UNIVERSITI PUTRA MALAYSIA

BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* 181

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BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* 181

By

LAITH ISSA YASSIN AL-ARAJI

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BIOSURFACTANT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* 181

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October 2004

Chairman : Professor Abu Bakar Salleh, Ph.D.
Faculty : Biotechnology and Biomolecular Sciences

This study involves the screening of biosurfactant producers that have been isolated from crude oil bacteria degraders. The bacteria were isolated by qualitative screening on cetyltrimethylammonium bromide (CTAB) agar plates and quantitative screening for biosurfactant production in liquid media. A biosurfactant producer identified as *Pseudomonas aeruginosa* 181 was selected for further analysis.

Maximum biosurfactant production by *Pseudomonas aeruginosa* 181 was achieved after 120 h incubation at pH 7.0 and 37°C. Static condition and 5.0% bacterial inoculum's gave the optimum biosurfactant yield. Culture medium containing glucose as the carbon source; and casamino acids as the organic nitrogen source gave the highest level of biosurfactant production. Corn steep liquor and ammonium nitrate on the other hand inhibited biosurfactant production. However, the addition of metal ions such as Fe, Mg and Mn maximized biosurfactant synthesis.

The biosurfactant produced by *Pseudomonas aeruginosa* 181 was purified to homogeneity by acid precipitation and ammonium sulphate precipitation. Biosurfactant produced by *Pseudomonas aeruginosa* 181 was stable and had a broad range of pH from 3.0 to 12.0 with the maximum activity (Surface Tension reduction and Emulsification Index (E24)) exhibited at pH 7.0. The purified biosurfactant had a broad range of temperature and exhibited optimum activity at 30°C. This biosurfactant had high activity compared to many commercial surfactants with 0.1 mg critical micelle concentration (CMC). The purified biosurfactant had a maximum emulsification index (E24) of 86% with hexadecane, followed by 80% with nonane, dodecane, tridecane, pentadecane, octadecane and o-Xylene.

Response surface methodology (RSM) was then used to study interactive effects of the parameters (pH, stirring rate, casamino acid concentration and incubation period) on the production of biosurfactants. Generally, simultaneously increasing surface tension reduction and emulsification index (E24) improved yields. Production carried out at larger volumes of 1L using Bioreactor under RSM-optimized conditions yielded 350.22 mg of products after purification by acid precipitation. Identities of isolated products were verified by using TLC, high performance liquid chromatography (HPLC), liquid chromatography–Mass spectrometry (LC-MS), mass spectrometry (MS-MS), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and infrared spectroscopy (FT-IR), from analysis carried out the rhamnolipids were monorhamnolipids and dirhamnolipids.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN BIOSURFAKTAN OLEH *PSEUDOMONAS AERUGINOSA* 181

Oleh

LAITH ISSA YASSIN AL-ARAJI

Oktober 2004

Pengerusi : Profesor Abu Bakar Salleh, Ph.D

Fakulti : Bioteknologi and Sains Biomolekul

Kajian ini melibatkan pemencilan dan penyaringan penghasil biosurfaktan daripada bakteria yang mendegradasikan minyak mentah. Bakteria ini pada awalnya dipencilkan nengikut penyaringan kualitatif di atas agar cetiltrimetilammonium bromida (CTAB) dan penyaringan kuantitatif di atas media cecair. Penghasil biosurfaktan dikenalpasti sebagai *Pseudomonas aeruginosa* 181 dan dipilih untuk analisis selanjutnya. Penghasilan biosurfaktan yang maksimum oleh *Pseudomonas aeruginosa* 181 adalah pada suhu 37°C selepas 120 jam pengeraman pada pH 7.0 keadaan statik dan 5 peratus inokulum memberikan keadaan optimum penghasilan biosurfaktan. Penghasilan biosufaktan tertinggi adalah dalam media yang mengandungi sumber karbon glukosa dan asid kasamino sebagai sumber nitrogen. Aktiviti biosurfaktan direncatkan oleh 'corn steep liquor' dan ammonium nitrat. Walau bagaimanapun, penambahan ion metal seperti Fe, Mg dan Mn memberikan sintesis biosurfaktan maksimum.

Penghasilan biosurfaktan oleh *Pseudomonas aeruginosa* 181 di tulenkan sehingga homogeniti melalui presipitasi asid dan presipitasi ammonium sulfat. Penghasilan biosurfaktan adalah stabil pada pH optimum 3.0 hingga 12.0 dan aktiviti maksimum (pengurangan ketegangan permukaan indeks imulsifikasi (E24)) pada pH 7.0. Penghasil

tulen menunjukkan aktiviti optimum pada 30^{o}C. Biosurfaktan ini mempunyai aktiviti yang sangat tinggi jika dibandingkan dengan kebanyakan surfaktan komersil dengan 0.1 mg kepekatan sel kritikal (CMC). Biosurfaktan tulen menunjukkan aktiviti indeks emulsifikasi (E24) maksimum pada 86% dengan heksadekana, diikuti 80% dengan nonana, dodekana, tridekana, pentadekana, oktadekana dan o-Xylena.

Seterusnya kaedah permukaan respon (RSM) digunakan untuk mengkaji kesan interaksi parameter untuk penghasil biosurfaktan (pH, kadar pengadukkan, kepekatan asid kasamino and masa pengeraman). Lazimnya, apabila pengurangan ketegangan permukaan dan indeks imulsifikasi (E24) meningkat menyebabkan penghasilan produktiviti meningkat. Penghasilan pada isipadu yang besar iaitu 1L menggunakan Bioreaktor dibawah keadaan optimum RSM menghasilkan 350.22 mg produk selepas ditulenkan menggunakan presipitasi asid. Identiti produk pencilan dipelbagaikan menggunakan TLC, kromatografi cecair tinggi (HPLC), spektrometri jisim-kromatografi cecair (LC-MS) spektrometri jisim (MS-MS), mikroskop elektron imbasan (SEM), mikroskop elektron transmisi (TEM) dan spektroskopi infra merah (FT-IR), hasil daripada analisis yang dijalankan rhamnolipid adalah monorhamnolipid and dirhamnolipid.

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I certify that an Examination Committee met on 19th October 2004 to conduct the final examination of Laith Issa Yassin Al-Araji on his Doctor of Philosophy thesis entitled "Biosurfactant Production by *Pseudomonas aeruginosa* 181" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of the Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as followed:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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LAITH ISSA YASSIN AL-ARAJI

Date:

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LIST OF ABBREVIATIONS

$CaCI_2$	Calcium chloride
cm	Centimetre
CMC	Critical micelle concentration
d	Day
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
g	Gram
g/L	Gram per liter
HCI	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
h	Hour
IR	Infrared
L	Liter
M	Molar
mg	Milligram
mL	Millilitre
mM	Millimolar
mM	Millimolar
mm	Minute
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	Revolutions per minute
RSM	Response surface methodology
TLC	Thin layer chromatography
TSB	Trypticase Soy Broth
µg	Microgram
µL	Microlitre
µm	Micrometer
v/v	Volume per volume
w/v	Weight per volume

CHAPTER I

INTRODUCTION

Biosurfactants, with both hydrophilic and hydrophobic structural moieties, seem to facilitate the uptake of hydrocarbons into cells. Wide spectra of microbial compounds, including glycolipids, lipopeptides, fatty acids, and polymeric biosurfactants, have been found to have surface activity. Such compounds are able to reduce the surface tension and interfacial tension between water and hydrocarbon phases (Morikawa *et al.,* 2000).

Biosurfactants have important advantages, such as biodegradability, low toxicity, and various possible structures, relative to chemically synthesized surfactants (Benincasa *et al.,* 2002). With environmental compatibility becoming an increasingly important factor in the selection of industrial chemicals, the use of biosurfactants in environmental applications, such as in bioremediation and the dispersion of oil spills, is increasing (Banat 1995a).

In addition, biosurfactants have other uses in the petroleum industry, such as in enhanced oil recovery (Kim *et al.,* 2000) and the transportation of crude oil. Other possible application fields are in the food, cosmetics, and pharmaceutical industries. In these industries, most biosurfactants are used as emulsifiers (Desai and Banat 1997). However, biosurfactants have not yet been employed extensively in industry because of the relatively high production and recovery costs involved.

Considerable attention has been given in the past to the production of the surface-active molecules of biological origin because of their potential utilization in food processing (Mata-Sandoval *et al.,* 1999) pharmacology, and oil industry. Although the type and amount of the microbial surfactants produced depend primary on the producer organism, factors like carbon and nitrogen, trace elements, temperature, and aeration also affected their production by the organism. Hydrophobic pollutants present in petroleum hydrocarbons and soil and water environment require solubilization before being degraded by microbial cells. Mineralization is governed by adsorptions of hydrocarbons from soil. Surfactants can increase the surface area of hydrophobic materials, such as pesticides in soil and water environment, thereby increasing their water solubility. Hence, the presence of surfactants may increase microbial degradation of pollutants. Use of biosurfactants for degradation of pesticides in soil and water environment has become important recently (Jennings and Tanner 2000). The worldwide surfactant market totals approximately 9.4 billion US$ per annum, and the demand for surfactants is expected to increase at a rate of 35% per annum (Desai and Banat 1997).

According to Karanth *et al.,* (1999), the type, quality and quantity of biosurfactant production is dependent on the culture conditions such as pH, temperature, agitation, dilution rate in continuous culture, the concentration of metal ions and the nature of the carbon source and nitrogen source in the medium. Moreover, the efforts were based on conventional optimisation methods where only one parameter is varied at any one time with the others being kept constant. As such, the interactions amongst these parameters are neglected, resulting in only an 'apparent' set of optimal conditions.