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# CRUDE OIL BIODEGRADATION BY PSEUDOMONAS J-45 THROUGH BIOSURFACTANT FORMATION

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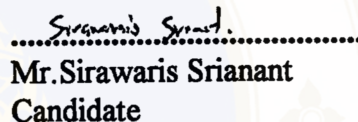

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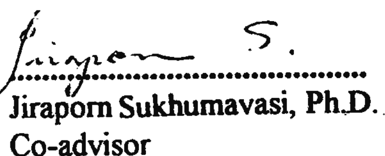

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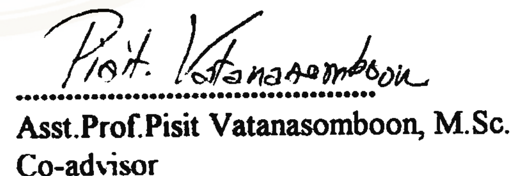

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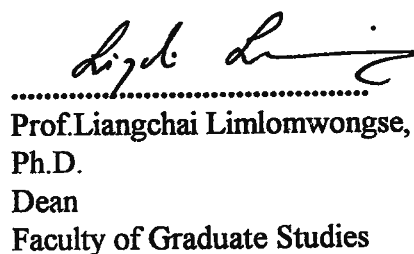

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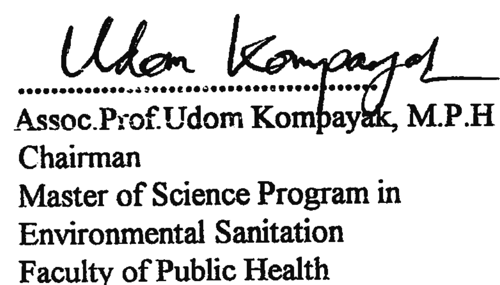

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SIRAWARIS SRIANANT: CRUDE OIL BIODEGRADATION BY PSEUDOMONAS J-45 THROUGH BIOSURFACTANT FORMATION. THESIS ADVISORS : UDOM KOMPAYAK, M.P.H., SHALASAI HUANGPRASERT, M.P.H., PISIT VATANASOMBOON, M.Sc., JIRAPORN SUKUMAVASRI, Ph.D. 157 P. ISBN 974-664-057-7

The objective of this research was to investigate a suitable condition for the biodegradation of crude oil by biosurfactant of *Pseudomonas* J-45 and to study properites of the produced biosurfactant by isolation, purification and identification of biosurfactant.

*Pseudomonas* J-45 is a bacterium isolated from oil contaminated sites in Thailand. It shows the ability to grow and degrade crude oil under various conditions, i.e., at temperature 25 to 37$^0$C, aeration rates 0.25 to 1.0 v.v.m. and when using of seawater as mineral source. The fermentation of 8 liters of bacterium broth containing 1 % crude oil in 10 liter fermenter showed that the optimum conditions to obtain the highest value of the surface tension reduction were 100 r.p.m. agitation and 1 v.v.m. aeration rate at 30$^0$C. After cultivation, bacterial cells were separated by refrigerated centrifugation at 8,000 r.p.m. at 4$^0$C for 20 min. Cells were dried by lyophilization. The biosurfactant (BS) content both in dry cells and in culture broth was detected. The BS content was found in both phases. It showed that the amount of BS in the broth is 10 times that of the dry cells. Therefore, the BS production for further study was aimed at the BS in culture broth only. The identification for the type of BS in cell and culture broth was attempted. The lipids in cell and broth were separated into 3 groups, neutrallipid, glycolipid and phospholipid by using the chromatographic technique. The BS activity in lipid was investigated using Emulsification Capacity (EC) test. The results revealed that only glycolipid. had EC activity while the other two groups of lipid did not. After that, the types of saccharides bound with lipids were investigated using thin layer chromatography. It showed that the saccharide found in BS is rhamnose. The comparison with the standard saccharides on chromatographic pattern showed that this biosurfactant of *Pseudomonas* J-45 is rhamnolipid type. The biosurfactant was stable over a wide range of pH from 6.0 to 12.0 with optimum activity at pH 8.0 with NaCl concentrations not higher than 5 % and temperature at 55 to 80$^0$C for 3 hours. The critical micelle concentration (CMC) and surface tension value at this point of partially purified biosurfactant were 148 mg/l and 34.5 mN/m, respectively. Preliminary analytical results indicated that biosurfactant was as effective as other commercial synthetic surfactants.

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ศิรวรินฐ์ ศรีอนันต์ : การย่อยสลายทางชีวภาพของน้ำมันดิบโดยแบคทีเรีย *Pseudomonas* J-45 ด้วยการสร้างสารลดแรงตึงผิวชีวภาพ (CRUDE OIL BIODEGRADATION BY PSEUDOMONAS J-45 THROUGH BIOSURFACTANT FORMATION). คณะกรรมการควบคุมวิทยานิพนธ์ : อุดม คมพยัคฆ์, M.P.H., ชลาศัย ห่วงประเสริฐ, M.P.H., พิศิษฐ์ วัฒนสมบูรณ์, M.Sc., จิราภรณ์ สุขุมาวาสี, Ph.D. 157 หน้า. ISBN 974-664-057-7

จุดประสงค์ของการศึกษานี้เพื่อศึกษาหาสภาวะที่เหมาะสมในการย่อยสลายน้ำมันดิบโดยสารลดแรงตึงผิวชีวภาพที่ผลิตจากแบคทีเรียสายพันธุ์ *Pseudomonas* J-45 รวมทั้งศึกษาคุณสมบัติของสารลดแรงตึงผิวชีวภาพที่ผลิตได้ โดยการแยก การทำให้บริสุทธิ์

*Pseudomonas* J-45 เป็นแบคทีเรียซึ่งแยกได้จากบริเวณที่มีการปนเปื้อนน้ำมันในประเทศไทย พบว่าแบคทีเรียนี้สามารถเจริญและย่อยสลายน้ำมันดิบได้ดีในสภาวะแวดล้อมต่างๆ เช่นที่อุณหภูมิตั้งแต่ 25-37 องศาเซลเซียส อัตราเติมอากาศ 0.25-1.0 v.v.m. และ โดยใช้น้ำทะเลเป็นแหล่งแร่ธาตุ จากการทดลองพบว่าสภาวะที่เหมาะสมในการย่อยสลายน้ำมันดิบ (1% ในอาหารเหลว 8 ลิตร ในถังหมักขนาด 10 ลิตร) ได้ดีที่สุด คือการกวนที่ 100 รอบต่อนาที ที่อุณหภูมิ 30 $^{o}$ซ และการเติมอากาศ 1.0 v.v.m. และที่สภาวะนี้แรงตึงผิวของอาหารเหลวจะมีค่าต่ำสุด หลังจากการเพาะเลี้ยง เซลแบคทีเรียจะถูกแยกออกจากอาหารเลี้ยงเชื้อโดยปั่นที่ 8,000 รอบต่อนาที ที่ 4 $^{o}$ซ เป็นเวลา 20 นาที และทำให้เซลแห้งโดย Lyophilization ต่อจากนั้นจึง นำทั้งเซล และน้ำใสไปแยกหาปริมาณสารลดแรงตึงผิว พบว่ามีอยู่ในส่วนที่เป็นน้ำใสมากกว่าในส่วนที่เป็นเซลประมาณ 10 เท่า ผลจากการศึกษาชนิดของสารลดแรงตึงผิวโดยใช้โครมาโตกราฟฟีเทคนิคและการสร้างอิมัลชั่น เพื่อพิสูจน์ว่าสารนี้อยู่ในกลุ่มของ นิวตรอนลิปิด กลัยโคลิปิด หรือ ฟอสโฟลิปิด พบว่าสารลดแรงตึงผิวนี้อยู่ในกลุ่มของกลัยโคลิปิด และจากการเปรียบเทียบกับน้ำตาลมาตรฐานพบว่ามี น้ำตาลแรมโนส เป็นส่วนประกอบ ดังนั้นจึงสรุปได้ว่า สารลดแรงตึงผิวชีวภาพนี้เป็น แรมโนลิปิด และสารลดแรงตึงผิวสามารถคงตัวได้ดีในช่วง pH กว้างตั้งแต่ pH 6.0 ถึง 12.0 โดยมีประสิทธิภาพสูงสุดที่ pH 8.0 และมีความคงตัวที่อุณหภูมิตั้งแต่ 55-80$^{o}$ซ ได้ 3 ชั่วโมง และมีค่า ซีเอ็มซี 148 มิลลิกรัมต่อลิตร โดยมีค่าแรงตึงผิว 34.5 มิลลินิวตันต่อเมตร ณ จุดนี้ จากผลการทดลองที่ได้รับทั้งหมดชี้ให้เห็นว่าสารลดแรงตึงผิวชีวภาพจากแบคทีเรียสารพันธุ์นี้มีประสิทธิภาพเทียบเท่ากับสารลดแรงตึงผิวเคมีสังเคราะห์ในท้องตลาดและสามารถใช้ทดแทนได้ดีกว่าเนื่องจากสามารถย่อยสลายได้ตามธรรมชาติ

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| r.p.m. | revolutions per minute |
| v.v.m. | volume of air per volume of broth per minute |
| mg/l | milligram per liter |
| mN/m | millinewton per meter |
| hr | hour |
| $^{0}C$ | degree of celsius |
| ASW | artificial seawater |
| SW | seawater |
| OD | optical density |
| $R_f$ | retention front value |
| v/v | volume per volume |
| w/v | weight per volume |
| g | gram |
| mol/l | molar per liter |

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# CHAPTER I

# INTRODUCTION

## 1.1 Background and Statement of Problem

Crude oil is a complex hydrocarbon mixture containing small amounts of non-hydrocarbon compounds, which depend on the refinery process. It has been used widely as an energy source and a starting material for the petrochemical society. However, when it is accidentally discharged into the environment, it will cause a severe ecological damage.

Tanker disaster and accident to off shore production facilities cause massive oil spills. The first major accident: Torrey Canyon incident, occurred in March 1967 when it run onto Seven stone rocks off the south west coast of England and 100,000 tons of crude oil was discharged into the ocean. Another accident was Exxon Valdez incident in 1989 which released 11 million gallon of crude oil into Alaska' exquisite Prince William Sound. Oil had drenched or spattered at least 1,200 miles of shoreline. Experts believed that about 100,000 birds had died and at least 1,000 sea otters had perished. Those caused concern about the effect of pollution in the environment and stimulated a large research effort in marine pollution in the following years (1).

The major process involved in petroleum in seawater degradation: spreading, evaporation, dissolution, emulsification, auto-oxidation, microbiological degradation, sinking and resurfacing (2). During the process, the film formed on water surface limits

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oxygen dissolved in water. As a result, marine organisms would die within only 5 minutes.

In order to remove oil spill, the methods of cleanup technology including physical, chemical, and biological methods have been applied. The use of buffles and booms are effective only in flowing water situations which the currents of less than 1 knot. Skimming machinery may also be effective where large an area of water surface has to be treated. While burning of oil on the ocean is not very successful due to the difficulty in igniting the oil. This method also results in the atmospheric pollution. Another popular method is the absorption of the oil by using straw, bark, clay, diatomaceous earth, sand and fly ash. After absorption, the materials are either picked up the burial or treatment, or allow sinking (3). However, the sunk oil may rise again and it was found that microbial degradation can hardly occurred due to the inhabitation of the absorbents used such as clay, Fuller's earth, and straw.

The use of chemical surfactant has been another popular method of treating contaminated areas. However, the use of detergents after the Torrey Canyon incident caused significant ecological damage as the detergents actually retarded the breakdown of oil by inhibiting oil-degrading bacteria (4).

A number of marine studies have related the toxic effects of chemical surfactant on the environment. Finding showed that in the case of marine organism, single surfactants were generally more toxic than oils and oil plus surfactant mixture although addition of surfactant to oils could reduce evaporation volatile. The effects of chemical surfactant and oil plus chemical surfactant mixture on microbial population were found to be variable and depended on whether the surfactant was biodegradable

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or possessed inhibitory, anti-microbial property (4). After the Torrey Canyon incident, the interest was paid on the role of microorganisms in removing oil from the environment. As a question raised whether hydrocarbon-utilizing ability is a characterize of a select groups of microorganisms or common to many microbial species, Zobell' s study indicated that more than 100 species of bacteria, yeasts, and fungi were capable to oxidizing hydrocarbons (5). It could also be said that the ability to utilize one or more hydrocarbons is a common characteristic of microorganisms.

Many microorganisms produced surface-active compounds (biosurfactant) when grown in media with hydrocarbons as a carbon source. These biocompounds can modify the interfacial and surface conditions. Microbes require a large contact area with the lipophilic phase in order to assimilate such water-insoluble substrates. The release of surface-active compounds promotes an emulsification of the hydrocarbon phase, rendering such lipophilic molecules available to the metabolic pathways of microorganisms (4).

Biosurfactants have several advantages over synthetic surfactants, such as: Biosurfactants present surface active properties differing in some cases from synthetic surfactants, providing new possibilities for industrial application; microbial surfactant have been shown to be more effective and specific than many conventional synthetic surfactants in specific applications; and they are usually non-toxic and biodegradable.

Several properties of these surface-active agents have been described, including surfactants, emulsifiers, and de-emulsifiers, flocculating agents, etc. Due to their high effectiveness, biosurfactants have been tested for some applications such as enhanced oil recovery and transport of heavy crude oils. Therefore, many applications

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of biosurfactants may be found in other fields, such as pharmacology, biocosmatics, textiles and food.

In Thailand, *Pseudomonas* J-45 an oil degrading bacteria, was isolated from an oil-contaminated site by Thailand Institute of Scientific and Technology Research (TISTR). Was found *Pseudomonas sp.* to be effective in oil degradable, It was shown to be able to grow better and produced higher crude oil degradable substances than the imported strain in environment and climate of Thailand, and was also demonstrated to be able to grow and degrade oil in various temperatures, salinity and pH (3). It can produce biosurfactant, which can reduce surface of water. Therefore, oil slick would be broken-down and combined with water, then become emulsion. In this form of emulsion, microorganisms would be able to utilize the small hydrocarbon molecules as a carbon source.

Biosurfactant could contribute to clean up to oil slicks by providing a factor for accelerating the naturally occurring process of conversion of oil pollutants to carbon dioxide and water soluble nontoxic organic materials and hope fully avoid effects caused by the toxicity of synthetic surfactant.

The study aimed at the suitable condition for the growth, crude oil degrading activity and biosurfactant formation from *Pseudomonas* J-45. This study was designed to isolation, purification, and identification to the type and properties of biosurfactant from *Pseudomonas* J-45. It is hopeful that the information generated from this study will be valuable in assessing the potential environment and industrial applications of this biosurfactant.

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## 1.2 Objectives of Study

**The objectives of this study were as follows:**

1. To study of the suitable condition to biodegradation of crude oil and biosurfactant formation by *Pseudomonas* J-45 in laboratory scale.

2. Isolation, purification and identification of biosurfactant produced by *Pseudomonas* J-45 in laboratory scale.

3. To study properties of the produced biosurfactant.

## 1.3 Variables of Study

### 1.3.1 Independent Variable

- Temperature
- Aeration rate
- Media

### 1.3.2 Dependent Variable

- Biomass
- Grease and oil value
- pH value
- Surface tension

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## 1.4 Definition of Terms

**Biodegradation**

Defined as destruction or breakdown of chemical compounds by the biological action of living micro-organisms and can be classified in to 3 levels as:

1. Primary change in the chemical structure of substance resulting in loss of a specific property of the substance.

2. Environmentally acceptable degradation to such an extent as to remove undesirable properties of the compound. This frequently corresponds to primary biodegradation. But may very depending on the circumstances under which the products are discharged to the environment.

3. Ultimate the complete breakdown of a compound to fully oxidized (aerobically) simple molecules (e.g. $CO_2$, $H_2O$, $NO_3^-$, $NH_4^-$) and the formation of new cells.

**Degradation**

Is any process by which the structure of a compound is simplified.

**Biosurfactant**

Biosurfactant are surface-active agents produced by certain types of microorganisms during growth on hydrophobic substrates.

**Critical Micelle Concentration (CMC)**

A concentration characteristic of a given surfactant at which certain solution properties change dramatically, indicating the formation of surfactant aggregates or micelles.

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**Surface Tension**

The property of a liquid was evidenced by the apparent presence of thin elastic membrane along the interface between the liquid and vapor phase, resulting in a contraction of the interface and reduction of the total interfacial area. Thermodynamically, the surface excess free energy per unit area of interface resulting from an imbalance in the cohesive force acting on liquid molecules at the surface.

**Interfacial Tension**

The property of a liquid/liquid interface exhibiting the characteristic of a thin elastic membrane acting along the interface in such a way as to reduced the total interfacial area by an apparent contraction process. Thermodynamically, the interfacial excess free energy resulting from an imbalance of forces acting upon molecules of each phase at near the interface.

**Petroleum Hydrocarbons**

Naturally occurring oily, flammable liquid is composed principally of hydrocarbons and constitute 50-98% of petroleum, and the remainder is composed chiefly of organic compounds containing oxygen, nitrogen, or sulfur and trace amounts of organo-matallic compounds.

**Cell Growth**

As mentioned earlier, bacteria can reproduce by binary fission, by as sexual mode, or by budding. Generally, they reproduce by binary fission (i.e., by dividing, the original cell becomes two new organisms). The time required for each fission, which is termed the generation time, can very from days to less than 20 mins.

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For example, if the generation times 30 mins, one bacterium would yield 16,777,216 bacteria after a period of 12 hrs.

## 1.5 Scope of the Study

1. To study optimum condition of crude oil degradation activity by *Pseudomonas* J-45 in 10 liters fermenter at various temperature, aeration rate and seawater.

2. To isolate and purify the biosurfactant produced by *Pseudomonas* J-45 in partially purified substance.

3. To identify type of the produced biosurfactant by chromatography technique and chemical reaction.

4. To investigate of properties of biosurfactant by *Pseudomonas* J-45 in the CMC value, Surface tension, interfacial tension and emulsification capacity evaluations at various condition and compare with synthetic surfactant.

## 1.6 Limitation of the study

Seawater sample was collected from Rayong province in the eastern of Thailand. However the crude oil biodegradation in seawater sample data available and how the data can not be applied for estimating crude oil biodegradation in seawater from other area of Thailand.

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**1.7 Expected outcome**

1. Biosurfactant used instead of chemical ones would reduce potential risks of residual chemical substances, which leads to a safe environment.

2. An efficient and effective biosurfactant would be recovered.

3. A number of imported chemical substances would be reduced.

4. A potential use of biosurfactant in industries would be considered as a better choice.

5. An ecological sound to aid oil removal would be developed.

**1.8 Conceptual Framework**

The conceptual framework in this study was showed in Figure 1.

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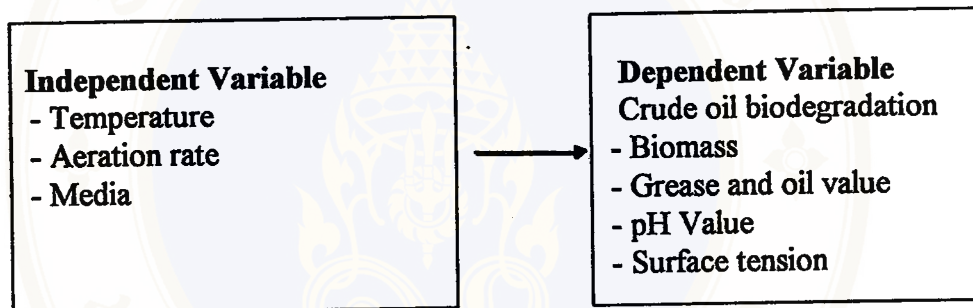


**Figure 1.** The conceptual frame work in this study

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# CHAPTER II

# LITERATURE REVIEW

## 1. Physical and Chemical Properties of Petroleum and related Hydrocarbon

Both crude oil and refined petroleum vary a complex mixture of hydrocarbons containing small amounts of other non-hydrocarbon compounds depending on their origins and the nature of refining process. Hydrocarbon in petroleum can be divided into four major classes: strength chain alkane (n-alkanes or n-paraffin), branched alkane (isoparaffin), cycloalkane (cycloparaffin), and aromatic. Molecular structures of these compounds are illustrated in Figure 2.

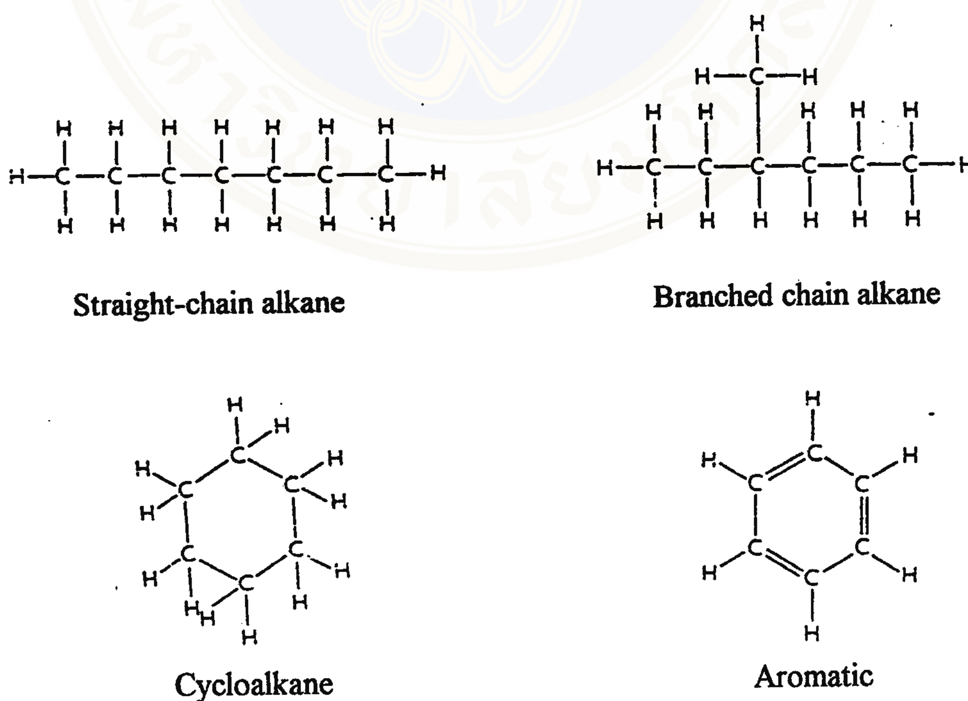


**Figure 2** Types of molecular structures found in petroleum, hydrogen atoms bound to carbon atoms are omitted (7,8)

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## 1.1 Type of Hydrocarbon Compounds form in Petroleum

### 1.1.1 Alkanes

Alkanes can be straight-chained, branched chains, or ring structure which are characterized by single chemical bounds between carbon atoms. The general formula of alkane is $C_nH_{2n+2}$. The most important characteristics of alkanes concerning are their volatility and biodegradability.

Table 1 lists certain physical constants for a number of the n-alkanes. As we Cannes, the boiling points and melting point rinse as the number of carbons increases. The process of boiling and melting require overcoming the intermolecular forces of a liquids and a solid: the boiling points and melting points rise because these intermolecular forces increase as the molecules get larger.

**Table 1** Alkanes (9)

| Name | Formula | M.p., °C | B.p., °C | Relative density (at 20 °C) |
|---|---|---|---|---|
| Methane | $CH_4$ | − 183 | − 162 | |
| Ethane | $CH_3CH_3$ | − 172 | − 88.5 | |
| Propane | $CH_3CH_2CH_3$ | − 187 | − 42 | |
| *n*-Butane | $CH_3(CH_2)_2CH_3$ | − 138 | 0 | |
| *n*-Pentane | $CH_3(CH_2)_3CH_3$ | − 130 | 36 | 0.626 |
| *n*-Hexane | $CH_3(CH_2)_4CH_3$ | − 95 | 69 | .659 |
| *n*-Heptane | $CH_3(CH_2)_5CH_3$ | − 90.5 | 98 | .684 |
| *n*-Octane | $CH_3(CH_2)_6CH_3$ | − 57 | 126 | .703 |
| *n*-Nonane | $CH_3(CH_2)_7CH_3$ | − 54 | 151 | .718 |
| *n*-Decane | $CH_3(CH_2)_8CH_3$ | − 30 | 174 | .730 |
| *n*-Undecane | $CH_3(CH_2)_9CH_3$ | − 26 | 196 | .740 |
| *n*-Dodecane | $CH_3(CH_2)_{10}CH_3$ | − 10 | 216 | .749 |
| *n*-Tridecane | $CH_3(CH_2)_{11}CH_3$ | − 6 | 234 | .757 |
| *n*-Tetradecane | $CH_3(CH_2)_{12}CH_3$ | 5.5 | 252 | .764 |
| *n*-Pentadecane | $CH_3(CH_2)_{13}CH_3$ | 10 | 266 | .769 |
| *n*-Hexadecane | $CH_3(CH_2)_{14}CH_3$ | 18 | 280 | .775 |
| *n*-Heptadecane | $CH_3(CH_2)_{15}CH_3$ | 22 | 292 | |
| *n*-Octadecane | $CH_3(CH_2)_{16}CH_3$ | 28 | 308 | |
| *n*-Nonadecane | $CH_3(CH_2)_{17}CH_3$ | 32 | 320 | |
| *n*-Icosane | $CH_3(CH_2)_{18}CH_3$ | 36 | | |
| Isobutane | $(CH_3)_2CHCH_3$ | − 159 | − 12 | |
| Isopentane | $(CH_3)_2CHCH_2CH_3$ | − 160 | 28 | .620 |
| Neopentane | $(CH_3)_4C$ | − 17 | 9.5 | |
| Isohexane | $(CH_3)_2CH(CH_2)_2CH_3$ | − 154 | 60 | .654 |
| 3-Methylpentane | $CH_3CH_2CH(CH_3)CH_2CH_3$ | − 118 | 63 | .676 |
| 2,2-Dimethylbutane | $(CH_3)_3CCH_2CH_3$ | − 98 | 50 | .649 |
| 2,3-Dimethylbutane | $(CH_3)_2CHCH(CH_3)_2$ | − 129 | 58 | .668 |

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The first four n-alkanes are gases, but as a result of the rise in boiling point and melting point with increasing chain length, the next thirteen ($C_5$-$C_{17}$) are liquids, and throes containing 18 or more are solids. The n-alkanes are only one groups of hydrocarbons able to from mixed crystals called "paraffin". Such crystals considerably increase the viscosity of an oil (10).

Branched chain alkanes are commonly occurred in petroleum. Examples of branched chain compounds, which have been isolated from crude oil, are 2,6,10,14-tetramethylpentadecane (pristane) and 3,5,11,15-tetramethylhexadecane (phytane). Both of these compounds are commonly cussed as market compounds during analysis of curdling oil because of their resistance to microbial degradation (11).

### 1.1.2 Cycloalkane

The cycloalkanes are saturated hydrocarbons. They can be monocyclic, bicyclic, substituted or unsubstituted. Their molecules have a ring form (10). Most of the cyclohexanes that found in crude oil are cyclopentane ($C_5H_{10}$) and cyclohexane ($C_6H_{12}$) (11). The structural formula of these compounds is shown in Figure 3.

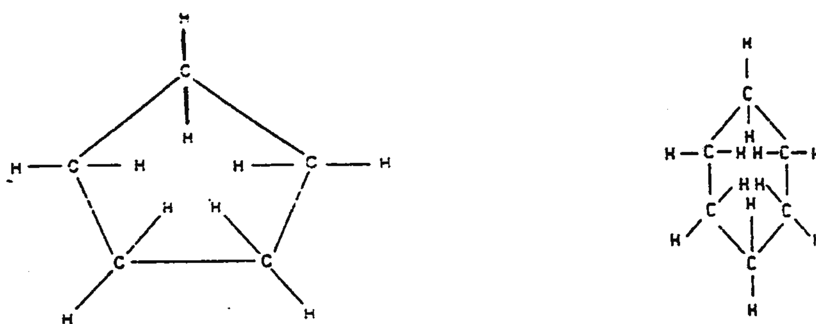

**Figure 3** Structural formula of cyclopentane and cyclohexane (10)

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In comparison with alkanes, cycloalkanes are similar to straight or branched chain alkanes in properties. Similar to alkanes, their lower molecular weights are toxic to microbial membranes. Their densities are less than 1, solubility's and vapor pressure decrease with increasing carbon atoms, and boiling points increase with the carbon atom (12). Generally, the density and boiling points of monocyclic alkanes are slightly higher than those of n-alkanes of the same carbon number (10).

#### 1.1.3 Aromatics

Aromatics hydrocarbon are compounds containing at least one benzene ring which dose not contain the maximum possible number of hydrogen atoms in the molecule. Thus, aromatics hydrocarbons are unsaturated compounds (10). This groups of major components of crude oil are benzene, toluene, the isomer of xylene, ethylbenzene, and 1,3,5-trimethylbenzene (11). Among these, benzene is the simplest one. The structural formula of benzene is show in Figure 4.

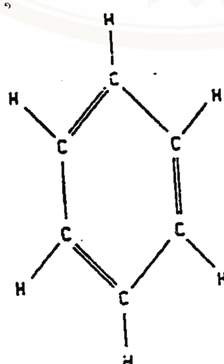

**Figure 4** Structural formula of benzene (10)

Each of these compounds has density less than one. Benzene is the most soluble of this class. It has a solubility of 1780 ppm at 20$^{0}$C, Whereas toluene of only

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515 ppm at the same temperature. The isomer of xylene has different solubility, i.e., 175 ppm for both ortho- and meta- xylene at 20$^0$C, and 198 ppm for para-xylene at 25$^0$C (12).

**1.1.4 Polycyclic aromatic hydrocarbon (PAH)**

Polycyclic aromatic hydrocarbons are aromatic hydrocarbons with two or more fused carbon rings. These compounds range from naphthalene ($C_{10}H_8$) to coronene ($C_{24}H_{12}$). PAH are present in small amounts in fuel and crude oils, and are a major component in creosote (13). Generally, PAH have low vapor pressure. Solubility and vapor pressure decrease with increase with molecular volume. PAH in the environment are formed by a process of combustion. The natural sources of PAH include forest and grass fires, oil seeps, and volcano (7). The example of PAH compounds are shown in Figure 5 and Figure 6.

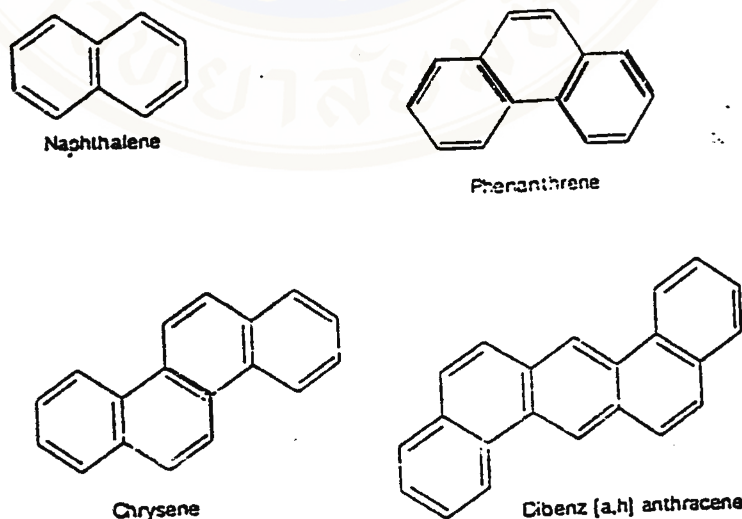


**Figure 5** Examples of PAH compounds without attached methyl groups or nitrogen, sulfur, and oxygen (8)

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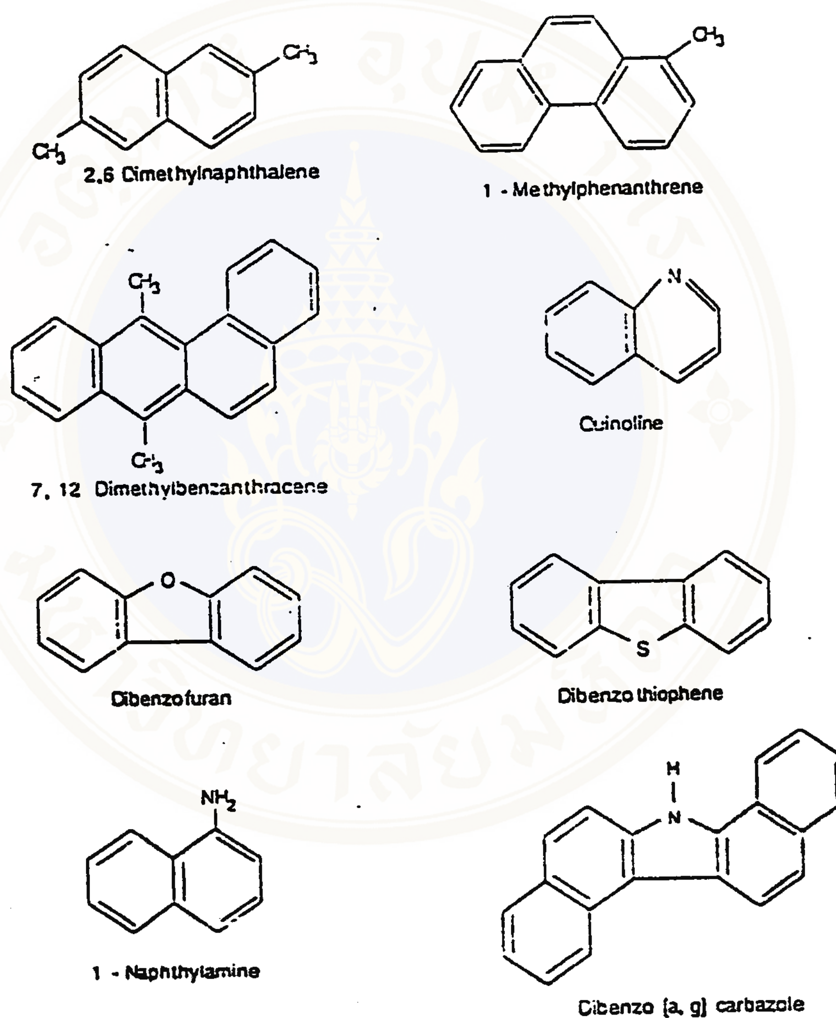


**Figure 6** Example of PAH compounds with attached methyl groups or nitrogen, sulfur, and oxygen (8)

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**2. The Fate of Spilled Oil in Aquatic Environment**

Petroleum in seawater is altered chemically by evaporation, dissolution, microbial action, chemical oxidation, and photochemical reactions, often collectively called weathering. The oil degradation degrades is markedly influenced by light, temperature, nutrients and inorganic substances, winds, tides, currents, and waves. They all affect the microbial degradation, evaporation, dissolution, dispersal, and sedimentation processes. Degradation rates appear to vary with the composition of the oil. The toxic fractions are generally less susceptible to microbial degradation. The heavy residuals that do not degrade may be deposited in sediments or they may float as tar lumps or tar ball (2).

The major process involved in the degradation of oil is as follows, as outlines in EPA report PB 213,880 (2).

**Spreading**: This process, the first to occur, thins the slick out to a few millimeters or less The rate dependent on several parameters, among them, viscosity of crude oil, surface tension of the oil and water, and time.

**Evaporation**: Evaporation is the process by which the low molecular weight compounds of relatively low boiling point are volatilized into the atmosphere. The rate of this process is also governed by many parameters, among them are viscosity of the oil, type of oil, and weather conditions, such as wind and sea state. The major loss due to evaporation occurs during the first few days.

**Dissolution**: Dissolution is the process by which the low molecular weight compounds and polar compounds are lost by the oil to the large volume of water under and around it. The rate of this process is also governed by many parameters including

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the type of oil, viscosity of the oil, the amount of oxidation the oil has undergone before, during and after the spill and the weather conditions such as wind. Although this process starts immediately, it is a long term one and continues throughout the duration of the total weathering process since the oxidation and microbiological degradation process constantly produce polar compounds which are finally dissolved in the water.

**Emulsification**: Emulsification is the process by which one liquid is dispersed into another immiscible liquid in droplets of optically measurable size. In the case of oil, The emulsion can be either an oil-in-water or a water-in-oil emulsion.

**Auto-oxidation**: Auto-oxidation is the light catalyzed reaction by which hydrocarbons react which atmospheric oxygen to from ketones, aldehydes, alcohols and carboxylic acids which are all polar component and, therefore, can either dissolve in the water or act as emulsifying agent or detergents.

**Microbiological degradation**: Microbiological degradation is a multifaceted process. Certain bacteria, actinomycetes, filamentous fungi, and yeasts utilize hydrocarbons and chemically oxidized hydrocarbon as food sources.

**Aerobic microbial oxidation**: Most of the microorganisms, which oxidize hydrocarbons, are require oxygen in either the free or dissolved from. When the oxidation of the oil occurs at the air-water interface there is usually sufficient oxygen to allow the maximum biological degradation to occur. However, areas of activity beneath the surface in the water column or in bottom mud are severally limited by the supply of oxygen.

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**Anaerobic microbial oxidation**: A few organisms are known which oxidize hydrocarbon when little or no dissolved or free oxygen is present. These utilize nitrate or sulfate as their oxygen source.

**Sinking**: Evaporation, dissolution and oxidation of lighter hydrocarbons may cause the oil to increase its density. When this happens to a sufficient degree the oil will sink to the bottom where anaerobic microbial oxidation will be the main process of degradation.

**Resurfacing**: If the density of oil mass is reduced to a sufficient degree by anaerobic oxidation, the oil will float again and the process above will again occur until the oil has either completely disappeared or has reached some land mass.

**3. Microbial Involvement in Biodegradation of Petroleum Hydrocarbons**

The ability of the microorganism to degrade hydrocarbons has been known since 1895 when Mioyoshi reported the microbial utilization of paraffin (14). Shortly thereafter in 1905 Suhngess and Kaseres described the microbial consumption of methane (14).

In 1969 ZoBell (5) in his review noted that more than 100 species representing 30 microbial genera had been shown to be capable of utilizing hydrocarbons. In a previous review, Bartha and Atlas (15) listed 22 genera of bacteria, 1 algae genus, and 14 genera of fungi which had been demonstrate to contain members which utilize petroleum hydrocarbon; all of these microorganism had been isolated from an aquatic environment. The most important (based on frequency of isolation) genera of hydrocarbon utilizes in aquatic environments were *Pseudomonas,*

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*Achromobacter, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Brevibacterium, Corynebacterium, Flavobacterium, Candida, Rhodotorula, and Sporobolomyces* (15). Bacteria and yeast appear to be prevalent hydrocarbon degrades in aquatic ecosystems. In polluted freshwater ecosystems, bacteria, yeasts, and filamentous fungi all appear to be important hydrocarbon degrades (16).

Jones and Eddington (17) found that isolates representing 11 genera of fungi and 6 genera of bacteria were the dominant microbial genera responsible for hydrocarbon oxidation in soil sample. They found that fungi played an important role in the hydrocarbon-oxidizing activities of the soil samples. Cerniglia and Perry (18) found that several fungi (*Penicillium* and *Cunninghamella sp.*) exhibited greater hydrocarbon biodegradation than bacteria (*Flavobacterium, Brevibacterium,* and *Arthrobacter sp.*).

Walker et al. (19) compared the abilities of bacteria and fungi to degrade hydrocarbons. The following genera were included in their study *Candida, Sporobolomyces, Hansenula, Aureobasidum, Rhodotorula, Cladosporium, Penicillium, Aspergillus, Pseudomonas, Vibrio, Acinetobacter, Leuothrix, Nocardia, and Rhizobium.* Bacteria and yeast showed decreasing abilities to degrade alkanes with increasing chain length.

Komagata et al. (20) examined almost 500 yeasts for their ability to degrade hydrocarbons and found 56 that could utilize hydrocarbons almost all of which were in the genus *Candida.*

Waker et al. (21) isolated *Vibrio, Pseudomonas,* and *Acinetobacter sp.* from oil-contaminated sediment and *Pseudomonas sp.* oil-free sediment. Microorganisms

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from the oil-free sediment produced greater quantities of polar compounds (asphaltics) after degradation. Whereas microorganisms from the oil-contaminated sediment provided greater degradation of saturated and aromatic hydrocarbons.

Walker et al. (22) also examined bacteria from water and sediment for their ability to degrade petroleum. Water samples contained a greater variety of bacteria species capable of degrading petroleum than sediment samples. Cultures from both water and sediment contained *Pseudomonas* and *Acinetobacter sp.* Bacteria present in the water samples yielded significantly greater degradation of two-, three-, four-, and five-ring cyclohexanes and mono-, di-, tri-tetra-, and penta-aromatics compared with bacteria from sediment samples.

Both temperature and chemical composition of a crude oil have been show to have a selective influence on the genera of hydrocarbon utilizes.

Some isolated thermopiles are obligate hydrocarbon utilizes and cannot grow on other carbon sources, The possible existence of obligate hydrocarbon utilizes is intriguing but perplexing, since the biochemical degradative pathways in dictate that hydrocarbon utilizes must also be capable of metabolizing fatty acid and alcohols (36).

It is now abundantly clear that the ability to utilize hydrocarbons is widely distributed among diverse microbial populations. Hydrocarbons are naturally occurring organic compounds. And it is not surprising that microorganisms have evolved the ability to utilize these compounds. When natural ecosystems are contaminated with petroleum hydrocarbons microbial communities likely to contain microbial populations of differing taxonomic relationships, which are capable of degrading the contaminating hydrocarbons (36).

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**Table 2 Genera of hydrocarbon-degrading bacteria, fungi, and yeasts (8)**

| Bacteria | Fungi | Yeasts |
|---|---|---|
| *Archomobacter*[a,b,c] | *Acremonium*[a] | *Candida*[c] |
| *Acinitobacter*[a,b,c] | *Allescheria*[b] | *Cryptococcus*[c] |
| *Actinomyces*[b,c] | *Aspergillus*[a,b] | *Debaryomyces*[c] |
| *Aeromonas*[b,c] | *Aureobasidium*[a,b] | *Endomyces*[c] |
| *Alcaligenes*[a,b,c] | *Beauveria*[a] | *Hansenula*[c] |
| *Arthrobacter*[a,b,c] | *Botrytis*[a,b] | *Mycotorula*[a] |
| *Bacillus*[a,b,c] | *Candida*[a,b] | *Pichia*[c] |
| *Beneckea*[b,c] | *Cephalosporium*[b] | *Rhodotorula*[c] |
| *Brevibacterium*[a,b,c] | *Chrysosporium*[a] | *Saccharomyces*[c] |
| *Chromobacterium*[a] | *Cladosporium*[a,b] | *Selenotila*[c] |
| *Corynebacterium*[a,c] | *Cochiobolus*[a] | *Sporidiobolus*[c] |
| *Coreneforms*[b] | *Cunninghamella*[b] | *Sporobolomyces*[c] |
| *Cytophaga*[a] | *Cylindrocarpon*[a] | *Torulopsis*[a] |
| *Erwinia*[a,b] | *Debaryomyces*[a,b] | *Trichosporon*[a] |
| *Flavobacterium*[a,b,c] | *Fusarium*[a,b] | |
| *Klebsiella*[b] | *Geotrichum*[a] | |
| *Lactobacillus*[b] | *Glaphium*[a] | |
| *Leucothrix*[b] | *Gliocladium*[a] | |
| *Methylobacter*[c] | *Gonytrichum*[b] | |
| *Methylobacterium*[c] | *Hansenula*[b] | |
| *Methylococcus*[c] | *Helminthosporium*[b] | |
| *Methylocystis*[c] | *Humicola*[a] | |
| *Methylomonas*[c] | *Monilia*[a] | |
| *Methylosinus*[c] | *Mucor*[b] | |
| *Micromonospora*[c] | *Oidiodendrum*[b] | |
| *Moraxella*[b] | *Paecilomyces*[a,b] | |
| *Mycobacterium*[a,c] | *Penicillium*[a,b] | |
| *Nocardia*[a,b,c] | *Phialophora*[b] | |
| *Peptococcus*[b] | *Phoma*[a] | |
| *Proteus*[a] | *Rhodotorula*[a,b] | |
| *Pseudomonas*[a,b,c] | *Rodosporidium*[b] | |
| *Sarcina*[a,b] | *Saccharomyces*[a,b] | |
| *Serratia*[a] | *Saccharomycopisis*[b] | |
| *Spherolilus*[b] | *Spicaria*[a] | |
| *Spirillum*[a,b,c] | *Tolypocladium*[a] | |
| *Streptomyces*[a,b] | *Trichoderma*[a,b] | |
| *Vibrio*[a,b,c] | *Trichosporon*[b] | |
| *Xanthomonas*[a,b] | *Verticillium*[a] | |

*Note: a = in soil, b = in marine, c = aliphatic degrader*

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**4. Biodegradation**

Biodegradation is natural process where by bacteria and other microorganisms alter and break down organic molecules into other substances, eventually producing fatty acids and carbon dioxide (23).

Biodegradation of hydrocarbon by indigenous microorganisms represents one of the primary mechanisms by which hydrocarbons are eliminated from the environmental. The microorganisms that consume the petroleum are "hydrocarbon oxidizers" that destroy petroleum molecules by adding oxygen to them. The newly formed oxygenated molecules are further consumed until only the final products, biomass and carbon dioxide, remain.

**4.1 Factors affecting Biodegradation of Hydrocarbons**

Biodegradation of petroleum hydrocarbon in the environment are determined by the populations of indigenous hydrocarbon degrading determined by the populations of indigenous hydrocarbon degrading microorganisms, the physiological capabilities of those population, and various abiotic factors that influence growth rates of those hydrocarbon-degrading microbial population. Rates of biodegradation under optimal population. Rates of biodegradation under optimal laboratory conditions have been reported to be as high as 2.5-100 kg/$m^3$ (24). Under *in situ* conditions, petroleum hydrocarbon biodegradation rates are in the order of lower magnitude, *In situ* natural rates have been reported in the range of 0.001-60 g/$m^3$ per day (6). Environmental variable greatly influences the rate of biodegradation.

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#### 4.1.1 Physical state of Oil

The concentration of soluble hydrocarbons is normally very low in water. Biodegradation of hydrocarbons occurs at the oil-water interface; thus availability of increased surface area of the oil droplets should accelerate biodegradation. Oil spreads in form of thin slick and disperses in form oil-in-water emulsions offering a very large surface area for bacterial attack and easy access to oxygen and dissolved nutrients. However, if a water-in-oil emulsion forms a thick "mousse", rates of oil biodegradation are slow. The degree of spreading is reduced at low temperature because of increased viscosity of the oil. Chemical dispersants can enhances biodegradation due to the increased surface areas, but some dispersants may contain chemicals, which cause mortality of flora and fauna (25). Many hydrocarbon-degrading microorganisms produce emulsifying agents that facilitate their abilities to degrade hydrocarbons. Emulsification helps the true dissolution of hydrocarbons in water and also provides an enlarged surface area for direct contact of microorganisms with liquid hydrocarbon droplets. However, if oil does not spread, there is limited surface area for microbial attack. Tarballs, which are large aggregates of weather and undegraded oil, also restrict access by microorganisms because of their limited surface area.

#### 4.1.2 Temperature

The temperature of most seawater is between -2$^0$C to 35$^0$C (26). Hydrocarbon biodegradation can occur in this entire temperature renege. Phychrotrophic, mesophilic, and thermophilic hydrocarbon utilizing microorganisms have been isolated. However, low temperatures generally suppress degradation rates

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by suppressing growth rates and metabolic rates of the microorganisms due to increase of toxic volatile compounds in the petroleum, since these compounds evaporate more slowly at low temperature. Nevertheless, Zobell (27) reported hydrocarbon degradation at temperature below $0^0$C. Higher temperatures increase the rates of hydrocarbon metabolism to a maximum, typically in the range of $30^0$C to $40^0$C, above which the toxicity of hydrocarbons is increased and biodegradation is slow down (28). However, Klug & Markovetz (29) reported hydrocarbon degradation at temperature as high as $70^0$C.

**4.1.3 Nutrient**

The major limitation in biodegradation of hydrocarbons is an available source of nitrogen and phosphorous. Several investigators have reported that the scarcity of mineral nutrients in seawater is often a limiting factor for petroleum biodegradation (30, 31).

Petroleum is rich in carbon and energy but deficient in those mineral nutrients, such as nitrogen, phosphorus, and iron, which are needed to support microbial growth. In addition, non-oil-degrading microorganisms (including Phytoplankton) also consume the mineral nutrients in competition with oil-degrading microorganisms. In theory, approximately 150 mg of nitrogen and 30 mg of phosphorous are consumed in the conversion of 1 g of hydrocarbon to cell material (32).

Cowell et al. (33) concluded that oil from the Metulla spill is degraded slowly in the marine environment, because of limitation by the relatively low concentrations of nitrogen and phosphorus available in seawater.

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In addition to N and P, in offshore seawater the low availability of iron was also found to limit hydrocarbon biodegradation, but the same limitation was not evident in sediment rich near shore seawater (34). The concentration of iron may limit biodegradation in non-polluted seawater, and when iron is precipitated out of the environment as ferric hydroxide, under alkaline condition (36).

#### 4.1.4 Oxygen

The initial steps in the biodegradation of hydrocarbons by bacteria and fungi involve the oxidation of the substrate by oxygenases for which molecular oxygen is required (31). Requirements of oxygen can be substantial 3-4 parts of dissolved oxygen are necessary to completely oxidize 1 part of hydrocarbon into carbondioxide normally do not exist in the upper levels of the water column in marine (26) and freshwater (35) environmental. When oxygen is less available, the rates of biodegradation decrease. Thus, oil sunk to the sea floor and covered by sediment tales much longer to be degraded. The availability of oxygen in soils, sediments and aquifers may be limited and dependent on the type of soil. There were been several reports of anaerobic conversion of hydrocarbon but at negligible rates (37). Anaerobic degradation of aromatic petroleum hydrocarbons by microorganisms has been reported by several investigators (38). Anaerobic biodegradation is several orders of magnitude slower than aerobic biodegradation.

#### 4.1.5 Salinity

There are few publications studying on the effects of salinity on the microbial degradation of hydrocarbons. Rates of metabolism of hydrocarbons decrease as salinity increase (36), and high salt concentrations inhibit emulsification of

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crude oil. The inability of the strains to emulsify some crude oil in 1.0 %, 1.5% w/v sodium chloride and seawater within the first 24 hours corresponding to the results of Zajic et al. (39).

Ward and Brock (40) showed the rates of hydrocarbon metabolism decreased with increasing salinity in the range 3.3-28.4 % and attributed the results to a general reduction in microbial metabolic rates.

#### 4.1.6 pH

Seawater is well buffered by the carbonate-bicarbonate-carbon dioxide system and pH around 8.5 is remarkable uniform throughout the oceans. It does not appear to have an important effect on biodegradation rates. In contrast to aquatic systems, soil pH is ranging from 2.5 in mine spoils to 11.0 in alkaline desert (28). Range of the original pH of soil from 6.0 to 7.8 increased the rate of hydrocarbon biodegradation (41). Verstaete et al. (42) reported a near doubling of rates of biodegradation of gasoline in an acidic (pH 4.5) soil by adjusting the pH to 7.4. The rates dropped significantly, however, when the pH was further raised to 8.5.

### 5. Uptake of n-Alkanes

The high degree of insolubility of n-Alkanes and other hydrocarbons in aqueous media contradicts their highly productive transformation to cell carbon material required the appropriate growth rates and yield. Three uptake mechanisms of alkanes have been proposed to explain the hydrocarbon assimilation (43).

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### 5.1 The Uptake of Dissolved Hydrocarbon

This methods only applies to short-chain hydrocarbons with chain lengths shorter than C-10 due to their limited solubility in aqueous media. However, it is sufficient to support growth by conventional uptake mechanisms. Since this method of uptake does not require the intermediary of a surfactant, it can be discounted for the purpose of this discussion.

### 5.2 Direct-contact Mechanism

Frequently observed phenomenon in hydrocarbon fermentations is the formation of flocs. These have been defined as complex associations of cell, hydrocarbon droplets, and entrapped air bubbles (44). The reason for floc formation is to increase the lipophilicity of the cell surface, induced by growth on hydrocarbons; thus the microbial cells may bind to droplets of the substrate. The assumptions is made, therefore that contact thereby achieved allows transport of hydrocarbon into the cell via the lipophilic laves of the cell envelope.

Supportive evidence shows that the phenomenon is not noticed in non-utilize of hydrocarbons or is much reduced in utilize cultivated on non-hydrocarbon substrate (45). For example, a mannan-fatty acid complex in the cell envelope of *Candida tropicalis* was described when yeast grew on n-Alkanes (46). This material was implicated in hydrocarbon uptake in when adherence and growth was much reduced as the mannan was masked with concanavalin A. Furthermore, the complex was absent when glucose was used as the principal growth substrate.

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**5.3 Uptake of Emulsified Hydrocarbon**

This uptake mechanism is an extension to that described in the previous section, which describes that, the hydrocarbons is taken up by direct contact between the substrate and cells. However, in this case the substrate is first converted into sub micrometer-sized droplets, a process termed "accommodation"or "pseudosolubilization" (47). It is proposed that this emulsifying step is achieved by means of extracellular agents who in most cases have been shown to posses powerful surfactant properties.

The way by which the pseudosolubilized alkane, in conjunction with a surfactant, will be taken up into the cell cytoplasm is still obscure. The variety of cellular adaptations to facilitate the uptake of hydrocarbons was reviewed by Finnerty and Singer (48). These include formation of a highly hydrophobic cell surface and extensive changes in membrane lipid composition during growth. Alterations in cell morphology are well documented in bacteria, yeasts and molds (49).

The microbial cell properties, such as the formation of hydrophobic complex of polysaccharide and fatty acids in the cell wall, play an important role in the uptake of alkane. Charged and neutral biosurfactants would render the charged cell surface hydrophobic, then attach and uptake alkanes into the cell, whilst ionic biosurfactant like negatively charged, would from micelles to increase its surface area. The pores/channels in the cell wall of yeast that hydrocarbons penetrate in to the cell membrane were revealed by microscopic studies (50).

A hypothesis for the action of biosurfactants in alkane uptake (Figure 7) which also includes the participation of a specific amphipathic receptor/channel was

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proposed by Ratledge (51). The alkane micelle or the directly attached alkane (52,53) would then be partitioned into the strong hydrophobic channel, which excludes biosurfactants, by size, hydrophobicity, or energetic considerations (interaction with other surfactant molecules to form micelles or reversed micelles). Due to the alkane's hydrophobicity, the alkane agglomeration inside the cell was detected by electron microscopy.

The restricted activities of biosurfactants with the microorganisms could be explained by specific interactions of the amphiphatic channel constituents and the biosurfactants to dissociate the biosurfactant monomer of the emulsion droplet or those of the micelles. Deposits of alkanes in the plasma membrane and within the cell wall of *Candida sp.* described by Scott (54).

Micro alkane uptake and the role of biosurfactants, including the extracellular high-molecular-weight polymeric materials, is a complex dynamic mechanism. The interaction of micelles or emulsified alkane droplets, the charged cell wall, and subsequent transport of the alkane into the cytoplasm cannot be completely explained by any of the three hypotheses of alkane uptake. There is no direct evidence for the role of biosurfactants in this process.

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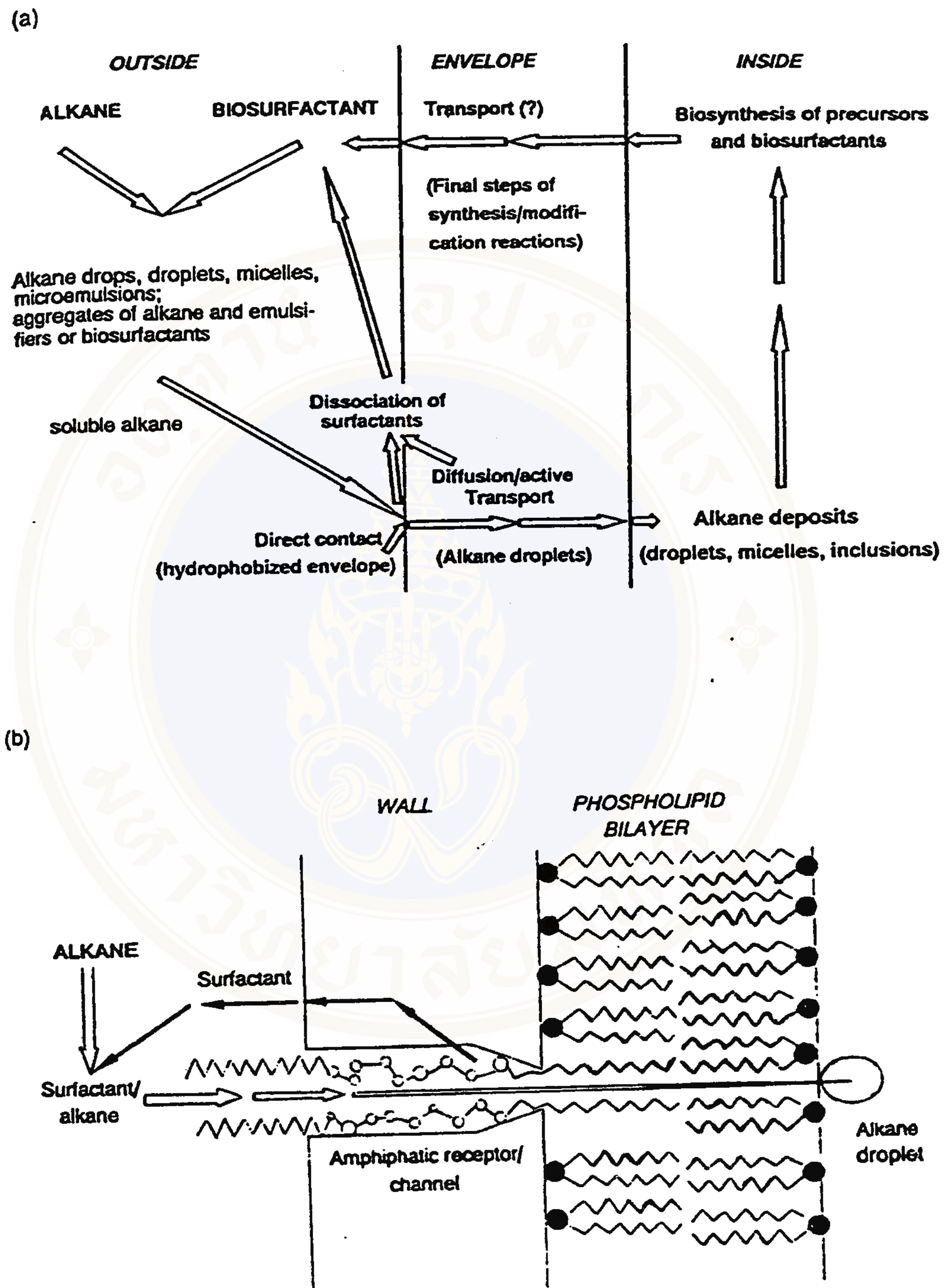


**Figure 7** Hypothetical scheme for surfactant-linked alkane uptake into a microbial cell (54). (a) General scheme; (b) Receptor channel concept

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## 6. Mechanism of Biodegradation of Petroleum Hydrocarbons

The actual mechanism of bacteria degradation of hydrocarbon contamination depends on the particular substrate metabolized and the type of microorganisms involved. However, the preferred pathway is via the electron transport chain using molecular oxygen as the terminal acceptor (55).

Degradation of aliphatic and aromatic compounds is realized in a stepwise fashion. Aliphatic terminal carbon oxidation is the first stage in the conversion, followed by a dehydrogenation reaction to the corresponding aldehyde. Oxidation continues in the third state conversion to the corresponding fatty acids, with then undergo oxidation to yield the fatty acids plus acetic acid. The acetic acid is then degraded further to yield carbon and energy for assimilatory purpose (Figure 8).

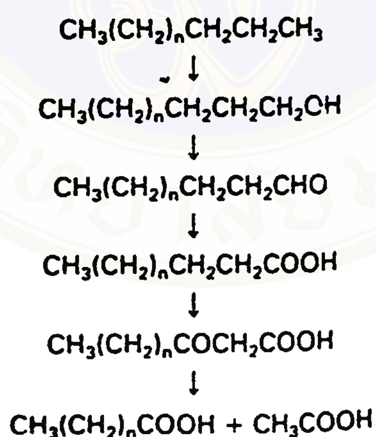


**Figure 8** Step of n-alkanes biodegradation (55)

The first phase aromatic metabolism is often the modification or removal of substituents on the benzene ring followed by stepwise conversion to catechol. Catechol is of primary importance as it represents the hydroxylated forms of benzene and phenol. Further on, aromatic rings such as toluene are assimilated via the extradial or

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Meta cleavage pathway, flowing identical R-group oxidation, as with the aliphatics (Figure 9).

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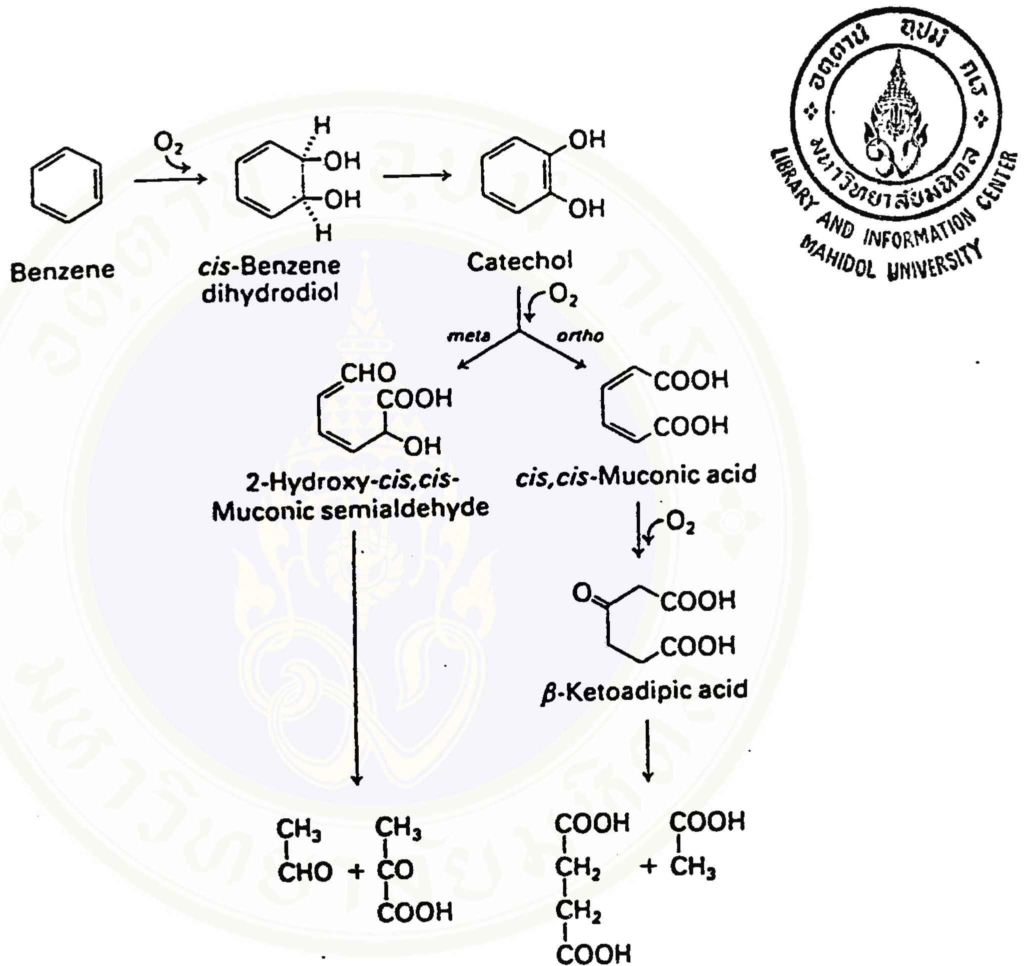


**Figure 9** Steps of benzene biodegradation (55)

Figure 10 illustrated the degradation of catecchol, with completes the bioconversion process generating organic acids used in TCA cycle. This in itself acts as an efficient receptor for the input of biochemical intermediates from catabolic pathways and is a principal source of metabolic energy in the form of adenosine triphosphate (ATP) (55).

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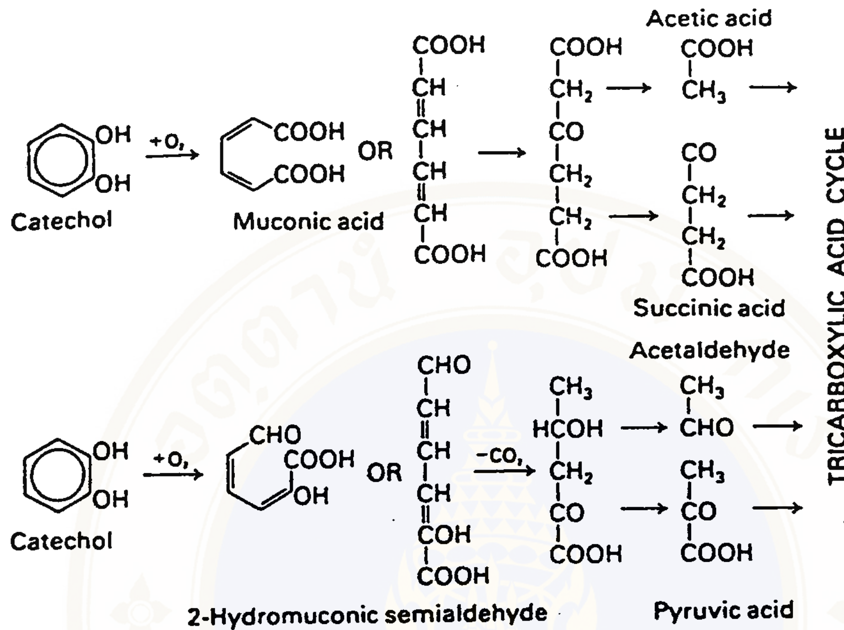


**Figure 10** Steps of catechol biodegradation (55)

**7. Biosynthesis of Biosurfactants**

Biosurfactants are amphiphilic compounds. They contain a hydrophilic and a hydrophobic molecule moiety. The polar molecule moiety can be a carbohydrate, an amino acid, a phosphate group, or some other compound. The nonpolar moiety is mostly a longchain fatty acid. In many cases the synthesis of hydrophilic and hydrophobic molecule moieties derives directly from the primary metabolism. This is reason that there are some common rules concerning the biosurfactant synthesis and its regulation in spilt of the wide spectrum of interfacial active compounds.

Concerning the biosynthesis of glycolipids, which constitute the greatest group among the biosurfactants, four different pathways are possible: (56)

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(1.) De novo synthesis of the carbohydrate and the lipid.

(2.) De novo synthesis of the carbohydrate moiety synthesis of the lipid moiety dependent on the chain length of the hydrocarbon substrate used for the glycolipid production.

(3.) De novo synthesis of the lipid moiety; synthesis of the carbohydrate moiety dependent on the substrate used for the glycolipid production.

(4.) Synthesis of carbohydrate and lipid moieties dependent on the substrates used for the glycolipid production.

The pathway of glycolipid formation depends on the microorganism. And example for the first group of glycolipid synthesis is the biosynthesis of anionic rhamnolipids by bacteria of the Pseudomonas *sp.* The rhamnolipids consist of one or two molecules of rhamnose and one or two molecules of beta-hydroxydecanoic acid (57). The biosynthesis of two of the rhamnolipids is well examined by experiments with enzyme extract and with resting cell by using different radioactivity labeled precursor (58).

Considering the results of those experiments, the scheme (Figure 11) shows the possible biosynthesis from n-alkanes as the sole C-source. Different cultivation conditions and the use of different C-sources influence the productivity and crude product composition (59), but the chain length of several hydrocarbon substrates has no influence on the chain length of the fatty acid of the glycolipids (60). The sugar moiety as well as the lipid moiety of the rhamnolipids is formed by de novo synthesis. Also other lipids, formed by *Pseudomonas aeruginosa* grown on different hydrocarbon substrates, are not affected by their chain length (61).

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Other examples for the de novo synthesis of biosurfactants are the cellobioselipids, formed by *Ustilago zeae* grown on glucose, and sophorolipids produced by resting and growing cells of *Torulopsis bombicola* from different lipophilic substrates (62).

The nonionictrehalose mono- and dicorynomycolates, formed by *Rhodococcus erythropolis,* are examples for the second group of glycolipid synthesis. While the sugar moiety of the molecule is formed by be novo synthesis, the chain lengths of lipid moieties are dependent on the hydrocarbon substrates used for trehalose-lipid production (52). Therefore the corvnomycolates are not formed by the de novo synthesis from C-2 units as the beta-hydroxydecanic acids of the rhamnolipids but by chain elongation.

Figure 12 shows a scheme of trehalose mono- and dicorynomycolate synthesis (63,64). Such a pathway can be observed also in the case of anionic trehalose tetraesters, formed by *Rhodococcus erythropolis* (59), mannosylorythnitollipids produced by *Candida sp.* (65). And extracellular glycolipids formed by *Torulopsis magnoliae* (66). The composition of the fatty acid portion varies with the carbon source used for glycolipid production. Different chain lengths of the formed lipids are also reported for *Nocardia erythropsis,* when different hydrocarbons are used as substrate (67). An influence of the substrate on the sugar moieties of the produced glycolipids is reported (69,70). This microorganism formed nonionic trehalose lipids when grown on n-alkanes as the C-source (72). *Arthrobacter sp.* Produced fructose lipids when the fructose was used as c-source (68), but with the sucrose, glucose and sucrose lipids were isolated from the culture broth (69).

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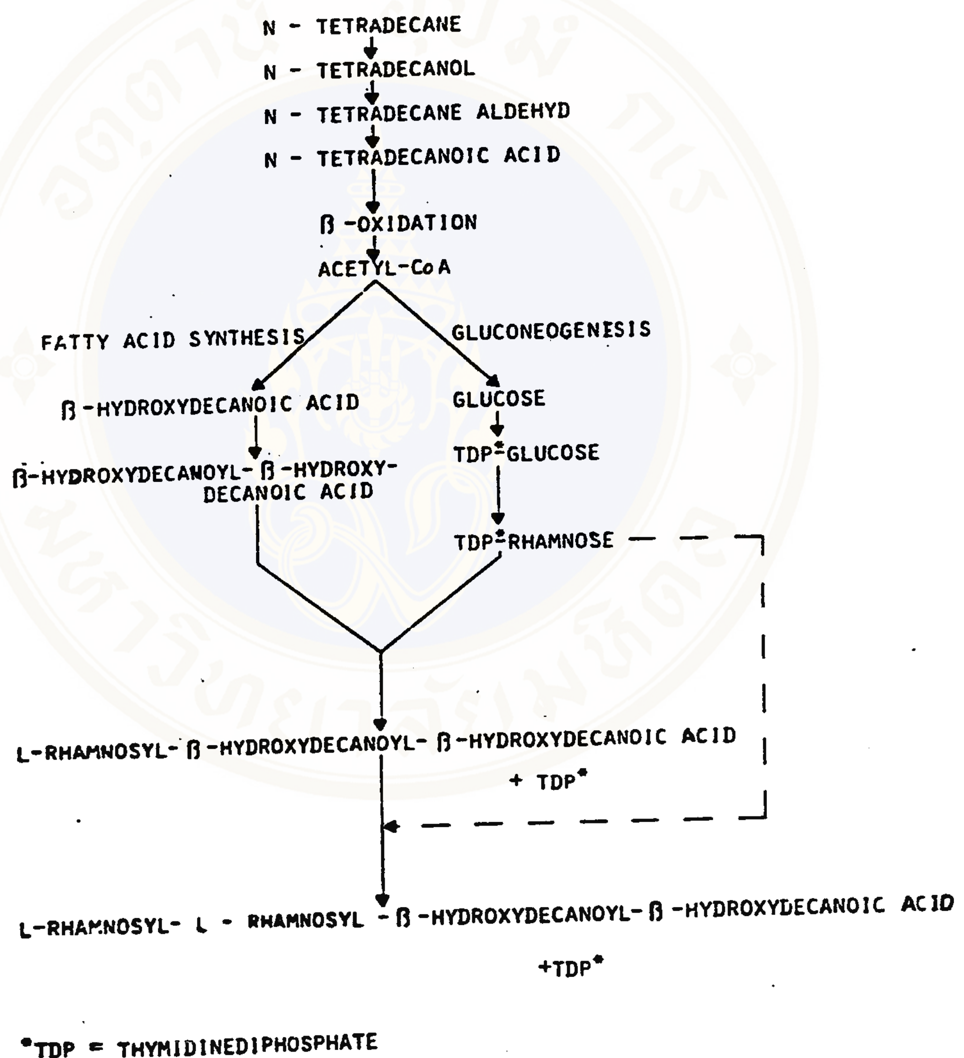


**Figure 11** Scheme of rhamnolipid biosynthesis by *Pseudomonas aeruginosa* from n-tertadecane (57)

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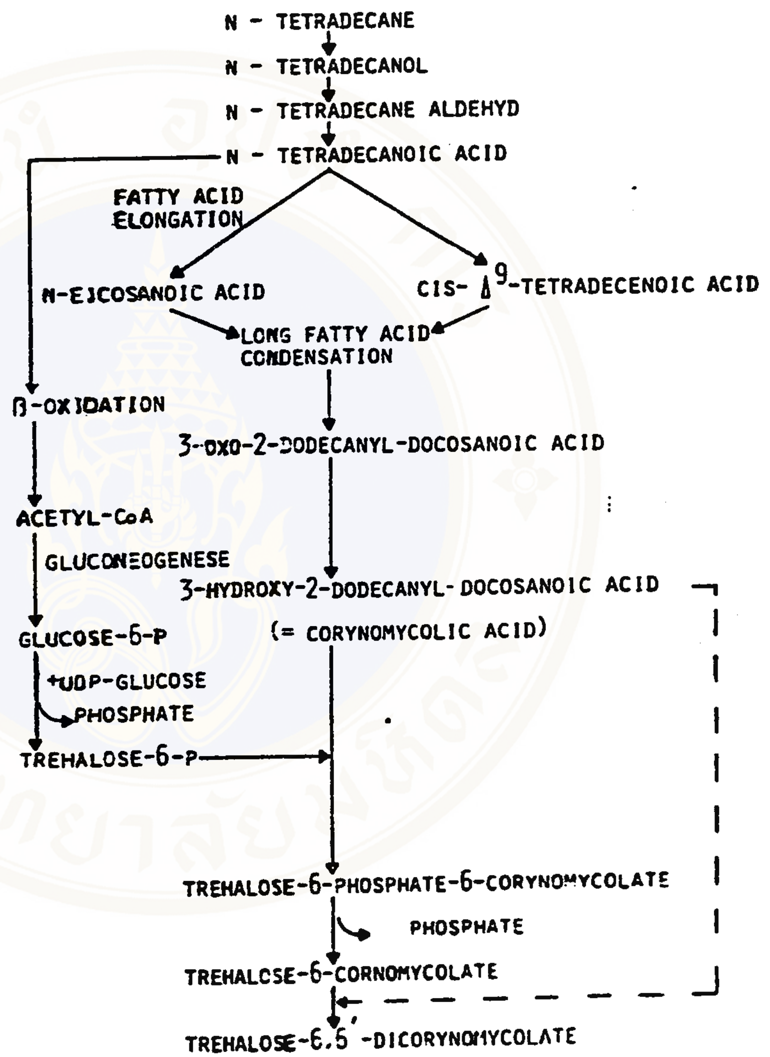


**Figure 12** Scheme of trehalose mono- and dicorynomycolate synthesis by *Rhodococcus erythropolis* from n-tetradecane (63,64)

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## 8. Source, Characteristics and Properties of Biosurfactant

Owing to the large surface-to-volume ratio and diverse biosynthetic capabilities, microbes are promising candidates in the search for enlarging our present range of surfactants. Many microbes appear to produce a complex mixture of biosurfactant, particularly during their growth on water-immiscible substrates. Among microbes, a majority of biosurfactants is found to be produced by bacteria. Generally, biosurfactants are microbial metabolites with typical amphiphilic structure of a surfactant, where the hydrophobic moiety is either a long-chain fatty acid, hydroxyl fatty acid, or alfa-alkyl-beta-hydroxy fatty acid and the hydrophilic moiety can be a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid, alcohol, etc. Physical and chemical properties, surface tension reduction, and stability of the emulsion formed are very important in the search for a potential biosurfactant. These properties are used in evaluating biosurfactant and in screening potential microorganisms of biosurfactant production.

Microbial surfactants are commonly differentiated on the basis or their biochemical nature the microbial species producing them. The important surfactant types and the producing microbial species are listed in Table 3. Major classes of biosurfactants include 1).Glycolipids, 2). Phospholipids and Fatty acid, 3). lipopeptide-lipoproteins, 4). Polymeric surfactants, 5). Particulate surfactant (71).

### 8.1 Glycolipids

Glycolipids, the most commonly isolated and studied biosurfactant, are carbohydrates in combination with long-chain aliphatic acids or hydroxyl aliphatic

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acids. Glycosyl diglycerides present in the cell membrane of a wide variety of bacteria are the most common glycolipids.

**Table 3 Major type of biosurfactants produced by microorganisms**

| Biosurfactant type | Producing microbial species |
|---|---|
| **A. Glycolipids** | |
| Trehalose mycolates | *Rhodococcus erythropolis* |
| | *Arthrobacter paraffineus* |
| | *Mycobacterium phlei* |
| Trehalose esters | *Mycobacterium fortitum* |
| | *Micromonospora* spp. |
| | *Mycobacteriun smegmatis* |
| | *Mycobacterium paraffinicum* |
| | *Rhodococcus erythropolis* |
| Mycolates of mono-, di-, and trisaccharide | *Corynebacterium diphtheriae* |
| | *Mycobacterium smegmatis* |
| | *Arthrobacter* spp. |
| Rhamnolipids | *Pseudomonas* spp. |
| Sophorolipids | *Torulopsis bombicola* |
| | *Torulopsis petrophilum* |
| | *Torulopsis apicola* |
| | *Candida* spp. |
| **B. Phospholipids and Fatty Acids** | |
| Phospholipids and fatty acids | *Candida* spp. |
| | *Corynebacterium* spp. |
| | *Micrococcus* spp. |
| | *Acinetobacter* spp. |
| Phospholipids | *Thiobacillus thiooxidans* |
| | *Aspergillus* spp. |
| **C. Lipopeptides and Lipoproteins** | |
| Gramicidens | *Bacillus brevis* |
| Polymyxins | *Bacillus polymyxa* |
| Ornithine–lipid | *Pseudomonas rubescens* |
| | *Thiobacillus thiooxidans* |
| Cerilipin | *Gluconobacter cerinus* |
| Lysin–lipid | *Agrobacterium tumefaciens* |
| | *Streptomyces sioyaensis* |
| Surfactin. subtilysin | *Bacillus subtilis* |
| Peptide–lipid | *Bacillus licheniformis* |
| **D. Polymeric Surfactants** | |
| Lipoheteropolysaccharide | *Arthrobacter calcoaceticus RAG-1* |
| Heteropolysaccharide | *A. calcoaceticus* A2 |
| Polysaccharide–protein | *A. calcoaceticus* strains |
| | *Candida lipolytica* |
| Manno–protein | *S. cerevisiae* |
| Carbohydrate–protein | *Candida petrophilum* |
| | *Endomycopsis lipolytica* |
| Mannan–lipid complex | *Candida tropicalis* |
| Mannose/erythrose–lipid | *Shizonella melanogramma* |
| | *Ustilago maydis* |
| Carbohydrate–protein-lipid complex | *Pseudomonas* spp. |
| | *Pseudomonas fluorescens* |
| | *Debaryomyces polymorphus* |
| **E. Particulate Biosurfactants** | |
| Membrane vesicles | *Acinetobacter* sp. H01-N |
| Fimbriae | *A. calcoaceticus* |
| Whole cells | Variety of microbes |

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The best examples of glycolipids studied from the point of view of surfactant characterization and properties are 1). Trehalose lipids, 2). Rhamnolipids, and 3). Sophorolipids.

**8.1.1 Trehalose lipids**

Several structure types of trehalose lipids are found to be widely distributed. Disaccharide trehalose linked at C6 and C6' to mycolic acids are associated with the cell wall structure of most species of the genera *Mycobacterium, Nocardia, and Corynebacterium.* Mycolic acid is long-chain α-branched β-hydroxyl fatty acids and the chain length synthesized is a characteristic of producing genera. Trehalose diester produced by *Rhodococcus erythropolis* and *Arthrobacter paraffineus* have been extensively studies by Rapp et al. (52) and Suzuki et al. (72). Production of novel nonionic trehaloselipid from *Rhodococcus erythropolis* have been isolated and characterized (73). Production of mono-, di-, trisaccharides has been reported using various species of *Corynebacterium, Mycobacterium,* and *Arthrobacter* (69,74).

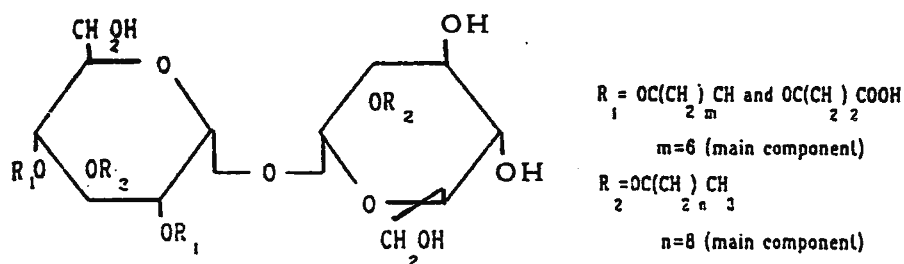


**Figure 13** Trehalose -2,3,4-2-tetraester produced by *Rhodococcus erythropolis* (73)

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Wagner and coworkers have extensively studied the surface and interfacial activities of treharoselipids. Trehalose lipid from *Rhodococcus erythropolis* has been shown to reduce surface tension to 25-30 mN/m and interfacial tension to 1 mN/m (75). Corynemycolates to various mono- and disaccharides from *Arthrobacter sp.* have also been reported to lower the surface tension to 33-44 mN/m and interfacial tension to 1-5 mN/m (75).

**8.1.2 Rhamnolipids**

Certain species of *Pseudomonas* are known to produce large amounts of glycolipids containing one or two molecules of rhamnose linked to one or two molecules of beta-hydroxydecanoic acid. Edward and Hyashi (76) and Hisatsuka et al. (77) have reported formation of glycolipid, type R-1, containing two rhamnose and two β-hydroxydecanoic acid units by *Pseudomonas aeruginosa*.

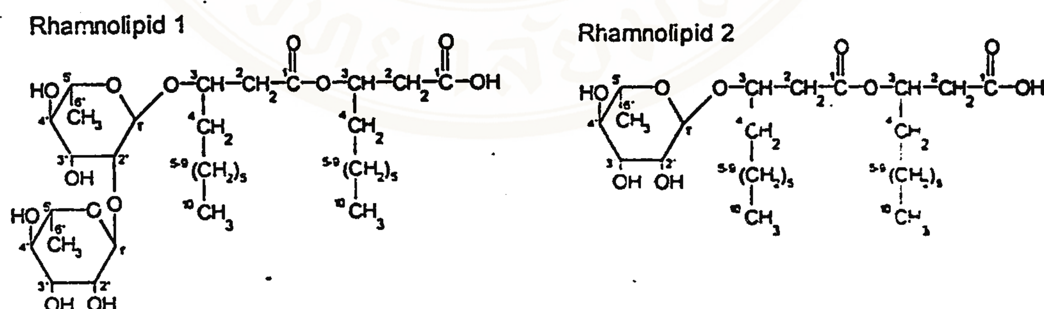


**Figure 14** Structure of the rhamnolipid from *Pseudomonas aeruginosa* (76)

Itoh *et al.* Later identified a second extracellular rhamnolipid (R-2), which was similar to R-1 but with only one rhamnose unit. It was postulated that R-2 was the precursor of R-1 (78).

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Hisatsuka et al. measured the surfactant properties of the R-1 lipid. The minimum surface tension of solution of R-1 in 0.1 M $NaHCO_3$ buffer was under 40 dyn/cm. The critical micelle concentration was about 0.006 %. The lipid was also found to be an excellent emulsion stabilizer. It produced very stable emulsion more efficiently than two commercial surfactant used for a comparison Tween 20 and Noigen EA 141. From properties, it was postulated that the function of the extracellular rhamnolipid was to act as an emulsifying for hydrocarbon substrate of *Pseudomonas aeruginosa* (77).

There are now several lines of evidences available to show that depending on the pH and salt concentration, pure rhamnolipids from *Pseudomonas sp.* can lower the interfacial tension against *n*-hexadecane to around 1 mN/m and surface tension to 25-30 mN/m (75,79,78).

**8.1.3 Sophorose lipids**

Production of biosurfactants heavier than water and consisting of the dimeric carbohydrate Sophorose linked to long-chain hydroxycarboxylic acids have been reported using *Torulopsis bombicola* (81), *Torulopsis petrophilum* (82), and *Torulopsis apicola* (66,83).

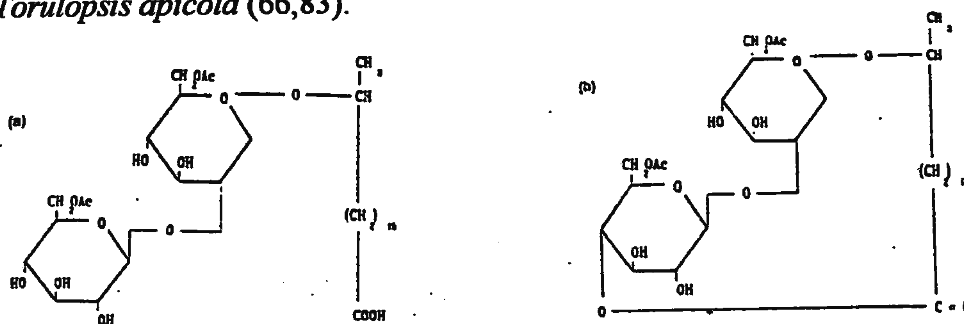


**Figure 15** Acidic (a) and lactonic (b) sophorose lipid of yeast *Torulopsis bombicola* (66)

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Species of the *Torulopsis* produce glycolipids that have some similarity to the bacterial rhamnolipids (83,84). Gorin *et al.*, were first to isolate these sophorose lipids from *Torulopsis magnoliae* (84). Like the R-1 lipid from *Pseudomonas aeruginosa,* these contain a disaccharide (i.e., sophorose) attached glycoside to the hydroxyl function of a hydroxycarboxycarboxylic acid.

Hommel et al. (85) have investigated the production of mixture of water-soluble sophorolipids from yeasts. Culter and light (86) showed that *Candida bogoriensis* Produces glycolipids in which sophorose is linked to docosanoic acid diacetate.

Although, sophorolipids lower surface and interfacial tension, they are not effective emulsifier agents (80). Both lactonic and acidic sophorolipids lowered the interfacial tension between n-hexadecane and water from 40 to 5 mN/m and showed a remarkable stability toward pH and temperature changes (75,82).

**8.2 Phospholipids and Fatty acids**

Phospholipids are found in very microorganism (87,88). In 1971 Beebe and Umbreit isolated both phosphatidylglycerol and phosphatidic acid from the broth. The best wetting agent, of all the lipids studied, was found to be phosphatidylethanolamine. This was not isolated from *Thiobacoillus Thiooxidans* cell-free medium but it present in the cell (89).

From *Corynebacterium lepus* a mixture of surface-active lipids was isolated that lowered the surface tension of distilled water to 49 dyn/cm. This mixture included several phospholipids, including phosphatidylglycerol, phosphatidylinositol,

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phosphatidylglycerol phosphate, cardiolipin, and a phosphatidylinositol mannoside (90).

The nature of the mixtures of phospholipids produced by microorganisms is influenced by the substrate. The amount of phospholipids produced by *Candida tropicalis* was much larger for cells grown on n-alkanes than for cells grown on glucose (91). Extracellular free fatty acid produced by microorganisms grown on alkanes also show surfactant activity. The important candidates are saturated fatty acid in the range of $C_{12}$ to $C_{14}$ and the complex fatty acids containing hydroxyl group and alkyl branches (92,93).

**8.3 Peptide and Amino acid containing Lipids**

Surfactin, a cyclic lipopeptide, reported first by Arima et al. (94) in *Bacillus subtilis* ATCC-21332, is one of the most effective biosurfactant known so far. It is capable of lowering the surface tension from 72 to 27.9 mN/m at a concentration as low as 0.005% (93). The ability of Surfactin to lyse red blood cells is of limited used, but this discovery has led to the development of quick method for the screening of biosurfactant-producing microbes (94). Production of surfactant by *Bacillus subtilis* (96) and lipopeptide surfactant, lichenysin by *Bacillus licheniformis JF-2* (97,98) with similar structural and physicochemical properties of Surfactin have been reported. *Bacillus licheniformis also* produce several other surface-active agents who act synergistically and exhibit excellent temperature, pH and salt stability (98).

A surfactant, BL-86 produced by *Bacillus licheniformis* 86, lowered surface tension of water to 27 dyne/cm and interfacial tension between water and n-

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hexadecane to 0.39 dynes/cm. The surfactant is stable to a wide range of pH, temperatures, and NaCl concentration (99).

**8.4 Polymeric Biosurfactants**

Height molecular weight biopolymers generally exhibit useful properties, such as high viscosity, tensile strength, and resistance to shear. It is, therefore, not surprising that polymeric biosurfactants have found a variety of industrial uses. The best studied of these biosurfactant are emulsan, liposan, and another polysaccharide-protein complexes.

**8.4.1 Emulsan**

*Acinetobacter calcoaceticus* RAG-1 has been shown to produce a potent extracellular polymeric bioemulsifier called emulsan (100). Emulsan has been characterized as a polyanionic amphipathic heteropolysaccharide (100). The heteropolysaccharide backbone contains repeating trisaccharide of N-acetyl-D-galactosamine, N-acetylgalactosamine uroic acid, and an unidentified N-acetyl amino sugar (101). In addition, fatty acid, which constitute about 10%-15% of the dry weight are shown to be covalently linked to the polysaccharide through O-ester linkage (101). Emulsan dose not appreciably reduce interfacial tension, but it is a very emulsifying agent for hydrocarbon in water even at a concentration as low as 0.001%-0.01%. It is one of the most powerful emulsion stabilizers known today and resists inversion even at a water-to-oil ratio of 1:4 (75).

**8.4.2 Liposan**

Extracellular water-soluble emulsifier, designated as liposan, is to be synthesized by *Candida lipolytica* (102,103). Ciriglino and Carmen recently

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purified and elucidated the structure of liposan. It is composed of 83% carbohydrate and 17% protein. The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid (103). The partially purified liposan stabilized the emulsion formed between many commercial vegetable oils and water (104).

**8.4.3 Other Polysaccharide Protein complexes**

Mannoprotein emulsifier production by *Sachcaromyces cerevisiae* has been reported by Cameron et al. (105). The purified emulsifier contains 44% carbohydrate (mannose) and 17% protein. This product emulsifiers many oils, alkanes, and organic solvent, and the emulsions are stable at extreme pH, temperatures, and salt concentrations. The emulsifiers from *Candida petrophilum* and *Endomycopsis lypolytica* also contain carbohydrate and protein (106,107). Hisatsuka et al. (108) isolated an emulsifying protein, called PA is approximately 14,300, and 51 out of 147 amino acids are serine and threonine (109).

Desai et al. reported the production of bioemulsifier by *Pseudomonas fluorescens* during growth on gasoline (110). The bioemulsifier is composed of 50% carbohydrate, 19.6% protein, and 10% lipid. A glycolipopeptide capable of emulsifying water-immiscible organophosphorus pesticides has been isolated from *Bacillus subtilis FE-2* (111).

**8.5 Particulate Biosurfactants**

Accumulation of extracellular membrane vesicles having 20-50 nm diameter and a buoyant density of 1.158 g/m$^3$ has been reported in *Acinetobacter* sp. HO1-N cells. The vesicles partition hydrocarbons in the form of micro-emulsion and

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play an important role in alkane uptake by the cells. The purified vesicles are composed of protein, phospholipid, and lipopolysaccharide. The vesicles have a phospholipids five times higher and a polysaccharide content 360-fold higher than that observed in the outer membrane of the same organism (112).

**9. Surface and Interfacial Tensions**

Regrading the molecules in a liquid, the attractive van der Waals forces between molecules are felt equally by all molecules except those in the interfacial region. This inequality in the van der Waals forces pulls the interfacial molecules toward the interior of the liquid. The interface thus has a tendency to contract spontaneously. For this reason, droplets of liquid and bubbles of gas tend to adopt a spherical shape, because this shape reduces the surface free energy. For two immiscible liquids, a similar situation applies that it may not be so immediately obvious the interface will tend to curve. There will still be an imbalance of intermolecular forces and a configuration that minimizes the interfacial free energy.

The surface free energy has units of millijoules per square meter ($1mJ/m^2$), reflecting the fact that area expansion requires energy. Surface free energies are usually described in terms of contracting forces acting parallel to the surface or interface. Surface tension, or interfacial tension, is the force per unit length around a surface, or the free energy required to create new surface area. Thus, the units of surface and interfacial tension are millinewtons per meter (1mN/m = 1 dyne/cm). These units for surface and interfacial tension are numerically equal to the surface free energy. Interfacial tensions are frequently intermediate between the values of the surface

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tensions of the liquids involved and are smallest when the liquids are the most chemically similar (for pure liquids) (113).

**10. Micelle Formation by Surfactant**

Micellization is the property that surface-active solutes have forming colloidal-sized clusters in solution. Micelle formation, or micellization, is an important phenomenon not only because a number of important interfacial phenomena, such as detergency and solubilization, depend on the existence of micelle in solution, but because it affects other interfacial phenomena, such as surface or interfacial tension reduction, that do not directly involves micelles.

In an aqueous media, when surfactants are at low concentration, the hydrophobic groups distort the structure of the water and increase the free energy of the system. To minimize the free energy of the system, surfactant monomers should orient so that their hydrophobic groups directed away from the solvent. Therefore surfactant monomers concentrate at the surface by replacing molecules of solvent at the interface, leading to surface tension reduction. If the surfactant concentration increase beyond the point at which the interface is completely covered with surfactant monomers, then the free energy of the system can be reduced by the aggregation of the surfactants monomers into cluster with their hydrophobic groups directed toward the interior of the clusters (micelles) and their hydrophobic groups directed toward the solvents. These cluster containing 50 to 200 monomers are called micelles. And his phenomenon is called micellization. At a specific concentration of monomers, the micellization begins to occur, is called Critical Micelle Concentration (CMC). Above

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the critical micelle concentration, the total monomer concentration still remains at the CMC. And if surfactants are added in the solutions, it forms micelles instead of monomers. At the critical micelle concentration, bulk properties are unusual. Changing in almost measurable physical property such as electrical conductivity, surface tension, light scattering and refractive index have been observed (114).

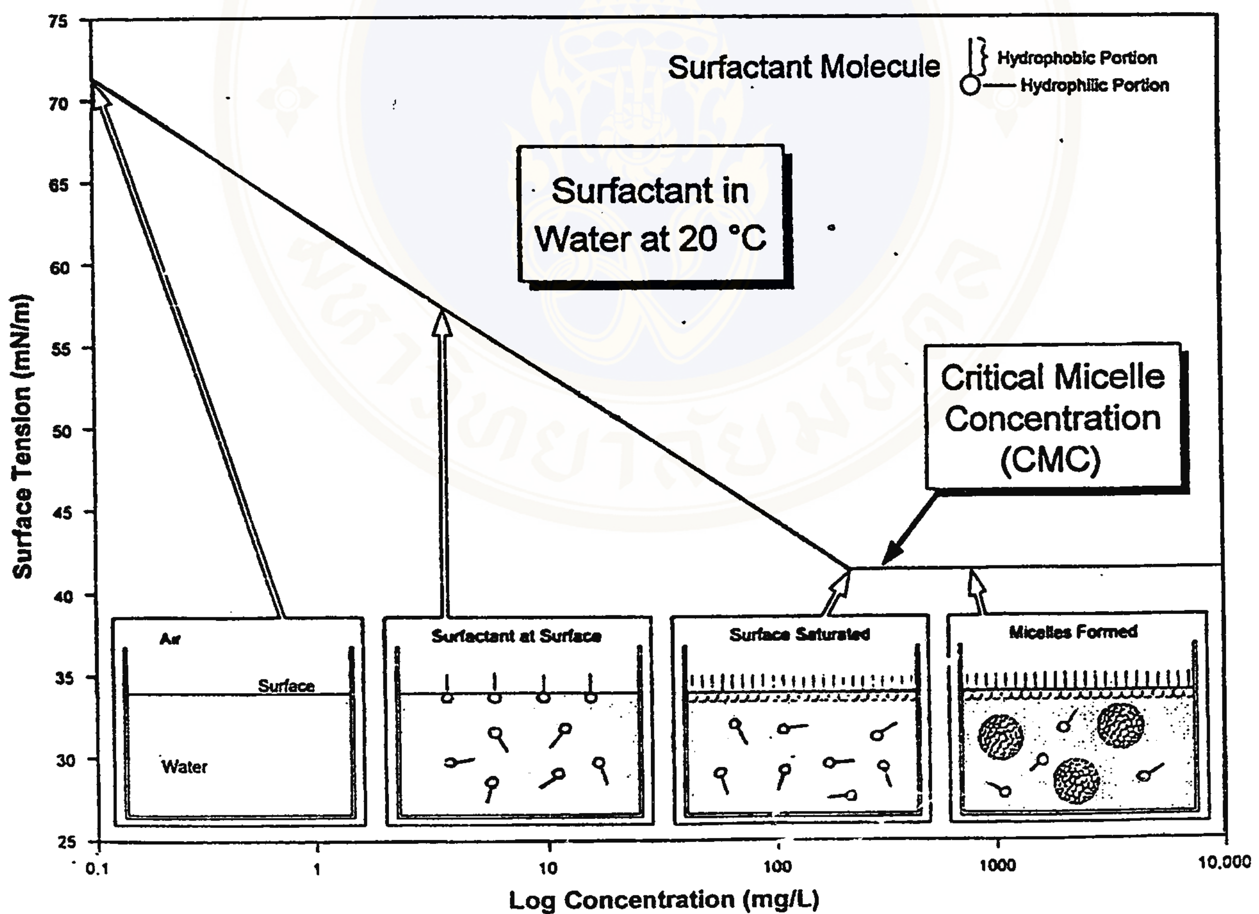


**Figure 16** The association behavior of surfactants in solution showing the Critical Micelle Concentration (CMC)

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The CMC is defined as the minimum concentration necessary to initiate micelle formation. There is little is further change in surface tension as any addition surfactant will be present in micelle from in the bulk phase. Efficient surfactant have very low critical concentration (i.e., less surfactant is necessary to decrease the surface tension).

Critical micelle concentration of biosurfactants (a measure of efficiency) range from 1 to 2000 mg/l. Surface and interfacial tensions (measure of effectiveness) of good biosurfactants are less than 30 and 1 mM, respectively. For example; a commercial surfactant has a CMC of 0.8 mM and costs $1/kg. However, if a biosurfactant has a CMC such as surfactin of 0.01 mM and costs $20/kg, it would be less expensive to use since less of the surfactant is required.

A comparison of biosurfactants and synthetic surfactants is shown in table 4 It is difficult to compare the properties of the biological with the synthetic, because the biological are poorly characterized due to mixtures of surfactants and interference from both components. For examples, the CMC of surfactin produced by *Bacillus subtilis* was determined initially by Cooper et al. to be 25-mg/L (115).

However, by measuring the concentration of the surfactin more accurately by acid hydrolysis of the compound and subsequent amino acid analysis, it was determined to be 11 mg/l (0.011 mM) (116).

Rhamnolipids produced by *Pseudomonas aeruginosa* have been measured by rhamnose content. This dose not indicated the actual amount present, because two rhamnolipids are produced and a ratio of the two is assumed (117). It makes it difficult to determined actual yields and properties. Once more accrete methods of measuring

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biosurfactant concentration are determined, it may be possible through genetic engineering, chemical modification, or culture conditions to alter the structure of the compounds so that they are more effective. The development cost, however, cannot be so high as to outweigh any gain in effectiveness.

**TABLE 4** Comparison of biosurfactant with synthetic surfactant.

| | Surface tension (mN/m) | Critical micelle concentration (mg/L) | 1990 Cost ($/kg)[a] |
|---|---|---|---|
| Producing organism | | | |
| *R. erythropolis* | 37 | 15 | 12.20 |
| *P. aeruginosa* | 29 | 15 | 5.90 |
| *T. bombicola* | 37 | 82 | 2.80 |
| *B. subtilis* | 27 | 11 (0.01 mM) | 20.32 |
| Anionic synthetics | | | |
| Detergent alkylate dodecylbenzene (LABS) | 47 | 590 (1.2 mM) | 1.03 |
| Sodium lauryl sulfate (SLS or SDS) | 37 | 2023–2890 (7–10 mM) | 0.95 (30%) 26.00 (98%) |

[a]Biosurfactant costs are calculated on the basis of raw material accounting for 35% of the total cost, assuming kerosene, molasses and soybean meal as substrates.
[b]Accurate Chemical and Scientific Corp., Westbury, NY.

Biosurfactant are produced as metabolic by-products by bacteria, yeast and fungi. They can produced extracellular or as part of the cell wall. They are not only potentially as effective but offer some distinct advantages over the highly used synthetic surfactant. Microbial surfactants exhibit high specificity and are consequently suited to new application. Effective physicochemical properties (low interfacial tensions and critical micelle concentration) and temperature stability are characteristic of these compounds. Other advantages include biodegradability, reduced toxicity, and a broad range of structures.

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Biosurfactants have been tested in enhanced oil recovery and the transportation of crude oil. They are effective in reducing the interfacial tension between oil and water in situ, reducing the viscosity of the oil removing water from emulsions prior to processing and releasing bitumen from tar sands. Its mean molecular weight is 9.9 x $10^5$, consisting of a polysaccharide backbone to with fatty acids and proteins are attached (118).

Application for other biosurfactants, in the future, will be in pharmacology, biocosmetics, textiles, food, pulp and paper, and coal benefaction. Since there are so many application and different sets of conditions exist for each, new products will always have to be developed with unique characteristics (119).

Biodegradability is also a requirement. It the surfactant is not toxic and is easily biodegradable, effluent treatment steps are not necessary before product release into the environmental or after treatment with the surfactants.

**11. Effects on Freshwater and Marine Inhabitants**

The crude oil contamination of rivers and marine ecosystems is a worldwide problem. To overcome these pollutants biosurfactants could be useful in the near future. In comparison to chemically synthesized detergents, a better biodegradability and a lower toxicity could be expected from microbial surface-active substances because of their biogenetic origin. Nevertheless toxicity testing for biosurfactants is also necessary. Known biological tests for these purpose are as follows;

(1.) Survival of fishes, daphnia, and algae.

(2.) Germination tests.

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(3.) Enzymatic tests.

(4.) Bioluminescence of bacteria.

Some application of these methods should be given. Zajic and Gerson tested crude microbial surfactants for toxicity using *Daphnia magna* (120). Table 5 shows that some microbial surfactants seem to be sources of nutrition to the *Daphnia* and death rates in the absence of surfactant exceeded those with biosurfactant (concentration near CMC). Results for several synthetic surfactants are included for comparative purposes.

Studying the effects of surfactants on special marine organisms burring tests to combat oil pollution in the European North Sea, found that some biosurfactants were superior to some synthetic detergents, stored for such purposes. In field tests, the brine shrimp *Corophium volutator* was more sensitive to synthetic detergents. In laboratory tests using larva of the brine shrimp *Artemia*, the results were similar (table 6) (121).

**Table 5** Toxicity of surfactant to *Daphnia magna*

| Surfactant | Source | Concentration[a] (ppm, w/v) | Mortality[b] |
|---|---|---|---|
| Microbial | | | |
| OSGB1 | *Corynebacterium* | 100 | -12 |
| ASPH-A1 | *Pseudomonas* | 200 | -4 |
| 5A | *Arthrobacter* | 300 | 17 |
| POSE | *Corynebacterium* | 500 | 25 |
| CD1 | *Corynebacterium* | 2000 | 65 |
| Synthetic | | | |
| Standamid | | 1050 | 100 |
| | | 105 | 55 |
| Unamide JJ-35 | | 100 | 100 |
| | | 10 | 100 |
| Petrostep A50 | | 900 | 100 |
| | | 90 | 100 |
| Span 20 | Atlas | 30 | 100 |
| | | 3 | 70 |

[a]1.0 or 0.1 × CMC.
[b]% mortality over controls in 48 h; negative numbers indicate greater survival than controls.

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**Table 6** Influence of surfactants on survival of marine organisms

| Crude oil + surfactant | Survival of *Corophium volutator* % | *Artemia*[a] $LC_{50}$/48 h (ppm) |
|---|---|---|
| Microbial | | |
| Trehalose-tetraester | 75 | 7,500 |
| Rhamnolipids | 72 | 3,000 |
| Synthetic | | |
| Finasol | 0 | 64 |
| Corexit | 5 | 440 |
| Without surfactant | 35 | n.d.[b] |

[a]Without crude oil.
[b]n.d. = not determined.

Recently Poramba et al. reported several toxicity testing series with marine microorganisms and their response to biosurfactant treatment. Bacteria growth *(A. caloaceticus, Photobacterium phosphoreum)* was slightly effects or stimulated, whereas that of algae and flagellates was reduced. Table 7 indicated a decreased multiplication of marine heterotrophic flagellates, especially in the case of some synthetic surfactants (122).

The highest sensitivity toward surfactants was found with the bioluminescence inhibition test (table 8). With the exception of GL, a new glucose lipid from the marine bacteria strain MMI, all surfactants decreased the bioluminescence of Photobacterium *phosphoreum* (123). Most EC values of synthetic surfactants were higher than those of biosurfactant (exception: the synthetic sucrose ester DK50 and DK160). Only special sophorose- and rhamnolipids (SL, RL) showed similar toxic effects. A subsequent

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ranking demonstrated that most biogenetic surfactants were less toxic than the synthetic ounces.

**Table 7** Growth inhibition of marine heterotrophic flagellates by surfactants. ($Ec_{fla-tox}$= surfactant concentration, in which on mass development over $10^5/cm^3$ occurred within 7 days)

| Surfactant | $EC_{fla-tox}$ (mg/L) |
|---|---|
| Biosurfactants | |
| TL-4 | >1000 |
| TL-2 | 500–1000 |
| GL | >1000 |
| LP | >1000 |
| Suc | >1000 |
| SL | 100–500 |
| SS | >1000 |
| RL | 25–50 |
| Emu | >1000 |
| Synthetic surfactants | |
| Finasol | 13–50 |
| Corexit | 50–100 |
| Pril | 25–50 |
| CTAB | 3–5 |
| E04,5 | 15–20 |
| E09 | 60–80 |
| DK 50 | >1000 |
| DK 160 | >1000 |

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**Table 8** Inhibition of the bioluminescence of *Photobacterium phophoreum* by surfactants. ; $E_{C5}0E_{C20}$ = Effective concentration at inhibition 50 (20) of luminescence; $EC_{max}$= maximal measured reduction of luminescence

| Surfactant | $EC_{50}$ (mg/L) | $EC_{20}$ (mg/L) | $EC_{max}$ (%) |
|---|---|---|---|
| Biosurfactants | | | |
| TL-4 | 286 | 33 | 24 |
| TL-2 | 49 | 7 | 43 |
| GL | | >3000 | 5 |
| LP | >3000 | 386 | 18 |
| Suc | 84 | 25 | 45 |
| SS | 141 | 12 | 54 |
| SL | 12 | 1 | 87 |
| RL | 50 | 6 | 100 |
| Emu | 202 | 10 | 50 |
| Synthetic surfactants | | | |
| Finasol | 7 | 1 | 100 |
| Corexit | 5 | 1 | 96 |
| Pril | 35 | 4 | 88 |
| CTAB | 0.5 | 0.3 | 100 |
| E04,5 | 79 | 38 | 45 |
| E09 | 78 | 7 | 57 |
| DK 50 | 67 | 27 | 20 |
| DK 160 | 334 | 88 | 17 |

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# CHAPTER III

# MATERIALS AND METHODS

This research work has been carried out in laboratory at Biotechnology laboratory, Thailand Institute of Scientific and Technology Research (TISTR). Framework of this experimental work shown in Figure 17 It was divided into three part namely suitable biodegradable condition of crude oil and biosurfactant formation by *Pseudomonas* J-45, Isolation, purification and identification type of biosurfactant produced by *Pseudomonas* J-45, and to study properties of the produced biosurfactant.

## 1. Experimental Procedure

An oil-degrading microorganism used in the present study was a *Pseudomonas* J-45 that obtained from the screening in Thailand that had been contaminated with crude oil for a period time. Thailand Institute of Scientific and Technology Research (TISTR) research team. The *Pseudomonas* J-45 was inoculated in a basal media called ASW medium (Artificial seawater medium, see the composition in Appendix) containing crude oil to examine for the ability to degrade crude oil.

### 1.1 Growth condition

The organism was grown for 24 h on Nutrient agar slant at $30^0$C. The bacterium were washed off in to 250 ml shake-flasks containing 100 ml ASW medium with 1 % sterilized crude oil and culture for 96 hours on a rotating shaker at 150 r.p.m. at room temperature. 800 ml of this culture was inoculated into 10 liter fermenter.

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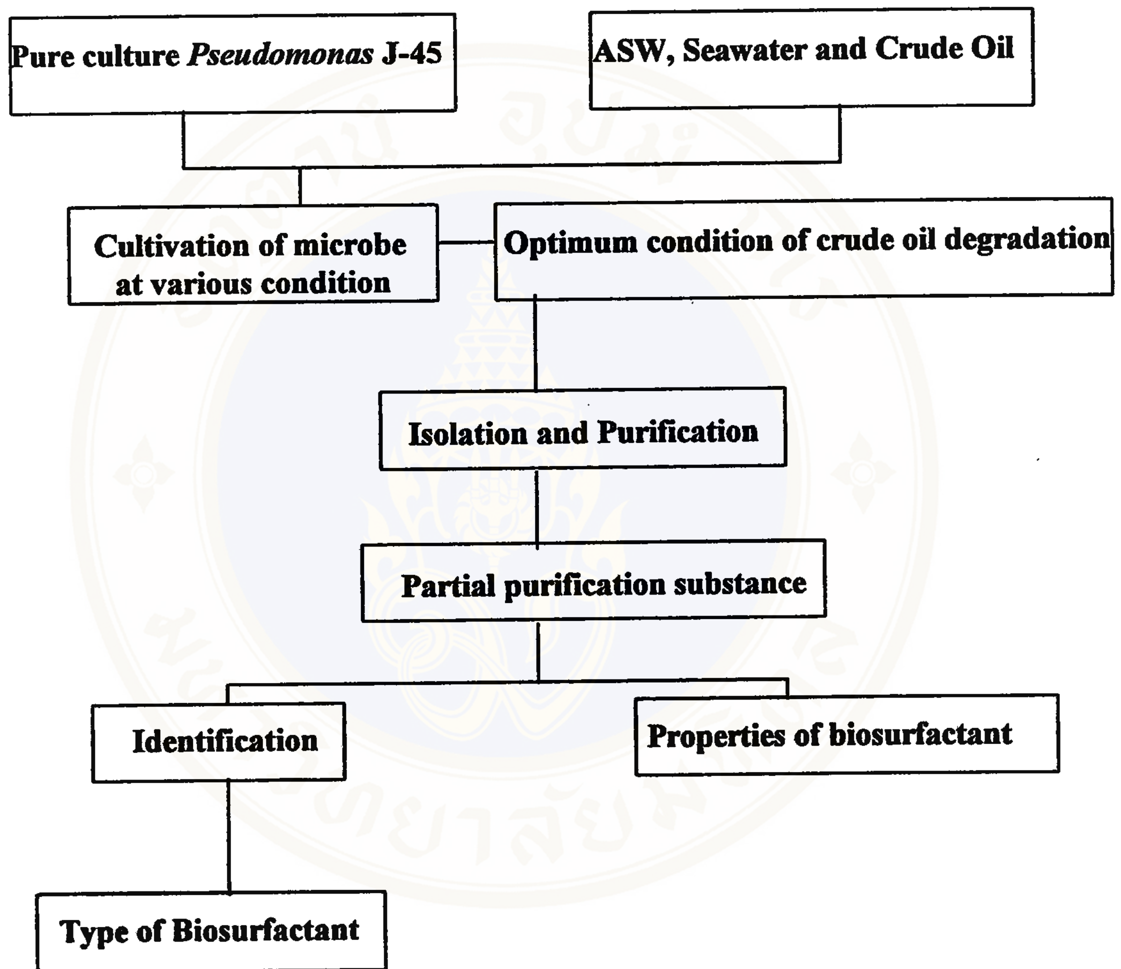


**Figure 17** Schematic diagram of experiment

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Experiments were also carried out in 10 liter New Brunswick Scientific Microferm Fermenter, using an 8 liter (ASW) with 1 % sterilized crude oil. The airflow rate was 1.0 v.v.m. the temperature was 30$^0$C, and two-flat-blade turbines were rotated at 100 r.p.m.. The sample was collected every 24 hours, 7 days. The cell growth, pH, Total petroleum hydrocarbon, surface tension were determined.

**1.2. Indicators for Growth and Oil Degrading activity**

**1.2.1 Cell growth**

Science the cell growth in hydrocarbon culture can not be estimated turbidimetrically, quantity of total protein in the culture broth was determined and used to express the cell growth a procedure modified from Suzuki et al. (72). The culture broth (1.0 ml) was treated with 1 N NaOH solution (1 ml) in a boiling water bath for 15 in and the determination of protein was carried out by the method of Lowry et al. (115) Bovine serum albumin was used for standardization.

**1.2.2 Total Petroleum Hydrocarbon**

Ten ml of the growing culture was transferred in a separator funnel, which had been previously rinsed with Tetrachloroethlene ($C_2Cl_4$). The sample tube was rinsed with 10 ml tetrachloroethylene and the washing solvent was pooled in the separator funnel. The mixture was shaked gently for 5 min. The tetrachloroethlene layer was then separated and drained through a funnel containing 1 g of anhydrous sodium sulfate on filter paper cone. The sample extract was determined value of total petroleum hydrocarbon with TPH-petroleum hydrocarbon analyzer.

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**1.2.3 Surface Tension**

The surface tension was measured to monitor biosurfactant production. Surface tension from the cell-free broth centrifuged at 8,000 r.p.m. For 20 min. at 4$^0$C with a ring tensiometer (K8, KRUSS, Hamburg, FRG). The surface tension was determined at a controlled liquid temperature (20$\pm$2$^0$C). Triplicate tests were performed for each measurement. The liquids were placed in a sample vessel (40 ml.). A DoNouy ring was carefully cleaned between each measurement by rinsing twice with acetone followed by the deionized and distilled water. The ring was then hung from the load sample vessel and lowered about 5 mm below the surface of the liquid. The ring was pulled from the surface and the apparent surface tension recorded. Care was taken to assure consistency in pulling the ring from the liquid for each measurement.

**1.3 Growth and Oil degrading activity of the *Pseudomonas* J-45 under Various conditions**

The crude oil was fermented with Pseudomonas J-45 was grown in 10 liter fermenter having medium with crude oil as one the raw material under various conditions, having in order to get the optimal conditions for growth, oil degrading activity, biosurfactant formation. The condition included temperature, aeration, and seawater.

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**1.3.1 Effect of Temperature**

It was carried out in 10 liter-fermenter (working volume 8 liter), operating at pH 7, and agitation speed 100 r.p.m. aeration rate 1.0 v.v.m. And various temperature at 25, 30, 37$^0$C respectively. The cultures were collected every 24 hr, 7 d. and analyzed for cell growth, pH, Total petroleum hydrocarbon, surface tension.

**1.3.2 Effect of Aeration**

It was carried out in 10 liter-fermenter (working volume 8 liter), operating at pH 7, and agitation speed 100 r.p.m. At temperature 30$^0$C and various aeration rate with 0.25 v.v.m. 0.5 v.v.m. and 1.0 v.v.m. respectively. The cultures were collected every 24 hr, 7 day and analyzed for cell growth, pH, Total petroleum hydrocarbon, surface tension

**1.3.3 Growth and Oil degrading activity of the *Pseudomonas* J-45 in Seawater**

The experiment was carried out in 10 liter-fermenter containing 8 liter seawater and 1 % sterilized crude oil was added. The seawater was sterilized by autoclaving at 121$^0$C, pressure 15 psi for 30 min., operating at temperature 30$^0$C, and agitation speed 100 r.p.m. aeration rate 1.0 v.v.m.. The culture were collected every 24 hr, 7 day and analyzed for cell growth, pH, total petroleum hydrocarbon, surface tension

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**1.4 Isolation, Purification and Identification of Biosurfactant produced by *Pseudomonas* J-45**

The experimental process was presented in Figure 18

Cultivated *Pseudomonas* J-45 at optimum condition.

Culture broth

Centrifuge at 8,000 r.p.m.
$4^{0}$C, 20 min

**Oil** | **Cell** | **Supernatant**

Cell:
- Lyophilize
- 1,000 mg
- Extract with Chloroform:Methanol (2:1) Floch (125)
- De-impurity with 0.88% KCl Floch (125)
- **Crude lipid (Crude biosurfactant)**

Supernatant:
- Filtered throughout 0.45 µm
- 1,000 ml
- Extract with ethyl acetate VanDyke (126) Evaporate to dryness
- Extract with Chloroform:Methanol (2:1) Floch (125)
- De-impurity with 0.88% KCl Floch (125)
- **Crude lipid (Crude biosurfactant)**

**Figure 18** Isolation of biosurfactant produced by *Pseudomonas* J-45

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**1.4.1 Production of Biosurfactant from *Pseudomonas* J-45**

The *Pseudomonas* J-45 was cultured in ASW medium containing 1 % crude oil in 10 liter fermenter. The fermentation process was performed with the agitation speed of 100 r.p.m. and the aeration rate of 1 v.v.m. at 30$^0$C for 96 hr. Afterwards, the cells were harvested by centrifuged at 8,000 r.p.m. for 20 min. at 4$^0$C. Discard the oil residue by suction. The bacteria cell was resuspend the cells in an equal volume of 0.85 % (w/v) NaCl, and centrifuge again. The cells were washed by centrifugation, and lyophilized them, stored in vial at -20$^0$C until use. Cell free supernatant was filtered through a Whatman membrane filter 0.45 micrometer. The supernatant fluid fraction was collected and stored at 4$^0$C.

**1.4.2 Isolation of Biosurfactant produced by *Pseudomonas* J-45**

**1.4.2.1 Extract of Biosurfactant from Supernatant**

Biosurfactant was recovery from culture supernatant after the remove cells by centrifugation at 8,000 r.p.m. for 20 min., 4$^0$C. Supernatant 1 l. was then extracted twice with 1 l. ethyl acetate (126). After evaporation of solvent to dryness the crude products were extracted with chloroform-methanol (2:1 v/v) three times (125). The organic solvent was evaporated on a rotary evaporator, After drying, and the yellowish oily residue was dissolved in chloroform-methanol (2:1 v/v) 10 ml. When storing lipids for many length of time, it is best to fill the container with as much solvent as possible, to seal under $N_2$ and store at -20$^0$C or less in the dark.

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**1.4.2.2 Extract of Biosurfactant from Lyophilized *Pseudomonas* J-45**

Biosurfactant from biomass was prepared by a modified method of Floch (116). Prepared One gram of lyophilized bacteria cells in glass bottle with 40 ml of chloroform-methanol (2:1 v/v) mixture. Shake the mixture at room temperature for another 30 min. Rapidly filter the extract through Whatman No.40 filter paper with slight suction. Transfer the filtrate to a glass separator funnel to allow separation of the layers. Re-extract the residue of cell material on the filter paper by shaking with 40 ml Chloroform:Methanol (2:1 v/v) mixture. Filter the mixture through Whatman No.40 filter paper, and rinse the filter with equal volume of Chloroform:Methanol (2:1 v/v) mixture. Add the combined filtrates to the chloroform layer from the first extraction, and evaporated them to dryness under reduce pressure at room temperature,. After drying, and the yellowish oily residue was dissolved in chloroform-methanol (2:1 v/v) 10 ml. When storing lipids for many length of time , it is best to fill the container with as much solvent as possible, to seal under $N_2$ and store at $-20^0$C or less in the dark.

**1.4.2.3 De-impurity of Crude biosurfactant**

For purified of crude total lipid (biosurfactant) was prepared by method of Folch (125). Dissolve of the dry crude total lipids (see preceding procedure) in to Chloroform:Methanol (2:1 v/v) mixture make up to 100 ml, transfer to separatory funnel. The crude extract is mixed thoroughly with 0.25 volume of 0.88% (w/v) KCl and the mixture is allowed to separate into two phase, with out

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interfacial fluff, either by standing. Collect and measure of total volume of chloroform phase, as much of the aqueous phase as possible removed. The chloroform phase was evaporated them to dryness under reduce pressure at room temperature, After drying, and the yellowish oily residue was transfer it to vials, and evaporated the solvents under reduced pressure at room temperature. After drying, weight the vial containing the dried lipid extract. Crude total lipid was dissolved in small amount of chloroform and store at -20$^0$C. Using known amounts of purified total lipids in the extraction and purification steps can check recovery of lipids.

**1.4.3 Partial purification of Biosurfactant from Total crude biosurfactant by Silica gel Column chromatography**

The experimental process was presented in Figure 19

For the extract of total crude biosurfactant from supernatant and dry cell bacteria. After evaporation of the solvent to dryness the crude products were initially separated using silica gel column chromatography using a procedure modified from Vorbeck and Marinetti (127): A slurry of 10 g of silica gel 60, 230-400 mesh (Merck, Darmstadt, FRG) in 50 ml of chloroform was poured into a glass column, 1.0 cm id. The final height of the adsorbent column was 20 cm. It was washed with 40 ml of chloroform and maximum flowrate 1.0 ml/min. The lipid sample, was applied to the column in 3-5 ml of chloroform and washed in with 5 ml portion of chloroform. Neutral lipids were eluted with 100 ml the glycolipid eluted with acetone, and were fractionated by use of increasing increments of acetone in methanol, and the phospholipids were eluted with 100 ml of methanol. Fractions (10 ml) were collected

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by means of an automatic fraction collector. Each tube from the column fractionation was analyzed for total carbohydrate and emulsification capacity. Assay total carbohydrate was by the anthrone reaction using the procedure of Hodge and Hofreiter (128). Emulsification capacity was determined by method of Banet et al. (129).

**1.4.4. Identification Type of the Biosurfactant produced by *Pseudomonas* J-45**

Identification of sugar moiety of partially purification glycolipids was as described by Suzuki et al (70). The experimental process was presented in Figure 20 The glycolipid isolated was dissolved in diethyl ether (10 ml) and treated with 1 N NaOH for 1 hr at $90^0C$ . After the addition of water (10 ml), the organic solvent layer was separated and the evaporated. The residue was extracted with diethyl ether. This procedure separated the fatty acid from sugars. The aqueous phase solution containing sugar was desalted by ion-exchange chromatography using a bed of Amberlite IR-120 (H-form). The sugar was identifying by thin-layer chromatography with solvent system (see below).

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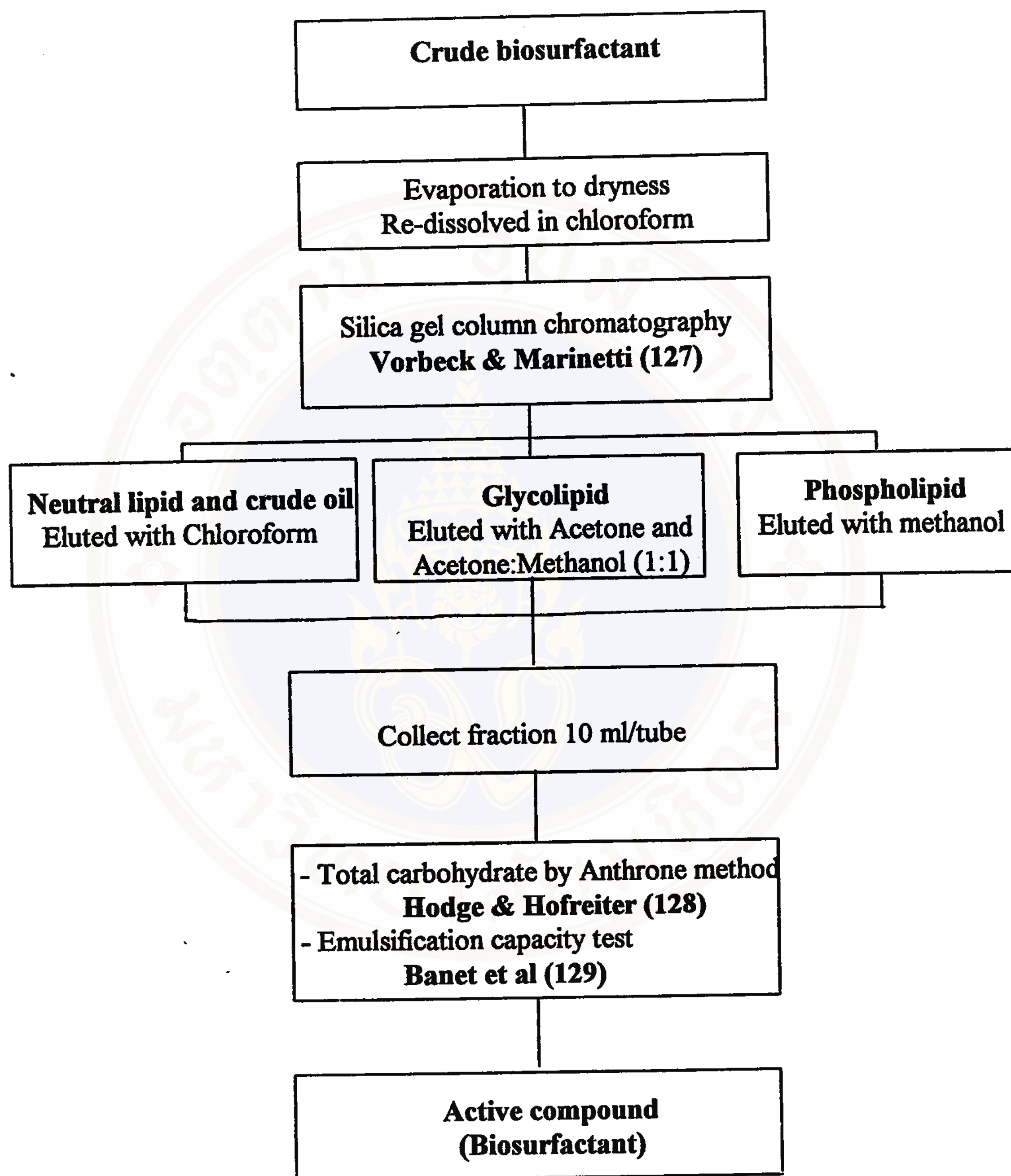


**Figure 19** Partial purification of glycolipid by silica gel column chromatography

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**1.4.5 De-Salting by Amberlite IR 120 (H-form) resin Column Chromatography**

Before use, Amberlite IR-120 (H-form) was washed with distilled water with stirring, until the intensive coloration of washing disappeared entirely was performed by the method described by Vladimir and Kveta (130). Amberlite IR-120 resin, was coveted into the H-form in portions of five grams in a 10 mm diameter column, by treatment with 4 N HCl solution 100 ml. Then the resin was washed with 1l of distilled water. Resin obtained in the H-form. The glycolipid hydrolysate was carefully added on the upper bed, just submerged in water. After sample soaked into the resin, 5 ml portions of water were added. These represented the initial portion of a total of 100 ml of water used for the elution. The maximum effluent rate was 1.0 ml/min. Collect of effluent with 5 ml/fraction by automatic fraction collector. Each fraction of elute was analyzed for total carbohydrate by the anthrone method. The fraction was positive anthrone reagent, lyophilized to dryness and used for thin-layer chromatography.

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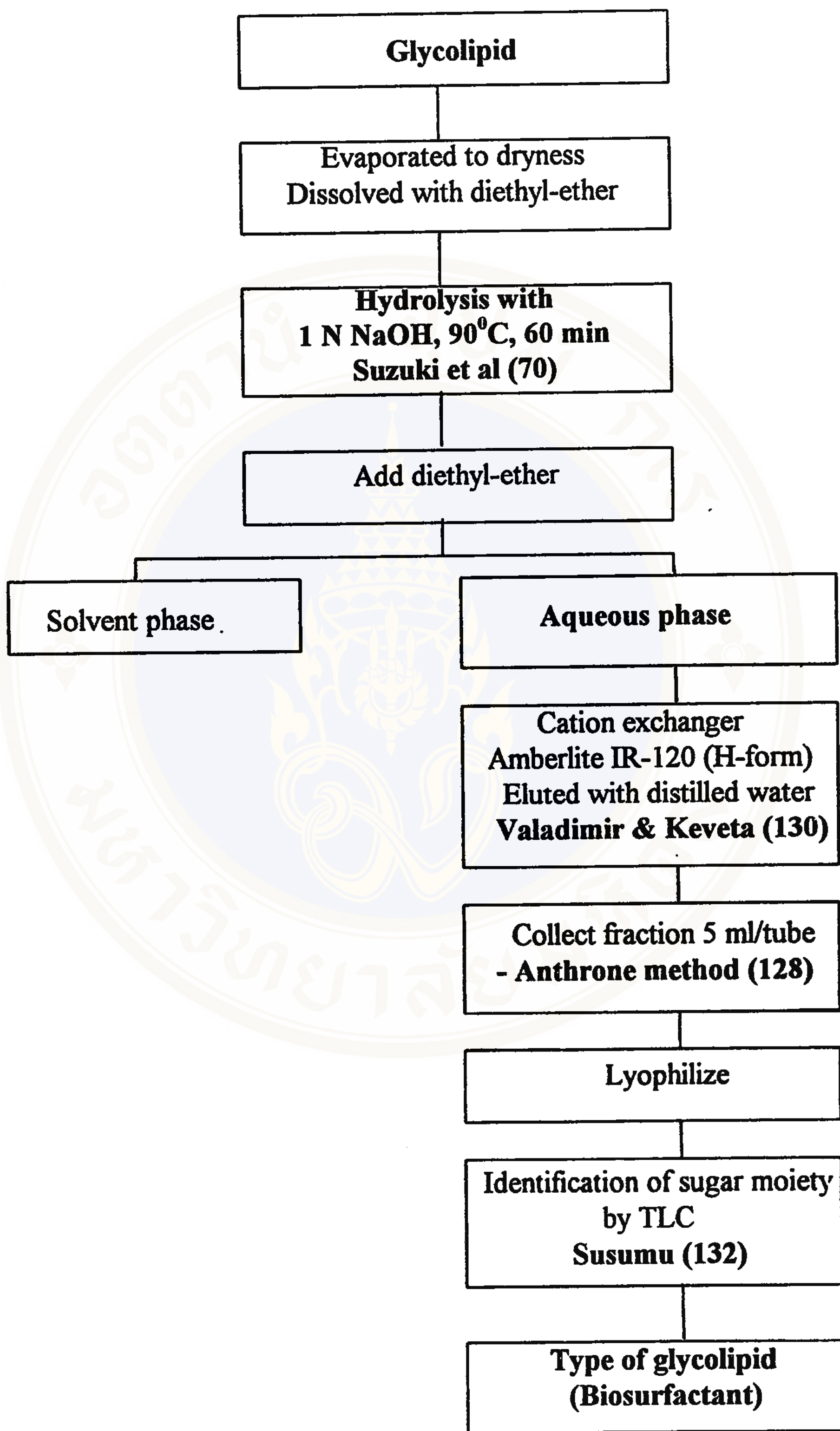


**Figure 20** Identification of sugar moiety of partially purification glycolipid (biosurfactant)

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**1.4.6 Thin-Layer Chromatography**

Thin-layer chromatography (t.l.c.) was performed on Merck silica gel 60 $F_{254}$ plates. The glycolipids, its derivatives and related compound were chromatography with the following solvent systems were by the method described by Fried (131): The glycolipid with solvent system A, Chloroform/methanol/acetic acid/water (25:15:4:2v/v.), solvent system B, chloroform/methanol (1:1v/v.), solvent system C, chloroform/methanol/water (65:25:4v/v), solvent system D, chloroform/ methanol/7 mol/l $NH_4OH$ (65:25:5v/v). Sugar, mono- and disaccharide's with solvent system E, propanol/water (85:15v/v) was determined by the methods of Susumu (132). Saturated and unsaturated polar lipid was detected by spraying with 2',7'-dichloroflorescein reagent. Glycolipid, sugar, mono- and disaccharide were detected by sparing with anthrone/$H_2SO_4$ reagent or phenol/$H_2SO_4$ reagent. Ninhydrin reagent detected lipid with free amino group.

**1.4.7 Determination of Total carbohydrate by Anthrone reaction**

Determination of total carbohydrate by anthrone reaction was assayed by the method of Hodge and Hoferiter (128). Anthrone reagent : add 200 mg of anthrone to 5 ml of absolute ethanol, and make up to 100 ml with 75% $H_2SO_4$. Preparê sample into the boiling tubes. Adjust the volume of all samples to 1.0 ml with distilled water. Prepare a blank of distilled water (1.0 ml) and standards containing 0.01-0.10 mg of glucose in 1.0 ml. chill the tubes and the anthrone reagent in an ice-water bath until cold. Add 5.0 ml of cold anthrone reagent, mix rapidly by vortex mixer, and continue the mixing in the ice-water bath for 5 min. Then transfer the tubes to a bath of boiling water for precisely 10 min. Return the tube to the ice water.

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Measure the color formed in each tube in a spectrophotometer at a wavelength of 625 nm. Determine the concentration of total carbohydrate in the samples from a standard curve prepared by plotting the absorbence of standards versus the concentration of glucose.

**1.4.8 Determination of Protein by Folin reaction**

Protein was determination by method of Lowry et al (124). Reagent A : 1% (w/v) $CuSO_4 5H_2O$, Reagent B: 2% (w/v) Sodium potassium tratrate, reagent C: 0.2 M NaOH, Reagent D: 4% (w/v) $NaSO_3$ . Reagent E : Just for use, mix reagent C 49 ml, reagent D 49 ml, reagent A 1 ml, reagent B 1 ml. Discard after 1 day. Reagent F: Diluted Folin-Ciocalteau reagent with water (1:1 v/v). Add up to 0.5 ml of a sample to a clean 10 ml test tube. Adjust the volumes of each sample to 0.5 ml with distilled water. Add 2.5 ml of reagent E, and mix well. Allow this mixture to stand for least 10 min. at room temperature. Add 0.25 ml of reagent F, and mix immediately. after 30 min. at room temperature, measure the absorbence at 750 nm in a spectrophotometer. Determine the concentration of protein in the samples from a standard curve prepared by plotting the absorbence of standards versus the concentration of Bovine serum albumin

**1.5. Properties of Biosurfactant produced by *Pseudomonas* J-45**

**1.5.1 Determination of Critical Micelle Concentration (CMC)**

The CMC is defined as the concentration of a surfactant necessary too initiate micelle formation. If more of the surfactant is percent, there will be no further decrease in surface tension (133). To obtain a measure of the CMC of

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biosurfactant solution sample, portions were diluted by various amounts, and the surface tension was obtained for each dilution. The CMC could then be estimated, from a plot of surface tension versus the log of the present dilution, as the dilution at which the surface tension starts to increase. The reciprocal of the CMC ($CMC^{-1}$) is proportional to the amount of surfactant present.

**1.5.2 Emulsification Capacity Test**

This study was carried out by procedure as described by Banet et al (129). Five ml of distill water were added to scrupulously clean 16 x 150 mm the tube to which 0.1 ml of sample and 0.1 ml of xylene were added just above the air/water interface. Using a clean 21 cm-long pasture pipette attached to a regulated air supply and flow meter, the liquid was aerated for 45 sec at 2.0 liter/min. with the pipette tip placed at the tube bottom. The mixture was allowed to set at room temperature for 20 min. without further agitation before the absorbance at 660 nm was read in the spectrophotometer. High absorbance indicated high-level surface-active compounds. On the contrary, low absorbance indicated low-level surface-active compounds.

**1.5.3 Effect of pH, NaCl and Temperature on Surface activity**

Biosurfactant was diluted with distilled water. To investigate the effects of pH, NaCl concentrations and temperatures on stability of biosurfactant J-45. The biosurfactant solution was adjusted with 1 N HCl or NaOH to pH 2 to 12. NaCl was added to the glycolipid solution to the final concentrations of 0-30 %. Biosurfactant solution was incubated for 20 min. at different temperatures (25-121$^0$C) and cooled to 20$^0$C. Surface tension and interfacial tension against hexadecane were

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performed via a DeNuoy ring tensiometer (K8, KRUSS, Germany) at 20$^0$C for all treated biosurfactant solutions.

#### 1.5.4 Effect of pH, NaCl, Temperature and Hydrocarbon on Emulsification Capacity

Biosurfactant was diluted to a final volume of 5 ml, with aqueous phase in various condition and 0.1 ml test hydrocarbon. To investigate the effects of pHs, NaCl concentrations and temperatures on stability of biosurfactant J-45, the aqueous solution was adjusted with 1 N HCl or NaOH to pHs 2 to 12, NaCl was added to the aqueous solution to the final concentrations of 0-30 %. Biosurfactant solution was incubated for 20 min. at different temperatures (25-121$^0$C) and cooled to room temperature. Emulsification capacity was measured as previous described .

## 2. Research Instruments and Equipments

1. Fermenter 10 litre. Microferm Fermenter, New Brunswick Scientific USA.
2. Refrigerated superspeed centrifuge. Sorvall RC-5B, DuPont Instruments.
3. Autoclave. Eyela Autoclave MAC-601, Eyela Tokyo rikakikai CO, LT Japan.
4. Temperature controller Julabo Paratherm III electronic, Juchheim labortechnik. West Germany.
5. Spectrophotometer. Milton Roy Spectronic 401, Milton Roy company USA.
6. Analytical balance. Sartorius Basic, Sartorius.

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7. Water bath. MemmertW760, Memmert.

8. Vortex mixer. MS-1, IKA-works Inc, USA.

9. Rotary evaporator. Rotavapor RE120, Buchi Switzerland.

10. Lyophilizer. Super Modulyo, Edwards England.

11. Rotary shaker. LED Obit Shaker, Lab-line Instruments, Inc.

12. Peristatic pump. Phamacia LKB-pump P-1, Phamacia Sweden.

13. Vacuum pump. Gast USA.

14. Vacuum oven. 6512 Vacuum oven, Forma Scientific

16. Incubator. Hotpack Incubator 351200, Hotpack corporation USA.

17. Fraction collector. Waters Japan.

18. Hot air oven. Memmert model 600

19. Tensiometer. KRUSS K8, KRUSS Germany.

20. Total petroleum hydrocarbon analyzer. TPH plus, GAC USA.

**3. Data analysis**

The obtained data will be analyzed and interpreted through qualitative data analysis as follow;

More specific, this study will employ need analysis, which uses experiment method to investigate the suitable biodegradation of crude oil and biosurfactant formation, Isolation, purification, identification of biosurfactant and to study properties of the produced biosurfactant.

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# CHAPTER IV

# RESULTS

## 1. Biodegradation of Crude oil by *Pseudomonas* J-45

The bacterial isolated *Pseudomonas* J-45 that was kindly provided by the Thailand Institute of Scientific and Technology Research (TISTR) team was reconfirmed for its growth and crude oil degrading activity. The cell growth was measured by protein increases (biomass), pH value, surface tension and oil degrading activity was determined by remaining grease and oil value after fermentation process. Table 9 and Figure 21 show the properties of bacterial after growing from o hr to 168 hr. It can be seen biomass of the oil degrading bacterium was increased exponentially. The biomass increased from 9.6 mg/l at 0 hr to 167.2 mg/l at 24 hr. and varied slight increase thereafter. While the grease and oil value decreased from 8420 mg/l at 0 hr to 819 mg/l at 168 hr. as well as the pH which drop from 6.99 at 0 hr to 4.69 within 24 hr. and maintain until the end of fermentation process. While the surface tension was gradually reduced from 62.6 mN/m at 0 hr to 32.5 mN/m at 168 hr.

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**Table 9** Change of each indicator by the *Pseudomonas* J-45 at different times

| Time (hr) | Biomass (mg protein/l) | Grease & oil (mg/l) | PH | Surface tension (mN/m) |
|---|---|---|---|---|
| 0 | 9.6 | 8420 | 6.99 | 63.6 |
| 24 | 167.2 | 3470 | 4.69 | 46.8 |
| 48 | 160 | 919 | 4.86 | 42.6 |
| 72 | 160 | 787 | 5.01 | 39 |
| 96 | 204 | 920 | 5.0 | 33 |
| 120 | 200 | 697 | 4.89 | 30.6 |
| 144 | 248 | 939 | 4.81 | 31.5 |
| 168 | 292 | 819 | 4.70 | 32.5 |

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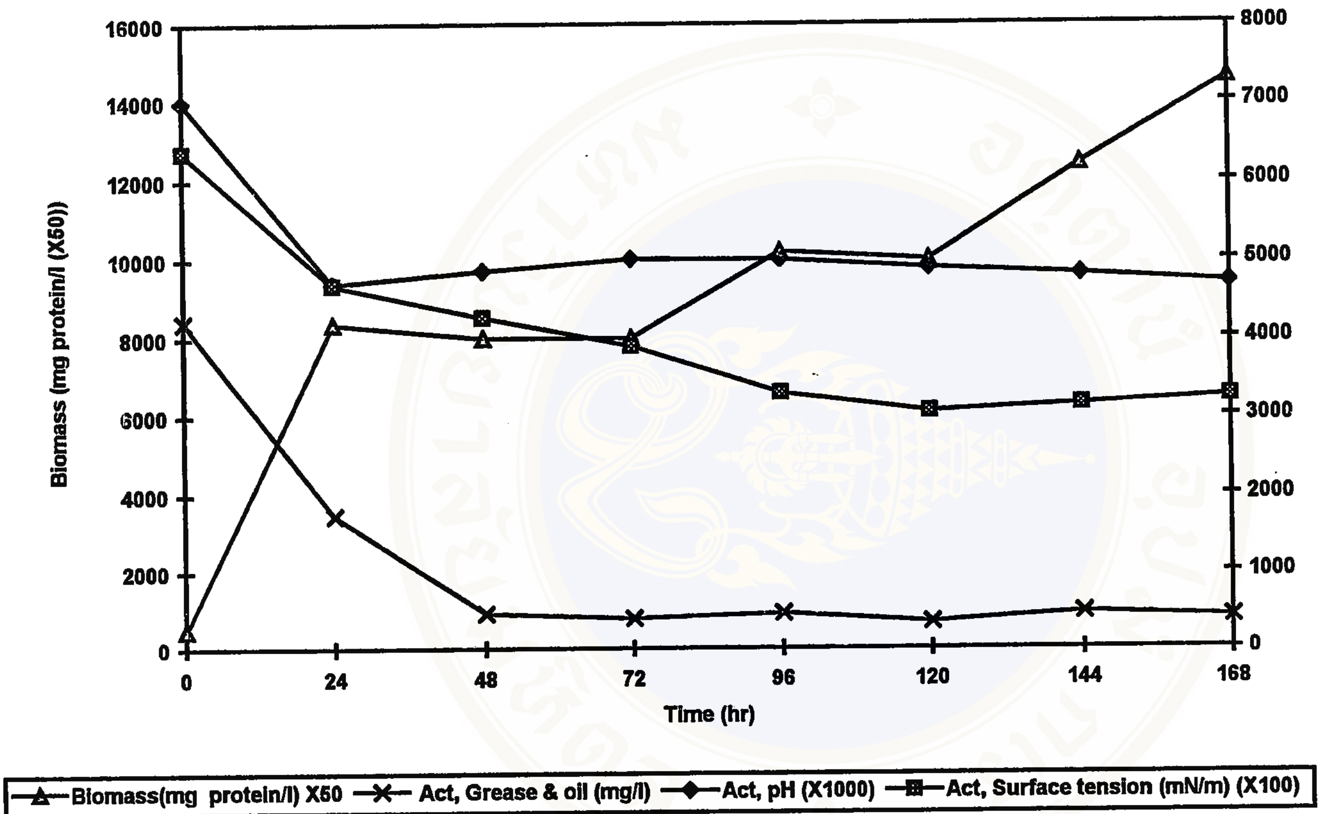


**Figure 21** Time-course of crude oil biodegradation by *Pseudomonas* J-45 with ASW medium + 1 % Crude oil at $30^0$C, aeration 1.0 v.v.m., agitation 100 r.p.m.

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**2. Growth and Oil degrading activity of Pseudomonas J-45 under Various condition**

**2.1 Effects of Temperatures on Crude oil degradation**

The effect of temperature on crude oil degradation by *Pseudomonas* J-45 grown in ASW medium are presented in Figure 22, 23, 24, 25, 26. In the case of temperature, the results showed that the highest level of degradation of crude oil (96 hr.) were obtain at 30$^0$C and the lowest level at 25$^0$C. From the results degradation of crude oil at 25$^0$C (65.99%), 30$^0$C (89.07%) and 37$^0$C (70.11%) were 2990, 920 and 2380 mg/l, receptively (Figure 23). The maximum growth was obtained at 37 C and the maximal crude oil degradation at 168 hr. (Figure 22). The pH in all group of temperature decreased rapidly in 72 hr. and maintains until the end of experiment (168 hr) (Figure 24). The surface tension of culture broth was lowest when incubated at 30$^0$C (33.8 mN/m) and was highest when incubated at 37$^0$C (38 mN/m) at end experiment are show in Figure 25. However, the surface tension of culture broth in all group of temperature decreases slowly to the end of experiment. Which correspond with the increasing of biomass.

The results in Figure 26 showed crude oil degrading activity by *Pseudomonas* J-45 in differ temperature at 96 hr. The results showed at 30$^0$C was best crude oil degrader in this study were grease and oil value at 96 hr of incubation was lowest (920 mg/l). When compare to other condition. However, the other condition, 250C and 30$^0$C could degrade crude oil at similar rates, i.e., 2990 and 2380 mg/l, respectively (Figure 26). The surface tension of culture broth at 96 hr. of incubation were 32.5 mN/m 34.5 mN/m and 38.5 mN/m at temperature 30$^0$C, 25 $^0$C and 37$^0$C, respectively.

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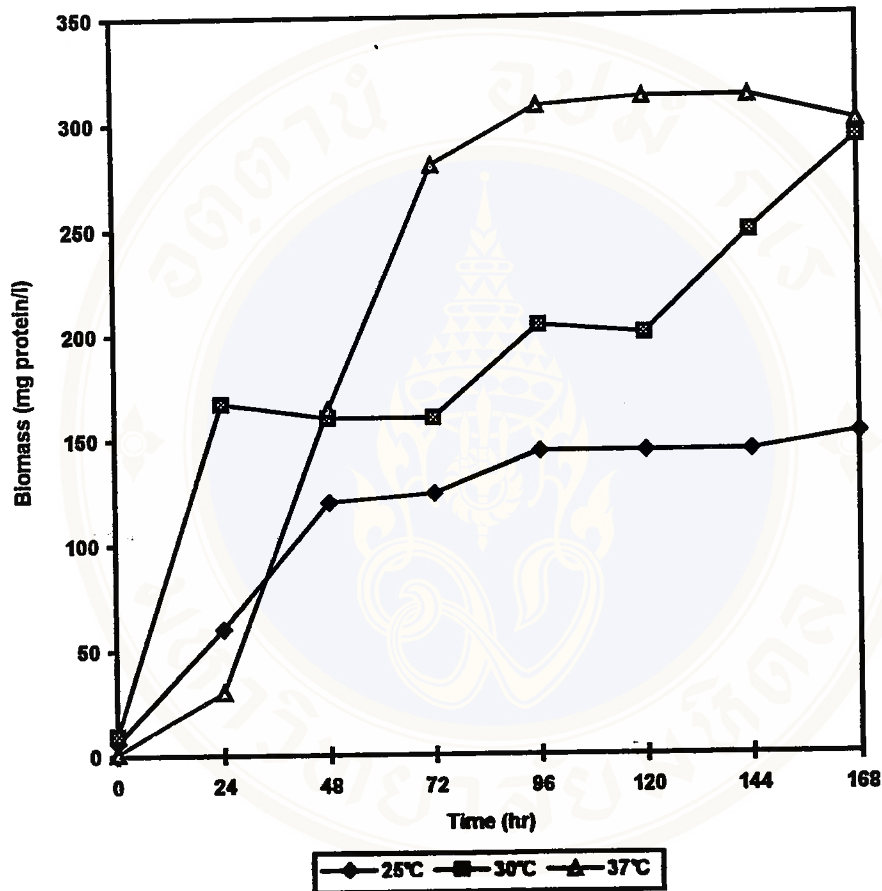


**Figure 22** Growth curves of *Pseudomonas* J-45 incubated with ASW + 1% crude oil in reactor at different temperatures (1.0 v.v.m., 100 r.p.m.)

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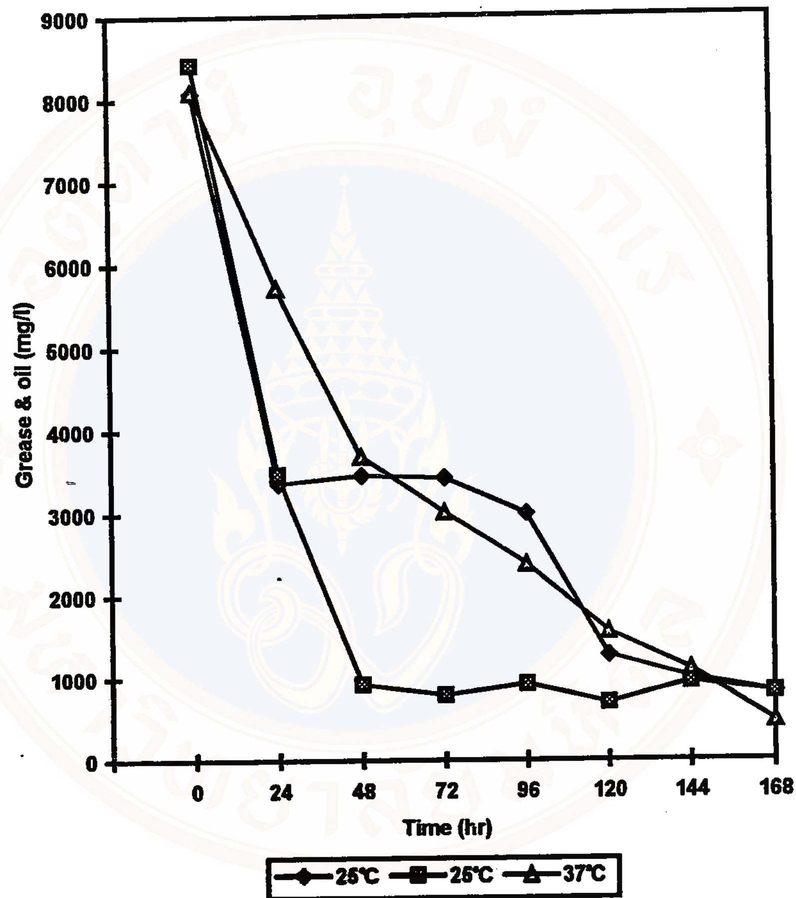


**Figure 23** Grease and oil value of *Pseudomonas* J-45 incubated with ASW + 1% crude oil in reactor at different temperatures (1.0 v.v.m., 100 r.p.m.)

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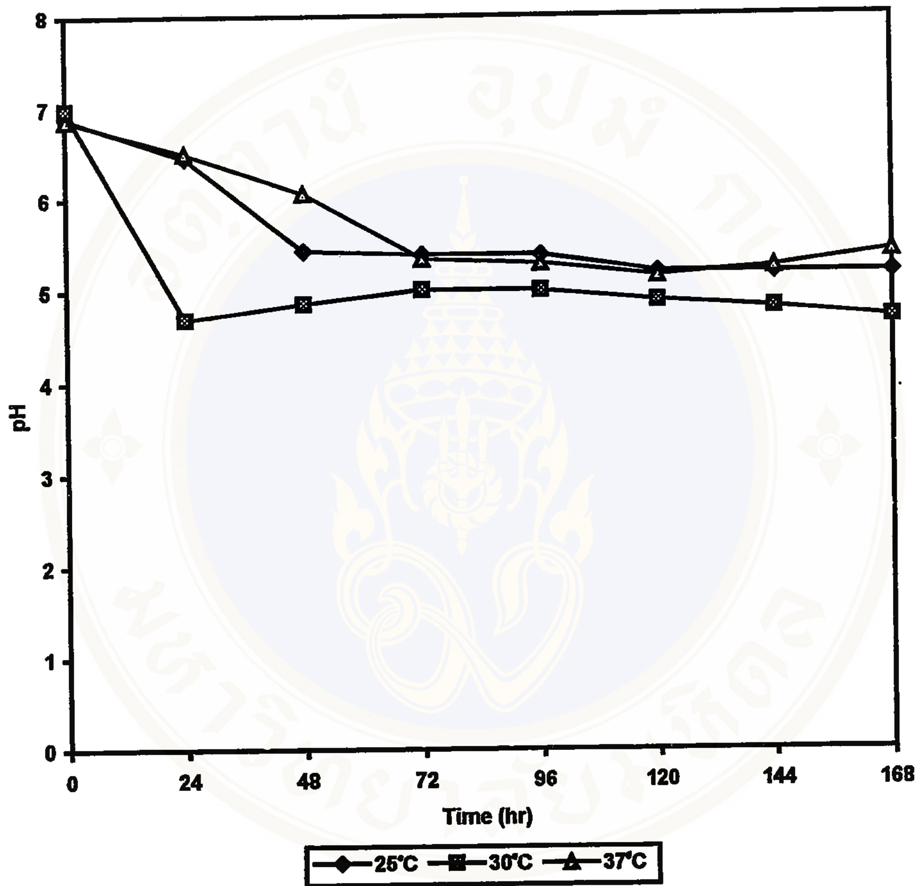


**Figure 24** pH value of *Pseudomonas* J-45 incubated with ASW + 1 % crude oil in reactor at different temperatures. (1.0 v.v.m.,100 r.p.m.)

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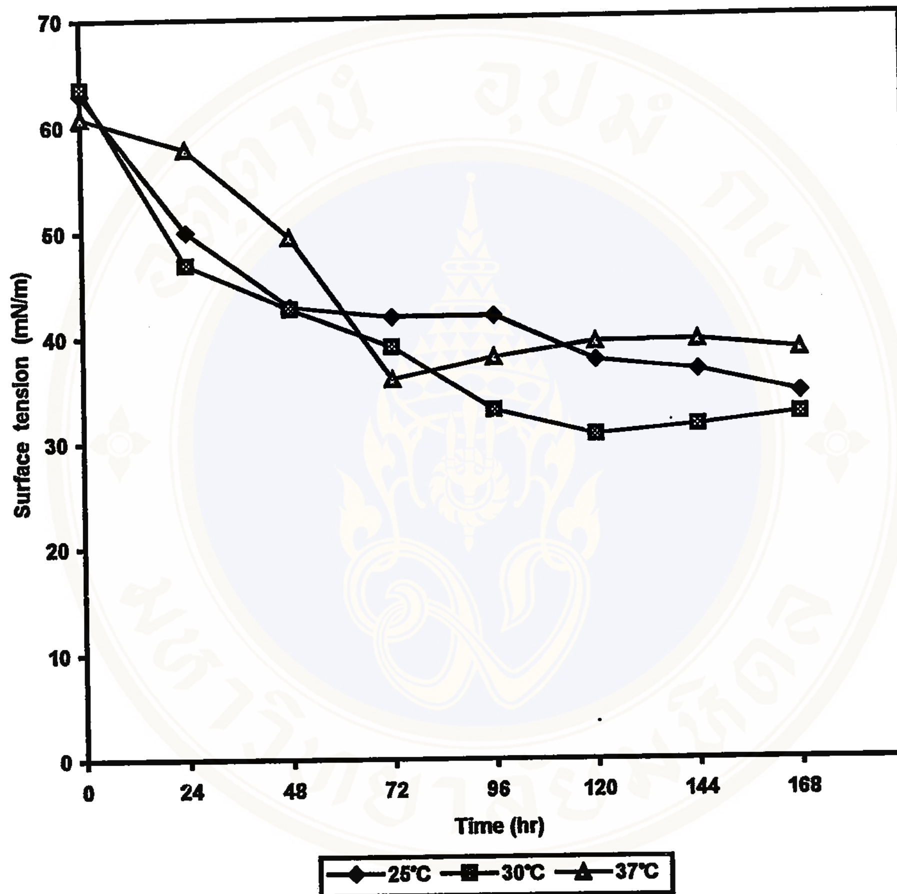


**Figure 25** Effect of various temperatures towards surface tension of *Pseudomonas* J-45 incubated with ASW + 1% crude oil in reactor (1.0 v.v.m., 100 r.p.m.).

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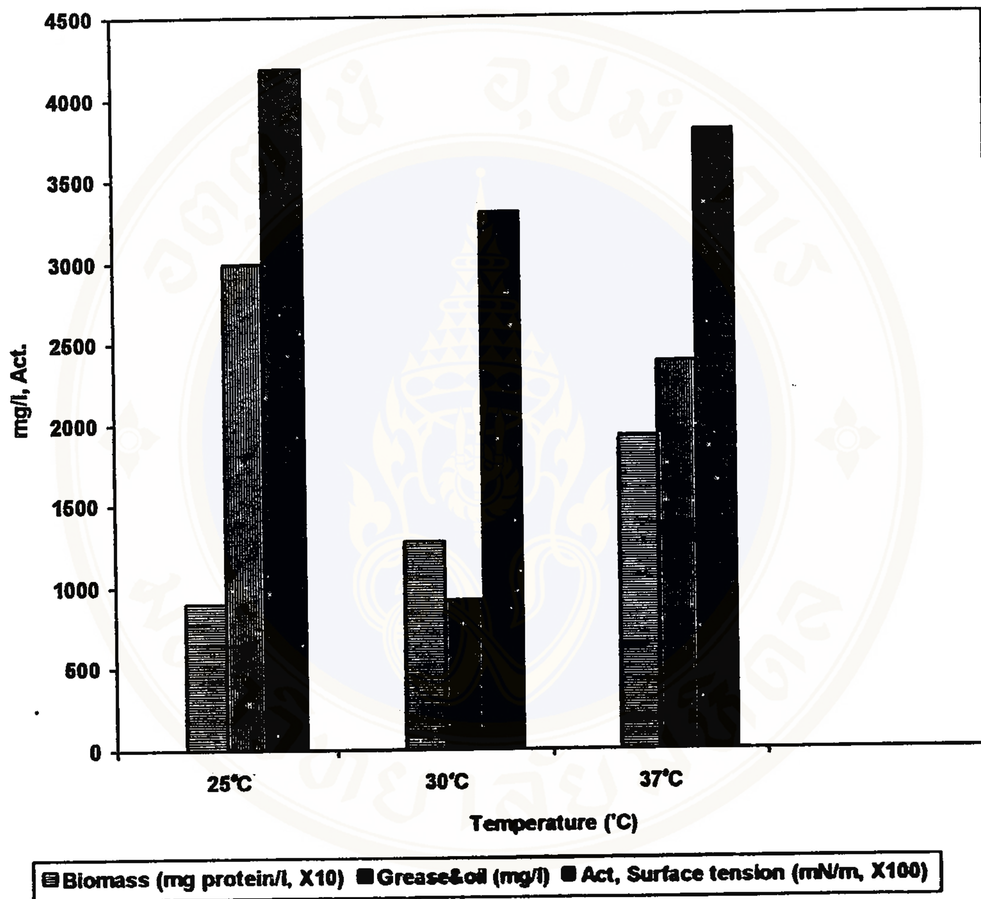


**Figure 26** Effect of various temperatures towards biodegradation activity of *Pseudomonas* J-45 at 96 hours

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**2.2 Effect of Aeration on Crude oil degradation**

The effect of aeration in crude oil degradation by *Pseudomonas* J-45 are illustrated in Figure 27, 28, 29, 30, 31. Figure 27 show the *Pseudomonas* J-45 remunerable growth in ASW medium with aerated ranging to 0.25 v.v.m., 0.5 v.v.m., and 1.0 v.v.m.. However, the growth rate *Pseudomonas* J-45 tended to higher in media with full rate aeration (1.0 v.v.m) and tends to be lower in media with low rate aeration (0.25 v.v.m. and 0.5 v.v.m.) (Figure 27). The crude oil was degraded fastest at 1.0 v.v.m. and decreased orderly from 0.5 v.v.m. to 0.25 v.v.m. (Figure 28). In the case of 1.0 v.v.m., pH value which drop from 6.99 to 4.69 in 24 hr. and maintain until the end of experiment, while the pH value in other aeration rate were higher than 1.0 v.v.m. (Figure 29).

The results in Figure 31 showed crude oil degrading activity by *Pseudomonas* J-45 in different aeration at 96 hr. The results showed at 1.0 v.v.m. was best crude oil degrading in this study. The grease and oil value was lowest (920 mg/l). When compare to other condition. However, 0.25 v.v.m. and 0.5 v.v.m. could degrade crude oil at similar rates, i.e., 1920 and 1120 mg/l, respectively (Figure 31). The biomass of *Pseudomonas* J-45 was 123, 135, and 1275 mg/l at 0.25 v.v.m., 0.5 v.v.m. and 1.0 v.v.m., respectively. The surface tension of culture broth at 96 hr. of incubation were 40.5 mN/m, 38 mN/m and 33 mN/m at 0.25 v/v/m, 0.5 v/v/m, and 1.0 v/v/m, respectively (Figure 31).

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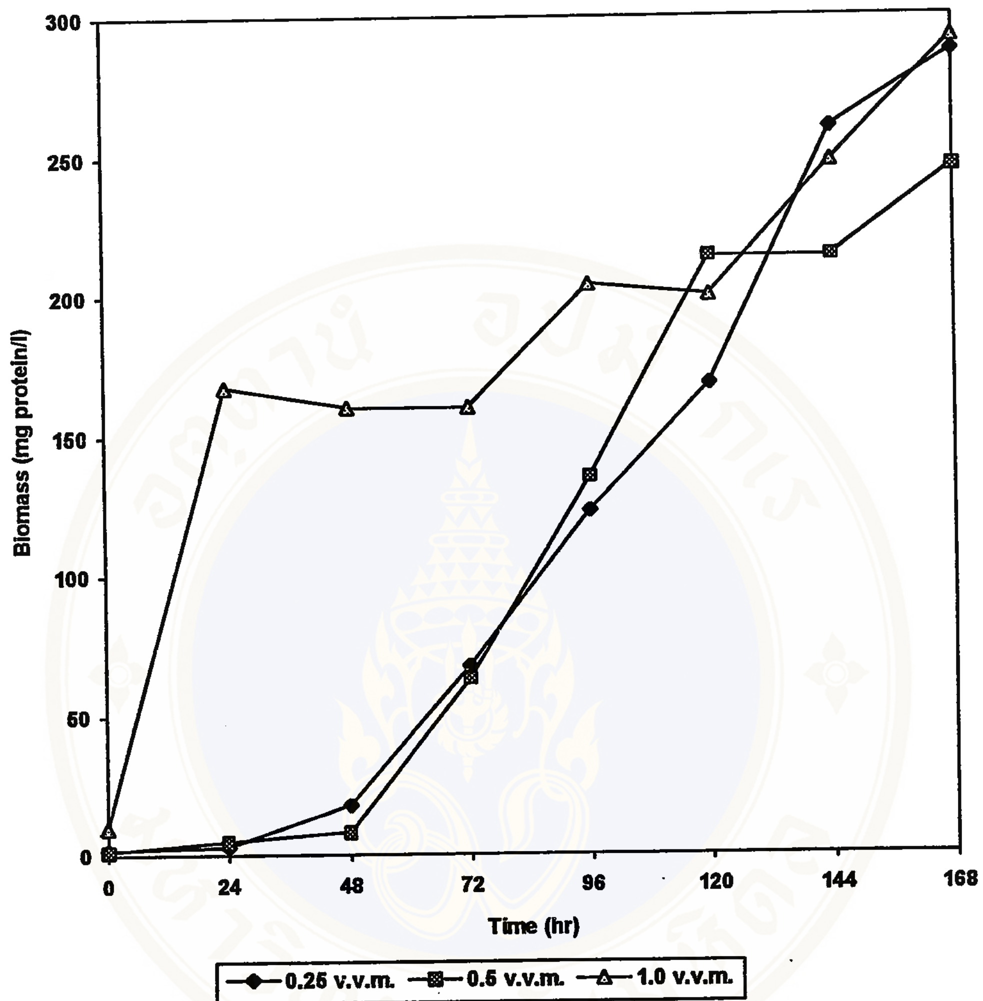


**Figure 27** Growth curves of *Pseudomonas* J-45 incubated with ASW + 1% crude oil in reactor at different aeration rates (30$^0$C, 100 r.p.m.)

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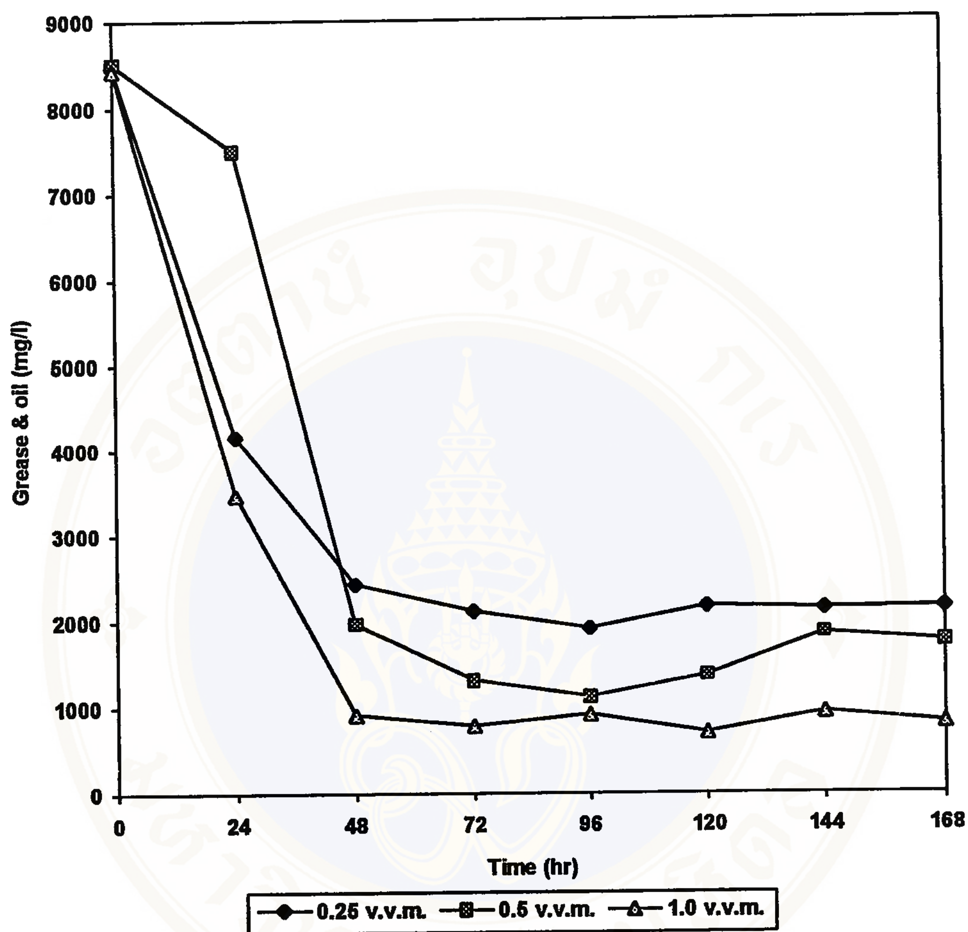


**Figure 28** Grease and oil value of *Pseudomonas* J-45 incubated with ASW + 1% crude oil in reactor at different aeration rates ($30^0$C, 100 r.p.m.)

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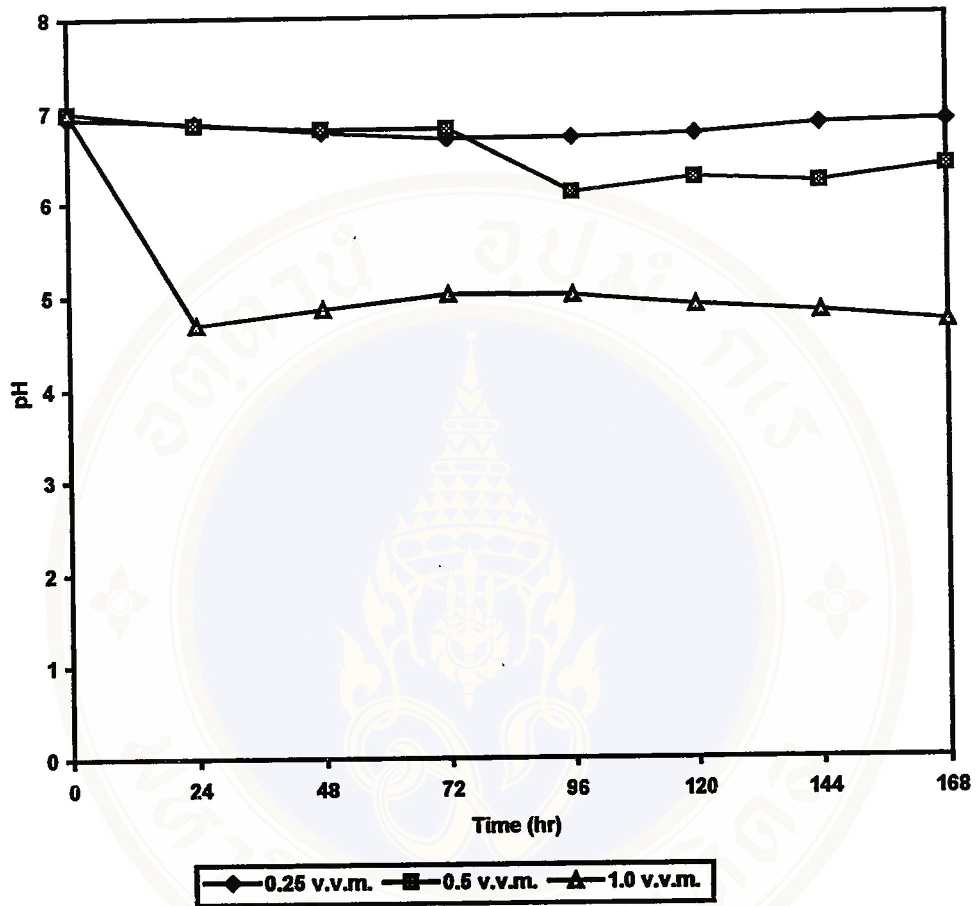


**Figure 29** pH value of *Pseudomonas* J-45 incubated with ASW + 1% crude oil in reactor at different aeration rates ($30^0$C, 100 r.p.m.)

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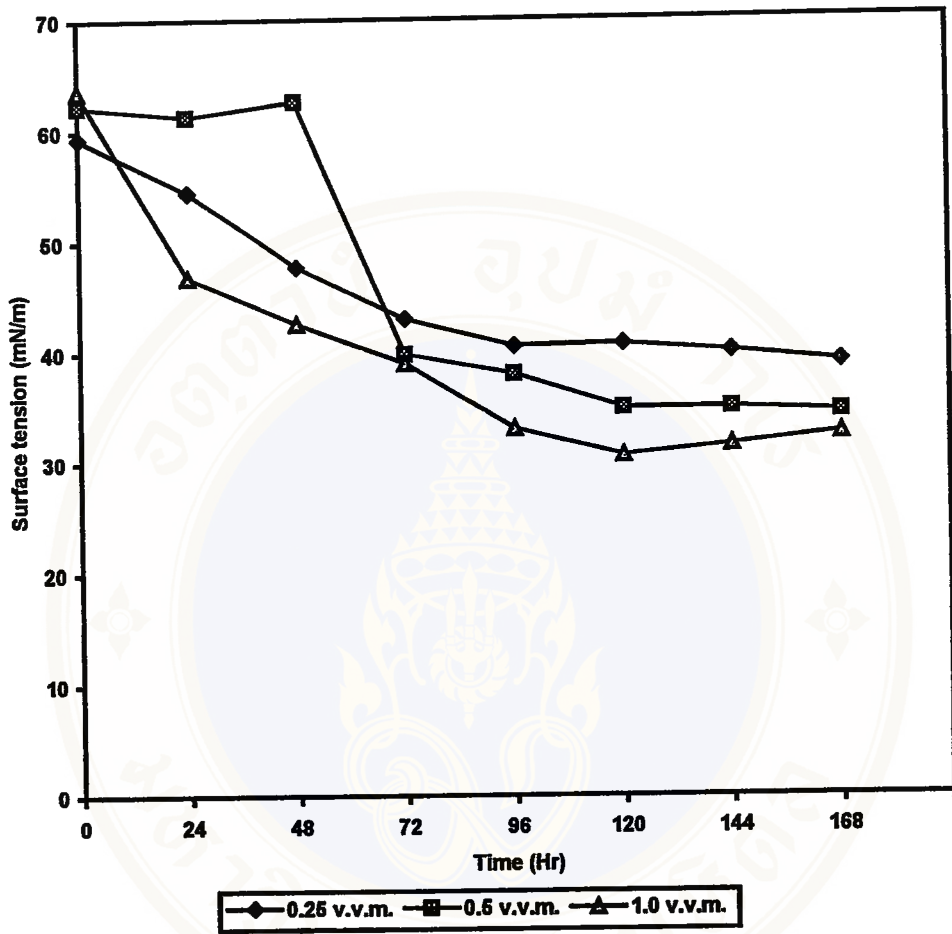


**Figure 30** Effect of various aeration rates towards surface tension of *Pseudomonas* J-45 incubated with ASW + 1% crude oil in reactor ($30^0$C, 100 r.p.m.)

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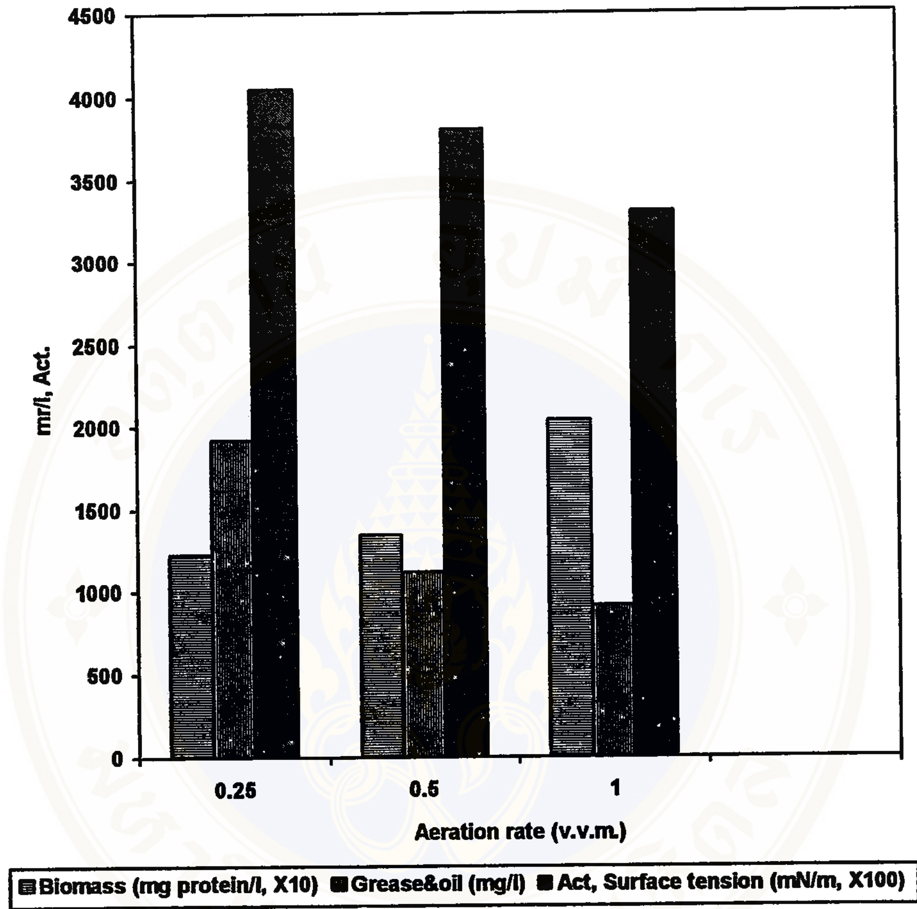


**Figure 31** Effect of various aeration rates towards biodegradation activity of *Pseudomonas* J-45 at 96 hour

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**2.3 Crude oil Degradation of *Pseudomonas* J-45 in Artificial Seawater Media (ASW) and Seawater (SW)**

The biodegradation of crude oil by *Pseudomonas* J-45 in ASW and SW at 30$^0$C, 1.0 v.v.m. is show in Figure 32, 33, 34, 35, and 36. In the case of SW, the results showed that the lower levels of degradation. The crude oil degradation by *Pseudomonas* J-45 were 90.27 % and 61.99 % in ASW and SW at 168 hr, respectively (Figure 34). The growth of *Pseudomonas* J-45 corresponded to the amount of crude oil degradation. The maximum growth of *Pseudomonas* J-45 was obtained in ASW medium and the maximal crude oil degradation (Figure 32, 33). In Figure 36 showed culture broth surface tension was reduced lowest in ASW (32.5 mN/m) and in SW surface tension was reduced slightly before 72 hr (58 mN/m) of incubation and decades rapidly at the end of the experiment (34.6 mN/m).

The results in Figure 36 show crude oil degrading activity by *Pseudomonas* J-45 in ASW and SW at 96 hr. The result showed *Pseudomonas* J-45 growth in ASW was more capable to degrade crude oil than SW and which corresponds with the increasing of biomass, and with the reduced of surface tension.

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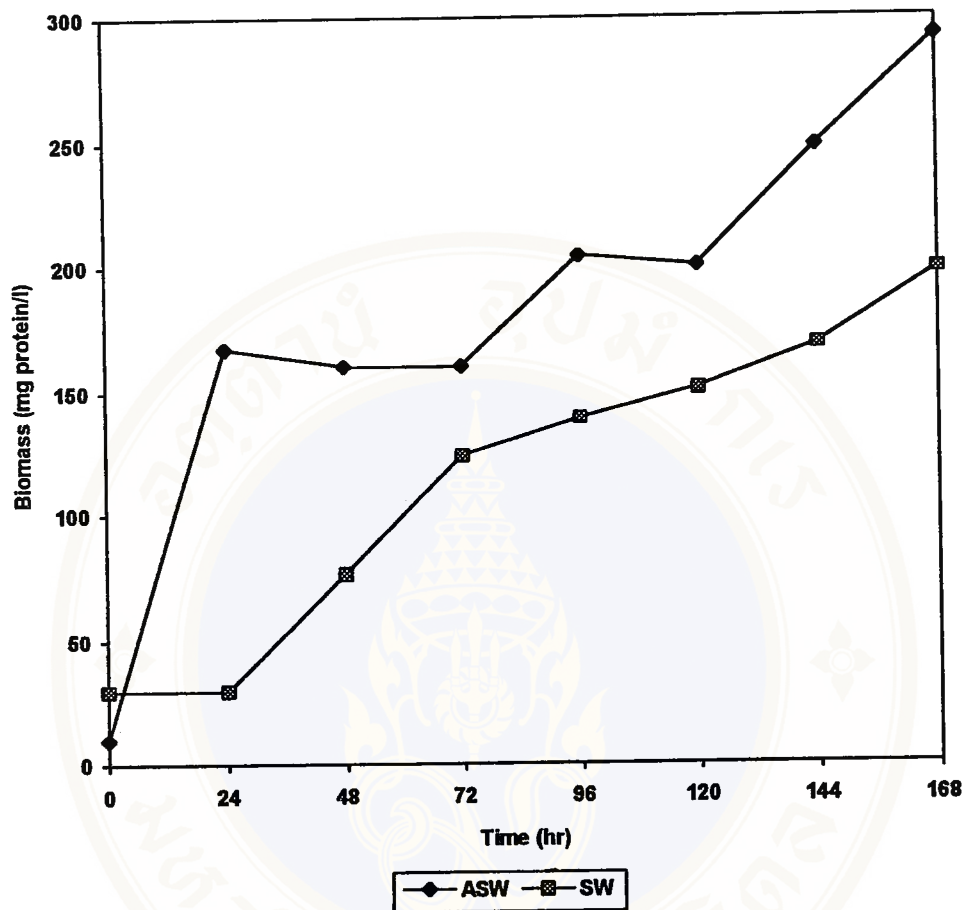


**Figure 32** Growth curves of *Pseudomonas* J-45 incubated with ASW and SW + 1% crude oil in reactor (30$^0$C, 1.0 v.v.m., 100 r.p.m.)

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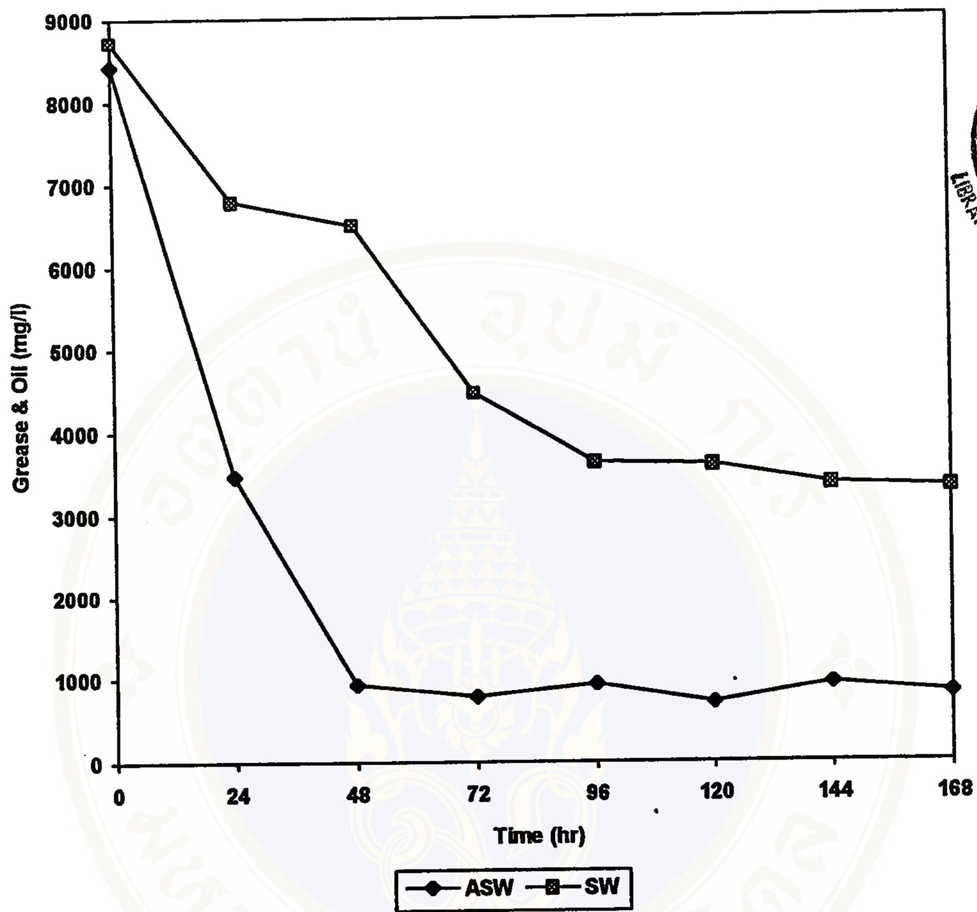


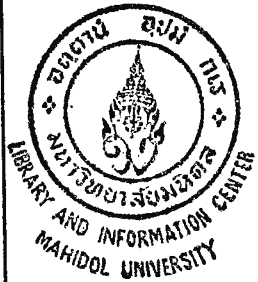

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**Figure 33** Grease and oil value of *Pseudomonas* J-45 incubated with ASW and SW + 1 % crude oil in reactor ($30^0C$, 1.0 v.v.m., 100 r.p.m.)

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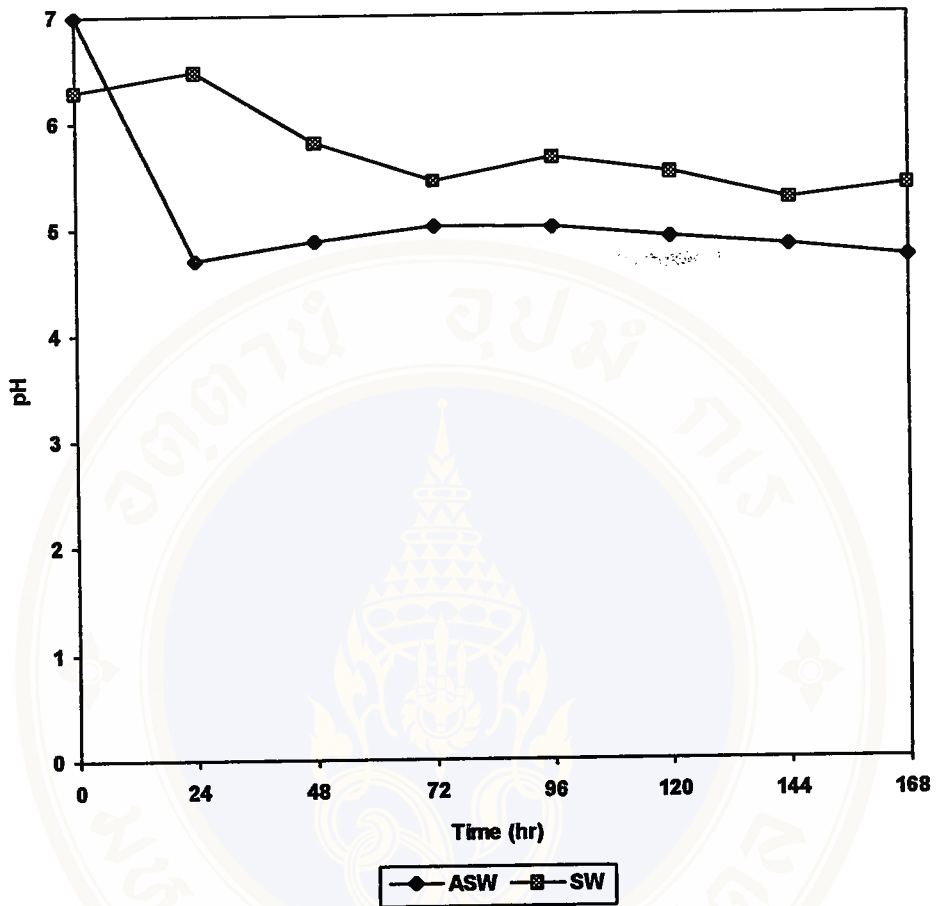


**Figure 34** pH values of *Pseudomonas* J-45 incubated with ASW and SW + 1% crude oil in reactor (30$^0$C, 1.0 v.v.m., 100 r.p.m.)

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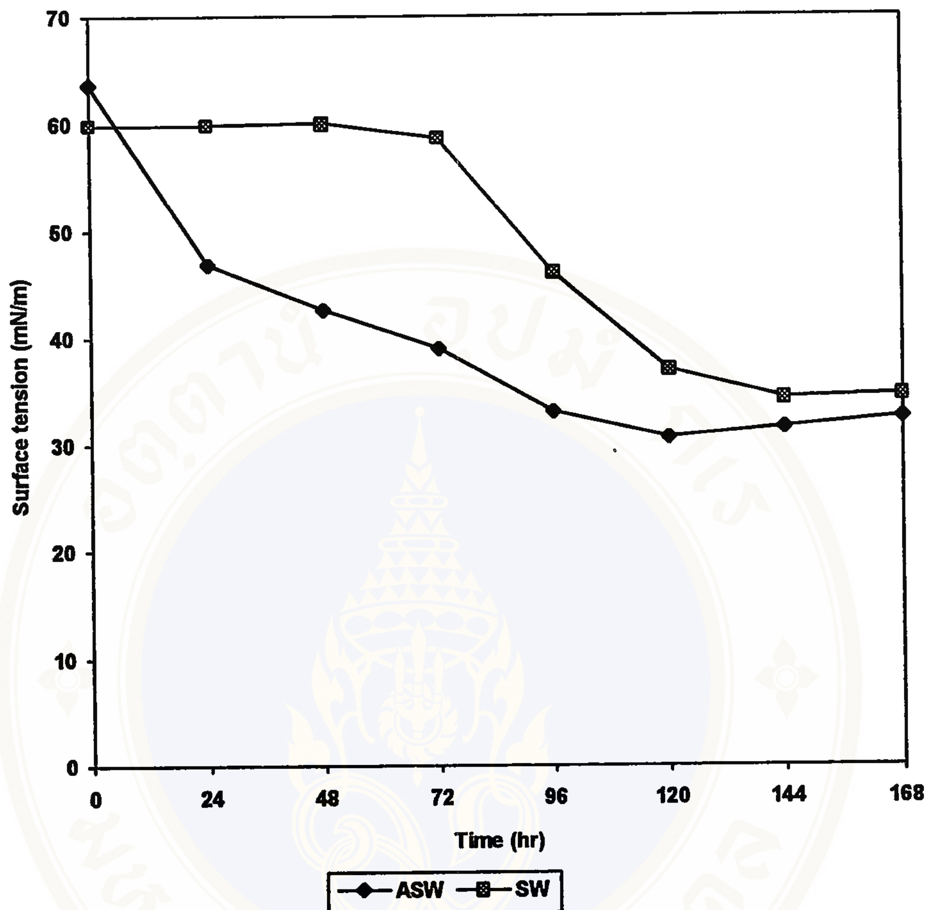


**Figure 35** Surface tension of *Pseudomonas* J-45 incubated with ASW and SW + 1% crude oil in reactor (30$^0$C, 1.0 v.v.m.,100 r.p.m.)

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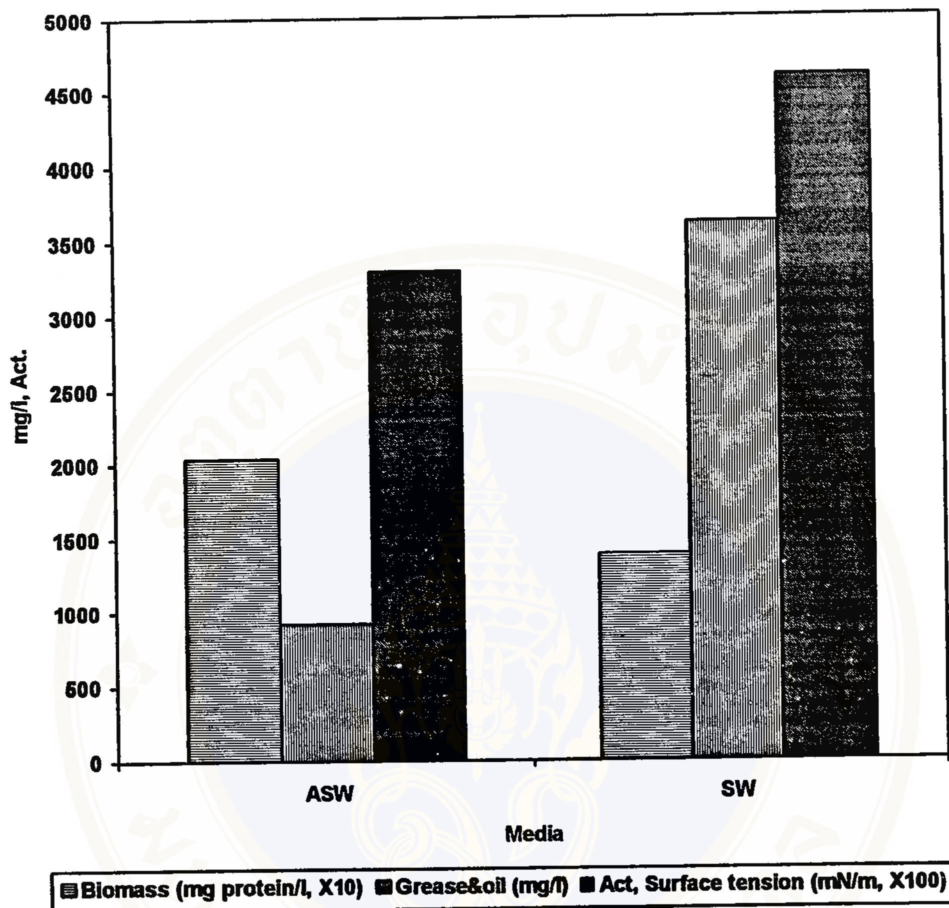


**Figure 36** Effect of differents media towards biodegradation activity of *Pseudomonas* J-45 at 96 hour (30$^0$C, 1.0 v.v.m., 100 r.p.m.)

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**3. Isolation, Purification and Identification of Biosurfactant produced by *Pseudomonas* J-45**

**3.1 Isolation and Purification of Biosurfactant from Lyophilized Cell *Pseudomonas* J-45**

The Chloroform:Methanol extracted crude lipid from 1.0 g dry cell *Pseudomonas* J-45 was purified by de-impurity proceed with 0.88% KCl solution was shown in Table 10. From, the total carbohydrate decreased by 63.9 % from its initial amount. The activity also decreased 82.98 %. The percentage of glycolipid produced (% yield) was found to be 82.98 %. However, its was shown that the glycolipid solution obtained from de-impurity process had it specific activity increased by 1.29 fold (the purity increased by 1.29 fold). Later the glycolipid solution was further purified by running through Silica gel 60 column chromatography.

The result from the column Chromatography showed that there was only one peak with a maximum emulsification capacity and total carbohydrate were eluted with Acetone:Methanol (1:1). The result of glycolipid purification is concluded in Figure 38. The result showed that the purification process crude isolate glycolipid from total lipid More over, the obtained glycolipid has 1 71 fold higher degree of purity with 23.43 % yield.

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**Table 10** Purification of glycolipids from lyophilized Cell*

| Procedures | Total Activity (OD660X1000) | Total carbohydrate (mg) | Specific activity | Yield (%) | Purity (Fold) |
|---|---|---|---|---|---|
| 1. Crude lipid | 1105 | 17.88 | 61.80 | 100 | 1 |
| 2.De-impurity (0.88%KCl) | 917 | 11.44 | 80.15 | 82.98 | 1.29 |
| 3.Silica gel 60 Column. | 259 | 2.45 | 105.71 | 23.43 | 1.71 |

* Lyophilized *Pseudomonas* J-45 1,000 mg.

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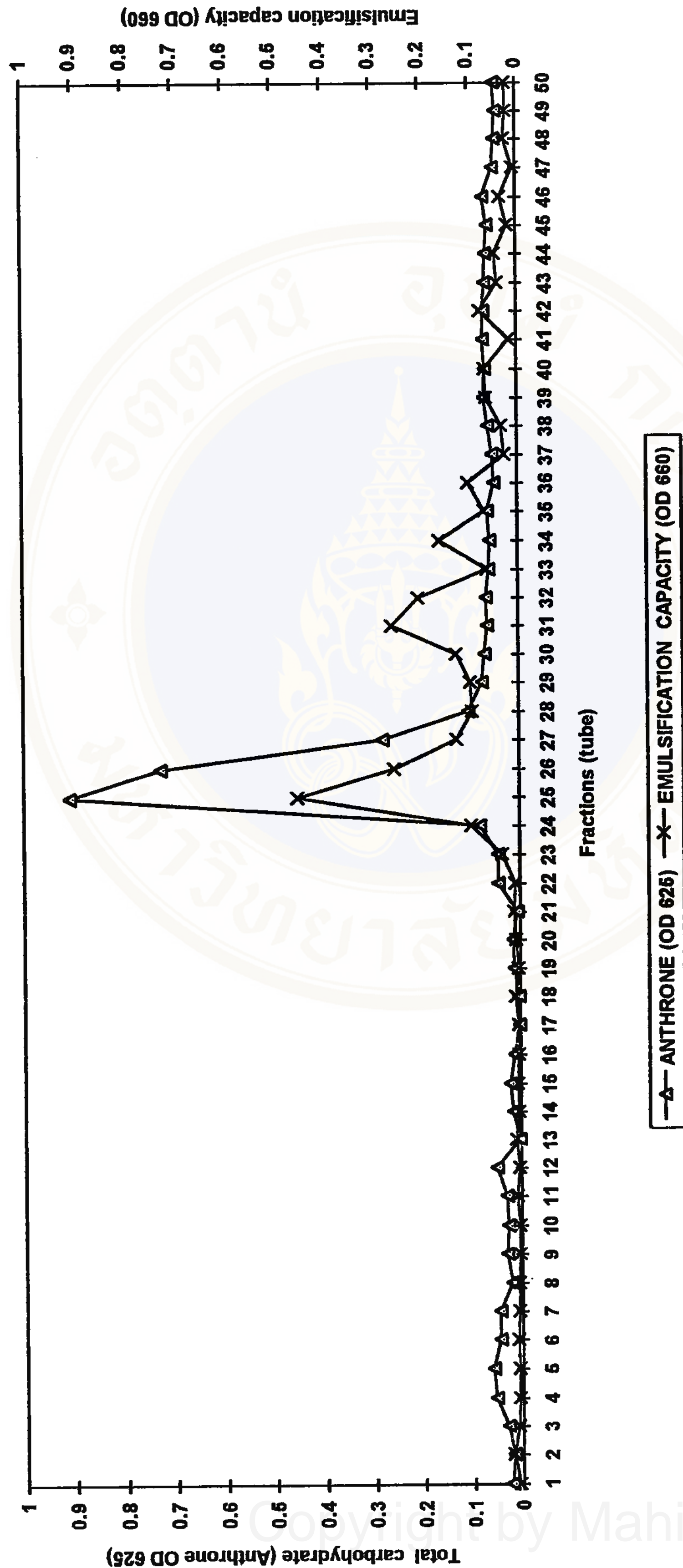


**Figure 37** Silica gel 60 column chromatography of a crude biosurfactant from lyophilized cell of *Pseudomonas* J-45.

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### 3.2 Isolation and Purification of Biosurfactant from Supernatant

The Chloroform:Methanol extracted crude lipid from supernatant 1,000 ml was purified by de-impurity proceed with 0.88% KCl solution was shown in Table 11 From, the total carbohydrate decreased by 57.83 % from its initial amount. The activity also decreased 82.94 %. The percentage of glycolipid produced (% yield) was found to be 82.94 %. However, its was shown that the glycolipid solution obtained from de-impurity process had it specific activity increased by 1.43 fold (the purity increased by 1.43 fold). Later the glycolipid solution was further purified by running through Silica gel 60 column chromatography.

The result from the column Chromatography showed that there was only one peak with a maximum emulsification capacity and total carbohydrate were eluted with Acetone:Methanol (1:1). The result of glycolipid purification is concluded in Figure 39. The result showed that the purification process crude isolate glycolipid from total lipid . More over, the obtained glycolipid has 2.11 fold higher degree of purity with 75.68 % yield.

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**Table 11** Purification of glycolipids from supernatant*

| Procedures | Total Activity (OD660X1000) | Total carbohydrate (mg) | Specific activity | Yield (%) | Purity (Fold) |
|---|---|---|---|---|---|
| 1. Crude lipid | 909 | 56 | 16.23 | 100 | 1 |
| 2.De-impurity (0.88% KCl) | 754 | 32.39 | 23.27 | 82.94 | 1.43 |
| 3.Silica gel 60 Column. | 688 | 20.06 | 34.29 | 75.68 | 2.11 |

* Supernatant (Free cell) from culture broth of *Pseudomonas* J-45 1,000 ml.

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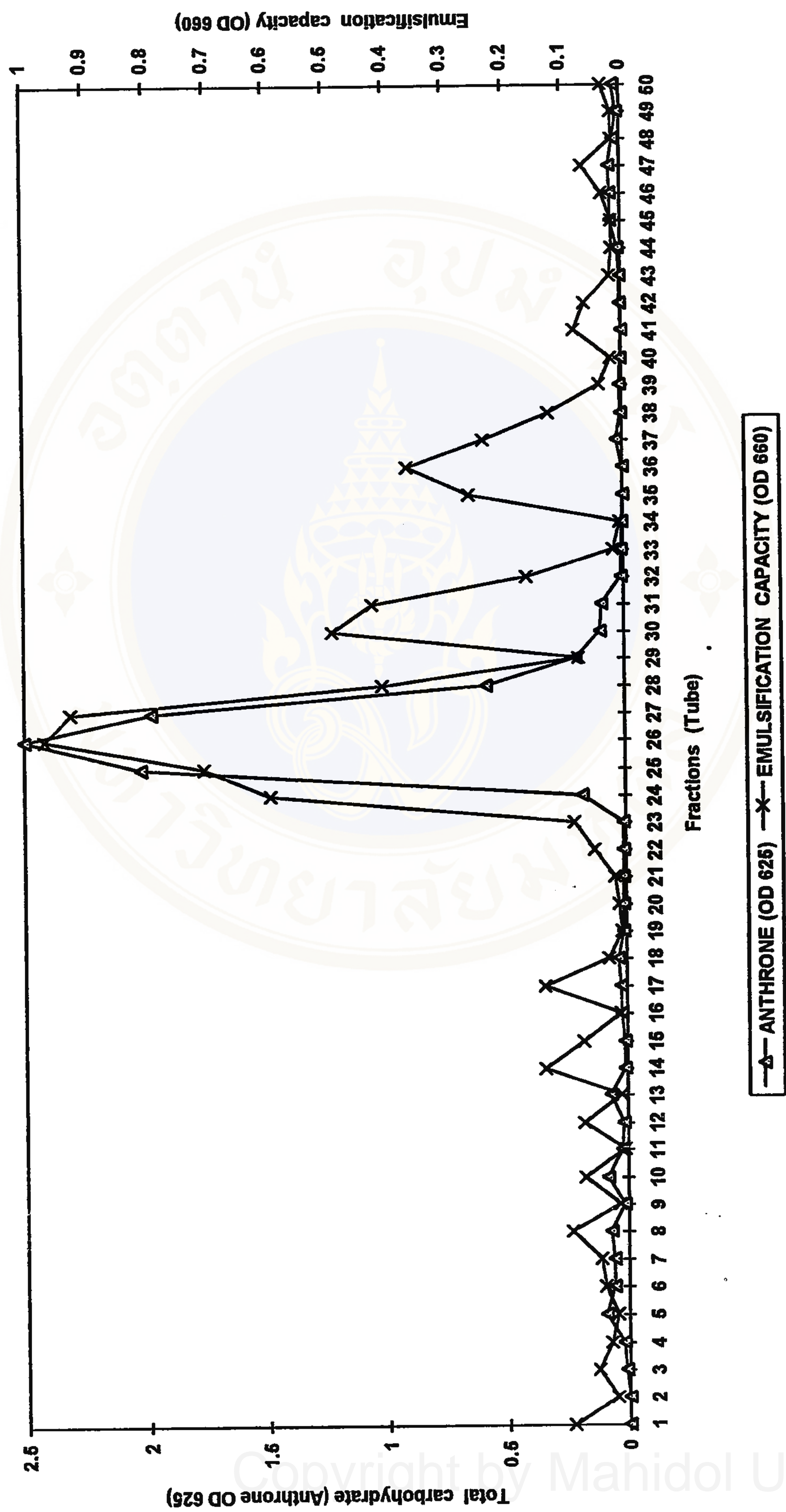


**Figure 38** Silica gel 60 Column chromatography of a crude biosurfactant from supernatant of *Pseudomonas* J-45

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### 3.3 Identification of Biosurfactant from *Pseudomonas* J-45

The glycolipid was isolated from supernatant and cell of *Pseudomonas* J-45 by extract with Chloroform:Methanol (2:1 v/v). Purification was carried out by column chromatography in Silica gel 60 using as solvent system Acetone:Methanol (1:1 v/v) was eluted. A preliminary structure study was carried out with thin layer chromatography on silica gel 60 plates with the solvent system. By spraying the plates with spray reagent, two dominant spots could be detected with a retention-front value ($R_f$) of 0.74 and 0.58 with solvent system C, 0.48 and 0.24 with solvent system D, examined by TLC with solvent system A, showed a single spot with an $R_f$ value of 1.0. A structure with a lipid moiety by Anthrone reagent (green spot) and fatty acid group by 2',7'-Dichlorofluorescein was assigned. The study with the Ninhydrin reagent gave negative results.

**Table 12** $R_f$ values of glycolipid separated in various solvent systems on silica gel

| | $R_f$ | | | | Spray reagent | | | |
|---|---|---|---|---|---|---|---|---|
| **Solvent system** | **A** | **B** | **C** | **D** | **1** | **2** | **3** | **4** |
| **Glycolipid A** | **1.0** | **0.64** | **0.74** | **0.48** | + | + | + | - |
| **Glycolipid B** | **1.0** | **0.52** | **0.58** | **0.24** | + | + | + | - |

**Solvent system**. A:Chloroform:Methanol:Acetic acid:Water (25:12:4:2 v/v), B:Chloroform:Methanol (1:1 v/v), C: Chloroform:Methanol:Water (65:25:4 v/v), D: Chloroform:Methanol:7 mol/l $NH_4OH$ (65:25:5 v/v).
**Spray reagent**. 1:Lipids test (2',7'-Dicholofluorescein), 2: Carbohydrate test (Anthrone reagent), 3:Sugar test (Phenol-Sulfuric acid), 4:Amino acid test. (Ninhydrin reagent).

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**3.4 Identification of Glycolipid was Isolated from Crude biosurfactant**

The positive reactivity of this lipid on anthrone reagent suggests that one of the carbohydrate. In an attempt to characterize the sugar moiety. The glycolipid was dissolved in diethyl ether and followed by saponification with 1 NaOH in a water bath at $90^0$C, 1 hr. The aqueous layer was separated and successively passed through a cation exchanger resin (Amberlite IRA-120 H-from).

The passage of Glycolipid hydrolysate is illustrated in Figure 39. The measurement of the total carbohydrate (anthrone method) in successive 5 ml fractions of the column effluent revealed that the initial two fractions did not contain any carbohydrate. The third 5 ml fraction its level sharply increased to 0.558 of the OD 625 (anthrone) passed through the column during the entire collection and declined markedly in the successive fourth portion (0.175, 0.081, 0.031, respectively). Starting with the 7 $^{th}$ fraction, the OD 625 stabilized in the range 0.02-0.01 (OD625) and remained unchanged in the following 13 fractions. After the fractions No. 2-6 was evaporation to dryness with lyophilize, TLC analyzed the fine white material for sugar component for carbohydrate.

The fine white materiel from lyophilize was dissolved in water and an aliquot transferred onto TLC, which was chromatograph in solvent system (Propanol-Water (85:15 v/v). Only one spot positive to phenol-$H_2SO_4$ reagent was detectable. It showed a sugar-positive spot with $R_f$ 7.0, identical with reference rhamnose. From the results of these experiments, it was consequently confirmed that the sugar moiety of the glycolipid was rhamnose (Table 13).

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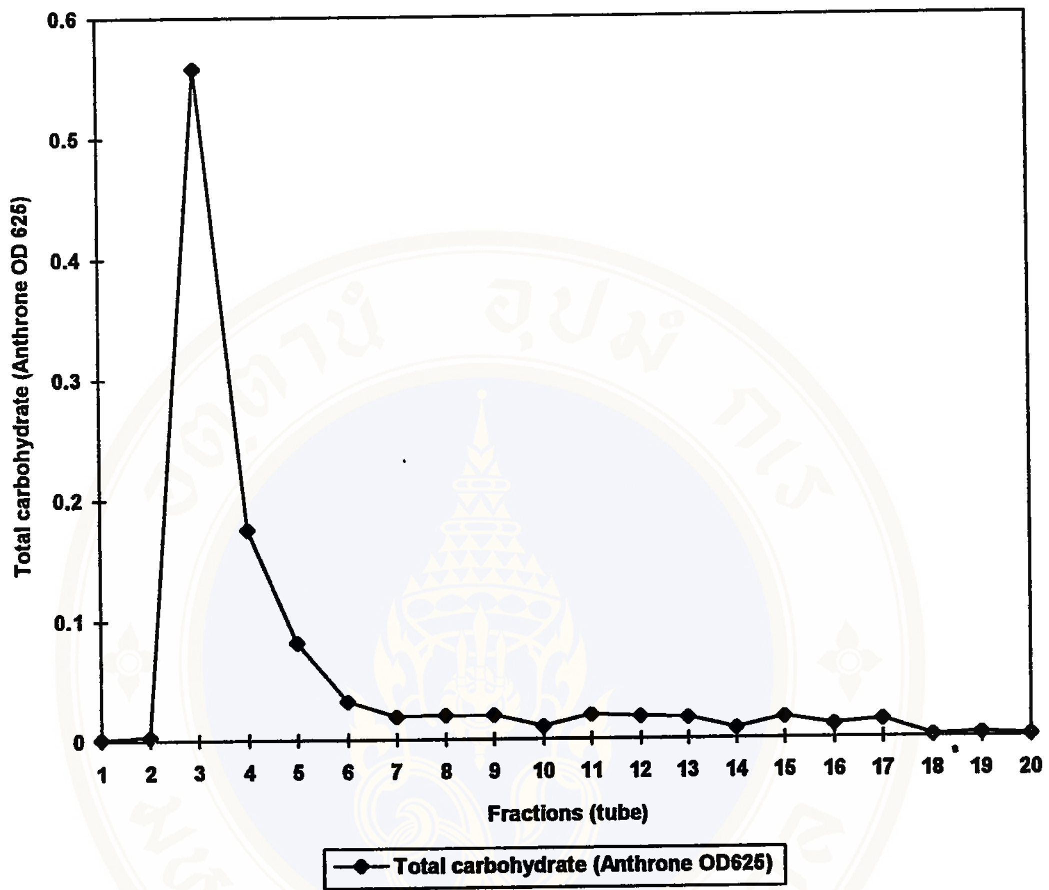


**Figure 39** Passage of glycolipid hydrolysate through Amberlite IRA-120 (H-form) column chromatography

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**Table 13** Thin layer chromatography identification of sugar moiety

| Sample | $R_f$ (X100) | Color |
|---|---|---|
| Glycolipid hydrolysate | 70 | Green |
| Rhamnose | 70 | Green |
| Mannose | 55 | Brown |
| Glucose | 51 | Brown |
| Galactose | 44 | Brown |
| Cellobiose | 39 | Brown |
| Trehalose | 39 | Black |

**Solvent system** : Propanol:Water (85:15 v/v)
**Spray reagent** : Phenol-$H_2SO_4$-Ethanol.

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**4. Properties of the Biosurfactant produced by *Pseudomonas* J-45**

**4.1 Determination of Critical Micelle Concentration (CMC)**

Figure 40 shows the dependence of surface tension on the biosurfactant concentration. Surface tension decreased rapidly from 72.5 to 35 mN/m with increased the biosurfactant concentration up to 139.2 mg of biosurfactant per liter. Further increase in the biosurfactant concentration only slowly reduced the surface tension from 35 to 34.5 mN/m. Once the surface tension reached 34.5 mN/m; further addition of rhamnolipid had no effect. The CMC was determined from a semilog plot of surface tension versus rhamnolipid concentration to be 148 mg/l.

**4.2 Surface active Properties of the Biosurfactant J-45**

**4.2.1 Effect of pH on the Surface activity**

A biosurfactant solution was prepared at a concentration of 0.0148 % (w/v). When the pH was lower than 6, the greatest decreases in the surface tension were observed (Figure 41). The interfacial tension against hexadecane of biosurfactant solution at increasing of pH was found to contrast the pattern as the surface tension. The interfacial tension reached a plateau at 5.9 mN/m at pH 8 to 8.8 mN/m at pH 2. Thus, natural to alkaline pH seemed to enhance the effectiveness of biosurfactant. Visually, the biosurfactant solutions were clear above pH 4 but turbid below this pH. Precipitation of biosurfactant was not apparent until the pH was increase above 4.

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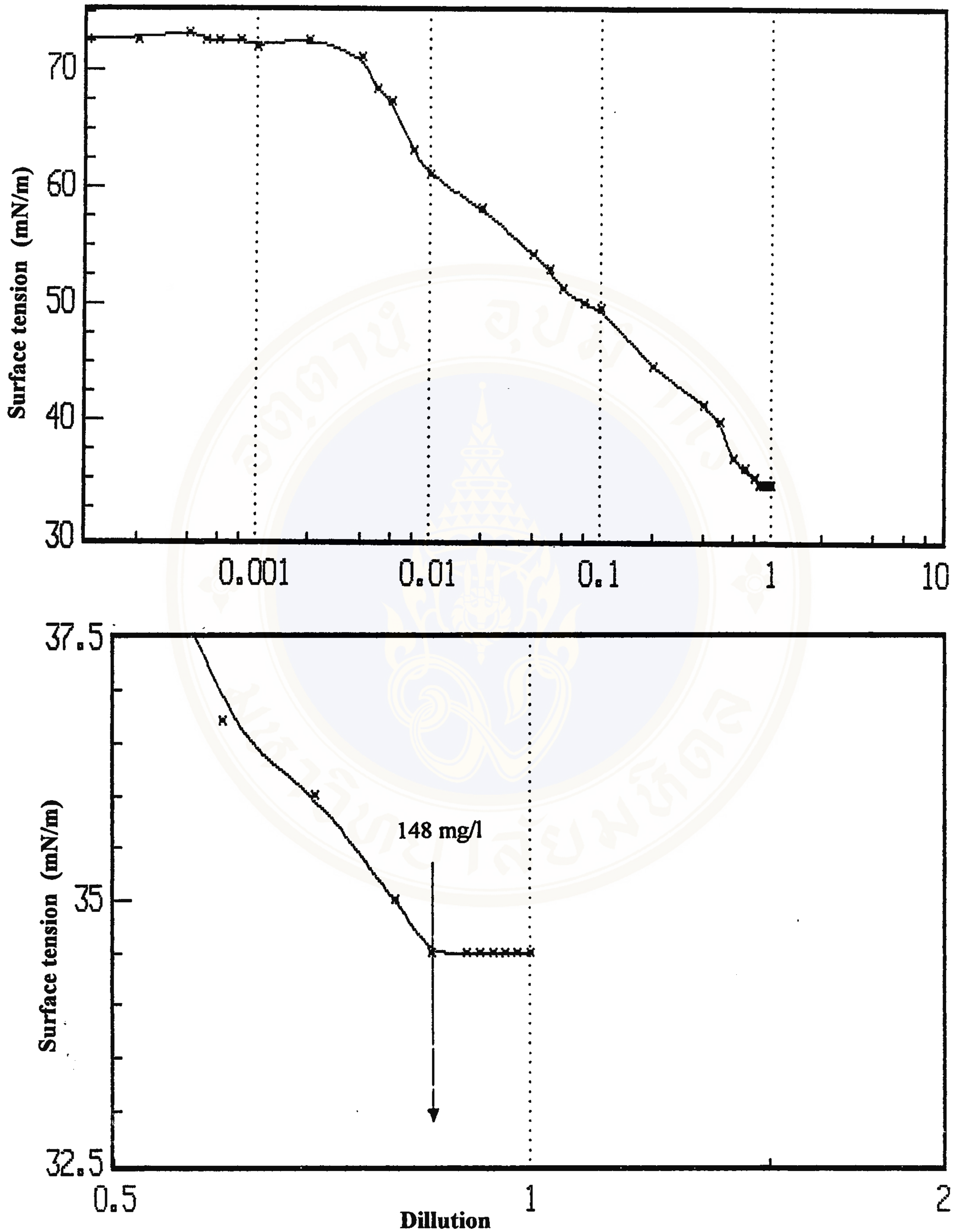


**Figure 40** Determination of Critical Micelle Concentration (CMC)

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**4.2.2 Effect of Temperature on Surface activity**

Biosurfactant solution (0.0148 %w/v) was treated for 20 min. at 20, 40, 60, 80, 100, 121 $^{0}$C. After the treatment, the solution was brought to 20$^{0}$C and the surface activity test was run as outlined previously. A significant loss in surface activity appeared when the biosurfactant solution was treated at a temperature higher than 80$^{0}$C (Figure 42). Surface tension decreased from 34.5 mN/m at 20$^{0}$C to 31.5 mN/m at 100$^{0}$C. And Interfacial tension against hexadecane increased from 6.1 mN/m at 20$^{0}$C to 6.3 mN/m at 100$^{0}$C

**4.2.3 Stability of Biosurfactant J-45 on Surface activity at Various Temperature**

The biosurfactant J-45 was stable in the range 55-80$^{0}$C for 3 hr but the interfacial tension against hexadecane began to rise after incubating at 100$^{0}$C for 2 hr (Figure 43).

**4.2.4 Effect of NaCl concentration on Surface activity**

Addition of 5% NaCl in the biosurfactant solution improved the surface activity of the biosurfactant but the ability to reduced surface tension will drop when NaCl concentration was presented at more than 5 %. A 30% NaCl solution brought the surface tension from 34.5 to 33.0 mN/m. As the interfacial tension increased from 5.8 mN/m at 0 % NaCl (w/v) to 20 mN/m at 30% NaCl (w/v), with interfacial tension with an increase in NaCl concentration (Figure 44).

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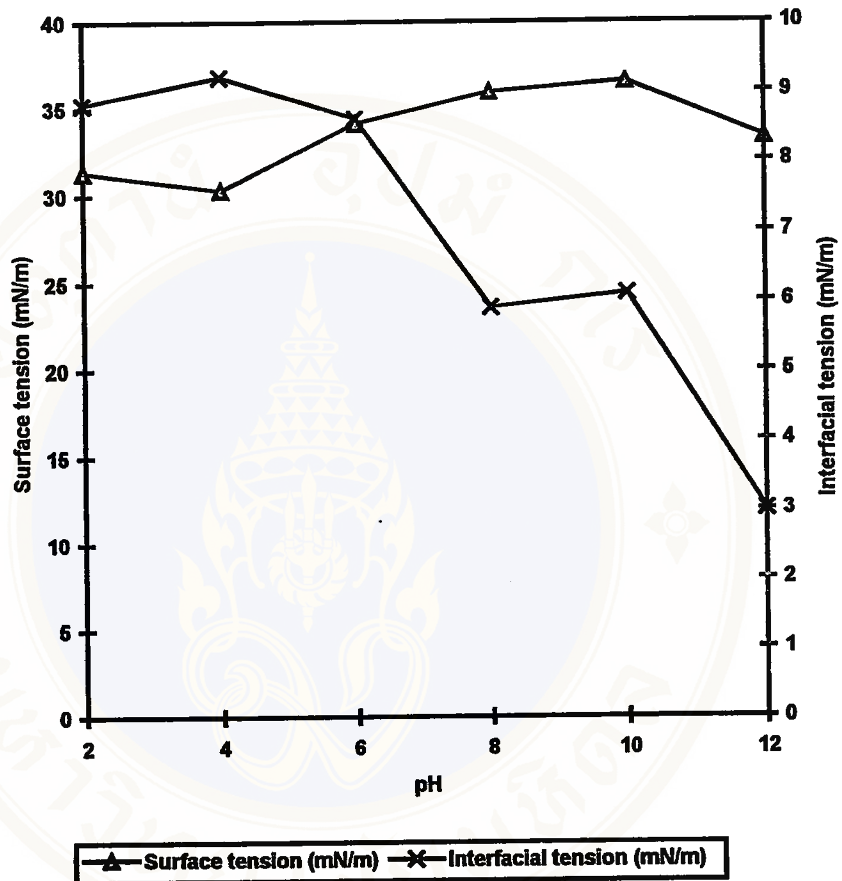


**Figure 41** Effect of the pH on surface activity of 0.0148 % (w/v) biosurfactant J-45

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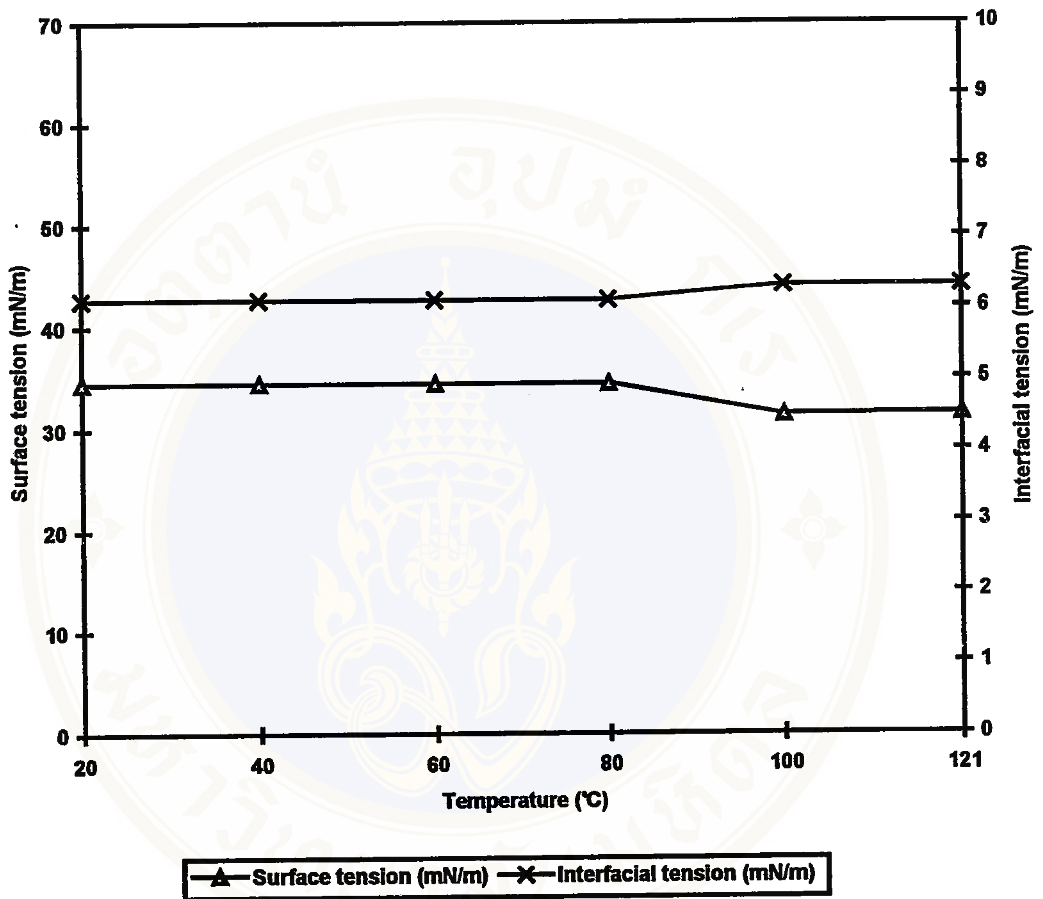


**Figure 42** Effect of the temperature on surface activity of 0.0148% (w/v) biosurfactant J-45

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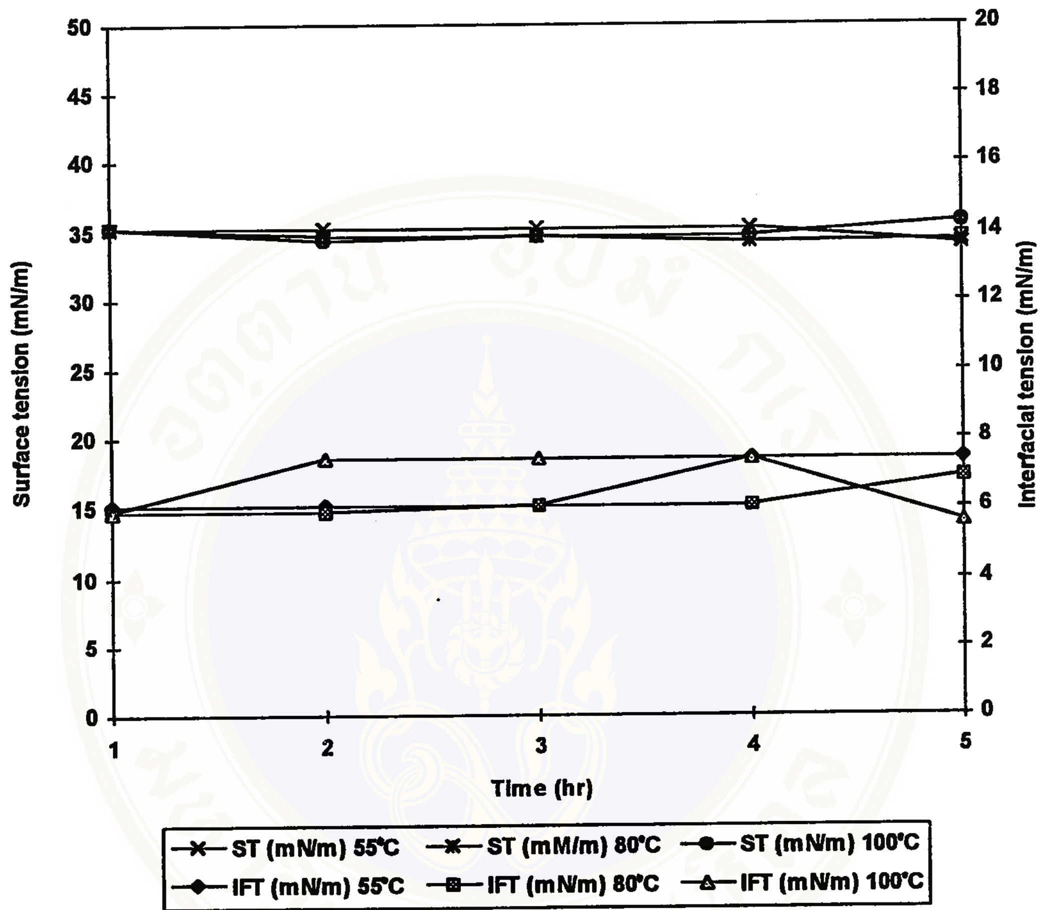


**Figure 43** Stability of biosurfactant J-45 on surface activity at various temperatures

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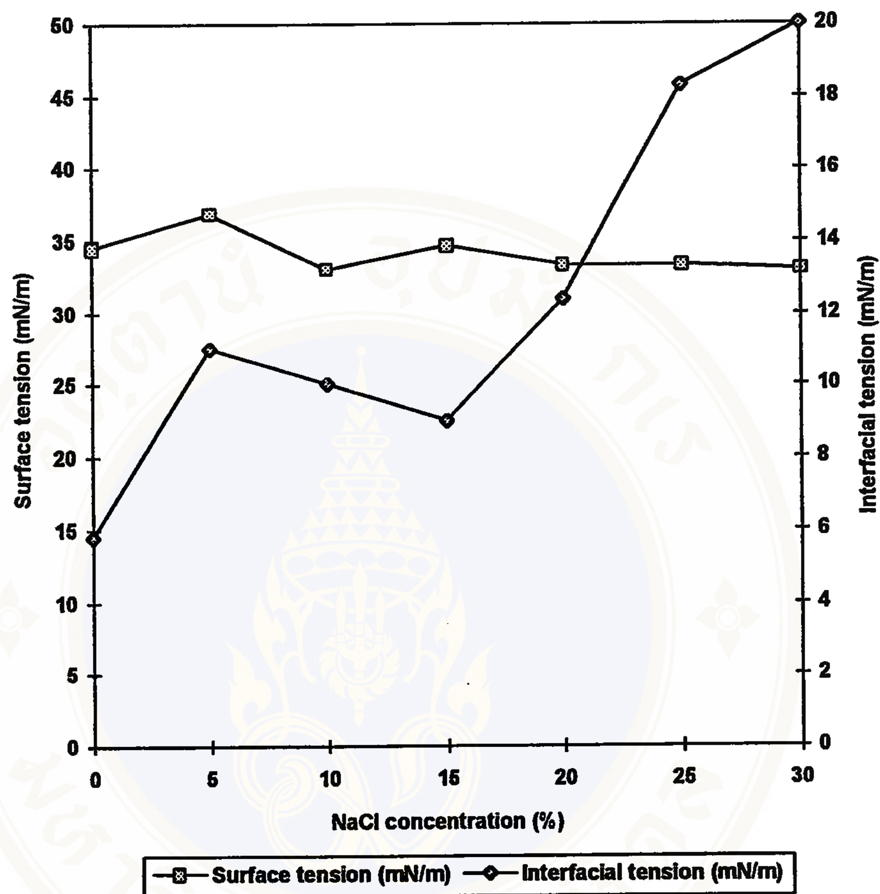


**Figure 44** Effect of the NaCl concentration on surface activity of 0.0148% (w/v) biosurfactant J-45

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## 4.3 Emulsification Properties of the Biosurfactant J-45

### 4.3.1 Effect of pH on the Emulsification Capacity

The Emulsification Capacity (EC) of biosurfactant solution was quite sensitive to pH (Figure 45). The EC of biosurfactant solution was highest at pH 8.0. As the pH was decreased above 8.0, there was a slight in EC that resulted in decreased Optical density (OD660) from 0.670 at pH 7 to 0.080 at pH 2. After increased to 0.991 at pH 8.0, as the pH was increase from 10 to 12 EC decreased significantly, resulting in decreased in OD 660 from 0.681 at pH 10 to 0.485 at pH 12.

### 4.3.2 Effects of Temperature on Emulsification Capacity

Biosurfactant solution was treated for 20 min. at 20,40,60,-80,100 and 121$^0$C. After treatment, the solutions were brought to room temperature and the EC test was run as out line previously. A significant loss in the EC property appeared when the biosurfactant solution was treated at temperature higher than 60$^0$C (Figure 46). EC decreased from 0.411 at 20$^0$C to 0.180 at 121$^0$C.

### 4.3.3 Stability of biosurfactant J-45 on Emulsification Capacity at Various Temperature

Biosurfactant solution were treated for 5 hr at 55$^0$C, 80$^0$C and 100$^0$C (Figure 47). After treatment, the solution were brought to room temperature are the EC test was run as outlined previously. The biosurfactant solution was stable in the range 55$^0$C-100$^0$C for 2 hr but the EC began to stable after incubating at 55$^0$C before 2 hr. A significant loss in the EC property appeared when the biosurfactant solution were treated at 55$^0$C, 80$^0$C and 100$^0$C from 2-5 hr. As the time was increased above 2 hr there was a slight decrease in OD 660 from 0.303 to 0.236 at

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55$^0$C, as the time from 2-5 hr EC decreased significantly, resulting in decreased in OD 660 from 0.311 to 0.146 at 80$^0$C and 0.303 to 0.149 at 100$^0$C.

#### 4.3.4 Effect of NaCl concentration on Emulsification Capacity

When NaCl was dissolved in the biosurfactant solution to the final concentration of 0-30 %. The EC of biosurfactant produced by *Pseudomonas* J-45 retained at 0-5 % and in the range 10-30 % NaCl a slight decrease in the EC. When NaCl was added, a higher concentration (5%) was necessary to get such an effect (Figure 48).

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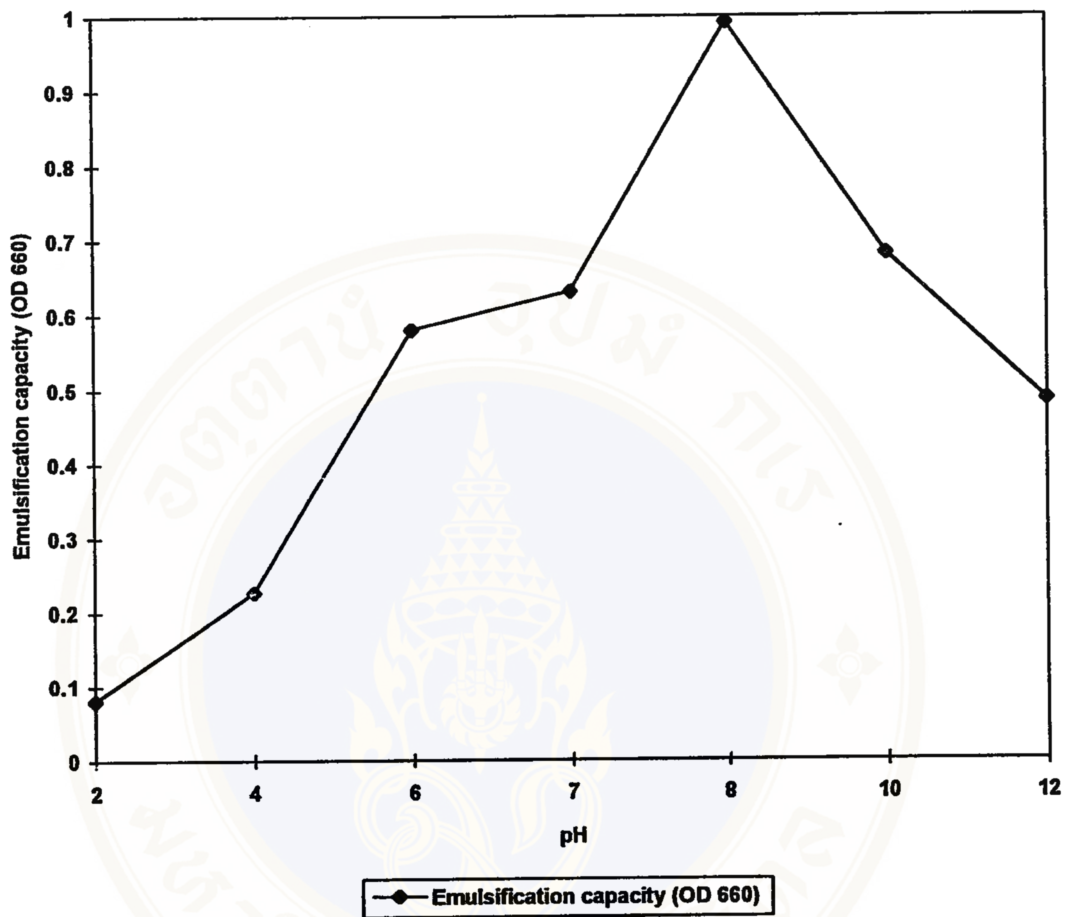


**Figure 45** Effect of pH on emulsification capacity of biosurfactant J-45

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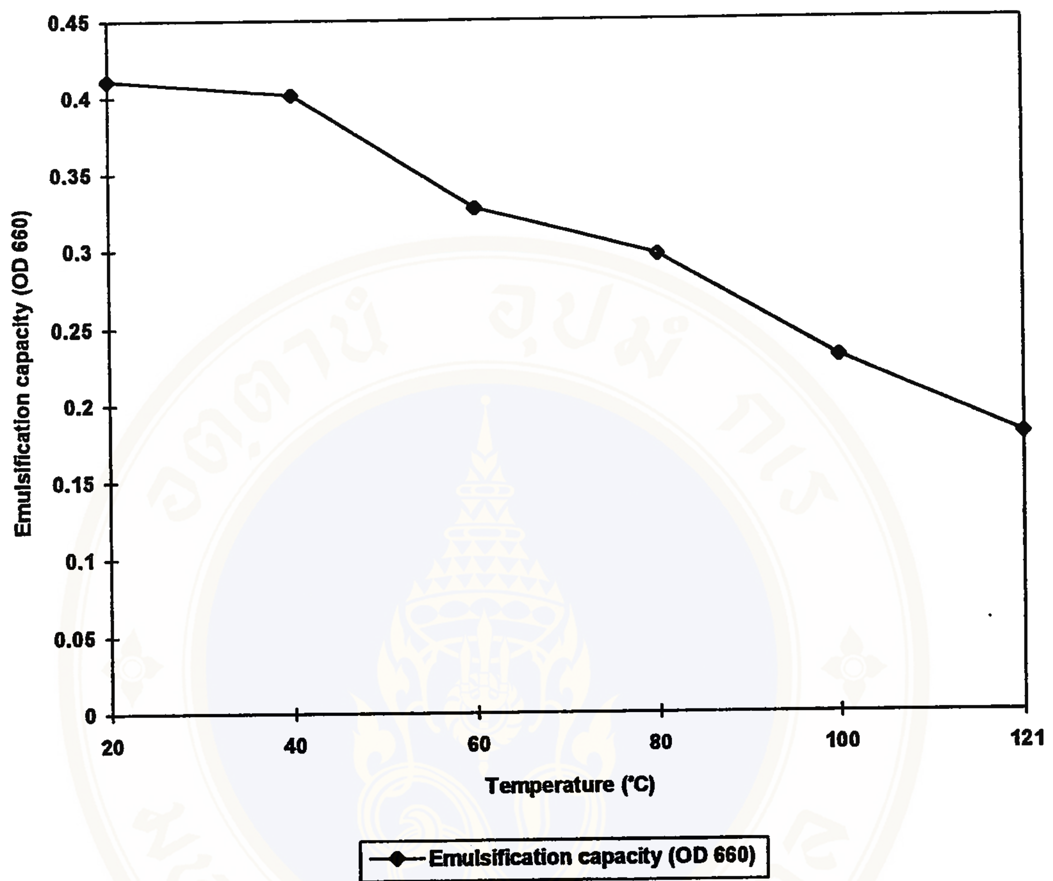


**Figure 46** Effect of temperature on emulsification capacity of biosurfactant J-45

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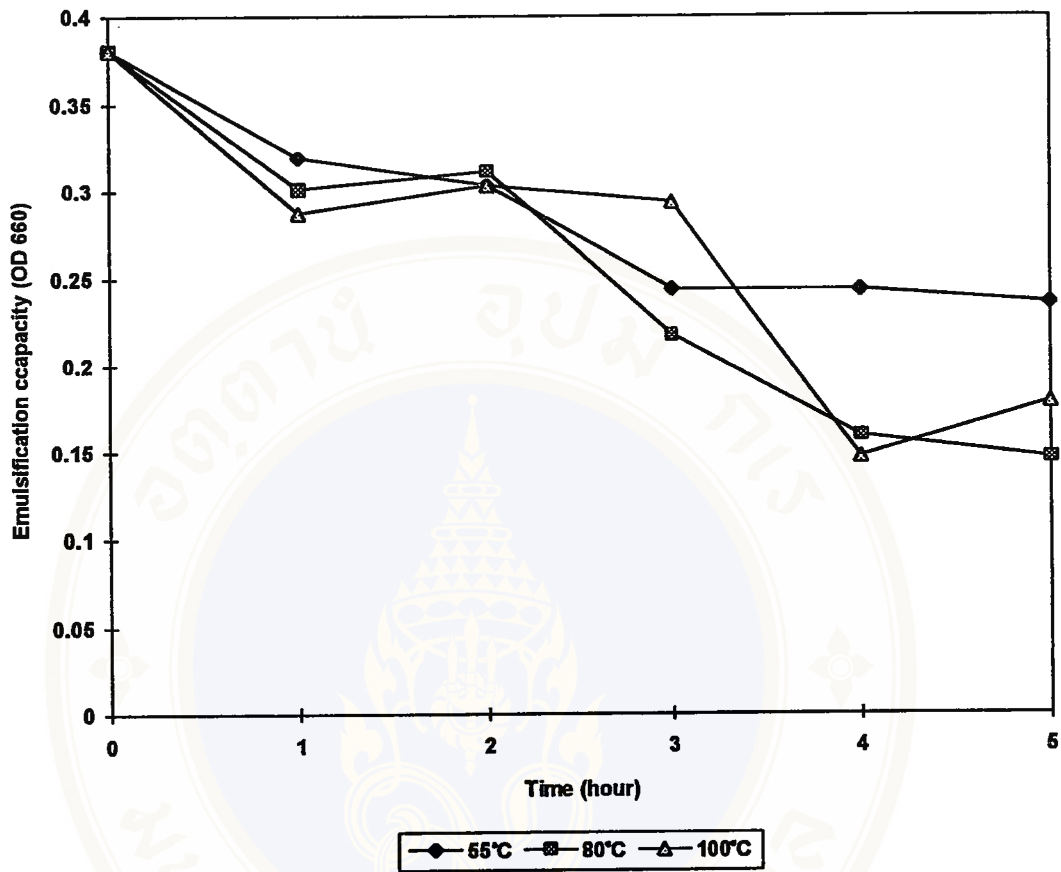


**Figure 47** Stability of emulsification capacity of biosurfactant J-45 at various temperatures

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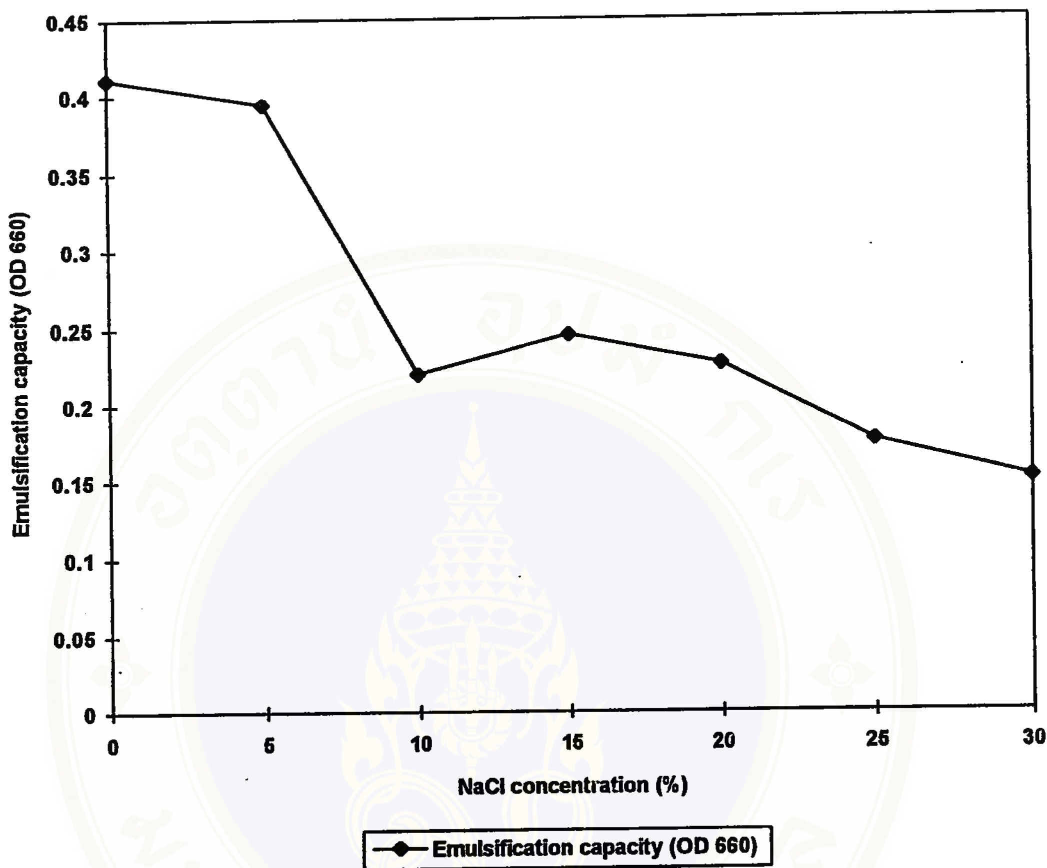


**Figure 48** Effect of NaCl concentration on emulsification capacity of Biosurfactant

J-45

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**4.4 Comparison of the Critical Micelle Concentration (CMC) of Biosurfactant J-45 with other Synthetic surfactants**

Table 14 shows critical micelle concentration (CMC) and surface tension of the biosurfactant from *Pseudomonas* J-45 in comparison with the chemical synthetic surfactant. It was found that the CMC of the biosurfactant J-45 was higher than Trion X-100 but lower than Tween 20, Tween 80, Sodium dodecyl sulfate (SDS), WIZARD, AQUAQUICK, and SUPERNATANT-25. The CMC of biosurfactant J-25, Tween 20, Tween 80, Sodium dodecyl sulfate (SDS), WIZARD, AQUAQUICK, and SUPERNATANT-25 were about 148, 150, 200,120, 900, 400, 800, 500 mg/l respectively. And was found that the surface tension of the biosurfactant J-45 was higher than Trion X-100, WIZARD and AQUAQUICK but lower than Tween 20, Tween 80, Sodium dodecyl sulfate (SDS), and SUPERNATANT-25. The surface tension of biosurfactant J-45, Tween 20, Tween 80, Sodium dodecyl sulfate (SDS), WIZARD, AQUAQUICK, and SUPERNATANT-25 were about 34.5, 42.5, 45, 31, 37, 32, 32, 35 mN/m respectively.

**4.5 Emulsification Capacity of Biosurfactant J-45 with various Hydro-carbon and Oils**

The ability of biosurfactant produced *Pseudomonas* J-45 to emulsify various hydrocarbons was tested. Biosurfactant efficiently emulsified aliphatic and aromatic hydrocarbons, petroleum product, and vegetable oil (Table 15). In the aliphatics and aromatics hydrocarbon series, EC was generally higher with longer-chain hydrocarbons than with the lower homologs. Very high EC value was obtained with Olive oil, Palm oil and Soybean oil respectively.

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**Table 14** Comparison of the properties of biosurfactant J-45 with other synthetic surfactant

| Surfactant | CMC (mg/l) | Surface tension (mN/m) |
|---|---|---|
| Biosurfactant J-45 | 148 | 34.5 |
| Tween 20 | 150 | 42.5 |
| Tween 80 | 200 | 45 |
| Trion X-100 | 120 | 31 |
| Sodium dodecyl sulfate | 900 | 37 |
| WIZARD* | 400 | 32 |
| AQUAQUICK* | 800 | 32 |
| SUPERNATANT-25* | 500 | 35 |

* Tread mark

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**Table 15 Emulsification of aliphatics, aromatics hydrocarbons and oils by biosurfactant J-45**

| Nonaqeous phase | Emulsification capacity (mean ± SD) |
|---|---|
| **Aliphatics hydrocarbons** | |
| Pentane (C-5) | 0.009 ± 0.008 |
| Hexane (C-6) | 0.002 ± 0.001 |
| Heptane (C-7) | 0.039 ± 0.003 |
| Decane (C-10) | 0.431 ± 0.024 |
| Hexadecane (C-16) | 0.387 ± 0.037 |
| **Aromatic hydrocarbons** | |
| Benzene (C-6) | 0.039 ± 0.044 |
| Toluene (C-7) | 0.048 ± 0.004 |
| Xylene (C-8) | 0.425 ± 0.034 |
| **Petroleum products** | |
| Crude oil | 0.314 ± 0.259 |
| n-paraffin | 0.124 ± 0.022 |
| Diesel fuel | 0.411 ± 0.016 |
| Gasoline (octane 95) | 0.429 ± 0.022 |
| Compressor oil (W-10) | 1.138 ± 0.031 |
| **Vegetable oil** | |
| Soybean oil | 1.264 ± 0.029 |
| Palm oil | 1.351 ± 0.012 |
| Olive oil | 1.410 ± 0.017 |

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# CHAPTER V

# DISCUSSION

## 1. Growth and Oil degrading activity of the *Pseudomonas* J-45 under Various conditions

The study growth and crude oil degrading activity of the *Pseudomonas* J-45, the cellmass were used as the indicators of the microbial growth. Indirectly, these indicators could also be used to show the crude oil degrading activity of the test bacteria due to their need to use carbon from crude oil, which was the only carbon source for growth. Another indicator, the surface tension, it indicated the crude oil degrading activity. Bacteria produce it varied to the amount of surfactant, while grease and oil value indicated the residual oil in the medium at each fermentation time. It was show by the residual oil remaining in the medium after metabolism process of the bacteria. There fore, it could be used as the indicator of the microbial growth and oil used by the microorganisms. If there were growth and oil degrading activity, the biomass would have increased whereas, grease and oil, surface tension of media would have decreased due to oil degrading activity of the microorganisms.

Hydrocarbon biodegradation can occur over a wide range of temperatures, and psychrotrophic, mesophilic, and thermophilic hydrocarbon-utilizing microorganisms have been isolated. Temperature can have a make effect on the rated of hydrocarbon degradation.

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Atlas (31) also studied the effects of temperature on the biodegradation of seven difference crude oils by using *Pseudomonas sp.* isolated from the arctic. He found that the percentages of biodegradation of all crude oils were higher at 20$^{o}$C than at 10$^{o}$C, and that the rates were different among seven types of crude oils. He also stated that the biodegradability of a variety of crude oils was found to be highly dependent on crude oil composition and temperature.

**1.1 Effect of Temperature**

In this study, the *Pseudomonas* J-45 that had crude oil biodegradation was temperature dependent. The percentage of total crude oil remove at 168 Hr. (Figure 23) was highest at 37$^{o}$C (94.19 %) and decreased orderly from 30$^{o}$C (90.27 %) to 25$^{o}$C (90.09 %). If we look at the crude oil removal (Figure 23) at 96 hr, % biodegradation at 30$^{o}$C (89.07%) was better than % degradation at 37$^{o}$C (70.11%) and 25$^{o}$C (62.99%). The growth rate curve of *Pseudomonas* J-45 were highest growth rate was obtained at temperature 37$^{0}$C which corresponded to crude oil degradation. Generally, *Pseudomonas* sp. are know to be pathogenic to man and animals, and pathogenic bacteria usually have optimal growth at body temperature.

Surface activity during growth of *Pseudomonas* J-45. Reduction in surface tension and interfacial tension would promoted emulsification of hydrocarbon and they by increase the interfacial area between hydrocarbon and water, and these interfacial surfaces are considered most efficient for cell adherence and growth. There are a number of reports on the close association of microbial growth on hydrocarbon with

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the reduction in surface tension and interfacial tension of the broth and the production of biosurfactant (90,134)

In this study, in Figure 25 show that in the case of good growth on crude oil at 25$^0$C, 30$^0$C and 37$^0$C, culture broth surface tension was reduced considerably to 34.5 mN/m, 32.5 mN/m and 38.6mN/m from a value of 64 mN/m uninoculated medium, indicating a relationship between growth and reduction in surface tension. The results indicated that cells acted as biosurfactant and primarily accounted for the reduction of surface tension in culture broth.

In consideration of the environmental in Thailand, the temperature, which is likely to be suitable for growth and oil dispersion activity of the microorganism, should range between 25-40$^0$C (3). In this study, *Pseudomonas* J-45 could grow and degrade oil at 25-37$^0$C.

**1.2 Effect of Aeration**

In this study the effects of aeration on the biodegradation of crude oil by *Pseudomonas* J-45. The percentage of grease and oil removal (Figure 28) was highest at 1.0 v.v.m (90.27%) and decreased orderly from 0.5 v.v.m (79.56%) to 0.25 v.v.m (74.58%). If we look at the grease and oil removal at 96 hr (Figure 31), % biodegradation at 1.0 v.v.m (89.07%) was better than % degradation at 0.5 v.v.m (86.90%) and 0.25 v.v.m (77.31%). The growth curve of Pseudomonas J-45 at 0.25 v.v.m (123 mg/l) and 0.5 v.v.m (135 mg/l) were almost identical. The growth rate patterns of *Pseudomonas* J-45 increase rapidly was obtained at 1.0 v.v.m (1275 mg/l).

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Oxygen as with nutrients, there has been controversy over whether oxygen is absolutely required for hydrocarbon biodegradation or whether hydrocarbon is subject to anaerobic degradation. In the case of *Pseudomonas* strain studied by Senez and Azoulay (135), the organisms consumed oxygen when growing on heptane even though in had an n-heptane dehydrogenase enzyme. The important of oxygen for hydrocarbon degradation is indicated by the fact that the major degradative pathways for both saturated and aromatic hydrocarbons, discussed earlier, involved oxygenases and molecular oxygen. The theoretical oxygen demand is 3.5 g of oil oxidized per g of oxygen and calculated that the dissolved oxygen in 3.2 x $10^5$ liters of seawater therefore would be required for the complete oxidation of 1 liter of oil (136).

In this study, in Figure 31 show that in the case of good growth on crude oil at 96 hr was highest at 1.0 v.v.m (1275 mg/l) and culture broth surface tension was reduced considerably to 33.0 mN/m and increased orderly from 0.5 v.v.m (135 mg/l, 38 mN/m) to 0.25 v.v.m (123 mg/l, 40.5 mN/m) from a value of 64 mN/m uninoculated medium, indicating a relationship between growth and reduction in surface tension. The results indicated that cells acted as biosurfactant and primarily accounted for the reduction of surface tension in culture broth.

Although the oxygen content is the main factor for the growth and capability to degrade crude oil, but many reported show groups of anaerobic or facultative anaerobic which are able to degrade oil (97).

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### 1.3 Growth and Oil degrading activity of the *Pseudomonas* J-45 in Seawater

In this study, the biodegradation of crude oil by *Pseudomonas* J-45 in Seawater. The percentage of crude oil removal (Figure 33) was higher in ASW medium (90.23%) and lower in SW medium (61.99%). The growth curve patterns were increased rapidly within 24 hr of incubation in ASW medium and increases slightly at the end of experiment. The growth curve of *Pseudomonas* J-45 growth in SW medium was increased slightly from 0 hr at end of experiment as well as the surface tension of culture broth in SW medium was maintain from 59.8 mN/m (0 hr) to 58.6 (72 hr) and decrease slightly to 32.5 mN/m at the end of experiment, which surface tension of culture broth in ASW medium was reduced considerably to 32.5 mN/m at end experiment. The results showed that the biodegradation of crude oil in Seawater was significantly lower than the degradation rate of crude oil in ASW.

Cowell et al. (33) concluded that Metula oil is degraded slowly in the marine environment, most probably because of limitations imposed by the relatively low concentrations of nitrogen and phosphorus available in seawater.

Atlas and Bartha (30) described as oleophilic nitrogen and phosphorus fertilizer, which could overcome limitations of nitrogen and phosphorus in seawater and stimulate petroleum biodegradation in seawater. The fertilizer consisting of paraffinized urea and octylphosphate supported degradation of oil in Seawater. Optimal C/N and C/P ratios were 10:1 and 100:1, respectively. In conjunction with the us Office of Naval research, they obtained a patent for use of fertilizers for stimulating oil degradation in seawater (137).

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Dibble and Bartha (34) examined the effect of iron on the biodegradation of petroleum in Seawater. Biodegradation of south crude oil and the effects of nitrogen, phosphorus and iron supplements on this process were compared in polluted and relatively clean littoral Seawater collected along the New Jersey coast. Without supplements, the biodegradation of crude oil was negligible in both Seawater samples. Addition of nitrogen and phosphorus allowed very rapid biodegradation; up to 73 % of the oil was degraded within 3 days in polluted seawater.

The above studies indicated that the available concentrations of nitrogen and phosphorus severely limit the extent of hydrocarbon degradation after most major oil spills. Rates of nutrient replenishment generally are inadequate to support rapid biodegradation of large quantities of oil. The addition nitrogen and Phosphorus containing fertilizers can be used to stimulated microbial hydrocarbon degradation.

## 2. Isolation, Purification and Identification of Biosurfactant produced by *Pseudomonas* J-45

The microorganisms were then grown in liquid culture and surface tension reductions were measured. The majority of the microbe achieved either on or small surface tension reduction (< 10 mN/m); these microorganisms were not identified as biosurfactant producers under the culture conditions defined herein. However, they may have been stimulated to produced biosurfactants given the proper nutrient and growth conditions (129). *Pseudomonas* J-45 produced large surface tension reductions indicating the high probability of biosurfactant production (Figure 25, 30, and 35).

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Further work in this laboratory will concentrate on identifying methods to enhance biosurfactant production by *Pseudomonas* J-45.

Biosurfactant have long been considered only with regard to microbial growth on hydrocarbon (139). One models discussed suggests that the excretion of biosurfactant increases the emulsification and therewith the available surface area as well as the solubility of the substrate (140).

The microorganisms are able to emulsify the hydrocarbon, thereby increasing surface area and making the hydrocarbon droplets more available for microbial attack, may have been better able to proliferate under conditions of added nutrient and oxygen. Most biosurfactants are synthesized by bacteria or yeast during growth on lipophilic substrate such as hydrocarbons. Trehalose (52) and rhamnolipids (141) have been reported in the literature. The *Pseudononas* J-45 studies in this work also produced surface- and interfacial-active compound when grown on crude oil. Suzuki and co-workers (69) also found glycolipids when *Arthrobacteria, Corynebacteria,* or *Nocardiae* were cultivated in excess sucrose or fructose. In all of these cases, the carbon source determined the type of sugar moiety, whereas the fatty acid remained constant.

*Pseudomonas* strain (142). These rhamnolipid compounds have also been described by several authors when strains of *Pseudomonas* were grown on different substrates; the structure of these rhamnolipids does not change with the carbon source used (141, 143, 144, 149). Guerra-santos (145), and Robert et al (142) have, extensively studied dependence of *Pseudomonas sp.* biosurfactant production on nutritional and environmental factors.

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In a preliminary study into the chemical nature of the biosurfactant produced, *Pseudomonas* J-45 cell growth with added crude oil was incubated in ASW medium. After centrifuge the cell was lyophilized and filtrate the supernatant to remove cell bacteria, extract with chloroform/methanol (2:1 v/v) and examined by thin layer chromatography on silica gel 60 plates with the solvent system D: chloroform/methanol/$NH_4OH$ (7mol/l) (65:25:5). By sparing the TLC plates with Anthrone, *Pseudomonas* J-45 produced two dominant of glycolipid compounds, which were be detected with a retention-font value ($R_f$) of 0.48 and 0.24, which corresponds to cationic glycolipid. For carbohydrate analysis , partially purified glycolipids were hydrolyzed with NaOH, the water-soluble fraction from hydrolyzed after cation exchange with Amberlite IR120 (H-form). A sugar having the same $R_f$ value as rhamnose was detected with anthrone reagent on TLC plate in solvent system E. Another sugar was not observed. *Pseudomonas* J-45 is know to produced large quantities of a chemically defined cationic glycolipid consisting of rhamnose, Which is called by the mane of Rhamnolipid (141).

*Pseudomonas* J-45 produced two dominant of glycolipid compounds, which were be detected with $R_f$ 0.74, 0.58 on TLC in solvent system C: chloroform/methanol/water(65:25:4). One anthrone positive spot with an $R_f$ value of 0.74 had the same $R_f$ values in TLC as described monorhamnolipid was identified by Parra (80). This study determined the type of biosurfactant J-45 is the same as those isolated from several other *Pseudomonas aeruginosa* strain (80,146).

The data on the biosurfactant purification of cell-free supernatant fluids indicated that many of the isolated had emulsification ability due, at least in part, to

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the whole cells themselves; emulsification was often reduced after remove of the cells, retained emulsifying ability after remove of cells indicating that extracellular surface-active compounds were excreted into the medium. Those isolated retaining emulsifying ability were the most promising biosurfacctant produced.

**3. Properties of Biosurfactant produced by *Pseudomonas* J-45**

Partially purified *Pseudomonas* J-45 rhamnolipid biosurfactants lowered the surface tension of water from 72.5 to 34.5 mN/m and achieved a CMC at 148 mg/l. These results are comparable to those of rhamnolipids produced by other *Pseudomonas* species, which have surfactant CMC values ranging from 0.01 to 1.0 g/l described by Gutnick and Minas(138).

The *Pseudomonas* J-45 biosurfactant is stable on surface activity under extremes of pH temperature and NaCl concentrations. The experimental data present also show in Figure 41, 42, 43, 44 that the minimum surface tension are constant within the different conditions, in contrast to interfacial tension, which did not show consistent surface properties under the different conditions

The biosurfactant shown to is resistance to 20 min. of incubation time at temperatures ranging from 20 to 80$^0$C. Biosurfactant J-45 retains activity at pH 6-10 and exhibited optimal at pH 8.0. The rhamnolipids emulsified in aqueous solutions are much stable at extreme at temperature values, e.g., 80$^0$C. and their surface activity is not reduced at temperatures exceeding 80$^0$C at 3 hr. A Loss in activity was observed at pH below 4.0 due to biosurfactant precipitation. The activity of biosurfactant solution is not inhibit by NaCl concentrations up to 5 %. The properties are similar to those of

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other rhamnolipids-type biosurfactant produced by *Pseudomonas sp.* (80,148,149) The stability over a wide range of pH, temperature and NaCl concentration are good criteria for commercial applications.

The minimum critical micelle concentration (CMC) and surface tension were parameters used to measure the efficiency of surfactant systems. The partial purification biosurfactant J-45 it was found glycolipids reduced the surface tension of water to 34.5 mN/m and the interfacial tension against n-hexadecane to 5.0 mN/m, respectively (Figure 40). Also the values for critical micelle concentration at 148 mg/l. The preliminary results indicated that J-45 biosurfactant was a potential surface-active agent with fairly good surface activity in comparison with other synthetic surfactants.

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# CHAPTER VI

# CONCLUSION

The present study was performed to illustrate the factor involved in biodegradation of crude oil and biosurfactant formation in artificial seawater (ASW). The experiment was conducted in closed reactor known as "fermenter", and tested factors were temperatures, aeration rate and comparison degradation activity between ASW and seawater were performed. The results showed that all of these factors affected the growth, degradation of crude oil and biosurfactant formation of the *Pseudomonas* J-45.

## 1. Study the suitable condition to biodegradation of crude oil and biosurfactant formation

The present study demonstrated that when *Pseudomonas* J-45 was grown in ASW and seawater medium. The fermentation of 8 l of bacterium broth containing 1% crude oil in 10 l fermenter showed that the optimum conditions to obtain the highest value of the surface tension reduction are at 100 r.p.m. agitation and 1 v.v.m. aeration rate at $30^0$C.

## 2. Isolation, purification and Identification type of Biosurfactant produced by *Pseudomonas* J-45

The identification for the type of biosurfactant *Pseudomonas* J-45 is know to

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produced large quantities in supernatant defined to extracellular product and

chemically defined to cationic glycolipid consisting of rhamnose, which is called by the mane of Rhamnolipids.

**3. Study Properties of the Biosurfactant J-45**

Biosurfactant J-45 was found to have good properties its stability over a wide range of pH, temperature and salt concentration. These properties are good criteria for commercial applications, they are interested to be studied and tended to replace the chemical surfactants, because of a number of advantages, especially the reduction of environmental pollution problem.

The increasing interest in the potential applications of biosurfactant in based on their broad range of functional properties that includes emulsification, wetting, foaming, solubilization and viscosity-reduction (e.g. of heavy crude oils). There are therefore, many areas of industrial application where chemical surfactant could be substituted by biosurfactants in fields as diverse as agriculture, the food and beverage industries, textiles, cosmetics, the pharmaceutical industry and, last but not least, petroleum and petrochemical industries.

The striking advantages of biosurfactant over chemically synthesized surface-active compounds include their broad range of novel structural characteristics and physical properties, their production on renewable substrates, their capacity to be modified (by genetic engineering, biological or biochemical techniques) and thus tailored to meet specific requirements, and (probably most important) their biodegradability. Many chemical surfactants cause environmental problems due to their

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resistance to biodegradation and their toxicity when allowed to accumulate in natural ecosystems.

The research contained several implications for future study of: Firstly, it was recommended that a study of genetic engineering technique to improve the strain to produce higher of the biosurfactant. Secondly, to study biodegradation of Rhammnolipid (Biosurfactant J-45). Thirdly, increasing efficiency of biosurfactant recovery process and structure analysis should be carried out.

It is hoped that future research will use this study as a basis for further improving our understanding of biosurfactant in general.

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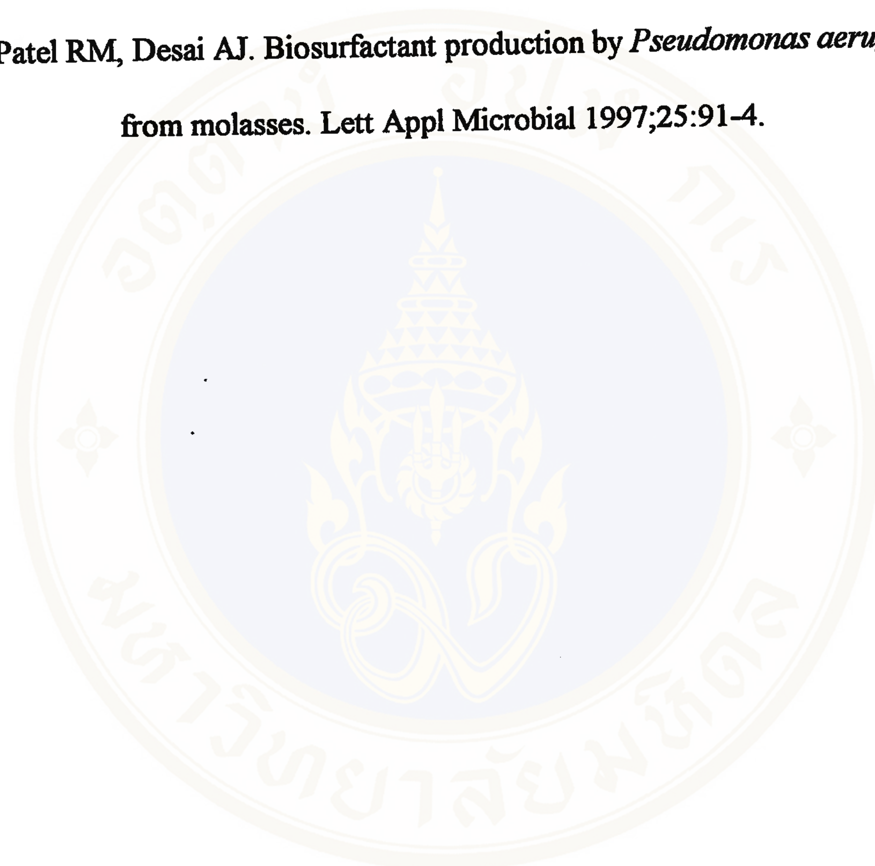

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# APPENDIX

## 1. Artificial Seawater medium (ASW medium)

| | |
|---|---|
| NaCl | 7.80 gm |
| KCl | 0.25 gm |
| $MgSO_4.H_2O$ | 2.33 gm |
| Distilled water | 1000 ml |

Dissolve NaCl 7.80 gm, KCl 0.25 gm and $MgSO_4.H_2O$ 2.33 gm in 1,000 ml of distilled water, mix thoroughly and adjust pH to 7.0.

## 2. Nitrogen-phosphate solution (NP)

| | |
|---|---|
| $K_2HPO_4$ | 70 gm |
| $KH_2PO_4$ | 30 gm |
| $NH_4NO_3$ | 100 gm |
| Distilled water | 1,000 ml |

Dissolve 70 gm $K_2HPO_4$, 30 gm $KH_2PO_4$ and 100 gm $NH_4NO_3$ in 1,000 ml distilled water, mix thoroughly.

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**3. Specific reagent for the Detection of Lipid and Carbohydrate on TLC plates.**

**Spray reagent 1 : Lipids (fatty acid test)**

Prepare 0.2 % 2′,7′-Dichloro-fluorescein in 95 % ethanol; spray the plate and observe in UV light. Results: Saturated and unsaturated polar lipids give green spots on purple background.

**Spray reagent 2 : Carbohydrate test.**

Dissolved 20 mg anthrone in 0.5 ml absolute ethanol and make up to 10 ml with 75 % $H_2SO_4$ ; spray the plate and then heat it in oven at $110^0$C for 10 min. Results, Carbohydrate and lipid with carbohydrate appear as green spots on a white background.

**Spray reagent 3 : Sugar test.**

Dissolved 1.5 gm of phenol in 2.5 ml concentrate $H_2SO_4$; Add 47.5 ml of ethanol; spray the plate and then heat it in oven at $100^0$C 10 min. Results, Sugar appear as dark spots on a white background.

**Spray reagent 4 : Amino acid test.**

Prepare a 0.2 % solution of ninhydrin in n-butanol; spray plates and then heat it in oven 100-$110^0$C. Results, Lipids with free amino groups show as red-violet spots.

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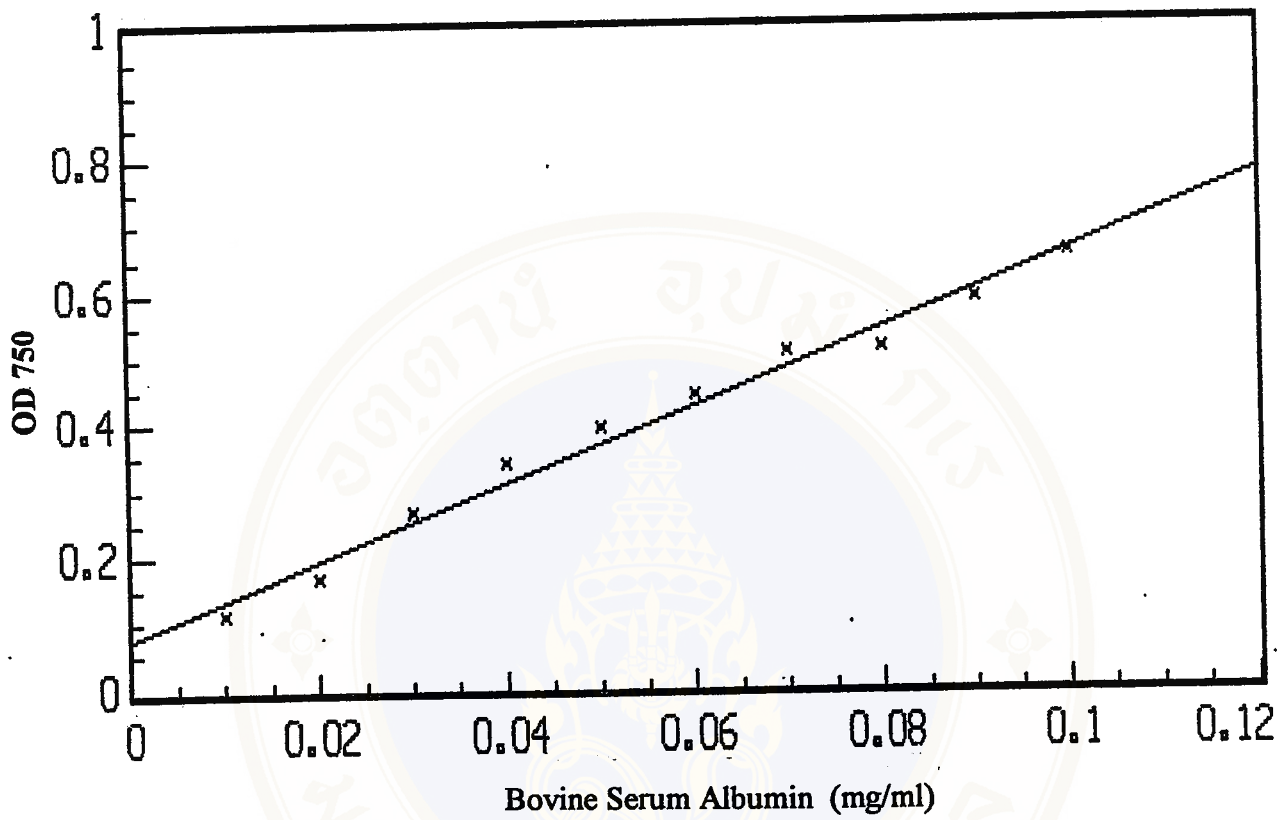
**4. Standard curve for the determination of protein by Lowry method.**

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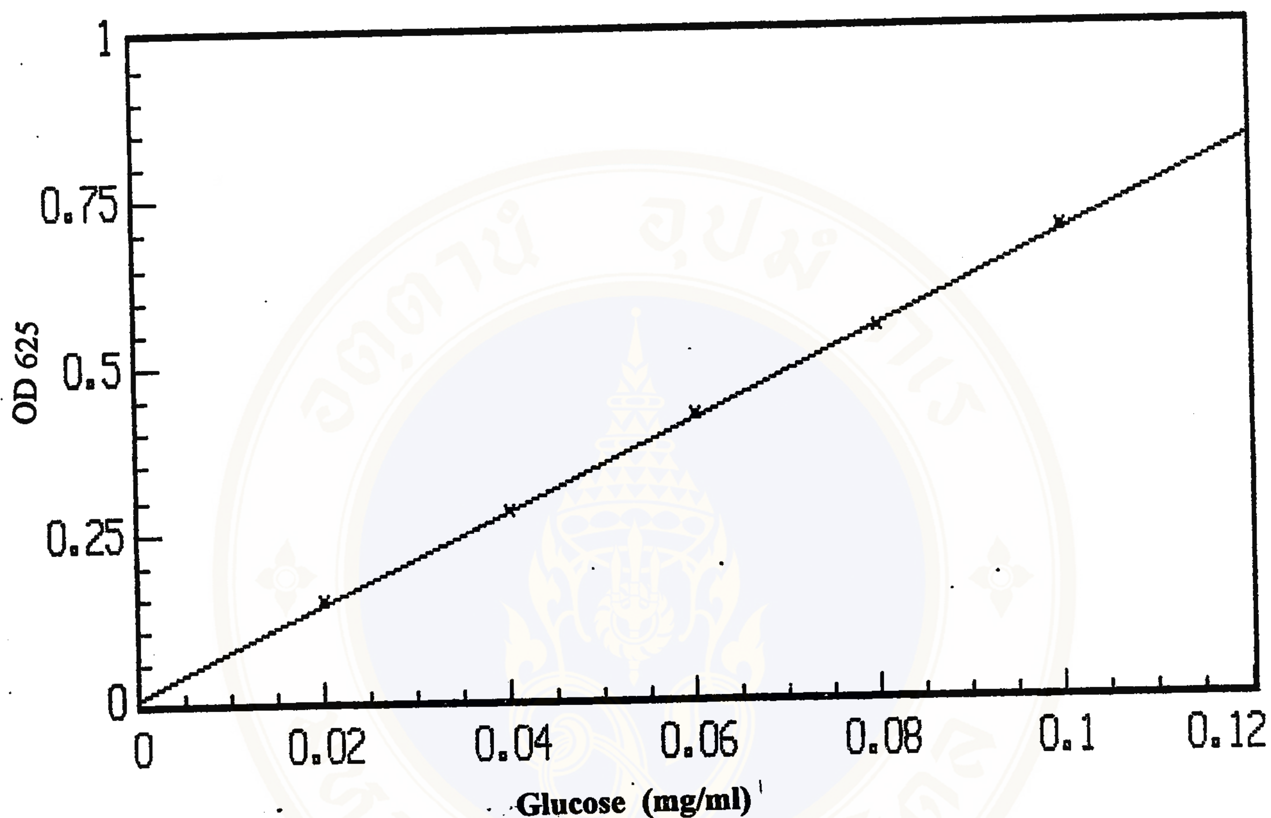
5. **Standard curve for the determination of total carbohydrate by Anthrone method**

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