

Optimized Conditions for Bioemulsifier production by Local *Streptomyces* sp. SS 20 isolated from hydrocarbon contaminated soil

Received for publication, October 15, 2013

Accepted, December 4, 2013

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Abstract

The present study was evaluated the bioemulsifier production and properties of *Streptomyces* sp. SS 20 isolated from hydrocarbons contaminated soil. Out of 90 isolates of *Streptomyces*; five isolates showed good bioemulsifier production and emulsification activity. *Streptomyces* sp. SS 20 produced maximum bioemulsifier activity ($E_{24\%} = 100\%$) and bioemulsifier stability 75% after 2 weeks, and showed good growth in malt yeast extract broth supplemented with 1% (v/v) of sun flower oil. The fermentation conditions were optimized, maximum growth and bioemulsifier production was at an initial pH 7, temperature 30 °C, yeast extract as best nitrogen source at (1% w/v), sucrose (1% w/v) was the best carbon source and time course of 3 days. Maximum bioemulsifier production was observed in a medium containing 1% (v/v) of kerosene as source of hydrocarbons. Corn oil at concentration of 3% (v/v) showed maximum growth and bioemulsifier production. Critical micelle concentration (CMC) of partial purified bioemulsifier was 300 mg/l and reduced the surface tension of the medium to 34.2 mN/m; the bioemulsifier obtained was viscous and had a glycolipid nature. The optimized fermentation process produced a bioemulsifier yield of 10.48 g/l. The bioemulsifier activity and stability was effective over a wide range of temperature (30 – 100), pH (3 – 7) and salt concentration of 3% (w/v) and liquid monoaromatic compounds was the best hydrocarbons for emulsion stability. The isolate *Streptomyces* sp. SS 20 have good properties to use them emulsion, mainly in the petroleum hydrocarbons industry, e.g. in enhanced oil recovery, and in bioremediation process, due to their emulsification properties, emulsion forming and stability.

Keywords: *Streptomyces* sp, Bioemulsifier, oils, Hydrocarbons, Emulsion stability.

Introduction

Microbial surface-active compounds are a group of structurally diverse molecules produced by different microorganisms and are mainly classified by their chemical structure and their microbial origin. They are made up of a hydrophilic moiety, comprising an acid, peptide cations, or anions, mono-, di- or polysaccharides and a hydrophobic moiety of unsaturated or saturated hydrocarbon chains or fatty acids (S. Lang [1]).

These structures confer a wide range of properties, including the ability to lower surface and interfacial tension of liquids and to form micelles and microemulsions between two different phases. These compounds can be roughly divided into two main classes: low-molecular-weight compounds called biosurfactants, such as lipopeptides,

glycolipids, proteins and high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins that are collectively called bioemulsans or bioemulsifiers (I. M. Banat & al. [2]). The former group includes molecules which can efficiently reduce surface and interfacial tension, while the latter are amphiphilic and polyphilic polymers which are usually more effective in stabilizing emulsions of oil-in-water but do not lower the surface tension as much (TJP. Smyth & al. [3]).

Biosurfactants are microbial surface active agents produced by microorganisms such as bacteria, yeasts and fungi during their growth phase. They may be extracellular or intracellular in nature (S.Y. Chen & al. [4]). Substrates for biosurfactant production are sugars, oils, alkanes and waste materials (S.C. Lin [5]). Biosurfactant are amphiphilic, non-toxic and biodegradable molecules with high specificity. They are highly stable at extremities of temperature, pH and salt concentration (C.M. Drouin, & D.G. Cooper [6]).

Although enormous literature appears on bioemulsifier production by bacteria like *Acinetobacter*, *Bacillus*, *Pseudomonas* (A. M. Shete & al. [7]), reports on production by actinomycetes are rare. Among various actinomycetes, glycolipids from *Rhodococcus erythropolis*, *Rhodococcus aurantiacus* and *Rhodococcus* sp. H13 are reported. *Nocardia erythropolis* has been studied for production of surface active lipids. These species are known to produce biosurfactants, however only few reports appear on actinomycetes producing bioemulsifiers such as, *Nocardia* sp. L-417 isolated from soil and *Streptomyces* spp. isolated from marine environment and bioemulsifier production by *Streptomyces* sp. S22 isolated from garden soil (J. P. Maniyar & al. [8] and A. Khopade & al. [9]).

However, biosurfactant production has been proven to be quite a challenge in industry. Problems such as low yield and high production cost of biosurfactant are important and cannot be ignored. These problems could be solved by searching for more effective biosurfactant and higher yield of biosurfactant from microorganisms. Therefore, when it comes to the production of biosurfactant for industrial application, Actinomycetes, gram-positive filamentous bacteria, are of good choice as biosurfactant producers because of their abundance in soil and their major roles in recycling of material in nature. Moreover, they have been found to produce many kinds of metabolites, including antibiotics, pigments, enzymes and biosurfactants (M. Oskay & al. [10]). Until recently, there have been only a limited numbers of reports published on biosurfactant producing actinomycetes. M. Richter & al. [11] reported production of extracellular hydrophobic peptide compounds, named streptofactin from *Streptomyces tendae* Tue 901/8c. This example highlights the potential of actinomycetes as biosurfactant producers.

The objective of the present study was optimizing the bioemulsifier production from local isolates of *Streptomyces*, including the effect of different carbon, oils and hydrocarbons sources on bioemulsifier production, and studies the stability of bioemulsifier at different pH, temperature, and salinity. Further more study, critical micelle concentration of bioemulsifier.

Materials and Methods

Isolation of Actinomycetes

For isolation of actinomycetes, samples were collected from four different places. Two soil samples were collected from petrol contaminated soil, one from garden soil and one from pond sediments, were used for isolation of actinomycetes. The soil and pond sediments samples were pretreated at 70°C for 2 h. Serial dilutions

of this pre-heated sample were prepared in sterile saline and 100 μ l of the dilutions was spread on sterile soybean agar plates consisting g/l, soybean 20g, manitol 20g and agar 30g, the pH was adjusted to 7.0. These plates were supplemented with 0.25% of nystatin. After incubation at 30°C for 7 days, plates were observed for growth of actinomycetes. Various *Streptomyces* isolates, based on their special morphological characteristics as different colored aerial mycelium with sitting colonies, were selected and purified by streaking (4-5) times on soybean agar plates (I. Thampayak & al. [12] and J. P. Maniyar & al. [8]). Pure isolates were maintained on soybean agar medium at 4 °C, until further use. Alternatively, suspensions of spores were stored in pretreated soil in oven for long term preservation.

Screening of Actinomycetes isolates

Biosurfactant activity was determined for pure culture of actinobacteria by four different methods:

(1) Lipase Production (2) Oil spreading method (3) Emulsification activity and Emulsification Index.

Lipase production test - For lipase activity Luria Bertani agar plates supplemented with (1%) of an olive oil emulsion were made (pH of the medium was adjusted 7.0) and spot inoculated with fresh culture of actinomycetes and incubated at 30°C for 7 days. After incubation, plates were observed for clear zone of hydrolysis around the colony (J. P. Maniyar & al. [8]). Positive isolates were screened for emulsification activity.

Screening of actinomycetes for bioemulsifier production- Species of actinomycetes positive for lipase were grown for 7 days in maltose yeast extract broth (MYE) consisting (g/l) distilled water, maltose (10 g), glucose (10 g), NaCl (5 g), and yeast extract (3 g) supplemented 1% of olive oil (C. R. Kokare & al. [13]). The broth cultures were incubated at 30 °C in a shaker incubator at 120 rpm for 5 days. Microbial cells were separated by centrifugation at 10000 g for 15 min at 10°C. The culture broth was then tested for the production of extracellular bioemulsifier with oil spreading method, emulsification activity, emulsification index (E24%) and E24 % stability.

Identification of Isolates

Identification of the Isolate SS 20: The potential isolate that was shown good bioemulsifier activity and stability was inoculated on soybean agar and the aerial spore mass color and reverse side and soluble pigment production was studied. The spore chain morphology of the selected isolate SS 20 of 14 day old was examined by light electron microscope. The colors of mature sporulating aerial mycelium and substrate mycelium were monitored for the 7, 14 and 21 day old cultures grown on the soybean agar (R. F. Laidi & al. [14]). The physiological and biochemical characteristics were determined according to the method of (J. G. Holt & al. [15] and D. Lakshmipathy & al. [16]). Cultures were incubated at 30 °C and examined after 7-14 days. Assimilation of various sugars such as arabinose, xylose, inositol, mannitol, glucose, fructose, rhamnose, sucrose and raffinose as sole carbon source was determined by inoculating the isolate in modified Benet's broth supplemented with the respective carbon source. After inoculation the tubes were incubated for 7 days at 30°C, and the color changes was observed.

Preparation of inocula

Preparation of inocula was carried out according to two methods: (1) for screening of isolates for bioemulsifier production, two plugs of (8 mm) of *Streptomyces* isolates mycelium, excised from the margin of 7 day old culture soybean plates, and placed in the centre of the

250 ml flasks containing 50 ml of MYE broth. (2) In optimized experiments, preparation of inocula was carried out according to the method described by P. Bhoria & al. [17] as follows: vegetative inocula of *Streptomyces* sp. SS 20 were used and prepared by inoculation of MYE broth with 2% of 48 h old seed culture of isolate.

Media and cultivation conditions

The isolates were grown in MYE broth for 48 h at 30 °C. This culture was used as stock culture inoculums at concentration of 2% (v/v). For optimum conditions of bioemulsifier production and surface active properties of produced biosurfactant, maltose yeast extract broth (MYE) with the following composition (g/L) distilled water was used, maltose (10 g), glucose (10 g), NaCl 5 g, and yeast extract (3 g) supplemented with 1% of sun flower oil. The pH of the medium was adjusted to 7.0. Carbon and nitrogen sources were added separately. Cultivations were performed in 250 mL flasks containing 50 mL medium at 30°C, and stirred in a rotary shaker incubator (Basal Switzerland) at 120 rpm.

Medium Optimization for Bioemulsifier Production

The medium optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed at a specific set of conditions. Bioemulsifier productions by *Streptomyces* sp SS 20 were tested at different physico-chemical conditions: Six factors were chosen aiming to obtain higher productivity of the bioemulsifier: Time course of biosurfactant production (incubation period), pH, temperature, nitrogen sources and carbon source and oils and hydrocarbons. For incubation period the isolate SS 20 was growing at different incubation period (1-7) days at pH 7 and 30°C in shaker incubator at 120 rpm. The effect of pH on bioemulsifier production and activity was studied. MYE medium was adjusted to different initial pH (5, 6,7,8,9 and 10), temperature used were (20 - 45°C) (A. Khopade & al. [9]). To evaluate the most appropriate organic and inorganic nitrogen sources for production of biosurfactant, NH₄Cl, NH₄SO₃, trypton, peptone, yeast extract beef extract and, alanine and urea were employed at a concentration of 1 %. All the experiments above amended with 1 % (v/v) of sun flower oil as source of carbon and energy except in the experiments of carbohydrate, oils and hydrocarbons test. For appropriate carbohydrate as carbon source for biosurfactant production, different carbohydrate sources (xylose, manitol, glucose, maltose, sucrose, starch and glycerol) were used at concentration of 1% (w/v). The effect of different hydrocarbons as a sole carbon source on bioemulsifier production was carried out by using propane, hexane, heptanes, xylene, toluene, kerosene, and gasoline and diesel oil at concentration of 1% (v/v). Optimization of different oil for bioemulsifier production was conducted. The different oils such as castor oil, lannised oil, corn oil, olive oil, sun flower oil, and seasam oil at concentration of (1% v/v) were used. For best concentration of oil source for bioemulsifier production, different concentration of corn oil (1% to 7%) was studied. In experiments above the biomass, Emulsification activity, E₂₄% and E₂₄ % stability was measured for bioemulsifier production.

Production and Partial Purification of Bioemulsifier

Study on growth of *Streptomyces* sp SS 20 and bioemulsifier production in MYE medium with corn oil as sole carbon source was carried out according to the method described by A. Khopade & al. [9] with some modification. The growth medium contained gm/ 100 ml: maltose 1.0 g; glucose 1.0 g; yeast extract 0.5 g; NaCl 0.5 g and corn oil (3% v/v). The pH of the medium was adjusted to 7.0 before sterilization. The medium (50 ml) contained in an Erlenmeyer flask (250) ml was inoculated with organisms and incubated at 30 °C with shaking at 120 rpm for 3 days. The culture broth was centrifuged (10.000 rpm, 15 min, 10 °C) to remove the cells. The bioemulsifier was extracted from culture after cell removal by using different solvent systems. A mixture of chloroform: methanol (2:1 v/v); hexane; acetone; diethyl ether and petroleum ether was added separately to the culture

medium, after being vigorously shaken, this was allowed to stand for 30 min until phase separation. The extract concentrated and then sodium sulphate anhydrous was added to remove water. The crud extract was obtained after removal of solvent and moisture by evaporation and drying in oven at 45 °C respectively for 24 h. The best solvent system which showed higher yield of biosurfactant was a mixture of chloroform: methanol (2:1 v/v), were used later for further extraction.

Biosurfactant Characteristics

Stability of bioemulsifier with different pH, temperature, salinity and hydrocarbons

To determine the effect of pH on bioemulsifier stability, pH of the cell free broth was adjusted to different values ranged from (3 to 12) using 1 N NaOH or 1 N HCl. The thermal stability of the bioemulsifier was determined by maintaining the supernatant at constant different range of temperature from 30 to 100 °C for 30 min and cooled at room temperature. The effect of addition of different concentration of NaCl on the activity of the bioemulsifier was studied; the specific concentration of NaCl was (1 to 9 % w/v). The effect of different hydrocarbons on bioemulsifier stability was investigated by using propane, hexane, heptanes, xylene, and toluene, kerosene, gasoline and diesel oil (Z. Zosim & al. [18]). After each experiment E24 % was measured and all the experiments were done in duplicate.

Critical Micelle Concentration (CMC)

The CMC, the minimum amount of surfactant required to cause the maximum decrease in surface tension, is an important measure of the surface activity and allows comparison with other surfactants (J. Souza & al. [19]). To determine the critical micelle concentration (CMC), the extract was dissolved in sterile Tris-Mg (20 mM) (pH 7.0) at concentrations of 0, 50, 100, 150, 200, 250, 300, 500, 750 and 1000 mg/l. Surface tension measurements were carried out with a K6 tensiometer (Kruss GmbH, Hamburg, and Germany) Du Nouy K6 ring method. Measurements were performed at 25°C, and sterile distilled water was used to calibrate the tensiometer and all the experiments were done in duplicate.

Analytical Techniques

Biomass measurement

The dry weight technique was used to quantify microbial growth as bacterial dry weigh. Biomass obtained after centrifugation at (10.000 g, 15 min), then the precipitate cells transferred to weighted container and dried overnight at 105°C and reweighed.

Oil Spreading Method

In oil spreading method, 50 ml of distilled water was added to the large petriplate followed by 20 µl of crude oil on the surface of the water. Ten micro liters of culture free cells samples was added onto the center of the oil film. The diameters of the clear zone on the oil surface were measured and compared with control using uninoculated medium (M. Morikawa & al. [20]).

Emulsification activity (EA)

Emulsification activity of the culture sample was determined according to the method described by G. Kumar & K. V. Road [21].

Emulsification index (EI24)

E24% of culture samples was determined by adding 2 mL of a hydrocarbon (toluene) to the same amount of culture free cells, mixing with a vortex for 2 min, and leaving to stand for 24 h. The E24 index is given as percentage of height of emulsified layer (mm) divided by the height of the hydrocarbon phase (mm) and multiplying to 100 (R.N. Patel & al. [22]).

Surface tension measurement

The surface tension of crude extract was determined according to the method described by A.A. Bodour & al. [23], using the du Nouy ring method. The values reported are the mean of two measurements.

Result and Discussion

Isolation and screening of isolates

A total of 90 colonies of *Streptomyces* isolates were selected for biosurfactant production. The selected isolates were screened for bioemulsifier production. Out of the 90 isolates, 45 showed lipase activity. All lipase positive isolates showed emulsification activity. Among 45 isolates, five isolates, showed maximum lipase and emulsification activity were selected for further screening. Among the five isolates tested, the isolate SS 20 showed maximum bioemulsifier activity according to the results of oil spreading, EA, E24% and bioemulsifier stability after two week (Table 1). Therefore the isolate SS 20 was selected for identification and for bioemulsifier production. The results agreed with the study of C. R. Kokare & al. [13] screened 80 *streptomyces* isolates for lipase activity, 56 showed positive lipase activity, and five strain of *Streptomyces* showed good bioemulsifier activity.

Table 1: Screening of isolates for bioemulsifier production in MYE broth.

No	Isolates	Source of isolates	Lipase activity (mm)	Oil displacement (mm)	EA	EI 24%	EI24% After 1 week	EI24% After 2 week
1	SS 20	Contaminated soil	+	8	0.535	100	75	60
2	SS 46	Contaminated soil	+	8	0.527	100	66	40
3	SS 151	Garden soil	+	7	0.500	100	70	41
4	SS 176	Garden soil	+	8	0.700	100	66	33
5	SS 220	Garden soil	+	8	0.660	100	13	8

Morphological and biochemical Characterization of Isolate SS 20

The selected isolate was examined for its morphology. The colonies of the isolate were large, regular, circular, raised, gray, and slimy on soybean agar surface. Dark brown pigmentation was observed on the reverse side of the colonies. The isolate was capable of assimilating all 9 sugars tested (Table 2). The morphological and cultural characteristics of the isolate were compared with known Actinomycetes species described in Bergey's Manual of determinative Bacteriology (J. G. Holt & al. [15]) and Bergey's Manual of Systematic Bacteriology (S. Williams & al. [24]) and was identified as *Streptomyces* sp.

Table 1: Morphological, cultural and biochemical characteristics of *Streptomyces* sp. SS 20

No.	Morphological and biochemical characteristics	<i>Streptomyces</i> SS 20
1.	Colony color and shape	Gray and circular
2.	Colony diameter	2 mm
3.	Presence of spores	Aerial mycelium

	Spore surface	slimy
	Aerial spore mass color	Goldy gray
4.	Pigmentation	
	a. Reverse side pigment	Brown
	b. Diffusible pigment	No pigment
	c. Melanin pigment	-
5.	Starch hydrolysis	+
6.	Growth at 4 °C	-
	Growth at 37 °C	+
	Growth at 45 °C	+
6.	Growth range pH 3-10	+
7.	Sugars	
	a. Arabinose	+
	b. Xylose	+
	c. Inositol	+
	d. Manitol	+
	e. glucose	+
	f. Fructose	+
	i. Rhamnose	+
	g. Sucrose	+
	k. Raffinose	+

Time Course and Kinetics of Bioemulsifier Production by *Streptomyces* sp. SS 20

It is very important to know that at which growth phase of the life cycle of *Streptomyces* produced maximum bioemulsifier. It was observed that maximum growth rate and bioemulsifier was produced during exponential growth phase after about 3 d of incubation, beyond which the production as well as growth decreased (Fig. 1a). These results indicate that the biosurfactant biosynthesis occurred predominantly during exponential growth phase, suggesting that the bioemulsifier is produced as primary metabolites with increase of cellular biomass formation (growth associated kinetics). These results was in agreement with finding of C. R. Kokare & al. [13] and J. P. Maniyar & al. [8] they shown that *Streptomyces* sp. produces maximum biomulsifier activity during late exponential phase after 96 h of incubation. The emulsification index E24% increased from 10% after 3 days of incubation to 100% at the end of the active growth phase, which suggests that the microorganisms assimilated oil source and produced bioemulsifier; the process can have acted according to two ways: either biosurfactant making cellular surface more hydrophobic or the biosurfactant enhanced aqueous solubilization and dispersion of the oil source. During the stationary phase, the E24% was stable after 7 days of incubation. Similarly, *Rhodococcus* strain produced maximum bioemulsifier activity (E24%=63%) at the end of exponential growth phase utilizing 3% (v/v) sun flower oil (Z. Sadouk & al. [25]).

Effect of Environmental factors on bioemulsifier production

Effect of pH

The pH of the medium is important characteristics for cell growth and metabolites production. The bioemulsifier production was affected by initial pH of culture medium. At pH 5, the bioemulsifier production was decreased. This low pH created unfavorable condition for growth and bioemulsifier production (Fig. 1b). Maximum growth and bioemulsifier production by isolate *Streptomyces* sp. SS 20 was at pH 7 (E24%= 100% and EA=0.30). A pH 7 was optimum for the activity and bioemulsifier production for a time period of 3 days. The bioemulsifier showed insignificant activity at pH 5 and 10. The results also indicated enhancement of bioemulsifier activity and production at alkaline pH. Similar results was observed by biosurfactant producer *Streptomyces* S1 (C. R. Kokare & al. [13]) and from marine *Streptomyces* sp. B3 (A. Khopade & al. [9]).

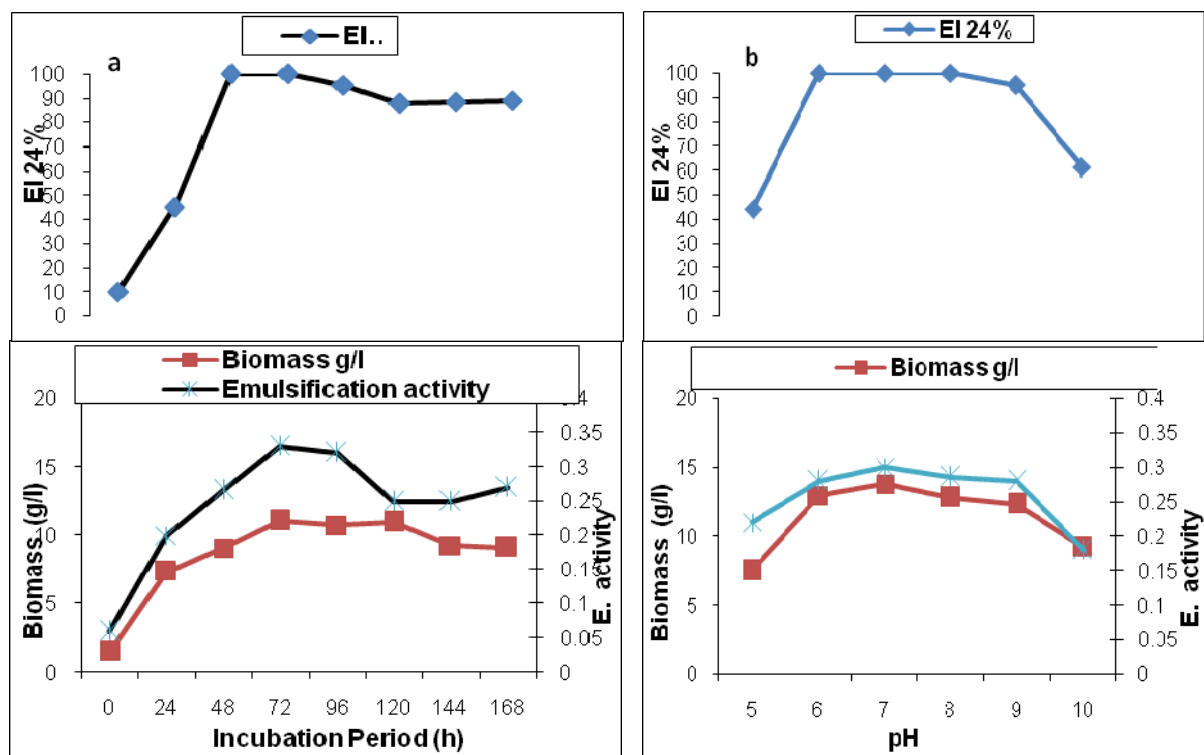


Figure 1: (a) Time course (growth kinetics) of *Streptomyces* sp. SS20 produced bioemulsifier, (b) Effect of pH on growth and bioemulsifier production by *Streptomyces* sp. SS20.

Effect of temperature

Temperature played an important role in the growth and biosurfactant production. The isolate *Streptomyces* sp. SS 20 showed good growth between the temperature ranged from 20 - 40 °C, the growth and bioemulsifier activity was decreased as temperature increased. The results in the present study revealed that the bioemulsifier activity reached highest value when the isolate was grown at 30 °C (E24%= 100% and EA= 0.44). (Fig. 2a), indicating clearly that the isolate is moderately thermostable. A 45% loss of activity of the produced bioemulsifier observed at 45 °C. Similarly *Streptomyces* sp. B3 isolated from marine environment shows maximum bioemulsifier production at 30 °C⁹ (A. Khopade & al. [9]). L. Deepika & K. Kannabiran [26] observed maximum bioemulsifier production and heavy metal resistance activity of *Streptomyces* spp. at 30 °C.

Effect of Nitrogen Sources on Bioemulsifier Production

The nature of nitrogen source and its limitation has been shown to play an important role in biosurfactant production. In present study among different organic and inorganic nitrogen source tested, yeast extract was found to be the best source of nitrogen for growth and biosurfactant synthesis (Fig. 2b). Maximum growth and emulsifying activity (0.30) and E24% (80%) were obtained in media contains yeast extract. Similar results mentioned by A. Khopade & al. [9], maximum emulsification activity (285 EU/ml) and minimum surface tension (30 mN/m) were obtained by isolate *Streptomyces* sp. in media supplemented with yeast extract.

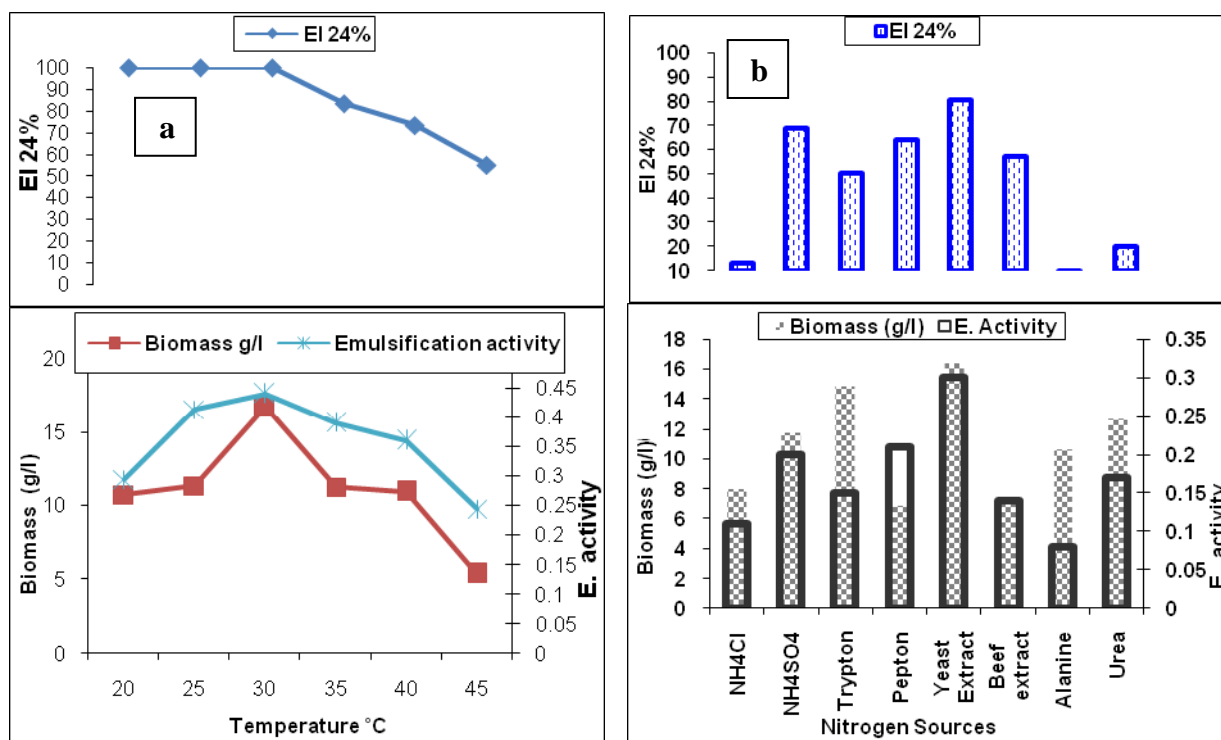


Fig.2: (a) Effect of temperature on growth and bioemulsifier production by *Streptomyces* sp. SS20, (b) Effect of nitrogen source on growth and bioemulsifier production by *Streptomyces* sp. SS20.

Effect of Carbon Sources

A number of carbon sources have been used by many researchers for biosurfactant production. The quality and quantity of produced biosurfactant are affected and influenced by the nature of the carbon substrate (L. H. Guerra-Santos & al. [27] and D.G. Cooper & D.A. Paddock [28]). The various carbon sources screened for bioemulsifier production in the current study, out of this sources, maltose, glucose and manitol were found best carbon source for growth and bioemulsion production (Fig. 3a). The highest bioemulsifier activity was obtained using sucrose at concentration of (1% w/v) as sole source of carbon, resulted in higher emulsifier activity (E24% = 100% and EA = 0.50). The results was agreement with the founding of A. Khopade & al. [9], they obtained maximum activity (E24%= 80%) when using sucrose as sole source of carbon by marine isolate *Streptomyces* sp. B3.

Effect of Oil and Hydrocarbons on Bioemulsifier Production

Fermentation was carried out with different oils and hydrocarbons. The production of bioemulsifier from *Streptomyces* sp. SS 20 was induced by addition of oils and hydrocarbons. Among the six oils tested as substrate for bioemulsifier production, corn oil was the best with maximum growth and emulsification activity (E24%= 100% and EA= 0.30) (Fig. 3b). The optimum concentration of corn oil for growth and bioemulsifier production was 3 % (v/v), the isolate has synthesized extracellular compounds which the E24% and EA of the culture was reached to 100% and 0.55 respectively (Fig. 4a). The results obtained indicated that the bioemulsifier production is a linear function of growth and substrate concentration. The results was agreement with J. R. Patil and D. A. Chopade [29] and C. R. Kokare & al. [13] confirmed that the production of bioemulsifier from *Acinetobacter junii* SC14 and *Streptomyces* sp. S1 have been induced by addition of oils and hydrocarbons. Z. Sadouk & al.

[25] observed maximum biosurfactant (fatty acid methyl esters) production by *Rhodococcus* strain using sun flower oil at concentration of 3% (v/v) with E24% activity up to 63%.

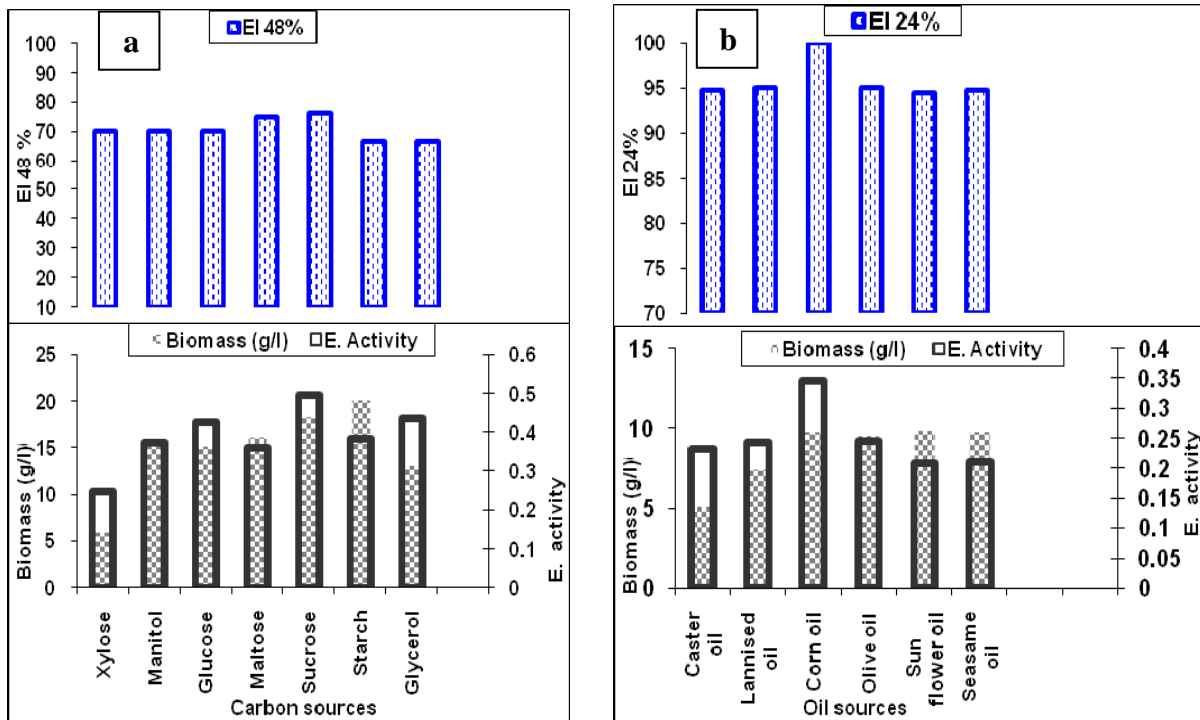


Fig.3: (a) Effect of carbon sources on growth and bioemulsifier production, (b) Effect of oil sources on growth and bioemulsifier production by *Streptomyces* sp. SS20.

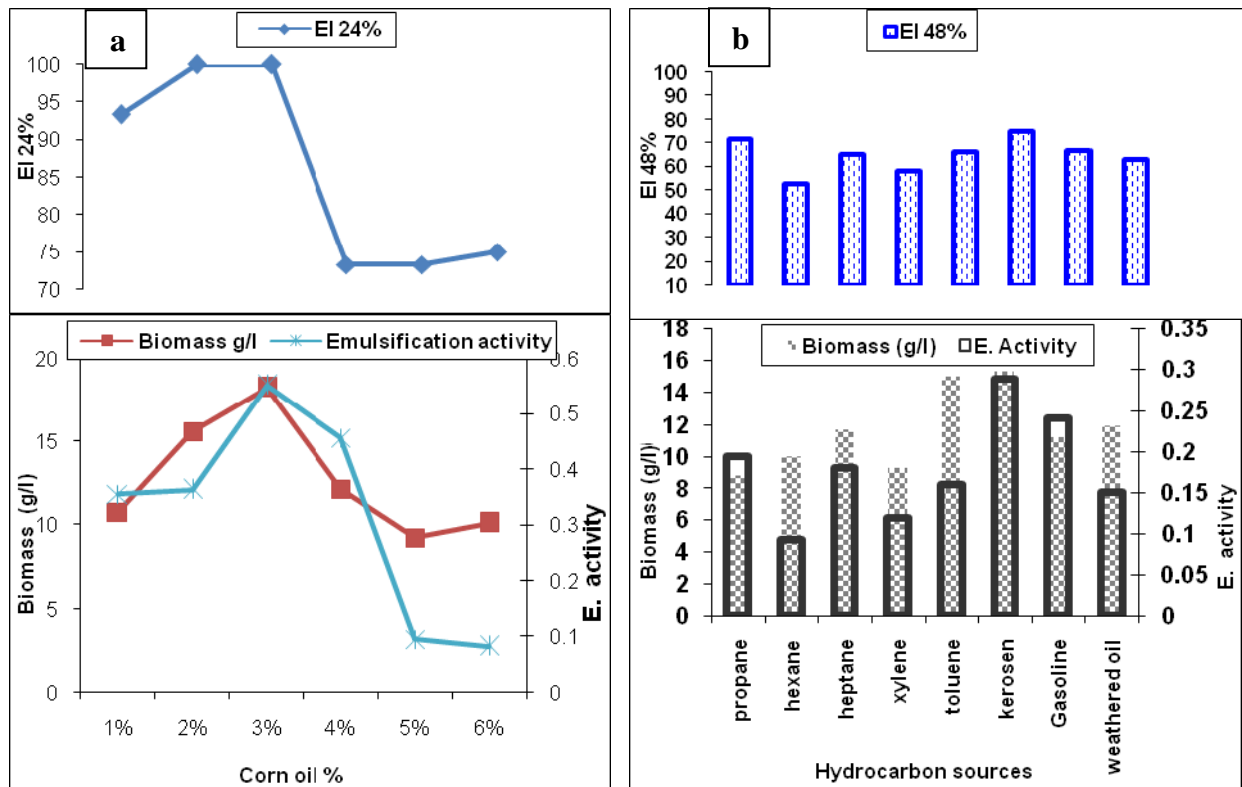


Fig.4: (a) Effect of different corn oil concentration on growth and bioemulsifier production, (b) Effect of hydrocarbons sources on growth and bioemulsifier production by *Streptomyces* sp. SS20.

A different hydrocarbon was tested as a substrate for bioemulsifier production. The results showed capability of isolate *Streptomyces* sp. SS 20 to utilize all the hydrocarbons as source of energy; growth was accompanied with bioemulsifier production. Maximum emulsification index (E24%=100) was observed with all hydrocarbons tested, while emulsification stability after 48 h (E48%) showed higher stability of emulsion with kerosene, retained up to 75% of the activity compared with remained hydrocarbons (Fig. 4b). Potential toxicity has been mentioned as possible reasons for the inability of most microorganisms to grow and emulsify hexane, xylene and toluene. Poor emulsification of some hydrocarbons may be due to the inability of the bioemulsifier to stabilize the emulsion with hydrocarbons.

Preliminary Characteristics of Bioemulsifier

Partially purified bioemulsifier was obtained from cell free supernatant of *Streptomyces* sp. SS20 grown in MYE broth. The produced bioemulsifier was dark brown, viscous, and was partially soluble in water with glycolipid nature (results not shown). Maximum yield of bioemulsifier was found to be 10.48 g/l when extracted with solvent system (chloroform: methanol 2:1 v/v). Bioemulsifier was stable at room temperature for more than 2 weeks and retained more than 75% of its activity. The bioemulsifier was partially soluble. Hence it can be used in formulation of pesticides, food and medicine (J. R. Patil and D. A. Chopade [29]). A. Khopade & al. [9] noticed during the composition analysis of bioemulsifier produced by *Streptomyces* B3 that the produced bioemulsifier possesses glycolipid nature consisting to lipid 58% (w/w) and 33% (w/w) carbohydrate and minor fraction of protein (8% w/w).

Stability of Bioemulsifier

Effect of Temperature

Application of biosurfactant in different fields depends on its stability at different pH, temperature and salinity. The stability of bioemulsifier was tested over a wide range of temperatures. The produced bioemulsifier was shown to be thermostable. Heating of bioemulsifier to 100 °C caused no significant effect on the bioemulsifier activity (Fig. 5). The results of emulsion activity (E24% = 100%) was stable at the temperature between 30 to 70 °C, but the loss of E24% beginning at 80 °C and retained 86% of its activity when heated to 100 °C. Therefore, it can be concluded that this bioemulsifier maintains its surface activity unaffected in the range of temperatures between 30 and 100 °C. The thermostability of produced bioemulsifier indicating, usefulness of bioemulsifier in oil industries, food, and pharmaceutical and cosmetics industries. The decrease in E24% activity at high temperature may be due to the effect of temperature, the composition of bioemulsifier, formed an immiscible of aqueous phase with hydrocarbon phase (toluene). The present study agreed with the study of M. Shafeeq & al. [30] found stability of glycolipid produced by *Pseudomonas* sp. at temperature reached 80 °C, and maximum E24% was at temperature between 60 and 70 °C. S. Navon-Venezia & al. [31] found increase in the emulsification activity of alasin by (2.5 to 3) times after heating to 100 °C.

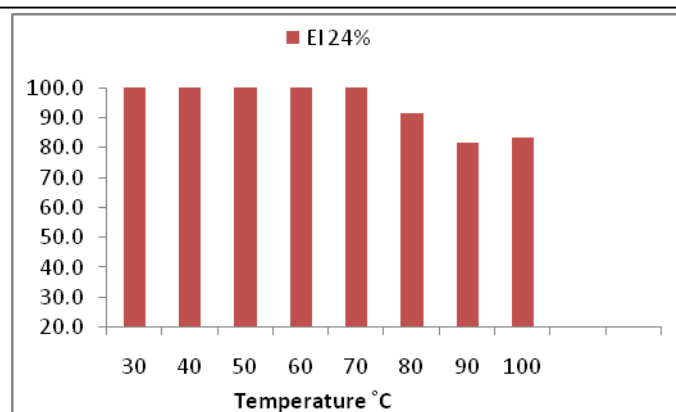


Fig. 5: Effect of temperature on bioemulsifier stability of *Streptomyces* sp. SS 20

Effect of pH

The results in (Fig. 6) showed that the produced bioemulsifier is remained stable at pH ranged from 5 – 7 (E24%=100%). These results indicates enhancement of bioemulsifier activity at acidic and neutral pH. This condition could be related to instability of bioemulsifier micelles in the presence of alkaline condition and the precipitation of primary metabolites at higher pH value. Similar results was observed from bioemulsifier produced by *Streptomyces* S1, they obtained stable bioemulsifier at neutral pH with 82% activity, while at pH 8 and 9 the bioemulsifier activity was decreased to 33 and 28% respectively (C. R. Kokare & al. [13]). Rhaminolipid produced by *Pseudomonas aeruginosa* showed more effectiveness and stability at neutral pH. While bioemulsifier produced by new strain *Candida glabrata* UCP 100 showed activity and stability at wide range of pH (2-12) (L. A. Sarubbo & al. [32]).

Effect of Salinity

Effect of sodium chloride was tested for bioemulsifier stability. Maximum emulsification stability (E24%=72) of bioemulsifier was observed at concentration of 3% (w/v) NaCl (Fig.7), as concentration of NaCl increased bioemulsifier production was decreased. At higher concentration of NaCl 9% (w/v), the bioemulsifier retains 57% of activity. Similar results was obtained by bioemulsifier producers *Rhodococcus ruber* Z 25 and *Streptomyces* sp. S1 they observed maximum bioemulsifier stability at NaCl concentration of 2.5 and 3% (w/v) respectively (C. Zheng & al. [33] and J. P. Maniyar & al.[8]).

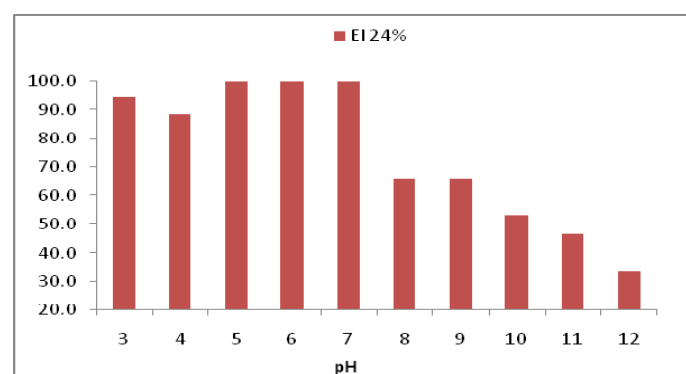


Fig. 6: Effect of pH on bioemulsifier stability of *Streptomyces* sp. SS 20

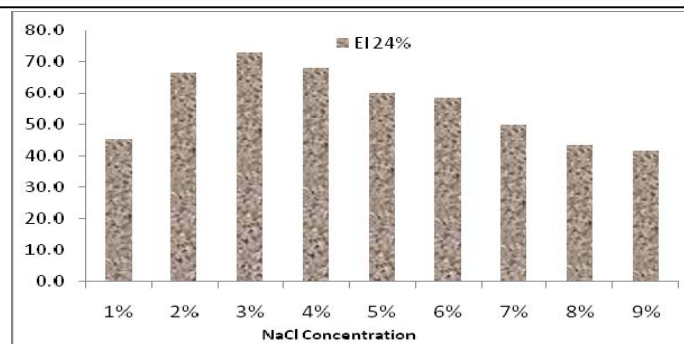


Fig. 7: Effect of NaCl concentration on bioemulsifier stability of *Streptomyces* sp. SS 20

Stability of Bioemulsifier with Hydrocarbons

The ability of isolate *Streptomyces* sp. SS 20 to emulsify different hydrocarbons was studied with aliphatic, aromatic and mixed hydrocarbons. The results in Figure 8 showed increase in emulsion stability with increasing number of carbon atoms and molecular weight of n- alkanes (propane, hexane and heptanes). Maximum emulsifying stability was obtained with liquid aromatic hydrocarbons (xylene and toluene) reached 86.7 and 100% respectively. While, the results showed less stability of emulsion with mixed hydrocarbons (kerosene, gasoline and diesel oil), and maximum stability (E24%=72.7) recorded with kerosene. Also J. D. Desai & I. M. Banat [34] showed that aliphatic alkanes revealed emulsion stability with rhamnolipid, and the stability increased with molecular weight of alkanes. F. C. Bicca & al. [35] observed maximum stability of glycolipid produced by *Rhodococcus* sp. with monoaromatic hydrocarbons (benzene, toluene and xylene) and reached more than 65%. Also they noticed less bioemulsifier stability with mixed hydrocarbons (E24%=60%), while they observed minimum emulsion stability with aliphatic hydrocarbons reached less than 30%.

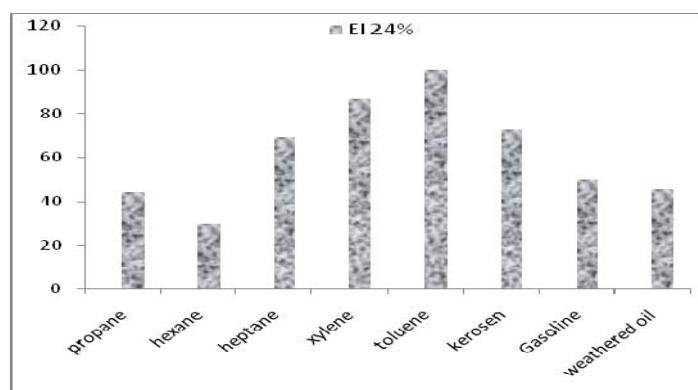
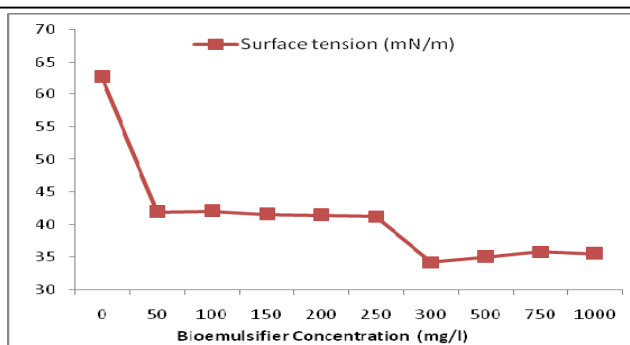


Fig. 8: Stability of bioemulsifier produced by *Streptomyces* sp. SS 20 with hydrocarbons.

Critical Micelle concentration of Bioemulsifier

The critical micelle concentration (CMC), which is a characteristics property of bioemulsifier, is the concentration at which micelles started to be formed: if the more of the surfactant present, there will be no further decrease in surface tension. To obtain the CMC value of the crude bioemulsifier, the crude extract from the bacterial culture was diluted in Tris-Mg (20 mM), and the surface tension was measured at various concentration of bioemulsifier (Fig.13). The addition of bioemulsifier to Tris-Mg solution reduced the surface tension value to 34.2 mN/m. Also the results indicated that the surface tension value remain quite constant with the increased in the bioemulsifier concentration; thus the CMC of the crude product was 300 mg/l and corresponds to the surface tension value = 34.20 mN/m.



F.g. 13: Critical micelle concentration of bioemulsifier produced by *Streptomyces* sp. SS20

From the results above it can be concluded that the biosurfactant produced by *Streptomyces* sp. SS 20 reached saturation condition at CMC, leads to change arrangement of hydrophobic moieties direction toward the medium, while hydrophilic moieties towards liquid solution resulted in stability of surface tension at this concentration (T. Barkay & al. [36]). Similarly, polysaccharide and lipid contained bioemulsifier for *Streptomyces* sp. S1 and bioemulsifier produced by *Streptomyces* sp. B 3 reduced the surface tension value of the medium to 42.6 mN/m at CMC = 300 mg/l and to 30 mN/m at CMC = 110 mg/l respectively (C. R. Kokare & al. [13] and A. Khopade & al. [9]).

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