Bioemulsifier production by *Streptomyces* sp. S22 isolated from garden soil

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Out of 45 actinomycetes isolated from garden soil, pond water and air; fifteen showed good emulsification activity. *Streptomyces* sp. S22 isolated from garden soil produced maximum bioemulsifier with 0.5% (v/v) sunflower oil during stationary phase at 37°C, pH 6 and 250 rev/min. Emulsification activity was maximum (320 EU/ml) with sunflower oil as substrate. Partially purified bioemulsifier from *Streptomyces* sp. S22 was a peptidoglycolipid containing lipid (51.25%), protein (30%), non-reducing sugar (17.75%) and reducing sugar (1%). The yield of partially purified bioemulsifier was 1.6 g/l and reduced the surface tension of water by 23.09 mN/m. The bioemulsifier produced by *Streptomyces* sp. S22 was stable at room temperature for seven days.

**Keywords:** Actinomycetes, Bioemulsifier, Emulsification activity, Surface tension, *Streptomyces* sp. S22

Two immiscible liquids can form an emulsion which may be described as unstable dispersion system of these two liquids by the action of an emulsifier. Emulsifiers are a subclass of surfactants that stabilize dispersions. Bioemulsifiers are amphipathic molecules secreted by microorganisms to facilitate uptake of insoluble substrates. Studies on bioemulsifier are fast gaining ground due to their widespread applications.

Bioemulsifiers could help in microbial enhanced oil recovery (MEOR), replacement of chlorinated solvents used in cleaning-up oil-contaminated pipes, vessels and machinery, transportation of heavy crude oil, bioremediation of oil-polluted soil and water and use in detergent industries, formulation of herbicides and pesticides, food and cosmetic industries. Although enormous literature appears on bioemulsifier production by bacteria like *Acinetobacter, Bacillus, Pseudomonas*, reports on production by actinomycetes are rare. Among various actinomycetes, glycolipids from *Rhodococcus erythropolis*, *Rhodococcus aurantiacus* and *Rhodococcus* sp. H13-A 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. With this background, the current study was undertaken to search for novel bioemulsifiers from actinomycete species. The present study describes bioemulsifier production by *Streptomyces* sp-S22 isolated from garden soil.

**Materials and Methods**

**Isolation and screening of actinomycetes for lipase production and haemolysis** — For isolation of actinomycetes, samples were collected from six different places. Three soil samples (two were collected from petrol contaminated soil and one from garden soil), two air samples and one pond water sample were used for isolation of actinomycetes. The soil and pond water samples were pretreated at 70°C for 2 h. Serial dilutions of this pre-heated sample were prepared in sterile saline and 0.1 ml of the dilutions was spread on sterile casein starch agar plates consisting per liter distilled water, soluble starch (2 g) K₂HPO₄ (4 g), KNO₃ (4 g), NaCl (4 g), casein (0.6 g), MgSO₄ (0.1 g), CaCO₃ (0.04 g), FeSO₄ (0.02 g) and agar (30 g). After incubation at 30°C for 7 days, plates were observed for growth of actinomycetes. Cultures were preserved on casein starch agar slants at 4°C, until further use.

All actinomycetes were tested for lipase production and lysis of red blood cells. For lipase activity Luria Bertani agar supplemented with an olive oil emulsion plates were made and spot inoculated with fresh

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culture of actinomycetes and incubated at 30°C for 7 days. After incubation, plates were observed for clear zone of hydrolysis around the colony. Hemolysis was carried out on blood agar plates [Nutrient agar containing 5% (v/v) fresh human blood]. Plates were spot inoculated and incubated at 30°C for 96 h. After incubation plates were observed for haemolysis. Positive strains were screened for emulsification activity.

Identification and screening of actinomycetes for bioemulsifier production — Species of actinomycetes positive for lipase and hemolysis were grown for 7 days in maltose yeast extract broth (MYE) consisting per liter distilled water, peptone (10 g), NaCl 5 g, glucose (10 g) and yeast extract (3 g). Microbial cells were separated by centrifugation at 8000 g for 15 min at 30°C. Cell free supernatant (3 ml) was vortexed vigorously with 0.5 ml test oil/hydrocarbon for 2 min and incubated at 30°C for 1 h for phase separation. Aqueous phase was removed carefully and its absorbance recorded at 400 nm. The blank was prepared similarly by replacing the cell free supernatant by sterile medium. An absorbance of 0.010 units at 400 nm multiplied by dilution factor, if any, was considered as one unit of emulsification activity per ml (EU/ml). Five strains of actinomycetes showing maximum activity were identified by slide culture technique and cell wall analysis. Streptomyces sp. S22 exhibiting highest activity was selected for further studies.

Oils and hydrocarbons used for emulsification — Edible oils such as sunflower oil, soybean oil (Gemini Mills Mumbai, India), sesame oil and mustard oil (United Pharmacy Pune, India), and hydrocarbons (petrol, toluene) were used.

Effect of oils and hydrocarbons on bioemulsifier activity and induction of bioemulsifier production — Emulsification of different oils and hydrocarbons was checked employing the emulsification assay. Induction of bioemulsifier production by sunflower oil (0.5, 1, 2, 3 and 4% v/v) by Streptomyces sp. S22 was checked in MYE broth supplemented with the oil. Cell-free supernatant was assayed for emulsification activity.

Bioemulsifier production by Streptomyces sp. S22 — Bioemulsifier production by Streptomyces sp. S22 was carried out by adding 5% (v/v) inoculum in 500 ml of MYE broth (supplemented with 0.5% v/v sunflower oil) and incubated at 37°C, 250 rev/min. Samples were tested for bioemulsifier production at intervals of 24 h employing emulsification assay. Growth was quantified by measuring absorbance at 660 nm.

Effect of physico-chemical factors on bioemulsifier production by Streptomyces sp. S22 — Effect of physico-chemical factors on bioemulsifier production by Streptomyces sp. S22 was studied in MYE medium. Effect of pH (5, 6, 7, 8 and 9), temperature (30°, 37°, 45° and 50°C), agitation (150, 200, 250 rev/min) and salts such as NaCl, CaCl2, and MgCl2 (0.5, 1, 2 and 3% w/v) was studied.

Partial purification of bioemulsifier from Streptomyces sp. S22 — Partial purification was carried out using 1 l of 96 h old broth of Streptomyces sp. S22 centrifuged at 8000 g for 20 min at 30°C. After centrifugation three volumes of chilled acetone was added in cell free broth and incubated at 4°C for 15 h. This was subjected to centrifugation at 8000 g for 30 min at 10°C and brown precipitate obtained was collected. This precipitate was dissolved in 3 ml of sterile distilled water and extensively dialysed against sterile distilled water at 10°C for 48 h (seamless cellulose tubing, width 40 mm, diameter 25 mm, retaining most proteins of molecular weight 12000 or more, Sigma Aldrich Chemie, GmbH, Seinheim, Germany). Distilled water was changed after every 12 h. The dialysate was then frozen at -20°C, lyophilized and stored at 30°C in air tight glass vials.

Chemical analysis of bioemulsifier — The crude bioemulsifier obtained was analysed chemically for protein, sugar and lipid content. Protein content was assayed using the method described by Reddy et al. Carbohydrates were quantified according to the protocol described elsewhere. Reducing sugar content was estimated using the dinitro salicylic acid method. Extraction and quantification of lipids was performed as per the method described by Reddy et al.

Stability of bioemulsifier — Stability of bioemulsifier was checked at different temperatures (10°, 30° and 40°C) by using the method as described earlier.

Determination of viscosity — Viscosity of partially purified bioemulsifier was tested by taking different aliquots (0.5-3 ml) of solution of the partially purified bioemulsifier (5 mg/ml). This was used to emulsify a fixed amount (6 ml) of sunflower oil. Viscosity was recorded by using Oswald’s standard viscometer at 30°C. Unemulsified sunflower oil served as control.
**Determination of surface tension and critical micelle concentration (CMC)** — Surface tension reduction ability of the partially purified bioemulsifier was checked by Dynamic Contact Angle Surface Tensiometer (DCAT 11, Dataphysics GmbH, Germany). Critical micelle concentration (CMC) of the partially purified bioemulsifier was estimated by addition of different concentrations of the partially purified bioemulsifier to distilled water till constant surface tension readings were obtained.

**Results and Discussion**

Total 45 strains of actinomycetes were isolated out of which 38 were soil isolates, five were from pond water and two were from air sample. Pre-heat treatment reduced number of contaminating bacteria and fungus. Lipase is the enzyme that acts at oil-water interface and is linked with bioemulsifier production. All 45 strains showed positive lipase and haemolytic activity, so all 45 strains were screened for bioemulsifier production. Out of these 45 strains 15 showed good emulsification activity. Five soil isolates showing maximum activity were identified by cell wall analysis; two were of *Actinopolyspora* genus and three were of *Streptomyces* genus. *Streptomyces* sp. S22 showing highest emulsification activity of 320 EU/ml was selected for further studies.

The production of bioemulsifier from *Streptomyces* sp. S1 and *Acinetobacter junii* SC14 have been induced by addition of hydrocarbons and oils, respectively. This is in agreement with our results where, bioemulsifier production from *Streptomyces* sp. S22 was induced by sunflower oil (Fig. 1). The bioemulsifier activity increased from 145 EU/ml to 320 EU/ml on addition of oil. The optimum concentration of sunflower oil was 0.5% (v/v). Among the four oils tested as substrates for bioemulsification, sunflower oil was the best with maximum emulsification (320 EU/ml) while sesame oil showed least emulsification (130 EU/ml; Fig. 2). Both toluene and petrol were emulsified to less extent. This is contrary to the findings of Kokare *et al.* who have found that the *Streptomyces* sp. S1 shows maximum activity with toluene and petrol. *Streptomyces* sp. S22 produced maximum bioemulsifier in stationary phase at 96 h after which production as well as growth decreased (Fig. 3). This is in agreement with the report by Kokare *et al.* who have shown that *Streptomyces* sp. S1 produces bioemulsifier during the stationary phase with a maximum activity of 361.2 EU/ml. Similarly, *Acinetobacter junii* SC14 produced bioemulsifier during stationary phase.

*Streptomyces* sp. S22 showed maximum bioemulsifier production at pH 6 (320 EU/ml). Bioemulsifier production at pH 5 (160 EU/ml) was least; but was significant at pH 7 (300 EU/ml). This result indicates that the bioemulsifier production is enhanced at acidic pH. On the contrary, *Candida glabrata* UCP 1002 shows activity at wide range of pH (2-12) and *Acinetobacter junii* SC14 shows
maximum bioemulsifier production at slightly alkaline pH (7.2) \(^{13}\). *Streptomyces* sp. S1 shows maximum bioemulsifier production at neutral pH \(^{10}\). *Streptomyces* sp. S22 showed maximum bioemulsifier production at 37°C (320 EU/ml) and minimum at 50°C (210 EU/ml). *Rhodococcus ruber* Z25 shows maximum biosurfactant production at 34°C \(^{23}\). *Streptomyces* sp. S1 isolated from marine environment shows maximum bioemulsifier production at 28°C \(^{10}\). It was found that as agitation increased, bioemulsifier production also increased correspondingly. *Streptomyces* sp. S22 showed maximum bioemulsifier production at 250 rev per min (320 EU/ml) and minimum at 150 rev per min (265 EU/ml). *Rhodococcus* sp. H13-A has shown bioemulsifier production at 300 rev/min \(^7\). NaCl (0.5% w/v) was found optimum, yielding maximum bioemulsifier production (320 EU/ml). As concentration of NaCl increased production of bioemulsifier decreased. Bioemulsifier production (120 EU/ml) was least at NaCl that is 3% w/v. *Streptomyces* sp. S1 and *Rhodococcus ruber* Z25 has optimal NaCl concentration of 3 and 2.5% (w/v), respectively \(^{10,23}\). However, CaCl\(_2\) and MgCl\(_2\) used separately caused inhibition of bioemulsifier production. Similarly, bioemulsifier from *Acinetobacter junii* SC14 was inhibited by CaCl\(_2\) and MgCl\(_2\) \(^{13}\).

Yield of partially purified bioemulsifier obtained from the cell free supernatant of *Streptomyces* sp. S22 was found to be 1.6 g/l. The bioemulsifier comprised protein (30%), non-reducing sugar (17.75%), reducing sugar (1%) and lipid (51.25%). The partially purified bioemulsifier was dark brown, hygroscopic and was partially soluble in water thus, indicating peptidoglycolipid nature of the bioemulsifier produced by *Streptomyces* sp. S22. *Pseudomonas aeruginosa* produces similar type of bioemulsifier \(^{24}\). While *Acinetobacter junii* SC14 and *Yarrowia lipolytica* NCIM 3589 produces glycolipopeptide type of bioemulsifier \(^{13,25}\). Bioemulsifier produced by *Streptomyces* sp. S1 is of protein polysaccharide nature \(^{10}\). Bioemulsifier produced by *Streptomyces* sp. S22 was stable at room temperature for more than seven days retaining 90% of its activity. The bioemulsifier was not stable at 10° and 50°C. Similarly, bioemulsifier from *Streptomyces* sp. S1 and *Acinetobacter junii* are stable at room temperature, but unstable at both, lower and higher temperatures \(^{10,13}\).

Bioemulsifier from *Streptomyces* sp. S22 led to a decrease in viscosity (60%) of sunflower oil. This is less as compared to the decrease in viscosity (72%) of cod-liver oil by bioemulsifier of *Streptomyces* sp. S1 and higher than that by bioemulsifier of *Acinetobacter junii* with a reduction in the viscosity of almond oil by 40.3% \(^{10,13}\). Partially purified bioemulsifier reduced surface tension of water by 23.09 mN/m (CMC, 4 mg/ml). Similarly, bioemulsifier from *Streptomyces* sp. S1 reduces the surface tension to 42.6 dynes/cm (CMC, 0.3 mg/ml) \(^{10}\). Contrary to this, bioemulsifier from *Nocardia* sp. L-417 and *Acinetobacter baumannii* A25 exhibit less reduction in surface tension \(^{9,12}\).

Bioemulsifier produced by *Streptomyces* sp. S22 was partially soluble in water, hence it could find potential applications in formulation of pesticides, food, pharmaceutical and medicine. Bioemulsifier produced by *Streptomyces* sp. S22 had good surface tension reduction property hence could be used as surfactant.

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**References**