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Isolation and identification of *Bacillus licheniformis* for biosurfactant production

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ABSTRACT

Biosurfactant is a structurally diverse group of surface-active molecule, Synthesized by microorganisms. They have capability of reducing surface and interfacial tension with low toxicity and high specificity and biodegradability. In the study, the soil sample were collected from the oil spilled areas of the automobile workshop and petrol pump. The sample was enriched in MSM agar incorporated with 2% Glucose. The organism was screened for the purpose of biosurfactant production by using the screening tests viz. oil spreading test, blood haemolysis test and emulsification test. The screened organism was identified by GC-FAME analysis using SHERLOCK MIDI software. The organism identified was *Bacillus licheniformis*. The biosurfactants were then extracted by centrifugation, supernatant obtained then added with chloroform: methanol, then sediment was obtained as result. The dry weight of the biosurfactant produced was calculated as 0.12g. The growth of *B.licheniformis* was tested in presence of hydrocarbon (toluene).

KEYWORDS

Bacillus licheniformis;
GC-FAME;
Biosurfactant;
Oil-spreading test.

INTRODUCTION

Surfactants a short form for "surface-active-agents", are basically chemical compounds which lower the surface tension of a liquid, the interfacial tension between two liquids, or that between a liquid and a solid. These surfactants are produced by a variety of microorganisms such as yeast, bacteria and filamentous fungi and thus are called Biosurfactants. Biosurfactants have differentproperties such as they act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants. These are usually organic compounds that are amphiphilic in nature, which contains both the hydrophobic and hydrophilic component. The hydrophobic (non-polar) part of the biosurfactant is insoluble in water and may have a long-chain of fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxy fatty acids. The hydrophilic (polar) end can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol[1].

The biosurfactants are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides etc. Biosurfactants lead to an increasing interest on these microbial products as alternatives to chemical surfactant. There are numbers of reports on the synthesis of various types of biosurfactants by microorganisms using water-soluble compounds such as glucose, sucrose, ethanol or glycerol as substrates[2].

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The properties of Biosurfactant allow their use and possible replacement of chemical surfactant in number of industrial application to their low concentration, easily production, the ability to synthesized renewable resources, higher foaming, specific activity at extreme temperature, pH and salinity their potential application in environmental protection and management. Biosurfactant reduce surface tension, critical micelle concentration (CMC) and interfacial tension in both aqueous and hydrocarbon mixture[3].

Bacillus licheniformis is a rod-shaped, Gram-positive bacterium. Its optimal growth temperature is around 30°C, though it can survive at much higher temperatures. It tends to form spores in soil which makes it desirable to be used for the industrial purposes such as the production of enzymes, antibiotics, and small metabolites[4].

The present study is focused on the isolation and identification of biosurfactant producer. The screening of the biosurfactant producer was done by oil-spreading test, emulsification test and blood haemolysis test. The screened bacteria was identified by GC-FAME analysis as *Bacillus licheniformis.*

MATERIALS AND METHOD

Sample collection

For the isolation of biosurfactant producing bacteria, the sample was collected from soil near automobile garage and petrol pump, where the oil is spilled in the soil. The soil sample was collected in a sterile polythene and stored at low temperature till use.

Isolation and enumeration of bacterial isolates from the sample

5 gram of the soil sample was inoculated in 50ml of MSM broth incorporated with 2% Glucose and incubated at 30^0C for 72 hours. After incubation this medium was serially diluted from 10^{-1} to 10^{-6} in sterile water. From the dilutions 10^{-1} to 10^{-6}, lml was transferred to sterile 20ml of MSM agar and was poured in plates. The plates were then inverted and incubated at 30^0C, for 48 hrs. After incubation morphologically different colonies were selected for further analysis.

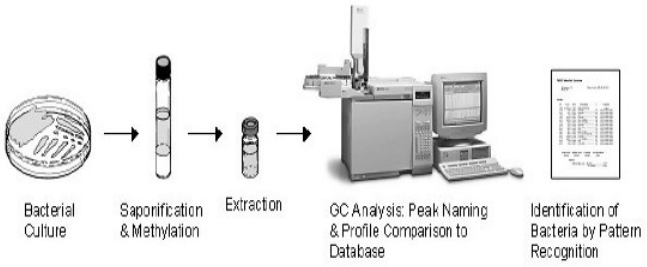

Figure 1 : Schematic diagram of FAME analysis

Screening of biosurfactant producing organisms

The isolated colonies were tested for their biosurfactant production by three methods.

1 Oil Spreading Technique
2 Blood haemolysis test
3 Emulsification stability test

Oil spreading technique

30ml of sterile distilled water was taken in the Petri Plates.1 ml of coconut oil was added to the centre of the plate containing distilled water. Then added 20µl of the supernatant (isolated culture) to the centre of Petri dish. The Plate were kept in static condition for 2-4 hrs. The biosurfactant producing organism can displace the oil and spread in the water.

Blood haemolysis test

The fresh single colonies were taken from MSM agar plate and streaked on Blood agar plates. The plates were incubated for 48-72 hours at 30^0C. The bacterial colonies observed for the presence of clear zone around the colonies. This clear zone indicates the presence of biosurfactant producing organisms.

Emulsification stability test [E24]

E24 of culture was determined by adding 2 ml of Oil to the same amount of culture, mixed with a vortex for 2 min and left to stand for 24 hours. The E24 index is given as percentage of height of emulsified layer (cm) divided by total height of the liquid column (cm).

$$E^{24} = \frac{hemulsion}{h\,total} x100\%$$

Identification of Bacteria by Gram's staining and FAME Analysis

The screened biosurfactant producer was characterized by Gram's staining and FAME analysis. For GC FAME analysis, the fatty acids were extracted by a procedure described in MIDI Sherlock Identification System Manual. The procedures consist of Saponification in dilute sodium hydroxide/methanol solution to give respective fatty acid methyl esters (FAME). The FAMEs were than extracted from the aqueous phase

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by the use of an organic solvent and the resulting extract was analysed by gas chromatography. The sample prepared were analysed by midi Sherlock microbial identification system.

Growth of bacteria in presence of hydrocarbons

1% hydrocarbon was spread on MSM agar plate and after its absorbtion the screened bacteria was spread on the plate. The plates were incubated at 30 ^{0}C for 48hrs and observed for growth.

Extraction of biosurfactants

The culture was inoculated in 50 ml of MSM broth with 1 ml of petrol. The culture was incubated at 25^{0}C for 7 days with shaking condition. After incubation the bacterial cells were removed by centrifugation at 5000rpm, 4^{0}C for 20 minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2, using 1N H_2SO_4. Now add equal volume of chloroform: methanol (2:1). This mixture was shaken well for mixing and left overnight for evaporation. White colored sediment was obtained as a result i.e. the "Biosurfactants"

Dry weight of biosurfactants

Sterile petriplate was taken and the weight of the plate was measured. Now the sediment was poured on the plates. They were placed in the hot air oven for drying at 100^{0}C for 30 minutes. After drying the plates were weighed again. The dry weight of the biosurfactants was calculated by the following formula:

Dry weight of Biosurfactant = Weight of the plate after drying – Weight of the empty plates.

RESULTS AND DISCUSSION

Morphologically different colonies were selected for screening of biosurfactant production. The colony characters of the screened bacteria are as follows (TABLE 1):

TABLE 1 : Colony characteristics of the biosurfactant producer

Size	shape	Elevation	Margin	Pigmentation	Gram Staining
Medium	Irregular	Entire	lobate	White	Gram positive

Screening of biosurfactant producing organism

Oil displacement test

For Oil spreading test, the culture was centrifuged and supernatant was added in to oil containing Plate. The Biosurfactant producing organism would displace oil and form a clear zone in the centre of the plate which indicates the ability of isolated organism to displace the oil (Figure 2).

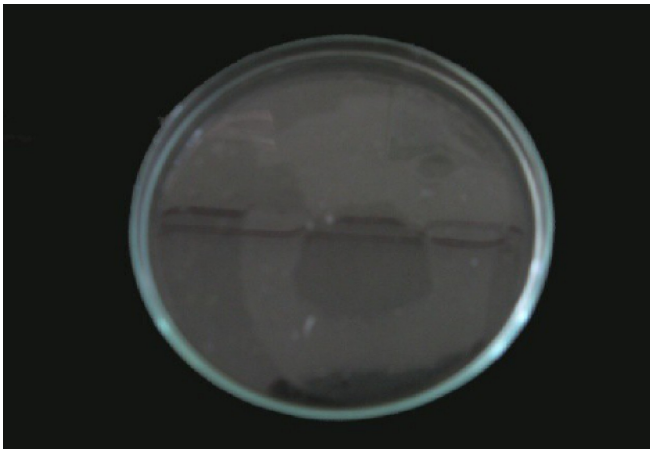

Figure 2 : Oil spreading test

B. Anandaraj et al in 2010[5] reported his five isolates obtained from soil were found to produce the biosurfactant. This was screened by the oil displacement method. The culture supernatant displaces oil by 5mm. The oil displacement method was also followed by[6] Kingsley Urum et al. in 2004 for the screening of biosurfactant producing microorganisms.

Blood haemolysis test

To test Blood Haemolysis, streak the microorganism on to the blood agar plate and after incubation check for clear zone around the growth, this clear zone indicates the presence of Biosurfactant production

Figure 3 : Blood haemolysis test

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(Figure 3).

Rashedi, *et al*., 2005[7] used blood haemolysis test for screening biosurfactant producing organism. The culture also showed haemolytic activity on blood agar plate. The culture showing haemolysis was able to produce biosurfactant. Bicca *et al*. In 1999[8] performed Blood Haemolysis test for Bacillus sp.

Emulsification stability test

By performing emulsification test the emulsifying

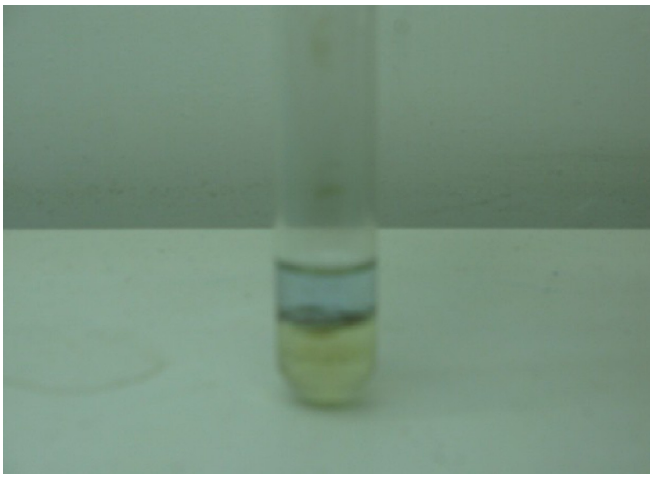


Figure 4 : Emulsification stability test

TABLE 2 : Result of emulsification test

Sr. No.	Length for 24 hrs	Length for 48 hrs	Result 24 hrs	Result 48 hrs
1. Length of mixture	2 cm	2cm	70%	76.5%
2. Length of Emulsified layer	1.4cm	1.53cm		

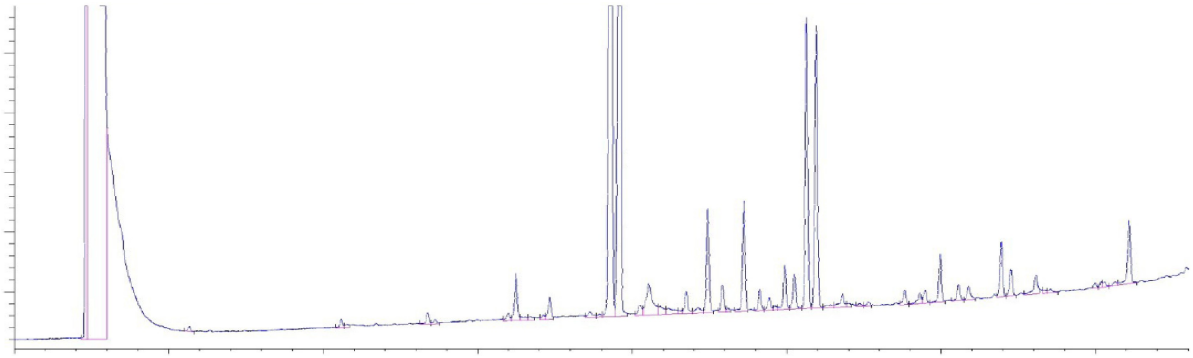
ability of microorganism for kerosene is checked. The microorganism has the ability to emulsifying the kerosene (Figure 4). The *Bacillus licheniformis* has Emulsification index of 70-80% (TABLE 2).

A. Tabatabaee *et al* in 2005[9] had performed Emulsification test for *Bacillus licheniformis*. Their 23 Strains of *Bacillus Licheniformis* had 70% Emulsification Ability. Screening of Microbial isolates was performed in order to check the abilities of crude oil Emulsifying Bacteria.

D. J. Mukesh Kumar *et al* 2012[10] reported the emulsification indices are differing based on the nature of the surfactant compound and source of the organisms. The emulsification activity of the biosurfactant produced from marine *Bacillus* spp. was tested with different hydrocarbons showed that the highest index was shown in corn oil followed by kerosene and sunflower oil.

Result of FAME analysis

Sim Index	Entry Name
0.430	**Bacillus-licheniformis (Bacillus subtilis group)**
0.398	Bacillus-subtilis
0.398	Staphylococcus-chromogenes
0.291	Bacillus-megaterium-GC subgroup A
0.289	Bacillus-pumilus-GC subgroup B
0.271	Staphylococcus-schleiferi
0.268	Bacillus-pumilus-GC subgroup A



Growth of bacteria in presence of hydrocarbons

Colonies of *Bacillus licheniformis* were observed on MSM agar plate with 1% hydrocarbon (Toluene) (Figure 5).

Extraction

In extraction process white coloured sediments had been observed in separating funnel which indicates the extraction of Biosurfactants (Figure 6).

Dry weight of biosurfactant

The dry weight of biosurfactant was calculated as 0.12g per 100 ml of production media. The similar study was reported by Anandaraj B[5].

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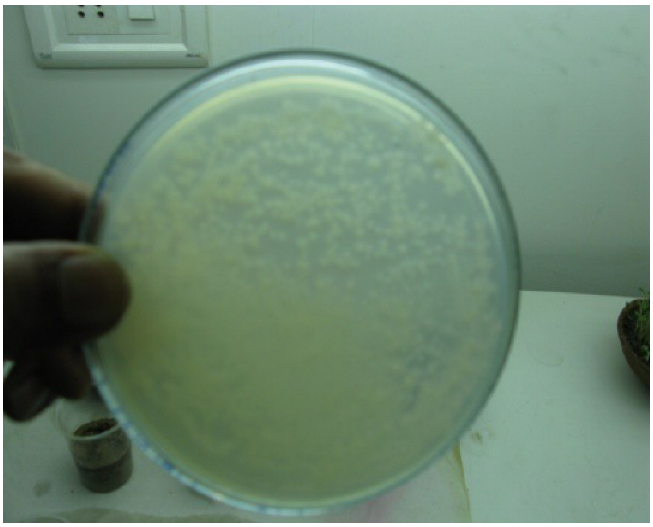

Figure 5 : Growth of *Bacillus licheniformis* on MSM agar plate + 1% Hydrocarbon (Toluene)

Figure 6 : Extraction of biosurfactant

TABLE 2a : Dry weight of biosurfactant

Weight of the empty Plate	Weight of Plate after drying	Dry weight of Bio surfactant
117.64 g	117.76 g	0.12 g

CONCLUSION

The identification of Bio surfactant producing organism was done by Gram's staining and GC-FAME analysis. The bacteria *Bacillus licheniformis* showed the growth in presence of hydrocarbon such as toluene.

The screening for the presence of Biosurfactant was done successfully by Oil displacement test, Blood Haemolysis test and Emulsification test. Clear zone was obtained during the oil displacement test and also clear zone around the growth of culture was observed in Blood agar plate. 70 and 75% of emulsification index was measured after 24 and 48hrs of emulsification activity respectively. The biosurfactant was extracted from the culture broth and dry weight was obtained as 0.12g per 100ml of the production media.

The bacteria *B.licheniformis* could be further studied for large scale biosurfactant production and field application.

REFERENCES

[1] R.C.Jaysre, S.Basu, P.Singh, T.Ghosal, P.Patra, Y.Keerthi, Rajendran; Isolation of Biosurfactant Producing Bacteria from Environmental sample. Pharmacologyonline, **3**, 1427-1433 **(2011)**.

[2] J.M.Ghayyomi, F.Forghani; Biosurfactant Production by Bacillus sp.Isolated from Petroleum Contaminated Soil of Sirri Island. American Journal of Applied Science, **9**, 1 **(2012)**.

[3] T.Priya, Usharani; Comparative Study for Biosurfactant Production by Using Bacillus subtilis and Pseudomanas aeruginosa. Botany Research International, **2(4)**, 284-287 **(2009)**.

[4] J.Thaniyavaran, N.Roongswang, T.Kameyama, M.Haruki, T.Imanaka; Production and characterization of Biosurfactants from Bacillus licheniformis F2.2. Biosci.Biotechnol.Biochem, **67(6)**, 1239-1244 **(2003)**.

[5] B.Anandaraj, P.Thivakaran; Isolation and Production of Biosurfactant Producing Organism from Oil Spilled Soil. J.Biosci.Tech, **1(3)**, 120-126 **(2010)**.

[6] U.Kigsley, T.Pekdemir; Evaluation of biosurfactants for crude oil contaminated soil washing. Chemosphere, **57**, 1139-1150 **(2004)**.

[7] H.Rashedi, M.M.Assadi, B.Bonakdarpour, E.Jamshidi; Environmental importance of rhamnolipid production from molasses as a carbon source. Int.J.Environ.Sci.Tech., **2**, 59-62 **(2005)**.

[8] F.C.Bicca, L.C.Fleck, M.A.Zachio; Production of biosurfactant by hydrocarbon degrading Rhodococcus rubber and Rhodococcus erythropolis. Rev.Microbiol., **30**, 231-236 **(1999)**.

[9] A.Tabatabaee, A.Mazaheri, A.Noohi, V.A.Sajadian; Isolation of Biosurfactant Producing Bacteria from Oil Reservoirs Iranian. J.Env.Health Sci.Eng., **2**, 6-12 **(2005)**.

[10] D.J.Mukesh Kumar, P.T.Kalaichelvan, C.R.Suresh Chander, T.Lohitnath; Production and characterization of biosurfactant from bacillus subtilis MTCC441 and its evaluation to use as bioemulsifier for food biopreservative. Advances in Applied Science Research, **3(3)**, 1827-1831 **(2012)**.