures. This process omits the need to freeze, and therefore eliminates the associated drawbacks. The boiling effect can be scaled up to deal with large volumes, whereas other similar evaporation techniques cannot be scaled up and take too long for them to be commercially viable. This technique has been reported to stabilize *L. acidophilus* and *L. lactis* subsp. *cremoris*, and also Gram negative species such as *E. coli*, *Bordetella bronchiseptica* at temperatures above 37°C with up to 40% loss of cell viability, to which is substantial maintain consistent cell viability at 37°C (Bronshtein, 2004; Morgan et al., 2006).

2.3.3 Spray drying

Spray drying can be used to produce large amounts of ingredients with a relatively low cost. It produces dry granulated powders from solution by atomizing the wet product at high velocity within a chamber. The air within the chamber is heated to increase temperature to ~200°C. The atomized droplets are then dried into granules before they hit the side of the chamber (Morgan et al., 2006). Several studies have considered at the possibility of using this relatively inexpensive for the starter culture preservation. Desmond, Ross, Callaghan, Fitzgerald, and Stanton (2002) found that the 100% survival rate of *L. paracasei* was achievable after 7 days storage at 4°C and 15°C. However, there was approximately 20-80% drop in cell viability at four weeks for both

temperatures. This recovery relied on a high 7×10^7 CFU/ml initial bacterial load, and the enumeration of the high cell concentrations gave results with up to 20% variation. Ying, Sun, Sanguansri, Weerakkody, and Augustin (2012) suggested that the incorporation of glucose into the encapsulant formulation prior to spray drying of protein-carbohydrate based *L. rhamnosus* GG formulations improves the cell viability during storage. Lian, Hsiao, and Chou (2002) showed that the *B. longum* B6 exhibited the least sensitivity to spray-drying, and showed the highest survival rate (82.6%) after drying with skim milk. However, the variable cells indicated that its cell injury can also occur during storage. In addition, to achieve long term storage of the dried starter culture, possible changes in the storage conditions should also be investigated, such as the storage temperature, relative humidity at the presence of oxygen (Morgan et al., 2006).

2.4 Bacterial cell function

2.4.1 Bacterial cell wall and function

Murein is a peptidoglycan in bacterial cell wall which consists of N-acetylglucosamine and N-acetylmuramic acid cross-linked by short chains of amino acids (peptide). Gram positive bacteria, the cell wall consists of several layers of peptidoglycan. As the peptidoglycan sheets is teichoic acids (Figure 2.10) which are polyol phosphate polymers, with either ribitol or glycerol connected by phosphodiester bonds. Substituent groups on the polyol chains can include D-alanine (ester linked), N-acetylglucosamine, N-acetylgalactosamine and glucose (Todar, 2014). Figure 2.11 shows highly negative charged polymers of the cell wall which can serve as a cation-sequestering mechanism.

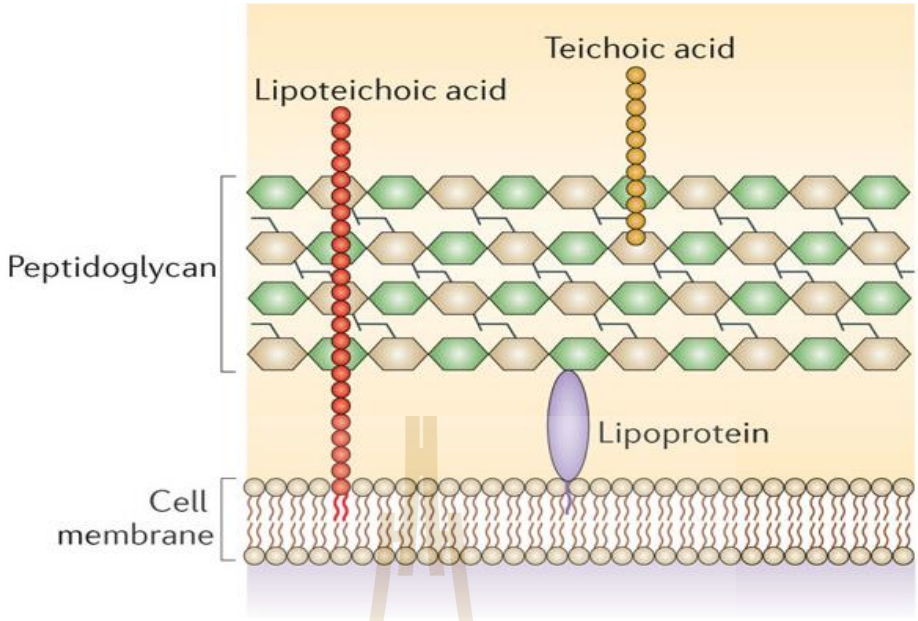

Figure 2.10 Structure of the gram positive bacterial cell wall (Brown, Wolf, Prados-Rosales, and Casadevall, 2015).

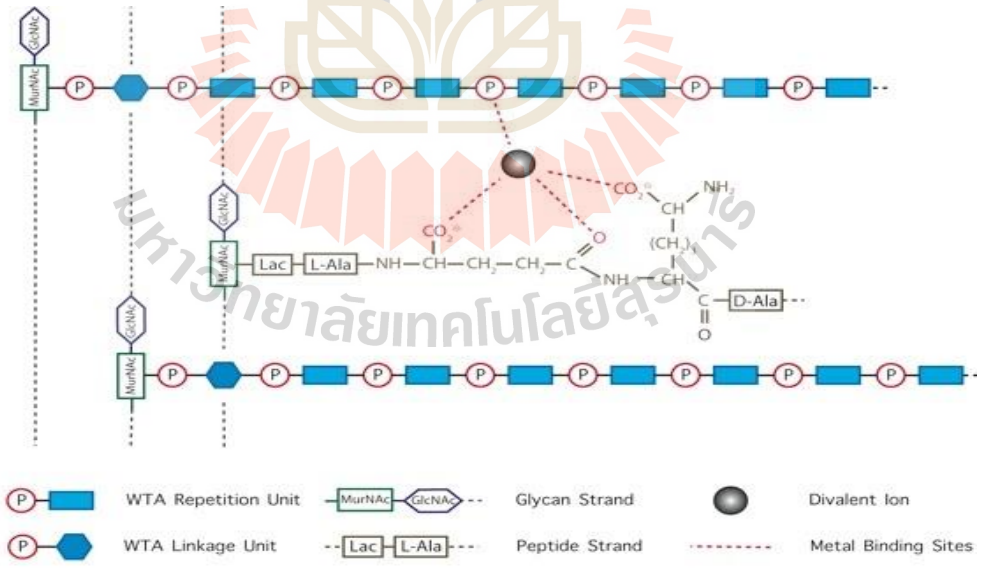

Figure 2.11 Schematic representation of the cation-mediated interaction between peptidoglycan and wall teichoic acids (WTA) in gram positive cell walls (Hediger et al., 2008).

Gram negative bacteria, the cell wall are composed of a single layer of peptidoglycan surrounded by a membranous structure which called the outer membrane. Since it contains a unique component, lipopolysaccharide, which is toxic to animals (Figure 2.12). The lipid A of LPS is inserted with phospholipids to create the outer substance of the bilayer structure. The lipid part of the lipoprotein and phospholipid form the inner leaflet of the outer membrane bilayer of gram negative bacteria (Todar, 2014).

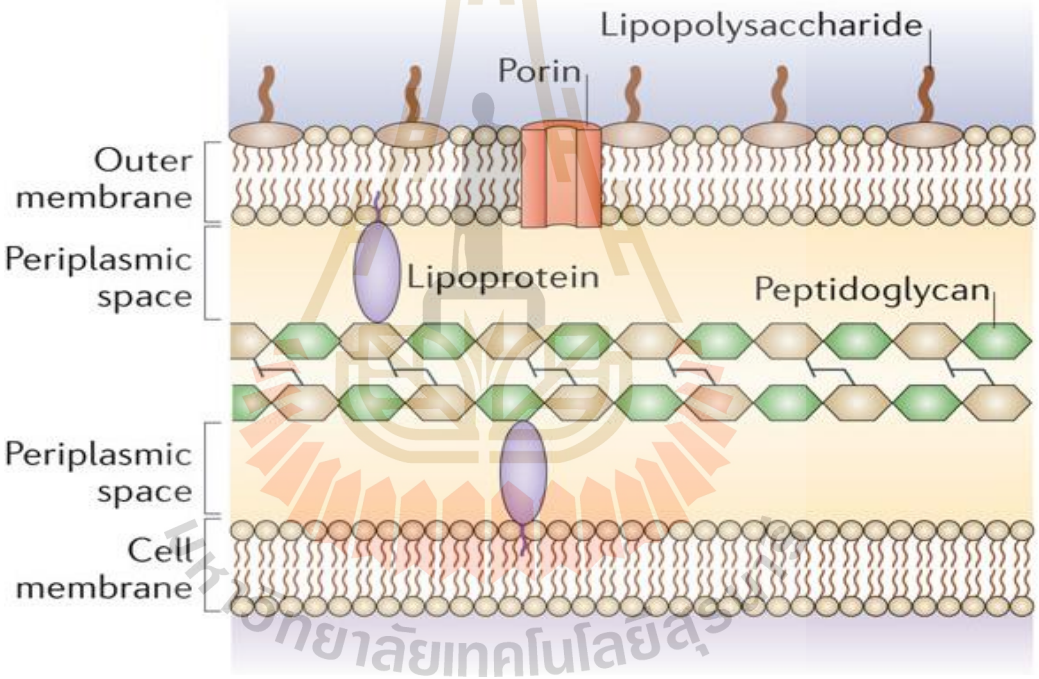

Figure 2.12 Structure of the gram negative bacterial cell wall (Brown et al., 2015).

2.4.2 *B. subtilis* spore structure and function

A spore is encased in layers of durable shells, has reduced water content, and is metabolically dormant. These characteristics are important to survival of the cell and resistance under environmental stress. However, the inert spore may be able to return

to active cells when nutrients are again available. Although dormant, spores are inactive part but they continuously monitor the environment for the renewed presence of nutrient. Once nutrient is enriched, a step known as germination will induce spores changing to vegetative cells (Driks, 2004).

Figure 2.13 shows the spore composition that the spore inside called the core, houses the chromosome, and is filled with small acid-soluble proteins (called SASP) that saturate the DNA and help to protect it. Surrounding this is a lipid membrane and then a thick layer of peptidoglycan. This layer called the cortex which differs from non-spore peptidoglycan both in cross linking and composition. The cortex keeps the spore core at low water activity, which is turn to spore resistance. Surrounding the cortex is a complex multilayered structure called the coat which plays roles in spore resistance, germination, and apparently possesses enzymatic activity that may possibly permit interactions with other organisms in the environment. A number of species, including *B. anthracis* and *B. thuringiensis* possess an additional layer as well which called the exosporium, whereas *B. subtilis* is not appeared. This structure does not call directly on the coat but is separated from it via a substantial gap. Consistent with a loose connection between the coat and exosporium since its shape varies from the size of spore (Driks, 2004).

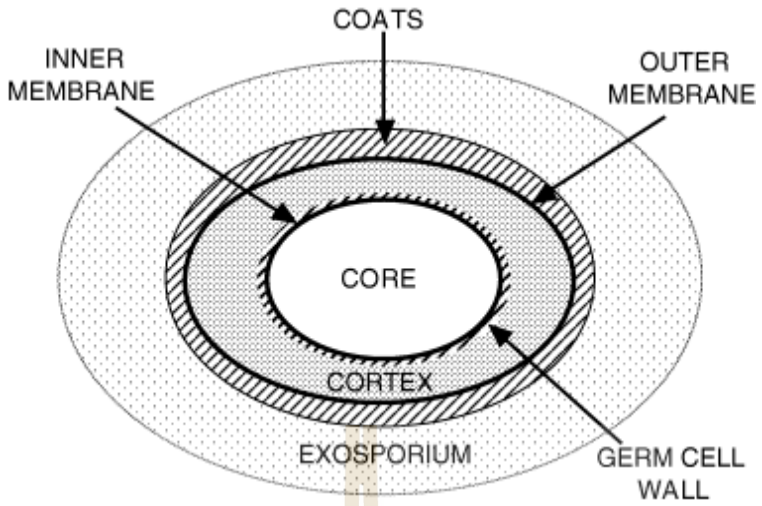

Figure 2.13 Spore structure (Setlow, 2006).

2.5 Cryoprotectant and microbial encapsulation mechanisms

The choice of an appropriate drying medium or cryoprotectant is thus very important in the case of microorganism. Its potential can maintain their survival rates during dehydration and subsequent storage. The compounds are differently permeable to the cells, which in turn affects the mechanism of their protective effect (Santivarangkna, Higl, and Foerst, 2008). Also, the examples of cryoprotectant and its mechanism in microbial encapsulation are revealed in the next section.

2.5.1 Starch

Starch is a polymer which consists of glucose units cross-linked by glycosidic bonds since its compositions are mainly amylose and amylopectin (Figure 2.14). In case of resistance starch, it can be prebiotic substance which protects probiotic bacteria under stress conditions because its potentiality is an ideal surface for the adherence of the bacterial cells to the starch granules, and can enhance viability and metabolic activity at the intestine (Burgain et al., 2011).

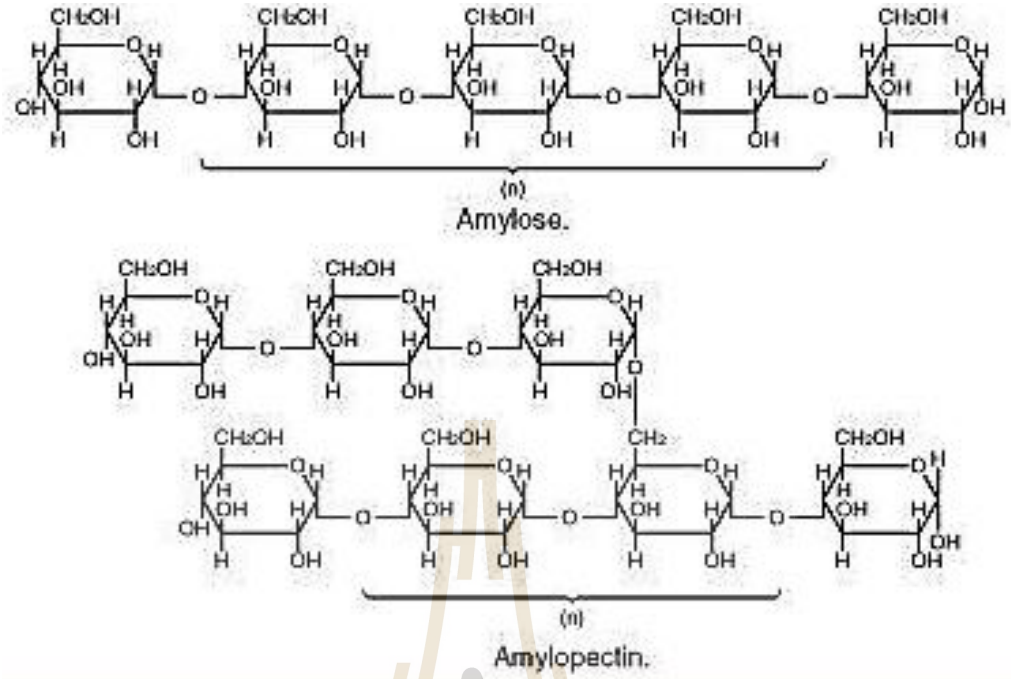

Figure 2.14 Amylose and amylopectin (Nippon starch chemical, 2012).

2.5.2 Sugars and sugar derivatives

Various sugars (glucose, fructose, lactose, mannose and sucrose), sugar alcohols (sorbitol and inositol) and non-reducing sugars (trehalose) have been tested for their protective effect during drying and subsequent storage. These compounds were in the most cases found to be effective toward protection of various lactic acid bacteria. Damage to biological systems derived from freeze-drying has been attributed to two primary causes: (i) changes in the physical state of the membrane lipids and (ii) changes in the structure of sensitive proteins in the cell. The mechanisms underlying sorbitol protection of dried cells would thus be: (i) prevention of damage to the membrane via interaction therewith and prevention of lipid oxidation owing to its anti-oxidant properties (ii) stabilization of the protein structure, and hence preservation of functionality associated with formation of sorbitol-protein complexes (iii) maintenance

of turgor, owing to accumulation additive (Carvalho, Silva, Ho, Teixeira, Malcata, and Gibbs, 2004).

The sugar effect of compatible solutes is believed to preferential exclusion solutes, which is the main mechanism of protecting macromolecules in organisms against moderate water loss. The preferential exclusion of solute can be defined as a situation where a macromolecule has higher affinity for water than that of the solute, and therefore water is presented in excess at the macromolecule surface. The protection of cells by preferential exclusion is shown in Figure 2.15. Santivarangkna, Naumann, Kulozik, and Foerst (2010) described that interaction between membrane phospholipids and sorbitol was observed from hydrogen-bonding sensitive C=O and P=O stretching bands by Fourier transform infrared spectroscopy (FT-IR). The position of the P=O band was shifted to the lower frequency in *Lactobacillus helveticus* dried cell with sorbitol, which reflects the hydrogen bonding interaction. In addition, the carbohydrate derivative did not affect to the morphology of the microbial capsules. For example of oligofructose-enriched inulin is the most appropriate prebiotic to be used as a partial replacement of reconstituted skim milk to microencapsulate *Bifidobacterium* BB-12, with a great potential as a functional ingredient to be applied in foods (Fritzen-Freire, Prudencio, Amboni, Pinto, Negrao-Murakami, and Murakami, 2012).

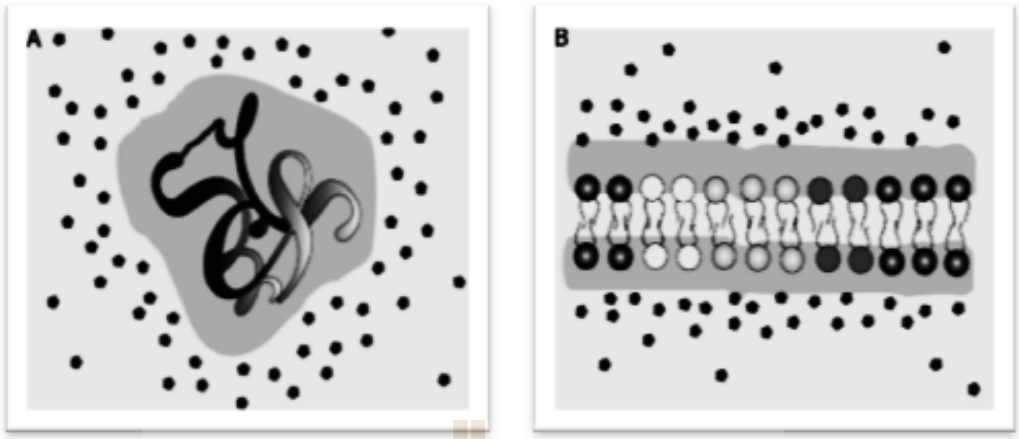

Figure 2.15 Preferential exclusion of sugars (closed pentagon) from the vicinity of a protein (A) and membrane (B) stabilizes them in their native conformations (Santivarangkna et al., 2008).

2.5.3 Soybean flour and soy protein isolate

Soy flour is generally made from dehulled, usually heat-processed whole soybeans or defatted soybean flakes, which are ground finely enough with a hammer mill so that 97% will pass through a 100-mesh screen and 95% will pass through a 200-mesh screen. Flour passing through the highest rated (finest mesh) screen has the highest protein content. The various types of soy flour are subjected to various amounts of heat processing for different end uses. Many research represent in the soybean flour molecular structures that consist of different polar and nonpolar amino acids (Table 2.2). Those amino acids present as an arginine (3.153 g/100g), lysine (2.706 g/100g) and histidine (1.097 g/100g) may be capable of reacting with a negatively charge crosslinking molecule (Iman and Maji, 2013; Shurtleff and Aoyagi, 2014; Soybean, 2014). Moreover, soy carbohydrates range from 33% to 35%, especially,

polysaccharide and dietary fiber, starch, hemicelluloses, cellulose, stachyose, raffinose, arabinose, glucose and sucrose are also contained (Esteves et al., 2010).

The soy protein isolate is a highly refined or purified form of soy protein with a minimum protein content of 90% on a moisture-free basis, since it made from defatted soy flour which removed the non-protein components, fats and carbohydrates. In addition, soy isolates are mainly used to improve the texture of meat products, but are also used to increase protein content, to enhance moisture retention, and as an emulsifier. Recently, Dianawati et al. (2013) demonstrated that the soy protein isolate provided the good protection on freezed-*B. longum* 1941, when it was combined by mannitol and maltodextrin.

Table 2.2 Content and molecular structure of amino acids in soybean flour and soy protein isolate.

Amino acids	**Content (g/100g) in soybean flour**	**Content (g/100g) in soy protein isolate**	**Molecular structure**
Tryptophan	0.591	1.1	Non polar
Threonine	1.766	3.3	Polar
Isoleucine	1.971	4.3	Non polar
Leucine	3.309	7.2	Non polar
Lysine	2.706	5.5	Polar positive charge
Arginine	3.153	6.7	Polar positive charge
Histidine	1.097	2.3	Polar positive charge
Phenylalanine	2.122	4.6	Non polar
Tyrosine	1.539	3.3	Non polar

Table 2.2 Content and molecular structure of amino acids in soybean flour and soy protein isolate (Continued).

Amino acids	Content (g/100g) in soybean flour	Content (g/100g) in soy protein isolate	Molecular structure
Valine	2.029	4.4	Non polar
Methionine	0.547	1.2	Non polar
Cysteine	0.655	1.1	Polar
Alanine	1.915	3.8	Non polar
Aspartic acid	5.112	10.2	Polar negative charge
Glutamic acid	7.874	16.6	Polar negative charge
Glycine	1.880	3.7	Non polar
Proline	2.379	4.5	Non polar
Serine	2.357	4.8	Polar

From: Fatih, 2010; Shurtleff and Aoyagi, 2014; Soybean, 2014; Chemipan product.

A Scientists described those principles base on the scientific evident of adhesion effect between soybean flour and negatively charged sites at the surfaces of glutaraldehyde agent and jute model (Figure 2.16) by FT-IR technique, they found that the peak intensities in the region 3400-3000 cm^{-1} assigned for -OH and -NH stretching vibrations were decreased. Furthermore, two peaks corresponding to 2230 and 1167 cm^{-1} assigned for –N-C=O and C-N stretching vibration were appeared. This might be attributed to the interaction of -OH and $-NH_2$ groups present in soybean flour with negatively charged sites at the surfaces of glutaraldehyde/juit (Iman and Maji, 2013). However, in term of cryoprotectant with microbial preservation and food

fementation, Gasaluck (2015) suggested that freeze-dried *B. subtilis* SB-MYP-1 cells with 10% (w/v) soybean flour are possibly used in Thua-nao fermentation.

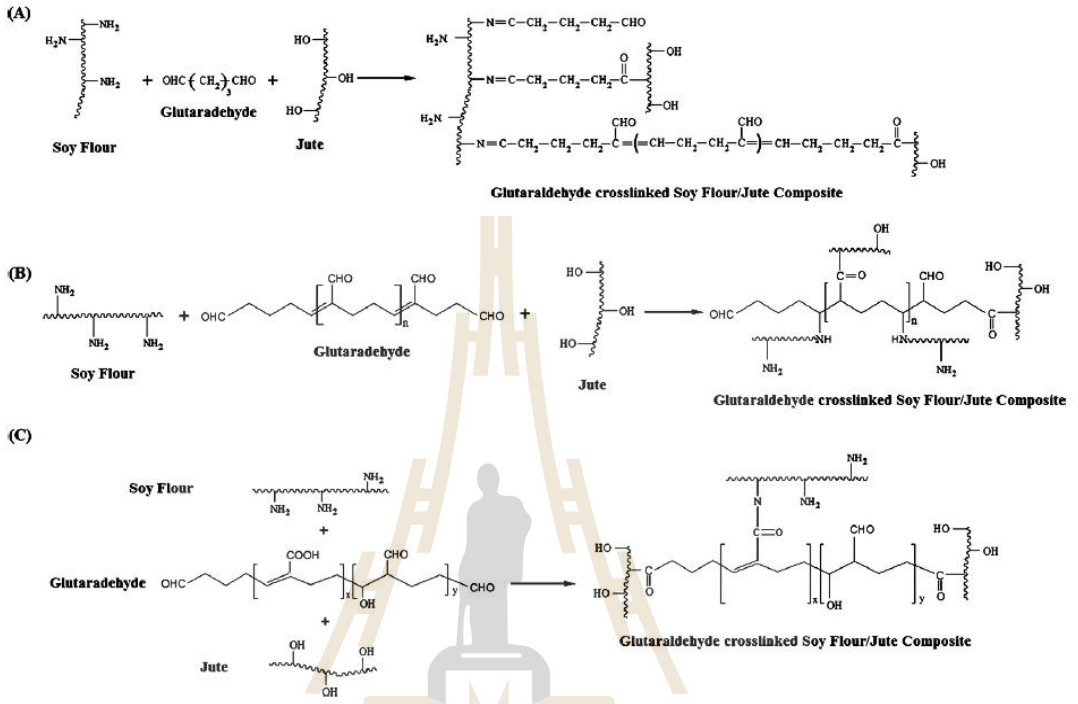

Figure 2.16 Schematic diagram of glutaraldehyde cross linked soy flour/jute composite (Iman and Maji, 2013).

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CHAPTER III

A NOVEL SOYBEAN FLOUR AS A CRYOPROTECTANT IN FREEZE-DRIED *BACILLUS SUBTILIS* SB-MYP-1

3.1 Abstract

Bacillus subtilis SB-MYP-1 isolated from Thai Thua-nao fermentation has already been demonstrated as a potential starter culture. The aim of this work was to reveal the efficiency order of soy protein isolate (SPI), soluble starch (ST), maltodextrin (MD), and Doikham soybean flour (SBF) for protecting *B. subtilis* SB-MYP-1 cells under sub-lethal stress of freeze-dried encapsulation. The combination criteria, including the survival rate, low water activity, and moisture content of freeze-dried cells with SBF, were approximately 63.72% (1.30×10^8 CFU/g as cell viability), 0.285, and 2.31% wet basis, respectively, which were relative to the standard quality of powdered starter culture. In addition, the cell membrane fluidity, metabolic retardation, activities of three intracellular enzymes (glutamate dehydrogenase, GDH; 2-oxoglutarate aminotransferase, GOGAT; and glutamine synthetase, GS), and *pgsB* gene expression of freeze-dried cells were maintained by SBF protection. Surprisingly, the mode of action of SBF could preserve the protein stabilization, phospholipid bilayer, and peptidoglycan surface in freeze-dried cells, whereas the partial cell surface and lipid bilayer of cells with SPI, ST and MD were destroyed after sub-lethal freeze

drying. This study revealed the novel application of SBF as a cryoprotective agent for further freeze-dried *B. subtilis* SB-MYP-1 preservation.

Keywords: freeze drying stress, soybean flour, cryoprotectant, encapsulation, *Bacillus subtilis*

3.2 Introduction

Freeze-dried bacteria, yeast, and mould have been commonly used as starter cultures in numerous fermented food industries because the freeze drying technique can control significant quality parameters, including the moisture content, water activity (a_w), and cell viability, based on the standard guidelines of ATCC and NCTC (Morgan, Herman, White, and Vesey, 2006). Moreover, the major problems of freeze-dried stress affect the sub-lethal conditions of cell targets, such as decreasing the cell viability, cell membrane fluidity and intracellular enzyme activity, while gene damage remains (Cavicchioli, Saunders, and Thomas, 2014; Li et al., 2011; Morichi and Irie, 1973; Wang, Yu, Xu, Aguilar, and Wei, 2016). Similarly, Li et al. (2011) reported that cold stress could directly damage the cell membrane fluidity, protein, and DNA function of *Lactobacillus reuteri* cells because of intracellular-ice crystallization in the presence of freeze-drying. Likewise, the cell wall of *L. helveticus* was broken after low vacuum at 100 mbar (Santivarangkna, Wenning, Foerst, and Kulozik, 2007). In addition, the low pressure induced low moisture stress, decreasing the intracellular enzyme activities of dried *L. reuteri* (Li et al., 2011).

For freeze-dried processing of a starter culture, it is generally recommended to use commercial cryoprotectants, such as soy protein isolate, soluble starch,

maltodextrin and its derivatives, to protect against freeze drying injuries, but those agents have high cost. A good cryoprotectant should have a low cost and the ability to be dried to the microbial cells during the freeze drying process. Additionally, it should also provide a good matrix for rehydration and stabilization. A suitable cryoprotectant was, therefore, considered for the freeze-dried starter culture, which was inoculated during food fermentation and did not affect the fermented food properties (Burgain, Gaiani, Linder, and Scher, 2011; Dianawati, Mishra, and Shah, 2013; Mahidsanan and Gasaluck, 2011; Pyar and Peh, 2011; Rajam and Anandharamakrishnan, 2015; Ray, Raychaudhuri, and Chakraborty, 2016; Santivarangkna, Higl, and Foerst, 2008).

The starter culture used in this study was *Bacillus subtilis* SB-MYP-1, isolated from fermented soybean (named Thua-nao in Thai), which significantly synthesizes the nutritional metabolite poly-γ-glutamic acid (PGA) during fermentation due to the consequence of *pgsB* gene expression and three intracellular enzyme (GDH, GOGAT, and GS) activities (Mahidsanan and Gasaluck, 2011; Najar and Das, 2015; Ruzal and Sanchez-Rivas, 2003; Shih and Van, 2001; Shih, Wu, and Shieh, 2005; Zeng, Chen, Wang, Zheng, Shu, and Liang, 2014). Gasaluck (2010) confirmed that the advantages of this starter culture were substantiated in Thua-nao fermentation, whose product properties consisted of the good pyrazine compound, nutritional values (calcium, ferric, phosphorus, and vitamin B12), and safety. *B. subtilis* SB-MYP-1 is, therefore, a beneficial starter culture to be preserved with the appropriate cryoprotectant.

Soybean flour (SBF), a Doikham product from The Royal Project Foundation, was an alternative cryoprotectant that consisted of protein (34.20 g/100 g), carbohydrate (36.00 g/100 g), and lipid (21.10 g/100 g) because the cost is lower than that of commercial cryoprotectants (Gasaluck, 2015). Freeze-dried *B. subtilis* SB-

MYP-1 encapsulated with SBF was initially reported by Gasaluck (2015). It was suggested that this dried culture could be directly inoculated into Thua-nao supplemented with PGA fermentation, and the organoleptic properties of the fermented product were not affected.

At present, there is no report of freeze-dried *B. subtilis* using the SBF mechanism. The main purposes of this study were, therefore, to validate an innovative SBF cryoprotectant for preventing *B. subtilis* SB-MYP-1 cell damage after freeze drying stress. Additionally, its viability, cell membrane fluidity, morphological properties, metabolic activity, activities of three intracellular enzymes and *pgsB* gene expression were compared with those of three different commercial cryoprotectants.

3.3 Materials and methods

3.3.1 Bacterial culture

B. subtilis SB-MYP-1 was obtained by isolation from fermented soybean (Thua-nao). *B. subtilis* biochemical identification was confirmed using API 50 CHB medium (Bio Merieux Inc), and a sample was kept as a certified stock at the School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand. Its cell suspension was prepared by transferring 0.5 mL of stock culture to 10 mL of nutrient broth followed by incubation in a shaker at 200 rpm and 37°C for 24 h. Cells were harvested by centrifugation (10000×g for 10 min at 4°C) and washed twice with 0.85% (w/v) sterile sodium chloride solution. The population of *B. subtilis* SB-MYP-1 in the suspension was approximately 10^8-10^9 CFU/mL.

3.3.2 Freeze drying method

The 10% (w/v) cell pellet was mixed with equal proportions of either sterile 10% (w/v) soy protein isolate (SPI; Chemipan product), 10% (w/v) soluble starch (ST; Carlo Erba product), 10% (w/v) maltodextrin (MD; Chemipan product) or 10% (w/v) soybean flour (SBF; Doikham Food Product Co., Ltd, Thailand) to reach a final of cell concentration of 10^8-10^9 CFU/mL. Those suspensions were frozen at -60°C overnight and then desiccated under a vacuum (0.001 mbar) in the freeze-drier (Christ Gamma 2-16 LSC) at a final temperature of 35°C for 24 h.

3.3.3 Enumeration

To quantify the viability of the cells, each sample was resuspended in an appropriate volume of 0.85% (w/v) sterile sodium chloride solution. After appropriate serial dilutions, the samples (0.1 mL) were spread on plate count agar and incubated at 37°C for 24 h. The survival rate for each sample tested was expressed as $(N/N_0)\times100$, where N_0 and N were the numbers of viable cells before and after freeze drying, respectively. All enumerations were performed in duplicate, and the plates containing 30-300 colonies were counted and calculated as CFU/g of freeze-dried culture or mL of solution.

3.3.4 Moisture content and water activity measurement

The moisture content of freeze-dried culture at the end of the freeze drying process was determined by the AOAC method (AOAC, 2000), and the water activity (a_w) was measured using an Aqua Lab CX-2 instrument at room temperature.

3.3.5 Morphological characterization

A fresh culture of *B. subtilis* SB-MYP-1 was washed twice with 0.1 M potassium phosphate buffer (pH 6.5) and immersed in glutaraldehyde (1 g/100 mL) at

4°C for 2 h. The fixative was removed by washing three times with 0.1 M potassium phosphate buffer, and the cells were subjected to dehydration with an increasing concentration of ethanol (0, 50, 70, 80, 90, 95 and 100 mL/100 mL) for 10 min. After complete evaporation of ethanol, the resulting sample was dried at 50°C overnight and then simultaneously mounted with all freeze-dried samples on carbon stumps and coated with a gold I sputtering device (JFC-1100E). The morphology of each sample was analysed by scanning electron microscopy (SEM) using a JEOL scanning electron microscope (JEOL JSM-6010LV) to observe the external appearance of all freeze-dried cultures.

3.3.6 Attenuated total reflectance infrared spectroscopy (ATR-FTIR)

ATR-FTIR experiments were recorded in a controlled chamber at 25°C. Freeze-dried and fresh culture samples were evaluated with the Platinum ATR accessory to a Bruker FTIR Tensor 27 Hyperion equipment (Bruker, Karlsruhe, Germany). All spectra of each sample were collected within the wavenumber range of 4000-400 cm^{-1} by averaging 32 scans at 4 cm^{-1} resolution. Analysis of the spectral data was performed using OPUS 7.0 software (Bruker, Karlsruhe, Germany).

3.3.7 Cell membrane fluidity

The cell membrane fluidity was determined by DPH probe fluorescence. Freeze-dried and fresh culture samples were rehydrated and washed twice with sterile 10 mM PBS. The DPH molecular probe (1,6-diphenyl-1,3,5-hexatriene, Sigma; 1 mM in methanol) was added to a suspension of cells to a final concentration of 0.004 mM. Staining of cells with DPH was performed in a dark incubator at 37°C for 30 min. Suspensions of stained cells were washed twice by centrifugation at 2700×g for 5 min and were then resuspended in sterile 10 mM PBS. Stained cells were pipetted into a

2-mL cuvette, and the fluorescence intensity of DPH was recorded with a spectrofluorometer. The excitation wavelength was 350 nm, and the emission wavelength was 425 nm (Modified from Herman et al., 2015; Liu, Duan, Wang, and Li, 2010).

3.3.8 Metabolic activity

The reduction of artificial electron acceptor INT to visible intracellular of INT-formazan was analysed. Freeze-dried and fresh culture samples were rehydrated in Ringer's solution (7.2 g NaCl, 0.37 g KCl and 0.17 g $CaCl_2$ in 1 litre of distilled water) for 90 min. Cultures were harvested by centrifugation (10000×g for 5 min at 4°C), washed twice and resuspended in 50 mM potassium phosphate buffer with glucose. Cell suspensions were mixed with 4 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT; ACROS) solution to a final concentration of 2 mM and incubated at 37°C for 2 h. The reduction of colourless INT to red formazan was detected by measuring the absorbance at 584 nm.

3.3.9 Cell extraction for *pgsB* intracellular enzyme assays

Freeze-dried and fresh culture samples were resuspended in 100 mM Tris-HCl buffer (pH 7.5). The cell suspension was then ultrasonically disrupted in an ice bath for 120 cycles of 5 s at 20 kHz (Sonics and Materials Inc). Cell debris was removed by centrifugation (10000×g for 60 min at 4°C), and the cell-free extracts (CFEs) were used for the intracellular enzymatic assays (Modified from Xu et al., 2014).

3.3.9.1 Glutamate dehydrogenase (GDH) activity

The mixture consisted of 1.5 mL of 3.6×10^{-4} mM Tris-HCl (pH 8.8) mixed with 1.6 mM NAD, 60 mM glutamic acid, and CFE. Control tubes were simultaneously incubated with all reagents, except for the substrates (glutamic acid).

The sample was measured at 340 nm at 25°C, and the change in the absorbance was recorded for 5 min. One unit of enzyme activity was defined as the amount of the enzyme that caused a change of 0.01 in the absorbance per minute, which was expressed in unit/h/g FW (Modified from Ruzal and Sanchez-Rivas, 2003; Xu et al., 2014).

3.3.9.2 2-oxoglutarate aminotransferase (GOGAT) activity

GOGAT activity was determined by measuring the decrease in the absorption at 340 nm due to the oxidation of NADH. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.5) with 0.1% (v/v) 2-mercaptoethanol, 1 mM EDTA, 18.75 mM 2-oxoglutarate, 75 mM L-glutamine, and CFE. Control tubes were simultaneously incubated with all reagents, except for the substrates (oxoglutarate and glutamine). The decrease in the absorbance was recorded for 5 min at 25°C. One unit of enzyme activity was defined as the amount of the enzyme that caused a change of 0.01 in the absorbance per minute, which was expressed in unit/h/g FW (Modified from Ruzal and Sanchez-Rivas, 2003; Xu et al., 2014).

3.3.9.3 Glutamine synthetase (GS) activity

The reaction mixture [1.5 mL samples, each containing 50 mM Tris-HCl buffer (pH 7.8) mixed with 20 mM $MgSO_4$, 0.05 mM EDTA, 60 mM glutamate, 10 mM hydroxylamine, 3 mM adenosine triphosphate (ATP), and CFE] and the blank control (without hydroxylamine) were simultaneously incubated for 30 min at 25°C. The reaction was terminated by adding 20% trichloroacetic acid. The precipitate was removed by centrifugation at 5000×g for 10 min, and the supernatant was measured at 540 nm. The GS activity was expressed in unit/h/g FW (Modified from Xu et al., 2014).

3.3.10 *pgsB* gene characterization by polymerase chain reaction (PCR)

The *pgsB* gene of *B. subtilis* was amplified by PCR using the specific forward primer (pgsB-f) 5'-ATGTGGTTACTCATTATAGCCTGTG-3' and reverse primer (pgsB-r) 5'-CTAGCTTACGAGCTGCTTAACCT-3' (Nucleic acid sequence location based on the GenBank accession number HQ599194.1; Yong et al., 2013). Total genomic DNA from the freeze-dried strains used in this study was isolated from overnight nutrient broth cultures using the CTAB method (Zhou, Bruns, and Tiedje, 1996). The extracted genomic DNA was amplified in a 10-µL reaction mixture containing 5X buffer (5X HOT FIREPol® Blend Master Mix), 10 pmol/µL of forward primer, 10 pmol/µL of reverse primer, sterile water and 50 ng/µL of DNA. Thermal cycling was performed at an initial PCR activation at 95°C for 15 min, which was followed by 35 cycles of 95, 55 and 72°C for 0.5, 0.5 and 1 min, respectively, and a final extension at 72°C for 5 min. The PCR products were separated by size using gel electrophoresis on 1.5% agarose gel, which was run at 135 volts for 40 min (Phamacia Biotech Model HE99x-15-1-5). A 100 bp DNA ladder was used as a size standard in each run. Gel patterns were visualized by ethidium bromide staining and photographs taken by Gel Documentation (MacroVue UVis-20 Hoefer).

3.3.11 Statistical analysis

All quantitative experiments were performed in duplicate, and the results were presented as the mean±standard deviation (SD). IBM SPSS statistics 22 (Armonk, New York, U.S.A) was used to perform all statistical analysis. One-way analysis of variance (ANOVA) was determined, which was followed by Duncan's multiple range test (DMRT) with an overall significance level set at 0.05.

3.4 Results and discussion

3.4.1 Viability, moisture content and water activity

The main factors of injury from freeze drying of bacterial cells are freezing and osmotic stress with the removal of bound water, which causes cell membrane damage, protein denaturation, and a decreased bacterial cell survival rate (Navarta, Calvo, Calvente, Benuzzi, and Sanz, 2011; Pyar and Peh, 2011; Zhan et al., 2011).

After a sub-lethal effect of freeze drying, the combination criteria, including viability (>10^7 CFU/g), moisture content (<7% wet basis) and a_w (<0.6), were evaluated for the most suitable efficiency of SBF to maintain *B. subtilis* SB-MYP-1 stability based on the standard guidelines of freeze-dried starter culture (Morgan et al., 2006). The results were compared with the potential of commercial SPI, ST and MD. As shown in Figure 3.1A and 3.1B, all freeze-dried *B. subtilis* SB-MYP-1 samples had parameters that significantly depended on the cryoprotectant efficiency ($P<0.05$). However, the survival rate, moisture content, and a_w of freeze-dried cells with SBF were approximately 63.72±14.47% (1.30×10^8 CFU/g as cell viability), 2.310±0.597% wet basis, and 0.285±0.020, respectively, while they were relevant to the standard quality of powdered starter culture. These results indicated that the SBF is an appropriate cryoprotectant that meets three significant criteria of freeze-dried *B. subtilis* SB-MYP-1 cells.

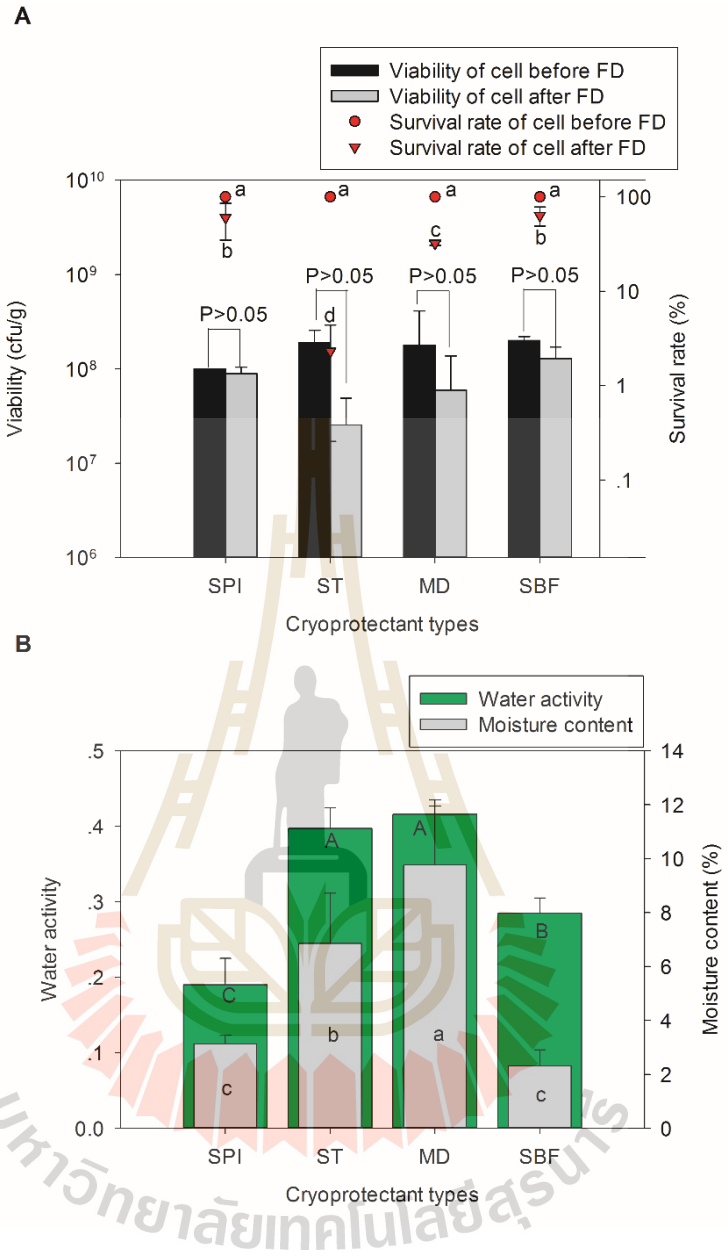

Figure 3.1 (A) Viability and survival rate of *B. subtilis* SB-MYP-1 before and after freeze drying (FD), (B) Water activity and moisture content of freeze-dried *B. subtilis* SB-MYP-1 with various cryoprotectants; SPI = soy protein isolate; ST = soluble starch; MD = maltodextrin; and SBF = Doikham soybean flour. The different letters, A, B, C, a, b, and c, between the scatter plots and groups indicate significant differences at $P<0.05$. $P>0.05$ indicates non significant differences within groups.

3.4.2 Morphological characterization and FT-IR spectroscopy

Morphological characterizations of freeze-dried *B. subtilis* SB-MYP-1 with commercial SPI, ST, MD, and SBF are shown in Figure 3.2B-E, respectively. Those dried cells were densely aggregated in a large cell group. Meanwhile, the lengths of cells (micrometres; μm) in Figure 3.2B-E are 3.67±0.58, 3.33±0.58, 3.67±1.15, and 4.00±1.00, respectively, and they are not significantly different ($P>0.05$) from the fresh cells in Figure 3.2A (4.00±1.00). The red arrow shows the potential action of cryoprotectant on a bacterial cell in which the *B. subtilis* cell embedded its polymer.

In addition, the FT-IR spectra were used to identify the mode of action between the cryoprotectant and bacterial cell surface due to the major component changes of fat, protein and carbohydrate after freeze drying stress (Figure 3.3). The FT-IR spectrum of fresh cells revealed distinct bands in a four-spectrum region (Figure 3.3A), namely, 3383 cm^{-1}, 1637-1224 cm^{-1}, 1070 cm^{-1} and 531 cm^{-1}, corresponding to the *B. subtilis* cell surface composition of fatty acid, protein mixed (α-helix and/or β-sheet of protein with phosphodiesters), carbohydrates and/or polysaccharide (peptidoglycan backbones), respectively (Filip, Herrmann, and Kubat, 2004; Ghosh et al., 2015; Maity et al., 2013). For the absorbance band of freeze-dried cells with SPI, ST and MD (Figure 3.3B, C, D, respectively), the intensities (1600-1200 cm^{-1}) decreased compared to those of the fresh cells for which the spectrum contributes to protein denaturation and carbohydrate deformation after freeze drying. Changes of those spectra in response to cold shock or freezing temperature may be associated with the cell membrane, cell wall or peptidoglycan sub-lethal injury, indicating abnormal cell surfaces. In the same manner, a region of phospholipid diesters (~1224 cm^{-1} peak; owing to P=O stretching of phosphodiesters) and protein structure (~1600-1200 cm^{-1} peak; owing to NH_2

bending, C=O stretching, C-N stretching and N-H bending) were decreased because a phosphate group and part of a phospholipid bilayer were destroyed (Ghosh et al., 2015; Li et al., 2011; Santivarangkna et al., 2007). Nevertheless, the intensities of freeze-dried cells with ST and MD (~1361-520 cm^{-1} peak) were clearly shifted compared to those of the fresh cells. The interaction between membrane phospholipids of bacterial cells and OH groups of carbohydrates/sugars can be described from these results based on the observed hydrogen bonding-sensitive C=O, P=O and C-OH stretching and P-O-C bonding (Bekhit, Sanchez-Gonzalez, Messaoud, and Desobry, 2016; Santivarangkna, Naumann, Kulozik, and Foerst, 2010).

Surprisingly, the mechanisms of SBF-protected cells were observed, as shown in Figure 3.3E. Four peaks at 3276, 3010, 2924 and 2854 cm^{-1} were assigned to asymmetric stretches of protein and methylene groups that are specific to lipid membranes. Additionally, the peak at ~1744 cm^{-1} was attributed to C=O stretching vibration of cell-phospholipid composition for stabilization of the protein and lipid bilayer. This might be interpreted as the interaction of a positive amino acid charge present in SBF with negatively charged sites on the bacterial cell as peptidoglycan surfaces via electrostatic interaction. Additionally, the spectral regions of 1631-524 cm^{-1} were shifted and had higher intensity compared to those of the fresh cells, which corresponds to potential hydrogen bonding between phosphate/ester groups of the cell membrane and polysaccharides/sugars of the SBF structure (Bekhit et al., 2016; Ghosh et al., 2015; Iman and Maji, 2013; Vodnar, Socaciu, Rotar, and Stanila, 2010). Consequently, the FT-IR results implied that the roles of polysaccharides/sugars and amino acids of SBF compositions could prevent freeze-

dried cell damage via the combination effects of the electrostatic interaction and hydrogen bonding between SBF and *B. subtilis* SB-MYP-1 cells.

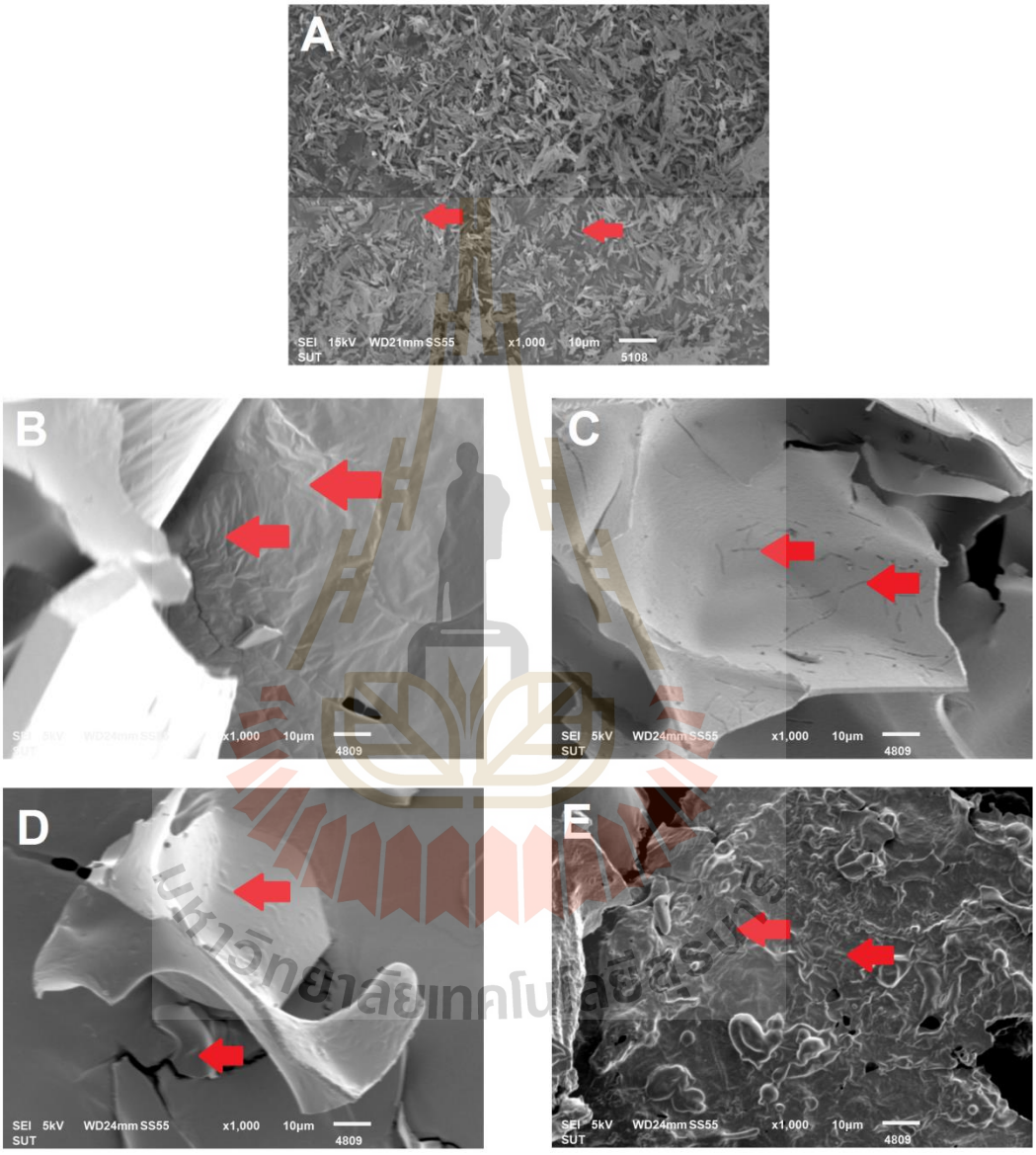

Figure 3.2 Scanning electron micrographs of fresh *B. subtilis* SB-MYP-1 cells (A) and freeze-dried *B. subtilis* SB-MYP-1 with (B) soy protein isolate, (C) soluble starch, (D) maltodextrin and (E) Doikham soybean flour.

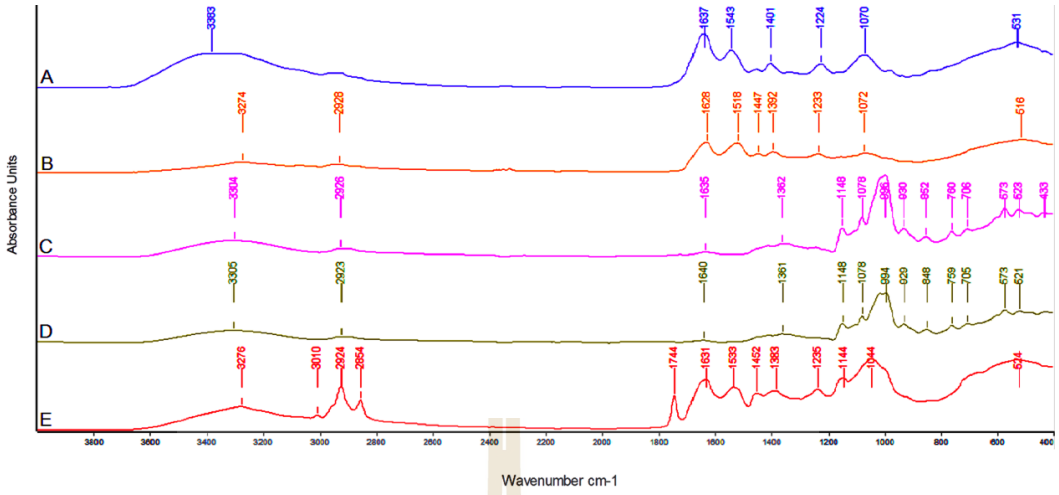

Figure 3.3 Comparative FT-IR spectra of fresh *B. subtilis* SB-MYP-1 cells (A) and freeze-dried *B. subtilis* SB-MYP-1 with (B) soy protein isolate, (C) soluble starch, (D) maltodextrin and (E) Doikham soybean flour.

3.4.3 Cell membrane fluidity

The DPH fluorescent probe can embed into the lipid bilayer and is sensitive to alterations in membrane fluidity. The fluidity of the cell membrane correlated to the probe fluorescence intensity; as the membrane fluidity decreased, the intensity increased (Denich, Beaudette, Lee, and Trevors, 2003; Hwang et al., 2011). Figure 3.4 shows the DPH probe fluorescence intensity of *B. subtilis* SB-MYP-1 as a result of different treatments for freeze drying. SPI obviously reduced the membrane fluidity compared to the fresh cells, ST, MD and SBF ($P<0.05$). These results are related to the FT-IR evidence that the sub-lethal effect of freeze drying destroyed the phospholipid bilayer of freeze-dried cells with SPI. In contrast, SBF application could maintain the cell membrane fluidity because its intensity is not significantly different from that of fresh cells ($P>0.05$).

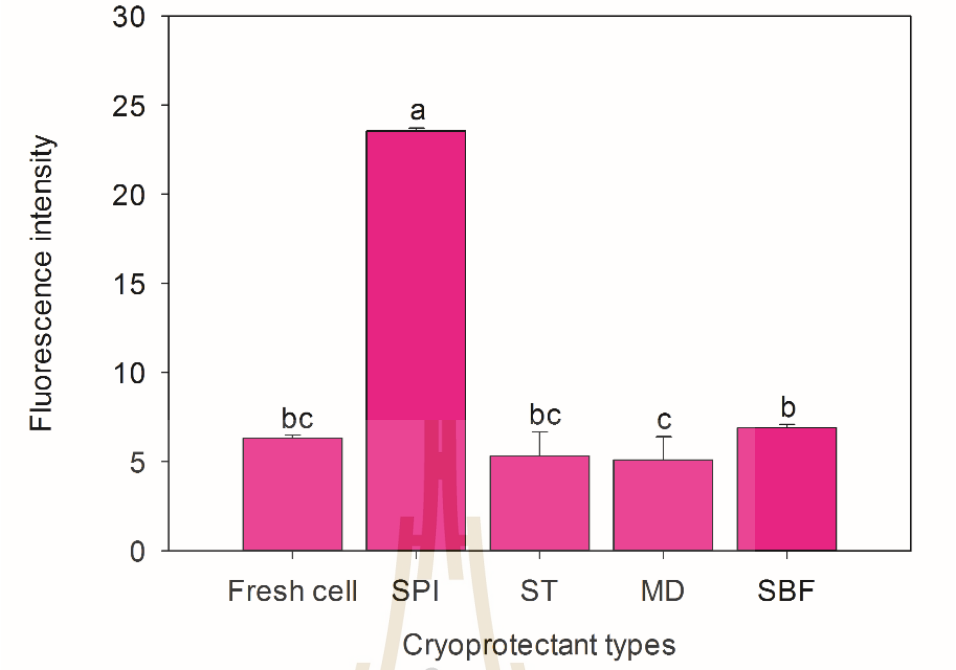

Figure 3.4 DPH fluorescence intensity of freeze-dried *B. subtilis* SB-MYP-1 with various cryoprotectants. The different letters, a, b, and c, between groups indicate significant differences at $P<0.05$.

3.4.4 Metabolic activity

Microorganisms can reduce the artificial electron acceptor INT to visible intracellular deposits of INT-formazan. A colorimetric assay of INT-formazan production was also used to assess dehydrogenase activity (Norton and Firestone, 1991). Figure 3.5 demonstrates the metabolic activity of freeze-dried cells with different cryoprotectants. Those results indicated that the relative metabolic activity (%) of freeze-dried cells was lower than that of fresh living cells ($P<0.05$). From this phenomenon, it can be suggested that the freeze drying process is a good alternative to retarding the catabolism and anabolism of the cell under low a_w/low moisture stress. However, the retention of their metabolically active cells and freeze-dried cells might

be different from the viability of cell survival, which was presented as our results and in other previous research (Santivarangkna et al., 2007; Ulmer, Ganzle, and Vogel et al., 2000).

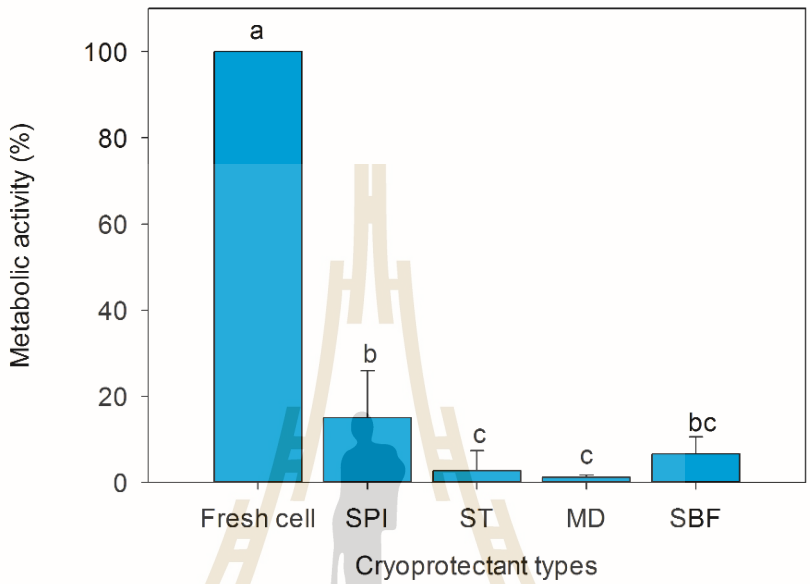

Figure 3.5 Metabolic activity of freeze-dried *B. subtilis* SB-MYP-1 with various cryoprotectants. The different letters, a, b, and c, between groups indicate significant differences at $P<0.05$.

3.4.5 *pgsB* gene-intracellular enzyme activity and its gene characterization

As mentioned in 3.4.1-3.4.4, SBF was demonstrated as a cryoprotectant with the highest cell survival rate as well as maintenance of cell membrane fluidity and metabolic retardation of *B. subtilis* SB-MYP-1 after freeze drying compared with three different commercial cryoprotectants. Nevertheless, the *pgsB* gene of *B. subtilis* plays a major role in controlling GDH, GOGAT, and GS activities, resulting in PGA productivity, which contributes to the significant quality of the Thua-nao product (Mahidsanan and Gasaluck, 2011; Najar and Das, 2015; Ruzal and Sanchez-Rivas,

2003; Shih and Van, 2001; Shih, Wu, and Shieh, 2005). The GDH, GOGAT and GS activities as well as *pgsB* gene expression of all freeze-dried *B. subtilis* SB-MYP-1 samples were demonstrated. As shown in Figure 3.6, the three intracellular enzyme activities of all freeze-dried treatments are not significantly different compared to those of the fresh cells (P>0.05). In addition, the expression of the *pgsB* gene was correctly detected in *B. subtilis* strains by PCR when using a specific primer for pgsB-f and pgsB-r (Yong et al., 2013). As seen in Figure 3.7, the *pgsB* gene characterization of all treatments was expressed by the PCR product size (~1182 bp). These results indicated that the freeze drying stress did not affect the *pgsB* gene of freeze-dried *B. subtilis* SB-MYP-1 that was treated with SBF.

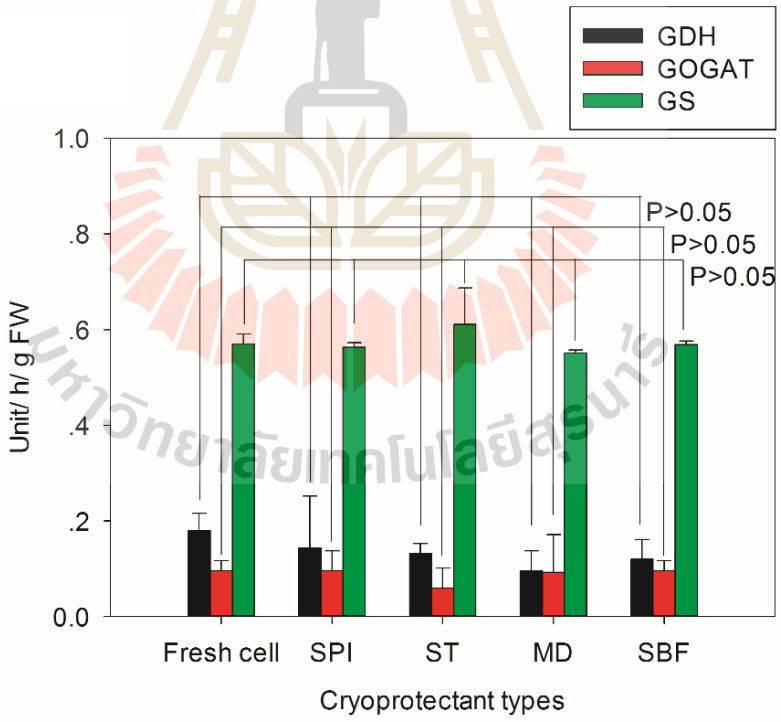

Figure 3.6 Intracellular enzyme activity for *pgsB* gene of freeze-dried *B. subtilis* SB-MYP-1 with various cryoprotectants, P>0.05 indicates non significant differences between groups.

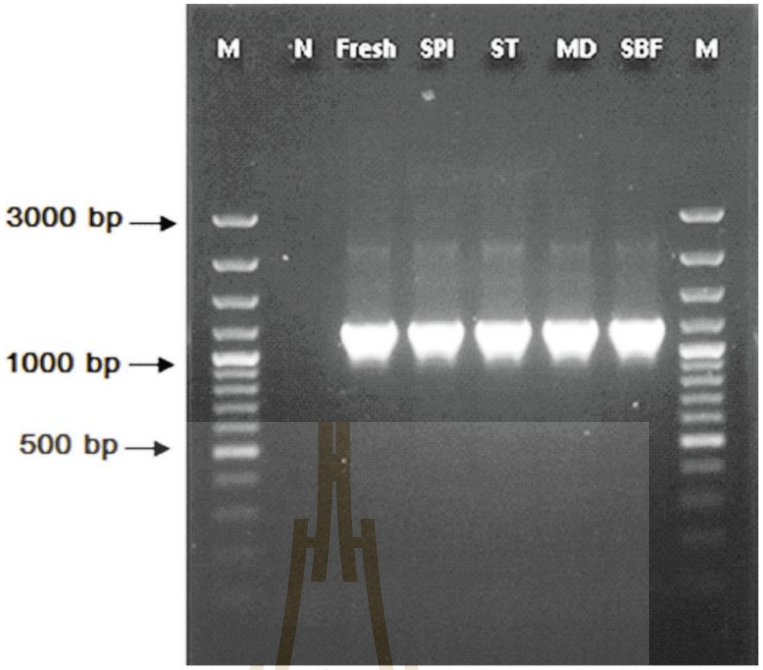

Figure 3.7 PCR band of *pgsB* gene of freeze-dried *B. subtilis* SB-MYP-1 with various cryoprotectants; M = marker 100 bp, N = negative control, and Fresh = Fresh cells as positive control.

3.5 Conclusions

The protective effects of each cryoprotectant on freeze-dried *B. subtilis* SB-MYP-1 targets, according to the viability, cell membrane fluidity, metabolic activity, activities of three intracellular enzymes, and *pgsB* gene, successfully revealed that the novel Doikham SBF is an inexpensive new cryoprotectant option for use in freeze-dried cell encapsulation. Additionally, these results demonstrated that fermented food industries can apply freeze-dried *B. subtilis* cells with SBF as a starter culture for specific Thua-nao production supplemented with PGA. Moreover, the properties of Thua-nao product from freeze-dried culture are not different from those of using fresh culture.

Further studies are required to monitor the stability of freeze-dried *B. subtilis* SB-MYP-1 with SBF during storage at different temperatures, while these conditions are an appropriate factor to preserve cell targets and to evaluate the potential of this dried starter culture throughout Thua-nao fermentation. As a result, the characteristics and properties of the Thua-nao product will not be changed.

3.6 Publication

Major content in this Chapter is recently published in the article:

Mahidsanan, T., Gasaluck, P., and Eumkeb, G. (2017). A novel soybean flour as a cryoprotectant in freeze-dried *Bacillus subtilis* SB-MYP-1. **LWT-Food Science and Technology.** 77: 152-159. http://dx.doi.org/10.1016/j.lwt.2016.11.015

3.7 References

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CHAPTER IV

PREDICTIVE MICROBIAL MODEL OF FREEZE-DRIED *BACILLUS SUBTILIS* SB-MYP-1 STABILITY DURING STORAGE

4.1 Abstract

The protective effect of SBF preserved the freeze-dried *Bacillus subtilis* SB-MYP-1 cell stress targets, including viability, cell membrane fluidity, metabolic activity, and intracellular enzyme of *pgsB* gene, was successfully reported. Surface attachment of SBF linked with peptidoglycan and cell membrane might be occurred. However, the long term preservation of cell is necessary. Control measures or key criteria; the highest cell viability (>10^7 CFU/g), moisture content (<7% wet basis), and water activity (<0.6), are an effective condition to maintain the stability of starter culture during storage. The purpose of this study revealed that predictive microbiological modelling using response surface methodology was an effective tool to assess the suitable point of control measure for persevering freeze-dried *B. subtilis* SB-MYP-1 with SBF under sub-lethal condition. The combination of extrinsic factors (times and temperatures) was performed to gain mathematical equations. These results demonstrated that the viability, cytoplasmic fluidity, metabolic activity, and activities of 2-oxoglutarate aminotransferase (GOGAT) and glutamine synthetase (GS) were significantly unstable affected by prolonging the preservation times and temperatures

their mathematical models. Regarding to the appropriate storage factors, achieved the freeze-dried *B. subtilis* SB-MYP-1 cell stress targets stability was at -25ºC for 90 days of which the combination criteria (viability, moisture content, and a_w) were accordingly to the standard quality of the powdered starter culture. In addition, the accurate predictive microbiological models might be applied to predict the stability of freeze-dried *B. subtilis* SB-MYP-1 cell stress targets during storage.

Keywords: freeze-dried *Bacillus subtilis* stress stability, model microbiological equation, response surface methodology, cell stability

4.2 Introduction

Starter cultures for food industries are commonly preserved by freeze-dried with cryoprotective pellets for long term during storage at freezing, chilling, or ambient temperature (Morgan, Herman, White, and Vesey, 2006; Pyar and Peh, 2011; Santivarangkna, Higl, and Foerst, 2008; Xu, Gagne-Bourque, Dumont, and Jabaji, 2016). Upon storage of powdered starter culture, the environmental factors, such as intrinsic factors (moisture, water activity), and extrinsic factors (temperature, vacuum package, atmosphere) may influence the viability, cell membrane fluidity, and intracellular activities via its stress targets (Cavicchioli, Saunders, and Thomas, 2014). Nevertheless, as previous researches had often been observed. Eratte, Wang, Dowling, Barrow, and Adhikari (2016) reported that the survival rate of freeze-dried *Lactobacillus casei* cell was significant decreased in the samples stored at 25 and 5°C owing to increasing the moisture content and oxygen during storage. Likewise, Poddar et al. (2014) confirmed that the water activity and moisture content during storage

conditions had influence on the stabilities of freeze-dried *L. paracasei*. Li, Zhang, Polk, Tomasula, Yan, and Liu (2016) found that the amount of *anti*-p40 antibody production from encapsulated *L. rhamnosus* GG was decreased as the storage time increased under -20°C and the ambient temperature. Li, Tian, Liu, Zhao, Zhang, and Chen (2011) suggested that the metabolic activity, cytoplasmic membrane fluidity and intracellular enzyme activities could be indicated to demonstrate the effects of intrinsic, extrinsic and implicit factors on starter culture stability.

Our previous research shows a novel strain of *B. subtilis* SB-MYP-1 which can be used as the starter culture in Thua-nao fermentation. Its functional abilities could produce amylase, protease, and bioactive-peptide as poly-γ-glutamic acid (PGA) which these potentialities were able to reduce undesirable aroma, and increase the nutritional values in fresh Thua-nao (Gasaluck, 2010). Since PGA is the nutritive composition of a sticky substance in Thua-nao, and has an efficient in health promoting compounds. However, its characteristic of *B. subtilis*-PGA production was controlled by *pgsB* gene expression to possess intracellular enzyme activities (glutamate dehydrogenase; GDH, 2-oxoglutarate aminotransferase; GOGAT, and glutamine synthetase; GS) resulting in threadlike characteristic of Thua-nao (Najar and Das, 2015; Shih and Van, 2001; Tanimoto, 2010). For this reason, the starter culture stabilities should be preserved for further its fermented food production. Gasaluck (2015) approved that the suitable technique of *B. subtilis* SB-MYP-1 preservation was freeze drying with 10% (w/v) SBF because this drying technique and its cryoprotectant could maintain the highest cell viability when compared with other methods used. In addition, it substantiated that this dried culture could be directly inoculated into Thua-nao supplemented with PGA fermentation, which whose organoleptic properties were not interfered.

Accordingly, predictive model of food microbiology, response surface methodology (RSM) is a statistical methodology generally used to analyze and predict the microbial responses that are influenced by one or multiple factors, namely, temperature or times, etc. (Behboudi-Jobbehdar, Soukoulis, Yonekura, and Fisk, 2013; Bevilacqua, Gallo, Corbo, and Sinigaglia, 2013; Khoramnia, Abdullah, Liew, Sieo, Ramasamy, and Ho, 2011; Nahr, Mokarram, Hejazi, Ghanbarzadeh, Khiyabani, and Benis, 2015; Schoug, Olsson, Carlfors, Schnurer, and Hakansson, 2006). In such case, there is few articles for reporting the RSM used to predict the stress target stability of freeze-dried starter culture during storage at the different temperatures. The appropriate condition could be specified to a control measure point which maintained stressed cells.

As mentioned above, the aim of this study was to demonstrate that the predictive model of RSM was an alternative technique to predict the stability of freeze-dried *B. subtilis* SB-MYP-1 stress targets, such as viability, cell membrane fluidity, metabolic activity and the three intracellular enzyme activities of *pgsB* gene under a change of extrinsic factors (times and temperatures). After that, a point of control measure for starter culture stability was gained to preserve freeze-dried cells.

4.3 Materials and methods

4.3.1 Bacterial culture

B. subtilis SB-MYP-1 was isolated from Thua-nao product, biochemically identified and confirmed its characteristics by using API 50 CHB medium (Bio Merieux Inc), kept as a certified stock at School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and Thailand Institute of Scientific and Technological Research (TISTR), Pathum

Thani, Thailand. *B. subtilis* SB-MYP-1 cell preparation was prepared by transferring 0.5 mL of stock culture into 10 mL of nutrient broth, and placed in a shaking incubator at 200 rpm, 37ºC for 24 h. The cultured nutrient broth suspension was done the centrifugation (10000×g for 10 min at 4°C), the cells was then harvested and washed twice with 0.85% (w/v) sterile sodium chloride solution. The number of *B. subtilis* SB-MYP-1 in the suspension was approximately 10^{8-9} CFU/mL.

4.3.2 Freeze drying method, starter culture preservation and experimental design

The cell pellet (from 4.3.1) was resuspended in sterile 10% (w/v) soybean flour (SBF; Doikham Food Product Co., Ltd, Thailand) to obtain a final cell concentration of 10^{8-9} CFU/mL, finally were frozen at -60ºC overnight and then desiccated under vacuum (0.001 mbar) in the freeze-drier (Christ Gamma 2-16 LSC) at 35ºC as final temperature for 24 h. Those freeze-dried starter cultures were placed in a laminate aluminum foil vacuum bag, and stored at the different temperatures and time that these independent factors, mathematical symbols and actual levels are shown in Table 4.1.

Table 4.1 Independent factors and their levels used in the experiments.

Type of independent factors (Unit)	**Mathematical symbol of variable**	**Level of storage time and temperature**		
		-1	**0**	**1**
Storage time (Days)	X_1	0	45	90
Temperature (ºC)	X_2	-25	0	25

The time and temperature of preservation could be modelled to prolong the stability of freeze-dried *B. subtilis* SB-MYP-1 with SBF using response surface methodology (RSM) as described in equation 1:

$$Y = \beta_0 + \Sigma_{i=1}^{k}\beta_i X_i + \Sigma_{i=1}^{k}\beta_{ii} X_{i^2} + \Sigma_{i=1}^{k}\Sigma_{j=i+1}^{k}\beta_i X_i X_j \quad (1)$$

Where Y is the response (4.3.3-4.3.7), β_0 constant, β_i the linear coefficient, β_{ii} the quadratic coefficient and β_{ij} the interaction coefficient. X_i and X_j are independent variables.

4.3.3 Enumeration

To enumerate the viability of freeze-dried cells during storage, each sample was resuspended in an appropriate volume of 0.85% (w/v) sterile sodium chloride solution. Appropriate serial dilutions were made. The samples (0.1 mL) were then spread on plate count agar and incubated at 37ºC for 24 h. All enumerations were performed in duplicate and the plates containing 30-300 colonies were counted and calculated as CFU/g of freeze-dried culture.

4.3.4 Moisture content and water activity measurement

The moisture content was determined by AOAC method (AOAC, 2000), and the water activity (a_w) was measured using an Aqua Lab CX-2 instrument at room temperature.

4.3.5 Cell membrane fluidity

The cell membrane fluidity was carried out by DPH probe fluorescence. Freeze-dried culture samples were rehydrated, and washed twice with sterile 10 mM PBS. The DPH molecular probe (1,6-diphenyl-1,3,5-hexatriene, Sigma; 1 mM in methanol) was added to the cell suspension with the final concentration of 0.004 mM, and further

placed in the dark incubator at 37°C for 30 min, washed twice by centrifuging at 2700×g for 5 min, and then resuspended in sterile 10 mM PBS. Stained cell suspensions were pipetted into a 2 mL cuvette, and the fluorescent intensity of DPH was recorded with a spectrofluorometer. The excitation wavelength was 350 nm, and the emission wavelength was 425 nm.

4.3.6 Metabolic activity

The reduction of artificial electron acceptor INT to visible intracellular of INT-formazan was analyzed. Freeze-dried and fresh culture samples were rehydrated to Ringer's solution (7.2 g NaCl, 0.37 g KCl and 0.17 g $CaCl_2$ in 1 liter of distilled water) for 90 min. Cultures were harvested by centrifugation (10000×g for 5 min at 4°C), washed twice and resuspended in 50 mM potassium phosphate buffer with glucose. Cell suspensions were mixed with 4 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT; ACROS) solution to a final concentration of 2 mM and incubated at 37°C for 2 h. The reduction of colorless INT to red formazan was detected by measuring the absorbance at 595 nm.

4.3.7 Cell extraction for *pgsB* intracellular enzyme assays

Freeze-dried cultures were resuspended in 100 mM Tris-HCl buffer (pH 7.5). The cell suspension was then disrupted ultrasonically in ice-bath for 120 cycles of 5 s at 20 kHz. Cell debris was removed by centrifugation (10000×g for 10 min at 4°C), and the cell-free extracts (CFEs) were used for the intracellular enzymatic assays.

4.3.7.1 Glutamate dehydrogenase (GDH) activity

The mixture consisted of 1.5 mL of 3.6×10^{-4} mM Tris-HCl (pH 8.8) mixed with 1.6 mM NAD, 60 mM glutamic acid, and CFE. Control tubes were simultaneously incubated with all reagents except for the substrates (glutamic acid).

The sample was measured at 340 nm at 25°C, and the change in absorbance was recorded for 5 min. One unit of enzyme activity was defined as the amount of the enzyme which caused a change of 0.01 in absorbance per minute and expressed unit/h/g FW.

4.3.7.2 2-oxoglutarate aminotransferase (GOGAT) activity

GOGAT activity was determined by measuring the decrease of absorption at 340 nm due to the oxidation of NADH. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.5) with 0.1% (v/v) 2-mercaptoethanol, 1 mM EDTA, 18.75 mM 2-oxoglutarate, 75 mM L-glutamine, and CFE. Control tubes were simultaneously incubated with all reagents except for the substrates (oxoglutarate and glutamine). The absorbance decreasing was recorded for 5 min at 25°C. One unit of enzyme activity was defined as the amount of the enzyme which caused a change of 0.01 in absorbance per minute and expressed unit/h/g FW.

4.3.7.3 Glutamine synthetase (GS) activity

The reaction mixture [1.5 mL samples, each containing 50 mM Tris-HCl buffer (pH 7.8) mixed with 20 mM $MgSO_4$, 0.05 mM EDTA, 60 mM glutamate, 10 mM hydroxylamine, 3 mM adenosine triphosphate (ATP), and CFE] and the blank control (without hydroxylamine) were simultaneously incubated for 30 min at 25°C. The stop reaction was done by adding 20% trichloroacetic acid, and centrifuged at 5000×g for 10 min, discarded the precipitate, and the supernatant was then measured by spectrophotometer at 540 nm. The GS activity was expressed unit/h/g FW.

4.3.8 Statistical analysis

A 3^2 full factorial design was used to further predict the change of freeze-dried *B. subtilis* SB-MYP-1 cell targets by means of the above experimental design. Analysis

of variance (ANOVA) of each response was performed to estimate the significance ($P<0.05$) of the main effects (linear and quadratic), their interactions (linear, linear-quadratic, and quadratic-quadratic), regression coefficients and their determination coefficients (R^2 values). Design-Expert® soft-ware version 7.0 was also used for analyzing the 3D graphical analysis of their experimental data. In addition, the predicted and actual values were compared and analyzed by paired-samples T-Test using IBM SPSS statistic 22 (Armonk, New York, U.S.) (StatSoft, Inc., USA).

4.4 Results and discussion

4.4.1 Equation model of freeze-dried cell viability, moisture content and a_w

As the Figure 4.1A shows that the viability of freeze-dried *B. subtilis* SB-MYP-1 cells decreased as the storage temperatures and times increased. This RSM modelling of viability significantly fitted to a Quadratic model ($P<0.05$) that can be expressed by the equation 2 ($R^2 = 0.9267$). The regression coefficients of temperatures and times affected the survival of freeze-dried cells during storage ($P<0.05$). In addition, the viability of freeze-dried cells preserved at -25°C for 90 days was approximately $>10^7$ CFU/g of which the standard requirements of dried starter culture are accordingly, but no cell viability was found at the 25°C for 90 days. Many researchers reported the similar stability phenomena of the starter culture preservation (Carvalho, Silva, Ho, Teixeira, Malcata, and Gibbs, 2004; Chen and Mustapha, 2012; Holkem et al., 2017; Jagannath, Raju, and Bawa, 2010; Wang, Yu, and Chou, 2004). Martin, Lara-Villoslada, Ruiz, and Morales (2013), for example, found that the storage temperature at -20°C after 45 days significantly provided the highest viability of freeze-dried

L. fermentum cells in alginate blended with starch, but the storage condition at 25°C was no viability. Based on the microbiological predictive modelling, the freezing temperatures (≤ -25°C) are applicable conditions or the significant extrinsic factors to maintain the viability of freeze-dried *B. subtilis* SB-MYP-1 for long term storage. At the same time, moisture content and a_w are also the significant intrinsic factors affecting the freeze-dried cell viability (Morgan et al., 2006; Santivarangkna, Kulozik, and Foerst, 2008). Two-factor interaction (2FI) model was significantly fitted ($P<0.05$) to the moisture content and water activity during storage (Figure 4.1B and C). The RSM models of moisture content and a_w of freeze-dried cells can be expressed by the equation 3 ($R^2 = 0.6499$) and 4 ($R^2 = 0.8731$), respectively. The regression coefficients of temperatures and times affected the moisture content and a_w of freeze-dried cells during storage ($P<0.05$), since moisture content and a_w were increased by the storage temperatures and times increased. The results of the those experiments corresponded to the research of Abe, Miyauchi, Uchijima, Yaeshima, and Iwatsuki (2009), which investigated that higher temperature, moisture content and a_w may induce lower survival rate, which was supported by the Arrhenius theory. It indicated that the natural logarithm of the inactivation growth rate constant showed an inverse dependence on absolute temperature at all a_w conditions.

$$\text{Cell viability} = + (3.58128\times10^{8}) - (8.55656\times10^{6}\times X_1) - (1.40120\times10^{6}\times X_2) - (10722.22222\times X_1X_2) + (50671.60494\times(X_1)^2) + (46492.00000\times(X_2)^2) \quad (2)$$

$$\text{Moisture content} = + 2.30750 + (0.024907\times X_1) + (0.018517\times X_2) + (1.01222\times10^{-3}\times X_1X_2) \quad (3)$$

$$a_w = + 0.27553 + (1.65741\times10^{-3}\times X_1) + (2.56667\times10^{-4}\times X_2) + (4.57778\times10^{-5} \times X_1X_2) \quad (4)$$

4.4.2 Equation model of cell membrane fluidity

The bacterial cytoplasmic membrane is a fluid, composed of phospholipids, cholesterol, proteins, and carbohydrates which is sensitively affected by various stress environmental factors (Cavicchioli et al., 2014; Santivarangkna, Kulozik, Kienberger, and Foerst, 2009). The cell membrane fluidity relative to the DPH probe fluorescence intensity; as the membrane fluidity decreased, the intensity increased (Denich, Beaudette, Lee, and Trevors, 2003; Hwang, Cho, Hwang, Jin, Woo, and Lee, 2011). Drawn from results, the cell membrane fluidity of freeze-dried cells gradually decreased by the increasing the storage temperatures and times (Figure 4.2A). Quadratic model fitted to fluorescence intensity was significant ($P<0.05$) that can be expressed by the following equation 5 ($R^2 = 0.7575$). The regression coefficients of temperatures and times, affected the cell membrane fluidity due to increasing a temperature and time throughout subsequent storage ($P<0.05$). Fatty acid of cytoplasmic membrane of dried starter culture, was injured due to lipid oxidation at ambient temperature stress. This phenomenon may decrease of the cell membrane fluidity which induces a change of cell function as well as cell death during storage (Santivarangkna et al., 2008; Fernandez-Sandoval, Ortiz-Garcia, Galindo, and Serrano-Carreon, 2012; Xu et al., 2016). In contrast, the cell membrane fluidity of freeze-dried cells during storage at -25°C was still maintained because the freezing temperatures may reduce the rate of fatty acid oxidation (Castro, Teixeira, and Kirby, 1995).

$$\text{Fluorescence intensity} = + (4.95620) - (0.020628 \times X_1) + (8.61717 \times 10^{-3} \times X_2) + (1.05692 \times 10^{-3} \times X_1X_2) + (5.69963 \times 10^{-4} \times (X_1)^2) + (4.12704 \times 10^{-3} \times (X_2)^2) \quad (5)$$

4.4.3 Equation model of metabolic activity

The relative metabolic activity (%) of freeze-dried cells decreased rapidly with increasing the storage temperatures and times (Figure 4.2B), comparing to the fresh cells (100%). Linear model fitted to metabolic activity was significant ($P<0.05$) that can be expressed by the following equation 6 ($R^2 = 0.6097$). Santivarangkna, Wenning, Foerst, and Kulozik (2007), presumed that decreasing the metabolic activity could be correlated to decreasing the cell viability due to temperature stress which damaged genetic level or in the cytoplasm. This effect indicated that stressed bacterial cells might not be able to recover on nutrient sources (Golden, Beuchat, and Brackett, 1988).

$$\text{Metabolic activity (\%)} = + (4.27642) - (0.037014 \times X_1) - (8.33933 \times 10^{-3} \times X_2) \quad (6)$$

4.4.4 Equation model of *pgsB* gene-intracellular enzyme activity

The three intracellular enzyme activities, such as GDH, GOGAT, and GS play an important role in the *B. subtilis*-pathway of nutritive PGA production throughout soybean fermentation (Shih and Van, 2001; Shih, Wu, and Shieh, 2005). Their activities of freeze-dried *B. subtilis* SB-MYP-1 cells, were monitored during storage. The unique fermented soybean quality, (Najar and Das, 2015), was demonstrated that Quadratic model fitted to GOGAT and GS was significant ($P<0.05$). Figure 4.3B and C, expressed by the following equation 7 ($R^2 = 0.9066$) and 8 ($R^2 = 0.9870$), respectively, except the model of GDH was not applicable for the predictive model because its activity of freeze-dried cells was not significant ($P>0.05$) during storage at

each temperature (Figure 4.3A). Nevertheless, GOGAT and GS were decreased by the storage temperatures and times increased ($P<0.05$), which similar to that of Dianawati and Shah (2011), demonstrated that the retentive activities of intracellular enzyme of freeze-dried *Bifidobacterium animalis* ssp. *lactis* Bb12 decreased after 10 weeks during storage at low water activity stress in a vacuum of aluminum foil.

$$\text{GOGAT (unit/h/g FW)} = +(0.12200) - (3.3333310^{-4}\times X_1)+(0.00\times X_2)-(2.40000\times10^{-5}\times X_1X_2) - (1.48148\times10^{-6}\times(X_1)^2) - (3.36000\times10^{-5}\times(X_2)^2) \quad (7)$$

$$\text{GS (unit/h/g FW)} = +(0.54465) - (0.016201\times X_1) - (9.01000\times10^{-4}\times X_2)-(3.24667\times10^{-5}\times X_1X_2) + (1.17556\times10^{-4}\times(X_1)^2) + (5.73600\times10^{-5}\times(X_2)^2) \quad (8)$$

4.4.5 The suitable condition for persevering freeze-dried *B. subtilis* SB-MYP-1 with SBF

As a result of control measure due to the extrinsic factors, at -25°C for 90 days, achieved accordingly to the standard qualities of dried starter cultures (the highest cell viability, moisture content, and a_w) (Morgan et al., 2006), as well as its activities of metabolic and three intracellular enzymes were successfully remained. Therefore, the validation of control measure at this condition was performed, by comparing the predictive time and temperature parameters for maintaining freeze-dried *B. subtilis* SB-MYP-1 with SBF to that of the actual during storage. Table 4.2 shows the not significant differences between the actual and predicted values of each parameters ($P>0.05$). Those parameters of the suitable condition were reliable to be applied for preserving freeze-dried *B. subtilis* SB-MYP-1 cells with SBF.

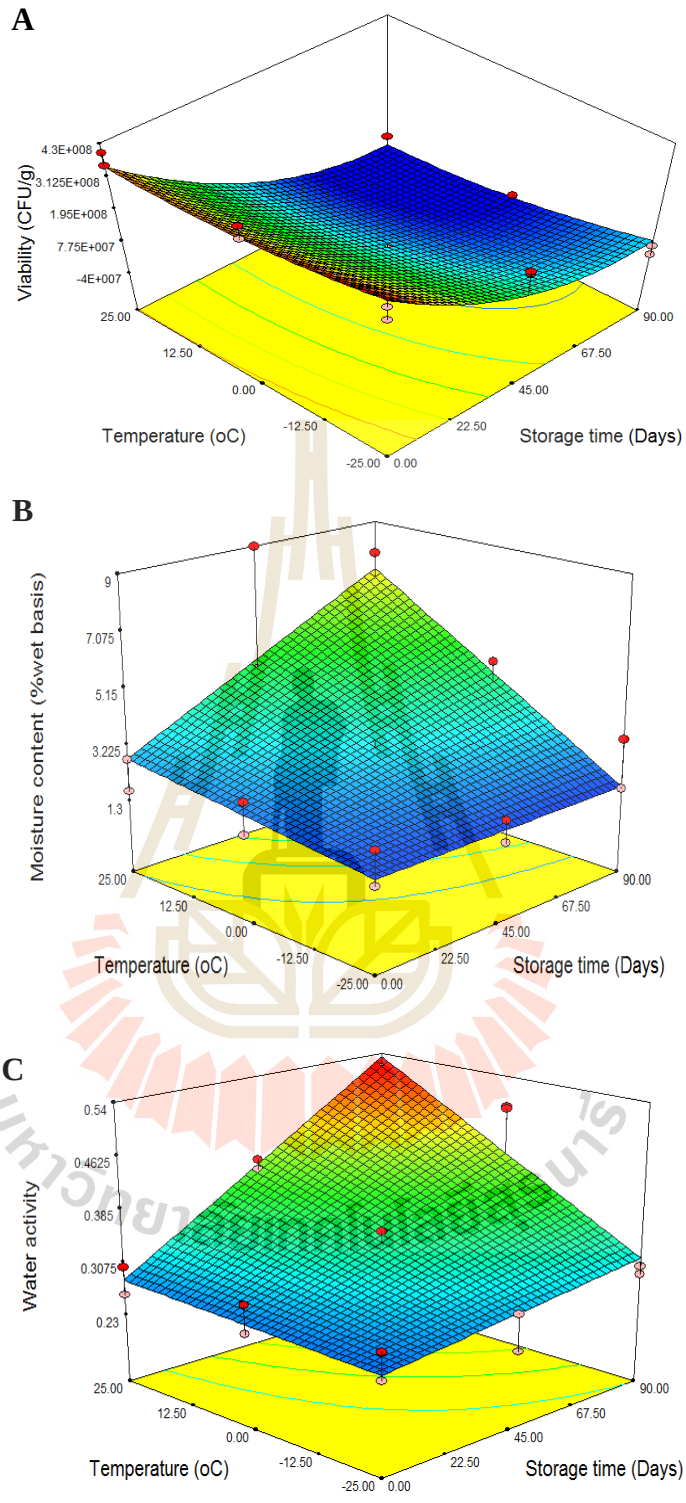

Figure 4.1 Combination criteria based on viability (A), moisture content (B), and water activity (C) of freeze-dried *B. subtilis* SB-MYP-1 with SBF relevant to storage times and temperatures.

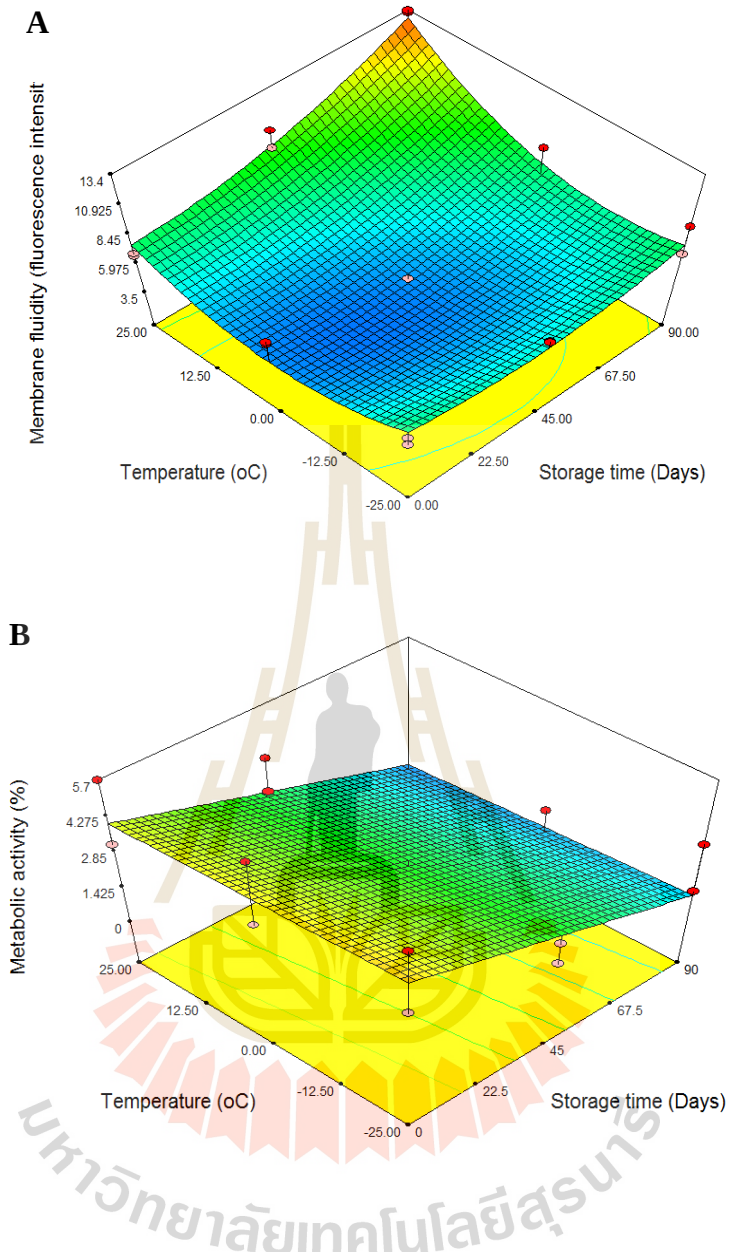

Figure 4.2 Membrane fluidity (A) and metabolic activity (B) change under storage times and temperatures in freeze-dried *B. subtilis* SB-MYP-1 with SBF.

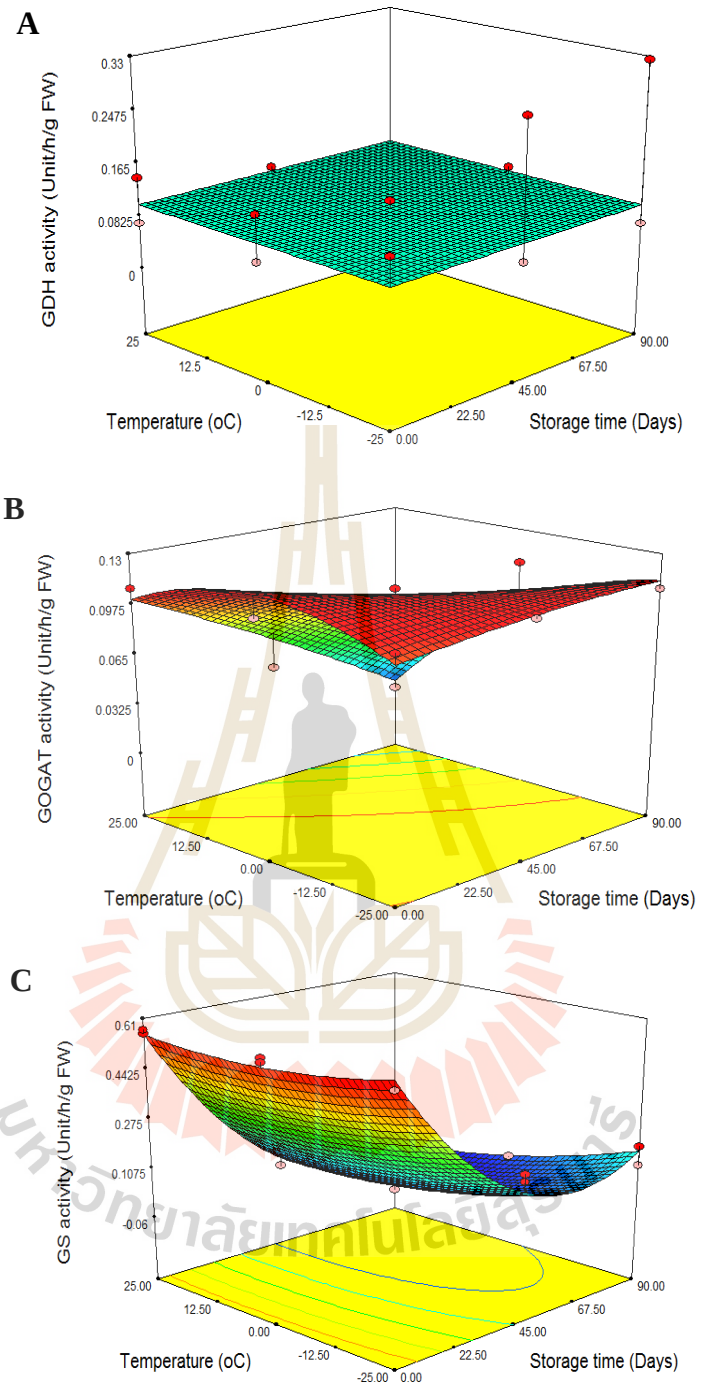

Figure 4.3 Intracellular enzyme activities of GDH (A), GOGAT (B), and GS (C) change under storage times and temperatures in freeze-dried *B. subtilis* SB-MYP-1 with SBF.

Table 4.2 The comparison of the results of actual and predicted values from freeze-dried *B. subtilis* SB-MYP-1 with SBF during storage at -25°C for 90 days.

Parameters / Values	Viability (CFU/g)	Moisture content (% wet basis)	Water activity	Membrane fluidity (fluorescence intensity)	Metabolic activity (%)	GDH activity (Unit/h/g FW)	GOGAT activity (Unit/h/g FW)	GS activity (Unit/h/g FW)
Actual values	$4.83\times10^{7}\pm2.37\times10^{7}$[ns]	2.59±1.21[ns]	0.30±0.01[ns]	8.10±1.66[ns]	2.27±1.38[ns]	0.20±0.18[ns]	0.11±0.00[ns]	0.15±0.04[ns]
Predicted values	$8.67\times10^{7}\pm0.00$[ns]	1.81±0.00[ns]	0.32±0.00[ns]	7.70±0.00[ns]	1.15±0.00[ns]	0.10±0.00[ns]	0.11±0.00[ns]	0.17±0.00[ns]

[ns] indicates the not significant differences between the actual and predicted values in columns (P>0.05).

4.5 Conclusions

A point of control measure, at -25ºC for 90 days could still maintain the combination criteria; the highest cell viability, moisture content, and a_w of freeze-dried cells with SBF which based on the standard qualities of powdered starter cultures. Those three parameters are also the key criteria to control the stability of stressed cell targets. In addition, those mathematical equations might be applied for monitoring the shelf life of freeze-dried cells throughout subsequent storage.

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CHAPTER V

POTENTIAL CHARACTERISTICS OF POWDERED *BACILLUS SUBTILIS* SB-MYP-1 IN THAI THUA-NAO FERMENTATION

5.1 Abstract

As proved previously in Chapter IV, *Bacillus subtilis* SB-MYP-1 cells were preserved under freeze-dried with 10% (w/v) soybean flour (SBF) at 0ºC for 45 days, and -25ºC for 45-90 days. Those conditions could maintain the stability of the stressed cell survival, cell membrane fluidity, metabolic retardation, intracellular enzyme activities of *pgsB* gene expression. Meanwhile, combination criteria of dried starter culture qualities, including viability (>10^7 CFU/g), moisture content (<7 % wet basis) and water activity (<0.6), were achieved. To verify the potential activities of starter cultures resulting in solid state fermentation of soybean (Thua-nao) characteristics (the highest amylase, protease, and PGA production), these characteristics of freeze-dried and fresh cell were, therefore, compared. The results revealed that the stimulations of amylase, protease and PGA in freeze-dried cells with SBF kept at -25ºC for 45-90 days were not significantly different (P>0.05), when compared to those of fresh cells at 36 h. Thus, these profiles confirmed that the storage temperature at -25ºC is a control factor for preserving the stability of freeze-dried *B. subtilis* SB-MYP-1 cell with SBF, which could be steady function for further specific Thua-nao fermentation.

Keywords: freeze-dried *Bacillus subtilis* potential, solid state soybean fermentation (Thua-nao), amylase, protease, poly-γ-glutamic acid

5.2 Introduction

Thai fermented soybean or Thua-nao is a traditional fermented food in northern Thailand (Chiang Mai, Chiang Rai, Mae Hong Son and Lampang). It has been produced by cooked soybean with/without starter culture inoculation throughout alkaline fermentation (Chantawannakul, Oncharoen, Klanbut, Chukeatirote, and Lumyong, 2002; Chukeatirote, 2015). In general, *B. subtilis* is the major starter culture used, because its amylase and protease activity bring about insoluble sugar, ammonia, bioactive peptide in the raw material of soybean while these activities led to the increasing of nutritional values for finish product (Inatsu et al., 2006; Visessanguan, Benjakul, Potachareon, Panya, and Riebroy, 2005; Sanjukta and Rai, 2016).

Freeze-dried *B. subtilis* SB-MYP-1 is a starter culture for Thua-nao production because its characteristics were the highest enzyme activities of amylase and protease, and bioactive-peptide as poly-γ-glutamic acid (PGA) at 36 h in alkaline fermentation to which were able to reduce undesirable aroma, and increase the nutritional values (calcium, ferric, phosphorus, and vitamin B12) in fresh Thua-nao (Gasaluck, 2010). Therefore, its activities of *B. subtilis* SB-MYP-1 should be preserved for further Thua-nao production. Gasaluck (2010) suggested that the freeze drying with cryoprotective SBF could maintain the viability of *B. subtilis* SB-MYP-1 cells, and implied in soybean fermentation without interfering during fermentation. Recently, Mahidsanan, Gasaluck, and Eumkeb, (2017) approved that SBF could preserve the protein stabilization, phospholipid bilayer, and peptidoglycan surface in freeze-dried

B. subtilis SB-MYP-1 cells, while the intracellular enzyme (glutamate dehydrogenase, 2-oxoglutarate aminotransferase, and glutamine synthetase) activities of *pgsB* gene expression were maintained. However, upon storage of powdered starter culture, some article revealed that dried starter cultures may demonstrate the loss of potential activity to produce a significant metabolite throughout fermentation. For example, Eratte, Wang, Dowling, Barrow, and Adhikari (2016) showed that the lactic acid production by freeze-dried *Lactobacillus casei* cells decreased with increase in the storage time and temperature. As same as the research of Ghandi, Powell, Broome, and Adhikari (2013) which represented that the dpH/dt of *Lactococcus lactis* ASCC930119 powders decreased with storage time increased, especially after 90 days. For this reason, the potentiality of dried starter culture should be concerned and monitored during subsequent storage.

As mentioned previously of Chapter IV indicated that freeze-dried *B. subtilis* SB-MYP-1 cells during storage at 0^{o}C for 45 days, and -25^{o}C for 45-90 days were accorded to standard qualities of dried starter culture. Thus, the aim of this study was to verify those dried starter culture potentials when inoculated in Thua-nao fermentation.

5.3 Materials and methods

5.3.1 Starter cultures

Freeze-dried *B. subtilis* SB-MYP-1 cells with SBF cryoprotectant, during storage at 0^{o}C for 45 days, and -25^{o}C for 45-90 days were considered based on standard qualities of powdered starter culture, including viability (>10^{7} CFU/g), moisture content (<7% wet basis) and a_w (<0.6). These freeze-dried cultures were verified to their potential activities during Thua-nao fermentation.

5.3.2 Solid state soybean (Thua-nao) fermentation

Soybeans were washed and soaked in water overnight at room temperature. The soaked beans were washed and get rid of water, autoclaved at 121°C for 15 min. One hundred and fifty grams of cooked soybean was equally added on to each plate. One gram of freeze-dried *B. subtilis* SB-MYP-1 cells with SBF cryoprotectant (concentration of cell powder was approximately 10^{7-8} CFU/g) was inoculated into cooked soybean (final concentration of cells was approximately 10^{5}-10^{6} CFU/g), further functioned under the solid state soybean alkaline fermentation at 37ºC for 72 h., whilst fresh cultures were compared as the control. The starter culture potential parameters; the total viable counts, pH values, relative activities of amylase and protease, and PGA production were then monitored.

5.3.3 Total viable number count and pH change measurement

All fermented soybean treatments were diluted with an appropriate volume of 0.85% (w/v) sterile sodium chloride solution. Further tenfold dilution was made with the same diluents. About 0.1 mL of those dilutions was plated out on plate count agar. The plates were incubated at 37°C for 24 h. Colonies were counted and calculated as log colony forming units per gram (log CFU/g) of the sample. The pH change was determined using the digital pH meter.

5.3.4 Enzymatic activity measurement and PGA production

Crude enzymes from fermented soybean were prepared. Those samples (10 g) were homogenized with 90 mL of 0.85% (w/v) sodium chloride solution. The soybean debris was then removed by centrifugation at 10000×g for 15 min at 4°C and then the supernatant was collected and kept on ice until use.

5.3.4.1 The activity of amylase and protease, and PGA production

The crude enzymes were initially incubated at 37°C for 5 min with McIlvaine buffer (pH 7.0), and started by adding 1% (w/v) starch solution. After that, it was performed at 37°C for 60 min, and stopped by boiling at 100°C in water bath. The amylase activity was then measured by determination amount of reducing sugar using DNS method. One unit of amylase activity was defined as amount of the enzyme which produced one micromole of glucose under its condition. For the protease activity, crude enzymes were incubated at 37°C with a mixture of 1% (w/v) azocasein and McIlvaine buffer (pH 7.0) for 60 min. The reaction was stopped by adding 50% (w/v) trichloroacetic acid (TCA). All samples were subsequently incubated in ice for 15 min, and centrifuged at 10000×g at 4°C for 15 min. The supernatant was transferred to a new test tube containing an equivalent volume (0.1 mL) of 10 N NaOH. The solution was mixed to measuring the absorbance at 450 nm. Both enzyme activities were subsequently expressed as percentage of relative activity (Adapted from Chukeatirote et al., 2006). As PGA production, crude supernatant from 5.3.4 was mixed with 0.1% (w/v) of safranin O, 0.06 M citrate buffer (pH 6.0) and 0.85% (w/v) sodium chloride, and then centrifuged at 2500 rpm, 10 min for 4°C. Supernatant was measured at the absorbance of 520 nm. The absorbance value defined as PGA standard (Tahara et al., 1998).

5.3.5 Statistical analysis

The fermentation profiles were considered at the 36 h, because of the suitable characteristics of fresh cell, as a control treatment of which providing the maximum significant metabolite based on previous research. All data were performed in duplicate, and the results were presented as the mean±standard deviation (SD). IBM SPSS statistic 22 (Armonk, New York, U.S.) was used to perform all statistical

analysis. One-way analysis of variance (ANOVA) was determined, followed by Duncan's multiple range test (DMRT) with an overall significance level set at 0.05.

5.4 Results and discussion

5.4.1 Total viable count and pH profiles

As shown in Figure 5.1, the total bacterial count during soybean fermentation of all treatments increased due to the fermentation time increasing which their microbial population rapidly increased within 12 h prior the beginning of stationary phase. Figure 5.2 shows the increasing of pH values to which relevant to the protease production of *B. subtilis* SB-MYP-1 starter as shown in Figure 5.4. Soybean protein was utilized, resulting in the present of strong ammonia concentration (~0.8-1.0% w/v) under alkaline fermentation (pH ~7.50-8.50) based on standard quality of Thua-nao (Allagheny, Obanub, Campbell-Plattc, and Owens 1996; Visessanguan et al., 2005).

5.4.2 Enzymatic activities and PGA production

Meanwhile, the amylase activities of those *B. subtilis* SB-MYP-1 were also observed (Figure 5.3), since it could be interpreted as the carbohydrates and simple sugars utilization for starter culture growth (Terlabie, Sakyi-Dawson, and Amoa-Awua, 2006). In addition, PGA concentration was occurred during soybean fermentation (Figure 5.5), because the *pgsB* gene expression of *B. subtilis* controls the PGA production using carbon and nitrogen sources of soybeans which this metabolite is unique nutritive value, slime-viscous, and thread-like properties of Thua-nao product (Mahidsanan et al., 2017; Najar and Das, 2015; Shih and Van, 2001; Tanimoto, 2010). On comparison, freeze-dried *B. subtilis* with SBF cryoprotectant at 0^{o}C for 45 days, and -25^{o}C for 45-90 days after subsequent storage, of which potentials demonstrated in

the Figure 5.1-5.5 comparing to those of the fresh cells. As to Thua-nao fermentation of fresh cells, the pH value gradually reached ~7.50-8.50 until 36 h, at the meanwhile, relative amylase and protease activities, and PGA concentration indicated the highest potentiality. In view of potential characterization of freeze-dried cultures kept at -25^{o}C for 45-90 days still provided the highest relative activities of amylase and protease (100%), and PGA concentration which these profiles were not significantly different ($P>0.05$) to the fresh cells at 36 h of Thua-nao fermentation. Except for freeze-dried cultures kept at 0^{o}C for 45 days, since its pH profile, relative amylase activity, and PGA production at 36 h, which were lower than those of other treatments ($P<0.05$). This phenomenon might be interpreted that the rate of ammonia and amylase production from *B. subtilis* SB-MYP-1 were retarded during Thua-nao fermentation due to the effect of decreasing metabolic activity of powdered starter culture. It implied that the stability and its activities of freeze-dried cells during storage at 0^{o}C were slightly reduced because the storage temperature induced the change of cell stress targets and/or sub-lethal injury which resulted a decrease of cell membrane fluidity as well as an increase of lipid oxidation (Holkem et al., 2017; Santivarangkna, Kulozik, Kienberger, and Foerst, 2009; Xu, Gagne-Bourque, Dumont, and Jabaji, 2016). Moreover, these results accorded with the research of Eratte, Wang, Dowling, Barrow, and Adhikari (2016), and Ghandi, Powell, Broome, and Adhikari (2013), revealed the potential characteristics of powdered starter culture under fermentation. It demonstrated that the longer storage time and temperature was the potential of metabolite production decreased.

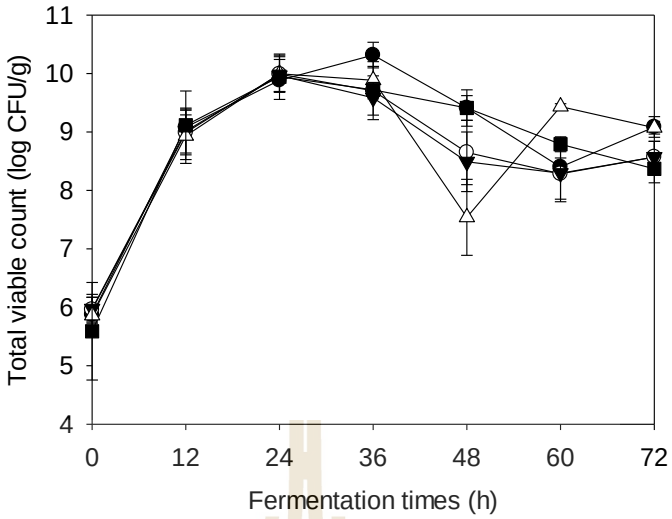

Figure 5.1 Total viable count during Thua-nao fermentation within 72 h. ● = fresh cell, ○ = freeze-dried cell storage 0 day, ▼ = freeze-dried cell storage at -25°C for 45 days, △ = freeze-dried cell storage at 0°C for 45 days, ■ = freeze-dried cell storage at -25°C for 90 days.

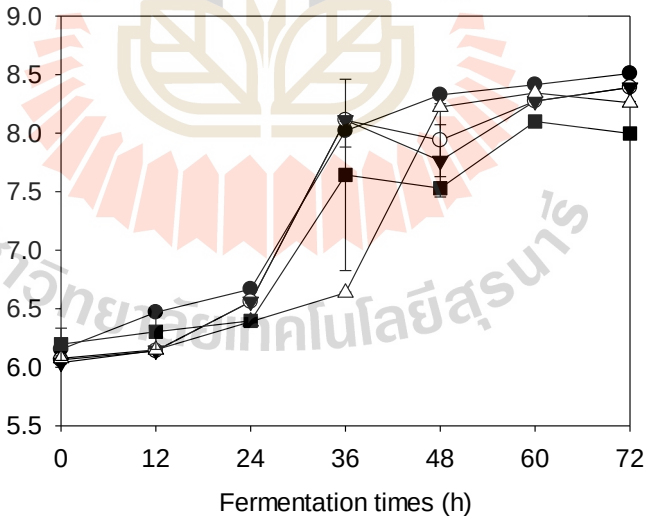

Figure 5.2 pH profiles during Thua-nao fermentation within 72 h. ● = fresh cell, ○ = freeze-dried cell storage 0 day, ▼ = freeze-dried cell storage at -25°C for 45 days, △ = freeze-dried cell storage at 0°C for 45 days, ■ = freeze-dried cell storage at -25°C for 90 days.

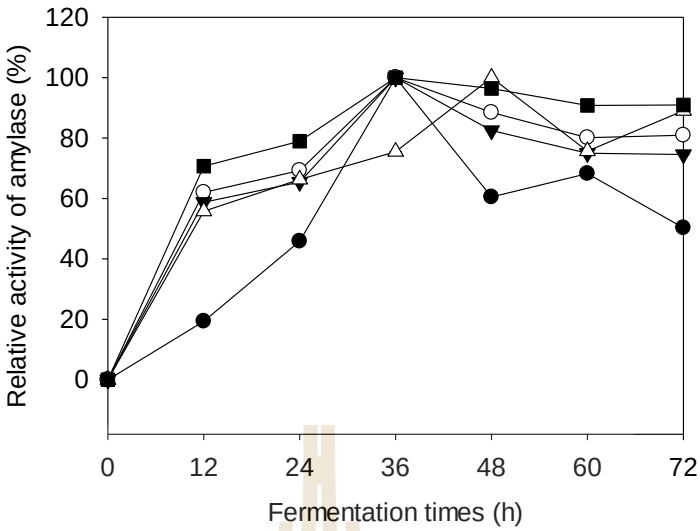

Figure 5.3 Relative activity of amylase during Thua-nao fermentation within 72 h. ◐ = fresh cell, ○ = freeze-dried cell storage 0 day, ▼ = freeze-dried cell storage at -25°C for 45 days, △ = freeze-dried cell storage at 0°C for 45 days, ■ = freeze-dried cell storage at -25°C for 90 days.

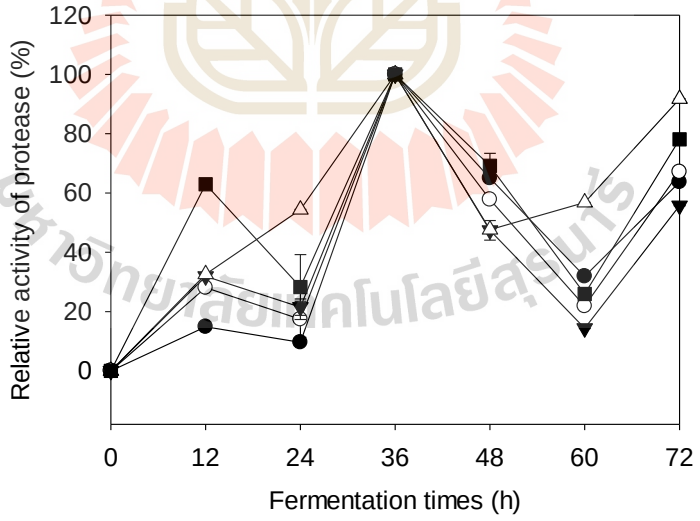

Figure 5.4 Relative activity of protease during Thua-nao fermentation within 72 h. ● = fresh cell, ○ = freeze-dried cell storage 0 day, ▼ = freeze-dried cell storage at -25°C for 45 days, △ = freeze-dried cell storage at 0°C for 45 days, ■ = freeze-dried cell storage at -25°C for 90 days.

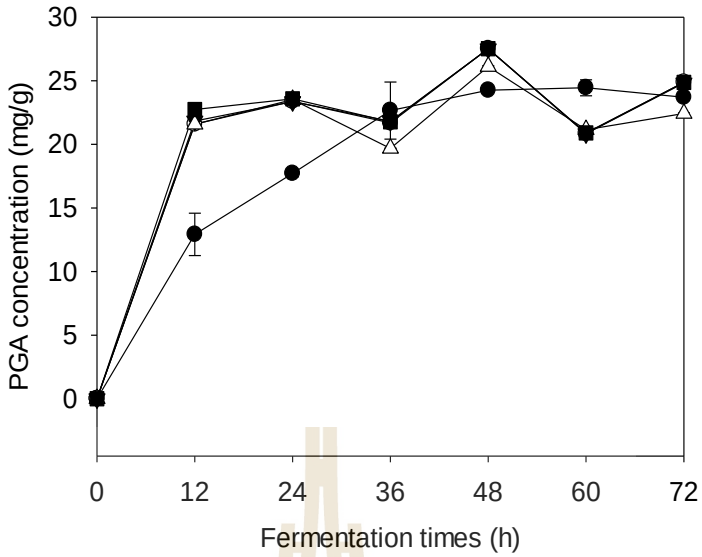

Figure 5.5 PGA concentrations during Thua-nao fermentation within 72 h. ● = fresh cell, ○ = freeze-dried cell storage 0 day, ▼ = freeze-dried cell storage at -25°C for 45 days, △ = freeze-dried cell storage at 0°C for 45 days, ■ = freeze-dried cell storage at -25°C for 90 days.

5.5 Conclusions

As the results of the freeze-dried starter culture verification, the potential characteristics of freeze-dried *B. subtilis* SB-MYP-1 cells with SBF cryoprotectant kept at -25°C, still stimulated the highest amylase, protease, and PGA production at 36 h during Thua-nao fermentation, when compared to that of fresh culture. Therefore, there is no doubt that, freeze-dried *B. subtilis* SB-MYP-1 cells with SBF cryoprotectant storaged at -25°C, still maintained the fermentation functionality and providing the meaningful specific combination criteria, accordance to that of fresh cell solid state soybean fermentation.

5.6 References

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CHAPTER VI

SUMMARY

Bacillus subtilis SB-MYP-1, starter culture for Thua-nao fermentation, preserved in freeze-dried powder with soybean flour (SBF) as a cryoprotectant. It revealed that SBF was the effective protective cryoprotectant from the stress condition, because of the function stability of the cell wall, cell membrane fluidity, metabolic retardation, activities of three intracellular enzymes (glutamate dehydrogenase, GDH; 2-oxoglutarate aminotransferase, GOGAT; and glutamine synthetase, GS), and *pgsB* gene expression. Furthermore, its combination criteria were accorded to standard qualities of dried starter culture; viability (>10^7 CFU/g), moisture content (<7% wet basis) and water activity (<0.6). In contrast, the partial cell wall and cell membrane of freeze-dried cells with three commercial cryoprotectants which were destroyed.

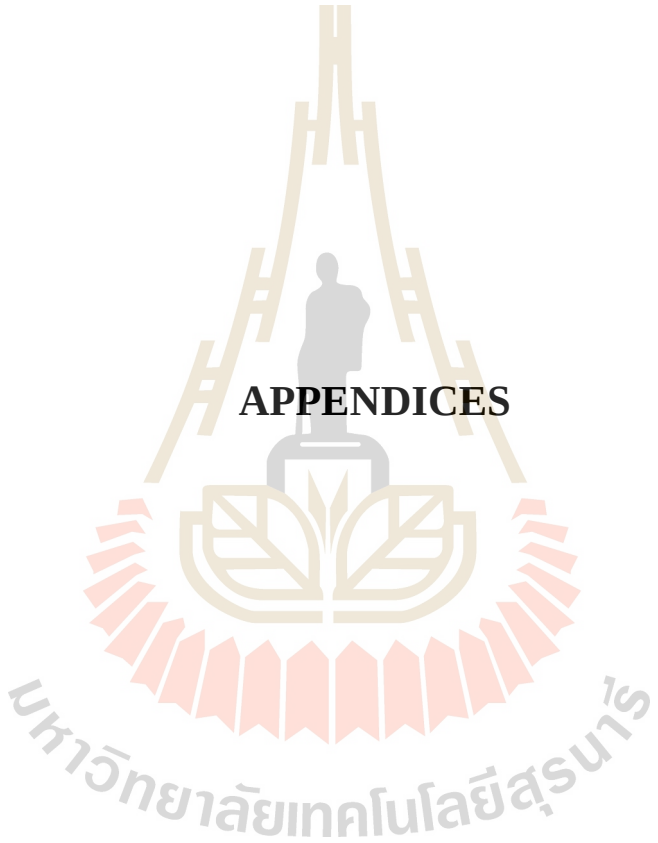
Upon the freeze-dried *B. subtilis* SB-MYP-1 with SBF preservation, under the combination extrinsic factors (temperatures and times), demonstrated that the stabilities of freeze-dried cell stress targets; the viability, cell membrane fluidity, metabolic activity, and activities of GOGAT and GS, were significant stable at -25^{o}C for 90 days, by means of predictive microbiological RSM models. Meanwhile, the mathematic equations of each combination parameters proved the reliability, of which being the applicable prediction for the stability of freeze-dried *B. subtilis* SB-MYP-1 cell stress targets during storage. The potential characteristics of freeze-dried starter culture with SBF were also verified. The freeze-dried cells kept at -25^{o}C usually remained the

highest amylase, protease, and poly-γ-glutamic acid (PGA) production at 36 h throughout fermentation to which its potential activities were not significant differences to those of the using fresh starter culture.

Therefore, SBF is an effective new cryoprotective agent for preventing *B. subtilis* cells sub-lethal stress under freeze drying and maintains its function stability during storage at ≤ -25ºC. Drawn from this dissertation revealed that, freeze-dried *B. subtilis* SB-MYP-1 cells with SBF as a starter culture can produce the specific fermented soybean product supplemented with PGA. The properties of Thua-nao product from freeze-dried culture are not different from those of using fresh culture. Furthermore, the prototype of soybean flour preserved freeze-dried *B. subtilis*, could be applied to spore-forming starter cultures preservation in fermented soybean industry.

At present, *B. subtilis* SB-MYP-1 is collected at Thailand Institute of Scientific and Technological Research coded TISTR 2397 for future interest of the fermented soybean industry.

APPENDICES



APPENDIX A

PREPARATION OF CULTURE MEDIA

1. Culture media

1.1 Plate count agar (PCA)

Pancreatic digest of casein	5 g
Yeast extract	2.5 g
Dextrose	1 g
Agar	15 g
Add distilled water to bring volume up to	1000 ml

pH 7.0±0.2

The medium was autoclaved at 121°C for 15 min.

1.2 Nutrient agar (NA)

Peptone	5 g
Yeast extract	3 g
Agar	15 g
Add distilled water to bring volume up to	1000 ml

pH 7.0±0.2

The medium was autoclaved at 121°C for 15 min.

1.3 Nutrient broth (NB)

Peptone	5 g
Yeast extract	3 g
Add distilled water to bring volume up to	1000 ml

pH 7.0±0.2

The medium was autoclaved at 121°C for 15 min.

APPENDIX B

THE EXAMPLES OF STATISTICAL ANALYSIS

Table 1B ANOVA of *Bacillus subtilis* SB-MYP-1 cell viability before and after freeze drying with various cryoprotectants.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	576103506750000000.000	7	8230050096428572.000	.965	.512
Within Groups	682580097000000008.000	8	8532251212500001.000		
Total	1258683603750000000.000	15			

Table 2B ANOVA of moisture content of freeze-dried cells with various cryoprotectants.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	107.484	3	35.828	16.466	.001
Within Groups	17.408	8	2.176		
Total	124.892	11			

Table 3B ANOVA of water activity (a_W) of freeze-dried cells with various cryoprotectants.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.100	3	.033	48.189	.000
Within Groups	.006	8	.001		
Total	.105	11			

Table 4B ANOVA of metabolic activity, membrane fluidity, activities of GDH, GOGAT, and GS of freeze-dried cells with various cryoprotectants.

		Sum of Squares	df	Mean Square	F	Sig.
Metabolic activity	Between Groups	21371.261	4	5342.815	170.033	.000
	Within Groups	314.222	10	31.422		
	Total	21685.483	14			
Membrane fluidity	Between Groups	752.803	4	188.201	255.381	.000
	Within Groups	7.369	10	.737		
	Total	760.172	14			
GDH	Between Groups	.012	4	.003	.859	.520
	Within Groups	.034	10	.003		
	Total	.045	14			
GOGAT	Between Groups	.003	4	.001	.351	.837
	Within Groups	.021	10	.002		
	Total	.024	14			
GS	Between Groups	.006	4	.002	1.198	.370
	Within Groups	.013	10	.001		
	Total	.019	14			

Table 5B The comparison of total viable count, pH value, relative activity of amylase and protease, and PGA concentrations in fermented soybean by each form of *B. subtilis* SB-MYP-1 fermentation at 36 h.

Starter culture form / Parameters	Total viable count (log CFU/g)	pH value	Relative activity of amylase (%)	Relative activity of protease (%)	PGA concentration (mg/g)
Fresh cells	10.32±0.22[a]	8.02±0.14[a]	100±0.00[a]	100±0.00[a]	22.66±2.24[a]
Freeze-dried culture storage 0 day	9.71±0.42[a]	8.11±0.01[a]	100±0.00[a]	100±0.00[a]	21.69±0.01[a]
Freeze-dried culture storage at -25°C for 45 days	9.59±0.37[a]	8.11±0.01[a]	100±0.00[a]	100±0.00[a]	21.81±0.01[a]
Freeze-dried culture storage at 0°C for 45 days	9.89±0.32[a]	6.64±0.01[b]	75.48±0.11[b]	100±0.00[a]	19.69±0.03[b]
Freeze-dried culture storage at -25°C for 90 days	9.70±0.13[a]	7.64±0.82[a]	100±0.00[a]	100±0.00[a]	21.77±0.07[a]

The different letters, a, and b, within the columns indicate significant differences at $P<0.05$.

Table 6B The comparison of viability, moisture content, water activity, membrane fluidity, metabolic activity and activities of GDH, GOGAT and GS in freeze-dried *B. subtilis* SB-MYP-1 with SBF during storage.

Starter culture form / Parameters	Viability (CFU/g)	Moisture content (% wet basis)	Water activity (a_w)	Membrane fluidity (fluorescence intensity)	Metabolic activity (%)	GDH (unit/h/g FW)	GOGAT (unit/h/g FW)	GS (unit/h/g FW)
Freeze-dried culture storage at -25ºC for 0 day	$3.78\times10^{8}\pm3.18\times10^{7a}$	2.19 ± 0.79^{b}	0.282 ± 0.028^{d}	6.376 ± 0.487^{cd}	4.469 ± 1.728^{a}	0.144 ± 0.00^{a}	0.108 ± 0.00^{a}	0.573 ± 0.00^{a}
Freeze-dried culture storage at 0ºC for 0 day	$3.78\times10^{8}\pm3.18\times10^{7a}$	2.19 ± 0.79^{b}	0.283 ± 0.029^{d}	6.808 ± 0.125^{bcd}	4.469 ± 1.728^{a}	0.108 ± 0.051^{a}	0.108 ± 0.00^{a}	0.566 ± 0.009^{a}
Freeze-dried culture storage at 25ºC for 0 day	$3.78\times10^{8}\pm3.18\times10^{7a}$	2.19 ± 0.79^{b}	0.281 ± 0.029^{d}	6.844 ± 0.175^{bcd}	4.469 ± 1.728^{a}	0.108 ± 0.051^{a}	0.108 ± 0.00^{a}	0.566 ± 0.009^{a}
Freeze-dried culture storage at -25ºC for 45 days	$2.35\times10^{8}\pm7.07\times10^{6b}$	1.75 ± 0.52^{b}	0.264 ± 0.038^{d}	6.907 ± 0.016^{bcd}	2.047 ± 0.595^{abc}	0.18 ± 0.153^{a}	0.108 ± 0.00^{a}	0.196 ± 0.017^{b}
Freeze-dried culture storage at 0ºC for 45 days	$4.95\times10^{7}\pm6.36\times10^{6c}$	2.44 ± 0.56^{b}	0.343 ± 0.017^{c}	4.157 ± 0.818^{d}	1.559 ± 0.825^{abc}	0.054 ± 0.076^{a}	0.108 ± 0.00^{a}	0.035 ± 0.001^{c}
Freeze-dried culture storage at 25ºC for 45 days	$6.95\times10^{5}\pm3.46\times10^{5c}$	6.81 ± 3.05^{a}	0.408 ± 0.011^{b}	9.642 ± 0.959^{b}	3.071 ± 0.946^{ab}	0.068 ± 0.056^{a}	0.054 ± 0.025^{b}	0.001 ± 0.00^{c}
Freeze-dried culture storage at -25ºC for 90 days	$4.83\times10^{7}\pm2.37\times10^{7c}$	2.59 ± 1.21^{b}	0.297 ± 0.008^{cd}	8.097 ± 1.664^{bc}	2.275 ± 1.381^{abc}	0.198 ± 0.178^{a}	0.108 ± 0.00^{a}	0.153 ± 0.045^{c}
Freeze-dried culture storage at 0ºC for 90 days	$5.30\times10^{6}\pm3.82\times10^{6c}$	3.57 ± 1.92^{b}	0.493 ± 0.003^{a}	6.89 ± 2.932^{bcd}	1.138 ± 0.691^{bc}	0.054 ± 0.076^{a}	0.09 ± 0.025^{a}	0.035 ± 0.001^{c}
Freeze-dried culture storage at 25ºC for 90 days	0.00 ± 0.00^{c}	7.14 ± 0.99^{a}	0.502 ± 0.02^{a}	13.32 ± 0.093^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}

The different letters, a, b, c, and d within the columns indicate significant differences at $P<0.05$.

APPENDIX C

pgsB gene, partial cds, GenBank: HQ599194.1

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VERSION	HQ599194.1
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ORGANISM	*Bacillus amyloliquefaciens* Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE	1 (bases 1 to 1179)
AUTHORS	Yong, X., Zhang, R., Zhang, N., Chen, Y., Huang, X., Zhao, J., and Shen, Q.
TITLE	Development of a specific real-time PCR assay targeting the poly-gamma-glutamic acid synthesis gene, *pgsB*, for the quantification of *Bacillus amyloliquefaciens* in solid-state fermentation
JOURNAL	Bioresour. Technol. 129, 477-484 (2013)
PUBMED	23266849
REFERENCE	2 (bases 1 to 1179)
AUTHORS	Yong, X., Shen, Q., and Yang, X.
TITLE	Direct Submission JOURNAL Submitted (09-NOV-2010) College of Resources and Environmental Science, Nanjing Agriculture University, Weigang Road, NanJing, JiangSu 210095, China

FEATURES Location/Qualifiers

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APPENDIX D

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR) SPECTRA

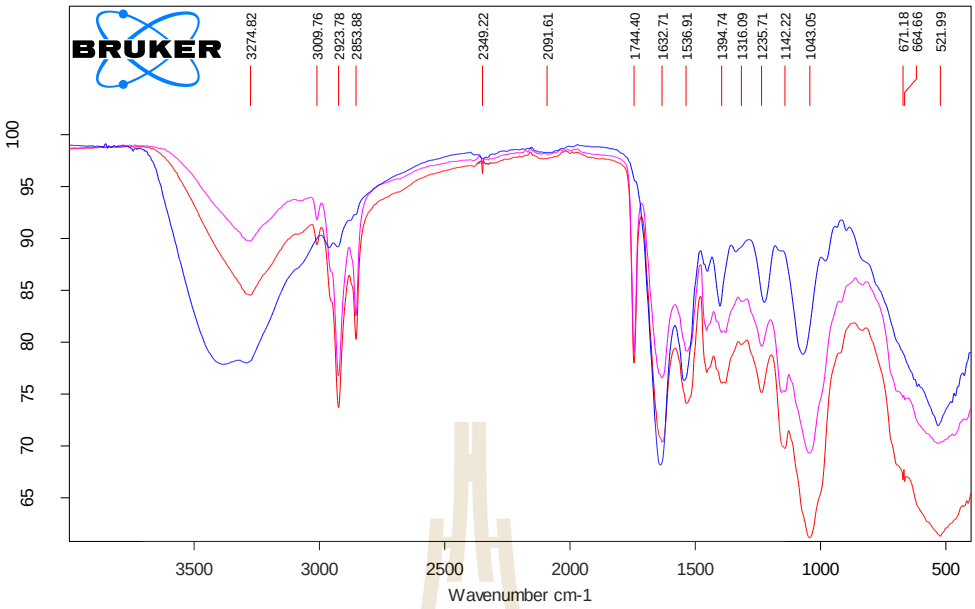

Figure 1D FTIR spectra of fresh cell (blue), freeze-dried SBF (pink), and freeze-dried cells with SBF (red).

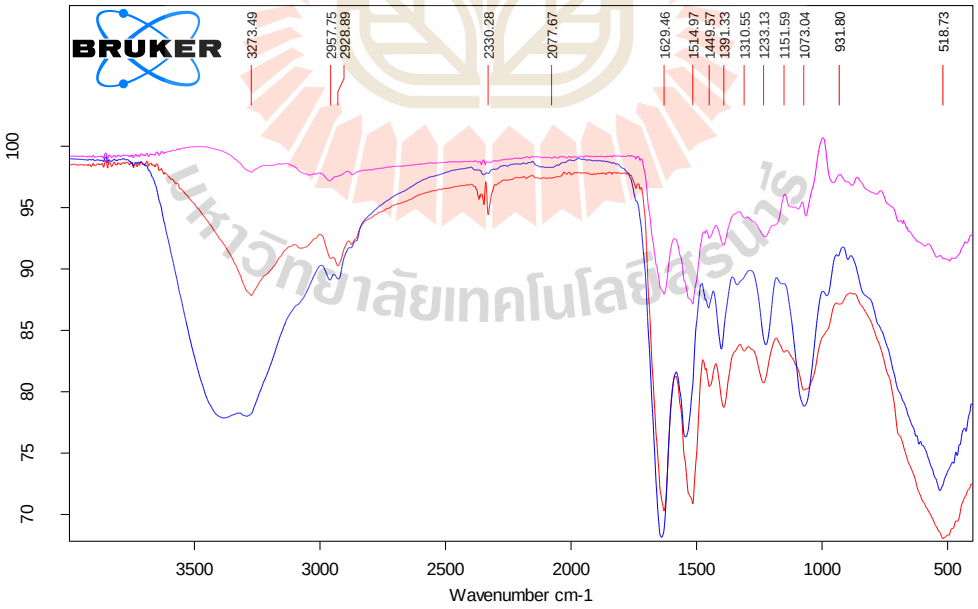

Figure 2D FTIR spectra of fresh cell (blue), freeze-dried SPI (pink), and freeze-dried cells with SPI (red).

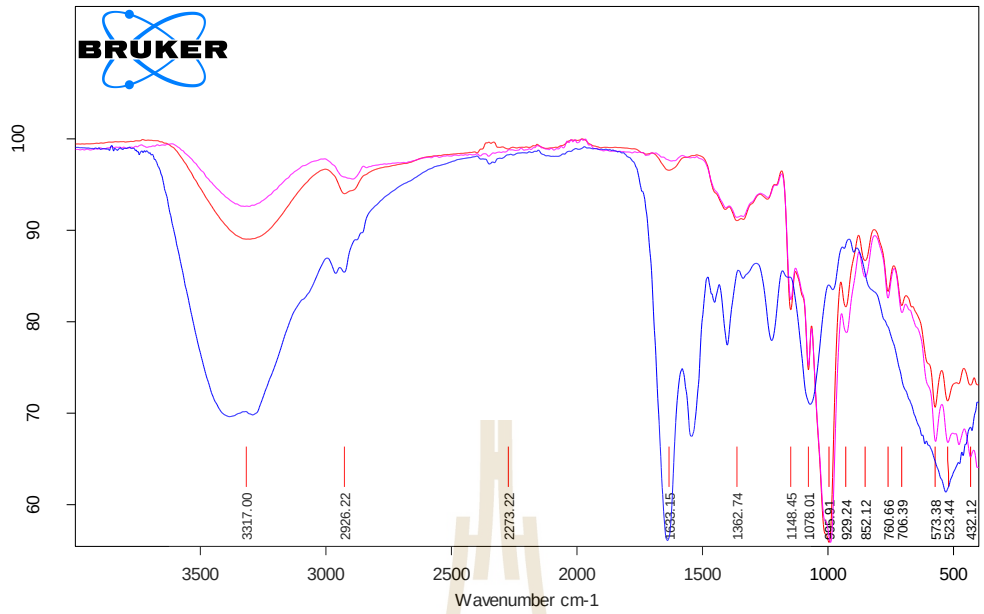

Figure 3D FTIR spectra of fresh cell (blue), freeze-dried ST (pink), and freeze-dried cells with ST (red).

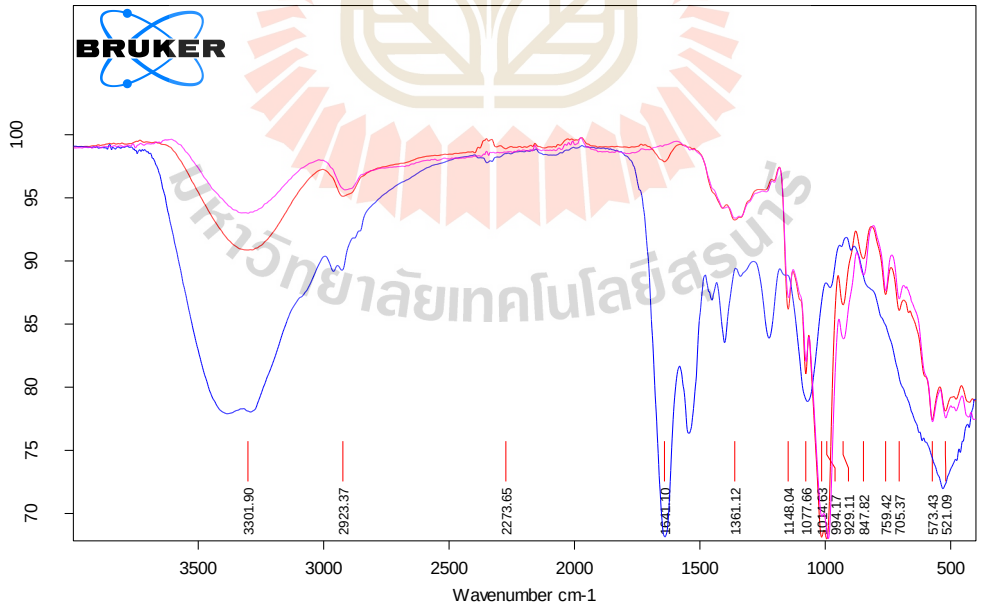

Figure 4D FTIR spectra of fresh cell (blue), freeze-dried MD (pink), and freeze-dried cells with MD (red).

BIOGRAPHY

Thitikorn Mahidsanan was born in June 11, 1988 in Nakhon Ratchasima province. In 2000-2006, he studied for his high school at Ratchasima Wittayalai School. He received Bachelor Degree in B.Sc. (Food science and Technology) with second class honor from Nakhon Ratchasima Rajabhat University (NRRU), Thailand in 2010. He received Master degree in M.Sc. (Food Technology) from Suranaree University of Technology (SUT), Thailand in 2012. In 2012-2017, he received SUT-OROG scholarship for his Ph.D. study. During graduate study, he worked as a teacher assistant of School of Food Technology, SUT in the course of Food Microbiology Laboratory I, Food Microbiology Laboratory II, and Food Fermentation Technology. In addition, he presented and published his research in several articles including:

Paper publication:

Mahidsanan, T., Gasaluck, P., and Eumkeb, G. (2017). A novel soybean flour as a cryoprotectant in freeze-dried *Bacillus subtilis* SB-MYP-1. **LWT-Food Science and Technology.** 77: 152-159.

Mahidsanan, T., and Gasaluck, P. (2016). Improvement of poly-γ-glutamic acid (PGA) producing *Bacillus subtilis* SB-MYP-1 by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis. **International Food Research Journal.** 23(2): 751-755.