DEVELOPMENT OF PROTECTIVE CULTURE PRODUCTION FROM *Bacillus subtilis* P5-6 AS FOOD BIO-PRESERVATIVE

Miss Hoang Truc Anh To

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การพัฒนากระบวนการผลิตโพรเทคทีฟคัลเจอร์จาก *Bacillus subtilis* P5-6 เพื่อใช้เป็นวัตถุกันเสียแบบชีวภาพในอาหาร

น.ส.ฮวง ตรุก แอน โต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	DEVELOPMENT OF PROTECTIVE CULTURE PRODUCTION FROM *Bacillus subtilis* P5-6 AS FOOD BIO-PRESERVATIVE
By	Miss Hoang Truc Anh To
Field of Study	Food Science and Technology
Thesis Advisor	Cheunjit Prakitchaiwattana

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..... Dean of the FACULTY OF SCIENCE

()

THESIS COMMITTEE

..... Chairman
(KITIPONG ASSATARAKUL)

..... Thesis Advisor
(Cheunjit Prakitchaiwattana)

..... Examiner
(SARN SETTACHAIMONGKON)

..... Examiner
(Sarisa Suriyarak)

..... External Examiner
(Pawinee Deetae)

ฮวง ตรุก แอน โต : การพัฒนากระบวนการผลิตโพรเทคทีฟคัลเจอร์จาก *Bacillus subtilis* P5-6 เพื่อใช้เป็นวัตถุกันเสียแบบชีวภาพในอาหาร. (DEVELOPMENT OF PROTECTIVE CULTURE PRODUCTION FROM *Bacillus subtilis* P5-6 AS FOOD BIO-PRESERVATIVE) อ.ที่ปรึกษาหลัก : ชื่นจิต ประกิตชัยวัฒนา

Bacilllus subtilis P5-6 คือแบคทีเรียไอโซเลทชนิดชอบเกลือที่มียีนสร้างแบคเทอริโอซิน (subtilin และ subtilosin) ซึ่งมีงานวิจัยก่อนหน้ารายงานว่าเป็นไอโซเลทที่สามารถพัฒนาเป็นโพรเทคทีฟคัลเจอร์เพื่อใช้ลดจำนวน *Staphylococcus aureus* และยืดคุณภาพความสดในชีสได้ งานวิจัยนี้มีวัตถุประสงค์เพื่อประมินสมบัติการสร้างแบคเทอริซินของ *Bacilllus subtilis* P5-6 และหาสภาวะและวิธีการที่เหมาะสมในการผลิตแบคเทอริโอซินเพื่อใช้เป็นวัตถุกันเสียชีวภาพในรูปแบบโพรเทคทีพคัลลเจอร์ เมื่อวิเคราะห์การแสดงออกของยีนใน P5-6 เมื่อเพาะเลี้ยงร่วมกับ *S. aureus* พบเพียงการแสดงออกของยีน subtilosin พร้อมกับ house keeping gene BA-rpoB เท่านั้น เมื่อประเมินสมบัติการยับยั้งจุลินทรีย์ในระบบอาหารเหลวพบว่า P5-6 ไม่แสดงฤทธิ์ยับยั้ง *S. aureus* แต่จะแสดงฤทธิ์ยับยั้งสูงในระบบอาหารแข็ง เมื่อประเมินการเพาะเลี้ยง P5-6 ด้วยอาหารเลี้ยงเชื้อต้นทุนต่ำ พบว่า P5-6 เจริญได้ดีที่สุดในอาหารที่เตรียมจากน้ำเกลือที่เหลือจากการดองผัก ปรับพีเอชเป็น 7 เสริมด้วย กลูโคส 0.04% และ $NH_4(SO_4)_2$ 0.01% (w/v) เมื่อเพาะเลี้ยงภายใต้อุณหภูมิห้องเขย่าด้วยความเร็วรอบ 150 rpm เป็นเวลา 16 ชั่วโมง P5-6 เจริญได้ถึง 9.14 log CFU/mL และแสดงฤทธิต้าน *S. aureus* ที่ตรวจวัดเส้นผ่านศูย์กลางของวงใส (IZ) ได้เท่ากับ 3.3 mm เมื่อผลิตโพรเทคทีฟคัลเจอร์จากคัลเจอร์ของ P5-6 พบว่าสภาวะที่ดีที่สุดคือ การใช้โมดิฟายสตาร์ชเป็นของแข็งยึดจับ โดยใช้คัลเจอร์ต่อโมดิฟายสตาร์ช ในอัตราส่วน 2:1 และทำแห้งแบบ 2 ขั้นตอน มีค่า a_w เท่ากัย 0.58 ซึ่งทำให้ได้โพรเทคทีฟคัลเจอร์ที่มีเซลล์ที่มีชีวิตมากที่สุดคือ 8.06 log CFU/ g และแสดงฤทธิ์ยังยั้ง *S. aureus* บนอาหารแข็ง (4 mg; 4 log CFU/mm^2) ที่มี IZ เท่ากับ 4.55 mm ในการประยุกต์ใช้โพรเทคทีพคัลเจอร์ในอาหาร (หมูดิบสด) โดยใช้โพรเทคทีฟคัลเจอร์ที่มีเซลล์มีชีวิต 6 log CFU เคลือบบนชิ้นหมูสด (4 log CFU/ cm^2) ที่เติมและไม่เติม *S.aureus* พบว่าเมื่อเก็บหมูที่อุณหภูมิแช่เย็นเป็นเวลา 24 ชั่วโมงโพรเทคทีฟแสดงฤทธิ์ยับยั้งการเจริญ *S.aureus* ทั้งที่ปนเปื้อนดั้งเดิมและที่เติมในหมูได้ แต่ไม่พบการแสดงฤทธิ์ฆ่า *S.aureus* ที่ชัดเจนภายใต้สภาวะดังกล่าว

สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต .....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Hoang Truc Anh To : DEVELOPMENT OF PROTECTIVE CULTURE PRODUCTION FROM *Bacillus subtilis* P5-6 AS FOOD BIO-PRESERVATIVE. Advisor: Cheunjit Prakitchaiwattana

Halophilic *Bacilllus subtilis* P5-6 contains bacteriocin encoding genes (subtilin and subtilosin). It has been reported as a potential strain developed to be a protective culture that could reduce viable count of *Staphylococcus aureus* and prolong the freshness when applied in cheese. The aim of this research was to evaluate bacteriocin generation property of P5-6 and to optimize the bacteriocin production methods for application as food bio-preservative in protective culture form. Through gene assay, only subtilosin gene expression was detected along with housekeeping gene BA-rpoB when co-cultured P5-6 with *Staphylococcus aureus*. The P5-6 showed low or no inhibitory effect on *S. aureus* when co-cultured in liquid medium but expressed higher effect on solid medium surface. Spent vegetable brine, adjusted to pH7 then supplemented with 0.04% (w/v) glucose as carbon source and 0.01% (w/v) $NH_4(SO_4)_2$ as nitrogen source was observed as optimal medium for the P5-6. Cultivation under room temperature at 150 rpm for 16 hours was optimal for this strain to maximally grow as well as inhibit *S.aureus* at 9.14 log CFU/ mL and 3.3 mm inhibition zone (IZ), respectively. Protective culture was produced from the formula using modified starch as a solid matrix and P5-6 culture at ratio 2:1, then dried with two-step process to have water activity at 0.58. It contained the highest viable count of P5-6 at 8.06 log CFU/ g. Four milligram of the protective culture showed inhibitory effect on *S.aureus* (4 log CFU/ cm^2 density) recorded at 4.55 mm IZ. Beyond 24 hours, when applied protective culture on fresh pork surfaces with non and spiked *S.aureus*, it was found that protective culture contained viable P5-6 at 6 log CFU (4 log CFU/ cm^2) could inhibit the growth of initially contaminated *S.aureus* in pork and spiked *S.aureus*. Bacteriocidal effect of protective culture on *S.aureus* were not clearly observed throughout the storage for 5 days in this food model.

Field of Study:	Food Science and Technology	Student's Signature .....
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CHAPTER 1. INTRODUCTION

Chemical preservatives have been considered as unhealthy for consumer so they have been gradually replaced by natural ones from plant or microorganism. According to Chhetri et al (2019), *Bacillus subtilis* P5-6 cultivated under salty conditions produced bacteriocin was manufactured as dried protective culture, which could significantly prolong the shelf life of cottage cheese and inactivate the growth of *Staphylococcus aureus.* This possesses potential as bio-control for food. In order to be applied widely as food preservative, *Bacillus subtilis* P5-6 should be able to be cultivated effectively under cost-effective process.

Kirt (1976) reported that spent brines (salted water) from fruit and/or vegetable pickles manufacturing factories accounted for 8% of the total hydraulic load and 59% of the salt generated in the manufacture of process pack products. Spent brines usually contain 9-15% of NaCl and the pH are between 3.2 - 3.5 (Etchells and Moore, 1971). They are interesting alternative to be used as main culture medium for cultivation of halophilic *Bacillus subtilis* P5-6 since it grows well under salty condition and bacteriocin production could be stimulated by varying salt concentration. This could be a solution to lower the production cost, add value to spent brine as well as ease the wastewater treatment burden.

Hectic lifestyles of consumers lead to the demand for the food products with longer shelf life. Besides, the increasing in their health awareness results in the requirement for clean food. Protective cultures are safe for consumption because they are derived from GRAS (generally recognize as safe) bacteria, which inhibit undesired pathogen or spoilage microbes (Young and Sullivan, 2011). Thus, protective cultures based on selected microorganism have been extensively applied in dairy and non-dairy products, sea foods and meat and poultry products lately. The global protective cultures market is forecasted to remain profitable in the upcoming years.

Therefore, the objective of this study was aimed to use spent brines to produce biomass containing bacteriocin from *Bacillus subtilis* P5-6 and develop as food bio-preservative.

1.1 Objective

i. To evaluate bacteriocin generation property of *Bacillus subtilis* P5-6 for application as food bio-preservative in protective culture form.

ii. To evaluate industrial spent pickle brines as cost-effective culture medium to produce biomass containing bacteriocin from *B. subtilis* P5-6.

iii. To evaluate the solid matrix for preparation of biomass containing bacteriocin from *B. subtilis* P5-6 as protective culture and application in food model.

CHAPTER 2 LITERATURE REVIEW

2.1 Food preservative

Chemical compounds have been used for a long time as food additives, which are added to prolong shelf-life, improve appearance and maintain the quality of product. Several compounds such as sodium benzoate, benzoic acid, nitrites, sulfites, sodium sorbate, potassium sorbate and so on are commonly known food preservatives (Alting and Velde, 2012). However, the supplementation of harmful preservatives to food is reported frequently (Williams et al. 2004). They cause several negative side effects on human health and environment. For example, nitrites and nitrates are added into cured meat to retard *C.botulinium* but high concentration of nitrates leads to malfunction of red blood cell (Nitrite Safety Council, 1980; Hotchkiss and Cassens, 1987). Using sulfites in dried fruit could cause severe respiratory syndrome to those who are susceptible (Stevenson and Simon, 1981; Schwartz, 1983). Therefore, people these days tend to use natural-based additives that cause no harm to handler and consumer, in order to improve their health and general well-being**.**

2.2 Natural food preservatives

Natural food preservatives are derived from natural based resources, considered as safe for consumer, which prolong shelf life of food by protecting them from deterioration caused by microorganism without generating any side effect. They are categorized into plant-derived products (herb and spices), certain antimicrobial constituents of other food, desirable microbe and their metabolites (Yadav and Singh, 2004).

The use of herb and spices in food has been recorded intensively. They not only enhance organoleptic properties but also preserve food due to their antimicrobial and antioxidant activities (Cattelan et al. 2013).

Some food naturally contains antimicrobial constituents such as egg (lysozyme, conalbumin, avidin), milk (lactoferrin) as mechanism to protect themselves against spoilage microbes. They could be considered as a potential source to be develop as food preservatives (Yadav and Singh, 2004).

There are several antimicrobials from microbial origin such as natamycin and nisin, which are approved to be used as food additives by the US and World Health Organization, respectively (Stark and Roller, 2003; Sobrino-Lopez and Martin-Belloso, 2008). In addition, live microorganisms have been commonly used as protective culture due to their GRAS status and antagonistic effect against undesired microbes in certain food (Castellano et al. 2008). They are either added or naturally occur in food product.

2.3 Microbial derived preservative

Microbial substances

There are many bioactive compounds synthesized by microbes including bacteriocin, organic acid, hydrogen peroxide, and enzymes (Castellano et al. 2008) that exert antagonistic effect against pathogens and spoilage microorganism. These compounds can be purified then applied in food matrix as natural-based preservatives to extend shelf-life of food products.

It was demonstrated that the produced bacteriocin from lactic acid bacteria associated with meat could be used as alternative of nisin to preserve meat product (Bromberg et al. 2004). Enterocin – a bacteriocin synthesized by *Enterococcus, which could control Lactobacillus sakei, Brochothrix thermosphacta*, and *Staphylococcus carnosus was* introduced to effectively preserve cooked ham (Baños et al. 2012).

Viable cells

Lactic acid bacteria is major sources of protective cultures (Gibson, 2008; Fliss et al. 2011). They frequently associate with diary and meat products. Two *Lactobacillus* isolates, which showed antagonistic effect against *Clostridium* spp could be used to substitute nitrite fermented pork meat (Gioia et al. 2016). It was demonstrated that living cells of lactic acid bacteria used in combination with modified atmosphere packaging (MAP) could considerably reduce the counts of *Listeria innocua* in sliced ready-to-eat pork loin after a short time (Vaz-Velho et al. 2015).

Although lactic acid bacteria and their bacteriocin, particularly nisin have been intensively used to preserve food, they have various limitation including high production cost, low productivity (de Vuyst, L. and Vandamme, 1992; Guerra et al. 2001; Qiao et al. 1997; Yu et al. 2002). Furthermore, several researches indicated that the antimicrobial activity of nisin is affected by factors such as pH, temperature, composition, structure, and natural microbiota of food (Zhou et al. 2013). It was proved to be inactive at alkaline pH (Featherstone, 2004). To minimize these troublesomes, *Bacillus subtilis* could be introduced as an ideal substitute for lactic acid bacteria since they could grow in low cost, large-scale fermentation with high growth efficiency (Riesenberg and Guthke, 1999; Schallmey et al. 2004; Westers et al. 2004). Moreover, *Bacillus* bacteriocins exert broader antimicrobial spectra than those of lactic acid bacteria (Pedersen et al. 2002; Riley and Wertz, 2002; Sumi et al. 2015). Additionally, subtilin – a nisin-like bacteriocin produced by *B.subtilis* strain was proved to be pH tolerance (Qin et al. 2019).

2.4 *Bacillus*

The genus *Bacillus* in general and *Bacillus subtilis* in particular, is a Gram positive, rod-shaped, spore-forming, aerobic or facultative anaerobic bacteria. They can grow from 5-15^{o}C to 40-50^{o}C, with optimum temperature ranging from 25^{o}C to 35^{o}C (Entrez Genome Project, NCBI). Many *Bacillus subtilis* strains were proved to have probiotic properties, which can be applied in food and feed production industry to improve the product quality. Through the formation of spore, *Bacillus subtilis* has significant advantages, which are heat stability and ability to survive the low pH of the gastric barrier making them be more preferred over other probiotic micro-organisms, specifically lactic acid bacteria (Mingmongkolchai and Panbangred, 2018). Several species in the genus *Bacillus* are known for antibiotic production.

Bacteriocins are ribosomally synthesized antibacterial peptides/ proteins that either kill or inhibit the growth of closely related bacteria (Ingolf et al. 2013). The aim of bacteriocin production is to gain competitive advantage for nutrients in the environment. According to Cotter et al (2013), they are categorized into two classes: those that undergo posttranslational modification and those that remain largely unmodified. *Bacillus subtilis* can synthesize more than 20 different bacteriocins such as: subtilin, subtilosin A, TasA, sublancin, chlorotetain, mycobacillin, rhizocticins, bacillanene, difficidin and so on (Stein, 2005). Yang et al (2014) stated that bacteriocins

are considered as safe food additives since their proteinaceous nature infers a putative degradation in the gastrointestinal tract.

2.5 *Bacillus* bacteriocin generation/mechanism

Classification

Despite several advantages over bacteriocin synthesized by lactic acid bacteria, *Bacillus* bacteriocins have not been well-classified and characterized. Abriouel et al (2011) proposed the classification of *Bacillus* bacteriocin as shown in table 1.

Table 1 Proposed classification of bacteriocins of *Bacillus* species

Class	Subclass/ Feature	Example
I. Post-translationally modified peptides	I.a. Single-peptide, elongated lantibiotics	Subtilin, ericin A
	I.b. Other single-peptide lantibiotics	Mersacidin, paenibacillin
	I.c. Two-peptide lantibiotics	Haloduracin, lichenicidin
	I.d. Other post-translationally modified peptides	Subtilosin A
II. Unmodified peptides	II.a. Pediocin-like peptides	Coagulin, SRCAM 1580
	II.b. Thuricin-like peptides	Thuricin S, thuricin 17, bacthuricin F4,
	II.c. Other linear peptides	Thuricin 439, cerein 7A, lichenin,
III. Large proteins	Unmodified, heat labile proteins (*Klaenhammer, 1993)*	Megacin A-19213, megacin A-216

Biosynthetic pathway

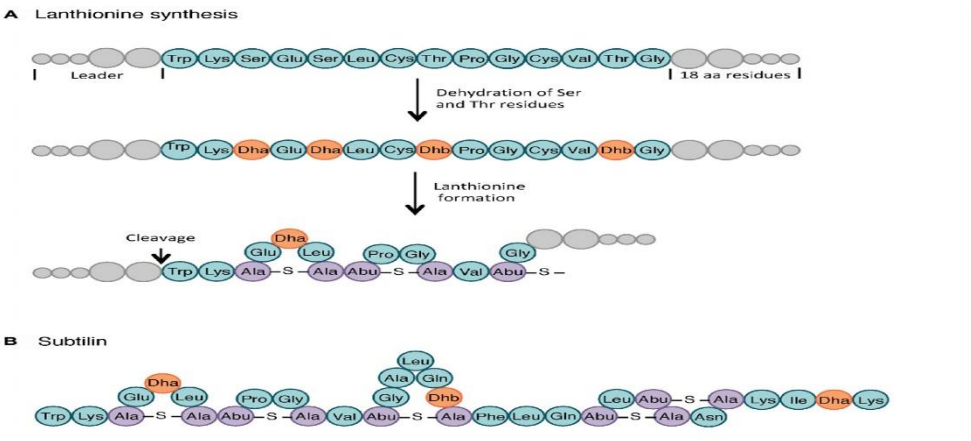

Figure 1. Lanthionine biosynthesis (Cotter et al. 2005; Spieß et al. 2015).

The general synthetic pathway of *Bacillus* bacteriocins involves many post-translational modifications, particularly the proteolytic cleavage of the leader peptide at the N-terminal end (McIntosh et al. 2009).

Class I bacteriocins undergo major post-translational modifications. Subtilin in subclass I.1, which has many similarities with nisin is the most characterized bacteriocins from the B. subtilis group (Guder et al. 2000; Abriouel et al. 2011). Subtilosin A in subclass I.4 is modified through different pathway due to its unique structure. It is a circular molecule of 35 amino acids, having an anionic nature, which is different from other membrane-targeting bacteriocins (Huang et al. 2009; Kawulka et al. 2003; Sutyak et al. 2008). According to Abriouel et al. (2011), class II includes non-modified peptides and class III are proteins, whose synthetic pathway have not been studied sufficiently.The bacteriocin class defines the modification, secretion and immunity of active peptides

Bacteriocin synthesis

The production of bacteriocin normally associate to specific cellular conditions such as stress response. For example, subtilin synthesis relies on cell density and increases under nutrient shortage condition (Abriouel et al. 2011). Its production is also regulated by quorum sensing (Kleerebezem, 2004). Quorum sensing (QS) is a cell–cell communication, which enables bacteria to exchange information regarding to cell density and response accordingly through gene regulation (Rutherford and Bassler, 2012).

QS systems are based on three basic principles. Firstly, AIs – signaling molecules are generated by the microorganism of the community. At low cell density (LCD), AIs diffuse away and no signal is detected. At high cell density (HCD), AIs is accumulated at high concentration, allowing detection and response (Kaplan and Greenberg, 1985). Second, there's receptor found in cytoplasm or in the membrance, which detects AIs. Third, aside from activating expression of genes needed for cooperative behaviors, detection of AIs results in activation of AI production (Novick et al. 1995; Seed et al. 1995). Thus, cell density could be one of important key associated to QS to initialize the bacteriocin production.

Bacteriocin are mainly generated as inactive biologically pro-forms, which contain an N-terminal leader sequence. During exportation from producer cell, this sequence is cleaved. This process is recognized by a specific bacteriocin exporting system or by a general secretory pathway.

Bacteriocin expression must be associated with immunity protein production as a mechanism of producing strains to resist it owns bacteriocin activity. Immunity proteins may act as an antagonistic receptor for specific bacteriocin, and/or as specialized ATP-binding cassette transporter systems, to pump bacteriocin from the cell of the producing strains (Peschel and Gotz, 1996; Havarstein et al. 1995). Other immunity mechanisms are re-exportation of bacteriocin through transporter system or the alteration of the targeted peptidoglycans bonds (Cotter et al. 2005; Dubois et al. 2009).

Mechanism of action

The difference in peptide, concentration, and bacterial strain results in different mode of action. Action including protoplasm vesicularization, pore formation or cell disintegration varies according to different bacteriocins (Sumi et al. 2015). Most of bacteriocins in class I and II target bacterial envelop because they are hydrophobic.

Subtilin – class I bacteriocin, has broad range of antimicrobial spectrum on Gram-positive bacteria (*Staphylococcus simulans*, *Bacillus subtilis*, and *Bacillus stearothermophilus*) (Linnett and Strominger, 1973; Parisot et al. 2008). It was reported to have dual mode of action including inhibiting cell wall synthesis and causing pore formation target cell, like nisin (Chatterjee et al. 2005; Cotter et al. 2005). Through in vitro demonstration, Thennarasu et al (2005) indicated that subtilosin caused pore formation on cell membrane of competitor strain. Mode of action of class III bacteriocin produced by *Bacillus* has not been elucidated.

2.6 Bacterial biomass and metabolite production

Biomass and metabolites of food-grade microbes have been used commonly in food industry. The major microbial metabolite, which has been mentioned frequently is bacteriocin. Since they can alternate harmful chemical additives, metabolites synthesized by microorganism have gained significant interest of both producer and consumer. Optimization of production process is required in order to satisfy the increasing demand.

2.6.1 Nutritive and other factors in culture medium

Several key factors associate to *Bacillus* growth in culture medium should be investigated to achieve optimum condition that favors the growth and bacterocin production of *Bacillus*.

2.6.1.1 Nutrient

Carbon source consumed by the cell is used as substrates of metabolism, in which they are broken down to build up amino acids and other components of cell (Wang et al. 2019). The major carbon sources used by microbe include sugar, organic acid, lipid, CO2 and so on (Nguyen and Bui, 2009). Although they are mostly used for cell growth and product formation, the carbon sources are also the key for secondary metabolites production that usually take place when carbon sources in the media are limited (Shu, 2007).

Nitrogen source make ups about 10 to 12% of cell dry weight. It can either be used in organic form (urea, peptides, amino acids, purine, pyrimidine) or inorganic form (ammonia, ammonium salts, nitrate) (Boze et al. 2008). Among various nitrogen sources, ammonia is the preferred nitrogen source of bacteria, which facilitates fast cell growth (Wang et al. 2016).

Other Elements: potassium, magnesium, and calcium are often required as enzyme cofactors. Calcium is required for cell wall synthesis in Gram positive bacteria, including Bacillus.

Trace Elements: many are used as enzyme cofactors and commonly found in tap water including iron, copper, molybdenum, zinc (Posten and Cooney, 2008). However, supplementation of trace elements together with multivitamin in cultivation medium of *Bacillus subtilis* strain recorded the decrease in bacteriocin activity (Sudha, 2021)

2.6.1.2. Physical and chemical parameters

In order to achieve optimum microbial growth and their metabolites, physical and chemical parameters applied for the target strain should be considered carefully because those parameters greatly affect microorganism. The small change in these factors may lead to the formation of unexpected product or major loss of production process.

Oxygen

Oxygen is a key factor in most of bioprocess of microorganisms. They have varying oxygen requirements and usage abilities and can be classified into obligate

aerobes, facultative anaerobes, obligate anaerobes, aerotolerant anaerobes (Engelkirk and Duben-Engelkirk, 2008), and certainly oxygen level required in each group depending upon microbial strains. For *Bacillus*, almost strains are aerobic but specific *Bacillus subtilis* isolates under anaerobic condition could form spore, as stated by Tam et al (2006).

Temperature

The growth rate of microorganism as well as all chemical reactions are affected by temperature. In general, microorganism grow from 25 to 30^{o}C. Based on prefered temperature under cultivation condition in liquid medium, microorganisms are devided into 4 groups: psychrophilic, mesophilic, thermophilic, and extreme thermophilic (VanMeter and Hubert, 2015). Generally, *Bacillus* genus is classified as mesophilic (20-45^{o}C) but the optimum temperature for growth varies according to particular strain (Harwood, 1989).

pH

pH- the measurement of ion H^+ activity, plays an important role in the growth of microorganism. Optimum pH of microbe ranges from 6.5 to 7.5, generally. pH value change response to the metabolism of microorganism. H^+ is generated when microorganism absorbs NH_4^+ and consumed during NO_3^- metabolism as well as when amino acid is used as carbon source (Posten and Cooney, 2008). Microorganisms are divided into 3 groups according to their optimum pH range: acidophiles, neutrophiles, alkaliphiles (Horikoshi, 1999; Baker-Austin and Dopson, 2007). *Bacillus* could growth in a wide range of pH but the optimum pH depends on each strain. For example, the studied *Bacillus thuringiensis* strain could survive from pH 4.9 to 8.0 with an optimal pH of 7.0 while other *Bacillus subtilis* isolates grew maximally at pH 8 (Sidorova et al. 2020).

Agitation

Microorganisms need oxygen for their propagation; otherwise, they cannot survive or switch to anaerobic growth and produce undesired products. The growth rate and metabolism of aerobic and facultative aerobic bacteria are depending on the amount of dissolved oxygen available. Oxygen does not dissolve very well in liquid so the agitation should be conducted to provide dissolved oxygen (Bates et al. 2011). Agitation was proved to have positive impact on cell growth and bacteriocin activity of producer strain. High bacteriocin was achieved when several *Bacillus subtilis*, *Bacillus thuringiensis* and *Bacillus mycoides* isolates were cultivated at 150 rpm under aerobic condition (Khochamit et al. 2015; Mathur et al. 2017 ; Phelan et al. 2013; Rea et al. 2010; Sharma et al. 2011). It is worthy to note that aerobic isolate may need high agitation rate in shake-flask cultivation, which ranges from approximately 180 to 200 rpm in compared with facultative anaerobe isolate. (Cladera-Olivera et al. 2004; Kayalvizhi and Gunasekaran, 2010; Liu et al. 2013; Somsap et al. 2016). However, agitation at high stirring speeds might cause disruption of free cells in the reactor which led to the poor mass transfer (Bakri et al. 2011).

Anti-foam agent

In bioprocess, foam occurs since gas is introduced into the culture medium then is stabilized by proteins produced by organisms in the culture (Etoc et al. 2006). Foaming is considered as undesirable and is an issue to various microbial fermentations. It reduces the productive volume, i.e. increasing process costs, and can lead to blockage of the outlets and threat the sterility of a fermenter. Therefore, antifoam agent is used

to minimize these problems. However, according to Routledge (2012), certain types of antifoam can affect the growth of either prokaryotic or eukaryotic organisms in addition to changing surface properties such as lipid content, resulting in changes to permeability. Several types of antifoaming agent have been used commonly such as industrial product from Brazil (Ind_B, Ind_C) and commercial product developed by Sigma-Aldrich, USA (Antifoam 204, Antifoam C, P2000).

2.6.1.3 Types of medium used for biomass production

Type of culture medium is critical factor for biomass and bioactive compounds production of bacteria. Each bacterial strain requires different types and amount of nutrient so the use of media must be studied intensively to obtain high biomass yield and desired metabolites. Nowadays, there are many synthetic media are available commercially. However, by-products could also be used as substitute, which also confer similar or higher effect compared to standard one as long as they are formulated properly.

Standard medium

Standard medium has been widely used for a long time ago for the growth of microorganism due to convenience. However, there are some limitations such as high cost, low efficiency and so on. *Bacillus* bacteria generally grow well on many different media including NB (Nutrient Broth), which is composed of meat extract, meat peptone; TSB (Tryptic Soy Broth) containing casein peptone, soya peptone sodium chloride and so on. Besides, LB (Luria broth) consisting of tryptone, yeast extract and sodium chloride is also a suitable medium support the growth of *Bacillus* (Merck).

Medium from by-product

There are various types of by-products, which can be formulated as culture medium for microorganism due to their high amount of certain nutrients, in order to not only minimize production cost but also reduce environmental pollution. Some common low-cost, waste products, which have been used widely are whey protein, molasses and so on.

Several reports have demonstrated the use of medium derived from food waste products for cultivation of *Bacillus*. The culture medium contained milled corn cobs and casein hydrolysate observed high spore formation of *Bacillus amyloliquefaciens* strain (Khardziani et al. 2017). The formulation using molasses as the sole carbon source and corn steep liquor as the principal nitrogen source to culture lactic acid bacteria achieved better cell growth in compare to synthetic medium (Lee et al. 2013). Molasses with soybean flour and cheese-whey with sucrose are industrial by-product, which highly facilitated cell growth of *Bacillus thuringiensis* strain (Salazar-Magallon et al. 2015). It was also demonstrated that *Bacillus subtilis* isolate could growth well and exert antimicrobial activity when citrus-juice waste was adopted as sole substrate in culture medium (Yoo et al. 2011).

2.6.1.4 Operation mode

Batch culture: microorganisms are inoculated to a medium in a fermenter, which was prepared in advance (Yang and Sha, 2019). With the growth of microbes, nutrients are gradually used and byproducts are accumulated. Microbes undergo lag phase, log phase, stationary phase and death phase. This process is operated without adding to and withdrawing from the culture of biomass, fresh nutrient medium and culture broth (exclude gas phase). At the end of the fermentation, broth is removed (Cinar et al.

2003). Although this mode could minimize the possibility of contamination due to the closed-system operation, the efficiency is relatively low.

Fed-batch culture is characterized by adding nutrient media gradually at different period, into the inoculated medium. This allows for controlling of several cellular process including cell growth, nutrient uptake and production of target metabolites. Because the used-up media are not removed until the end of the process, the culture increases continuously (Cinar et al. 2003). Fed-batch culture has higher efficiency than batch culture but there is higher chance of contamination.

Continuous operation: this technique is used to prolong the exponential phase by adding essential nutrient continuously together with the withdrawal of culture at certain rate and time throughout the process (Moslamy, 2019). This method could support the scaling up the production to apply in industry. However, there are several drawbacks such as high chance of infection, variation from batch to batch, operation difficulties.

2.7 Protective cultures

Protective cultures are preparations contain live microorganisms (pure cultures or culture concentrates), which are supplemented to foods to minimize the risks caused by pathogenic or toxigenic microorganisms and eventually extend shelf-life of food product (Vogel et al. 2011). They are selected from conventional starter cultures, traditionally fermented foods or newly isolated strains groups that have GRAS status. There are many benefits of using microorganisms as protective cultures since they also generate other molecules including organic acids, carbon dioxide, ethanol, hydrogen peroxide and diacetyl, whose antimicrobial action is well known. Therefore, they can contribute to the flavor, texture and nutritional value of food (Amareswari et al. 2018). Protective cultures applied in food may confer an advantage over the use of semi-purified/purified bacteriocins because the antibacterial activity is more stable (Célia et al. 2018). The global protective culture can be classified based on product form, target microorganism, application and region. Table 2 listed several commercial protective cultures mostly developed from lactic acid bacteria, from different manufacturers.

Table 2. Commercial protective culture

Name	Protective culture composition	Form	Food product	Target microbes	How to apply	Manufacturer

Pediococcus acidilactici B-LC-20	*Pediococcus acidilactici* B-LC-20 produces a class II bacteriocin	Powdered culture	Fermented sausage products	*L.monocytogenes*	Adding on top of the normal starter culture	Chr. Hansen (Denmark)
Lactobacillus curvatus B-LC-48	*Lactobacillus curvatus* produce the bacteriocin curvatin.	Frozen pelletized culture	Ready-to-Eat meat product which are packed under vacuum or modified atmosphere and cold stored	*L.monocytogenes*	- Dipping, dripping or spraying a culture suspension onto the surface after cooking - Mixing into product together with the dry ingredients	Chr. Hansen (Denmark)
Lactococcus lactis ssp. *lactis* BioSafe-10	*Lactococcus lactis* ssp. Lactics produce nisin	Frozen pelleted culture	Cheese	*Clostridium sp*. and *Bacillus sp*	Direct inoculation	Chr. Hansen (Denmark)
Lactobacillus rhamnosus LRB	*Lactobacillus rhamnosus* No information on bacteriocin production	Powdered culture	Fermented milk products and cheese	*Listeria* spp., yeasts, and molds in fermented milk and cheese products	Inoculating as non-starter culture Adding to the liquid used to pack Mozzarella cheese	Sacco Inc (Italy)
Carnobacterium ssp. Lyofast CNBAL	*Carnobacterium* species produce bacteriocin	Freeze-dried culture	Fermented milk products and cheese products	*L.monocytogenes* and generally *Listeria* spp	Directly inoculating or surface treating	Sacco Inc (Italy)
Hafnia alvei B16	*Hafnia alvei*	Frozen pelleted culture	Cheese	Gram-negative organisms in cheese	Directly inoculating	Lallemande (France)

Staphylococcous xylosus XF01	*Staphylococcous xylosus*	Powdered culture	Cheese	Gram positive organisms in cheese	Directly inoculating	Lallemande (France)

Aside from lactic acid bacteria, which were comphrehensively developed as protective culture. Non-lactic acid bacteria, namely *Bacillus* is starting to draw interest of researcher, manufacturer because they have some properties which could solve the limitation of lactic acid bacteria. For example, *B. amyloliquefaciens ssp. plantarum* strains were reported to be potential protective stater culture of Hibiscus alkaline fermented seeds (Compaoré, 2013).

2.8 Application of protective culture in food

Using food-grade bacteria as bio-preservatives in fruit and vegetable products has gained interest of consumers because they can prolong shelf-life, retain the freshness and other nutritional value as well as reduce food waste caused by pathogen and spoilage microbes. However, there is little information available regarding to this practice.

Russo et al. (2014) investigated the potential of using lactic acid bacteria as preservative for fresh cut pineapple. Pieces of fresh-cut pineapple were dipped in prepared buffer containing *Lactobacillus plantarum* and *Lactobacillus fermentum* then air-dried and packed in bag made of polypropylene. There was no change in sensory properties of final product was observed during storage. These bacteria were further found to have inhibitory effect against *L.monocytogenes* and *Escherichia coli* O157:H7.

In another research, commercial fresh soybean sprouts were immersed in enterocin solution produced by *E. faecalis* A-48-32 and stored at 10^{o}C. The result suggested that the presence of bacteriocin interfered the natural microflora in bean sprout such as pathogens, spore formers (Cobo-Molinos et al. 2009).

The efficacy of nisin and pediocin combined with other reagents was investigated in terms of suppressing populations of *L. monocytogenes* in fresh-cut cabbage, broccoli, and mung bean sprouts. The considerable reduction of that pathogen was recorded upon application (Bari et al. 2005).

In the study of Narsaiah et al. (2014), papaya pieces were dipped in alginate solution containing pediocin from *Pediococcus pentosaceus* then packed in LDPE package and stored at 4^{o}C. After storage time, microbial count of coated treatment was much lower than that of the control and those key physical-chemical properties of papaya product were maintained.

Chhetri et al. (2019) investigated the potential of halophilic *B.subtilis* P5-6 as protective culture in food. *B.subtilis* P5-6 showing inhibitory effect against *S.aureus* was prepared in skim milk powder and applied in cheese. During 16 days of storage under 4-6^{o}C, the viable count of *S.aureus* was significantly reduced resulting in the prolong freshness of the cheese over without interfering its physicochemical properties and pH.

In order to inhibit *L.monocytogenes* contaminated in strawberries, raspberries, and blackberries, Cobo-Molinos et al. (2008) proposed washing treatment, in which fruits were immersed in distilled water containing enterocin AS-48. This method helped

to reduce *L.monocytogenes* in raw whole fruit, especially in fruits having higher pH, which is more susceptible to this bacterium.

Jacobsen et al. (2003) applied *Leuconostoc carnosum* as bio-preservative of cooked meat product. The living *L.carnosum* was prepared to 10^7 CFU/g then sprinkled on the surface of each slice of meat product using nozzle. Counts of *L. monocytogenes* never exceeded 10 CFU/ g during 4 weeks of storage at 10°C.

2.9 *Staphylococcus aureus* – a halotolerance pathogen

Staphylococcus aureus is Gram-positive, cocci-shaped, aerobic or facultative anaerobic and non-spore former. Its temperatures for growth ranges from 18°C to 40°C (Taylor and Unakal, 2020). *S.aureus* can tolerate up to 10% salt and survive well in dessication condition (Chaibenjawong and Foster, 2011). It has ability to grow at low water activity (a_w = 0.86) (Scott, 1953). This organism usually presents on soil, skin and other environment (Dwivedi and Tomar, 2016). *It* is responsible for one of the most common foodborne illness, which is Staphylococcal food poisoning (SFP) (Martin et al. 2001). *S. aureus* causes contamination in food products due to improper handling, preparation and processing (Kadariya et al. 2014). Foods including meat and meat products, poultry and egg products, dairy products are most likely to be contaminated with *S.aureus* (Tamarapu et al. 2001; Wieneke et al. 1993). Therefore, using P5-6 isolate, which is a halophilic strain to develop as food preservative targeting *S.aureus* could be a promising practice to preserve food as well as protect consumer's health.

CHAPTER 3 METHODOLOGY

3.1 MATERIALS AND INSTRUMENTS

3.1.1 Culture Media

i. Bair-Paker agar (Himedia, India)
ii. MYP agar (Merck, Germany).
iii. Nutrient agar (Himedia, India)
iv. Nutrient broth (Himedia, India)

3.1.2 Sole substrate

i. Spent mango brine
ii. Spent vegetable brine

Aproximate analysis results of the two spent brines was in appdendix D2 and D3, respectively

3.1.3 Instruments

i. Autoclave (Tommy SX-700, Meditop, Thailland)
ii. Biosafety cabinet (Class II cabinet, Telstar, Thailand)
iii. Bio spectrometer (Eppendorf®, Germany)
iv. Centrifuge (Hermle Z36HK, HERMLE Labortechnik GmbH, Germany)
v. Centrifuge (Mikro 22R, Germany)
vi. Colony Counter (Gallenkamp, England)
vii. Deep freezer (Sanyo Biomedical freezer, Japan-Thailand)
viii. DGGE chamber (BioRad^{TM} Universal Mutation detector, Singapore)
ix. Electronic balance (Mettler Toledo, Switzerland)
x. Electrophoresis gel chamber (HU413L, United Kingdom)
xi. Electrophoresis power supply (Amersham pharmacia, Bitech, Sweden)
xii. Gel documentation chamber (SYNGENE Bio-imaging, USA)
xiii. Hot air Oven (Heraeus, Germany)
xiv. Hot air Oven (Memmret, Germany)
xv. 100-1000 µL Micropipette (Thermo Fisher Scientific, Mexico)
xvi. 20-200 µL Micropipette (Thermo Fisher Scientific, Mexico)
xvii. Micro plate Reader (ASVS, Biochrom, England)
xviii. pH meter (CyberScan® pH 1000 meter, Eutech instruments, Netherland)
xix. Stomacher (AES Labotorie, France)
xx. Shaker (Innova^{TM} 2000, New Brunswick Scientific, Thaoland)
xxi. Refrigerator (Mitsubishi, Thailand)
xxii. Thermal Cycler (BioRad T100^{TM} Singapore)
xxiii. UV transluminator (Vilber Lourmat, France)
xxiv. Vortex (Vortex Genie 2, Scientific Industries, Thailand)

3.1.4 Chemicals and Reagents

i. Agarose basic (Applichem, Spain)
ii. Ammonium Sulfide (*KemAus* / Australia)
iii. D-Glucose (*KemAus* / Australia)
iv. Sodium hypochlorite (Sigma-Aldrich, Germany)
v. Gelatin powder (Ajax Finechem, New Zealand)
vi. Sodium Chloride (QReC®, New Zealand)
vii. Sodium Hydroxide (*KemAus* / Australia)
viii. Tris (Vivantis, Malaysia)

3.1.5 PCR Reagents

i. DNA Tag polymerase (Vivantis, Malaysia)
ii. dNTPs (Vivantis, Malaysia)
iii. Ethidium Bromide (Applichem, Spain)
iv. Ladder (Vivantis, Malaysia)
v. Magnesium Chloride (Vivantis, Malaysia)
vi. Total RNA extraction kit (Vivantis, Malaysia)
vii. Buffer (Vivantis, Malaysia)

3.1.6 Primers

Table 3. List of primers

Primer	Primer Sequence	Reference
Subtilin (SpA)	F CAAGTTCGATGATTTCGATTTGYGATGT	Klein et al (1992)
	R GCAGTTACAAGTTAGTGTTTGAAGGAA	
Subtilosin (SbO)	F CGCGCAAGTAGTCGATTTCTAACA	Stein et al (2004)
	R CGCGCAAGTAGTCGATTTCTAAC	
Housekeeping gene BA-rpoB	F5'-GAC GAT CAT YTW GGA AAC CG-3'	Ko et al (2004)
	R5'-GGN GTY TCR ATY GGA CAC AT-3'	
Bacterial V3 region of 16S rDNA	338F ACT CCTACG GGA GGC AGC AG	Wei et al (2013)
	518R ATTACC GCG GCT GCT GG	

3.1.7 Bacterial culture

i. *Bacillus subtilis* P5-6 isolated from Plara (Thai traditional salt fermented fish) (Chhetri et al. 2019).
ii. *Staphylococcus aureus* ATCC 25923 (Faculty of Science, Chulalongkorn University).

3.2. Methodology

3.2.1 Investigation of *Bacillus subtilis* P5-6 growth, bacteriocin gene and expression

Bacillus subtilis P5-6 was the bacteriocin producing strain used in this study. It was stored at −80°C in Nutrient Broth (NB) medium supplemented with 5% (w/v) NaCl, containing 25% (v/v) glycerol as cryoprotectant. A loopful of cell culture on nutrient agar supplemented with 5% (g/v) NaCl was inoculated on 100 mL of nutrient broth, combined with 5% (g/v) NaCl and incubated on a rotary shaker at 37℃ for 16 hours at 150 rpm. Then 5% (v/v) of culture broth with OD (600 nm) of 0.1 was inoculated into studied media for further experiment.

Stapphylococcus aureus ATCC 25923 was the target pathogen, which was used to investigate the inhibitory effect of bacteriocin producing strain. The culture of this

strain was prepared in the same manner as *B.subtilis* but without the present of NaCl in culture medium.

Growth of *Bacillus subtilis* P5-6 and its bacteriocin genes expression under single culture and co-culture with *Staphylococcus aureus* were investigated. Inhibitory effect of cell culture and cell-free supernatant on *Staphylococcus aureus* relative to the gene expression were also assayed as following.

Bacillus subtilis P5-6 was grown in NB supplemented with 5% NaCl in shaking incubator at 37ºC, 150 rpm for 24 hours. Cell population in the culture was determined every one hour to construct the growth curve. The viable population was done by cultural plating as described by Sander (2012). Cell suspension was diluted in serial dilution. One hundred µL of desired dilution was subjected onto agar plate then spread evenly until agar surface turns dry properly. Plates were incubated at 37ºC for 24 hours. The colonies appear on plate were counted then calculated to get the cell population in sample.

The cell culture of *B.subtilis* P5-6 in NB supplemented with 5% (v/v) NaCl was subjected to DNA extraction using commercial kit (GF01-1, Vivantis, Malaysia). Primers of subtilin (spa) and subtilosin (sbo) encoding genes were used to perform PCR. Gel electrophoresis was followed by running PCR products on 1.5% agarose gel stained with ethium bromide (Velho et al. 2013).

Co-culture of P5-6 and *S.aureus* was conducted by co-inoculation of both strains with the same initial cell concentration in NB supplemented with 5% NaCl in shaking incubator at 37ºC, 150 rpm for 16 hours. The co-culture at 16^{th} hours was subjected to RNA extraction using commercial kit (GF-1, Vivantis, Malaysia). The obtained RNA were converted into cDNA. Primers of subtilin (spa) and subtilosin (sbo) encoding genes were used to perform PCR. Gel electrophoresis was followed by running PCR products on 1.5% agarose gel stained with ethium bromide (Velho et al. 2013). The procedure was conducted as mentioned in section 3.8.4

Antimicrobial activity of P5-6 against *S.aureus* was determined by using spot on lawn assay as described by Tagg and Mc Given (1971). *Stapphylococcus aureus* ATCC 25922 was prepared at selected cell population in peptone water 0.5%. One mL of each cell suspension was spreaded onto Nutrient agar plate. Twenty µL of *B.subtilis* P5-6 cell suspension was spotted directly on each seeded plate. Inhibitory effect was qualitatively determined by measuring the clear zone generated on each lawn. After 24 hours. The diameter of inhibition zones were determined by measuring from the edge of colony spot to the edge of the clear zone.

3.2.2 Determination of inhibitory effect of *Bacillus subtilis* P5-6 on *Staphylococcus aureus* in liquid medium

Bacillus subtilis P5-6 and *Staphylococcus aureus* with initial concentration 8 log CFU/ mL were co-cultured in 5 mL NB supplemented with 5% NaCl. The inoculum size of target pathogen was fixed at 10% (v/v) while *B.subtilis* P5-6 inoculum size was varied at 2, 10 and 20% (v/v). The mixed cultures were incubated for 8 hours before counting population of each bacterium by spreading plate technique on Nutrient agar (NA) (Sander, 2012).

3.2.3 Determination of inhibitory effect of *Bacillus subtilis* P5-6 on *Stapphylococcus aureus* on solid medium

Inhibitory action in solid medium was investigated based on spot on lawn assay. *Staphylococcus aureus* lawn on plate were prepared from 0.1 mL of the cultures

containing 6, 7 and 8 log CFU/ mL then spreaded onto nutrient agar contains 5% (w/v) NaCl and without NaCl . *Bacillus subtilis* P5-6 cell suspension was diluted to obtain 1 to 7 log CFU/ mL then spot 0.1 mL on the *S.aureus* lawn. Inhibitory effect was qualitatively determined by measuring the inhibition zone on each lawn. Diameter of inhibition zone was measured from the edge of cell suspension to the edge of clear zone.

3.3 Culture medium formulation

3.3.1 Screening of spent brines for optimal culture medium

Bacillus subtilis P5-6 was cultivated in NB supplemented with NaCl at 37°C, 150 rpm overnight to obtain cell culture with OD_{600} = 0.8 (approximately 8 log CFU/ mL) then inoculated 5% (v/v) into each treatment as described in table below. Those treatments were incubated at room temperature for 16 hours, at 150 rpm in open air shaker. Culture medium was varied by adjusting mango spent brine, vegetable spent brine and mixture of them at 1:1 ratio to pH 7 and supplemented with minimal nutrients as shown in table 4 then incubated at room temperature in rotary shaker with 150 rpm speed. Mid-stationary phase at around 16^{th} hour was chosen to harvest bacterial cell (Malheiros et al. 2015).

Table 4. Varying the culture medium

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Control
Mango brine	100 mL	-	50 mL	100 mL	-	50 mL	-
Vegetable brine	-	100 mL	50 mL	-	100 mL	50 mL	-
Distilled water	-	-	-	-	-	-	100 mL
Commercial glucose	-	-	-	0.4 g	0.4 g	0.4 g	0.4 g
NH_4Cl	-	-	-	0.1 g	0.1 g	0.1 g	0.1 g
B.subtilis P5-6	5% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)

3.3.2 Investigation of carbon and nitrogen supplementation

The effect of different concentration at 2, 3, 4% (w/v) of glucose (Juan et al. 2014) as carbon sources supplemented in brine medium (chosen from 3.4.1) were investigated.

Commercial glucose was added to vegetable brine that initially contained 0.05% (w/v) carbohydrate, to make up 2, 3, 4% (w/v).

The effect of different concentration at 0.67, 1, 1.5% (w/v) of $(NH_4)_2SO_4$ as nitrogen sources (Tabinda et al. 2016) supplemented in brine medium (chosen from 3.3.1) with glucose at three concentration aforementioned were investigated.

$(NH_4)_2SO_4$ was added in vegetable brine, which initially contained 0.67% (w/v) protein, to make up 1, 1.5% (w/v).

The bacterial cultivation was performed for 16 hours, under room temperature and inoculum size of 5% (v/v) (initial population was 8 log CFU/ mL), at 150 rpm for 16 hours. All experimental units were performed using factorial design with 2 factors, 3 levels. The basal medium, which was vegetable brine with minimal nutrient supplementation served as control. The cell population was determined at the end of cultivation (16^{th} hour). The antimicrobial activity of culture was also determined at 16^{th} hour. The effect of each carbon and nitrogen sources were evaluated based on cell growth and antimicrobial activity.

3.3.3 Investigation of agitation rate

Cultivation in terms of agitation rate was investigated. *Bacillus subtilis* P5-6 was cultivated in brine medium (chosen from 3.3.2) and incubated at 100 and 150 rpm (Kamoun et al. 2009) under room temperature for 16 hours.

The cell population were determined at 16^{th} hour. The antimicrobial activity of culture was also determined at 16 hours.

The optimum agitation rate was chosen according to cell population and inhibitory effect of *B.subtilis* P5-6 against target pathogen.

3.4 Protective culture preparation

Three solid matrices including whey protein, maltodextrin and modified starch were chosen to investigate their capability in protection of viable cell and antimicrobial activity from drying process.

Post-cultivation medium was mixed with each solid matrix with 1:1 and 2:1 (liquid: solid) ratio to form a semi-dried powder. The powders were dried under 2 steps process with 50°C for 30 minutes -then changed to 55°C for 120 minutes (Rogers, 1914) to obtain water activity lower than 0.6 and finally grinded into fine powder. The effectiveness of solid matrices was evaluated based on viability of the studied strain and inhibitory effect of protective powder against target pathogen.

3.5 Application of *Bacillus* protective culture in food model

Pork loin was purchased from local supermarket. Initial microbial load of meat was reduced by modified method of GonÇalves and others (2005), as described by Tananuwong et al (2012). Approximately 200 g of silced pork was soaked into 200 mL of NaOCl solution, containing 200 ppm of free chlorine, for 2 min. Those slices was then soaked in sterile water for 15 s to wash of NaOCl residue. After pretreatment, initial microbial population in meat sample reduced from 6 log CFU/ mL to 5 log CFU/ mL, determined by total plate count. *Staphylococcus aureus* was cultivated in rotary shaker at 37°C, 150 rpm for 24 hours to reach 8 log CFU/ mL. One mL of protective culture solution was evenly spread on surface of 25 g piece of meat sample (4x5x1 cm). Calculated volume of *S.aureus* were inoculated onto meat surface to achieve theoretical load of 2, 3 and 5 log CFU/ g. Samples were then treated with protective powder at calculated volume. Pork meat without spiked *S.aureus* and protective culture was used as negative control while positive one was slice inoculated with 5 log CFU/ mL then treated with protective culture. Meat sample contained only spiked *S.aureus* was used to monitor the behavior of this strain upon inoculation. Each 25g slice was kept in sterile petri dish and sealed in ziplock bag then stored in domestic refrigerator (8-10°C). All treatments were prepared in duplicate. The viability of *S.aureus*, *B.subtilis* P5-6 from protective culture and total plate count were monitor on 0, 1 and 5 days after. Meat

sample was taken out aseptically then homogenized in 225 mL peptone water 0.1% followed by serial dilution. Total plate count was enumerated on NA while *S.aureus* was detected using Baid-parker (Merck, Germany) and *B.subtilis* P5-6 was counted on MYP agar (Merck, Germany).

3.6 Statistical analysis

Data were presented as mean ± standard deviation from experiments conducted in triplicates. Analysis of variance (ANOVA) and multiple comparisons by Duncan's test were performed using IBM-SPSS statistics package version 22 (SPSS Inc., Chicago, IL, USA). Statistically significant difference was calculated at significant level of $p < 0.05$.

3.7 Analytical methods

3.7.1 DNA extraction

Bacillus subtilis P 5-6 were cultured on NA plate supplemented with 5% (v/v) NaCl and grown at at 37ºC for 24 hours. Bacterial DNA was extracted using commercial kit (GT-100, Bacterial DNA extraction kit, Vivantis, Malaysia).

3.7.2 RNA Extraction

In order to study the bacteriocin gene expression of *B.subtilis* P5-6, the bacterial RNA were directly extracted from the post-cultivation medium and purified using commercial kits (GT-1, Total RNA extraction, Vivantis, Malaysia) (Appendix A1). The purified RNA were converted to complementary DNA (cDNA) by reverse transcriptase reaction (Total RNA extraction kit, Vivantis, Malaysia). The first set of reverse transcription reaction was performed using thermal cycler (BioRad T100^{TM}, Singapore) and standard PCR tubes, containing a total of 10 µL mixture with dNTPs (10U), Oligo d (40 µm), hexamer (50 ng) and 2 µL (10-50 ng/ µL) programed initially at 65ºC, 5 min. Then followed by 42ºC, 60 min with addition of 10X buffer, reverse transcriptase (200 U) and RNA free water making the total volume up to 20 µL.

3.7.3 Nucleotide sequencing analysis

The nucleic acid sequences were chosen from the conversed regions of 16S rDNA. PCR was performed using primer set 338F/ 519R (Mao et al. 2012), in 50 µL reaction mixtires using 2 µL DNA (10-50ng/ µL), 0.1 mM of each primer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs mix and 2 U of DNA Tag polymerase (Vivantis, Malaysia). Samples were subjected to an initial cycle of denature (94ºC for 2 min), followed by 35 cyclesof denaturation (94ºC for 30 s), annealing (55ºC for 30 s) and elongation (72ºC for 30 s), ending with extension at 72ºC for 7 min, in a DNA thermal cycler (Biorad T100^{TM} Singapore). Ten µL of PCR products were directly applied onto 1.5% (w/v) agarose gel in 1% TAE buffer containing 2M Tris base, 1M Glacial acetic acid, 0.5M EDTA, pH 8.0 to 1000 mL distilled water. Electrophoresis gel chamber; HU413L, United Kingdom and Electrophoresis power supply, Amersham pharmacia, Bitech, Sweden). After electrophoresis, gel was stained with 1% ethidium bromide (Applichem, Spain) and photographed under UV transluminator (Vilber Lourmat, France). Thus, this 16S rDNA region amplified PCR product was sent to commercial manufacturer for sequencing analysis (Macrogen, Korea) after cleaning. The sequencing data was analyzed with nucleotide BLAST program of NCBI.

3.7.4 Gene encoding bacteriocin analysis

Extracted DNA was examined for gene encoding bacteriocin. Subtilosin and/ or subtilin bacteriocin genes were amplified using primer Sbo/ Spa for PCR and run on

1.5% agarose gel strained with ethidium bromide. PCR was performed using subtilin (Spa) and subtilosin (SboA) (Sutyak et al. 2008) encoding primers (primer sequence as mentioned in table 3), in 50 µL reaction mixtures using 2 µL (10-50ng/ µL) DNA, 0.1 mM of each set primers, 1.5 mM $MgCl_2$, 0.1 mM dNTPs mix and 2 U of DNA Tag polymerase (Vivantis, Malaysia). Samples were subjected to an initial cycle of denature (94^{o}C for 2 min), followed by 35 cycles of denaturation (94^{o}C for 30 s), annealing (55^{o}C for 30 s) and elongation (72^{o}C for 30 s), ending with extension at 72^{o}C for 7 min, in a DNA thermal cycler (Biorad T100^{TM} Singapore). The test was repeated in duplicate.

3.7.5 Gene expression assay

Bacillus subtilis P5-6 containing bacteriocin encoding gene was evaluated for the gene expression under several cultivation conditions. RNA was extracted and converted into cDNA as describe above. The gene encoding for bacteriocin (subtilin and subtilosin) production was confirmed by PCR along with housekeeping gene BA-rpoB (F: 5'-GAG GAT CAT YTW GGA AAC CG-3'; R:5'-GGN GTY TCR ATY GGA CAC AT-3') (Ko et al. 2004). The PCR amplicon was measured on 1.5% agarose gel electrophoresis, respectively.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Growth property of *Bacillus subtilis* P5-6 in standard medium

Since P5-6 is halophilic and this study aims to develop culture medium containing salt, it is nescessary to determine growth properties of this isolate in high salt concentration. Based on previous study, which isolated P5-6 strain, it was reported that

5% (w/v) of NaCl was the optimum condition that maximally supported bacteriocin production of P5-6. Thus, this condition was adopted for further investigation.

In evaluation of *Bacillus subtilis* P5-6 growth property in standard medium, it is clearly shown in figure 2 that during cultivation, significant increase in bacterial population as log phase ranged from 1 to 10th hour and then changed to stationary phase at the 10th to 25th hour. According to Oakley (2017), who demonstrated that the production of secondary metabolites, particularly antimicrobial substances which were not necessary for bacterial growth would be generated in the mid-stationary phase. Therefore, from this observation, in order to investigate the bacteriocin production and or inhibitory properties of *B.subtilis* P5-6, it was cultivated for total 16 hours.

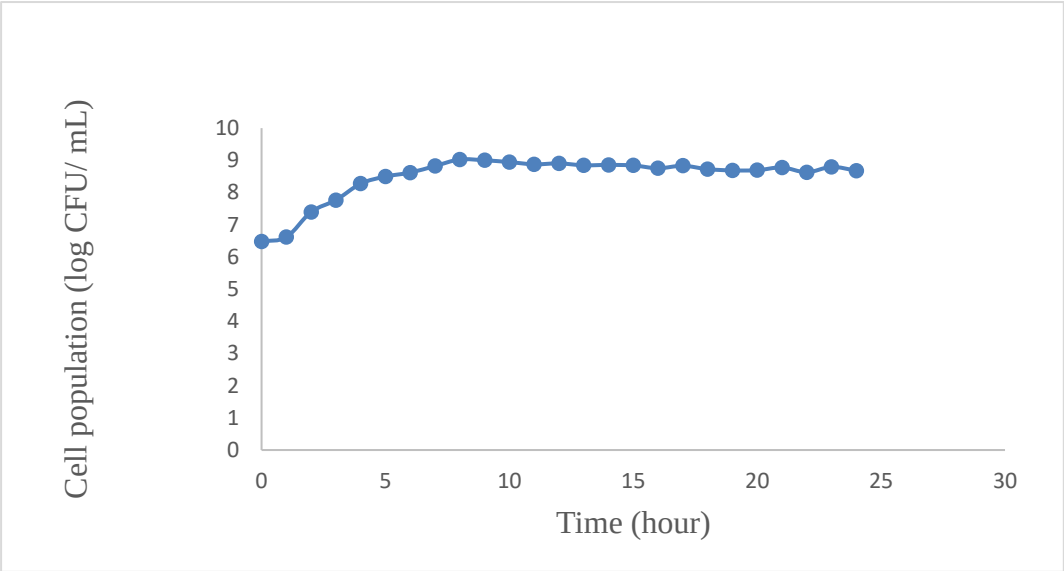

Figure 2. Growth curve of *Bacillus subtilis* P5-6 in NA supplemented with 5% NaCl (w/v) under 37ºC, 25 hours

4.2 Bacteriocin generation properties

Two target bacteriocins including sboA (734 bp) encodes subtilosin and spaS (566 bp) encodes subtilin were well characterized, which could inhibit several Gram positive and Gram negative bacteria. According to figure 3a, both genes were observed in *B.subtilis* P5-6. However, when the gene expression during cultivation at 16th hour tested, only the sboA was observed (figure 3b). This result was different from the one obtained from previous research (Chhetri et al. 2019) using the same strain, in which expression of both genes were detected. The difference could be resulted from the different investigation method. In Chhetri et al (2019) study, P5-6 was co-culture with three *S.aureus* isolates for 24 hours under 37ºC while in this study, only one *S.aureus* strain was co-cultured with P5-6 then incubated for 16 hours under the same temperature. Thus, the subtilosin might be the key bacteriocin playing inhibitory effect on the competitive/specific microbes. Based on this understanding, appropriate condition and method was developed to achieve the optimum activity of subtilosin.

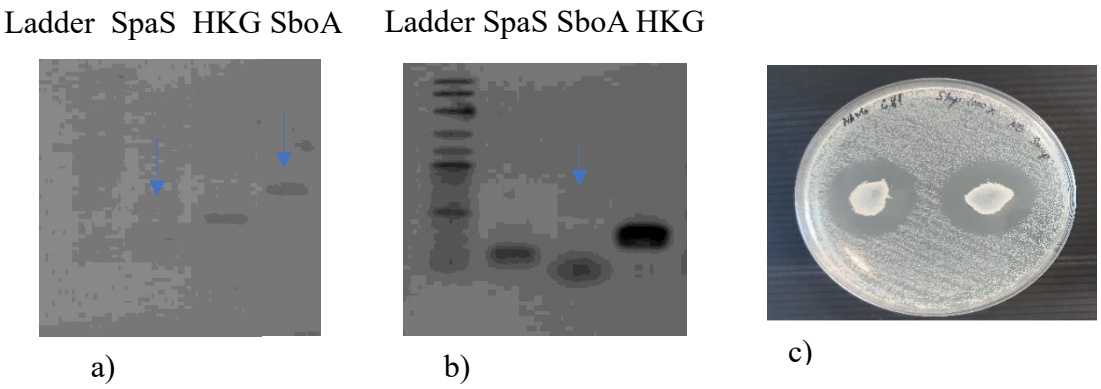

Figure 3. Gene detected from PCR (a) RNA expression during cultivation (b) and (c) spot on lawn of *B.subtilis* P5-6 cell suspension (2 replicates) on *S.aureus* lawn

Subtilosin A was reported to be more effective against gram-positive bacteria than gram-negative bacteria (Shelburne et al. 2007)**.** In this study, the spaS gene expression was not detected the *B.subtilis* P5-6 . It could be because this gene did not function properly and/or the cultivation condition in this study might not favor the expression of spaS gene. Accordingly, antimicrobial test demonstrated that cell suspension of *B.subtilis* P5-6 of this period could effectively inhibit *S.aureus* (Fig 3c). Based on the gene expression result, this inhibitory effect could be due to an activity of subtilosin encoded by sboA gene as stated above. However, when bacterial cells were removed, cell free supernatant did not show any inhibitory effect against *S.aureus*. It could be because of the (i) low concentration of bacteriocin secreted in broth or (ii) being diluted, or (ii) bacteriocin produced might attach to *B.subtilis* P5-6 cell surface. Therefore, when cells were removed, bacteriocin was also taken out, resulting in no inhibition zone on *S.aureus* lawn. When cell suspension was spotted on lawn, bacteriocin generated during cultivation might still attach to protect the cell from *S.aureus* without regenerating. It might not generate at too high concentration in broth system to prevent self-inhibitory effect (to kill its cells). In solid matrix, bacteriocin generated could diffuse through the solid matrix and was re-genrated to futher kill *S.aureus*. Since the bacteriocin would migrate away from bacterial cell, the self-inhibitory effect is unlikely to occur on this medium state.

The hypothesis about bacteriocin attached to producer cell was proposed based on preliminary lab result (not included), in which 10 minutes sonicated cell suspension of P5-6 showed relatively small inhibition zone on *S.aureus* lawn compared to fresh cell suspension but cell free supernatant of both did not perform inhibitory effect against target pathogen. It could be intepreted that when sonicated cell culture was spot on *S.aureus* lawn, only bacteriocin bound to P5-6 cell took action, created relatively small clear zone on lawn *S.aureus*. Sonicated cells were dead or majorly injured so they could not generate bacteriocin. In non-sonicated cell, the inhibition zone of P5-6 isolate against *S.aureus* was much larger than sonicated one because membrane attached bacteriocin performed antimicrobial activity against *S.aures* first, then viable cell continued to produce more bacteriocin, leading to larger inhibition zone. Removing cell out of the suspension means removing bacteriocin, resulting in no inhibitory effect

against *Saureus* recorded. This hypothesis has not been mentioned or elucidated by previous study so it requires further investigation.

4.3 Inhibitory effect of *Bacillus subtilis* P5-6 on *Staphylococcus aureus* in liquid medium

According to the growth curve, mid-stationary phase at 16th hour, which involves bacteriocin production was chosen to harvest the cultivation broth. Yan and research group (2016) indicated that inhibition was affected by both the growth stage and the amount of *B. subtilis* cells presented in cultivation broth. Thus, to evaluate the factor associated to bacteriocin generation, *B.subtilis* was co-cultivated with various cell density of *S.aureus*. The viability of both bacteria was then assayed. The results as shown in table 5. In co-culture for 16 hours, *S.aureus* could not grow well relative to in single culture. This demonstrated that the presence of *B.subtilis* could interfere the growth of *S.aureus*. The inhibition of *B. subtilis* on neighboring *S. aureus* could be a function of the lipopeptide bacteriocin released. In addition, when co-culture with *S.aureus*, the amount of those peptides secreted would be increased due to microbial competition (Gonzalez et al. 2011). Thus, *B.subtilis* was co-cultured with *S.aureus* at different ratio in order to induce bacteriocin production in broth. Results as shown in table 5, in co-culture of 2% (v/v) *B.subtilis* with 10% (v/v) of *S.aureus* for 16 hours, although inoculum size of *B.subtilis* was 5 times less than *S.aureus*, they both reached equal cell counts after 16 hours. When *B.subtilis* P5-6's inoculum size was increased from 2 to 10% (v/v), the change in *S.aureus* population was still not much different. *Bacillus subtilis* at 10% of inoculum size was nearly equal to that in single culture. When doubled the inoculum size of *B.subtilis* P5-6 from 10 to 20% (v/v), *S.aureus* population decreased 1 log CFU/ mL (from 8.414 to 7.929 log CFU/ mL) and it's 1 log CFU/ mL less than that in single culture (8.854 log CFU/ mL). It seemed like *B.subtilis* P5-6 could dominate in the co-culture system. Because its initial population was double *S.aureus*, it could occupy more nutrient and grow faster than its competitor rather than playing inhibitory effect. Thus, to test the inhibitory effect of bacteriocin of the *Bacillus* that might present, the cell free-supernatant culture were spotted on *S.aureus* lawn. There were no inhibition zone observed (Fig 4). This observation proved that the P5-6 might have no inhibitory effect on *S. aureus* in the liquid culture.

Inoculum size % (v/v) of 8 log CFU/ mL		Cell population (log CFU/ mL)	
B.subtilis P5-6	*S.aureus*	*B.Subtilis* P5-6	*S.aureus*
2 (6 log CFU/ mL)	10 (7 log CFU/ mL)	8.304 ±0.18[b]	8.497 ±0.48[b]
10 (7 log CFU/ mL)	10 (7 log CFU/ mL)	8.769 ±0.09[a]	8.414 ±0.12[b]
20 (>7 log CFU/ mL)	10 (7 log CFU/ mL)	8.825 ±0.04[a]	7.929 ±0.03[c]
10 (7 log CFU/ mL)		8.729 ±0.32[a]	ND
	10 (7 log CFU/ mL)	ND	8.854 ±0.09[a]

Table 5 Bacterial population in co-culture after 16 hours of cultivation in Nutrient broth supplemented with 5% NaCl (*statistical analysis was based on the same column*)*

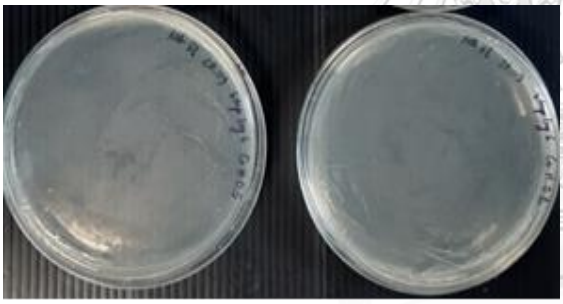

Figure 4. Spot-on-lawn of co-culture cell-free supernatant on *S.aureus* lawn (6 log CFU/ mL) (ISO 16187, 2013) *Cell density of *S.aureus* lawn: 4 log CFU/ cm^2

4.4 Inhibitory effect of *Bacillus subtilis* P5-6 on *Stapphylococcus aureus* on solid medium

According to bacteriocin production properties and the result of co-culture of *B.subtilis* P5-6 and *S.aureus*, antimicrobial activity in liquid culture was not significant Supposedly,-bacteriocin produced might attach to cell membrane of this *Bacillus* strain to protect itself from *S.aureus* located near its cell. . The liquid culture could disperse *B.subtilis* P5-6 and *S.aureus* cells a part and also dilute bacteriocin, if it was secreted into broth. Therefore, to evaluate the inhibitory activity of bacteriocin from P5-6 on *S.aureus*, cultivation on solid medium model were subjected to be tested by spot on lawn technique. The cell concentrations of two strains were varied for screening a proper cell concentration of *B.subtilis* P5-6 that effectively inhibit *S.aureus*.

The results in table 6, which were measured from figure 5, showed that at the same population of *S.aureus*, the inhibition zone increased as *B.subtilis* P5-6 population increased. It relatively cleared that on solid medium, *Bacillus subtilis* P5-6 could inhibit *S.aureus* even when its population was much lower than *S.aureus* population. However,

at low population, this *Bacillus* isolate displayed no significant inhibitory activity (IZ < 0.8 mm). This could be due to the mechanism of quorum sensing-signalling molecules. It has been known when cell density is low, the signalling molecules generated from inducing cell will diffuse away so there is no detection and response between cell (Kaplan and Greenberg, 1985). Thus, at the low amount of *B.subtilis* P5-6 cell, signal molecules (autoinducers) were insufficient for them to communicate with each other, to produce antimicrobial agent against *S.aureus*.

In the presence of 5% (w/v) NaCl (table 6), the inhibition zones were detected at second lowest cell concentration of P5-6 isolate (2 log CFU/ mL) among tested concentration whereas no inhibitory effect was observed at the same cell concentration when there was no NaCl supplemented. Because *B.subtilis* P5-6 is halophile while *S.aureus* is halotolerant, the supplementation of NaCl could stimulate *B.subtilis* P5-6 growth and inhibit the growth of *S.aureus*.

Table 6 Inhibitory effect of different concentration of *B.subtilis* P5-6 on different concentration of *S.aureus* on solid medium (*statistical analysis was based on the same column)

Bacillus subtills P5-6	*Stapphylococcus aureus*					
	6 Log CFU/ mL on plate *Cell density of *S.aureus* lawn: 4 log CFU/ cm²		7 Log CFU/ mL on plate *Cell density of *S.aureus* lawn: 5 log CFU/ cm²		8 Log CFU/ mL on plate *Cell density of *S.aureus* lawn: 6 log CFU/ cm²	
Population (Log CFU/ mL)	Inhibition zone on NA w/o NaCl (mm)	Inhibition zone on NA+5% NaCl (mm)	Inhibition zone on NA w/o NaCl (mm)	Inhibition zone on NA+5% NaCl (mm)	Inhibition zone on NA w/o NaCl (mm)	Inhibition zone on NA+5% NaCl (mm)
7	6.06±0.47^{a}	6.20±0.17^{a}	5.58±0.33^{a}	5.13±0.31^{a}	4.32±1.36^{a}	4.55±0.13^{a}
6	4.45±0.13^{b}	5.75±0.35^{a}	4.50±0.56^{b}	4.07±0.38^{b}	3.03±0.45^{b}	3.70±0.01^{b}
5	3.88±0.53^{b}	4.75±0.08^{b}	3.27±0.33^{c}	3.22±0.28^{c}	1.90±0.25^{c}	2.73±0.32^{c}
4	2.43±0.40^{c}	3.63±0.20^{c}	2.18±0.10^{d}	2.25±0.34^{d}	0.87±0.27^{cd}	2.45±0.22^{c}
3	1.68±0.35^{d}	2.73±0.51^{d}	1.25±0.17^{e}	1.43±0.05^{e}	ND	1.12±0.12^{d}
2	ND	1.20±0.03^{e}	ND	0.80±0.34^{ef}	ND	0.93±0.0^{d}
1	ND	ND	ND	ND	ND	ND

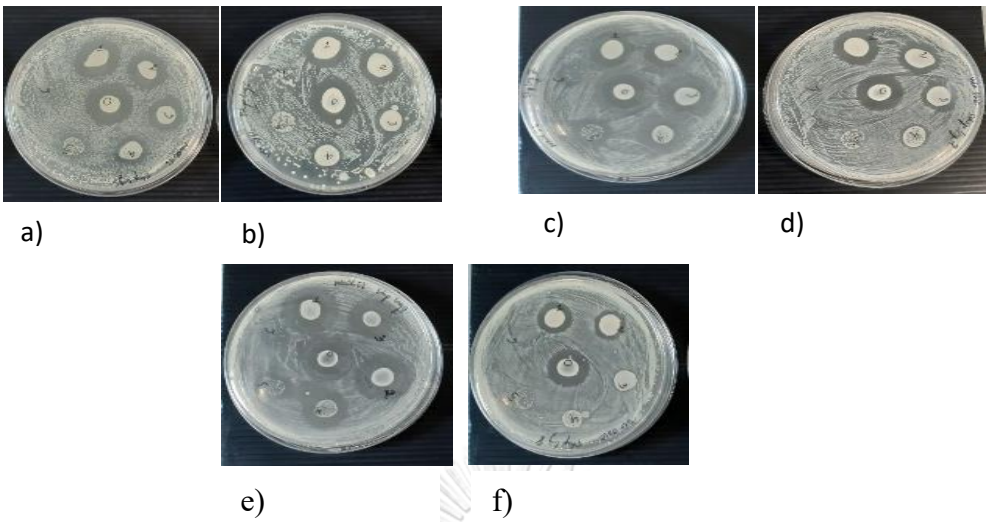

Figure 5. Spot on lawn on different population of *B.subtilis* on *S.aureus* lawn 6 log CFU/ mL on NA with 5% NaCl (a), and without NaCl (b), 7 log CFU/ mL on NA with 5% NaCl (c) and without NaCl

It was shown that in solid medium, *B.subtilis* P5-6 could exert higher antagonistic action against *S.aureus* relative to liquid medium. In liquid culture, because bacteriocin was supposed to be generated with low amount or still attached to cells of producer strain, in order to observe a considerable inhibition effect, *B.subtilis* P5-6 must be used in the same population and double inoculum size in compared with that of *S.aureus*. On the other hand, solid culture might facilitate cell-cell contact between two bacteria, which might induce the antimicrobial activity of *B.subtilis*. In addition, antimicrobial substance produced by *B.subtilis* P5-6 could be more concentrated than that in liquid culture. Because the solid medium might support the migration of bacteriocin, they could diffuse away from cell and be regenerated. The concentration of bacteriocin was higher in compared with that in liquid culture but it was not harmful for producer strain because it did not attach to its cells. Thus, inhibitory effect could be detected at low population of *B.subtilis* P5-6.

4.5 Culture medium formulation

4.5.1. Screening of spent brines for optimal culture medium

In order to develop cost effective culture medium for P5-6 isolate, spent brines from mango containing 2.11% (w/v) carbohydrate, 0.2% (w/v) nitrogen and vegetable factories containing 0.05% (w/v) carbohydrate, 0.67% (w/v) nitrogen as shown in Appendix D2 and D3, respectively were used as basal medium formulated with supplementary agent as varied in table 7. The growth and inhibitory effect on *S.aureus* shown in table 7 demonstrated that treatment 5, which was vegetable brine, originally contained 4.92% (w/v) NaCl, supplemented with minimal nutrient ((0.04% (w/v) glucose and 0.01% (w/v) $(NH_4)_2SO_4$) recorded maximal growth for the studied isolate at 8.98 log CFU/ mL and inhibition zone at 2.9 mm.

Table 7. Bacterial growth in different brine media (*statistical analysis was based on the same row)

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Control
Mango brine	100 mL		50 mL	100 mL		50 mL	
Vegetable brine		100 mL	50 mL		100 mL	50 mL	
Distilled water							100 mL
Commercial glucose				0.4g	0.4g	0.4g	0.4g
NH_4Cl				0.1g	0.1g	0.1g	0.1g
B.subtilis P5-6	5% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)
Cell population (Log CFU/ mL)	8.46 ± 0.084^{b}	8.5 ± 0.035^{b}	8.54 ± 0.063^{b}	8.2 ± 0.459^{bc}	8.98 ± 0.084^{a}	8.49 ± 0.049^{b}	7.94 ± 0.014^{c}
Inhibition zone (mm)	1.6 ± 0.007^{de}	1.9 ± 0.014^{bc}	2.1 ± 0.014^{b}	1.3 ± 0.00^{e}	2.9 ± 0.021^{a}	1.7 ± 0.021^{cd}	0.7 ± 0.035^{f}

because preservative was included in mango brine according to the manufacturing process, it might inhibit the bacterial cell growth to some certain extend. Thus, this mango brine condition could not be an alternative medium for *B.subtilis* P5-6. The population obtained when cultivated in the optimum spent brine medium (treatment 5) was approximately equal to cell population when cultivated in standard medium, which were 8.98 and 8.75 (from growth curve) log CFU/ mL, respectively. In compared with the non-supplemented vegetable brines (treatment 2), minimal supplementation (treatment 5) showed better result. Because nutrient retained in spent brine might be insufficient for bacterial proliferation, so the minimal supplementation of carbon and nitrogen source, which was 0.04% (w/v) glucose and 0.01% (w/v) $(NH_4)_2SO_4$, respectively could help to increase cell population in this culture medium.

Supposely, higher amount of nutrient added would help to promote higher cell population and inhibitory effect of P5-6 against *S.aureus*, so the formula of treatment 5 as *B.subtilis* P5-6 cultivation medium was used to further optimized the carbon and nitrogen supplementation.

4.5.2 Investigation of carbon and nitrogen supplementation

Vegetable brine supplemented with minimal nutrient was recorded to be the optimum basal medium in section 4.5.1. It was used to further vary the carbon and nitrogen supplementation to improve the growth and antagonistic effect of P5-6 strain on *S.aureus*. Statistical analyses of table 8, figure 6,7 results based on Duncan test after ANOVA analyses, showed that there was no significant difference in cell population when adding more nutrient in compared with that in treatment supplemented with minimal nutrient. Similar pattern was observed in inhibitory effect of P5-6 on *S.aureus*, in which the inhibition zone of minimal supplementation was not significantly different from other higher supplementation level. The results were in agreement with those finding of Guerra et al (2001) and de Rojas et al (2007) that the production of bacteriocin was increased as glucose increased up to certain level. However, the high glucose concentration did not facilitate bacteriocin production because of the saturation of glucose transport in cell. Panda et al (2017) also demonstrated that the excess of glucose negatively affected the growth of *B.subtilis*. It was reported that bacteriocin activity reduced due to substrate inhibition when high glucose was added in culture media (Khay et al. 2013). Anderson and Jayaraman (2003) stated that further increasing in carbon and nitrogen source beyond the optimum level would cause decrease in cell density. Therefore, the result of this experiment could be interpreted that the amount of nutrient presented in vegetable brine together with added glucose and ammonium sulfate was sufficient for microbial activity of this bacterial strain. Thus, the treatment of vegetable brine supplemented with minimal nutrient included 0.04% (w/v) glucose and 0.01% $(NH_4)_2SO_4$ was chosen for next experiments.

Table 8. Bacterial cell population and inhibitory effect in different culture media (*statistical analysis was based on the same column)

Treatment	Cell population (Log CFU/ mL)	Inhibition zone* (mm)
2%C + 0.67%N	8.91 ± 0.398^{ab}	1.30 ± 0.045^{a}
2%C + 1%N	9.46 ± 0.398^{a}	1.23 ± 0.015^{a}
2%C + 1.5%N	8.99 ± 0.343^{ab}	2.03 ± 0.005^{a}
3%C + 0.67%N	9.42 ± 0.167^{a}	2.15 ± 0.187^{a}
3%C + 1%N	9.56 ± 0.109^{a}	1.71 ± 0.148^{a}
3%C + 1.5%N	9.28 ± 0.336^{a}	2.40 ± 0.03^{a}
4%C + 0.67%N	8.86 ± 0.557^{ab}	1.43 ± 0.01^{a}
4%C + 1%N	8.98 ± 0.506^{ab}	1.45 ± 0.004^{a}
4%C + 1.5%N	9.12 ± 0.345^{ab}	2 ± 0.25^{a}
Control (minimal nutrient supplemented)	9.14 ± 0.329^{a}	2.70 ± 0.056^{a}

*Diameter of inhibition zone was measured from the edge of bacterial cell colonized to the edge of clear zone at 3 different areas.

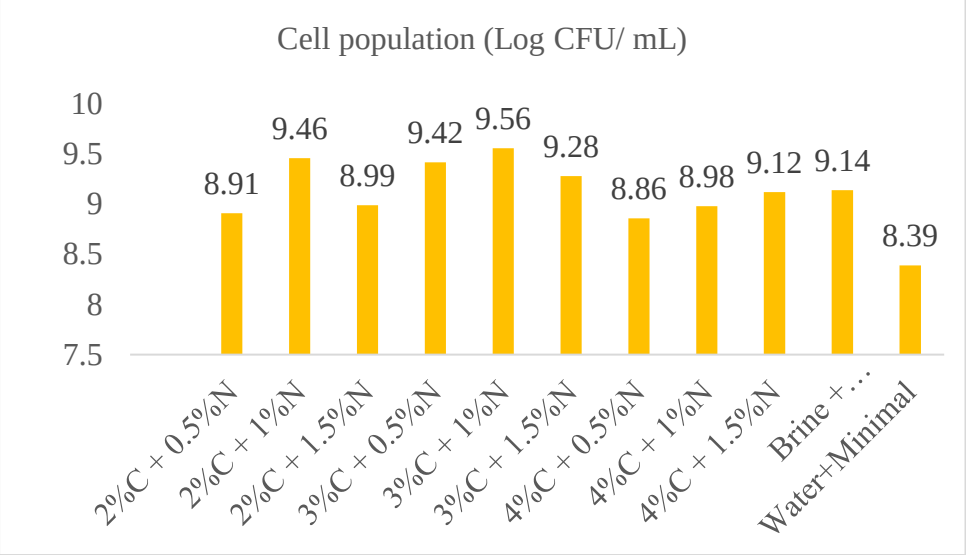

Figure 6. Impact of glucose and ammonium sulphate on bacterial growth

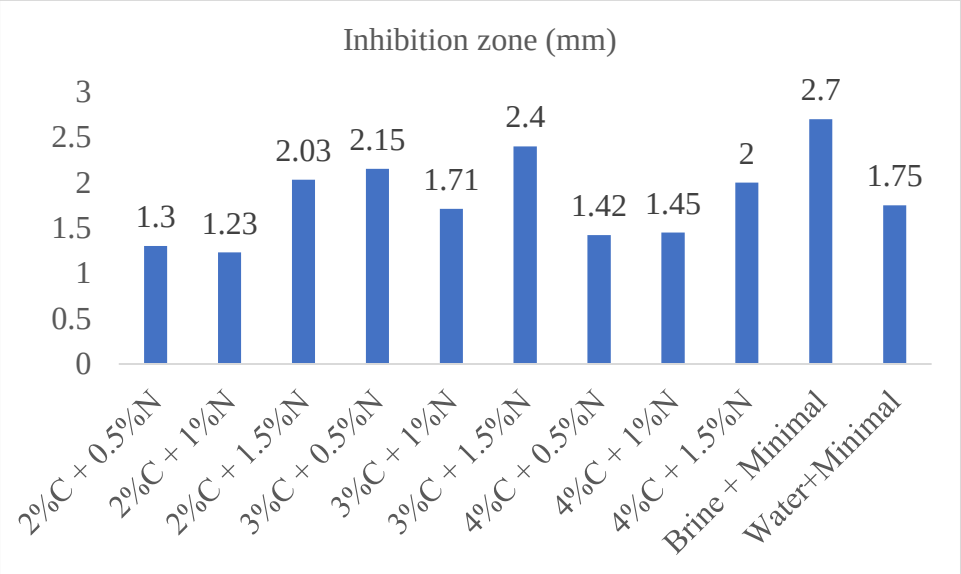

Figure 7. Impact of glucose and ammonium sulphate on inhibitory effect of *B.subtilis* P5-6 against *S.aureus*

4.5.3 Investigation of agitation rate

The optimized culture medium obtained from previous section, which was vegetable brine supplemented with minimal nutrient including 0.04% (w/v) glucose and 0.01% (w/v) $(NH_4)_2SO_4$ was used to investigate the cultivation condition. According to preliminary lab result (not included), the cell population and inhibition zone of culture incubated at room temperature (30°C) and 37°C as obtained in this study were not different. In addition, there were some reports demonstrated impacts of cultivation condition on *Bacillus* metabolize profiles, particularly, temperature. It was reported that the growth profile *Bacillus* strain under the range of 30°C and 37°C were relatively

similar (Ayed et al. 2015) but the antimicrobial activity against *S.aureus* achieved at 30ºC was better than that at 37ºC (Abd and Luti, 2017). Thus, room temperature was chosen as cultivation temperature to minimize production cost and enhance efficiency of bacteriocin production throughout this research without re-investigation.

Agitation rate has various effects on the growth of microbe including cell wall disruption, changes in growth morphology, variations in growth rate and production rate of the desired product (Purwanto et al. 2009). It favors microbial activity by supporting the transfer of oxygen and nutrient in culture broth (Fonseca et al. 2007). However, there was another finding that strong agitation rate did not favor bacteriocin production (Kamoun et al. 2009). It has also been reported that cultivation under too high/severe agitation rate could interfere gene expression and decompose some metabolite as well as bacteriocin causing an activity reduction (Parente et al. 1997). As a result, agitation rate of 200 rpm, which was mentioned in proposal was excluded from this experiment. Vegetable brine with minimal supplementation of nutrient was cultivated under room temperature at 100 and 150 rpm of agitation rate to investigate the optimum cultivation condition.

Table 9 and figure 8 recorded that when cultivated *B.subtilis* P5-6 at 100 rpm and 150 rpm, cell population and inhibition zone of culture at 150 rpm were considerably higher than those at 100 rpm. The result indicated that at higher agitation rate, *B.subtilis* grew better with higher inhibitory effect on *S.aureus*. There are some findings, which were different from the observation in P5-6. It was noted that static condition recorded highest cell population and bacteriocin production of LAB bacterial isolate while the lowest value of these parameters were observed at 250 rpm (Danial et al. 2016; Verluyten et al. 2003) indicated that bacteriocin of lactic acid bacteria was active under limited or reduced oxygen in the medium. The differences between this study and the previous one could be due to different producer strains used. While *B.subtilis* P5-6 is aerobic or facultative an aerobic, lactic acid bacterial strain anaerobic, they required different oxygen level for their growth and metabolite production.

Table 9 . Bacterial cell population and inhibitory effect against *S.aureus* at different agitation rate

Treatment	Cell population (Log CFU/ mL)	Inhibition zone (mm)
100 rpm	8.30 ±0.165	2.2±0.2
150 rpm	9.14 ±0.527	3.3±0.2

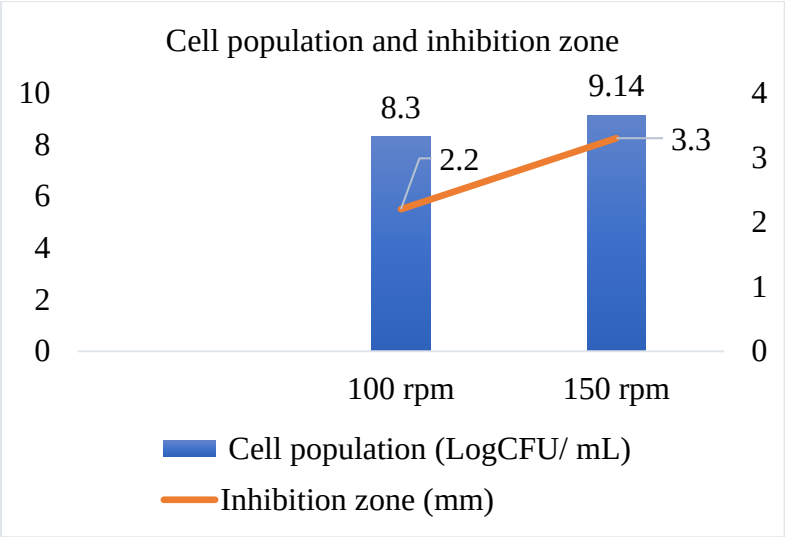

Figure 8. Cell population and inhibitory effect of *B.subtilis* P5-6 against *S.aureus* at agitation rate of 100 and 150 rpm.

4.6 Protective culture preparation

Vegetable brine supplemented with minimal nutrient was used to cultivate *B.subtilis* P5-6 under room temperature for 16 hours at 150 rpm agitation rate, which was optimized from section 4.5.3.

Whey is a by-product of the dairy industry containing high nutrients including lactose, soluble proteins and minerals salts (Abbasiliasi et al. 2017). It has been suggested that maltodextrin could retain water, stabilize enzyme, prevent cellular injuries, offer good oxidative stability and overcome the stickiness (Bhandari et al. 1993). DePaz et al (2002) also indicated that maltodextrin could prevent protein unfolding during drying due to its amourphous form. Modified starches had been reported to be used as coating materials for microencapsulation. They have excellent absorbent capacity because of large surface area (Zhang et al. 2012). Those pores in modified starch granules serve as an expandable space that could be used as a protective agent for microbial encapsulation (Li et al. 2016). Thus solid matrices including whey protein, modified starch and maltodextrin were evaluated for protective culture preparation. The post-cultivation broth was mixed with different solid matrix at certain ratio then underwent two-step drying to become protective culture in powder form. From table 10 and figure 9, it can be observed that both maltodextrin and modified starch similarly protected the viable cell of *B.subtilis* P5-6 as well as inhibitory effect against *S.aureus*.

At ratio 1:1 (liquid: solid), the mixture employing modified starch achieved highest viable cell of P5-6 isolate at 7.95 log CFU/ mL. Mixtures contained whey protein and maltodextrin showed lower viable count of isolated strain. In terms of inhibitory effect, inhibition zone of protective powder made of modified starch and maltodextrin on *S.aureus* were 2.4 and 2.8 mm, respectively, which were not significantly different from each other. It was worth to note that protective powder prepared from whey protein did not exert any inhibitory effect again target pathogen.

At ratio 2:1 (liquid: solid), modified starch upon mixing with P5-6 culture could retain greatest cell count at 8.06 log CFU/ mL, followed by mixture contained whey protein and maltodextrin, respectively. The maxium inhibition zone on *S.aureus* lawn was observed from modified starch mixture at 4.55 mm followed by maltodextrin

mixture. This observation was in compatible with the research of Vásconez et al (2009) tapioca starch contained in chitosan edible film could exert antimicrobial effect to protect food agianst spoilage microbe. Usmiati and Noor (2011) also proved that maltodextrin mixed with skim milk as matrix for bacteriocin powder shown great antimicrobial activity on several pathogens. The treatment using whey protein as carrier material did not possessed any inhibitory effect against *S.aureus*.

Although maltodextrin was comparatively potential as carrier material as modified starch, it took much longer time to dry and required grinding step to be turned into powder form. The longer drying time and heat generated during grinding may affect bacteriocin activity. Moreover, its water activity was quite high after drying process. Zhu et al (2013) indicated that an increase in aw of protective powder led to a quicker decrease in viable cell content during storage, regardless of state of that powder.

Whilst, modified starch was dried and reached water activity lower than 0.6 within set time then only needed mild grinding or mixing to transform into powder. According to Li et al (2016), modified starch containing various pores enhanced initial microbial loading efficiency and protection capability under unfavored conditions compared to cell in free form. Thus, modified starch was more feasible than maltodextrin to be developed as protective culture for further application in the following section.

The difference in viable cell count in each mixture could also be due to difference in glass transition temperatures (T_g), resulting in different grade of protection against cell damage (Carvalho et al. 2004). Sinha and Ranganathan, (1974) also stated that carbohydrate was capable of protect bacterial cells due to the difference in their glass-forming tendencies, which could be expressed through their glass transition temperatures (Tg). Based on the research of Tantratian and Pradeamchai (2020), the encapsulation of lactic acid bacterial strain by spray drying, which employed carrier materials having Tg higher than 100^{o}C offered a higher number of viable cells. Nevertheles, that finding was different from the result of this research because modified starch having Tg at 88~91^{o}C, which was lower than 100^{o}C but still protected bacterial cell better than maltodextrin and whey protein, having Tg at 75~120 and 160^{o}C, respectively (Zhou and Labuza, 2007; Tran et al. 2007; Pradeamchai et al. 2012). Different drying process could be the reason of the different result.

On the other hand, mixture contained whey protein did not perform any antagonistic effect against *S.aureus* so it could not be used as carrier material in protective powder. This finding was in disagreement with the study of Storia et al (2020) that biofilm contained whey protein could protect *Lactobacillus* cell and performed good antimicrobial activity against *Listeria monocytogenes*. The active whey protein eadible film incoporated with lactic acid bacteria showed potential antifungal activity on cheese (Guimarães et al. 2020). The varied result could be due to difference in bacterial strain studied, target pathogen and protective culture preparation technique, particularly microencapsulation, mixing, drying method.

Table 10. Cell population and inhibitory effect of *B.subtilis* P5-6 against *S.aureus* of different solid matrix (*statistical analysis was based on the same column)

Solid matrix	Ratio (Liquid:solid)	Cell population (log CFU/ mL)	Inhibition zone (mm)	Water activity
Whey protein	1:1	6.24±0.08^{c}	0	0.75
	2:1	6.51±0.04^{b}	0	0.8
Modified starch	1:1	7.95±0.07^{a}	2.4±0.14^{b}	0.50
	2:1	8.06±0.03^{a}	4.55±0.63^{a}	0.58
Maltodextrin	1:1	6.17±0.03^{c}	2.8±0.28^{b}	0.9
	2:1	6.23±0.04^{c}	3.1±0.14^{b}	0.98

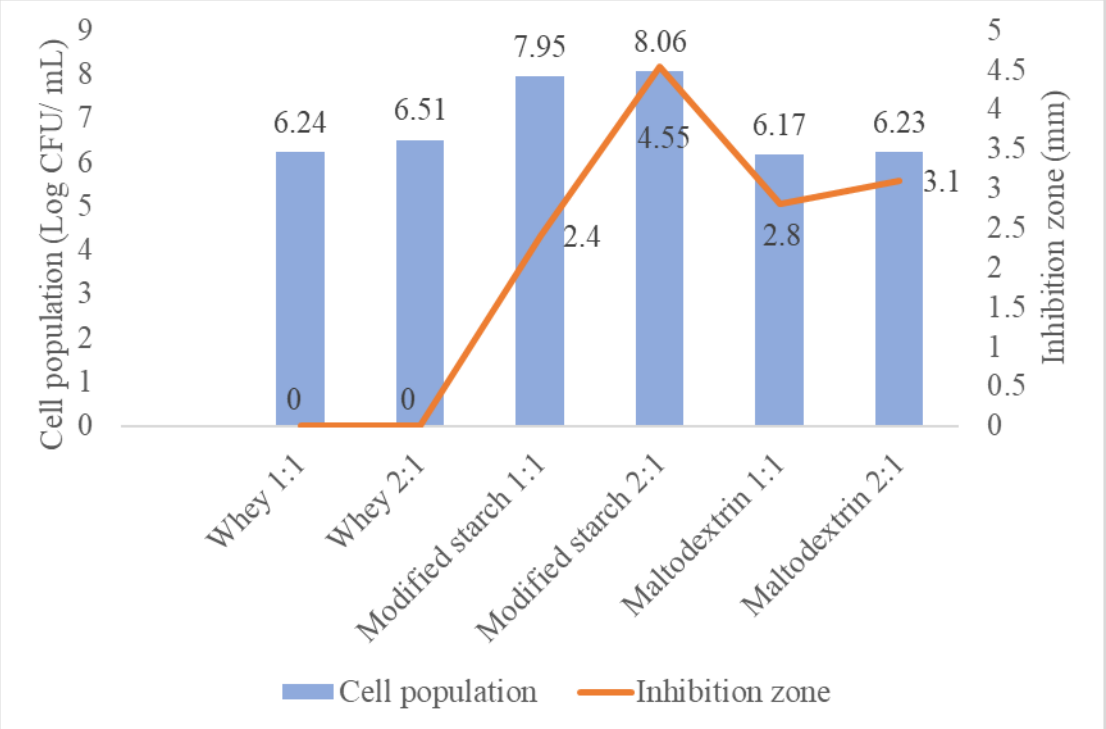

Figure 9. Cell population and inhibition effect of *B.subtilis* P5-6 against *S.aureus* of different solid matrix

4.7 Application in food model

Raw meat normally contaminated with several kinds of microorganism including *S.aureus*, which shorten shelf-life and reduce market value of product. By applying protective culture in powder form, fresh meat product could be stored for longer time with improved sensory properties. This could help to increase consumer demand and raise profit of animal husbandry sector as well as reduce food waste. In

this study, the purchased fresh pork loin was treated with chemical reagent to lower initial microbial load. The treatment were divided into 6 treatments as listed in Table 11. Bacterial cell count in pork sample during storage (*statistical analysis was based on the same row)

Treatment		**Bacterial count (Log CFU/ mL)**		
		Day 0	**Day 1**	**Day 5**
Control 1 (Pork slice only)	TPC	4.61±0.007^{c}	5.84±0.014^{b}	7.32±0.028^{a}
	S.aureus	2.68±0.035^{c}	3.39±0.035^{b}	4.32±0.021^{a}
Control 2 (Pork slice + Protective culture)	TPC	6.33±0.014^{b}	6.20±0.035^{c}	7.03±0.042^{a}
	S.aureus	2.84±0.091^{a}	2.74±0.056^{a}	2.48±0.021^{b}
	B.subtilis	6.32±0.028^{a}	5.67±0.042^{b}	3.70±0.007^{c}
Control 3 Pork slice + spiked *S.aureus* 5 Log CFU/ mL	TPC	5.66±0.049^{a}	6.06±0.028^{b}	7.11±0.007^{c}
	S.aureus	5.55±0.042^{a}	5.58±0.035^{a}	4.24±0.176^{b}
Treatment 1 Meat + Protective culture + spiked *S.aureus* 2 Log CFU/ mL	TPC	6.30±0.261^{b}	6.02±0.134^{b}	7.00±0^{a}
	S.aureus	3.69±0.014^{a}	3.55±0.078^{a}	3.30±0.028^{b}
	B.subtilis	6.01±0.410^{a}	5.83±0.728^{a}	4.32±0.240^{b}
Treatment 2 Pork slice + Protective culture + spiked *S.aureus* 3 Log CFU/ mL	TPC	6.06±0.028^{b}	6.38±0.282^{b}	7.02±0.028^{a}
	S.aureus	4.95±0.021^{a}	4.89±0.035^{a}	3.72±0.021^{b}
	B.subtilis	5.79±0.098^{a}	5.74±0.360^{a}	4.87±0.120^{b}
Treatment 3 Pork slice + Protective culture + spiked *S.aureus* 5 Log CFU/ mL	TPC	6.23±0.352^{ab}	5.91±0.289^{b}	7.00±0^{a}
	S.aureus	5.28±0.028^{a}	5.31±0.070^{a}	4.2±0.353^{b}
	B.subtilis	5.93±0.212^{a}	6.28±0.395^{a}	3.39±0.127^{b}

table 11. The first two control group including untreated pork and pork with protective culture were subjected to investigate the impact of protective culture on the original contaminated *S. aureus* and TPC in pork. The other 4 treatments were subjected to investigate the impact of protective culture on spiked *S. aureus*. *Staphylococcus*

aureus was spiked on pork at 2, 3, 5 log CFU/ mL, which represented treatment 1, 2, 3, respectively and followed by evenly spreading of diluted liquid protective culture on each meat surface. Each treatment was kept in sterile petri dish and sealed in ziplock bag then stored in domestic refrigerator (8-10^{o}C). Microbial count mentioned in section 3.8.3 was examined after day 0, 1 and 5 to check for the efficiency of protective culture.

Pork samples was originally contaminated with *S.aureus* at 2 log CFU/ g. In control 1, without protective culture, *Staphylococcus aureus* population increased 1 and 2 log CFU/ g after 1 and 5 days of storage, respectively. Total plate count (TPC) in the same sample increased from 4-5 log CFU/ g to 7 log CFU/ g (table 11). In pork treated with protective powder containing log 6 CFU/g of *B.subtilis* P5-6 (control 2), *S.aureus* population remained unchange after 24 hours. This indicated the bacteriostatic effect of protective powder on *S.aureus*. The result was in agreement with the finding of Chhetri et al (2019) that when adding protective culture into cottage cheese, the contaminated *S.aureus* reduced 1 log CFU/ mL after 4 days due to the antimicrobial compound produced from *Bacillus* isolate.

In control 3 containing spiked *S.aureus* at 5 log CFU/ g, the population of this target pathogen did not change after 24 hours. Moreover, it reduced from the initial population for 1 log CFU/ g at the last storage day. The reduction in *S.aureus* population could be due the high cell density relative to control 2. Because the more cells present, the more metabolite generated and the less nutrient available resulting in death phase of bacterial cell. This decrease in cell population was aligned with the result of Patarata et al (2020) that spiking *S.aureus* in normally contaminated meat resulted in lower survival of this pathogen. The same pattern can be seen in the treatment included both *S.aureus* 5 log CFU/g and protective powder. Notably, *S.aureus* population in both control and treatment were not significantly different from each other, which implies that *B.subtilis* P5-6 and its bacteriocin could not possess any inhibitory effect against *S.aureus* 5 log CFU/ g presents in pork slice. The result is different from the antimicrobial activity of *B.subtilis* P5-6 on *S.aureus* on solid medium, in which 6 log CFU/ mL of *B.subtilis* P5-6 could inhibit up to 8 log CFU/ mL of *S.aureus*.

Spiking 2 and 3 log CFU/ mL of *S.aureus* resulting in 3 and 4 log CFU/ g in meat sample, respectively. They both showed bacteriostatic effect of isolate P5-6, in which the growth of *S.aureus* was retardered after 1 day. On the 5th day of storage, *S.aureus* population detected was considerably lower than that on the 1st day, which could be due to the high cell density of *S.aureus* that may inhibit themselves as previously discussed above.

According to the different effect of protective powder on different population of *S.aureus* in meat sample, it could be concluded that protective powder gave bacteriostatic effect when there was 3 and 4 log CFU/ g of *S.aureus* contaminated in meat. The higher *S.aureus* population, the less effective protective powder is. The meat model containing 5 log CFU/g of *S.aureus* was probably not suitable for describing inhibitory effect of protective powder because normally, *S.aureus* is not contaminated up to 5 log CFU/ mL in fresh meat. Additionally, too high cell density leads to reduction in bacterial population and it could be mistaken as antimicrobial activity from protective powder.

Along with *S.aureus* count, Total plate count (TPC) was also investigated to evaluate the effeciency of protective powder. Day 0 and 1 reflected the dominant population, which was *B.subtilis* P5-6, means that this isolate could compete with other

microbes contaminated in meat and retard their growth for those first two days upon applying protective powder. However, the number of *B.subtilis* P5-6 cell continued to reduce along storage time and other microbes became dominant in meat sample. TPC on day 5 in the control and in sample treated with protective powder were not significantly different from each other, which indicates that *B.subtilis* P5-6 could no longer inhibit other microbes in meat sample. This result aligned with the finding of Le et al (2019) that no significant difference was observed between control and pork meat treated with bacteriocin in total bacterial count at the end of storage time.

Since *B.subtilis* P5-6 is halophilic, the presence of NaCl in food model could facilitate its growth and inhibitory effect against *S.aureus.* Upon being applied in food model containg salt approximately at 0.8 to 4.4% such as cottage cheese, bamboo shoot pickle, green chili pickle, the bacteriocidal effect on *S.aureus* was well-observed. However, food model in this research was fresh pork, which did not contain salt so P5-6 population reduced along storage time and it was gradually dominated by TPC.

Data from the application of protective culture on food model illustrated that protective culture from *B.subtilis* P5-6 could inhibit the growth of *S.aureus*. Bacteriostatic effect was shown after day 1. In comparision with the spot on lawn result, the same population of *B.subtilis* P5-6 was used and these two systems both achieved bacteriostatic effect after 24 hours. In order to obtain bacteriocidal effect on 24 hours, the concentration of protective culture applied should be increased. Bacteriocin activity should be quantificated to obtained the optimum dosage of protective culture, which can be used to effectively protect food against spoilage microbes. Protective powder developed from this halopihilic P5-6 should be specifically used for salt containing food in order to maximally excert protective effect. Moreover, more salt shall be supplemented into solid matrix during preparation process at appropriate amount, which will not intefere the organoleptic properties of food, in order to support the growth of bacteriocin producing strain during application.

Structure and characteristic of bacteriocin should be further investigated to develop a better formulation of protective culture, which contains appropriate agents that support better cell growth and inhibitory effect against unwantted microbes.

Additionally, the effect of food matrix on protective culture including bacterial cell and bacteriocin should be examined. Normally, the natural agent assayed from in vitro test needs to be used up to 10 times concentrated to show positive effect upon applying in food matrix. Because it may react with components in food matrix leading to the reduction in inhibitory activity. For example, heat sensitive bacteriocin could be inactivated under high temperature process or extreme pH could lower activity of the target antimicrobial peptides. Lipid content or enzyme present in food may also be factors that inhibit bacteriocin (DeVuyst, 2000). Subtilosin is relatively stable at extreme pH and unfavored temperature. Its antimicrobial activity was completely retained after 60 min underwent the pH range from 2 to 10 at 100^{o}C. Antimicrobial activity of subtilosin was only reduce because of the presence pepsin and proteinase K (Flühe et al. 2012). Meanwhile, pH of pork ranges from pH5.7 to 6.1 so it could not alter the efficiency of subtilosin (Kim et al. 2016). The lipid oxidation if occur upon application could not perform any negative effect on subtilosin activity.

CHAPTER 5 CONCLUSION

Bacillus subtilis P5-6 showed no or less inhibitory effect on *S.aureus* when co culture in liquid medium. In solid medium, the P5-6 significantly expressed an inhibitory effect on *S.aureus* even when its population was 2 log CFU/ mL lower than that of target pathogen strain. The results obtained in this study demonstrated that P5-6 could express the subtilosin gene having an inhibitory effect on *S.aureus*. The solid

state cultivation with supplementation of NaCl could enhance production and/or activity of bacteriocin of P5-6 as well as bacterial cell growth.

Culture medium, which is vegetable brine at pH 7 supplemented with minimal nutrient was optimized. Besides, cultivation at room temperature, 150 rpm of agitation was the most favorable for P5-6 isolate in terms of cell growth and inhibitory effect against *S.aureus*. The mixture of modified starch and post-cultivation medium at ratio 1:2, undergone two-step drying was the most effective formula to prepare protective culture, which could be applied as food bio-preservative.

In application of protective culture, fresh raw pork was used as a food model. Protective culture contained viable P5-6 at 7 log CFU (5 log CFU/ cm^2) could inhibit the growth of initially contaminated *S.aureus* in pork and spiked *S.aureus* after storage for 24 hours under chilling temperature. Bacteriocidal effect of protective culture on *S.aureus* were not clearly observed throughout the storage for 5 days in this food model. This observation could help to develope the technology for production and application of protective culture from P5-6 isolate to protect food against *S.aureus* – one of the most common cause of foodborne disease.

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APPENDIX

APPENDIX A
Molecular Procedure

APPENDIX A1

Total RNA Extraction protocol (Vivantis, Malaysia)

1. Bacterial cell (10^{7-8} CFU/ mL)
2. Centrifuge 6,000 x g for 5 min at 4°C, discard the supernatant completely
3. Resuspend the bacteria pellet in 100µl of
a) 0.5mg/ml lysozyme for Gram-negative bacteria strains. . Mix by vortexing and incubate at room temperature for 3 - 5 min.
b) 5mg/ml lysozyme for Gram-positive bacteria strains. Mix by vortexing and incubate at room temperature for 5 - 10 min.
3. Add 350µl Buffer TR to the sample.
4. Transfer the lysate into a Homogenization column assembled in a collection tube. Centrifuge at maximum speed for 2 min. Save the flow-through
5. Add 300µl absolute ethanol to the flow-through. Mix thoroughly by pipetting.
6. Transfer the sample precipitate into a RNA Binding Column. Centrifuge at 10,000 x g for 1 min. Discard the flow-through.
7. Add 500µl of Wash Buffer and centrifuge at maximum speed for 1 min. Discard flow through.
8. Pipette 70µl of DNase I Digestion Mix into RNA Binding Column and incubate at room temperature for 15 min. Prolong the incubation time if necessary.
9. Add 500µl of Inhibitor Removal Buffer and centrifuge at maximum speed for 1 min. Discard flow through.
10. Add 500µl of Wash Buffer and centrifuge at 10,000 x g for 1 min. Discard flow-through.
11. Repeat wash with 500µl of Wash Buffer and centrifuge at 10,000 x g for 1 min. Discard flow-through.
12. Centrifuge the column at 10,000 x g for 1 min to remove traces of buffer.
13. Place the column into a new microcentrifuge tube. Add 40-60µl of RNase-free Water directly onto the membrane and stand for 1 min. Centrifuge at 10,000 x g for 1 min. Convert into cDNA

APPENDIX A2
Total DNA extraction (Vivantis, Malaysia)

1. Bacterial cell (10^{7-8} CFU/ mL)
2. centrifugation at 6,000 x g for 2 min at room temperature. Discard supernatant completely.
3. Resuspension of pellet Add 100µl Buffer R1 to the pellet and resuspend the cells completely by pipetting up and down.
4. Lysozyme treatment

For Gram-negative bacteria strains, add 10µl lysozyme (50mg/ml) into the cell suspension. For Gram-positive bacteria strains, add 20µl lysozyme (50mg/ml) into the cell suspension. Mix thoroughly and incubate at 37°C for 20 min.

5. Centrifugation Pellet digested cells by centrifugation at 10,000 x g for 3 min. Discard the supernatant completely.

6. Resuspend pellet in 180 µl of Buffer R2 and add 20 µl of Proteinase K. Mix thoroughly. Incubate at 65°C for 20 min in a shaking waterbath or with occasional mixing every 5 min. Mix and incubate at 37°C for 5 min.

7. Homogenization Add 2 volumes (~400 µl without RNase A treatment, ~440 µl with RNase A treatment) of Buffer BG and mix thoroughly by inverting tube several times until a homogeneous solution is obtained. Incubate for 10 min at 65°C.

8. Addition of Ethanol Add 200 µl of absolute ethanol. Mix immediately and thoroughly.

9. Loading to column Transfer the sample (max.650µl) into a column assembled in a clean collection tube (provided). Centrifuge at 10,000 x g for 1 min. Discard flow through.

10. Wash the column with 650 µl of Wash Buffer and centrifuge at 10,000 x g for 1 min. Discard flow through.

11. Centrifuge the column at 10,000 x g for 1 min to remove residual ethanol.

12. DNA elution Place the column into a clean microcentrifuge tube. Add 50 - 100 µl of preheated Elution Buffer, TE buffer or sterile water directly onto column membrane and stand for 2 min. Centrifuge at 10,000x g for 1 min to elute DNA. Store DNA at 4°C or -20°C.

APPENDEIX A3

PCR reagent preparation

Reagent	Concentration	Volume	Thermal cycling program

	Stock	Working	Work 50 µl	Total reaction	
Vi buffer	10X	1X	5		Lid: 80°C Volume: 50 µL 1. 94°C, 2:00 min 2. 94°C, 0:30 sec 3. 55°C, 0:30 sec 4. 72°C, 0:30 sec 5. Go to STEP 2, 35X 6. 72°C, 7:00 min 7. 4°C, ∞
$MgCl_2$	50mM	1.5mM	1.5		
dNTPs	10 µM	0.1mM	0.5		
Primer	F-10 µM	0.1mM	0.5		
	R-10 µM	0.1mM	0.5		
Tag DNA	5U/ µL	2U	0.4		
H_2O	-	-			
Template	-	10-50 ng/ µL	0.4		

Reagent (Reverse Transcriptase)	Concentration	Volume		Thermal cycling program
		Work 10µL	Total Reaction	
dNTPs	10U	1		Lid: 80°C Volume: 10 µL 1. 65°C, 5 min 2. 4°C ∞
Oligo d	40µM	1		
Hexamer		1		
RNA free H_2O	-	5		
Template	10-50ng/ µL	2		
10X buffer		2		Lid: 80°C Volume: 20 µL 1. 42°C, 60 min 2. 4°C, ∞
Reverse Transcriptase		0.5		
RNA free H_2O	-	7.5		

Reagents	8% Denaturation gel (DGGE)	
	20	40
40% Acrylamide/ Bis	3 mL	3 mL
50XTAE	0.3 mL	0.3 mL
Formamide	1.2 mL	2.4 mL
Urea (g)	1.26 g	2.52 g
Glycerol	0.3 mL	0.3 mL
RNA free H_2O	10.2 mL	9 mL
TEMED	0.0154 mL	0.0154 mL
10% APS	0.154 mL	0.154 mL

APPENDIX B
Culture Media Preparation

B1. Baird-Paker agar: dissolve 63 g in 1000 mL of distilled water. Sterile at 121^{o}C for 15 minutes. Cool down to 47^{o}C then aseptocally add 50 mL Egg Yolk Tellurite Emulsion. Mix well and pour into sterile petri dishes.

B2: MYP agar: suspend 21.5 g in 450 ml of purified water. Heat in boiling water and agitate frequently until completely dissolved. Autoclave 15 minutes at 121 °C. At 47-50 °C mix in 50 ml of a sterile Egg-yolk emulsion (article number 103784). Mix well and pour to sterile petri plates.

B3. Nutrient agar: suspend 28 g in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Sterilize at 15 lbs pressure (121^{o}C) for 15 minutes. Cool to 45-50^{o}C. Pour to sterile petri plates.

B4. Nutrient Broth: suspend 13 g in 1000 mL distilled water. Sterile at 121^{o}C for 15 minutes.

B5. 0.5% Peptone water: dissolve 5 g of peptone powder in 1000 mL of distilled water. Heat to boiling to dissolve the medium completely. Sterile at 121^{o}C for 15 minutes.

APPENDIX C

Determination of bacterial population (Sander, 2012)

1. Take 1 mL of sample into 9 mL 0.5% peptone water
2. Make serial dilution to obtain desired concentration
3. Pipette 100 µl diluted sample onto agar plate and spread evenly
4. Incubate at 37oC for 24 – 48 hours
5. Count colony formed

APPENDIX D1

Nucleotide sequencing analysis

Code	Nucleotide sequence	Spp.ID	%Homogeneity	Accession no.
P5-6	GTAATTTCGCATGGAGAAGTCTGACG GAGCACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAACTCTGTTGTTAG GGAAGAACAAGTACCGTTCGAATAGG GCGGTACCTTGACGGTACCTAACCAG AAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAAT	*B.subtilis*	99	GU434356.1

APPENDIX D2

Proximate analysis of spent mago brine

Food Research and Testing Laboratory
Faculty of Science Chulalongkorn University
Floor 16th Maharaknat Building Phayathai Road,
Pathumwan, Bangkok 10330, Thailand

Report No. : C 0516/20
Issued Date : 17 August 2020
Sample ID : 202735
Page 1 of a total of 1 page

---------- begin report ----------

Test Report

Client Name : CHEUNJIT PRAKITCHAIWATTANA
Client Address : FOOD TECHNOLOGY, FACULTY OF SCIENCE, CHULALONGKORN UNIVERSITY

Sample Description : Mango brine Lot No. 060820
(yellow liquid packed in plastic bag sealed, volume 250 mL

Sampling by : Client
Date Sample Received : 6 August 2020
Date Analyzed : 13 August 2020

Test Results

Test Item	Test Results	Test Method
Moisture	94.01 g/100g	ASEAN Manual of Food Analysis (2011) p.1-2
Ash	3.68 g/100g	ASEAN Manual of Food Analysis (2011) p.38-39
Total fat	0.00 g/100g	In-house method based on AOAC (2019) 922.06
Protein (N x 6.25)	0.20 g/100g	In-house method TC 014 based on AOAC (2019) 991.20

Remark : -

---------- end report ----------

Approved By

(Phayoonhong, Wilai)
Technician Manager,
Chemical Laboratory

(Tantratian, Sumate Assoc. Prof. Dr.)
Deputy Director

(Kokpol, Sirirat Prof. Dr.)
Director

APPENDIX D3

Proximate analysis of spent vegetable brine

Food Research and Testing Laboratory
Faculty of Science Chulalongkorn University
Floor 16th Mahamakut Building Phayathai Road,
Pathumwan, Bangkok 10330, Thailand

Report No. : C 0667/20
Issued Date : 4 November 2020
Sample ID : 203702
Page 1 of a total of 1 page

begin report

Test Report

Client Name : HOANG TRUC ANH TO
Client Address : -

Sample Description : Vegetable brine Lot No. 021120
/ turbid green liquid packed in plastic bag sealed, weight 250 g

Sampling by : Client
Date Sample Received : 2 November 2020
Date Analyzed : 4 November 2020

Test Results

Test item	Test Results	Test Method
Total carbohydrate	0.05 g/100g	Method of Analysis for Nutrition Labeling. Virginia : AOAC International ; 1993, p. 8
Moisture	93.64 g/100g	ASEAN Manual of Food Analysis (2011) p.1-2
Ash	5.57 g/100g	ASEAN Manual of Food Analysis (2011) p.38-39
Total fat	0.07 g/100g	In-house method based on AOAC (2019) 922.06
Protein (N x 6.25)	0.67 g/100g	In-house method TC 014 based on AOAC (2019) 991.20

Remark : -

end report

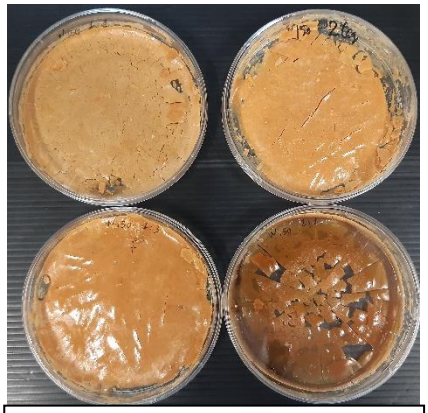
Approved By

(Phayoonhong, Wilai)
Technician Manager,
Chemical Laboratory

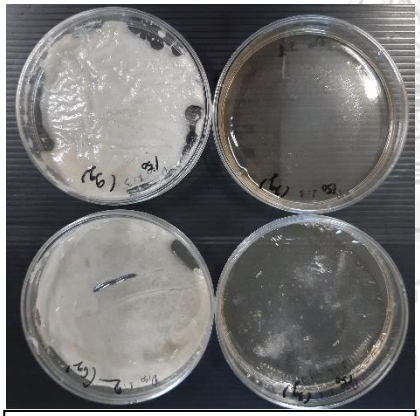
(Tantratian, Sumate Assoc. Prof. Dr.)
Deputy Director

(Kokpol, Sirirat Prof. Dr.)
Director

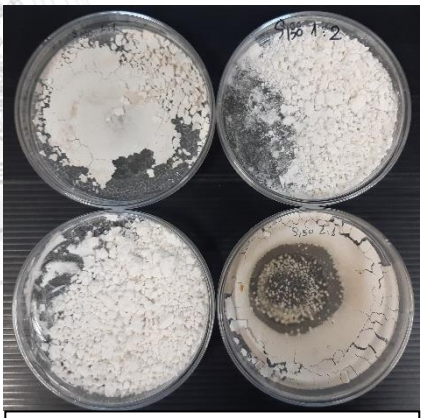
APPENDIX 4
PROTECTIVE CULTURE PREPARATION
FROM DIFFERENT SOLID MATRICES



Whey protein + P5-6 culture (1:1) and (1:2)



Maltodextrin + P5-6 culture (1:1) and (1:2)



Modified starch + P5-6 culture (1:1) and (1:2)

APPENDIX D5
PROTECTIVE CULTURE APPLICATION ON PORK MEAT

Pork loin purchased at local supermarket

	Day 0	Day 1	Day 5
Control 1			
Meat contains 3 log CFU/ g + protective powder			

VITA

NAME	Hoang Truc Anh To
DATE OF BIRTH	02 January 1992
PLACE OF BIRTH	Can Tho, Vietnam
INSTITUTIONS ATTENDED	Can Tho University
HOME ADDRESS	59 Le Thi Hong Gam, Binh Thuy ward, Can Tho city, Vietnam