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Screening and Optimization of *Achromobacter xylosoxidans* GSMSR13B Producing Bacteria

Golamari Siva Reddy, Botlagunta Mahendran and Ronda Srinivasa Reddy
Center for Bioprocess Technology, Department of Biotechnology,
Koneru Lakshmaiah Education Foundation (Deemed to be University), Green fields,
Vaddeswarm, 522502 Guntur, Andhra Pradesh, India

Abstract: Biosurfactants are amphiphilic mixes created by microorganisms as optional 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, petroleum refining and pharmaceutical industries and in environmental applications. In this study 25 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) vegetable oil as carbon source. The presence of biosurfactant was determined by the blood hemolysis, drop collapse tests, emulsification assay, Emulsification index (E24), foaming activity, lipase activity, haemolytic assay, oil spreading and tilted glass slide. Microplate analysis and surface tension measurement. Only 1 isolate, *Achromobacter xylosoxidans* GSMSR13B was found to be positive for all the qualitative and qualitative tests and reducing the surface tension of the medium to 47.8 dynes/cm with emulsification index of 28.7 %. This isolate produced biosurfactant optimally at pH 8.0 and incubation temperature of 37°C. Furthermore, *Achromobacter xylosoxidans* GSMSR13B when grown in MSM with addition of 1% (v/v) glycerol and 1.5 g/L NH_4NO_3 C/N ratio 16:1 produced biosurfactant with percentage of surface tension reduction at 56% or 28.6 dynes/cm with $\%EI_{24}$ of 36%. This percentage of surface tension reduction represents an increasing reduction in surface tension of medium by 33% over the value before optimization. This study showed that *Achromobacter xylosoxidans* GSMSR13B has the ability to biodegrade hydrocarbon and concurrently produce bio surfactant.

Key words: *Achromobacter xylosoxidans* GSMSR13B, bacteria, optimization, surface tension, emulsification index, quantitative and qualitative analysis, MATLAB

INTRODUCTION

Biosurfactants are exacerbates that deliver surface-dynamic and emulsifying exercises and are themselves created by microorganisms, for example, microscopic organisms, yeast and growths. For instance, *Achromobacter xylos* has been accounted for to deliver the biosurfactant glycolipid (Cameotra and Makkar, 2004) 4while *Bacillus subtilis* is known to create surfactin (Pornsunthorntawee *et al*., 2008).

Biosurfactants are amphiphilic, comprising of 2 sections, a polar (hydrophilic) moiety and a non-polar (hydrophobic) gathering. The hydrophilic gathering comprises of mono-, oligo- or polysaccharides, peptides or proteins while the hydrophobic moiety for the most part contains immersed, unsaturated and hydroxylated unsaturated fats or greasy alcohols (Pacwa-Plociniczak *et al*., 2011). Biosurfactants assume various parts including expanding the surface region and bioavailability of hydrophobic water-insoluble substrates, authoritative of substantial metals, majority detecting and biofilm arrangement (Rodrigues *et al*., 2006). Contrasted and manufactured surfactants, biosurfactants have higher surface action, bring down poisonous quality, higher biodegradability and better natural similarity (Wei *et al*., 2007). With their high surface action and ecological similarity, biosurfactants are broadly utilized as a part of natural applications, for example, for improvement of oil corruption (Pacwa-Plociniczak *et al*., 2011) as cancer prevention agents as antimicrobials in the beauty care products industry (Williams, 2009) and as hostile to cements against a few microbes and yeasts in medicinal applications (Rodrigues *et al*., 2006).

In oil handle, a few, however, not all powerful oil-debasing microscopic organisms create extracellular biosurfactants to encourage microbial oil take-up and

Corresponding Author: Ronda Srinivasa Reddy, Center for Bioprocess Technology, Department of Biotechnology, Koneru Lakshmaiah Education Foundation (Deemed to be University), Green fields, Vaddeswarm, 522502 Guntur, Andhra Pradesh, India

debasement by emulsifying the hydrocarbon (Morikawa *et al*., 2000). Surfactants and biosurfactants can fabricate the pseudo-dissolvability of oil portions in water (Pekdemir *et al*., 2005). Also, biosurfactants can be as compelling as manufactured concoction surfactants because of 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 AND METHODS

Bacterial isolates: Hydrocarbon-degrading bacteria were obtained from the Environmental Microbiology Laboratory culture collection of the Department of Biotechnology at KL University Andhra Pradesh, India. These isolates a were able to degrade hydrocarbons (Mukred *et al*., 2008; Bakar, 2010).

Media: This study used Mineral Salt Medium (MSM) (Zajic and Supplisson, 1972) containing 0.1% (v/v) vitamins and trace elements (Bouchez *et al*., 1995) supplemented with 1% (v/v) vegetable crude oil as carbon source. The pH of the medium was adjusted to 7.0 with 1 M 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 vegetable 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) vegetable 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 blood hemolysis, drop collapse tests, emulsification assay, Emulsification index (E24), foaming activity, lipase activity, haemolytic assay, oil spreading and tilted glass slide, microplate analysis 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 bio surfactant 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, vegetable 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*., 2007a, b). 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 seen utilizing a sponsorship sheet of paper with framework. A positive outcome was recorded when there was no optical contortion of the framework.

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

The Emulsification Index (%EI24) was determined according to Cooper and Goldenberg (1987). The same volume of supernatant and vegetable crude oil in a ratio of 1:1 were mixed in a glass test tube (125×15 mm). Then, the mixture was vortexed for 2 min and left to stand for 24 h. The %EI24 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 pressure was measured utilizing a surface tensiomat model 21 tensiometer (fisher scientific) by the Du Nouy Ring method. For the calibration of the instrument, the surface tension of pure water was measured. The model utilized for choosing biosurfactant-delivering segregates was the emulsification and lessening of the surface strain of the medium to underneath 40 dynes/cm (Bodour and Miller-Maier, 1998).

Haemolytic activity: This is a subjective screening test for the identification of biosurfactant makers (Satpute *et al*., 2010). Nutrient Agar (NA) supplemented with 5% (v/v) fresh blood was used according to Banat (1993) and Carrillo *et al*. (1996). The plates were incubated at 37°C for 24 h. After incubation, the plates were then observed for the presence of clear zone around the colonies.

Lipase activity by Tributyrin Clearing Zone (TCZ): Lipolytic activity was observed directly by changes in the appearance of the substrate, tributyrin and triolein which were emulsified mechanically in various growth media poured into petri dish. The secludes were screened for lipolytic movement on mineral salt agar containing 1% tributyrin (w/v). The pH of the medium was adjusted to 7.3±0.1 using 0.1 M of HCl and incubated at 35°C for 3 days. The plates were examined for zones of clearance around the colonies as described by Gandhimathi *et al.* (2009).

Emulsification assay: Culture broths were centrifuged at 10,000 rpm for 15 min/RT. About 3 mL of supernatant were mixed with 0.5 mL hydrocarbon and vortexed vigorously for 2 min. This was left undisturbed for 1 h to separate the aqueous and hydrocarbon phases. Un-inoculated broth was used as blank. The absorbance of the aqueous phase was measured with a spectrophotometer at 400 nm (Patil and Chopade, 2001).

Tilting glass slide test: This technique is effectively a modification of the drop collapse method (Satpute *et al.*, 2010). Isolates were grown for 24 h on nutrient agar plates. A sample colony was mixed with a droplet of 0.85% NaCl at one end of the glass slide. The slide was tilted and droplet observed. Biosurfactant producers were detected by the observation of droplet collapsing down.

Drop-collapsed assay: The assay was carried out as described by Jain *et al.* (1991). A glass plate was covered with a thin layer of mineral oil and a drop of free cell broth was placed on the hydrocarbon surface. Drop collapse in less than a minute indicated the presence of biosurfactant in the culture medium. Water was used as a negative control.

Foaming activity: For each 100 mL of nutrient broth medium taken in 250 mL Erlenmeyer flasks add the freshly isolated strains and incubate at 200 rpm, 37°C for 72 h. Observe the foam activity, foam height and foam shape in the graduated cylinder. After screening, candidate biosurfactant-producing bacteria were selected for optimization of the physical and nutrient parameters for optimum biosurfactant production.

Optimizationof physical and nutrientparameters: For all the experiments below, the following standard procedure was used: 10% (v/v) of standardized inoculums was inoculated into 250 mL conical flask containing 50 mL of MSM, supplemented with 1% (v/v) vegetable 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):

$$\text{Percentage of surface tension measurement} = \frac{(\gamma_m - \gamma_c)}{\gamma_m} \times 100$$

γ_m = The surface tension of the control (medium without inoculation)

γ_c = The surface tension of the test supernatant

pH and temperature: For determination of optimal pH, the standardized inoculums 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 inoculums was inoculated in MSM at optimized pH to which was added different carbon sources comprising dextrose, fructose, glucose, glycerol, starch, sucrose, maltose and vegetable crude oil at 1% (v/v) and 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 bio surfactant, 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 inoculums was inoculated in MSM at optimized pH with added different nitrogen sources, namely, aspiragine ($C_4H_8N_2O_3$), NH_4NO_3, peptone, urea (CH_4N_2O) and yeast extract ($C_{19}H_{14}O_2$), then incubated on an orbital shaker at 150 rpm and at the predetermined optimized temperature for 5 days. The nitrogen source that prompted the largest amount of biosurfactant generation as showed by the most minimal surface pressure action was additionally decided for variety in various focuses, running from 0.5-4.96 g/L.

Statistical analysis: The means of the results were analysed statistically using the MATLAB R2013a (Version 8.1.0.604). 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 bio surfactant-producing bacteria: Out of 25 isolates screened, only *Achromobacter xylosoxidans* GSMSR13B showed a positive result in the drop-collapse test. The other six isolates (*Achromobacter denitrificans* GSMSR1B, *Achromobacter pulmonis* GSMSR2B, *Achromobacter* sp. FBHYA2 GSMSR3B, *Bordetella petrii* GSMSR8B, *Alcaligenes* sp. BZC5 GSMSR10B and *Achromobacter xylosoxidans* GSMSR13B) showed positive result in the microplate analysis while eight isolates (*Achromobacter denitrificans* GSMSR1B, *Achromobacter pulmonis* GSMSR2B, *Achromobacter* sp. FBHYA2 GSMSR3B, *Bordetella petrii* GSMSR8B, *Alcaligenes* sp. BZC5 GSMSR10B, *Achromobacter xylosoxidans* GSMSR13B, *Achromobacter* sp. *A3* GSMSR2C and *Achromobacter insuavis* GSMSR5C) were detected positive by the oil-spreading technique. The other seven isolates (*Achromobacter* sp. FBHYA2 GSMSR3B, *Achromobacter anxifer* GSMSR7B, *Bordetella petrii* GSMSR8B, *Alcaligenes* sp. BZC5 GSMSR10B, *Achromobacter xylosoxidans* GSMSR13B, *Achromobacter denitrificans* GSMSR4C, *Achromobacter insuavis* GSMSR5C) showed positive results in the foaming activity while 7 bacterial isolates (*Achromobacter denitrificans* GSMSR1B, *Achromobacter pulmonis* GSMSR2B, *Achromobacter* sp. FBHYA2 GSMSR3B, *Alcaligenes* sp. BZC5 GSMSR10B, *Betaproteobacteria bacterium* GSMSR11B, *A. xylosoxidans* gene GSMSR12B and *Achromobacter xylosoxidans* GSMSR13B) were detected positive by the tilting glass technique as shown in Table 1 and 2.

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 (%EI24) 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 and Miller-Maier, 1998) and/or maintained at least 50% of the original emulsion volume 24 h after formation of emulsification (Willumsen and Karlson, 1996). *Achromobacter xylosoxidans* GSMSR13B showed positive results in all qualitative tests and in the quantitative evaluation produced a higher reduction in surface tension (47.8±0.09 dynes/cm) and a

Table 1: Screening of potential biosurfactant production using lipase test, oil spreading test, emulsification assay, emulsification index and foaming activity

Bacterial isolates	Lipase test (mm)	Oil spreading test (mm)	Emulsification assay (@400 nm)	Emulsification index (E_{24} %)	Foaming activity
Achromobacter denitrificans GSMSR1B	15±2	+	0.6135±0.0034	13.1±2.0	-
Achromobacter pulmonis GSMSR2B	10±2	+	0.6185±0.0024	43.5±1.0	-
Achromobacter sp. FBHYA2 GSMSR3B	18±2	+	0.4330±0.0020	28.1±2.0	+
Achromobacter sp. XRF-1 GSMSR4B	0	-	0.4326±0.0010	29.6±0.8	-
Achromobacter ruhlandii GSMSR5B	11±2	-	0.4318±0.0023	39.8±0.9	-
Alcaligenes sp. GSMSR6B	22±1	-	0	0	-
Achromobacter anxifer GSMSR7B	0	-	0.4568±0.0029	35.7±2.2	+
Bordetella petrii GSMSR8B	0	+	0.4423±0.0019	31.9±2.8	+
Bacterium strain TLSY-1 GSMSR9B	0	-	0.5067±0.0024	30.6±3.1	-
Alcaligenes sp. BZC5 GSMSR10B	0	+	0.4786±0.0025	32.3±2.6	+
Betaproteobacteria bacterium GSMSR11B	15±2	-	0	0	-
Achromobacter xylosoxidans gene GSMSR12B	12±1	-	0	0	-
Achromobacter xylosoxidans GSMSR13B	13±1	+	0.5030±0.0030	28.7±00	+
Achromobacter sp. Ir-12.2 gene GSMSR14B	14±2	-	0	0	-
Achromobacter sp. Ir-1 gene GSMSR1C	0	-	0	0	-
Achromobacter sp. A3 GSMSR2C	0	+	0.5102±0.0031	37.8±1.5	-
Achromobacter sp. BV11 GSMSR3C	0	-	0	0	-
Achromobacter denitrificans GSMSR4C	20±2	-	0.6783±0.0029	32.9±2.8	+
Achromobacter insuavis GSMSR5C	21±1	+	0	0	+
Achromobacter sp. CanL-53 GSMSR6C	23±2	-	0.6876±0.0034	33.7±3.2	-
Alcaligenes sp. TG19 GSMSR7C	0	-	0.6912±0.0015	40.9±2.9	-
Distilled water	0	-	0	0	-
MSM +1% (v/v) Vegetable oil	0	-	0	0	-
1% (w/v) SDS	25±3	+	0.9993±0.0048	53.95±2.4	+

Table 2: Screening of potential biosurfactant production using blood hemolysis, drop collapsed test, tilting glass, haemolytic, micro plate analysis and surface tension

Bacterial isolates	Blood hemolysis	Drop collapse test	Tilting glass	Haemolytic	Micro plate analysis	Surface tension
Achromobacter denitrificans GSMSR1B	γ	-	+	5±2.0	+	61±0.32
Achromobacter pulmonis GSMSR2B	γ	-	+	14±2.0	+	67.6±0.05
Achromobacter sp. FBHYA2 GSMSR3B	γ	-	+	3±0.6	+	60.25±0.06
Achromobacter sp. XRF-1 GSMSR4B	γ	-	-	6±1.6	-	55.1±1.48
Achromobacter ruhlandii GSMSR5B	γ	-	-	2±0.6	-	56.4±1.09
Alcaligenes sp. GSMSR6B	γ	-	-	4±0.8	-	60.7±0.25
Achromobacter anxifer GSMSR7B	γ	-	-	13±1.9	-	62.7±0.74
Bordetella petrii GSMSR8B	γ	-	-	16±2.1	+	61.4±3.80
Bacterium strain TLSY-1 GSMSR9B	γ	-	-	9±1.6	-	65.0±1.99
Alcaligenes sp. BZC5 GSMSR10B	γ	-	+	11±2.2	+	60.8±0.71
Betaproteobacteria bacterium GSMSR11B	γ	-	+	12±2.4	-	63.1±7.08
A. *xylosoxidans* gene GSMSR12B	γ	-	+	18±2.7	-	59.5±0.09
Achromobacter xylosoxidans GSMSR13B	γ	+	+	7±2.0	+	47.8±0.09
Achromobacter sp. Ir-12.2 gene GSMSR14B	γ	-	-	13±2	-	68.7±1.84
Achromobacter sp. Ir-1 gene GSMSR1C	γ	-	-	17±2.9	-	68.3±0.85
Achromobacter sp. A3 GSMSR2C	γ	-	-	0	-	69.6±4.24
Achromobacter sp. BV11 GSMSR3C	γ	-	-	20±3.2	-	69.2±0.64
Achromobacter denitrificans GSMSR4C	γ	-	-	23±3.5	-	69.8±0.21
Achromobacter insuavis GSMSR5C	γ	-	-	25±3.9	-	65.9±1.06
Achromobacter sp. CanL-53 GSMSR6C	γ	-	-	0	-	57.6±0.64
Alcaligenes sp. TG19 GSMSR7C	γ	-	-	0	-	58.8±0.05
Distilled water	-	-	-	0	-	79.1±0.14
MSM +1% (v/v) Vegetable oil	-	-	-	0	-	59.6±0.07
1% (w/v) SDS	γ	+	+	30±4.4	+	41.0±0.00

higher percentage of emulsification at 24 h (28.7±00) than did the positive control SDS with 41.0 dynes/cm and a %EI24 of 54%.

Isolates *Achromobacter pulmonis* GSMSR2B, *Bordetella petrii* GSMSR8B and *Alcaligenes* sp. BZC5 GSMSR10B, 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: *Achromobacter xylosoxidans* GSMSR13B grow and produced biosurfactant at a wide range of pH from 6.5-9.0. Although, statistical analysis showed no significant difference between the production of biosurfactant at pH8.0 and pH8.5, pH8.0 was selected as the best pH for biosurfactant production because it produced the highest surface tension reduction at 39.5 dynes/cm or 42.55±2.15% reduction as compared with medium without inoculation (Fig. 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

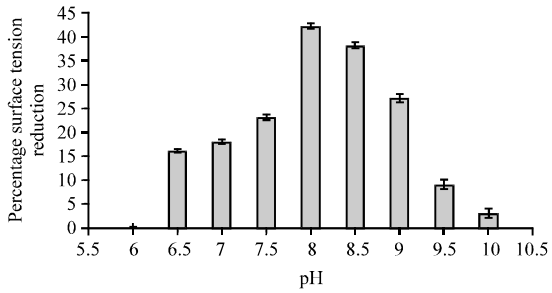

Fig. 1: Effects of different pH on bio surfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, incubated at 37°C and shaken at 150 rpm for 5 days

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).

Achromobacter xylosoxidans GSMSR13B grown in MSM at pH8.0 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 38.1 dynes/cm representing a 41% reduction in surface tension (Fig. 2). When the incubation temperature increased to 40°C, bacterial growth and biosurfactant production were totally inhibited, indicating that the biosurfactant produced by *Achromobacter xylosoxidans* GSMSR13B was temperature-dependent.

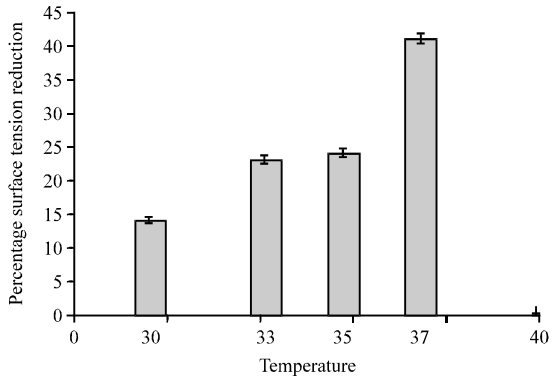

Fig. 2: Effects of different growth temperatures on biosurfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH8.0, incubated at different temperatures, shaken at 150 rpm for 5 days

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*., 2007a, b), 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.

Optimization of nutrient parameters

Carbon and nitrogen: The ability of *Achromobacter xylosoxidans* GSMSR13B to utilize various types of carbon sources for biosurfactant production was tested with dextrose, fructose, glucose, glycerol, starch, sucrose, maltose and vegetable 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 39.0 dynes/cm representing a 55% reduction, followed by vegetable oil with a surface tension of 41.7 dynes/cm for a 53% reduction. While there was no significant difference between glycerol and vegetable oil as carbon sources, glycerol was selected as the optimal carbon source, since, it produced the highest percentage reduction in surface tension (Fig. 3). When grown at different concentrations of glycerol, *Achromobacter xylosoxidans* GSMSR13B effected the highest percentage of reduction in surface tension with glycerol 1% (v/v) (Fig. 4). However, statistical analysis showed no significant difference between the 1% (v/v) and 10% (v/v) levels of glycerol.

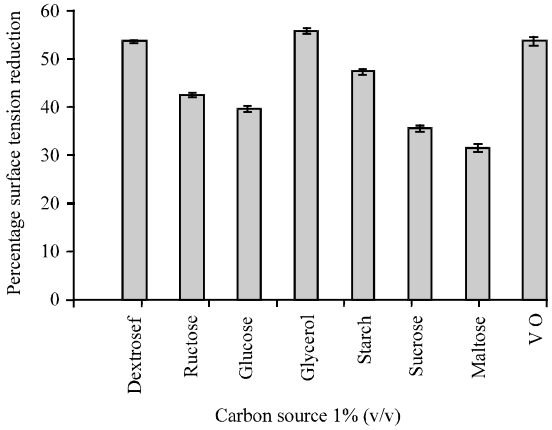

Fig. 3: Effects of different carbon sources added to MSM on bio surfactant production by *Xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH8.0, incubated at 37°C, shaken at 150 rpm for 5 days. (V.O = Vegetable Oil)

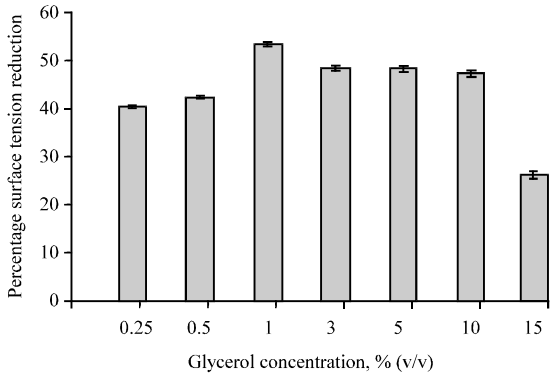

Fig. 4: Effects of different glycerol concentrations added to MSM on biosurfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH8.0, incubated at 37°C, shaken at 150 rpm for 5 days

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*. (2007, 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 straightforward unsaturated fat antecedent with high solvency in medium, so, it is effortlessly used by microscopic organisms for their carbon and vitality source.

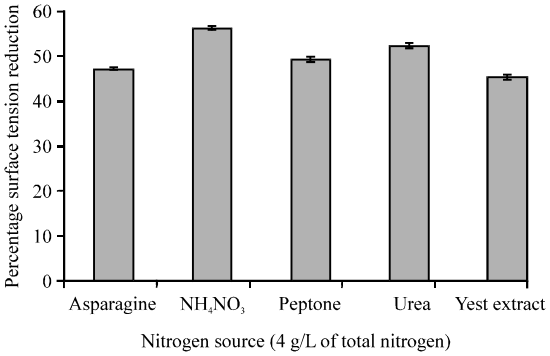

Fig. 5: Effects of different nitrogen sources added to MSM on bio surfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH8.0, incubated at 37°C, shaken at 150 rpm for 5 days

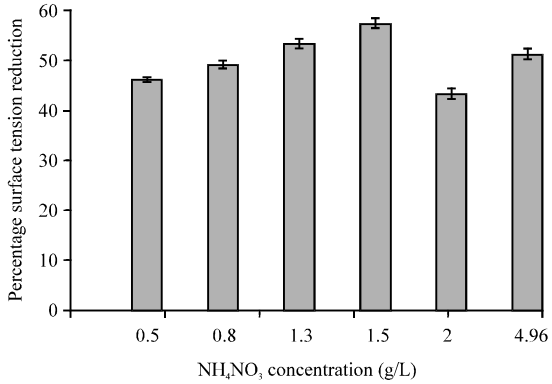

Fig. 6: Effects of different ammonium sulphate concentrations added to MSM on bio surfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH 8.0, incubated at 37°C shaken at 150 rpm for 5 days

Achromobacter xylosoxidans GSMSR13B was able to utilize all types of nitrogen sources tested containing ammonium salt or nitrate or both; Aspiragine ($C_4H_8N_2O_3$), NH_4NO_3, peptone, urea (CH_4N_2O) and yeast extract $C_{19}H_{14}O_2$ at 4 g/L of total nitrogen added to the MSM together with 1% (v/v) glycerol. Statistical analysis showed no significant difference between aspiragine ($C_4H_8N_2O_3$), peptone, NH_4NO_3 and urea (CH_4N_2O) except for yeast extract $C_{19}H_{14}O_2$ for biosurfactant production (Fig. 5). However, in this study NH_4NO_3 was chosen as the optimal nitrogen source since it produced the lowest surface tension (35 dynes/cm) and the highest percentage of reduction in surface tension (56%).

In this study, addition of NH_4NO_3 at 1.5 g/L with a C/N ratio of 16:1 to MSM which is itself high in nitrogen content (C/N ratio of < 20) showed highest percentage of reduction in surface tension 57% for a low surface tension of 37.9 dynes/cm instead of a C/N ratio more than 20:1 which is the amount of nitrogen in limiting condition (Fig. 6). Although, there is no significant difference among the different concentrations of NH_4NO_3 tested, the percentage of reduction in surface tension can be increased by reducing the amount of nitrogen content from 4.96-1.5 g/L in MSM. However, Prieto *et al.* (2008) reported that *P. 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/Nratio 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. Catalysts for biosurfactant creation were delivered amid the living being's exponential development stage when it is in a non dynamic frame. 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 *Achromobacter xylosoxidans* GSMSR13B when grown in MSM medium containing 1% (v/v) of glycerol and 1.5 g/L of NH_4NO_3 with a C/N ratio of 16:1 at pH8.0, incubated at 37°C and shaken at 150 rpm for 5 days. The biosurfactant produced reduced surface tension to 37.9 dynes/cm, representing a 57% reduction in surface tension with Emulsification Index (%EI_{24}) of 47.4% while before optimization, the biosurfactant produced only reduced surface tension to 47.8 dynes/cm, representing a 20% reduction in surface tension with (%EI_{24}) 28.7%.

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REFERENCES

Bakar, N.F.A., 2010. Biodegradation study of Tapis crude oil and condensate Terengganu by selected bacteria. MSc. Thesis, University Kebangsaan Malaysia, Bangi, Malaysia.

Banat, I.M., 1993. The isolation of a thermophilic biosurfactant producing *Bacillus* sp. Biotechnol. Lett., 15: 591-594.

Bodour, A.A. and R.M. Miller-Maier, 1998. Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. J. Microbiol. Methods, 32: 273-280.

Bouchez, M., D. Blanchet and J.P. Vandecastelle, 1995. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: Inhibition phenomena and cometabolism. Applied Microbiol. Biotechnol., 43: 156-164.

Cameotra, S.S. and R.S. Makkar, 2004. Recent applications of biosurfactants as biological and immunological molecules. Curr. Opin. Microbiol., 7: 262-266.

Carrillo, P.G., C. Mardaraz, S.I. Pitta-Alvarez and A.M. Giulietti, 1996. Isolation and selection of biosurfactant-producing bacteria. World J. Microbiol. Biotechnol., 12: 82-84.

Chen, C., S.C. Baker and R. Darton, 2007a. The application of a high throughput analysis method for the screening of potential biosurfactants from natural sources. J. Microbiol. Method, 70: 503-510.

Chen, S.Y., W.B. Lu, Y.H. Wei, W.M. Chen and J.S. Chang, 2007b. Improved production of biosurfactant with newly isolated *Pseudomonas aeruginosa* S2. Biotechnol. Progr., 23: 661-666.

Cooper, D.G. and B.G. Goldenberg, 1987. Surface-active agents from 2 *Bacillus* species. Applied Environ. Microbiol., 53: 224-229.

Gandhimathi, R., G.S. Kiran, T.A. Hema, J. Selvin and T.R. Raviji *et al*., 2009. Production and characterization of lipopeptide biosurfactant by a sponge-associated marine actinomycetes *Nocardiopsis alba* MSA10. Bioprocess Biosyst. Eng., 32: 825-835.

Hamzah, A., A. Rabu, R.F.H.R. Azmy and N.A. Yussoff, 2010. Isolation and characterization of bacteria degrading Sumandak and South Angsi oils. Sains Malaysiana, 39: 161-168.

Jain, D.K., D.L. Collins-Thompson, H. Lee and J.T. Trevors, 1991. A drop-collapsing test for screening surfactant-producing microorganisms. J. Microbiol. Methods, 13: 271-279.

Kosaric, N., 1993. Biosurfactants: Production, Properties and Application. Marcel Dekker, New York, USA.,.

Lotfabad, T.B., M. Shourian, R. Roostaazad, A.R. Najafabadi, M.R. Adelzadeh and K.A. Noghabi, 2009. An efficient biosurfactant-producing bacterium *Pseudomonas aeruginosa* MR01, isolated from oil excavation areas in South of Iran. Colloids Surf. B: Biointerfaces, 69: 183-193.

Morikawa, M., Y. Hirata and T. Imanaka, 2000. A study on the structure-function relationship of lipopeptide biosurfactants. Biochim. Biophys. Acta BBA. Mol. Cell Biol. Lipids, 1488: 211-218.

Mukred, A.M., A.A. Hamid, A. Hamzah and W.M. Wan Yusoff, 2008. Enhancement of biodegradation of crude petroleum-oil in contaminated water by the addition of nitrogen sources. Pak. J. Biol. Sci., 11: 2122-2127.

Pacwa-Piociniczak, M., G.A. Piaza, Z. Piotrowska-Seget and S.S. Cameotra, 2011. Environmental applications of biosurfactants: Recent advances. Intl. J. Mol. Sci., 12: 633-654.

Patil, J.P. and B.A. Chopade, 2001. Distribution and in vitro antimicrobial susceptibility of *Acinetobacter* species on the skin of healthy humans. National Med. J. India, 14: 204-208.

Pekdemir, T., M. Copur and K. Urum, 2005. Emulsification of crude oil-water systems using biosurfactants. Proc. Saf. Environ. Prot., 83: 38-46.

Plaza, G.A., I. Zjawiony and I.M. Banat, 2006. Use the different methods for detection of thermophillic biosurfactant-Producing bacteria from hydrocarbon-contaminated and bioremediated soils. J. Petrol. Sci. Eng., 50: 71-77.

Pornsunthorntawee, O., N. Arttaweeporn, S. Paisanjit, P. Somboonthanate and M. Abe *et al*., 2008. Isolation and comparison of biosurfactants produced by *Bacillus* subtilis PT2 and *Pseudomonas aeruginosa* SP4 for microbial surfactant-enhanced oil recovery. Biochem. Eng. J., 42: 172-179.

Praveesh, B.V., A.R. Soniyamby, C. Mariappan, P. Kavithakumari and M. Palaniswamy *et al*., 2011. Biosurfactant production by *Pseudomonas* sp. from soil using whey as carbon source. N. Y. Sci. J., 4: 99-103.

Prieto, L.M., M. Michelon, J.F.M. Burkert, S.J. Kalil and C.A.V. Burkert, 2008. The production of rhamnolipid by a *Pseudomonas aeruginosa* strain isolated from a Southern Coastal Zone in Brazil. Chemosphere, 71: 1781-1785.

Rodrigues, L., I.M. Banat, J. Teixeira and R. Oliveira, 2006. Biosurfactants: Potential applications in medicine. J. Antimicrobial Chemotherapy, 57: 609-618.

Satpute, S.K., A.G. Banpurkar, P.K. Dhakephalkar, I.M. Banat and B.A. Chopade, 2010. Methods for investigating biosurfactants and bioemulsifiers: A review. Crit. Rev. Biotechnol., 30: 127-144.

Silva, S.N.R.L., C.B.B. Farias, R.D. Rufino, J.M. Luna and L.A. Sarubbo, 2010. Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. Colloids Surf. B. Biointerfaces, 79: 174-183.

Wei, Y.H., C.C. Lai and J.S. Chang, 2007. Using Taguchi experimental design methods to optimize trace element composition for enhanced surfactin production by *Bacillus* subtilis ATCC 21332. Process Biochem., 42: 40-45.

Wei, Y.H., C.L. Cheng, C.C. Chien and H.M. Wan, 2008. Enhanced di-rhamnolipid production with an indigenous isolate *Pseudomonas aeruginosa* J16. Process Biochem., 43: 769-774.

Williams, K.J., 2009. Biosurfactants for cosmetic application: Overcoming production challenges. MMG Basic Biotechnol., 5: 78-83.

Willumsen, P.A. and U. Karlson, 1996. Screening of bacteria, isolated from PAH-contaminated soils, for production of biosurfactants and bioemulsifiers. Biodegradation, 7: 415-423.

Wu, J.Y., K.L. Yeh, W.B. Lu, C.L. Lin and J.S. Chang, 2008. Rhamnolipid production with indigenous *Pseudomonas aeruginosa* EM1 isolated from oil-contaminated site. Bioresour. Technol., 99: 1157-1164.

Yin, H., J. Qiang, Y. Jia, J. Ye and H. Peng *et al*., 2009. Characteristics of biosurfactant produced by *Pseudomonas aeruginosa* S6 isolated from oil-containing wastewater. Process Biochem., 44: 302-308.

Youssef, N.H., K.E. Duncan, D.P. Nagle, K.N. Savage, R.M. Knapp and M.J. McInerney, 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Methods, 56: 339-347.

Zajic, J.E. and B. Supplisson, 1972. Emulsification and degradation of Bunker C fuel oil by microorganisms. Biotechnol. Bioeng., 14: 331-334.

Zhang, G., Y. Wu, X. Qian and Q. Meng, 2005. Biodegradation of crude oil by *Pseudomonas aeruginosa* in the presence of rhamnolipids. J. Zhejiang Univ. Sci. B, 6: 725-730.