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PRODUCTION AND CHARACTERIZATION OF DI-RHAMNOLIPID PRODUCED BY *Pseudomonas aeruginosa* TMN

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Abstract - *Pseudomonas aeruginosa* TMN was used to produce rhamnolipid (RL) from a variety of carbon and nitrogen substrates. The most favorable carbon sources for RL production were glucose and glycerol (both at 40 g/L), giving a RL yield of 0.3 and 0.25 g/L, respectively. Meanwhile, sodium nitrate appeared to be the preferable nitrogen source, resulting in a RL production of 0.34g/L. Rhamnolipid production from *P. aeruginosa* TMN was affected by temperature, pH and agitation rate, with 37 °C, pH 7 and 200 rpm agitation favorable for rhamnolipid production. Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and electro spray ionization–mass spectrometry (ESI–MS) analyses indicated that the purified product contained one type of commonly found rhamnolipid, which is L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate. The rhamnolipid product can reduce the surface tension of water to 34 mN/m with a critical micelle concentration of nearly 18.75 mg/L and emulsified kerosene by 46%. *P. aeruginosa* TMN strain is a potential source of rhamnolipid biosurfactant, which could be used for the development of bioremediation processes in the marine environment.
Keywords: Rhamnolipid; *Pseudomonas aeruginosa*; Optimization; Purification; Glucose.

INTRODUCTION

Environmental pollution caused by petroleum hydrocarbons represents a great risk to ecosystems. Biodegradation is an effective way to overcome this problem in which microbes utilize the contaminants as a carbon source, leading to the breakdown of the pollution components into low molecular weight compounds (Pirôllo *et al.,* 2008; Zhang *et al.,* 2005).

Synthetic surfactants are unsuitable for bioremediation applications since they may cause toxic effects to the environment or result in secondary pollution. Thus, biosurfactants, natural products of a variety of microorganisms (Providenti *et al*., 1995; Rahman *et al*., 2002a), appear to be preferable for environmental applications. Biosurfactants have recently received much more attention due to their potential to become an environment-friendly alternative to conventional synthetic surfactants. Advantages of biosurfactants over their synthetic counterparts include lower toxicity, biodegradability, better environmental compatibility, higher foaming, high selectivity, and specific activity at extreme temperatures, pH and salinity (Desai and Banat, 1997; Makkar and Cameotra, 1999; Makkar and Cameotra, 2002; Ilori *et al*., 2005; Raza *et al*., 2007; Abouseoud *et al*., 2008; Fontes *et al.*, 2012).

The factors restricting commercial application of

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biosurfactants have been the low yield and high production cost. Therefore, there is an urgent need to develop an efficient and cost-effective bioprocess for the production of biosurfactants. Biosurfactants are a leading group of valuable microbial natural products with unique biochemical properties. From a biotechnology prospective, the production of biosurfactants is important owing to their vast applications in food, cosmetics, pharmaceuticals, and the agricultural and petrochemical industries (Robert *et al.,* 1989; Pruthi and Cameotra, 2003; Abouseoud *et al.,* 2008; Nguyen *et al*., 2008). They are interesting amphiphilic biomacromolecules with various biological functions/ properties such as; enhancer of polycyclic aromatic hydrocarbon bioavailability to microorganisms and, accordingly, their biodegradation, reducer of the interfacial tension by partitioning preferentially at interfaces, effective activity on surfaces, oil recovery, and so on (Desai and Banat, 1997; Pruthi and Cameotra, 2003; Joshi *et al*., 2008; Nguyen *et al*., 2008). Microbial surfactants, which are secreted by different groups of bacteria, are composed of lipid, phospholipids, polysaccharide, protein and other biological macromolecules and contain various functional groups including carboxyl, amino and phosphate groups (Kosaric, 1992; Desai and Banat, 1997; Christofi and Ivshina, 2002).

Pseudomonas aeruginosa, a Gram-negative bacterium, secretes rhamnolipids when grown under the appropriate conditions (Jarvis and Johnson, 1949). Rhamnolipids are a group of biosurfactants of glycolipid nature, composed of a hydrophilic head formed by one or two rhamnose molecules, known respectively as monorhamnolipid (monoRL) and dirhamnolipid (diRL), and a hydrophobic tail which contains one or two fatty acids. The composition of rhamnolipidic homologues is related to many parameters; most importantly strain, media composition, culture conditions, and culture age (Deziel *et al*., 1999; Mata-Sandoval *et al*., 1999; Monteiro *et al*., 2007). Previous work showed that *P. aeruginosa* is able to produce six types of rhamnolipids, which possess similar chemical structure and surface activity and have an average molecular weight of 577 (Torrens *et al*., 1998). Rhamnolipid can reduce the surface tension of water from 72 to 30 mN/m (Abalos *et al.,* 2001) with a critical micelle concentration of 27–54 mg/l (Miller-Maier and Bodour, 1998). Although rhamnolipid is not the strongest biosurfactant available, it is well suited for applications in bioremediation of oil pollutants due to having high emulsification activity and minor antibiotic effects (Mulligan, 2005).

The specific objectives of this study were: to examine the influence of nutritional requirements; to optimize the environmental conditions for the production of rhamnolipid by a newly characterized *Pseudomonas aeruginosa* TMN strain and the purification process; to perform a structural characterization and determine some associated physicochemical properties, including the critical micelle concentration and the characterization of the bioemulsifier produced, based on its solvent specificity and stability.

MATERIALS AND METHODS

Isolation of Biosurfactant-Producing Microorganisms

Biosurfactant-producing microbial strains were isolated from compost pile waste collected from El-Sharkia region in Delta, Egypt using Cetrimide agar (Difo, U.S.A). The isolated bacterial strains were characterized depending on their morphological, biochemical characteristics by following Bergey's Manual of Determinative Bacteriology, API 20NE kit (Biomérieux, Mercy, France) and 16S rRNA gene analysis.

Extraction of DNA and PCR

For molecular identification, the selected bacterial isolate was referred for 16S rRNA gene sequence analysis. DNA extraction was performed from 2 mL of bacterial cultures collected at the mid-exponential growth phase using Roche Kit (Germany) according to the manufacturer's instructions and run in triplicate through the polymerase chain reaction (PCR). Three sets of primers are listed in Table 1.

V3 and V6 primers were used to amplify V3 and V6; the most two relevant hyper variable regions of the bacterial 16S rRNA gene, giving products of 203 and 124 bp, respectively (Chakravorty *et al*. 2007). The reaction was carried out in a 25 µl volume containing 1x PCR buffer, 1.5 mM MgSO4, 2mM dNTP mixture, 1 µM of each primer, 1 µL of *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany) and 1 ng of template DNA. PCR amplification was performed as follows: initial denaturation at 95 °C for 5 min, followed by 25 cycles each of 94 °C for 1 min, 55 °C of annealing for 45 s, and a 45 s extension at 72 °C. The 203 and 124 bp PCR products amplified from the bacterial isolates which appeared as a single band were purified using a High Pure PCR product purification kit (Roche Applied Science, Germany) and sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA).

Table 1: List of primers used in this study.

Name		Sequence	Size (bp)	Reference
27	27f	5′-AGAGTTTGATCCTGGCTCAG-3′		(Lane, 1991)
1492	1492r	5′-TACGGTTACCTTGTTACGACTT-3′		
V3	V3F	5′-CCAGACTCCTACGGGAGGCAG-3′	203	(Chakravorty *et al*. 2007)
	V3R	5′-CGTATTACCGCGGCTGCTG-3′		
V6	V6F	5′-TCGATGCAACGCGAAGAA-3′	124	(Chakravorty *et al*. 2007)
	V6R	5′-ACATTTCACaACACGAGCTGACGA-3′		

Sequence homologies were examined using BLAST version 2.2.12 of the National Center for Biotechnology Information (Altschul *et al*. 1990). Multiple sequence alignments were carried out using ClastalW and a consensus neighbor-joining tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software (version 4.0) (Tamura *et al*. 2007). Finally potent biosurfactant producing isolates were maintained on nutrient agar slants for further studies.

Preparation of Culture Medium

The strain from nutrient agar slants was streaked on a Cetrimide agar plate, which was then incubated at 37 °C for 14–16 h. After that, a single colony was taken from the plate and transferred into 50 mL of liquid broth (LB) to prepare the seed culture. The cultivation condition for the seed culture was 37 °C, 200 rpm, and 14–16 h of incubation time.

Fermentation Medium and Condition

For liquid fermentation, the seed culture (5% inoculum) was inoculated into a 500-ml flask containing 150 mL of mineral salts (MS) medium consisting of (g/L): NH_4NO_3, 4; KH_2PO_4, 4.08; Na_2HPO_4, 5.68; $CaCl_2$, 7.77×10^{-4}; $MgSO_4.7H_2O$, 0.2; sodium EDTA, 1.49×10^{-3}; $FeSO_4 \cdot 7H_2O$, 5.56×10^{-4} (Wei and Chu, 1998). In general, the MS medium was amended with 40 g/L glucose as the sole carbon substrate (designated as GMS medium) (Wei and Chu, 1998; Wei *et al*., 2005). The culture temperature and agitation rate were 37 °C and 200 rpm, respectively. The pH of the medium was initially adjusted to 6.8 with 1.0 M HCl.

Optimization of Biosurfactant Production

Carbon Source Optimization

For the experiments exploring the effect of carbon substrates on rhamnolipid production, the carbon source in GMS medium (i.e., glucose) was replaced by glycerol (40 g/L), sucrose (40 g/L), hexane (40 g/L), olive oil (80 g/L) and oleic acid (80 g/L) (Wu *et al*., 2008).

Nitrogen Source Optimization

The nitrogen source in GMS medium (i.e., NH_4NO_3) was also replaced by NH_4Cl (50 mM), $NaNO_3$ (50 mM), urea (50 mM), and yeast extract (10 g/L) to investigate the effect of nitrogen source on rhamnolipid production (Wu *et al*., 2008).

Optimization of pH and Temperature

The optimum pH of MS medium for growth and biosurfactant production of the tested bacterial isolates was investigated. The pHs tested were 4, 5, 6, 7, and 8. The pH of the medium was adjusted using a solution of HCl or NaOH (1 M), with the aid of a Sentron 2001 pH meter, and the inoculated medium was amended with optimum carbon substrate and optimum nitrogen source.

The growth and biosurfactant production of the isolated microorganisms were monitored at a range of temperatures to determine the optimum one for each isolate. The tested temperatures were 25, 30, 35, 37, 40 and 45 °C. The inoculated medium was adjusted to the optimum pH and supplied with the optimum carbon substrate and optimum nitrogen source.

Agitation Rate

The growth and biosurfactant production of the isolated microorganisms were observed at different ranges of agitation rate to determine the optimum one for each isolate. The tested rpms were 100, 150, 200, and 250 rpm. The inoculated medium was adjusted to the optimum pH and supplied with the optimum carbon substrate and optimum nitrogen source and adjusted at optimum temperature.

Dry Weight Cell Determination

After cultivation for 7 days, the collected fermentation broth was first centrifuged at 9000 g for 15 min to remove bacterial cells. 50 mL of the filtrate was taken to detect surface tension, emulsification index, and oil displacement test, which will be described later. The removed bacterial cells were collected and placed in an oven at 110 °C for 12 h to obtain a dried weight, reported in terms of g/L, which is used to express the microbial concentration.

Extraction and Purification of the Produced Biosurfactants

The pH of the remaining supernatant was adjusted to pH 2.0 with 1 N HCl to precipitate rhamnolipid. The precipitate was harvested by centrifugation (9000 g, 20 min) and was then extracted three times with ethyl acetate at room temperature. The organic phase was collected and the solvent was removed in a rotary evaporator, allowing the yield of viscous honey-colored rhamnolipid product (Chen *et al*., 2005; Wei *et al*., 2005).

The extracted viscous honey-colored rhamnolipid product was collected for further purification using chromatographic procedures. A sample of viscous honey-colored rhamnolipid was dissolved in chloroform, mixed with a small amount of silica gel 60 and dried on a rotary evaporator. The column (1.5 cm×35 cm) was packed with silica gel in hexane and not allowed to dry. The sample was applied on the surface of the column and eluted with hexane. After that, it was eluted with solvents of gradually increasing polarity: hexane > acetone > chloroform > chloroform: methanol (2:1, v/v) >methanol (Darvishi *et al*., 2011).

Each fraction was evaporated on a rotary evaporator and its oil displacing area was measured as indicated blow. The fractions that demonstrated the oil displacement test were further separated by thin-layer chromatography (TLC) using aluminum silica gel 60 F254 plates and a chloroform:methanol:20% aqueous acetic acid (65:15:2) solvent system (Koch *et al*., 1991). Rhamnolipids can be visualized using the orcinol test (Koch *et al*., 1991) or Molisch test, with reagents specific for sugars or fatty acids or with reagents that are used to reveal most organic compounds on TLC such as the "ceric dip" (Mechaly *et al*., 1997).

Structural Characterization

The active fraction was analyzed by Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and electro spray ionization–mass spectrometry (ESI–MS).

Fourier Transform Infrared Spectroscopy (FTIR)

FT-IR spectra of the dried biosurfactants were recorded on a Bruker 113V FT-IR spectrometer equipped with a mercury–cadmium–telluride (MCT) detector cooled with liquid N_2. About 2mg of dried biomaterial was milled with 200mg of KBr to form a very fine powder. This powder was then compressed into a thin pellet which could be analyzed by FT-IR in the wave number range of 4000–400 cm^{-1}. The analysis of IR spectra was carried out by using OPUS 3.1 (Bruker Optics) software.

Nuclear Magnetic Resonance Analysis (^{1}HNMR)

The ^{1}HNMR spectra of fractions obtained from the fractionation step were recorded on a 500 mHz FT NMR spectrometer (JEOL, JNM-A500), using deuterated methanol as solvent.

Electro Spray Ionization–Mass Spectrometry (ESI–MS)

The analyses were performed with a triple Quadrupole Quattro LC (Micromass, Manchester, UK) in negative-ion mode. 2 mg of rhamnolipid fraction was dissolved in 1 mL chloroform: methanol (1:1, v/v), and aliquots of 0.1 mL were removed and diluted in 1.9 mL of acetonitrile–water (7:3, v/v), which was introduced by direct infusion with a syringe pump at a flow rate of 10 μL min^{-1}. ESI tandem mass spectra were acquired by mass-selecting the target ion using a quadrupole mass analyzer. The conditions of the analyses were: F1 – capillary 2.03 kV, cone 15V and collision energy 18V for collision induced dissociation (CID); F2 – capillary 2.03 kV, cone 27 V and collision energy 45 V for CID.

Physicochemical Characterization

Oil Displacement Test

The oil displacement test is a method used to determine the surface activity by measuring the diameter of the clear zone, which occurs after dropping a surfactant-containing solution on a thin layer of oil on water. The binomial diameter allows an evaluation of the surface tension reduction efficiency of a given biosurfactant (Rodrigues *et al*., 2006). The oil displacement test was done by adding 40 mL of distilled water to a Petri dish with a diameter of 15 cm.

After that, 15 μL of crude oil was dropped to form a thin oil layer on the surface of the water, and then 10 μL of a test solution was dropped onto the surface of oil. The test was conducted at room temperature (25–27 °C). The maximum diameter of the clear zone was observed under light and measured. The larger the diameter of the clear zone, the higher the surface activity of the test solution.

Surface Tension Measurement

The surface tension of the aqueous solution at different surfactant concentrations was measured by the Du-Nouy ring method (Wei *et al*., 2005) with a Kruss Tensiometer (Kruss, Hamburg, Germany).The surface tension measurement was carried out at 25 ± 1 ℃ after dipping the platinum ring in the solution for a while in order to attain equilibrium conditions. The measurement was repeated three times and an average value was obtained. The critical micelle concentration (CMC) was then determined from the break point of the surface tension versus log of bulk concentration curve. For the calibration of the instrument, the surface tension of the pure water was measured before each set of experiments.

Measurement of the Critical Micelle Concentration (CMC)

The CMC is an important parameter during the evaluation of activity of biosurfactant. The surface tension of a surfactant reaches the lowest value at its CMC. Above this concentration, no further effect can be observed on the surface activity. Measuring surface tension of serially diluted biosurfactant solution, the CMC was determined by plotting the surface tension versus concentration of biosurfactant in the solution. The determination of CMC was performed by several dilutions of crude biosurfactant. These experiments were conducted in three independent replicates and the results presented were the average data.

Determination of the Emulsification Index

A mixture of 2 mL supernatant and 3 mL kerosene (or diesel) was vertically stirred for 2 min and the height of the emulsion layer was measured after 24 h to determine the emulsification index (Cooper and Goldenberg, 1987). The equation used to determine the emulsification index (E24 (%)) is as follows:

$$\text{E24 (\%)} = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100\%$$

RESULT AND DISCUSSION

Isolation and Identification of Selected Biosurfactant-Producing Bacterial Strain

The bacterial isolate was examined based on its morphological and biochemical characteristics. The isolated strain produced a diffusible green colored fluorescent pigment with excessive amounts of foam when it was grown on glucose-containing MS medium. The bacterial strain was a non-spore forming-gram negative, rod-shaped, motile bacterium. The catalase and oxidase tests were positive, growth on triple sugar iron was neutral bottom and neutral slant, and there was no production of hydrogen sulfide (H_2S) when grown on MacConkey agar. It could not use lactose as a carbon source (Lotfabad *et al*., 2009; Abbasi *et al*., 2012). Preliminary results indicated that the strain was a member of the genus *Pseudomonas*.

Further identification was performed based on the 16S rRNA gene sequence analysis. Partial 16S rRNA sequence alignment revealed that *P. aeruginosa* TMN strain was closely related to the species in genus *Pseudomonas*. *P. aeruginosa* TMN Strain also exhibited the highest similarity (98%) to *P. aeruginosa*.

Effects of Carbon Sources on Rhamnolipid Production

This study started with the investigation of carbon sources on rhamnolipid production and the results are presented in Table 2. The results showed that glucose and glycerol were effective carbon substrates for rhamnolipid production. After cultivation for 7 days, the culture with glucose and glycerol produced 0.3±0.01 and 0.25±0.02 g/L of rhamnolipid, respectively. Meanwhile, the dry cell weights for glucose and glycerol were 2.9±0.02 and 1.35±0.01 g/L, respectively and this result is according with the literature (Lotfabad *et al*., 2009; Wei *et al*., 2005; Wu *et al*., 2008). *P. aeruginosa* TMN was unable to utilize sucrose efficiently, resulting in a poor rhamnolipid yield and dry cell weight of 0.02 and 0.91 g/L, respectively. It is likely that the strain may lack the enzyme (i.e., invertase) for sucrose hydrolysis (Lotfabad *et al*., 2009; Wu *et al.,* 2008). Boulton and Ratledge (1987) mentioned that the use of vegetable oil as carbon sources to produce rhamnolipids seemed to be an interesting and low cost alternative, but *P. aeruginosa* TMN strain attained a lower rhamnolipid yield from olive oil than that from glucose and glycerol.

Table 2: Effect of carbon source on rhamnolipid production (nitrogen source: 50 mM NH_4NO_3).

Carbon Source	Dry cell weight (g/L)	Rhamnolipid Yield (g/L)	Surface Tension (dyne/cm)	P-value
Glucose (40 g/l)	2.9±0.02	0.3±0.01	34±1.2	**0.0***
Glycerol (40 g/l)	1.35±0.01	0.25±0.02	46±0.99	**0.0***
Sucrose (40 g/l)	0.91±0.03	0.02±0.01	51±0.87	**0.0***
Hexane (40 g/l)	0.2±0.01	0.07±0.02	51±0.54	**0.0***
Oleic acid (80 g/l)	5.0±0.06	0.13±0.01	35±0.23	**0.0***
Olive oil (80 g/l)	5.07±0.04	0.19±0.03	36±0.45	**0.0***

Data were statistically described in terms of mean and standard error (±S.E.);*, represent highly significant difference (P<0.001).

The rhamnolipid production and dry cell weight were 0.19±0.03 and 5.07±0.04 g/L for olive oil, respectively. The data showed that there seems to be no clear trend between cell yield and rhamnolipid yield, which were, however, strongly dependent on the carbon source used. Santa Anna *et al*. (2002) showed that glycerol gave much more rhamnolipid yield than different oils. Use of oleic acid as the carbon source did not improve rhamnolipid production, suggesting that hydrolysis of the oils was not the bottle-neck step and this agrees with the literature (Wu *et al*., 2008). Some reports showed that vegetable oils were more efficient substrates in rhamnolipid production from *P. aeruginosa* when compared with glucose, glycerol, and hydrocarbons (Maier and Chavez, 2000; Mata-Sandoval *et al*., 2001).

Moreover, hexane was also inefficient in cell growth and rhamnolipid production; resulting in a low rhamnolipid yield and cell dry weight of only 0.07±0.02 and 0.2± 0.01 g/L, respectively, probably due to its poor biodegradability (Chayabutra *et al*., 2001).

P. aeruginosa TMN used in this work showed a different trend, as glucose and glycerol were superior to the olive oils tested in terms of both rhamnolipid yield and productivity (Lotfabad *et al*., 2009; Anyanwu and Chukwudi, 2010). This suggests that the carbon source preference for rhamnolipid production seems to be strain dependent (Panesar *et al.,* 2009).

On the other hand, the surface tension measurement for the biosurfactants produced from different carbon sources indicated that glucose was the best carbon source. The biosurfactant had a surface tension of 34 dyne/cm and achieved an emulsification index of over 46% for kerosene when $NaNO_3$ was used as nitrogen source (Wu *et al*., 2008).

Effects of Nitrogen Sources on Rhamnolipid Production

Five different organic and inorganic nitrogen sources used in this study are shown in Table 3. It was found that $NaNO_3$ was the most efficient nitrogen source for *P. aeruginosa* TMN, followed by ammonium nitrate, for producing rhamnolipid, giving a high rhamnolipid yield of 0.34±0.01 and cell dry weigth of 0.3±0.01 g/L, respectively. The observation that nitrate was the better inorganic nitrogen source than ammonium ion for rhamnolipid production is consistent with the findings of comparable studies in the literature (Santos *et al.,* 1984; Arino *et al*., 1996; Wei and Chu, 1998; Anna *et al*., 2002; Santos *et al*., 2002; Jeong *et al*., 2004). Moreover, using urea and yeast extract as the organic nitrogen sources led to a rhamnolipid yield of 0.3±0.01 and cell dry weight of 0.25±0.01 g/L, respectively. Despite giving rise to better cell growth, these organic nitrogen sources had poorer performance than $NaNO_3$ in terms of rhamnolipid yield. In fact, it has been reported that an organic nitrogen source could help cell growth, but was unfavorable for production of glycolipid biosurfactant (Kim *et al*., 2006).

Table 3: Effect of nitrogen source on rhamnolipid production (carbon source: 40 g/l glucose).

Nitrogen Source	Dry cell weight (g/L)	Rhamnolipid Yield (g/L)	Surface Tension (dyne/cm)	P-value
NH_4Cl (50 mM)	3.61±0.1	0.04±0.01	37±1.0	**0.0***
$NaNO_3$ (50 mM)	4.2±0.03	0.34±0.01	34±0.69	**0.0***
Urea (50 mM)	3.89±0.02	0.3±0.01	47±0.47	**0.0***
Yeast extract (1%)	2.43±0.11	0.25±0.01	40±0.34	**0.0***
NH_4NO_3 (50 mM)	2.9±0.08	0.3±0.01	36±0.22	**0.0***

Data were statistically described in terms of mean and standard error (±S.E.); *, represent highly significant difference (P<0.001).

Surface tension measurements for the biosurfactants produced from different nitrogen sources indicated that $NaNO_3$ was the best nitrogen source. The best biosurfactant had a surface tension of 34 dyne/cm and achieved an emulsification index of over 46% for kerosene and this agrees with the literature (Wu *et al*., 2008). Hence, in the following experiments, $NaNO_3$ was chosen as the nitrogen source and glucose as the carbon source to achieve a higher rhamnolipid yield.

Effect of pH

The yield of biosurfactant, investigated with pH varied from 4 to 8, reached the highest at pH 7; however, when the pH reached 8, the biosurfactant yield declined and reached its lowest point. Thus, it can be inferred that *P. aeruginosa* TMN strain excreted biosurfactant more effectively under neutral conditions, as shown in Figure 1. These values agree with those previously reported for rhamnolipid by the authors Priya and Usharani (2009). They showed maximum production of rhamnolipid at pH 7 using *Pseudomonas aeruginosa*. Temperature and pH were two environmental factors that have a major effect on the biological activities of prokaryotes (Yakimov *et al*., 1995).

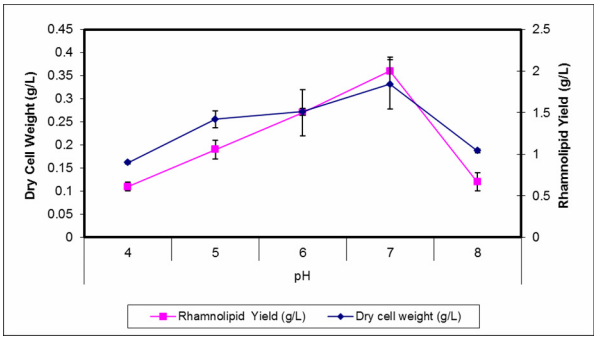

Figure 1: Effect of different pHs on Rhamnolipid Yield and Dry cell weight.

Effect of Temperature on Rhamnolipid Production

P. aeruginosa TMN strain was grown in GMS medium at 25–45 °C to explore the influence of culture temperature on rhamnolipid production. As indicated in Figure 2, rhamnolipid production increased with temperature from 25 to 37 °C, and decreased slightly when the temperature was further increased to 45 °C. *P. aeruginosa* was unable to grow at 47 °C, leading to negligible rhamnolipid production at that temperature. These results suggest that the optimal temperature for rhamnolipid production with *P. aeruginosa* TMN was in the range of 37–40 °C. This result is consistent with the findings of comparable studies in the literature (Wei *et al*., 2005; Priya and Usharani, 2009), which showed maximum biosurfactant production at 37 °C.

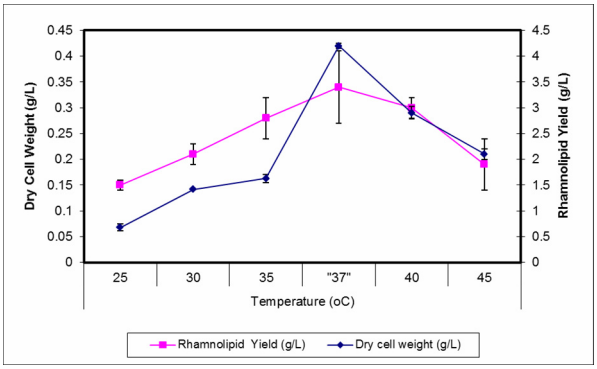

Figure 2: Effect of different temperatures on Rhamnolipid Yield and Dry cell weight.

Effect of Agitation Rate on Rhamnolipid Production

Agitation rate affects the mass transfer efficiency of both oxygen and medium components and is considered crucial to the cell growth and biosurfactant formation of the strictly aerobic bacterium *P. aeruginosa*, especially when it was grown in a shake flask. Results from batch fermentation under different agitation rates (100–250 rpm) show that, as the agitation rate increased from 100 to 200 rpm, rhamnolipid production increased and dry cell weight was also improved from 0.14±0.01 and 0.94±0.05 to 0.3±0.01 and 1.8±0.03 g/l respectively as shown in Figure 3. The rhamnolipid production and dry cell weight are slightly decreased to 0.15±0.02 and 1.59±0.06 g/L, respectively, at 250 rpm. This can be attributed to the severe foaming when the flask was shaken at 250 rpm (Wei *et al*., 2005). The heavy foaming caused by emulsification of rhamnolipid during vigorous shaking (e.g., 250 rpm) may decrease the transfer efficiency of oxygen gas into the liquid medium, and is thus unsuitable for rhamnolipid production in shake-flask cultures. As a result, an agitation rate of 200 rpm (rather than 250 rpm) was used in the rest of the experiments. It should be pointed out that the heavy foaming during biosurfactant fermentation is not necessarily a negative factor. Some researchers collected the biosurfactant-rich foams from the fermenter for continuous recovery of biosurfactant to avoid product inhibition and to simplify downstream processing (Davis *et al*., 2001).

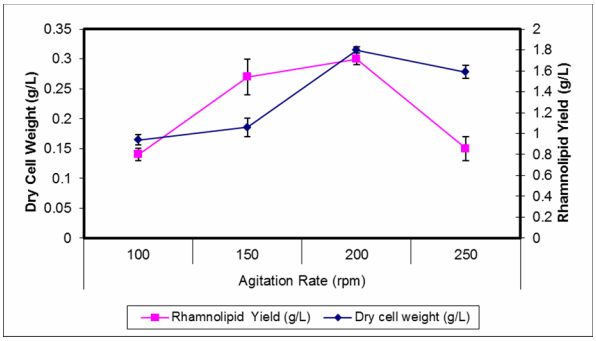

Figure 3: Effect of different agitation rates on rhamnolipid Yield and Dry cell weight.

Structural Characterization

Thin Layer Chromatography (TLC)

The formation of various rhamnolipids was checked by TLC. The crude rhamnolipids contained both monorhamnolipid and di-rhamnolipids, but the most active fragment is di-rhamnolipid, which gave a diameter of 10.5 cm in the oil displacement test.

Fourier Transform Infrared Spectroscopy (FTIR)

The infrared spectrum of purified biosurfactant indicated a broad peak at 3429 cm^{-1}, characteristic of O–H stretching vibrations. Absorption around 2922 cm^{-1} is assigned to the asymmetric C-H stretch of CH_2 and CH_3 groups of aliphatic chains. The corresponding symmetric stretch is seen at 2853 cm^{-1}. Also, a weak symmetric stretching peak at 1715 cm^{-1} indicated the presence of ester carbonyl groups (C=O in COOH) in the biosurfactant. The ester carbonyl group was indicated by the band at 1321 cm^{-1} which corresponds to C- O deformation vibrations, although other groups also absorb in this region.

The presence of the carboxylic acid functional group in the molecule was also confirmed by the medium intensity bands in the region 1455–1386 cm^{-1} for bending of the hydroxyl (O–H). The absorption peak around 1051cm^{-1} was also reported as C–O–C stretching in rhamnose (Pornsunthorntawee *et al*., 2008). The α-pyranyl II sorption band at 834 cm^{-1} showed the presence of di-rhamnolipid in the mixture. These characteristic adsorption bands taken together demonstrate that both have chemical structures identical with those of rhamnolipids, which have rhamnose rings and long hydrocarbon chains. Thus, according to the results of the IR spectra, the rhamnolipids produced by *P. aeruginosa* TMN belong to the glycolipid group, which is made up of aliphatic acids and esters. The adsorption bands obtained are consistent with the report of Guo *et al*. (2009) showing the presence of rhamnose rings and hydrocarbon chains. In the FTIR spectrum, we could observe only a minor shoulder; this might be because of the di-rhamnolipid-rich biosurfactant produced by *P. aeruginosa* TMN (Rahman *et al*., 2002b). The results obtained are consistent with the structure reported by Stanghellini and Miller (1997) consisting of aliphatic acid and the glycolipid moiety.

Nuclear Magnetic Resonance (NMR)

The structures of purified rhamnolipids were confirmed by ^{1}H-NMR and the results are shown in Table 4. The chemicals shifts were comparable to previous reports (Ramana and Karanth, 1989; Sim *et al.,* 1997; Wei *et al*., 2005) and the results were in accordance with the structures as shown by ESI-spectra, Figure 4.

Table 4: ^{1}H-NMR chemical shift data for rhamnolipid components.

^{1}H chemical shift (ppm)	Multiplicity	Assignment
0.882	Singlet	$–CH_3$ (on β-hydroxyfatty acids)
1.185, 1.204	Doublet	$–CH_3$ (on rhamnose moiety)
1.276	Multiplet	$–(CH_2)_5–$ (on β-hydroxyfatty acids)
1.574	Multiplet	$–(CH_2)–CH(O)–CH_2COO$ (on β-hydroxyfatty acids)
2.545	Multiplet	$–CH(O)–CH_2COO$ (on β-hydroxyfatty acids)
3.372	Multiplet	$–(CH_2)–CH(O\text{-}Rha)–CH_2COO$ (on β-hydroxyfatty acids)
3.335	Multiplet	–CH–OH (on rhamnose moiety)
4.130	Multiplet	$–(CH_2)–CH(–O–C=O)–CH_2COO$ (on β-hydroxyfatty acids)
4.938	Singlet	–CH–OH (on rhamnose moiety)
5.275, 5.295	Doublet	–CH–O–C (on rhamnose moiety)

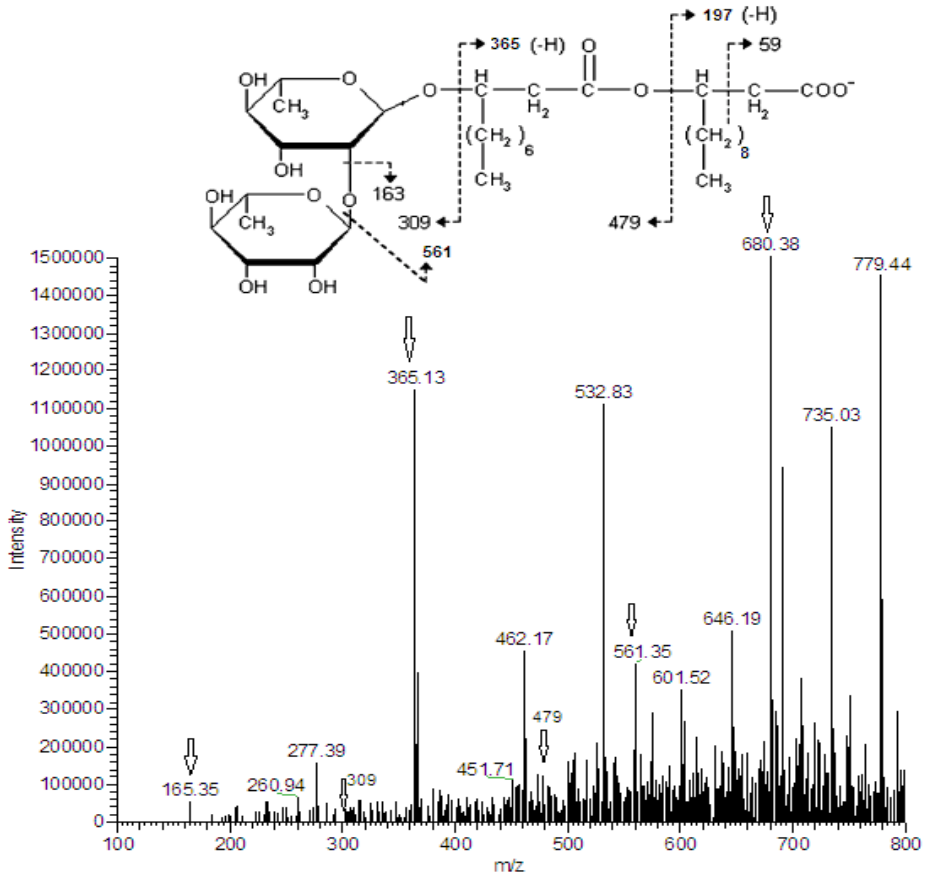

Figure 4: ESI mass spectrum and chemical structure of Rha-Rha-C_{10}–C_{12} rhamnolipid and its fragments.

Electrospray Ionization–Mass Spectrometry (ESI–MS)

In this study, the highest intensity rhamnolipid found was the di-rhamnolipid homologues which correspond to Rha-Rha-C_{10}–C_{12} with m⁄z of 680; their fraction at m⁄z 365 is shown in Figure 4. This work is in agreement with previous reports dealing with production of rhamnolipid surfactant mixtures in which di-rhamnolipids were the predominant species (Arutchelvi and Doble, 2010). On the contrary, few reports (Arino *et al.,* 1996; Sim *et al.,* 1997) describe a rhamnolipid mixture in which monorhamnolipid is the predominant component.

Physicochemical Characterization

Oil Displacement Test

The Oil displacement test is an indirect measurement of the surface activity of a surfactant sample tested against oil; a larger diameter represents a higher surface activity of the testing solution (Pornsunthorntawee *et al.,* 2008). The oil spreading method is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample (Płaza *et al.,* 2006). It can be applied when the activity and quantity of biosurfactant is low. Plaza *et al.* (2004) and Youssef *et al*. (2006) demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms. The assay was also applied for screening by Huy *et al*. (1999).

Figure 5 lists the diameters of the clear zones on the oil surface obtained from oil displacement testing with the crude biosurfactant produced from different carbon and nitrogen sources by *P. aeruginosa* TMN.

Critical Micelle Concentration (CMC)

The CMC also represent the surface activity of a surfactant. A lower CMC of the surfactant means that the concentration required for this surfactant to form micelles is also lower. Therefore, the surfactant can

change the surface properties and exhibit functions such as emulsification, solubilization and foaming even at a relatively low concentration. The surface tension of purified biosurfactant decreased as its concentration increased, until reaching the lowest value, 34 mN m^{-1}. At this point, the concentration of biosurfactant, namely the CMC, was 18.75 mg/L. This CMC was much lower compared with some synthetic surfactants. For instance, sodium dodecyl sulfate (SDS) has a CMC value of 2100 mg/L (Chen *et al.,* 2006). Nitschke and Pastore (2006) reported that the CMC of biosurfactant obtained from *Bacillus subtilis* was 33 mg/L. Li *et al*. (1984) showed that the CMC of rhamnolipid fermentation liquor was 386 mg/L (Daoshan *et al*., 2004).

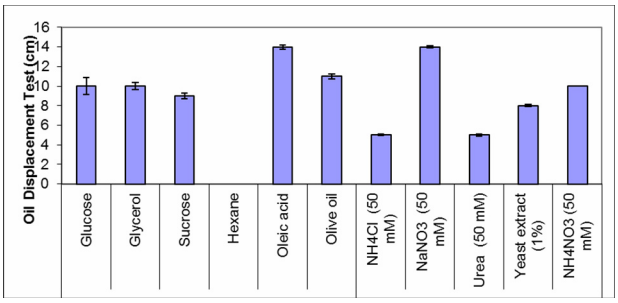

Figure 5: Diameters of the clear zones on the oil surface obtained from oil displacement testing with the crude biosurfactant produced from different carbon and nitrogen sources by *Pseudomonas aeruginosa* TMN.

Estimation of Emulsification Activity

Emulsification of rhamnolipids depends on the carbon sources used to produce rhamnolipids (Pornsunthorntawee *et al*., 2008). Six carbon sources, i.e., glucose, glycerol, sucrose, hexane, oleic acid, and olive oil were examined for their effectiveness of biosurfactant production. The results of various carbon sources for emulsification activity under the above mentioned conditions are shown in Figure 6. As evident from the figure, *P. aeruginosa* TMN was able to grow in a medium containing oleic acid and olive oil with maximum emulsification activity. Other carbon sources (glycerol, glucose, sucrose and hexane) used, showed less emulsification activity, hexane being the lowest, as compared to other carbon sources. This suggested that there is a carbon source preference of the strain for biosurfactant production, which seems to be strain dependent (Wu *et al*., 2008). Most microbial surfactants were substrate specific, solubilizing or emulsifying different hydrocarbons at different rates.

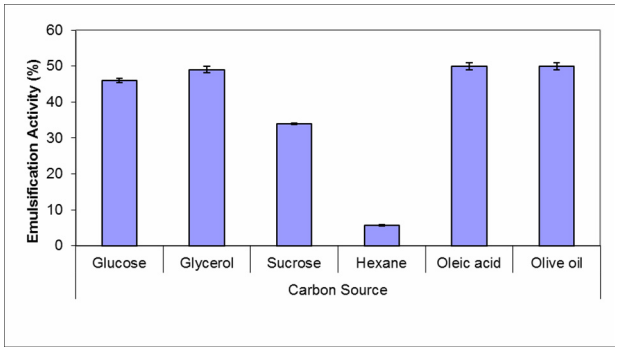

Figure 6: Emulsifying activity (E24%) of biosurfactants obtained from different carbon sources against kerosene.

Poor emulsification of other hydrocarbons might be due to the inability of the biosurfactant to stabilize the microscopic droplets. Also, an inefficient oxygen supply in the flask cultures may be responsible for poor growth with other carbon sources, as biodegradation of these oils is known to be an oxygen intensive metabolic event (Panesar *et al*., 2009). On other hand, oleic acid and olive oil are the carbon sources which are taken up very easily compared to others. Among the six carbon sources tested, oleic acid and olive oil were the best carbon sources, with an emulsification activity of 50%.

Nitrogen sources such as sodium nitrate, ammonium chloride, urea, yeast extract, and ammonium nitrate were added to the fermentation media. Sodium nitrate and ammonium nitrate were found to be the most effective amongst these nitrogen sources, as shown in Figure 7. Other nitrogen sources (urea, ammonium chloride and yeast extract) gave low emulsification activity. Among the nitrogen sources, sodium nitrate and ammonium nitrate gave similar results, but, considering the cost factor, sodium nitrate was the most effective.

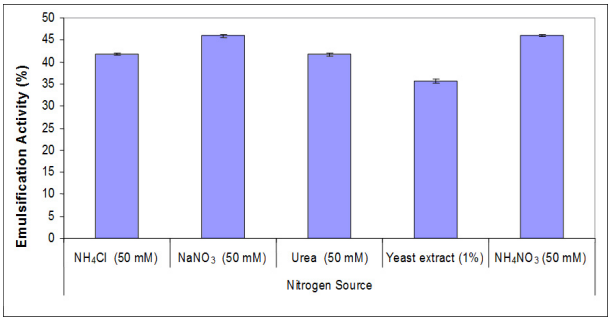

Figure 7: Emulsifying activity (E24%) of biosurfactants obtained from different nitrogen sources against kerosene.

CONCLUSIONS

P. aeruginosa TMN capable of effectively producing rhamnolipid from various carbon sources was successfully isolated. *P. aeruginosa* TMN can degrade vegetable oils to produce biosurfactant and emulsify kerosene. Hence, the strain itself or its biosurfactant product have the potential to be applied in bioremediation of oil pollutants. Among the six carbon substrates and five nitrogen sources examined, glucose and glycerol were the most efficient carbon sources, while $NaNO_3$ was the best nitrogen source for rhamnolipid production. Rhamnolipid production was optimal in batch cultures when the temperature, pH and agitation rate were controlled at 37 °C, pH 7 and 200 rpm, respectively. FTIR, NMR and ESI-MS analysis showed that the purified product contained L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate rhamnolipid. In conclusion, the biosurfactant produced by *P. aeruginosa* TMN strain was the preferable surface-active substance, which can be used for potential application in bioremediation of crude oil contamination.

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