การพัฒนาแบคทีเรียสูตรน้ำของ *Exiguobacterium* sp. AO-11 เพื่อการบำบัดทางชีวภาพน้ำทะเลที่ปนเปื้อนน้ำมันดิบ

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บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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DEVELOPMENT OF LIQUID BACTERIAL FORMULATION OF
Exiguobacterium sp. AO-11
FOR BIOREMEDIATION OF CRUDE OIL CONTAMINATED SEAWATER

Mr. Sysouvanh Boubpha

A Thesis Submitted in Partial Fulfillment of the Requirements
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สีสุวัน บุบผา : การพัฒนาแบคทีเรียสูตรน้ำของ *Exiguobacterium* sp. AO-11 เพื่อการบำบัดทางชีวภาพน้ำทะเลที่ปนเปื้อนน้ำมันดิบ (DEVELOPMENT OF LIQUID BACTERIAL FORMULATION OF *Exiguobacterium* sp. AO-11 FOR BIOREMEDIATION OF CRUDE OIL CONTAMINATED SEAWATER) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.อรฤทัย ภิญญาคง, 137 หน้า.

การบำบัดทางชีวภาพเป็นวิธีที่มีประสิทธิภาพ ต้นทุนต่ำ และเป็นเทคนิคที่เป็นมิตรต่อสิ่งแวดล้อม ซึ่งเหมาะสำหรับการบำบัดสิ่งแวดล้อมที่ปนเปื้อนน้ำมันดิบ งานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาแบคทีเรียพร้อมใช้สูตรน้ำของแบคทีเรียย่อยสลายปิโตรเลียมไฮโดรคาร์บอน*Exiguobacterium* sp. AO-11 ซึ่งคัดแยกจากดินตะกอนทะเล เพื่อบำบัดทางชีวภาพในน้ำทะเลที่ปนเปื้อนน้ำมันดิบ ผลการทดลองพบว่าแบคทีเรียสายพันธุ์ AO-11 มีประสิทธิภาพในการย่อยสลายน้ำมันดิบในสภาวะต่างๆ โดยสามารถย่อยสลายน้ำมันดิบได้ในสภาวะที่มีการแปรผันค่าความเป็นกรดด่างที่ 6-9 ที่ความเค็ม 8-45.4 ppt ที่อุณหภูมิในช่วง 25-37°C และความเข้มข้นของน้ำมันดิบ 0.25-1.5% (v/v) จากนั้นได้ใช้วัสดุเหลือใช้จากอุตสาหกรรมและน้ำทะเล ที่ความเข้มข้นแตกต่างกันเพื่อลดค่าใช้จ่ายในการผลิตแบคทีเรียสูตรน้ำ โดยพบว่ากากตะกอนน้ำทิ้งจากโรงงานน้ำมันถั่วเหลือง 15% (w/v) SB ในน้ำทะเลที่เจือจาง 1:4 เป็นสารตั้งต้นที่เหมาะสมในการผลิตอาหารเลี้ยงเชื้อเพื่อเพิ่มมวลชีวภาพของแบคทีเรียสายพันธุ์ AO-11 ซึ่งสามารถเพิ่มปริมาณเซลล์แบคทีเรียจาก 6.07±0.06 Log CFU/มล. เป็น 8.6 ± 0.02 log CFU/มล. ในระยะเวลา 9 ชั่วโมง และในการเตรียมแบคทีเรียสูตรน้ำ พบว่าฟอสเฟตบัฟเฟอร์เป็นสารละลายที่เหมาะสมสำหรับการแขวนลอยแบคทีเรีย โดยสามารถรักษาการรอดชีวิตของแบคทีเรียสายพันธุ์ AO-11 ได้ถึง 86.7 ± 1.4% ในระยะเวลา 30 วัน และยังรักษาประสิทธิภาพการย่อยสลายน้ำมันดิบในอาหารเหลวไว้ได้มากกว่า 80% นอกจากนี้เพื่อเพิ่มการรอดชีวิตของ AO-11 ในระยะยาว ได้ใช้ PEG 1% เป็นสารปกป้องเซลล์ ซึ่งสามารถรักษาการรอดชีวิตของ AO-11 ได้ถึง 72.4±0.2% และ 58.7±0.7% ที่อุณหภูมิ 4°C และ 30°C ในระยะเวลา 60 วันตามลำดับ โดยมีต้นทุนเบื้องต้นในการผลิตแบคทีเรียสูตรน้ำ 68 บาท/ลิตร ซึ่งแบคทีเรียพร้อมใช้สูตรน้ำ AO-11 ที่ผ่านการเก็บรักษาไว้ 30 วัน มีประสิทธิภาพการย่อยสลายน้ำมันดิบ 0.5% (v/v) ในน้ำทะเลได้ถึง 61 ± 5.2% ภายในระยะเวลา 15 วัน จากผลงานวิจัยนี้แสดงให้เห็นแนวโน้มการใช้วัสดุเหลือใช้จากอุตสาหกรรมการเกษตรในการเพิ่มจำนวนเซลล์สำหรับการเตรียมแบคทีเรียสูตรน้ำ และได้การพัฒนาแบคทีเรียพร้อมใช้สูตรน้ำโดยสามารถรักษาประสิทธิภาพการบำบัดทางชีวภาพในน้ำทะเลที่ปนเปื้อนน้ำมันดิบได้

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SYSOUVANH BOUBPHA: DEVELOPMENT OF LIQUID BACTERIAL FORMULATION OF *Exiguobacterium* sp. AO-11 FOR BIOREMEDIATION OF CRUDE OIL CONTAMINATED SEAWATER. ADVISOR: ASSOC. PROF. ONRUTHAI PINYAKONG, Ph.D., 137 pp.

Bioremediation is an effective, low cost and environmental friendly technique to clean up crude oil-contaminated environment. This study aimed to develop the ready to use liquid bacterial formulation of petroleum hydrocarbon-degrading *Exiguobacterium* sp. AO-11 which was isolated from marine sediment, for bioremediation of crude oil contaminated-seawater. Strain AO-11 was evaluated for the degradation of crude oil in various environmental conditions. The results revealed that this strain could degrade crude oil in various environmental conditions including pH 6 to 9, salinity 8 to 45.4 ppt, temperature 25 to 37°C and crude oil concentration 0.25 to 1.5% (v/v). In order to reduce the cost of bacterial formulation, agro-industrial byproducts, soybean oil mill dry sludge 15% (w/v) (SB) in 1:4 diluted seawater based medium was used for bacterial cultivation and it could enhance bacterial cell growth from 6.07±0.06 Log CFU/ml up to 8.6±0.02 Log CFU/ml in 9 hours. For preparation of liquid formulation, phosphate buffer was selected as a suitable suspension solution which could preserve AO-11 survival at 86.7±1.4% in 30 days and maintain crude oil degradation in liquid cultivation at more than 80%. Protective agent (PEG 1%) was selected for prolonged storage and it could preserve AO-11 up to 72.4±0.2% and 58.7±0.7% at 4°C and 30°C in 60 days, respectively. The preliminary estimation of the costs of AO-11 liquid formulation was at 68 baht/L. Moreover, 30-day stored AO-11 formulation could degrade 0.5% (v/v) crude oil in seawater up to 61±5.2%. This study revealed the potential of using agro-industrial waste based cultivation medium for bacterial cell production. Furthermore, liquid bacterial formulation was developed and it has potential to be used for bioremediation of crude oil-contaminated seawater.

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Field of Study:	Microbiology and Microbial Technology	Advisor's Signature	....
Academic Year:	2016		

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LIST OF ABBREVIATIONS

H	Hour
min	Minute
CFU	Colony forming unit
L	Liter
ml	Milliliter
g	Gram
mg	milligram
ppm	Part per million
ppt	Part per thousand
v	Volume
w	Weight
SB	Soybean oil mill dry sludge
CM	Coconut meal (milk) residue
LB	Luria-Bertan

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CHAPTER I

INTRODUCTION

1.1 Statement of problem

Petroleum is naturally found in marine environment on the subsurface reservoirs and other underground formations. The initial substance of petroleum is normally known as crude oil and it is used to refine to other petroleum products. Crude oil is a complex composition of aliphatic and aromatic hydrocarbons, including volatile components of gasoline, lubricating oil, kerosene petrol and solid asphaltene residues (Agarry and Ogunleye, 2012). As the increasing tendency of global energy demand leads to increase the offshore drilling dependency for extracting petroleum hydrocarbon including oil and natural gas in areas such as the marine shelf, along with regions in deep water (Skogdalen et al., 2011). Furthermore, the assurance for consistent delivery of petroleum through vessels and pipelines is critical transportation due to these are economically routes. Therefore, oil extraction, oil field installations, refining, transportation, liquid fuel distribution, utilization of petroleum, storage devices and illegal drillings in pipelines can be significant effects related to accidental discharge of oil into soil and marine environments in developed and developing countries (Auta et al., 2014).

Petroleum hydrocarbons are persistent pollutants which have recalcitrant nature to biodegradation, bioaccumulation in the environment and immense health effects associated with its exposure lead to long term harmful effect on the living organisms (Kumar et al., 2014). The oil contamination causes negative impacts on human health as well as industries, including tourism and fisheries. There are new technologies in locating, extracting and exporting oil by reducing the zone of seafloor disturbance, altering drilling fluids with mineral oils and synthetic fluids and introducing double hulled vessels, which have reduced environmental impacts (Ball et al., 2012). However, the risk of oil spills in marine ecosystems is still widespread around the world. The Gulf of Thailand is one of the major routes for transportation in this area

which has long been under threat from oil spills such as in 2013 around 50,000 liters of oil spilled into the sea caused largely effect to Ao Phrao beach, Samet island (Johanson, 2013). Thus, effective remediation treatments are needed to clean up this pollutant. Remediation technologies should be simplicity in application and be economic to source, to ensure their application in a global context (Simons et al., 2012). After an oil spill, conventional methods, physical and chemical techniques are normally conducted for removing oil from contaminated areas. However, these methods cannot completely remove the oil from contaminated sites and can be potentially toxic to treated ecosystem (Nikolopoulou and Kalogerakis, 2011; Sheppard et al., 2014; Zahed et al., 2011)

Bioremediation is microbial utilizing technique used to degrade or transform contaminants to less toxic or nontoxic compounds (Ghaly et al., 2013). This method was considered to be beneficial over physical and chemical treatments to clean up petroleum hydrocarbon contaminated sites because of its cost effectiveness along with environmental friendly nature (Wang et al., 2012). Bacteria, yeast, fungi and plant can be used in bioremediation to remove crude oil from contaminated area. Many studies have reported the discovery of crude oil degrading bacteria isolated from environment such as *Pseudomonas putida* (Vinothini et al., 2015), *Agromyces* sp. (Navarre, 2014), *Sphingopyxis* sp. (Amini et al., 2015). The success of bioremediation technologies applied to hydrocarbon-polluted environments highly depends on the biodegrading capabilities of native microbial populations or exogenous microorganisms used as inoculants (Venosa and Zhu, 2003).

Bioaugmentation is a technique in which microbes with the desired qualities are added exogenously in a remediation processes (Auta et al., 2014). The suitable microbes for bioaugmentation must exhibit fast growth, be easy to culture, resistant to high concentration of pollutants and can survive in the environment of the remediation area (Cunliffe et al., 2006; Mrozik and Piotrowska-Seget, 2010). The preparation of microbial inoculant for the bioremediation of crude oil contaminated areas by bacterial consortium is more difficult than single strain. Therefore, pure culture bacterium is appropriate for easy and inexpensive preparation.

Exiguobacterium sp. AO-11 may be suitable for these criterions due to it has capability to degrade more than 90% of crude oil from initial concentration 0.25% (v/v) in 10 days. It also contains genes which encode for alkane degrading enzymes such as *alkM*, $alkB_1$, *alkB-1* and CYP 153 (Srisuwankarn, 2015). This strain was isolated from crude oil-contaminated sediment of Ao Phrao beach, Samet Island, Rayong province, Thailand by Srisuwankarn (2015).

There are many techniques that can be used to preserve interested bacteria in ready to use formulation. These methods can be grouped into 2 major types, solid and liquid formulations (Mishra and Arora, 2016).

Liquid formulation is an interesting approach in order to storage bacteria as ready to use form in the case of pollutant contamination due to high efficacy and cost effective. This technique can preserve commercial inoculums prior to use in various applications such as bioaugmentation. Liquid formulation of bacteria, especially single strain bacteria, has advantages over other storage techniques in which it is easy to prepare and apply while also being cost-effective (Nopcharoenkul et al., 2011). The preparation of liquid formulations is performed by increasing bacterial cell density follow by suspending bacterial cells in an appropriate solution. Some protective agents such as glutamate, sorbitol, glucose, lactose, trehalose, glycerol polyvinyl alcohol, gum arabic, polyethylene glycol (PEG), sucrose and carboxymethyl cellulose have been added into liquid bacterial formulation as mediation to prolong cell storage (Jha and Saraf, 2012; Liu et al., 2009; Nita et al., 2012; Nopcharoenkul et al., 2011).

A good liquid formulation should maintain high bacterial survival and retain the effectiveness of biological activity after long-term storage (Nopcharoenkul et al., 2011). It has been demonstrated that growing bacterial strain in different media as inoculum pretreatment can affect the survival, metabolic activity and catabolic gene expression of the bioaugmented bacteria in contaminated site (Cunliffe et al., 2006). Despite the high concentration of initial cells, survival of bacteria and production cost are the critical points for making ready to use bacteria. To decrease the price while increase the bacterial cells, chemical and agro-media have been used as substrates for cultivation medium to enhance bacterial growth for high density (Poopathi and Archana, 2012).

Coconut milk residue and soybean oil mill effluent are agro-industrial byproducts in food processing in Thailand. These wastes have been reported from early studies that could be used as substrate for carbon and energy sources in cultivation media in order to grow bacteria (Kanmani et al., 2015; Wichaidit, 2014; Poopathi et al., 2013). In this case, the optimized agro-industrial wastes are interested as substrates for bacterial cultivation media in order to reduce cost. Thailand is predominantly an agricultural country which may support to good opportunity for utilizing them as reusable organic matters along with reducing wastes.

In addition, suitable preservation conditions for the long-term storage of bacterial cells which provide high survival while maintaining the biodegrading activity against hazardous compounds should be concerned (Nopcharoenkul et al., 2011). While, the preservation condition may depend on type of bacteria and protective agent as shown in the previous research (Nita et al., 2012). Moreover, degradation of crude oil contaminated seawater samples should be monitored to confirm the efficacy of bacterial formulation.

Therefore, this study has developed low cost and high efficient ready to use liquid bacterial formulation of *Exiguobacterium* sp. AO-11 on bioremediation of crude oil contaminated seawater.

1.2 Objectives

The main objectives of this research are to develop the ready to use liquid bacterial formulation of *Exiguobacterium* sp. AO-11 on bioremediation of crude oil contaminated seawater. Subordinate objectives are listed as follows:

1 To evaluate the capability of strain AO-11 for crude oil degradation on various environmental conditions and degradation of specific petroleum hydrocarbon compounds.

2. To select low cost substrate for bacterial cultivation of liquid bacterial formulation preparation.

3. To develop of liquid bacterial formulation for crude oil biodegradation.

4. To determine capability of liquid bacterial formulation on biodegradation of crude oil contaminated seawater.

1.3 The benefit of the study

Low cost and easy preparation liquid bacterial formulation was developed and it could be used for bioremediation of crude oil contaminated seawater with non-toxic to native sea microorganisms.

CHAPTER II

LTERATURE REVIEW

2.1 Crude oil

Petroleum crude oil is a dark sticky fluid complex mixture compounds contain variety of molecular weight hydrocarbons and other organic substances found below the earth's surface. Crude oil consists of more than 17,000 different chemical components analyzed by ultrahigh resolution mass spectrometry (Marshall and Rodgers 2003). It can be classified into four major fractions: the alkanes, the aromatics, the nitrogen-sulfur-oxygen compounds (NSO) and the asphaltene fraction as shown in Figure 2.1 (Bertrand et al., 2015).

Saturated hydrocarbons are major constituents of petroleum hydrocarbons including alkanes (paraffin) and cycloalkanes (naphthalene) (Margesin and Schinner, 2001). These fractions can be ranged from methane to compounds with carbon chain lengths of 40 or more which occur as straight-chain or branched-chain compounds (Scullion, 2006). Aliphatic compounds ranged from C_5-C_{12} are the most volatile and C_{13}-C_{18} aliphatic ranges are considered as “semi-volatile.”, while aliphatic compounds with greater than 18 carbon atoms are not volatile (Brewer et al., 2013). Aromatic hydrocarbons have one (mono aromatic hydrocarbon) or more aromatic rings (polycyclic aromatic hydrocarbon: PAHs) with or without alkyl substitution(s).

Resins and asphaltenes are nonhydrocarbon polar compounds with complex chemical structures (Harayama et al., 2004). Crude oil also contains elements less than 3% (v/v) such as nitrogen, sulfur and oxygen, and some trace constituents less than1% (v/v), including phosphorus and heavy metals such as vanadium and nickel (Hassanshahian et al., 2013). Light oils normally contain high proportion of saturated and aromatic hydrocarbons, with smaller level of resins and asphaltenes. While heavy oils have lower content of saturated and aromatic hydrocarbons and a higher level of polar chemicals (resins and asphaltenes) (Kaushik, 2015).

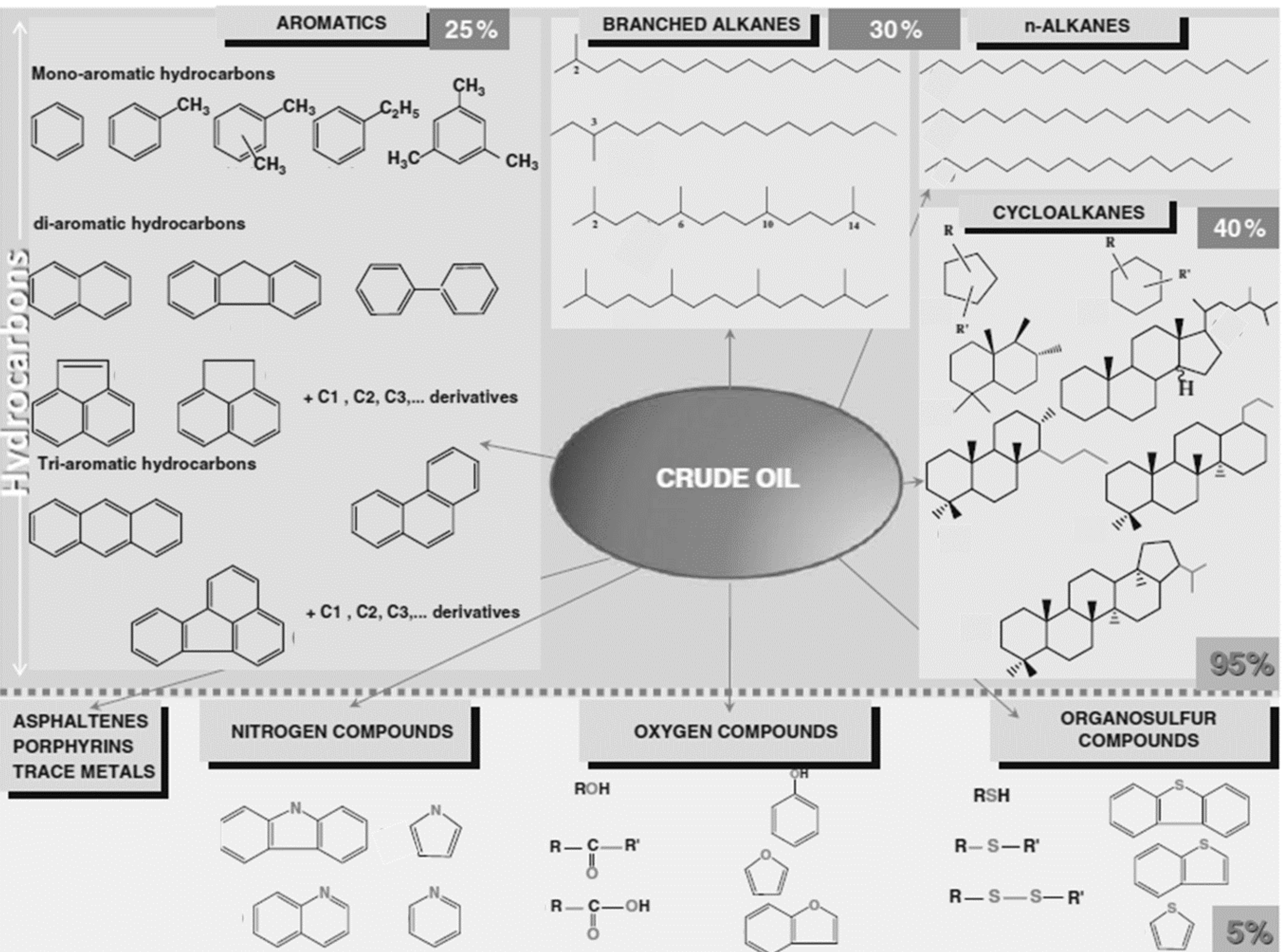

Figure 2. 1 Chemical composition of the crude oil “Arabian light (w/w %) from Persian Gulf (Bertrand et al., 2015).

2.1.2 Crude oil contamination

Petroleum-based products are the main source of energy use in industry and daily life. Crude oil was produced around 3,857,747,231tons in 2014 (OECD, 2014) and it was predicted to increase 1.4% each year from 2015 to 2017. The global crude-oil shipments was reached to 55.3 million barrel per day in 2012 (UNCTAD, 2013). Therefore, it is difficult to prevent oil spill due to human activities cause an accidental or incidental release of liquid petroleum hydrocarbon into marine environment. Around 1.7- 8.8 x 10^6 tons of petroleum hydrocarbons are annually being released to the marine and estuarine ecosystems (McKew et al., 2007). There are 4 main routes of oil spill, consumption, natural seeps, production and transportation as shown in Figure 2.2

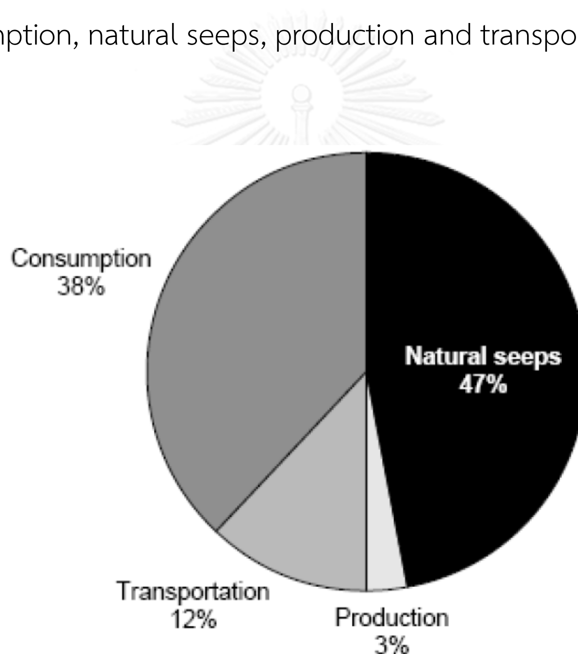

Figure 2. 2 Four main routes of oil spill (Hassanshahian et al., 2013).

2.1.2 History of oil spill

There were many oil spilled accidents in the marine environment since the explosion of oil. Some incidents have been showed as in the Table 2.1

Table 2. 1 Some heavy oil spilled incidents world-wide

Well/ship/company name	Location	Date	Tons	References
Deepwater Horizon	Macondo Prospect, Gulf of Mexico, US	Apr. 2010	686,000	(Ivshina et al., 2015)
Singapore	Singapore	Jan. 2015	4,500	(ITOPF, 2016),
Louis Cristal	Gallipoli coast, Turkey	Jun. 2015	1,400	(ITOPF, 2016)
Hebei Spirit	Taean, Republic of Korea	Dec. 2007	11,000	(ITOPF, 2016)
Prestige	Off Galicia, Spain	Nov. 2002	63,000	(ITOPF, 2016)
Southern Star VII	Shela River, Bangladesh	Dec. 2014	311.5	(Mijanur Rahman and Rakhimov, 2015)
Plains Midstream Canada	Little Buffalo, Alberta, Canada	Apr. 2011	3,920	(Steele, 2011)
North Dakota pipeline	Tioga, North Dakota, US	Sep. 2013	2,810	(Gebrekidan, 2013)
Mid-Valley Pipeline	Mooringsport, Louisiana, US	Oct. 2014	546	(Maykuth, 2014)
Cushing storage terminal	Cushing, Oklahoma, US	May 2013	340	(Sider, 2013)

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2.1.3 Impact of oil spills

Flora and fauna in the topography of surrounding areas which oil spills are the most affected from oil contamination (Kaushik, 2015). Animals may be affected by oil and killed or seriously injured quickly after contact with oil. However, the effects of oil spills are more sensitivity and long lasting. Aquatic animals live close to the shore such as turtle, dolphin seal and walrus, endanger themselves when they consume oil-contaminated prey (Kaushik, 2015).

Birds and aquatic animals normally use kelps and sea grasses as food, shelter, and nesting. Their reproductive cycle and nursing of the young are affected due to,

kelps and sea grasses are destroyed by oil contamination. The loss of their insulating properties is caused by direct physical contact of oil with fur of mammals leading to hypothermia induced death. Feathers also lose their architecture when in contact with oil as well as their insulating properties, which help birds in keeping warm, flying, and floating. The death of the embryo can occur in resulting of oil spills onto the surface of eggs which seals their pores and prevents gaseous exchange.

Aquatic mammals and birds often die by starving due to refuse to eat oil-stained unpleasant-smelling prey (EPA, 1999). The nurseries for fingerlings of fish as coral reefs are often smothered in oil and risk exposure to toxic substances in oil. The exposure of tidal flats, sheltered beaches, salt marshes, and mangrove forests harbor rich biodiversity to oil, gets disturbed, damaged, and destroyed (EPA, 1999).

Oil spills are also effects to human health/activities and industries like fishing, aquaculture, recreational activities and tourism industry. Fishing and shellfish fishing are often strict to prevent catching oil-contaminated fish. Tourism industry and operators of recreational activities are the causes of severe economic losses like scuba diving, angling, and boating. The cooling of nuclear desalination, and power plants are risked to intake of oiled water into their piping and machinery. Moreover, the inhaling or touching oil products and eating of oil contaminated fish and shellfish can cause personnel ill health (ITOPF, 2013).

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2.2 Bioremediation of crude oil contaminated environments

Bioremediation is the method in which microorganisms have been used to degrade the environmental pollutants into less toxic forms. It associated with the use of bacteria and fungi or plants to degrade or detoxify hazardous compounds to human health and the ecosystem (Sonawdekar, 2012). This technique is inexpensive and environmental friendly by using renewable sources.

Bioremediation of crude oil contaminated environments can be enhanced by the two complementary approaches: biostimulation and bioaugmentation.

2.2.1 Biostimulation

Biostimulation is a method used to stimulate the indigenous oil degrading bacteria naturally present within the contaminated site by modifying environmental conditions, addition of growth nutrients and other cosubstrates to the contaminated environment (Kaushik, 2015; Kouzuma and Watanabe, 2011). So, the applying of P and N-based fertilizers such as ammonium phosphate, nitrates, phosphates, and urea, to alleviate nutrient limitation can stimulates the growth of oil degrading bacteria (Kaushik, 2015).

As previous reports, the removal of main petroleum hydrocarbons contaminants was up to 98% after aeration for 3 months by stimulating for reawakening of allochtonous aerobic obligate marine hydrocarbonoclastic bacteria through an in situ oxygenation to degrade the oil, result in decreasing toxicity of sediments after treatment (Genovese et al., 2014). Meanwhile, the addition of inorganic or organic nutrients to contaminated beach sand microcosms could enhance native microorganisms to degrade crude oil more than 90% in the treatments (Nikolopoulou et al., 2013).

2.2.2 Bioaugmentation

Bioaugmentation of crude oil is the application of native or allochthonous or genetically modified desirable microorganisms into the oil spill site or bioreactors in order to enhance the oil biodegradation. The groups of microbes that utilize hydrocarbon as sole carbon and energy sources, are called hydrocarbonoclastic bacteria. Oil degrading bacteria can be used as pure or consortium by liquid, immobilized or pellet formulation. Inoculating strains which are efficient in degrading target pollutants, bioaugmentation could effectively remove oil form contaminated area (Ma et al., 2009). The consortium contains some bacterial species that can degrade toxic compounds better than pure culture due to single species can metabolize only a limited range of hydrocarbon substrates, while consortium contains many different species, with variety of enzymatic capacities for oil degradation (Röling et al., 2002). Ibrahim et al (2013) have conducted experiment by inoculating *Serratia marcescens* for crude oil degradation, it could degrade crude oil up to 90% in 20 days. Previous

research had been used *Alcanivorax borkumensis* strain $SK2^T$ in an oil polluted mesocosm simulation experiment, this strain could degrade 95% *n*-alkanes of crude oil in 20 days while bacterial consortium between $SK2^T$ and *Thalassolituus oleivorans* strain MIL-1T were able to degrade only 70% of crude oil (Hassanshahian et al., 2014a). This consequence may be due to an unfavorable interaction between the two bacterial strains. Moreover, the preparation of bacterial consortium is more difficult and also expensive than the single one. Therefore, the use of single bacterial strain is easy to prepare and be able to degrade crude oil efficiency.

2.2.3 Factors influencing bioremediation of petroleum hydrocarbon

1) Temperature

Temperature is a critical factor to rule the metabolic activity of the degrading microorganisms as well as physical and chemical nature of hydrocarbons (Tyagi et al., 2011). It is important due to at low temperatures, molecules move relatively slowly, and colliding molecules do not always bring about a reaction (Cappello et al., 2007). It is been found that the microbial enzyme activity increases at the mesophilic and thermophilic range of temperatures which helps in increasing the rate of hydrocarbon degradation.

Normally, the most suitable temperature for mesophilic bacteria is 30-40°C and sometimes 60°C for thermophile. At low temperatures the viscosity of oil increases which suppresses the spreading of oil on surface causes difficult degradation. Moreover, there are more variety of organisms in the mesophilic range can be available for degradation. Therefore, mesophilic or thermophilic temperatures are the better option for bioremediation (Obuekwe et al., 2001).

2) pH

pH is influence to microorganisms to be used for the oil degradation in distinct levels. Microbial biodegradation processes can be inhibited by extreme pH condition (Tyagi et al., 2011). The degradation rate of bacteria is decreased with low pH. Naturally, degrading bacteria can habitat in pH 4.0-9.0 (Boszczyk-Maleszak et al., 2006). In contrast, the most suitable pH value for bacterial growth ranged from 6.5-8.0 are certain bacteria that are alkaliphiles found in alkaline lakes at pH 7.5-10 (Vidali, 2001).

3) Salinity

Many bacterial strains are able to grow at salinity comparable in sea water. The salinity concentration is significant factor for hydrocarbon degradation ranged from 0.1-2 M salt, with the maximum 0.4 M which almost equivalent to natural sea water. However, the degradation rate was decreased with higher salinity level (Sonawdekar, 2012).

4) Oxygen

It is one of the fundamental requirements for the biodegradation of bacteria metabolisms. Despite, the use of oxygen concentration depends on type of microorganisms. Oxygen is an important factor for the major pathways in aerobic hydrocarbon degradation on both saturates and aromatic hydrocarbons relates to molecular oxygen or oxygenases (Cappello et al., 2007). The oxygen requirement of aerobic bacteria is stoichiometrically 3.1 mg/ml for the degradation of 1 mg/ml hydrocarbons without taking into consideration the total mass of bacteria. Therefore, varying with increasing or decreasing mass of bacteria may require different oxygen level (Curtis and Lammey, 1998).

5) Nutrients

Nutrients are required to support the biological activity, and hence bioremediation. The requirement of carbon, nitrogen and phosphorous are commonly needed for microorganisms on degradation of hydrocarbons. The limitation of oil degradation is low availability of nitrogen and phosphorus as essential nutrients for microbial growth, while high carbon content of oil. Consequently, the use of inorganic fertilization with N and P can be used to enhance the growth of hydrocarbon-degrading bacteria and hydrocarbon. The C:N:P ratio is maintained as 120:10:1 in the majority of treatments (Sonawdekar, 2012).

6) Chemical composition of petroleum

Petroleum hydrocarbons compose of four different types for degradation: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and resins (pyridines, quinolines, carbazoles, sulfoxides, and amides). In general, the decreasing susceptibility of hydrocarbon biodegradation have been ranked in the

following order: n-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes, with high molecular weight aromatics and polar compounds being extremely recalcitrant (Sonawdekar, 2012).

7) Petroleum concentrations

The concentrations of petroleum are directly affect microbial activity. When too high concentrations of oil may be toxic effects on the present bacteria. Result in slow and longtime biodegradation, it also effects the change of bacterial community. In contrast, bacterial degradation enzymes may be prevented by induction of low contaminant concentration (Adams et al., 2015).

8) Contaminant bioavailability

The major challenge in bioremediation is low bioavailability of recalcitrant hydrocarbons. Bacteria can well degrade in high bioavailability, in contrast biodegradation is decreased in low bioavailability due to bacteria cannot attach to the oil and use it as carbon and energy sources. To overcome the low bioavailability of the pollutants, surfactants are the powerful tool in which it can reduce the interfacial tension, improve the emulsification of hydrophobic pollutants, and increase the solubility of hydrocarbon (Collina et al., 2007).

2.2.4 Crude oil-degrading bacteria

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Hydrocarbon-degrading bacteria have been studied for almost a century, and the most recent list includes almost 200 bacterial, cyanobacterial, algal and fungal genera (Yakimov et al., 2007). Normally bacteria in sediment are much more than other places. It was estimated that around 3.8×10^{30} bacterial cell in the unconsolidated subsurface sediments (Whitman, 1998). Because, as a result of organic matter precipitation as mixed animal and plant, these are essential nutrients for bacteria growth and reproduction. Moreover, sediments also accumulate organic and inorganic pollutant from natural and human activities including oil pills. The influx of oil in a marine site causes population densities of marine hydrocarbon-degrading bacteria increase up to 90% of the total microbial community (Yakimov et al., 2007).

Table 2. 2 Bacteria in crude oil and petroleum product degradation.

Bacteria	Substrate	concentration	Reduction	Incubation (days)	Reference
Acinetobacter sp. LS-1	Crude oil	1%	70.3%	7	(Liu et al., 2014)
Bacillus subtilis	Crude oil	0.2%	76.7%	28	(Al-Wasify and Hamed, 2014)
Pseudomonas aeruginosa	Crude oil	0.2%	77.8%	28	
Acinetobacter lwoffi	Crude oil	0.2%	74.3%	28	
Pseudomonas putida	Crude oil	2%	65%	7	(Vinothini et al., 2015)
Achromobacter sp. HZ01	diesel	2%	95.6%	10	(Deng et al., 2014)
Shewanella haliotis BHA35	Crude oil	2.5%	73.45%	15	(Bayat et al., 2015)
Bacillus methylotrophicus	Crude oil	2%	92%	14	(Chandankere et al., 2014)
Corynebacterium variabile PG-Z	Crude oil	1%	82%	7	(Hassanshahian et al., 2014b)
Sphingomonas paucimobilis	Crude oil	2%	90%	20	(Ibrahim et al., 2013)
Bacillus subtilis YB7	waxy crude oil	2%	80%	10	(Sakthipriya et al., 2015)

Bacteria are the most active agents in petroleum hydrocarbon degradation, and they are primary degraders of crude oil contaminated environments. Several bacteria are known as obligate hydrocarbonoclastic bacteria (OHCB). Many bacterial species could degrade crude oil and petroleum product as shown in Table 2.2. Marine bacteria

25 genera are classified as hydrocarbon degrading bacteria that have the efficacy for petroleum biodegradation ranged from 0.003% to 100% (Das and Chandran, 2011).

2.3 **Crude oil-degrading bacterium *Exiguobacterium* sp. AO-11**

The genus *Exigoubacterium* is a Gram-positive facultative anaerobic bacterium which belong to the low GC (guanine–cytosine content) phyla of Firmicutes. This genus has been found in a wide ranges of environment such as pollutant contaminated sites, Greenland glacial ice, hot springs at Yellowstone National Park, the rhizosphere of plants, and the environment of food processing plants (Pandey and Bhatt, 2015; Vishnivetskaya et al., 2009). The bioremediation capability of bacteria in the genus *Exiguobacterium* have been so far reported including reducing arsenic and Cr [VI] pollutant, neutralizing highly alkaline, pesticide removal, diesel and PAH degradation (Jeswani and Mukherji, 2013; Kulshreshtha et al., 2010; Kumar et al., 2006; Lopez et al., 2005; Mohanty and Mukherji, 2008; Okeke et al., 2007; Pandey and Bhatt, 2015).

Exiguobacterium sp. AO-11 is Gram-positive, rod shaped, non-spore forming bacteria with orange, circular, convex, entire margin colony as shown in Figure 2.3.

This strain was isolated from crude oil-contaminated sediment of Ao Phrao beach, Samet Island, Rayong province, Thailand by Srisuwankarn (2015). The most similar bacterial species based on 16S rRNA gene sequencing comparison to GenBank database is *Exiguobacterium indicum* with 99% similarity. It could degrade up to 84% (v/v) of crude oil from initial concentration 0.25% (v/v) in 10 days. Strain AO-11 contains many genes which encode for alkane degrading enzymes such as *alkM* (C_{12}-), *alkB*$_1$ (nonspecific), *alkB-1* (C_{13}-C_{23}) and CYP 153 (C_8-C_{16}). Therefore, this strain is interested for application in crude oil degradation and it was used in this experiment.

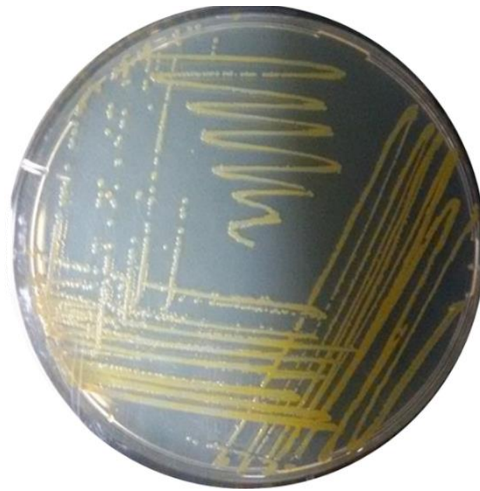

Figure 2. 3 *Exiguobacterium* sp. AO-11 on Luria-Bertani (LB) agar plate

2.4 Microbial formulation

The application of microorganisms is increasingly drawing for biodegradation of various natural and synthetic substances by reducing the level of hazards. Microbial bioremediation possess a wide variety of benefit potentials from both an environmental and an economic standpoint. Bioremediation and biotransformation methods have been applied to utilize the natural microbial metabolic ability to degrade, transform, or accumulate toxic compounds including hydrocarbons, heterocyclic compounds, pharmaceutical substances, radionuclides, and toxic metals (Karigar and Rao, 2011).

Microbial application is normally in the form of ready to use microorganisms by adding microbial formulation as single strain or consortium onto contaminated sites. Microbial formulation can be separated into 2 major groups which have different advantages and disadvantages as shown in table 2.3.

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Table 2. 3 Comparison of liquid and solid microbial formulation on production and application.

formulation	Liquid	Solid	References
Type	Suspension, Concentrates (SCs) , Oil-Miscible Flowable Concentrate (OF), Ultralow Volume (ULV), Suspension (SU), Oil Dispersion (OD)	Granules (GR), Microgranules (MG), Wettable powders (WP)/water-dispersible granules (WG, WDG), Dusts, Encapsulation	(Arora et al., 2016)
Procedure and application	Few processes for production, no need expensive and complex technology, less time consuming, easy to prepare and apply	Many processes for production, some formulations need high technology, time consuming, messy and difficult for large quantity production	(Arora et al., 2016; Melin et al., 2006; Melin et al., 2011; Sivasakthivelan and Saranraj, 2013)
Storage condition	Some microbial species require cold conditions for long-term storage to maintain their efficiency and cell viability	Ambient temperature	(Arora et al., 2016)
Properties	High cell count low contamination, longer shelf life, greater protection against environmental stress and increased field efficacy	Shorter shelf life, poor quality, high contamination and low field performance	(Liu et al., 2009; Sivasakthivelan and Saranraj, 2013; Tittabutr et al., 2007; Vendan and Thangaraju, 2006)

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Production cost and transportation	Low cost while difficult transportation	High cost and easy tansportation	(Arora et al., 2016; Melin et al., 2006; Melin et al., 2011)

Bioformulation of single microbial strain is easy preparation and cost effective due to less process, save time and easy to control cell density. Although, the effective oil degradation of many single strains have been reported that can degrade crude oil more than 80%, for instance *Achromobacter* sp. HZ01 (Deng et al., 2014), *Pseudomonas* sp. 4M12 (Mansur et al., 2015), *Bacillus methylotrophicus* (Chandankere (Chandankere et al., 2014). There are some forms of ready to use microorganisms used to degrade oil as liquid (free cell), immobilized cell and powder as shown in Table 2.4.

The immobilized cell and powder can keep cell for long time but they have many steps, taking long time and difficult preparation. Whilst, the process of liquid form is easy and low cost despite liquid bacterial formulation can keep cell for some period. Even though, some reports revealed that types of liquid inoculants have been demonstrated the long term storage more than 6 months such as liquid formulation of *Acetobacter diazotrophicus* L1 and *Herbaspirillum seropedicae* J24 (Nita et al., 2012), *Pseudoxanthomonas* sp. RN402 (Nopcharoenkul et al., 2011).

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Table 2. 4 Microbial formulation for petroleum hydrocarbon degradation

Bacterial formulation	Bacteria	Type of petroleum degradation	References
Immobilized cell	Consortium	Phenanthrene (PHE) in wastewater	(Partovinia and Naeimpoor, 2014)
Powder (Petro-Clear F10)	Consortium	Benzene, diesel, lubricant and BTEX contaminated Soil and water	(Nichiporowich, 2011)
Liquid (Liquid Remediact[MT])	Consortium	Oil contaminated Soil and water	(Envirologic, 2011)
Liquid	*Pseudoxanthomonas* sp. RN402	pyrene-contaminated soil	(Nopcharoenkul et al., 2011)
Immobilized cell	*Pseudoxanthomonas* sp. RN402	Diesel contaminated water	(Nopcharoenkul et al., 2013)

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2.5 Liquid bacterial formulation

Liquid microbial formulations are a developed derivative of “noformulation” inoculants. Normally, they are microbial cultures or suspensions modified with substances that may improve stickiness, stabilization, and surfactant and dispersal abilities (Singleton et al., 2002).

Liquid bacterial formulations are to keep bacterial cell in suitable solution or buffer for long term storage along with activity preservation. The easy handle is the main advantage of these inoculants over solid inoculants. They can be used easily in applying to contaminated sites or adapt to other forms of bacteria (e.g. immobilized cell). The long shelf life of liquid bacterial inoculant depended on cultivation medium,

protective addition, temperature and initial cell density (Nopcharoenkul et al., 2011). The addition of nutrients, protectants can be done to improve the performance of liquid formulations.

Further, it is claimed that the advantage of a liquid inoculant over solid carrier-based formulation is its long shelf life up to 2 years when compared to shelf life of common solid inoculant ~ 6 months (Bashan et al., 2014). Moreover, liquid inoculants have been reported as no contaminated formulation, greater protection against environmental stresses, and increased field efficacy (Singleton et al., 2002).

The process for creating liquid bacterial formulation consists of increasing bacterial cell density, suitable solution and protective agent addition to prolong shelf life and preserve bacterial activity in appropriate temperature (Nopcharoenkul et al., 2011).

2.5.1 Medium for increasing bacterial cell density

The first step of ready to use bacterial formulation is to increase high density of bacterial cell for liquid bacterial formulation in laboratory which normally enhanced by conventional cultivation media such as Nutrient broth and Luria-Bertani. Despite these media are expensive for enhancing high cell density in large scale production. Low cost bacterial cultivation media are necessary for industrial level. In attempting to reduce cost of cultivation media, researchers have been studied on low cost substrates for replacement of high price components or all composition by using agro-industrial products and agro-industrial byproducts such as soybean powder, glucose, sugarcane molasses, soybean molasses, bagasse, coconut cake powder, neem oil cake, groundnut oil cake (Lee et al., 2013; Letti et al., 2012; Poopathi and Archana, 2012; Vohra and Satyanarayana, 2004). The microbial density could be increased by these agro-industrial media as similar as or more than conventional components.

2.5.2 Solution and role of protective agent in cell protection

For development of bacterial formulation, some solution and buffer have been used to preserve bacterial cell such as carbon free mineral medium (CFMM), 0.1 LB, potassium phosphate buffer and based preservation buffer (Nita et al., 2012; Nopcharoenkul et al., 2011). The cell survival after storage in these solutions may be

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depended on temperature, bacterial strain and type of solution. After selection suitable solution, the most appreciate protective agent has to be supplemented in bacterial solution for long term preservation of bacterial formulation.

The desirable protective agent should preserve bacterial formulation for high cell survival as much as possible in long term storage. There were some protective agents used in liquid formulation such as trehalose, galactose, glycerol, polyvinyl alcohol, gum arabic, polyethylene glycol (PEG), polyvinylpyrrolidon (PVP) and others as shown in Table 2.5. These protective agents have been used as osmoprotectants, antioxidants, additional nutrient source, stabilizing agent, desiccation agents and temperature tolerant agents in microbial formulation to prevent microorganisms from adverse environmental effect and to inhibit microbial metabolisms resulting in improvement survival of inoculant (Liu et al., 2009; Manikandan et al., 2010; Nita et al., 2012; Rivera et al., 2014).

The preservation of bacterial cell may depend on type of protective agent, temperature and bacterial species. As previous study, the viability of *Acetobacter diazotrophicus* L1 and *Herbaspirillum seropedicae* J24 liquid inoculants with gum arabica (5% w/v) and PEG 300 (5% w/v) maintained 80% and 76% at 4°C after 7 months, respectively while they also retained efficacy of plant growth promotion (Nita et al., 2012).

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The bacterial viability of variety species in liquid formulation may be optimized by different concentrations of different osmolytes. The previous study of liquid inoculants of *Azotobacter* sp., *Azospirillum* sp., *Acinetobacter* sp., *Bacillus* sp., and *Pseudomonas* sp. with different osmolytes and their concentration has shown that each organism responds variably to different concentrations of protectants. High viability of *Pseudomonas* sp. and *Bacillus* sp. were found in PVP at 2% concentration. While PEG 4000 at 2% concentration was the best for *Acinetobacter* sp. *Azotobacter* sp. was found higher population density in 2% glycerol. The density of *Azospirillum* sp. was higher in both 1 % and 2 % of PVP and PEG (Dayamani, 2010).

Temperature is one of the main environmental effect on microbial survival during inoculant storage, due to poor level of microbial metabolic activity at low

temperature (Aguilera et al., 2007). For example, the survival of *Rhodopseudomonas palustris* strain PS3 of liquid based formulation were higher at 4°C around 4.1–8.7 log CFU/ml compared to 40°C around 2.7–5.3 log CFU/ml after 30 days storage (Lee et al., 2016).

Table 2. 5 Protective agent addition in bacterial liquid formulation

Bacterial species	Protective agents	References
Acetobacter diazotrophicus L1	Trehalose, sucrose, glutamate, L-cysteine, carboxy methyl cellulose, glycerol, PEG, and gum arabica	(Nita et al., 2012)
Herbaspirillum seropedicae J24		
Pseudoxanthomonas sp. RN402	Sorbitol, Glycine, Proline, galactose, trehalose, Lactose, PEG, and glycerol	(Nopcharoenkul et al., 2011)
Azotobacter sp., *Azospirillum* sp., *Acinetobacter* sp., *Bacillus* sp., and *Pseudomonas* sp.	PEG, PVP, glycerol	(Dayamani, 2010)
Rhodopseudomonas palustris strain PS3	Alginate, PEG, PVP, glycerol, glucose, and horticultural oil	(Lee et al., 2016)
Rhizobium sp. G58	Polyvinyl alcohol, Carbomer-Carbopol 940, Sodium alginate, PEG, PVP and Hydroxypropyl methyl cellulose-HPMC	(Rivera et al., 2014)
Azospirillum brasilense	PVP, glycerol, gum arabica, trehalose, PEG, and polyvinyl alcohol	(Kumaresan and Reetha, 2011)
Rhizobium	PVP	(Girisha et al., 2006)

2.6 Agro-industrial byproduct for bacterial cultivation media

Cultivation medium is very important for increasing high microbial cell density in order to achieve in many applications. Normally, conventional cultivation media has been used such as Luria-Bertani (LB) broth, yeast mannitol broth (YMB), nutrient broth

(NB), tryptic soy broth (TSB). Even though, the compositions of these media are not appropriate for large scale or industrial production due to cost inefficiently. To overcome the cost of cultivation medium, alternative substrates including industrial and agricultural by-products (e.g. cheese whey, malt sprouts) are interested due to their containing growth factors such as nitrogen and carbon for supporting bacterial growth (Rebah et al., 2007). Other agro-industrial wastes such as bagasse, molasses and molasses may be useful material in bacterial cultivation media. Moreover, wastewater sludge, a worldwide recyclable waste has shown good potential for bacterial formulation production as a growth medium and as a dehydrated sludge carrier (Rebah et al., 2007).

2.6.1 Byproduct of sugarcane process

Sugarcane is agro-industrial plant that widely planted in Thailand. Total production of sugarcane was 107,000,000 metric tons which it was mainly utilized to produce sugar 101,000,000 metric tons (USDA, 2016). While, sugar industrial processes generate large quantities of organic solid waste and by-products for instance leaves from cane, molasses derived from final crystallization, press mud, bagasse fiber, mud and soil arriving from plant with the raw material, and lime solids from the juice clarification as shown in Figure 2.4. High quality waste can provide chances for reprocessing of otherwise discarded raw materials into commercially viable by-products for example paper making and particle board manufacturing (IFC, 2007). Especially, bagasse and molasses have been largely studied and used as substrates of fermentation and unconventional microbial media through pre or non-pretreatment.

Many products have been produced from whole-bagasse or treated-bagasse via several processes for instance enzymes, ethanol, and single cell protein (SCP) production (Pandey et al., 2000). Moreover, non-pretreatment bagasse was reported as agro substrate of bacterial cultivation medium that could increase the bacterial density (Poopathi et al., 2013). While molasses has been used in many batch and large scale production as low cost substrate that could produce high yield of microbial biomass for instance the biomass of *Pichia anomala* was increased higher in molasses

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based medium compared to synthetic glucose–beef extract medium (Vohra and Satyanarayana, 2004).

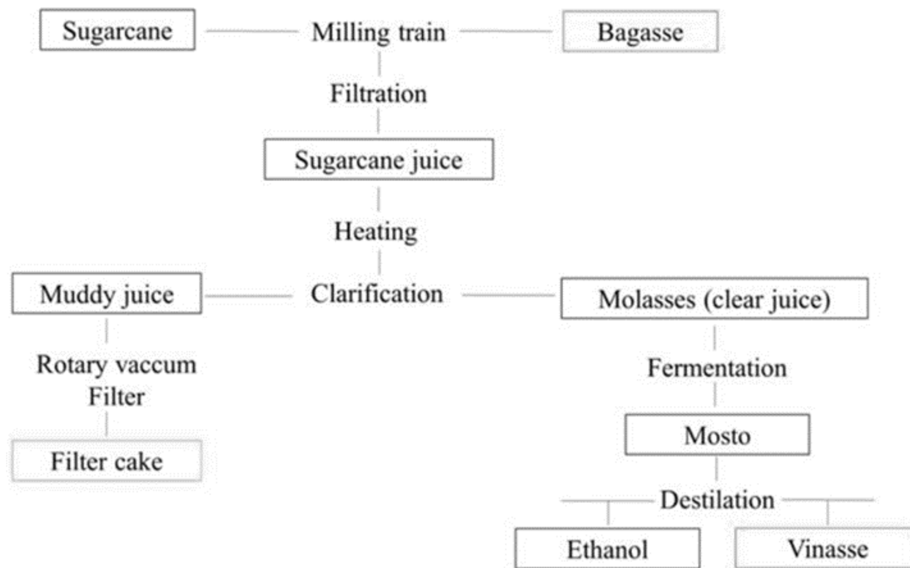

Figure 2. 4 Byproducts generated during the sugarcane processing (Botellho et al., 2014).

2.6.2 Residues from soybean oil processing industry

Soybean oil is one of the most demands for healthy life due to containing variety of nutrient and being precursors of Omega-3, Omega-6 and Vitamin E (Karasulu et al., 2011). These properties lead to high production of soybean oil and other soy products worldwide. Thailand become a large soybean oil production in ASEAN countries and widely use soybean in agricultural sector by importing soybean around 2.2 MMT during 2015 to 2016 (USDA, 2016). Even though, almost of soybean oil processed byproduct can be used as feed for livestock and generate into other products but the effluent from this process has to be treated to prevent from environmental damage.

The refine of oil process is one of the major issue of environmental problem in developing countries for several decades, as a result of oil refinery release high organic content waste which can serious threat to ecosystem especially aquatic life (Sharma et al., 2014). In contrast, dry sludge of soybean oil mill effluent can be used

as carbon and energy source for bacterial growth in order to produce biosurfactant (Wichaidit, 2014). On the other hand, after soybean oil production, soybean meal can be used to produce some products including protein concentrate, and the byproduct of the process is soy molasses as shown in Figure 2.5.

“Soy molasses” or “soybean molasses” is characterized as a brown viscous syrup with bittersweet flavor from a concentrated, desolventized, aqueous alcohol extract of defatted soybean flakes, a by-product of “traditional” aqueous alcohol soy protein concentrate production. It is low cost product from soybean oil processing which has been used in feeding livestock. This residue has also been reported in biosurfactant production due to high amount of sugar that useful for promoting microbial growth (Solaiman et al., 2007). Soybean molasses (dry mass) contains 57.3% carbohydrates (include 28.4% sucrose and 18.6% stachyose and 9.68% raffinose), 9.44% proteins and 21.2% lipids (Siqueira et al., 2008). The high quantity composition of sugar, lipids and protein in soybean molasses are able to utilize as nitrogen and carbon source for sustaining bacteria and yeast growth in ethanol production (Letti et al., 2012; Siqueira et al., 2008).

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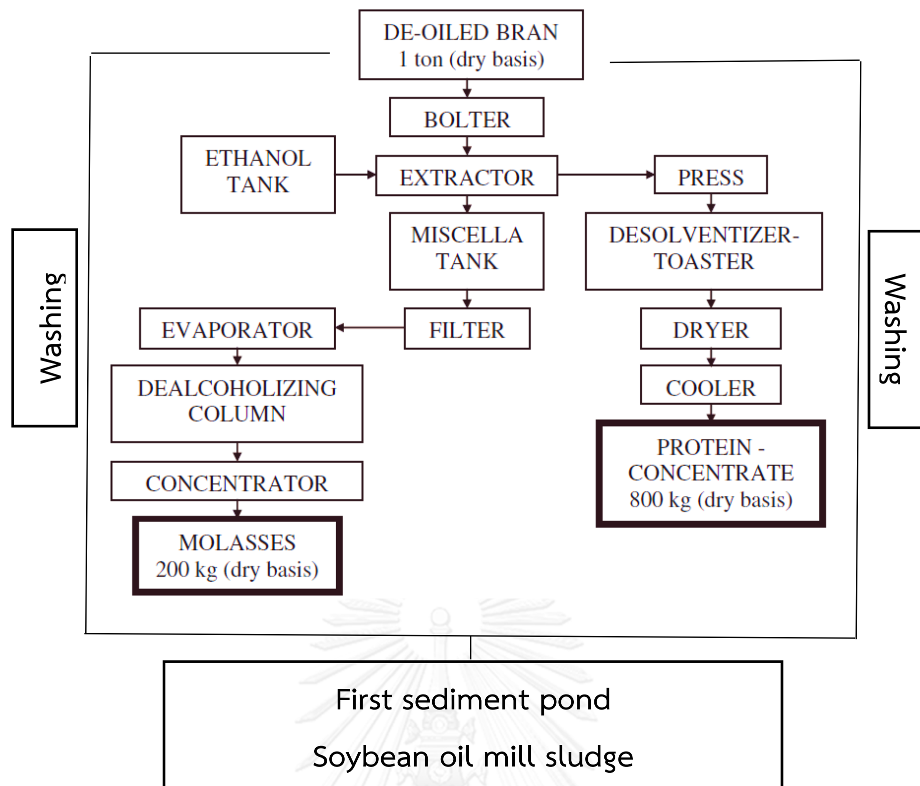

Figure 2. 5 soybean oil byproducts produced from de-oil soybean meal modified from Siqueira et al., (2008).

The alternative carbon source from soybean oil dry effluent and soy molasses are quite interesting since, the large soybean oil production in Thailand. The oil refining process generated great amount of wastewater as a thick brownish liquid that contains high solids, oil and grease while the direct discharge without treatment of effluent can adversely affects the ecosystem (Sharma et al., 2014). Therefore, this waste from oil refining process should be converted into valuable products or alternative substrate for microbial cultivation medium which can reduce the cost of treatment and prevent environmental damage.

2.6.3 Coconut milk processing byproducts

Coconut milk is white liquid extracted from the grated fresh coconut kernel that used for traditional and healthy foods. Coconut is widely planted in Thailand as a major production in Asian and pacific countries in which around 218 metric tons copra produced each year (FAO, 2014). The process of coconut milk and oil production

created a lot of residue, coconut shell, water, coconut milk residue, effluent and others. The residues of coconut milk extraction from grated or shredded coconut kernel is called “coconut milk residue” as shown in Figure 2.6.

It represents about 25–50% of the weight of fresh kernel, based on the use of coconut milk extraction process (Bawalan, 2011). Normally, this residue is used for animal feeding or discarding as waste in most Pacific households. The composition of dried coconut milk residue was determined by the Philippine Food and Nutrition Research Institute (FNRI) revealed that it contained 51% carbohydrates, 32% dietary fiber, 38% fat, 5% protein, 4% moisture and 2% ash (Bawalan, 2011). The high nutrient composition of coconut milk byproduct is interesting for reusing it as alternative carbon and energy sources for microbial growth. The utilization of coconut product and byproducts as nutritional source for microbial growth have been studied in past several years in order to reduce cost production. For instance, coconut oil mill waste, coconut water and coconut milk were used as alternative carbon sources for substrate based medium for bacterial growth in lipase and cellulose production (Hungund et al., 2013; Kanmani et al., 2015). Coconut oil cake and tender coconut water based media could also increase high concentration of bacterial cell density (Poopathi and Archana, 2012; Sekar et al., 2013). Hence, coconut milk residue may be suitable to apply as inexpensive alternative nutritional source for increasing bacterial cell density in the process of ready to use bacteria.

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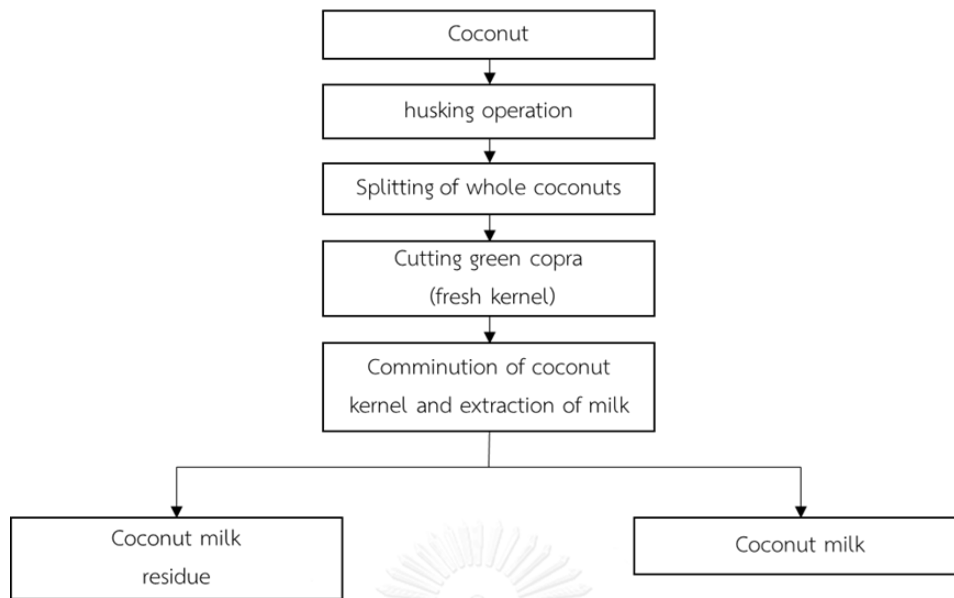

Figure 2. 6 Coconut milk residue produced from coconut milk process (Bawalan, 2011).

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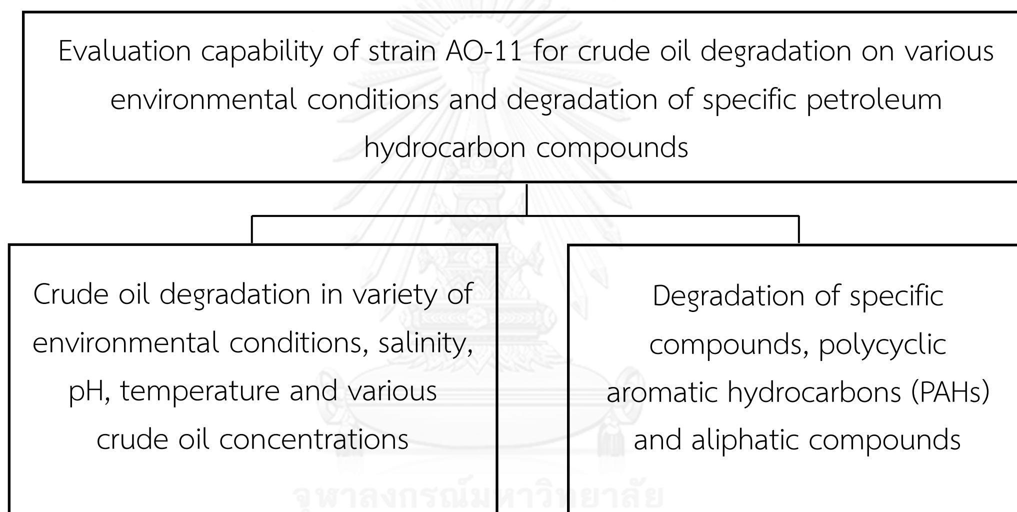
CHAPTER III

METHODOLOGY

3.1 Flow chart of experimental procedure

The experiment was conducted in 4 phases as show in the flow chart below:

Phase 1 Evaluation capability of strain AO-11 on petroleum hydrocarbon degradation



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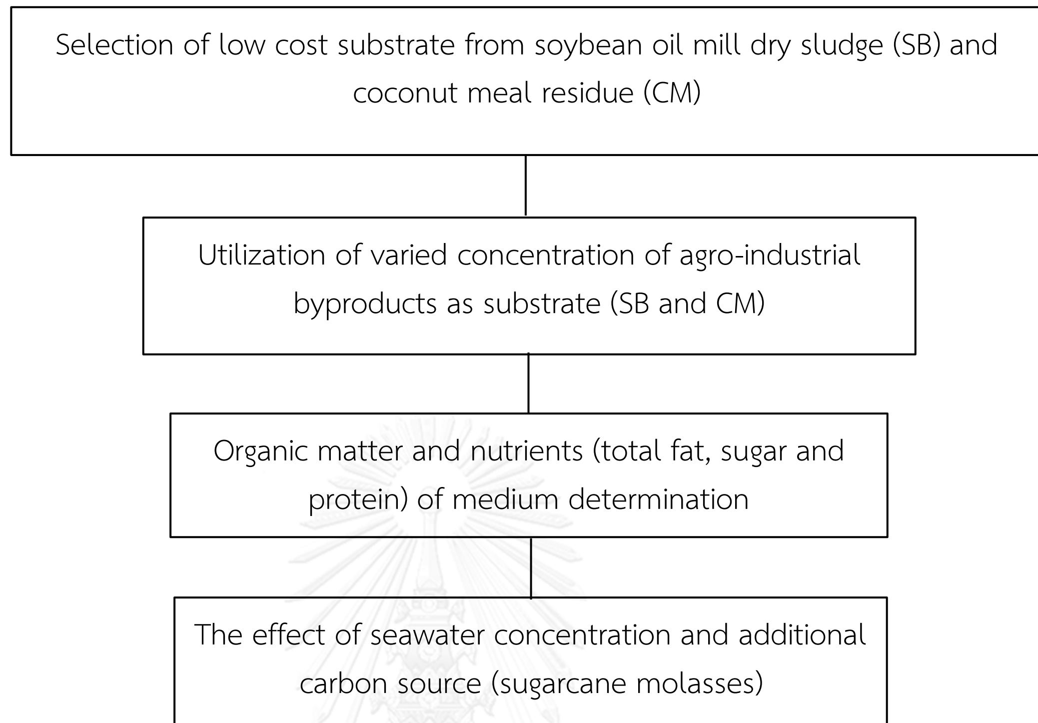
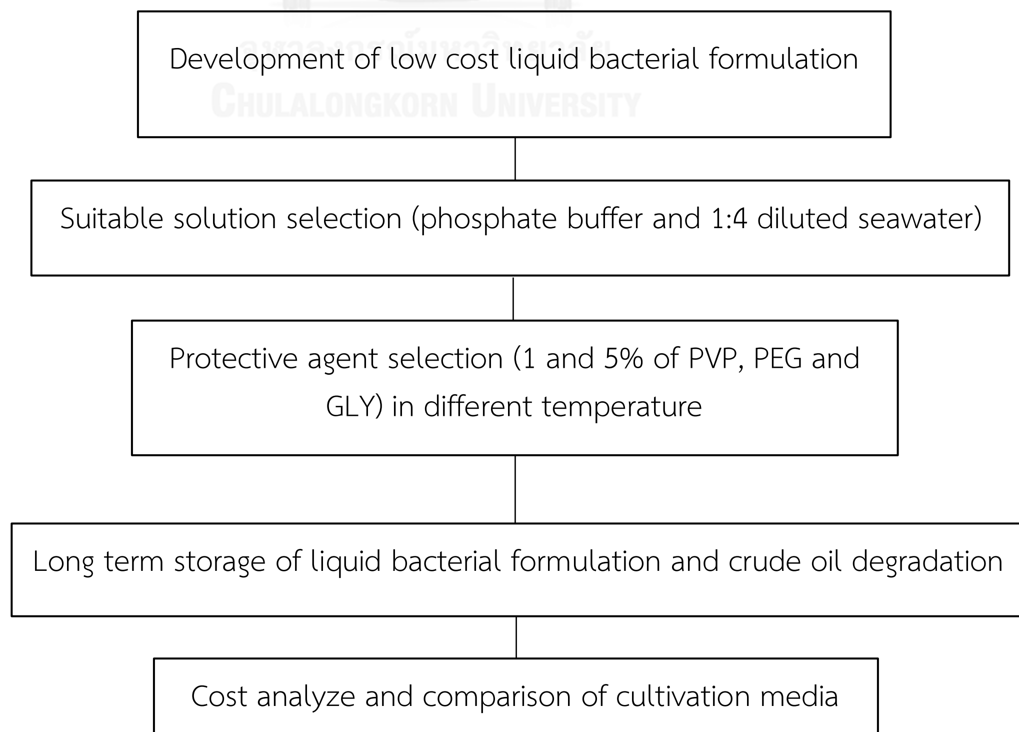
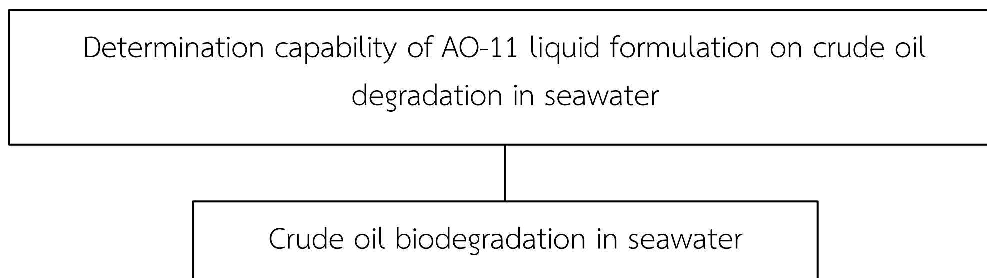
Phase 2 Selection of low cost substrate for bacterial cultivation of liquid bacterial formulation



Phase 3 Development of liquid bacterial formulation for crude oil biodegradation



Phase 4 Determination capability of liquid bacterial formulation on crude oil biodegradation



3.2 Chemicals, substrates and equipment

3.2.1 Chemicals

1. The mixture between Arab Extra Light (AXL) and Arab Light (ARL) crude oil was obtained from Petroleum Authority of Thailand (PTT), Thailand

Table 3. 1 Crude oil composition in this research (PTT)

Composition	**Arab Extra Light (AXL) Yield (% wgt)**	**Arab Light (ARL) Yield (% wgt)**
LPG	1.64	2.22
Light Naphtha	9.18	5.45
Naphtha	14.08	10.18
Kerosene	24.06	19.54
Gasoil	18.39	18.30
Waxy	24.06	28.86
Short residue	8.59	15.70
Total	100.00	100.00

1. Yeast extract, Difco Laboratories, USA
2. Tryptone, Difco Laboratories, USA
3. Sodium chloride (NaCl), Merek, Germany

4. Potassium dihydrogen phosphate (KH_2PO_4), Merek, Germany
5. Dipotassium hydrogen phosphate (K_2HPO_4), Merck, Germany
6. Hydrochloric acid (HCl), BDH Chemical, Australia
7. Tetradecane, Fluka, Germany
8. Phenanthrene, Sigma, USA
9. Pyrene, Sigma, USA
10. Hexadecane, Sigma,USA
11. Ammonium nitrate (NH_4NO_3), Merck, Germany
12. Sodium hydroxide (NaOH), Merck, Germany
13. Hydrochoric acid (HCl), BDH Chemicals, Australia
14. Bacto agar, Difco, USA
15. Glycerol, Research organics. Inc., USA
16. Hexane, J.T.Baker, USA
17. Methanol, Fisher Scientific, UK
18. N,N dimethyl formamide, Ajax Finechem, Australia
19. Polyvinylpyrrolidone (PVP), Chemipan, Thailand
20. Polyethyl Glycol (PEG), Chemipan, Thailand
21. Zobell Marine Broth 2216, HiMedia Laboratories, India
22. Zobell Marine Agar 2216, HiMedia Laboratories, India
23. Chemical Oxygen Demand Reagent HI 93754C-25 HR, Hanna Instruments, USA
24. Seawater, Choumpon province, Thailand

3.2.2 Substrates

1. Coconut meal (milk) residue, Samyarn market, Thailand
2. Soybean oil mill dry sludge, Thai Vegetable Oil Public Company Limited (TVO), Thailand
3. Sugarcane molasses, Banpong Sugar Co., Ltd., Thailand

3.2.3 Equipments

1. ISSCO laminar flow, International Scientific Supply, Japan

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2. Incubator (30°C), model BE800, Memmert, Germany
3. Oven, Contherm Scientific, New Zealand
4. Balance, model P2002-S and AG285, mettle Toledo, Switzerland
5. Vortex mixer, model Genie 2, Scientific Industries, USA
6. Autoclave, model ES 315, Tomy Kogyo, Japan
7. Spectrophotometer, model UV-160A, Shimadzu, Japan
8. Micropipette (20, 200, 1,000 and 5,000 µl) from Gilson, France
9. pH meter, model SevenEasy™ S20, Mettler-Toledo AG, Switzerland
10. Gas chromatography - Flam Ionization Detector (GC-FID)
11. Gas chromatography, model 6890N, Agilent Technology, USA
12. HP-5 column (30 m x 90.25 mm x 9 0.25 µm), Agilent Technology, USA
13. Flam Ionization Detector, , Agilent Technology, USA
14. Mixed Cellulose Esters Membrane (0.22 µm GSWP), Merk Miillipore, Ireland
15. High Speed Refrigerated Centrifuge, Model 6500, Kubota, Japan
16. Innova platform shaker, model 2300, New Brunswick Scientific, USA
17. Innova refrigerated incubator shaker, model 4330, New Brunswick Scientific, USA
18. C-MAG hotplate stirrers, model HS 7, IKA, Germany
19. Multiparameter Photometer, model HI 83214, Hanna Instruments Inc., USA
20. COD reactor, Model 45600, Hach Company, USA
21. ISSCO Laminar flow, model HT-122.5, International Scientific, USA
22. Oven, Conthem Scientific, New Zealand
23. Hot air oven, model D06063, Memmert, Germany
24. Incubator 30°C, model BE800, Memmert, Germany
25. Incubator 25°C, Thailand

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3.3 Methods

3.3.1 Bacterial strain and inoculum preparation

Crude oil-degrading bacterium, *Exiguobacterium* sp. AO-11 was isolated from crude oil-contaminated sediment, Aoprao Bay, Samet Island, Rayong province, Thailand (Srisuwankarn, 2015). It was collected in culture collection as number MSCU0807, at Department of Microbiology, Faculty of Science, Chulalongkorn University. It is sub-cultured in NSW medium (Appendix A) with 0.25 % crude oil every 10 days. The old transferred bacterial culture 10 ml was added into 90 ml 0.25xMarine broth or 0.1xLB (Appendix A) to increase cell density around 18 hours on 200 rpm rotary shaker, room temperature followed by centrifugation to get cell pellet. Then cell pellet was washed 2 times with 1% NaCl solution and suspended in the same solution. Cell suspended solution was measured the absorbance by spectrophotometer OD 600 nm, value 1 (cell density approximately 1×10^8 CFU/ml) after that, one night resting cell was conducted on 200 rpm rotary shaker, room temperature. This inoculum was used in petroleum hydrocarbon degradation. For optimization of agro-industrial byproduct media, AO-11 inoculum was prepared by centrifugation of old bacterial culture and suspending cell in 1% NaCl solution with OD 600 nm at 0.1 (cell density approximately 1×10^7 CFU/ml). It was also streaked/spread and dropped on LB or Marine agar for checking pure colony and measurement bacterial growth.

3.3.2 Extraction and detection of remaining petroleum hydrocarbon compound

The mixture between Arab Extra Light (AXL) and Arab Light (ARL) was used in this research. These crude oil densities are 0.8229 and 0.8549 (g/cc) at 15°C, respectively. The mixed Arab crude oil composition was measured by Thai oil Public Co., Ltd. as shown in Table 3.1.

In this study, the remaining petroleum hydrocarbon compounds including crude oil, pyrene, phenanthrene, hexadecane, tetradecane and docosane in test tubes were similar extracted as described in Nopcharoenkul et al., (2013). Hexane 5 ml was added into experimental tube (5 ml). Then it was mixed gently 2 min by vortex machine. The separation of hexane layer was performed by keeping the sample in -

20°C for 24 hours. The hexane mixture on the upper layer was transferred into new tube. After that, hexane was evaporated at 150°C on hot plate. Hexane 1 ml was introduced into remaining petroleum hydrocarbon compounds in test tube and mixed gently 1 min. The mixture was filtered through 0.2 µm PTFE filter into GC vial. Then remaining crude oil was detected by GC-FID 6890N (Nopcharoenkul et al., 2013). The temperature of detector was set to 320°C. It was operated in splitless mode. The conditions of detection process were set as follows: a 2 min hold at 40°C, increased from 40 to 320°C at 10°C min^{-1}. Percent of petroleum hydrocarbon compound degradation was calculated based on chromatographic peak areas as following formula.

$$\text{Percent of petroleum hydrocarbon degradation} = \frac{\text{Peak area of control day (N) - Peak area of day (N)}}{\text{Peak area of control day (N)}} \times 100$$

3.3.3 Evaluation capability of strain AO-11 for petroleum hydrocarbon degradation

3.3.3.1 Effect of environmental conditions on crude oil degradation

The environmental condition including salinity, pH, temperature and crude oil concentration are important factors on crude oil degradation. In order to evaluate the effect of these factors, the prepared culture from 3.3.1 (0.5 ml) was added into NSW medium (Appendix A) in these experiments. The environmental conditions as following:

The effect of pH of NSW medium ranged from 6, 7, 8 and 9 with 0.25% (v/v) crude oil at room temperature.

The effect of salinity concentration ranged from 9.1, 18.2, 27.3, 36.4 and 45.4 ppt ppt with 0.25% (v/v) crude oil at room temperature.

The effect of crude oil concentration was also varied from 0.25, 0.5, 1 and 1.5% (v/v) at room temperature.

The effect of temperature ranged from 25, 30 and 37 °C with 0.25% v/v crude oil.

Then these test tubes were incubated on rotary shaker 200 rpm for 10 days. After incubation, the samples were extracted and detected remaining crude oil as shown in 3.3.2. The control experiments were conducted by adding crude oil into 5 ml NSW medium without inoculum. All experiments were performed in triplicate.

3.3.3.2 Capability of strain AO-11 on degradation of specific hydrocarbon compounds

The major compositions of light crude oil are polycyclic aromatic hydrocarbons (PAHs) and aliphatic compounds. Biodegradation of these substances might indicate the ability of bacteria in crude oil degradation. Therefore, evaluation the capability of AO-11 on specific petroleum hydrocarbon degradation are essential for using it as ready to use bacteria in bioremediation of crude oil contaminated environments. To evaluate the capability of strain AO-11 on specific compounds, polycyclic aromatic hydrocarbons including pyrene and phenanthrene (Appendix B) were added into 4.5 ml NSW medium with 0.5 ml inoculum (3.3.1), with both initial concentration 50 ppm. The aliphatic compounds including hexadecane ($C_{16}H_{34}$), tetradecane ($C_{14}H_{30}$) and docosane ($C_{22}H_{46}$) (Appendix B) with the initial concentration 1000, 1000 and 100 ppm, respectively were also used to evaluate the ability of AO-11 in NSW medium 4.5 ml with 0.5 ml inoculum. Then all experimental tubes were incubated on 200 rpm rotary shaker at room temperature for 10 days. The control experiments were conducted by adding specific compound into 5 ml NSW medium without inoculum. After incubation, the samples were extracted and detected remaining crude oil as shown in 3.2.2. All experiments were performed in triplicate.

3.3.4 Optimization of agro-industrial byproduct media

3.3.4.1 Effect of agro-industrial byproduct concentration on AO-11 growth

Three agro-industrial wastes including coconut milk residue (CM) and soybean oil mill dry sludge (SB) were used as substrates for cell growth as shown in Figure 3.1. The preparation of agro-industrial residues medium was performed by adapting process from previous study (Poopathi and Archana, 2012). The concentrations of these wastes were varied from 1-20% (w/v) in seawater to get the optimal concentration for cell growth. The mixtures were boiled (autoclaved) for 15 min, 121°C. After cooling, the liquid phase was filtered through cotton sheet, and the pH of the filtrate was adjusted

(pH 7.8±1). Then it was centrifuged to separate unfiltered particles or grease for 10 min at 4°C. The extracts of these residues (45 ml) were dispensed separately into Erlenmeyer flasks (vol. 250 ml) for culturing strain AO-11. The agro-industrial byproduct culture media were sterilized at 121°C, 15 min. Then the prepared inoculum (5 ml) from 3.3.1 was added into each medium. These media with inoculum were incubated on rotary shaker 200 rpm at room temperature for 48 hours. The samples were collected at 3, 6, 9, 12, 15, 18, 24, 30, 36, 48 hours and dropped plate by dilution technique on LB agar for determining AO-11 growth. LB medium was used as control medium in this experiment. All experiments were performed in triplicate. From the above test of culture media, the medium which showed maximum density of strain AO-11 growth was selected for further experiments.

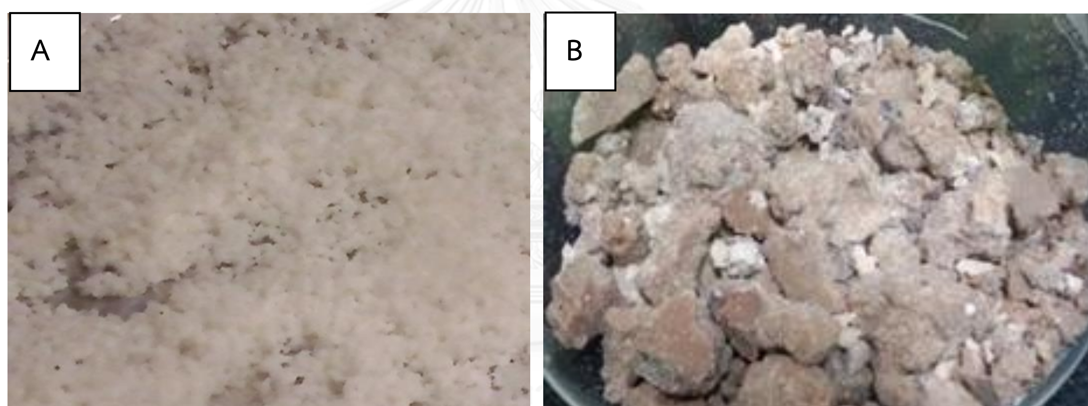

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Figure 3. 1 Agro-industrial byproducts: A. Coconut milk residue (CM) and B. soybean oil mill dry sludge (SB).

3.3.4.2 Determination of organic matter and nutrients of optimized agro-industrial byproduct media

Organic matter and nutrients were determined by using 15% CM and SB media. Chemical oxygen demand (COD) was determined to evaluate organic matter (biomass) of media by Hanna Instruments. The green color HI 93754C-25 HR: COD high range (0 to 15000 mg/L) reagent was used in this study. The sample 0.2 ml was added into the vial, while keeping the vial at a 45 °C. Then, the sample was mixed by inverting the

vials a couple of times. After that, the vials were inserted into the reactor and heat them for 2 hours at 150°C. They were allowed to cool about 120°C around twenty minutes, fellow by inverting each vial several times while they were warm. After they were cooled down at room temperature, colorimetric COD of the samples were determined by HI 83214 multiparameter bench photometer. Distilled water was used instead of samples as blank. COD measurement was conducted in duplicate.

The total protein, sugar and fat were investigated to evaluate nutrient contents in available forms of each medium by Central Laboratory (Thailand) Co., Ltd.

3.3.4.3 Effect of seawater concentration on AO-11 growth

The selected medium from 3.2.4.1 was used to evaluate the effect of seawater concentration on bacterial growth. Seawater concentration were varied in distilled water with the proportion 1:4, 2:3, 3:2, 4:1 (seawater: distilled water), to optimize cell density in variety of salt concentration. The process of this experiment is the same as in 3.3.4.1. Samples were collected at 3, 6, 9, 12 hours. The most suitable concentration of seawater for bacterial growth was selected for further assays.

3.3.4.4 Effect of sugarcane molasses concentration on AO-11 growth

The selected medium from 3.3.4.3 was utilized to examine the effect of sugarcane molasses concentration as alternative additive carbon source for bacterial growth. Different concentration of molasses (5, 10, 20, 30 and 50 g/L) was added into selected medium before the process of pH adjustment as shown in 3.3.4.1. Incubation techniques are similar to 3.3.4.1. Samples were collected at 3, 6, 9, 12 hours. The variety concentration of sugarcane molasses concentration on AO-11 growth was compared to medium without molasses. The most suitable medium based on composition and easy preparation was selected as production medium for further experiments.

3.3.5 Suitable solution selection for suspending cell on crude oil degradation

3.3.5.1 Suitable solution selection

Pure colony on LB agar was added into flasks containing selected production medium from 3.3.4. They were incubated on rotary shaker at 200 rpm, room temperature for 12 - 15 hours. After that, the grown medium was centrifuged at 8000

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rpm, 4°C and washed with 1% salt solution for 2 times. Then, the cell pellet was added into phosphate buffer (0.05 M PB) and 1:4 diluted seawater (SW) with distilled water with initial cell approximately 10^9 CFU/ml. Cell-suspended solutions (50 ml) were added into 120 ml plastic bottles. They were stored in room temperature (26.8±3.6°C) for 30 days. The samples were collected each 10 days to evaluate bacterial survival rate by drop plate technique. The experiments were conducted in triplicate. Percent of bacterial survival could be calculated by following formula (Nopcharoenkul et al., 2011).

$$\text{Percent survival} = 100 - \frac{(\text{Log}_{10}\ \text{CFU/ml day 0} - \text{Log}_{10}\ \text{CFU/ml day (N)}) \times 100}{\text{Log}_{10}\ \text{CFU/ml day 0}}$$

N is the day that samples were collected.

3.3.5.2 Crude oil degradation of cell suspended solution

Bacterial suspension of each 10 day sample (0.5 ml) was added into test tube containing 4.5 ml sterile NSW (initial cell concentration approximately 1 x 10^7 CFU/ml) with 0.25% v/v crude oil. Abiotic control was set up in test tube containing 5 ml sterile NSW without inoculum with 0.25% v/v crude oil. The experiment was conducted for 10 days on rotary shaker at room temperature, 200 rpm. Bacterial samples and controls were performed in triplicate. Remaining crude oil was examined by gas chromatography-flame ionization detector as explained in 3.3.2.

3.3.6 Protective agent selection in different temperature for liquid bacterial formulation

Polyvinylpyrrolidone (PVP), glycerol (Gly), polyethylene glycol 6000 (PEG) were used as protective agents. Two concentrations of three protective agents 1 and 5% (w/v) of PVP and PEG, and 1 and 5% (v/v) of Gly were added into selected solution from 3.2.5 and sterilized at 121°C for 15 min. Each liquid bacterial formulation 100 ml was added into 120 ml plastic bottle as shown in Figure 3.1. All of them were stored at 30°C and room temperature (31±1) with initial cell concentration approximately 1 x 10^9 CFU/ml. They were also stored at 4 and 25°C with initial cell concentration approximately 1 x 10^{10} CFU/ml. Phosphate buffer with bacterial cell was performed as

control. The samples were collected each 10 days to evaluate bacterial survival by drop plate technique. Percent of bacterial survival could be calculated by formula as shown in 3.3.5. All experiments were conducted in triplicate. The most suitable concentration of protective agent in two different temperatures which had high bacterial survival and low cost was selected for long term storage of liquid bacterial formulation.

Figure 3. 2 liquid bacterial formulation 100 ml in 120 ml plastic bottle.

3.3.7 Extension of stored liquid bacterial formulation for crude oil degradation

Bacterial cell was prepared by adding old culture into the production medium in the proportion 1:10 in Erlenmeyer flask. Then it was incubated on rotary shaker 200 rpm, room temperature for 15 hours. After that, it was centrifuged and washed 2 times with 1% salt solution to get cell pellet. The selected formulations from 3.3.6 contained initial cell concentration approximately 1 x 10^{10} CFU/ml, 50 ml in 120 ml plastic bottle were stored at both 4 and 30°C for 60 days. The samples were collected each 30, 45 and 60 days to evaluate bacterial survival by drop plate technique. PB with bacterial cell was set as control in this experiment.

3.3.8 Cost analyze and comparison of liquid bacterial formulation

The cost of liquid bacterial formulation was calculated based on electricity use and medium composition. It was compared to other liquid commercial and previous

study formulation for petroleum hydrocarbon degradation to confirm inexpensive of own liquid bacterial formulation.

3.3.9 Determination capability of liquid bacterial formulation on crude oil biodegradation in seawater

3.3.9.1 Characterization of seawater

Seawater was collected from gulf of Thailand at Chumphon Province, Thailand in 2016. It was stored in closed plastic bottle and kept in 4°C. Environmental conditions of seawater including salinity concentration and pH were determined before crude oil degradation due to their might effect on crude oil degradation.

3.3.9.2 Crude oil biodegradation in seawater

The bacterial liquid formulation at 4°C after 30 day storage from 3.2.7 was used to evaluate capability of crude oil degradation in seawater. The experiments were carried out in 250 ml Erlenmeyer flasks by adapting from (Kok Kee et al., 2015). Erlenmeyer flasks contained 100 ml of sterilized seawater with AO-11 initial cell concentration (AO-11+STSW) approximately 1 x 10^7 CFU/ml were performed to indicate the capability of AO-11 formulation on crude oil degradation. Seawater with AO-11 (AO-11+SW) was done to determine bioaugmentation in simulating situation. Sterilized seawater with crude oil was set as negative control. Two set of experiments were received 0.25% and 0.5% (v/v) crude oil, respectively. While seawater with crude oil was set as positive control for 0.5% (v/v) crude oil degradation. All the flasks of 0.25% crude oil were incubated on rotary shaker 200 rpm at room temperature for 10 days. For 0.5% (v/v) crude oil, the samples were collected in 10 and 15 days. These experiments was conducted in triplicate.

3.3.9.3 Extraction and detection of residual crude oil

The residual crude oil was extracted from treated and control seawater samples as previous report from (Kok Kee et al., 2015). Five milliliter of *n*-hexane was introduced into samples and mixed vigorously. Then the mixture was move to a separation funnel. It was rested for 10 min for separation of the organic and aqueous phases. The organic *n*-hexane part was transferred into test tube with closure. The extraction process was repeated twice. After that, hexane was evaporated at 150°C on

hot plate. Then remaining crude oil was diluted 10 folds in test tube. Detection of remaining crude oil was conducted as shown in 3.3.2.

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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Capability of *Exiguobacterium* sp. AO-11 on petroleum hydrocarbon degradation

Exiguobacterium sp. AO-11 was evaluated for its capability on crude oil biodegradation in various environmental conditions; and degradation of polycyclic aromatic hydrocarbons (PAHs) and aliphatic compounds in liquid cultivation for 10 days with initial concentration of: 0.25 % (v/v) crude oil, pyrene (50 ppm), phenanthrene (50 ppm), tetradecane (1000 ppm), hexadecane (1000 ppm) and docosane (100 ppm).

4.1.1 Effect of environmental conditions on crude oil degradation

The examination of crude oil degradation by strain AO-11 in the different concentration of crude oil revealed that strain AO-11 could degrade crude oil up to 84.5±6%, 38.1±10%, 32.1±6 and 21.8±0.6% with concentration of 0.25% (v/v), 0.5% (v/v), 1% (v/v) and 1.5% (v/v) of crude oil, respectively as shown in Figure 4.1.

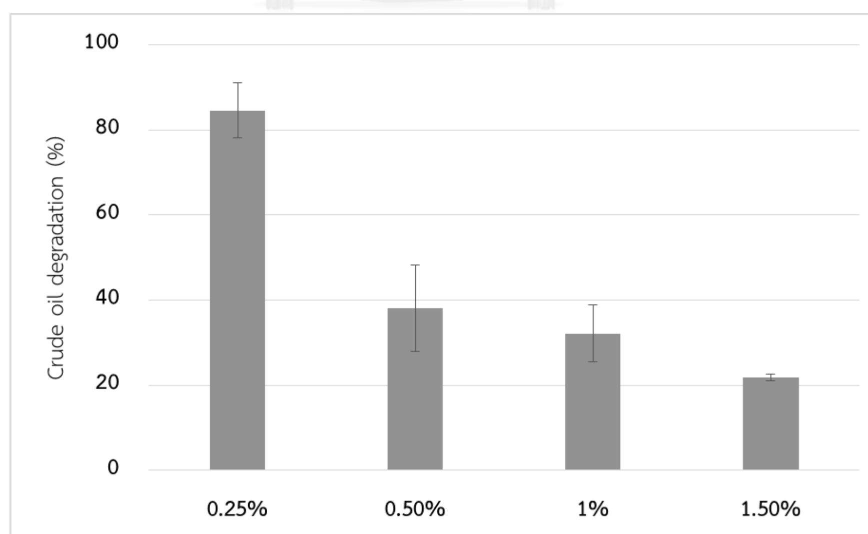

Figure 4. 1 Crude oil degradation by strain AO-11 on various oil concentrations at pH 7, salinity 8 ppt, room temperature (31±1°C) for 10 days. Note, controls were shown in Appendix F.

The result indicated that strain AO-11 (with initial amount of 2.8±0.5 x 10^7 CFU/ml) was able to degrade wide range of crude oil concentration despite lower percent degradation in higher volume of crude oil in 10 days with final cell density 9.3±0.5 x 10^6, 1.5±0.6 x 10^7, 7.3±0.5 x 10^6 and 7.3±0.5 x 10^6 CFU/ml in 0.25%, 0.5%, 1% and 1.5% respectively, indicating that the strain still survived in all concentration of crude oil and prolonged incubation may increase percent degradation. This study is agreement with the previous research shown that the percent biodegradation of crude oil decreased in high concentration (Sathishkumar et al., 2008). The concentrations of petroleum may directly affect microbial activity when too high concentrations of oil which may be toxic effects on the present bacteria (Adams et al., 2015).

The examination of effect of pH from 6 to 9 on crude oil degradation in 10 days showed that the highest 0.25% (v/v) crude oil degradation was achieved about 82.1±4.1% at pH 7. Final cell density was 1.6±1.1 x 10^6, 2.3±1.5 x 10^6, 6.6±5 x 10^6 and 2±0.6 x 10^6 CFU/ml at pH 6, 7, 8 and 9, respectively. The tendency of crude oil degradation was decreased at pH 9 as shown in Figure 4.2. This phenomenon was explained by previous research that microbial biodegradation processes can be inhibited by extreme pH condition (Tyagi et al., 2011).

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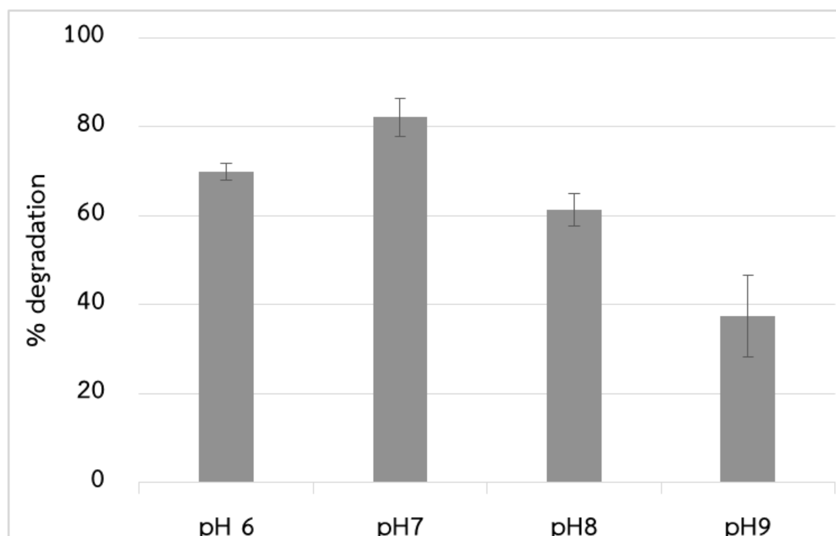

Figure 4. 2 Crude oil degradation (0.25% (v/v)) by strain AO-11 on pH 6 to 9 at pH 7, salinity 8 ppt for 10 days at room temperature (31±1°C). Note, controls were shown in Appendix F.

The crude oil biodegradation in variety of temperature revealed that the optimal temperature for strain AO-11 on degradation of 0.25% (v/v) crude oil was at 30°C which achieved 82.1±4.1% degradation; while, the degradation was lower than 60% at 25°C as shown in Figure 4.3. Final cell density was 7.6±3.2 x 10^6, 2.3±2 x 10^6 and 1±0.2 x 10^6 CFU/ml at 25, 30 and 37°, respectively. Temperature is ranged as a critical factor to rule the metabolic activity of the degrading microorganisms as well as physical and chemical nature of hydrocarbons (Tyagi et al., 2011). The result indicated that low temperature at 25°C can cause declining in crude oil degradation. The similar result was observed in earlier study which showed that *Achromobacter* sp. HZ01 could degrade 2% (w/v) evaporated diesel oil up to 95.6% for 10 days at 28°C and decrease to 21.1% at 16°C (Deng et al., 2014).

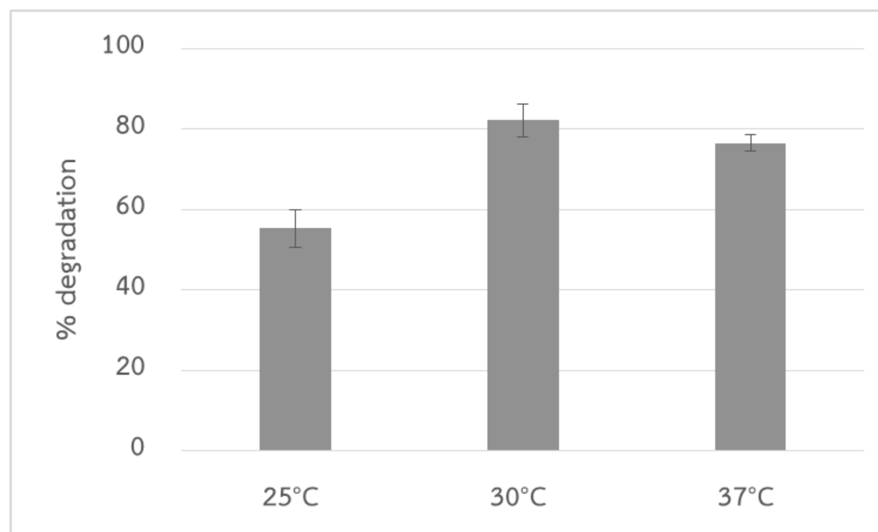

Figure 4. 3 Crude oil degradation (0.25% (v/v)) by strain AO-11 at temperatures 25, 30 and 37°C with pH 7, salinity 8 ppt, for 10 days. Note, controls were shown in Appendix F.

The examination of effect of salinity concentration on crude oil degradation showed that this strain could degrade 0.25% (v/v) crude oil high up to 78.5±2.3% with salinity 8 ppt followed by 9.1, 18.2, 27.3, 36.4 and 45.4 ppt, respectively, as shown in Figure 4.4. Final cell density was $4.2\pm2.3 \times 10^6$, $9\pm1 \times 10^6$, $2.3\pm2.3 \times 10^5$, $6.6\pm6 \times 10^5$, $4.39\pm1.5 \times 10^5$ and $7.6\pm2 \times 10^4$ CFU/ml at 8, 9.1, 18.2, 27.3, 36.4 and 45.4 ppt, respectively. This result revealed that crude oil biodegradation decrease in high salt concentration. It is agreement with previous report that *Achromobacter* sp. HZ01 could degrade 2% (w/v) evaporated diesel oil more than 85% for 10 days in 10 ppt salt concentration while the degradation was decreased less than 55% in 10 ppt salt (Deng et al., 2014).

The results indicated that strain AO-11 has the capability to degrade crude oil in various environmental conditions in laboratory experiments. Therefore, this strain was used for further experiment in degradation of specific petroleum hydrocarbon compounds.

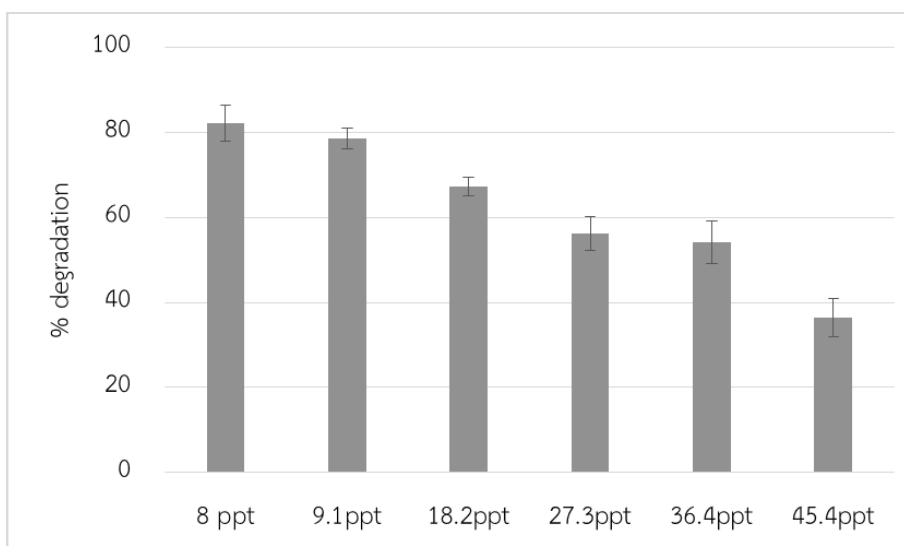

Figure 4. 4 Crude oil degradation (0.25% (v/v)) by strain AO-11 on various salinity concentrations from 8 ppt to 45.4 ppt at pH 7, room temperature (31±1°C) for 10 days. Note, controls were shown in Appendix F.

4.1.2 Capability of *Exiguobacterium* AO-11 on degradation of specific petroleum hydrocarbon compounds

The capability of strain AO-11 on degradation of PAHs and aliphatic compounds was determined with different initial concentration; pyrene (50 ppm), phenanthrene (50 ppm), hexadecane (1000 ppm), tetradecane (1000 ppm) and docosane (100 ppm).

The result showed that short chain *n*-alkane, tetradecane ($C_{14}H_{30}$) was better degraded up to 91.8±0.4% followed by docosane and hexadecane as shown in Figure 4.5. While little degradation of PAHs by strain AO-11 was observed. Final cell density of the experiment was 6.3±4.1 x 10^5, 5.6±2.5 x 10^5, 6.6±1.5 x 10^6, 4±3 x 10^6 and 3.3±1.5 x 10^6 CFU/ml at pyrene, phenanthrene, tetradecane, hexadecane and docosane, respectively. The low activity on degradation of PAHs may be due to low composition of PAHs in the enrichment culture which crude oil was used as substrate for bacterial isolation and PAHs may be toxic to bacterial cell.

Another study demonstrated that *Exiguobacterium aurantiacum* was good in degradation of diesel containing *n*-alkanes (C9–C26) and was also capable of degrading pristane (Mohanty and Mukherji, 2008).

This result accorded with earlier report that strain AO-11 contains many genes which encode for alkane degrading enzymes such as *alkM* (C_{12}-), *alkB*$_1$ (nonspecific), *alkB-1* (C_{13}-C_{23}) and CYP 153 (C_8-C_{16}) (Srisuwankarn, 2015). The high activity on degradation of aliphatic compounds may be due to strain AO-11 was enriched and cultured with Arab light crude oil which contains high composition of aliphatic compounds.

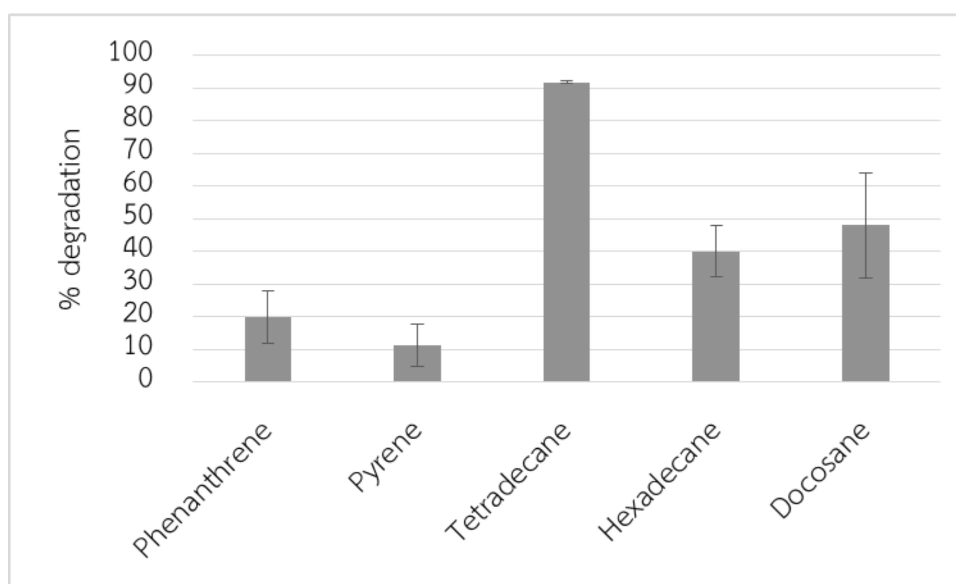

Figure 4. 5 Biodegradation of PAHs and aliphatic compounds by strain AO-11 at pH 7, salinity 8 ppt, room temperature (31±1°C) for 10 days. Note, controls were shown in Appendix F.

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Therefore, *Exiguobacterium* sp. AO-11 is suitable for further application of crude oil degradation. It was then used for developing bacterial liquid formulation for degradation of crude oil.

4.2 Optimization of agro-industrial byproduct media for bacterial cultivation for liquid bacterial formulation preparation

4.2.1 Effect of agro-industrial byproduct concentration on strain AO-11 growth

The growth of strain AO-11 was examined in different concentration media prepared from 2 substrates; coconut meal (milk) residue (CM) and soybean oil mill dry sludge (SB). The results showed that concentration of CM from 5 to 20% could increase

bacterial density as shown in Figure 4.6. The CM concentration of 15 and 20% media could increase high bacterial cell from 6.07±0.06 Log CFU/ml to 8.3±0.1 and 8.4±0.1 Log CFU/mL in 9 hours, respectively. The similar growth trends were observed at 15 and 20% concentration. The growth order of CM media was presented as CM 20% >15% > 10% > 5% > 1%. Similar agro-waste medium of coconut oil cake was reported that could increase *Bacillus thuringiensis* from 50 μg/L to 6.18±0.2 g/L in 72 hours (Poopathi and Archana, 2012). This CM may be rich in nutrient that can enhance AO-11 growth. As it was reported that dried composition of coconut milk residue consists of 51% carbohydrates, 32% dietary fiber, 38% fat, 5% protein, 4% moisture and 2% ash (Bawalan, 2011). Carbohydrate (sugars), protein and lipid are the main energy sources for heterotrophic microorganisms (Baron, 1996).

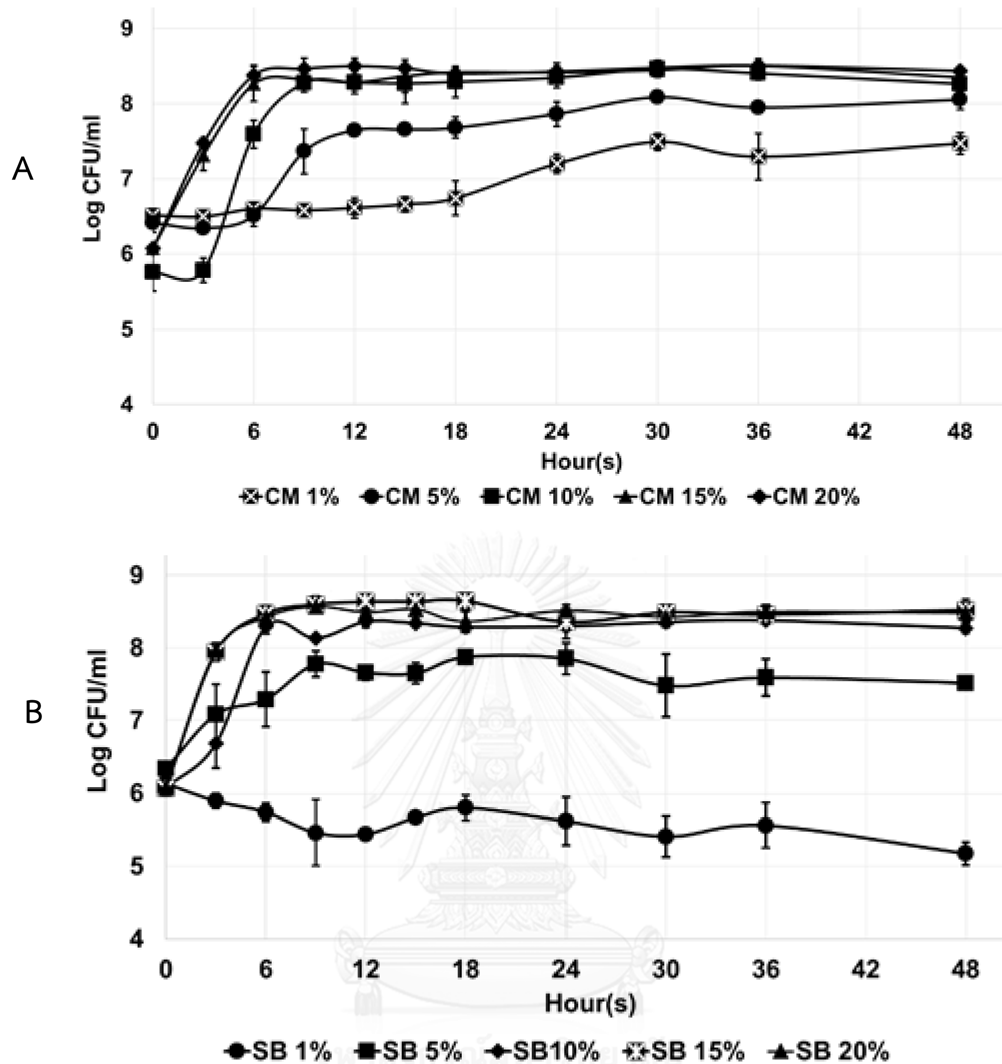

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Figure 4. 6 Growth pattern of *Exiguobacterium* sp. AO-11 produced from different concentration of (A) coconut milk residue (CM) and (B) soybean oil mill dry sludge (SB) in seawater.

Strain AO-11 could also be increased by SB media with high growth trend in SB 15% and 20%. They were able to increase bacterial cell from 6.07±0.06 Log CFU/mL to 8.6±1 and 8.5±0.01 Log CFU/mL, respectively, in 9 hours. The tendency of SB concentration on bacterial growth could be ranked as SB 15% > 20% > 10% > 5% > 1% as shown in Figure 4.6 B. The similar result of soybean residue was also reported by previous study, soybean molasses medium could enhance *Zymomonas mobilis* from 1.0 x 10^6 CFU/mL to 1.1 x 10^7 CFU/mL in 16 hours (Letti et al., 2012). This residues has been reported in biosurfactant production due to high amount of sugar that useful

for promoting microbial growth (Solaiman et al., 2007). Soybean molasses (dry mass) contains 57.3% carbohydrates (include 28.4% sucrose and 18.6% stachyose and 9.68% raffinose), 9.44% proteins and 21.2% lipids (Siqueira et al., 2008). The high quantity composition of sugar, lipids and protein in soybean molasses were able to utilize as nitrogen and carbon source for sustaining bacteria and yeast growth in ethanol production (Letti et al., 2012; Siqueira et al., 2008).

The results showed that coconut milk residue and soybean oil mill dry sludge can be used as carbon and energy sources for bacteria growth in comparison of conventional LB medium 9.1 Log CFU/mL as shown in (Appendix D).

4.2.2 Determination of organic matter and nutrients of optimized agro-industrial byproduct media

The agro-residue media, 15% CM and SB could enhance high growth of AO-11 which means that these substrates may contain valuable nutrient for bacterial cell. To clarify this assumption, chemical oxygen demand of agro-byproduct media was determined to demonstrate total organic matter that may be used for bacterial growth. The 15% CM medium contained higher COD value followed by 15% SB, respectively as shown in Table 4.1.

Then, total protein, sugar and fat were measured as nutrient in available forms to ensure that organic matter indicated available nutrient form for bacterial growth. As previous report revealed that carbohydrate (sugars), protein and lipid are nutritional forms for heterotrophic microorganisms (Baron, 1996). The measurement revealed that 15% CM and SB seawater media contained some amount of protein and fat as shown in Table 4.1. These results indicated that CM and SB contained nutrient in available form for bacterial growth. The SB (15%) seawater medium was therefore selected for further experiments due to it be able to increase high bacterial cell and ease preparation.

Table 4. 1 Total organic matter and available nutrient compositions of agro-industrial byproduct media

Media	COD mg/L	Total nutrients mg/L		
		Fat	Sugar	Protein
15% CM	541±4.24	<10	0	60
15% SB	328±53.74	10	0	50

4.2.3 Effect of seawater concentration and sugarcane molasses concentration on strain AO-11 growth

The diluted concentration of seawater with distilled water (seawater : distilled water) was varied in order to examine the effect of salt concentration on bacterial growth. The seawater concentration was set in the proportion from 4:1, 2:3, 3:2, 1:4 distilled water equal 25 ppt, 19 ppt, 13 ppt and 7 ppt, respectively.. The results showed that the growth of strain AO-11 was maintained at 8.6±0.02 Log CFU/mL in 1:4 diluted seawater in 9 hours as shown in Figure 4.7. This could imply that strain AO-11 was able to grow in variety of salt concentration. It was reported that 1:4 diluted seawater could increase 10% biomass of *Bacillus thuringiensis* in 72 hour at 30°C (Ghribi et al., 2007). In addition, crude oil-degrading bacteria isolated from sediment could grow in variety concentration of salt from 10 to 30 g/L (Liu et al., 2016). This bacterial strain entered to stationary phase after 9 hours in 15% SB medium with diluted and non-diluted seawater. Therefore, 15% SB with 1:4 diluted seawater was selected as bacteria production medium for further experiment in addition of sugarcane molasses as additional substrate to improve bacterial growth.

Sugarcane molasses concentration was varied in 1:4 SW 15% SB medium in order to increase bacterial cell growth. After incubation for 9 hours, bacterial density was increased from 6.6±0.2 Log CFU/mL to 8.9±0.1, 8.9±0.1, 8.8±0.1, 8.8±0.1 and 8.4 Log CFU/mL in the molasses concentration of 5, 10, 20, 30 and 50 g/L, respectively as

shown in Figure 4.8. The result revealed similar bacterial growth pattern with those of without addition of sugarcane molasses and 1:4 SW 15% SB medium.

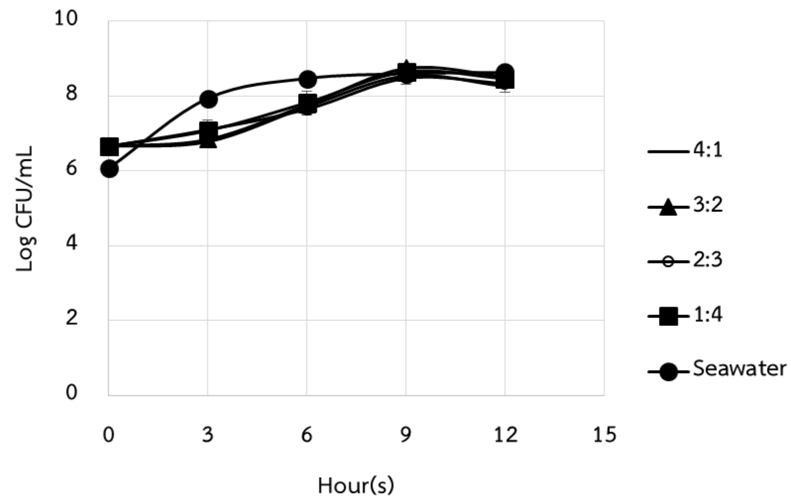

Figure 4. 7 Growth pattern of *Exiguobacterium* sp. AO-11 on variety of diluted seawater concentration in 15% SB. Note, 1:4, 2:3, 3:2, 4:1 are diluted seawater with distilled water (seawater:distilled water).

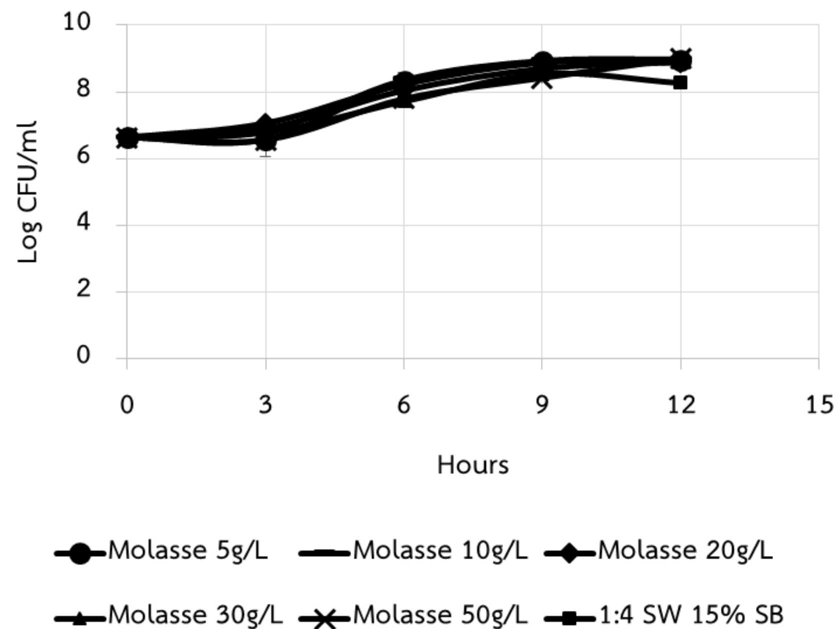

Figure 4. 8 Growth pattern of *Exiguobacterium* sp. AO-11 on variety of sugarcane molasses concentration in 1:4 SW 15% SB medium.

Therefore, in case of cost production and easy preparation 1:4 SW 15% SB medium without molasses was selected as production medium.

4.3 Development of liquid bacterial formulation for crude oil biodegradation

4.3.1 Selection of suitable solution for suspending cell

Phosphate buffer and 1:4 diluted seawater (salinity 7 ppt) were determined the capability to preserve bacterial cell for month due to inexpensive and simple preparation. Phosphate buffer showed higher survival at 86.7±1.4% of bacterial cell compared to that of 1:4 diluted seawater which gave 71.7±1.7% bacterial survival in 30 days as illustrated in Fig 4.9. These results indicated that suspending cell in non-carbon source solution (PB) at room temperature gave the high bacterial survival. This could assume that the metabolic rate of cell was reduced in carbon starving condition, which allows them to survive after a month storage. As previous report shown that phosphate buffer could preserve *Pseudoxanthomonas* sp. RN402 up to 94±1.5% for 30 days at 30°C (Nopcharoenkul et al., 2011).

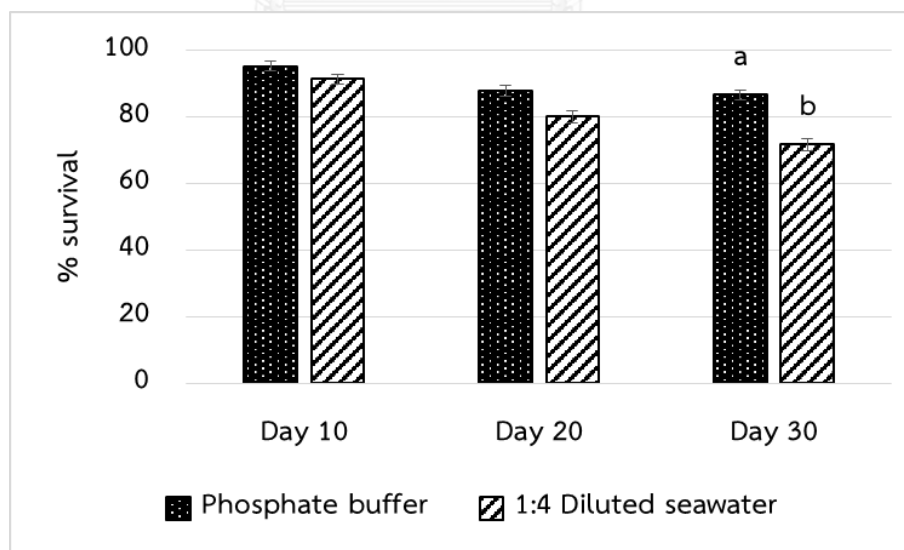

Figure 4. 9 Percent survival of *Exiguobacterium* sp. AO-11 suspended in two solutions at room temperature (26.8±3.6°C). The error bar with alphabets a and b indicate the significant difference between bacterial survival at day 30 at $P<0.05$.

The efficacy of strain AO-11 on crude oil degradation was examined during storage. It has been found that suspended cell in phosphate buffer could retain percentage of crude oil degradation more than 80% in 10 days as illustrated in Table 4.2. Moreover, the results indicated that stored AO-11 had higher efficacy than that of fresh cell preparation before storage. The starvation phenomenon could be used to explain this result. It has been shown that survival of cells was maintained by starving carbon source of a bacterial inoculum, and it also promoted the ability of bacteria to degrade pollutant (Watanabe et al., 2000). Therefore, the improvement of the stored AO-11 efficacy might result from the carbon starvation conditions of bacteria. This result is in agreement with previous study which indicated that phosphate buffer could preserve *Pseudoxanthomonas* sp. RN402 survival and retain degradation of pyrene 93.9 ± 9.2%, diesel 89.02±12%, crude oil 83.2± 6.8%, *n*-tetradecane 92.5±1.1% and *n*-hexadecane 65.5±5% (Nopcharoenkul et al., 2011, 2013).

Table 4. 2 Crude oil degradation (0.25% (v/v)) by strain AO-11 after 30-day storage in different suspending solution at room temperature for 10 days.

Storage time (days)	Crude oil degradation (%)	
	Cell suspended in phosphate buffer	Cell suspended in 1:4 Seawater
0	84.5±6.5	84.5±6.5
10	94.8±4.4	72.8±1.5
20	88.1±8.5	88.8±0.4
30	89.4±3	87.4±1.8

4.3.2 Protective agent selection in different temperature for liquid bacterial formulation

Three protective agents including glycerol (Gly), polyvinylpyrrolidone (PVP) and polyethylene glycol 6000 (PEG) with two concentrations (1 and 5%) were added into

phosphate buffer in attempting to increase AO-11 survival in liquid formulation in 4°C, 25°C, 30°C and room temperature (31±1°C). The results of survival of AO-11 in all liquid formulation were shown in Figure 4.10. At 4°C, all liquid formulation could preserve bacterial survival more than 80% after 30 days. Similar result was reported in previous study that the viability of *Acetobacter diazotrophicus* L1 and *Herbaspirillum seropedicae* J24 liquid inoculants with gum arabica (5% w/v) and PEG 300 (5% w/v) maintained 80% and 76% at 4°C after 7 months, respectively (Nita et al., 2012).

In contrast, survival of AO-11 was lower than 60% at 25°C and 30°C in 30 days in phosphate buffer supplemented with Gly (1%) while bacterial survival in phosphate buffer supplemented with PB, PVP (1%), PEG (1% and 5%) were higher than 60%. The result indicated that low bacterial survived in high temperature in 25°C, 30°C and room temperature compared to 4°C. Earlier report also revealed that temperature is one of the major factors that effects on bacterial survival in liquid formulation. For example, liquid formulations of *Rhodopseudomonas palustris* PS3 stored at 4°C could sustain cell survival more than 70% in one month while bacterial survival decreased less than 60% at 25°C (Lee et al., 2016). This phenomenon may be clarified that low temperature may inhibit microbial metabolism which can lead to high survival. The increase of temperature or unfavorable conditions may increase bacterial metabolic activity resulted in accumulation of microbial wastes or toxins (Lee et al., 2016). Moreover, oxidative stress and high cell concentration can induce a rapid toxic compounds accumulation (Patiño-Vera et al., 2005).

The result showed that bacteria in liquid formulations in phosphate buffer supplemented with PEG (1%) and PVP (1 and 5%) were the highest survival in 30°C and room temperature. The similar result has been reported previously that liquid inoculant containing PVP and PEG could support the survival of *Bradyrhizobium japonicum* USDA110 and *Azorhizobium caulinodans* IRBG23 cell concentration higher than 10^8 cells/mL up to 5 months (Tittabutr et al., 2007). The additive agents including PVP and PEG are cryoprotective additives grouped in cryoprotective additives not penetrating even cell wall; they can be adsorbed on the microbial surface where they form a viscous layer, cause partial efflux of water from the cell (Hubálek, 2003). These

osmoprotectants are able to prevent the influx of water that causes cells to burst, thus protecting against the harmful effects of pore-forming antimicrobials and the excessive viscosity PEG solutions could hinder bacterial growth (Smith et al., 2015). Polyvinylpyrrolidone may reduce enzyme dehydrogenase activity lead to slow down microbial activity as dextran as macromolecular crowder (Schneider et al., 2015).

Furthermore, polyvinylpyrrolidone could protect cells against toxic factors and has property as colloidal stabilization which protects the bacteria in colloids (Surendra and Baby, 2016). Both of these polymeric additives are soluble in water and in other polar solvents; their capacity to bind polar and hydrophobic molecules, function as complexing agents can reduce toxicity of compounds and could be used to create high osmotic potential in liquids (Dayamani and Brahmaprakash, 2014). Their osmoprotectant properties, high water binding capacity and viscous nature may be slow the drying process of the bioinoculants (Kumaresan and Reetha, 2011). Based on result and price of substance, polyethylene glycol was selected for further experiment in extension storage of liquid bacterial formulation.

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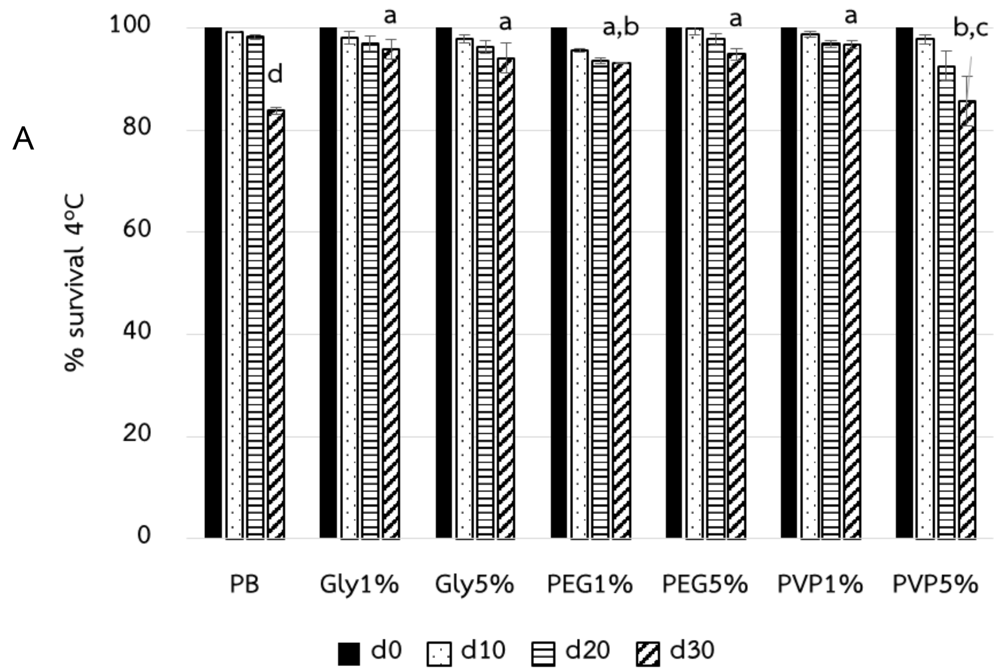

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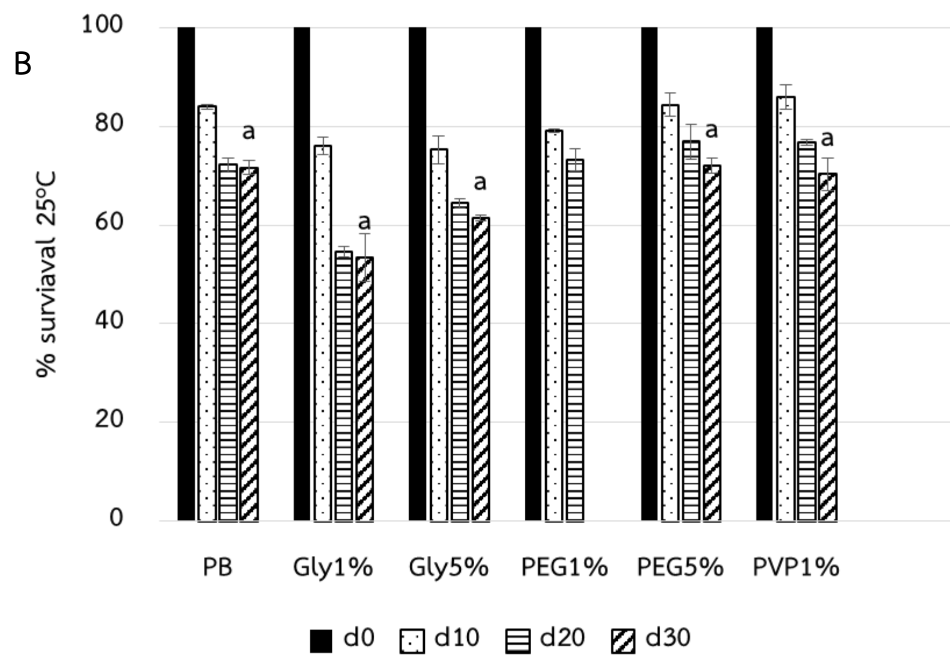

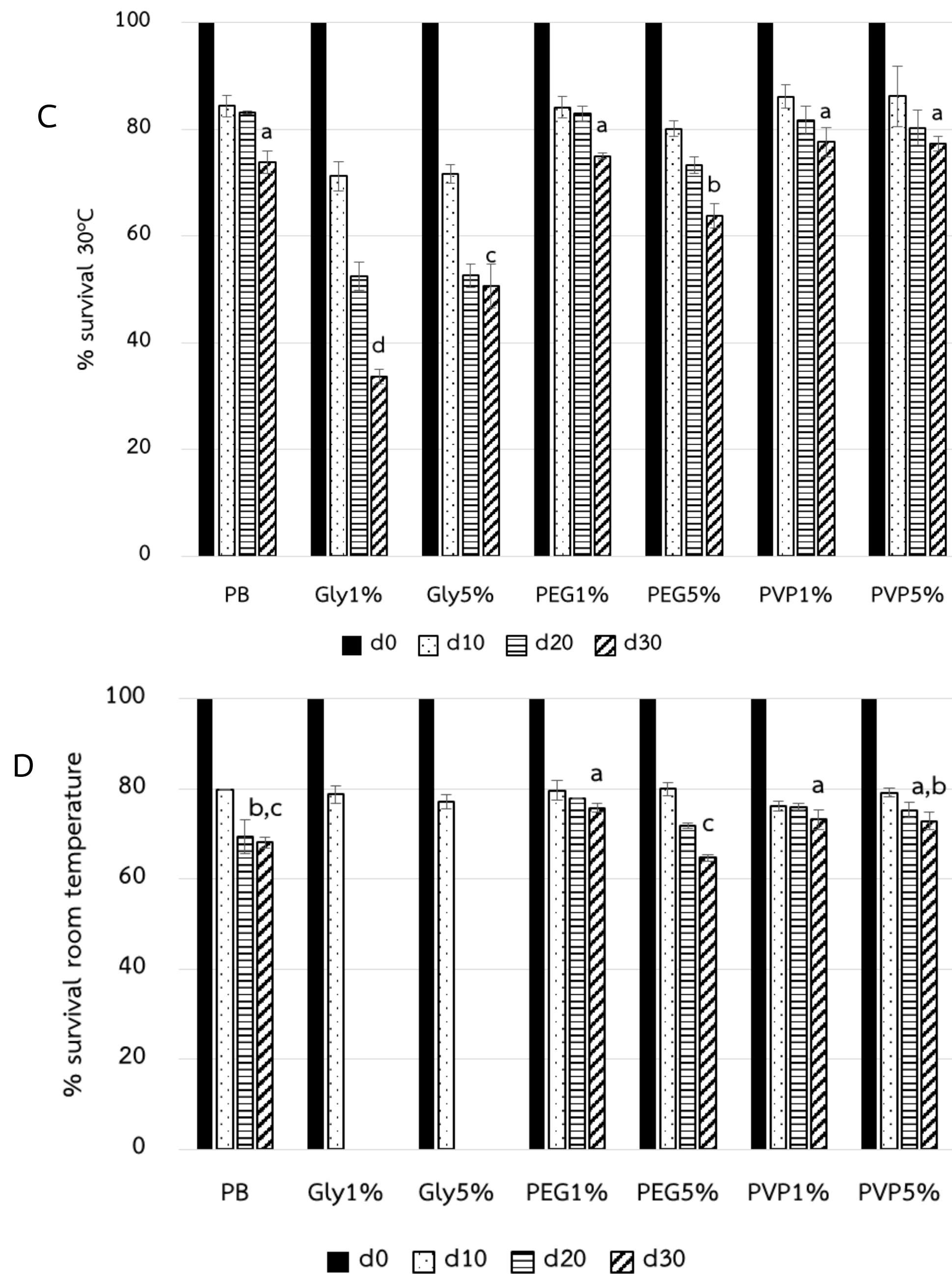

Figure 4. 10 Percent survival of AO-11 in liquid formulation with PB, and 1 and 5% Gly, PEG and PVP for 30 days: A. 4°C, B. 25°C, C. 30°C and D. room temperature. Without column is contamination. The error bar with alphabets a, b and c indicate the significant difference between bacterial survival at day 30 at P<0.05.

4.3.3 Extension of stored liquid bacterial formulation for crude oil degradation

4.3.3.1 Extension of stored liquid bacterial formulation

The liquid formulation of AO-11 with PEG (1%) and PB as controls in 4 and 30°C were conducted to determine bacterial survival in 60 days. The results showed that survival of strain AO-11 was 77.7±1.4% and 72.4±0.2% at 4°C in PB and PEG (1%), respectively as shown in Figure 4.11. While survival at 30°C was lower than that stored in low temperature 58.3±1.8% and 58.7±0.7%, respectively. It can be explained that cold temperature is great in protection bacterial survival. The different result was observed in PEG (1%) and PB in the short extension which PEG (1%) may be inappropriate for protecting strain AO-11 in liquid formulation for long term storage.

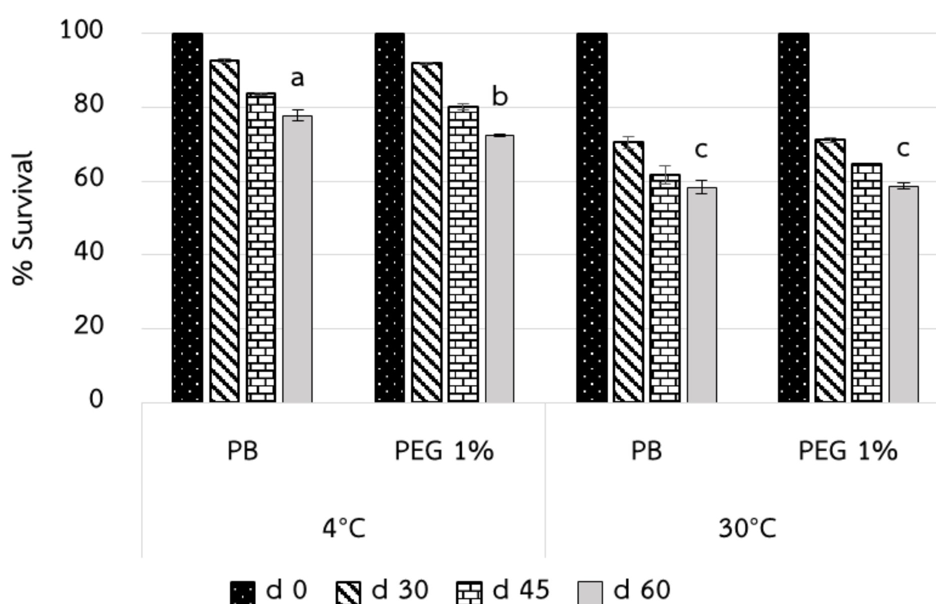

Figure 4. 11 Percent survival of AO-11 in liquid formulation with PB and PEG (1%) at 4°C and 30°C for 60 days. The error bar with alphabets a, b and c indicate the significant difference between bacterial survival at day 60 at $P<0.05$.

4.3.4 Cost analysis and comparison of liquid bacterial formulation

The cost of liquid bacterial formulation with PEG (1%) was calculated based on method described in Nopcharoenkul et al. (2011). The cost of one liter of AO-11 liquid

formulation with cell concentration 10^{10} CFU/mL was 68.22 Baht as shown in Table 4.3.

Production cost of AO-11 liquid formulation was assessed in the price table of products in market and in other studies for petroleum hydrocarbon degradation as shown in Table 4.4. It revealed that our liquid formulation is cheap in cost production. The similar cost production was observed in previous study from Nopchaleunkul et al. (2011).

This can indicate that our bacterial liquid formulation is low cost production in laboratory scale while has capability to degrade crude oil. This information suggested that further large scale and commercial production may be possible.

Table 4. 3 Detail cost of one liter of AO-11 liquid formulation with cell concentration 10^{10}CFU/ml.

List		Quantity	Unit	Baht/Unit	Total (Baht)
Medium	Distilled water	25	Liter	1	25
	Electricity	1.05	Kwh	2.8	2.94
Distilled water		1	Liter	1	1
K_2HPO_4		2.7	g	1.6	4.32
KH_2PO_4		4.658	g	1.6	7.4528
PEG 6000		10	g	0.243	2.43
Electricity		3.6	Kwh	2.8	10.08
Plastic bottle		1	piece	15	15
Total					68.2228

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Table 4. 4 Cost comparison of AO-11 liquid formulation with commercial products and other study.

Bacterial formulation	Bacteria	Type of petroleum degradation	Price (Baht)	References
Powder (Petro-Clear F10)	Consortium	Benzene, diesel, lubricant and BTEX contaminated Soil and water	21,300 Baht/25 ponds	(Nichiporowich, 2011)
Liquid (Liquid Remediact MT)	Consortium	Oil contaminated Soil and water	1,311 Baht/L	(Envirologic, 2011)
Liquid	*Pseudoxanthomonas* sp. RN402	pyrene-contaminated soil	62.88 Baht/L	(Nopcharoenkul et al., 2011)
Liquid	*Exiguobacterium* sp. AO-11	Crude oil contaminated seawater	68.22 Baht/L	This study

4.4 Determination capability of liquid bacterial formulation on crude oil biodegradation in seawater

The capability of liquid bacterial formulation on crude oil biodegradation in seawater was examined in 250 mL flasks. The salinity and pH determination of collected seawater were 31 ppt and 7.63, respectively. As previous study reported about seawater's properties in the central Gulf of Thailand showed that salinity was 32.66 ppt, pH was 8.07 and dissolved oxygen was 5.43 mg/L (Jusiripongkul et al., 2007). The results revealed that AO-11 with initial concentration ($3\pm2 \times 10^7$ CFU/ml) could degrade 0.25% (v/v) crude oil up to 67.2±0.4% with final cell density $2.3\pm1.5 \times 10^6$ CFU/ml . While degradation of crude oil was high at 84.9±8.6% by the strain AO-11 with initial native (7.6±2.5 x 10 CFU/ml) microbial seawater (AO-11+ SW) in 10 days,

and final cell density of seawater microbial increased up to 8.6±2 x 10^6 CFU/ml with strain AO-11 2.6±1.5 x 10^6 CFU/ml.

At crude oil concentration of 0.5% (v/v), strain AO-11 (3±2 x 10^7 CFU/ml) with sterilized seawater (AO-11+ STSW) could degrade 39±2.7% and 61±5.2% of initial crude oil in 10 and 15 days with final cell density 5.6±2 x 10^6 and 8.6±2 x 10^7 CFU/ml, respectively. While higher crude oil degradation was observed in the experiment containing strain AO-11 with seawater (30 days stored AO-11+SW) up to 83.5±12% and 98.3±0.8% of crude oil in 10 and 15 days as shown in Figure 4.12. The final cell density of native seawater microorganisms increased up to 9.6±4 x 10^6 CFU/ml with strain AO-11 2.3±1.1 x 10^6 CFU/ml in 10 days, while these bacterial density decreased to 2.5±0.5 x 10^4 CFU/ml with AO-11 4.6±2 x 10^4 CFU/ml at 15 days in 0.5 % (v/v) crude oil degradation by strain AO-11 with seawater (30 days stored AO-11+SW). Biodegradation of crude oil by native seawater microorganisms showed that it could degrade 54.8±15.2% and 74±4.5% with final cell density 1.1±0.3 x 10^7 CFU/ml and 7.3±1.5 x 10^5 CFU/ml in 10 and 15 days, respectively. The results indicated that AO-11 and seawater microorganisms have effective capability to degrade crude oil in seawater.

This phenomenon observed in the experiment containing both strain AO-11 and native seawater microorganisms that gave high activity on degrading crude oil may be due to native seawater microorganism could help to enhance crude oil degradation with final seawater microbial concentration 9.6±4 x 10^6 CFU/ml and 2.5±0.5 x 10^5 CFU/ml in 10 and 15 days respectively. As previous study reported that the synergistic relationship between *Dietzia cinnamea* KA1 and *Dietzia cinnamea* AP in consortium could degrade crude oil components, including poisonous and carcinogenic compound in a short time (Kavyanifard et al., 2016). It has been prior reported that around 25 marine bacterial genera are classified as hydrocarbon degrading bacteria that have the efficacy for petroleum biodegradation ranged from 0.003% to 100% (Das and Chandran, 2011). Therefore, seawater sample might contain crude oil degrading bacteria that could enhance crude oil degradation.

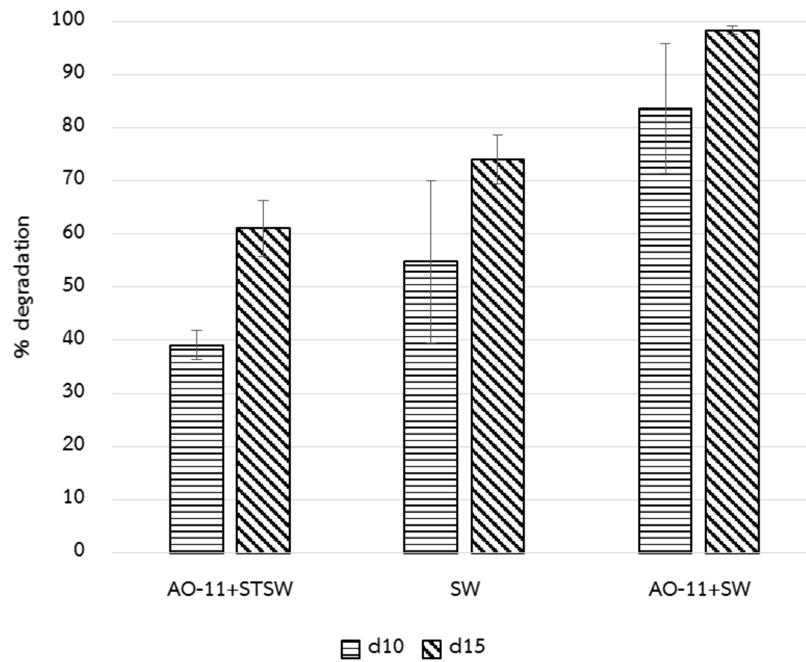

Figure 4. 12 Degradation of 0.5% (v/v) crude oil in seawater by 30-day stored AO-11 in 10 and 15 days. Controls were shown in Appendix F. Note, STSW: sterilized seawater, and SW: non-sterilized seawater.

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CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

In this study, *Exiguobacterium* sp. AO-11 could degrade crude oil in different environmental conditions including pH, salinity, temperature and crude oil concentration. Even though, percent degradation of crude oil in different environmental factors are distinct which mean that these factors have impact on bacterial degradation process. Strain AO-11 could also degrade specific aliphatic compounds which are the main component of Arab light crude oil. This strain was suitable for using as bioremediation agent for crude oil degradation as ready to use bacterial formulation.

Agro-industrial wastes, soybean oil mill dry sludge (SB) 15% (w/v) with 1:4 diluted seawater was utilized as alternative source for low cost cultivation medium to increase AO-11 cell density in order to decrease production cost. The SB production medium contains some amount of protein which can be used for bacterial growth and it also easier preparation among three agro-industrial wastes including coconut milk residue (CM) and soybean oil mill dry sludge (SB).

For liquid formulation development, phosphate buffer was the appropriated solution for preserving high AO-11 survival in one month than that of 1:4 diluted seawater. The stored AO-11 suspension could efficiently degrade crude oil in 10 days. To improve higher survival of AO-11, three protective agents including PVP, PEG and GLY with 1% and 5% concentrations were added into phosphate buffer and storage in different temperatures. High bacterial survival was observed at low temperature (4°C). Protectant, PEG 1%, was selected as additive protective agent in liquid bacterial formulation due to inexpensive and could preserve high survival cell for prolonged storage. The production cost of liquid formulation was low at laboratory level. Further development for large scale production and commercial application are then possible.

The stored liquid formulation could degrade crude oil in seawater. Low cost liquid bacterial formulation was developed in this study and its crude oil degradation could decrease toxic of crude oil to microorganisms in seawater.

5.2. Recommendations

1. *Exiguobacterium* sp. AO-11 could highly degrade crude oil at concentration 0.25% (v/v) but the degradation was decreased in higher concentrations. This phenomenon was also occurred in extreme environmental conditions as shown in this study. Therefore, this strain should be used in suitable condition to receive desirable results. The combination of AO-11 with other PAH degrading stains as consortium is recommended in order to completely degrade many types of petroleum hydrocarbon and its products.

2. Production medium may be modified by adding nitrogen sources or other substance to increase higher cell concentration. The optimized conditions of cultivation medium on shaker speed, pH and temperature should be performed for AO-11 growth.

3. The survival of AO-11 in liquid formulation with PEG 1% protective agent was lower than formulation without protective addition similar in 60 days. Other protective agents should be apply such as trehalose, glycerol polyvinyl alcohol, gum arabic, sucrose and carboxymethyl cellulose for improvement of AO-11 survival and storage time should be extended more than 6 months in order to get the most suitable protective agent for preserving AO-11 in long term storage.

4. *Exiguobacterium* sp. AO-11 may be applied as other formulation such as immobilization and other formulation which may be reusable for long time degradation. The application of AO-11 formulation on degradation of other oil contaminated samples should be conducted for determination efficacy of this strain in various oil contaminated environments such as soil and fresh water.

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APPENDIX

APPENDIX A

Medium preparation

Nutrient seawater medium (NSW)

Dipotassium potassium hydrogen phosphate (K_2HPO_4)	0.02g
Ammonium nitrate (NH_4NO_3)	1g
Ferric citrate	0.02g
Yeast extract	0.5g
Seawater	200 mL
Distilled water	800 mL

It was mixed gently and sterilize by autoclaving with pressure 15 lb/inch^2 at 121 °C for 15 minutes.

Marine Broth (MB)

Zobell Marine Broth 2216	42.5g
Distilled water	1000 ml

It was mixed gently and sterilize by autoclaving with pressure 15 lb/inch^2 at 121 °C for 15 minutes.

LB broth

Yeast extract	5g
Tryptone	10g
NaCl	5g
Distilled water	1000 ml

It was mixed gently and sterilize by autoclaving with pressure 15 lb/inch^2 at 121 °C for 15 minutes.

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LB agar

LB broth	1000 ml
Agar	20g

It was mixed gently and sterilize by autoclaving with pressure 15 lb/inch^2 at 121 °C for 15 minutes.

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APPENDIX B

Chemical preparation

Phosphate Buffer (PB) 0.05 M pH 6.6

- Solution A

Dipotassium potassium hydrogen phosphate (K_2HPO_4) 8.709g

Distilled water 1000 ml

Mix gently

- Solution B

Potassium dihydrogen phosphate (KH_2PO_4) 6.8045g

Distilled water 1000 ml

Mix gently

Then solution A 381 ml and 619 ml of solution B were mixed gently and sterilized by autoclaving with pressure 15 lb/inch^2 at 121 °C for 15 minutes.

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NaCl solution (1%)

NaCl 10g

Distilled water 1000 ml

Solution was sterilized by autoclaving with pressure 15 lb/inch^2 at 121 °C for 15 minutes.

Resazurin solution

Dissolving buffer

Solution A

Potassium dihydrogen phosphate ($KH_2PO_4.H_2O$)	13g
Dipotassium potassium hydrogen phosphate ($K_2HPO_4.H_2O$)	8.2g
Sodium acetate	2g
Deionized water	1000ml

Dissolving buffer was mixed gently and sterilized by autoclaving with pressure 15 lb/inch^2 at 121 °C for 15 minutes.

Aliquot solution to dissolve 2g of glucose. Filter glucose solution back to solution A through filter paper pour size 0.45 μm.

Resazurin dye solution

Resazurin	0.005g
Dissolving buffer	100ml

Note that resazurin dye solution has to be prepared freshly before use.

1N NaOH

NaOH	4g
Deionized water	100 ml

70% Ethanol

99% Ethanol	700 ml
Sterilized deionized water	300 ml

PAHs preparation (10.000 ppm)

PAH	500 mg
Dimethylformamide	50 ml

It was mixed gently and filtered through 0.22 μm PTFE filter. Then it was stored at -20°C.

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Docosane preparation

Docosane 500 mg

Dichloromethane 50 ml

It was mixed gently and filtered through 0.22 µm PTFE filter. Then it was stored at -20°C.

APPENDIX C

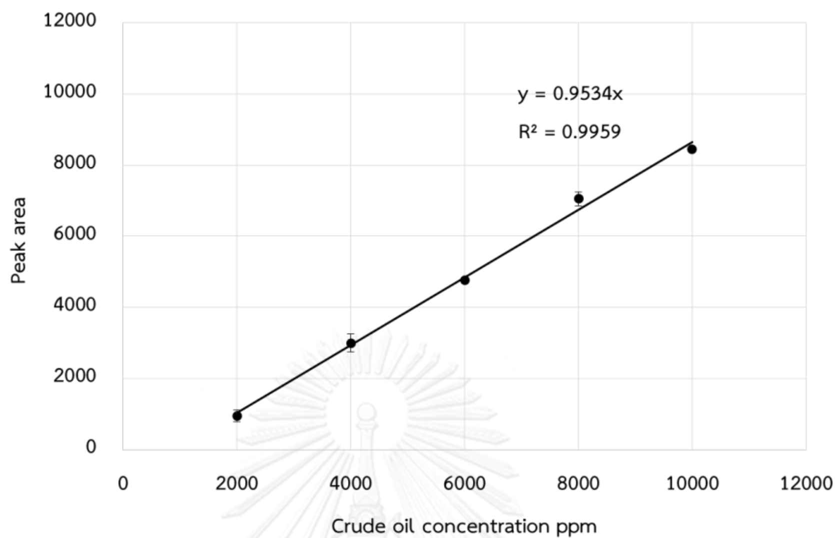

Figure C.1 Standard curve of Arab crude oil from GC-FID. Each data point was averaged from triple spot on chromatorods.

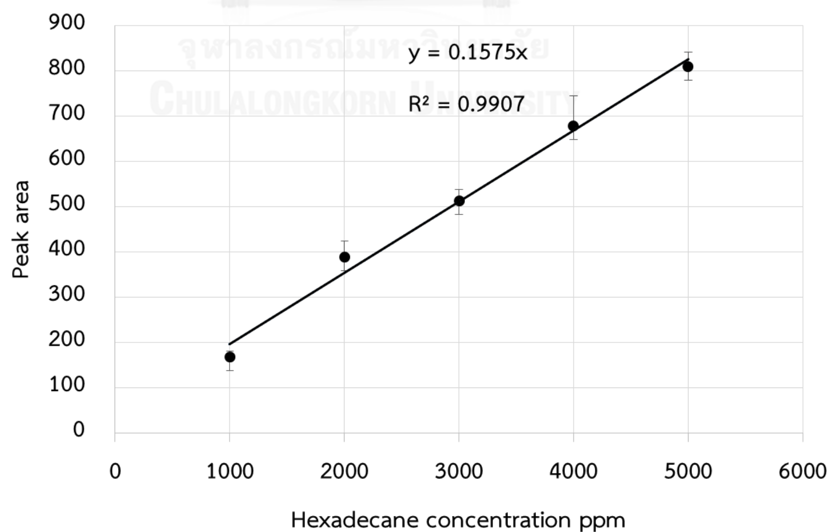

Figure C.2 Standard curve of hexadecane from GC-FID. Each data point was averaged from triple spot on chromatorods.

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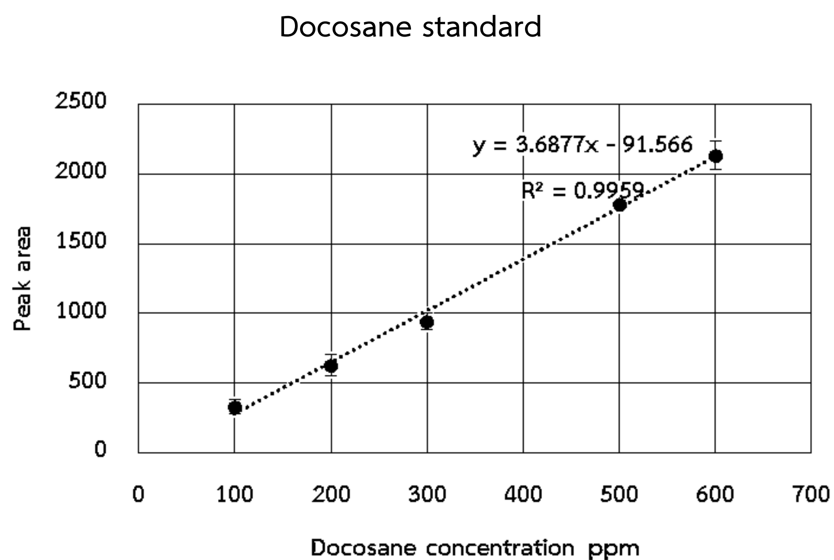


Figure C.3 Standard curve of docosane from GC-FID. Each data point was averaged from triple spot on chromatorods.

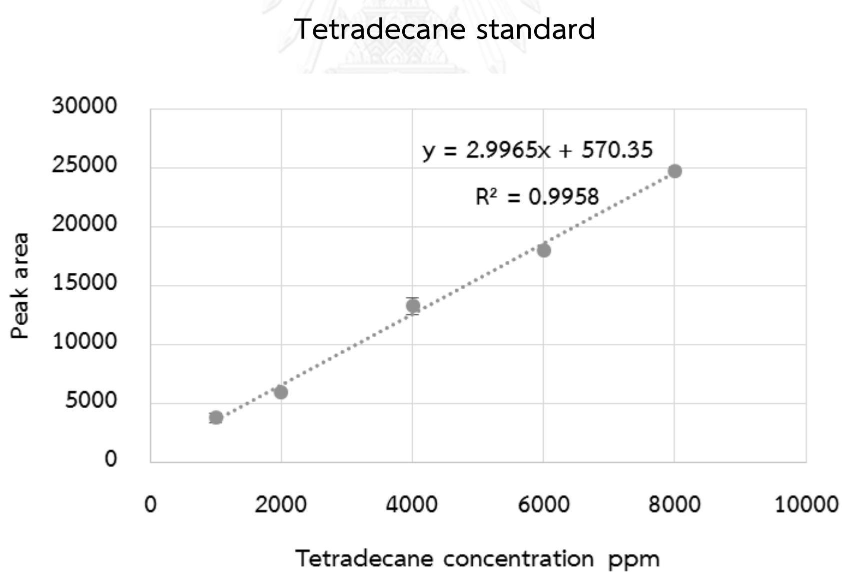


Figure C.4 Standard curve of tetradecane from GC-FID. Each data point was averaged from triple spot on chromatorods.

APPENDIX D

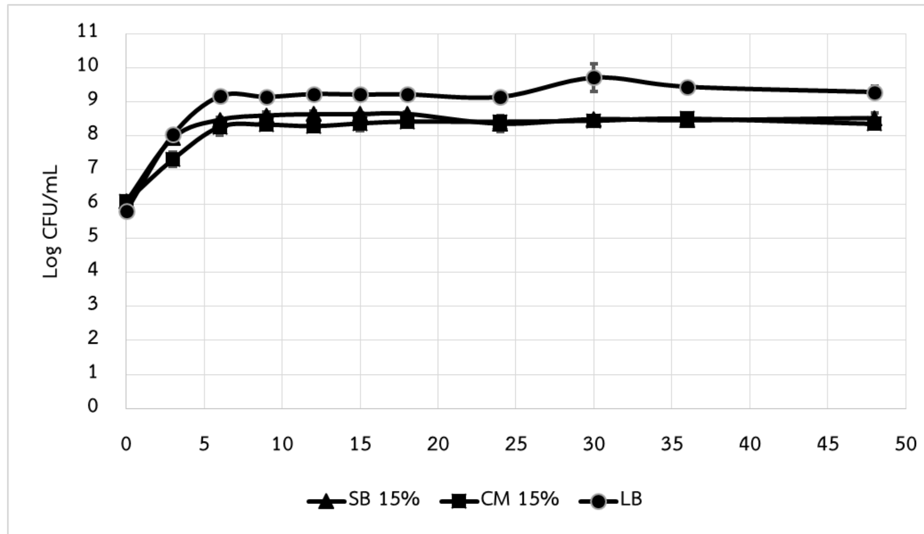


Figure D.1 Comparison of CM and SB with LB medium. Each data point was averaged from triplicate samples.

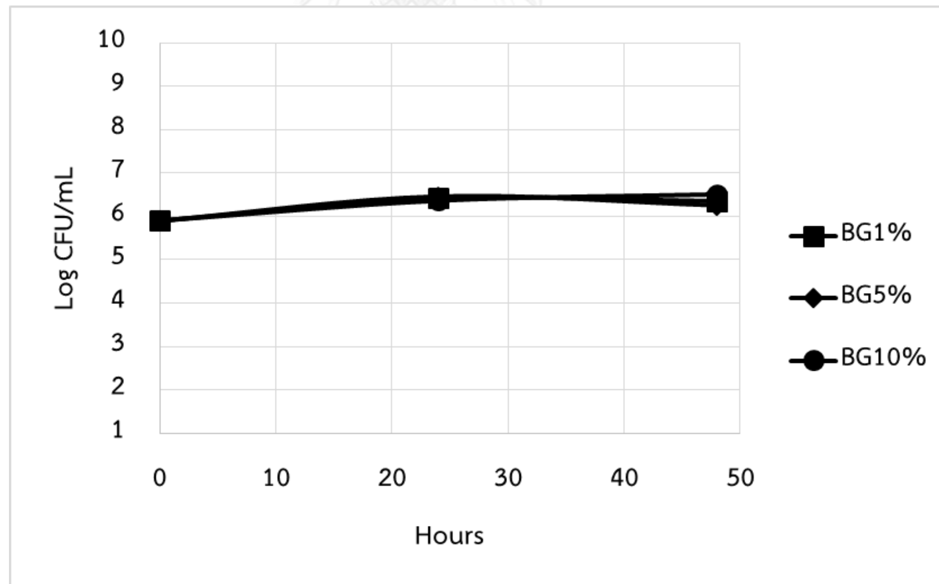


Figure D.2 Growth pattern of *Exiguobacterium* sp. AO-11 produced from 3 concentrations of bagasse.

APPENDIX E

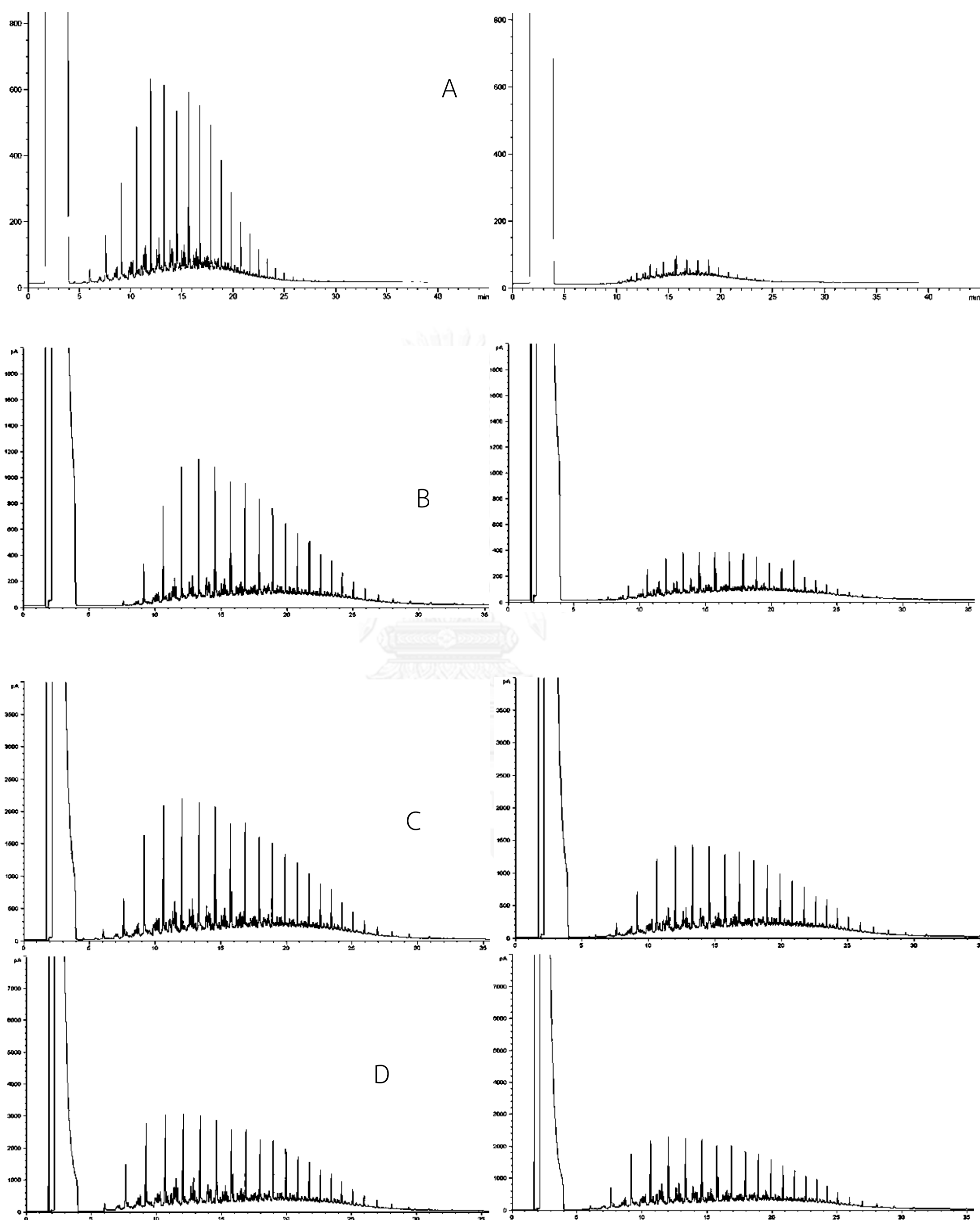
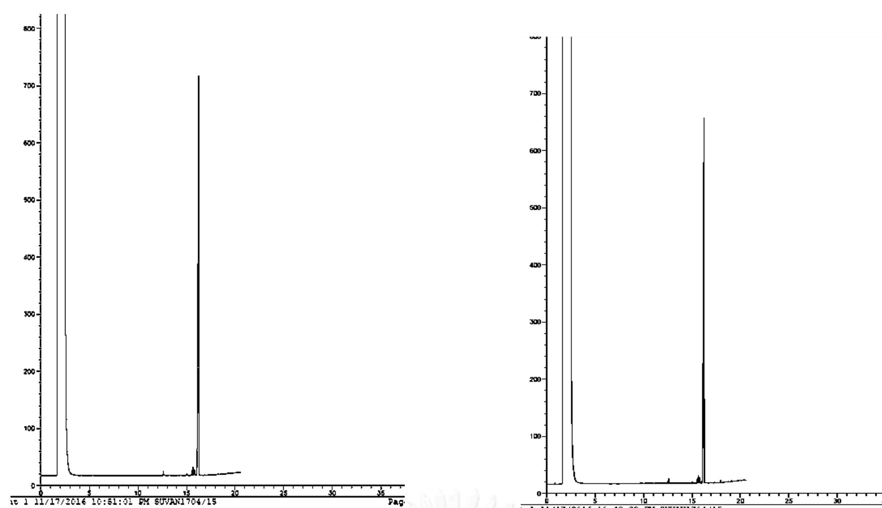
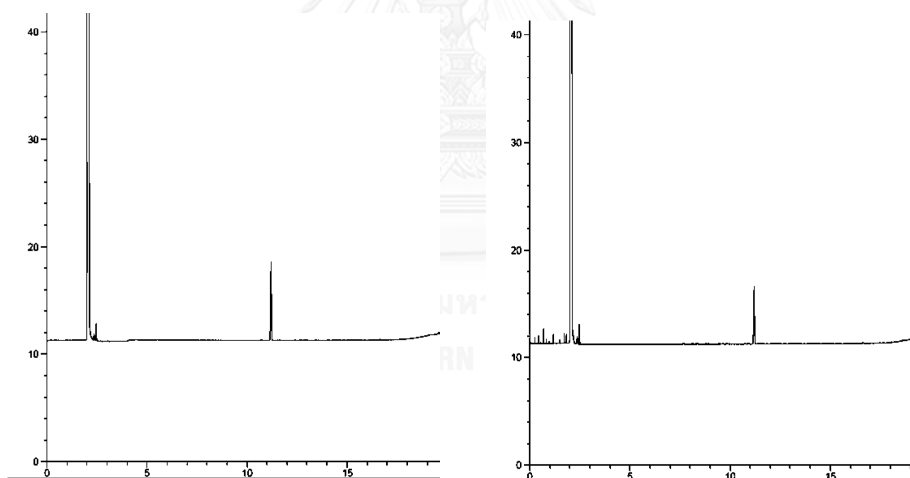


Figure E.1 Chromatograms of degradation of variety concentration of crude oil GC-FID (OILSTD method). A: 0.25% (v/v), B: 0.5% (v/v), C: 1% (v/v) and D: 1.5% (v/v). Note: left side chromatogram is a control in each concentration.



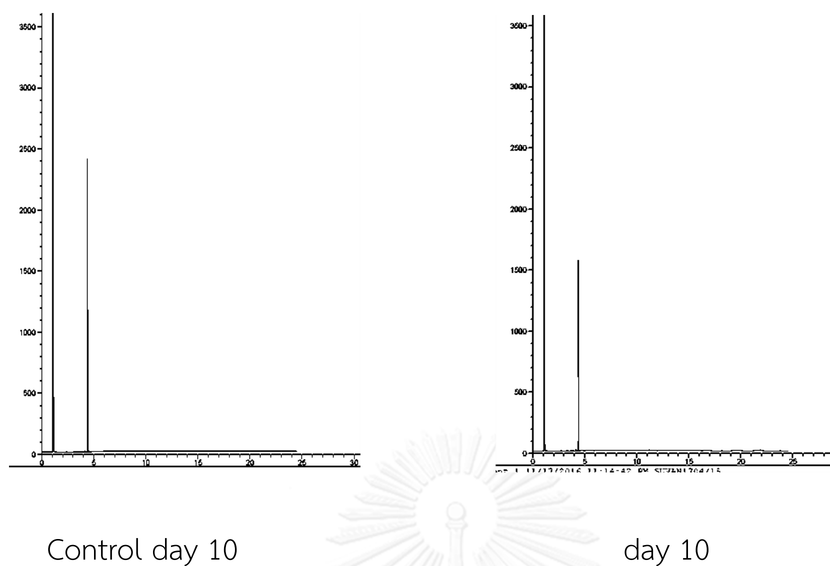
Control day 10 day 10

Figure E.2 Chromatograms of degradation of pyrene in 10 days GC-FID (OILSTD method).



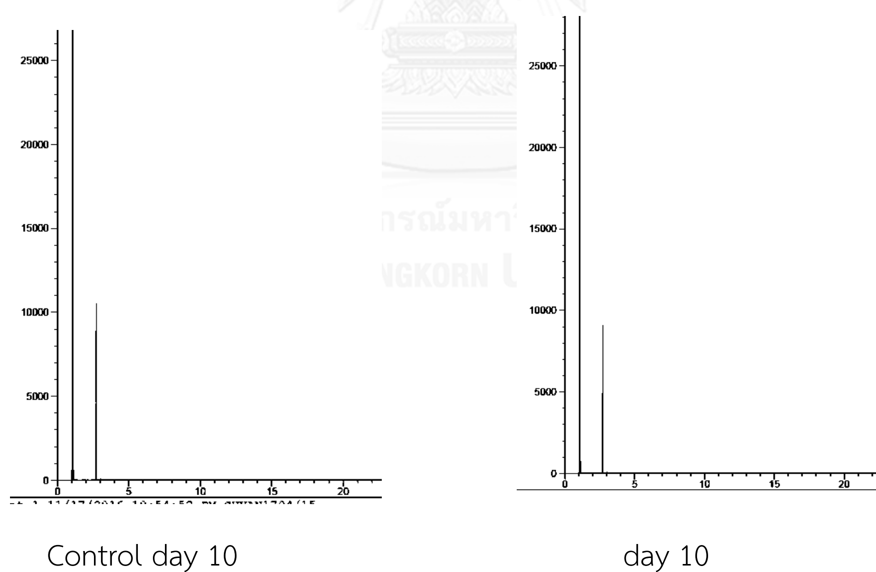
Control day 10 day 10

Figure E.3 Chromatograms of degradation of phenanthrene in 10 days GC-FID (OILSTD method).



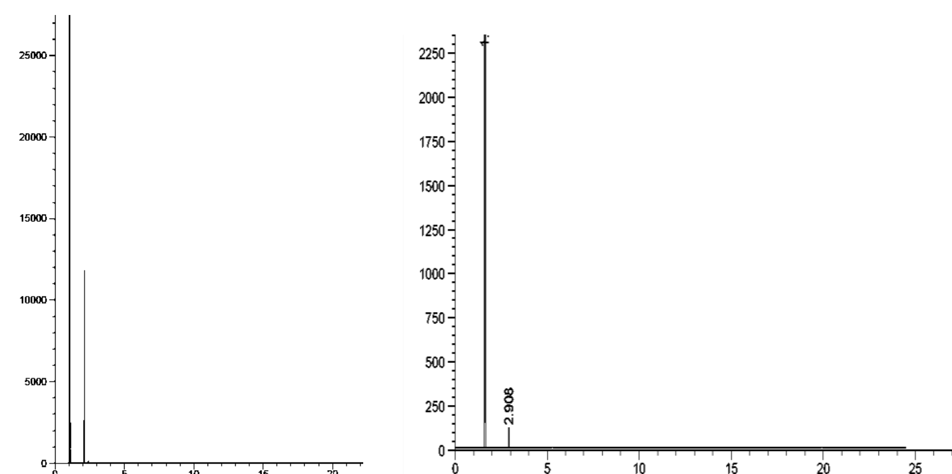
Control day 10 day 10

Figure E.4 Chromatograms of degradation of docosane in 10 days GC-FID (OILSTD method).



Control day 10 day 10

Figure E.5 Chromatograms of degradation of hexadecane in 10 days GC-FID (OILSTD method).



Control day 10 day 10

Figure E.6 Chromatograms of degradation of tetradecane in 10 days GC-FID (OILSTD method).

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APPENDIX F

Raw Data

Table F.1 Tetradecane degradation by strain AO-11 for 10 days

	Tetradecane d10 degradation			
sample	Control d0	d10	Control d10	% degradation
1	120086.5	9915.2	114146.4	91.313
2	118561.3	8637.4	105836.4	91.838
3	112631.7	7873.7	102164.4	92.293
Mean				91.815
SD				0.490

Table F.2 Docosane degradation by strain AO-11 for 10 days

	Docosane d10 degradation			
sample	Control d0	control d10	d10	% degradation
1	1803.52	1736.92	699.87	59.706
2	2025.93	1965.04	1250.3	36.372
3	2136.34	2053.95	1067.99	48.003
Mean				48.039
SD				16.499

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Table F.3 Hexadecane degradation by strain AO-11 for 10 days

	Hexadecane degradation			
sample	Control d0	d10	control d10	% degradation
1	192502.5	109513.7	183066.4	40.178
2	202815.9	1.19131.4	181766.4	34.459
3	290817.2	150050.4	275547.4	45.544
Mean				40.001
SD				7.838

Table F.4 Pyrene and phenanthrene degradation by strain AO-11 for 10 days

	Pyrene degradation					Phenantrene degradation		
	Control d0	control d10	d10	% degradation	Control d0	d10	control d10	
d10-1	4235.44	5007.54	4184.98	16.426	27.13553	19.149	26.055	26.506
d10-2	4235.44	4397.1	3795.07	13.691	21.11348	15.353	20.072	23.509
d10-3	4235.44	3795.07	3641.88	4.036	21.75218	18.988	21.374	11.164
Mean				11.384				20.393
SD				6.509				8.132

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Table F.5 Crude oil degradation by strain AO-11 on various pH 6 to 9

pH			pH6		pH7		pH8		pH9	
	Control d0	Control d10	d10	% degradation	d10	% degradation	d10	% degradation	d10	% degradation
	860.806	845.42	275.42	72.07	102.89	86.964	372.36	62.24	527.85	37.563
	802.721	789.35	248.9	68.456	199.759	79.746	359.66	57.456	567.215	28.141
	1003.72	986.27	259.76	69.273	172.15	79.637	279.2	64.62	526.923	46.574
Average				69.935		82.115		61.443		37.42
SD				1.897		4.199		3.652		9.217

Table F.6 Crude oil degradation by strain AO-11 on various salinity concentration

Salinity		10 ppt		20 ppt		30 ppt		40 ppt		50 ppt	
	Control d10	d10	% degradation	d10	% degradation	d10	% degradation	d10	% degradation	d10	% degradation
	845.42	185.09	78.10	278.73	67.03	370.28	53.09	426.86	49.50	290.36	39.72
	789.35	185.57	76.48	273.53	65.34	385.99	60.86	364.25	53.85	509.61	33.30
	986.27	185.84	81.15	299.96	69.58	382.81	54.71	400.04	59.43	657.77	36.51
Average			78.58		67.32		56.22		54.26		36.51
SD			2.36		2.13		4.09		4.97		4.53

Table F.7 Crude oil degradation by strain AO-11 on various temperature

Temperature		25			30			37	
	Control d10	d10	% degradation	Control d10	d10	% degradation	Control d10	d10	% degradation
	977.90	391.71	59.94	845.42	102.89	86.96	838.42	204.09	75.65
	986.27	486.66	50.65	789.35	199.75	79.74	794.35	197.64	75.11
	982.08	439.19	55.27	986.27	172.15	79.63	986.27	209.57	78.75
Average			55.29			82.11			76.50
SD			4.64			4.19			1.96

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Table F.8 Preliminary test of SB and BG

Hour	0	24	48
SB10%	5.845	8.23	8.079
	6.008	8.041	8.255
	5.845	8.146	8.278
average	5.899333	8.139	8.204
SD	0.094108	0.094694	0.108862
BG 1%	5.845	6.38	6.204
	6.008	6.462	6.447
	5.845	6.397	6.397
average	5.899333	6.413	6.349333
SD	0.094108	0.043278	0.128321
BG 5%	5.845	6.414	6.23
	6.008	6.431	6.255
	5.845	6.531	6.278
average	5.899333	6.458667	6.254333
SD	0.094108	0.063217	0.024007
BG 10%	5.845	6.342	6.643
	6.008	6.38	6.462
	5.845	6.361	6.414
average	5.899333	6.361	6.506333
SD	0.094108	0.019	0.120766

Table F.9 Growth of *Exiguobacterium* sp. AO-11 from different concentration of soybean oil mill dry sludge (SB).

SB 1%											
Hour	0	3	6	9	12	15	18	24	30	36	48
Log CFU/ml	6.113	5.954	5.602	5	5.477	5.698	5.903	5.602	5.38	5.301	5
	6.176	5.954	5.778	5.477	5.397	5.698	5.903	5.954	5.146	5.477	5.23
	6.113	5.778	5.845	5.903	5.447	5.602	5.602	5.301	5.698	5.903	5.301
Mean	6.134	5.895 333	5.741 667	5.46	5.440 333	5.666	5.802 667	5.619	5.408	5.560 333	5.177
SD	0.036 373	0.101 614	0.125 508	0.451 74	0.040 415	0.055 426	0.173 782	0.326 832	0.277 063	0.309 531	0.157 344
SB5%											
Log CFU/ml	6.361	6.698	7.11	7.602	7.602	7.518	7.903	7.602	7.845	7.778	7.477
	6.322	7.518	7.724	7.954	7.602	7.806	7.845	7.954	7.602	7.698	7.477
	6.342	7.041	7.041	7.778	7.778	7.633	7.874	8	7	7.301	7.602
Mean	6.341 667	7.085 667	7.291 667	7.778	7.660 667	7.652 333	7.874	7.852	7.482 333	7.592 333	7.518 667
SD	0.019 502	0.411 821	0.375 998	0.176	0.101 614	0.144 97	0.029	0.217 725	0.435 025	0.255 453	0.072 169
SB 10%											
Log CFU/ml	6	6.301	8.176	8.176	8.278	8.361	8.23	8.342	8.342	8.38	8.204
	6.113	6.845	8.342	8.079	8.361	8.278	8.38	8.23	8.301	8.38	8.342
	6.113	6.903	8.361	8.146	8.447	8.38	8.255	8.322	8.431	8.38	8.278
Mean	6.075 333	6.683	8.293	8.133 667	8.362	8.339 667	8.288 333	8.298	8.358	8.38	8.274 667
Standard Deviation	0.065 241	0.332 09	0.101 769	0.049 662	0.084 504	0.054 243	0.080 364	0.059 733	0.066 461	0	0.069 06
SB5%											
Log CFU/ml	6	7.845	8.531	8.681	8.591	8.643	8.69	8.113	8.491	8.579	8.643
	6.113	7.903	8.397	8.477	8.724	8.662	8.672	8.397	8.568	8.477	8.361
	6.113	8.079	8.491	8.643	8.591	8.612	8.579	8.568	8.414	8.322	8.568
Mean	6.075 333	7.942 333	8.473	8.600 333	8.635 333	8.639	8.647	8.359 333	8.491	8.459 333	8.524
SD	0.065 241	0.121 858	0.068 79	0.108 487	0.076 788	0.025 239	0.059 573	0.229 827	0.077	0.129 408	0.146 058
SB20%											
Log CFU/ml	6	8.041	8.301	8.556	8.414	8.462	8.204	8.531	8.38	8.447	8.591
	6.113	7.903	8.591	8.579	8.447	8.579	8.414	8.518	8.342	8.462	8.342

	6.113	7.945	8.38	8.579	8.612	8.556	8.477	8.491	8.556	8.591	8.518
Mean	6.075333	7.963	8.424	8.571333	8.491	8.532333	8.365	8.513333	8.426	8.5	8.483667
SD	0.065241	0.070739	0.149923	0.013279	0.10608	0.061987	0.142944	0.020404	0.114175	0.079164	0.128001

Table F.10 Growth of *Exiguobacterium* sp. AO-11 produced from different concentration of coconut milk residue (CM)

CM 1%											
Hour	0	3	6	9	12	15	18	24	30	36	48
Log CFU/ml	6.544	6.414	6.633	6.531	6.748	6.602	6.845	7.14	7.544	6.95	7.397
	6.431	6.531	6.568	6.633	6.612	6.602	6.903	7.113	7.568	7.554	7.38
	6.568	6.544	0	6.579	6.477	6.778	6.477	7.342	7.361	7.38	7.633
Mean	6.514333	6.496333	6.6005	6.581	6.612333	6.660667	6.741667	7.198333	7.491	7.294667	7.47
SD	0.07316	0.071598	0.045962	0.051029	0.1355	0.101614	0.231035	0.125149	0.113221	0.31091	0.141418
CM 5%											
Log CFU/ml	6.278	6.342	6.672	7.672	7.633	7.643	7.77	8.049	8.041	7.903	8.146
	6.447	6.342	6.43	7.079	7.681	7.633	7.748	7.792	8.176	7.903	7.903
	6.518	6.342	6.43	7.342	7.623	7.69	7.521	7.748	8.041	8.041	8.113
Mean	6.414333	6.342	6.510667	7.364333	7.645667	7.655333	7.679667	7.863	8.086	7.949	8.054
SD	0.12329	0	0.139719	0.29713	0.031005	0.030436	0.137849	0.162576	0.077942	0.079674	0.131807

CM 10%											
Log CFU /ml	5.954	5.903	7.579	8.38	8.176	8	8.491	8.477	8.477	8.431	8.176
	5.845	5.845	7.414	8.301	8.301	8.531	8.079	8.361	8.342	8.301	8.361
	5.477	5.602	7.785	8.146	8.361	8.278	8.301	8.204	8.568	8.477	8.2555
Mean	5.758667	5.783333	7.592667	8.275667	8.279333	8.269667	8.290333	8.347333	8.462333	8.403	8.264167
SD	0.249945	0.159695	0.185877	0.119039	0.094384	0.265598	0.206207	0.137012	0.113712	0.09128	0.092804
CM 15%											
Log CFU /ml	6	7.447	8	8.38	8.361	8.505	8.342	8.361	8.431	8.556	8.431
	6.113	7.414	8.342	8.204	8.38	8.477	8.462	8.38	8.414	8.397	8.342
	6.113	7.079	8.447	8.397	8.113	8.113	8.447	8.505	8.491	8.556	8.278
Mean	6.075333	7.313333	8.263	8.327	8.284667	8.365	8.417	8.415333	8.445333	8.503	8.350333
SD	0.065241	0.203608	0.233737	0.10686	0.148971	0.218687	0.065383	0.078233	0.040452	0.091799	0.07684
CM 20%											
Log CFU /ml	6	7.477	8.414	8.414	8.623	8.447	8.397	8.505	8.568	8.505	8.462
	6.113	7.544	8.491	8.623	8.462	8.518	8.361	8.477	8.491	8.477	8.447
	6.113	7.397	8.23	8.361	8.414	8.447	8.447	8.301	8.38	8.544	8.397

Mean	6.075333	7.472667	8.378333	8.466	8.499667	8.470667	8.401667	8.427667	8.479667	8.508667	8.435333
SD	0.065241	0.073596	0.134106	0.138524	0.109473	0.040992	0.04319	0.110586	0.094511	0.03365	0.034034

Table F.11 Growth of *Exiguobacterium* sp. AO-11 on LB medium

LB											
Hour	0	3	6	9	12	15	18	24	30	36	48
Log CFU/ml	5.77	7.9	9.23	9.113	9.176	9.176	9.301	9.079	9.414	9.447	9.079
	5.69	8	9.146	9.146	9.204	9.204	9.204	9.176	10	9.431	9.397
	5.95	8.2	9.113	9.176	9.301	9.255	9.146	9.176		9.447	9.38
Mean	5.803333	8.033333	9.163	9.145	9.227	9.211667	9.217	9.143667	9.707	9.441667	9.285333
SD	0.133167	0.152753	0.060324	0.031512	0.065597	0.040054	0.078313	0.056003	0.414365	0.009238	0.178892

Table F.12 Growth of *Exiguobacterium* sp. AO-11 on variety of seawater concentration in 15% SB.

Hour	0	3	6	9	12
4=1 diluted seawater					
Log CFU/mL	6.60206	6.90309	7.60206	8.414973	8.447158
	6.60206	6.60206	7.69897	8.653213	8.146128
	6.778151	6.845098	7.845098	8.556303	8.20412
average	6.660757	6.783416	7.715376	8.541496	8.265802

SD	0.101666	0.159713	0.122347	0.119808	0.159713
3=2 diluted seawater					
Log CFU/mL	6.60206	6.845098	7.69897	8.724276	8.447158
	6.60206	6.778151	7.69897	8.70757	8.447158
	6.778151	6.90309	7.90309	8.792392	8.672098
average	6.660757	6.842113	7.76701	8.741413	8.522138
	0.101666	0.062523	0.117849	0.044932	0.129869
2=3 diluted seawater					
Log CFU/mL	6.60206	7.176091	7.69897	8.447158	8.342423
	6.60206	7.176091	7.778151	8.643453	8.39794
	6.778151	6.954243	7.477121	8.342423	8.278754
average	6.660757	7.102142	7.651414	8.477678	8.339705
SD	0.101666	0.128084	0.156048	0.152818	0.05964
1=4 diluted seawater					
1=4 Log CFU/mL	6.60206	7.146128	7.60206	8.653213	8.531479
	6.60206	6.778151	7.69897	8.623249	8.230449
	6.778151	7.322219	8.176091	8.681241	8.643453
average	6.660757	7.082166	7.825707	8.652568	8.46846
SD	0.101666	0.277616	0.307286	0.029001	0.213592
SW					
Log CFU/ml	6	7.845	8.531	8.681	8.591
	6.113	7.903	8.397	8.477	8.724
	6.113	8.079	8.491	8.643	8.591
Mean	6.075333	7.942333	8.473	8.600333	8.635333
Standard Deviation	0.065241	0.121858	0.06879	0.108487	0.076788

Table F.13 Growth of *Exiguobacterium* sp. AO-11 on variety of sugarcane molasses concentration in 1:4 SW 15% SB medium.

Sugarcane molasses	hours	0	3	6	9	12
5g/L	Log CFU/ml	6.477	6	8.301	8.903	8.903
		6.477	5.954	8.113	8.778	9.204
		6.954	5.778	8.518	9.041	8.698
	Average	6.636	5.910667	8.310667	8.907333	8.935
	SD	0.275396	0.117172	0.202673	0.131554	0.254513
10g/L	Log CFU/ml	6.477	6.698	8.146	9.113	8.845
		6.477	6.954	8.591	8.778	9.146
		6.954	6.826	8.322	8.903	8.954
	Average	6.636	6.826	8.353	8.931333	8.981667
	SD	0.275396	0.128	0.224114	0.169288	0.152395
20g/L	Log CFU/ml	6.477	7.041	8.255	8.778	8.954
		6.477	7	8.397	8.778	8.778
		6.954	7.146	8.041	9.079	8.903
	Average	6.636	7.062333	8.231	8.878333	8.878333
	SD	0.275396	0.075302	0.179209	0.173782	0.090556
30g/L	Log CFU/ml	6.477	7.041	7.845	8.477	8.954
		6.477	6.698	8.204	8.778	8.778
		6.954	6.954	8.079	8.845	8.954
	Average	6.636	6.897667	8.042667	8.7	8.895333
	SD	0.275396	0.178304	0.182237	0.196008	0.101614
50g/L	Log CFU/ml	6.477	6.778	7.602	8.342	8.778
		6.477	6.602	8	8.477	9.041
		6.954	6.301	7.778	8.447	9.204
	Average	6.636	6.560333	7.793333	8.422	9.007667
	SD	0.275396	0.241214	0.199443	0.070887	0.214947

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Table F.14 Survival of *Exiguobacterium* sp. AO-11 suspended in two solutions at room temperature.

PB				
	d0	d10	d20	d30
Log CFU/ml	9.322	9	8.322	8
	9.301	8.903	8.204	8
	9.278	8.698	8	8.204
Mean	9.300333	8.867	8.175333	8.068
SD	0.022008	0.154185	0.162903	0.117779
	1=4 diluted seawater			
Log CFU/ml	8.69897	8.079181	7.146128	6.301
	9.278754	8.380211	7.361728	6.477
	9.176091	8.361728	7.253928	6.698
Mean	9.227422	8.273707	7.253928	6.492
SD	0.30939	0.168717	0.152452	0.198925

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Table F.15 Survival of AO-11 in liquid formulation with PB, and 1 and 5% GLY, PEG and PVP: at 4°C.

Survival of on liquid bacterial survival at 4°C				
liquid formulation	d0	d10	d20	d30
PB	100	99.54243	97.78151	83.61728
	100	99.41441	98.73839	84.62219
	100	99.21441	98.25292	83.31966
average	100	99.39041	98.25761	83.85304
SD	0	0.16532	0.478454	0.682517
Gly 1%	100	99.46867	98.5214	98.15202
	100	97.30015	95.57891	94.80493
	100	97.45474	96.68914	94.54411
average	100	98.07227	96.92894	95.829

SD	0	1.209838	1.485936	2.011969
Gly 5%	100	97.30015	96.80015	94.80493
	100	98.86119	94.80493	90.91517
	100	97.74742	97.30015	96.80015
average	100	97.96959	96.30175	94.17342
SD	0	0.803882	1.320167	2.992885
PVP 1%	100	98.90354	97.43554	97.43554
	100	98.15202	97.30015	96.80015
	100	99.54243	96.0206	96.0206
average	100	98.866	96.91876	96.7521
SD	0	0.69596	0.780774	0.708693
PVP 5%	100	98.18381	95.57392	80.58447
	100	96.72906	89.38943	85.96389
	100	98.52152	92.57285	90.45539
average	100	97.81146	92.51207	85.66792
SD	0	0.952478	3.092691	4.942115
PEG 1%	100	95.19141	92.8415	92.8415
	100	96.1729	94.03931	93.2778
	100	95.73283	94.03931	93.2778
average	100	95.69905	93.64004	93.13237
SD	0	0.491614	0.691559	0.251902
PEG 5%	100	98.70971	98.0584	93.81928
	100	101.1519	98.8734	96.22749
	100	100	96.67991	94.36545
average	100	99.95388	97.87057	94.80407
SD	0	1.221762	1.108744	1.262601

Table F.16 Survival of AO-11 in liquid formulation with PB, and 1 and 5% GLY, PEG and PVP: at 25°C.

Survival of on liquid bacterial survival at 25°C				
liquid formulation	do (10^9)	d10 (107)	d20(106)	d30 (106)
PB	100	84.31364	72.78754	72.78754
	100	84.43554	73.26271	72.16059

	100	83.48866	70.58057	70.23568
average	100	84.07928	72.21027	71.72793
SD	0	0.515111	1.431224	1.329807
	100	81.80697	72.63499	72.63499
	100	74.30818	55.53267	40.52733
Gly 1%	100	77.75067	54.96581	50.86893
	100	76.4143	53.4834	69.0953
average	100	76.15772	54.66063	53.49719
SD	0	1.735531	1.058174	14.4642
	100	#DIV/0!	#DIV/0!	#DIV/0!
Gly 5%	100	74.30818	63.31219	60.64367
	100	78.60254	65.4807	61.99555
	100	73.08693	64.53343	61.59094
average	100	75.33255	64.44211	61.41005
SD	0	2.896979	1.087138	0.693852
	100	81.80697	72.74688	72.70994
	100	86.5859	77.09191	74.27717
PVP 1%	100	83.16809	77.3436	68.09309
	100	88.13107	76.03608	68.7567
average	100	85.96169	76.82387	70.37565
SD	0	2.539687	0.693752	3.395062
	100	#DIV/0!	#DIV/0!	#DIV/0!
	100	#DIV/0!	#DIV/0!	#DIV/0!
	100	#NUM!	#NUM!	#NUM!
PVP 5%	100	#NUM!	#NUM!	#NUM!
	100	#NUM!	#NUM!	#NUM!
average	100	#NUM!	#NUM!	#NUM!
SD	0	#NUM!	#NUM!	#NUM!
	100	#DIV/0!	#DIV/0!	#DIV/0!
	100	#DIV/0!	#DIV/0!	#DIV/0!
	100	#DIV/0!	#DIV/0!	#DIV/0!
	100	78.76421	75.85358	#NUM!
PEG 1%	100	79.39335	71.90964	#NUM!
	100	79.39335	71.90964	#NUM!

average	100	79.18364	73.22429	#NUM!
SD	0	0.363229	2.277036	#NUM!
PEG 5%	100	84.63059	72.74346	71.0303
	100	86.79257	79.09872	71.22959
	100	82.07511	79.09872	73.78327
	100	84.49942	76.9803	72.01439
	0	2.361464	3.669211	1.535134

Table F.17 Survival of AO-11 in liquid formulation with PB, and 1 and 5% GLY, PEG and PVP at 30°C.

Survival of on liquid bacterial survival at 30°C				
liquid formulation	d	d10	d20	d30
PB	100	83.94171	83.32576	75.90173
	100	82.76443	82.76443	73.99752
	100	86.66967	83.4084	71.5248
average	100	84.45861	83.1662	73.80802
SD	0	2.003277	0.350387	2.194613
Gly 1%	100	72.11924	50.3421	35.41881
	100	68.04761	55.45775	33.05272
	100	73.44086	51.97769	32.6701
average	100	71.20257	52.59251	33.71178
SD	0	2.811049	2.612654	1.488861
Gly 5%	100	73.59505	54.98255	54.98255
	100	70.59059	52.50324	46.62667
	100	70.68571	50.41775	50.41775
average	100	71.62378	52.63451	50.67566
SD	0	1.707831	2.285229	4.183903
PVP 1%	100	88.75654	81.73353	79.89829
	100	84.8373	79.17114	74.42286
	100	84.85058	84.41382	78.58038
average	100	86.14814	81.77283	77.63384
SD	0	2.258951	2.621562	2.857799
PVP 5%	100	88.72102	82.07672	79.04911

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	100	79.6522	76.49809	76.49809
	100	90.41721	82.40871	76.49117
average	100	86.26348	80.32784	77.34612
SD	0	5.788007	3.320811	1.474833
PEG 1%	100	84.59358	82.40871	74.92703
	100	81.92189	81.92189	74.48441
	100	85.96758	84.63815	75.69684
average	100	84.16102	82.98958	75.03609
SD	0	2.057239	1.448302	0.613528
PEG 5%	100	78.49233	72.32859	66.45408
	100	81.63157	75.06	63.13236
	100	80.36784	72.57803	61.97511
average	100	80.16391	73.32221	63.85385
SD	0	1.579521	1.510129	2.325018

Table F.18 Survival of AO-11 in liquid formulation with PB, and 1 and 5% GLY, PEG and PVP at room temperature (31±1.5)

Survival of on liquid bacterial survival at room temperature				
liquid formulation	D0	d10	d20	d30
PB	100	79.77322	65.1882	67.10138
	100	80.10835	71.06826	69.59032
	100	79.5829	72.18421	67.83329
average	100	79.82149	69.48022	68.175
SD	0	0.266034	3.758648	1.279174
Gly 1%	100	81.09053	0	0
	100	78.58038	0	0
	100	76.92708	0	0
average	100	78.866	0	0
SD	0	2.096372	0	0
Gly 5%	100	75.39334	0	0
	100	78.50968	0	0

	100	77.64054	0	0
average	100	77.18119	0	0
SD	0	1.60815	0	0
PVP 1%	100	77.55537	76.03208	75.39334
	100	76.30333	76.80539	71.06826
	100	75.31279	75.31279	73.35622
average	100	76.3905	76.05009	73.27261
SD	0	1.123828	0.746464	2.163751
PVP 5%	100	78.62439	74.1547	72.75138
	100	79.82646	76.53512	73.24378
	100	80.75117	73.02039	69.69092
average	100	79.22542	75.34491	72.99758
SD	0	1.066399	1.793804	1.924911
PEG 1%	100	77.29086	77.96608	76.91318
	100	80.56252	77.67419	75.10578
	100	81.48246	77.97158	75.39334
average	100	79.77861	77.87061	75.8041
SD	0	2.203012	0.170135	0.971193
PEG 5%	100	78.19232	71.15244	65.51722
	100	80.94156	72.38004	64.82791
	100	80.88603	72.18421	63.9079
average	100	80.00664	71.90556	64.75101
SD	0	1.57149	0.65953	0.807412

Multiple Comparisons of solution selection between PB and 1:4 SW at room temperature for 30 days

	(I) solution	(J) solution	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Tukey HSD	PB	1:4 sw	15.01798*	1.32597	.000	10.9495	19.0864
		20 sw	6.58175*	1.32597	.006	2.5133	10.6502
	1:4 sw	PB	-15.01798*	1.32597	.000	-19.0864	-10.9495

		20 sw	-8.43623*	1.32597	.002	-12.5047	-4.3678
	20 sw	PB	-6.58175*	1.32597	.006	-10.6502	-2.5133
		1:4 sw	8.43623*	1.32597	.002	4.3678	12.5047
Scheffe	PB	1:4 sw	15.01798*	1.32597	.000	10.7653	19.2707
		20 sw	6.58175*	1.32597	.008	2.3290	10.8345
	1:4 sw	PB	-15.01798*	1.32597	.000	-19.2707	-10.7653
		20 sw	-8.43623*	1.32597	.002	-12.6890	-4.1835
	20 sw	PB	-6.58175*	1.32597	.008	-10.8345	-2.3290
		1:4 sw	8.43623*	1.32597	.002	4.1835	12.6890
LSD	PB	1:4 sw	15.01798*	1.32597	.000	11.7735	18.2625
		20 sw	6.58175*	1.32597	.003	3.3372	9.8263
	1:4 sw	PB	-15.01798*	1.32597	.000	-18.2625	-11.7735
		20 sw	-8.43623*	1.32597	.001	-11.6808	-5.1917
	20 sw	PB	-6.58175*	1.32597	.003	-9.8263	-3.3372
		1:4 sw	8.43623*	1.32597	.001	5.1917	11.6808

*. The mean difference is significant at the 0.05 level.

Homogeneous subsets of solution selection between PB and 1:4 SW at room temperature for 30 days.

	solution	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	1:4 sw	3	71.7442		
	20 sw	3		80.1804	
	PB	3			86.7622
	Sig.		1.000	1.000	1.000
Scheffe[a]	1:4 sw	3	71.7442		
	20 sw	3		80.1804	
	PB	3			86.7622
	Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Multiple Comparisons of bacterial survival of liquid formulation at 4°C for 30 days

Dependent Variable: survivalrate4

	(I) liquidformulation	(J) liquidformulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Tukey HSD	PB	GLY 1%	-11.98064*	1.95316	.000	-18.6499	-5.3114
		GLY 5%	-10.32037*	1.95316	.002	-16.9896	-3.6512
		PEG 1%	-9.27932*	1.95316	.004	-15.9485	-2.6101
		PEG 5%	-10.95103*	1.95316	.001	-17.6202	-4.2818
		PVP 1%	-12.89905*	1.95316	.000	-19.5683	-6.2298
		PVP 5%	-1.81487	1.95316	.961	-8.4841	4.8543
	GLY 1%	PB	11.98064*	1.95316	.000	5.3114	18.6499
		GLY 5%	1.66027	1.95316	.975	-5.0089	8.3295
		PEG 1%	2.70132	1.95316	.802	-3.9679	9.3705
		PEG 5%	1.02961	1.95316	.998	-5.6396	7.6988
		PVP 1%	-.91841	1.95316	.999	-7.5876	5.7508
		PVP 5%	10.16577*	1.95316	.002	3.4966	16.8350
	GLY 5%	PB	10.32037*	1.95316	.002	3.6512	16.9896
		GLY 1%	-1.66027	1.95316	.975	-8.3295	5.0089
		PEG 1%	1.04105	1.95316	.998	-5.6282	7.7103
		PEG 5%	-.63066	1.95316	1.000	-7.2999	6.0386
		PVP 1%	-2.57868	1.95316	.832	-9.2479	4.0905
		PVP 5%	8.50550*	1.95316	.009	1.8363	15.1747
	PEG 1%	PB	9.27932*	1.95316	.004	2.6101	15.9485
		GLY 1%	-2.70132	1.95316	.802	-9.3705	3.9679
		GLY 5%	-1.04105	1.95316	.998	-7.7103	5.6282
		PEG 5%	-1.67171	1.95316	.974	-8.3409	4.9975

		PVP 1%	-3.61973	1.95316	.537	-10.2889	3.0495
		PVP 5%	7.46445*	1.95316	.024	.7952	14.1337
	PEG 5%	PB	10.95103*	1.95316	.001	4.2818	17.6202
		GLY 1%	-1.02961	1.95316	.998	-7.6988	5.6396
		GLY 5%	.63066	1.95316	1.000	-6.0386	7.2999
		PEG 1%	1.67171	1.95316	.974	-4.9975	8.3409
		PVP 1%	-1.94802	1.95316	.946	-8.6172	4.7212
		PVP 5%	9.13616*	1.95316	.005	2.4669	15.8054
	PVP 1%	PB	12.89905*	1.95316	.000	6.2298	19.5683
		GLY 1%	.91841	1.95316	.999	-5.7508	7.5876
		GLY 5%	2.57868	1.95316	.832	-4.0905	9.2479
		PEG 1%	3.61973	1.95316	.537	-3.0495	10.2889
		PEG 5%	1.94802	1.95316	.946	-4.7212	8.6172
		PVP 5%	11.08418*	1.95316	.001	4.4150	17.7534
	PVP 5%	PB	1.81487	1.95316	.961	-4.8543	8.4841
		GLY 1%	-10.16577*	1.95316	.002	-16.8350	-3.4966
		GLY 5%	-8.50550*	1.95316	.009	-15.1747	-1.8363
		PEG 1%	-7.46445*	1.95316	.024	-14.1337	-.7952
		PEG 5%	-9.13616*	1.95316	.005	-15.8054	-2.4669
		PVP 1%	-11.08418*	1.95316	.001	-17.7534	-4.4150
Scheffe	PB	GLY 1%	-11.98064*	1.95316	.002	-20.0541	-3.9072
		GLY 5%	-10.32037*	1.95316	.008	-18.3939	-2.2469
		PEG 1%	-9.27932*	1.95316	.019	-17.3528	-1.2058
		PEG 5%	-10.95103*	1.95316	.005	-19.0245	-2.8775
		PVP 1%	-12.89905*	1.95316	.001	-20.9725	-4.8256
		PVP 5%	-1.81487	1.95316	.987	-9.8884	6.2586

GLY 1%	PB	11.98064*	1.95316	.002	3.9072	20.0541
	GLY 5%	1.66027	1.95316	.992	-6.4132	9.7338
	PEG 1%	2.70132	1.95316	.916	-5.3722	10.7748
	PEG 5%	1.02961	1.95316	.999	-7.0439	9.1031
	PVP 1%	-.91841	1.95316	1.000	-8.9919	7.1551
	PVP 5%	10.16577*	1.95316	.009	2.0923	18.2393
GLY 5%	PB	10.32037*	1.95316	.008	2.2469	18.3939
	GLY 1%	-1.66027	1.95316	.992	-9.7338	6.4132
	PEG 1%	1.04105	1.95316	.999	-7.0324	9.1145
	PEG 5%	-.63066	1.95316	1.000	-8.7042	7.4428
	PVP 1%	-2.57868	1.95316	.932	-10.6522	5.4948
	PVP 5%	8.50550*	1.95316	.036	.4320	16.5790
PEG 1%	PB	9.27932*	1.95316	.019	1.2058	17.3528
	GLY 1%	-2.70132	1.95316	.916	-10.7748	5.3722
	GLY 5%	-1.04105	1.95316	.999	-9.1145	7.0324
	PEG 5%	-1.67171	1.95316	.992	-9.7452	6.4018
	PVP 1%	-3.61973	1.95316	.746	-11.6932	4.4538
	PVP 5%	7.46445	1.95316	.080	-.6090	15.5379
PEG 5%	PB	10.95103*	1.95316	.005	2.8775	19.0245
	GLY 1%	-1.02961	1.95316	.999	-9.1031	7.0439
	GLY 5%	.63066	1.95316	1.000	-7.4428	8.7042
	PEG 1%	1.67171	1.95316	.992	-6.4018	9.7452
	PVP 1%	-1.94802	1.95316	.982	-10.0215	6.1255
	PVP 5%	9.13616*	1.95316	.022	1.0627	17.2097
PVP 1%	PB	12.89905*	1.95316	.001	4.8256	20.9725
	GLY 1%	.91841	1.95316	1.000	-7.1551	8.9919
	GLY 5%	2.57868	1.95316	.932	-5.4948	10.6522
	PEG 1%	3.61973	1.95316	.746	-4.4538	11.6932
	PEG 5%	1.94802	1.95316	.982	-6.1255	10.0215
	PVP 5%	11.08418*	1.95316	.005	3.0107	19.1577
PVP 5%	PB	1.81487	1.95316	.987	-6.2586	9.8884
	GLY 1%	-10.16577*	1.95316	.009	-18.2393	-2.0923

		GLY 5%	-8.50550*	1.95316	.036	-16.5790	-.4320
		PEG 1%	-7.46445	1.95316	.080	-15.5379	.6090
		PEG 5%	-9.13616*	1.95316	.022	-17.2097	-1.0627
		PVP 1%	-11.08418*	1.95316	.005	-19.1577	-3.0107
LSD	PB	GLY 1%	-11.98064*	1.95316	.000	-16.1697	-7.7915
		GLY 5%	-10.32037*	1.95316	.000	-14.5095	-6.1313
		PEG 1%	-9.27932*	1.95316	.000	-13.4684	-5.0902
		PEG 5%	-10.95103*	1.95316	.000	-15.1401	-6.7619
		PVP 1%	-12.89905*	1.95316	.000	-17.0882	-8.7100
		PVP 5%	-1.81487	1.95316	.369	-6.0040	2.3742
	GLY 1%	PB	11.98064*	1.95316	.000	7.7915	16.1697
		GLY 5%	1.66027	1.95316	.410	-2.5288	5.8494
		PEG 1%	2.70132	1.95316	.188	-1.4878	6.8904
		PEG 5%	1.02961	1.95316	.606	-3.1595	5.2187
		PVP 1%	-.91841	1.95316	.645	-5.1075	3.2707
		PVP 5%	10.16577*	1.95316	.000	5.9767	14.3549
	GLY 5%	PB	10.32037*	1.95316	.000	6.1313	14.5095
		GLY 1%	-1.66027	1.95316	.410	-5.8494	2.5288
		PEG 1%	1.04105	1.95316	.602	-3.1481	5.2302
		PEG 5%	-.63066	1.95316	.752	-4.8198	3.5584
		PVP 1%	-2.57868	1.95316	.208	-6.7678	1.6104
		PVP 5%	8.50550*	1.95316	.001	4.3164	12.6946
	PEG 1%	PB	9.27932*	1.95316	.000	5.0902	13.4684
		GLY 1%	-2.70132	1.95316	.188	-6.8904	1.4878
		GLY 5%	-1.04105	1.95316	.602	-5.2302	3.1481
		PEG 5%	-1.67171	1.95316	.406	-5.8608	2.5174
		PVP 1%	-3.61973	1.95316	.085	-7.8088	.5694

	PVP 5%	7.46445*	1.95316	.002	3.2753	11.6536
PEG 5%	PB	10.95103*	1.95316	.000	6.7619	15.1401
	GLY 1%	-1.02961	1.95316	.606	-5.2187	3.1595
	GLY 5%	.63066	1.95316	.752	-3.5584	4.8198
	PEG 1%	1.67171	1.95316	.406	-2.5174	5.8608
	PVP 1%	-1.94802	1.95316	.336	-6.1371	2.2411
	PVP 5%	9.13616*	1.95316	.000	4.9471	13.3253
PVP 1%	PB	12.89905*	1.95316	.000	8.7100	17.0882
	GLY 1%	.91841	1.95316	.645	-3.2707	5.1075
	GLY 5%	2.57868	1.95316	.208	-1.6104	6.7678
	PEG 1%	3.61973	1.95316	.085	-.5694	7.8088
	PEG 5%	1.94802	1.95316	.336	-2.2411	6.1371
	PVP 5%	11.08418*	1.95316	.000	6.8951	15.2733
PVP 5%	PB	1.81487	1.95316	.369	-2.3742	6.0040
	GLY 1%	-10.16577*	1.95316	.000	-14.3549	-5.9767
	GLY 5%	-8.50550*	1.95316	.001	-12.6946	-4.3164
	PEG 1%	-7.46445*	1.95316	.002	-11.6536	-3.2753
	PEG 5%	-9.13616*	1.95316	.000	-13.3253	-4.9471
	PVP 1%	-11.08418*	1.95316	.000	-15.2733	-6.8951

*. The mean difference is significant at the 0.05 level.

Homogeneous subsets of bacterial survival of liquid formulation at 4°C for 30 days

	liquidformulation	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	PB	3	83.8530		
	PVP 5%	3	85.6679		
	PEG 1%	3		93.1324	
	GLY 5%	3		94.1734	
	PEG 5%	3		94.8041	

	GLY 1%	3		95.8337	
	PVP 1%	3		96.7521	
	Sig.		.961	.537	
Scheffe[a]	PB	3	83.8530		
	PVP 5%	3	85.6679	85.6679	
	PEG 1%	3		93.1324	93.1324
	GLY 5%	3			94.1734
	PEG 5%	3			94.8041
	GLY 1%	3			95.8337
	PVP 1%	3			96.7521
	Sig.		.987	.080	.746

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Multiple Comparisons of bacterial survival of liquid formulation at 25°C for 30 days

Dependent Variable: survival25

	(I) liquidformulation	(J) liquidformulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Tukey HSD	PB	1% PVP	18.23075*	4.63266	.020	2.4121	34.0494
		5% PVP	10.31788	4.63266	.341	-5.5007	26.1365
		1% GLY	1.35228	4.63266	1.000	-14.4663	17.1709
		5% GLY	71.72793*	4.63266	.000	55.9093	87.5465
		1% PEG	71.72793*	4.63266	.000	55.9093	87.5465
		5% PEG	-.28645	4.63266	1.000	-16.1051	15.5322
	1% PVP	PB	-18.23075*	4.63266	.020	-34.0494	-2.4121
		5% PVP	-7.91287	4.63266	.622	-23.7315	7.9057
		1% GLY	-16.87847*	4.63266	.033	-32.6971	-1.0599

	5% GLY	53.49719*	4.63266	.000	37.6786	69.3158
	1% PEG	53.49719*	4.63266	.000	37.6786	69.3158
	5% PEG	-18.51720*	4.63266	.017	-34.3358	-2.6986
5% PVP	PB	-10.31788	4.63266	.341	-26.1365	5.5007
	1% PVP	7.91287	4.63266	.622	-7.9057	23.7315
	1% GLY	-8.96560	4.63266	.491	-24.7842	6.8530
	5% GLY	61.41005*	4.63266	.000	45.5914	77.2287
	1% PEG	61.41005*	4.63266	.000	45.5914	77.2287
	5% PEG	-10.60433	4.63266	.313	-26.4229	5.2143
1% GLY	PB	-1.35228	4.63266	1.000	-17.1709	14.4663
	1% PVP	16.87847*	4.63266	.033	1.0599	32.6971
	5% PVP	8.96560	4.63266	.491	-6.8530	24.7842
	5% GLY	70.37565*	4.63266	.000	54.5570	86.1943
	1% PEG	70.37565*	4.63266	.000	54.5570	86.1943
	5% PEG	-1.63873	4.63266	1.000	-17.4573	14.1799
5% GLY	PB	-71.72793*	4.63266	.000	-87.5465	-55.9093
	1% PVP	-53.49719*	4.63266	.000	-69.3158	-37.6786
	5% PVP	-61.41005*	4.63266	.000	-77.2287	-45.5914
	1% GLY	-70.37565*	4.63266	.000	-86.1943	-54.5570
	1% PEG	.00000	4.63266	1.000	-15.8186	15.8186
	5% PEG	-72.01439*	4.63266	.000	-87.8330	-56.1958
1% PEG	PB	-71.72793*	4.63266	.000	-87.5465	-55.9093
	1% PVP	-53.49719*	4.63266	.000	-69.3158	-37.6786

		5% PVP	-61.41005*	4.63266	.000	-77.2287	-45.5914
		1% GLY	-70.37565*	4.63266	.000	-86.1943	-54.5570
		5% GLY	.00000	4.63266	1.000	-15.8186	15.8186
		5% PEG	-72.01439*	4.63266	.000	-87.8330	-56.1958
	5% PEG	PB	.28645	4.63266	1.000	-15.5322	16.1051
		1% PVP	18.51720*	4.63266	.017	2.6986	34.3358
		5% PVP	10.60433	4.63266	.313	-5.2143	26.4229
		1% GLY	1.63873	4.63266	1.000	-14.1799	17.4573
		5% GLY	72.01439*	4.63266	.000	56.1958	87.8330
		1% PEG	72.01439*	4.63266	.000	56.1958	87.8330
Scheffe	PB	1% PVP	18.23075	4.63266	.067	-.9186	37.3801
		5% PVP	10.31788	4.63266	.568	-8.8315	29.4673
		1% GLY	1.35228	4.63266	1.000	-17.7971	20.5017
		5% GLY	71.72793*	4.63266	.000	52.5785	90.8773
		1% PEG	71.72793*	4.63266	.000	52.5785	90.8773
		5% PEG	-.28645	4.63266	1.000	-19.4358	18.8629
	1% PVP	PB	-18.23075	4.63266	.067	-37.3801	.9186
		5% PVP	-7.91287	4.63266	.808	-27.0623	11.2365
		1% GLY	-16.87847	4.63266	.104	-36.0279	2.2709
		5% GLY	53.49719*	4.63266	.000	34.3478	72.6466
		1% PEG	53.49719*	4.63266	.000	34.3478	72.6466
		5% PEG	-18.51720	4.63266	.061	-37.6666	.6322
	5% PVP	PB	-10.31788	4.63266	.568	-29.4673	8.8315
		1% PVP	7.91287	4.63266	.808	-11.2365	27.0623
		1% GLY	-8.96560	4.63266	.709	-28.1150	10.1838
		5% GLY	61.41005*	4.63266	.000	42.2607	80.5594
		1% PEG	61.41005*	4.63266	.000	42.2607	80.5594
		5% PEG	-10.60433	4.63266	.538	-29.7537	8.5451
	1% GLY	PB	-1.35228	4.63266	1.000	-20.5017	17.7971
		1% PVP	16.87847	4.63266	.104	-2.2709	36.0279

		5% PVP	8.96560	4.63266	.709	-10.1838	28.1150
		5% GLY	70.37565*	4.63266	.000	51.2263	89.5250
		1% PEG	70.37565*	4.63266	.000	51.2263	89.5250
		5% PEG	-1.63873	4.63266	1.000	-20.7881	17.5107
	5% GLY	PB	-71.72793*	4.63266	.000	-90.8773	-52.5785
		1% PVP	-53.49719*	4.63266	.000	-72.6466	-34.3478
		5% PVP	-61.41005*	4.63266	.000	-80.5594	-42.2607
		1% GLY	-70.37565*	4.63266	.000	-89.5250	-51.2263
		1% PEG	.00000	4.63266	1.000	-19.1494	19.1494
		5% PEG	-72.01439*	4.63266	.000	-91.1638	-52.8650
	1% PEG	PB	-71.72793*	4.63266	.000	-90.8773	-52.5785
		1% PVP	-53.49719*	4.63266	.000	-72.6466	-34.3478
		5% PVP	-61.41005*	4.63266	.000	-80.5594	-42.2607
		1% GLY	-70.37565*	4.63266	.000	-89.5250	-51.2263
		5% GLY	.00000	4.63266	1.000	-19.1494	19.1494
		5% PEG	-72.01439*	4.63266	.000	-91.1638	-52.8650
	5% PEG	PB	.28645	4.63266	1.000	-18.8629	19.4358
		1% PVP	18.51720	4.63266	.061	-.6322	37.6666
		5% PVP	10.60433	4.63266	.538	-8.5451	29.7537
		1% GLY	1.63873	4.63266	1.000	-17.5107	20.7881
		5% GLY	72.01439*	4.63266	.000	52.8650	91.1638
		1% PEG	72.01439*	4.63266	.000	52.8650	91.1638
LSD	PB	1% PVP	18.23075*	4.63266	.001	8.2947	28.1668
		5% PVP	10.31788*	4.63266	.043	.3818	20.2539

	1% GLY	1.35228	4.63266	.775	-8.5838	11.2883
	5% GLY	71.72793*	4.63266	.000	61.7919	81.6640
	1% PEG	71.72793*	4.63266	.000	61.7919	81.6640
	5% PEG	-.28645	4.63266	.952	-10.2225	9.6496
1% PVP	PB	-18.23075*	4.63266	.001	-28.1668	-8.2947
	5% PVP	-7.91287	4.63266	.110	-17.8489	2.0232
	1% GLY	-16.87847*	4.63266	.003	-26.8145	-6.9424
	5% GLY	53.49719*	4.63266	.000	43.5611	63.4332
	1% PEG	53.49719*	4.63266	.000	43.5611	63.4332
	5% PEG	-18.51720*	4.63266	.001	-28.4533	-8.5811
5% PVP	PB	-10.31788*	4.63266	.043	-20.2539	-.3818
	1% PVP	7.91287	4.63266	.110	-2.0232	17.8489
	1% GLY	-8.96560	4.63266	.073	-18.9017	.9705
	5% GLY	61.41005*	4.63266	.000	51.4740	71.3461
	1% PEG	61.41005*	4.63266	.000	51.4740	71.3461
	5% PEG	-10.60433*	4.63266	.038	-20.5404	-.6683
1% GLY	PB	-1.35228	4.63266	.775	-11.2883	8.5838
	1% PVP	16.87847*	4.63266	.003	6.9424	26.8145
	5% PVP	8.96560	4.63266	.073	-.9705	18.9017
	5% GLY	70.37565*	4.63266	.000	60.4396	80.3117
	1% PEG	70.37565*	4.63266	.000	60.4396	80.3117
	5% PEG	-1.63873	4.63266	.729	-11.5748	8.2973
5% GLY	PB	-71.72793*	4.63266	.000	-81.6640	-61.7919
	1% PVP	-53.49719*	4.63266	.000	-63.4332	-43.5611

	5% PVP	-61.41005*	4.63266	.000	-71.3461	-51.4740
	1% GLY	-70.37565*	4.63266	.000	-80.3117	-60.4396
	1% PEG	.00000	4.63266	1.000	-9.9361	9.9361
	5% PEG	-72.01439*	4.63266	.000	-81.9504	-62.0783
1% PEG	PB	-71.72793*	4.63266	.000	-81.6640	-61.7919
	1% PVP	-53.49719*	4.63266	.000	-63.4332	-43.5611
	5% PVP	-61.41005*	4.63266	.000	-71.3461	-51.4740
	1% GLY	-70.37565*	4.63266	.000	-80.3117	-60.4396
	5% GLY	.00000	4.63266	1.000	-9.9361	9.9361
	5% PEG	-72.01439*	4.63266	.000	-81.9504	-62.0783
5% PEG	PB	.28645	4.63266	.952	-9.6496	10.2225
	1% PVP	18.51720*	4.63266	.001	8.5811	28.4533
	5% PVP	10.60433*	4.63266	.038	.6683	20.5404
	1% GLY	1.63873	4.63266	.729	-8.2973	11.5748
	5% GLY	72.01439*	4.63266	.000	62.0783	81.9504
	1% PEG	72.01439*	4.63266	.000	62.0783	81.9504

*. The mean difference is significant at the 0.05 level.

Homogeneous subsets of bacterial survival of liquid formulation at 25°C for 30 days

	liquidformulation	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	5% GLY	3	.0000		
	1% PEG	3	.0000		

		N	1	2	3
	1% PVP	3		53.4972	
	5% PVP	3		61.4101	61.4101
	1% GLY	3			70.3757
	PB	3			71.7279
	5% PEG	3			72.0144
	Sig.		1.000	.622	.313
Scheffe[a]	5% GLY	3	.0000		
	1% PEG	3	.0000		
	1% PVP	3		53.4972	
	5% PVP	3		61.4101	
	1% GLY	3		70.3757	
	PB	3		71.7279	
	5% PEG	3		72.0144	
	Sig.		1.000	.061	

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Multiple Comparisons of bacterial survival of liquid formulation at 30°C for 30 days

Dependent Variable: survival

	(I) liquidformulation	(J) liquidformulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	PB	1% PVP	-3.82583	1.96789	.704	-11.9602	4.3086
		5% PVP	-3.53810	1.96789	.770	-11.6725	4.5963
		1% GLY	40.09414*	1.96789	.000	31.9597	48.2286
		5% GLY	23.13236*	1.96789	.000	14.9979	31.2668
		1% PEG	-1.22807	1.96789	.999	-9.3625	6.9063
		5% PEG	9.95417*	1.96789	.012	1.8198	18.0886
	1% PVP	PB	3.82583	1.96789	.704	-4.3086	11.9602
		5% PVP	.28772	1.96789	1.000	-7.8467	8.4221

	1% GLY	43.91997*	1.96789	.000	35.7856	52.0544
	5% GLY	26.95819*	1.96789	.000	18.8238	35.0926
	1% PEG	2.59775	1.96789	.932	-5.5367	10.7322
	5% PEG	13.77999*	1.96789	.001	5.6456	21.9144
5% PVP	PB	3.53810	1.96789	.770	-4.5963	11.6725
	1% PVP	-.28772	1.96789	1.000	-8.4221	7.8467
	1% GLY	43.63224*	1.96789	.000	35.4978	51.7667
	5% GLY	26.67046*	1.96789	.000	18.5360	34.8049
	1% PEG	2.31003	1.96789	.960	-5.8244	10.4444
	5% PEG	13.49227*	1.96789	.001	5.3579	21.6267
1% GLY	PB	-40.09414*	1.96789	.000	-48.2286	-31.9597
	1% PVP	-43.91997*	1.96789	.000	-52.0544	-35.7856
	5% PVP	-43.63224*	1.96789	.000	-51.7667	-35.4978
	5% GLY	-16.96178*	1.96789	.000	-25.0962	-8.8274
	1% PEG	-41.32221*	1.96789	.000	-49.4566	-33.1878
	5% PEG	-30.13997*	1.96789	.000	-38.2744	-22.0056
5% GLY	PB	-23.13236*	1.96789	.000	-31.2668	-14.9979
	1% PVP	-26.95819*	1.96789	.000	-35.0926	-18.8238
	5% PVP	-26.67046*	1.96789	.000	-34.8049	-18.5360
	1% GLY	16.96178*	1.96789	.000	8.8274	25.0962
	1% PEG	-24.36043*	1.96789	.000	-32.4948	-16.2260
	5% PEG	-13.17819*	1.96789	.001	-21.3126	-5.0438
1% PEG	PB	1.22807	1.96789	.999	-6.9063	9.3625
	1% PVP	-2.59775	1.96789	.932	-10.7322	5.5367
	5% PVP	-2.31003	1.96789	.960	-10.4444	5.8244
	1% GLY	41.32221*	1.96789	.000	33.1878	49.4566
	5% GLY	24.36043*	1.96789	.000	16.2260	32.4948
	5% PEG	11.18224*	1.96789	.005	3.0478	19.3167
5% PEG	PB	-9.95417*	1.96789	.012	-18.0886	-1.8198
	1% PVP	-13.77999*	1.96789	.001	-21.9144	-5.6456
	5% PVP	-13.49227*	1.96789	.001	-21.6267	-5.3579
	1% GLY	30.13997*	1.96789	.000	22.0056	38.2744

		5% GLY	13.17819*	1.96789	.001	5.0438	21.3126
		1% PEG	-11.18224*	1.96789	.005	-19.3167	-3.0478
LSD	PB	1% PVP	-3.82583	1.96789	.072	-8.0465	.3949
		5% PVP	-3.53810	1.96789	.094	-7.7588	.6826
		1% GLY	40.09414*	1.96789	.000	35.8734	44.3149
		5% GLY	23.13236*	1.96789	.000	18.9116	27.3531
		1% PEG	-1.22807	1.96789	.543	-5.4488	2.9926
		5% PEG	9.95417*	1.96789	.000	5.7335	14.1749
	1% PVP	PB	3.82583	1.96789	.072	-.3949	8.0465
		5% PVP	.28772	1.96789	.886	-3.9330	4.5084
		1% GLY	43.91997*	1.96789	.000	39.6993	48.1407
		5% GLY	26.95819*	1.96789	.000	22.7375	31.1789
		1% PEG	2.59775	1.96789	.208	-1.6230	6.8185
		5% PEG	13.77999*	1.96789	.000	9.5593	18.0007
	5% PVP	PB	3.53810	1.96789	.094	-.6826	7.7588
		1% PVP	-.28772	1.96789	.886	-4.5084	3.9330
		1% GLY	43.63224*	1.96789	.000	39.4115	47.8530
		5% GLY	26.67046*	1.96789	.000	22.4498	30.8912
		1% PEG	2.31003	1.96789	.260	-1.9107	6.5307
		5% PEG	13.49227*	1.96789	.000	9.2716	17.7130
	1% GLY	PB	-40.09414*	1.96789	.000	-44.3149	-35.8734
		1% PVP	-43.91997*	1.96789	.000	-48.1407	-39.6993
		5% PVP	-43.63224*	1.96789	.000	-47.8530	-39.4115
		5% GLY	-16.96178*	1.96789	.000	-21.1825	-12.7411
		1% PEG	-41.32221*	1.96789	.000	-45.5429	-37.1015
		5% PEG	-30.13997*	1.96789	.000	-34.3607	-25.9193
	5% GLY	PB	-23.13236*	1.96789	.000	-27.3531	-18.9116
		1% PVP	-26.95819*	1.96789	.000	-31.1789	-22.7375
		5% PVP	-26.67046*	1.96789	.000	-30.8912	-22.4498
		1% GLY	16.96178*	1.96789	.000	12.7411	21.1825
		1% PEG	-24.36043*	1.96789	.000	-28.5811	-20.1397
		5% PEG	-13.17819*	1.96789	.000	-17.3989	-8.9575

1% PEG	PB	1.22807	1.96789	.543	-2.9926	5.4488
	1% PVP	-2.59775	1.96789	.208	-6.8185	1.6230
	5% PVP	-2.31003	1.96789	.260	-6.5307	1.9107
	1% GLY	41.32221*	1.96789	.000	37.1015	45.5429
	5% GLY	24.36043*	1.96789	.000	20.1397	28.5811
	5% PEG	11.18224*	1.96789	.000	6.9615	15.4030
5% PEG	PB	-9.95417*	1.96789	.000	-14.1749	-5.7335
	1% PVP	-13.77999*	1.96789	.000	-18.0007	-9.5593
	5% PVP	-13.49227*	1.96789	.000	-17.7130	-9.2716
	1% GLY	30.13997*	1.96789	.000	25.9193	34.3607
	5% GLY	13.17819*	1.96789	.000	8.9575	17.3989
	1% PEG	-11.18224*	1.96789	.000	-15.4030	-6.9615

*. The mean difference is significant at the 0.05 level.

Homogeneous subsets of bacterial survival of liquid formulation at 30°C for 30 days

	liquidformulation	N	Subset for alpha = 0.05			
			1	2	3	4
Scheffe[a]	1% GLY	3	33.7139			
	5% GLY	3		50.6757		
	5% PEG	3			63.8539	
	PB	3				73.8080
	1% PEG	3				75.0361
	5% PVP	3				77.3461
	1% PVP	3				77.6338
	Sig.		1.000	1.000	1.000	.704

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Multiple Comparisons of bacterial survival of liquid formulation at room temperature for 30 days.

Dependent Variable: survival RT

	(I) liquidfrmulation	(J) liquidfrmulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Scheffe	PB	PVP 1%	-5.09761*	1.24464	.030	-9.7400	-.4552
		PVP 5%	-3.72036	1.24464	.138	-8.3628	.9220
		PEG 1%	-7.62910*	1.24464	.002	-12.2715	-2.9867
		PEG 5%	3.42399	1.24464	.188	-1.2184	8.0664
	PVP 1%	PB	5.09761*	1.24464	.030	.4552	9.7400
		PVP 5%	1.37725	1.24464	.867	-3.2652	6.0196
		PEG 1%	-2.53149	1.24464	.436	-7.1739	2.1109
		PEG 5%	8.52160*	1.24464	.001	3.8792	13.1640
	PVP 5%	PB	3.72036	1.24464	.138	-.9220	8.3628
		PVP 1%	-1.37725	1.24464	.867	-6.0196	3.2652
		PEG 1%	-3.90874	1.24464	.113	-8.5511	.7337
		PEG 5%	7.14435*	1.24464	.003	2.5020	11.7868
	PEG 1%	PB	7.62910*	1.24464	.002	2.9867	12.2715
		PVP 1%	2.53149	1.24464	.436	-2.1109	7.1739
		PVP 5%	3.90874	1.24464	.113	-.7337	8.5511
		PEG 5%	11.05309*	1.24464	.000	6.4107	15.6955
	PEG 5%	PB	-3.42399	1.24464	.188	-8.0664	1.2184
		PVP 1%	-8.52160*	1.24464	.001	-13.1640	-3.8792
		PVP 5%	-7.14435*	1.24464	.003	-11.7868	-2.5020
		PEG 1%	-11.05309*	1.24464	.000	-15.6955	-6.4107
LSD	PB	PVP 1%	-5.09761*	1.24464	.002	-7.8708	-2.3244
		PVP 5%	-3.72036*	1.24464	.014	-6.4936	-.9471
		PEG 1%	-7.62910*	1.24464	.000	-10.4023	-4.8559
		PEG 5%	3.42399*	1.24464	.020	.6508	6.1972
	PVP 1%	PB	5.09761*	1.24464	.002	2.3244	7.8708
		PVP 5%	1.37725	1.24464	.294	-1.3960	4.1505
		PEG 1%	-2.53149	1.24464	.069	-5.3047	.2417
		PEG 5%	8.52160*	1.24464	.000	5.7484	11.2948

	PVP 5%	PB	3.72036*	1.24464	.014	.9471	6.4936
		PVP 1%	-1.37725	1.24464	.294	-4.1505	1.3960
		PEG 1%	-3.90874*	1.24464	.011	-6.6820	-1.1355
		PEG 5%	7.14435*	1.24464	.000	4.3711	9.9176
	PEG 1%	PB	7.62910*	1.24464	.000	4.8559	10.4023
		PVP 1%	2.53149	1.24464	.069	-.2417	5.3047
		PVP 5%	3.90874*	1.24464	.011	1.1355	6.6820
		PEG 5%	11.05309*	1.24464	.000	8.2799	13.8263
	PEG 5%	PB	-3.42399*	1.24464	.020	-6.1972	-.6508
		PVP 1%	-8.52160*	1.24464	.000	-11.2948	-5.7484
		PVP 5%	-7.14435*	1.24464	.000	-9.9176	-4.3711
		PEG 1%	-11.05309*	1.24464	.000	-13.8263	-8.2799

*. The mean difference is significant at the 0.05 level.

Homogeneous subsets of bacterial survival of liquid formulation at room temperature for 30 days

	liquidfrmulation	N	Subset for alpha = 0.05		
			1	2	3
Scheffe[a]	PEG 5%	3	64.7510		
	PB	3	68.1750	68.1750	
	PVP 5%	3		71.8954	71.8954
	PVP 1%	3			73.2726
	PEG 1%	3			75.8041
	Sig.		.188	.138	.113

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Descriptive of Multiple Comparisons of liquid formulation extension for 45 days between PB and PEG 1%

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
PB	3	83.7107	.35777	.20656	82.8219	84.5994	83.49	84.12
PEG 1%	3	80.0203	.86131	.49728	77.8807	82.1599	79.10	80.80
Total	6	81.8655	2.10562	.85961	79.6557	84.0752	79.10	84.12

Multiple Comparisons of bacterial survival of liquid formulation at room temperature for 60 days.

Dependent Variable: survival

	(I) liquidformulation	(J) liquidformulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Tukey HSD	PB day 60 4 degree	PEG1% day 60 4degree	5.27788*	.99264	.003	2.0991	8.4567
		PB day 60 30 degree	19.33083*	.99264	.000	16.1520	22.5096
		PEG1% day 60 degree	19.02098*	.99264	.000	15.8422	22.1998
	PEG1% day 60 4degree	PB day 60 4 degree	-5.27788*	.99264	.003	-8.4567	-2.0991
		PB day 60 30 degree	14.05295*	.99264	.000	10.8742	17.2317
		PEG1% day 60 degree	13.74310*	.99264	.000	10.5643	16.9219
	PB day 60 30 degree	PB day 60 4 degree	-19.33083*	.99264	.000	-22.5096	-16.1520

		PEG1% day 60 4degree	-14.05295*	.99264	.000	-17.2317	-10.8742
		PEG1% day 60 degree	-.30985	.99264	.989	-3.4886	2.8689
	PEG1% day 60 degree	PB day 60 4 degree	-19.02098*	.99264	.000	-22.1998	-15.8422
		PEG1% day 60 4degree	-13.74310*	.99264	.000	-16.9219	-10.5643
		PB day 60 30 degree	.30985	.99264	.989	-2.8689	3.4886
Scheffe	PB day 60 4 degree	PEG1% day 60 4degree	5.27788*	.99264	.005	1.8109	8.7448
		PB day 60 30 degree	19.33083*	.99264	.000	15.8639	22.7978
		PEG1% day 60 degree	19.02098*	.99264	.000	15.5540	22.4879
	PEG1% day 60 4degree	PB day 60 4 degree	-5.27788*	.99264	.005	-8.7448	-1.8109
		PB day 60 30 degree	14.05295*	.99264	.000	10.5860	17.5199
		PEG1% day 60 degree	13.74310*	.99264	.000	10.2762	17.2100
	PB day 60 30 degree	PB day 60 4 degree	-19.33083*	.99264	.000	-22.7978	-15.8639
		PEG1% day 60 4degree	-14.05295*	.99264	.000	-17.5199	-10.5860
		PEG1% day 60 degree	-.30985	.99264	.992	-3.7768	3.1571
	PEG1% day 60 degree	PB day 60 4 degree	-19.02098*	.99264	.000	-22.4879	-15.5540
		PEG1% day 60 4degree	-13.74310*	.99264	.000	-17.2100	-10.2762
		PB day 60 30 degree	.30985	.99264	.992	-3.1571	3.7768

LSD	PB day 60 4 degree	PEG1% day 60 4degree	5.27788*	.99264	.001	2.9888	7.5669
		PB day 60 30 degree	19.33083*	.99264	.000	17.0418	21.6199
		PEG1% day 60 degree	19.02098*	.99264	.000	16.7319	21.3100
	PEG1% day 60 4degree	PB day 60 4 degree	-5.27788*	.99264	.001	-7.5669	-2.9888
		PB day 60 30 degree	14.05295*	.99264	.000	11.7639	16.3420
		PEG1% day 60 degree	13.74310*	.99264	.000	11.4541	16.0321
	PB day 60 30 degree	PB day 60 4 degree	-19.33083*	.99264	.000	-21.6199	-17.0418
		PEG1% day 60 4degree	-14.05295*	.99264	.000	-16.3420	-11.7639
		PEG1% day 60 degree	-.30985	.99264	.763	-2.5989	1.9792
	PEG1% day 60 degree	PB day 60 4 degree	-19.02098*	.99264	.000	-21.3100	-16.7319
		PEG1% day 60 4degree	-13.74310*	.99264	.000	-16.0321	-11.4541
		PB day 60 30 degree	.30985	.99264	.763	-1.9792	2.5989

*. The mean difference is significant at the 0.05 level.

Homogeneous subsets of bacterial survival of liquid formulation at room temperature for 30 days

survival

	liquidformulation	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	PB day 60 30 degree	3	58.3917		
	PEG1% day 60 degree	3	58.7015		
	PEG1% day 60 4degree	3		72.4446	
	PB day 60 4 degree	3			77.7225
	Sig.		.989	1.000	1.000
Scheffe[a]	PB day 60 30 degree	3	58.3917		
	PEG1% day 60 degree	3	58.7015		
	PEG1% day 60 4degree	3		72.4446	
	PB day 60 4 degree	3			77.7225
	Sig.		.992	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Degradation of 0.25% v/v crude oil in seawater

	Control d0	Control d10	d10	% degradation
In+stsw	1878.061	1782.982	573.719	67.591
	1858.035	1770.252	589.262	66.95
	1872.047	1776.617	588.304	66.767
Average				67.27
SD				0.452
AO+sw	1878.061	1782.982	158.151	91.066
	1858.035043	1770.252	377.27	78.84
	1872.04775	1776.617	282.97	84.015
Average				84.953
SD				8.644

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Degradation of 0.5% v/v crude oil in seawater

	Control d0	Control d10	d10	% degradation	Control d 15	d15	% degradation
AO+stsw	981.136	960.956	611.61	36.354	948.185	266.214	72.296
	973.735	948.362	551.284	41.869	946.362	404.828	57.312
	978.566	952.185	581.447	38.935	956.007	335.521	64.762
Average				39.053			61.037
SD				2.759			5.268
AO+sw	981.136	960.956	240.717	74.95	948.185	21.430	97.769
	973.735	948.362	73.742	92.22	946.362	9.991	98.946
	978.566	952.185	182.16	83.526	956.007	21.427	97.749
Average				83.567			98.358
SD				12.214			0.83
	981.136	960.956	601.06703	37.45	948.185	198.27	79.26
	973.735	948.362	372.96824	60.67	946.362	273.09	71.14
	978.566	952.185	321.08558	66.27	956.007	268.41	71.69
Average				54.8			74.03
SD				15.28			4.53

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