PRODUCTION OF BIOSURFACTANT FROM LOCALLY ISOLATED BACTERIA

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MASTER OF ENGINEERING IN (BIO-PROCESS)
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We certify that the thesis entitled "Production of Biosurfactant from Locally Isolated Bacteria" is written by Rihab Hussein Jawad. We have examined the final copy of this thesis and in our opinion; it is fully adequate in terms of scope and quality for the award of the degree of *Master of Engineering in Bioprocess. We herewith recommend that it be accepted fulfilment of the requirements for the degree *Master of Engineering specializing in Bioprocess.

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I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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To my husband, my sons and my parents

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ABSTRACT

In this study, a total of 176 isolates was obtained from two coastal sampling locations. Out of this total, 124 (70.4%) isolates were obtained from the seawaters of the coast of Kertih, Terengganu while the remaining 52 isolates (29.5 %) were from Kuantan, Pahang. Five bacterial strains previously isolated were selected for the screening of biosurfactant producer(s) via three different characterization tests for biosurfactant; (i) surface tension measurements, (ii) emulsification activity, and (iii) cetyltrimethylammonium bromide assay (CTAB) test. One isolate coded KRT-142 identified as *Pseudomonas aeruginosa* was chosen to be the best candidate for biosurfactant production. Biosurfactant productions by isolated bacteria were found to be growth-associated in all the conditions tested. Microbiological properties of strain KRT-142 were investigated. It was found that strain KRT-142 produces water soluble, greenish yellow fluorescent pigments on a nutrient agar plate. It is an aerobic, gram negative, straight rods, motile bacteria, and not surrounded by sheaths. Ethanol as a carbon source was found to support the highest growth (as measured by whole cell protein) followed by glycerol and glucose. Slight growth was also observed with crude oil. Decreasing growth was observed with tetradecane, 1-propanol, 1-butanol, sucrose and maltose. Ethanol yielded maximum biosurfactant production, reducing the surface tension to 43.3 mN/m. It was followed by glycerol, hexadecane and crude oil with surface tension reduction to 44.5, 49 and 53.5 mN/m, respectively. The highest emulsifying activity was 56% at 7h and 52.7% at 14h for ethanol. In the study of organic nitrogen sources, soytone supported the highest growth followed by peptone, meat extract, yeast extract, tryptone and casamino acid, Soytone yielded the highest biosurfactant production, followed by meat extract and tryptone. At the optimum conditions (35^{o}C, 4% inoculum size, 100 rpm and pH 7.2), the surface tension reached a minimum of 30.76 mN/m, after 6h in the stationary growth phase. Stable and compact emulsification index (E24) was observed after 2h of cultivation, reaching a maximal value of 86% at 6h of incubation.

ABSTRAK

Dalam kajian ini sebanyak 176 isolat telah diperolehi dari 2 lokasi pesisir pantai. Daripada jumlah tersebut, 124 (70,4%) isolat diperolehi dari pesisir Kertih, Terengganu manakala 52 isolat (29,5%) lagi di ambil dari pantai berdekatan Kuantan, Pahang. Lima jenis bakteria yang telah dipencilkan dipilih untuk proses saringan bagi penghasilan biosurfaktan melalui tiga kaedah pencirian biosurfaktan iaitu (i) pengukuran ketegangan permukaan (ii) aktiviti pengemulsian, dan (iii) ujian assay cetiltrimetilammonium bromida (CTAB). Isolat kod KRT-142 yang dikenalpasti sebagai *Pseudomonas aeruginosa* dipilih sebagai calon terbaik bagi penghasilan biosurfaktan. Penghasilan biosurfaktan oleh isolat bakteria didapati berkait rapat dengan pertumbuhannya pada semua keadaan yang diuji. Kajian terhadap sifat mikrobiologi strain-142 KRT juga telah dijalankan. Strain KRT-142 didapati menghasilkan pigmen fluorescent kuning-hijau yang larut air di atas nutrien agar. Ia merupakan bakteria aerobik, gram negatif, berbatang lurus dan motil. Ia juga tidak dikelilingi oleh selubung. Etanol, sebagai sumber karbon, didapati menyokong pertumbuhan paling tinggi apabila protein sel keseluruhan diukur. Ini diikuti oleh gliserol dan glukosa. Sedikit pertumbuhan juga didapati apabila menggunakan minyak mentah. Penurunan pertumbuhan didapati dengan penggunaan tetradekana, 1-propanol, 1-butanol, sukrosa dan maltosa. Etanol menghasilkan pengeluaran biosurfaktan tertinggi, mengurangkan tegangan permukaan kepada 43.3 mn/m. Ini diikuti oleh gliserol, minyak mentah dan heksadekana, dengan pengurangan ketegangan permukaan 44.5, 49 dan 53.5 mn/m masing-masing. Untuk etanol, Aktiviti pengemulsian paling tinggi, iaitu sebanyak 56% didapati pada 7 jam, dan 52.7% pada 14 jam. Untuk kajian sumber nitrogen organik, soyton didapati menyokong pertumbuhan paling tinggi, diikuti oleh pepton, ekstrak daging, ekstrak ragi, trypton dan asid kasamino. Soyton menghasilkan pengeluaran biosurfaktan paling tinggi, diikuti oleh ekstrak daging dan trypton. Pada keadaan optima (35^{o}C, 4% saiz inokulasi, 100 rpm dan pH 7.2), ketegangan permukaan mencecah ke tahap minima, iaitu 30.76 mN/m, selepas 6 jam berada dalam fasa pertumbuhan malar. Indeks pengemulsian (E24) stabil dan padat dicapai setelah kultivasi selama 2 jam, dan mencapai nilai maksimum 86% bagi tempoh pengeraman 6 jam.

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LIST OF ABBREVIATIONS

$CaCI_2$	Calcium chloride
cm	Centimetre
CMC	Critical micelle concentration
d	Day
dH_2O	Distilled water
g	Gram
g/L	Gram per liter
HCI	Hydrochloric acid
h	Hour
L	Liter
M	Molar
mg	Milligram
mL	Millilitre
mM	Millimolar
mm	Millimeter
NaC1	Sodium chloride
Na_2HPO_4	Disodium hydrogen orthophosphate
NaOH	Sodium hydroxide
SDS	Sodium dodecyl sulphate
PAHs	Polycyclic-Aromatic-Hydrocarbons
R^2	Coefficient of determination
Rpm	Round per minute

TLC	Thin layer chromatography
TSB	Trypticase Soy Broth
µg	Microgram
µL	Microlitre
µm	Micrometer
v/v	Volume per volume
w/v	Weight per volume

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUNDS OF SURFACTANT AND BIOSURFACTANT

Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface (Mulligan, 2005). It contains a hydrophobic moiety with little affinity for the bulk medium and a hydrophilic portion that is attracted to the bulk medium. Surfactants have been used industrially as flocculating, wetting, foaming agents, adhesives and deemulsifiers, lubricants and penetrants (Mulligan and Gibbs, 1993). The ability to reduce surface tension is a major characteristic of surfactant. Because of their amphiphilic nature, surfactants tend to accumulate at interfaces (air-water and oil-water) and surfaces. As a result, surfactants reduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily (Bodour and Miller-Maier, 2002). Due to the presence of surfactant, less work is required to bring a molecule to the surface, and the surface tension is reduced. It is obvious that their surface and membrane-active properties play an important role in the expression of their activities. Commercially, Surfactants are key ingredients used in detergents, shampoos, toothpaste, oil additives and a number of other consumers and industrial products..

Biosurfactant is a structurally diverse group of a surface-active molecule synthesized by microorganisms. Their capability to reduce surface and interfacial tension with low toxicity and high specificity and biodegradability, lead to an increasing interest on these microbial products as alternatives to chemical surfactants (Banat et al., 2000). Hester (2001) estimated that biosurfactants could capture 10% of the surfactant market by the year 2010 with sales of $US200 million.

However, up to now, biosurfactants is still unable to compete with the chemically synthesized surfactants in the surfactant market. This could be due to their high production costs in relation to inefficient bioprocessing method available, poor strain productivity and the need to use expensive substrates (Cameotra and Makkar, 1998; Deleu and Paquot, 2004).

The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential applications in such areas as the environmental protection. The uniqueness with unusual structural diversity, the possibility of cost-effective ex-situ production and their biodegrability are some of the properties that make biosurfactant a promising choice for use in environmental application (Hua et al., 2003). Initial focus of industrial interest towards biosurfactants concentrates on the microbial production of surfactants, cosurfactants and so on for the application on microbial-enhanced oil recovery (MEOR)(Thomas, 2008). The applications of biosurfactants however, are still at the developmental stage of industrial level. The development of biosurfactant application in industries is focused mainly on high biosurfactant production yield and the production of highly active biosurfactants with specific properties for specific applications.

Majority of surfactants produced today are of petrochemical origin beside from renewable resources like fats and oils (Deleu and Paquot, 2004). Amongst the renewable raw materials, oleochemical products represent half of the total surfactant production. The petrochemical industry is one of the important sectors in Malaysia, with investments totalling RM34.8 billion as at the end of 2008 (MIDA, 2009). Unfortunately, industrial wastewater from petroleum-related industries has been identified as one of the major sources of pollution in Malaysia. The biodegradation of a petroleum pollutant and its related compound is limited by poor availability to the microorganisms, due to their hydrophobicity and low aqueous solubility. This suggests that by applying biosurfactants to influence the bioavailability of the contaminant can possibly enhance the solubility of these compounds. Due to their biodegradability and low toxicity, they are in demand to be use in remediation technologies (Mulligan, 2004).

Biosurfactants plays an important application in petroleum-related industries which such as enhanced oil recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludges (Daziel et al., 1996, Bertrand et al., 1994). These industries are known to be the potential target for the application of these compounds. This is due to the ability of biosurfactant-producing microorganisms to use petroleum or its' products as substrates as well as the properties of the biosurfactant which require less rigorous testing than chemical surfactant, there are numbers of reports on the synthesis of various types of biosurfactants by microorganisms using water-soluble compounds such as glucose, sucrose, ethanol or glycerol as substrates (Desai and Banat, 1997).

Sea water was found to be a great potential in producing a microorganism that may produce biosurfactants (Maneerat and Phetrong, 2007). Hence, there could probably be a potential chance of producing biosurfactants using locally isolated bacteria originated from sea water available in this country. It has been focused here that improving the method of biosurfactant production and characterizing the major properties of the biosurfactant are highly important in the commercial application of biosurfactant.

1.2 PROBLEM STATEMENT

Many factors affecting on the production of the surface-active molecules of biological origin, such as the type and amount of the microbial surfactants produced, which depend primary on the producer organism, factors like carbon and nitrogen, trace elements, temperature, and aeration also affected their production by the organism.

Many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically; however, much effort in process optimization and at the engineering and biological levels have been carried out. In addition, legal aspects such as stricter regulations concerning environmental pollution by industrial activities and health regulations will also strongly influence the chances of biodegradable biosurfactants replacing their chemical counterparts. Aiming at the final

biosurfactant cost reduction, the development of economical alternatives for its production has been investigated. Thus, the use of low-cost raw matter appears as a natural choice to generate an overall economy.

Pollution of the sea, especially by crude oil, which is caused by stranding of tankers, is one of the serious environmental problems over the world. The operations of the ships also produce wastes, this waste must be managed properly to avoid environmental pollution. Biodegradation by marine microorganisms is overburdened due to the additional hydrocarbons, especially large oil spills. Therefore, the use of biosurfactants can be playing an important role by emulsifying the polluted oils prior to biodegradation. Due to the long coasts of South China Sea, Strait of Malacca and Strait of Johor, the importance biodiversity in the sea has been recognized. However, no information regarding the biosurfactant-producing marine bacteria has been reported in Malaysia.

1.3 OBJECTIVES

The objective of this study is to:

1- Screening for biosurfactant producing bacteria
2- Characterizing the selected bacteria producing biosurfactant.
3- Production of biosurfactant by bacteria isolated for a potential biosurfactant production.
4- Optimization of biosurfactant productions where only one parameter is varied at any one time with the others being kept constant and interactions the parameters to set optimal conditions.

1.4 SCOPES

The principal scope of the experimental work was therefore, to develop optimize, and purify the biosurfactant production by local marine bacteria. Such a programme of product and process developments entailed several stages, which are:

1- Screening test and characterization of the potential biosurfactant-producing microbes from sea water samples using various screening methods.
2- Examining the effect of nutritional and physical parameters on the biosurfactant production by isolated bacteria.
3- Chosing the best substrate for comorcial production.
5- Obtaining a set of optimal conditions for the production.
6- Conducting optimized production.
7- Recovery of biosurfactant production.
8- Analysis of biosurfactant production by thin layer chromatography (TLC).

1.5 RESEARCH CONTRIBUTIONS

This study investigated the potential production of biosurfactants using locally isolated bacteria originating from sea water. It has been focused here that improving the optimizatin of biosurfactant production and the major factor's effect on production, which were highly important in the commercial production of biosurfactant.

1.6 THESIS ORGANIZATIONS

This thesis consists of five main chapters, including an introduction in Chapter 1. The literature related to classification, chemical nature of biosurfactant, factors affecting biosurfactant production, recovery and applications of biosurfactants are discussed in Chapter 2 while, the methodology, apparatus and equipment for experimental work are discussed in Chapter 3. In addition, the experimental results are discussed in Chapter 4, and the conclusion and recommendations are summarized in last chapter, which is Chapter 5. This thesis is completed with references and appendices.

CHAPTER 2

LITERATURE REVIEW

A review of previous studies relevant to biosurfactants production was conducted. The classifaction and chemical nuture of surfactant are decumenated with different type of microorganisms related to production of biosurfactants. The biosurfactant production study was an introduction of this chapter followed by classification and chemical nature of biosurfactants and the factors such as nutritional and physical affecting on production. Finally, the potential applications of microbial surfactant discussed in detail in this chapter.

2.1 INTRODUCTION

All living cells produce amphipathic molecules. These molecules which consist of both hydrophilic and hydrophobic moieties are called surface-active compounds or surfactants. In many cases, they exhibit surface-active characteristics such as dramatic lowering of surface tension at the air/water interface, lowering interfacial tension at the oil/water interface, and micelle or pseudomicelle formation (Haddad et al., 2008). Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which make surface-active compound, some of the most versatile process chemicals (Greek, 1991).

Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon (CxHy) microorganism facilitate their diffusion into the cell by producing a variety of substances, the biosurfactants. Some bacteria excrete ionic surfactant, which emulsify hydrocarbon substrates in the growth medium. The exact reason why some microorganisms produce surfactant is unclear (Deziel et al., 1996). Biosurfactants produced by various microorganisms together with their properties are listed in Table 2.1.

Table 2.1: Structural Types of Microbial Surfactants

Biosurfactant	Source
Glycolipids	
Trehalolipids	*Rhodococcus erythropolis, Nocardia erythropolis*
Trehalose Dimycolates	*Mycobacterium sp., Nocardia sp.*
Trehalose dicorynemycoaltes	*Arthrobacter sp., Corynebacterium sp.*
Rhamnolipids	*Pseudomonas aeruginosa Pseudomonas sp.*
Sophorolipids	*Torulopsis bombicola, Torulopsis Apicola, Torulopsis petrophilum Torulopsis sp.*
Cellobiolipids	*Ustilago zeae, Ustilago maydis*
Aminoacid-lipids	*Bacillus sp.*
Lipopeptides and lipoprotein	*Streptomyces sp., Corynebacterium sp., Mycobacterium sp.*
Peptide-lipid	*Bacillus licheniformis*
Serrawettin	*Serratia marcescens*
Viscosin	*Pseudomonas fluorescens*
Surfactin	*Bacillus subtilis*
Subtilisin	*Bacillus subtilis*
Gramicidins	*Bacillus brevis*
Polymyxins	*Bacillus polymyxa*
Ornithine-lipid	*Pseudomonas sp., Thiobacillus sp. Agrobacterium sp., Gluconobacter sp.*
Phospholipids	*Candida sp., Corynebacterium sp. Micrococcus sp., Thiobacillus sp.*
Fatty acids /Natural lipids	*Acinetobacter sp.,Pseudomonas sp., Micrococcus sp., Mycococcus sp., Candida sp., Penicillium sp., Aspergillus sp.*
Polymeric surfactants	
Emulsan	*Arethrobacter calcoaceticus*
Biodispersan	*Arethrobacter calcoaceticus*
Mannan-lipid-protein	*Candida tropicalis*
Liposan	*Candida lipolytica*
Carbohydrate-protein-lipid	*Pseudomonas fluorescens Debaryomyces polymorphis*
Protein PA	*Pseudomonas aeruginosa*
Particulate biosurfactants	
Vesicles and fimbriae Whole cells	*Arthrobacter calcoaceticus*

(Muthusamy et al., 2008)

2.2 CLASSIFICATION AND CHEMICAL NATURE OF BIOSURFACTANTS

Biosurfactants are categorised mainly by their chemical composition and their microbial origin. The microbial surfactants are complex molecules covering a wide range of chemical types, including glycolipids, peptides, fatty acid, phospholipids, antibiotics and lipopiptides. Microorganisms also produce surfactants that are in some cases' combination of many chemical types referred to as the polymeric microbial surfactants. . A broad classification of biosurfactants is given in Table 2.2.

2.2.1 Glycolipids

The low molecular weight biosurfactants are generally glycolipids or lipopeptides (Table 2.1). The best studied glycolipid bioemulsifiers, rhamnolipids, trehalolipids and sophorolipids, are disaccharides that are acylated with longchain fatty acids or hydroxy fatty acids (Rosenberg, 2006). The constituent monosaccharides, disaccharides, trisaccharides and tetrasaccharides include glucose, mannose, galactose, glucuronic acid, rhamnose, and galactose sulphate. The fatty acid component usually has a composition similar to that of the phospholipids of the same microorganism. The glycolipids can be categorized as:

i. Trehalose lipids

The serpentine growth seen in many members of the genus *Mycobacterium* is due to the presence of trehalose esters on the cell surface. A succinoyl trehalose lipid produced by *Rhodococcus* sp. behaves as a biological surfactant and also displays various interesting biological activities (Zaragoza et al., 2010**).** Yields of trehalose lipids were increased to 4 g/liter when the bacteria were grown on 10% (w/v) n-alkanes and the trehalose lipids were continuously extracted. The yield of rhamnolipids was increased to 24.3 g/liter in media containing 6% canola oil (Sim et al., 1997). Trehalose mycolates reduced the surface tension of water from 72 to 26 mN/m (Lang and Philip, 1998).

Table 2.2: Classification of Biosurfactant

Biosurfactant	Type
1-Glycolipids	Trehalose lipids Sophorolipids Rhamnolipids
2-Fatty acids	
3-Phospholipids	
4- Lipopeptides antibiotics	Gramicidin Polymixins Surfactine
5-Polymeric microbial surfactants	Emulsan from *Acinebacter calcoacceticus* RAG-1 (ATCC 31012). The polysaccharide protein complex of *Acinebacter Calcoaceticus* BD4. Other *Acinetobacter* sp. Emulsifiers Emulsifing protein from *Pseudomonas aeruginosa.* Emulsifying and solubilizing factors from *Pseudomonas* sp. PG-1. Bioflocculant and emulcyan from the filamentous *Cyanobacterium phormidium* J-1.
6-Particulate surfactant	Extracellular vesicles from *Acinetobacter* sp. HO1-N. Microbial cell with high cell surface hydrophobicities.

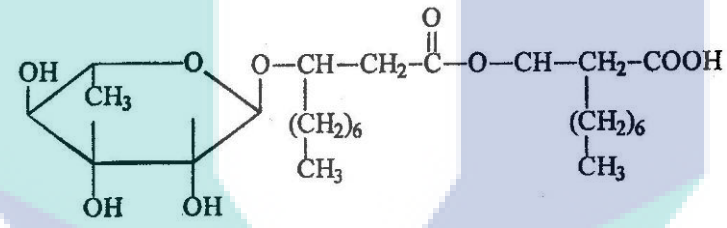
(Muthusamy et al., 2008)

ii. Sophorolipids

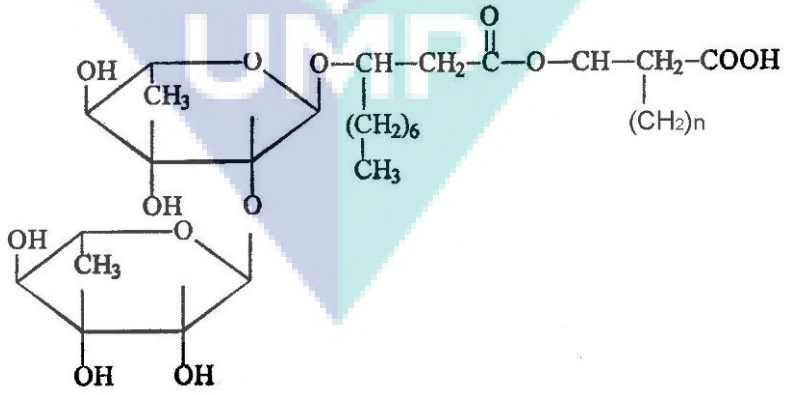
These are produced by different strains of the yeast, the sugar unit is the disaccharide sophorose which consists of two β-1, 2-linked glucose units, the 6 and 6' hydroxy groups are generally acetylated. *Candida apicola* and *Candida bombicola* produced extracellular sophorolipids biosurfactant which was a mixture of acidic and lactonic forms (Thaniyavarn et al., 2008). The sophorolipids reduce surface tensions between individual molecules at the surface, although they are effective emulsifying agents (Hirata et al., 2009). The sophorolipids of *Torulopsis* have been reported to stimulate, inhibit and have no effect on growth of yeast on water-insoluble substrates. The yields have improved to over 150 g/liter (Davila et al., 1997).

iii. Rhamnolipids

Some *Pseudomonas* sp. produces large quantities of a glycolipids consisting of one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid (Cameotra and Singh, 2009). While the OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, the OH group of the second acid is involved in ester formation (Figure 2.1). Since one of the carboxylic acids is free, the rhamnolipids are anion above pH 4.0 Rhamnolipids are reported to lower surface tension, emulsify hydrocarbon, and stimulate growth of *Pseudomonas* on *n*-hexadecane (Desai and Banat, 1997). The pure rhamnolipid lowered the interfacial tension against *n*-hexadecane in water and had a critical micellar concentration depending on the pH and salt conditions. More than 100 g/liter rhamnolipids were produced from 160 g/liter soybean oil (Lang and Wullbrandt, 1999).



Monorhamnolipid ($RhaC_{10}C_{10}$)



Dirhamnolipid ($RhaRhaC_{10}C_n$)

n= 8, 10, 12 (Muthusamy et al., 2008)

Figure 2.1: Structures of Rhamnolipids

2.2.2 Fatty acids

Surfactin's structure consists of a peptide loop of seven aminoacids (L-asparagine, glycine, two L-leucine, L-valine and two D-Leucines) and anhydrophobic fatty acid chain thirteen to fifteen carbons long (Priya and Usharani, 2009). Besides the straight-chain acids, microorganisms produce complex fatty acids containing OH groups and alkyl branches, some of these complex acids, for example corynomucolic acids are surfactants (Rodrigues et al., 2006).

2.2.3 Phospholipids

Phospholipids are major components of microbial membranes. When certain hydrocarbon degrading bacteria or yeast are grown on alkane substrates, the levels of phospholipids increase greatly (Rosenberg, 2006). Phospholipids from hexadecane grown *Acinetobacter* sp. have potent surfactant properties. Phospholipids produced by *Thiobacillus thiooxidans* are responsible for wetting elemental sulphur, which is necessary for growth (Moussa et al., 2006).

2.2.4 Lipopeptides antibiotics

Several lipopeptide antibiotics show potent surface active properties. Leclere, et al., 2006 demonstrated that in the addition to surfactin, the lipopeptide mycosubtiln was involved in the spreading mechanism and the role in this process consist of the decrease of surface tension of the liquid medium and the increase of the wettability of the sold medium.

Bacillus subtilis produces a cyclic lipopeptide called surfactin or subtilysin (Deleu, 2008), which is reputedly the most active biosurfactant. The amphipathic nature of surfactin may contribute to some of its interesting biological properties, such as the formation of ion-conducting pores in membranes (Grau et al., 1999). *Streptomycestendae* produces an extracellular hydrophobic peptide referred to as

streptofactin (Thampayak et al., 2008). Streptofactin is a mixture of structurally related peptides ranging in molecular mass from 1,003 to 1,127 Da. Streptofactin reduced the surface tension of water from 72 mN/m to 39.4 mN/m and had a critical micelle concentration of 36 mg/liter. Interestingly, streptofactin restored the ability of mutants defective in aerial mycelium formation to develop normally.

2.2.5 Polymeric Microbial Surfactants: Surfactants from *Pseudomonas* PG-1

Pseudomonas PG-1 is an extremely efficient hydrocarbon-solubilizing bacterium. It utilizes a wide range of hydrocarbon, including gaseous volatile and liquid alkanes, alkenes, pristane, and alkyl benzenes (Mazzola et al., 2007).

2.2.6 Particulate surfactants

i. Extracellular Vesicles from *Acinetobacter* sp. HO1-N

Acinetobacter sp. when grown on hexadecane accumulated extracellular vesicles of 20 to 50 mm diameter with a buoyant density of 1.158 g/cm$^{3.}$ These vesicles appear to play a role in the uptake of alkanes by *Acinetobacter* sp. HO1-N (Muthusamy et al., 2008).

ii. Microbial Cells with High Cell Surface Hydrophobicities

Most hydrocarbon-degrading microorganisms, many nonhydrocarbon degraders, some species of *Cyanobacteria* (Mohamed et al., 2006), and some pathogens have a strong affinity for hydrocarbon-water and air-water interfaces (Prpich et al., 2009). The best studied are the bioemulsans produced by different species of *Acinetobacter* (Rosenberg and Ron, 1998). Several reported biosurfactants are effective at a high temperature, including the protein complex from *Methanobacterium thermoautotrophium* (De Acevedo et al., 1996) and the proteinpolysaccharide- lipid

complex of *Bacillus stearothermophilus* ATCC 12980 (Gunjar et al., 1995). Rocha et al., 2006 reported that biosurfactant produced by *Acinetobacter calcoaceticus* achieved amaximum emulsion index of 80% with kerosene.

2.3 FACTORS AFFECTING BIOSURFACTANT PRODUCTION

Biosurfactants are amphiphilic compounds containing a hydrophobic and hydrophilic moiety. The polar moiety can be a carbohydrate, an amino acid, a phosphate group, or some other compounds. The non polar moiety is mostly a long –carbon-chain fatty acid. Although the various biosurfactants possess different structures, there are some general phenomena concerning their biosynthesis. For example, hydrocarbons or other water-insoluble substrates can induce biosurfactants production (Radwan and Sorkhoh, 1993).

2.3.1 Carbon Source

Water-soluble carbon sources such as glycerol, glucose, mannitol, and ethanol were all used for rhamnolipid production by *Pseudomonas sp.* Biosurfactant product. However, was inferior to that obtained with water-immiscible compounds such as *n*-alkanes and olive oil (Raza et al, 2006). Rodrigues et al., (2006) showed that using glucose or lactose as a carbon source gave 0.693 g/L and 1.054 g/L of biosurfactant production respectively by *Lactococcuse lactis* 53. Parthasarathi and Sivakumaar (2009) demonstrated that although different carbon sources in the medium affected the composition of biosurfactant production in *Pseudomonas sp.*, substrates with different chain lengths exhibited no effect on the chain length of fatty acid moieties in glycolipids.

Jagtap (2010) showed evidence for qualitative variation, reflecting the carbon number of alkane for biosurfactant production in *Acinetobacter* sp. Mulligan (2005), reported that the presence of large amounts of biosurfactant bound to *Corynebacterium*

lepus cells when grown on glucose, and addition of hexadecane facilitated the release of surfactant from cells.

Banat (1995a) observed little biosurfactant production when cells were growing on a readily available carbon source, only when all the soluble carbon was consumed and when water-immiscible hydrocarbon was available was biosurfactant production triggered, Davila et al., (1992) demonstrated a high yield of sophorose lipids by overcoming product inhibition in *Candida bombicola* CBS6009 through the addition of ethyl esters of rape seed oil fatty acids in D-glucose medium. Lee and Kim (1993) reported that in batch culture, 37% of the carbon input was channelled to produce sophorolipid by *Torulopsis bombicola.* However, in fed batch cultures, about 60% of the carbon inputs were incorporated into biosurfactant, increasing the yield. The availabling of carbon source, particularly the carbohydrate used, has a great bearing on the type of biosurfactant produced (Muthusamy et al., 2008).

2.3.2 Nitrogen Source

Medium constituents other than a carbon source also affect the production of biosurfactants. Among the inorganic salts tested, ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus*, whereas nitrate supported maximum surfactant production by *Pseudomonas aeruginosa* (Pansiripat et al., 2010) and *Rhodococcus sp.* (Abu-Rawaida et al., 1991a). Raquel et al., (2008) showed that using 0.2% yeast extract as nitrogen sources gave maximum emulsifier activity within 40h of cultivation by *Candida lipolytica*.

Biosurfactant production by *Arthrobacter paraffineus* is increased by the addition of amino acid such as aspartic acid, glutamic acid, asparagine and glycine to the medium. Rashedi et al., (2005), and Abu-Ruwaida et al., (1991a) observed nitrate to be the best source of nitrogen for biosurfactant production by *Pseudomonas aeruginosa* and *Rhodococcus* strain ST-5 growing on olive oil and paraffin, respectivly. Similarly, nitrogen limitation caused increased biosurfactant production in *Candida tropicalis* IIP-4 (Singh et al., 1990), and *Nocardia* strain SFC-D (Kosaric et al., 1990).

Rismani et al., (2006), showed that nitrogen limitation not only caused overproduction of biosurfactant but also changed the composition of the biosurfactant produced. Franzetti et al., (2009), showed maximum rhamnolipid production after nitrogen limitation at a C:N ratio of 16:1 to 18:1 and no surfactant production below a C:N ratio of 11:1, where the culture was not nitrogen limited. According to Lotfabad et al., (2009), it was the absolute quantity of nitrogen and not its relative concentration that appeared to be important for optimum biomass yield, while a concentration of hydrophobic carbon source determines the conversion of carbon available to the biosurfactant.

2.3.3 Environmental Factors

Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability affect biosurfactant production through their effects on cellular growth or activity. Zulfigar et al., (2007) showed that with pH 7 the biosurfactant production by *Pseudomona aeruginosa mutant was* 8.5 g/L. The pH of the medium plays an important role in sophorolipid production by *Torulopsis bombicola* (Hirata et al., 2009). Rhamnolipid production in *Pseudomonas sp*. was at its maximum at a pH rang from 6 to 6.5 and decrease sharply above pH 7(Nitschke et al., 2005). In addition, surface tension and critical micelle concentrations of a biosurfactant product remained stable over a wide range of pH values, whereas emulsification had a narrower pH range (Abu-Rawaida et al., 1991b).

Pal et al., (2009) showed that emulsification actinity by *Rhodococcuse erthropolis* MTCC 2794 was 65.20% at 30^{o}C. In *Arthrobacter paraffineus* and *Pseudomonas* sp. strain DSM-2874 (Mukherjee et al., 2006), temperature caused alteration in the composition of the biosurfactant produced. A thermophilic *Bacillus sp*. grew and produced biosurfactant at a temperature above 40^{o}C. Heat treatment of some Biosurfactant caused no appreciable change in biosurfactant properties such as the lowering of surface tension and interfacial tension and the emulsification efficiency, all

of which remained stable after autoclaving at 120 ^{o}C for 15 minutes (Abu Rawaida et al., 1991b).

Namir et al., (2008) showed that highest reduction of surface tension was achieved with *Brevibacilis brevis* HOB1 at 150 rpm. Wang and Wang (1990) revealed that the cell-bound polymer/dry-cell ratio decrease as the shear stress increase. In *Pseudomonas aeruginosa* FR strain the rotation velocities from 100-150 rpm provided free-cell fermented media with the lowest surface tension 33 mN/m (Oliveira et al., 2006). On the other hand, in yeast, biosurfactant production increases when the agitation and aeration rates increased. Sheppard and Cooper (1990) had concluded that oxygen transfer was one of the Key parameters for the process optimization and scale-up of surfactin production in *Bacillus subtilis.* Salt concentration also affected biosurfactant production depending on its effect on cellular activity. Some biosurfactant products, however, were not affected by salt concentrations up to 10% (w/v), although a slight reduction in the critical micelle concentrations was detected (Abu Rawaida et al., 1991b).

2.4 BIOSURFACTANT RECOVERY

Downstream processing in many biotechnology processes is responsible for up to 60 % of the total production cost. Due to economic consideration, most biosurfactant would have to involve either whole- cell spent culture broths or other crude preparations; In addition, biosurfactant activity may be affected by other materials present in these preparations. Soumen et al., (2009) reported a simple turbidometric method for quantification of crude biosurfactants based on their property to become insoluble at low pH value. The most widely used techniques are extraction with chloroform-menthanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, and ether, in the recovery of Trehalose lipids of *Mycobacterium sp*. (Guidry et al., 2007), trehalose corynomycolates and tetraesters of *Rhodococcus erythropolis* (Tuleva et al., 2008), mono-, die-, and pentasaccharide lipids of *Arthrobacter paraffineus* (Husain, 2008) and *Nocardia sp.* L-417 (Adebusoye et al.,

2008), cellobiolipids of *Ustilago sp.*, sophorolipids of several yeast species (Satpute et al., 2010).

Liposan from *Candida lipolytica* and rhamnolipids of *Pseudomonas spp*. are some of the well-known example of biosurfactant recovery by solvent extraction (Cameotra and Singh, 2009). Glycolipids produced by *Candida bombicola* (Felse et al., 2007), *Torulopsis petrophilum* and *Torulopsis apicola* (Muthusamy et al., 2008) are extracted by chilled ethyl acetate after adsorption on charcoal. Biosurfactant from *Pseudomonas aeruginosa* had also been recovered in a similar way, except that extraction was carried out in acetone (Deka and Das, 2009). Both the glycolipids produced by *Ustilago zeae* and the mannosylerythritol lipid produced by *Candida spp.* are sedimented as heavy oils upon centrifugation and then extracted in either ethanol or methanol. Glycolipids from *Pseudomonas aeruginosa* and *Ustilago zeae* have also been recovered by acid precipitation at a low temperature. Other glycolipids from a mixed microbial population and rhamnolipids from both *Pseudomonas aeruginosa* and *Pseudomonas aeruginosa* RB 28 have been recovered by acidification followed by extraction in chloroform-methanol mixture (Sifour et al., 2007). Leppchen et al., (2006) extracted a cell-bound bioemulsifer from *Saccharomyces cerevisiae* at 121^{o}C in a buffer containing potassium metabisulfite followed by precipitation in an ethanol-acetic acid mixture.

Ammonium sulphate precipitation has also been successfully used in isolation emulsion and biodispersan from *Acinetobacter calcoaceticus* A2 as well as a bioemulsifier from an unidentified gram-negative bacterium (Satpute et al., 2010). Surfactin and surfactin-like biosurfactants produced by *Bacillus subtilis* and *Bacillus licheniformis* PTCC 1595 (Noudeh et al., 2010), respectively, have been recovered by acid precipitation, whereas other biosurfactant from *Pseudomonas sp.* (Goswami and Singh, 1991), *Candida tropicalis* and *Debaryomyces polymorphus* were best recovered by acetone precipitation(Yang et al., 2008).

In other developments, continuous removal of biosurfactant during fermentation by different techniques has increased the cell density in the reactor and eliminated product inhibition, resulting in a several fold net increase in biosurfactant yield (Neu

and Poralla, 1990). One of the successful techniques involved a continuous in situ removal of surfactin from fermentation broth by foam fractionation. In this technique, foam was collected and acidified to pH 2 with concentrated HCl, and the precipitated surfactin was extracted in dichloromethane (Chen et al., 2006). Neu and Poralla (1990) in contrast, recovered a *Bacillus* biosurfactant by blowing the foam out of the fermentor to be collected, centrifuged, and extracted by acetone precipitation.

The ability of biosurfactants to aggregation has also been used to retain on high-molecular-weight-cut off membranes. For example, Amicon XM-50 membrane (a-5000 molecular-weight-cut off membrane) has been successfully used for 98% recovery of 97% pure surfactin and rhamnolipid, while YM-10 membrane (a-10000-molecular-weight-cutoff membrane) gave 92% rhamnolipid recovery (Bertrand et al., 1994). Thus, this technique can be successfully used by selecting the membrane with molecular weight cut off for most surfactants present in the fermentation broth at a level higher than the critical micelle concentration.

2.5 POTENTIAL COMMERCIAL APPLICATIONS

The structural analysis of biosurfactants has also opened possibilities for their chemical synthesis. Most is their environmental acceptability, because they are readily biodegradable and have lower toxicity than synthetic surfactants. A number of industrial applications of biosurfactants were envisaged (Fiechter, 1992). One of the potential uses is in the oil industry with minimum purity specification so that whole - cell broth could be used (Banat et al., 2000). Compared with chemical surfactants, they are very selective, required in small quantities, effective under broad ranges and reservoir conditions (Sepahy et al., 2005).

2.5.1 Applications of Biosurfactants in Pollution Control

The potential applications of microbial surfactant are:

i. Microbial Enhanced Oil Recovery

Increased interest in oil biotechnology has stimulated attempts to isolate novel microorganisms to exploit in various fields such as enhance oil recovery (Perfumo et al., 2006). Thomas (2008) reported that nearly 2.0 × 1012 barrels (0.3 × 1012 m^3) of conventional oil and 5.0 × 1012 barrels (0.8 × 1012 m^3) of heavy oil will remain in reservoirs worldwide after conventional recovery methods have been exhausted. *Bacillus licheniformis* JF-2, an isolated from oilfield injection water which, in addition to producing the most effective biosurfactants has other properties such as being anaerobic, halotolerant, and thermotolerant, makes biosurfactants that are potentially useful for in situ microbially enhanced oil recovery (Desai and Banat, 1997).

ii. Biosurfactants in Oil Storage Tank Clean-up

Biosurfactant production by *Pseudomonas putida* not only resulted increased emulsification of the oil but also change the adhesion of the hydrocarbon to the cell surface of other bacteria (Kumar et al., 2006). Banat et al., (1991) have demonstrated the use of a biosurfactant for dislodging of a crude oil storage tank for Kuwait Oil Company and achieved 90% recovery of the trapped in the sludge by using biosurfactant-producing strain. Field tests, utilising biosurfactants produced from a bacterial strain (Pet 1006), were performed to test their ability to clean oil storage tanks and to recover hydrocarbons from the emulsified sludge (Banat et al., 1991). Such a clean-up process is highly desirable as it is economically rewarding, environmentally sound and is less hazardous for the persons involved than the conventional process (Lillienberg et al., 1992).

iii. Hydrocarbon Degradation

Microbial remediation of hydrocarbon and crude oil-contaminated soils is an emerging technology involving an application of biosurfactants (Banat, 1995a, 1995b). Chrzanowski et al., (2006) reported that the yeast strain *Candida maltose* EH15was used as a biological agent in the hydrocarbon and emulsified hydrocarbon biodegradation. Rhamnolipid from *Pseudomonas aeruginosa* has removed substantial quantities of oil from contaminated Alaskan gravel from the Exxon Valdez oil spill. In a large-scale experiment, the effectiveness of in situ bioremediation on the Exxon Valdez oil spill has been demonstrated by Bragg et al., (1994). In another experimente, Van Dyke et al., (1993) demonstrated a 25 to 70% and 40 to 80% increase in the recovery of hydrocarbons from contaminated sandy-loam and silt-loam soil, respectively, by rhamnolipid from *Pseudomonas aeruginosa*. Similarly, 56% of the aliphatic and 73% of the aromatic hydrocarbons was recovered from hydrocarbon-contaminated sandy-loam soil by treatment with *Pseudomonas aeruginosa* biosurfactant (Scheibenbogen et al., 1994). A stimulatory effect of different rhamnolipids on the degradation of hexadecan and octadecane by seven *Pseudomonas* strains has been demonstrated (Zhang and Miller, 1994, Zhang and Miller, 1995).

iv. Hydrocarbon Degradation in the Soil Environment

Degradation is dependent on the presence of soil hydrocarbon-degrading microorganisms, hydrocarbon composition, oxygen availability, water, temperature, pH and nutrients. Jayashree et al., (2006) reported that the potential of biosurfactant produced by *Bacillus sutilis* for inhancing the release of endosulfan from contaminated agricultural soils. Addition of synthetic surfactants or microbial surfactant resulted in increased mobility and solubility of hydrocarbon, which is essential for effective microbial degradation (Lal et al., 2010). Hamamura et al., (2008) used mixed soil population to assess hydrocarbon degradation in model oil. Addition of biosurfactants, such as some sophorolipids, increased both extent of degradation and final biomass yield (Oberbremer et al., 1990). Berg et al., (1990) by using the surfactant from *Pseudomonas aeruginosa* UG2, reported an increase in the solubility of

hexachlorobiphenyl added to soil slurries, which resulted in a 31% recovery of the compound in the aqueous phase.

v. Hydrocarbon Degradation in Aquatic Environment

When oil is spilled in an aquatic environment, the lighter hydrocarbon components volatilise while the polar hydrocarbon components dissolve in water. However, because of low solubility (less than 1ppm) of oil, most of the oil components will remain on the water surface. The primary means of hydrocarbon removal is photooxidation, evaporation, and microbial dagradation. Since hydrocarbons degrading organisms are present in the seawater, biodegradation may be one of the most efficient methods of removing pollutants (Barathi and Vasudevan, 2001). Shete et al., (2006) reported that an emulsifier produced by *Pseudomonas aeruginosa* SB30 was able to quickly disperse oil into fine droplets; therefore, it may be useful in removing oil from contaminated beaches.

vi. Biosurfactant and Hexa-chlorocyclohexane

Hexa-chlorocyclohexane is still the highest-ranking pesticide used in many countries. The use of technical hexa-chlorocyclohexane, which is a mixture of isomers, will continue used in the Indian market because of their all-time availability with good insecticide efficiency and at a price is 10-12 times less than that of the pure gamma hexa-chlorocyclohexane (Anu Appaiah and Karanth, 1995). In the presence of biosurfactants, hexa-chlorocyclohexane was converted through the involvement of isomerase and dechlorinase to tetrachlorohexenes and then to chlorophenols (Robles-González et al., 2008).

2.5.2 Biosurfactants Applications in Food Industry

Biosurfactants show potential applications in many sectors of food industry, Associated with emulsion forming and stabilization, antiadhesive and antimicrobial activities are some properties of biosurfactants, which could be explored in food processing and formulation (Nitschke and Costa, 2007). Lecithin and its derivatives, fatty acid esters containing glycerol, sorbitan, or ethylene glycol, and ethyoxylated derivatives of monoglycerides, including a synthesised oligopeptide are currently in use as emulsifiers in the food industries world wide. A novel bioemulsifer from *Candida utilis* has shown potential use in salad dressing (Shephord et al., 1995).

2.5.3 Biosurfactants Effects on Freshwater and Marine Inhabitants

The crude oil contamination of rivers and marine ecosystems is a world-wide problem. To overcome these pollutants, biosurfactants could be useful in a future, in comparison to chemically synthesised detergents, a better biodegradability and a lower toxicity could be expected from microbial surface-active substance because of their biogenetic origin. Gustafsson et al., (2009) reported that very low biosurfactant concentrations (5 μg mL^{-1}) decreased both the photosynthesis efficiency and the cell viability in four harmful algal blooms (HAB) species. Poremba et al., (1991) reported several toxicity testing series with microorganisms and their response to biosurfactant treatment, bacterial growth (*A. calcoaceticus*, *S. marinorubra*, *Photobacterium phosphoreum*) was slightly effected or stimulated, whereas that of algae (e.g., *Dunaliella tertiolecta*) and flagellates were reduced.

2.5.4 Microbial Biosurfactants and Enzyme-Synthesized Surfactants

There are many commercial processes, which exploit hydrolases in organic solvent media for bioorganic synthesis and some of the processes benefit from the availability at low cost of bulk industrial hydrolytic enzymes such as proteases and lipases (Singh et al., 2007). The isolated enzymes that catalysed hydrolysis, alcoholysis,

condensation, and acylation or esterification reactions have been employed for the preparation of various surfactants, including monoglycerides, phospholipids, glycolipids and amino acid-based surfactants, from relatively inexpensive raw material such as fats and plant oil (Sarney and Vulfson, 1995). Compared with the conventional chemical synthesis, these enzymatic methods have the advantages of low energy requirement, minimal thermal degradation, high biodegradability and high regioselectivily and, therefore, have emerged as promising substitutes for conventional approaches for the production of surfactants (Lin, 1996).

2.5.5 Other Biosurfactants Applications

The usefulness of biosurfactants in other fields is emerging, especially in personal and health care and as therapeutic agents (Banat et al., 2000). Biosurfactants are potential candidates in the search for functionally different products, as they meet the requirements of functional food additives. Biosurfactants are also very attractive in the health care and cosmetic industries (Klekner and Kosaric, 1993). Antibiotic effects and inhibition of growth of human immunodeficiency virus in leukocytes by biosurfactants have been noted (Kitamoto et al., 1993).

Other areas of applications are in the pulp and paper, coal, textile and uranium ore-processing industries (Mclnerney et al., 1990). A heteropolysaccharide containing mannuronic acid and guluronic acid from *Macrocystis pyrifeu* (kelp), *Azotobacter vinelandii* and polyglutamic acid from *Bacillus licheniformis* have been used as effective dispersants in the ceramic processing industry (Pellerin et al., 1992). Biosurfactants are expected to be among the most versatile process chemicals for use in a future.

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter manily describes the procedures, apparatus and equipments used for sampling, isolation, of the microorganisms, screening for biosurfactant production and identification of microorganisms. Series of laboratory experiments were performed to study the effect of nutritional and physical factors on the biosurfactant production. These experiments consisted of different carbon, nitrogen and metal ions, the experiments of physical factors include different temperatures, inoculum size, agitation rate, and initial pH. The main purpose of the nutritional and physical factors experiments were to obtain the optimal conditions which to be used in procedure of optimization and growth gurve. This chapter also provides a detail description of each material used during the study of recovery and thin layer chromatography (TLC) of biosurfactant production. All the experiments summarised in (Figure 3.1).

3.2 MATERIALS

Table 3.1 shows all the chemicals used in this study. Abbreviated names of suppliers are included. The reagents used were of analytical grade.

3.3 PREPARATION OF MICROORGANISMS

Seawater samples were collected from Kuantan, Malaysia and Kertih Port, Terengganu, Malaysia.

Table 3.1: The list of chemicals and instruments

Materials		Manufacturers
Bushnell and Hass	Media	Difco, USA
Casamino acids	Media	Difco, USA
Corn Steep Liquor	Media	Sigma, USA
Malt extract	Media	Oxoid, UK
Meat Extract	Media	Oxoid, UK
Nutrient Broth	Media	Oxoid, UK
Peptone	Media	Oxoid, UK
Soytone	Media	Difco, USA
Trypticase Soy Broth	Media	Difco, USA
Tryptone	Media	Oxoid, UK
Yeast Extract	Media	BBL, USA
D (+) Glucose	Carbohydrate	Merck, Germany
Glycerol	Carbohydrate	BDH, UK
Maltose	Carbohydrate	Sigma, USA
Sucrose	Carbohydrate	Ajax, Australia
Starch	Carbohydrate	BDH, UK
Bovine Albumin	Protein	Sigma, USA
Acetic Acid	Chemical	Merck, Germany
Ammonium Chloride	Chemical	Sigma, USA
Ammonium Nitrate	Chemical	Sigma, USA
Ammonium Sulphate	Chemical	Merck, Germany
Calcium Chloride	Chemical	Merck, Germany
Cetyl trimethylammonium bromide	Chemical	Sigma, USA
Commassie Brilliant Blue R 250	Chemical	BioRad, USA
Crude Oil	Chemical	Esso, Malaysia
Di-Ammonium hydrogen phosphate	Chemical	BDH, UK
Di-Ammonium sulphate	Chemical	Fluka, Switzerland
Di-Potassium monohydrogen phosphate trihydrate	Chemical	BDH, UK
Di-Potassium monohydrogen phosphate	Chemical	Ajax, Australia
Di-Sodium Hydrogen Phosphate	Chemical	Merck, Germany
Ferric Chloride	Chemical	BDH, UK
Hydrochloric Acid	Chemical	Merck, Germany
Iodine Crystals	Chemical	Merck, Germany
Magnesium Chloride	Chemical	Ajax, Australia
Magnesium Sulphate heptahydrate	Chemical	BDH, UK
Manganese sulphate	Chemical	BDH, UK
Methylene blue	Chemical	Sigma, USA
Mono-Potassium di-Hydrogen phosphate	Chemical	Ajax, Australia
Orthophosphoric acid	Chemical	Ajax, Australia
Potassium Di-Hydrogen Phosphate	Chemical	Merck, Germany
Potassium nitrate	Chemical	Sigma, USA

Table 3.1: continue

Materials		**Manufacturers**
Sodium Carbonate	Chemical	BDH, UK
Sodium chloride	Chemical	Merck, Germany
Sodium Dihydrogen Phosphate	Chemical	Merck, Germany
Sodium dodecyl sulphate SDS	Chemical	Merck, Germany
Sodium Hydrogen Carbonate	Chemical	BDH, UK
Sodium Hydroxide	Chemical	Merck, Germany
Sodium Nitrate	Chemical	Fisher, USA
Zinc Chloride	Chemical	Merck, Germany
Acetone	Solvent	J.T.Baker
Benzene	Solvent	BDH, UK
Butanol	Solvent	Ajax, Australia
Chloroform	Solvent	Ajax, Australia
Ethanol	Solvent	Merck, German
Heptadecane	Solvent	Sigma, USA
Heptane	Solvent	Sigma, USA
1-Heptanol	Solvent	Sigma, USA
Hexane	Solvent	Sigma, USA
n-Hexadecane	Solvent	Sigma, USA
Methanol	Solvent	BDH, UK
1-Propanol	Solvent	Merck, German
n – Tetradecane	Solvent	Sigma, USA
Autoclave	Instrument	HIRAYAMA, HVE 50 Japan
Centrifuge	Instrument	Eppendorf, 5810R,USA
UV-vis Spectrophotometer	Instrument	HITACHI, V-1800
Incubators	Instrument	INFFORS/MULTITRON II HEIDOLPH
Rotary Evaporator	Instrument	BUCHI R200
Shaker Bath	Instrument	JEIO TECH BS-21
pH meter	Instrument	METROHM 827
Surface Tensiomat	Instrument	SEO DST 60
Vortex	Instrument	VELP Scientifica
Filter membrane		WHATMAN
Silica Gel 60		Merck, Germany

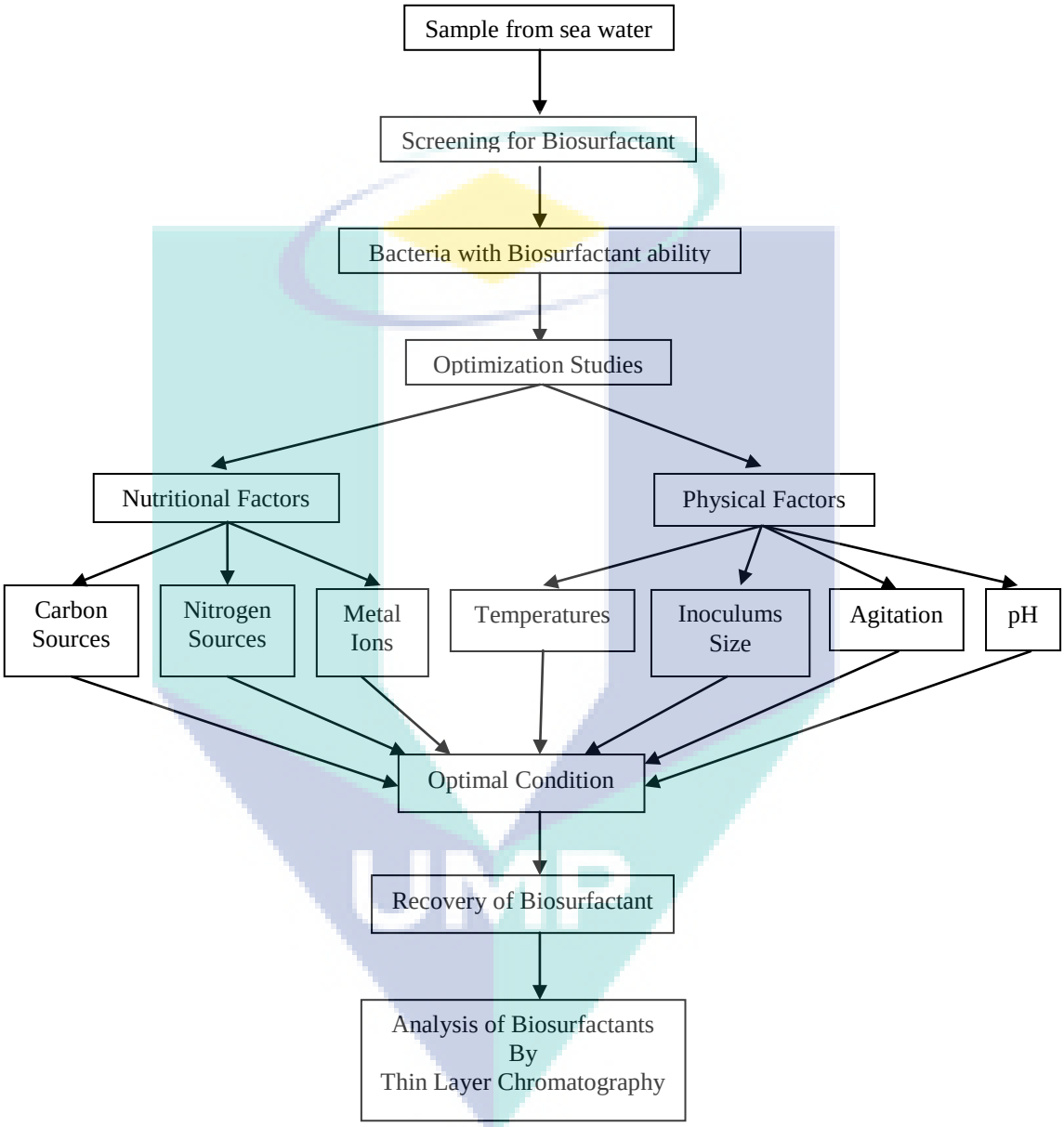


Figure 3.1: Flow chart of all experiment and test used in the study

3.3.1 Procedure of Bacterial Isolates: Route of Isolation

Seawater Samples were subjected to enrichment Ramsay liquid medium (Maneerat, 2005) with continuous shaking (100 rpm) at 37ºC, for up to two days and then 10 µL streaked onto nutrient agar and Ramsay's agar and incubated at 37ºC, for 1-2 days. Plates were examined and preliminary identification of isolates made based on colony morphologies and cell characteristics and different bacteria were selected for biosurfactant screening. Isolates were purified by repeated single colony isolation and purity of cultures checked periodically by streaking on to nutrient agar and Ramsay agar. In addition, the cell suspensions of isolated strains, were tested for the presence of surfactant by using cetyl trimethylammonium bromide (CTAB), the surface tension reduction and emulsification activity.

3.4 MEDIA PREPARATION

3.4.1 Procedure of Ramsay Liquid Medium

A Ramsay medium was used for growth of bacteria isolates consisted of (g/L): 2.0g NH_4NO_3, 0.5g KH_2PO_4, 1.0g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.01g $CaCl_2.2H_2O$, 0.1g KCl and 0.06g yeast extract in 1000 mL of distiller water. The pH was adjusted to 6.5-6.8 before autoclaving at 120ºC and 15 psi for 20 minutes. A 3 to 10mM glucose was added into the medium prior to inoculation. Glucose stock solution (1M) was prepared by adding 49.54g glucose in 250mL distilled water and was filtered sterilized using 0.2µm nylon membrane.

3.4.2 Procedure of Nutrient Agar

Nutrient Agar (NA) was used for growth and maintenance of isolated bacteria from the sea water. NA (2% w/v) was suspended in 1000mL distilled water before autoclaving at 120ºC and 15 psi for 20 minutes. The medium was then cooled to approximately 50ºC prior to pour (~20mL) into sterile Petri dishes. The molten agar was

left to cool and gel at room temperature. The medium can be used directly following preparation or stored in room temperature for up to one week.

3.4.3 Procedure of Ramsay Agar

The preparation of Ramsay's agar was similar to that of the Ramsay liquid medium, except that agar (2% w/v) was added as gelling agent.

3.5 PROCEDURE OF PRELIMINARY SCREENING STUDIES

3.5.1 Procedure of Selective Media

i. Mineral Media

Mineral media composed of (g/L); $16K_2HPO_4$ $3H_2O$, $7.14KH_2PO_4$, $4(NH_4)_2SO_4$, $0.2MgSO_47H_2O$, dissolved in 1.0 L deionized water stirrer with magnetic bar and adjusted to pH 7 by adding NaOH, adding ethanol 2%(v/v) as a carbon source (Maneerat, 2005).

ii. Selective Media

A semiquantitive selective medium that has been used consisted of (g / L); 0.2 cetyl trimethylammonium bromide (CTAB), 0.005 methylene blue and five nutrient agar in 1000 mL mineral media, This medium was autoclaved at 121^{o}C, 15 psi for 15 minutes. The medium was poured to the plate and dried at room temperature by methods of Lin et al., (1998).

3.6 IDENTIFICATION OF MICROORGANISM

Microbiological properties and characteristics of selected bacteria were investigated by morphological and biochemical tests. The results were further confirmed by using Microlog Microbial Identification System manufactured by Biolog Automated Microstation System, Biolog Inc., USA.

3.7 PROCEDURE OF GROWTH AND BACTERIAL ISOLATES

3.7.1 Inoculum Preparation

A fresh single pure colony of each bacterial isolates was transferred aseptically from an agar plate into Ramsay's medium using a sterile wire loop and incubated for 18 h at 37^{o}C in an orbital shaker (150 rpm). The cells were harvested by centrifugation at 8,600 x *g* for 10 minutes at 4^{o}C and the bacterial pellet was dissolved in sterile physiological saline 0.85% (w/v) NaCl to give an absorbance reading 0.5 at wavelength 540 nm to use as inoculum.

3.7.2 Procedure of Culture Storage

All pure isolates were maintained in liquid and solid media. They were regularly subcultured into a fresh medium for short-term storage. Stock cultures of all pure isolate were prepared in a ‘protect mixed bacterial preserver beads’ at –80ºC, according to manufacture instructions for a long-term maintenance.

3.8 DETERMINATION OF BACTERIAL BIOMASS

3.8.1 Procedure of Optical Density

Bacterial biomass was determined by measuring the culture optical densities at 540nm. Optical densities of the samples removed from cultures were read against a blank of salin throughout this study for growth determination.

3.8.2 Procedure of Growth Measurement

The growth was measured in terms of whole cell protein by methods of Patel and Desai (1997) with modification. The culture was centrifuged at 8,600 x *g* for 10 minutes at 4^{o}C. The pellet was mixed with one mol l^{-1} sodium hydroxide (NaOH), followed by boiling in water bath for 15 minutes.

3.8.3 Procedure of Protein Assay

The protein was estimated by the methods of Bradford (Patel and Desai, 1997) to measure the growth of the isolated bacteria. The pellet which was prepared as in biomass section above, where 1.0 mL of pellet with 1.5 mL distilled water plus 5 mL Bradford's solution vortexing for one minute, and absorbance measurements at 595 nm.

3.8.4 Preparation and Procedure of Bradford Reagent

Bradford's reagent was prepared by dissolving 100 mg of Brilliant Blue R 250 in 100 mL mixture of 85% orthophosphoric acid and 50 ml 95% ethanol. After the dye had completely dissolved, the volume was brought to 1.0 litre with cold distiller water.

Table 3.2: Preparation of Albumin Bovine Standard Curve

Tube	Amount of AB (µg)	Volume of AB standard stock solution (mL)	Volume of Distilled water (mL)	Bradford reagent (mL)
0	0	0	2.5	5.0
1	10	0.1	2.4	5.0
2	20	0.2	2.3	5.0
3	30	0.3	2.2	5.0
4	40	0.4	2.1	5.0
5	50	0.5	2.0	5.0
6	60	0.6	1.9	5.0
7	70	0.7	1.8	5.0
8	80	0.8	1.7	5.0
9	90	0.9	1.6	5.0
10	100	1.0	1.5	5.0

3.8.5 Procedure of Standard Curve

Calibration curve was plotted using albumin bovine standard solutions in the range of 0 - 100 µg. Different albumin bovine concentration was prepared to use AB standard solution (100 µg /mL) as follows (Table 3.2).

3.9 PROCEDURE OF EMULSIFICATION INDEX (E24) MEASUREMENT

Emulsification index (E24) was estimated by vortexing equal volumes (1.0 mL) of the culture supernatant with hexadecane. Hexadecane was used as it had good emulsion form properties in the test and provided a very sensitive and highly reducible assay. The mixture was placed in a glass test tube, vortexed thoroughly at room temperature for 4 minutes. The resulting uniform emulsion was allowed to settle for 24 h, after which it was visually examined for the percentage of volume occupied by the emulsion. The height of the emulsion layer and total height were measured after 24 hours. The emulsion index (E 24) was the height of the emulsion layer, divided by the total height, multiplied by 100.

3.10 PROCEDURE OF SURFACE TENSION MEASUREMENT

Surface tension of the culture supernatant was measured with Automatic SEO Digital Surface Tension Analyzer 60. The DuNouy ring was cleaned by dipping the ring in a benzene solution (to remove hydrocarbons) and then in acetone (to remove the benzene). The DuNouy ring was then air- dried, the cleaned ring was allowed hanging on a hanger inside the balance. The culture supernatant (20 mL) was poured into a clean disposable beaker and was placed on the sample stage. The sample stage was adjusted until it was directed beneath the DuNouy ring. The stage was then raised until the ring was immersed in the broth, the distance between the immersed ring and the sample surface approximately not less than 3mm. While, the sample stage was moving automatically up or down, the balance measures the maximum weight when the ring gets out of the sample. Then, the result of the tension was displayed. Each time before SEO Digital Surface Tension Analyzer 60 was used; it was first calibrated with distilled water to give a reading of 72 mN/m for three times.

3.11 STATISTICAL ANALYSES

The regression analyses and statistical significance were tested using (ANOVA) of the software Design Expert Version 6.0.4 manufactured by Stat-Ease Inc., Statistics Made Easy, Minneapolis, MN, USA.

3.12 PROCEDURE OF NUTRITIONAL AND PHYSICAL FACTORS

Throughout the study on nutritional factors affecting the growth and biosurfactant production, the general procedures for cultivation were as follows: 1.0 mL of 18 h bacterial inoculum (OD_{540} = 0.5) was inoculated into 50 mL of a culture medium and incubated at 37°C for 21 hours. The basal medium (BM) was Bushnell Hass containing (g/L): 0.05 $FeCl_2$, 0.02 $CaCl_2$, 1.0 KH_2PO_4, 1.0 $(NH_4)_2SO_4$, 1.0 KNO_3 and 0.2 $MgSO_4$. The culture was harvested from the medium by centrifugation at 8,600 x *g* at 4°C for 10 minutes. The supernatant was then filtered with a cellulose

acetate membrane filter (pore size, 0.20 μm). The biomass and the biosurfactant activity (surface tension reduction, emulsification index (E24)) were assayed by the method of Patel and Desai (1997) with modification. Each experiment was done in triplicates. Unless otherwise stated, the cultivation parameters and assay procedures remained the same throughout this project.

3.12.1 Procedure of Carbon Sources Experiments

A series of experiments was conducted at 37^{o}C under shaking condition at 150 rpm for 21 hours with different types of carbon sources supplied at 2% (v/v) in the medium. Hexadecane, tetradecane, glycerol, crude oil, ethanol, 1-propanol, 1-butanol, glucose, maltose, starch and sucrose were separately sterilized by membrane filtration (0.20 μm membrane filter).

3.12.2 Procedure of Nitrogen Sources Experiments

Biosurfactant production was then tested on various nitrogen sources at 2% (w/v), Yeast extracts, meat extracts, malt extracts, soytone, peptone, tryptone, corn steep liquor and casamino acid were organic nitrogen sources, separately autoclaved at 120^{o}C of 15 psi for 20 minutes. The samples were incubated at 37°C for 21 hours.

3.12.3 Procedure of Different Concentration of Ethanol Experiments

The different concentration of ethanol acid was tested at 1%, 1.5%, 2%, 2.5%, 3%, and 3.5% (v/v). The cultures were incubated at 37°C for 21 hours.

3.12.4 Procedure of Different Metal Ions Experiments

To determine the effect of metal ions on the biosurfactant production the various concentration of (g/L); (0.0, 0.05, 0.2, 0.4) $MgSO_4$, (0.0, 0.01, 0.05, 0.1) $FeCl_2$, (0.0, 0.01, 0.02, 0.04) $CaCl_2$, (0.0, 0.05, 0.1, 0.2) $MnSO_4$ and $ZnSO_4$ were tested in the basal medium.

3.12.5 Procedure of Temperature Experiments

The ability of bacteria to grow and produce biosurfactant at elevated temperatures, in the range of 5°C to 50°C was investigated. The samples were incubated at 5°C, 30°C, 35°C, 40°C, 45°C and 50°C.

3.12.6 Procedure of Inoculums Size Experiments

To investigate the effect of inoculum sizes (O.D 540nm = 0.5) on biosurfactant activity, the biosurfactant assay was carried out at various inoculum sizes, in the range of 2% (v/v) to 16% (v/v). The cultures were incubated at 37°C for 21 hours.

3.12.7 Procedure of Agitation Rate Experiments

Bacterial cultivation was carried out at various agitation rates, i.e.:0, 50, 100, 150, 200 and 250 rpm at 37°C for 21 hours.

3.12.8 Procedure of Initial pH Experiments

The initial pH of the medium was adjusted by HCl and NaoH to pH values, i.e: 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, and incubated at 37°C for 21 hours.

3.13 PROCEDURE OF GROWTH CURVE IN OPTIMIZED MEDIUM CONDITION FOR BIOSURFACTANT PRODUCTION

The growth and biosurfactant production was investigated in an optimized growth medium (1.5% ethanol, basal medium, 35^{o}C, 4% inoculum size, agitation rate 100 rpm, pH 7.2). Samples were withdrawn at 1.0 day intervals for determination of biosurfactant production, surface tension reduction, emulsification index (E24), pH of culture medium and biomass.

3.14 PROCEDURE OF RECOVERY BIOSURFACTANT PRODUCTION

3.14.1 Maier Method

The first method was as described by Maier (2003) with modification. Cells were removed from the culture fluid by centrifugation at 15,300 x g for 15 minutes. Crude biosurfactant from the supernatant were then isolated by adding concentrated hydrochloric acid to the supernatant until the pH reached to <2.0. The resulting flocculate were separated from the solution by centrifuging at 15,300 x g for 15 minutes then the resulting pellet obtained was dissolved in 10:1 chloroform to methanol, followed by removing the organic phase and recovering the rhamnolipids by removing the solvent by rotoevaporation. The remaining aqueous phase was further extracted twice using the same chloroform/methanol mixture. The residue was dissolved in $NaHCO_3$ and the biosurfactant estimated by the cysteine hydrochloride reaction by methods of Patel and Desai (1997).

3.14.2 Daniels Method

The second method was as described by Daniels et al., (1990). Cells were removed from the culture fluid by centrifugation at 6000 x *g* for 45 minutes. Crude biosurfactant from the supernatant was then isolated by adding concentrated sulphuric acid to the supernatant until the pH reached to 2.5 and allowed to stand overnight at 4^{o}C. The resulting precipitate was separated from the solution by centrifuging at 6000 x

g for 45 minutes. The pellet was dissolved in $NaHCO_3$. The biosurfactant was estimated by the cysteine hydrochloride reaction by methods of Patel and Desai (1997).

3.14.3 Mixich Method

The third method was as described by Mixich et al., (1997). The culture was acidified by adding 12M sulphuric acid until the pH reached 3.0 and then heated to 100^{o}C and kept at this temperature for 60 minutes. Subsequently, the solution was cooled down to 20^{o}C and centrifuged at 3200 x *g* for 15 minutes. This centrifugation lead to the formation of two phases (upper & lower) which clearly separated from each other and the pellet (lower phase) was then dissolved in $NaHCO_3$. The mixture was then filtered through 0.22 μm filters membranes and the biosurfactant estimated by the cysteine hydrochloride reaction by methods of Patel and Desai (1997).

3.14.4 Zahang and Millar Method

The fourth method was as described by Zhang & Millar (1992). Rhamnolipid was recovered from the culture supernatant after the removal of the cells by centrifugation at 6,800 x *g* for 20 minutes. Rhamnolipid was then precipitated by acidification of the supernatant to pH 2.0 by adding hydrochloric acid and centrifugation at 12,000 x *g* for 20 minutes. The precipitate was dissolved in 0.05 M bicarbonate, reacidified, and recentrifuged at 12,000 x *g* for 20 minutes. Following centrifugation, the precipitate was extracted with chloroform-ethanol (2:1) three times. The organic solvent was evaporated on a rotary evaporator. The residue was dissolved in $NaHCO_3$ and the biosurfactant estimated by the cysteine hydrochloride reaction by methods of Patel and Desai (1997).

3.15 ANALYSIS OF BIOSURFACTANTS PRODUCTION

3.15.1 Preparation of the Samples Prior to Thin Layer Chromatography (TLC)

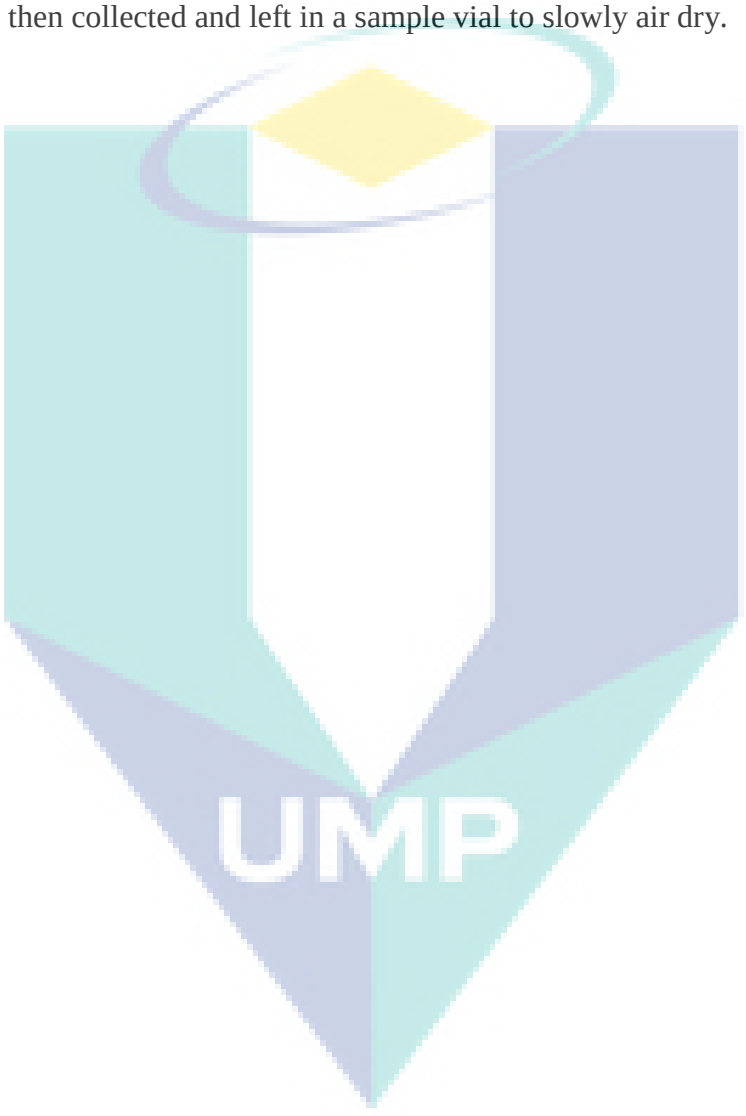
Cells were removed from the culture fluid by centrifugation at 15,300 x *g* for 10 minutes. Crude biosurfactant from the supernatant was then isolated by adding concentrated hydrochloric acid to the supernatant until the pH reached to <2.0. The resulting flocculate was separated from the solution by centrifuging at 15,300 x *g* for 10 minutes. The resulting pellet obtained was dissolved in 10:1 chloroform to methanol, followed by removing the organic phase and recovering the rhamnolipids by removing the solvent by rotoevaporation. The remaining aqueous phase was extracted more than twice using the same chloroform/methanol mixture. The solvent was decanted off and the precipitate was dried under vacuum. Initial and final weights of the centrifuge tubes were noted to calculate recovery.

3.15.2 Procedure of Thin Layer Chromatography (TLC)

The respective dried acidified precipitates were dissolved in distilled water before being spotted onto plastic sheets coated with a 0.2mm thick layer of silica gel 20 X 20 cm plates manufactured by Kieselgel 60, Merck Cat. No. 1.05735. Ten µL of various concentrations of dissolved precipitates were applied as tight spots 1-1.5 cm from the bottom edge. The plates were then developed using the following solvent systems: 80% ethyl acetate, 10% methanol and 10% acetic acid for 20 minute and then air dried. Detection and visualization were achieved by transferring the plate into a chamber saturated with iodine vapour for resolved spots, by methods of Deziel et al., (2000). The positions of the separated spots on developed thin layer chromatograms were described by the R_f value of each substance and “a” spot of product, where

$$R_{fa} = \frac{\text{distance traveled by the spot centre of solute “a”}}{\text{distance traveled by the solvent front}}$$

The reproducibility of R_f values depends on many factors such as humidity and ambient temperature, hence, the R_f values serve only as a guide and are not absolute. The portions of the plates were scraped, corresponding to the bands visualized in the iodine area. The silica gel scrapings were collected, and the rhamnolipids extracted twice with 8 ml of chloroform: methanol (1: 2 v/v). The extraction involved vortexing the solvent-scrapings mixture for one minute and centrifuging down the silica gel for 10 minutes and then collected and left in a sample vial to slowly air dry.



CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

In this chapter, the sampling, isolation, of the microorganisims, various methods for the screening of biosurfactant producing bacteria such as CTAB, surface tension reduction and emulsification activity (E24) and identification of microorganisims were invistegated. Surface tension measurement was reported as a primary method used as to indicate the ability of microbes to produce biosurfactant, though other methods as a comparison for a better selection of biosurfactant producer. As cited in the literature, many factors affecting on the production of the biosurfactant, such as the type and amount of the microbial surfactants produced, which depend primary on the producer organism. For that reason, this study was carried out to explore the new biosurfactants in various conditions. The selected organisms identified as *Pseudomonas aeruginosa* KRT-142 were investigated in different carbon, nitrogen, metal ions, temperatures, inoculum size, agitation rate, and initial pH for biosurfactant production.

In general, the selected biosurfactant were recovered by acidified of culture using different acids concentrated hydrochloric acid, concentrated sulphuric acid and 12M sulphuric acid, then the precipitate was extracted with a different ratio of chloroform, and methanol and estimated by the cysteine hydrochloric reaction. The biosurfactant production was analyzed using thin layer chromatography (TLC).

4.2 SCREENING OF BIOSURFACTANT-PRODUCING BACTERIA

A total of 176 isolates were obtained from two sampling points (Table 4.1). Out of this total 124 (70.4%) isolates obtained from the Kertih, Terengganu location while the remaining 52 isolates (29.5 %) were from location of the Kuantan, Pahang.

4.2.1 Preliminary Screening Studies

This assay was developed based on the property that the concentration of anionic surfactants in aqueous solutions can be determined by the formation of insoluble ion pairs with various cationic substances. The formation of an insoluble ion pair precipitates in the agar plate containing methylene blue exhibited blue colour against the light blue background. The cationic chemical selected in the assay was a cetyltrimethylammonium bromide. A large dark colour formed from isolated bacteria, which indicated biosurfactants production. The diameter of the dark region has previously been shown to be semiquantitatives proportional to the concentration of the anionic biosurfactants (Siegmund and Wagner, 1991). Shulga et al., (1993) described a method for determining anionogenic bacterial peptidolipid biosurfactants based on the ability of the anionic surfactants to form a coloured complex indicator methylene blue.

An isolated bacteria KRT-142 shows the highest biosurfactants activity by reducing the surface tension of the culture broth to 46.6 mN/m, followed by KRT-93 (51.2 mN/m), KU-76 (54.3 mN/n), KU114 (58.1 mN/m) and KRT-21 (59.9 mN/m), at 21 hours respectively. Its emulsification index (E24) in Ramsay medium was 36% followed by KRT-93 (13%), KU-76 (11%), KRT-21 (9%) and KU-114 (8%) at 21 hours respectively (Table 4.1). Selection was made based on the results obtained from all screening methods used in this study, for the most potential bacteria capable of producing biosurfactant which was isolated KRT-142. Table 4.1 summarized the results for the screening of biosurfactant-producing bacteria using three different methods commonly used and described elsewhere.

Table 4.1: Screening of biosurfactant-producing bacteria using three different methods using Ramsay medium at pH 7.0, 37^{o}C for 21hours.

Isolates	CTAB	Emulsification Index (E24) %	Surface Tension (mN/m)
KU-114	-	8	58.1
KU-76	-	11	54.3
KRT-93	-	13	51.2
KRT-21	-	9	59.9
KRT-142	+	36	46.6

4.2.2 Identification of Bacteria

Strain KRT-142 produced water – soluble, yellow- green fluorescent pigments on a nutrient agar plate (Figure 4.1). It is an aerobic, gram negative (Figure 4.2), straight rods and motile bacteria. It does not produce prosthecae and is not surrounded by sheaths. Bacterial growth on solid foods is in the form of microcolonies, in general the ability of cells to slide along a solid surface optimizes the exposure of the cell surfaces to air and nutrients. It was proposed that linear radial growth represent the "steady state growth phase of bacterial colonies "and was comparable to the exponential growth phase in liquid culture. The concentration of agar in the present study was 1.0% (w/v). Stecchini et al., (2001) found that at a lower agar concentration (1.0% w/v), *Bacillus cereus* cells could create a more sliding colony type than that found at higher agar content (7.0% w/v).

The ability of the dividing cells to push neighbours aside rather than piling in one location could be due to the presence of a liquid film, such as found at 1.0% (w/v) agar surface, which could reduce the surface friction at a higher concentration.

Table 4.2: Characteristics of *Pseudomonas aeruginosa* KRT-142

Morphological Characteristics	
Shape	Straight rods
Cell dimension (µm)	1x1.4 µm
Motility	Motile
Biochemical and Cultural Conditions	
Growth temperature at : 5^{o}C	Negative
: 30^{o}C	Positive
: 40^{o}C	Positive
Optimum growth pH	7.0
Oxidase activity	Positive
Gram Staining	Negative
Catalase activity	Positive
Indole Test	Negative
Methyl Red	Negative
Voges-Proskauer	Negative
Gelatine Liquefaction	Positive
Nitrate Reduction	Positive
Hydrogen Sulfide Production	Positive
Production of Pigment	Yellow-green
Starch Hydrolysis	Negative
β-Calactosidase (ONPG)	Positive
Carbon Sources for growth:	
D- Xylose	Positive
D-Glucose	Positive
D-Fructose	Positive
D-Galactose	Negative
Maltose	Negative
Sucrose	Negative
Lactose	Negative
D-Manose	Negative
D-Mannitol	Positive
L-Arabinose	Negative

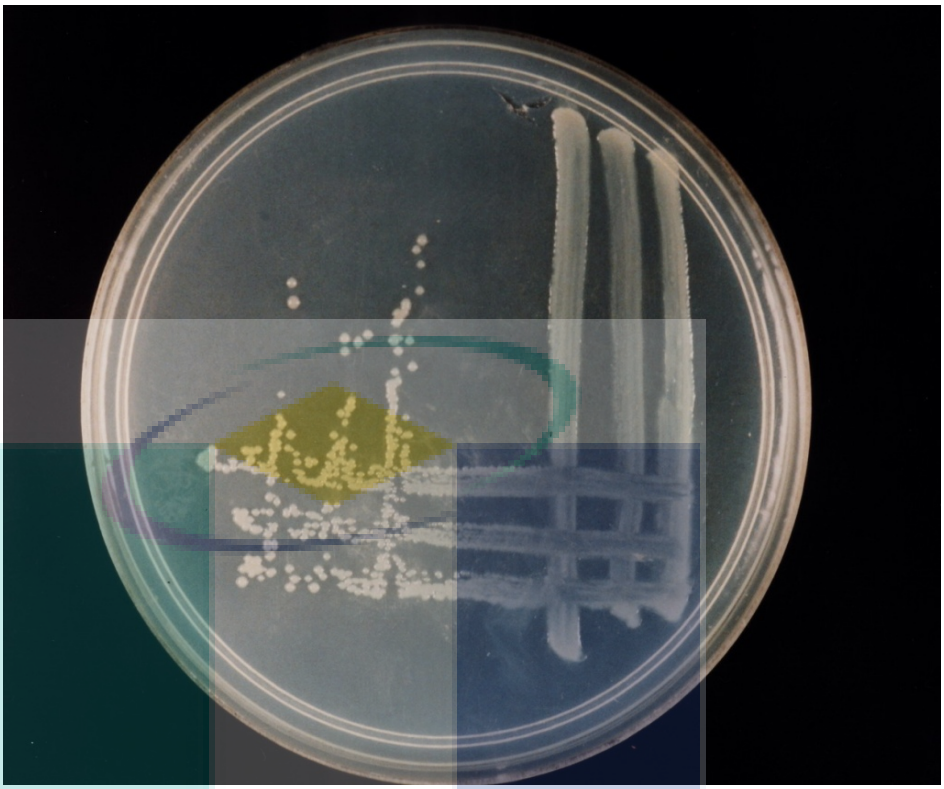


Figure 4.1: Colonies of *Pseudomonas aeruginosa* KRT-142 on a Nutrient Agar Plate. *Pseudomonas aeruginosa* KRT-142 was grown on nutrient agar plate containing (g/L): 10 nutrient agar, and incubated at 37°C for 24 h.

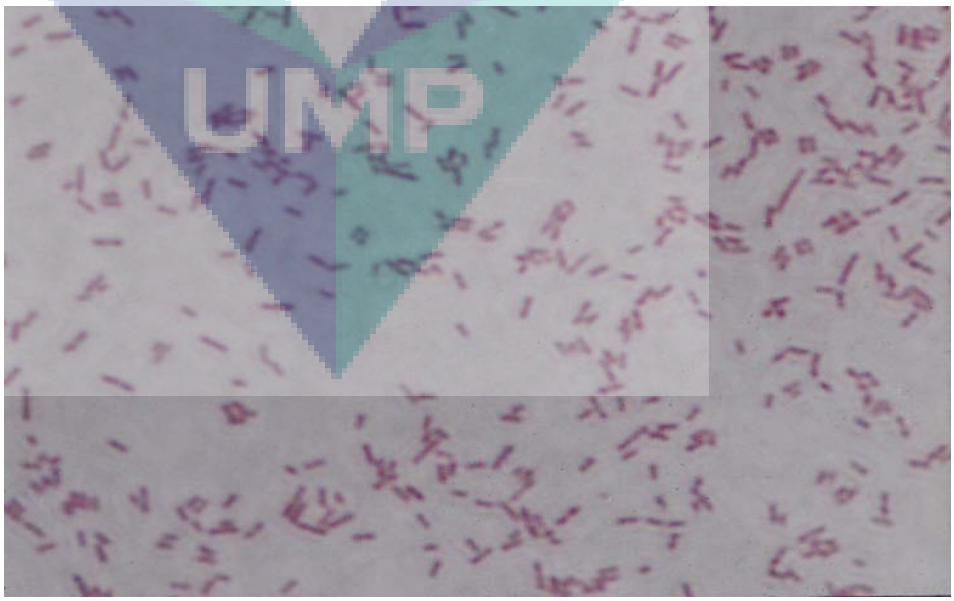


Figure 4.2: Gram Staining of *Pseudomonas aeruginosa* KRT-142, *Pseudomonas aeruginosa* KRT-142 an aerobic, gram negative and short rods bacteria.

Based on its morphological, biochemical and cultural characteristics listed in Table 4.2 isolate KRT-142 was identified as *Pseudomonas aeruginosa*. This result was further confirmed by using MicroLog Microbial Identification System.

Pseudomonas aeruginosa (family *Pseudomondaceae)* has a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. In some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. It is oxidase and catalase positive. *Pseudomonas aeruginosa* has very simple nutritional requirements and can survive in almost any environment, lives primarily in water, soil and on vegetation. It was grown at neural pH and at temperatures in the mesophilic range.

Pseudomonas aeruginosa is among the most commonly isolated microorganism in petroleum contaminated soils and groundwater (Ridgway et al., 1990). It is known that *Pseudomonas aeruginosa* mineralizes aliphatic compounds. *Pseudomonas aeruginosa* strains attack aromatic, polyaromatic hydrocarbons and are typically active denitrifies and produces biosurfactant (rhamnolipids) when they are grown on hydrophobic substrates. Rhamnolipids of these strains are actively synthesis during the first stage of proposed sequential bioremediation process and contribute to mobilize and solubiliz the contaminants during the subsequent mineralization stage. Therefore, the versatility and unique metabolic characteristics are the most suitable species for initial studies of sequential hydrocarbon metabolism (Chayabutra and Ju, 2000).

4.3 OPTIMIZATION STUDIES

Microbial surfactants are of considerable importance for commercial application (Zaragoza et al., 2010). However, their yields in production are generally very low. It is essential that the surfactant producing bacteria are provided with optimal growth conditions to increase biosurfactant production. The biosurfactants production has been shown to be influenced by the culture conditions (Parthasarathi and Sivakumaar, 2009). Therefore, with a view to develop an economically feasible technology, research efforts have been focused on:

(i) Evaluation of the effect of various carbon and nitrogenous nutrients as cost effective substrates in the yields of biosurfactant.
(ii) Requirement of metal ions in the culture media.
(iii) Optimization of environmental and the production parameters' media.

The optimization of medium composition is done to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of the production. There is no defining medium that can promote maximum production of surfactants for different microbial strains. Each organism or strain has its own special conditions for maximum enzyme production (Kumar and Takagi, 1999).

4.3.1 Effect of Nutrients Supplements

i. Effect of Carbon Sources

The result shows that ethanol supported the highest growth as measured by whole cell protein (1.34 g/ L) after seven hours (Figure 4.3), followed by glycerol and glucose. Slight growth was observed with crude oil. Decreasing growth was observed with tetradecane, 1-propanol, 1-butanol, sucrose, maltose and starch. Ethanol yielded the highest biosurfactant production, reducing the surface tension to 43.3 mN/m after seven hours. It was followed by glycerol, glucose and hexadecane with surface tension reduction to 44.5, 49 and 50.9 mN/m after 21 hours, respectively. The highest

emulsifying activity was 56% for ethanol after seven hours, 52.7% and 42% for ethanol and glycerol, respectively after 14 hours. Hexadecane gave a significant emulsification index (E24) only after 21 hours (35.8%), which correlated well with the increase in biomass. This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (0.042) for whole cell protein, (453.3844) for surface tension, (73.0044) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses, The computed F-values of 13.12 (whole cell protein), 21.75 (surface tension) and 447.90 (emulsification index (E24)) were higher than their tabular value implying which significant at $P< 0.01$ level (Appendix B1, B2 and B3).

The result was in agreement with Matsufuji et al., (1997), which reported that no growth was observed for *Pseudomonas aeruginosa* IFO 3924 with 1-propanol and 1-butanol. The physiology of a microorganism is not exclusively determined by its genetic information, but is also a function of the nutritional and physical condition, In all cases, the microorganisms tend to produce some metabolite products, which facilitate the uptake of the water-insoluble substrates such as hydrocarbon into a cell.

This justifies for the wide spectrum of different substances produced by microorganisms, which are called biosurfactants. Hence, emulsification of insoluble substrate is not the only reason for the production of biosurfactants by microbes. In an attempt to maximize the biosurfactants production from *Pseudomonas aeruginosa* KRT-142, the effects of several carbon sources on growth performances and biosurfactants production in a time course study were examined. Hexadecane was used slowly by the bacteria, growth was observed only after 21 hours, and the delay may be due to the adaptation of the bacteria to the carbon source. This delay was expected since a number of different biochemical reactions are involved in alkane utilization including their terminal hydroxylation and the β-oxydation (Witholt et al., 1990). Similar observation by Tuleva et al., (2002) which reported that *Pseudomonas putida* 21BN showed delayed growth with 2% hexadecane. The result suggested that the biosurfactant production of *Pseudomonas aeruginosa* KRT-142 when grown in ethanol was growth dependent. However, glucose though supported good growth but biosurfactant activity was lower compared to hexadecane.

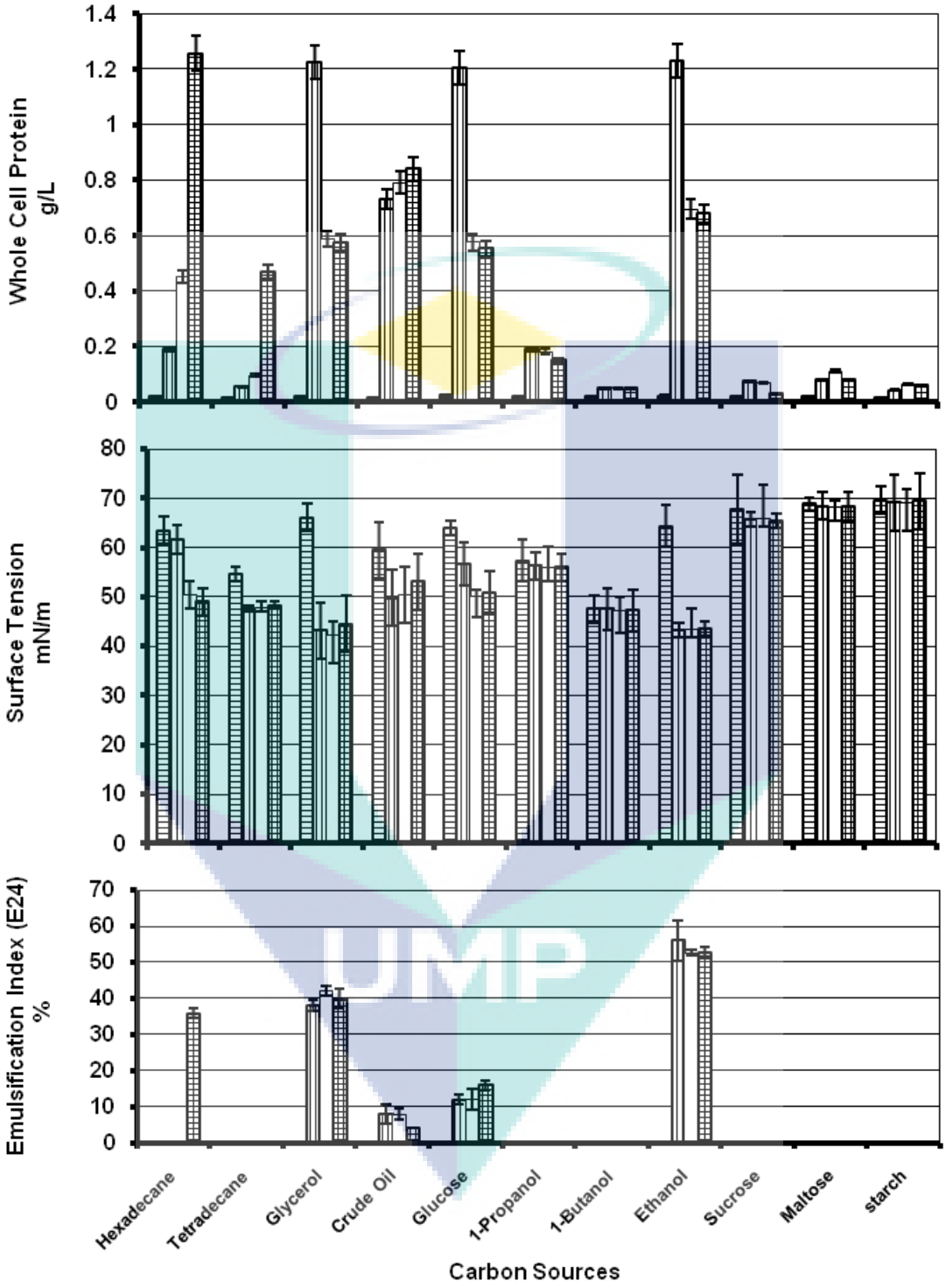


Figure 4.3: Effect of Carbon Sources on Bacterial Growth and Biosurfactant production by *Pseudomonas aeruginosa* KRT-142 as Determined by Whole Cell Protein, Surface Tension Reduction and Emulsification Index (E24). Different Carbon Sources:2.0% (v/v) of Hexadecane Tetradecane, Glycerol, Crude oil, Ethanol, 1-Propanol, 1-Butanol, Glucose, Sucrose, Maltose, Starch were added into Basal Media and at pH 7.0, 37^{o}C for 0 (▤) 7 (▥), 14 (□) and 21 hour (▦).

On the other hand, some microorganisms produce biosurfactants only when hydrophobic carbon sources such as hydrocarbons or vegetable oils are used, while others require carbohydrates, and several other carbon sources, either in combination or individually (Mulligan and Gibbs, 1993). The presence of biosurfactant indicated the reduction in the surface tension of the growth medium. Thus, by monitoring the surface tension of the cell-free culture fluid, it reflects the production of biosurfactant (Carillo et al., 1996).

The experimental results showed that the surface tension dropped rapidly while emulsification capacity continued to increase over the entire period of the experiment. This contrast may be due to the biosurfactant reaching its critical micelle concentration, beyond which constant surface tension is observed. Emulsification capacity, however, would continue to increase with further biosurfactant accumulation. Abu-Ruwaida et al., (1991b) reported that 3% of glucose reduced the surface tension to 46.3 mN/m from *Rhodococcus* sp. ST-5, on the other hand, decreased the emulsification index (E24) ranged between (0-5%). Crude oil induced biosurfactant production by strain KRT-142, and so far no report was available on *Pseudomonas* sp producing biosurfactant with crude oil. Hexadecane inhibited the biosurfactant production by *Bacillus subtilis* C9 when it was used as the sole carbon source (Kim et al., 1997a). Espuny et al., (1996) found that the growth of *Rhodococcus sp* 51T7 was observed with ethanol and glycerol but a little surfactant was produced. Arino et al., (1998) demonstrated that glycerol when added to the culture medium contained n-alkanes the biosurfactant production was lower than culture contained glycerol alone, possibly because oxygen transfer was decreased by the presence of n-alkanes.

Under experimental conditions the results showed that when a parallel culture grown on a water-immiscible carbon source such as tetradecane, the percentage of the emulsifying activity was negligible due to the poor growth. Biosurfactant production, like cell growth, depends on the availability of substrates. Thus, when the cells were grown on insoluble polycyclic aromatic hydrocarbons (PAHs), such as naphthalene, it resulted in poor growth and hence poor biosurfactant production (Arino et al., 1996). In fact, at high cell density, the availability of poorly soluble substrates becomes limiting

because PAHs are utilized only in the dissolved state (Volkering et al., 1992). The rationale behind biosurfactants production on PAHs is that it should stimulate itself by enhancing the substrate availability. Tiehm (1994) reported that microorganisms would adapt to PAHs degradation when the culture was subcultured several times on PAHs.

Zhang and Miller (1994) pointed out that high cell hydrophobicities enhance the contact between insoluble substrates and cells. Rhamnolipids have been claimed to be involved in growth on water-insoluble substrates and some mutants unable to produce rhamnolipids were found to be deficient in the capacity to assimilate n-alkanes (Koch et al., 1991, Ochsner et al., 1994). Cells exhibiting high cell hydrophobicities enhance the contact between insoluble substrates and cells, which are classified as the faster hydrocarbon degraders (Zhang and Miller, 1994). Although water-soluble substrates are less suitable for the induction of biosurfactant, the production of surface –active compounds on water soluble substrates do offers some advantages when compared with hydrocarbons.

Biosurfactant is secondary metabolites, the production of secondary metabolites classically exhibits a two stage process, namely trophophase (or growth phase) and idiophase (or production phase), usually when growth has slowed down or ceased to proceed. As secondary metabolites, most of the biosurfactants began to be released into media containing a water-soluble carbon source throughout the exponential phase. For strain KRT-142 maximum level was reached at the stationary phase. However, production of biosurfactant for another *Bacillus sp.* occurred earlier in growth phase (Melor, 1999).

In contrast, production of many secondary metabolites by bacilli which produced surfactant was induced when the cells have exhausted one or more essential nutrients, biosurfactant production (surfactin) was stimulated by actively grown cells with a post exponential synthesis. Competition with cellular growth in probably one of the reasons for the rather disappointing products yield, the cost of using hydrocarbons as a growth substrate will prohibit the large-scale use of biosurfactants, since hydrocarbons require more power input and more sophisticated equipment to achieve adequate dispersion of insoluble substrates. Furthermore, more heat of reaction is

produced during cultivation on hydrocarbons, thus requiring extensive cooling surfaces within the bioreactor system. In addition, residual hydrocarbon may have negative influence on downstream processing.

Considering the limited number of known biosurfactants producing bacteria, which are able to produce a significant biosurfactant that uses water soluble substrates as a carbon and energy source, thus *Pseudomonas aeruginosa* KRT-142 is a potential candidate for large scale products. These observations suggest that the overall rate of biosurfactant synthesis may be subjected to some control mechanisms, such as O_2 availability, the rate of utilization of products of carbohydrate catabolism (Jenny et al., 1993) and the factors regulating lipid formation (Mulligan and Gibbs, 1993). The kinetics of biosurfactant production exhibit many variations among various systems and only a few generalizations can be derived. It was grouped into the following types:

1- Growth-associated production
2- Production under growth-limiting conditions
3-Production by resting or immobilized cells
4- Production with precursor supplementation

Production of biosurfactant by strain KRT-142 appeared to be growth associated similar to that shown in the production of biosurfactant from *Pseudomonas putida* (Tuleva et al., 2002). A number of studies indicted that the type of media and growth conditions can influence the type and yield of the biosurfactant. The carbon sources influence the biosurfactant synthesis either via induction or repression. In some cases, addition of water-immiscible substrates results in induction of biosurfactant production (Cameotra and Makkar, 1998).

Makkar and Cameotra (1997) found that, a 2% concentration of sucrose as carbon sources was optimal for biosurfactant formation from *B. subtilis* MTCC 2423. While, Melor (1999) reported that, when *B. macerans* and *B. subtilis* were grown on a different carbon source, they were shown to produce different components of biosurfactant

Such a wide variation in surface tension for the alkanes (Tetradecane and Hexadecane), might be attributed to possible differences in the nature of the biosurfactants produced due to the differences in chain length of the substrates. Similar observations were reported by Das et al., (1998) with *Micrococcus* species grown on alkanes and sugars. Synthesis of many lipopeptide of biosurfactants is also regulated by induction (Besson and Michel, 1992, Ullrich et al., 1991). Smaller amount of glycolipids was produced from phenanthrene than naphthalene, since it is much less water soluble than naphthalene, it is probable that cell required more time to adapt themselves to the utilization of this poorly accessible polycyclic aromatic hydrocarbon (Deziel et al., 1996). These suggest that the medium component could play an important role in determining the type, amount and efficiency of biosurfactant produced. The production of surfactant in the present study was expected to be above their critical micelle concentration level.

The exact reason why some microorganisms produce surfactants is unclear. However, biosurfactant-producing bacteria are found in higher concentrations in hydrocarbon contaminated areas (Margesin and Schinner, 2001). Biosurfactant producer *B. macerans* was isolated from a high hydrocarbon environment at a Petronas refinery plant (Melor, 1999).

ii. Effect of Nitrogen Sources

Nitrogen represents another important element in bacterial metabolism. To evaluate the influence of nitrogen sources on the growth and biosurfactants production by *Pseudomonas aeruginosa* KRT-142, batch cultures were conducted in the media containing 2% of various organic nitrogen sources (Figure 4.4). There was low growth observed, in the presence of ammonium phosphate ($(NH_4)_2PO_4$) 0.103 g/L and potassium nitrate (KNO_3) 0.110 g/L in the basal medium. When these two compounds were omitted from the medium and replaced with ammonium nitrate (NH_4 NO_3) and urea, no growth and biosurfactant production was observed. This may be due to the lack of a cofactor that served as an active transport system, which was involved in the assimilation of the inorganic nitrogen. Similarly, Matsufuji et al., (1997) reported that

no growth was observed with ammonium nitrate in *Pseudomonas aeruginosa*. *Bacillus subtilis* ATCC21332 utilized ammonium in preference to nitrate in the early stage of cultivation (Davis et al., 1999). Espuny et al., (1996) reported that ammonium sulphate decreased the pH of the medium and cell growth became inhibited after 48 hours from *Rhodococcus sp.* 51T7. This phenomenon might be due to the repression of enzyme synthesis by rapid metabolism of ammonium ion concentrations in the basal medium (Giesecke et al., 1991). In the study of organic nitrogen sources soytone supported the highest growth (1.35 g / L) followed by peptone, meat extracts, yeast extracts, tryptone and casamino acid after seven hours (Figure 4.4).

The results showed that malt extract was used slowly by the bacteria; growth was gradually observed the delay may be due to the adaptation of the bacteria to the nitrogen source. Corn steep liquor was ineffective as a nitrogen source to promote the growth and biosurfactant production of *Pseudomonas aeruginosa* KRT-142. Low bacterial growth of only 96 mg / L and 56 mg / L was observed at 14 hour and 21 hour, respectively (Figure 4.5). Apart from serving as a nitrogen source, corn steep liquor also provided several micronutrients, vitamins and growth-promoting factors. However, its use was limited by its seasonal and inters batch variability factors (Malathi and Chakraborty, 1991).

Soytone yielded the highest biosurfactant production, reducing the surface tension to 43.1 mN/m after 14 hours. It was followed by meat extract and tryptone; they reduced the surface tension to 46.23 and 48.5 mN/m after 21 hours respectively. Malt extracts, peptone, yeast extracts and casamino acid reduced the surface tension to 51.2 mN/m, 55.2 mN/m, 56.5 mN/m and 57.5 mN/m respectively, after 21 hours. The highest emulsifying activity was 56 % and 52% for soytone and meat extract respectively after 21 hours. Casamino acid (48%), yeast extracts (34%), malt extracts (34%), peptone (34%) and tryptone 27%, gave a significant emulsification index (E24) which correlated with the increased in biomass.

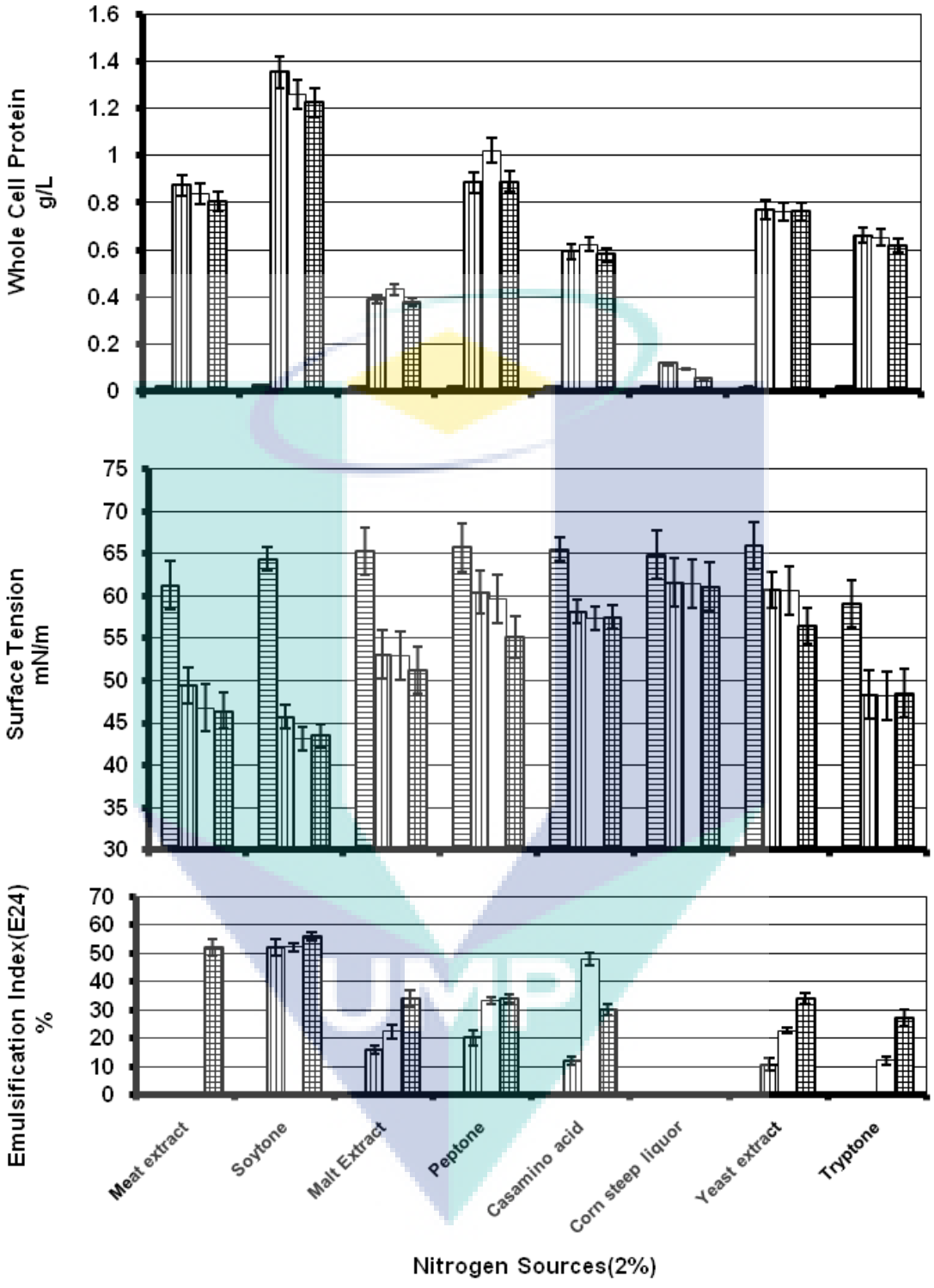


Figure 4.4: Effect of Nitrogen Sources on Bacterial Growth and Biosurfactant production by *Pseudomonas aeruginosa* KRT-142 as Determined by Whole Cell Protein, Surface Tension Reduction and Emulsification Index (E24). Different Nitrogen sources: 2.0% (w/v) of Yeast Extract, Tryptone, Soytone, Corn steep liquor, Casamino acid, Malt Extract, Peptone and Meat Extract were added into basal media at pH 7.0, 37^{o}C for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

This data was obtained based on three-time repetitions of exiperimental work which the sum of squres of pure errors is (1.371) for whole cell protein, (255.03) for surface tension, (72.0032) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses, The computed F-values of 321.05 (whole cell protein), 13.41 (surface tension) and 328.87 (emulsification index (E24) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix C1, C2 and C3).

The results suggested that the biosurfactant production of *Pseudomonas aeruginosa* KRT-142 when grown in soytone and meat extract was growth dependent (Figure 4.5). However, casamino acid and yeast extract through supported good growth but biosurfactant activity was lower to malt extract which gave 34 % emulsification index (E24) and reduced the surface tension of the medium from 66.1 mN/m to 51.2 mN/m after 21 hours.

Various nitrogen sources were reported to support and enhanced the production of biosurfactant by Ghurye et al., (1994) and Matsufuji et al., (1997). In most of the studies, the levels of biosurfactant increase are marginal and are probably due to a difference in the process condition, i.e. physicochemical and engineering parameters or nutritional factors. A nitrogen source was also understood to regulate secondary metabolite (Marwick et al., 1999; Haba et al., 2000). Banat (1995b) reported that using 3.5 % of yeast extract reduced the surface tension of culture-broth to 42 mN/m on *Rhodococcus* bacterium strain ST5. Abu-Ruwaida et al., (1991b) reported that the addition of yeast extracts and trypticase soy broth generally had no effect on a biomass production but effect on biosurfactant production by increasing the emulsification index values and lower surface tension from *Rhodococcus* sp. ST-5. Kitamoto et al., (1993) using *Candida antarctica* reported that among different organic nitrogen sources, polypepton and beef extract only stimulated cell growth and the highest yield of biosurfactant (Mannosylerythritol lipids) was obtained with yeast extract. With soytone, most of the biosurfactants was produced by *Pseudomonas aeruginosa* KRT-142 at the late exponential growth phase and the maximum level was obtained at the stationary growth phase.

iii. Effect of Different Concentrations of Ethanol

Since ethanol is a crucial source for biosurfactant production, it was important to study the effect of different concentration of ethanol on the growth and biosurfactants production. The results show that the ethanol at 1.5% (1.243 g/L) gave the highest growth followed by 2%, 1%, 2.5%, 3% and 3.5% in seven hours (Figure 4.5). Ethanol at 1.5% yielded the highest biosurfactant production, reducing the surface tension of the culture medium to 40.3 mN/m in seven hours. Ethanol at 3% reduced the surface tension of the culture medium to 59.13 mN/m. Concentration at 1% reduced the surface tension to 54.26 mN/m in seven hours. The highest emulsifying activity at 1.5% (64%), Ethanol concentration at 3% gave 34.43% emulsification index (E24), concentration at 1% gave 46% in 14 hours. The decrease in biosurfactant production at a high concentration of ethanol may be due to ethanol deficiency, which became significant below 2% v/v conversely the decrease also may result from inhibition by ethanol concentration.

The data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (3.800) for whole cell protein, (144.7424) for surface tension, (134.00) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses. The computed F-values of 322.15 (whole cell protein), 37.99 (surface tension) and 214.56 (emulsification index (E24)) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix D1, D2 and D3).

Kim et al., (1997b) demonstrated that the soybean oil concentration varied from 20 g/L to 100 g/L in medium containing 100 g/L glucose, the sophorose lipid yield from soybean oil and the production rate of sophorose lipid were maximum at 40 g/L and 80 g/L respectively, by *Torulopsis bombicola*. Adamczak and Bednarski (2000) demonstrated that *Candida antarctica* synthesised a surface-active mannosylerythritol lipids at maximum by adding 80 g / L soybean oil to the medium.

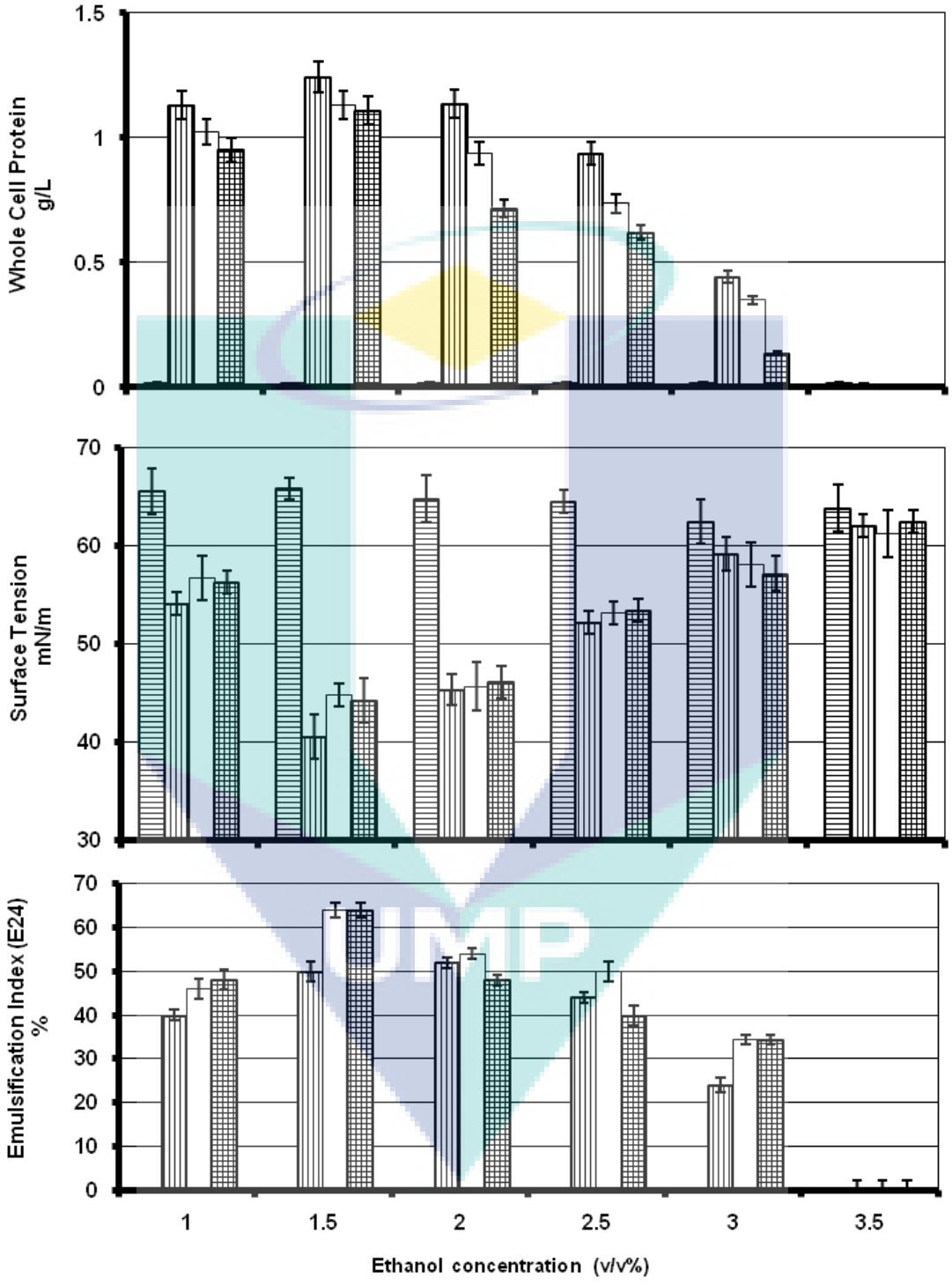


Figure 4.5: Effect of Different Ethanol Concentrations on Bacterial Growth and Biosurfactant production by *Pseudomonas aeruginosa* KRT-142 as Determined by Whole Cell Protein, Surface Tension Reduction and Emulsification Index (E24). Ethanol was added into basal media at pH 7.0, 37^{o}C for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

Biosurfactant production by *Pseudomonas sp*. which were dependent on nutritional and environmental factors has been previously studied by Robert et al., (1991) and Mercade et al., (1993). Patel and Desai (1997) reported that the biosurfactant production by *Pseudomonas aeruginosa* GS3 increased with the increase of molasses concentration, and maximum production occurred when 7% (v/v) of molasses were used; further increase in the concentration of molasses decreased the surfactant production significantly.

On the other hand, the biomass increased with the increase in the concentration of molasses, as evident from the whole cell protein. Arino et al., (1996) demonstrated that the glycosides produced by *Pseudomonas* species increased with the increase in the concentration of peptone, and maximum production occurred when 5 g/L of peptone was used, and further increase in the concentration of peptone decreased the surfactant production. Mercade et al., (1993) demonstrated that the addition of different amounts of sodium nitrite (1-6 g / L), led to the greatest drop in surface tension of a culture medium, indicating the highest concentration of rhamnolipids. Davila, et al., (1997) reported that *Candida bombicola* CBS 6009 produced at a maximum rate of sophorose lipid when the concentration of glucose solution was increased from 0.6 g / L to 500 g / L.

iv. Effect of Different Metal Ions

The basal medium employed for this study was Bushnell Hass, which consisted of (g/L); 0.2 $MgSO_4$, 0.02 $CaCl_2$, 1.0 $KHPO_4$, 1.0 $(NH_4)_2$ PO_4, 1.0 KNO_3, 0.05 $FeCl_3$. Different concentrations of selected metal ions, different concentrations of zinc sulphate and manganese sulphate were tested to optimize the basal medium.

iv.1 Effect of Different Concentrations of Ferric Chloride

Luxuriant growth was observed in all concentrations (0 - 0.1 g / L) of ferric chloride. The presence of ferric chloride ions was not necessary for the production of biosurfactant from *Pseudomonas aeruginosa* KRT-142. However, increasing the

concentrations (0 - 0.01 g / L) of the ion in the media was enhanced the biosurfactant yield proportionally. A concentration of 0.05 g / L of ferric chloride maximized the biosurfactant production, whereas higher concentrations considerably repressed the biosurfactant synthesis (Figure 4.6). This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (2.825) for whole cell protein, (122.0016) for surface tension, (132.5016) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses. The computed F-values of 199.68 (whole cell protein), 14.99 (surface tension) and 164.30 (emulsification index (E24)) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix E1, E2 and E3).

Pseudomonas aeruginosa KRT-142 exhibited a similar growth pattern in all concentrations, of ferric chloride, and it started to produce biosurfactant at the late-exponential phase and maximum productionwas reached at the stationary phase (Figure 4.6). Currently, the molecular mechanism underlying metal ion regulation remains unknown. Moreover, many researchers have found that biosurfactant liberated in the presence of iron salts was more active and stable. Therefore, based on the cost effectiveness, availability, and stability, stimulating effects on surfactant actions contributed to optimize the biosurfactant of *Pseudomonas aeruginosa* KRT-142 by adding the ferric chloride in the basal medium.

Persson et al., (1990) reported that when the supplement of iron to the medium was omitted, the product yield increased, and the culture turned to yellow green due to siderophore production. Zenaitis and Cooper (1994) showed that with no iron in the medium, the average production of tetracycline per cycle was 6.6 mg/L., in media contained varying amounts of $FeSO_4$ $7H_2O$. An addition of 0.2 ppb of iron resulted in a slightly lower production of tetracycline (5.4 mg/L), but for iron concentrations of 0.4 ppb and greater, no tetracycline was detected from *Streptomyces aureofaciens*.

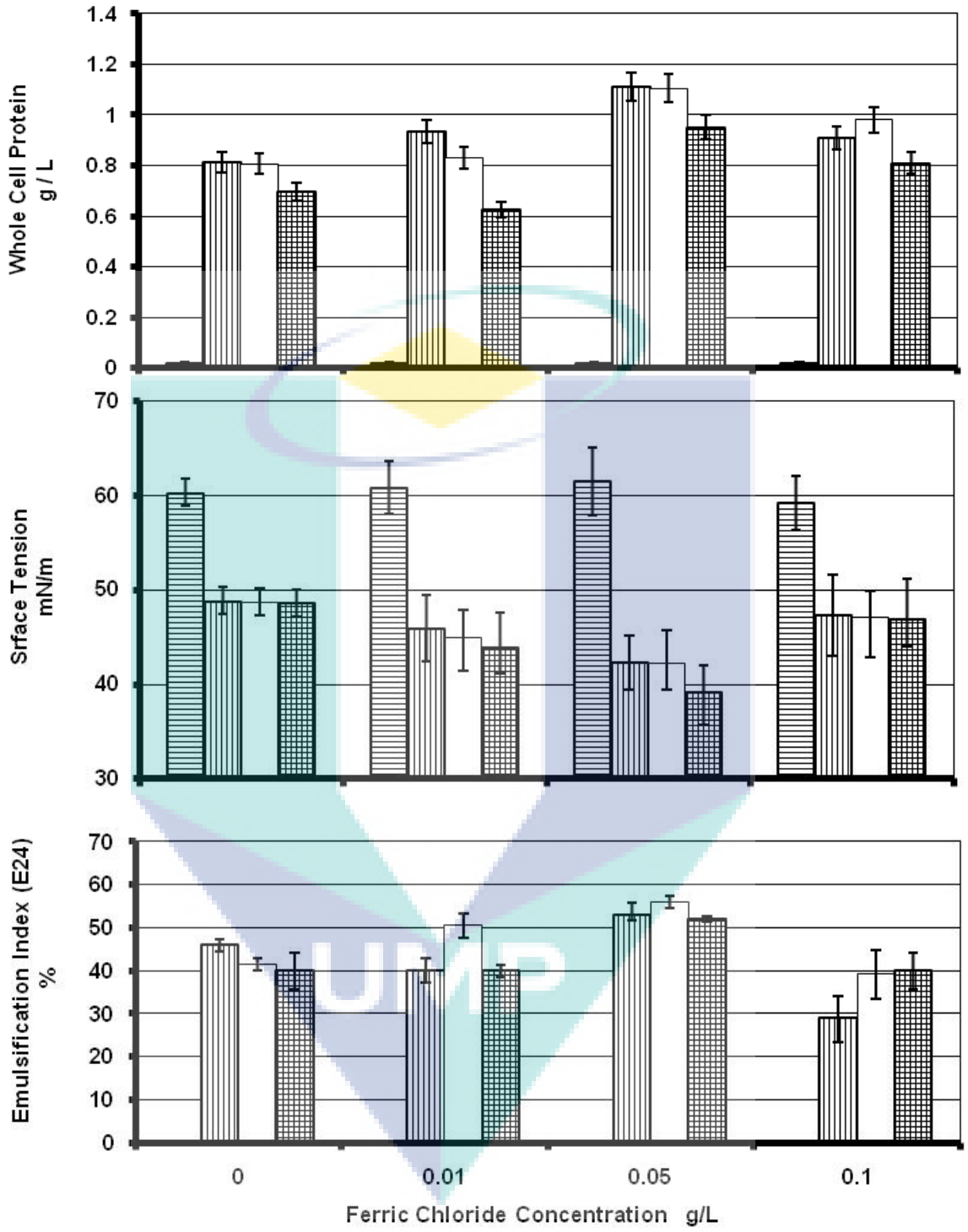


Figure 4.6: Effect of Different Ferric Chloride Concentrations on Bacterial Growth and Biosurfactant production by *Pseudomonas aeruginosa* KRT-142 as Determined by Whole Cell Protein, Surface Tension Reduction and Emulsification Index (E24). Ethanol, was added into basal media at pH 7.0, 37^{o}C for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

iv.2 Effect of Different Concentrations of Magnesium Sulphate

The possible effect by different concentrations of magnesium sulphate was also examined (Figure 4.7). Maximum biomass observed at 0.2 g / L of magnesium sulphate. However, increasing the concentrations (0 - 0.2 g / L) of the magnesium sulphate in the media was shown to enhance the biosurfactant yield proportionally. Biosurfactant was produced initially at the late-exponential phase, and the maximum production was reached at the stationary phase. A concentration of 0.2 g / L of magnesium sulphate maximized the biosurfactant production, whereas higher concentrations considerably repressed the biosurfactant synthesis (Figure 4.7). This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (2.310) for whole cell protein, (122.00) for surface tension, (116.00) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses. The computed F-values of 278.27 (whole cell protein), 17.60 (surface tension) and 127.37 (emulsification index (E24)) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix F1, F2 and F3). The results were in agreement with Patel and Desai (1997) who showed that 0.2 g/L of magnesium sulphate was the optimum concentration for biosurfactant produced by *Pseudomonas aeruginosa* GS3.

The optimum magnesium concentration was demonstrated by many researchers. Deziel et al., (2000) reported that optimum concentration of magnesium sulphate was 0.4 g /L for rhamnolipids produced by *Pseudomonas aeruginosa* 57RP. For glycolipid produced by *Pseudomonas species* the optimum concentration of magnesium sulphate was 0.1 g/L (Arino et al., 1996).

iv.3 Effect of Different Concentrations of Calcium Chloride

This experiment was carried out by supplementing the basal medium with various concentrations of calcium chloride. The results indicated that bacterial growth and biosurfactant activity decreased dramatically in the absent of calcium chloride (Figure 4.8).

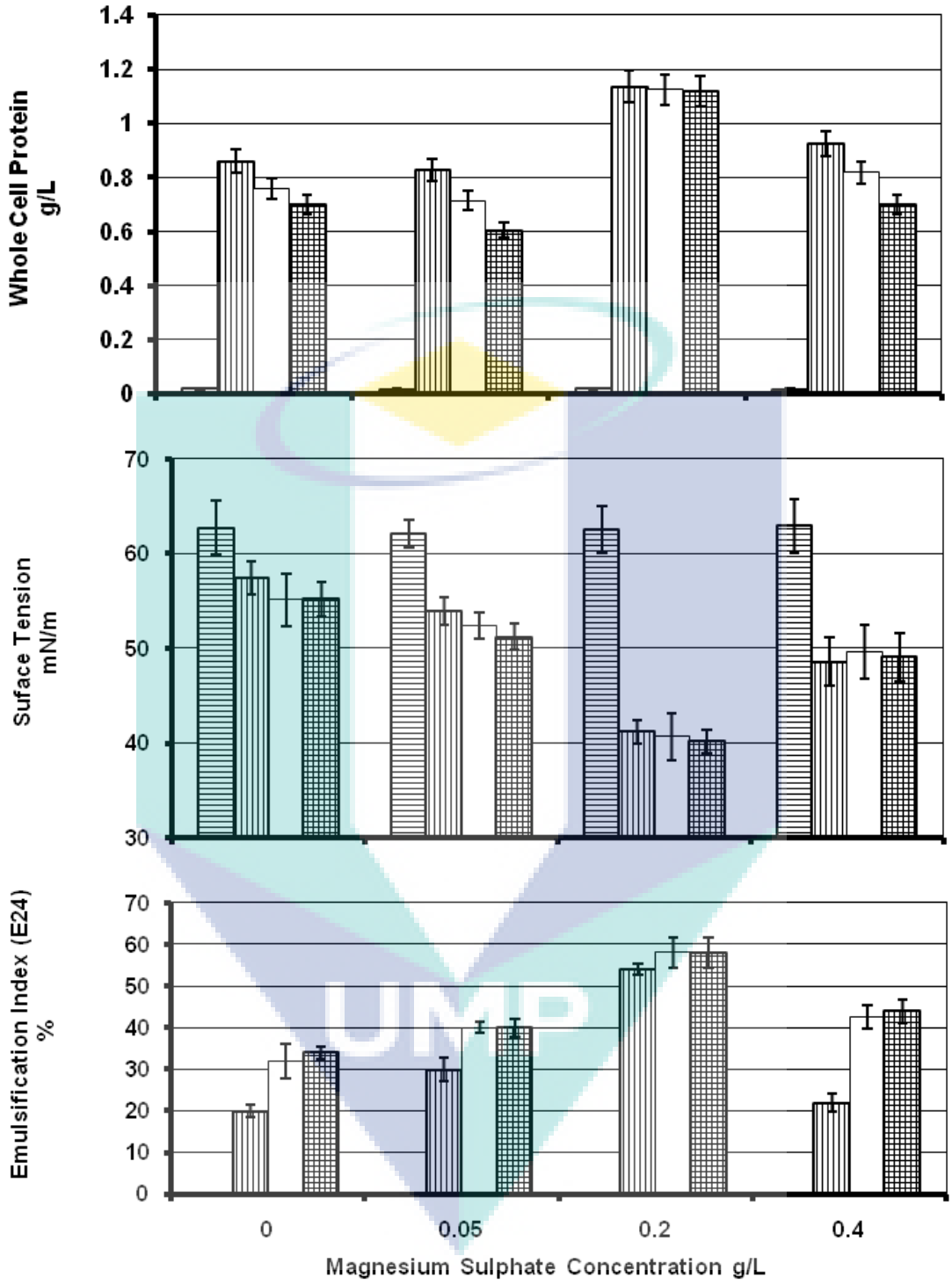


Figure 4.7: Effect of Different Magnesium Sulphate Concentrations on Bacterial Growth and Biosurfactant production by *Pseudomonas aeruginosa* KRT-142 as Determined by Whole Cell Protein, Surface Tension Reduction and Emulsification Index (E24). Ethanol, was added into basal media at pH 7.0, 37^{o}C for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

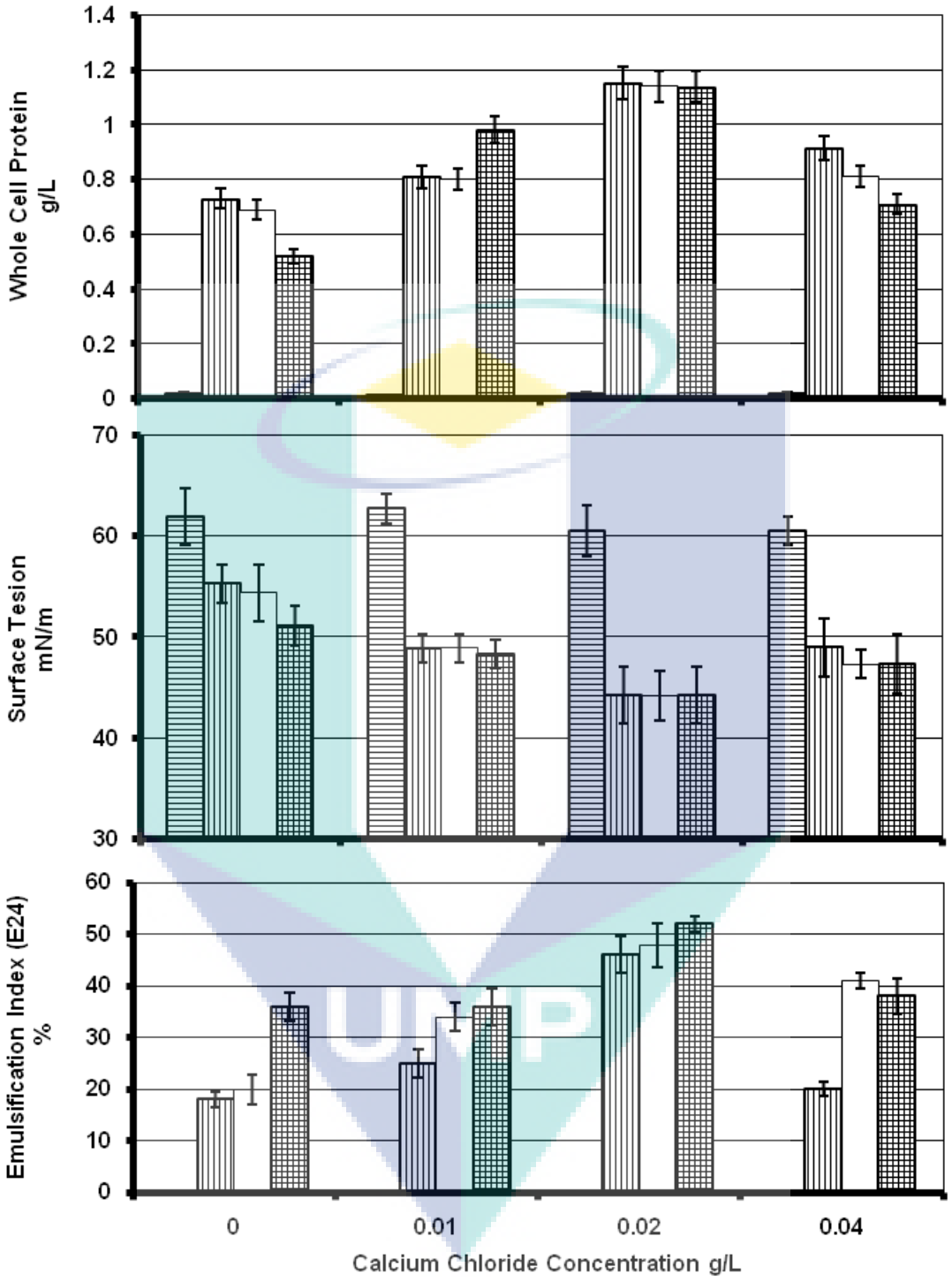


Figure 4.8: Effect of Different Calcium Chloride Concentrations on Bacterial Growth and Biosurfactant production by *Pseudomonas aeruginosa* KRT-142 as Determined by Whole Cell Protein, Surface Tension Reduction and Emulsification Index (E24). Ethanol, was added into basal media at pH 7.0, 37^{o}C for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

The calcium chloride at 0.02 g / L supported the highest growth followed by 0.04 g / L and 0.01 g / L. Calcium chloride at 0.02 g / L yielded the highest biosurfactant production, reducing the surface tension to 44.2 mN/m followed by 0.04 g / L and 0.01 g/L.

The maximum emulsifying activity (52%) was obtained at 0.02 g / L of calcium chloride. A concentration at 0.04 g / L and 0.01 g / L gave 38% and 36% emulsifying activity respectively. This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (2.450) for whole cell protein, (89.00) for surface tension, (92.00) for emulsification and the probability are less than1% (P< 0.0001) for all responses, The computed F-values of 279.12 (whole cell protein), 16.15 (surface tension) and 193.20 (emulsification index (E24) were higher than the values shown in the statistical tables which are significant at P< 0.01 level (Appendix G1, G2 and G3).

Tuleva et al., (2002) reported that the maximum biosurfactant production by *Pseudomonas putida* was achieved at 2 mg / L of calcium chloride. *Bacillus subtilis* C9-BS retained all of its biosurfactant activity (surface tension reduction) at a concentration up to 1.10 g / L calcium chloride and the maximum emulsification index (E24) was obtained at a concentration of 1.10 g / L calcium chloride (Kim et al., 1997a). The effect of calcium is an important consideration for field applications because precipitation can cause major losses of surfactant, which can in turn affect the performance of the surfactant system (Bai et al., 1998).

iv.4 Effect of Different Concentrations of Zinc Sulphate and Manganese Sulphate

Biosurfactant production was detected in a medium containing different concentrations of zinc sulphate and manganese sulphate individually. The results indicated that bacterial growth and biosurfactant activity decreased dramatically in the absent from zinc sulphate. Among the different concentrations of zinc sulphate tested, the bacterial growth was also detected in a medium incorporated with concentration of zinc sulphate at 0.05 g / L, and gave 52% emulsification index (E24) after 21 hour incubation compared with the basal medium at zero times (Figure 4.9).

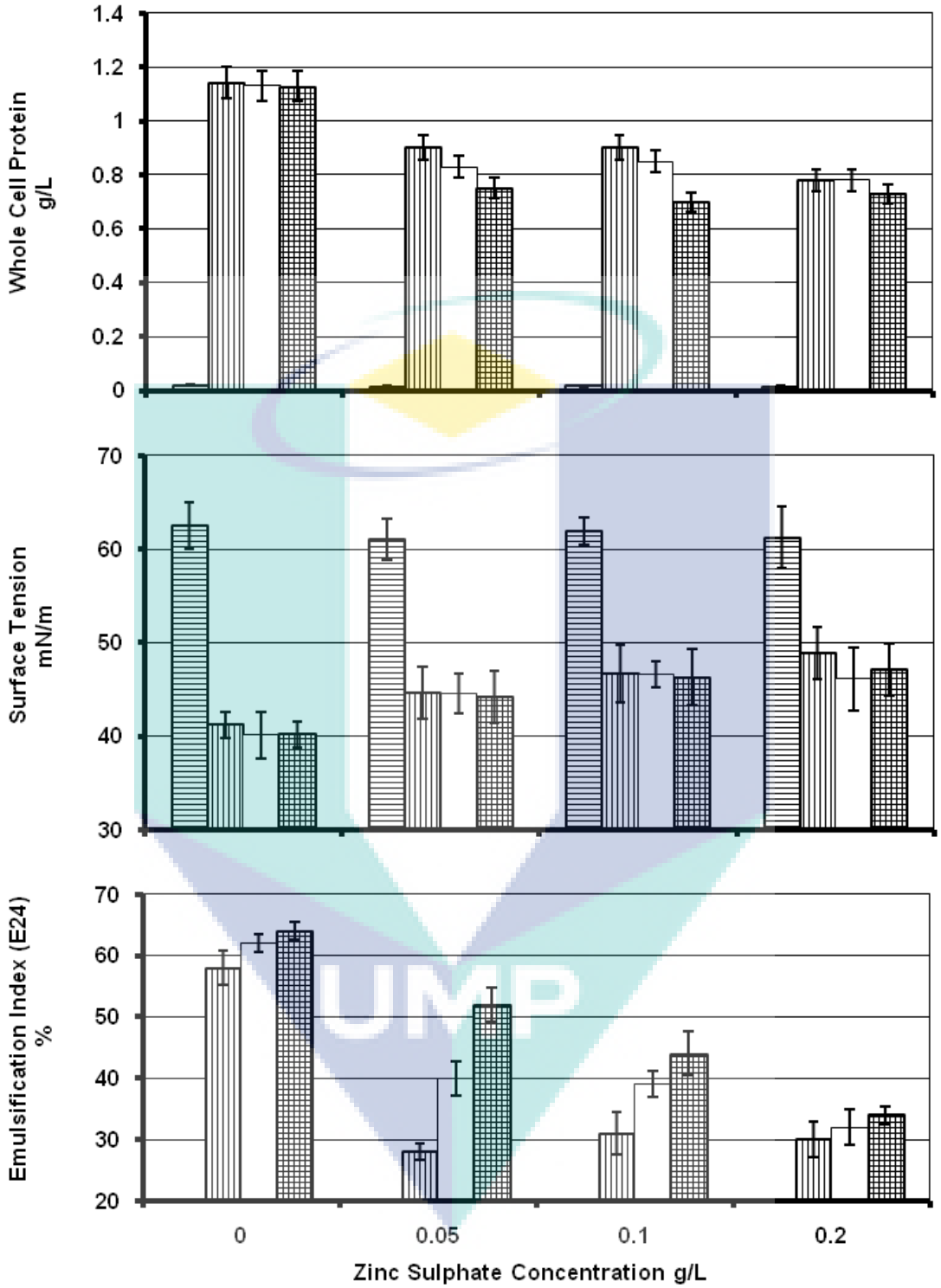


Figure 4.9: Effect of Different Zinc Sulphate Concentrations on Bacterial Growth and Biosurfactant Production by *Pseudomonas aeruginosa* KRT-142 as Determined by Whole Cell Protein, Surface Tension Reduction and Emulsification Index (E24). Ethanol, was added into basal media at pH 7.0, 37^{o}C for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

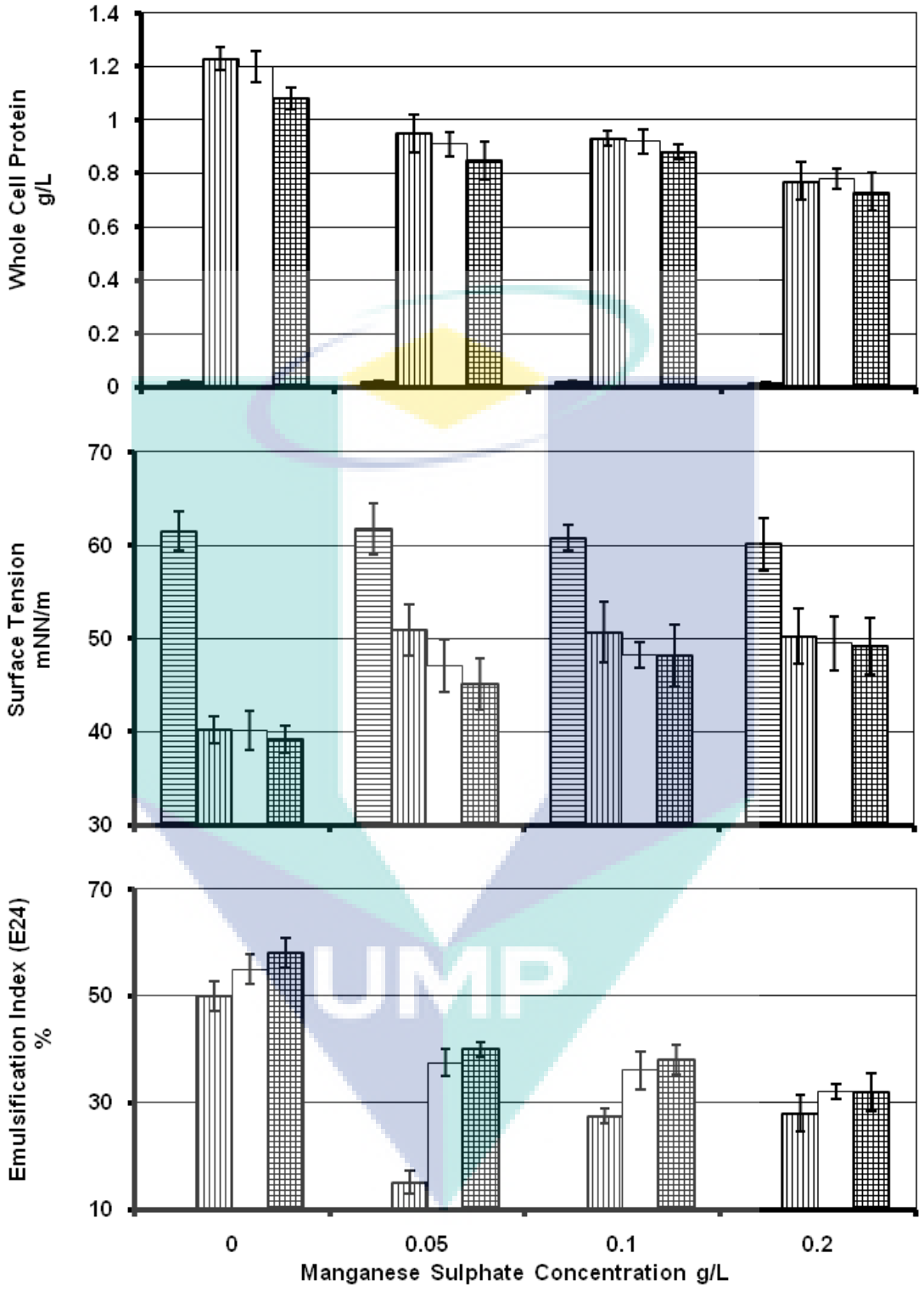


Figure 4.10: Effect of Different Manganese Sulphate Concentrations on Bacterial Growth and Biosurfactant Production by *Pseudomonas aeruginosa* KRT-142 as Determined by Whole Cell Protein, Surface Tension Reduction and Emulsification Index (E24). Ethanol, was added into basal media at pH 7.0, 37oC for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

The surface tension activity in the presence of 0.05 g/L, 0.1 g/L and 0.2 g/L zinc sulphate after seven hour incubation was 44.7 mN/m, 46.9 mN/m and 48.5 mN/m respectively. This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (3.780) for whole cell protein, (128.00) for surface tension, (78.50) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses, The computed F-values of 152.99 (whole cell protein), 18.01 (surface tension) and 211.81 (emulsification index (E24)) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix H1, H2 and H3).

Supplementation of a basal medium with various concentrations of manganese sulphate reduced the biosurfactant activity. At a concentration of 0.05 g / L the emulsification index (E24) was 28% after seven hours but after 21 hours of incubation it increased to 52% emulsification index (E24). The cell growth values obtained with various concentrations of manganese sulphate were considerably lower than those observed in a basal medium (Figure 4.10). The results showed that a surface tension activity at different concentrations of manganese sulphate was 45.2 mN/m, 48.3 mN/m and 49.3 mN/m at 0.05 g / L, 0.1 g / L and 0.2 g / L respectively after 21 hour incubation. This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (3.012) for whole cell protein, (118.34) for surface tension, (82.00) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses. The computed F-values of 145.07 (whole cell protein), 18.30 (surface tension) and 240.55 (emulsification index (E24)) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix I1, I2 and I3).

Deziel et al., (2000) demonstrated that maximum rhamnolipids production by *Pseudomonas aeruginosa* 57RP were obtained in the absence of zinc and manganese salts. Lower levels of biosurfactant were observed when zinc sulphate and manganese chloride were used in the medium (Kosaric et al., 1990). This observation suggested that zinc and manganese sulphate were not essential for growth and biosurfactant production by *Pseudomonas aeruginosa* KRT-142.

4.3.2 Effect of Physical Factors

i. Effect of Temperature

Investigations on the effect of cultivation temperatures on cell growth and biosurfactant production at different incubation temperatures: 5, 30, 35, 40, 45 and 50°C have been carried out in basal media. Growth and biosurfactant production by *Pseudomonas aeruginosa* KRT-142 was detected at various temperatures within the range of 30 to 40°C, with 35°C being the optimum temperature. Higher temperatures at 40^{o}C caused a decreased on surface tension reduction and emulsification index (E24) (Figure 4.11).

Lower or higher temperatures generally had a depressing effect on both growth and biosurfactant production as detected by lower cell yields and biosurfactant production determined by surface tension reduction and emulsification index (E24). This effect was more evident at the higher temperatures, the biomass decreasing from 1.241 g/L at 35^{o}C to 0.314 g/L as the cultivation temperature was increased to 40^{o}C. This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (2.945) for whole cell protein, (216.06) for surface tension, (52.00) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses, The computed F-values of 283.46 (whole cell protein), 11.05 (surface tension) and 520.40 (emulsification index (E24)) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix J1, J2 and J3). The biosurfactant properties were affected with the changing of cultivation temperatures. Lower than 35^{o}C (5^{o}C and 30^{o}C) or higher than 35^{o}C (40^{o}C, 45^{o}C and 50^{o}C) lead to change in the microbial metabolism and had a depressing effect on the biosurfactant production which was indicated by the lower emulsification index (E24) and lesser surface tension reduction. No bacterial growth and biosurfactant activity were observed at 5°C or at 45°C or at higher temperatures. Hence, based on the optimum temperature, *Pseudomonas aeruginosa* KRT-142 can be classified as a mesophilic organism.

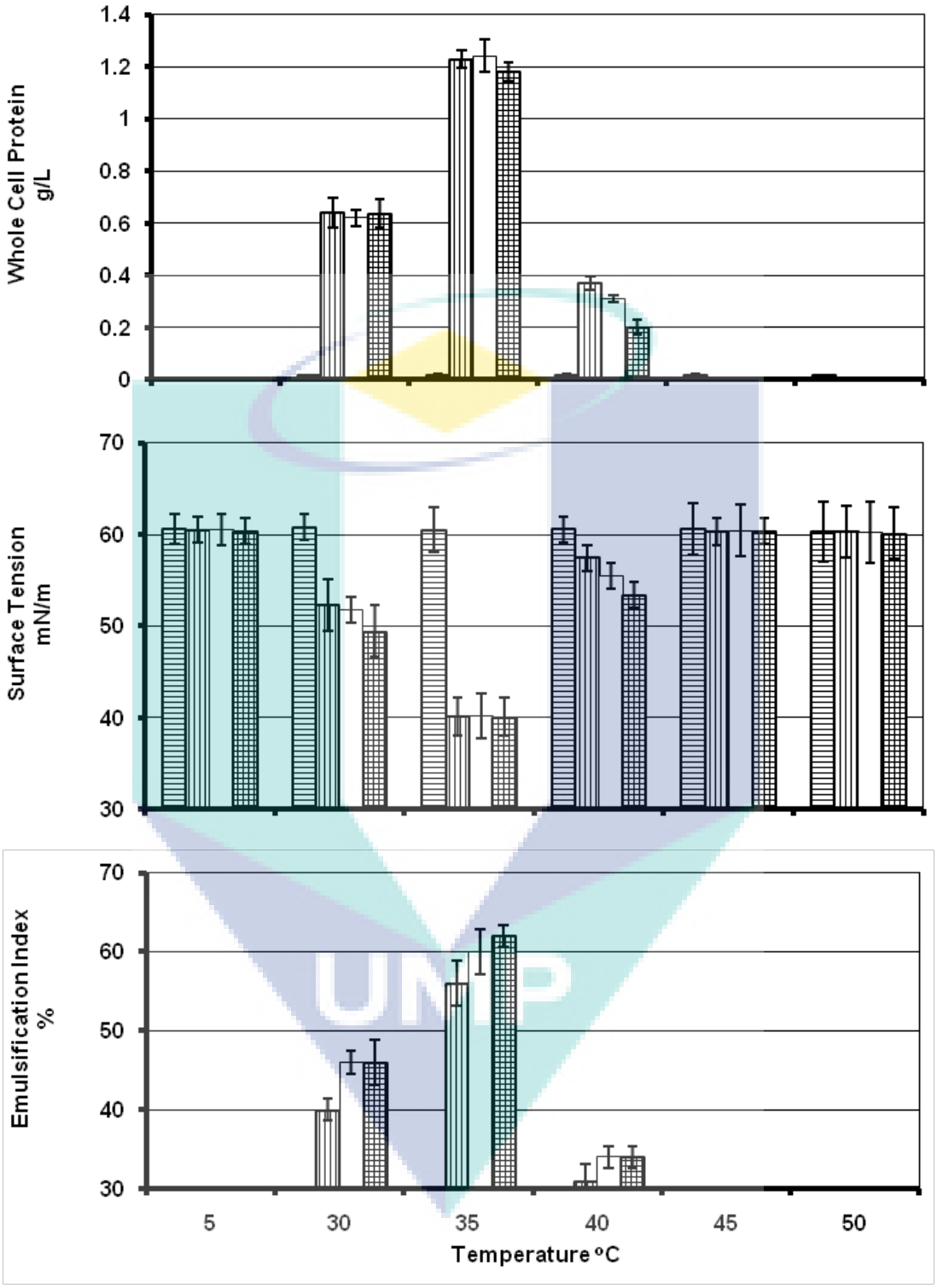


Figure 4.11: Effect of Temperature on the Growth and Biosurfactant Produced by *Pseudomonas aeruginosa* KRT-142 as Determined by Surface Tension and Emulsification Index (E24). Ethanol, was added into basal media at pH 7.0 for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

Abu-Ruwaida et al., (1991b) who worked on *Rhodococcus* sp. ST-5 reported that optimal growth and biosurfactant production were obtained at 37^{o}C, which gave 40% emulsification index (E24). The optimal temperature for biosurfactant production by *Bacillus subtilis* was 37°C (Peypoux et al., 1999). Rocha and Infante (1997) demonstrated that the optimal biodegradation of an oily sludge by a microbial tensio-active agent isolated from *Pseudomonas aeruginosa* USB-CSI was 30°C. On the other hand, Kendrick and Ratledge (1992) reported that the percentage of polyunsaturated fatty acids (PUFA) increased proportionally from 18 to 27% (w/w) of the total fatty acids when the growth temperature was decreased from 30 to 20°C by the oleaginous fungus *Entomophthora exitalis*. The highest performance for sophorose lipid by *Candida bombicola* was at 25°C (Davila et al., 1997).

ii. Effect of Inoculums Size

The amount of inoculum used to culture the bacteria affect on the biosurfactant production of *Pseudomonas aeruginosa* KRT-142. The results indicated that, bacterial growth and biosurfactant production (Figure 4.12) were optimum when 4.0% (v/v) of bacterial inoculum was used and the culture was incubated for 21 hours. A reduction in whole cell protein (from 1.56 g / L to 0.68 g / L) at seven hours was observed in bacterial growth if the inoculum size was increased to 16%. Biosurfactant production with 4% (v/v) of bacterial inoculum reduced the surface tension to 46.2 mN/m at 14 hours and emulsification index (E24) obtained was 60% 21 hours. Higher inoculums size at 8.0% (v/v), 12.0% (v/v) and 16.0% (v/v) gave 54%, 45% and 42% (E24), respectively (Figure 4.13). High inoculums sizes might not necessarily give higher biosurfactant yield or cell growth. On the contrary, higher inoculum sizes could result in the lack of oxygen and nutrient depletion in the culture media. This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (4.900) for whole cell protein, (176.00) for surface tension, (112.00) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses, The computed F-values of 148.79 (whole cell protein), 13.99 (surface tension) and 240.75 (emulsification index (E24)) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix K1, K2 and K3).

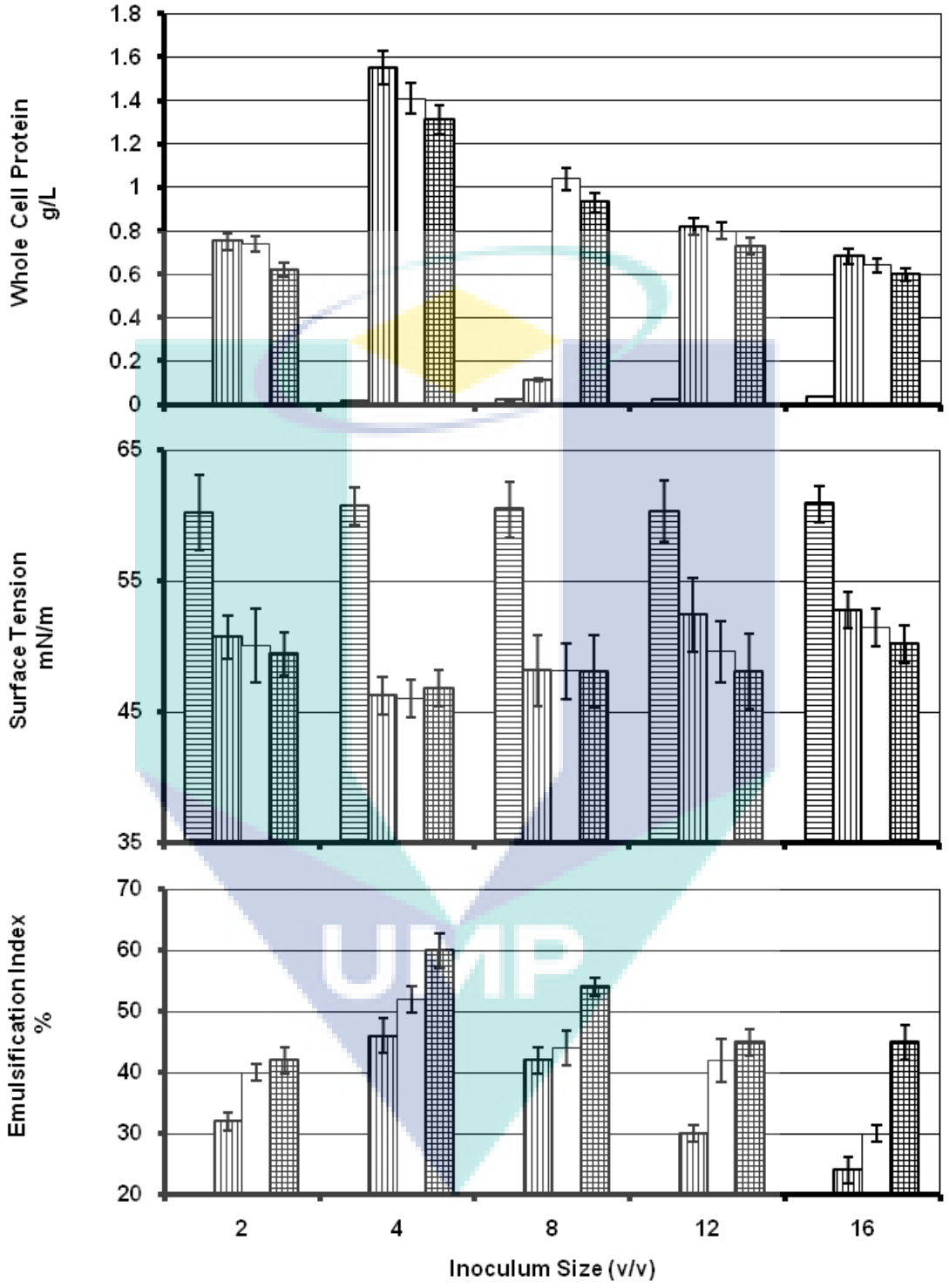


Figure 4.12: Effect of Inoculum Size on the Growth and Biosurfactant Produced by *Pseudomonas aeruginosa* KRT-142 as Determined by Surface Tension and Emulsification Index (E24). Ethanol, was added into basal media at pH 7.0, 37°C for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

Different optimum inoculum sizes have been reported by other workers for bacteria, 1.0% (v/v) for *Pseudomonas aeruginosa* IFO 3924 (Matsufuji et al., 1997), 2% for *Bacillus subtilis* (Makkar and Cameotra, 1997), 5.0% (v/v) for *Pseudomonas aeruginosa* UW-1 (Sim et al., 1997), 10.0% (v/v) for *Pseudomonas putida* (Tuleva et al., 2002) and 15% for *Nocardia sp.* L-417 (Kim et al., 2000). Therefore, microorganisms required different percentages of inoculum sizes for maximum biosurfactant yield.

iii. Effect of Agitation Rate

Agitation rates influenced the biosurfactant yield and cell growth of *Pseudomonas aeruginosa* KRT-142. Maximum bacterial growth and biosurfactant production were obtained when the culture medium was grown at 100 rpm condition (Figure 4.13). In general, shaking the cultures at various shaking rates increased the growth of *Pseudomonas aeruginosa* KRT-142 when changing the cultural condition from static (0.65 g/L) to 50 rpm (0.94 g/L). When this bacterium was grown and agitated at 150 rpm the growth increased to 1.39 g/L (Figure 4.13). Similarly, the biosurfactant production yield was increased at 21 hour incubation Agitation rate of 50 rpm increased the emulsification index (E24) to 32% and 52% at shaking rates above 200 rpm. This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (7.765) for whole cell protein, (186.04) for surface tension, (82.00) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses, The computed F-values of 213.74 (whole cell protein), 22.31 (surface tension) and 410.09 (emulsification index (E24)) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix L1, L2 and L3).

Production of biosurfactant was highest in cultures agitated at 130 rpm for *Pseudomonas putida* (Tuleva et al., 2002). Thus, comparing the effect of shaking condition with a non-shaking condition on the biosurfactants production by this bacterium showed that shaking increased the yield of biosurfactants production.

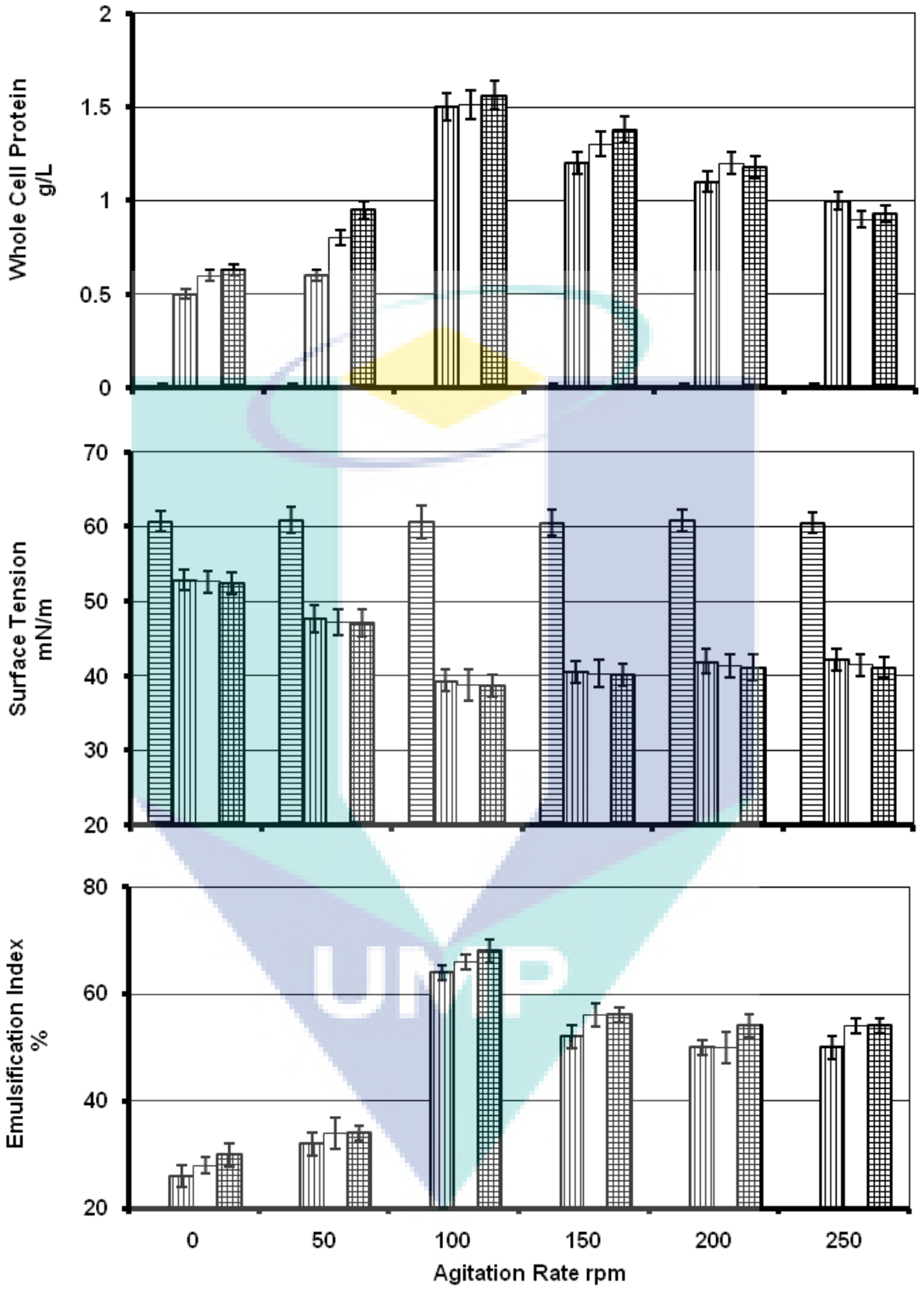


Figure 4.13: Effect of Agitation Rates on the Growth and Biosurfactant Produced by *Pseudomonas aeruginosa* KRT-142 as Determined by Surface Tension and Emulsification Index (E24) Ethanol, was added into basal media at pH 7.0, 37°C for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

iv. Effect of Initial pH

The effect of initial pH on growth and biosurfactant production or activity is shown in Figure 4.14. Luxuriant growth by *Pseudomonas aeruginosa* KRT-142 was detected at pH ranging from 6.2 to 7.8 with the maximum cell growth exhibited at pH 7.2. Extracellular biosurfactant was detected over a broad pH ranging from 6.2 to 7.8, with optimum production exhibited at pH 7.2. No biosurfactant activity was detected when the bacteria were grown at pH 6.0 and pH 8.0 even though there was a low bacterial growth observed (Figure 4.14).

Biosurfactant production, as determined by surface tension and emulsification index (E24) measurements was less influenced by the cultivation pH, since the biosurfactant production or activity was highest within a wide pH range (6.6 to 7.6). This data was obtained based on three-time repetitions of experimental work which the sum of squares of pure errors is (4.904) for whole cell protein, (278.50) for surface tension, (182.50) for emulsification and the probability are less than1% ($P< 0.0001$) for all responses, The computed F-values of 23.10 (whole cell protein), 14.71 (surface tension) and 256.18 (emulsification index (E24)) were higher than their tabular value, which is significant at $P< 0.01$ level (Appendix M1, M2 and M3).

These findings suggest that the culture, although maximum growth occurs within a narrow pH range, can still produce biosurfactant effectively in a wider pH range, which is useful for large-scale production where unexpected changes in pH can occur. In this study, maximum yield of *Pseudomonas aeruginosa* KRT-142 was achieved at pH 7.2. It was also proposed that different types of biosurfactant could be regulated by varying the pH of the media. Similar results have been reported by Arino et al., (1996) who found that the biosurfactant from *Pseudomonas species* decreased rapidly at higher and lower of pH 7.0.

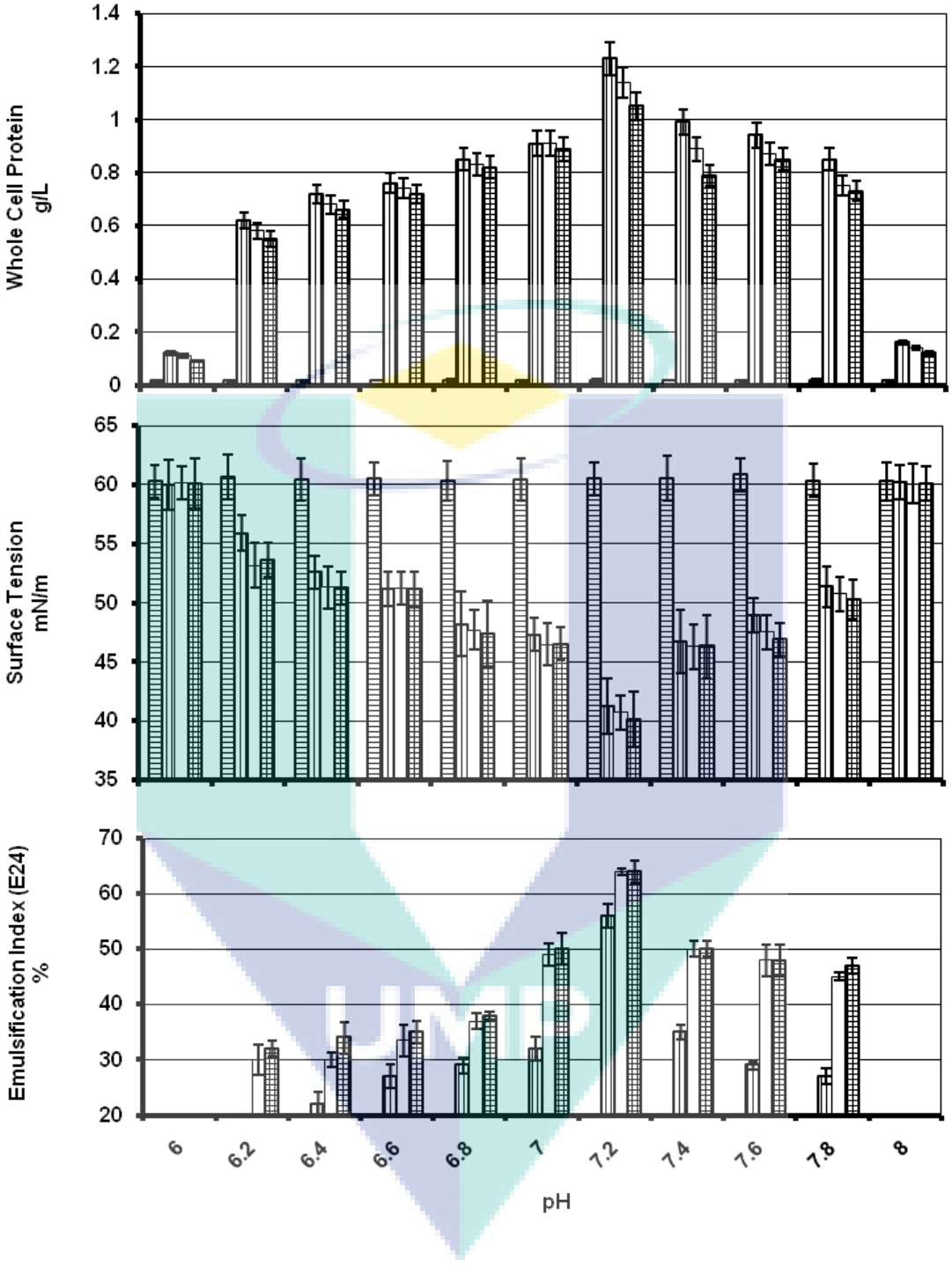


Figure 4.14: Effect of Initial pH on the Growth and Biosurfactant Produced by *Pseudomonas aeruginosa* KRT-142 as Determined by Surface Tension and Emulsification Index (E24). Ethanol, was added into basal media at 37°C for 0 (▤) 7 (▥), 14 (□) and 21h (▦).

Production of high biosurfactant by *Bacillus subtilis* was observed in a neutral medium (Makkar and Cameotra, 1997). Abu-Ruwaida et al., (1991b) reported that highest growth and biosurfactant production was obtained at pH 6.5 and 6.8 by *Rhodococcus* sp. ST-5. In contrast, maximum production of biosurfactant by *Pseudomonas species* was reported at pH 13.5 (Morikawa et al., 2000), pH 3.3 for *Candida bombicola* (Daniel et al., 1998).

4.4 GROWTH CURVE IN OPTIMIZED MEDIUM CONDITION

The optimization studies have defined a medium composition and conditions which were 1.5% ethanol, basal medium (metal ions), 35^{o}C, 4% inoculums size, agitation rate 100 rpm and pH 7.2 for maximum biosurfactants production by *Pseudomonas aeruginosa* KRT-142. It was established specifically for optimum biosurfactant production by this strain, and it might not be suitable for other strains or microorganisms. Each organism or strain has its own defined medium and special conditions for maximum enzyme production (Kumar and Takagi, 1999).

A time course study was performed to determine the growth and biosurfactant production in an optimized growth medium. Figure 4.15 shows the different parameters determined at 24-hour intervals. Maximum growth and whole cell protein of this bacterium were achieved after six-hour incubations. In an optimized medium, the logarithmic phase commenced at first hour and continued for another hour. Biosurfactant production was detected in the logarithmic phase (after two hour), and it increased until its optimum production after six-hour incubations. Since biosurfactants are secondary metabolites, maximal biosurfactants production was obtained when the cells reached the stationary phase of growth.

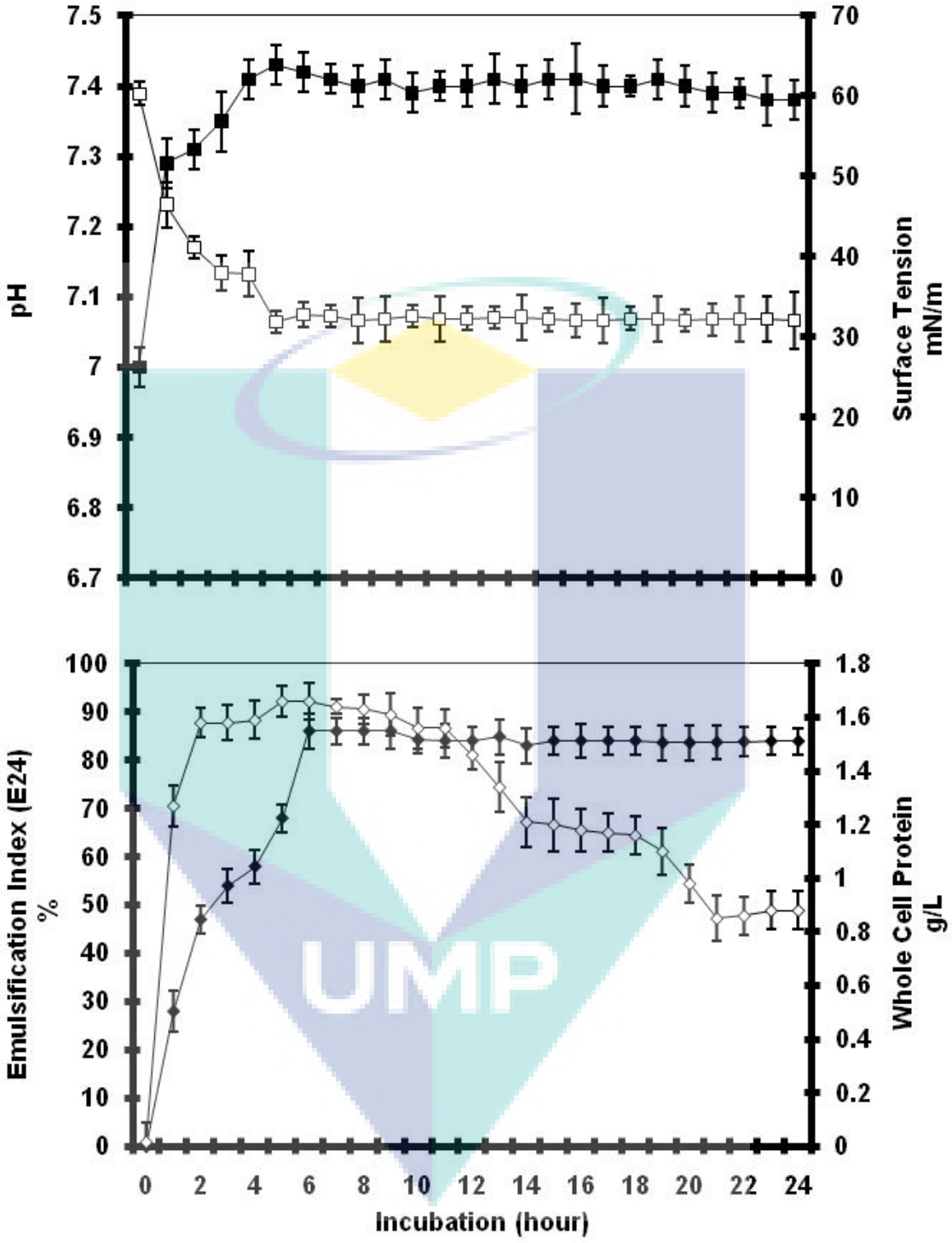


Figure 4.15: Growth Curve and Biosurfactant Production by *Pseudomonas aeruginosa* KRT-142 in Optimized Medium (1.5% ethanol, basal medium, 35^{o}C, 4% inoculums size, agitation rate 100 rpm) at pH 7.2. Samples were withdrew at 24h interval and Biosurfactant Determined by Surface Tension (□), Emulsification Index (E24)(◆), pH of culture medium (■) and Whole Cell Protein (◇).

This result showed that *Pseudomonas aeruginosa* KRT-142 biosurfactant was growth associated. The production of biosurfactant normally occurs in the logarithmic phase of growth, when the cell density is high. Biosurfactants secreted caused a drop in the surface tension to 46 mN/m even after 2 h of incubation. The surface tension reached a minimum of 30.76 mN/m after six hours in the stationary growth phase and did not decline further. Stable and compact emulsification index (E24) of hexadecane with the supernatant fluid of the culture was observed after two hour of cultivation reaching maximal value of 86% at six hours of incubation. Biosurfactant production increased progressively and maximal values were reached in the stationary phase. Tuelva et al., (2002) reported that maximal glycolipids production (expressed as rhamnose equivalents) was reached in the stationary growth phase. The isolated biosurfactant of *Candida lipolytica* reduced the surface tension to 32 mN/m (Rufino et al., 2007)

Biosurfactants activity remained in the medium for 24 hour indicating that this surfactant was quite stable. The very rapid drop of surface tension may be partially explained by the fact that ethanol utilization by the cells was enhanced by the concomitant production of biosurfactants in the growth medium. Moreover, the inoculum culture fluid may have contained diffusible auto inducers, which regulate rhamnolipids synthesis in *Pseudomonas aeruginosa* (Ochsner and Reiser, 1995). The pH of a culture medium increased gradually to pH 7.41 after 5h. The rise of pH could be due to utilization of ethanol or production of alkaline compounds during this period of time. However, further evidence is required to confirm this assumption.

4.5 RECOVERY AND QUANTIFICATION OF BIOSURFACTANT

The process for purifying rhamnolipids was based on an extraction, crystallization and chromatographic process. Same degree of purification from culture solution can be achieved by acidification. Four methods were used to purify the product; based on acidification, but with two different acids, hydrochloric acid and sulphuric acid.

The results show (Table 4.3) that acidification using hydrochloric acid gave the highest yield of pure rhamnolipids (9.44 g/L.), while using two different concentration of sulphuric acid (Daniels methods and Mixich methods) gave 3.81 g/L and 2.74 g/L of rhamnolipids respectively. On the other hand, purifying rhamnolipid from the culture solution by acidifying the culture solution and subsequently cooling the culture by Daniel's methods or heating the culture solution by Mixich methods did not give a high yield of rhamnolipid as compared to a procedure using centrifugation of the culture by Maier method and Zhang & Millar method.

These results compare favorably with those reported for other biosurfactant-producing bacteria (Mata Sandoval et al., 1999), which were purified using sulphuric acid. It may be possible to employ Maier's method for both small scale and large scale purification. Acid precipitation (Maier method) showed the most appropriate recovery method where it gave the highest yield (9.44 g/L) compared to the other three, indicating by its ability to reduce the surface tension. The isolated biosurfactant by *Candida lipolytica* corresponds to ayield of 4.5 g/L (Rufino et al., 2007)

In this study based on an amount of recovery, the most suitable method for recovery of biosurfactant was acid precipitation with a mixture of chloroform and methanol (10:1), which facilitates adjustment of the polarity of extraction agent to the product. A wide variety of organic solvents (e.g. methanol, ethanol, diethyl ether, pentane, acetone, chloroform, dichioromethane) have been used, either singly or in combination, for biosurfactant extraction (Desai and Banat, 1997).

Table 4.3: The Amount of Purified Rhamnolipids Produced by *Pseudomonas aeruginosa* KRT-142 from Four Different Methods

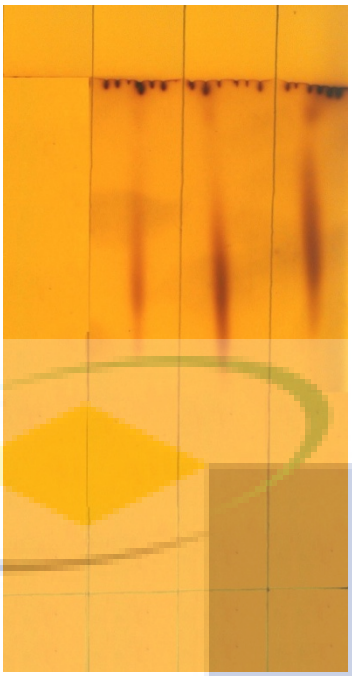
Method	**Amount of Product (g/L)**
1-Maier (2003)	9.44
2-Daniels et al., (1990)	3.81
3-Mixich et al., (1997)	2.74
4-Zhang & Millar (1992)	4.92

Most effective are the mixtures of chloroform and methanol in various ratios. Kuyukina et al., (2001) reported that higher extraction yield obtained using a mixture of chloroform and methanol (1:1) by *Rhodococcus rubber* IEGM 231, while Patel and Desai (1997) used (2:1) ratio of chloroform and methanol to extracted rhamnolipid produced by *Pseudomonas aeruginosa* GS3. The main problem in biosurfactant studies is the extraction and recovery of the product. Biosurfactant recovery depends mainly on its ionic charge, water solubility, and location such as intercellular, exteracellular or cell bound. Therefore, there are no general rules for the isolation of biosurfactants (Desai and Banat, 1997).

4.6 ANALYSIS OF BIOSURFACTANT BY THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was first performed to check the purity of the products isolated and purified from the medium at larger volumes. When subjected to the detection system (Figure 4.16), clearly faint spots at Rf values of 0.65 for products (Lanes 1-3) were detected for products from the medium.

The purified products as shown were relatively pure because no other spots were detected. Control experiments undergoing the same treatment showed no product spot. Arino et al., (1996) identified four types of rhamnolipid R1, R2, R3 and R4 produced by *Pseudomonas* strain GL1, the Rf values of these rhamnolipids were 0.72, 0.40, 0.32, 0.13 respectively. Schenk et al., (1995) reported that the Rf values of two type rhamnolipid (RL-1 and RL-2) produced by *Pseudomonas aeruginosa* DSM 2659 were 0.74 and 0.36 respectively.



1 2 3

Figure 4.16: Thin Layer Chromatogram of Purified Products. Solvent: ethyl acetate / methanol /acetic acid (80/10/10, v/v/v) Detection System: iodine vapour. 1-3 Different Concentration of the product. Lane 1, 12.5 *m*g / mL; Lane 25 *m*g / mL; Lane 3, 50 *m*g /mL

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

Five bacterial strains isolated from sea water were selected for the screening of biosurfactant producer via three different characterizations of biosurfactant (i) surface tension measurements, (ii) emulsification activity, and (iii) cetyltrimethylammonium bromide assay (CTAB) test. One mesophilic bacteria coded KRT-142, isolated from Kertih, Terengganu. From the results of the biochemical tests and their morphological characteristics, it was possible to suggest that the isolates KRT-142 might belong to *Pseudomonas aeruginosa*. These biosurfactant producers were selected to be studied further for biosurfactant production.

The production of biosurfactant by isolates was found to be growth associated at all conditions tested. Linear relationship between biosurfactant production and cell growth indicated that the biosurfactant which produced was a secondary metabolite. The efficiency of isolate to produce biosurfactant was indicated by the value of productivity and surface tension reducing an ability in the medium containing different carbon, nitrogen sources and metal ions (basal medium). This study also showed that ethanol at 1.5% gave the highest growth, highest biosurfactant production and highest emulsifying activity.

Biosurfactant production was less influenced by the cultivation pH, since the biosurfactant production or activity was high within a wide pH range (6.6 to 7.6). At the optimum conditions (35^{o}C, 4% inoculums size, 100 rpm and pH 7.2), the production of biosurfactant by isolated strain KRT-142 in the first phase of biosurfactant production occurred during the exponential phase of cell growth followed by the production at stationary growth phase. The maximum biosurfactant production was obtained during the first six hours. No addition of biosurfactant production was observed during the following 18 hours. The surface tension reached a minimum of 30.76 mN/m after six hours in the stationary growth phase and did not decline further. Stable and compact emulsification index (E24) of hexadecane with the supernatant fluid

of the culture was observed after two hours of cultivation reaching a maximal value of 86% at six hours of incubation.

5.2 RECOMMENDATION

The present study has generated much important information on the trend of biosurfactant production by the strain's isolated and their physicochemical properties of the biosurfactant produced. However, the available information is still limited on biosynthetic mechanism and structural characteristics. Thus, further studies from different fields are required in order to promote the research and development of biosurfactants. The production of biosurfactant can also be studied in a large scale, fed-batch or continuous fermentation process. The fed-batch culture would maintain the low level of the residual substrate concentration in the system. This could avoid the toxic effects of a medium component, thus enhance the production of biosurfactant. In the continuous culture, the exponential growth phase might be prolonged by the addition of a fresh medium into the fermentation system. The loss of cells in the system could be balanced by the formation of new biomass.

The structural elucidation of biosurfactant can be determined by high resolution 1H and 13C Nuclear Magnetic Resonance (NMR) Spectroscopy, High Performance Liquid Chromatography (HPLC) and Fast Atom Bombardment (FAB) Mass Spectroscopy. By these techniques, the structure of biosurfactant can be determined in a relatively short time, with a very small amount of sample. Once the structure of biosurfactant had been determined, the biosynthetic pathway of biosurfactant production could be proposed by enzymatic or radioactively labelled precursors.

Research can also be carried out on the bioavailability of biosurfactant and their effects on biodegradation of a contaminant. The study can be done by looking at the interaction of the biosurfactants and the crude oil contaminant, relationship of biosurfactant structure and the treatment of petrochemical waste, scale-up of biosurfactant production and finally, the cost reduction efforts for ex-situ production of biosurfactant.

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APPENDIX A

FORMULAE FOR STATISTICAL ANALYSIS (COCHRAN AND COX, 1992; DESIGN EXPERT VERSION 6.0.4 USER'S GUIDE)

G= Grand Total

N= Number of Points

y_{lu}= Responses at Center Points

y_i= Mean

n_1 = Total Number of Center Points

n_2= Total Number of Axial and Fractional Points

k= Degree of Freedom of First Order Coefficients

Model represents the terms estimating factor effects.

Residual are the unexplained variation seen as the difference between the observed response and the value predicted by the model for a particular design point. It is used to estimate experimental error.

Lack of Fit compares the residual error mean square (MS) to the pure error MS and represents the variation of data around the fitted model. It diagnoses how well each of the full models fit the data.

Pure Error or experimental error is the normal variation in the response which appears when an experiment is repeated.

Corrected Total is the total sum of squares corrected for the mean. It is the sum of the squared differences between the individual observations and the overall average.

Sum of Squares (SS) is the sum of the squared distances from the mean due to an effect.

Model SS is the total sum of squares for terms in the model.

Residual SS is the sum of squares for all the terms not included in the model.

Residual SS = Corrected Total SS - Model SS

Lack of Fit SS is the residual SS after removing the pure error SS.

Lack of Fit SS = Corrected Total SS - Pure Error SS - Model SS

Pure Error SS is the pure error SS for replicated points.

Pure Error SS = $\sum (y_{1u}-y_1)^2$

Corrected Total SS is the sum of squared deviations of each point from the mean.

Corrected Total SS = $\sum_{u=1}^{N} y_u^2 - G^2 / N$

Degree of Freedom (d.f.) is the number of independent comparisons available to estimate a parameter.

Model d.f is the d.f. for the model which comprises the number of model terms including the intercept minus one.

Model d.f.= $k + \frac{k(k+1)}{2}$

Residual d.f

Residual d.f. = Corrected Total d.f. - Model d.f.

Lack of Fit d.f is the amount of information available after accounting for model terms and pure error. -

Lack of Fit d.f. = $n_2 - \frac{k(k+3)}{2}$

Pure Error d.f. is the amount of information available from replicated points.

Pure Error d.f. = $n_1 - 1$

Corrected Total d.f is the total degrees of freedom for the experiment, minus one for the mean.

Corrected Total d.f. = $n_1 + n_2 - 1$

Mean Square (MS) is the sum of squares divided by the number of degrees of freedom and is used to estimate the variance.

Model MS is the estimate of model variance.

$$\text{Model MS} = \frac{\text{Model SS}}{\text{Model d.f.}}$$

Residual MS is the estimate of process variance.

$$\text{Residual MS} = \frac{\text{Residual SS}}{\text{Residual d.f.}}$$

Lack of Fit MS is the estimate of lack of fit.

$$\text{Lack of Fit MS} = \frac{\text{Lack of Fit SS}}{\text{Lack of Fit d.f.}}$$

Pure Error MS is the estimate of pure error variance.

$$\text{Pure Error MS} = \frac{\text{Pure Error SS}}{\text{Pure Error d.f.}}$$

F-value is a probability distribution used to compare variances by examining their ratio. If they are equal, then the F-value would equal one.

F-value of Model compares model variance with residual variance.

$$\text{Model F-value} = \frac{\text{Model MS}}{\text{Residual MS}}$$

F-value of Lack of Fit compares lack of fit variance with pure error variance.

$$\text{Lack of Fit F-value} = \frac{\text{Lack of Fit MS}}{\text{Pure Error MS}}$$

Prob>F relates the risk of falsely rejecting a given hypothesis. It is the probability of seeing the observed F-value if the null hypothesis is true (there is no factor effect). Small probability values call for rejection of the null hypothesis.

Coefficient of Determination (R^2) is an estimate of the function of overall variation in the data around the mean accounted for by the model. A value of 1.00 represents the ideal case at which 100% of the variation in the observed value can be explained by the chosen model.

$R^2 = 1 - [\text{Residual SS} / (\text{Model SS} + \text{Residual SS})]$

APPENDIX B1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF CARBON SOURCES ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	0.49	43	0.011	13.12	< 0.0001	significant
A	0.29	10	0.029	30.39	< 0.0001	
B	0.055	3	0.018	19.35	< 0.0001	
AB	0.15	30	4.891E-003	5.16	< 0.0001	
Pure Error	0.042	44	9.476E-004			
Cor Total	0.53	87				
Std. Dev.	0.031	**R-Squared**		0.9215		
Mean	0.062	Adj R-Squared		0.8449		
C.V.	49.46	Pred R-Squared		0.6862		
PRESS	0.17	Adeq Precision		11.623		

A= Carbon Sources (Hexadecane, Tetradecane, Glycerol, Crude oil, Glucose, 1-Propanol, 1-Butanol, Ethanol, sucrose, Maltose and Starch)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX B2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF CARBON SOURCES ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	9151.49	43	212.83	21.75	< 0.0001	significant
A	6198.44	10	619.84	60.15	< 0.0001	
B	1271.98	3	423.99	41.15	< 0.0001	
AB	1681.08	30	56.04	5.44	< 0.0001	
Pure Error	453.38 44	10.30				
Cor Total	9604.88	87				
Std. Dev.	3.21	**R-Squared**		0.9528		
Mean	55.66	Adj R-Squared		0.9067		
C.V.	5.77	Pred R-Squared		0.8112		
PRESS	1813.53	Adeq Precision		15.098		

A= Carbon Sources (Hexadecane, Tetradecane, Glycerol, Crude oil, Glucose, 1-Propanol, 1-Butanol, Ethanol, sucrose, Maltose and Starch)
B= Reading Time (0, 7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX B3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF CARBON SOURCES ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	31233.01	43	726.35	447.90	< 0.0001	significant
A	19099.76	10	1909.98	1151.22	< 0.0001	
B	2905.47	3	968.49	583.75	< 0.0001	
AB	9227.78	30	307.59	185.40	< 0.0001	
Pure Error	73.00 44		1.66			
Cor Total	31306.01	87				
Std. Dev.	1.29	**R-Squared**		0.9977		
Mean	9.48	Adj R-Squared		0.9954		
C.V.	13.59	Pred R-Squared		0.9907		
PRESS	292.00	Adeq Precision		69.346		

A= Carbon Sources (Hexadecane, Tetradecane, Glycerol, Crude oil, Glucose, 1-Propanol, 1-Butanol, Ethanol, sucrose, Maltose and Starch)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX C1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF NITROGEN SOURCES ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.41	31	0.013	321.05	< 0.0001	significant
A	0.15	7	0.021	501.64	< 0.0001	
B	0.20	3	0.066	1546.42	< 0.0001	
AB	0.064	21	3.038E-003	70.88	< 0.0001	
Pure Error	1.371E-003	32	4.286E-005			
Cor Total	0.41	63				
Std. Dev.	6.547E-003		**R-Squared**	0.9967		
Mean	0.11		Adj R-Squared	0.9935		
C.V.	5.71		Pred R-Squared	0.9868		
PRESS	5.486E-003		Adeq Precision	53.758		

A= Nitrogen Sources (Meat Extract, Soytone, Malt Extract, Peptone, Casamino Acid, Corn Steep Liquor, Yeast Extracr and Tryptone)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX C2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF NITROGEN SOURCES ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	3041.53	31	98.11	13.41	< 0.0001	significant
A	782.81	7	111.83	14.03	< 0.0001	
B	1660.19	3	553.40	69.44	< 0.0001	
AB	598.53	21	28.50	3.58	0.0006	
Pure Error	255.03	32	7.97			
Cor Total	3296.56	63				
Std. Dev.	2.82	**R-Squared**		0.9226		
Mean	47.67	Adj R-Squared		0.8477		
C.V.	5.92	Pred R-Squared		0.6906		
PRESS	1020.10	Adeq Precision		14.142		

A= Nitrogen Sources (Meat Extract, Soytone, Malt Extract, Peptone, Casamino Acid, Corn Steep Liquor, Yeast Extracr and Tryptone)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX C3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF NITROGEN SOURCES ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	22234.28	31	717.23	328.87	< 0.0001	significant
A	9693.40	7	1384.77	615.45	< 0.0001	
B	7659.91	3	2553.30	1134.80	< 0.0001	
AB	4880.97	21	232.43	103.30	< 0.0001	
Pure Error	72.00 32	2.25				
Cor Total	22306.28	63				
Std. Dev.	1.50	**R-Squared**		0.9968		
Mean	16.63	Adj R-Squared		0.9936		
C.V.	9.02	Pred R-Squared		0.9871		
PRESS	288.00	Adeq Precision		52.797		

A= Nitrogen Sources (Meat Extract, Soytone, Malt Extract, Peptone, Casamino Acid, Corn Steep Liquor, Yeast Extracr and Tryptone)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX D1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT ETHANOL ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	0.11	23	4.941E-003	322.15	< 0.0001	significant
A	1.687E-003	5	3.373E-004	21.31	< 0.0001	
B	0.11	3	0.037	2318.71	< 0.0001	
AB	1.813E-003	15	1.209E-004	7.63	< 0.0001	
Pure Error	3.800E-004	24	1.583E-005			
Cor Total	0.11	47				
Std. Dev.	3.979E-003		**R-Squared**	0.9967		
Mean	0.10		Adj R-Squared	0.9935		
C.V.	3.91		Pred R-Squared	0.9867		
PRESS	1.520E-003		Adeq Precision	46.914		

A= Concentration of Ethanol (1, 1.5, 2, 2.5, 3 and 3.5 v/v)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX D2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT ETHANOL ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	3869.25	23	168.23	37.99	< 0.0001	significant
A	263.80	5	52.76	8.75	< 0.0001	
B	3474.12	3	1158.04	192.01	< 0.0001	
AB	131.34	15	8.76	1.45	0.2017	
Pure Error	144.74 24	6.03				
Cor Total	4013.99	47				
Std. Dev.	2.46	**R-Squared**		0.9639		
Mean	49.12	Adj R-Squared		0.9294		
C.V.	5.00	Pred R-Squared		0.8558		
PRESS	578.98	Adeq Precision		16.487		

A= Concentration of Ethanol (1, 1.5, 2, 2.5, 3 and 3.5 v/v)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX D3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT ETHANOL ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	26256.27	23	1141.58	214.56	< 0.0001	significant
A	585.64	5	117.13	20.98	< 0.0001	
B	25349.38	3	8449.79	1513.40	< 0.0001	
AB	321.25	15	21.42	3.84	0.0017	
Pure Error	134.00	24	5.58			
Cor Total	26390.27	47				
Std. Dev.	2.36	**R-Squared**		0.9949		
Mean	39.60	Adj R-Squared		0.9901		
C.V.	5.97	Pred R-Squared		0.9797		
PRESS	536.00	Adeq Precision		38.304		

A= Concentration of Ethanol (1, 1.5, 2, 2.5, 3 and 3.5 v/v)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX E1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT FERRIC CHLORIDE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.050	15	3.347E-003	199.68	< 0.0001	significant
A	4.428E-003	3	1.476E-003	83.59	< 0.0001	
B	0.044	3	0.015	829.76	< 0.0001	
AB	1.830E-003	9	2.033E-004	11.51	< 0.0001	
Pure Error	2.825E-004	16	1.766E-005			
Cor Total	0.050	31				
Std. Dev.	4.202E-003		**R-Squared**	0.9944		
Mean	0.083		Adj R-Squared	0.9892		
C.V.	5.06		Pred R-Squared	0.9776		
PRESS	1.130E-003		Adeq Precision	38.705		

A= Concentration of Ferric Chloride (0, 0.01, 0.05 and 0.1 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX E2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT FERRIC CHLORIDE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	1588.37	15	105.89	14.99	< 0.0001	significant
A	35.89	3	11.96	1.57	0.2358	
B	1517.58	3	505.86	66.34	< 0.0001	
AB	34.91 9	3.88	0.51	0.8475		
Pure Error	122.00 16	7.63				
Cor Total	1710.37	31				
Std. Dev.	2.76	**R-Squared**		0.9287		
Mean	47.32	Adj R-Squared		0.8618		
C.V.	5.84	Pred R-Squared		0.7147		
PRESS	488.00	Adeq Precision		10.822		

A= Concentration of Ferric Chloride (0, 0.01, 0.05 and 0.1 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX E3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT FERRIC CHLORIDE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142
RESPONSE: E24

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	19154.38	15	1276.96	164.30	< 0.0001	significant
A	676.51	3	225.50	27.23	< 0.0001	
B	18127.56	3	6042.52	729.66	< 0.0001	
AB	350.32	9	38.92	4.70	0.0035	
Pure Error	132.50 16	8.28				
Cor Total	19286.88	31				
Std. Dev.	2.88	**R-Squared**		0.9931		
Mean	39.60	Adj R-Squared		0.9867		
C.V.	7.27	Pred R-Squared		0.9725		
PRESS	530.00	Adeq Precision		30.469		

A= Concentration of Ferric Chloride (0, 0.01, 0.05 and 0.1 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX F1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT MAGNESIUM SULPHATE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	0.058	15	3.872E-003	278.27	< 0.0001	significant
A	6.053E-003	3	2.018E-003	139.75	< 0.0001	
B	0.050	3	0.017	1147.74	< 0.0001	
AB	2.310E-003	9	2.567E-004	17.78	< 0.0001	
Pure Error	2.310E-004	16	1.444E-005			
Cor Total	0.058	31				
Std. Dev.	3.800E-003		**R-Squared**	0.9960		
Mean	0.086		Adj R-Squared	0.9923		
C.V.	4.42		Pred R-Squared	0.9842		
PRESS	9.240E-004		Adeq Precision	44.291		

A= Concentration of Magnesium Sulphate (0, 0.05, 0.2 and 0.4 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX F2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT MAGNESIUM SULPHATE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	1886.98	15	125.80	17.60	< 0.0001	significant
A	595.75	3	198.58	26.04	< 0.0001	
B	1084.11	3	361.37	47.39	< 0.0001	
AB	207.11	9	23.01	3.02	0.0260	
Pure Error	122.00	16	7.62			
Cor Total	2008.98	31				
Std. Dev.	2.76	**R-Squared**		0.9393		
Mean	52.56	Adj R-Squared		0.8823		
C.V.	5.25	Pred R-Squared		0.7571		
PRESS	488.00	Adeq Precision		12.153		

A= Concentration of Magnesium Sulphate (0, 0.05, 0.2 and 0.4 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX F3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT MAGNESIUM SULPHATE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	12753.22	15	850.21	127.37	< 0.0001	significant
A	1905.84	3	635.28	87.62	< 0.0001	
B	9954.84	3	3318.28	457.69	< 0.0001	
AB	892.53	9	99.17	13.68	< 0.0001	
Pure Error	116.00	16	7.25			
Cor Total	12869.22	31				
Std. Dev.	2.69	**R-Squared**		0.9910		
Mean	29.41	Adj R-Squared		0.9825		
C.V.	9.16	Pred R-Squared		0.9639		
PRESS	464.00	Adeq Precision		30.463		

A= Concentration of Magnesium Sulphate (0, 0.05, 0.2 and 0.4 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX G1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT CALCIUM CHLORIDE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.062	15	4.119E-003	279.12	< 0.0001	significant
A	7.851E-003	3	2.617E-003	170.91	< 0.0001	
B	0.051	3	0.017	1109.86	< 0.0001	
AB	2.955E-003	9	3.283E-004	21.44	< 0.0001	
Pure Error	2.450E-004	16	1.531E-005			
Cor Total	0.062	31				
Std. Dev.	3.913E-003		R-Squared	0.9961		
Mean	0.088		Adj R-Squared	0.9923		
C.V.	4.44		Pred R-Squared	0.9842		
PRESS	9.800E-004		Adeq Precision	48.609		

A= Concentration of Calcium Chloride (0, 0.01, 0.02 and 0.04 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX G2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT CALCIUM CHLORIDE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	1255.51	15	83.70	16.15	< 0.0001	significant
A	193.85	3	64.62	11.62	0.0003	
B	986.06	3	328.69	59.09	< 0.0001	
AB	75.60	9	8.40	1.51	0.2261	
Pure Error	89.00	16	5.56			
Cor Total	1344.51	31				
Std. Dev.	2.36	**R-Squared**		0.9338		
Mean	50.14	Adj R-Squared		0.8717		
C.V.	4.70	Pred R-Squared		0.7352		
PRESS	356.00	Adeq Precision		11.327		

A= Concentration of Calcium Chloride (0, 0.01, 0.02 and 0.04 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX G3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT CALCIUM CHLORIDE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	15792.00	15	1052.80	193.20	< 0.0001	significant
A	2940.00	3	980.00	170.43	< 0.0001	
B	11308.00	3	3769.33	655.54	< 0.0001	
AB	1544.00	9	171.56	29.84	< 0.0001	
Pure Error	92.00	16	5.75			
Cor Total	15884.00	31				
Std. Dev.	2.40	**R-Squared**		0.9942		
Mean	28.50	Adj R-Squared		0.9888		
C.V.	8.41	Pred R-Squared		0.9768		
PRESS	368.00	Adeq Precision		36.566		

A= Concentration of Calcium Chloride (0, 0.01, 0.02 and 0.04 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX H1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT ZINC SULPHATE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	0.051	15	3.376E-003	152.99	< 0.0001	significant
A	9.207E-003	3	3.069E-003	129.91	< 0.0001	
B	0.039	3	0.013	545.54	< 0.0001	
AB	2.762E-003	9	3.069E-004	12.99	< 0.0001	
Pure Error	3.780E-004	16	2.363E-005			
Cor Total	0.051	31				
Std. Dev.	4.861E-003		**R-Squared**	0.9926		
Mean	0.077		Adj R-Squared	0.9856		
C.V.	6.34		Pred R-Squared	0.9704		
PRESS	1.512E-003		Adeq Precision	37.243		

A= Concentration of Zinc Sulphate (0, 0.05, 0.1 and 0.2 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX H2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT ZINC SULPHATE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	2149.03	15	143.27	18.01	< 0.0001	significant
A	180.77	3	60.26	7.53	0.0023	
B	1842.66	3	614.22	76.78	< 0.0001	
AB	125.60	9	13.96	1.74	0.1589	
Pure Error	128.00	16	8.00			
Cor Total	2277.03	31				
Std. Dev.	2.83	**R-Squared**		0.9438		
Mean	47.83	Adj R-Squared		0.8911		
C.V.	5.91	Pred R-Squared		0.7751		
PRESS	512.00	Adeq Precision		12.645		

A= Concentration of Zinc Sulphate (0, 0.05, 0.1 and 0.2 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX H3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT ZINC SULPHATE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	14844.47	15	989.63	211.81	< 0.0001	significant
A	2189.59	3	729.86	148.76	< 0.0001	
B	11551.59	3	3850.53	784.82	< 0.0001	
AB	1103.28	9	122.59	24.99	< 0.0001	
Pure Error	78.50	16	4.91			
Cor Total	14922.97	31				
Std. Dev.	2.22	**R-Squared**		0.9947		
Mean	31.97	Adj R-Squared		0.9898		
C.V.	6.93	Pred R-Squared		0.9790		
PRESS	314.00	Adeq Precision		39.585		

A= Concentration of Zinc Sulphate (0, 0.05, 0.1 and 0.2 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX I1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT MANGANESE SULPHATE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	0.038	15	2.560E-003	145.07	< 0.0001	significant
A	4.012E-003	3	1.337E-003	71.04	< 0.0001	
B	0.033	3	0.011	587.06	< 0.0001	
AB	1.229E-003	9	1.365E-004	7.25	0.0003	
Pure Error	3.012E-004	16	1.883E-005			
Cor Total	0.039	31				
Std. Dev.	4.339E-003		**R-Squared**	0.9922		
Mean	0.075		Adj R-Squared	0.9849		
C.V.	5.79		Pred R-Squared	0.9689		
PRESS	1.205E-003		Adeq Precision	34.191		

A= Concentration of Manganese Sulphate (0, 0.05, 0.1 and 0.2 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX I2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT MANGANESE SULPHATE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	1908.06	15	127.20	18.30	< 0.0001	significant
A	217.83	3	72.61	9.82	0.0007	
B	1592.39	3	530.80	71.77	< 0.0001	
AB	97.84	9	10.87	1.47	0.2402	
Pure Error	118.34	16	7.40			
Cor Total	2026.40	31				
Std. Dev.	2.72	**R-Squared**		0.9416		
Mean	49.15	Adj R-Squared		0.8869		
C.V.	5.53	Pred R-Squared		0.7664		
PRESS	473.36	Adeq Precision		12.257		

A= Concentration of Manganese Sulphate (0, 0.05, 0.1 and 0.2 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX I3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF DIFFERENT MANGANESE SULPHATE CONCENTRATION ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142
RESPONSE: E24

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	17746.50	15	1183.10	240.55	< 0.0001	significant
A	181.75	3	60.58	11.82	0.0002	
B	17019.75	3	5673.25	1106.98	< 0.0001	
AB	545.00	9	60.56	11.82	< 0.0001	
Pure Error	82.00	16	5.13			
Cor Total	17828.50	31				
Std. Dev.	2.26	**R-Squared**		0.9954		
Mean	39.38	Adj R-Squared		0.9911		
C.V.	5.75	Pred R-Squared		0.9816		
PRESS	328.00	Adeq Precision		37.482		

A= Concentration of Manganese Sulphate (0, 0.05, 0.1 and 0.2 g/l)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX J1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF TEMPERATURE ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	0.077	23	3.354E-003	283.46	< 0.0001	significant
A	0.056	5	0.011	912.12	< 0.0001	
B	3.158E-003	3	1.053E-003	85.78	< 0.0001	
AB	0.018	15	1.202E-003	97.96	< 0.0001	
Pure Error	2.945E-004	24	1.227E-005			
Cor Total	0.077	47				
Std. Dev.	3.503E-003		**R-Squared**	0.9962		
Mean	0.032		Adj R-Squared	0.9926		
C.V.	11.04		Pred R-Squared	0.9848		
PRESS	1.178E-003		Adeq Precision	50.263		

A= Temperature (5, 30, 35, 40, 45 and 50 ^{o}C)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX J2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF TEMPERATURE ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	2268.07	23	98.61	11.05	< 0.0001	significant
A	1441.34	5	288.27	32.02	< 0.0001	
B	326.87	3	108.96	12.10	< 0.0001	
AB	499.86	15	33.32	3.70	0.0022	
Pure Error	216.06	24	9.00			
Cor Total	2484.13	47				
Std. Dev.	3.00	**R-Squared**		0.9130		
Mean	56.11	Adj R-Squared		0.8297		
C.V.	5.35	Pred R-Squared		0.6521		
PRESS	864.24	Adeq Precision		9.761		

A= Temperature (5, 30, 35, 40, 45 and 50 ^{o}C)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX J3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF TEMPERATURE ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	25429.92	23	1105.65	520.40	< 0.0001	significant
A	15514.42	5	3102.88	1432.10	< 0.0001	
B	4690.92	3	1563.64	721.68	< 0.0001	
AB	5224.58	15	348.31	160.76	< 0.0001	
Pure Error	52.00	24	2.17			
Cor Total	25481.92	47				
Std. Dev.	1.47	**R-Squared**		0.9980		
Mean	17.04	Adj R-Squared		0.9960		
C.V.	8.64	Pred R-Squared		0.9918		
PRESS	208.00	Adeq Precision		59.568		

A= Temperature (5, 30, 35, 40, 45 and 50 ^{o}C)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX K1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF INOCULUM SIZE ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	0.065	19	3.398E-003	148.79	< 0.0001	significant
A	0.017	4	4.282E-003	174.79	< 0.0001	
B	0.039	3	0.013	527.54	< 0.0001	
AB	8.659E-003	12	7.216E-004	29.45	< 0.0001	
Pure Error	4.900E-004	20	2.450E-005			
Cor Total	0.065	39				
Std. Dev.	4.950E-003		**R-Squared**	0.9925		
Mean	0.074		Adj R-Squared	0.9853		
C.V.	6.69		Pred R-Squared	0.9699		
PRESS	1.960E-003		Adeq Precision	42.000		

A= Inoculum Size (2, 4, 8, 12 and 16 v/v)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX K2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF INOCULUM SIZE ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	2154.91	19	113.42	13.99	< 0.0001	significant
A	510.51	4	127.63	14.50	< 0.0001	
B	1452.24	3	484.08	55.01	< 0.0001	
AB	192.16	12	16.01	1.82	0.1142	
Pure Error	176.00	20	8.80			
Cor Total	2330.91	39				
Std. Dev.	2.97	**R-Squared**		0.9245		
Mean	50.14	Adj R-Squared		0.8528		
C.V.	5.92	Pred R-Squared		0.6980		
PRESS	704.00	Adeq Precision		10.054		

A= Inoculum Size (2, 4, 8, 12 and 16 v/v)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX K3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF INOCULUM SIZE ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	24541.10	19	1291.64	240.75	< 0.0001	significant
A	3300.60	4	825.15	147.35	< 0.0001	
B	20063.50	3	6687.83	1194.26	< 0.0001	
AB	1177.00	12	98.08	17.51	< 0.0001	
Pure Error	112.00	20	5.60			
Cor Total	24653.10	39				
Std. Dev.	2.37	**R-Squared**		0.9955		
Mean	38.15	Adj R-Squared		0.9911		
C.V.	6.20	Pred R-Squared		0.9818		
PRESS	448.00	Adeq Precision		44.821		

A= Inoculum Size (2, 4, 8, 12 and 16 v/v)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX L1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF AGITATION RATE ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.15	23	6.588E-003	213.74	< 0.0001	significant
A	0.024	5	4.715E-003	145.74	< 0.0001	
B	0.12	3	0.040	1227.97	< 0.0001	
AB	8.768E-003	15	5.845E-004	18.07	< 0.0001	
Pure Error	7.765E-004	24	3.235E-005			
Cor Total	0.15	47				
Std. Dev.	5.688E-003		**R-Squared**	0.9949		
Mean	0.10		Adj R-Squared	0.9900		
C.V.	5.60		Pred R-Squared	0.9796		
PRESS	3.106E-003		Adeq Precision	44.753		

A= Agitation Rate (0, 50, 100, 150, 200 and 250 rpm)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX L2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF AGITATION RATE ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: SURFACE TENSION

Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	3415.74	23	148.51	22.31	< 0.0001	significant
A	619.88	5	123.98	17.71	< 0.0001	
B	2596.09	3	865.36	123.59	< 0.0001	
AB	199.77	15	13.32	1.90	0.0775	
Pure Error	168.04	24	7.00			
Cor Total	3583.78	47				
Std. Dev.	2.65	**R-Squared**		0.9531		
Mean	47.92	Adj R-Squared		0.9082		
C.V.	5.52	Pred R-Squared		0.8124		
PRESS	672.16	Adeq Precision		11.865		

A= Agitation Rate (0, 50, 100, 150, 200 and 250 rpm)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX L3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF AGITATION RATE ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	31511.25	23	1370.05	410.99	< 0.0001	significant
A	1435.75	5	287.15	84.04	< 0.0001	
B	29516.25	3	9838.75	2879.63	< 0.0001	
AB	559.25	15	37.28	10.91	< 0.0001	
Pure Error	82.00	24	3.42			
Cor Total	31593.25	47				
Std. Dev.	1.85	**R-Squared**		0.9974		
Mean	42.63	Adj R-Squared		0.9949		
C.V.	4.34	Pred R-Squared		0.9896		
PRESS	328.00	Adeq Precision		56.617		

A= Agitation Rate (0, 50, 100, 150, 200 and 250 rpm)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX M1

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF PH ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: WHOLE CELL PROTEIN

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.11	43	2.452E-003	23.10	< 0.0001	significant
A	0.045	10	4.504E-003	40.41	< 0.0001	
B	0.044	3	0.015	131.04	< 0.0001	
AB	0.017	30	5.528E-004	4.96	< 0.0001	
Pure Error	4.904E-003	44	1.115E-004			
Cor Total	0.11	87				
Std. Dev.	0.011	**R-Squared**		0.9556		
Mean	0.056	Adj R-Squared		0.9121		
C.V.	19.00	Pred R-Squared		0.8222		
PRESS	0.020	Adeq Precision		15.271		

A= pH (6, 6.2, 6.4, 6.6, 6.8, 7, 7.2, 7.4, 7.6, 7.8 and 8)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX M2

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF PHS ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-14 RESPONSE: SURFACE TENSION

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	3703.76	43	86.13	14.71	< 0.0001	significant
A	1459.13	10	145.91	23.05	< 0.0001	
B	1733.91	3	577.97	91.31	< 0.0001	
AB	510.72	30	17.02	2.69	0.0014	
Pure Error	278.50	44	6.33			
Cor Total	3982.26	87				
Std. Dev.	2.52		**R-Squared**	0.9301		
Mean	52.92		Adj R-Squared	0.8617		
C.V.	4.75		Pred R-Squared	0.7203		
PRESS	1114.00		Adeq Precision	12.131		

A= pH (6, 6.2, 6.4, 6.6, 6.8, 7, 7.2, 7.4, 7.6, 7.8 and 8)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B

APPENDIX M3

ANOVA FOR SELECTED FACTORIAL ANALYSIS OF VARIANCE TABLE EFFECT OF pHs ON BACTERIAL GROWTH AND BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* KRT-142 RESPONSE: E24

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	43888.95	43	1020.67	256.18	< 0.0001	significant
A	15313.20	10	1531.32	369.20	< 0.0001	
B	22672.05	3	7557.35	1822.05	< 0.0001	
AB	5903.70	30	196.79	47.45	< 0.0001	
Pure Error	182.50	44	4.15			
Cor Total	44071.45	87				
Std. Dev.	2.04	**R-Squared**		0.9959		
Mean	25.98	Adj R-Squared		0.9918		
C.V.	7.84	Pred R-Squared		0.9834		
PRESS	730.00	Adeq Precision		44.442		

A= pH (6, 6.2, 6.4, 6.6, 6.8, 7, 7.2, 7.4, 7.6, 7.8 and 8)
B= Reading Time (0,7, 14 and 21 hours)
AB= Intercept A X B