POTENTIAL BIOSURFACTANT AS A SANITIZER AND THEIR EFFECTS ON *Listeria monocytogenes* STRESS IN FRESH PRODUCE DURING STORAGE

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สารลดแรงตึงผิวชีวภาพที่มีศักยภาพเพื่อใช้เป็นสารฆ่าเชื้อและมีผลต่อความเครียดของ *Listeria monocytogenes* ในผลิตภัณฑ์ผักผลไม้สด (fresh produce) ระหว่างการเก็บรักษา

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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ปริยดา สิทธิศาสตร์ : สารลดแรงตึงผิวชีวภาพที่มีศักยภาพเพื่อใช้เป็นสารฆ่าเชื้อและมีผลต่อความเครียดของ *Listeria monocytogenes* ในผลิตภัณฑ์ผักผลไม้สด (fresh produce) ระหว่างการเก็บรักษา (POTENTIAL BIOSURFACTANT AS A SANITIZER AND THEIR EFFECTS ON *Listeria monocytogenes* STRESS IN FRESH PRODUCE DURING STORAGE) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.ปิยะวรรณ กาสลัก, 126 หน้า.

วัตถุประสงค์ของการศึกษาวิทยานิพนธ์นี้คือ เพื่อประเมินศักยภาพของแบคทีเรียแลคติกในการผลิตสารลดแรงตึงผิวชีวภาพ ที่สามารถใช้แหล่งคาร์บอนและไนโตรเจนจากมะม่วงน้ำดอกไม้ (*Mangifera indica* Linn) สุกงอมได้ สารสกัดหยาบของสารลดแรงตึงผิวชีวภาพที่ผลิตได้นี้ ต้องพิสูจน์อิทธิพลต่อการทำลายเซลล์เป้าหมายต่าง ๆ ของ *Listeria monocytogenes* รวมถึงสามารถทำลายยีน *sigB* ตลอดจนสามารถยืนยันถึงความปลอดภัยทางด้านจุลินทรีย์ตลอดการเก็บรักษาผักผลไม้สด

จากผลการคัดแยกแบคทีเรียแลคติก จากมะม่วงน้ำดอกไม้สุกงอม พบว่าทั้งหมด 5 สายพันธุ์ แสดงลักษณะเฉพาะของสารลดแรงตึงผิวชีวภาพที่ยับยั้ง *L. monocytogenes* โดย *Lactobacillus plantarum* MGL-8 มีศักยภาพที่ดีที่สุดในการใช้แหล่งคาร์บอนและไนโตรเจนจากอาหารเลี้ยงเชื้อ MRS ที่เติมเสริมสาร (1% (w/v) glycerol and 2% (w/v) sucrose) อัตราการเจริญสูงสุดพบชั่วโมงที่ 36 ซึ่งปัจจัยเหล่านี้ทำให้ได้สารลดแรงตึงผิวชีวภาพที่มีค่าแรงตึงผิว 39.14 mN/m ดังนั้นเมื่อนำ *L. plantarum* MGL-8 ผลิตภายใต้สภาวะการหมักแบบ spontaneous ในถังหมัก พบว่า กล้าเชื้อแบคทีเรียแลคติกสามารถปรับตัวใช้สารตั้งต้นใหม่ (น้ำมะม่วงสุกงอม) ร่วมกับจุลินทรีย์ประจำถิ่นชนิดอื่น ซึ่งสามารถผลิตสารที่มีคุณสมบัติลดแรงตึงผิว เท่ากับ 36.8 mN/m ในเวลาการหมักที่ 120 ชั่วโมง มีค่าดัชนีการเกิดอิมัลชัน (E24) สูงถึง 40.38% และสามารถยับยั้ง *L. monocytogenes* ที่มีประสิทธิภาพดีที่สุด ในสภาวะที่มีการควบคุมปัจจัยในถังหมัก (ความเร็วใบพัด 100 rpm อัตราการให้อากาศ 1 vvm และอุณหภูมิ 35ºC) ร่วมกับการใช้ *L. plantarum* MGL-8 เป็นกล้าเชื้อในการหมักน้ำมะม่วงสุกงอม จะช่วยเพิ่มศักยภาพในการผลิตสารลดแรงตึงผิวชีวภาพที่มีปริมาณสารสกัดหยาบ 4.22 g/L หลังจากนั้นยืนยันประสิทธิภาพของการเป็นสารฆ่าเชื้อในการล้างผักผลไม้สด พบว่าที่ความเข้มข้น 350 400 และ 450 µg/mL ที่เวลารับสัมผัสนาน 20 20 และ 10 นาที ตามลำดับ ส่งผลต่อความเครียดของ *L. monocytogenes* (*sigB* gene; ทนต่อสารฆ่าเชื้อ) ซึ่งทำให้เกิดการตอบสนองต่อกลไกการยับยั้งผ่านทางเยื่อหุ้มเซลล์

ดังนั้นจึงนำสารลดแรงตึงผิวชีวภาพไปใช้เพื่อล้างผักผลไม้สดที่ความเข้มข้น 400 และ 450 µg/mL ด้วยการแช่นาน 20 และ 10 นาที ตามลำดับ ส่งผลให้ยีน *sigB* ถูกทำลาย และเพียงพอที่จะควบคุมอัตราการอยู่รอดของ *L. monocytogenes* ที่จำลองการปนเปื้อนเกินมาตรฐานในผักคะน้าสด ในระหว่างการเก็บรักษาที่ 12 และ 5ºC เป็นเวลา 14 วัน เช่นเดียวกับการล้างด้วยสารละลายแคลเซียมไฮโปคลอไรต์ ($CaClO_2$) เข้มข้น 75 ppm กระบวนการล้างนี้สามารถเป็นต้นแบบสำหรับมาตรฐานขั้นตอนการปฏิบัติงานด้านสุขลักษณะ (SSOP) ได้ต่อไปในระดับอุตสาหกรรมอาหาร

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา ชัชฎา ศิทธิสาสตร์

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา

PRIYADA SITTISART : POTENTIAL BIOSURFACTANT AS A SANITIZER AND THEIR EFFECTS ON *Listeria monocytogenes* STRESS IN FRESH PRODUCE DURING STORAGE. THESIS ADVISOR : ASSOC. PROF. PIYAWAN GASALUCK, Ph.D., 126 PP.

BIOSURFACTANT CHARACTERISTICS/INDIGENEOUS MICRO-FLORA FERMENTATION/BIOSURFACTANT PRODUCING PROFILE/*Listeria monocytogenes*/STRESS RESISTANT CELL TARGET/FRESH-PRODUCE SAFETY DURING STORAGE

The objective of this dissertation was to evaluate the potential native Lactic acid bacteria (LAB) isolates for biosurfactant (BSF) production, which is capable to use the overriped Nam Dok Mai mango (*Mangifera indica* Linn) juice as carbon and nitrogen source. It was proved that the efficiency of crude BSF as a sanitizer lethal to *sigB* gene damage with different cell targets of *Listeria monocytogenes*, during fresh-produce storage for ensuring the microbiological safety.

A total of 5 strains of LAB isolated from overriped Nam Dok Mai mango provided specific BSF characteristics and *L. monocytogenes* inhibition. The best was BSF producing strain *Lactobacillus plantarum* MGL-8, which utilized MRS medium with an extra supplement (1% (w/v) glycerol and 2% (w/v) sucrose), and a maximum growth rate at 36 h of which provided the highest BSF production at a surface tension value 39.14 mN/m. BSF produced by the potential strain resulted in spontaneous fermentation in the fermenter. It was found that *L. plantarum* MGL-8 co-culture adapted to the new substrate (overriped mango juice), and produced positive effects in reducing the surface tension to 36.8 mN/m, the percentage of E24 increased to the

highest level approximately 40.38% at 120 h, whereas antimicrobial properties against *L. monocytogenes* was the most effective. Suggesting that under optimum conditions (agitation speed 100 rpm, aeration rate 1 vvm and temperature 35ºC) combined with *L. plantarum* MGL-8 in mango juice fermented as a starter culture for producing dried crude BSF was enhanced to 4.22 g/L. Afterward, the appropriate dried crude BSF was verified as a sanitizing agent for washing fresh produce, resulting in a BSF stress concentration at 350, 400 and 450 µg/mL with an exposure time of 20, 20 and 10 min, respectively. Of which influence its antimicrobial action by the major function via cell membrane composition of *L. monocytogenes* (containing of *sigB* gene; sanitizer resistant gene).

Thus, BSF was subjected to fresh-produce, soaking 400 and 450 µg/mL BSF, for 20 and 10 min, respectively, resulting in damage to the *sigB* gene, and sufficient to control the survival rate of *L. monocytogenes* challenged Chinese kale produce during storage at 12 and 5ºC for 14 days as well as 75 ppm calcium hypochlorite solution ($CaClO_2$). So far, this washing procedure prototype would practically imply SSOP (sanitation standard operating procedure) in food safety management in the food industry.

School of Food Technology

Academic Year 2019

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Advisor's Signature

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Priyada Sittisart

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α	=	Alpha
ANOVA	=	Analysis of variance
bp	=	Base pair
β	=	Beta
BSF	=	Biosurfactant
CTAB	=	Cetyl trimethylammonium bromide
CFU	=	Colony forming unit
CIP	=	Clean-in-place
COP	=	Clean out of place
^{o}C	=	Degree celsius
DNA	=	Deoxyribonucleic acid
et al.	=	et alia (and others)
γ	=	Gamma
h	=	Hour
kHz	=	Kilohertz
(m, μ, p) g , L, mol, M	=	(milli, micro, pico) Gram, Liter, Mole, Molar
μm	=	Micrometre
min	=	Minute
mN/m	=	Millinewtons per meter
MMT	=	Million metric tons
MC-MJ	=	Multi-strain cultures in mango juice
MJ	=	Mango juice
sec	=	Second

LIST OF ABBREVIATIONS (Continued)

nm	=	Nanometre
%	=	Percentage
cm^{-1}	=	Reciprocal centimeter
×g	=	Relative centrifugal force
rpm	=	Revolutions per minute
vvm	=	Gas volume flow per unit of liquid volume per minute
RTE	=	Ready-To-Eat
%E24	=	Emulsification Index
% (w/w)	=	Percent mass/mass
% (w/v)	=	Percent mass/volume
% (v/v)	=	Percent volume/volume
FSMS	=	Food Safety Management System
SSOP	=	Standard Operating Procedure
ST	=	Surface tension
GMP	=	Good Manufacturing Practice
HACCP	=	Hazard Analysis and Critical Control Points

CHAPTER I

INTRODUCTION

1.1 Introduction

Due to emphasize the post-harvest safety of fresh cut produces, the most 47% of recall food products are recently microbiological contamination. In the year 2018, much information have reported the 99.2% of impact recalls of FDA-regulated foods, resulting in bacterial contamination, especially the high risk of *Listeria monocytogenes* remains a significance of foodborne illness. It causes colonize or contaminate vegetables and fruits at all steps of the food chains. Its contamination causes microbial spoilage and human listeriosis infections. In addition, the safety system of post-harvest management emphasize that unsanitary operating procedure and maintenance/repair practices occasionally cause the *Listeria* survival which induce the viable but non-culturable phase (Hardin et al., 2017; Gorny and McEntire, 2017; Rodríguez-López et al., 2018; Zhu et al., 2017). This phenomenon is the microbiological hazard to develop the *Listeria* biofilm formation and to enhance the cell recovery of injured *Listeria* that called sub-lethal grown in fresh cut products. To indicate the risk of *Listeria*, its physiological characteristics are tolerance under cold stress conditions, especially in chilling (0 to 5^{o}C) and freezing (-20 and-10^{o}C), led to cell survival because *sigB* gene expression stimulates its cell adaptation. Fatty acid compositions of bacterial cell membranes can be changed into length, saturation, and branching under stress response, these affect the membrane fluidity and the cell

stability in the surrounding environmental factors (Tasara and Stephan, 2006; Abram et al., 2008; Utratna et al., 2014).

As mentioned previously, the washing with detergent at the step sanitizer treatment and rinsing of fresh cut produces, in order to control pathogens and heavy metal (fig. 1.1), of which lead to *L. monocytogenes* stress response resulting in cell survival. The step of packaging, labeling and coding may also associate the oxidative stress. Therefore, the microbial requirement for fresh produce standard safety, BC standard is implemented by the preventive and/or corrective action based on risk management practices (Alimentarius Commission, 2007; Yaktine and Pray, 2009; Ricci et al., 2017).

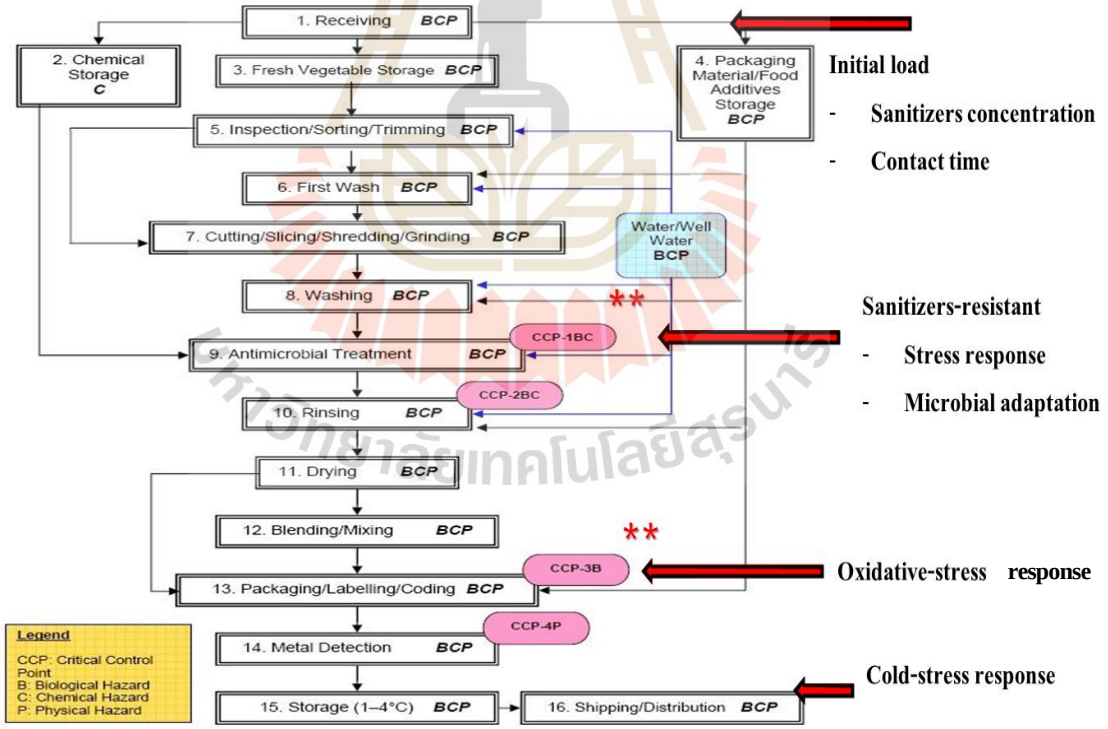

Figure 1.1 Diagram for fresh produces.

Modified from Canadian Food Inspection Agency (2014).

In fact, the problem of chemicals reagents (detergent, surfactant, and sanitizer) in the food production line has been improved for their concentration and exposure time to eliminate the cell includes biofilm. In fresh produce and other food products required the low temperature (4°C and 5°C) storage to which induce the growth characteristic of *L. monocytogenes* (Ziegler et al., 2018). Fresh produce packaging should be well considered for controlling *L. monocytogenes* recovery.

The biosurfactants (BSF) are secondary metabolites produced from microorganisms which are high effectiveness to decrease surface tension and have important characteristic, such as emulsification, solubilization, detergency, lubrication and phase dispersion (Campos et al., 2013; Nitschke and Silva, 2018). Significant interest in BSF has been demonstrated by cosmetic, pharmaceutical, and food industries because of their low toxicity, and sustainable technology. BSFs are generally produced by indigenous micro-flora bacteria, yeast and mold via their fermentation. In the aspect of isolation and screening of microbial BSF producing presented, based on hydrocarbon-contaminated sites are the most significance for BSF producing strains (Walter, Syldatk and Hausmann, 2010).

Recently, fruits and vegetables are processed for value added products, namely the product from food products. In some fruit as agro-wastes of the discarded portion are very high such as orange 30-50%, mango 30-50%, and banana 20%, those wastes contain various composition of significant carbohydrates, minerals and vitamins, which are carbon and nitrogen sources of micro-flora fermentation. It means that the high value waste of mango (*Mangifera indica* L.), a climacteric fruit, containing mainly carbohydrate ranged between 90.1 and 93% and a dietary fiber level between 3.85 and 12.64% (Bello-Perez, Garcia-Suarez and Agama-Acevedo, 2007). Moreover,

a by-product from mango waste fermentation process contains a lot of phytochemicals or bioactive compounds, including antimicrobial and antioxidant (Vega-Vega et al., 2013; Percival et al., 2014) Anyhow, the isolation of indigenous micro-flora or co-cultures plays an important role to produce biological compounds, and mango-waste is potentially developed for industrial BSF production as key performance factors. Likewise Gasaluck (2010) demonstrated that BSF from indigenous fermented sour cherry could be used as a prototype of sanitizer because it could affect the growth potential of *Staphylococcus aureus*, *Salmonella* spp, *Bacillus cereus*, and *Escherichia coli*. They also confirmed that their BSF had non-ionic BSF (0.3 mg/100ml) and decreased the heavy metal such as arsenic, cadmium, mercury and lead.

In summary, utilization agro-mango waste as a carbon and nitrogen source produced BSF by potential co-culture isolates under spontaneously fermentation in fermenter was proved for influencing the crude BSF efficiency as a sanitizer lethal to *sigB* gene expression and or damage of *L. monocytogenes*, during fresh-produce cold storage for ensuring the microbiological safety.

1.2 Research objectives

1. To evaluate the efficiency of native isolates for BSF production.

2. To gain the crude BSF characteristics from potential co-culture isolates, using agro-mango waste as carbon and nitrogen source, under spontaneously fermentation in fermenter.

3. To determine crude BSF as sanitizer effects on *L. monocytogenes* stress on *sigB* gene expression and/or damage during fresh-produce cold storage for ensuring safety.

1.3 Research hypothesis

1. Potential co-culture isolates capably produce crude BSF to which compatible commercial sanitizer.

2. Carbon and nitrogen source from agro-mango waste can enhance the co-culture to produce specific characteristics of BSF under simultaneously fermentation in fermenter.

3. Crude BSF influence the sanitizer resistant *sigB* gene of *L.monocytogenes* lethal during fresh-produce cold storage.

1.4 Scope and limitation

At the first part of this study, the isolation and screening of potential BSF-producing indigenous strains and the formulation of carbon and nitrogen ratio, based agro-waste (mango) were evaluated by three characteristics; (1) indigenous micro-flora are screened by light blue mineral salts agar containing the surfactant cetyl trimethyl ammonium bromide, (2) antimicrobial properties (3) BSF curve production yield and growth under broth condition.

Furthermore, over-ripe Nam Dok Mai mango was applied as a carbon and nitrogen source in fermentation process. Pulp and peel of under grade mango came from the same source. The mango juice was done under the control of pH (4.85±0.3) and %Brix (19.6±3.05), of which the value can be use cover in a range of 10% safety factor. The BSF from mango-juice fermentation was produced according to fermenter conditions, mixing with the co-cultures; lactic acid bacteria, yeast and molds. The kinetic rate and environment parameter measurements were determined by growth rate, pH, and surface tension, emulsification index (%E24), antimicrobial properties,

its characteristics, and yield of dry crude BSF were then proved.

Finally, evaluation of the mode of action against different *L. monocytogenes* cell targets. Implementation dried crude BSF treated *L. monocytogenes* on fresh Kale produces would be proved under soaking and storage conditions. These key performance parameters are lethal rate profile, cell membrane lipid composition, and *sigB* gene expression.

1.5 Expected results

1. The potential co-culture isolates could utilize glucose and convert to hydrophilic moiety, while hydrophobic is a fatty acid.

2. Improvement and efficacy of crude BSF production, using potential co-culture isolates from agro-mango waste as a carbon and nitrogen source under simultaneously fermentation in fermenter are revealed.

3. Drawn from BSF production resulting in *sigB* gene to lethal level of *L. monocytogenes* during fresh-produce cold storage is assured for the safety system implementation.

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CHAPTER II

LITERATURE REVIEWS

2. 1 Potential biosurfactants and its characteristics

Microbial-surfactant or biosurfactants (BSF) are surface active compounds from microbiological sources, usually extracellular, produced by bacteria, fungi, which have gained attention due to their natural characteristics and environmental factors (Nitschke and Silva, 2018). BSF can replace synthetic surfactants in a great variety of industrial applications as detergents, solubilizers, emulsifiers, wetting agents and foaming. Nowadays, natural substance additives are into safety health, and environmental concerns creating have demand for new "green" additives in the food industry, due to surface-active properties. BSF have been played a role of antimicrobial and biofilm damage against food pathogens in line production. Therefore, BSFs can be classified as an additives or ingredients for food processing. In addition, it can be defined as natural categories of surface-active compounds, these are amphiphilic molecules comprising hydrophilic (typically sugars or amino acids) and hydrophobic moiety showing the ability to reduce interfacial tension (Nitschke, Ferraze and Pastore, 2004). BSF can be classified to different criteria because of their molecular weight (high molecular and low molecular weight BSF), ionic charges (cationic, anionic, non-ionic and neutral BSF) and secretion type (intracellular, extracellular and adhered to microbial cells), for example, glycolipids biosurfactant is a one type and the most popular in food industry.

In view of safety and application, they indicated the glycolipid type BSF most popular to develop in pharmaceutical, cosmetic and food industry. In fact, having the ability to induce pore and ion channels, they destabilize biological membranes disturbing their integrity and permeability permitting their use as antibacterial, antifungal, antiviral, and hemolytic agents. Also, having the ability to inhibit the bio-adhesion of pathogenic bacteria and disruption of biofilm formation (Nitschke and Silva, 2018).

2.1.1 Classification and biosurfactants characteristics

Various microorganisms could produce specific kind of BSF, of which depends on mainly the molecular composition of the type of BSF produced. In case of, non-degrade hydrocarbon plays a role produce BSF groups is *Lactobacillus* sp. As mentioned, BSF can be classified into seven categories namely such as glycolipids, phospholipids, lipopeptides, neutral lipids, polymeric, particulate compounds and fatty acid (Nitschke and Silva, 2018). However, the type of glycolipid biosurfactant is a majority producing by bacteria, that have amphiphilic molecule or surface-active molecule with main function characteristics, but the carbohydrate moiety were different. Therefore, it can be classified based on carbohydrate moiety such as rhamnolipids, sophorolipids, trehalolipids and mannosylerythritol lipids (Mnif and Ghribi, 2015), of which structure and characteristic and properties as follow.

Glycolipids: It composes of carbohydrates (galactose, glucose, mannose, rhamnose, etc.) in combination with long-chain aliphatic acids. Among the glycolipids, are the trehalolipids, sophorolipids and especially rhamnolipids (Chen et al., 2007). It is regulatory used in food industry as biopesticides to reduce the plant pathogenic bacteria as well as phytopathogenic fungi. Especially, antimicrobial

activity against gram-positive and gram-negative bacteria pathogens, including the ability to decrease the surface and interfacial tension that supported the main mechanisms of biofilm removing that significant problem in the washing process and storage at the low temperature (Mnif and Ghribi, 2016).

Rhamnolipids: Bacteria of the genus *Pseudomonas* are known to produce glycolipid surfactant containing rhamnose and 3-hydroxy fatty acids (Rahman et al., 2006). Rhamnolipids produced by *P. aeruginosa* strains are among the most effective surfactants especially used for oil removal, and decontaminated soils, found that the surface tension range of 30-32 mN/m reveal high effective to oil removal.

Sophorolipids: It was produced by *Torulopsis* sp., the main structure contains with the dimeric sugar (sophorose) and a hydroxyl fatty acid, linked by a β-glycosidic bond (Hu and Ju, 2001).

Trehalolipids: A kind of glycolipids, which can be produced by *Mycobacterium*, normally presence of trehalose esters on the cell-bound, resulting in trehalolipids from different microorganisms that differ in the structure and molecular weight of mycolic acid was different as well. For example, trehaloselipids from *Rhodococcus erythropolis* and *Arthrobacter* sp. were found to the surface and interfacial tension characteristics in synthetic medium from 25-40 and 1-5 mN/m, respectively.

Lipoproteins and Lipopeptides: Lipopepetides in generally called surfactin is produced by *Bacillus* sp., which containing several amino acids linked to a carboxyl and hydroxyl groups of a 14-carbon acid. Its characteristic reduces surface tension from 72 to 27 mN/m with concentrations as low as 0.005%, and they are a few

low toxicities to the human cells (Shaligram and Singhal, 2010).

Fatty acids: which produced from alkanes as a result of microbial oxidations have been considered as surfactants. In addition to the straight-chain acids, micro-organisms produce complex fatty acids containing OH groups and alkyl branches.

Polymeric biosurfactants: For example, emulsan, liposan, mannoprotein and polysaccharide-protein complexes, which are known to be applied as food emulsifiers and stabilizers (Nitschke and Silva, 2018).

2.1.2 Biosurfactant production by lactic acid bacteria

Pseudomonas, *Candida*, and other genus are commonly reported for the BSF production, but due to opportunistic pathogenic, resulting in their implementation is limited in the food industry till to pharmaceutical and cosmetic. The molecules are still questionable due to non-acceptable of safety and toxicity. Recently, a number of studies have been reported the potential of *lactobacilli* as BSF producers (Ghasemi et al., 2020; Ghasemi et al., 2018). Lactic acid bacteria (LAB) derived BSF indicated a complex mixture of different composition including carbohydrates, proteins, and glycolipids (Morais et al., 2017). Supporting, Sharma et al (2014) suggest that *Lactobacilli* was isolated from traditional soft Churpi cheese found positive for biosurfactant production, which reduced the surface tension from 72.0 to 39.5mN/m, and their presence of glycolipid type of biosurfactant closely similar to xylolipids. The performance of starter culture, LAB, resides in their “generally regarded as safe” (GRAS). Those microorganisms do not affect the risks of toxicity or its pathogenicity, which allows in the food industries. However, the biosurfactant its limits to commercial production because substrate such carbon and nitrogen source were high

cost and purify process as well. Therefore, strategy of using waste for biosurfactant production, which is the important to reduce cost production.

2.1.3 Utilization of agro-waste for the production of biosurfactant

At present, the society is emphasized by an increase in expenditures to reuse materials and environmental concerns. The implementation of green environmental led to the reuse of various agro-wastes, which is valid for the food industry (Banat et al., 2014). The selection of waste products should be guaranteed for the for the adequate nutrient for supporting and BSF production. Principally, the total carbohydrates and total lipids were correlated to a substrate in fermentation process. Therefore, the agricultural and food industry waste were crucial implanting for the feasible BSF production (Makkar, Cameotra and Banat, 2011). The information of those substrates are (1) Olive oil mill effluent, it also contains rich of nitrogen compositions and organic acids; (2) Animal fat and lard could be achieved for the maximum glycolipid production by *C. lipolytica*; (3) Frying oil, the combination of glucose and canola oil has been used for the successful production of a BSF by *C. lipolytica*, and especially (4) Molasses and sugar cane were high sugar content around 48%-56% makes by *Lactobacillus paracasei* subsp. (Hippolyte et al., 2018)

In order, to reduce production costs, the inexpensive method, including the selective criteria of waste substrates involved. Thus, tropical sour fruit such as mango, banana, and mangosteen were major exported product, including mass processed. Of which resulting in tropical fruits waste and under grade of over-ripe fruits were bundle. The mango waste are carbohydrate-rich and contains 4.5% crude proteins, 10.12% fiber, total carbohydrate 71.93% and a few of total lipid (Arora et al., 2018). In addition, the metabolites produce from mango, that reveal antimicrobial

activity which is the important BSF characteristics (Vega-Vega et al., 2013). Therefore, it was interesting to apply for the production of BSF by spontaneously fermentation and/or co-culture fermentation.

2.1.4 Factors affecting biosurfactant production

The production of BSF can be spontaneously produced the potential BSF, which can induce and control by the variation parameters such as temperature, pH, aeration, and agitation speed. These physicochemical factors are shown below.

2.1.4.1 Carbon and nitrogen source

The carbon and nitrogen source are used for the microbial growth and production of BSF. A very low yield was found to be either glucose or vegetable oil and the BSF was produced *T. bombicola*, but the yield increased to 70 g/L, when both glucose and soybean oil were achieved for 80 and 40 g/L, respectively. However, industrial waste was used for the production of a biosurfactant by *C. lipolytica*, the yield of the protein-lipid-carbohydrate complex was 4.5 g/L. A high production of BSF was obtained from *C. lipolytica* when supplemented 1.5% glucose (w/v) (Bednarski et al., 2004). Mouafo et al (2018) reported that *Lactobacillus plantarum* G88, *Lactobacillus delbrueckii* N2 and *Lactobacillus cellobiosus* TM1 utilized sugar cane molasses and glycerol would reassure the cell growth and production, while the excessive nitrogen lead to synthesis of cellular material and limits the buildup products (Vecino et al., 2017; Kim et al., 2006). The high-level nitrogen of which accumulated during BSF synthesis, will stimulate growth rather than biosurfactant production, Saikia et al (2014) demonstrated the suitable C/N ration for microbial producing BSF could be approximately C/N ratio of 55 to 22.

2.1.4.2 Growth conditions

The environmental factors during fermentation such as temperature, pH, agitation speed oxygen and specific microorganisms, also influence BSF production. For example, produce maximum BSF yields in a wide pH range, *Candida* sp. SY16, pH 5.0, but pH 6.0 for *C. batistae* (Sarubbo, Farias, Campos-Takaki, 2007). Different microbial processes are affected by even a small change in temperature. In view of LAB, Ghasemi et al (2018) proved that BSF production *Lactobacillus rhamnosus* PTCC 1637 within bioreactor and control follow agitation speed at 120 rpm, temperature 37˚C, and maintain the pH value approximately 6.7 by 2.5 N of sodium hydroxide solution, gave these BSF characteristics, 6.0 mg/ml with a minimum surface tension value of 39.00 mN/m and a maximum emulsifying index of 42%. In addition, BSF *producing Pediococcus dextrinicu*s SHU1593 strain using MRS-Lac, syrup and molasses media, under 35-37˚C, agitation speed at 120 rpm and the initial pH value 6.7, revealed surface tension from 72.80 ± 0.10 to 39.01 ± 0.32 mN/m, and equal ratio of lipids and proteins (Ghasemi et al., 2020). That correlative function or implicit microbial growth incorporated liquid medium to cell. Whenever oxygen decreased, bacteria cell and BSF compound are decreasing. Thus, gained the aeration rate at least 0.5 vvm combine with a few of agitation speed can be linked to solubility of hydrophobic and hydrophilic substrates in medium and, consequently, facilitate, result in increasing of opportunity cell producing biosurfactant.

2.2 Metabolic Pathways

2.2.1 Biosurfactant fermentation metabolic pathways

Generally, microorganism growth and produce a hydrophilic moiety as water-soluble substrates like carbohydrate groups, while hydrophobic substrates like fats and oils are used to build up the hydrophobic portion of BSF. Various precursors of BSF production, are produced via various metabolic pathway deposited in culture medium. For example, the carbon flow will be regulated by both lipogenic pathway and the development of hydrophilic moiety via glycolytic pathway, that are restrained by microbial metabolism (fig 2.1).

A soluble substrate such as glucose, fructose, sucrose and glycerol are broken down to the intermediate; glucose-6-phosphate (G6P) via glycolytic pathway, which is one of the major precursors of carbohydrates found in the hydrophilic part of a biosurfactant. A series of enzymes are used to catalyze G6P on route to synthesizes various forms of hydrophilic moieties in the biosurfactant. In part of the hydrophobic moiety (lipid), such as glucose is oxidized to pyruvate which then transformed into acetyl-CoA that synthesizes malonyl-CoA combined with oxaloacetate, which be then converted into fatty acid (precursors) for lipid production (Nurfarahin, Mohamed and Phang et al., 2018). In addition, there were report of the potential *L. cellobiosus* TM1, *Lactobacillus delbrueckii* N2 and *L. plantarum* G88, expressed BSF with higher lipid content were produced when using glycerol and sugar cane molasses as a substrate (Mouafo, Mbawala and Ndjouenkeu, 2018).

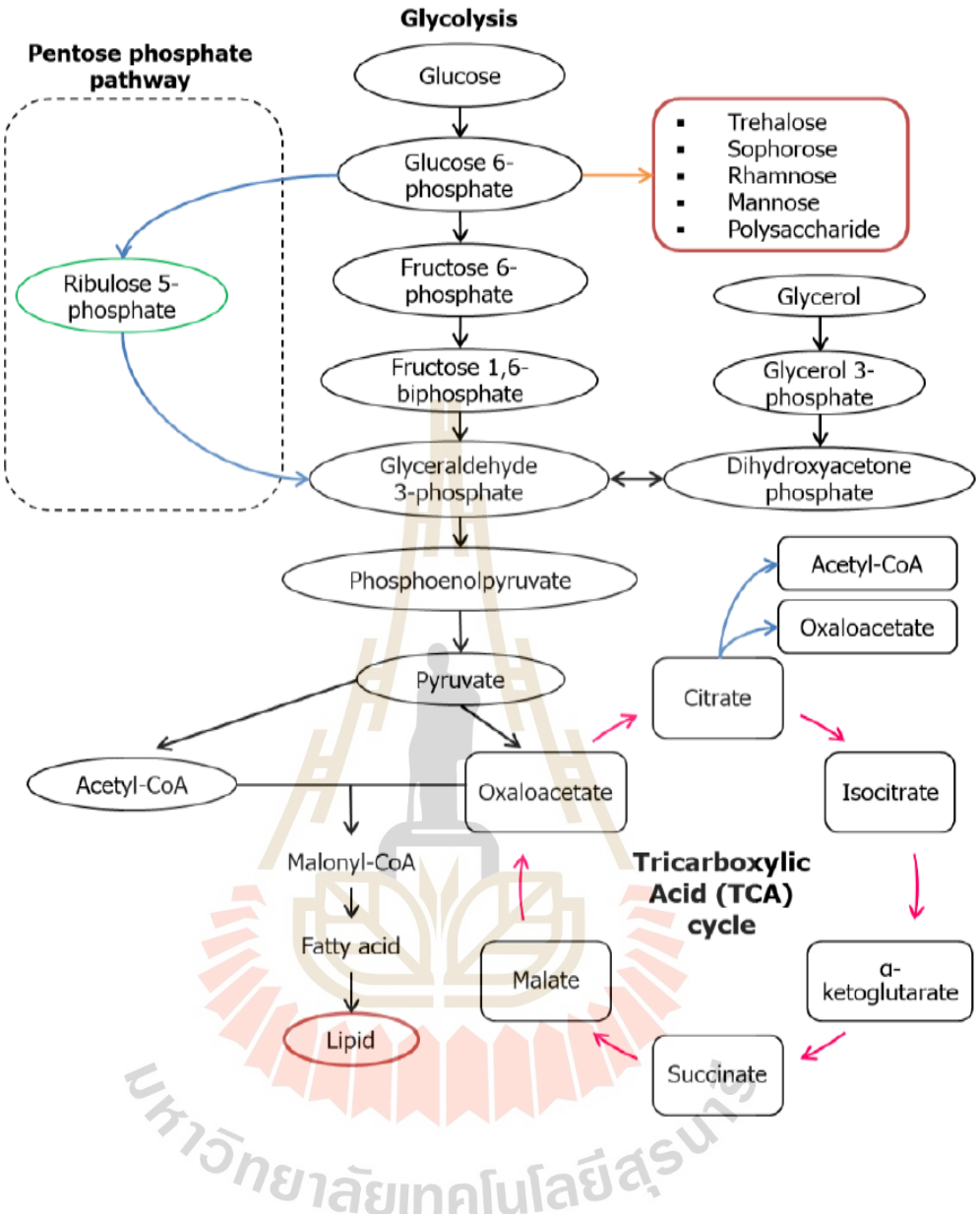

Figure 2.1 Synthesis of biosurfactant of microbial with the use of carbohydrates as substrate or water-soluble substrate.

Source: Nurfarahin, Mohamed and Phang (2018).

2.2.2 Quorum sensing (QS):co-culture in spontaneous fermentation

Quorum sensing (QS) is the mechanism by which bacteria engage in cell-to-cell communication using diffusible molecules based on a critical cell density.

In view of LAB that safety microbial use in food, described the mechanism of action of bacteriocins production in food, must be understood as a dynamic process during different interactions are always changing, resulting in food preservation. It is regulated by quorum-sensing mechanisms (Kareb and Aïder, 2019). They can be described as follow, also know the LAB dominant microorganism native in sour fruit, when applied as a substrate for production under acid conditions fermentation that were supported to rapid growth and till to the LAB population density increases, consequence to the concentration of autoinducer molecules in the environment also rises (Albuquerque et al., 2020). When the LAB multi-strain secreting auto-inducing peptides (AIP) in the surroundings that induced QS system activated, which the plays a role in complex processes such as biofilm formation, production of bacteriocin and, BSF and other antimicrobial compound, including especially activities that would not be possible to survival in stress condition (agitation speed, aeration rate in fermenter) that beneficial in low population density as well as cell injured (Papadimitriou et al., 2016; Sharma and Saharan, 2016). This is an important system to control the quality of product when producing by co-worker of native microorganisms in waste and potential culture in fermenter condition.

2.3 Properties and function of biosurfactant

Biosurfactant (BSF) is an alternative natural based sanitizer applied in food industry, because easily biodegradable and non-mutagenic, the friendly nature and low acute mammalian toxicity, which were approved by FDA (Nitschke & Costa, 2007). Some characteristics are common to the majority of BSF and have advantages over conventional surfactants, as described; (a) Surface and interfacial activity, efficiency

is measured by the CMC, whereas effectiveness is related to surface and interfacial tensions, the best effect which should be approximately 35 mN/m. (b) Tolerance to a broad temperature and pH; (c) Biodegradability, the BSF are easily degraded by microorganisms in water and soil, making these compounds adequate for bioremediation and waste treatment. (d) Low toxicity, of which allows the use of BSF in foods, cosmetics and pharmaceuticals. (e) Emulsion forming and/or breaking, it can be either emulsifiers or de-emulsifiers (Nitschke and Silva, 2018). Moreover, in view of antimicrobial activity and anti-adhesion activity, several BSF have shown antimicrobial against bacteria and fungi. For example, the concentration 50- 100 ppm of lipopeptide iturin from *B. subtilis* showed potent antifungal activity, and reduction on the microflora present in stored grains of cottonseeds, corn and peanuts and cottonseeds. Besides, the glycolipid was revealed antimicrobial and antiadhesive activity against several food pathogenic, such as trehalose lipid showed significant antiadhesive properties against *Escherichia coli* and *C. albicans* on polystyrene and silicone surface (Janek et al., 2018). Of which properties and function made it more interested to application in food industry as release and/or combine with chemical synthetic surfactant.

2.4 Biosurfactants and its application in fresh produces washing

Fresh produce is currently produced in minimally processed packaging as the convenience food categories to purchase, especially leafy vegetables have been demanded as the valuable qualities of long shelf life and safety (Luo et al., 2018). However, many researches have recently reported that the microbial contamination fresh produce especially *L. monocytogenes* contaminated (Machado-Moreira et al.,

2019). As mentioned, a broad antimicrobial characteristic of BSF was interested approach and can be applied use in food, moreover, that low toxicity was proved. For instance, especially BSF derived from *Lactobacillus* may represent safer alternatives for food industry can be good strategies to solve this problem. BSF produced by *L. jensenii* and *L. rhamnosus* were able to inhibit the growth *Escherichia coli* and methicillin resistant *Staphylococcus aureus* (Sambanthamoorthy et al., 2014). Magalhães and Nitschke (2013) reported the combined effect of nisin and rhamnolipid was evaluated against *L. monocytogenes*, sensitive to rhamnolipid (MIC156.2 μg/mL) a, that show antimicrobial activity against *L. monocytogenes* about 90%. Thus, some of research try to use BSF as a sanitizer in washing fresh produce. Dilarri et al (2016) reported washing fresh fruit by the different solution such as rhamnolipids solution produced by *P. aeruginosa*, tap water and electrolyzed water. The results showed rhamnolipids solution were the most efficient.

2.5 Role of microbial stress response in food safety

Some microorganisms can induce adaptive responses to environmental stresses, like in food processing, which there are a variety of processes, such as heat treatment (sterilization and pasteurization), high pressure, a_w decrease, radiation, fermentation and sub-lethal processing or preservation treatments may evoke bacterial coping mechanisms that alter gene expression, specifically and broadly, resulting in enhance their tolerance to these stresses and may promote persistence under adverse conditions. Thus, stress responses of microorganism especially foodborne pathogens can have profound effects on their survival in foods. Additionally, exposure to one sub-lethal stress may produce a spectrum of adaptive responses, cross-protecting

microorganisms against multiple stresses (Horn and Bhunia, 2018). For example, most *L. monocytogenes*, is of particular concern to the Ready-To-Eat (RTE) food industry, it has a variety of genetically encoded survival mechanisms to withstand environmental stressors such as heat, cold, salt, and acidic conditions. Moreover, this organism is ubiquitous and can be found in soil, water, and on food processing equipment. More importantly, this pathogen has a very high case-fatality rate, *L. monocytogenes* is a growing issue in fresh sprouts. Stress responses of foodborne pathogens have a much greater impact on food safety than has already been recognized. As the consumers demand foods with higher food quality, the food industry is applying cumulative mild processing steps for the control of pathogens in foods. On the other hands, this trend facilitates more frequent exposure of foodborne pathogens to sub-lethal stresses, potentially compromising food safety through inducing resistance responses and cross-protection. Therefore, at present, many of institutions and agencies related to food were collected the outbreak, virulence, epidemiology and the phenomenon of *L. monocytogenes* stress response in food products (NicAogáin and O'Byrne, 2016).

2.6 *L. monocytogenes* characteristics and its stress responses

2.6.1 Characteristics and occurrence

The capacity of *L. monocytogenes* to survive and multiply under a wide range of environmental-stress conditions appears to be critical for foodborne transmission of the pathogen. Harmful factors affecting the growth of microorganisms in a negative way are called as "stress". The reactions occurring in bacteria against stress are the form of long-term adaptation or immediate (shock) response. In many cases immediate and long-term adaptation responses are generated through similar

shock proteins. The synthesized proteins may be effective against a single stress factor or multiple stress factors. Moreover, resistance mechanisms such as detergent, sanitizer, chlorine compound, acid and cold resistant, that are activated against the stress factor may be different or same (Krawczyk, Balska and Markiewicz, 2016).

As mentioned, *L. monocytogenes* is found in a variety of raw foods especially fresh produce including RTE, that become contaminated after processing. For example, in the U.K. a study of pre-packaged RTE mixed salads, reported the detection high level of *L. monocytogenes.* Pre-packaged mixed salads were contaminated with *Listeria* spp. and L. monocytogenes, and more frequently detected when stored above 5 to 8°C. Machado-Moreira et al (2019) reported the indicator of minimally processed leafy vegetables, which detected *L. monocytogenes* around 53.1% of all samples analyzed. Thus, demonstrated *L. monocytogenes* presence in a wide variety of fresh produce samples and other minimally processed foods, as shows in table 2.1 that are capable of growing in a wide range of temperatures (1.5 to 45°C). This endows the organism with a unique capacity to survive food processing and food storage conditions. However, optimal growth of the organism occurs between 30-37°C, the bacteria motile at low temperatures (20°C), although some *Listeria* strains are non-motile at 37°C because they lack the expression of the flagellin proteins at this temperature. Of which reason why *L. monocytogenes* is a high risk, and it necessary to eradicate. Guidelines in the United States advise that should not be present (<1 CFU 25 g/L) in RTE foods that support the growth of bacteria and should not be equal to or above 100 CFU /g for foods that do not support the growth of that.

Table 2.1 Factors identified to have an impact on the growth and survival of *L. monocytogenes*.

Factor	Can grow			Can survival (no growth)
	Lower limit	Optimum	Upper limit	
Temperature (°C)	1.5 to +30	30.0 to 37.0	45.0	-18.0
pH	4.2 to 4.3	7.0	9.4 to 9.5	3.3 to 4.2
Water activity (a_w)	0.90 to 0.93	0.99	> 0.90	<0.90
Salt concentration (%)	<0.5	0.7	12-16	≥ 20
Atmosphere	Facultative anaerobe (it can grow in the presence or absence of oxygen, e.g. in a vacuum or modified atmosphere package)			
Heat treatment during food processing	A temperature/time combination e.g. of 70°C and 2 min is required for a D6 (i.e. 106 or 6 decimal) reduction in numbers of *L. monocytogenes* cells. Other temperature/time combinations may also provide the same reduction.			

Adapted from: Lennox et al (2017).

As mentioned above, contamination and occurrence outbreak most found in fresh produce especially lettuces and leafy brassicas (kale, spinach, chines kale etc.), which account for a significant proportion of the worldwide market and are considered 'high-risk' in terms of bacterial contamination because of their leaf structures and proximity to the ground. The fresh leafy produce supply chain (FLPSC), from farm to fork, is complex and contains a diverse range of environments where *L. monocytogenes* can be detected during routine sampling of fresh leafy produce throughout low temperature stored. Especially, strain 10403S, serotype 1/2a and strain containing *sigB* gene, that are capable response, tolerant and adaptation to stress environmental such as sanitizer/detergent/disinfectant-resistant, nisin-resistant,

acid-resistant and cold-resistant (Machado-Moreira et al., 2019; NicAogáin and O'Byrne, 2016).

2.6.2 *L. monocytogenes*: detergent/surfactant stress response

In case of minimally process including fresh produce, that stress of *L. monocytogenes* occurs with that process such as disinfectant sensitivity response including acid, alkali, surfactant, detergents and nisin which were relevant of washing process. These stressors will influence the physiology, function, and activity of microorganisms that are found in foods. Based on the magnitude of the stress involved, stress can be differentiated as either "sub-lethal" or "lethal or severe". Exposure to detergent/sanitizer stresses (oxidative stress) of the L. *monocytogenes* stress response, mediated by the alternative sigma factor B which regulates several stress, virulence and transporter-associated genes (e.g. lmo2230, ltrC, ctc, inlA, inlB and opuC operon) and related proteins. Sigma factors are subunits of prokaryotic RNA polymerase responsible for the recognition of particular DNA sequences in promotor sites. Sigma factors responsible for the baseline expression of genes during normal conditions are called housekeeping sigma factors, and sigma factors with an affinity to promoters for genes needed to respond to "non-normal" conditions are classified as "alternative sigma factors". *L. monocytogenes*, the sigma factor B modulates around 140 genes associated with stress responses to a great number of adverse environmental conditions, such as exposure to acid, low temperatures, oxidative stresses from sanitizer (Melo, Andrew and Faleiro, 2015). Therefore, fresh-produce, Sigma B play role to the detergent. Ryan, Gahan and Hill (2008) reported that Sigma B is essential for resistance at lethal levels of benzalkoniumchloride (BC), (cetylpyridinium chloride) CPC and (sodium docecyl sulfate) SDS, analysis revealed

the induction of *sigB* by sub-lethal levels of surface-active agents. Figure 2.2 represents the mechanisms of environmental stress on *L. monocytogenes* relevant with *sigB* gene expression, σ^B activity is tightly controlled both transcriptionally and post transcriptionally. In this section if a *sigB* in a stress condition, that will be showed in the regulation of bacterial cell wall components.

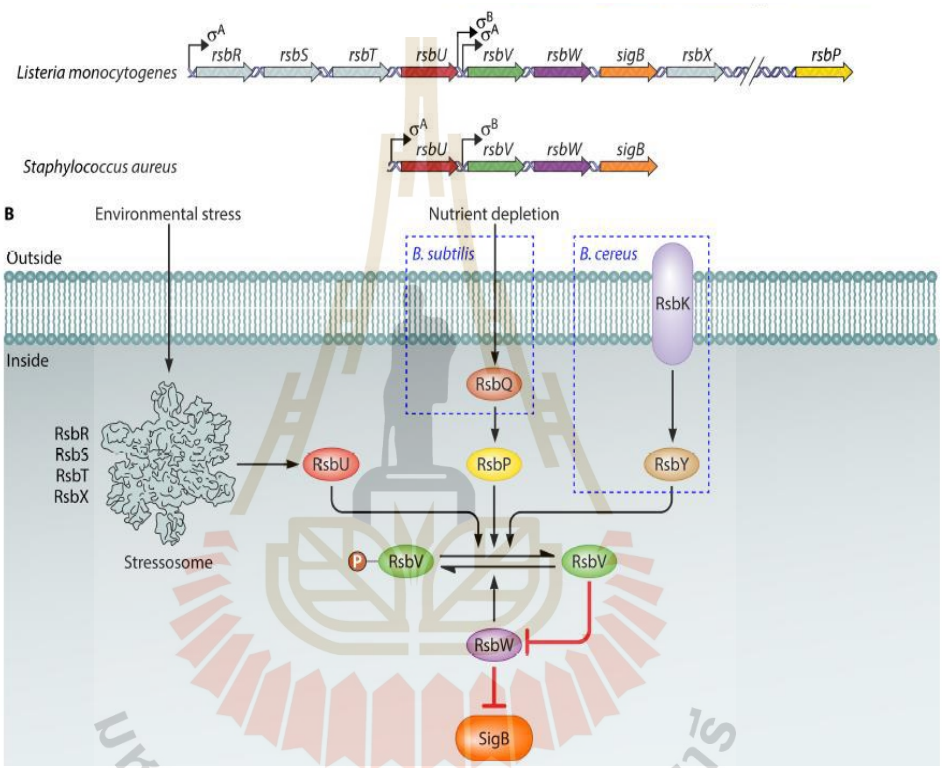

Figure 2.2 Regulation of *sigB* expression and activity, conservation of *sigB* genes and operon structures across gram-positive bacteria.

2.6.3 *L. monocytogenes*: cold-stress response

Cold-stress is the importance index of food product that request to store at low temperature or refrigerator especially, RTE, salad and fresh-produce. These "hurdles" can introduce varying degrees of "stress" in different bacteria. In view of

L. monocytogenes, the response mechanisms described so far involve maintenance cell-membrane lipid fluidity, intracellular uptake of compatible solutes, and production of several cold stress proteins (Csps and Caps). Under the low temperatures, *L. monocytogenes* response to induced maintenance of lipid fluidity in cell membranes, the correct physical state of the membrane lipids is crucial to optimal structural and functional integrity of cell membranes. Thus, the molecular adaptation measures adopted in cell-membrane lipids include (i) a change in the fatty acid chain lengths, (ii) an alteration in the degree of fatty acid unsaturation, and (iii) a change in the type of branching at the methyl end of the fatty acids (NicAogáin and O'Byrne, 2016). Of which, were related to the role of *sigB* gene and more cross protective by biofilm formation gene during sublethal under cold stress stored condition that related with *inlB* gene expression.

2.7 Sanitizing agent against *L. monocytogenes* applied in fresh produce

The current tolerance to disinfectants in *L. monocytogenes* has been a topic of concern in the context of the food industry and public health regarding foodborne pathogens. The presence of high bacterial concentrations and the interference with organic matter due to insufficient cleaning prior to disinfection diminishes the activity and thus the efficacy of disinfectants commonly used in industrial premises. However, anthropologic factors such as failure in dosage or inadequate rinsing are also responsible for the generation of tolerances due to the formation of areas in which sub-lethal concentrations of the disinfectant are present. Additionally, it has also been

stated that tolerance to certain disinfectants may contribute to the persistence of *L. monocytogenes* in the food industry.

2.7.1 Chlorine-based compounds

Different chlorine-based compounds such as sodium hypochlorite, chlorine dioxide gas or aqueous chlorine dioxide have been proven to be active against *L. monocytogenes*. Due to their fast-oxidising nature, they interact with cellular membranes or penetrate directly into the cell forming N-chloro groups that react with the cellular metabolism due to the interference with key enzymes. With this regard, Valderra et al (2009) found a *L. monocytogenes* reduction of about 4 log CFU/mL treated with 3 mg/mL chlorine dioxide with just 90s contact time. Nevertheless, in *L. monocytogenes*, proper chlorine efficacy seems to be age-dependent since the thickness of the cell wall in young cultures is higher, thus protecting the cells from these sanitizers. Tolerance development against chlorine-based sanitizers has been described so far to be unlikely in *L. monocytogenes* cell suspensions (Bansal et al., 2018).

2.7.2 Acid compounds and other sanitizers

L. monocytogenes is able to adapt to low pH environments generated by natural processes (e.g., lactic fermentation) or artificially induced (e.g., acidification of water for cleaning systems) by means of different mechanisms. Additionally, it has been demonstrated that sub-lethal acid adaptation deeply alters the intracellular protein pattern expression, being more evident as the pH decreases, and that this differential pattern is strain-dependent. *L. monocytogenes* can adapt to acidic conditions, all of them focused on the maintenance of the intracellular homeostasis. Among them, the glutamate decarboxylase (GAD) system is considered one of the

major mechanisms (Bucur et al., 2018). This involves the GAD enzyme, which promotes the irreversible conversion of cytosolic glutamate to a neutral compound, the aminobutyrate (GABA), by irreversible decarboxylation. The synthesis of GABA has a dual protective role: firstly, it consumes an intracellular proton during the process, with the subsequent increase of the pH inside of the cell (Ryan, Hill and Gahan, 2008). Accompanying strains, such as lactic acid bacteria, can exert a protective effect to *L. monocytogenes* in mixed-species biofilms, increasing its tolerance to acidic sanitizers. There is no doubt that the recalcitrance of *L. monocytogenes* in foodstuffs is greatly influenced by the ubiquitous presence of its resistant gene and biofilm among food contact and non-food contact surfaces within food-processing premises.

Additionally, to biocontrol as presented previously, one choice is microbial surfactant or biosurfactant compound (BSF), which is a strategy to apply as a sanitizer within RTE or fresh-produce to eliminate bacteria and/or effective lethal level to protect that regrowth during storage.

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CHAPTER III

ISOLATION AND SCREENING OF *Lactobacilli* ASSOCIATED WITH POTENTIAL BIOSURFACTANT PRODUCTION

3.1 Abstract

Lactic acid bacteria (LAB) are sour fruit microflora and well-known commensal bacteria for the food industry due to their GRAS (generally recognized as safe) statute. Therefore, the objective of this work was to gain the specific BSF characteristic-isolates from over-ripe Nam Dok Mai mango (*Mangifera indica* Linn), collected from Saraburi province, Thailand. Of which using 1% (w/v) glycerol and 2 % (w/v) sucrose as carbon source in fermentation were able to produce antimicrobial metabolites. Fifty-two out of sixty-six isolates LAB were screened BSF producing strains based on morphological, biochemical, physiological characteristics, API CHL 50 system, cetyltrimethylammonium bromide (CTAB Agar Plates) and antimicrobial against *Listeria monocytogenes* (contain significant *sigB* gene). It was found five isolates (*Lactobacillus fermentum*; MGL-1, *Leuconostoc mesenteroides*; MGL-3, *Lactococcus lactis* ssp. lactis; MGL-11, *Lactobacillus plantarum*; MGL-8 and *Lactobacillus plantarum* MGL-12) were crude BSF producing effected on *L. monocytogenes*. Those of five isolates were further evaluated the optimum growth rate and crude biosurfactant production in MRS condition with 1% (w/v) glycerol and 2% (w/v) sucrose. The maximum growth rate at 36 h of *L. plantarum* (MGL-8)

provided the highest crude biosurfactant production at surface tension value 39.14 mN/m.

Keywords: fruit-waste, predominant microorganisms, lactic acid bacteria, microorganism characteristics, biosurfactant production, antimicrobial

3.2 Introduction

Fruits and vegetables have a crucial role in our diet and human life, and as a result of the growing world population and the changing dietary habits, these food commodities have, therefore, significantly increased (Vilariño, Franco and Quarrington, 2017). Especially, fruits produced globally were 124.73 million metric tons (MMT) of citrus, 114.08 MMT of bananas, 84.63 MMT of apples, 74.49 MMT of grapes, and 45.22 MMT of mangoes (FAO, 2017). The higher consumer demand, the greater countries export and must be controlled the quality and condition of commodities by fruit grade standards in the marketplace. Lacking of the proper handling methods such as harvesting, transport to packing houses, storage, classification and grading, then high accumulate of undergrads, over-ripe and/or wastes respectively, exist. Nowadays, fruit wastes around 46% of the global solid wastes, making up the largest proportion (Sadh, Duhan2and Duhan1, 2018), which is one of the reasons to utilize fruite wastes including over-ripe, also indicates the positive prospects of its utilization in the future. Fruit waste has been implemented as the low-cost substrates for the bio-based products i.e. biofuels, biorefinery including crude BSF (Red, Corn, Fatemi and Engelberth, 2018). Recently, increasing global environmental awareness has led to much more interest in microbial surfactants (biosurfactants) compared to their chemical counterparts. The features that make them

commercially promising alternatives to chemically synthesized surfactants are their lower toxicity, higher biodegradability, antimicrobial properties, milder production conditions, environmental acceptability, lower critical micelle concentration, and greater stability toward temperature and pH (Santos, Rufino, Luna, Santos and Sarubbo, 2016). Barracuda Mango or Nam-Dok-Mai mango (*Mangifera indica* Linn.) is one of the most exceptional mango variety in the appearance and the taste. In 2009 until recent years, Thai mango exports has been climbing steadily, at an annual rate of 15 to 25 per cent, and increase of 62 percent, due to huge demand from the Korean market. Higher demand along with grade A quality that must meet each of national standards resulting in accumulate of over-ripe (undergrad) and waste from food industry. Agro-industrial wastes including mango with high contents of carbohydrates and lipid meet the requirements for carbon substrates for biosurfactant production (ThaiFarmFresh, 2019). In addition, vegetables and fruits have a microbial population whose composition depends on the characteristics of each fruit matrix as well as geographical origin. In case of mango and/or source fruit containing natural microflora, which are beneficial microorganisms, including yeasts (*Saccharomyces, Pichia, Candida, Torulaspora genera*), mold (*Rhizopus* spp.), aerobic (*Bacillus* spp. and *Acetobacter* spp.) and particularly facultative anaerobe (lactic acid bacteria, LAB). LAB from several genera, including *Lactobacillus, Streptococcus,* and *Leuconostoc* are predominant in fermented food, and bioproduct agent such as bacteriocin, bioemulsifiler, organic acid and BSF (Bartkiene et al., 2017; Carvalho et al., 2019; Ghasemi et al., 2019). The LAB counts in these can range from 10^2 to 10^7 colony-forming units (CFU/g), which spontaneous ferment raw vegetables and fruits correspond with preservation and stability (Torres, Verón, Contreras and Isla, 2020).

LAB are generally regarded as safe microorganisms (GRAS, the United States Food and Drug Administration), recognized for their use in medicine and food industries. Due to their non-pathogenicity, many researches use the indigenous LAB isolates from food material for improving the quality characteristics, functional properties, and some species are capable of surface-active molecules biosynthesis or commonly known as biosurfactant (BSF) (Yien Ong et al., 2012). LAB characteristics could provide an antimicrobial in food, and play a role in the lack of a respiratory chain causes them to exhibit a fermentative metabolism, which depending on how they ferment hexoses. The microflora mainly functions in various food fermentation, especially vegetables and sour fruit is predominate LAB, of which metabolites serve as preservatives by lowering the pH to 4 (due to lactic acid formation), and thereby inhibiting the growth of most other microorganisms. In addition, a part of hetero-fermentative LAB uses the 6-phosphogluconate/phosphoketolase pathway resulting in lactic acid, carbon dioxide, ethanol (or acetic acid) as the major end products and including aroma compounds, exo-polysaccharides, bacteriocins and BSF (Alkan, Erginkaya, Konuray and Turhan 2019). BSF are effective at extreme temperatures and extreme pH values. The process of BSF production was fermentation and/or hydrophilic substrates of which primarily used by microorganisms for cell metabolism and the synthesis of the polar moiety. Where lipid substrates are used exclusively for the production of the hydrocarbon portion of the BSF (Almeida et al., 2016). Gerba (2015) reported the main target to damage bacteria should be made disruption in the phospholipid bilayer and subsequent cellular content leakage and then eventual bacterial death.

At present, there is a few reports of lactic acid bacteria producing biosurfactant

and implementation in food industry. The main purposes of this study were, therefore, to evaluate the efficiency of native LAB isolates for BSF production from agro-mango over-ripe. Additionally, characterization of the LAB isolates, by performing a biological, physiological test, CHL identification and growth curves, by examining biosurfactant production through the surfactension test and antibacteria against *L. monocytogest* contain *sig*B gene were conducted.

3.3 Materials and methods

3.3.1 Microbial source test

Listeria monocytogenes strain DMST17303 was obtained from Department of Medical Science, Thailand, is considered to be the surrogate pathogen throughout the experiment. It was strored at 4°C and sub-cultured every two weeks onto tryptone soy agar (TSA, Hi-media, Mumbai, India) slant. The test microorganisms used for screening of antimicrobial activity-producing LAB, and propagated in TSB broth at 37°C. The optical density was adjusted to the McFarland No.1 (about 10^8 CFU/mL) to determine detergent resistant gene (*sigB* gene) by Polymerase chain reaction (PCR).

3.3.2 *L. monocytogenes sigB* gene detection by PCR

The *sigB* gene of *L. monocytogenes* was amplified by PCR method using the specific forward primer (sigB-fwd) 5'-AAT ATA TTA ATG AAA AGC AGG TGG AG-3' and reverse primer (sigB-rev) 5'-ATA AAT TAT TTG ATT CAA CTG CCT T-3' (Nucleic acid sequence location based on the GenBank accession number EU161935.1;). Cells were grown to late exponential phase in TSB broth. This cell suspension was kept at room temperature for 30 min prior to DNA extraction. DNA extraction and purification of the chromosomal DNA by CTAB method were

carried out as described Zhou et al., 1996. The extracted genomic DNA was amplified in 20 µL reaction mixture containing 5X buffer (5X HOT FIREPol® Blend Master Mix), 10 pmol/µL of forward primer, 10 pmol/ µL of revese primer, 7 µL of PCR Qualified Water, and 50 ng/µL of DNA. Thermal cycling was performed at an initial PCR activation at 95ºC for 3 min, followed by 35 cycles of 95, 50 and 72ºC for 0.5, 0.5 and 1 min, respectively, and a final extension at 72ºC for 5 min. The PCR products were separated by gel electrophoresis, 1% 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 pattern were visualized by ethidium bromide staining and photographs taken by Gel documentation (Macro Vue UVis-20 Hoefer).

3.3.3 Isolation and screening of lactic acid bacteria

LAB was isolated from the samples collected from local Nam Dok Mai mango (*Mangifera indica* Linn) farm at Saraburi province, Thailand. Ten-gram mango subjected into 250 mL flask containing 100 ml of enrichment basal medium (BM) with 1% (w/v) glycerol and glucose 10% (w/v) as a carbon source. The enriched samples were incubated at 35ºC, 180 rpm for 5 days (Gámez et al., 2017; Aziz et al., 2014). One mL of the culture was transferred to 100 mL new medium BM medium and incubated at 35ºC, 180 rpm for 5 days. Diluted cell were plated onto Man, Rogosa and Sharpe (MRS) agar to determine the best medium for the LAB growth. The isolation for the specific indigenous microflora from the previous enriched cultures was spread on Nutrient agar (NA), and potato dextrose agar (PDA) with 10% (v/v) tartaric acid. Colonies with different morphological characteristics were purified by the streak method onto MRS agar media. The purified isolates were kept on MRS slant agar at 4°C for working isolates and the identical isolates were in 30% (v/v)

glycerol at -20°C for stock culture.

3.3.4 Morphological, physiological characteristics and biochemical test

All LAB isolates were performed to study the morphological, biochemical, and physiological characteristics (Bergey's Manual of Systematic Bacteriology, 1986). The LAB isolated strains were, therefore, examined by gram staining, catalase production and oxidation/fermentation test.

3.3.4.1 Gram's stain test

LAB are known to be gram positive, it means that they give blue-purple color by gram staining. The gram reaction of the isolates was determined by light microscopy after gram staining. Cultures were steaked on MRS agar plates and grown at 35°C for 24 h under anaerobic conditions, isolates were done the gram staining and examined microscopically for morphology and phenotype (Acharya, 2015).

3.3.4.2 Catalase test

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme. Overnight cultures of isolates were grown on MRS agar at 35°C for 24 h under anaerobic conditions. After 24 h 3% hydrogen peroxide solution was dropped onto randomly chosen colony. The isolates, which did not give gas bubbles, were choosed. Since, LAB are known as catalase negative.

3.3.4.3 Oxidation/Fermentation test (O/F test)

The nutrient broth solution (100 mL) was prepared in conical flask and 1 ml phenol red was then added. It was autoclaved at 121°C for 15 min and

cooled at room temperature. A syringe filter sterilized solution of 1% glucose was prepared under aseptic conditions. Five ml of the broth and 100 µl of the glucose solution was done. These test tubes were then kept at room temperature for 24 h to check the contamination. All samples were inoculated with fresh grown bacterial culture and incubated at 37°C for 48 h. In case of homo-fermentation, acid would be produced along with the change in color of the medium from red to yellow, and in heterofermentation there would be gas production in Durham tube alongside the change in the color.

3.3.5 CTAB agar plate method for screening potential isolate producing biosurfactant

The 30 µL cell-free supernatant of LAB isolates was dropped onto a light blue mineral salts agar plate, containing the surfactant, cetyltrimethylammonium bromide (CTAB) and the basic dye methylene blue, and incubated at 37ºC for 7 days. In this experiment used rhamnolipids (90%, Standard, Sigma-Aldrich, catalog number: R90) as a positive control. An metabolite surfactant is a specific glycolipid compound, insoluble ion pair with cetyltrimethylammonium bromide and dark blue colony with a halo (Shoeb et al., 2015). Positive strains were kept at 5ºC in MRS agar slant for further experiment.

3.3.6 Antimicrobial effectiveness

The disk diffusion method was adapted from Mostafa et al (2018). LAB isolates from 3.3.5 were sub cultured into in 25 mL of MRS broth, then collected supernatant by centrifugation at 4°C 10,000xg for 10 min, and sterilized by sterile syringe filter pore size 0.2 µm. The surrogate pathogen, *L. mocytogenes* were cultured in TSB, incubated at 37°C for 24 h., then collected the cell precipitate by

centrifugation at 4°C 10,000xg for 10 min. The optical density of cell suspension was adjusted to 10^6 cells/mL, swabbed cell suspension on Mueller-Hilton agar (MHA) plates. After that, loaded over sterile filter paper discs (8 mm in diameter) on MHA plate, and drop 30 µL of each isolate cell free supernatant at equal distance with positive control (5% v/v of SDS and rhamnolipids), negative control (sterile distilled water). The plates were incubated at 37°C for 24 h, the presence of inhibition zone was then measured by Vernier caliper and considered as indication for antibacterial activity.

3.3.7 Identification of LAB strain glycolipid-biosurfactant production

All strains were screened the producing biosurfactant and antibacterial according to the criteria of Bergey's Manual of Determinat on Bacteriology, and identified on API 50 CHL medium and API 50 CH strips (bioMerieux, Marcy-I'Etoile, Frence). The API 50 CHL system was used for the identification of *Lactobacilli, Lactococci, Leuconostoc, Pediococcus*, and *S. thermophilus* strains, based on sugar fermentation. The inoculum was prepared according to the manufacturer's instruction by aseptically transferring pure culture of LAB isolated from MRS agar into the API Suspension Medium ampoule (2.0 mL) using sterile swab. The suspension was mixed and transferred 350 µL to a second API Suspension Medium ampoule (5.0 mL) to reach turbidity equivalent to McFarland standard No. 2. The final inoculum was prepared by transferring 700 µL from the initial bacterial suspension (API Suspension Medium ampoule, 2.0 mL) into an API 50 CHL Medium (10.0 mL). The suspension was mixed and 150 µL (inoculated API 50 CHL medium suspensions) was measured into the well using sterilized micropipette and covered with 50 µL mineral oil. The strips were incubated (Memmert, Germany) at 37°C for

48 h (Pyar and Kok., 2019). The indication of a positive or negative result was determined from the color change from a scale of 1 to 5, from purple (1) to green (3) to yellow (5) at the 48-h mark, whereby a value of ≥3 was considered positive. The results were then cross-referenced to the API® databases using APIweb™.

3.3.8 Optimization of growth condition for biosurfactant-producing strains

3.3.8.1 Growth condition

The experiments with pure LAB strains cultures in MRS broth (cell concentration 10^8 CFU/mL) were carried out in 2000 mL flasks with a volume of 1000 mL MRS broth without tween 80, adding 1%(w/v) glycerol and 2% (w/v) sucrose (from the previous study), 10% (v/v) was then inoculated. The flasks were incubated at 35ºC under shaking incubator 180 rpm, and growth curves were observed until the end of stationary phase. To determine the optimum growth rate of five strains, all test samples would be collected at the interval 12 h for 72 h. All done in tandem with the control experiment were five strains of culture in MRS broth, in the same condition. Thereafter, viable cell count was performed by the pour plating techniques on bilayer MRS agar medium in sterile disposable petri plates, incubated in an anaerobic jar at 35°C for 48 h. The count was expressed in CFU/mL. The cultures were centrifuged (20 min, 4°C, 10,000xg), filtered (0.22μm; Millipore, Sartorius Stedim, Germany), and stored for the determination of biosurfactant quality and antimicrobial properties.

3.3.8.2 Surface tension (ST) measurement

ST is a property of the liquid surface which behaves as an elastic sheet, also used as a criterion for primary screening of biosurfactant producing isolates using tensiometric analysis. The aliquot aqueous solution (3.3.7.1) was

measured by using a Du Nouy ring type tensiometer. It was carried out at 25ºC after dipping the platinum ring (4 cm diameter) in the solution for a while in order to attain equilibrium conditions. The measurement was done in triplicate, and MRS medium was used as a control.

3.3.9 Statistical analysis

All quantitative data were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD). IBM SPSS statistics 26 (Armonk, New York, U.S.A.) was applied to perform all statical analysis. One-way analysis of variance (ANOVA) was determined, which was followed by Duncan's multiple rang test (DMRT) at significant level of 0.05.

3.4 Results and discussions

3.4.1 Isolation of predominant microflora from over-ripe mango

A total of 108 microflora isolates was evaluated at the growth in 1% (w/v) of glycerol and glucose as a carbon source with basal mineral medium condition. The effect of different carbon source in growth of isolates was shown in Figure 3.1. The major of 66 isolates was LAB, 26 and 16 isolates were fungi and aerobic bacteria, respectively. Applying the enrichment medium, basal mineral medium, containing abound of mineral could enhance either a structural or functional role in the cells, and cell growth rate supplemented with 1% (w/v) glycerol as well as glucose at the same concentration (Al-Wahaibi et al., 2014; Gudiña, Teixeira and Rodrigues, 2011). However, several bacterial strains and fungi from over-ripe mango were possibly found. Since, the natural acidity of mango (pH 3.38-4.46) also serves a preventive factor of microbial spoilage, meanwhile, it supports the growth of LAB and

its survival (Ruiz Rodríguez et al., 2019). Thus, all 66 LAB isolates were selected for further identification based on biochemical, morphological, and physiological characteristics (Bergey's Manual of Systematic Bacteriology).

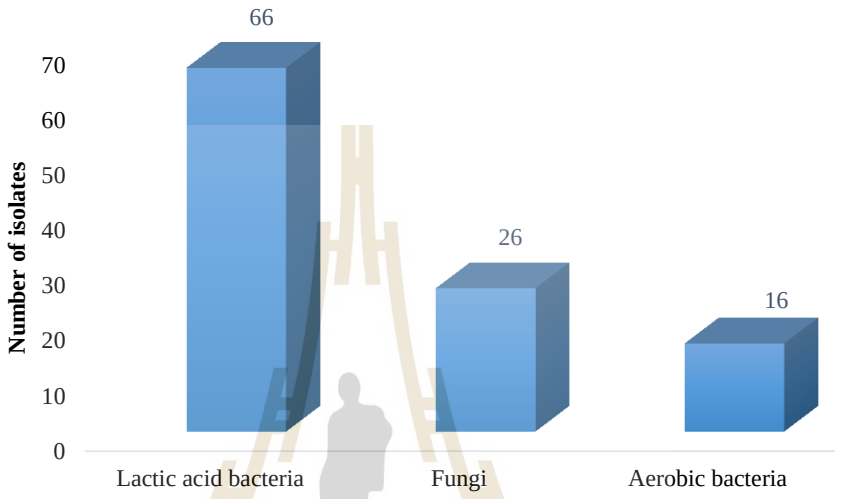


Figure 3.1 The types of microbial isolates in over-ripe Nam Dok Mai mango from Saraburi Province, Thailand.

3.4.2 Presumptive LAB characterization

A total of 66 isolates from over-ripe Nam Dok Mai mango using MRS agar was characterized base on morphological and physiological properties. Table 3.1 showed the isolates of which was classified into 4 groups. Considering the main characterization of LAB which are the catalase-negative, glucose fermentation, and growth ability at low pH at 4.4, group I, II and III were the suspected LAB but group IV. However, it was shown as a remarkable growth at the higher pH 9.6 and temperature at 45ºC, confirming a broad range of growth environment. Therefore, 52 out of 66 isolates were considered to the LAB, and kept for further experiment.

3.4.3 Screening for biosurfactant and antimicrobial metabolites production

Initially, the 52 LAB isolates were screened for BSF production by blue agar plates treated with cetyltrimethylammonium bromide (CTAB) and methylene blue. Extracellular glycolipid biosurfactant production were monitored by the presence of dark blue colonies with halos (Bhat et al., 2015). However, the potential BSF production expressed the metabolite for antimicrobial effectiveness to against *L. monocytogenes*. Table 3.2 shows that 13 out of 52 LAB isolates demonstrated antimicrobial activity against *L. monocytogenes* (containing a severity gene of *sigB*). Nevertheless, 5 of those 13 isolates were potential glycolipid- biosurfactant producing strains ($P<0.05$), which were MGL-1, MGL-3, MGL-8, MGL-11 and MGL-12. Many researchers reported a large number of antimicrobial compounds, such as H_2O_2, organic acid, diacetyl enzymes and bacteriocins, but not all LAB strains could produce BSF (Sharma, and Saharan, 2014; Deegan, Cotter, Hill and Ross., 2006). Therefore, the identification of LAB species and the optimum growth of 5 potential biosurfactant strains were further conducted.

3.4.4 Identification species of LAB potential biosurfactant production

Based on biochemical, morphological and physiological characteristics of carbohydrate fermentation by the API 50 CHL system (Table 3.3), 5 isolates identified as *Lactobacillus fermentum* was MGL-1, *Leuconostoc mesenteroides* was MGL-3, *Lactococcus lactis* ssp. lactis was MGL 11 and the rest two isolates were *Lactobacillus plantarum*; MGL-8, MGL-12. Both species of *Lactobacillus plantarum* showed the evidence of different lactose fermentation. According to starter culture

characteristic, which should be grown in wide range condition, different five strains were, therefore, considered to the optimum growth for the best condition.

Table 3.1 Morphological and physiological characteristics of LAB isolates from over-ripe mango cultured in MRS agar plates.

Characteristics	**Group I 14 isolates**	**Group II 26 isolates**	**Group III 12 isolates**	**Group IV 14 isolates**
Gram straining	+	+	+	+
Cell shape	Rod	Rod	Coccobacilli	Rod
Catalase test	-	-	-	+
O/F test	+/+	+/+	+/+	-/-
Motility	-	-	-	-
Growth temperature and pH				
10ºC	+	+	+	ND
25ºC	+	+	+	ND
30ºC	+	+	+	ND
35ºC	+	+	+	ND
45ºC	+	+	+ [a]	ND
pH 4.4	+	+	+	ND
pH 9.6	+	+ [a]	+	ND

[a] slight growth; + : growth positive; - : negative; ND: not detected.

Table 3.2 The biosurfactant production and antimicrobial against *L.monocytogenes* of LAB strains from over-ripe mango.

LAB isolates	**Biosurfactant production (zone-production, mm)**	**Antimicrobial activity (zone-inhibition, mm)**
MGL-1	12.7±0.6^{b}	11.1±0.2^{def}
MGL-3	14.3±0.6^{a}	12.5±0.5^{b}
MGL-4	ND	11.2±0.7^{def}
MGL-8	14.1±0.2^{a}	14.1±0.3^{a}
MGL-11	14.1±0.4^{a}	12.3±0.6^{bc}
MGL-12	11.7±0.6^{c}	11.1±0.1^{def}
MGL-14	ND	10.9±0.2^{ef}
MGL-16	ND	12.3±0.3^{cd}
MGL-21	ND	11.7±0.4^{bcde}
MGL-48	ND	12.0±0.9^{bcd}
MGL-52	ND	11.5±0.5^{cdef}
MGL-57	ND	10.7±0.6^{f}
MGL-63	ND	10.7±0.3^{f}

Different upper letters within a column indicated significant differences ($P<0.05$), and values are expressed as mean of dimeter±standard deviation. Rhamnolipids (90%, Standard form Sigma-Aldrich) is a representative glycolipid-biosurfactant was used as positive control. For the antimicrobial activity was used 20 mM of sodium dodecyl sulfate (SDS; chemical surfactant).

Table 3.3 Identification of biosurfactant producing LAB isolates with API CHL 50 system.

Potential isolates	(0) Control	(1) Glycerol	(2) Erythritol	(3) D-arabinose	(4) L-arabinose	(5) Ribose	(6) D-xylose	(7) L-xylose	(8) Andonitol	(9) β-methyl-D-xyloside	(10) Galactose	(11) Glucose	(12) Fructose	(13) Mannose	(14) Sorbose	(15) Rhamnose	(16) Dulcitol	(17) Inositol	(18) Mannitol	(19) Sorbitol	(20) Methyl-D-mannoside	(21) Methyl-D-glucoside	(22) N-acetyl-glucosamine	(23) Amygdalin	(24) Arbutin	(25) Esculin	(26) Salicin	(27) Cellobiose	(28) Maltose	(29) Lactose	(30) Melibiose
MGL-1	-	-	-	-	-	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+
MGL-3	-	-	-	-	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+
MGL-8	-	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+
MGL-11	-	-	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-
MGL-12	-	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	-	+

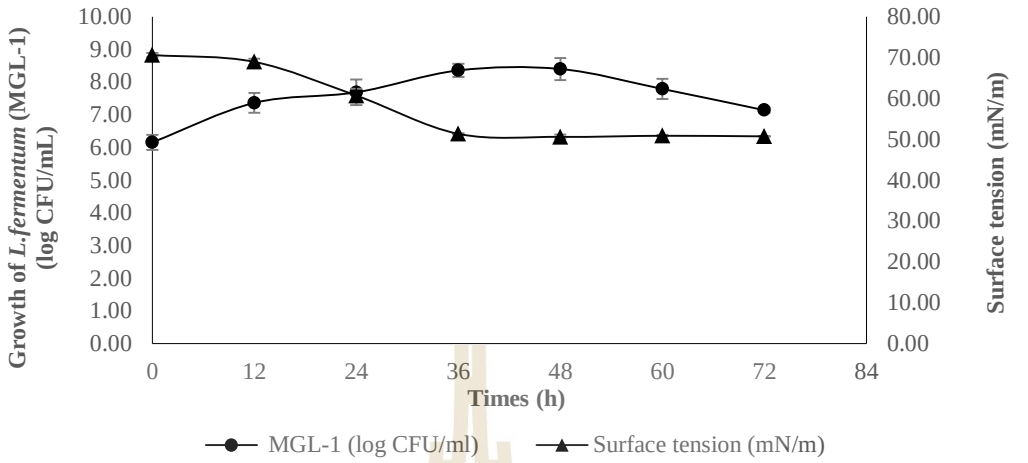
Potential isolates	(31) Sucrose	(32) Trehalose	(33) Inulin	(34) Melezitose	(35) Raffinose	(36) Starch	(37) Glycogen	(38) Xylitol	(39) β-gentiobios	(40) D-turanose	(41) D-lyxose	(42) D-tagatose	(43) D-fucose	(44) L-fucose	(45) D-arabitol	(46) L-arabitol	(47) Gluconate	(48) 2-kato-gluconate	(49) 5-keto-gluconate	Identified species	% of similarity
MGL-1	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*Lactobacillus fermentum*	94.1%
MGL-3	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	*Leuconostoc mesenteroides*	99.9%
MGL-8	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	*Lactobacillus plantarum*	99.9%
MGL-11	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	*Lactococcus lactis* ssp *lactis*	98.5%
MGL-12	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	*Lactobacillus plantarum*	99.9%

+ : positive reaction, - : negative reaction

3.4.5 The potential biosurfactant producing LAB isolates

The results in Fig 3.2 demonstrated the viable cell growth (log CFU/ml) of each potential LAB strains and surface tension of the MRS medium with glycerol 1% (w/v) and sucrose 2% (w/v) as carbon source. BSF production was directly proportional to cell growth as the surface tension decreased with increasing cell density. Especially, *Lactobacillus plantarum* MGL-8 (Fig 3.2 C) indicated the lowest surface tension (39.14 mN/m) and highest cell viability (9.5 log CFU/mL) was obtained at 36 h. Finally, suggested that the increase in extra-cellular biosurfactant concentration might be the result of the cell division of LAB at the end of logarithmic phase to the stationary phase, and including cell-bound biosurfactant molecules released into the broth medium. Moreover, support data was described by Mouafo et al (2018), indicated the potential of three indigenous bacterial strains (*Lactobacillus delbrueckii* N2, *Lactobacillus cellobiosus* TM1, and *Lactobacillus plantarum* G88) for the production of biosurfactants using sugarcane molasses or glycerol as substrates was investigated high surface tension reduction from 72mN/m to values ranged from 47.50±1.78 to 41.90±0.79mN/m. Therefore, *L. plantarum* MGL-8 showed the prototype model of 1% glycerol and 2% sucrose as a carbon source for fermentation at 36 h. was sufficient for biosurfactant production and could be applied for over-ripe mango as a majority substrate.

A



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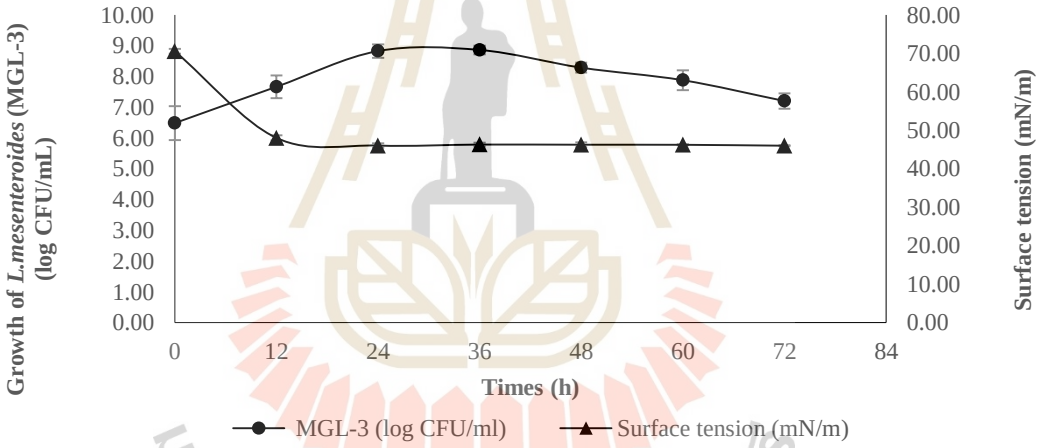

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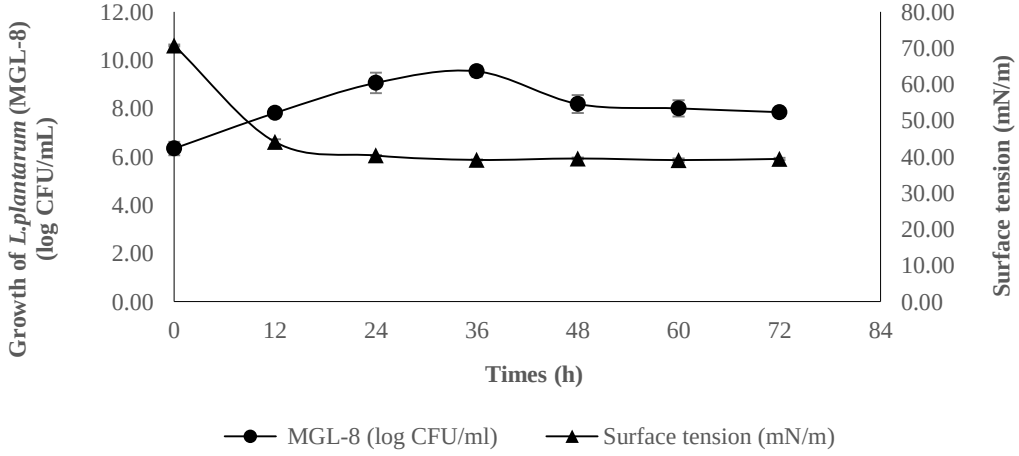

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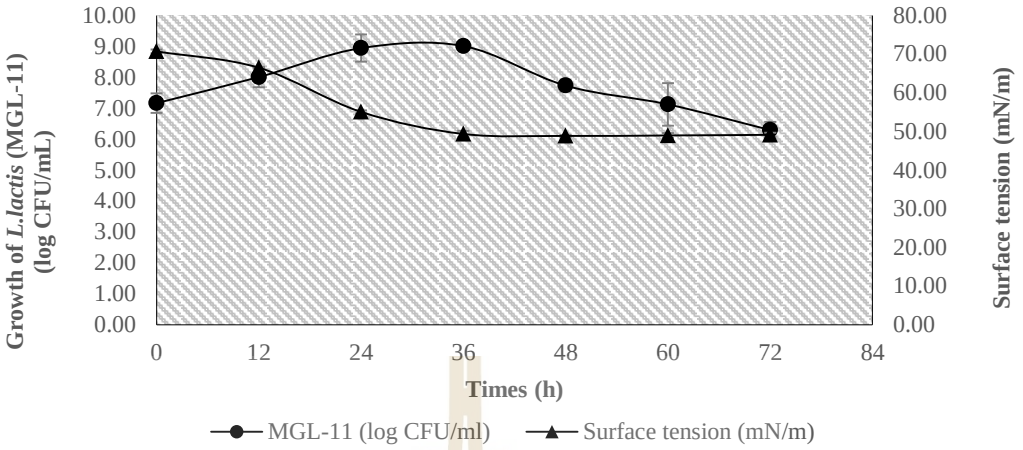

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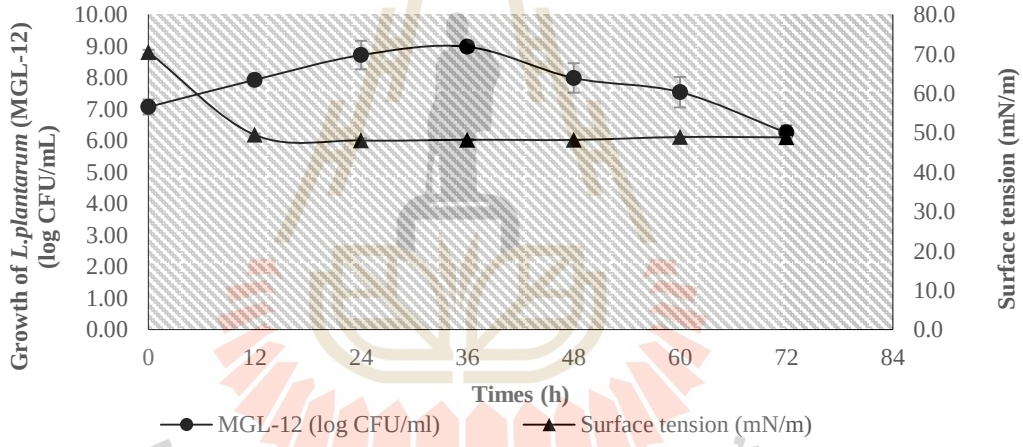

Figure 3.2 Temporal changes in surface tension during growth of *L. fermentum* MGL-1 (A), *L. mesenteroides* MGL-3 (B), *L. plantarum* MGL-8 (C), *L. lactis* MGL-11 (D) and *L. plantarum* MGL-12 (E), in MRS condition supplemented with glycerol and sucrose as a carbon source. Data represent means±standard deviations of quantitative triplicate quantitative determinations from each sample at a different period fermentation.

3.5 Conclusions

The biosurfactant-producing strain LAB was isolated from indigenous over-ripe Nam Dok Mai mango. At present, isolate (*L. plantarum* MGL-8) can be produced BSF and antibacterial against *L. monocytogenes*, thereby decreasing the surface tension of the culture medium to 39.14 mN/m after 36 h of its growth. Moreover, it was able to utilize glycerol and sucrose as a carbon source for promoting the growth. Thus, biosurfactant-producing *L. plantarum* MGL-8 processed characteristics of *L. monocytogenes* inhibition, would be taken to prototype model condition (1% glycerol and 2% sucrose) for upscale in fermenter using over-ripe mango substrate.

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CHAPTER IV

Lactobacillus plantarum MGL-8 GROWTH AND BIOSURFACTANT PRODUCTION PROFILE IN OVER-RIPE MANGO JUICE AS A SUBSTRATE IN SUBMERGE FERMENTATION

4.1 Abstract

The BSF producing *L. plantarum* MGL-8 was isolated from over-ripe Nam Dok Mai mango, which was a major substrate in the fermentation process. In order to produce a good quality and effective product, a must controlling of significant key factors which were agitation speed (100 rpm), aeration rate (1 vvm) and temperature (35ºC), including substrate in ratio 1:3 (over-ripe mango juice), whereof adding glycerol (1% w/v) and sucrose (2% w/v) with a minimal ratio for achieving the effective biosurfactants. Those conditions had affected LAB co-culture adapted to the new substrate and producing positive effects in reducing the surface tension to 36.8 mN/m from its initial of 71.25 mN/m. At the late exponential state in fermenter, the percentage of E24 increased to over 27% and reach the high level approximately 40.73% at 96 h. as well as antimicrobial properties against *L. monocytogenes* indicated non-significant difference during fermentation time at 96 and 120 h. Therefore, under optimum conditions combined with *L. plantarum* MGL-8 fermented as a starter culture for producing BSF was enhanced from 1.82 g/L to 4.22 g/L. In

consequence, the optimization procedure by LAB was expected to improve the biosurfactant production in upscale and its implementation and this BSF could be used as a sanitizer in washing fresh produce to minimize *L. monocytogenes* hazard.

Keywords: LAB fermentation, implicit growth factors, biosurfactant producing profile, kinetic growth

4.2 Introduction

A BSF is an amphipathic molecule that can be classified into two different classes, low and high molecular weight BSF. Low molecular weight are generally glycolipids, such as rhamnolipids or lipopeptides, whereas the high molecular weight BSF encompass amphipathic polysaccharides, lipopolysaccharides, proteins, and lipoproteins (Vijayakumar and Saravanan, 2015). Recently, BSF was more interesting than synthetic surfactant, because of the broad antimicrobial properties including low toxicity which could be applied in pharmaceutical, cosmetic and food industry (Vecino, Cruz, Moldes and Rodrigues, 2017). Synthetic surfactant process such as fermentation medium synthesis, extraction, recovery and downstream process are high cost. Nowadays, the use of cheaper, renewable substrates from various industries such as agricultural (sugars, molasses, plant oils, oil wastes, starchy substances, lactic whey), industries have been reported and reviewed thoroughly by several researchers (Marchant, Funston, Uzoigwe, Rahman and Banat., 2014). However, large scale production for the most microbial surface-active agents have not reached a satisfactory economic level due to the low yields.

Generally, three types of carbon sources being commonly used in BSF productions; starch, plant sugar-based carbohydrates and simple sugar. In view of

glucose, is the typical example of the carbon source which can easily be metabolized by microorganisms through the glycolysis pathway for the generation of energy and is commonly reported to give higher yield of product (Ghribi and Ellouze-Chaabouni., 2011; Tomar and Srinikethan., 2016). For example, *P. aeruginosa* MTCC 7815 utilized glucose much better than glycerol, fructose, and starch, to yield higher amounts of BSF, and emulsification index (E24) approximately 76.77%, and exhibited 34.53 mN/m of surface tension. While produced by *Klebsiella* sp. RJ-03, that shown the sucrose was to be used as a carbon source, which provided the BSF the highest yield (Tomar and Srinikethan., 2016). In term of performance, *L. paracasei* produced the BSF with the highest surface tension reduction of water (27.3 mN/m), when grown in lactose-based medium with vineyard pruning waste (Vecino et al., 2017). When industrial waste was applied for a carbon and nitrogen source, it found that the yield of the protein-lipid-carbohydrate complex was 4.5 g/L, with a reduction in the surface tension of distilled water from 71 to 32 mN/m. A very low yield was found when only either glucose or vegetable oil was used for the BSF production by *T. bombicola*, but the yield increased to 70 g/L while both carbon sources were provided together (Sarubbo, Farias and Campos-Takaki, 2007). It suggested that carbon source plays an important role in the growth and production of BSF by microorganisms, while the nitrogen source is the second supplement which the excessive nitrogen leads to the synthesis of cellular material and limits the buildup of products. Depending upon the nature of the BSF and the producing microorganisms, several patterns of biosurfactant production by fermentation are possible. For exceptional, the BSF released by lactobacilli is maximum for cells at the stationary phase and be stimulated by growing the microbial cells under growth-limiting conditions (Rodrigues, Moldes, Teixeira and

Oliveira, 2006). Thus, the implicit factors can be either spontaneous or induced by the variations of aeration, agitation speed and temperature, to which stress on cell differently. In the case of multiple-strain culture fermentation, the physicochemical characteristics of BSF were related in the use of those strain under growth-limiting conditions including specific carbon and nitrogen supplementation. A consequence of the environmental factors, being the chemical composition complexity derived from LAB off the qualitative and quantitative of the final product. The comprise of different components depending on the type of strain: glycolipids, glycoproteins or multi-component mixtures of proteins and polysaccharides associated with phosphate groups (Sharma, Saharan and Kapil, 2016; Sambanthamoorthy, Feng, Patel, Patel and Paranavitana, 2014). Nonpahogenic and safe microorganisms, in the principle, are used in BSF production. In fact, the exploitation in large-scale industrial processes are troublesome of pathogenic microorganisms. In addition, cheap substrates are needed, but high production yield, in order to decrease the overall costs fermentative processes. In other words, effective BSF production could develop economic system of which making low-cost materials and controllable to produce the most functionality and high yield in finish product.

To achieve the high quality and quantitative of BSF, the alternative substrate for the potential fermentative LAB to utilize carbon and nitrogen source under fermentation measurement control were performanced. The over-ripe mango was considered to be the natural substrates implemented with specific carbon and nitrogen sources for enhancing the potential functional characteristics of LAB. Therefore, the antimicrobial against *L. monocytogenes* and some structure properties of the synthesized BSF were determined.

4.3 Materials and Methods

4.3.1 Sampling and preparation of LAB fermentation

To produce biosurfactant by *L. plantarum* MGL-8, over ripe Nam Dok Mai mango, represent are agro-waste substrate was applied from the Khlong Yang Ltd., Part., Saraburi province, Thailand. Washing was done twice with tap water, soaking for 5 min following by air dried for 45 min. After, separated into pulp and peels, then did do the size reduction and blended to become mango paste, weigh in ratio 1:3 w/v (mango paste: deionized water), the mixture called mango juice (MJ) was transferred aseptically in fermenter.

4.3.2 Inoculum preparation of *L. plantarum* MGL-8

Inoculum was prepared from a subcultured *L. plantarum* MGL-8 in MRS broth and incubated for 36 h at 35ºC. The inoculum size 10 % (v/v) consisted of approximately 10^8 CFU/mL. The inoculum was transferred into sterilized fermenter (BioFlo 115 fermenter, New Brunswick Scientific; USA).

4.3.3 Batch submerged fermentation

To gain the biosurfactant production by using overriped mango juice (MJ) from 4.3.1 as a substrate, and enhanced by 1% (w/v) glycerol and 2% (w/v) sucrose without inoculum, but multi-strains (microflora) culture **(MC-MJ)**. In case of BSF production from *L. plantarum* MGL-8, all conditions were as the same as MC-MJ condition but inoculum 10% (v/v) approximately 10^8 CFU/mL *L. plantarum* MGL-8 **(L-MJ)**. And for the positive control condition was used MRS broth with adding 1% (w/v) glycerol and 2% (w/v) sucrose, and inoculum 10% (v/v) approximately 10^8 CFU/mL *L. plantarum* MGL-8 **(L-MRS)**. All conditions were carried out by fermenter, and controlled parameters at 35ºC, agitator speed 100 rpm,

aeration rate 1 vvm and dissolved oxygen rate; DO rate 100%, fermentation times for 5 days. The control parameter of fermenter used in this experiment, as evidenced by previous studies.

4.3.4 Kinetic growth rate and metabolites of *L. plantarum* MGL-8

The kinetic growth and environment parameters measurement were determined as follows: enumeration of lactic acid bacteria, total bacteria, yeast & mold, surface tension, emulsification index, and antimicrobial properties. Ten milliliters of each samples were collected at the interval 24 h for 120 h.

4.3.4.1 Enumeration of LAB

Ten milliliters of each sample were made by the tenfold dilution with 0.1% peptone (pH 7.2) decimal dilutions were prepared and transferred to plates with a specific MRS agar plates (pH 6.5). The plates were incubated at 35ºC for 48 h under anaerobic jar (BAM, 2001).

4.3.4.2 Enumeration of total viable bacteria

The samples of multi-strain cultures in mango juice (MC-MJ) and inoculated *L. plantarum* MGL-8 (L-MJ) were determined for total viable counts (TVC), from diluted samples (from 4.3.4.1). An aliquot of 0.1 mL of each the diluted samples was spread onto NA plates, and incubated at 37°C for 24 h. After incubation, colonies were enumerated as colony forming units per milliliter (CFU/mL).

4.3.4.3 Enumeration of Yeast & Mold

The diluted samples (4.3.4.1) were analyzed for total yeast and mold numbers. An aliquot of 0.1 mL of diluted samples (MC-MJ and L-MJ, respectively) were spread onto PDA with 10% (v/v) tartaric acid**,** all the plates were incubated at 25ºC for 72 h.

4.3.5 Quantitative test of biosurfactant

4.3.5.1 Surface tension

Biosurfactants produced by *L. plantarum* MGL-8 and multi-strains cultures fermentation conditions (MC) were determined by measuring the surface tension (ST) of the cultured supernatant. In case of the excreted BSF, the fermentation medium was centrifuged (10,000 g, 20min, 4ºC) and filtered using a Syringe filter 0.2 µm (Thermo Scientific™ Nalgene™ 25mm Syringe Filters, Sterile and non-pyrogenic). ST of sample was measured by the Ring method (Kim et al., 2000) using a Tensiometer equipped with the platinum ring (4 cm diameter) De Nouy platinum ring at the same condition in 3.3.7.2. About 10 mL sample was withdrawn every 24 h and surface tension was measured.

4.3.5.2 Emulsification assays

The emulsification index (E24) was calculated as an indicator for BSF production, according to Abouseoud et al (2008) method. The E24 was determined by adding 2 mL of hydrocarbon (palm oil), then add 2 mL of the sample was prepared according to surface tension measurement (4.3.5.1). The mixture was then vigorously vortexed for 2 min, and allowed to stand for 24 h without any further shaking. After 24 h, emulsification index (%E24) was calculated as the ratio of the height of the emulsion layer and the total height of liquid by measuring the emulsion layer form follow the formula below. Distilled water and a SDS 5% (w/v) were used as negative and positive control, respectively.

$$\text{Emulsification index (\%E24)} = \frac{\text{volume of emulsified (cm)}}{\text{total liquid volume (cm)}} \times 100$$

4.3.5.3 Antimicrobial properties

The disk diffusion method was used to evaluate antimicrobial activity of each supernatant, according to 3.3.5.2, collected supernatant by centrifugation at 4°C 10,000 x g for 20 min, and sterilized by sterile syringe filter pore size 0.2 µm. The surrogate pathogen, *L. mocytogenes* cultured in TSB, then collected the cell precipitate by centrifugation at 4°C 10,000xg for 10 min. The optical density of cell suspension was adjusted to 10^6 cells/mL, and swabbed cell suspension on Mueller- MHA plates. After that, loaded over sterile filter paper discs (8 mm in diameter) on MHA plate, and drop 30 µL of each isolate cell free supernatant isolates at equal distance with positive control (5% v/v of SDS and rhamnolipids), negative control (sterile distilled water). The plates were incubated at 37°C for 24 h, the presence of inhibition zones was measured by Vernier caliper, recorded and considered as indication for antibacterial activity.

4.3.6 Extraction of biosurfactant

The metabolites were extracted from the supernatants using the acid precipitation according to the method described by Morais et al. (2017). Harvesting and collection the metabolites in supernatant was done by centrifugation at 4°C 10,000 x g for 20 min, and sterilized by sterile syringe filter pore size 0.2 µm. The pellet (1L) was adjusted to pH 2.0 with 1 M HCl, the acidified samples were further incubated at 4°C for 2 h., then the precipitates were collected by centrifugation (10,000×g for 15 min at 4°C), and washed twice with the distilled water. The precipitates were dissolved in distilled water and adjusted to pH 7.0 with 1 M NaOH. It was, then, frozen at -50ºC overnight and desiccated under a vacuum (0.001 mbar) in the freeze-drier (Christ Gamma 2-16 LSC) at the final temperature 35ºC for 48 h.

Finally, the dried crude BSF was collected, weighed, and stored at room temperature.

4.3.7 Statistical analysis

All quantitative data were done in triplicate, and the results were expressed as the mean ± standard deviation (SD). IBM SPSS statistics 26 (Armonk, New York, U.S.A.) was used to perform all statical analysis. Data were analyzed by one-way ANOVA using Duncan's Multiple Range Test (DMRT) at a significance level of $p<0.05$.

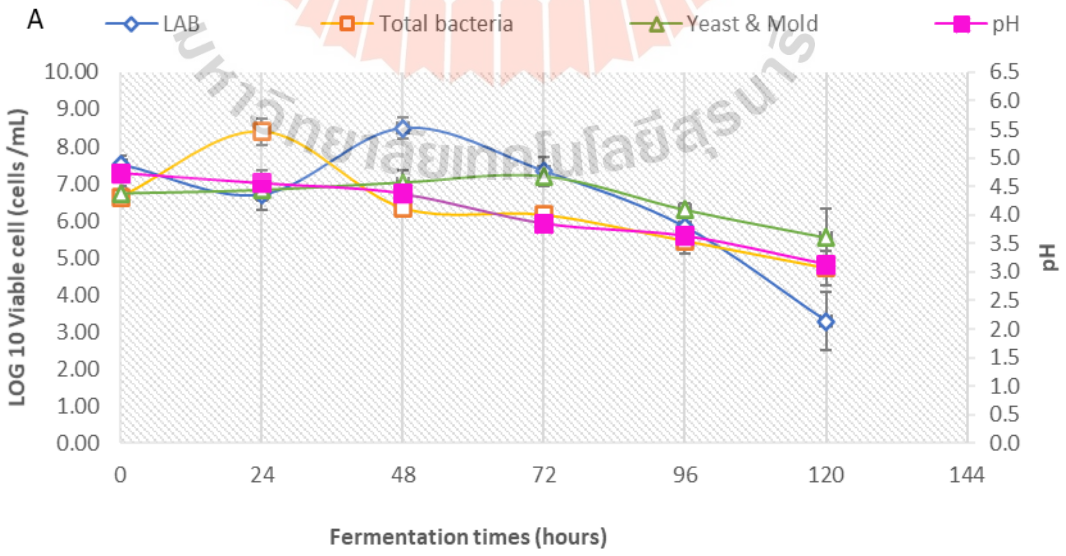
4.4 Results and discussions

4.4.1 *L. plantarum* MGL-8 kinetic growth and biosurfactant production

Growth kinetics were obtained from the three conditions of biosurfactant-producing multiple-strain cultures in mango juice (MC-MJ), *L. plantarum* MGL-8 in MRS (L-MRS) and *L. plantarum* MGL-8 in mango juice (L-MJ). Figure 4.1 B showed, the maximum growth was found at 24 h with approximately 9.5 log CFU/mL. However, a small number dropped at 48 h about 8.7 log CFU/mL, and decreased reaching counts between 4 and 5 log CFU/mL within 120 h. It indicated that *L. plantarum* MGL-8 still growing quite well as the same condition as previous proved. It was, then, would be the growth reference for considering of which MC-MJ (Fig 4.1 A) and L-MJ (Fig 4.1 C) did. Thus, fig 4.1 (A) shows multi-strains (LAB, total bacteria and yeast & mold) provided different maximum growth at independent fermentation period. However, the wild-type LAB showed the maximum growth at 48 h with the approximately 8.5 log CFU/mL, in which *L. plantarum* MGL-8 growth in MRS did (Fig 4.1 C), the count number 9 log CFU/mL at 24 h. Furthermore, the acid production and other second metabolites increased after 48 h to

120 h, which pH was the range of 3.5-4.5. The pH at the 120 h of *L. plantarum* MGL-8 in all conditions were closed value, pH 2.95. It was no doubt that the predominant microbes in overriped mangoes was LAB, and its survival was higher than yeast and mold. Then, the metabolites from the mango juice fermentation come from the synergism or antagonistic relationship between microorganisms concerned. Furthermore, the corresponding to the adaptation phase of the current physical-chemical composition would occur at the stationary phase, which observed the pH during fermentation up to 120 h., gradually decreased of 3.1, 2.95 and 3.6 of MC-MJ, L-MJ and reference LAB in MRS (L-MRS) respectively.

This indicates that the multi-strains cultures were stressed and adapted to the alternative substrate and promoted the biosynthesis of essential compounds to the growth, and produced positive effects in reducing the surface tension correlated with BSF concentration. According to Rufino et al. (2008), each microorganism adapted a specific pH of each type of BSF.



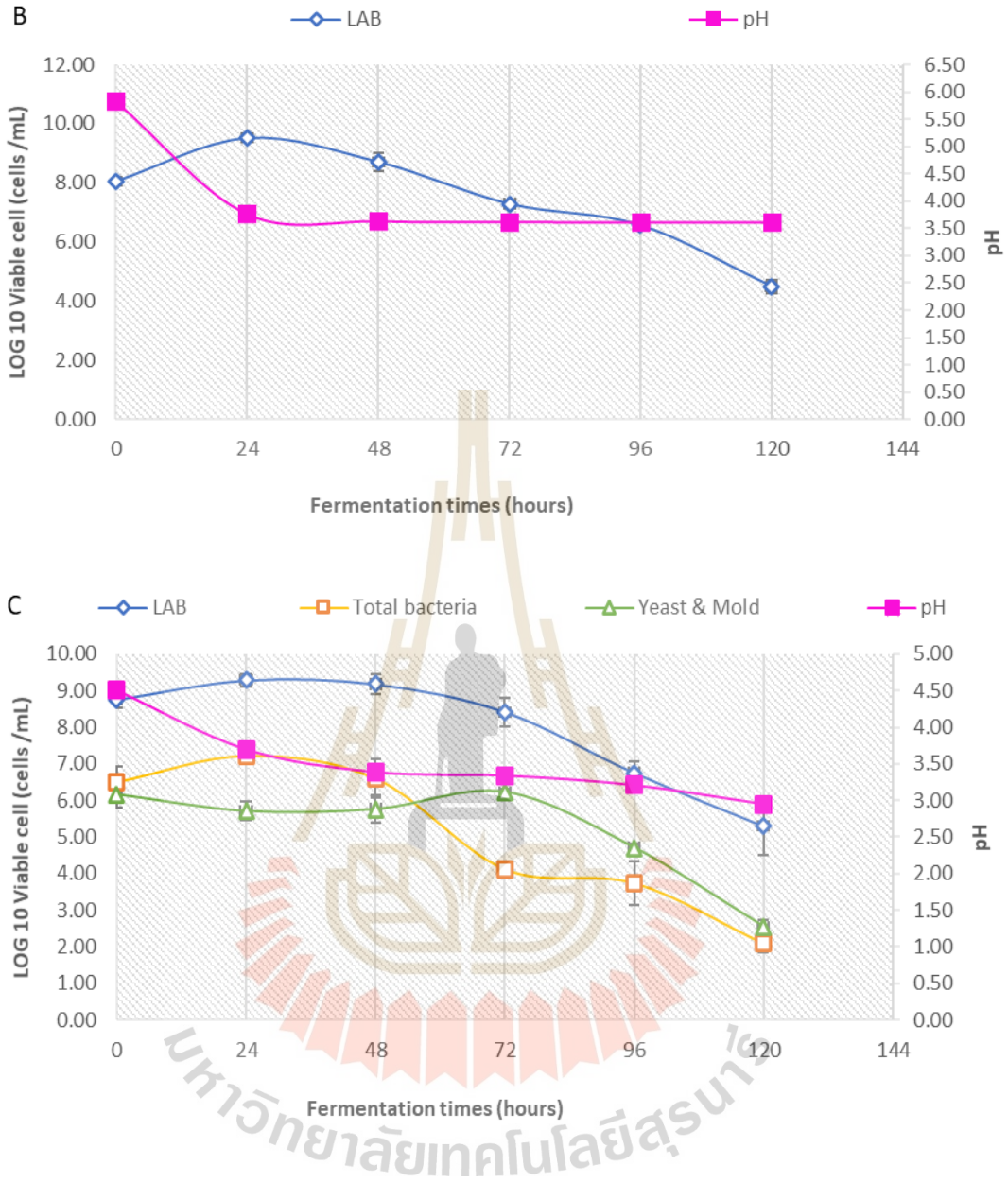


Figure 4.1 Comparison effect growth kinetic pattern of multi-strain cultures in mango juice (MC-MJ) (A), *L. plantarum* MGL-8 in MRS broth (L-MRS) growth control (B) and *L. plantarum* MGL-8 in mango juice (L-MJ) (C). All control and treatment medium were supplemented with 1% (w/v) glycerol and 2% (w/v) sucrose. All data points represented the mean of triplicate values, and expressed as the mean ± standard deviation (SD).

4.4.2 The relation of growth kinetic and biosurfactant characteristics

4.4.2.1 Surface tension

In this work, medium (over-ripe mango juice) was trialed, which hopefully expected low cost and high yield of BSF compared with the synthetic medium. Whereas using the least amount of supplements synthetic carbon sources such as glycerol and sucrose would enhanced production. It found that under stressed conditions in the mango juice substrate with the extra carbon source, the predominant LAB multi-strains exhibited survival response activity, which affect the physiochemical change such as microbial population, growth rate, acid production accumulated and/or pH, and biosurfactant compound. These factors resulted in supported producing positive effects in reducing the surface tension, correlated with BSF concentration as shown in Fig 4.2. When compared the growth curve as a previous study, it found that after maximum growth rate at 24 h, the surface tension value of *L. plantarum* MGL-8 in MRS broth (L-MRS) was reduced significantly, the value gradually changed and stable at 120 h., was approximately 38.2 mN/m. In view of *L. plantarum* MGL-8 in mango juice (L-MJ) showed that the surface tension was reduced to 36.8 mN/m (at 120 h.) from it initial of 71.25 mN/m ($p<0.05$), and gradually decreased and stable at the end of the fermentation process. In addition, *L. plantarum* MGL-8 in mango juice (L-MJ) revealed the significant surface tension reduction at 120 h. approxymately 36.8 mN/m, which lower than that of 96 h. (ST approxymately 39.8 mN/m). The low surface tension corresponds to the high accumulate of excreted surfactant into the medium. Therefore, under the stressed under a new substrate (mango juice) due to adaptation phase of the current physical-chemical composition, resulting metabolites synthesis delayed and became of crude BSF accumulated. Thus, the high yield of BSF production

might be correlated with the reduction of surface tension.

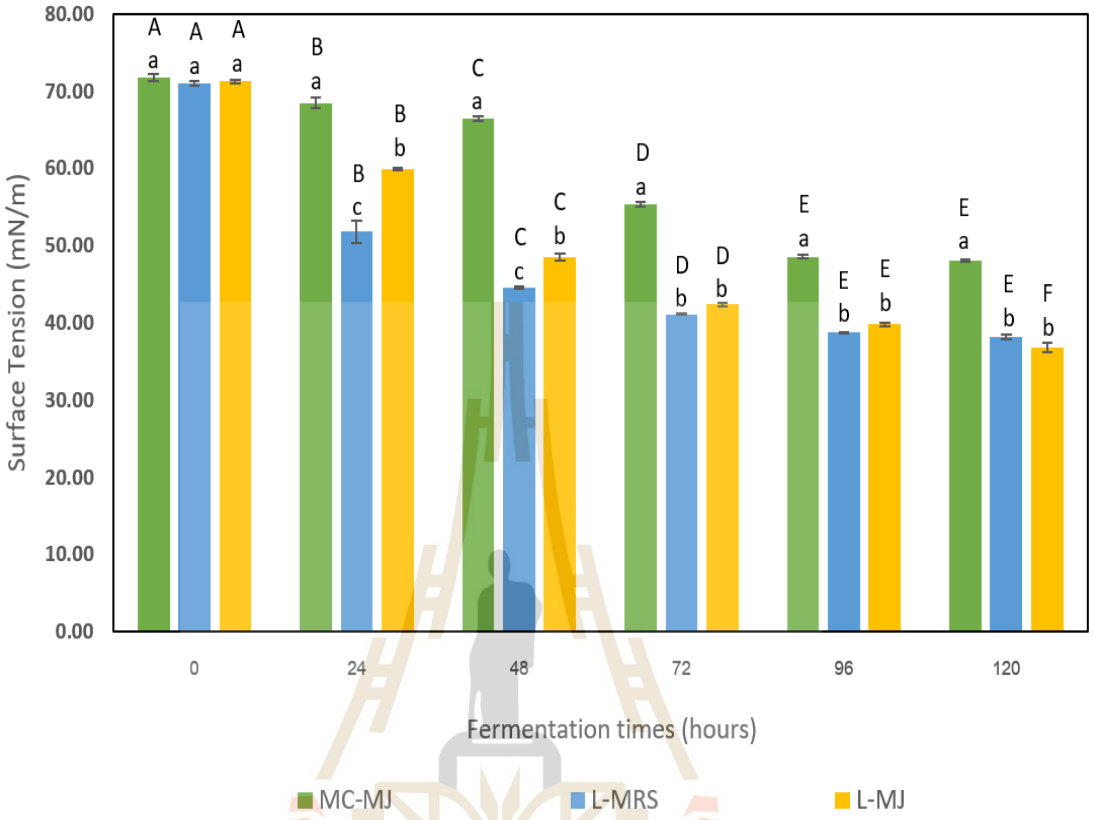


Figure 4.2 The surface tension variation during fermentation carried out with multi-strain cultures in mango juice (MC-MJ), *L. plantarum* MGL-8 in MRS (L-MRS), and *L. plantarum* MGL-8 in mango juice (L-MJ). All control and treatment medium were supplemented with 1% (w/v) glycerol and 2% (w/v) sucrose. Values are expressed as the mean ± standard deviation (SD), and the different big letters A, B, C, D, E, and F, at the same conditions (MC-MJ, L-MRS, and L-MJ respectively) within different period fermentation indicate significant differences ($p<0.05$). While small letters, a, b and c, at the same fermentation time indicate significant differences ($p<0.05$).

4.4.2.2 Emulsification activity

The emulsification activity was determination biosurfactant production and their characteristics (Harshada, 2014). As previously studied indicated the MC-MJ and L-MJ gave the best surface tension value at 120 h. When considering the %E24 (emulsification activity) exhibited the best value of %E24 at 40.73% in L-MJ, which tendency like the surface tension value was observed at fermentation 120 h. Therefore, it confirmed that the *L. plantarum* MGL-8 properties for using mango juice as a new substrate. Moreover, when considering L-MJ, these metabolites cumulative production since 72 to 120 h indicated the %E24 were non-different significantly ($P>0.05$). Demonstrated the relationship between the ability to reduce the surface tension and emulsification activity increased, while a high percentage of emulsification activity represented the capacity of emulsification with a hydrocarbon surface-active molecule. Thus, it could be confirmed the *L. plantarum* MGL-8 properties for using mango juice as a new substrate, along with the using mango juice as the main substrate, it was found BSF produced similarly fermentation time as the same L-MRS (positive synthetic medium), but the BSF yield would increase accordingly. In conclusion, LAB wild type synergism along with yeast and mold, to where occurred the stress response, and the LAB potential producing BSF with a small number when compared to the inoculated *L. plantarum* MGL-8 in mango juice (L-MJ). For the reason, a new substrate mango juice, extra as a carbon and nitrogen source could be a potential producing BSF within spontaneous fermentation.

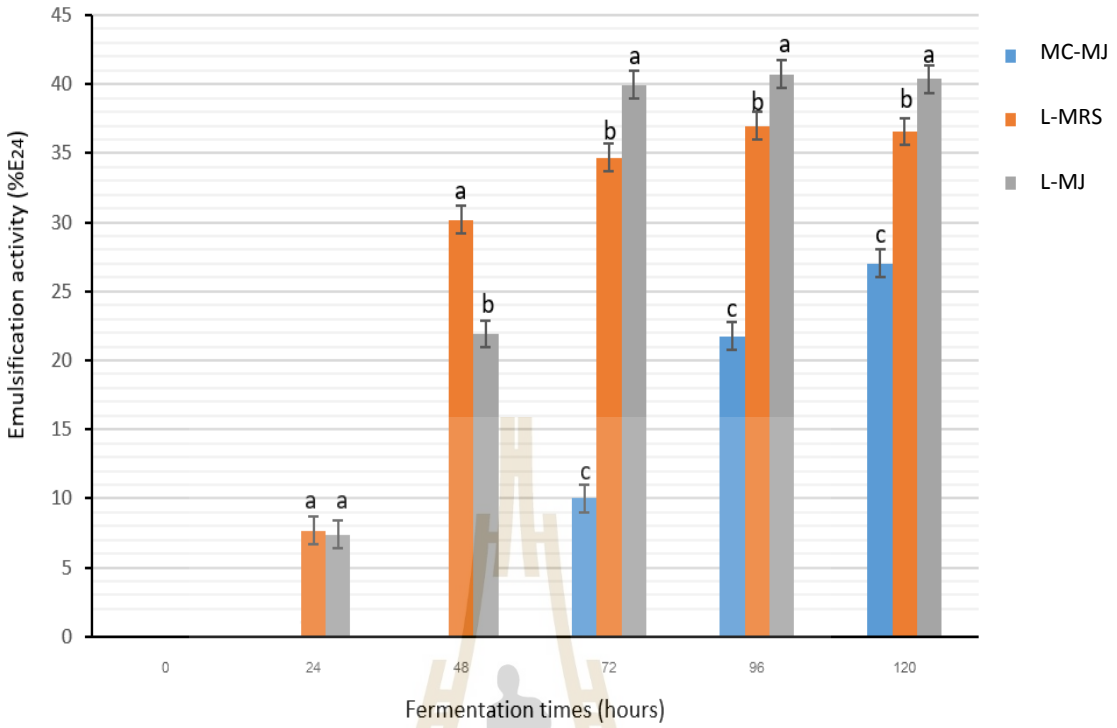


Figure 4.3 The percentage of emulsification activity during fermentation carried out with multi-strain cultures in mango juice (MC-MJ), *L. plantarum* MGL-8 in MRS (L-MRS), and *L. plantarum* MGL-8 in mango juice (L-MJ). All control and treatment medium were supplemented with 1% (w/v) glycerol and 2% (w/v) sucrose. Results represented the average of three independent experiments and the different letters at the same fermentation time indicate significant differences ($p<0.05$).

4.4.2.3 Antimicrobial properties

The *L. monocytogenes* obtained significant *sigB* gene (detergent-resistant) inhibition efficiency of the BSF, produced different during fermentation, as shown in Fig 4.4. Results of producing BSF characteristic by L-MRS and L-MJ were capable for antimicrobial activity, of which it can be observed since

48 h to 120 h. For L-MRS indicated at the 96-120 h fermentative providing BSF performance against inhibitory with non-different significantly. While L-MJ suggested mainly inhibition zone in highly effective against *L. monocytogenes* found at 120 h. Therefore, the results demonstrated surface tension, emulsification activity and antimicrobial properties of L-MJ, it would be considered at 120 h.

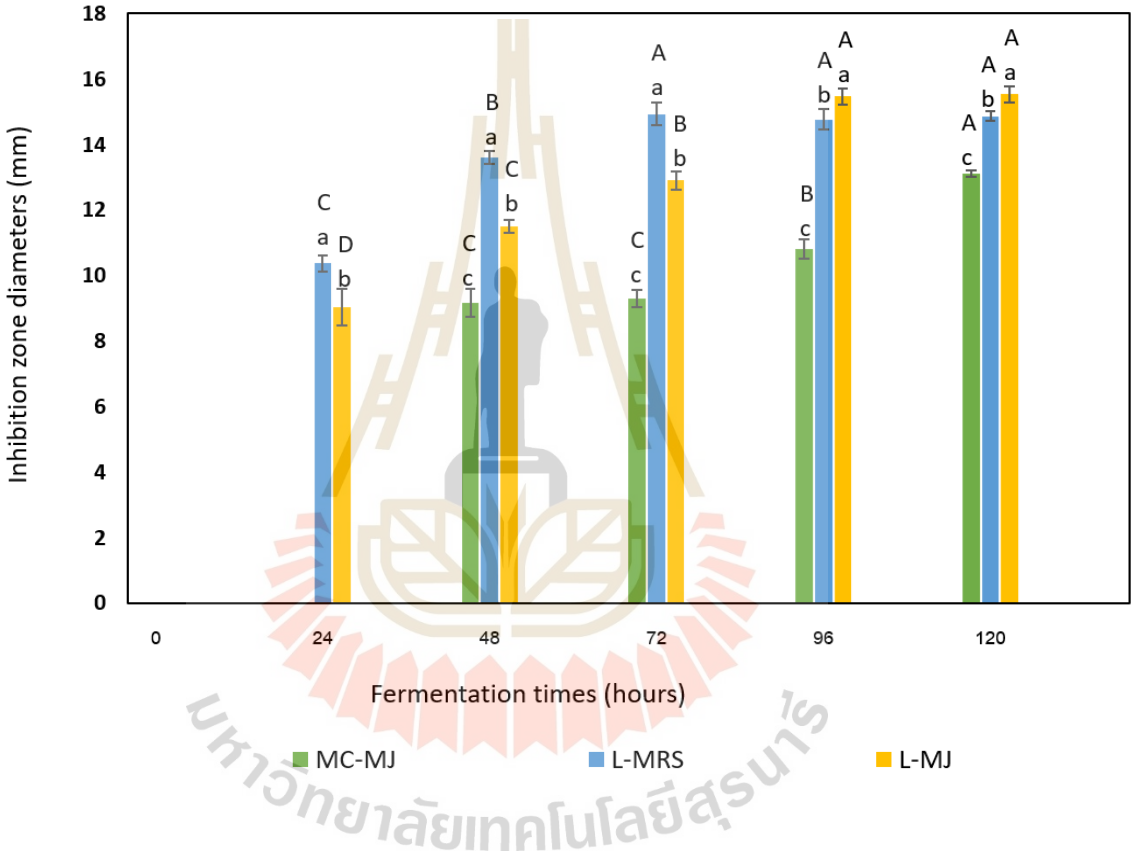


Figure 4.4 Antimicrobial activity against *L. monocytogenes* carried out with multi-strain cultures in mango juice (MC-MJ), *L. plantarum* MGL-8 in MRS (L-MRS), and *L. plantarum* MGL-8 in mango juice (L-MJ). All control and treatment medium were supplemented with 1% (w/v) glycerol and 2% (w/v) sucrose. Values are expressed as the mean ± standard deviation (SD), and the different big letters A, B and C, at the same conditions (MC-MJ, L-MRS, and L-MJ respectively) within different period fermentation

indicate significant differences ($p<0.05$). While small letters, a, b and c, at the same fermentation time indicate significant differences ($p<0.05$).

4.4.3 Crude biosurfactant

Overall, submerged fermentation for BSF production by *L. plantarum* MGL-8 at 120 h, those conditions were an optimized for producing BSF, table 4.1 reveals the actual dried crude BSF approximately 4.22 g/L of SFL production (submerged fermentation using *L. plantarum* MGL-8), which was found to be more prominent than 1.82 g/L of CSF (conventional submerged fermentation), and the difference was statistically significant ($p<0.05$). According, this condition will be preliminary condition for upscale in fermenter by using mango substrate.

Table 4.1 Crude biosurfactant production of *L. plantarum* MGL-8 in mango juice with the supplement and selective medium (MRS medium).

Biosurfactant producing condition	Dry crude (g/L)
Multi-strain cultures in mango juice (MC-MJ)	1.82 ± 0.03^{c}
L. plantarum MGL-8 in MRS broth (L-MRS)	3.08 ± 0.12^{b}
L. plantarum MGL-8 in mango juice (L-MJ)	4.22 ± 0.13^{a}

All control and treatment medium were supplemented with 1% (w/v) glycerol and 2% (w/v) sucrose. Results of three replicates were presented as average values ± standard deviation. Average values in the column with different superscript letters were significantly different ($p<0.05$).

4.5 Conclusions

These studies demonstrated that the by *L. plantarum* MGL-8, can utilized for over-ripe mango as an alternative substrate for the biosurfactant production and enhanced by the addition of 1% (w/v) glycerol and 2% (w/v) sucrose under suitable control measure parameters in fermenter (at 35ºC, agitator speed 100 rpm, aeration rate 1 vvm and dissolved oxygen rate 100%). Moreover, *L. plantarum* MGL-8 could be a potential BSF producing starter culture within submerged fermentation at 120 h., since its characteristic of which survival in the low pH, resulting in positive production of BSF and antimicrobial against *L. monocytogenes*.

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CHAPTER V

THE INFLUENCES OF CRUDE BIOSURFACTANT STRESS ON *Listeria monocytogenes* TARGETS AND WASHING IMPLEMENTATION DURING FRESH PRODUCE STORAGE

5.1 Abstract

As proved previously in chapter IV, dried crude BSF producing strain *L. plantarum* MGL-8 in mango juice (L-MJ) was to determine the effect of dried crude BSF induced sublethal oxidative stress till to adaptations in *L. monocytogenes* (sanitizer resistant; *sigB* gene), and evaluated the mode of action at different targets, including to assess the washing effectiveness of crude BSF as a sanitizer. The result indicated exposure to the sublethal stress induced by crude BSF (350 µg/mL; MBC Normal cell), and adapted cell was 450 µg/mL (MBC; Adapted cell), this illustrated the dried crude BSF function to stress a resistance and tolerance in *L. monocytogenes*. Therefore, those concentrations combination with exposure time was conducted, resulting in 350, 400 and 450 µg/mL BSF with exposure time for 20, 20 and 10 min, respectively. It was demonstrated the responsibility of its antimicrobial action by the major function via cell membrane composition of *L. monocytogenes*. The BSF molecules attached to reduce the surface tension of lipid bilayer, loss of membrane fluidity made cell membrane disintegration due to engendering higher cell

permeability leading, lysis and death. Accordingly, all BSF conditions were washing implementation, suggested that soaking with 400 and 450 μg/mL BSF for 20 and 10 min, damaged *sigB* gene, and sufficient to control the survival rate of these pathogens on Chinese kale produce during storage at 12 and 5ºC for 14 days as well as 75 ppm $CaClO_2$ (commercial sanitizer). Furthermore, this washing procedure prototype would further practically imply to SSOP (sanitation standard operating procedure) in food safety management.

Keywords: crude biosurfactant as sanitizer, microbial hazard risk, fresh produce-washing, cold stress response, *sigB* gene, *Listeria monocytogenes*, storage safety

5.2 Introduction

Sanitizing agents have been used for the cleaning and washing of raw food materials and agricultural produce, as well as clean in place (CIP) and clean out of place (COP), which occur at all food industries. Their substance properties should include being water soluble, non-corrosive and non-toxic, while enabling antimicrobial activity, the disinfection of food-borne pathogens, and the reduction of undesirable substances. Thus, FDA has reported the sanitizers that are allowed to be used in food such as sodium hydroxide, acetic acid, polyethenoxyethers condensates and proteolytic enzymes. Parish et al. (2003) presented that the synthetic cleaning agents of hypochlorite (50-100 ppm) and acidified sodium (500-1200 ppm) were used in fruit and vegetable washing, but these are still considered carcinogenic for humans. In view of synthetic surfactants, which a popular trend applied in the food industry as a sanitizer, the compounds that are amphiphilic, containing both hydrophobic groups

and hydrophilic groups and can be categorized as anionic, cationic, nonionic, amphoteric and hydrotropes (Falbe, 2012). As mentioned, surfactants structure may enhance contact between the sanitizer and microorganisms, thus render improving microbial inactivation (Huang and Nitin, 2017). Moreover, Salgado et al (2014), suggested amphiphilic molecule of sodium dodecyl sulfate (SDS) can be enabled to gain to crevices and cracks in the lettuce, and especially the SDS did not degrade quality compared to chlorine. In addition, poor washing procedure and management system were encouraged the survival of *Listeria* in lettuce to be greater in cold condition (Oliveira, Vinas, Usall, Anguera and Abadias, 2012). For this reason, *L. monocytogenes* has the highest spread contamination in various freshly and ready to eat (RTE) such as Lone et al. (2016) reported the 33-case mortality of patients from Listeriosis disease resulted from the consumption of *L. monocytogenes* contaminated cantaloupes.

Focusing on the *L. monocytogenes* was a frequent detected from the food processing line and the most problematic in fresh produce, RTE and chilled food. Because the ability of *L. monocytogenes* to resist antimicrobial compounds, sanitizer, detergent and to tolerate acidic, high-salt conditions, of which that due to form biofilms, to grow at temperatures below refrigeration temperatures (Van Coillie, Werbrouck, Heyndrickx, Herman and Rijpens, 2004; Little, Sagoo, Gillespie, Grant and McLauchlin, 2009). Studies have shown that the expression of *inlB* gene linked to biofilm formation in *L. monocytogenes* during low temperature, as a result, bacteria can be survived under chilling temperatures (Piercey, Hingston and Hansen, 2016). Similarly, under food processing environments differ in their relative capacity to adapt to sanitizer oxidative stress and cold temperatures, that result in different adaptation,

of which correlated with *sigB* gene expression. The role key *sigB,* encodes the major stress response regulator, in *L. monocytogenes* static and continuous-flow biofilm formation and resistance of biofilm-grown cells to the disinfectants benzalkonium chloride. Moreover, role important of *sigB* gene that correlated to investigating stress response activator biofilm formation and disinfectant resistance as well as peracetic acid, SDS surfactant and nisin (Van der Veen and Abee, 2010). Thus, *sigB* gene is the significant gene and should be invested by the role of this major stress response in *L. monocytogenes* treated with disinfectant and/or sanitizer resistance.

Therefore, the washing system and the correspondent concentration of antimicrobial treatment, including sanitizing agent were used to influence the ability of a given product washing system for preventing cross-contamination and could damaged by sanitizer resistance gene. However, the distribution in the environment of surfactant such as fatty alcohol polyoxyethylene ether (AEO), which may be cause serious pollution and pose a threat to human health (Wang et al., 2015). Dehghan-Noudeh, Housaindokht and Bazzaz (2005) reported SDS, BC (benzalkonium chloride), were maximum hemolytic and toxicity more than biosurfactant produced by *B. subtilis* ATCC 6633.

Biosurfactants are therefore the natural choice for such processes as they possess a lot of advantages over synthetic surfactants. Moreover, they are generally produced from waste and low cost agricultural raw materials by microorganism fermentation, while surfactant properties are also applied in anti-adhesive agents, emulsion forming and emulsion breaking (George and Jayachandran, 2009). Furthermore, especially biosurfactant has demonstrated antimicrobial activity against several microorganisms such as *Staphylococcus aureus, Clostridium perfringens,*

Salmonella Typhimurium, Escherichia coli, and the fungi (Sha, Jiang, Meng, Zhang and Song, 2011). Antimicrobial properties which are the important function to applied as a sanitizing agent for washing fresh produce. The significant mode of action against pathogens cell as follow; Ortiz et al., (2006) explained that the role of rhamnolipid antimicrobial activity related to hypothetical site action with the cell membrane, as they possess an amphipathic nature that allows its interaction with phospholipids. In addition, it suggested that biosurfactant increases in membrane permeability with consequent alteration of this barrier causing cell damage of gram-positive bacteria (Sotirova et al., 2008). In a recent study, they demonstrated that the mechanism of biosurfactant action against *L. monocytogenes* is not yet elucidated but, it is supposed that the cell membrane is the target, because these molecules can increase the permeability of microbial cells (Magalhães and Nitschke, 2013). However, when considered their amphiphilic compound of biosurfactant may demonstrate the effect of adsorbing and diffuse to the cell wall until that leakage and intracellular material denature until final cell death. Therefore, it is possible to apply these as sanitizing agents for washing fresh produce to be safety.

The aim of this study was to prove the efficacy of biosurfactant produce from *L. plantarum* MGL-8 as a sanitizer, in order to observe the mode of action with different cell target including that possibility to damage *sigB* gene (sanitizer resistant gene) of *L. monocytogenes.* To evaluate the possible implementation for washing Chinese kale to minimize the hazardous risk of *L. monocytogenes*, and coss-protective during storage at low-temperature, finally to achieve a good practice prototype of fresh produce washing procedure.

5.3 Material and methods

5.3.1 Antimicrobial properties assay and crude BSF induced stress adaptation in *L. monocytogenes* with significant *sigB* gene

5.3.1.1 Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) determination

The antimicrobial activity was determined by MIC and MBC method (Lana, Carazza and Takahashi, 2006) and performed in TSB medium. The exponential phase of *L. monocytogenes* culture was diluted in TSB to obtain a final concentration of 10^6 CFU/mL for MIC assay. The total available dried crude BSF concentration range of 1000-20 µg/mL were prepared to determine MIC across 10 dilutions (1000, 800, 600, 400, 200, 100, 80, 60, 40, 20 µg/mL), of which added 100 µL of 10^6 CFU/mL into each concentration, all treatments were incubated at 37ºC for 24 h. *L. monocytogenes* inoculum without BSF and with TSB alone were used as a positive and negative control, respectively. Bacterial growth was indicated by turbidity at 600 nm, absence of bacterial growth was interpreted as antibacterial activity, and MIC was taken as the lowest concentration of the crude BSF in broth showing no visible bacterial growth. Consequently, for the MBC was determined by aliquots of 100 µL from previously without visible growth of *L. monocytogenes* were spread plated triplicate onto TSA to determine the minimum bactericidal concentration (concentration of available crude BSF that kills all *L. monocytogenes* cells after 24 h, incubation at 37ºC).

5.3.1.2 Crude BSF induce stress adaptation in *L. monocytogenes*

The sanitizer resistant bacteria and stress-induced adaptation of crude BSF were assessed by the modification method of Bansal et al. (2018). The

L. monocytogenes with BSF resistant was achieved by using the MIC concentration from previous, to evaluate the sublethal by BSF which induced resistance and tolerance. Bacteria cell were prepared follow as 3.5.1.1, then aliquot cell suspension 100 µL, was added to 10 ml of TSB with the initial 200 µg/mL (concentration was obtained from MIC studies) crude BSF and incubated at 37ºC for 24 h, this is modified sublethal BSF adaptation condition. Subsequently 100 µL cell viable with BSF concentration 200 µg/mL after incubated 24 h, it was transferred into fresh TSB with the gradually increasing crude BSF concentrations of 50 µg/mL. Therefore, *L. monocytogenes* adapted cells from ten different sublethal stress levels were obtained at final concentration; at 200, 250, 300, 350, 400, 450, 500, 550, 600 and 650 µg/mL. All concentrations incubated at 37ºC for 24 h, and MIC was taken as the lowest concentration of the crude BSF in broth showing no visible bacterial growth, while the MBC was determined by without visible growth of *L. monocytogenes* were spread plated onto TSA.

5.3.2 Evaluation of the exposure time of BSF on *L. monocytogenes* different stress targets.

To evaluate the suitable condition the inhibit and effect on cell targets to BSF, based on our previous study (Sittisart, Mahisanan and Gasaluck, 2016) the exposure for 10, 20, 30 min were conducted in the present experiment.

5.3.2.1 Cell lysis determination

L. monocytogenes cells were grown overnight in TSB medium at 37°C under shaking conditions at 200 rpm, which cell concentration approximately 10^8 cfu/mL. Subsequently aliquot 1.5 mL (each treatment) to harvesting cell by centrifugation (4,500 xg, 15 min, 4ºC), cells were washed twice and resuspended in

peptone buffer (pH 7.4) with equal volume (1.5 mL). The effects of crude BSF at various concentration 350, 400 and 450 µg/mL (cell untreated use for control experiment) were then added to the cell suspension and incubated at 37ºC for different exposure time 10, 20, 30 min. Afterward, harvesting cell damage by centrifugation (13,400 g, 15 min, 4ºC) and measurement OD260 absorbance were done as an indicator of the released UV-absorbing materials and cell lysis (Sitohy et al., 2013).

5.3.2.2 Crystal violet determination

The cell membrane permeability was detected by the crystal violet assay (Halder et al., 2015), where a high percentage uptake of crystal violet, indicates bacterial membrane function was a loss. An a approximately 10^8 cfu/mL of *L. monocytogenes* cells were grown overnight as the same as 5.3.2.1, was centrifuged at 4,500xg, 5 min, 4ºC, the cells precipitate were corrected and washed twice with phosphate buffered saline solution (pH 7.4), resuspend cell suspension with the same buffer and original volume. The tested died crude BSF of various concentrations were added to the cell suspension but control and incubated at 37ºC as same as the 5.3.2.1 method. Afterward, cells were harvested by centrifugation at 9,300xg for 5 min, then suspended in phosphate buffered saline solution (pH 7.4) containing 10 µg/mL of crystal violet, all treated were incubated at 37ºC for 10 min. After incubation, cell pellets were removed by centrifugation at 13,400xg for 15 min, then supernatant were measured at a wavelength of 590 nm using spectrophotometer (JASCO UV-VIS, Model name V-670). The percent uptake of crystal violet was calculated as the formula given below:

$$\text{Uptake}(\%) = \left(\frac{\text{Abs value of sample}}{\text{Abs value of the crystal violet solution}}\right) \text{x} 100$$

5.3.2.3 Cell membrane fluidity determination

The cell membrane fluidity was determined by DPH probe fluorescence, the protocol was adapted from Liu et al. (2010) All samples were prepared according to the topic of 5.3.2.1, rehydrated and washed twice with sterile 10 mM PBS. The DPH (1,6-Diphenyl-1,3,5-hexatriene; 1 mM in methanol) molecular probe was added to cell suspension to a final concentration of 0.004 mM. Then staining cell was incubated with DPH solution at 37ºC for 30 min, and washed twice by centrifugation at 2700×g for 5 min. Afterward, suspended in sterile 10 mM PBS. The measurement of DPH fluorescence intensity was described by Herman et al. (2015).

5.3.2.4 DNA release

The release of cellular nucleic acids was detected by measuring A260 (Diao, Hu, Zhang, and Xu, 2014). The samples were centrifuged at 13,000×g for 15 min at 4^{o}C, and supernatant was measured DNA concentration release by using a Nano-drop 2000c spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA.). Nucleic acid concentrations were calculated with the Nanodrop 2000 software.

5.3.2.5 Enumeration of viable *L. monocytogenes*

A viable-but-nonculturable (VBNC) *L. monocytogenes* state in response to environmental stresses such as chlorine, was enumerated of a differential medium chromogenic listeria agar ISO (Oxoid CM1084, UK). The cells suspension of *L. monocytogenes* (from 5.3.2.1), aliquot cell suspension 1 mL of each sample into buffered Listeria (Oxoid CM0897, UK), final volume 10 mL. Then subsequently applied 100 µL onto chromogenic listeria agar iso (mixed OCLA (ISO) differential

supplement and brilliance listeria) by spreading plate technique and incubated at 37ºC for 48 h. Countable the blue colony, which indicates that phosphatidylcholine phospholipase C (PCPLC) activity could be detected, and collected cells viable counts to calculate in value of percentage reduction as the formula given below:

$$\text{Percent Reduction (\%)} = \left(\frac{A - B}{A}\right) x100$$

Where:

A is the number of viable microorganisms before treatment

B is the number of viable microorganisms after treatment

5.3.3 The implementation in washing fresh produce using crude BSF and its stress under BSF sublethal condition during storage

5.3.3.1 Chinese kale preparation and inoculation

Chinese kale (*Brassica oleracea* var. *alboglabra*) was obtained from Talad Thai (produce wholesale market, Bangkok, Thailand), and stored at 5ºC, the *L. monocytogenes* challenging. The Chinese kale were trimmed the damage out and aseptically with sterile water for several time, then drain out the excess water for 45 min. The inoculum size of *L. monocytogenes* (*sigB* gene) at approximately 10^8 CFU/mL in 0.1% peptone water, was randomly over all deposited of kale (1 Kg) in 2 L per each, with a final concentration *L. monocytogenes* approximately 10^6 CFU/g of Chinese kale. Allow the inoculum absorb to the leaf surface for 60 min, after that, transfer to the washing process.

5.3.3.2 Wash BSF as a sanitizer preparation and produce washing

Wash solutions containing targeted dried crude BSF concentrations 350, 400 and 450 µg/mL were prepared using dissolve in sterile water,

and these experiments were operated control under wash water quality by using water without chlorine (eliminate the effectiveness from chlorine), before each wash.

To evaluate the effect of actual dried crude BSF concentration and suitable soaking time for *L. monocytogenes* reduction, with sublethal stress, through the practical procedure, adapted and descried by FDA, US (2008). It was recommend 75 ppm calcium hypochlorite solution ($CaClO_2$), soaking at least 3 min to eliminate microorganisms contamination. This concentration, therefore, was used as positive control. The procedure as the follows; A) Pre-washing step, the inoculated Chinese kales (1 Kg; 10^6 CFU/g *L. monocytogenes*) all treatments were rinsed with cleaned water for 100 sec and drained off water at ambient temperature for 45 min, B) Washing the tank with sanitizer agent, subsequently. Each sample was soaked in various sanitizer concentrations (BSF suitable conditions from 5.4.2, Concentration and exposure times of crude BSF effects on different bacteria cell targets), 1 kg of Chinese kale soaked in 5 liters of sanitizer solution, C) Rinsing through to remove sanitizer residue, and washed twice by cleaned water for twice times for 35 min. After, draining aseptically for 45 min, and then packed the sample in sterile plastic bags (Linear low-density polyethylene, LLDPE). Samples before and after washing were enumerated for *L. monocytogenes*, and storage safety at 35ºC, 12ºC and 5ºC for further studies.

5.3.3.3 Sampling and enumeration of *L. monocytogenes*

To assess the effectiveness of sanitizing BSF whether it can remove *L. monocytogenes* contaminated in Chinese kale or not. That enumeration of *L. monocytogenes* survived were, therefore, conducted as previously described (5.3.2.5). Whereas, to enumerate cell stress that survival in wased water, conducted

the washed water of each sample from step B: washing tank with sanitizer agent (5.3.3.2). The survival rate was expressed as (N/N0) x 100, where N0 and N were the numbers of viable cells before and after treated, respectively.

5.3.3.4 *sigB* gene characterization by polymerase chain reaction (PCR)

The objective of this study was to evaluate the sublethal-BSF induced stress effect deducting in the damage significant *sigB* gene. The method used in this experiment was descried by Hasegawa et al, (2003) the 100 mL of washed water from 5.3.3.2 (step B; washing tank with sanitizer agent), *L. monocytogenes* cell (cell size on the average 0.3 µm) passed through a 0.45-µm pore size filter (Thermo Scientific™, syringe filter sterilized). The cell solution was centrifuged at 10,000xg, 10 min, 4ºC. Then, stress cell pellet was resuspended into TSB broth, and 10-fold dilution was made. Afterward, 100 µL aliquots were spread on TSA plate, and incubated at 37°C for 24h. Single colony were subcultured in 25 mL TSB broth, incubated at 37°C for overnight. *L.mococytogenes* cell stress was performed for the further *sigB* gene characterization analysis, by PCR method as previously described (3.3.2)

5.3.3.5 Storage treatment of Chinese kale

Both unwashed and washed with virous sanitizers (5.3.3.2) were packed in sterile plastic bags (Linear low-density polyethylene, LLDPE) and stored at 5°C, 12°C and RT (35°C) in lab-scale refrigerated incubators. Enumeration of the L. monocytogenes count of the sample at selected sampling point were done approximately 1 packed/each treatment (250g of Chinese kale) at 7 and 14 days

storage according to method presented in 5.3.2.5., the survival rate of all samples were done.

5.3.4 Statistical Analysis

All washing treatment were done in triplicate and data presented are mean values ± standard deviation (SD). IBM SPSS statistics 26 (Armonk, New York, U.S.A.) was used to perform all statical analysis. One-way analysis of variance (ANOVA) was determined the difference between group and within group, which was followed by Duncan's multiple range test (DMRT) with an overall significance level at 0.05 ($p<0.05$).

5.4 Results and discussions

5.4.1 Inhibitory effects of crude BSF and stress adaptation

The sensitivity of *L. monocytogenes* with BSF as sanitizer-resistant to various BSF concentration was quantified by the MIC and MBC methods. The data showed (table 5.1) that MIC and MBC of the crude BSF on *L. monocytogenes* was 200 µg/mL and 400 µg/mL, respectively. Indicated that the inhibitory concentration was significantly different from bacteria-killing concentration. It was remarkable that inhibitory concentration was significantly different from bacterial-killing concentration. Considering the MIC of normal cell to sublethal cell and that of MBC, the later were higher induced. In order to achieve killing, the MBC (450 µg/mL) stress *L. monocytogenes* was focus.

Since, the result of exposure to the sublethal stress induced ad by BSF (350 µg/mL; MBC Normal cell), and adapted cell was 450 µg/mL. Thus, designed an experiment to evaluate the sanitizer function to stress a resistance and tolerance in

L. monocytogenes. It was, thereby, suggested that the concentration of dried crude BSF to till the resistance and tolerance *L. monocytogenes* could be applied with a range 350-450 µg/mL.

Table 5.1 Minimum inhibitory and bactericidal concentrations of crude BSF on *L. monocytogenes* with detergent-resistant gene adapted to sublethal concentrations of BSF.

Crude BSF tolerance	*L. monocytogenes*	Concentration (µg/mL)
MIC (µg/mL)	Normal cell	200±0.00
	Adapted cell	400±0.00
MBC (µg/mL)	Normal cell	350±0.00
	Adapted cell	450±0.00

Results of three replicates are presented as average values ± standard deviation.

5.4.2 Concentration and exposure times of crude BSF effects on different bacteria cell targets

5.4.2.1 Mode of action of crude BSF on the membrane cell surface

The main damage target and induced oxidative stress of *L. monocytogenes* were treated with sanitized and detergents, which cause cell membrane damage, cellular ion exchange, membrane fluidity, degradation of proteins, and nucleic acids due to a decreased bacteria cell survival rate (Falk et a., 2019; Pietrysiak, Kummer, Hanrahan and Ganjyal, 2019). After a sub-lethal effect of crude BSF treated with different exposure times for 10, 20 and 30 min, indicated all treatments were significant ($p<0.05$) effects on cell lysis (Fig 5.1), while at the 350 to

450 µg/mL for 20 and 30, respectively, were same level effective. Moreover, initial exposure times for 10 min such as cell lysis with the high rate, especially at 450 µg/mL BSF, treated induced cell lysis (approximately 2.27) at exponential rate. When considered under short exposure time, the consequences, rendered membrane permeation was lost, and the high rate cell lysis of 450 µg/mL for 10 min, and 350, 400 µg/mL BSF for at least 20, respectively, could induced cell membrane distortion, that leading to up take (%) of crystal violet increased (Fig 5.2). Moreover, the DPH probe can embed into the lipid bilayer and is sensitive to alterations in membrane fluidity; as the membrane fluidity decreases, while the intensity increases, which the target result shown in fig 5.3., resulting in all BSF treatment (350, 400 and 450 µg /mL) that interrupted of membrane fluidity phospholipid bilayer of *L. monocytogenes* during exposure time 10-30 min, result in the intensity increased. These results are related to the membrane disruption by cell lysis and it might be the interaction of hydrophobic active site of crude BSF.

5.4.2.2 DNA release effectiveness

Although, crude BSF is thought to kill bacteria by membrane damage followed by cell lysis, but genomic DNA release is an important indicator of bacterial lysis. Supported by Yuk et al. (2007) as described the mode of action of ozone depended on singlet oxygen which acts on oxidized double bonds in the cell due to DNA damage. Therefore, to further detect the integrity of cell membranes after exposure with crude BSF induce cell adapted. Figure 5.4 shows, the DNA concentration release increased with an increase of crude BSF concentration and related with exposure time ($p<0.05$). However, 450 µg/mL results in non-significant

between in 20 and 30 min exposure, indicated concentration has a direct effect on destruction due to their release, but must be under the exposure for at least 10 min.

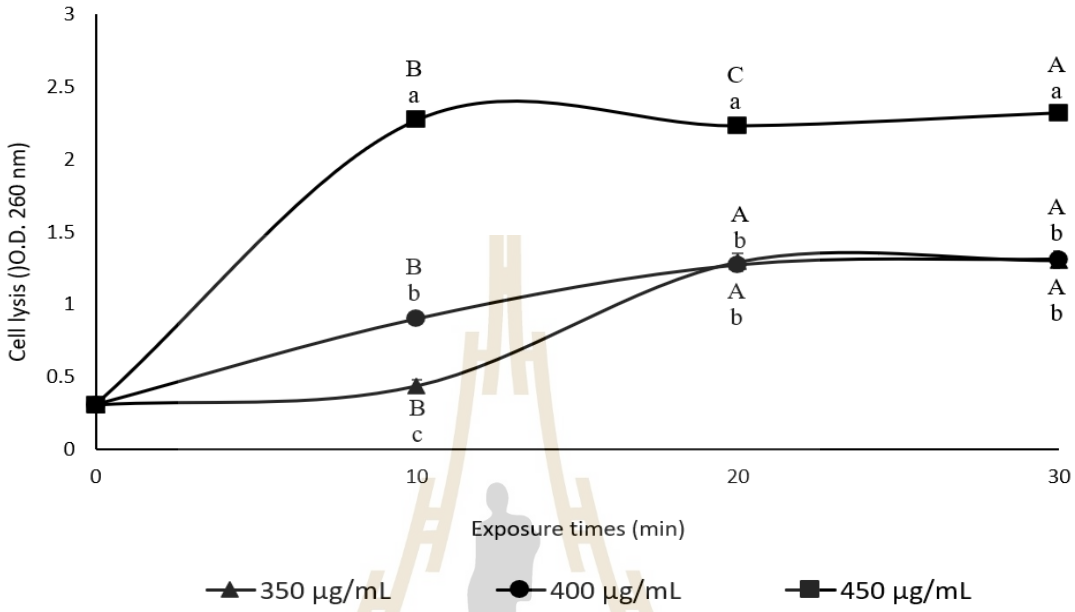

Figure 5.1 Cell lysis of *L. monocytogenes* treated with various BSF concentrations and exposure times. Data represented in three replicates. The different big letters represented at the same concentration at the different exposure times indicate significant differences at $p<0.05$., while small letters were different concentrations but the same exposure time indicate significant differences at $p<0.05$.

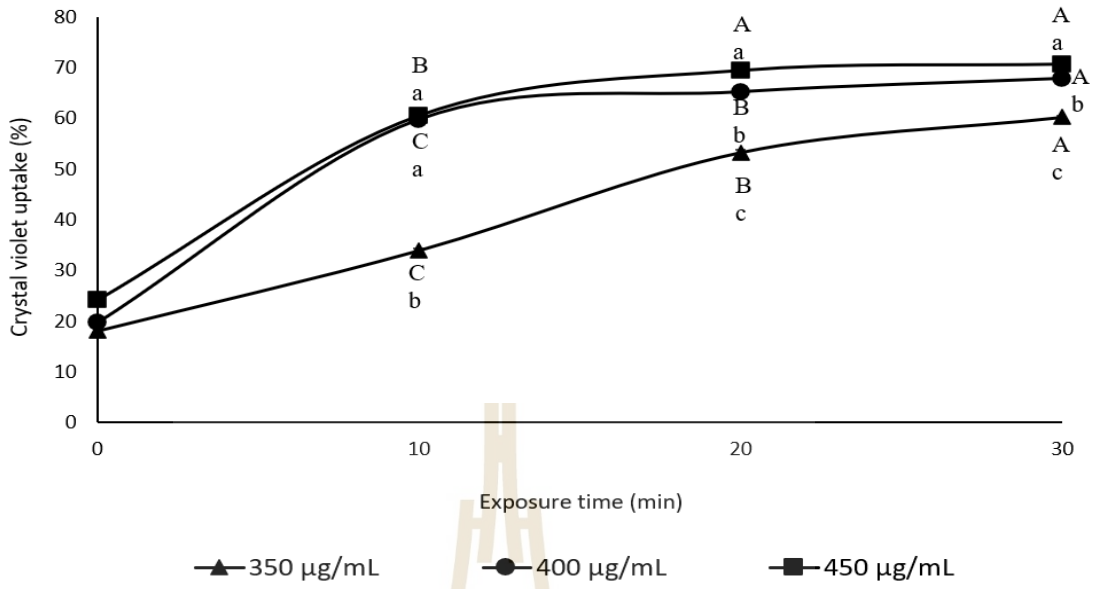

Figure 5.2 Cell permeation of *L. monocytogenes* treated with various BSF concentrations and exposure times. Data represented in three replicates. The different big letters represented at the same concentration at the different exposure times indicate significant differences at $p<0.05$., while small letters were different concentrations but the same exposure time indicate significant differences at $p<0.05$.

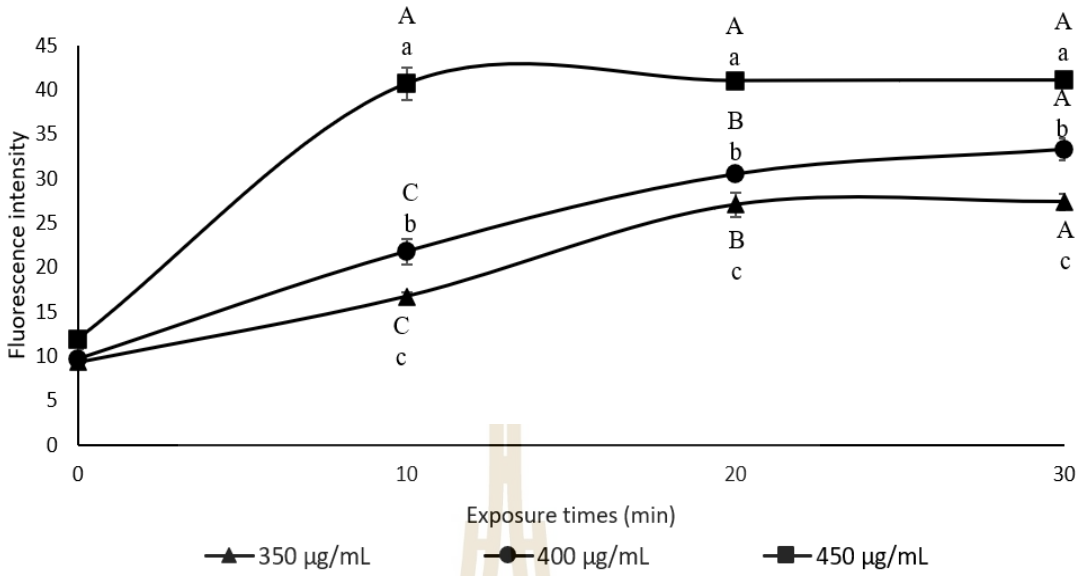

Figure 5.3 DPH fluorescence intensity of *L. monocytogenes* treated with various concentrations and exposure times. Data represented in three replicates. The different big letters represented at the same concentration at the different exposure times indicate significant differences at $p<0.05$., while small letters were different concentrations but the same exposure time indicate significant differences at $p<0.05$.

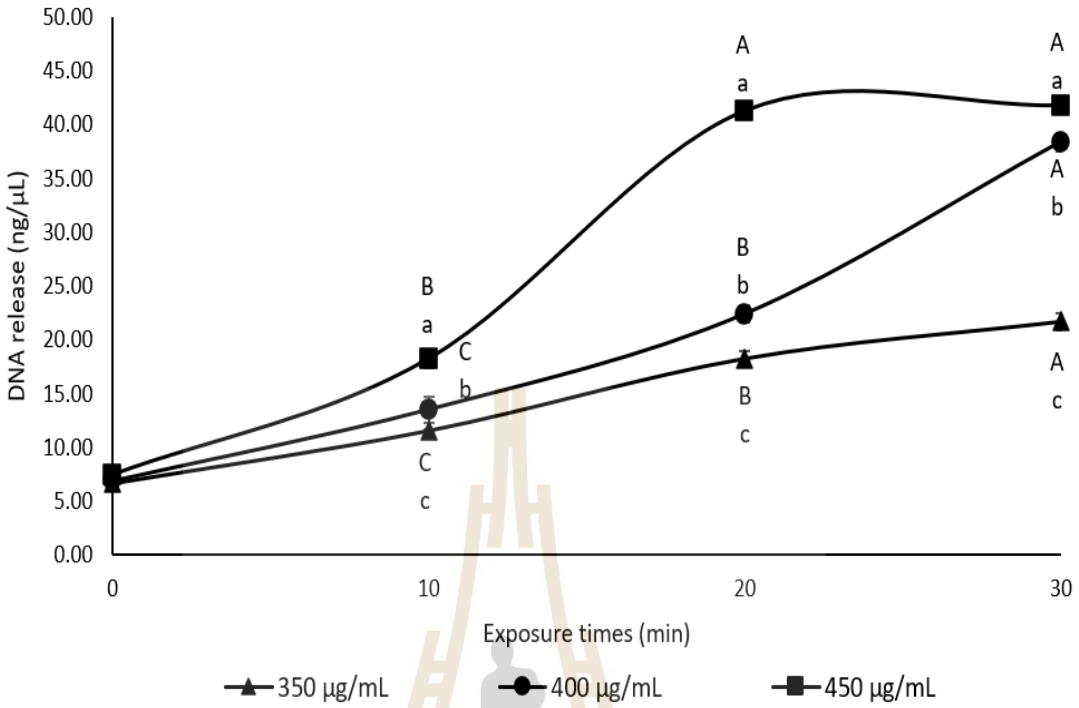

Figure 5.4 DNA release of *L. monocytogenes* treated with various BSF concentrations and exposure times. Data represented in three replicates. The different big letters represented at the same concentration at the different exposure times indicate significant differences at $p<0.05$., while small letters were different concentrations but the same exposure time indicate significant differences at $p<0.05$.

5.4.2.3 *L. monocytogenes* viable cell reduction effectiveness

Generally, *L. monocytogenes* is resistant to detergent, sanitizer, antimicrobial and benzalkonium chloride, of which were related to the role of *sigB* gene, and more cross-protective by biofilm formation gene during sublethal under cold stress condition such as *inlB* gene. For the reason supported that this bacterium is sensitive and tolerant to a broad chemical substance including nisin from

Lactobacillus and some of rhamnolipid from *Pseudomonas,* these induced resistant cells possessed a modified phospholipid composition, finally making it difficult to eradicate and reduction them during washing and/or cleaning procedures (Van der Veen and Abee, 2010; Tamburro et al., 2015). Thus, our research expected that a new BSF produced from *L. plantarum* MGL-8 could be a gap analysis compound with effectiveness to *L. monocytogenes* reduction. After a sublethal effect of BSF exposure, the combination criteria resulting in the percentage of *L. monocytogenes* reduction was increased with an increase crude BSF concentration ($P<0.05$). It was suggested that the concentration at 350 µg/mL, at minimum 20 min for exposure was required to sufficiently reduce the *L. monocytogenes* approximately 30%, as the same as that of 400 µg/mL of the 73% reduction. While at the highest BSF effective concentration was 450 µg/mL with the maximum rate approximately 74% within 10 min (fig 5.5). Indicates, concentration was the first criterion for effective crude BSF and exposure time respectively. In conclusion, *L. monocytogenes* strain obtain *sigB* gene (detergent resistant gene) had BSF sensitivity and tolerance. However, the BSF sensitivity range at 350, 400, and 450 µg/mL, within at least the exposure time for 20, 20, and 10 min respectively, could be applied in the washing fresh produce process.

5.4.3 Implementation washing and stress in fresh Chinese kale produce.

5.4.3.1 Efficacy of BSF on *L. monocytogenes* after washing

Survival rate of *L. monocytogenes* on Chinese kale produce after washing were shown in Table 5.2. Indicated the efficacy each of $CaClO_2$ and crude BSF which were statistically different ($p<0.05$), when compared with the challenged *L. monocytogenes* Chinese kale (unwashed treatment). Moreover, considered the 400 and 450 µg/mL of BSF, they displayed the high performance against

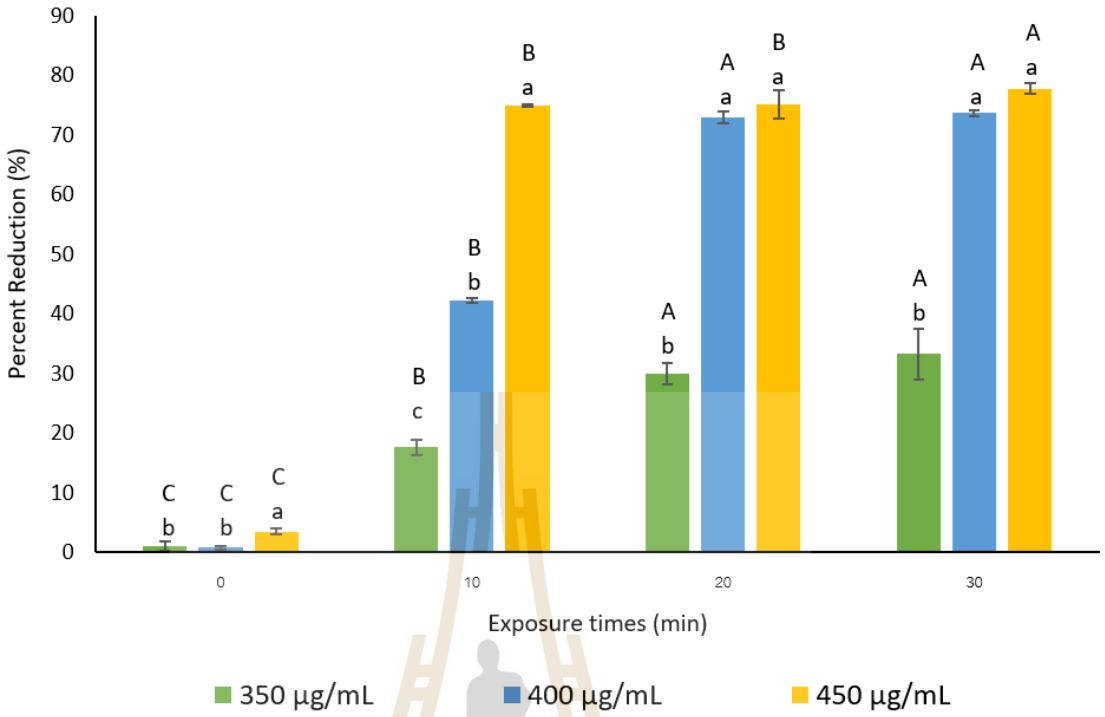

Figure 5.5 Percent reduction of *L. monocytogenes* treated with various concentrations (BSF-350, BSF-400 and BSF-450 µg/mL) and exposure times. The different big letters represented at the same concentrations at the different exposure times indicate significant differences at $p<0.05$., while small letters were different concentrations but the same exposure time indicated significant differences at $p<0.05$.

the survival rate of *L. monocytogenes* as the same as 75 ppm of $CaClO_2$. Regarding to the exposure time as extrinsic factor of BSF during soaking, revealed that the exposure up to 10 min for BSF concentration 450 µg/mL, initial *L. monocytogenes* survival significantly decreased after washing with rather than all 350 and 450 µg/mL BSF. However, at 350 and 400 µg/mL needed longer exposure time to 20 min. Chinese kale were substantially, reduced the survival rate 3.41 and 2.44 log CFU/g, respectively. It

might be suggested that the exposure time was impacting over the cell growth until survival under the BSF stressed.

Anyhow, in case of water-washed samples showed the survival rate corresponding with the concentration, exposure time 10 up to 20 min in order which necessary to lethal cell. Thus, observed the all BSF concentration with various exposure time at 20 and 10 min, were capable to reduce the survival rate in water-washed samples. Therefore, soaking with the sanitizer was also the key performance factors to ensure food safety, of which concerned the relationship between concentration, exposure time, including standard operating procedure (SOP).

Table 5.2 Microbial quality of Chinese kale after washing and the sub-lethal survival rate in water-washed Chinese kale with calcium hypochlorite ($CaClO_2$) and various BSF concentrations.

Washing treatment	**After washing**	
	Cell survival in Chinese kale (log CFU/g)	**Cell survival in water-washed (log CFU/ml)**
Unwashed Chinese kale	6.55 ± 0.09^{a}	NE
$CaClO_2$ 75 ppm	2.16 ± 0.19^{c}	3.13 ± 0.07^{a}
BSF-350 µg/mL	3.41 ± 0.37^{b}	2.5 ± 0.02^{b}
BSF-400 µg/mL	2.44 ± 0.09^{c}	2.24 ± 0.61^{b}
BSF-450 µg/mL	2.33 ± 0.08^{c}	2.08 ± 0.22^{b}

The value of maximum reduction means the decrease in *L. monocytogenes* survival; Chinese kale unwashed (negative control treatment), $CaClO_2$ 75 ppm for 3 min (positive control treatment), BSF 350 µg/mL for 20 min, BSF 400 µg/mL for 20 min and BSF 450 µg/mL for 10 min. Data of three replicates are presented as average values±standard deviation. Different superscripted letters indicate significant differences ($P<0.05$) within the same column.

5.4.3.2 Evaluation of BSF sub-lethal stressed on *sigB* gene characterization

The significant *L. monocytogenes* is an important a foodborne pathogen that contaminated in freshly, and more especially the strain containing a severity gene of *sigB* which have a unique mechanism protects the cell from detergent and/oxidative stress that lead to persistence (Lennox, Etta, John and Henshaw, 2017). Thus, when susceptibility of *L. monocytogenes* to BSF in produce were soaked in water (water-washed), of which potential induction of viable but non-culturable state (sub-lethal state). Resulting in *sigB* gene expression, thereby, *sigB* gene characterization should be determined of all treatments were expressed by the PCR product size (~780 bp). Figure 5.6, indicated that crude BSF at the concentrations 350, 400 and 450 µg/mL with exposure time for 20, 20 and 10 min, respectively, were effect to *sigB* gene damaged.

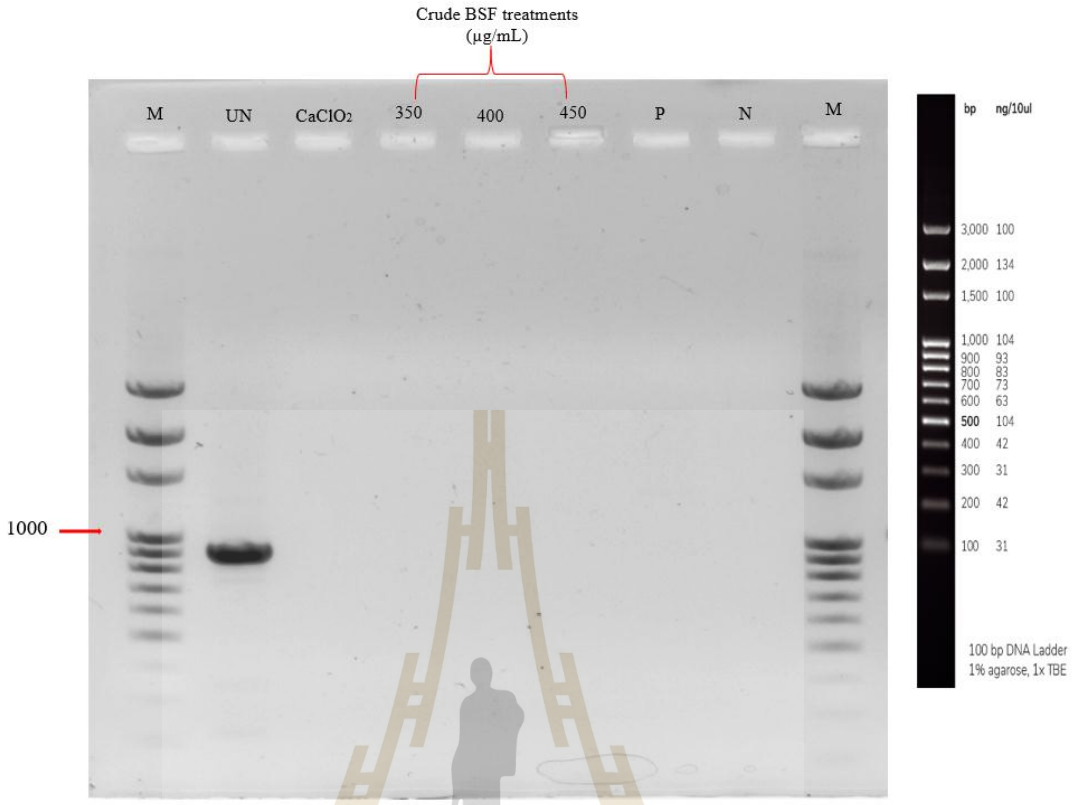

Figure 5.6 The results of PCR product 1% Agarose Gel Electrophoresis, the PCR amplification of *sigB* gene of *L. mococytogenes* cell stress (water-washed Chinese kale) with various sanitizers; $CaClO_2$ = 75 ppm treatment, and 350, 400 and 500 µg/mL BFS treatments, and M= marker 100 bp (Thermo Scientific™), UN= untreated control cells, N = negative control (load only sterile deionized water), P = positive control (20 mM SDS).

5.4.4 Evaluation of the washing effectiveness procedure of BSF during storage

The important role of factor sigma B under the low-temperature condition, of which induced ribosomes to stress some with a higher abundance with the relevance of the overexpression of chaperone proteins such as cold shock proteins

(Santos, Viala, Chambon, Esbelin and Hébraud, 2019). Thus, *L. monocytogenes* is notable for its ability to grow at refrigeration temperatures, thereby, after the washing process was required to monitor the actual survival growth during storage low temperature at 35ºC (to represent ambient temperature), 12ºC and 5ºC.

In general, after storage a week (at 7 days), *L. monocytogenes* grew rapidly on the unwashed under 35ºC, including low temperature at 12 and 5ºC, reaching survival rate about of 8.76 and 7.09 log CFU/g, respectively, which were increased after washing around 2-1 log CFU/g (table 5.2). Except for Chinese kale produce, washed with each of $CaClO_2$ and crude BSF, shown in Fig 5.7 (A). Indicated all crude BSF concentrations and CaClO2 were significantly different ($p<0.05$) with in the same storage temperature. Especially, during storage at 12 and 5ºC of 400 and 450 µg/mL BSF respectively, were a small grew by increased survival rate lower than 1 log CFU/mL. Of which observed survival rate of 75 ppm $CaClO_2$ treatment at 5ºC approximately 2.82 log CFU/g, but 450 µg/mL BSF treatment approximately 2.37 log CFU/g. The consequences for 14 days storage as shown in Fig 5.7 (B), the data was revealed that those crude BSF concentrations 400 and 450 µg/mL at 5ºC, could be the selective effectiveness of growth inhibitory until to *sigB* gene damage (Succeed from 5.4.3.2). It was remarkable that sanitized produce with 400 and 450 µg/mL BSF had a small survival rate of bacteria after washing procedure till storage at 14 days, which result was a similar level with 75 ppm $CaClO_2$.

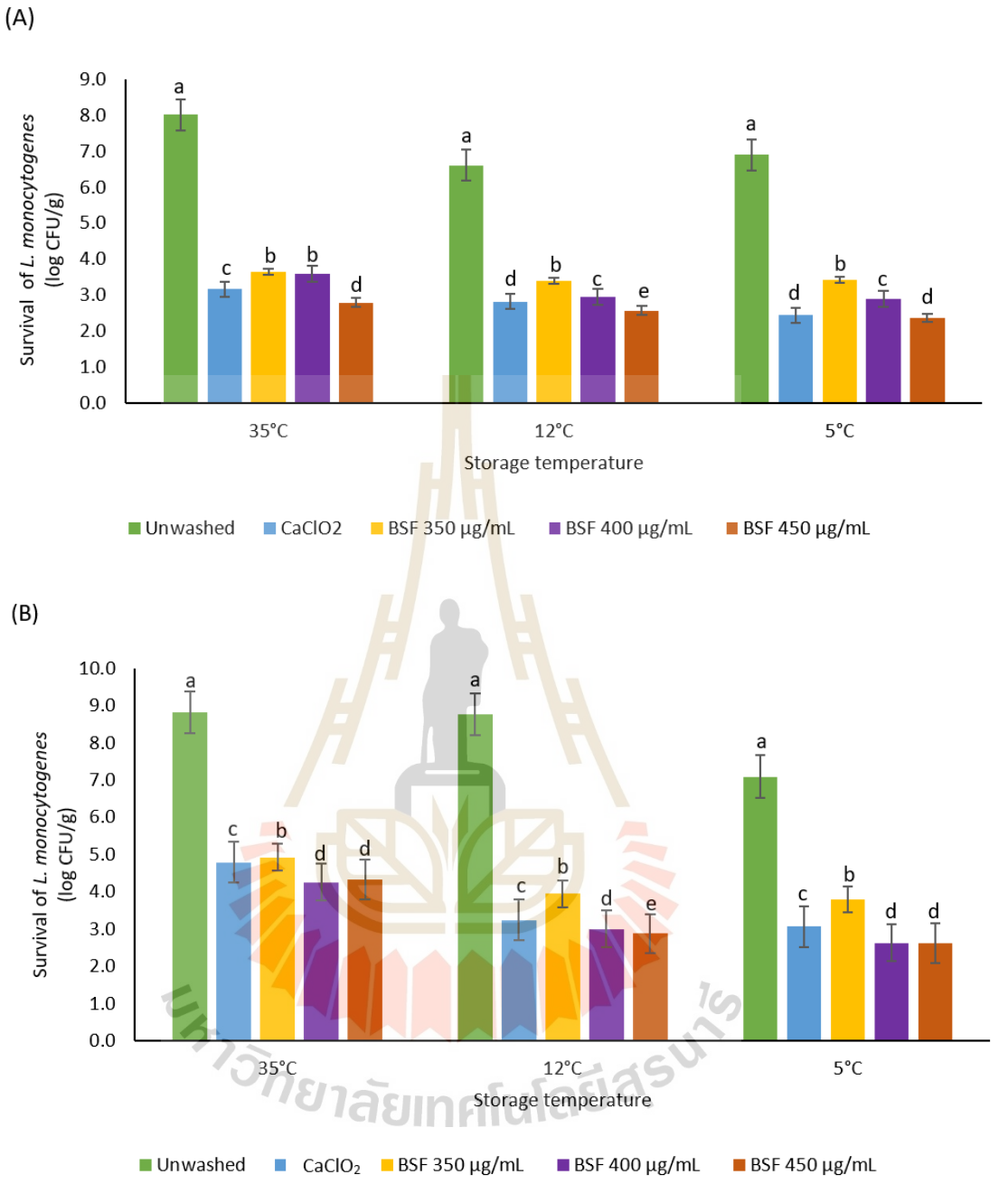

Figure 5.7 The survival of *L. monocytogenes* in washed and unwashed Chinese kale during storage for 7 days (A), and 14 days (B), at 35ºC (represent ambient temperature), 12ºC and 5ºC. All treatments were performed in triplicated; error bars refer to standard deviation, and different label on a bar at which specifically temperature represents significant difference ($p<0.05$).

5.5 Conclusions

In conclusion, this study demonstrated the potential of the use of dried crude BSF concentration 350, 400, and 450 µg/mL combined with exposure time 20, 20, and 10 min, respectively, of which still be the inhibitory and killing action on *L. monocytogenes.* The mode of action against *L. monocytogenes* target was BSF oxidative stress, the action of crude BSF may be with the oscillating random motion of BSF molecules attached to the cell walls and membranes may cause them to reduce the surface tension of lipid bilayer, producing cell leak, and till to cell membrane disintegration. Of which engendering higher cell permeability leading, finally to cell emptiness, lysis, DNA release with a high rate, and death. By the successful scientific data on the high risk hazard (*L. monocytogenes*) damage, it could be approved to imply for sanitizing agent in Chinese kale contaminated *L. monocytogenes*, by optional concentration of 400 and 450 µg/mL BSF, soaked for 20 and 10 min, at when damaged *sigB* gene (detergent resistant gene), and increased *L. monocytogenes* reduction. In addition, by these concentrations of crude BSF for at least 10 min, was sufficient to control the survival rate of these pathogens on fresh produce during storage at 12 and 5ºC for 14 days.

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CHAPTER VI

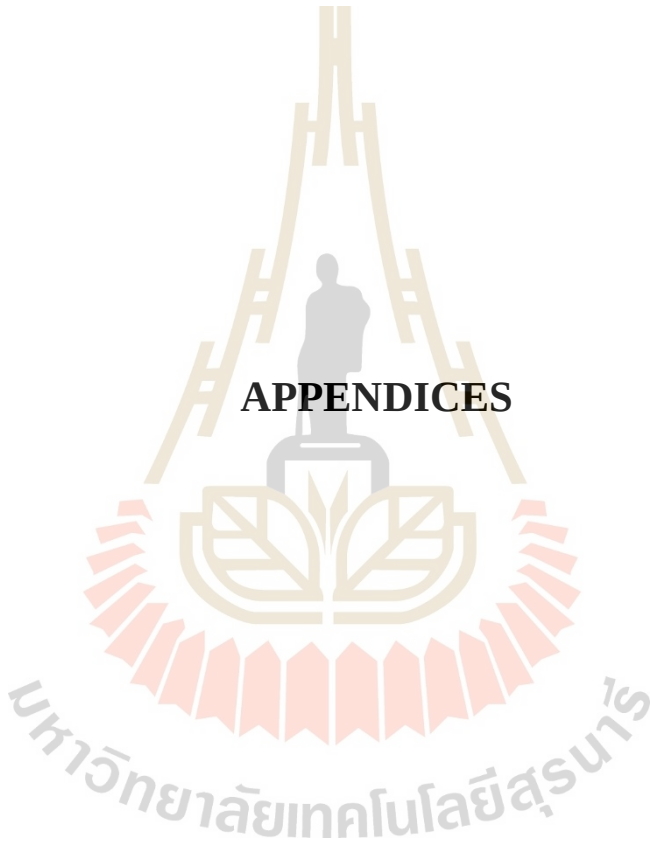
SUMMARY

Lactobacillus plantarum MGL-8 was isolated from indigenous overriped Nam Dok Mai mango, representative specific LAB strain which potential produce BSF with antimicrobial activity against *L. monocytogenes* characteristics. In addition, strain can produce BSF, thereby decreasing the surface tension of the culture medium to 39.14 mN/m after 36 h of growth. Of which the time corresponding to the maximum growth curve using MRS broth and 1% (w/v) glycerol and 2% (w/v) sucrose supplement. *L. plantarum* MGL-8 with the success conditions, thus, considered for produce BSF with use low-cost substrate such as overriped Nam Dok Mai mango by spontaneous fermentation.

Upon the *L. plantarum* MGL- 8 potential producing BSF provide a higher at 36 h under synthetic medium condition was applied for overriped mango juice (ratio 1:3, mango paste: deionized water), which is the alternative cheapens substrate. Of which were carried out by fermenter, and controlled parameters at 35ºC, agitator speed 100 rpm, aeration rate 1 vvm and dissolved oxygen rate; DO rate 100%. Those factors provided *L. plantarum* MGL-8 in mango juice (L-MJ), supplemented with 1% (w/v) glycerol and 2% (w/v) sucrose. Resulting in the fermentation times at 120 h., it sufficient to provide BSF characteristics as follows; surface tension value 36.8 mN/m, %E24 approximately 40.73%, effective to inhibitory *L. monocytogenes*, and these metabolites come from the synergistic or antagonistic relationship (implicit factors)

which potential to controlled another microorganism. Finally, in the actual dried crude BSF approximately 4.22 g/L of SFL production (submerged fermentation using *L. plantarum* MGL-8 or L-MJ). To gain the actual concentration for applied in the washing process, consequently, assess determine the mechanism against inhibitory till to killing of *L. monocytogenes*. Resulting in sublethal stress induced by crude 350 µg/mL BSF (MBC Normal cell) and 450 µg/mL (MBC; Adapted cell) were applied for evaluated suitable the exposure time. With 350, 400 and 450 µg/mL BSF with exposure time for 20, 20 and 10 min respectively, demonstrated the responsibility of its antimicrobial against *L. monocytogenes* by the major functions via cell membrane, results in loss of membrane fluidity, cell membrane disintegration, higher cell permeability leading, lysis and death. When conducting these conditions for washing Chinese kale, found the damage of *sigB* gene, and sufficient to control the survival rate *L. monocytogenes* on Chinese kale produce during storage at 12 and 5ºC for 14 days as well as 75 ppm $CaClO_2$ (commercial sanitizer). These key performance factors or control measurement, could control the final level of *L. monocytogenes* and would be further a good practice as a SSOP, implemented in food safety management systems (FSMSs).

APPENDICES



APPENDIX A

MEDIA AND REAGENT PREPARATION

1. Culture media

1.1 *Lactobacillus* MRS Broth (MRS Broth)

Proteose peptone	10	g
Peptone	10	g
Yeast extract	5	g
Dextrose (Glucose)	20	g
Polysorbate 80 (Tween 80)	1	g
Ammonium citrate	2	g
Sodium acetate	5	g
Magnesium sulphate	0.1	g
Manganese sulphate	0.05	g
Dipotassium hydrogen phosphate	2	g
Distilled water	1000	ml

Final pH (at 25°C) 6.5±0.2

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

1.2 *Lactobacillus* MRS Agar (MRS Agar)

Proteose peptone	10	g
HM Peptone	10	g
Yeast extract	5	g
Dextrose (Glucose)	20	g
Polysorbate 80 (Tween 80)	1	g
Ammonium citrate	2	g
Sodium acetate	5	g
Magnesium sulphate	0.1	g

Manganese sulphate	0.05	g
Agar	15	g
Dipotassium hydrogen phosphate	2	g
Distilled water	1000	ml

Final pH (at 25°C) 6.5±0.2

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

1.3 Nutrient agar (NA)

Peptone	5	g
Yeast extract	3	g
Agar	15	g
Distilled water	1000	g

pH 7.0±0.2

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

1.4 Nutrient broth (NB)

Peptone	5	g
Yeast extract	3	g
Distilled water	1000	ml

pH 7.0±0.2

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

1.5 Potato dextrose broth (PDB)

Potato starch	4	g
Dextrose	20	g
Tataric acid 10%(w/v)	10	ml
Distilled water	1000	ml

pH 5.2±0.2

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

1.6 Potato dextrose broth (PDA)

Potato starch	4	g
Dextrose	20	g
Agar	15	g
Tataric acid 10%(w/v)	10	ml
Distilled water	1000	ml

pH 5.2±0.2

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

1.7 Mueller Hinton Broth

HM infusion B	300	g
Acicase	17.5	g
Starch	1.5	g
Distilled water	1000	ml

Final pH (at 25°C) 7.4±0.1

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

1.8 Mueller Hinton Agar

HM infusion B	300	g
Acicase	17.5	g
Starch	1.5	g
Agar	17	g
Distilled water	1000	ml

Final pH (at 25°C) 7.4±0.1

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

1.9 Soyabean Casein Digest Medium (Tryptone Soya Broth)

Tryptone	17	g
Soya peptone	3	g
Sodium chloride	5	g
Dextrose (Glucose)	2	g
Dipotassium hydrogen phosphate	2.5	g
Distilled water	1000	ml

Final pH (at 25°C) 7.3±0.2

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

1.10 Soyabean Casein Digest Medium (Tryptic Soya Agar)

Tryptone	17	g
Soya peptone	3	g
Sodium chloride	5	g
Dextrose (Glucose)	2.5	g
Dipotassium hydrogen phosphate	2.5	g
Agar	15	g
Distilled water	1000	ml

Final pH (at 25°C) 7.3±0.2

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

1.11 CTAB-Methylene blue agar medium

glucose	20	g
peptone	10	g
beef powder	1	g

CTAB	0.78	g
methylene blue	0.002	g
yeast extract	0.5	g
Agar	17	g
Add distilled water to bring volume up to	1000	ml

Final pH (at 25°C) 7.2±0.2

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

1.12 Basal Mineral Medium

Ammonium chloride	0.8	g
Dipotassium phosphate	0.7	g
Magnesium sulphate heptahydrate	0.01	g
Disodium EDTA	0.0092	g
Ferrous sulphate heptahydrate	0.007	g
Calcium sulphate,dihydrate	0.002	g
Boric acid	0.000	g
Zinc sulphate, heptahydrate	0.0001	g
Manganese sulphate, quadrahydrate	0.00002	g
Cobalt nitrate	0.00001	g
Sodium molybdate dihydrate	0.00001	g
Copper sulphate.pentahydrate	0.0005	g
Add distilled water to bring volume up to	1000	ml

Final pH (at 25°C) 7.2±0.2

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

1.13 Buffered *Listeria* broth

Tryptone soya broth	30	g
Yeast extract	6	g
Potassium di-hydrogen orthophosphate	1.35	g
Disodium hydrogen orthophosphate	9.60	g
Distilled water	500	ml

Final pH (at 25°C) 7.3±0.2

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

Reconstitute one vial as directed, and add the contents to 500 ml of Buffered Listeria broth. Cool to 50°C and mix well before distributing into sterile containers in volumes as required.

1.14 Chromogenic *Listeria* Agar (ISO)

Enzymatic digest of animal tissues	18	g
Enzymatic digest of casein	6	g
Sodium pyruvate		g
Glucose	2	g
Magnesium glycerophosphate	1	g
Magnesium sulphate (anhydrous)	0.5	g
Sodium chloride	5	g
Yeast extract	10	g
Lithium chloride	10	g
Disodium hydrogen phosphate (anhydrous)	2.5	g
X-glucoside chromogenic mix	0.05	g
Agar	12	g

Distilled water	1000	ml

Final pH 7.2 ± 0.2 @ 25°C

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

Cool the medium to 46°C and add one vial of Chromogenic *Listeria* Selective Supplement (ISO), reconstituted as directed and one vial of Brilliance *Listeria* Differential Supplement. Mix well and pour into sterile Petri dishes.

2. Preparation of Gram stain reagents

2.1 Crystal Violet

Crystal violet powder	2	g
Ammonium oxalate powder	0.8	g
Absolute ethanol	20	ml
Distilled water	80	ml

Store at room temperature in a brown bottle

Filter before use

2.2 Safranin O

Safranin O powder	1.25	g
95% ethanol	50	ml

Dilute 50 ml stock solution with 450 ml of distilled water

Distilled water	450	ml

Store at room temperature in a brown bottle

Filter before use

2.3 Acetone alcohol

Acetone	500	ml
95% ethanol	475	ml
Distilled water 25 ml	25	ml

2.4 Iodine solution

Iodine	1	ml
Potassium iodide	2	ml
Distilled water	300	ml

Store at room temperature (25°C) in a foil-covered bottle (to protect solution from light).

2.5 Hydrogen peroxide

Hydrogen peroxide	3	ml
Distilled water	97	ml

APPENDIX B

sigB GENES, PARTIAL CDS, GENBANK

GenBank: EU161935.1

LOCUS	EU161935 711 bp DNA linear BCT 26-JUL-2016
DEFINITION	Listeria monocytogenes strain LMO188 SigB (sigB) gene, partial cds.
ACCESSION	EU161935
VERSION	EU161935.1
KEYWORDS	.
SOURCE	*Listeria monocytogenes*
ORGANISM	*Listeria monocytogenes*
	Bacteria; Firmicutes; Bacilli; Bacillales; Listeriaceae; Listeria.
REFERENCE	1 (bases 1 to 711)
AUTHORS	Martinez,A.J., Salazar,C. and Vanegas,M.C.
TITLE	Characterization of Listeria monocytogenes by partial sequencing Of *sigB* and actA genes
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 711)
AUTHORS	Martinez,A.J., Salazar,C. and Vanegas,M.C.
TITLE	Direct Submission
JOURNAL	Submitted (18-SEP-2007) Biological Sciences, Universidad de los Andes, Cra 1 No 18 A 10 J209, Bogota, Cundinamarca, Colombia
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/strain="LMO188"

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/gene="sigB"

CDS <1..>711

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/note="sigma factor B"

/codon_start=1

/transl_table=11

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/protein_id="ABX80461.1"

/translation="KVYIWIAAYQENGDQDAQYNLVVHYKNLVESI
ARKYSQGKSFHEDLVQVGNIGLLGAIRRYDATFGKSFEAFA
VPTIVGEIKRFLRDKTWSVHVPRRIKELGPKIKNAVEELTRE
LQSSPQISDIADFIGVTEEEVLEAMEMGKSYQALSVDHSIEA
DSDGSTITLLDVVGGTDDGFERVNQRMLLEKVLPVLDEREQ
KILQFTFIENRSQKETGELLDISQMHVSRIQRQAIKKLR"

ORIGIN

1 aaagtctata tatggattgc cgcttaccaa gaaaatggcg atcaagacgc ccaatataat

61 ttagttgttc attacaaaaa cctagtagag tctattgctc gcaaatattc ccaaggtaaa

121 tcttttcatg aagatttagt tcaagttgga aatattggct tacttggagc tattagaaga

181 tatgatgcga cgtttgggaa aagctttgaa gcatttgctg taccaaccat tgttggcgaa

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421 gagatgggaa aaagctacca agcgctttct gtagatcatt cgattgaagc tgattcggat

481 ggaagtacaa ttaccttatt agatgttgtt ggtggtacgg atgatggatt tgaacgtgtg

541 aatcaacgta tgctcttaga gaaagttctc cctgttttag atgagcgaga acagaagatt

601 ttgcaattta catttattga aaatcgcagt caaaaagaaa cgggtgaact actcgatata

661 tcacaaatgc atgtgtctag aatccaacgt caagctatta aaaaacttcg a

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BIOGRAPHY

Priyada Sittisart was born on 27th September 1988 in Yasothon province, Thailand. She received Bachelor Degree in B.Sc (Food Technology) from Suranaree University of Technology, Thailand in 2010, and a Master Degrees in M.Sc. (Food Technology) from Suranaree University of Technology, Thailand in 2015. In 2016-2020, she received funding source from KHLONG YANG LTD., PART, Thailand, and SUT-OROG scholarship for this Ph.D study. During graduate study, she worked as a teacher assistant of School of Food Technology, SUT in the course of Food Microbiology Laboratory I, and Food Microbiology Laboratory II. In addition, she in the process of filing petty patent (Manufacturing process of biosurfactant from mangoes as sanitizing agent in fresh-produce washing, Application No; 2003002200, 2020).

Paper publication:

Sittisart, P., Mahisanan, T., and Gasaluck, P. (2016). Decontamination and Quality Safety Control of Chili (*Capsicum annuum* L.) after Post-harvest Using Washing Process with Biosurfactant, **Agricultural Science Journal**, 47:3 (Suppl.), 43-46.

Oral presentation:

Young Investigator Award

Priyada Sittisart and Gasaluck, P. (2015). Cleaning effectiveness of biosurfactant for

Sclerotium rolfsii contamination level controlling of fresh cut Alpinia product. **The 6th International Conference on Food Factors** (ICoFF 2015), South Korea.

Patent:

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