MULTIFUNCTIONAL PROPERTIES OF BACILLUS CELL AUTOLYSATE AS ANTIMICROBIAL AND COLORING AGENTS IN FOODS

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สมบัติเชิงหน้าที่ร่วมจากออโตไลเซตของเซลล์บาซิลลัสเป็นสารต้านจุลินทรีย์และสารให้สีในอาหา
ร

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Thesis Title	MULTIFUNCTIONAL PROPERTIES OF BACILLUS CEL L AUTOLYSATE AS ANTIMICROBIAL AND COLORIN G AGENTS IN FOODS
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เบคติ วูลาน สาริ : สมบัติเชิงหน้าที่ร่วมจากออโตไลเซตของเซลล์บาซิลลัสเป็นสารต้านจุลินทรีย์และสารให้สีในอาหาร. (MULTIFUNCTIONAL PROPERTIES OF BACILLUS CELL AUTOLYSATE AS ANTIMICROBIAL AND COLORING AGENTS IN FOODS) อ.ที่ปรึกษาหลัก : ชื่นจิต ประกิตชัยวัฒนา, อ.ที่ปรึกษาร่วม : สาริศา สุริยรักษ์

งานวิจัยมีวัตถุประสงค์เพื่อประเมินความเป็นไปได้ในการใช้รงควัตถุจากบาซิลลัสชอบเกลือเพื่อใช้เป็นสารให้สีร่วมกับการมีแอคติวิตีทางชีวภาพในอาหาร โดยบาซิลัสคัดแยกได้จากตัวอย่างปลาร้าและจำแนกชนิดด้วยการวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ 16SrDNA ไอโซเลทที่ได้มีทั้งหมด 5 สายพันธุ์ ประกอบด้วย *Bacillus sp.* 81-2; 13-3; 77-3 เป็นตัวแทนกลุ่มที่สร้างรงควัตถุที่มีเฉดสีชมพู-ส้ม *Bacillus amyloliquefaciens* 34-12 เฉดสีเหลือง, and *Bacillus vietnamensis* 39-23 เฉดสีเหลือง-ส้ม ทุกสายพันธุ์จัดเป็นสายพันธ์ชอบเกลือปานกลาง เมื่อสกัดรงควัตถุและทดสอบแอคติวิตีในการต้านจุลินทรีย์ (200 มก./มล.) และต้านอนุมูลอิสระ (50 มก./มล.) ด้วยวิธี disc diffusion และการวิเคราะห์ค่า DPPH ตามลำดับ พบว่ารงควัตถุสกัดของทุกสายพันธุ์แสดงฤทธิ์ต้าน *Escherichia coli, Salmonella Typhimurium* และ *Bacillus cereus* โดยมีเพียง *Bacillus sp*. 81-2 และ *B. amyloliquefaciens* 34-12 ที่แสดงฤทธิ์ต้าน *Staphylococcus aureus* ค่า % scavenging จาก DPPH ของรงควัตถุที่สกัดจาก *Bacillus* sp. 13-3; 77-3; 81-2, *B. vietnamensis* 39-23; and *B. amyloliquefaciens* มีค่าเท่ากับ 18.716±1.782; 15.750±0.520; 11.543±1.171; 9.628±5.835, และ13.907±0.091 ตามลำดับ เมื่อจำแนกรงควัตถุด้วยวิธี UV spectrophotometry, thin layer chromatography (TLC) และ fourier transform infrared spectroscopy (FTIR). ผลจาก spectrophotometry และ TLC พบว่ารงควัตถุที่สกัดจากทุกสายพันธุ์คล้ายกับแคโรทีนอยด์ (carotenoid) จาก การวิเคราะห์ด้วย FTIR บ่งชี้ว่ารงควัตถุสีชมพู-ส้ม จาก *Bacillus sp*. 81-2 ประกอบด้วยสารไลโคปีน (lycopene) และกลุ่มสีเหลืองและเหลือง-ส้มประกอบด้วยแซนโทฟิล (xanthophyll) จากผลการทดลองเลือก *Bacillus* sp. 81-2 ไปศึกษาสภาวะในการผลิตรงควัตถุและความปลอดภัยต่อไป พบว่าสภาวะที่เหมาะสมคือเพาะเลี้ยงในอาหารนิวเทรียนเหลวเติมเกลือเข้มข้นร้อยละ 3 เขย่า 150 rpm ที่อุณหภูมิห้องเป็นเวลา 72 ชั่วโมง รงควัตถุที่ได้มีรูปแบบของพีคจากการวัดด้วย UV spectrophotometry พบว่าพีคที่ 481 นาโนเมตร สูงกว่าพีครูปแบบปรกติ สมบัติการยับยั้ง *S. aureus* มีค่าความเข้มข้นต่ำสุดในการยับยั้งการเจริญ (MIC) และค่าความเข้มข้นต่ำสุดในการฆ่าแบคทีเรีย (MBC) เท่ากับ 100 และ 200 มก./มล. ตามลำดับ เมื่อเติมรงควัตถุลงในน้ำสลัดที่ความเข้มข้น 200 mg/ml ทำให้น้ำสลัดมีสีชมพู-ส้มที่ดีพร้อมแนวโน้มแสดงแอคติวิตีทางชีวภาพ จากการศึกษาบ่งชี้ได้ว่าของรงควัตถุที่ประกอบด้วยแคโรทีนอยด์จากบาซิลลัสมีศักยภาพที่สามารถประยุกต์ใช้เป็นสารให้สีที่มีแอคติวิตีทางชีวภาพร่วมในอาหารซึ่งช่วยเพิ่มคุณภาพและความปลอดภัยของอาหารได้

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This research aimed to evaluate feasibility of pigments from halophilic *Bacillus* as coloring agent having multi-bioactivity in foods. Those *Bacillus* were isolated from Thai traditional salty fermented fish (plara) and identified by 16S rDNA sequencing analysis. Five strains including *Bacillus* sp. 81-2; 13-3; 77-3, *Bacillus amyloliquefaciens* 34-12, and *Bacillus vietnamensis* 39-23, with pink-orange, yellow, and yellow-orange pigmentation were selected as representative of each pigment color shade. All of strains were classified as slight halophilic based on salt tolerance determination. Pigment was extracted and subjected to test for antimicrobial (200 mg/ml) and antioxidant activities (50 mg/ml) through disc diffusion and DPPH assay, respectively. Crude extracts of all strains had inhibitory effect on *Escherichia coli*, *Salmonella* Typhimurium, and *Bacillus cereus*, while only extracts from *Bacillus* sp. 81-2 and *B. amyloliquefaciens* 34-12 exhibited inhibitory action against *Staphylococcus aureus.* DPPH assay showed % scavenging activities of extracts from *Bacillus* sp. 81-2; 13-3;77-3; *B. vietnamensis* 39-23; and *B. amyloliquefaciens* 34-12 as 11.54±1.17; 18.72±1.78; 15.75±0.52; 9.63±5.84; 13.91±0.09, respectively. Pigment extracts were further characterized using UV spectrophotometry, thin layer chromatography (TLC), and fourier transform infrared spectroscopy (FTIR). From spectrophotometric and TLC assay, pigment of all isolates showed similar properties to carotenoid. The FTIR analysis suggested that pink-orange pigment of *Bacillus* sp. 81-2 comprised lycopene, whereas yellow and yellow-orange pigment of *Bacillus* sp. 13-3; 77-3, *B. amyloliquefaciens* 34-12 , and *B. vietnamensis* 39-23 were xanthophylls. Based on color shade and bioactivities, *Bacillus* sp. 81-2 was selected for further evaluation including safety evaluation and pigment production. The optimal condition for pigment production was nutrient broth supplemented with 3 % NaCl under room temperature for 72 hours, yielded pigment 0.22 gram per gram of wet cell. Addition of carbon and nitrogen sources gave no impact on both bacterial growth and pigment production. Spectrometric profile of pigment extract showed higher peak of 481 nm than usual profile and its minimum inhibitory concentration (MIC) and/or minimum bactericidal concentration (MBC) against *S. aureus* were 100 mg/ml and 200 mg/ml, respectively. Addition of 200 mg/ml pigment generated well-incorporated pink-orange pigment in salad dressing matrix with potential expression of bioactivities. This study demonstrated a potential *Bacillus* pigment containing carotenoid group to be applied into food as colorant having multifunctional properties that could enhance both food quality and safety.

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

Food quality and safety according to Food and Agriculture Organization (FAO) are both important issues regarding food control system. Food quality which related to sensorial properties, particularly color, increases consumer preference by providing better appearance. The other one, food safety, an issue associated with hazard and injury term helps consumer to prevent risk of foodborne illness. Assuring both of issues are necessary to protect consumer health (FAO, 2008). Hence, providing safe-qualified product becoming a challenge for food industries.

As a strategy for improving quality attribute of food product, industries develop proper colorant in comply with safety aspect for consumer. Thus, various color sources, especially natural colors which are less toxic and environmental friendly over artificial colors gain more attention nowadays. Microorganisms are one of natural pigment sources as well as plants. However, microbial pigments deliver more benefit than plant pigments. These pigments produced in numerous color without seasonal variation, obtained from fast process also high-productivity fermentation, and simple extraction. Moreover, microbial pigments production can be altered by doing strain improvement to gain high yield and utilizing agricultural waste to minimize production cost. In order to get different kind of pigment, isolation and development of new strain are possible to be carried out (Venil et al., 2013, Malik et al., 2012). Lately, several microbial pigments have been accepted by food industries and launched on market as food grade pigment. Those are Arpink Red from *Penicillium oxalicum* which allowed by Codex Alimentarius Commission meeting on 2002 to be added on meat also milk product with maximum amount of 100 mg/kg and 150 mg/kg, red pigment from *Monascus* for sausage, riboflavin from *Ashbya gossypii* for instant dessert (Dufossé, 2006), and crystalline β-carotene from co-fermentation of non-pathogenic *Blaskeslea trispora* DS 30627 and DS 3062 (EU, 2000).

Over than that, microbial pigments as secondary metabolite have been reported to possess broad-ranging pharmacological activities. According to several studies, microbial carotenoids revealed multi-biological activities. As examples, both

antimicrobial and antioxidant activities were found on pigment-identified as carotenoid from *Micrococcus* spp. (Mohana et al., 2013), while 3 kinds of biological activities including antimicrobial, antioxidant, and antitumor have been shown by pigment extracted from *Micrococcus roseus* and *Rhodotorula glutinis*, potential producers of carotenoid (Rostami et al., 2016). Microbial pigments containing biological activities besides food colorant show their potential as alternative substance to be applied on food in order to enhance food quality and safety by providing better sensorial properties and extending shelf life. Halophilic *Bacillus* group is ubiquitous source of pigment, especially diverse color of carotenoids (Khaneja et al., 2010) in which their potential on biological activities and application on food have not been considerably studied. Therefore, this study would investigate ability of *Bacillus* isolate pigments as antimicrobial against food pathogens and coloring agents in food matrix.

1.1 Objectives

i. To screen and characterize pigments from *Bacillus* isolates and assay their bioactivities including antimicrobial and antioxidant properties.
ii. To optimize pigment production condition from selected *Bacillus* strains.
iii. To evaluate *Bacillus* pigment as coloring agents with multi-bioactivities in food matrix.

CHAPTER 2
LITERATURE REVIEW

2.1 Plara

Plara is well known-traditional fermented fish product among every region of Thailand, particularly in northern and north-eastern part. Plara can be made from fresh water fish *Channa striata*, *Trichogaster* sp., Cyclocheilichthys sp., and *Labiobarbus* (Sangjindavong et al., 2008). In order to prepare plara, fish is scaled, followed by excrements removal, and then washed thoroughly in clean water before drying process. When drying process is completed, fish is then mixed with rock salt (4:1.5) to be further kept on basket 24 hours, prior addition of roasted rice powder. To facilitate the fermentation, the mixture is put on the jar, covered with plastic and leave for at least 6 months under the shade (Udomthawee et al., 2012). Generally, plara properties are yellowish brown or dark brown liquid having salty and sour taste also strong flavor (Chotechuang, 2012). Since plara is a high salted-protein-rich fermented product, Plara fermentation is therefore dominated and conducted by proteolytic-halophillic bacteria, such as *Pediococcus halophilus* (Tanasupawat and Komagata, 1995).

2.2 Halophilic microorganism

Halophilic microorganisms are those microorganisms which require salt for their growth (illustration shown in Figure 1). This kind of microorganisms are divided into 3 classification which are slightly, moderate, and highly halophilic. The term of slightly and moderate halophilic are defined for microorganisms that grow optimally on 2-5% salt concentration and 5-20% salt, respectively. Moderate halophilic are often found in environment containing higher salt concentration than sea, such as salty food and/or salty soil. Finally, halophilic microorganisms that grow optimally on of 20-30% salt are assigned as extreme halophilic group in which halo-bacteria is well known model of this group (Larsen, 1986).

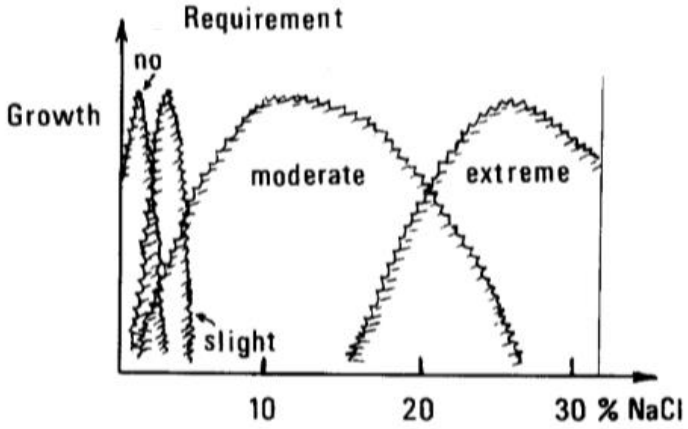


Figure 1. Halophilic microorganisms illustration

2.3 Pigments

2.3.1 Definition and history of pigments

The word “pigment” is derived from latin word *pingere* (to paint) and firstly defined as solid materials added on medium in order to generate color or improve appearance. Later this definition is getting widespread into colored materials extracted from vegetables, plants, and animals used for same purpose in many others media (Luescher, 1993). Pigments can be found in white, colored, or fluorescent particle which are usually insoluble in applied medium. Thus, those alteration on appearance occur by scattering a light (Gürses et al., 2016).

The history of pigments began around 2600-1900 BC when Indus Valley civilization still took place. The existence of color in this era was proved by finding of colored garment and trail of madder dye in Mohenjodaro and Harappa, so that both color and textile were said as the same old. Primitive technique such as rubbing crushed pigment into cloth in earliest era was known around 2600 BC in China, followed by sophisticated and more advance technique, such as boiling crushed fruit or pigment on fabric to perform colors. For long period, natural dye was famous as the only color source which applied into fabric, fur, leather, cosmetic product, ink, and watercolor. In 1856, synthetic color was introduced by Perkin which followed by several convenient and cheaper technique on pigment synthesize. The existence of synthetic color in that period replaced the use of natural color, supported by advance organic chemistry to produce greater amount

of synthetic one. Application of synthetic color was then getting widely in industry such as leather industry especially in tanning process also textile and paper industry. This application was also adopted in agricultural and food technology. It was around 1960, when environmental activist demonstrated and criticized against application of synthetic additives on food, followed by campaign regarding advantages of natural color, such as nutritional characteristic and pharmacological properties. Those benefits were then generated consumer preference to natural color over synthetic color (Venil et al., 2013).

2.3.2 Classification of pigments

Pigments can be classified into numerous ways based on their origin, structural characteristic, and biological type. Based on the origin, pigments are classified as natural and synthetic pigments (Malik et al., 2012). Natural pigments are produced by organisms such as plant, animal, and microorganism, while synthetic pigments or known as artificial color are produced chemically in laboratory (Korumilli, 2012). Riboflavin, carotenoid, canthaxanthin, prodigiosin, violacein, and pyocyanin are examples of naturally occuring pigments, whereas azo-dyes mentioned as an example of synthetic pigment (Mortensen, 2006, Korumilli and Susmita, 2014).

Biological pigments, biochrome, are define as a component produced by organisms that exhibit color due to the particular color absorption. Biological pigments based on their source are organized as plant pigment, animal pigment, and microbial pigment (Korumilli and Susmita, 2014). Anthocyanins, carotenoids, and betalains are examples of pigment delivered from plant (Delgado-Vargas et al., 2000), canthaxanthin is orange pigment delivered from salmon, shrimp, and flamingos (Mapari et al., 2005); while carotenoids from *Micrococcus* spp. (Mohana et al., 2013) and prodigiosin from *Serratia marcescens* (Williams et al., 1971) are examples of microbial pigment. Another organization of pigment based on their structural characteristic is shown in Table 1.

Table 1. Organization of pigment based on structural characteristic (Delgado-Vargas et al., 2000)

Group of pigment	Characteristics	Example
Tetrapyrrole derivatives	Characterized by four pyrrol structure	Chlorophylls
Isoprenoid derivatives	Most of the compound are polymers	Carotenoids
N-heterocyclic compounds	Characterized by presence of nitrogen	Purines
Benzopyran derivatives	Characterized by oxygenated heterocyclic compound	Anthocyanins and other flavonoid
Quinones	Characterized by quinone functional group	Benzoquinone
Melanins	Polymeric structures from Nitrogen with monomer	Eumelanins

2.4 Microbial pigments

2.4.1 Source of microbial pigments

Microbial pigments are divided into bacterial, fungal, and algal based on their origin or their source (Pankaj and Kumar, 2016) that released in different type with various color (Malik et al., 2012). A number of bacterial pigments, such as violet violacein, red prodigiosin, and blue-green pyocyanin which produced by *Chromobacterium violaceum, Serratia marcescens, Pseudomonas aeruginosa,* respectively, have been reported. Other than that, fungi *Blakeslea trispora* as β-carotene source and yeast *Rhodotorula* sp. as orange-red torularhodin source have also denoted. The list of pigment producing microorganisms are shown in Table 2.

Table 2. Pigment producing microorganisms
(Khaneja et al., 2010, Malik et al., 2012)

Microorganism	Species	Pigment	Color
Bacteria	*Chromobacterium violaceum*	Violacein	Purple
	Bacillus clausii	β -carotene	Orange
	Pseudomonas aureuginosa	Pyocyanin	Blue-Green
	Flavobacterium sp.	Zeaxanthin	Yellow
	Serratia marcescens	Prodigiosin	Red
	Xanthophyllomyces dendrorhous	Astaxanthin	Pink-Red
Fungi	*Blakeslea trispora*	β -carotene	Cream
	Monascus sp.	Ankaflavin	Yellow
	Blakeslea trispora	Lycopene	Red
	Ashbya gossypi	Riboflavin	Yellow
Yeast	*Rhodotorula sp.*	Torularhodin	Orange-Red
Algae	*Dunaliella salina*	β-carotene	Red

2.4.2 Factor affecting microbial pigments production

A promising pigment producing microorganisms should perform moderate grow, capable to use wide range of carbon (C) and nitrogen (N), show tolerance to pH and temperature as physical parameter and also mineral. They should be easy to be separated from cell mass, provide reasonable color yield, and need to fulfill safety term: non-toxic and non-pathogenic (Joshi et al., 2003). In order to obtain higher production of microbial pigments, there are physiological, nutritional and biological factor that play substantial role on it. Those factors not only induce growth kinetic of microbes, but also modulate metabolic processes in which it can promote yield of pigment (Kirishna et al., 2014). Factor affecting microbial pigments are as follow:

2.4.2.1 Physiological factor

i. Temperature

Microbial pigment production is affected by temperature of incubation, depending on the type of microorganism. As examples, *Monascus* sp. requires 25-28^{o}C (Joshi et al., 2003) for pigment production, while *Serratia marcescens* needs 25-30^{0}C. Additionally, *Serratia* sp. does not produce pigment at 38^{0}C (Williams et al., 1971).

ii. pH

Each microbe has different optimal pH in which pigment could be produced greatly. Optimum pH for *Monascus* sp. and *Bacillus cereus* are 5.5-6.5 and 7 , respectively. pH changing may also change the pigment color shade (Banerjee et al., 2011, Joshi et al., 2003).

iii. Fermentation type

There are 2 types of fermentation which are solid state and submerged fermentation. Solid state fermentation gives more benefit than submerged such as having resemblance with natural habit environment, having higher productivity, producing higher end-concentration product and product stability, and showing lower catabolic repression (Hölker et al., 2004).

2.4.2.2 Nutritional factor

Carbon (N) and nitrogen (N) sources

Pigment production also affected by C and N source, depending upon the type of microorganisms also utilization of C and N regarding their metabolism. As C source, glucose and its oligosaccharides serve better nutrition for producing pigment. Generally, sugar type gives impact on pigment shade. In fungi such as *Monascus*, C source affects to the mycelial growth (Joshi et al., 2003), while nitrogen source, especially ammonium group affected on different type of pigmentation (Pisareva and Kujumdzieva, 2014).

2.5 Biological activities of microbial pigments

It has been reported in some studies that certain microbial pigments contained biological activities such as antimicrobial, antioxidant, and anticancer. Some microbial pigments exhibit multi-biological properties as antibacterial and antioxidant, as examples red prodigiosin from *Serratia marcescens* KH1R KM035849, red carotenoid from *Micrococcus roseus*, and yellow carotenoid from *Micrococcus luteus* (Mohana et al., 2013, Vora et al., 2014). Several effects that caused by addition of prodigiosin into *E. coli* cells are reducing growth rate of *E. coli*, inducing outer membrane leakage, holding up cell division, and causing impaired metabolic activity on cell such as

decreasing the respiration activity also RNA synthesis inhibition (Danevcic et al., 2016).

Study conducted by Kavitha et al. (2010) revealed that prodigiosin of *Serratia marcescens* KJ 1307 contained anticancer activity. This prodigiosin exhibits inhibition to cell proliferation or cell growth in human cervix carcinoma. In other word, it can induce the cell line to undergo apoptosis or programmed cell death. Prodigiosin from broth culture of *Serratia* which acted as polyclonal proliferation inhibitor was described by Nakamura in 1986 (Pandey et al., 2007). Pandey et al. (2007) also mentioned that prodigiosin has ability to be anticancer therapy since it could reduce tumor cell by inducing apoptosis and suppressing cell proliferation.

2.6 *Bacillus*

Bacillus species are rod shaped Gram-positive bacteria and endospore-forming aerobic or facultatively anaerobic. *Bacillus* spores are resistant to radiation, heat, cold, desiccation, also disinfectants. The presence of *Bacillus* in nature is ubiquitous since many species of the genus have wide range physiologic capability which allow them to live in every natural environment. In addition, many sectors take advantage on the wide range physiologic characteristic of *Bacillus* and their ability to produce host of enzyme and other metabolites, such as nattokinase production using *Bacillus* natto. However, some *Bacillus* spp. are associated with infections of wound, meningitis, and also a well known *Bacillus cereus* that causing food poisoning syndrome (Mahajan et al., 2010, Turnbull, 1976).

2.6.1 *Bacillus* pigments

Apart from pigment examples on Table 2, research literatures reported that *Bacillus* is a potential pigment production. Some *Bacillus* species produce well characterized pigment such as orange/ yellow as carotenoid (Khaneja et al., 2010), black&brown as melanin (Drewnowska et al., 2015), blue as pyocyanin (Jordan, 1899), and other variety of *Bacillus* pigments that demonstrated by many studies. Table 3 represents various pigments with different color obtained from *Bacillus* group.

Table 3. Pigmented *Bacilli* and their pigments

Species/strain	Pigment	Color	Reference
B. firmus	Carotenoid	Red/ deep pink	Khaneja et al. (2010)
B. aquimaris	Carotenoid	Orange	
B. indicus	Carotenoid	Yellow	
B.megaterium QMB1551	Carotenoid	Red (forespore); yellow (cell compartment)	Mitchell et al. (1986)
B. pyoceaneus	Pyocanin	Blue	Jordan (1899)
Bacillus weihenstephanensis	Melanin	Black & brown	Drewnowska et al. (2015)
B. cereus TS	Pulcherrimin	Red	Canale-Parola (1963)

2.6.2 *Bacillus* as bacterial carotenoid

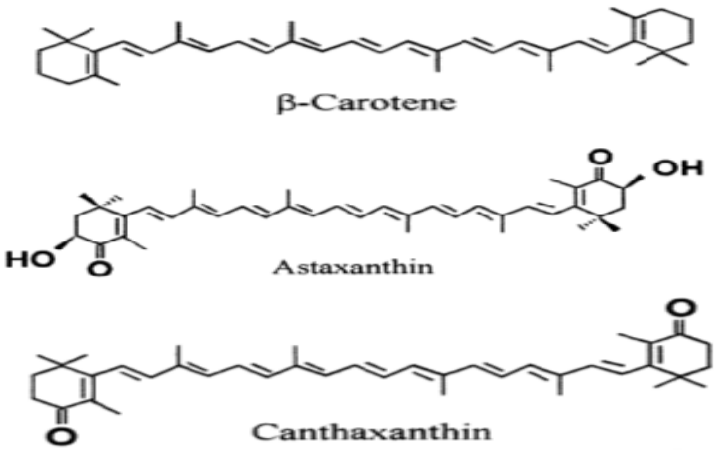


Figure 2. Chemical structure of β-carotene, astaxanthin, and canthaxanthin (Delgado-Vargas et al., 2000)

Bacillus pigmentation as a result from the presence of carotenoids is found abundantly (Khaneja et al., 2010). Those pigments express carotenoid biosynthetic pathway which have similarity with general carotenoid biosynthetic or carotenogenic pathway (Khaneja et al., 2010, Duc le et al., 2006). Carotenoid is naturally occuring pigment with lipid-soluble character that produced in various color from yellow to red in the wavelength range of 300-600 nm. The basic structure of carotenoid is built of tetraterpene with series of conjugated double bonds (Cardoso et al., 2017). This pigment can be classified based on its chemical structure which divided into 2 groups. The first is carotene as pigment that constituted by carbon and hydrogen, and the second is xanthophylls such as astaxanthin and canthaxanthin that also contain oxygen other than carbon and hydrogen (Delgado-Vargas et al., 2000).

In addition, Delgado-Vargas et al. (2000) explained that generally, carotenoid was synthesized within photosynthetic and non-photosynthetic organisms. It occurs in photosynthetic system of higher plants, phototropic bacteria, algae, and in non-photosynthetic organisms such as animal, non-photosynthetic bacteria, yeast, and mold. The existence of carotenoid in plant and microorganism come from de novo synthesize, whereas in animals it synthesized by either modification during metabolism or accumulation in tissues. Other than plant as the most common source of natural carotenoid, Cardoso et al. (2017) mentioned that microorganism biomass was becoming more common source of these subtances. Some species of *Bacillus* containing carotenoid are *Bacillus sp., Bacillus clausii, and Bacillus subtilis.*

The most common pathway of carotenogenic is the condensation of geranyl geranyl-phyrophosphate (GGPP) units that come from isoprene biosynthesis. Based on Figure 3, GGPP forms to prephytoene diphosphate and then condense to phytoene, a 40-carbon polyunsaturated precursor. When subsequent desaturation occurs completely, neurosporene will be formed. Neurosporene can be converted into lycopene or β-zeacarotene and then into several derivatives such as β-carotene (Cardoso et al., 2017).

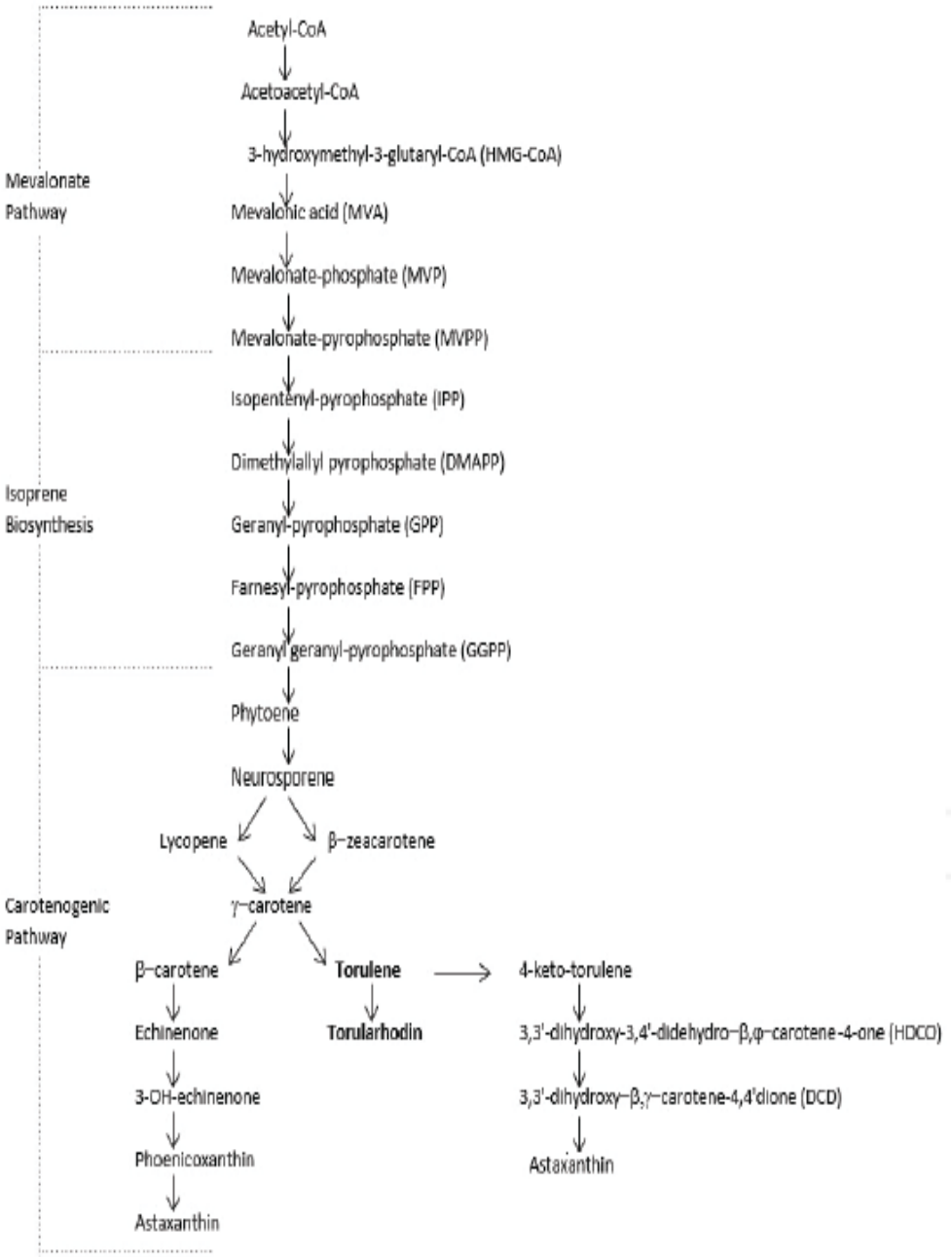


Figure 3. General carotenoids biosynthesis pathway
(Cardoso et al., 2017)

According to a study on *Bacillus* pigment characterization by Duc le et al. (2006), carotenoids from *B. indicus* HU36 vegetative cell was indicated by presence of 1-HO-demethylspheroidene (ODMS), the end product of carotenoid putative pathway. The occurrence of ODMS as carotenoid derivatives started from 2 unit of GGPP condensed into phytoene and formed neurosporene (acyclic carotenoid) via subsequent desaturation (Figure 4, solid square line). From other *Bacillus* pigment investigation which obtained from spores, Khaneja et al. (2010) also detected a condensation product of 2 units GGPP carotenoids precursor called as diapophytoene. However, environmental conditions and cultural stress during bacterial growth affect on carotenoid biosynthesis pathway, especially on subsequent desaturation of phytoene. Since desaturation is a multistep process which varies between microbe, it may lead to different product formation. The most common process is the desaturation which leads to the formation of lycopene (Vachali et al., 2012).

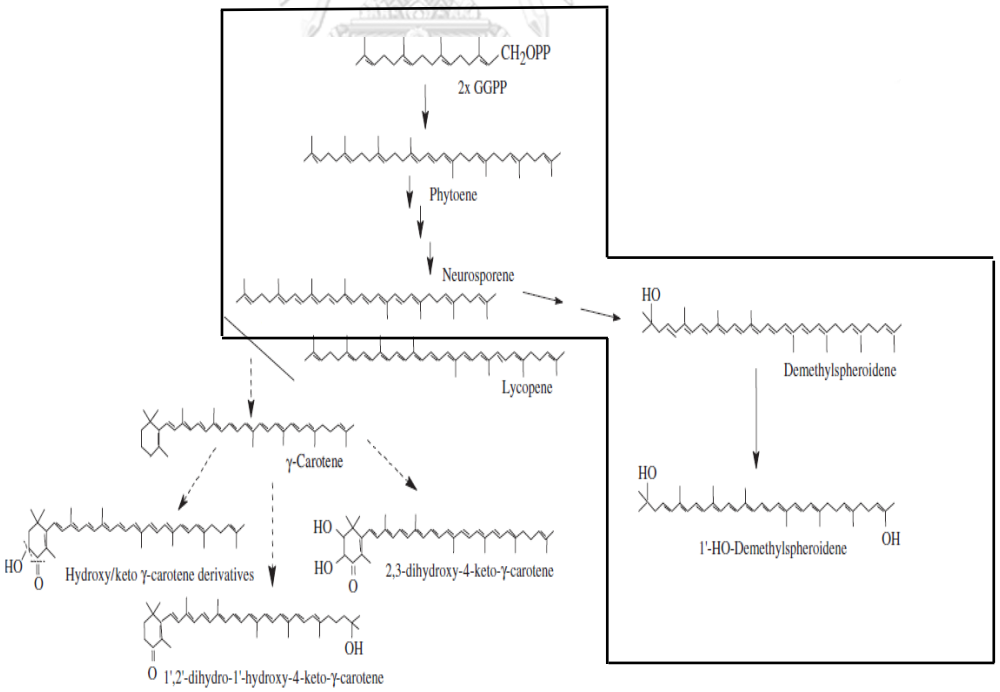


Figure 4. Putative pathway involved in carotenoid during vegetative growth of *Bacillus*
(Duc le et al., 2006)

2.7 Applications of microbial pigments

2.7.1 Microbial pigments as food colorants

Recently, some food grade pigments from microorganisms have been launched on market. There are *Monascus* pigment, Arpink Red from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, and β-carotene from *Blakeslea trispora*. Riboflavin as yellow colorant can be applied in beverages, ice cream, and dessert, whereas red colorant or angkak from *Monascus* cultivation has been used as food ingredient. This red colorant is easy to be found in Japan or Southern China oriental food, lately this colorant comes to processed food such as sausage also fish product such as surimi and fish paste (Dufossé, 2006). Crystalline β-carotene produced from co-fermentation of nonpatogenic *Blaskeslea trispora* DS 30627 and DS 30628 has been confirmed by EU (2000) that it has similar quality as the one from chemically synthesis and acceptable to be applied as food coloring agent. To obtain β-carotene from this mold, there are several steps starting from initial fermentation of seed to produce biomass, followed by recovery process by converting biomass in order to isolate β-carotene efficiently. After the biomass is extracted, it is then purified, and concentrated so that crystallin ß-carotene will be formed.

Lycopene is other type of carotenoid which produced by *Blakeslea trispora.* Lycopene from biosynthesized *Blakeslea trispora* produced through similar method as lycopene produced in the tomato. Lycopene derived from *Blakeslea trispora* preparation intended for use in foods, including food supplements are formulated in the form of suspension in edible oils, direct compressible or cold water-dispersible powders (CWD). In order to prevent oxidative changes in such formulation, this lycopene should be protected by sufficient anti-oxidative protection. Hence, tocopherol in methylene chloride as an antioxidant is used (EFSA, 2008). Based on European Food Safety Authority (EFSA) journal published in 2008, there are several steps for producing lycopene from *Blakeslea trispora* started from extraction from biomass, purification and crystallization of the extract, followed by addition of tocopherol in methylene chloride. In order to get homogeneous emulsion, an octenyl succinic anhydride (OSA)-starch solution was mixed. After drying process, dark red powder as final

product would be obtained. EFSA (2008) also explained value of lycopene exposure estimation in miligram each day. Lycopene exposure estimation for average intake are 0.5-5; 2-6; 1.3-3.5 (mg/day) from naturally occuring lycopene, food color, and enriched food CWD, respectively, whereas for high intake are 8-20; 11-23; 4-8 (mg/day) from naturally occuring lycopene, food color, and enriched food CWD, respectively.

Arpink Red by *Penicillium oxalicum* in crystalline form is obtained from centrifugation of liquid red colorant. It is then precipitated, dissolved in ethyl acohol, and filtered. According to Codex Alimentarius Commission meeting on 2002, there is no prohibition of Arpink Red as coloring agent in meat product with maximum amount of 100 mg/kg, alcoholic beverages with maximum amount 200 mg/kg, and milk product and ice cream with maximum amount 150 mg/kg (Dufossé, 2006).

Apart from those colorant that have launched on market, a study conducted by Venil et al. (2015) tried to developed microbial pigment violacein in the form of powder as food colorant. As an violet pigment, violacein is supposed to give color on product in order to enhance appearance as sensorial property. Violet pigment obtained by fermentation of *Chromobacterium violaceum* UTM5 in bioreactor was concentrated and encapsulated with gum arabic as core material with ratio 1 for pigment and 3 for core material (v/v), then subjected to spray dryer in order to get powder. Various concentration of violet pigment ranging from 0.25;0.5;1% were applied into jelly and white yoghurt. The result showed that intense color for both jelly and white yoghurt were obtained along with increasing concentration of the pigment. Moreover, the stability of this pigment as colorant in both food model system was found for 1 month of storage.

2.7.2 Application of microbial pigments on textile and other product

The efficiency of prodigiosin from *Serratia marcescens* and violacein from *Chromobacterium violaceum* as dye or coloring agent on textile and other product were evaluated by Ahmad et al. (2012). In the term of textile colorant, violacein could be used in silk satin and pure rayon due to the presence of intense color on it, whereas prodigiosin could be applied on acrylic. Other result indicated that addition of red prodigiosin into melted commercial candle resulted in more

intense of red colorant in translucent candle than fluted candle. Apart from that application, red prodigiosin and violet violacein could act as ballpoint ink.

2.7.3 Application of microbial pigments on pharmaceutical industry

Many researchers have developed compounds which have potential clinical treatment. Some investigation regarding microbial pigments with their property such as anticancer, antibiotic, and other properties is further boosted lately (Venil et al., 2013). Prodigiosin was potential candidate for pharmaceutical term as immunosuppressant and anticancer agent (Pandey et al., 2007). Violacein from *Chromobacterium violaceum* also showed its potential as antioxidant since it works efficiently against oxygen and nitrogen reactive species (Konzen et al., 2006). Other microbial pigments such as carotenoid from *Micrococcus spp.* were also reported to have biological activities as antibacterial and and antioxidant (Mohana et al., 2013).

2.8 Pigment as coloring agent and its biological activity in food

Less report and information regarding biological activities of microbial pigments which combined with the colorant itself in food model. Biological activity combined with colorant for food from natural source, turmeric (*Curcuma longa*) has been reported by Gul and Bakht (2015). They studied antimicrobial activity of turmeric extracted from rhizome which applied on chicken and potato based cooked meal. (1) Total bacterial count (TBC) of turmeric treated sample, (2) consumer acceptance through organoleptic test for sensorial properties (color, odor, and taste of product), and (3) antimicrobial activity were determined. The results showed that turmeric extract had antimicrobial activity against some pathogen, gave desirable color, as well as preservation for odor and taste on chicken and potato based cooked meal.

CHAPTER 3
MATERIALS AND METHODS

The instruments, materials, chemicals, PCR reagents, and primers used for this research are listed below :

3.1 Materials and instruments

3.1.1. Cultural Medias

i. Nutrient agar/NA (HiMedia, India)
ii. Nutrient broth/NB (HiMedia, India)
iii. Mueller-Hinton agar/MHA (Himedia, India)
iv. Sheep blood agar (Scharlau, Spain)
v. Compact Dry X-SA (Nissui Pharmaceutical Co., LTD)

3.1.2 Instruments

i. Autoclave (Tommy SX-700, Meditop, Thailand)
ii. Biosafety cabinet (Class II cabinet, Telstar, Thailand)
iii. Bio spectrometer (eppendrop®, Germany)
iv. Centrifuge (Universal 320 R, Germany)
v. Chroma meters (CR-400, Konica Minolta, Japan)
vi. Deep frezeer (Sanyo Biomedical freezer, Japan-Thailand)
vii. Electronic balance (Mettler Toledo, Swizerland)
viii. Fourier Transform Infrared Spectroscopy/ FTIR (Perkin Elmer, USA)
ix. Fumehood
x. Hot air oven (Heraus, Germany)
xi. Hot air oven (Memmret, Germany)
xii. Micropipette (Pipet-LiteXLS, Rainin, Mettler Toledo, Thailand)
xiii. Microplate reader (ASVS, Biochrom, England)
xiv. Refrigerator (Mitsubishi, Thailand)
xv. Rotary evaporator (Buchi Rotavapor R200, Thailand)
xvi. Shaker (Innova™2000, New Brunswick Scientific, Thailand)
xvii. Sonicator (Bandelin, Sonorex digitec, Germany)

xviii. Vortex (Vortex Genie 2, Scientific Industries, Thailand)

3.1.3 Chemichal and reagents

i. Acetone (RCI Lab Scan Limited, Thailand)
ii. Ammonium chloride (Ajax Finechem, Thermo Fisher Scientific, Australia)
iii. Ammonium sulfate (Ajax Finechem, Thermo Fisher Scientific, Australia)
iv. L-Ascorbic acid (Ajax Finechem, Thermo Fisher Scientific, Australia)
v. Chloroform (RCI Lab Scan Limited, Thailand)
vi. Dimethyl sulfoxide /DMSO (Sigma-Aldrich, Laborchemikalien GmbH, Germany)
vii. Fructose (Ajax Finechem, Thermo Fisher Scientific, Australia)
viii. Methanol (RCI Lab Scan Limited, Thailand)
ix. Sodium Chloride (Carlo Erba Reagents, France)
x. Silica plate (TLC Silica gel 60G $F_{254,}$ Merck, Germany)
xi. Sucrose (Ajax Finechem, Thermo Fisher Scientific, Australia)
xii. Tetracycline (Thermo Fisher Scientific, China)
xiii. Petrolium ether (RCI Lab Scan Limited, Thailand)
xiv. 2,2-diphenyl-1-picrylhydrazyl/ DPPH (Sigma-Aldrich, Chemie GmbH, Germany)

3.1.4 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)

3.1.5 Primers

Table 4. List of primers

Primer (BA)	Primer Sequence	Reference
Histamine	JV16HC, 367bp AgA Tgg TAT TgT TTC TTA Tg	Jeune et al. (1995)
	JV17HC AgA CCA TAC ACC ATA ACCTT	
	HDC3, 435 bp gAT ggT ATT gTT TCK TAT gA	Coton et al. (2010)
	HDC4 CCA AAC ACC AgC ATC TTC	
Tyramine	TD2, 1100 bp ACA Tag TCA ACC ATR TTg AA	Coton et al. (2010)
	TD5 CAA ATg gAA gAA gAA gTA gg	
	TDC1, 720 AAC TAT CgT Atg gAT ATC AAC g	Fernandez et al. (2007)
	TDC2 Tag TCA ACC ATA TTg AAA TCT gg	
	TDC-F, 825 bp Tgg YTN gTN CCN CAR ACN AAR CAY TA	de Las Rivas et al. (2005)
	TDC-R ACR TAR TCN ACC ATR TTR AAR TCN gg	
Putrescine	PUT1-F, 1440 bp TWY MAY gCN gAY AAR CAN TAY YYT Gt	de Las Rivas et al. (2005)
	PUT1-R ACR CAN AGN ACN CCN gNg gRT ANg g	
	PUT2-F, 624 bp ATH WgN TWY ggN AAY ACN ATH AAR AA	
	PUT2-R gCN ARN CCN CCR AAY TTN CCD ART C	

3.2. Methods

3.2.1 Microorganism and culture medium preparation

The bacterial code numbers 81-2, 13-3, 77-3, 39-23, and 34-12 isolated from plara were obtained from collection of Food Microbiology Laboratory, Department of Food Technology, Chulalongkorn University. They were grown in Nutrient broth (NB) medium with addition of 3% NaCl at room temperature (27-28^{0}C), 150 rpm orbital shaker, during 72 hours for general cell- and 96 hours for pigment- production, modified from Khaneja et al. (2010). Four reference strains of food pathogens used including *Escherichia coli* ATCC25922; *Salmonella* Typhimurium ATCC1331; *Staphylococcus aureus* ATCC25923; and *Bacillus cereus* ATCC6633 were also obtained from the same collection source. The references bacteria were prepared up to 10^8 CFU/ml, based on standard condition (Ortez, 2005).

3.2.2 Bacterial identification and salt tolerance determination

3.2.2.1 Bacterial identification

The isolates were identified by DNA sequencing analysis. Isolates were grown under general condition stated above, followed by refrigerated centrifugation at 10,000 rpm for 2 minutes. DNA was extracted from pellets of bacterial cells based on Dashti et al. (2009) (see Appendix A), whereas the nucleic acid sequences were chosen from the conserved regions of the 16S rRNA. PCR was performed using primer set 338F/517R (338F: 5′-ACT CCT ACG GGA GGC AGC AG-3′) and 517R (5′-ATT ACC GCG GCT GCT GG-3′) (Mao et al., 2012). PCR condition was: denaturation at 94°C for 2 mins, annealing at 55°C for 30 sec, elongation at 72^{0}C for 30 sec, and extension at 72°C for 7 min in an initial cycle. After that, denaturation time was 30 sec and amplification was done for 35 cycles. The last extension was prolonged for 10 mins. A volume of 10 µl of PCR product were applied on 1.5% (w/v) agarose gel in 1% TAE buffer involving Tris base (2M), glacial acetic acid (1M), EDTA (0.5M), pH 8.0 to 1000 ml distilled water. Electrophoresis was set as 200 V for 30 minutes (Electrophoresis, gel chamber, HU413L, United Kingdom and Electrophoresis power supply, Amersham pharmacia, Bitech, Sweden). After electrophoresis completed, gel was stained with ethidium bromide 1% (Applichem, Spain) and

visualized by exposure to UV transilluminator (Vilber Lourmat, France). The PCR amplicon was cleaned and then sent to commercial sequencing facility (Macrogen, Korea). For further confirmation of each bacterial identity, the sequences data were ran on nucleotide BLAST program of NCBI.

3.2.2.2 NaCl tolerance

NaCl tolerance was investigated by cultivating each isolate in NB supplemented with various concentration of NaCl (3, 5, and 10%) and NB without addition of NaCl, following Tanasupawat et al. (2002) with some modifications. Those were examined based on visual inspection and assigned to +++, ++, +, - for best, moderate, low and no growth.

3.2.3 Screening of biological activities, pigment characterization, and safety evaluation

3.2.3.1 Pigment extraction

Extraction process was done by following Khaneja et al. (2010) with some modifications. Isolates were grown under general condition stated above, followed by centrifugation at 10,000 rpm for 15 minutes. The cell pellet obtained were washed twice and froze at least 24 hours. Frozen cell pellet (200 mg) was thawed, suspended in 1 M NaOH (500 µl), and treated with sonication for 5 minutes at room temperature. Those mixture was then re-centrifuged to remove NaOH and added with methanol (250 µl), chloroform (500 µl) and water (250 µl). As the phase separation was created, lower phase as organic layer was collected, whereas upper phase or aqueous layer re-extracted with chloroform until the debris was colorless. Finally, organic layer was concentrated in rotary evaporator (60^0C) to get dried pigment extract. Those pigment extracts were collected and stored in amber bottle at -21^0C until further analysis. Prior to analysis, dried extract were re-dissolved on specific solvent based on each analysis.

3.2.3.2 Antimicrobial activity determination

Antimicrobial activity of dried pigment extract was checked using disc diffusion method (Mohana et al., 2013). Sterile paper disk was impregnated on 20 µl dimethylsulfoxide (DMSO) solution of pigment extracts at the concentration of 200 mg/ml, then put on the surface of pre-inoculated Mueller-Hinton agar (MHA) containing 10^8 CFU/ml of reference food pathogens mentioned before.

The presence of Inhibition Zone (IZ) in milimeter (mm) which indicated inhibitory effect to pathogen was measured after 24 hours incubation at 37^0C. Positive and negative controls used were tetracycline and DMSO, respectively.

3.2.3.3 Antioxidant activities determination

Antioxidant activities of pigment extracts were assayed through 2,2-Diphenyl-1picrylhydrazyl (DPPH) assay adapted from Brand-Williams et al. (1995) with some modifications. Each pigment was prepared as 50 mg/ml solution in methanol, whereas DPPH solution was prepared as 0.025 mg/ml in the same solvent. A volume of 0.5 ml of pigment was mixed with 5 ml of DPPH solution and incubated for 30 minutes in the dark room. The absorbance was recorded at 517 nm using UV-vis spectrophotometer. DPPH solution, ascorbic acid, and methanol were used as control, standard, and blank, respectively. DPPH radical scavenging activities (% inhibition) was calculated formula as follow:

$$I\% = \frac{(A_c - A_s)}{A_c} \times 100$$

where A_c is absorbance of control and As is absorbance of sample.

3.2.3.4 Pigment characterization

Each dried pigment extracts obtained from 5 isolates were studied on their pigment properties using UV-vis spectrophotometer, thin layer chromatography (TLC), and FTIR.

i. Spectrophotometric analysis

Prior to analyze the absorbance (λmax) on UV-vis spectrophotometer, pigment extracts were dissolved in DMSO, following Popova (2017) with some modifications. For preparation of pigment solution, each extract (0.1 gram) was dissolved in 1 ml of DMSO, which then transferred to 1 cm-cuvette. Absorption spectrum of each pigment was recorded on spectral region of 350-600 nm at room temperature.

ii. TLC assay

Pigment property was further analyzed by TLC based on Perez-Fons et al. (2011) with modification. One miligram of dried extract dissolved in 200 µl solvent containing chloroform : methanol 1:1 by volume. TLC separation of the pigment was developed on a system consist of silica gel as stationary phase and

acetone (35%) in petroleum ether as mobile phase. In order to help visual identification, solution of paraffin in petroleum ether was sprayed on silica plate before ultraviolet (UV) light applied. TLC result was expressed as retention factor (Rf) by following formula :

$$Rf = \frac{\text{distance traveled by compound}}{\text{distance traveled by solvent}}$$

iii. FTIR analysis

Pigments were sent to Scientific and Technological Research Equipment Centre, Chulalongkorn University to be analyzed their functional groups through FTIR. FTIR spectra from each pigment was recorded on a PerkinElmer FTIR spectrophotometer (USA), in which its frequency for functional groups detection ranging from 515-4000 cm^{-1}. For pigment samples preparation, a method from Li et al. (2017) was used. Approximately 10 mg of pigment sample was ground with a hundred milligram of KBr powder. The grounded-mixture then pressed into pellets for allowing infrared spectroscopy transmission process.

3.2.3.5 Safety evaluation of selected isolate

The safety of selected isolate was evaluated for several potential production of allergen such as biogenic amine, hemolysin test, and acute toxicity test (in vitro against cell line).

i. Biogenic amine test

In order to investigate biogenic amines (histamine, tyramine, putrescine) occurrence, PCR was employed. The bacterial DNA extracted was subjected to PCR using 0.1 mM specific primers. Specific primer used for histamine were JV16HC (367bp)/ JV17HC (Jeune et al., 1995) and HDC3 (435 bp)/HDC4; for tyramine were TD2 (1100 bp)/ TD5 (Coton et al., 2010), TDC1/TDC 2 (Fernandez et al., 2007), and TDC-F (825 bp)/TDC-R (de Las Rivas et al., 2005); whereas for putrescine were PUT1-F(1440 bp)/PUT1-R and PUT2-F (624 bp)/ PUT2-R (de Las Rivas et al., 2005). The PCR was performed in total volume of 50 µL with 1.5 Mm MgCl2, 0.1 mM dNTPs mix and 1 U of DNA Tag polymerase (Vivantis, Malaysia). Samples were subjected to condition : initial cycle of denaturation (94^0C for 2 min), followed by 34 cycles of

denaturation (94^{0}C for 30 s), annealing (55^{0}C for 30 s), and elongation (72^{0}C for 30 s), ending with extension at 72^{0}C for 7 min, in a DNA thermal cycler (BioRadT100^{TM} Singapore). The test was repeated with duplicate. The PCR amplicon was applied on 1.5% agarose gel electrophoresis comparing to positive control.

ii. Hemolytic activity

Hemolytic activity was determined by streaking selected isolate on sheep blood agar and incubated for 48-72 hours at 37^{0}C. The zone of hemolytic was recorded as β–haemolysis (clear zone hydrolysis around colony), α-haemolysis (partial hydrolysis and greenish zone), or γ–haemolysis if there is no reaction (Pisano et al., 2014).

iii. Cytotoxicity test

Cytotoxicity study is preliminary test for evaluating toxicity of tested compound. This assay is employed considerably especially for a substance intended to be used on pharmaceuticals field in which "non-toxic" term should be convinced (McGaw et al., 2014). Human cell namely human caucasian colon adenocarcinoma or Caco2 cell is one of in vitro cell line model's mostly used for investigating absorption of a compound in intestine during screening level (Turco et al., 2011). Furthermore, Caco2 cell line is an intestinal model which directly contact with food (Er et al., 2015). Due to pigment from this study further tested on food matrix, cytotoxicity assay was tested against Caco2 cell. In order to conduct in vitro study, pigment extract was sent to BIOTEC, Rangsit, Thailand and performed using Resazurin Microplate Assay (REMA). Previously, pigment extract was prepared using the same method for pigment extraction mentioned in 3.2.3.1

3.2.4 Optimization of *Bacillus* pigment

Optimum condition for pigment production from selected isolate was evaluated through 3 type of nutritional factor, including NaCl concentration, Carbon (C), and Nitrogen (N) sources. Effect of various salt concentration and different combination of Carbon (C) and Nitrogen (N) were investigated as follows:

3.2.4.1 NaCl optimization on pigment production

Isolate was grown in 250 ml flask containing 150 ml NB supplemented with 3 various salt concentrations (1%, 3%, and 5%). The cultivation was conducted under room temperature (27-28^{o}C), orbital shaking at 150 rpm for 96 hrs. The culture was sampled to spectrophotometric analysis every 24 hours. The cell culture (25 ml) was transferred into tube, centrifuged (10,000 rpm, 15 minutes), and washed with normal saline. After that, mixture was suspended in 1 M NaOH, sonicated 5 minutes, and re-centrifuged. Cell pellet was suspended in methanol, vortexed, and ran on centrifuge at 3,500 rpm for 20 minutes to collect supernatant (Kirishna et al., 2014).

Prior optimization, λ max of pigment extract in methanolic solution was measured, and then used to record pigment production following Popova (2017) with some modifications. For preparation of pigment solution, each extract (0.1 gram) was dissolved in 1 ml of methanol, which then transferred to 1 cm-cuvette. Absorption spectrum of each pigment was recorded on spectral region of 350-600 nm at room temperature. Full factorial design was used and all treatments were performed as 2 replications. NaCl concentration with maximum pigment production was selected for C and N sources optimization.

3.2.4.2 C and N sources optimization on pigment production

Two types of C sources (sucrose and fructose) and N sources (ammonium chloride and ammonium sulfate) were supplemented in NB with salt concentration selected from section 3.2.4.1. C was supplemented in the amount of 1%, whereas N was added 0.5% (Pandey et al., 2018, Chatterjee and Bhowal, 2016). The cultivation condition were conducted same as 3.2.4.1 with full factorial design and 2 replications. Pigment generations were monitored every 24 hours using the same method as mentioned in 3.2.4.1. C and N sources that generated highest production of pigment was selected for further study.

3.2.5 *Bacillus* pigment tested in food matrix

Prior to the testing of selected *Bacillus* pigment into food matrix, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of pigment against *Staphylococcus aureus* were investigated. MIC and MBC were performed according to Mohana et al. (2013).

In order to investigate MIC, 90 µl different concentration of DMSO solution of pigment in NB (200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml) were prepared and transferred separately into sterile 96-well microtiter plate. Each of them was inoculated with 10 µl of bacterial culture (10^8 CFU/ml of *S. aureus*) and incubated 37^0C for 24 hours. After incubation, turbidity of each well was determined at OD_{600} using microtiter plate reader and clear culture well was further picked with loop and streaked on NA, incubated 37^0C for 24 hours. MIC was defined as the lowest pigment concentration that inhibited the visible growth of bacteria, while MBC was considered as lowest concentration of the pigment required to kill particular bacteria (Mohana et al., 2013, Andrews, 2001)

For further test in food, oily salad dressing prepared from 2 types of dressing was used as food matrix. Those dressing were bought from weekend market, Chulalongkorn University. Briefly, creamy salad dressing with thick-oily characteristic containing oil, salt, sugar, and other ingredients was mixed with basic dressing containing sugar solution, which had lighter characteristic. Three gram of dressing mixture was mixed with pigment crude extract (based on MBC obtained before). The stability of color in salad dressing was evaluated by measurement of color shade using chroma meters (L*a*b* value) every 2 days during storage for 6 days under refrigerated temperature (5^0C). Antimicrobial activity of pigment was evaluated by total plate count (TPC), *S. aureus*, and yeast & mold count (see detail methods in Appendix B).

3.2.6 Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS 22.0). Every statistical significance was defined as *P* value < 0.05.

CHAPTER 4
RESULT AND DISCUSSION

4.1 Bacterial identification and NaCl tolerance determination

This study was preceded by isolation of halophilic-pigmented bacteria obtained from plara. Five isolates with code number 81-2, 13-3, 77-3, 34-12, and 39-23 having pink-orange, yellow, and yellow orange pigmentation were selected as representative from each color shade. From DNA sequencing analysis, isolates number 81-2, 13-3, 77-3 were identified as *Bacillus* sp., whereas number 39-23 and 34-12 were identified as *Bacillus vietnamensis* and *Bacillus amyloliquefaciens*, respectively (Table 5).

Since those *Bacillus* strains were isolated from plara which known as salt containing foods (Tanasupawat and Komagata, 1995), salt tolerance determination was done to investigate their physiological characteristics. Based on Table 6, all of *Bacillus* strains could survive on medium containing 3-10% NaCl, also on medium without NaCl supplementation. Among 3 different NaCl concentration, 3% NaCl supported better growth for all strains than others. Therefore, all strains were classified as slight halophilic (DasSarma and Arora, 2001). Some studies reported that high salt concentration that being used on cultivation would reduce pigment production. Chadni et al. (2017) stated that pigment production of *Talaromyces verruculosus* was higher on salt concentration below 4%, while Kirishna et al. (2014) mentioned that salt concentration higher than 8% causing a decrease on pigment yield of halophilic *Vibrio* sp. Thus, in this present study, 3% salt concentration was chosen as NB supplementation for further analysis.

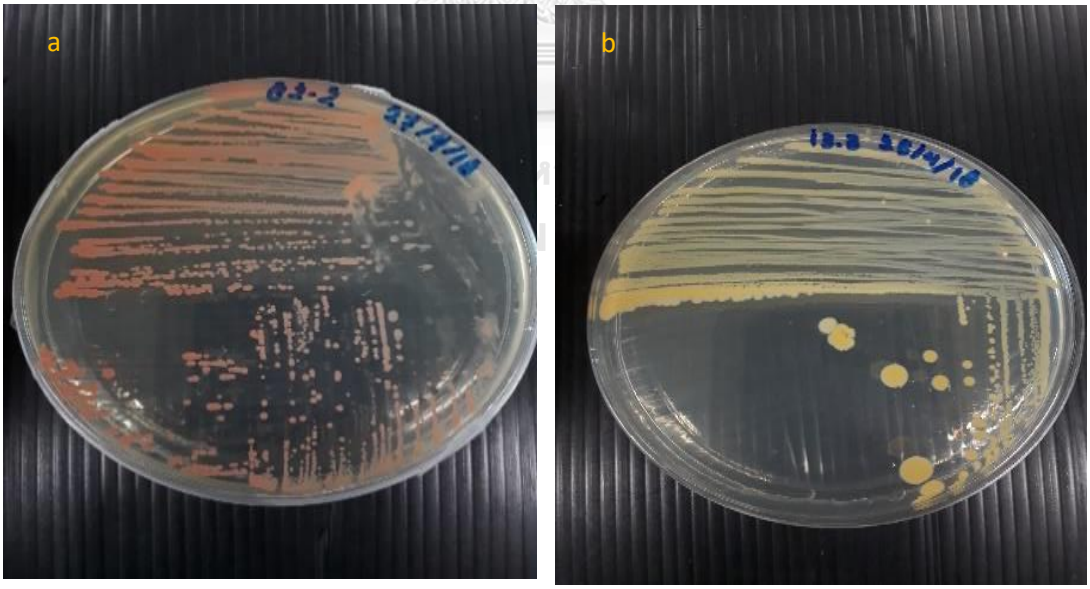
Table 5. Identification of isolates

Isolate	Colony color	Sequencing (16S RNA)	Homology (%)
81-2	Pink-orange	*Bacillus* sp.	94%
13-3	Yellow	*Bacillus* sp.	86%
77-3	Pale yellow	*Bacillus* sp.	99%
39-23	Yellow to orange	*B. vietnamensis*	99%
34-12	Pale yellow	*B. amyloliquefaciens*	99%

Table 6. Salt tolerance determination of isolates

	Salt tolerance			
Isolate	0%	3%	5%	10%
81-2	++	+++	++	+
13-3	+++	+++	+++	++
77-3	+++	+++	++	++
39-23	++	+++	+++	++
34-12	++	+++	+++	++

note : +++ = best growth; ++ = moderate growth ; + = low growth



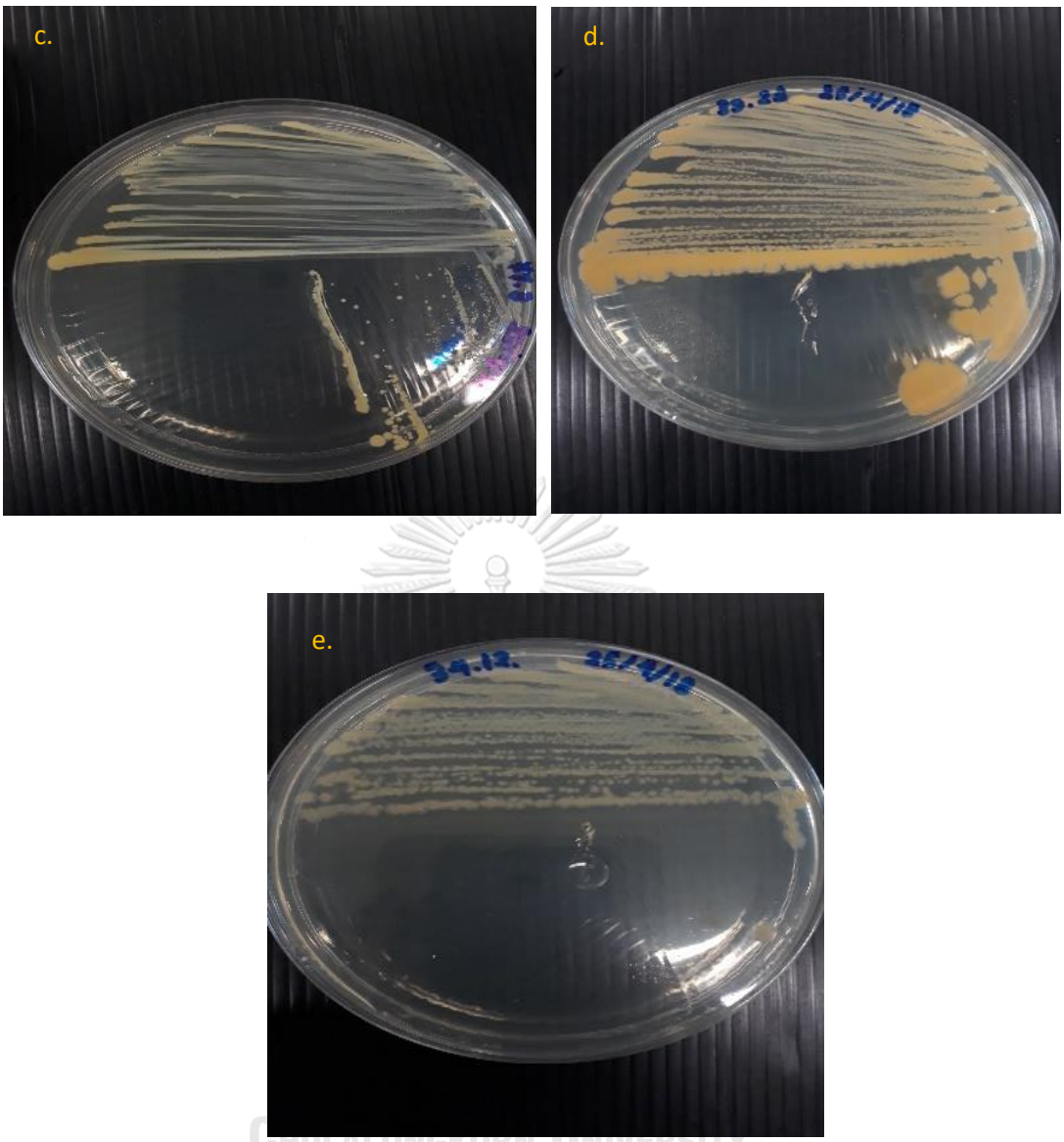


Figure 5. *Bacillus* spp. grown in NA with 3% salt

(a.) *Bacillus* sp. 81-2, (b.) *Bacillus* sp. 13-3 ,(c.) *Bacillus* sp. 77-3 ,(d) *Bacillus vietnamensis* 39-23 ,(e.) *Bacillus amyloliquefaciens* 34-12

4.2 Screening of biological activities, pigment characterization, and safety evaluation

4.2.1 Biological activities

Antimicrobial activity of pigments in this study were shown in Table 7. All of pigment extracts had inhibitory effect against *E. coli*, *S.* Typhimurium, and *B. cereus*. However, for *S. aureus* only pink-orange and pale yellow pigments from *Bacillus* sp. 81-2 and *B. amyloliquefaciens* 34-12 which had inhibitory against, respectively. Standard tertracycline showed IZ value from 13.5 up to 19.5, whereas antimicrobial activities of pigments in this study ranging from 0.25-4.000 mm of IZ. Thus, those pigments were categorized as pigments having weak antimicrobial activity Mohamed et al. (2017).

As comparison with other study, antimicrobial activity of 1-10 mg/ml carotenoid pigment from *M. roseus* exhibited antimicrobial against *S. aureus* with 6.5-12.5 mm of IZ (Mohana et al., 2013). Antimicrobial activity of 0.5-5 mg/ml pigment extracted from *Rhodotorula glutinis* resulted in 9.5-17.9 mm IZ against *B. cereus*, 7.9-13.1 IZ against *E. coli*, and 8.8-13.3 mm IZ against *S. aureus* (Rostami et al., 2016). In this study, all of pigment had lower antimicrobial activities which might caused by source of pigment obtained. Pigments of our study were obtained from vegetative cell of *Bacillus*. Based on Duc le et al. (2006), *Bacillus* produce carotenoid through putative biosynthetic pathway on both vegetative cell and spores with different quantity and quality. In addition, pigmentation from spores contained higher amount of carotenoid and had different pigment composition that may produce different kind of carotenoid end product.

According to Table 7, no inhibition action revealed by negative control DMSO against *E. coli* and *S. aureus.* Nevertheless, DMSO showed IZ against *S.* Typhimurium and *B. cereus* with value of 2.000 and 0.500, respectively. The IZ revealed by DMSO in this antimicrobial test might caused by different susceptibility of *S.* Typhimurium and/or *B. cereus* with 2 others pathogens toward DMSO solvent. Study conducted by Wadhwani et al. (2008) found that a strain of *Salmonella, Salmonella paratyphi A* was the most sensitive against 4-6% DMSO and their study denoted that each bacteria had different susceptibility

toward DMSO. Further mentioned by Wadhwani et al. (2008), the use of DMSO as solvent in several bioassay was preferred since it had exceptional property, such as high polarity. However, DMSO was reported as a solvent having antimicrobial effect which might interfere antimicrobial assay.

Table 7. Antimicrobial activity of crude pigment extracts

Strain	Antimicrobial activity of 200 mg/ml crude extract (IZ in mm)			
	E. coli	*S. aureus*	*S.* Typhimurium	*B. cereus*
Bacillus sp. 81-2	2.500±0.707^{abc}	1.000±1.414^{a}	3.500±1.414^{a}	3.875±1.945^{a}
Bacillus sp. 13-3	1.500±2.121^{abc}	0.000±0.000^{a}	3.625±1.237^{a}	3.250±1.061^{a}
Bacillus sp. 77-3	3.375±0.177^{ab}	0.000±0.000^{a}	3.500±0.000^{a}	2.375±0.177^{a}
Bacillus amylolique-faciens 34-12	4.000±0.707^{a}	0.250±0.354^{a}	2.875±0.884^{a}	3.750±2.475^{a}
Bacillus vietnamensis 39-23	1.250±0.354^{bc}	0.000±0.000^{a}	3.500±0.354^{a}	2.250±1.768^{a}
DMSO	0.000±0.000^{c}	0.000±0.000^{a}	2.000±0.000^{a}	0.500±0.707^{a}

note :different superscripts within a column indicate significant differene ($p<0.05$)

Based on Table 8, the highest scavenging activity was shown by *Bacillus* sp. 13-3 which had 18.716 % inhibition. Even though this highest value was lower than scavenging activity of ascorbic acid, it showed significant difference to pigments with low antioxidant activities (pigments from *Bacillus* sp. 81-2, *B. amyloliquefaciens* 34-12, *B. vietnamensis* 39-23). In line with antimicrobial activity, our finding on antioxidant activity was lower than other studies. Antioxidant activity of 1-10 mg/ml carotenoid from *M. roseus* resulted in 32.8-98.5 % inhibition, while 1-10 mg/ml carotenoid from *M. luteus* resulted in 29.1-98.5 % inhibition. As previously mentioned, low antioxidant capacity might caused by pigmentation that have not yet been obtained from an appropriate production.

Table 8. Antioxidant activity of crude pigment extracts (50 mg/ml)

Strain	% Inhibition of crude extract
Bacillus sp. 81-2	11.543 ± 1.171^{cd}
Bacillus sp. 13-3	18.716 ± 1.782^{b}
Bacillus sp. 77-3	15.750 ± 0.520^{bc}
B. vietnamensis 39-23	9.628 ± 5.835^{d}
B. amyloliquefaciens 34-12	13.907 ± 0.091^{cd}
Ascorbic acid	95.579 ± 0.251^{a}

note: different superscripts indicate significant difference ($p<0.05$)

4.2.2 Pigment characterization

Characterization of all 5 pigments in this present study were performed by 3 analytical methods including UV-vis spectrophotometric analysis, TLC assay, and FTIR. The use of 3 analytical methods were aimed to determine and identify bioactive compound in which each characteristic obtained from different analytical method could support each other. UV-vis spectrophotometer is carried out as preliminary study on identification also quantification of compound based on the characteristic of absorption maximum (Gunasekaran et al., 2008). Mostly, crude extracts consist of many type of bioactive compound having dissimilar polarity. It is therefore, separation of the compound should be performed. TLC is simple, cheap, and rapid technique which help to separate mixture of a compound based on its Rf and further compared to Rf of known compound (Sasidharan et al., 2011). To support identification, the use of more sensitive and accurate technique, such as FTIR should be employed. IR spectrum of each molecule is typical and unique which then can be used to interpret the molecule (Griffiths and De Haseth, 2007).

Table 9 shows pigment properties based on spectrophotometric analysis and TLC assay. All of pigments revealed λmax of 447-526 nm. Those peak profile had similarity with carotenoid profile from *Bacillus* spp. pigment (Khaneja et al., 2010). Due to the absence of available authentic standard, Rf obtained from TLC result was compared to Rf value of *Bacillus* pigment from literature. TLC result suggested that pink-orange pigment of *Bacillus* sp. 81-2 with Rf of 0.31 was

lycopenoate according to previous study of purified *Bacillus* pigment having Rf value of 0.34 (Perez-Fons et al., 2011). Pigment from *Bacillus* sp. 13-3 showed lower polarity with Rf value of 0.66 (Figure 6).

As stated before, TLC helped to separate a mixture of crude extract. However, TLC result obtained from this study revealed that only 1 spot of each pigment could be visualized. This might caused by unsuitable solvent system. Additionally, 3 pigments including yellow pigment from *Bacillus* sp. 77-3 and *B. amyloliquefaciens* 34-12, also yellow-orange pigment from *B. vietnamensis* 39-23 could not be detected through TLC. This phenomenon might caused by low color intensity of their band to be seen on TLC plate. To support pigment properties, those pigments were subjected to FTIR analysis.

FTIR absorption of all of pigments were shown more detail on Table 10 and Figure 7. All yellow pigments and orange-yellow pigment exhibited close spectrum which had absorption frequencies responsible for C-H aromatic, alkyl chain CH_3- and $–CH_2$-, C=C alkene, CH_3- and -CH_2- bending vibration, also carboxylic acid of yellow-orange pigment only, and it found the same pattern of Ramachandran et al. (2014) that suggested to be xanthophylls. However, the pink-orange pigment showing characteristic of the CH_2 stretching, C-CH_3 stretching vibration, C=O conjugated/carbonyl group, C=C stretching, CH_2 bending, and CH_3 bending vibration, showed similar pattern and characteristic of lycopene according to Afra et al. (2017).

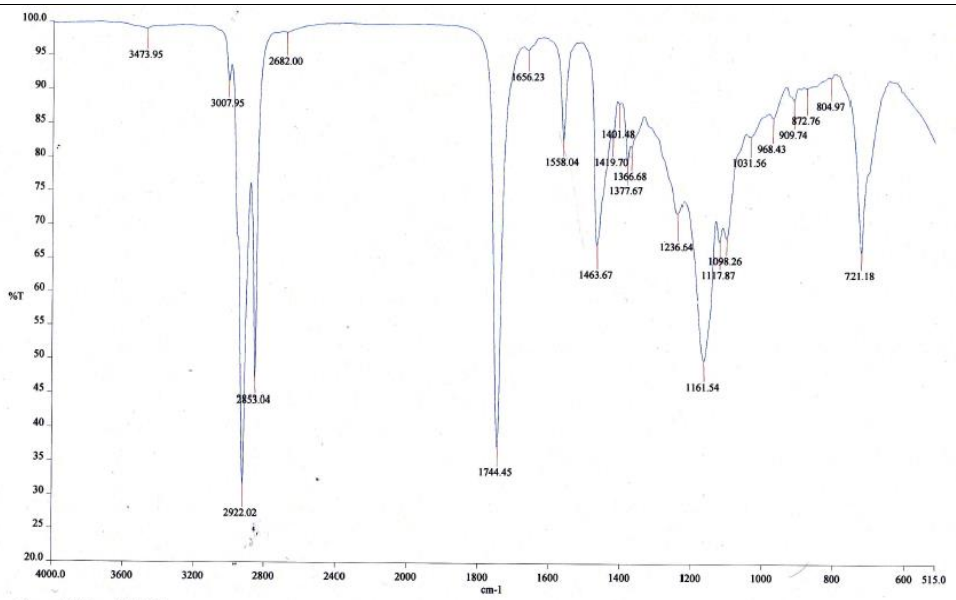
Table 9. Pigment properties based on spectrophotometric analysis and TLC assay

Isolate	Visible color		Spectro-metric analysis (λmax)	TLC assay (Rf)
Bacillus sp. 81-2	pink-orange		479 nm; **498 nm;** 526 nm	0.31
Bacillus sp. 13-3	yellow		**458** nm; 487 nm	0.66
Bacillus sp. 77-3	pale yellow		447 nm; **458** nm	u
B. vietnamensis 39-23	yellow-orange		486 nm	u
B. amyloliquefaciens 34-12	pale yellow		**457nm**; 486 nm	u

note: u is responsible for unidentified result

Figure 6. TLC assay for (a.) pink-orange pigment *Bacillus* sp. 81-2, (b.) yellow pigment from *Bacillus* sp. 13-3.

a.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cm-1
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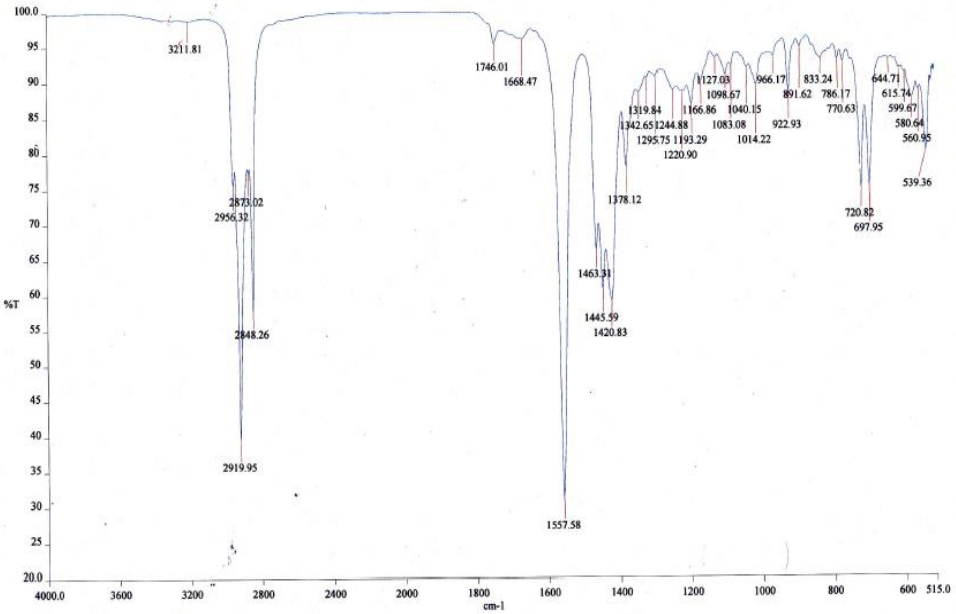
3007.95
2682.00
1656.23
1558.04
1401.48
1419.70
1366.68
1377.67
1463.67
1236.64
1098.26
1117.87
1031.56
968.43
909.74
872.76
804.97
721.18
1161.54
2853.04
2922.02
1744.45
Pigment 81.2.sp - 7/15/2018

b.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1127.03
1098.67
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1342.65
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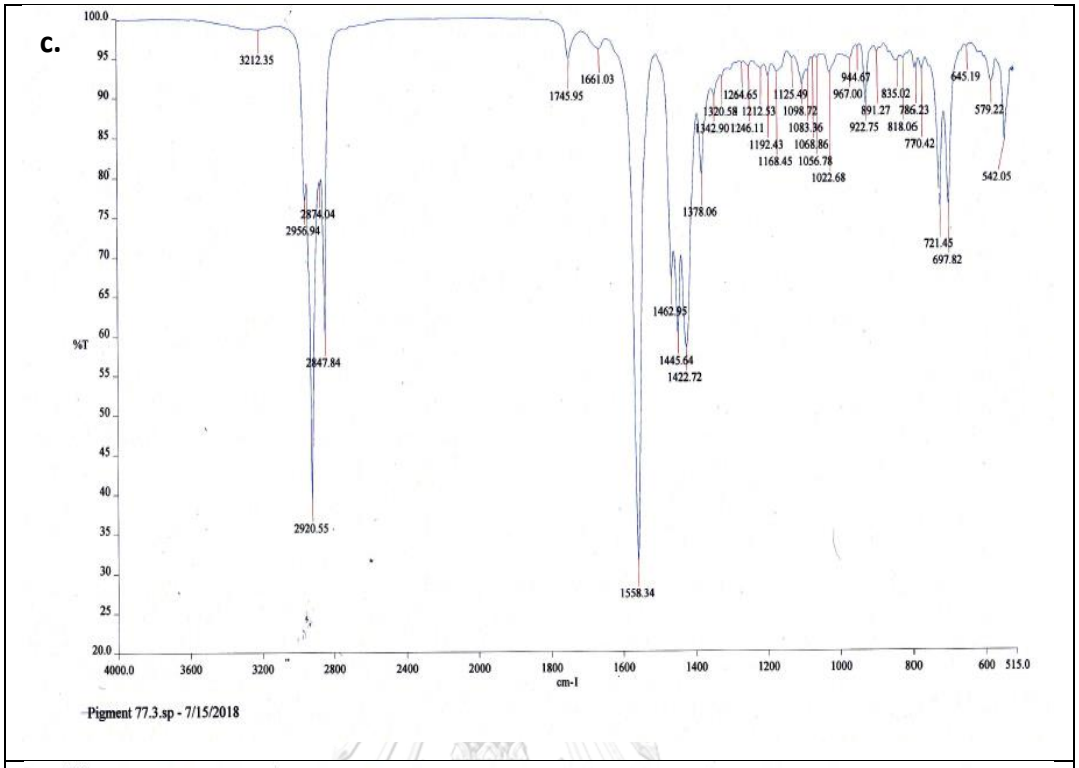
1166.86
1083.08
1040.15
966.17
922.93
891.62
833.24
786.17
770.63
644.71
615.74
599.67
580.64
560.95
539.36
1295.75
1193.29
1220.90
1014.22
1378.12
2873.02
2956.32
1463.31
1445.59
1420.83
2848.26
2919.95
1557.58
720.82
697.95
Pigment 13.3.sp - 7/15/2018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1422.72
1378.06
1264.65
1320.58
1212.53
1342.90
1246.11
1125.49
1098.72
1083.36
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1068.86
1168.45
1056.78
1022.68
944.67
967.00
891.27
922.75
835.02
786.23
818.06
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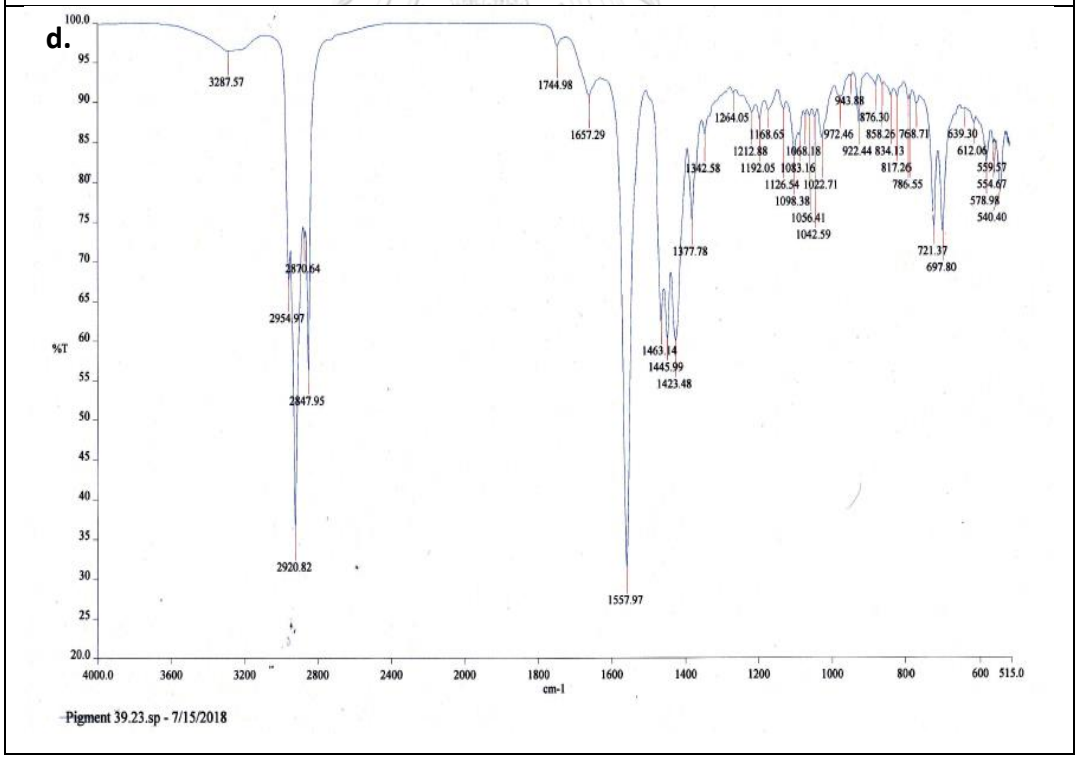
579.22
542.05
721.45
697.82
%T
cm-1
Pigment 77.3.sp - 7/15/2018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1445.99
1423.48
1377.78
1342.58
1264.05
1212.88
1192.05
1168.65
1126.54
1098.38
1083.16
1068.18
1056.41
1042.59
1022.71
943.88
876.30
858.26
834.13
817.26
786.55
768.71
721.37
697.80
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559.57
554.67
578.98
540.40
%T
cm-1
Pigment 39.23.sp - 7/15/2018

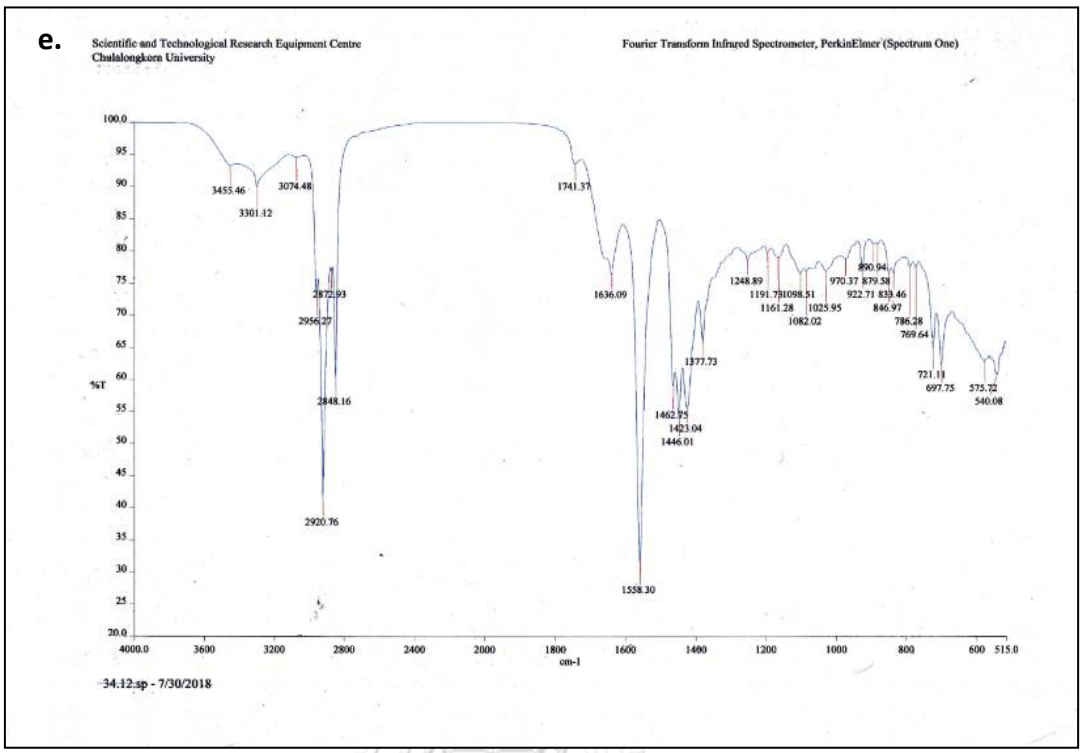


Figure 7. FTIR spectrum of *Bacillus* spp.

(a.) *Bacillus* sp. 81-2 pink-orange pigment ; (b) *Bacillus* sp. 13-3 yellow pigment; (c) *Bacillus* sp. 77-3 yellow pigment ; (d.) *B. vietnamensis* 39-2 yellow-orange pigment; (e.) *B. amyloliquefaciens* 34-12 yellow pigment

Table 10. Pigment properties based on FTIR

Isolate	Identifying functional group from FTIR	Tentative identification
Bacillus sp. 81-2	2922.02 and 2853.04 (CH_2 stretching); 2682.00 (C-CH_3 stretching vibration); 1744.45 (C=O conjugated/carbonyl group); 1656.23 (C=C stretching); 1463.67 and 1419.70 (CH_2 bending); 1377.67 an 1366.68 (CH_3 bending vibration)	Lycopene
Bacillus sp. 13-3	2956.32 (C-H aromatic);2919.95 and 2848.26 (alkyl chain CH_3- and –CH_2-); 1668.47 (C=C alkene); 1445.59 (CH_3- and -CH_2- bending vibration)	xanthophyll
Bacillus sp. 77-3	2956.94 (C-H aromatic); 2920.55 and 2847.84 (alkyl chain CH_3- and –CH_2-); 1661.03 (C=C alkene); 1445.64 (CH_3- and -CH_2- bending vibration)	xanthophyll
Bacillus vietnamensis 39-23	3287.57 (carboxylc acid); 2954.97 (C-H aromatic); 2920.82 and 2847.95 (alkyl chain CH_3- and –CH_2-); 1657.29 (C=C alkene); 1445.99 (CH_3- and -CH_2- bending vibration)	xanthophyll
Bacillus amyloliquefa-ciens 34-12	2956.27 (C-H aromatic); 2920.76 and 2848.16 (alkyl chain CH_3- and –CH_2-); 1636.09 (C=C alkene); 1446.01 (CH_3- and -CH_2- bending vibration)	xanthophyll

After passing biological activities assay, especially antimicrobial test, *Bacillus* sp. 81-2 pigment exhibited inhibitory action against all pathogen tested. Other than that, pink-orange pigment color shade from this *Bacillus* strain was the most intense among 5 pigments. Therefore, pink-orange pigment from *Bacillus* sp. 81-2 was selected for further study.

4.2.3 Safety evaluation of selected isolate

The joint working group of Food and Aid Organization/World Health Organization have construct guidelines for assessing a candidate strain to be further develop as food grade, as an example for the use of probiotics in food. Other than strain identity assessment, safety of the strain is also included as the minimum assessment. Several safety assessment that often used are hemolytic activity and cytotoxicity test (Kesen and Aiyegoro, 2018). In this present study, pigment from *Bacillus* sp. 81-2 was investigated for biogenic amines or allergens, hemolysis activity, and cytotoxicity test against Caco2 cell line.

4.2.3.1 Biogenic amine evaluation (BAs)

BAs are organic,- basic, nitrogenous compounds, generally formed by an amino acids decarboxylation. BAs can be found in many kinds of food which occasionally accumulate in high concentration. Consuming high amount of BA can induce toxicological effect, such as nausea, intestinal problem, blood pressure changing, headaches etc. Therefore, it is important for doing early detection of BA formation in food industries to prevent from the risk of amino formation. Nowadays, methods for detecting BA have been developed. PCR approaches allowed fast and reliable result through determination genes associated to potential biogenic amine production (Spano et al., 2010).

The selected culture from this study was verified for its BA producing capability based on determination of BAs-encoding genes namely histamine, tyramine and putrescine, whose generally detected in food and beverage (de Las Rivas et al., 2005). Two reactions of Multiplex PCR indicated the safety of *Bacillus* 81-2 culture based on histamine, tyramine and putrescine formation ability as none specific DNA band of BA-producing genes were shown in Figure 8. The result revealed that there were no production of those BAs generated by pigment producing *Bacillus* sp. 81-2. In accordance with Eom et al. (2015), their study showed that there were no detection of gene encoding histidine and tyrosine decarboxylase in several *Bacillus* strain. Therefore, pigmented *Bacillus* 81-2 could be further used in food since there was no potential regarding BAs formation.

Figure 8. Genes associated to potential biogenic amine production (Band from left to right = control, mix primer, HDC3/4, JV16/JV17, PUT2R/2F, PUT1R/F, TD2/5, TDCF/R, TDC1/2)

4.2.3.2 Hemolysin assay

Hemolysins are molecules that have ability to lyse red blood cell (RBCs). Hemolysins divided into β–hemolysin and α-hemolysin. α-hemolysin is the type of hemolysin that causes partial lysis of RBCs which indicated by darkening media around a colony, whereas β–hemolysin causes completely lysis of RBCs which shown by clear zone around the colony. The term of γ–hemolysin is used when there is an absence of hemolysis production (Vesper and Jo Vesper, 2004, Pisano et al., 2014). Through this analysis, result in Figure 9 showed no clear zone around colony of *Bacillus* sp. 81-2 when compared to positive control (a strain of *Bacillus* that produces β–hemolysin). Therefore, *Bacillus* sp. 81-2 was classified as γ –hemolysin which was consider as safe for further application on food.

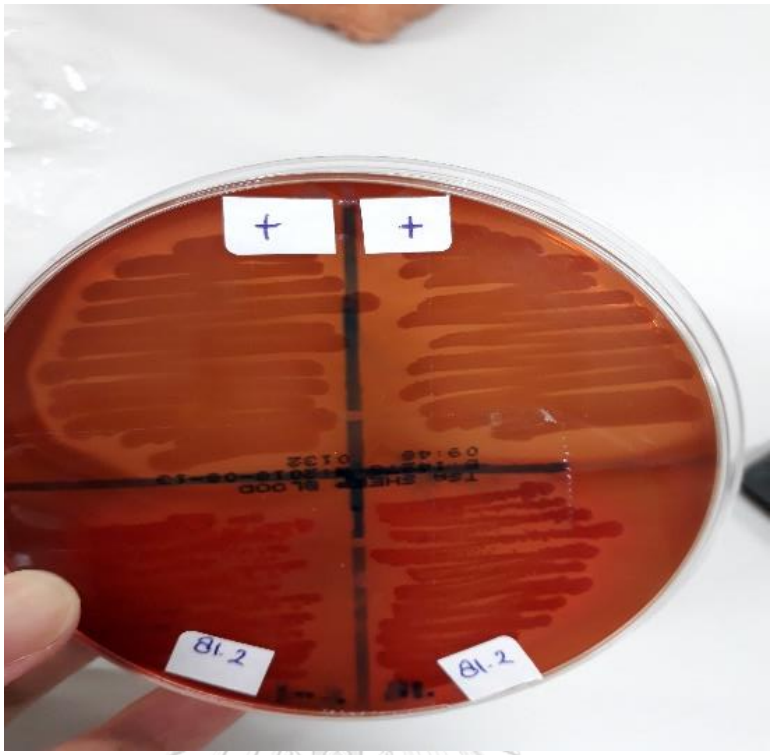


Figure 9. Hemolysin production in blood agar

4.2.3.3 Cytotoxicity testing

In vitro study against Caco2 cell result through REMA method was shown in Appendix C. Basically, REMA method are based on reduction oxidized blue dye into resorufin having pink fluorescent color, by a number of living cell (McGaw et al., 2014). The result obtained from BIOTEC and Department of Medical Sciences indicates that pigment from selected isolate *Bacillus* sp. 81-2 was consider as non-toxic against intestinal model Caco2. This pigment was compared to ellipticine as positive standard which delivered a toxicity against Caco2 at concentration of 20 µg/ml, when it reached IC_{50}. Since in vitro test against intestinal model Caco2-food related showed non-toxic result, the pigment was fulfilled to be incorporated as food grade and safe for food consumption.

Corresponding to overall result of safety evaluation on this study, pink-orange pigment from selected strain *Bacillus* sp. 81-2 having biological activities was safe and potential to be further tested on food matrix.

4.3 Optimization conditions for *Bacillus* pigment production

According to Popova (2017), wavelength position of the main absorption peak of carotenoid group was affected by water content of solvent. Additionally, wavelength position also depended on refractive index of the solvent which defined red-shift of linear polyenes. Prior optimization, λ max of pigment extract in methanolic solution was measured, and then used to record pigment production as shown in material and method section 3.2.4.1. This pigment was obtained from selected strain *Bacillus* 81-2 cultivated in NB with 3% salt and harvested on 96 hours cultivation, which generated pigment yield of 0.18 gram per gram of wet cell. Based on Figure 10, peak recorded were on the wavelength of 467 nm, 481 nm, and 516 nm, with the absorbance of 0.107; 0.112; and 0.085, respectively. Those peaks were close to peak profiles of this isolate previously determined in section 4.2.2 (Table 9). Thus, this method could be used to quantify pigment generated from tested strains during optimization processes.

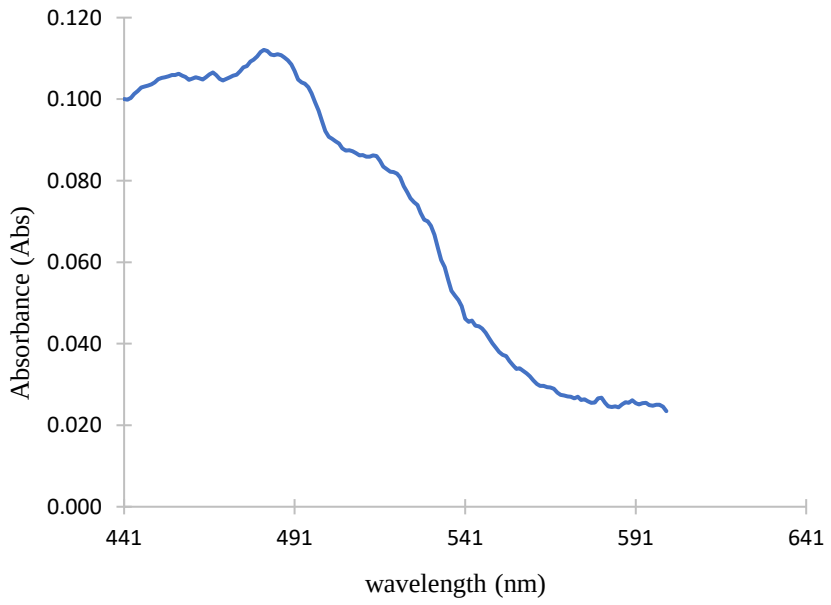


Figure 10. λmax measurement of pigment methanolic extract

4.3.1 Optimal NaCl concentrations

Halophilic microorganisms are microorganisms which require salt for their growth. Salt concentration also associated to cell activities including generation of their specific metabolites. Thus this study also evaluated an effect of salt concentrations on the *Bacillus* 81-2 growth and pigment generation in term

of both quality and quantity. The results as shown in Figure 11 and Figure 12. *Bacillus* 81-2 growth patterns in NB contained salts 1, 3 and 5% as shown in Figure 11 showed the same trend. Logarithmic phase were reached on 48 hours of incubation, followed by late logarithmic phase for culture grown in NB with 3 % and 5% salt, and/or stationary phase for culture grown in NB with 1% salt. The highest optical density was 0.251 (Log 8.4) at late logarithmic phase when cultured in NB with 3% salt. The lowest optical density was observed when bacteria was cultured on NB with 1% salt during 96 hours incubation time. These results indicated that 3% salt concentration could induce bacterial growth rather than the other concentrations. As discussed previously, based on salt tolerance determination, this bacteria was classified as slight halophilic in which their salt requirement for optimum growth was around 3-5%. One percent salt in this case might not sufficient to induce *Bacillus* sp. 81-2 to uptake oxygen or to support metabolism, such as enzyme activation. Furthermore, optimal NaCl was important for halophilic bacteria to protect themselves from changing internal and external osmotic pressure, also from deformation and bacterial lysis (Kirishna et al., 2014).

In determination of pigment production, identical trend on pigment production was shown in bacteria cultured on 3% and 5% salt. Pigment production recorded in 3 wavelengths showed fluctuating result. The pigment generation increased on 24 hours incubation, and gradually reached the highest absorbance on 72 hours of cultivation then the absorbance decreased on the last day of cultivation.

Fluctuating trend of pigment that found in NB with 3% and 5 % salt might associated with bacterial growth. As shown in Figure 11 (3% and 5% salt), *Bacillus* was in the early period of stationary phase on 48 hours of cultivation. According to Maier (2009), during stationary phase the number of cell growth and cell died start to be equivalent, meaning there is an absence of net growth. In the case of this optimization, during early stationary phase, *Bacillus* growth started to be balanced by the death rate. Some died cell containing pigment might be washed away during washing process. Therefore, only pigment from living cell that detected and resulted in lower absorbance. However, during the middle

of stationary phase (72 hours), pigment accumulation occurred, then lead to higher absorbance detection, even reached to the highest absorbance. The drop of pigment absorbance on last day of cultivation was caused by death phase of *Bacillus* in which cell underwent to lyse. Pigment generated by bacteria cultured on medium with 1% salt was lower than others. Again, low pigment generation on NB supplemented with 1 % salt might caused by insufficient salt to support metabolism, as previously stated.

Highest pigment concentration with OD value 1.394 recorded at 481 nm was obtained from the cell harvested from NB added-3% salt at 72 hours incubation. It was denoted from this optimization that wavelength of 481 nm often revealed highest pigment absorbance in every treatment (1-5% salt), especially on 72 hours of incubation. Thus, based on this result, 3% salt was selected for further pigment optimization.

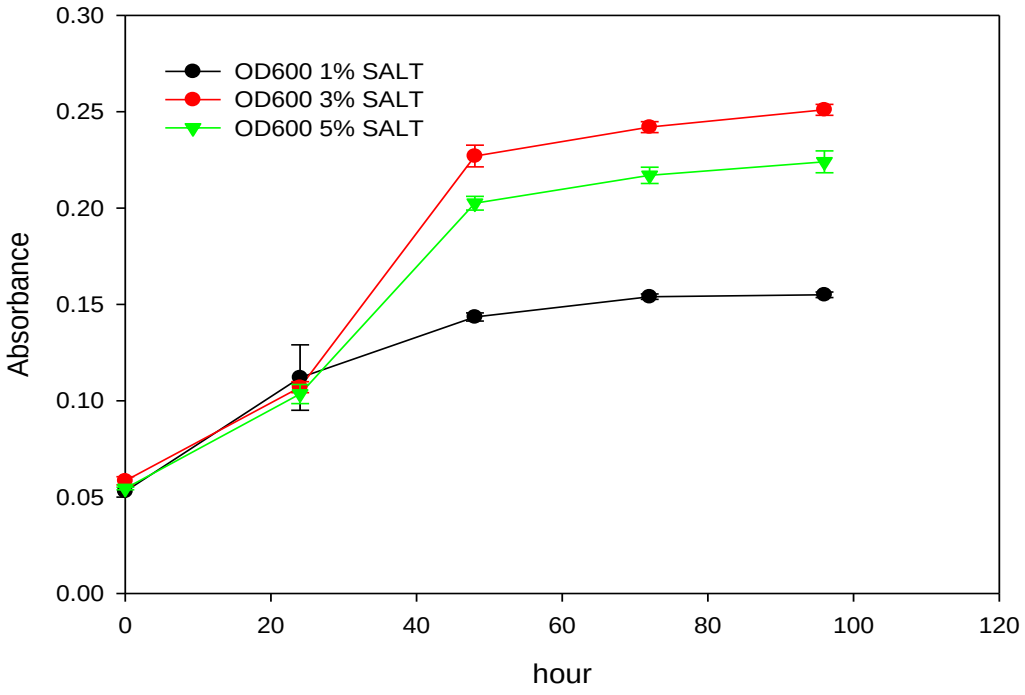
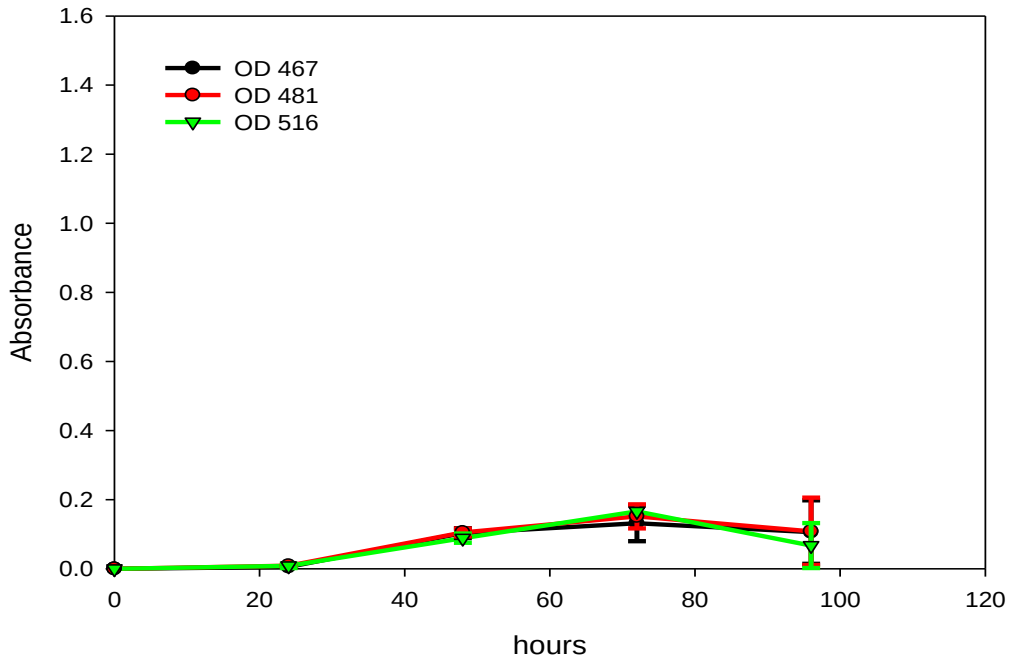
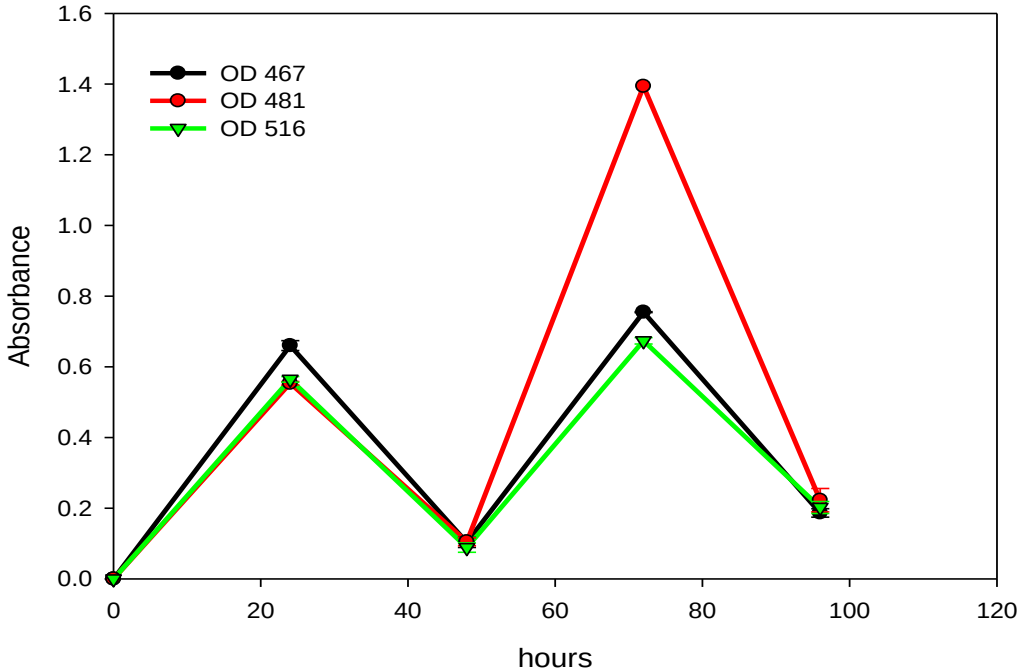


Figure 11. Relationship between cultivation time and bacterial density (OD 600) in NB with 1 % salt; 3%, and 5% salt

a.


OD 467
OD 481
OD 516
Absorbance
hours
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
20
40
60
80
100
120

b.


OD 467
OD 481
OD 516
Absorbance
hours
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
20
40
60
80
100
120

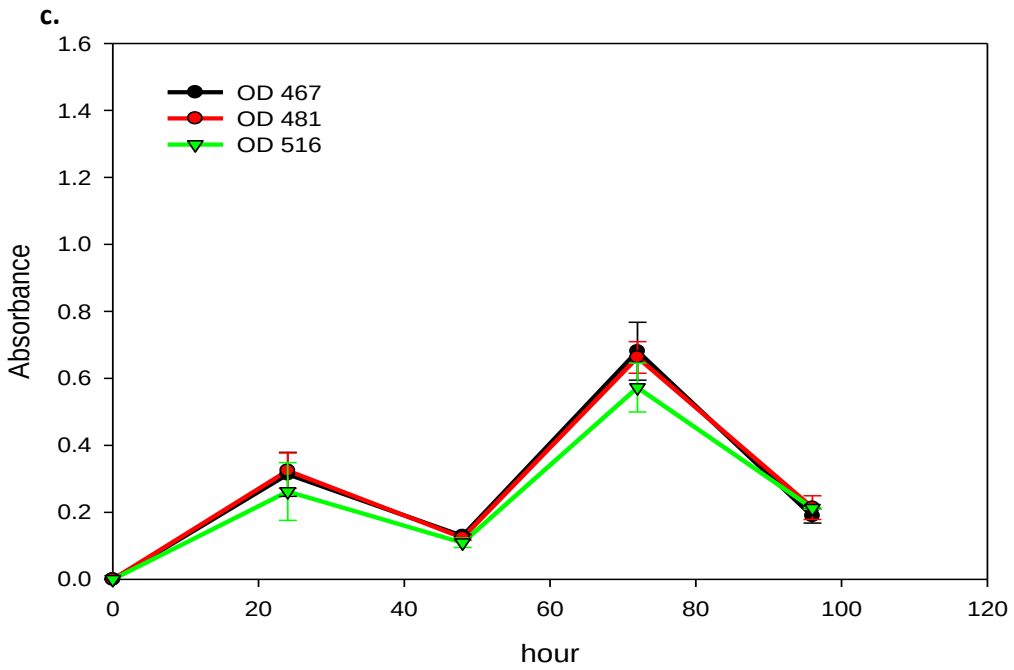


Figure 12. Relationship between cultivation time (h) and pigment (467, 481, 516 nm) in NB supplemented with (a.)1%; (b.)3%; and (c.) 5 % salt

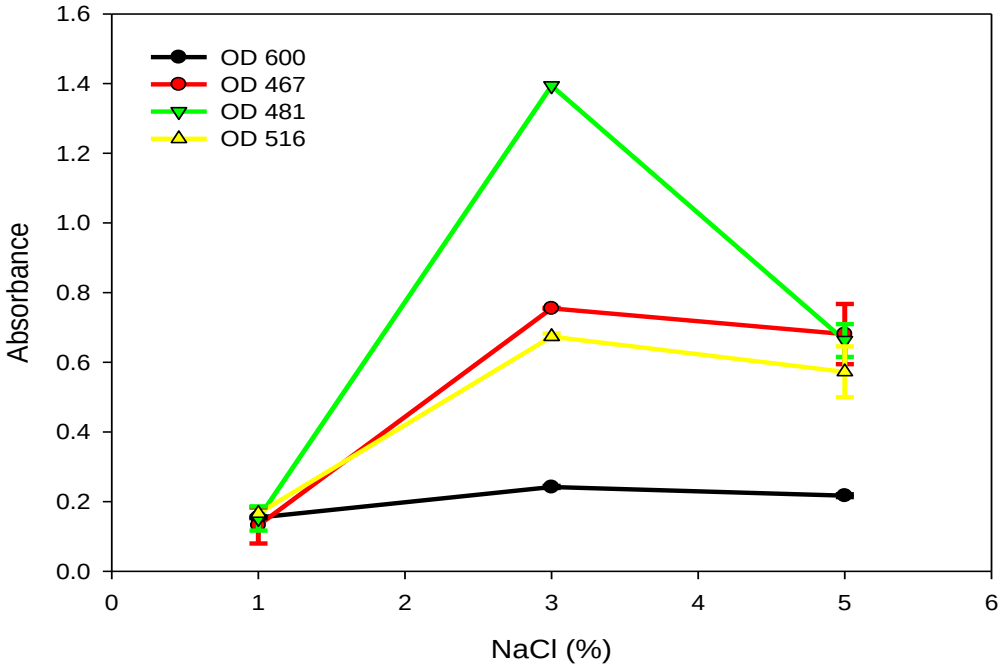


Figure 13. Effect of NaCl concentration on bacterial density (OD 600) and pigment (467, 481, and 516) at 72 hours incubation

4.3.2 Optimal Carbon (C) and Nitrogen (N) sources

In order to obtain higher production of microbial pigments, there are physiological, nutritional and biological factor that play substantial role on it. Those factors not only induce growth kinetic of microbes, but also modulate metabolic processes in which it can promote yield of pigment (Kirishna et al., 2014). As mentioned previously, pigment production also affected by C and N source, depending upon the type of microorganisms also utilization of C and N regarding their metabolism.

According to Singh et al. (2017), there are 2 fundamental things that caused an importance of doing C and N optimization or medium optimization. First, C and N source optimization on production medium were crucial factor prior conducting a larger scale metabolic production. Second, suitable type and concentration of nutritional factor should be provided to supply essential nutrition for bacterial growth in which less supply lead to restriction of cell growth and product formation, such as pigment formation.

There are 3 technique regarding on medium optimization based on Singh et al. (2017), which are removal, supplementation, and replacement technique (Figure 14). Removal technique means there is a removal of a component in medium, whereas supplementation is defined as addition of new composition in medium, such as supplementation of C and N without removal of other component. Both technique are evaluated their effect on secondary metabolic formation after desired incubation period. Third model of optimization is replacement technique or known as medium formulation in which formulated- C and N sources having impact on production of secondary metabolic replace former C and N for further production.

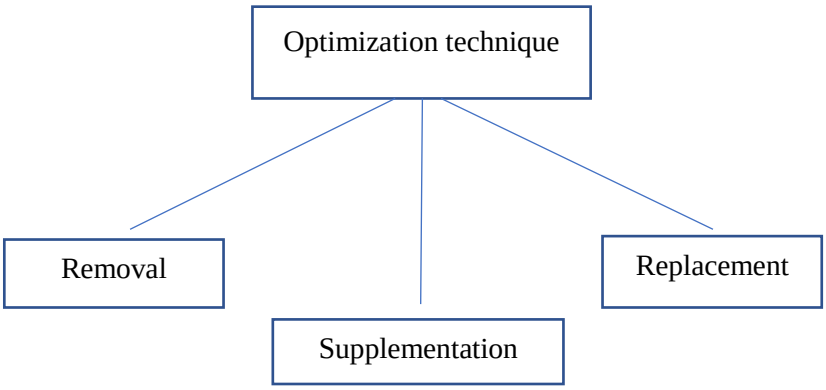


Figure 14. Various technique on medium optimization (Singh et al., 2017)

In this present study, optimization of C and N was performed using supplementation technique. Impact of C source including sucrose and fructose (1%) and N source including ammonium sulfate and ammonium chloride (0.5%) on pigment production of *Bacillus* 81-2 were evaluated. NB supplemented with 3 % salt was used as based medium to assay impact of C in-combination with N sources. The use of sucrose and fructose as C sources was based on several previous study. The use of sucrose could induced pigment production of bacteria isolated from soil (Chatterjee and Bhowal, 2016), while the use of fructose seemed to increase pigment production of *Penicillium* sp. (Pandey et al., 2018). Additionally, glucose and its oligosaccharides serve better nutrition for producing pigment and gives impact on pigment shade (Pisareva and Kujumdzieva, 2014).

The selection of C source for optimization on this study was also supported by a study from Warda et al. (2016) and Tariq et al. (2016) which mentioned ability of various *Bacillus* strain including *Bacillus* isolated from food product to utilize fructose and sucrose. For N selection, ammonium were used since ammonium affected to pigmentation of bacteria (Pisareva and Kujumdzieva, 2014). Supplementation of C was 1%, higher than N concentration (0.5%) since C was the most important energy source, cell growth, also several secondary metabolic generation of bacteria (Singh et al.,

2017). Other than that the use of C sources 2 times higher than nitrogen sources was also reported by Pandey et al. (2018).

According to Figure 15, the highest bacterial density was shown by medium supplemented with sucrose and ammonium chloride (SAC), followed by sucrose and ammonium sulfate (SAS), fructose and ammonium chloride (FAC), and the lowest was fructose with ammonium sulfate (FAS). Sucrose in combination with ammonium chloride seemed to be potential sources that could induce the *Bacillu*s 81-2 to generate more pigment (absorbance = 0.1515 on the wavelength of 481 nm). This observation corresponded to Mazmira et al. (2012) that mentioned the use of various saccharides for *Bacillus thuringiensis*. Among various saccharides, sucrose significantly increased the growth (CFU/ml) with specific growth rate (h^{-1}) than fructose. Mazmira et al. (2012) also demonstrated that the capability of *B. thuringiensis* to grow on disaccharide (sucrose) might caused by secretion of sucrase for degrading disaccharide into monosaccharide. This mechanism could help the bacterial cell to utilize and/or uptake more diverse C sources for its growth and particularly for pigmentation.

Although addition of sucrose and ammonium chloride as C and N source demonstrated an increase of pigment absorbance in this optimization, this value which was 0.1515 (Figure 17) still lower relative to pigment obtained in NB supplemented only 3% salt. It was shown that under that previous condition, 3 wavelength had highest absorbance of pigment on 72 hours of cultivation when 3% salt was added. In detail, 467 nm; 481 nm; and 516 nm were recorded OD value of 0.755, 1.394, and 0.689.

Our result was in contrary with previous study mentioned before. Addition of C and N did not impact both bacterial growth and pigment production (Figure 18). This phenomenon might due to the occurring of carbon catabolite repression (CCR). CCR is a mechanism of bacteria adapting to their environment containing different carbon sources. During the presence of multiple carbon sources, bacteria select sole carbon which they prefer and obstruct the expression of enzyme that catabolize undesired carbon sources (Stülke and Hillen, 1999). In this study, without addition of new formulated C source, NB itself contained peptone and/or yeast extract as C and N source. Supplementation of sucrose or fructose might be

a potential cause of CCR. Due to there was no impact of C and N supplementation, cultivation condition selected for production of pigment from *Bacillus* 81-2 was NB supplemented only with 3% NaCl, cultivated on room temperature (27-28^{0}C) with 150 rpm orbital shaker, and harvested on 72 hours.

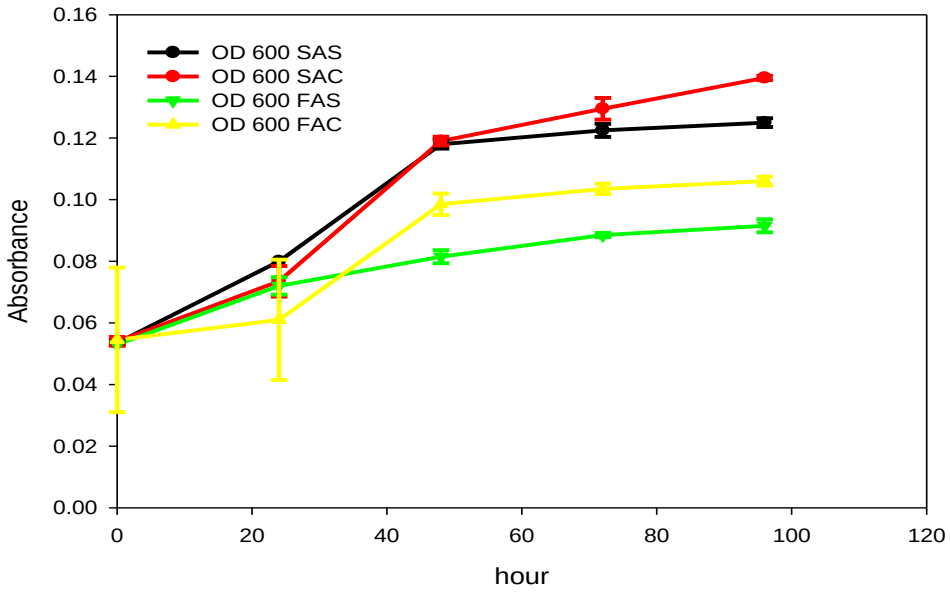
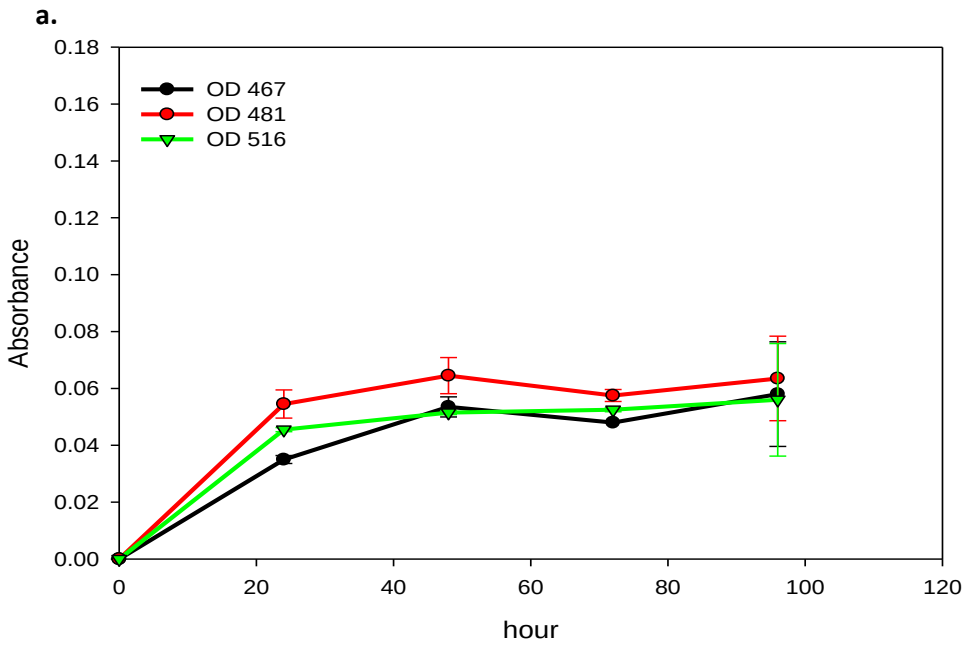
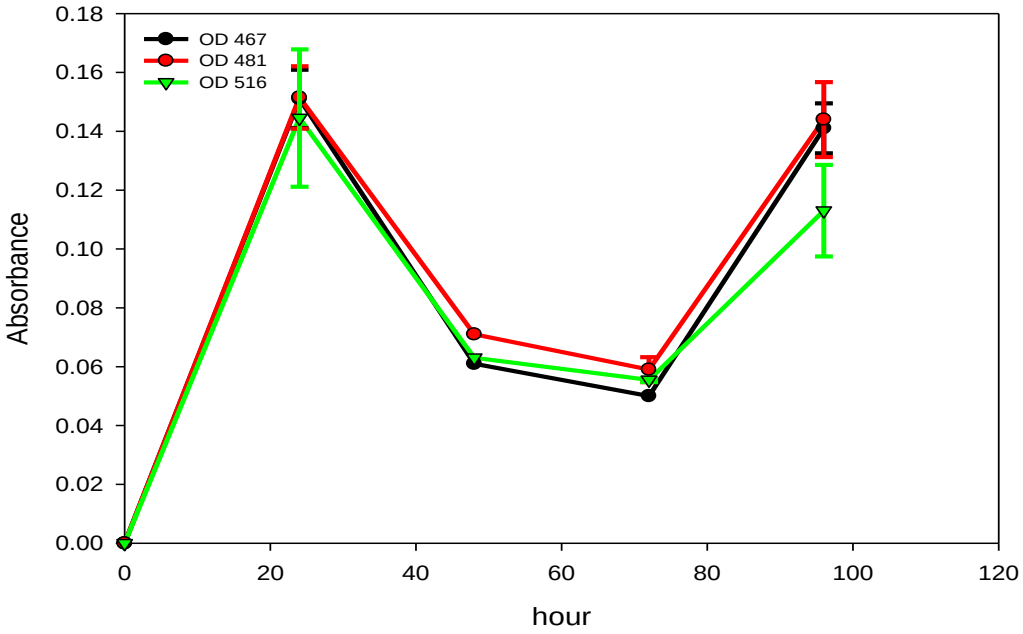


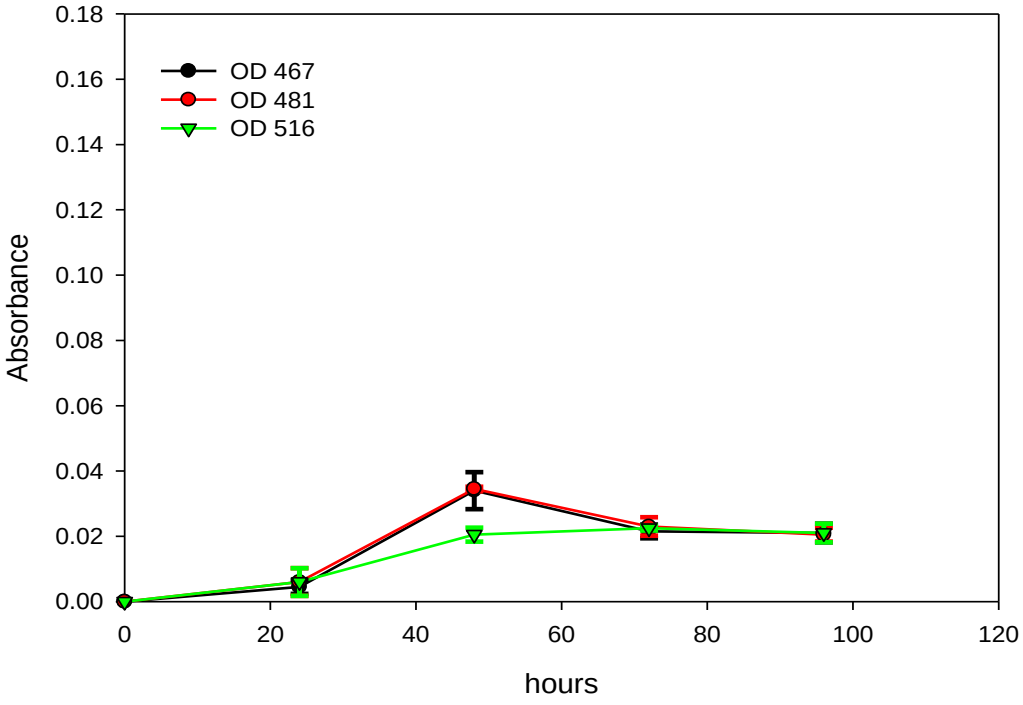
Figure 15. Relationship between cultivation time (h) and bacterial density (OD 600) in NB with SAS; SAC, FAS, and FAC



b.


OD 467
OD 481
OD 516
Absorbance
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
20
40
60
80
100
120
hour

c.


OD 467
OD 481
OD 516
Absorbance
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
20
40
60
80
100
120
hours

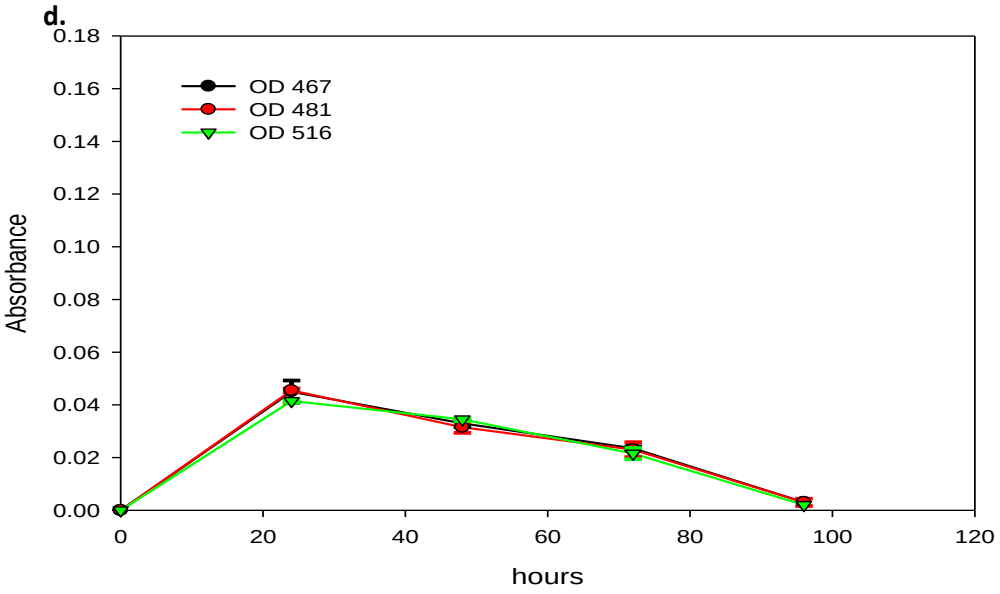


Figure 16. Relationship between cultivation time (h) and pigment (467, 481, 516 nm) in NB supplemented with (a.) SAS, (b.) SAC, (c.) FAS, (d.) FAC

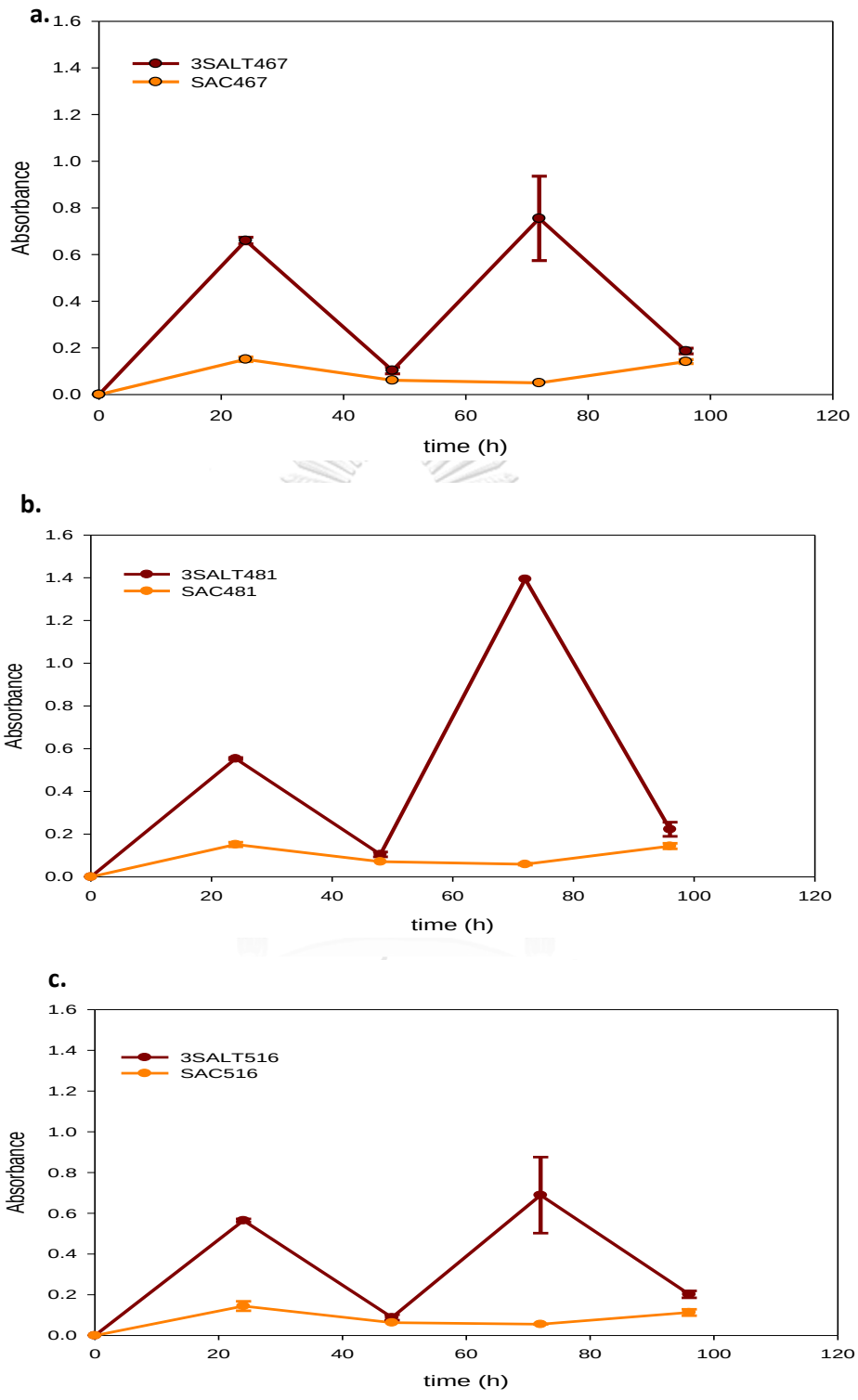


Figure 17. Pigment generation between medium with 3% salt and medium with 3% salt + addition of SAC on wavelength of (a.) 467, (b.) 481, (c.) 516 nm

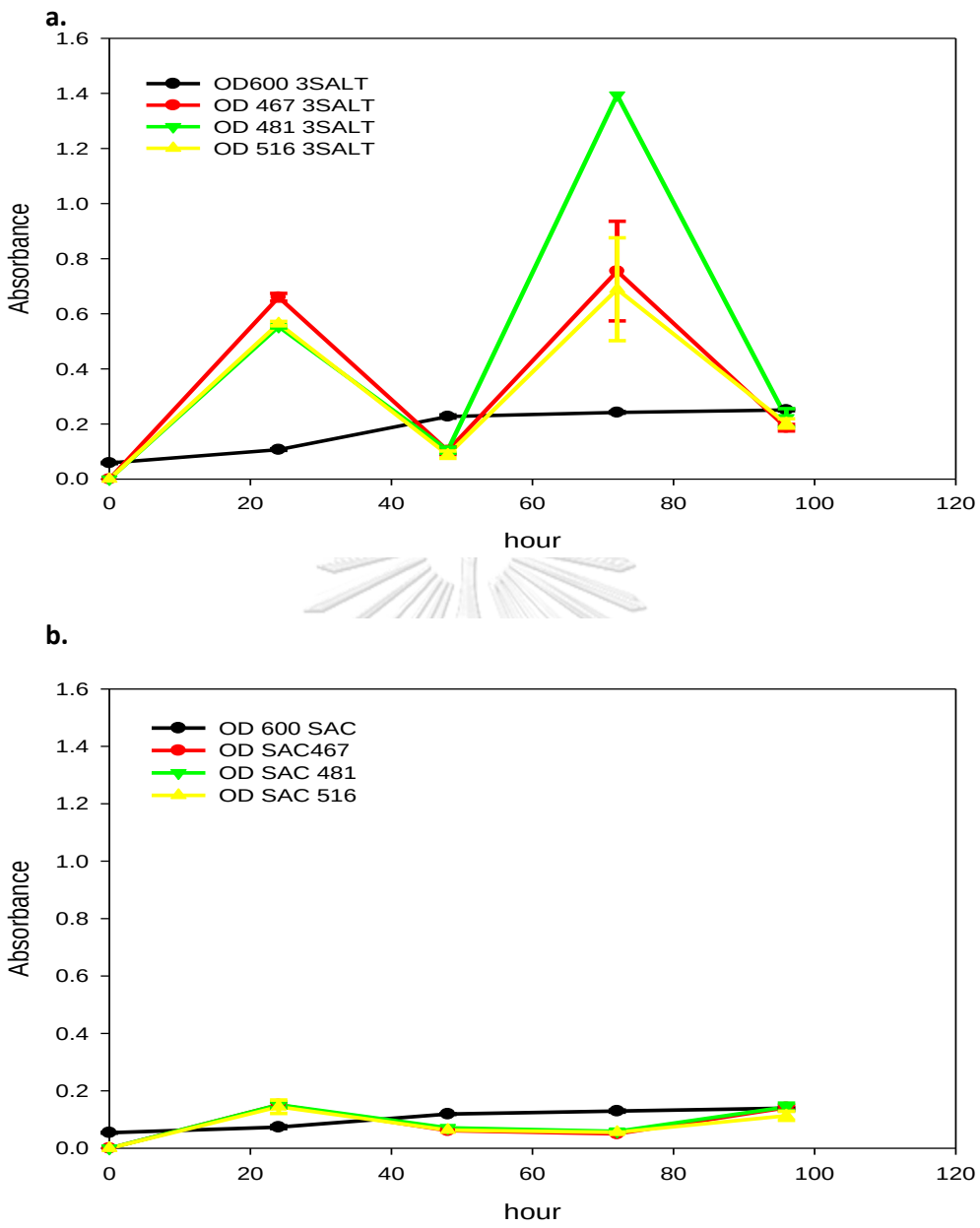


Figure 18. Bacterial density (OD600) and pigment generation (467, 481, 516) of (a.) NB with 3% salt and (b.) NB 3% salt with addition of SAC

4.4 *Bacillus* pigment as coloring agent tested in food matrix

Bacillus pigment was produced under optimum cultivation condition. Amount of 0.22 g of dried pigment extracts were obtained from 1 g of fresh cell. In order to test in food matrix, MIC and MBC were determined to estimate the amount of pigment to be added. *Staphylococcus aureus* that was most tolerance to the pigments as tested on section 4.2 was selected for this test. MIC was determined by comparison of cell density of *Bacillus* treated with the pigment to control (*Bacillus* without pigment addition). The minimum concentration with lower cell density than control would be assigned as MIC. Thus, based on this determination, the MIC of *Bacillus* sp. 81-2 pigment was found at 100 mg/ml (Table 11). For MBC as shown in Figure 19, this pigment had MBC at 200 mg/ml. MBC was considered as the lowest concentration of the pigment required to kill particular bacteria. It could be concluded that concentration of 200 mg/ml was the lowest concentration of this pink-orange pigment to kill particular bacteria.

Table 11. MIC of *Bacillus* sp. 81-2 pigment

Sample	Absorbance
Pigment (200 mg/ml)	0.132
Pigment (100mg /ml)	**0.125**
Pigment (50mg/ml)	0.501
Pigment (25 mg/ml)	0.641
Pigment (12.5 mg/ml)	0.644
Pigment (6.25 mg/ml)	0.651
Control	0.650

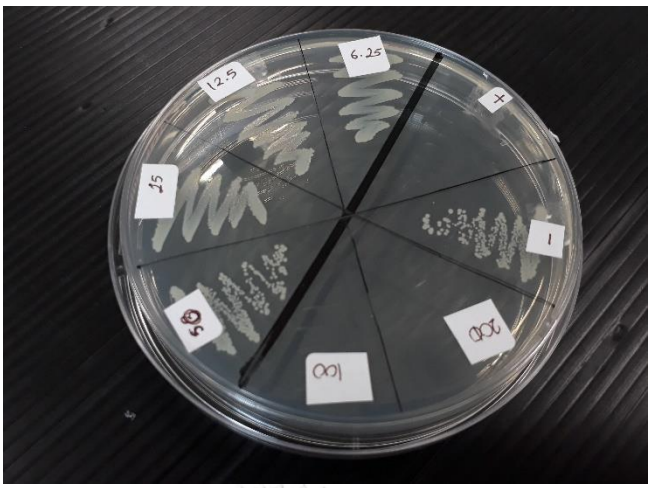


Figure 19. MBC result of *Bacillus* sp. 81-2 pigment extract

Since this carotenoid pigment had lipophilic property and easily dissolved in oil, oily salad dressing was used as food matrix in this test. This oily salad dressing was prepared according to section 3.2.5 and resulted in slight turbid dressing (turbidity= +). In order to further test in food matrix, control salad dressing without addition of pigment was prepared along with sample salad dressing treated with pink-orange crude pigment extract. Crude pigment extract was added in the amount of 200 mg/ml, following the MBC. The color of salad and microbiological properties were monitored in every 2 days during storage for 6 days. The results as shown in Table 12 and 13, also Figure 20.

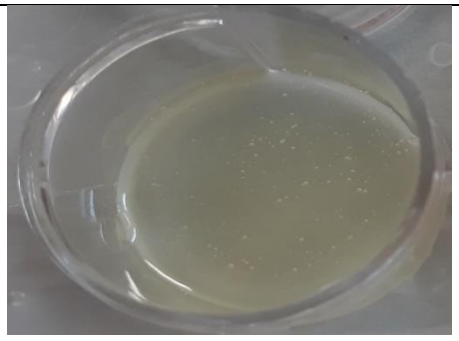
Control	Sample (salad + pigment)
	

Figure 20. Color property of control: salad without pigment and sample : salad + pigment

Salad dressing with, and without pigment addition were shown in Figure 20 Based on visual observation, the pigment changed the salad dressing to have intense

pink-orange color. For further color determination, salad dressing with pigment was subjected to colorimeter measurement. Through this determination, there are 3 three terms including L* value for indication of lightness and darkness differentiation, a* for redder or greener differentiation, and b* for yellower and bluer differentiation. Based on these 3 values, the salad with pigment had close to lighter (+L*), red (+a*), and blue (-b*).

Table 12. Observation of salad color property during 6 days

Day of observation	L*	a*	b*
0	56.330 ± 2.679^{a}	11.810 ± 1.410^{b}	-12.883 ± 2.024^{a}
2	55.707 ± 0.679^{a}	11.383 ± 0.467^{b}	-12.097 ± 1.207^{a}
4	62.387 ± 0.376^{b}	9.527 ± 0.626^{a}	-8.917 ± 1.192^{b}
6	64.517 ± 0.248^{b}	10.270 ± 0.678^{ab}	-8.407 ± 0.115^{b}

note:different superscripts indicate significant difference ($p<0.05$)

Generally, results obtained from color measurement in this pink-orange salad dressing were close to color of surimi after addition of 100 mg/kg-200 mg/kg bacterioruberin, a carotenoid produced from *Halobacterium salinarum* (Yachai, 2009). Bacterioruberin addition in study conducted by Yachai (2009) changed surimi to have pink color with L*, a*,b * ranging from 51.2-67.6, 15-7-31.7, 15-7-23.5, respectively. Only b* value of pink-orange salad dressing in this present study that showed slight different, since b* value of surimi reached to 157-23.5 which meant its yellow color was more intense.

During 6 days of storage under 5^{0}C, a* values of salad dressing were not significantly changes while L* values significantly changed to be lighter and b* increased. Consistence of a* value found through storage demonstrated a stability of some compounds in this carotenoid pigment. These compounds might have some specific properties for use as more stable coloring agent that are being commercial need relative to the other natural sources. This requires further investigation, particularly characterization of this special feature for valued added applications. The change of L* value to lighter term in this study was similar with L* value changing of surimi-added bacterioruberin after 7 days of storage.

Apart from coloring agent, the multi-bioactivity of this pigment playing as anti-microbial and antioxidant agents in food was also proposed. Thus, the concentration of pigment added to salad dressing was estimated from MBC values to allow active compounds playing inhibitory action on microbes contaminated in food matrix along with giving color as described above. The results as shown in Table 13 , the viable counts of all determinations (TPC, yeasts & mold and *Staphylococcus aureus*) showed below 1000 CFU/ml, excepted TPC of control sample from Day 1, the TPC was 1000 CFU/ml. These results could not demonstrate the inhibitory effect of the pigment on microorganisms in salad dressing matrix. There might be some technical error regarding to some steps of microbial determination. For instance, microbial present in oil matrix might not disperse during serially diluted in diluent water and/or might be bound in oil that could not expose to agar surface to grow. Furthermore, during salad dressing making process, food preservative such as antimicrobial agent usually added to extend shelf-life by prevent spoilage caused by pathogen, including *S. aureus.* Some examples of food preservative added on salad dressing are vinegar, salt, and sorbic acid (Inetianbor et al., 2015). The presence of one of those compounds might protect salad dressing so that there were no microorganism detected on microbial count during 6 days storage.

Based on visual observation, it was found that the control salad dressing turned turbid during days 4 and 6 of storage while sample with pigment was still clear (Figure 21). This could reflect that the turbidity as observed in control might be generated from either oxidation or microbial activity. According to Yachai (2009), color change of product during storage was one of result from protein decomposition. This phenomenon could demonstrate the action of the pigment that might either play inhibitory effect on oxidation reaction or microbes in the salad matrix. However, ability of this pigment to act as antioxidant need to be further studied, for example using thiobarbituric acid reactive subtances (TBARS).

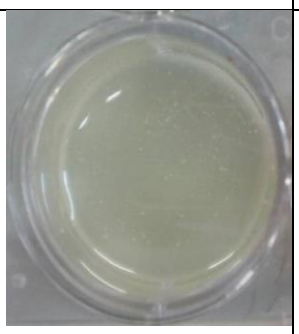
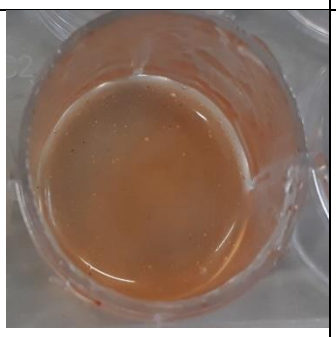
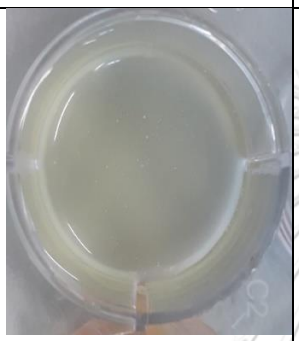
Day	Control	Turbidity	Salad + pigment	Turbidity
2		++		+
4		+++		+
6		+++		+

Figure 21. Observation of control and salad dressing added pigment during 6 days

Table 13. Microbial analysis in salad dressing and control during storage

Day of observation	TPC (CFU/ml)		PDA (CFU/ml)		*S. aureus* (CFU/ml)	
	Control	Sample	Control	Sample	Control	Sample
1	1×10^3	$<10^3$	$<10^3$	$<10^3$	$<10^2$	$<10^2$
2	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^2$	$<10^2$
3	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^2$	$<10^2$
4	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^2$	$<10^2$

Although pink-orange pigment in this study could well-incorporated on salad dressing, its potential regarding multifunctional properties as food colorant having biological properties was less compared to natural colorant on food. The use turmeric (*Curcuma longa*) as natural colorant in food having biological activities has been reported by Gul and Bakht (2015). Addition of 1% turmeric (*Curcuma longa)* on autoclaved chicken-potato based food product resulted in longer shelf-life. There was no detection of fungi and bacteria up to 90 days. Furthermore, corresponding to sensory evaluation on color, flavor, and taste, the autoclaved chicken-potato meal treated with 1% turmeric got the highest score of panelist acceptance.

CHAPTER 5
CONCLUSIONS

5.1. Conclusions

1. Five of pigmented bacteria code number 81-2, 13-3, 77-3, 34-12, and 39.23 were identified as *Bacillus* group. They were *Bacillus* sp. 81-2 with pink-orange pigment, *Bacillus* sp. 13-3 and 77-3 with yellow pigment, *Bacillus vietnamensis* 39-23 having yellow-orange pigment, and *Bacillus amyloliquefaciens* 34-12 with yellow pigment. All of them classified as slight halophilic which showed optimal growth on 3-5% salt. Low antimicrobial activity from 200 mg/ml pigment crude extracts were found against *E. coli, S.* Typhimurium, and *Bacillus cereus.* Additionally, inhibition against *S. aureus* was also shown by pink-orange pigment and yellow pigment of *Bacillus* sp. 81-2 and *B. amyloliquefaciens* 34-12. Antioxidant in this study was prepared as 50 mg/ml pigment crude extract which resulting in weak antioxidant activity.
2. Spectrophotometric and TLC assay showed that all pigments had similar characteristic with carotenoid group. Further analysis on FTIR showed that lycopene was responsible on pink-orange pigmentation, whereas xanthophylls were responsible for yellow color.
3. Optimal condition for pigment production which resulted in highest absorbance was NB medium supplemented with 3 % NaCl. Highest absorbance was recorded on wavelength of 481 nm.
4. Pigment obtained from *Bacillus* sp. 81-2 could play function as coloring since addition 200 mg/ml generated pink-orange color and well-incorporated on salad dressing as matrix. This pink-orange pigment had potential expression of bioactivities as antioxidant and/or antimicrobial in food matrix tested.

5.2 Recommendations

1. Pigment characterization through advance technique, such as HPLC and/or LC-MS should be employed to know the main compound of pigment.
2. Detail study on antioxidant, such as TBARS on food matrix added with pigment should be considered.

3. Optimum condition for pigment production of *Bacillus* sp. 81-2 need to be validated.

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APPENDICS

APPENDIX A (Dashti et al., 2009)

1. Transfer 1000 µl of cell suspension
2. Centrifuge at 10,000 rpm for 15 minutes, transfer cell pellets into sterile micro vials
3. Mix with 500µl lysis buffer (50 mM Tris, 10 Mm EDTA, 2% SDS, pH 8.0)
4. Incubate at 60^0C for 1 hour
5. Add 500 µl of phenol-chloroform (1:1), mixed
6. Centrifuge at 10,000 rpm for 15 minutes
7. Transfer the supernatant, add equal volume of chloroform
8. Centrifuge at 10,000 rpm for 15 minutes
9. Transfer supernatant, add equal volume of isoproponal, then mix
10. Keep in an ice water-bath for 30 minutes
11. Centrifuge at 10,000 rpm for 20 minutes
12. Wash pellet with ice-cold 70% ethanol.
13. Centrifuge at 10,000 rpm for 15 minutes
14. Air dry
15. Dissolve in 50 µl TE (Tris-HCL 10mM pH 7.6, EDTA 1 mM pH 8.0)
16. DNA sample was stored -18^0C.

APPENDIX B

Microbial analysis on salad dressing sample

Salad dressing was prepared in 100 µl, transferred into sterile tube, and diluted with 900 µl normal saline (1:10). After vortexed, serial dilutions of 10^{-2}, 10^{-3} and/or appropriate dilution were prepared for every parameter as follow:

B1. TPC (Maturin and Peeler, 2001)

A volume of 0.1 ml of sample was transferred aseptically into triplicate NA plate and incubated 37^0C for 24 hours.

B2. Yeast & Mold count (Tournas et al., 2001)

A volume of 0.1 ml of sample was transferred aseptically into triplicate potato dextrose agar plate and incubated 28^0C for approximately 120 hours (5 days).

B3. *S. aureus* detection

A volume of 1 ml of sample was transferred aseptically into Compact Dry X-SA, and incubated at 37^0C for 24 hours.

APPENDIX C

BIOTEC
a member of NSTDA

BIOASSAY TEST REPORT

Customer name:	พิชญาภา คณานุรักษ์
Address :	ภาควิชาเทคโนโลยีทางอาหาร คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
Test:	Cytotoxicity against human caucasian colon adenocarcinoma (Caco2) ATCC HTB-37
Method:	Resazurin Microplate assay (REMA)
IC_{50} of positive control:	Ellipticine = 14.11 µg/ml
Reported date (dd/mm/yy):	21/11/2018
Total number of sample:	1

Item	Screening code	Sample code	Final concentration (µg/ml)	Fluorescence unit		% Cyto toxicity	Activity	IC_{50} (µg/ml)
				Average	SD			
	Negative	Cell+DMSO	1% DMSO	23489	1560	0.00	-	-
	Positive1	Ellipticine	40.00	4356	486	81.45	Cytotoxic	
			20.00	6142	542	73.85	Cytotoxic	14.11
			10.00	18804	1608	19.95	Non-cytotoxic	-
			5.00	20804	1732	11.43	Non-cytotoxic	-
			2.50	22506	1295	4.19	Non-cytotoxic	-
			1.25	25304	1741	-7.73	Non-cytotoxic	-
1	VA2091*	pigment 81.2	100.00	29458	2597	-25.41	Non-cytotoxic	-
			50.00	27893	2939	-18.75	Non-cytotoxic	-
			25.00	27687	2015	-17.87	Non-cytotoxic	-
			12.50	29775	2822	-26.76	Non-cytotoxic	-
			6.25	30362	2656	-29.26	Non-cytotoxic	-
			3.13	30894	2663	-31.52	Non-cytotoxic	-

Remark:	* Partially soluble in 100% DMSO	**Interpretation**	
		% Cytotoxicity	**Activity**
		< 50%	Non-cytotoxic
		≥ 50%	Cytotoxic

Assayed by

(Pattiyaa Laksanacharoen)
(21/ 11 /18)

Approved by

(Kannawat Danwisetkanjana)
(22/ 11 /18)

National Center for Genetic Engineering and Biotechnology (BIOTEC) 113 Phaholyothin Road, Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand
Tel. 02-5646629, Email: bioassayservice@biotec.or.th, www.biotec.or.th/bioassay

APPENDIX D

Cultural media preparation

D1 Nutrient agar (NA)

Dissolve 28 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize at 15lbs pressure for 15 minutes. Cool to 45-50^{0}C. Mix well and pour into sterile petri plates.

D2 Nutrient broth (NB)

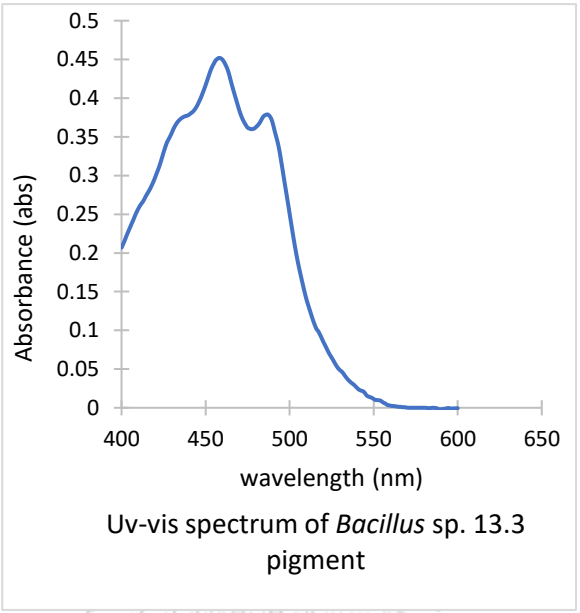
Dissolve 13 grams into 1000 ml distilled water. Mixed thoroughly without heat. Sterilize at 15lbs pressure for 15 minutes.

D3 Potato dextrose agar (PDA)

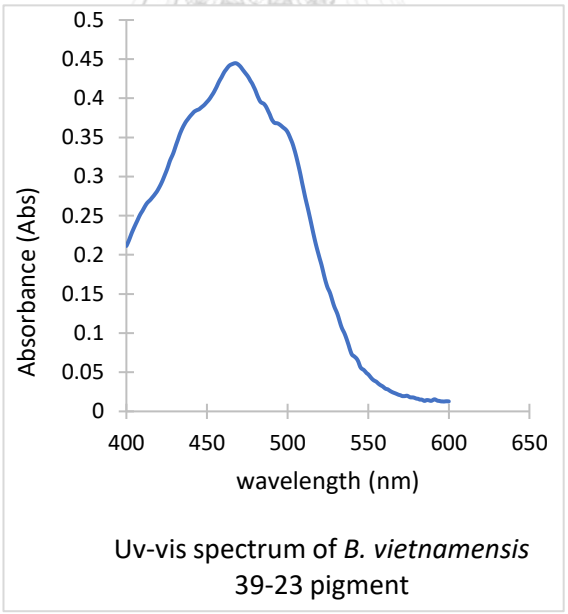
Suspend 39 grams into 1000 ml distilled water. Heat to boiling with agitation to completely dissolve. Sterilize by autoclaving at 121°C for 15 minutes.

APPENDIX E

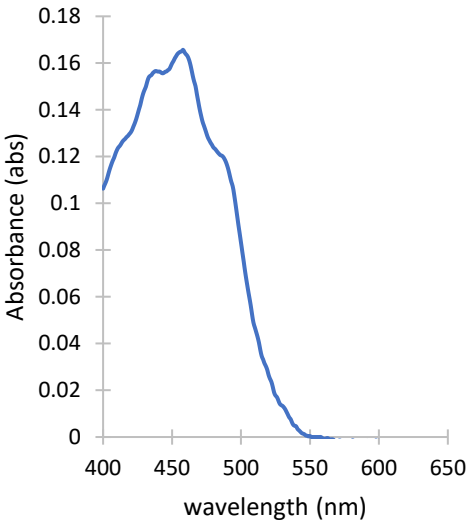
UV-vis spectrum of *Bacillus*



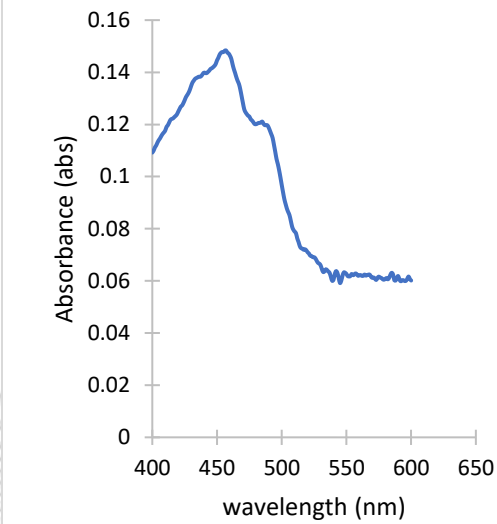
Uv-vis spectrum of *Bacillus* sp. 13.3 pigment



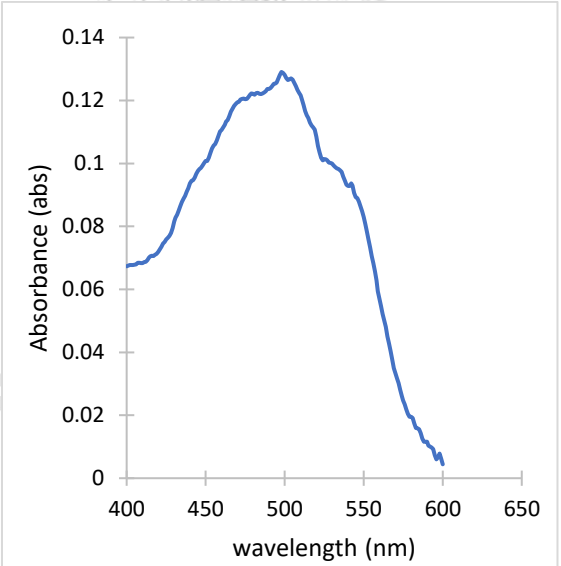
Uv-vis spectrum of *B. vietnamensis* 39-23 pigment



Uv-vis spectrum of *Bacillus* sp. 77.3



UV-vis spectrum of
B. amyloliquefaciens 34.12



UV-vis spectrum of *Bacillus* sp. 81-2
pigment

APPENDIX F

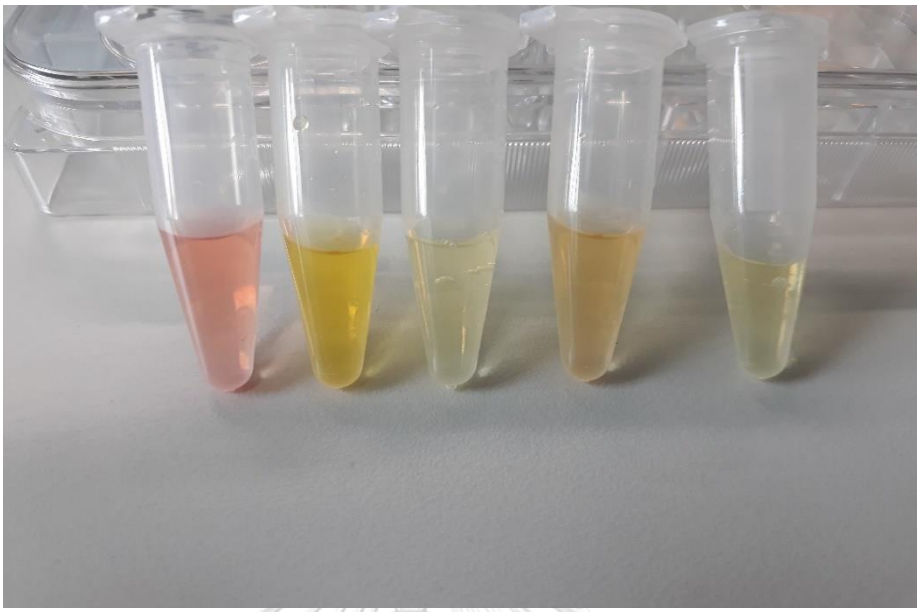


Figure F1. Pigment crude extract on DMSO solution.
From left to right: pigment from *Bacillus amyloliquefaciens* 34-12, *Bacillus vietnamensis* 39-23, *Bacillus* sp. 77-3, *Bacillus* sp. 13-3, and *Bacillus* sp. 81-2

APPENDIX G

Standard curve of bacterial population

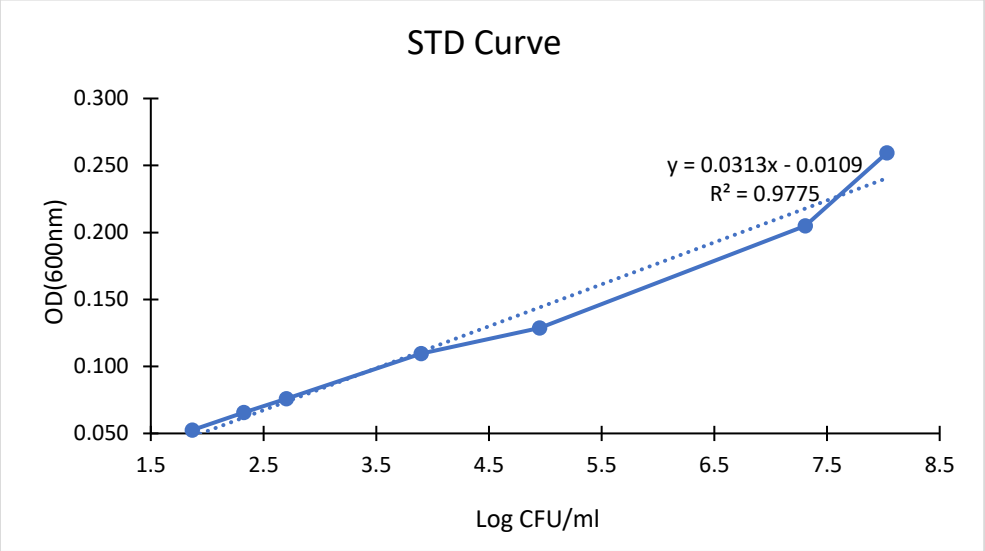


Figure G1. Relationship between bacterial population (log 10 CFU/ml) and absorbance value (OD_{600})

VITA

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AWARD RECEIVED	-