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A study on isolation, extraction, purification and characterization of surfactin by *Bacillus subtilis* from oil contaminated soils

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ABSTRACT

Biosurfactants are microbial surface active agents produced by variety of microorganism and it has great potential advantages over chemical surfactant. Surfactin is a type of biosurfactant produced by *Bacillus subtilis*, its production kinetics under different conditions and their efficient activity are examined in this study. Oil contaminated soil samples were collected from petroleum industry in north Chennai, India and *Bacillus subtilis* is isolated for screening of surfactin production. The growth parameters such as size of the inoculum, period of inoculation, concentration of glucose substrate were optimized for better yield of surfactin. Thus the microbial synthesized surfactin is screened for surfactant activity by emulsification test, oil displacement test, drop collapsing test and chemically characterized by thin layer chromatography. The stability of the surfactin was determined by various parameters (temperature, pH and salinity). A better yield of 1.8g/l surfactin was obtained, when the glucose concentration of 40g/l were used along with 2% v/v size of inoculum at 72 hours of incubation. The surfactin activity was found to be high for diesel when compared to petrol. The chemical characterization of surfactin revealed red spots with retention factor of 0.53. The surfactin thus obtained was found to be thermo stable and active at the pH range of 7 to 9 and upto the salt concentration of 10% w/v. Thus the surfactin obtained can be used in various industries including petroleum, food, pharmaceuticals, cosmetics etc.

KEYWORDS

Surfactin;
Emulsification test;
Oil displacement test;
Drop collapsing test;
TLC.

INTRODUCTION

Naturally occurring surface-active compounds derived from microorganisms, also called biosurfactants (Microbial surface active agents), are attracting attention in recent years because they offer several advantages over chemical surfactants, such as low toxicity, inherent good biodegradability, ecological acceptability, selectivity, specific activity at extreme temperatures, pH and salinity, the possibility of their production

through fermentation, their potential applications in environmental protection.

Research in the area of biosurfactants has expanded quite a lot in recent years due to its potential use in different areas, such as the food industry, agriculture, pharmaceutics, the oil industry, petro chemistry and the paper and pulp industry amongst others. In petroleum industry surfactants are used in enhanced oil recovery process from oil fields. In food and pharmaceuticals it is utilized as emulsifiers, foaming agents, solubilizers, wetting agents, cleansers in formulation of cream, tablets and gels. Cosmetic products using surfactants includes insect repellents, antacids, contact lens solution, hair colours and care products, deodorants, nail care, lipsticks, lip makers, eye shades, soap, tooth pastes and polishes, denture cleansers, adhesives, baby products, shampoos, conditioners, shampoos, conditioners, shave and depilatory products, moisturizers, health and beauty pro-ducts.

Even though interest in biosurfactants is increasing, these compounds do not compete economically with synthetic surfactants. To reduce production costs, different routes could be investigated such as the increase of yields and product accumulation; the development of economical engineering processes, and the use of cost-free or cost-credit feedstock for microorganism growth and surfactant production. The choice of inexpensive raw materials is important to the overall economy of the process because they account for 50% of the final product cost and also reduce the expenses with wastes treatment.

Surfactin is a very powerful surfactant produced by the Gram-positive endospore-forming bacteria Bacillus subtilis and it is lipoprotein in nature. In our study the production optimization involving varying substrate concentration, size of inoculum and incubation time are examined for better yield of surfactin. Glucose is used as sole carbon source which is a cost effective and also easily available source. The surfactin activity was studied by various test which includes Emulsification test, Oil displacement test, Drop collapsing test and chemically characterized by Thin layer chromatography. Finally the stability of surfactin was determined from Emulsification test at various temperature, pH and salt concentration.

MATERIALS AND METHODS

Microorganism and culture conditions

Oil contaminated soil samples are collected near the petrochemical industry in north Chennai, with a clean spatula. The soil was then diluted and plated on nutrient agar. Staining techniques and biochemical test (Bergey's manual of microbiology) are carried out with reference to the strain ATCC 21332 to isolate the pure culture of *Bacillus subtilis*.

The basal mineral salt medium of composition (g/l) KH_2Po_4 - 0.5, K_2HPo_4 - 1, Kcl - 0.1, $MgSo_4.H_2O$ - 0.5, Yeast Extract - 0.1, $cacl_2$ - 0.02 supplemented with trace elements solution was used in this study. Glucose at various concentration (10,20,30,40,50) g/l was used as carbon source and urea 1g/l is used as nitrogen source along with basal mineral salt medium.

The medium is inoculated with *Bacillus subtilis* of different inoculum size (1%,2%,3%,4%,5%) and incubation time (24h, 48h,72h,96h) were studied.

Extraction and purification of surfactin

Each culture were centrifuged at 8000 rpm, 4^0 c for 10 minutes to harvest the cells. The culture supernatant was taken. pH of the culture supernatant was lowered to 2 with 5 M Hcl and incubated at 4^0 c for 24 hours. The precipitate was separated by centrifugation at 8000 rpm for 20 minutes. This white precipitate formed culture was taken. The precipitate was then extracted with chloroform-ethanol (2:1), kept un disturbed for 1 hour it is then concentrated by evaporation at room temperature to obtain a biosurfactant concentrate. Following the evaporation, the biosurfactant concentrate is dissolved in 25ml of 0.05M bicarbonate (pH 8.6) to carry out screening process.

Biomass and protein estimation

For biomass determination, the samples were centrifuged (10,000*g) for 15 min at 4^0 c. The pellet was dried at 105^0 c for 24h. Protein concentration of the sample was determined by Bradford method, using bovine serum albumin as blank

Activity characterization

(a) Emulsification activity (E_{24})

Equal volumes of oil and surfactin in test tube were

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vortexed at high speed for 2 min and allowed to stand for 24h. Petrol and Diesel are the two oils studied in this test. The E_{24} index is calculated as

E_{24} = (Height of emulsion formed / Total height of solution) X 100

(b) Oil displacement test (ODT)

The 50ml of distilled water was added to a large Petri dish, then 20µl of crude oil is then added to the surface of water and 10µl of culture supernatant broth is then poured in center of the oil film. Zone of displacement is visualized and measured.

(c) Drop collapsing test

Two microliter of crude oil was added to each well of plate lid. The lid was equilibrated for 1 hour at room temperature and then 5 microliter of the surfactin was added to surface of oil. The shape of the drop on the drop on the oil surface was inspected after 1 minute. Biosurfactant producing cultures giving flat drops were taken as positive. Those cultures that have rounded drop were taken as negative indicating the lack of surfactin activity.

Chemical characterization by thin layer chromatography

A thin uniform layer of stationary phase (silica gel) was made on a glass plate. The plate was air dried for 15 minutes and then over-dried for 10-15 minutes and 100^0C. 20 microliter of surfactin were spotted on a line drawn about 1.5-2.0 cm from the bottom. The TLC plate was plated gently in a mobile phase {CH_3Cl_3: Ch_3OH: H_2O (70: 10: 0.5 v/v)} contained in a chromatographic tank and allowed for solvent development, then the plate was removed and air dried. The plate was sprayed with the spraying reagent (ninhydrin) and treated at 100^0 C for 10 minutes. The retention factor was determined as,

$$R_f = \frac{\text{Dist moved by solute}}{\text{Dist moved by solvent}}$$

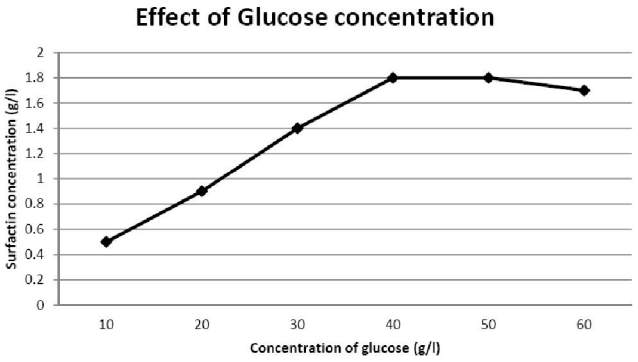
Stability test

The effect of temperature (10,20,30,40,50,60 ^{0}c), pH (4,5,6,7,8,9,10) and salt concentration (Nacl-5,10,15,20 %w/v) on the emulsification activity of the surfactin was examined.

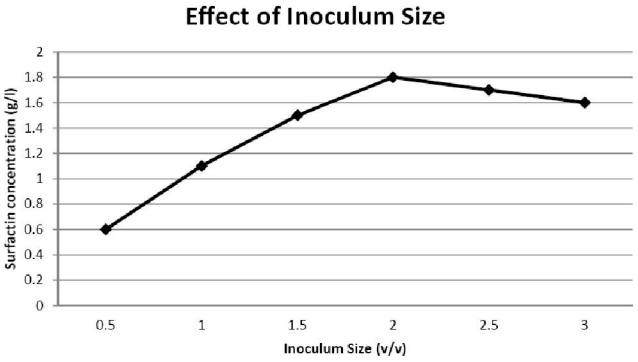
RESULTS AND DISCUSSION

Surfactin of high yield can be produced at optimized conditions from *Bacillus subtilis,* when glucose is used as a sole carbon source.

The surfactin production can be optimized to obtain maximum yield from Bacillus subtilis species. The maximum yield of 1.8 g/l of surfactin were produced, when 40 g/l of glucose was used as a carbon source with 2% v/v inoculum size and 72 hrs incubation time as shown in graph. This shows that the rate of production of surfactin increases as the substrate concentration increases upto optimum level (40 g/) after that the production decreases appropriately.



Graph 1 : Effect of glucose concentration on surfactin production

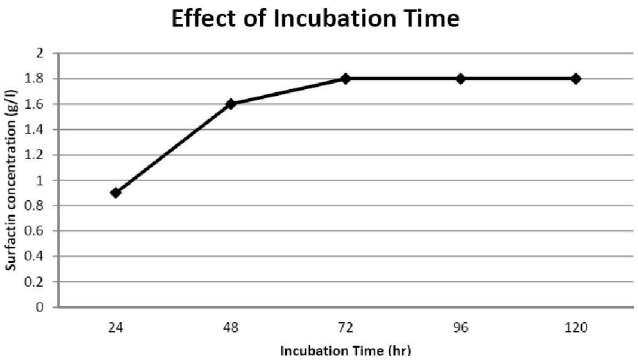


Graph 2 : Effect of inoculum size on surfactin production

At the maximum production condition, the biomass concentration was found to be 0.38g/g of glucose and protein concentration by Bradford method was 25.1 mg/g of glucose.

The emulsification activity of surfactin over petrol and diesel was found to be 54% and 58% which shows that the sufactin has more reactivity and emulsifying power towards diesel compared to petrol.

The zone of displacement by surfactin for petrol and diesel was 14mm and 17mm revealing that the die-

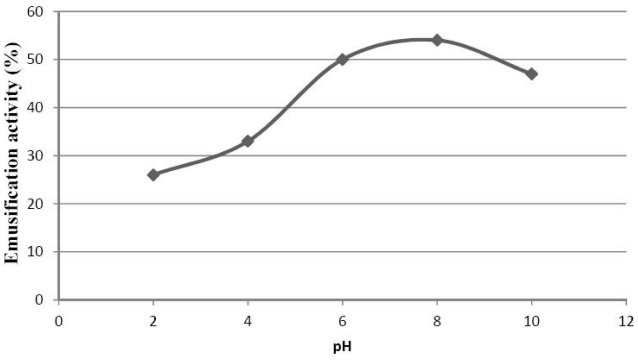


Graph 3 : Effect of incubation time concentration on surfactin production

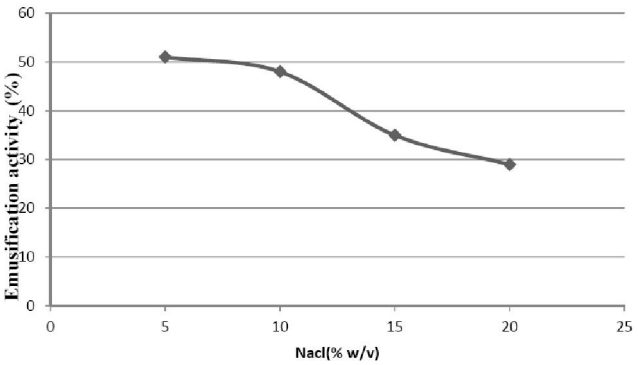
sel has high zone formation and high surfactant activity as shown in TABLE 1.

TABLE 1 : Activity of surfactin over petrol and diesel

Oil sample	E_{24} (%)	ODT (mm)
Petrol	54	14
Diesel	58	17



Graph 4 : Effect of pH on emulsification activity



Graph 5 : Effect of Salt on surfactin activity

Flat drops are observed when surfactin was placed on the oil coated surface which shows positive surfactant activity.

Chemical characterization of surfactin by Thin layer chromatography revealed with red spots for surfactin and R_f value was found to be 0.53.

Emulsification activity of surfactin was not affected by the gradient of temperature of 4°C-100°C, which shows it is thermostable. Surfactin in 50 mMTris-HCl at pH 7-9 exhibited best emulsification activity, while a little change in the pH has significant effect on the surfactin activity as shown in the graph. The addition of Nacl upto 10%w/v does not have high impact on surfactin activity.

Based on the experimental results, it can be concluded that the maximum surfactin production can be achieved by using glucose as a cheap and sole carbon source at the optimized condition of 40g/l glucose, 2% v/v inoculum at 72 hours of incubation. The activity characterization f surfactin shows high emulsifying and oil displacement property which can be used in enhanced oil recovery in petroleum industry and other fields such as food, cosmetics, pharmaceuticals etc.

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