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Advances in Applied Science Research, 2014, 5(6): 159-164

Pelagia Research Library

ISSN: 0976-8610
CODEN (USA): AASRFC

Effect of carbon and nitrogen source on biosurfactant production by biosurfactant producing bacteria isolated from petroleum contaminated site

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ABSTRACT

Biosurfactant reduces the surface tension of both aqueous solutions and hydrocarbon mixtures. In this study, isolation and identification of biosurfactant producing strain were assessed. Soil samples from petrol pumps in Kalyan and Dombivli area was collected and 36 strains were isolated. Emulsification assay, E_{24} test and surface tension measurement tests were performed to confirm the ability of isolates to produce biosurfactant, Among water soluble and insoluble carbon source sodium acetate and diesel were found to be the best substrate for production of biosurfactant. Ammonium nitrate supported as best nitrogen source. The TLC analysis revealed that isolate no. 10 (Bacillus sp) biosurfactant component was lipoprotein in nature. The biosurfactant efficiently emulsified hydrocarbons like kerosene, petrol, hexadecane and diesel. This ability of the isolate can be utilized for cleaning up of hydrocarbon contaminated sites.

Key words: Biosurfactant, emulsification, lipopeptide, surface tension, TLC

INTRODUCTION

Biosurfactants are biological amphiphilic compounds consisting of hydrophilic and hydrophobic domains. The hydrophilic domain can be carbohydrate, amino acid, phosphate group or some other compounds whereas the hydrophobic domain usually is a long chain fatty acid [1]. The majority of known biosurfactants are synthesized by microorganisms grown on water immisible hydrocarbons, but some have been produced on water soluble substrate such as glucose, glycerol and ethanol [2]. Microorganisms have been reported to produce several classes of biosurfactants such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants [3, 4, 5]. A number of studies have reported the potential of *Bacillus* species as biosurfactant producers and they produce lipopeptide type of biosurfactant [6]. Lipopeptides represent a class of microbial surfactant with remarkable surface properties and biological activities, such as surplus crude oil recovery, food-processing, de-emulsification, antimicrobial and antitumor, antiviral, antiadhesive activities, etc. [7,8] . Production of an effective lipopeptide type biosurfactant, surfactin, was first reported for a strain of *Bacillus subtilis* [9]. *Bacillus sp.* are the best known bacteria capable of utilizing hydrocarbons as carbon and energy sources for producing biosurfactants which enhance the uptake of immisible hydrophobic compounds [10,11,12,13]. Chemically synthesized surfactants have been used in the oil industry to aid clean up of oil spills as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment. Biosurfactant have special advantage over their commercially manufactured counterparts because of their lower toxicity, biodegradable nature and effectiveness at extreme temperature, pH, salinity and ease of synthesis [7, 14].

This study describes the isolation and screening of biosurfactant producing microorganism from petrol contaminated sites, the effect of various carbon and nitrogen source on growth and biosurfactant production along with surface tension reduction and its emulsification properties.

MATERIALS AND METHODS

Isolation and screening of biosurfactant producing bacteria

Biosurfactant producing bacteria were isolated by successive enrichment culture technique from the petroleum contaminated soil using mineral salt medium. The mineral salt media used consist of (g/L^{-1}): KNO3 (0.3 %), Na2HPO4 (0.22 %), KH2PO4 (0.014 %), NaCl (0.001 %), MgSO4 (0.06 %), CaCl2 (0.004 %), FeSO4 (0.002 %) and 0.1 ml of trace element solution containing (g/L): 2.32 g ZnSO4·7H2O, 1.78 g MnSO4·4H2O, 0.56 g H3BO3, 1.0 g CuSO4·5H2O, 0.39 g Na2MoO4·2H2O, 0.42 g CoCl2·6H2O, 1.0 g EDTA, 0.004 g NiCl2·6H2O and 0.66 g KI [14] and 1% crude oil as carbon source. The isolation was done on solidified mineral salt medium where oil was introduced in vapour phase transfer technique as described by Raymond *et al.*, [15]. Incubation was carried out at room temperature for 5 days. The isolates obtained were grown in liquid medium with diesel (1% w/v) for 5 days on shaker at room temperature. The cell free broth obtained after centrifugation (10,000 rpm for 30 min) was then studied for surfactant property by oil emulsification assay and $E_{24\%}$.

Determination of Emulsification activity

2.0 ml cell free supernatant obtained after removal of cells by centrifugation at 10,000 rpm for 30 min were added to a screw capped tube containing 15 ml of 0.2 M Tris buffer (pH 8.0) and 0.2 ml diesel. After vigorous agitation on vortex for 5 min, tubes were allowed to stand for 30 min. Absorbance was measured at 540 nm. Emulsification activity was defined as the measured optical density [16] .

Emulsification Index ($E_{24\%}$)

$E_{24\%}$ of cell free supernatant was determined by adding 2 ml of fuel oil to the same amount of cell free supernatant, mixing with a vortex for 2 min, and leaving it undisturbed for 24 hr. The E_{24} index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) [17] .

Surface tension

The surface tension of the cell free supernatant was measured by KRUSS Processor Tensiometer K-12.

Identification of best biosurfactant producer

The bacterial isolate in this work was selected amongst other bacteria because of its higher emulsification and better surface tension reducing ability. The strain was examined on the basis of morphological, cultural and biochemical characteristics as described by Holt *et al.*, [18].

Extraction of biosurfactant

Biosurfactant in cell-free broth was precipitated by adjusting the pH of the broth to 2.0 using 6 N HCl and holding the solution at 4^0C overnight. The precipitate thus obtained was pelleted at 12,000 rpm for 20 min, redissolved in distill water adjusted to pH 7, freeze dried and weighed. The further purification was done by extracting the freeze dried acid precipitate with chloroform and methanol (65:15). The extract was then evaporated by rotator evaporator under vaccum [19].

Analysis of component of biosurfactant by Thin layer Chromatography

The biosurfactant extract was separated by TLC using aluminium sheets silica gel plates with chloroform: methanol: acetic acid (65:15:2) as solvent system. Ninhydrin reagent (0.5 g ninhydrin in 100 ml anhydrous acetone) was used to detect lipopeptide biosurfactant and anthrone reagent (1 g anthrone in 5 ml sulphuric acid mixed with 95 ml ethanol) to detect glycolipid biosurfactant.

Effect of carbon sources on growth and surface tension

The effect of water soluble carbon sources like glucose, xylose, ethanol and sodium acetate was investigated. Similarly among the water insoluble carbon source petrol, diesel, hexadecane and kerosene were used by adding 2% of soluble and insoluble carbon source to mineral salt medium. The incubation was carried out on shaker at room temperature for 5 days.

Effect of nitrogen sources on growth and surface tension

The effect of nitrogen sources on growth and biosurfactant production was investigated by adding 1% of NH_4NO_3, $NaNO_3$, urea and peptone to mineral salt medium. The incubation was carried out on shaker at room temperature for 5 days.

Emulsification properties of biosurfactant

The emulsification index (E_{24}) of biosurfactant using different hydrocarbons like kerosene, petrol, diesel and hexadecane was tested.

Table 1: Screening the ability of several strains to produce biosurfactant

Isolate	Emulsification activity (OD at 540 nm)	E_{24} (%)
1	0.11	31.2
2	0.19	42.4
3	0.08	31.3
4	0.16	40.2
5	0.18	43.6
6	0.12	34.0
7	0.10	31.0
8	0.13	32.6
9	0.12	39.0
10	0.54	52.5
11	0.09	34.0
12	0.10	33.0
13	0.12	40.8
14	0.13	43.9
15	0.10	35.5
16	0.12	38.5
17	0.18	39.0
18	0.15	36.8

Table 2: Screening the ability of isolates to reduce surface tension

Isolate	Surface tension (mN/m)
2	38
4	38.9
5	39.4
6	38
8	38.8
9	39.6
10	29.85
13	37.5
14	38
16	39.4
17	38.8
18	37.0

Table 3: Emulsification activity of the produced biosurfactant

Hydrocarbon	Emulsification index	Emulsification activity
Kerosene	68.14	0.61
Petrol	51.42	0.33
Diesel	45.71	0.24
Hexadecane	42.34	0.21

RESULTS AND DISCUSSION

The biosurfactant screening procedure resulted in isolation of 36 bacterial isolates that could grow in presence of crude oil as the only source of carbon. Among them, 18 isolates showed emulsification activity. Selected strains were further screened by performing E_{24} test (Table-1). Of them, 12 showed the ability to reduce surface tension of culture broth below 40 mN/m (Table-2). The isolate no. 10 reduced the surface tension to the lowest. Based on morphology, cultural characteristics and biochemical tests the isolate no.10 was identified as *Bacillus sp.* The growth measured in terms of dry cell weight and reduction in surface tension using tensiometer showed sodium

acetate as the best carbon source. This was followed by ethanol that gave highest biomass yield. Among immisible carbon sources kerosene gave highest biomass as well as better reduction in surface tension as compared to petrol and diesel (Fig-1). Medium concentration other than carbon sources also affect the production of biosurfactant. Among the inorganic salts tested ammonium nitrate was the preferred nitrogen source for growth as well as reduction in surface tension (Fig-2).

Fig-1 Effect of Carbon source on biosurfactant production

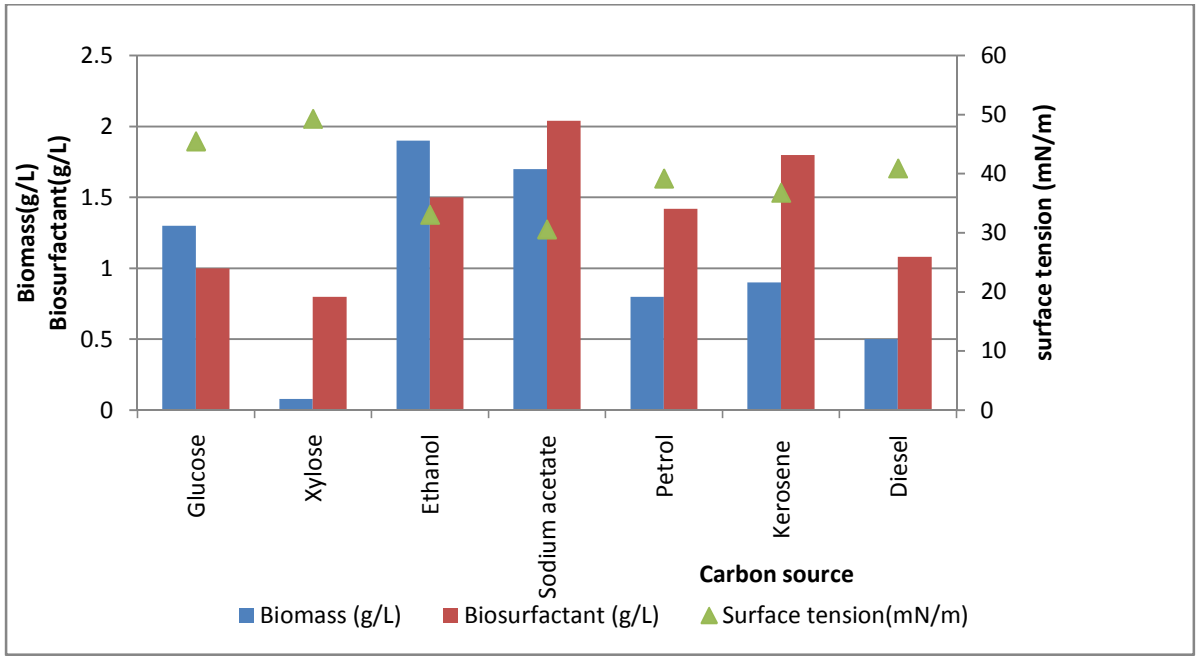
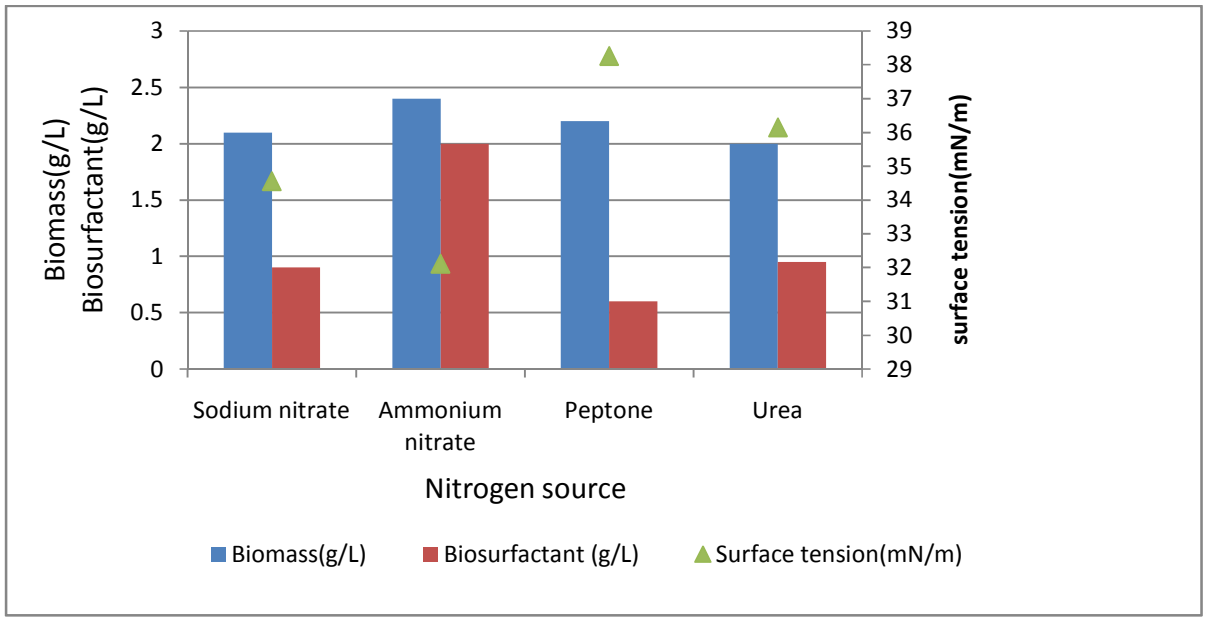


Fig-2 Effect of Nitrogen source on biosurfactant production



The produced compound was purified with acid precipitation and solvent extraction. Thin layer chromatography on silica gel plate showed purple spot when developed with ninhydrin reagent indicating that the biosurfactant is a

lipopeptide. The emulsification index ($E_{24\%}$) and emulsification activity of the produced biosurfactant was tested with different hydrocarbon and all the hydrocarbons were emulsified efficiently (Table-3). In order to increase their interfacial area for improved uptake of insoluble substrates most of the hydrocarbon degrading microorganisms have been reported to produce emulsifying agents. In this study, soil continuously exposed to petroleum oil served as an excellent source for isolation of biosurfactant producers. Biosurfactant producing bacteria are found in higher concentration in hydrocarbon contaminated sites. These surface active agent increase the bioavailability of hydrocarbons to the microbial cells by increasing the area at the aqueous hydrocarbon interface. This increases the rate of hydrocarbon dissolution and their utilization by microorganisms [20]. Amongst the thirty six isolates obtained, isolate no.10 was selected because of its better emulsification activity and reduction in surface tension over the others. This isolate produced biosurfactant when grown in presence of 2% kerosene as well as 2% sodium acetate. The surface tension reduction ability of the biosurfactant was found to be 29.85 mNm^{-1}. Surface tension reduction is the choice of method to quantify biosurfactant production. Reduction of surface tension was indicative to the production of biosurfactant by the microbe. Measuring the reduction in surface tension by isolated bacteria from Kalyan region petrol pump indicates the production of surface-active compounds. Similar results obtained by Banat *et al*., [21]. They isolated several bacteria which showed the ability to reduce culture broth surface tension below 40 mN/m. Earlier studies [22,23] focused primarily on surface tension reduction analysis for the characterization of biosurfactant and have not reported the emulsification activity of these products. Here we show that biosurfactant produced by *Bacillus sp* is good in both surface tension reduction and emulsifying activity. The majority of known biosurfactant are synthesized by microorganisms grown on water immisible hydrocarbons [24], but there are many reports of biosurfactant production on water- soluble substrates such as glucose, glycerol and ethanol [25] . In the present study we found sodium acetate a water soluble substrate as a better carbon source for growth as well as for reduction in surface tension over the water immisible substrates. Nitrogen plays an important role in the production of surface-active compounds by microorganisms. *Arthrobacter paraffineus* ATCC 19558 preferred ammonium to nitrate for biosurfactant production. Similar to our finding, Ilori *et al*.,[26], found that for production of biosurfactant by *Aeromonas* sp ammonium nitrate was preferred. Syldatk *et al*., [27], showed that nitrogen limitation increased the production of some biosurfactants but also changed the composition of the biosurfactants. Many researchers have reported that biosurfactant possess the ability to emulsify hydrocarbons [28,16, 29]. Similarly the biosurfactant produced by isolate no.10 could form stable emulsion with various hydrocarbons.Thus the ability of isolate no.10 (*Bacillus sp*) to produce biosurfactant with efficient emulsification properties suggests its potential application in bioremediation procedures.

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