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Screening and Optimization of Biosurfactant Production by the Hydrocarbon-degrading Bacteria

(Penyaringan dan Pengoptimuman Biosurfaktan yang Dihasilkan oleh Bakteria Pendegradasi-hidrokarbon)

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ABSTRACT

Biosurfactants are amphiphilic compounds produced by microorganisms as secondary metabolite. The unique properties of biosurfactants make them possible to replace or to be added to synthetic surfactants which are mainly used in food, cosmetics and pharmaceutical industries and in environmental applications. In this study twenty hydrocarbon-degrading bacteria were screened for biosurfactant production. All of the bacterial isolates were grown in mineral salt medium (MSM) with addition of 1% (v/v) Tapis crude oil as carbon source. The presence of biosurfactant was determined by the drop-collapse test, microplate analysis, oil spreading technique, emulsification index (%EI_{24}) and surface tension measurement. Only one isolate, Pseudomonas aeruginosa *UKMP14T, was found to be positive for all the qualitative tests and reducing the surface tension of the medium to 49.5 dynes/cm with emulsification index of 25.29%. This isolate produced biosurfactant optimally at pH9.0 and incubation temperature of 37°C. Furthermore,* P. aeruginosa *UKMP14T when grown in MSM with addition of 1% (v/v) glycerol and 1.3 g/L ammonium sulphate with C/N ratio 14:1 produced biosurfactant with percentage of surface tension reduction at 55% or 30.6 dynes/cm with %EI_{24} of 43%. This percentage of surface tension reduction represents an increasing reduction in surface tension of medium by 39% over the value before optimization. This study showed that* P. aeruginosa *UKMP14T has the ability to biodegrade hydrocarbon and concurrently produce biosurfactant.*

Keywords: Biosurfactant; hydrocarbon-degrading bacteria; optimization; surface tension

ABSTRAK

Biosurfaktan adalah sebatian ampifilik yang dihasilkan oleh mikroorganisma sebagai metabolit sekunder. Ciri-ciri biosurfaktan yang unik membolehkan mereka menggantikan atau ditambahkan kepada surfaktan sintetik yang kebanyakannya digunakan dalam industri makanan, kosmetik dan farmaseutikal dan diaplikasi dalam sekitaran. Dalam kajian ini sebanyak 20 pencilan bakteria-pencurai hidrokarbon disaring untuk penghasilan biosurfaktan. Kesemua pencilan bakteria dihidupkan di dalam medium garam mineral (MSM) yang ditambah dengan 1% (v/v) minyak mentah Tapis sebagai sumber karbon. Kehadiran biosurfaktan ditentukan dengan ujian titisan-runtuh, analisis mikroplat, teknik sebaran minyak, indeks emulsifikasi (%EI_{24}) dan pengukuran ketegangan permukaan. Hanya satu pencilan iaitu Pseudomonas aeruginosa *UKMP14T yang memberikan hasil positif ke atas semua ujian kualitatif dan mengurangkan ketegangan permukaan medium sehingga 49.5 dynes/cm dengan indeks emulsifikasi 25.29%. Pencilan ini menghasilkan biosurfaktan yang optimum pada pH9.0 dan suhu eraman 37°C. Selain itu, apabila* P. aeruginosa *UKMP14T dihidupkan di dalam MSM dengan penambahan 1% (v/v) gliserol dan 1.3 g/L ammonium sulfat dengan nisbah C/N 14:1, ia menghasilkan biosurfaktan dengan peratus pengurangan ketegangan permukaan sebanyak 55% atau 30.6 dynes/cm dengan %EI_{24}, 43%. Peratus pengurangan ketegangan permukaan ini mewakili peningkatan pengurangan dalam ketegangan permukaan medium sebanyak 39% jika dibandingkan sebelum pengoptimuman. Kajian ini menunjukkan bahawa* P. aeruginosa *UKMP14T mempunyai keupayaan untuk biodegradasi hidrokarbon serta menghasilkan biosurfaktan.*

Kata kunci: Bakteria pencurai-hidrokarbon; biosurfaktan; ketegangan permukaan; pengoptimuman

INTRODUCTION

Biosurfactants are compounds that produce surface-active and emulsifying activities and are themselves produced by microorganisms, such as bacteria, yeast and fungi. For example, *Pseudomonas aeruginosa* has been reported to produce the biosurfactant rhamnolipid (Cameotra & Makkar 2004) while *Bacillus subtilis* is known to produce surfactin (Pornsunthorntawee et al. 2008).

Biosurfactants are amphiphilic, consisting of two parts, a polar (hydrophilic) moiety and a non-polar (hydrophobic) group. The hydrophilic group consists of mono-, oligo-, or polysaccharides, peptides or proteins while the hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols (Pacwa-Plociniczak et al. 2011). Biosurfactants play a number of roles including increasing the surface area and

bioavailability of hydrophobic water-insoluble substrates, binding of heavy metals, quorum sensing and biofilm formation (Rodrigues et al. 2006). Compared with synthetic surfactants, biosurfactants have higher surface activity, lower toxicity, higher biodegradability and better environmental compatibility (Wei et al. 2007). With their high surface activity and environmental compatibility, biosurfactants are widely used in environmental applications such as for enhancement of oil degradation (Pacwa-Plociniczak et al. 2011), as antioxidants, as antimicrobials in the cosmetics industry (Williams 2009) and as anti-adhesives against several bacteria and yeasts in medical applications (Rodrigues et al. 2006).

In oil fields, some but not all effective oil-degrading bacteria produce extracellular biosurfactants to facilitate microbial oil uptake and degradation by emulsifying the hydrocarbon (Morikawa et al. 2000). Surfactants and biosurfactants can increase the pseudo-solubility of petroleum components in water (Pekdemir et al. 2005). Moreover, biosurfactants can be as effective as synthetic chemical surfactants due to their high specificity and their biodegradability. The objectives of this study were to screen local hydrocarbon–degrading bacteria for their ability to produce biosurfactants and to optimize the physical and nutrient parameters to enhance bacterial production of biosurfactants.

Materials & Methods

Bacterial Isolates

Hydrocarbon-degrading bacteria were obtained from the Environmental Microbiology Laboratory culture collection of the School of Biosciences and Biotechnology at Universiti Kebangsaan Malaysia. These isolates a were able to degrade hydrocarbons (Mukred et al. 2008; Nur Faizah 2010).

Media

This study used mineral salt medium (MSM) (Zajic & Supplison 1972) containing 0.1% (v/v) vitamins and trace elements (Bouchez et al. 1995) supplemented with 1% (v/v) Tapis crude oil as carbon source. The pH of the medium was adjusted to 7.0 with 1M sodium hydroxide (NaOH) or 1M hydrochloric acid (HCl). The medium was autoclaved at 121°C, 15 psi for 15 min and then cooled in a water bath to 45°C before added with Tapis crude oil.

Screening for Biosurfactant-Producing Bacteria

Preparation of Culture Medium

A standardized inoculum of each isolate was prepared as described by Hamzah et al. (2010). Then, 10% (v/v) of this standardized inoculum was inoculated into 250 mL conical flask containing 50 mL of MSM with added 1% (v/v) Tapis crude oil and incubated at 37°C on an orbital shaker with agitation speed 150 rpm for 5 days.

Next, the culture medium was centrifuged at 8022 g (RC5C Sorvall Centrifuge Instrument) at 4°C for 30 min. The supernatant was collected and used for preliminary screening for biosurfactant present using a drop-collapse test, microplate analysis, oil spreading technique, calculation of the emulsification index ($\%EI_{24}$) and surface tension measurement. Distilled water and MSM without inoculation were used as negative control, while 1% (w/v) sodium dodecyl sulphate (SDS) was used as positive control.

Screening of Biosurfactant Producer

Qualitative Methods. The drop-collapse test was performed according to Plaza et al. (2006). In this method, supernatant from each bacterial isolate was pipetted onto a microplate lid (12.7 × 8.6 cm^2, Corning Incorporated 3790, USA). Then, Tapis crude oil was added onto the surface of the supernatant. If the drop of oil on the supernatant became flat 1 min after adding the oil, the result was taken to be positive. If the drops remained beaded, the result was scored as negative.

Microplate analysis was carried out according to Chen et al. (2007). The surfactant activity of each bacterial isolate was determined using a microwell plate. The supernatant from each bacterial isolate was added to a 96-microwell plate (12.7 × 8.6 cm^2, Corning Incorporated 3790, USA). The plate was then viewed using a backing sheet of paper with grid. A positive result was recorded when there was no optical distortion of the grid.

The oil spreading technique was carried out as described by Youssef et al. (2004). Briefly, distilled water was added to the Petri dish (90 mm × 15 mm) followed by addition of Tapis crude oil to the surface of water. Then, 10 μL supernatant for each bacterial isolate was dropped onto the Tapis crude oil surface. The diameter of the clear zone on the oil surface was measured and compared with those on the negative and positive controls.

The emulsification index ($\%EI_{24}$) was determined according to Cooper and Goldenberg (1987). The same volume of supernatant and Tapis crude oil in a ratio of 1:1 were mixed in a glass test tube (125 mm × 15 mm). Then, the mixture was vortexed for 2 min and left to stand for 24 h. The $\%EI_{24}$ is given as percentage yielded by dividing the height of the emulsified layer (mm) by the total height of the liquid in the glass test tube (mm), then multiplying by 100. A higher emulsification index indicates a higher emulsification activity of the tested surfactant.

The surface tension was measured using a surface tensiomat model 21 tensiometer (Fisher Scientific) by the Du Noüy Ring method. For the calibration of the instrument, the surface tension of pure water was measured. The criterion used for selecting biosurfactant-producing isolates was the emulsification and reduction of the surface tension of the medium to below 40 dynes/cm (Bodour & Miller-Maier 1998).

After screening, candidate biosurfactant-producing bacteria were selected for optimization of the physical and nutrient parameters for optimum biosurfactant production.

Optimization of Physical and Nutrient Parameters

For all the experiments below, the following standard procedure was used:

Ten percent (v/v) of standardized inoculum was inoculated into 250 mL conical flask containing 50 mL of MSM, supplemented with 1% (v/v) Tapis crude oil and incubated in an orbital shaker at agitation speed of 150 rpm for 5 days. The negative control in these tests was MSM without inoculation.

MEASUREMENT OF PARAMETERS

For both physical and nutrient parameters, after 5 days of incubation, the culture was centrifuged at 8022 g at 4°C for 30 min. The supernatant was collected and the surface tension was read using a tensiometer; results were expressed in dynes/cm. The surface activity of the bacteria-produced biosurfactant was also expressed as a percentage of the reduction in surface tension calculated using the following equation (Pornsunthorntawee et al. 2008):

$$\text{Percentage of surface tension reduction} = \frac{(\gamma m - \gamma c)}{\gamma m} \times 100,$$

γm is the surface tension of the control (medium without inoculation) and γc is the surface tension of the test supernatant.

pH AND TEMPERATURE

For determination of optimal pH, the standardized inoculum was inoculated in MSM at different pH (6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0), then incubated at 37°C on an orbital shaker at 150 rpm.

After optimal pH had been determined, the bacteria were grown in MSM at optimized pH and incubated at different temperatures (30, 33, 35, 37 and 40°C) on an orbital shaker at 150 rpm for 5 days.

CARBON SOURCES

Bacterial inoculum was inoculated in MSM at optimized pH to which was added different carbon sources comprising glycerol, palm oil, soy bean oil, olive oil and Tapis crude oil at 1% (v/v), then incubated on an orbital shaker at 150 rpm and at the predetermined optimized temperature for 5 days.

The carbon source that induced the highest biosurfactant production demonstrated by showing the lowest surface tension was subsequently chosen for variation in different concentrations of carbon starting from 0.25, 0.5, 1, 3, 5, 10 and 15% (v/v).

NITROGEN SOURCES

To determine the best nitrogen source for optimized production of biosurfactant, the total amount of nitrogen in MSM which was contained in $NH_4Cl \approx 4$ g/L was replaced with the same amount of total nitrogen. Standardized bacterial inoculum was inoculated in MSM at optimized pH with added different nitrogen sources, namely, ammonium sulphate ($(NH_4)_2SO_4$), sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl) and ammonium nitrate (NH_4NO_3), then incubated on an orbital shaker at 150 rpm and at the predetermined optimized temperature for 5 days. The nitrogen source that induced the highest level of biosurfactant production as demonstrated by the lowest surface tension activity was further chosen for variation in different concentrations, ranging from 0.5-4.96 g/L.

STATISTICAL ANALYSIS

The means of the results were analysed statistically using the SPSS software (version 17.0). The means were compared using one-way ANOVA and the Tukey test to indicate any significant difference among parameters and the variables. The result was considered significant if $p<0.05$.

Results and Discussion

SCREENING OF BIOSURFACTANT-PRODUCING BACTERIA

Out of 20 isolates screened, only *Pseudomonas aeruginosa* UKMP14T showed a positive result in the drop-collapse test. The other five isolates (*Bacillus flexus* UKMP3T, *Rhodococcus* sp. UKMP5T, *Rhodococcus* sp. UKMP7T, *Acinetobacter baumannii* UKMP12T and *Pseudomonas aeruginosa* UKMP14T) showed positive result in the microplate analysis while nine isolates (*Rhodococcus* sp. UKMP1T, *Rhodococcus* sp. UKMP2T, *Bacillus flexus* UKMP3T, *Rhodococcus ruber* UKMP4T, *Rhodococcus* sp. UKMP5T, *Rhodococcus* sp. UKMP7T, *Acinetobacter calcoaceticus* UKMP9T, *Acinetobacter baumannii* UKMP12T and *Pseudomonas aeruginosa* UKMP14T) were detected positive by the oil-spreading technique (Table 1).

These results suggested that the oil-spreading technique is more sensitive than the other methods for biosurfactant detection in the supernatant from a culture medium. According to Youssef et al. (2004), the drop-collapse method is not as sensitive as the oil-spreading technique in detecting low levels of biosurfactant production. Similarly, microplate analysis was unable to detect the presence of surfactant at low levels.

Quantitative analysis including emulsification index ($\%EI_{24}$) and surface tension measurement was found to be a more reliable method for quantification of the soluble biosurfactant in the medium. An isolate was selected as a biosurfactant-producer if it reduced the surface tension below 40 dynes/cm (Bodour & Miller-Maier 1998) and/or maintained at least 50% of the original emulsion volume 24 h after formation of emulsification (Willumsen & Karlson 1997). *P. aeruginosa* UKMP14T showed positive results in all qualitative tests and in the quantitative evaluation produced a higher reduction in surface tension (49.5 ± 0.07 dynes/cm) and a higher

percentage of emulsification at 24 h (25.49 ± 0.00%) than did the positive control SDS with 40.0 dynes/cm and a $\%EI_{24}$ of 53%.

Isolates *Rhodococcus* sp. UKMP5T and *Acinetobacter baumannii* UKMP12T although did not produce biosurfactant, are bioemulsifier producer because their emulsification index was the highest among others. This study showed that quantitative analyses were more reliable for detection of the presence of biosurfactant in the medium by bacterial isolates.

Optimization of Physical Parameters

pH and Temperature

P. aeruginosa UKMP14T grew and produced biosurfactant at a wide range of pH from 6.5 to 9.0. Although statistical analysis showed no significant difference between the production of biosurfactant at pH8.5 and pH9.0, pH9.0 was selected as the best pH for biosurfactant production because it produced the highest surface tension reduction at 40.3 dynes/cm or 38.85 ± 3.75% reduction as compared with medium without inoculation (Figure 1). At an acidic pH (6.0) and extreme alkaline pH (10.0), this isolate produced lower levels of biosurfactant.

Different species of *Pseudomonas* have been found to produce biosurfactant at different pH. For example, *P. aeruginosa* S6 isolated from sludge containing oil produced biosurfactant when grown in MSM with added 5.0 g/L of glucose at pH9.0 reducing surface tension to 33.9 dynes/cm (Yin et al. 2009). Meanwhile, *Pseudomonas* sp. isolated from oil-contaminated soil produced maximum biosurfactant at pH7.0 when grown in medium with 3% (v/v) when added (Praveesh et al. 2011).

P. aeruginosa UKMP14T grown in MSM at pH9.0 produced maximum biosurfactant when incubated at temperature 37°C, significantly different from cultures grown at 30, 33, 35 and 40°C, while producing the lowest surface tension at 40.3 dynes/cm representing a 39% reduction in surface tension (Figure 2). When the incubation temperature increased to 40°C, bacterial growth and biosurfactant production were totally inhibited, indicating that the biosurfactant produced by *P. aeruginosa* UKMP14T was temperature-dependent.

P. aeruginosa MR01 isolated from oil excavation areas in the south of Iran (Lotfabad et al. 2009) and *P. aeruginosa* S2 isolated from diesel-contaminated soil (Chen et al. 2007), both produced optimum biosurfactant when grown at 37°C. Another study by Praveesh et al. (2011) showed *P. aeruginosa* sp. produced the maximum rhamnolipid at 35°C while at 40°C, bacterial growth and biosurfactant production were inhibited. Different strains of *P. aeruginosa* have different optimum pH and *P. aeruginosa* was shown to be a mesophilic bacterium that cannot survive at temperature more than 40°C.

TABLE 1. Screening of potential biosurfactant production using several methods

Bacterial isolates	'Drop-collapse'	Microplate analysis	Oil spreading technique	Emulsification index ($\%EI_{24}$)	Surface tension (dynes/cm)
Rhodococcus sp. UKMP1T	-	-	+	15.68 ± 0.00	60.0 ± 0.28
Rhodococcus sp. UKMP2T	-	-	+	24.51 ± 1.39	66.1 ± 0.07
Bacillus flexus UKMP3T	-	+	+	23.53 ± 0.00	57.7 ± 2.76
Rhodococcus ruber UKMP4T	-	-	+	25.49 ± 0.00	60.2 ± 0.00
Rhodococcus sp. UKMP5T	-	+	+	48.53 ± 2.08	55.1 ± 1.48
Stenotrophomonas sp. UKMP6T	-	-	-	0	55.4 ± 1.06
Rhodococcus sp. UKMP7T	-	+	+	15.69 ± 0.00	59.5 ± 0.21
Acinetobacter calcoaceticus UKMP9T	-	-	+	6.86 ± 1.39	61.4 ± 0.64
Bacillus subtilis UKMP10T	-	-	-	0	60.4 ± 3.40
Streptomonas maltophylia UKMP11T	-	-	-	0	64.0 ± 1.84
Acinetobacter baumannii UKMP12T	-	+	+	32.35 ± 0.00	59.8 ± 0.70
Acinetobacter jejuni UKMP13T	-	-	-	0	62.1 ± 6.08
Pseudomonas aeruginosa UKMP14T	+	+	+	25.49 ± 0.00	49.5 ± 0.07
Eriguobacterium lactigenes UKMP1G	-	-	-	0	58.7 ± 1.84
Bacillus megaterium UKMP2G	-	-	-	0	58.3 ± 0.85
Pseudomonas aeruginosa UKMP5G	-	-	-	0	59.6 ± 4.24
Bacillus cereus UKMP6G	-	-	-	0	65.2 ± 0.64
Bacillus flexus UKMP7G	-	-	-	0	59.8 ± 0.21
Acinetobacter sp. TDA4.2	-	-	-	0	61.9 ± 1.06
Neisseria sp. WD2	-	-	-	23.53 ± 0.00	56.6 ± 0.64
Distilled water	-	-	-	0	72.1 ± 0.14
MSM + 1% (v/v) Tapis crude oil	-	-	-	0	58.6 ± 0.07
1% (w/v) SDS	+	+	++++	52.94 ± 0.00	40.0 ± 0.00

'-' : No biosurfactant produced**; '+' :** Biosurfactant produced

Oil spreading technique: '+' clear zone diameter 0.5-0.9 cm; '++' clear zone diameter 1.0-1.5 cm; '+++' clear zone diameter >1.5-<2.1 cm;'++++' clear zone diameter >2.1 cm and <3.0cm

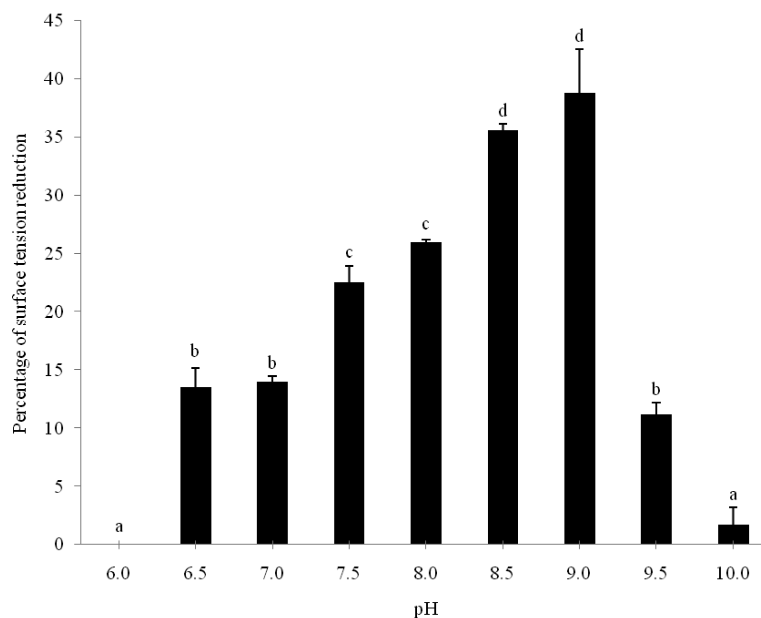

FIGURE 1. Effects of different pH on biosurfactant production by *P. aeruginosa* UKMP14T. The bacteria was grown in MSM, incubated at 37°C and shaken at 150 rpm for 5 days. Different letters represent significance differences ($p< 0.05$) among different pH

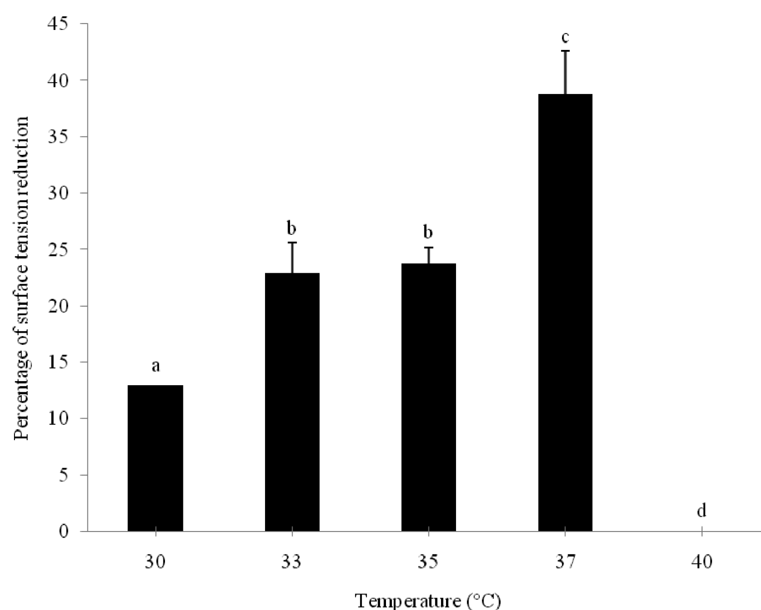

FIGURE 2. Effects of different growth temperatures on biosurfactant production by *P. aeruginosa* UKMP14T. The bacteria was grown in MSM, pH9.0, incubated at different temperatures, shaken at 150 rpm for 5 days. Different letters represent significant differences ($p< 0.05$)

OPTIMIZATION OF NUTRIENT PARAMETERS

CARBON AND NITROGEN

The ability of *P. aeruginosa* UKMP14T to utilize various types of carbon sources for biosurfactant production was tested with glycerol, Tapis crude oil, palm oil, soy bean oil and olive oil at 1% (v/v). Among these carbon sources tested, glycerol produced the lowest surface tension and the highest percentage of reduction in surface tension at 32.0 dynes/cm representing a 48% reduction, followed by Tapis crude oil with a surface tension of 40.3 dynes/cm for a 39% reduction. While there was no significant difference between glycerol and Tapis crude oil as carbon sources, glycerol was selected as the optimal carbon source since it produced the highest percentage reduction in surface tension (Figure 3). When grown at different concentrations of glycerol, *P. aeruginosa* UKMP14T

effected the highest percentage of reduction in surface tension with glycerol 1% (v/v) (Figure 4). However, statistical analysis showed no significant difference between the 1% (v/v) and 10% (v/v) levels of glycerol.

Zhang et al. (2005) found that the highest biosurfactant production was obtained when *P. aeruginosa* was grown in 30 g/L glycerol rather than in glucose, vegetable oil and paraffin oil. This amount is 3 times higher than the levels found in this study. Another study by Wei et al. (2008) found that *P. aeruginosa* J16 when grown in medium with 0.32 M glycerol produced a higher production of rhamnolipid than did soy bean oil, sunflower oil and mannitol. Silva et al. (2010) also used 3% (v/v) glycerol and 0.6% (w/v) $NaNO_3$ for biosurfactant production by *P. aeruginosa* UCP0092. Glycerol is a simple fatty acid precursor with high solubility in medium, so it is easily utilized by bacteria for their carbon and energy source.

P. aeruginosa UKMP14T was able to utilize all types of nitrogen sources tested containing ammonium salt or nitrate or both; $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3 and $NaNO_3$

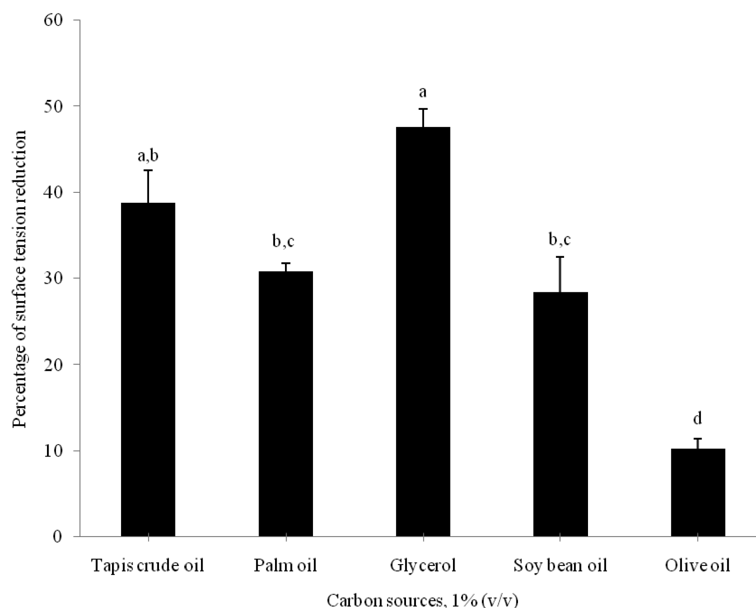

FIGURE 3. Effects of different carbon sources added to MSM on biosurfactant production by *P. aeruginosa* UKMP14T. The bacteria was grown in MSM, pH9.0, incubated at 37°C, shaken at 150 rpm for 5 days. Different letters represent significant differences ($p < 0.05$) among different carbon sources

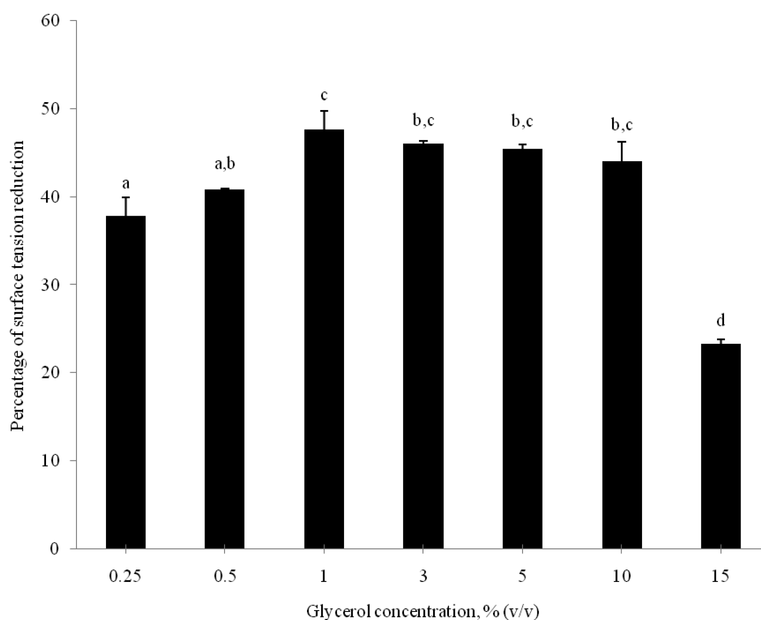

FIGURE 4. Effects of different glycerol concentrations added to MSM on biosurfactant production by *P. aeruginosa* UKMP14T. The bacteria was grown in MSM, pH9.0, incubated at 37°C, shaken at 150 rpm for 5 days. Different letters represent significant differences ($p < 0.05$) among different glycerol concentrations

at 4 g/L of total nitrogen added to the MSM together with 1% (v/v) glycerol. Statistical analysis showed no significant difference between $(NH_4)_2SO_4$, NH_4Cl and $NaNO_3$ except for NH_4NO_3 for biosurfactant production (Figure 5). However, in this study $(NH_4)_2SO_4$ was chosen as the optimal nitrogen source since it produced the lowest surface tension (32 dynes/cm) and the highest percentage of reduction in surface tension (51%).

In this study, addition of $(NH_4)_2SO_4$ at 1.3 g/L with a C/N ratio of 14:1 to MSM, which is itself high in nitrogen content (C/N ratio of less than 20) showed highest percentage of reduction in surface tension 55% for a low surface tension of 30.6 dynes/cm instead of a C/N ratio more than 20:1, which is the amount of nitrogen in limiting condition (Figure 6). Although there is no significant difference among the different concentrations of ammonium sulphate tested, the percentage of reduction in surface tension can be increased by reducing the amount of nitrogen content from 4.96 g/L to 1.3 g/L in MSM. However, Prieto et al. (2008) reported that *P.*

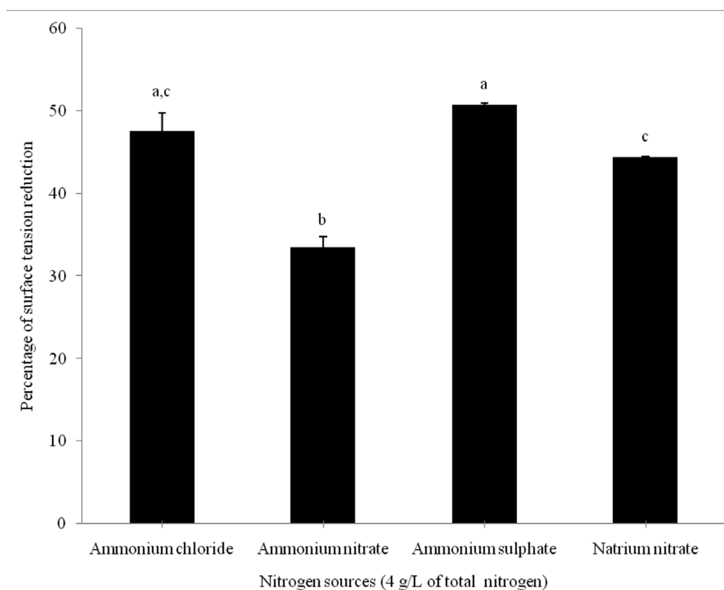

FIGURE 5. Effects of different nitrogen sources added to MSM on biosurfactant production by *P. aeruginosa* UKMP14T. The bacteria was grown in MSM, pH9.0, incubated at 37°C, shaken at 150 rpm for 5 days. Different letters represent significance differences ($p < 0.05$) among different nitrogen sources

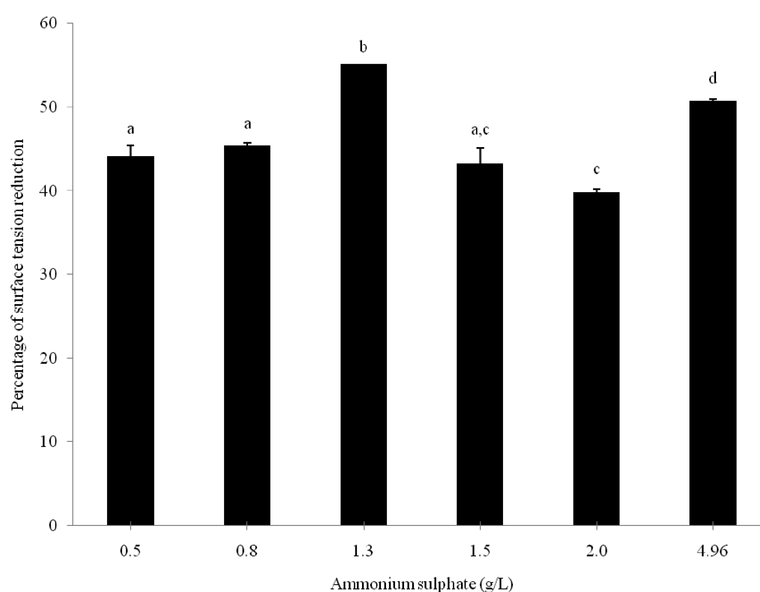

FIGURE 6. Effects of different ammonium sulphate concentrations added to MSM on biosurfactant production by *P. aeruginosa* UKMP14T. The bacteria was grown in MSM, pH9.0, incubated at 37°C shaken at 150 rpm for 5 days. Different letters represent significant differences ($p < 0.05$) among different ammonium sulphate concentrations

aeruginosa isolated from a southern coastal zone in Brazil produced optimum biosurfactant production when grown in medium containing soy bean oil and sodium nitrate as carbon and nitrogen sources, with a C/N ratio of 100:1. Another study by Wu et al. (2008) used glycerol and sodium nitrate with a C/N ratio of 52:1 as the best carbon and nitrogen sources for growth of *Pseudomonas* sp. to enhance production of biosurfactant with nitrogen limiting condition.

Some yeasts, fungi and bacteria are able to utilize triglycerides including glycerol and fatty acid for growth and synthesis of glycolipids during their stationary phase. Enzymes for biosurfactant production were produced during the organism's exponential growth phase when it is in a nonactive form. Upon change of one or more environmental parameters such as nutrient, temperature and pH, growth is inhibited and enzymes for biosurfactant synthesis are switched on. Since carbon, hydrogen and oxygen are the only important elements for the development of molecular structure of glycolipids, biosurfactant production does not need any additional nitrogen-containing salts and the production of biosurfactant continues as long as the carbon source and oxygen are available (Kosaric 1993).

Conclusion

Optimum levels of biosurfactant were produced by *P. aeruginosa* UKMP14T when grown in MSM medium containing 1% (v/v) of glycerol and 1.3 g/L of ammonium sulphate with a C/N ratio of 14:1 at pH9.0, incubated at 37°C and shaken at 150 rpm for 5 days. The biosurfactant produced reduced surface tension to 30.6 dynes/cm, representing a 55% reduction in surface tension with emulsification index ($\%EI_{24}$) of 44.3% while before optimization, the biosurfactant produced only reduced surface tension to 49.5 dynes/cm, representing a 16% reduction in surface tension with ($\%EI_{24}$) 25.49%.

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