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Screening and Optimization of *Achromobacter xylosoxidans* GSMSR13B Producing Bacteria

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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 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 (E_{24}), foaming activity, lipase activity, haemolytic assay, oil spreading and tilted glass slide, microplate analysis and surface tension measurement. Only one 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 mineral salt medium 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 biosurfactant.

Keywords: *Achromobacter xylosoxidans* GSMSR13B, Bacteria, Optimization, Surface tension, Emulsification index.

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 xylosoxidans* has been accounted for to deliver the biosurfactant glycolipid [1] while *Bacillus subtilis* is known to create surfactin [2].

Biosurfactants are amphiphilic, comprising of two 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 [3]. 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 [4]. Contrasted and manufactured surfactants, biosurfactants have higher surface action, bring down poisonous quality, higher biodegradability and better natural similarity [5]. 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 [3], as cancer prevention agents, as antimicrobials in the beauty care products industry [6] and as hostile to cements against a few microbes and yeasts in medicinal applications [4].

In oil handle, a few however not all powerful oil-debasing microscopic organisms create extracellular biosurfactants to encourage microbial oil take-up and debasement by emulsifying the hydrocarbon [7]. Surfactants and biosurfactants can fabricate the pseudo-dissolvability of oil portions in water [8]. 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.

EXPERIMENTAL

Bacterial isolates: Hydrocarbon-degrading bacteria were obtained from the Environmental Microbiology Laboratory culture collection of the Department of Biotechnology at K L University, Andhra Pradesh, India. These isolates were able to degrade hydrocarbons [9,10].

Media: This study used mineral salt medium (MSM) [11] containing 0.1 % (v/v) vitamins and trace elements [12] supplemented with 1 % (v/v) vegetable crude oil as carbon source. The pH of the medium was adjusted to 7.0 with 1 M NaOH or 1 M 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.* [13]. 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 (E_{24}), 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 biosurfactant producer

Qualitative methods: The drop-collapse test was performed according to Plaza *et al.* [14]. 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.* [15,16]. 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.* [17]. Briefly, distilled water was added to the Petri dish (90 mm × 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 [18]. The same volume of supernatant and vegetable 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 %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 Noüy 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 [19].

Haemolytic activity: This is a subjective screening test for the identification of biosurfactant makers [20]. Nutrient agar (NA) supplemented with 5 % (v/v) fresh blood was used according to Banat [21] and Carrillo *et al.* [22]. 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.* [23].

Emulsification assay: Culture broths were centrifuged at 10,000 rpm for 15 min/RT. 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 [24].

Tilting glass slide test: This technique is effectively a modification of the drop collapse method [20]. 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 [20].

Drop-collapsed assay: The assay was carried out as described by Jain *et al*. [25]. 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.

Optimization of physical and nutrient parameters: For all the experiments below, the following standard procedure was used:

Ten percent (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 [2].

$$\text{Surface tension measurement (\%)} = \frac{(\gamma_m - \gamma_c)}{\gamma_m} \times 100$$

γ_m is the surface tension of the control (medium without inoculation) and γ_c is 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 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 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 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 analyzed statistically using the Mat lab 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 biosurfactant-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* GSMSR 13B, *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 seven 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 Tables 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.* [17], 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 [19] and/or maintained at least 50 % of the original emulsion volume 24 h after formation of emulsification [26]. *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 higher percentage of emulsification at 24 h (28.7 ± 00) than did the positive control SDS with 41.0 dynes/cm and a $\%EI_{24}$ 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 to 9.0. Although statistical analysis showed no significant difference between the production of biosurfactant at pH 8.0 and pH 8.5, pH 8.0 was selected as the best pH for biosurfactant production because it produced the highest

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	+	61.00 ± 0.32
Achromobacter pulmonis GSMSR2B	γ	–	+	14 ± 2.0	+	67.60 ± 0.05
Achromobacter sp. FBHYA2 GSMSR3B	γ	–	+	3 ± 0.6	+	60.25 ± 0.06
Achromobacter sp. XRF-1 GSMSR4B	γ	–	–	6 ± 1.6	–	55.10 ± 1.48
Achromobacter ruhlandii GSMSR5B	γ	–	–	2 ± 0.6	–	56.40 ± 1.09
Alcaligenes sp. GSMSR6B	γ	–	–	4 ± 0.8	–	60.70 ± 0.25
Achromobacter anxifer GSMSR7B	γ	–	–	13 ± 1.9	–	62.70 ± 0.74
Bordetella petrii GSMSR8B	γ	–	–	16 ± 2.1	+	61.40 ± 3.80
Bacterium strain TLSY-1 GSMSR9B	γ	–	–	9 ± 1.6	–	65.00 ± 1.99
Alcaligenes sp. BZC5 GSMSR10B	γ	–	+	11 ± 2.2	+	60.80 ± 0.71
Betaproteobacteria bacterium GSMSR11B	γ	–	+	12 ± 2.4	–	63.10 ± 7.08
A. xylosoxidans gene GSMSR12B	γ	–	+	18 ± 2.7	–	59.50 ± 0.09
Achromobacter xylosoxidans GSMSR13B	γ	+	+	7 ± 2.0	+	47.80 ± 0.09
Achromobacter sp. Ir-12.2 gene GSMSR14B	γ	–	–	13 ± 2.0	–	68.70 ± 1.84
Achromobacter sp. Ir-1 gene GSMSR1C	γ	–	–	17 ± 2.9	–	68.30 ± 0.85
Achromobacter sp. A3 GSMSR2C	γ	–	–	0	–	69.60 ± 4.24
Achromobacter sp. BV11 GSMSR3C	γ	–	–	20 ± 3.2	–	69.20 ± 0.64
Achromobacter denitrificans GSMSR4C	γ	–	–	23 ± 3.5	–	69.80 ± 0.21
Achromobacter insuavis GSMSR5C	γ	–	–	25 ± 3.9	–	65.90 ± 1.06
Achromobacter sp. CanL-53 GSMSR6C	γ	–	–	0	–	57.60 ± 0.64
Alcaligenes sp. TG19 GSMSR7C	γ	–	–	0	–	58.80 ± 0.05
Distilled water	–	–	–	0	–	79.10 ± 0.14
MSM + 1 % (v/v) Vegetable oil	–	–	–	0	–	59.60 ± 0.07
1 % (w/v) SDS	γ	+	+	30 ± 4.4	+	41.00 ± 0.00

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) and extreme alkaline pH (10), this isolate produced lower levels of biosurfactant.

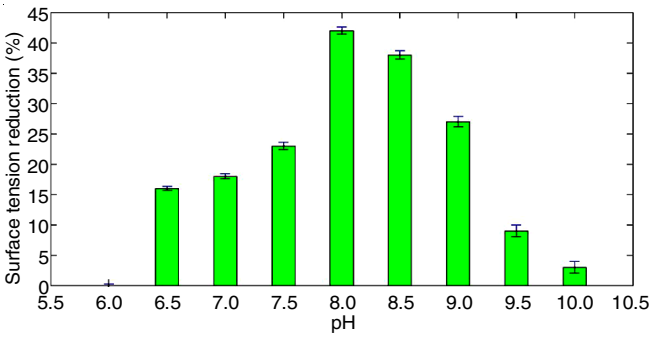

Fig. 1. Effects of different pH on biosurfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, incubated at 37 °C and shaken at 150 rpm for 5 days

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 pH 9.0 reducing surface tension to 33.9 dynes/cm [27]. Meanwhile, *Pseudomonas* sp. isolated from oil-contaminated soil produced maximum biosurfactant at pH 7.0 when grown in medium with 3 % (v/v) when added [28].

Achromobacter xylosoxidans GSMSR13B grown in MSM at pH 8.0 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.

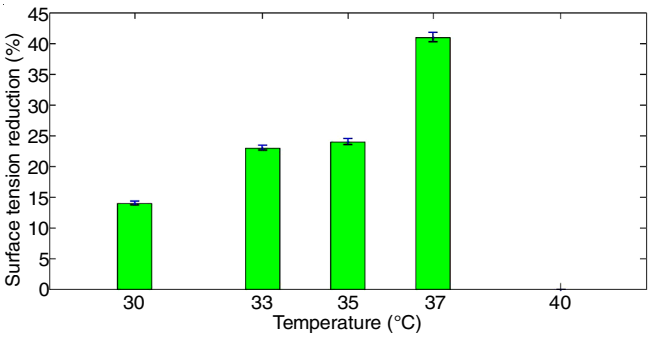

Fig. 2. Effects of different growth temperatures on biosurfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH 8.0, incubated at different temperatures, shaken at 150 rpm for 5 days

P. aeruginosa MR01 isolated from oil excavation areas in the south of Iran [29] and *P. aeruginosa* S2 isolated from diesel-contaminated soil [3], both produced optimum biosurfactant when grown at 37 °C. Another study by [28] 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.

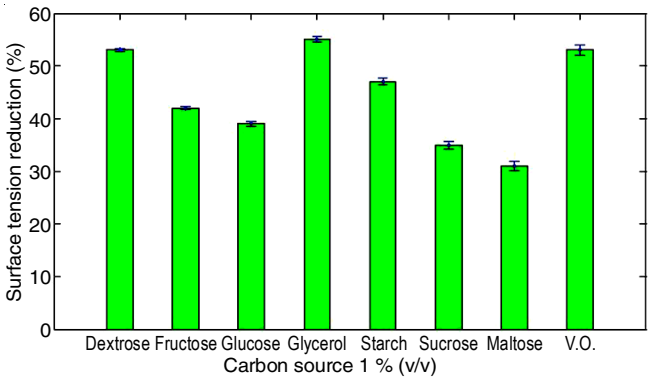

Fig. 3. Effects of different carbon sources added to MSM on biosurfactant production by *xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH 8.0, incubated at 37 °C, shaken at 150 rpm for 5 days (V.O. = Vegetable oil)

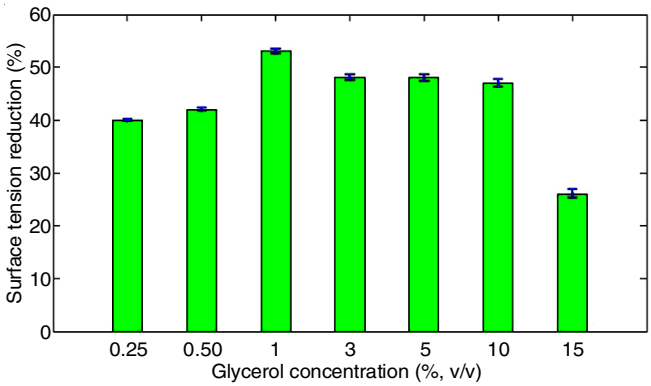

Fig. 4. Effects of different glycerol concentrations added to MSM on biosurfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH 8.0, incubated at 37 °C, shaken at 150 rpm for 5 days

Zhang *et al*. [30] 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*. [31] 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*. [32] 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.

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 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, peptone, NH_4NO_3 and urea except for yeast extract 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 %).

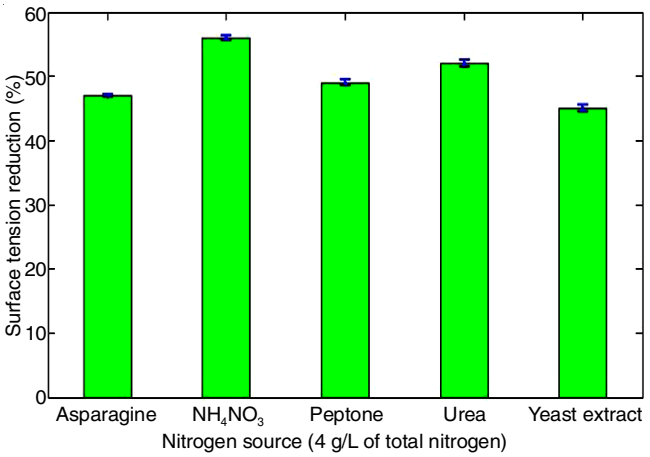

Fig. 5. Effects of different nitrogen sources added to MSM on biosurfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH 8.0, incubated at 37 °C, shaken at 150 rpm for 5 days

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 less than 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 g/L to 1.5 g/L in MSM. However, Prieto *et al*. [33] 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/N ratio of 100:1. Wu *et al*. [34] 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.

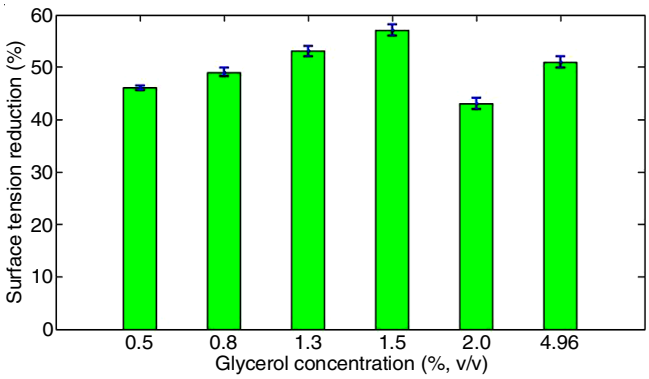

Fig. 6. Effects of different ammonium sulphate concentrations added to MSM on biosurfactant production by *Achromobacter xylosoxidans* GSMSR13B. The bacteria was grown in MSM, pH 8.0, incubated at 37 °C shaken at 150 rpm for 5 days

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 [35].

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 pH 8.0, incubated at 37 ºC 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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