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Research Article

ISOLATION, OPTIMIZATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCING BACTERIAL STRAIN FROM THE CONTAMINATED SOIL OBTAINED FROM ADYAR PETROL BUNK

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ABSTRACT

Naturally occurring microorganisms are having ability to produce the various enzymes and compounds. Indeed, it is boon of god because nowadays most of these compounds are industrially important and human welfare. Biosurfactant is one of the important compounds involved in bioremediation, which can be produced by various microorganisms. Many species of microorganisms are known to produce biosurfactant by the by the utilization of hydrocarbon. The biosurfactant producing bacterial strain isolated from petrol bunk sample (ADYAR, Chennai). The isolated bacterial strain was identified as *Bacillus licheniformis*. In the production optimization studies, the bacterial strain needs temperature around 37°C,PH 7, glycerol as carbon source, incubation period of 36 hrs and initial glucose concentration of 3mM as optimum parameter for better production of biosurfactant. The biosurfactant produced by above organism was acid precipitated and extracted as partially purified one using ethyl acetate extraction technique. The extracted partially purified biosurfactant characterized through TLC and the presence of corresponding amino-acid (Glu, Asp, Val, Leu, Ile) was detected whose having Rf value of 0.83.When studied with application, biosurfactant inhibited the growth of *Bacillus subtilis* thus forming clear zone of lysis around the well. Moreover it seemed to having antibacterial effect against 5 gram positive bacteria and no effect against 2 gram negative bacteria tested. The systematic position of *Bacillus licheniformis* BIOS PTK1 was determined based on 16S rRNA sequence. BLAST homology analysis revealed that the sequence of *Bacillus licheniformis* BIOS PTK1 showed 100% sequence identity with more than 60 *Bacillus licheniformis.*

INTRODUCTION

Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface. They contain a hydrophobic moiety with little affinity for the bulk medium and a hydrophilic portion that is attracted to the bulk medium. Surfactants have been used industrially as adhesives, de-emulsifiers, flocculating, wetting and forming agents, lubricants and penetrants [1]

Because of their amphiphilic nature, surfactants tend to accumulate at interfaces (air-water and oil-water) and surfaces. As a result, surfactants reduce the forces of repulsion between unlike phases at interfaces or surfaces and allow the two phases to mix more easily [2]

Several microorganisms are known to synthesize surface-active agents; most of them are bacteria and yeasts. When grown on hydrocarbon substrate as the carbon source, these microorganisms synthesize a wide range of chemicals with surface activity, such as glycolipid, phospholipid, rhamnolipid, etc., .These chemicals are apparently synthesized to emulsify the hydrocarbon substrate and facilitate its transport into the cells. Biosurfactants are also involved in a group motility behavior called swarming motility. Generally the structure of biosurfactants includes a hydrophobic moiety composed of aminoacids or peptides, anions or cations or mono or di or polysaccharides. The hydrophobic portion is often made up of saturated, unsaturated or hydroxylated fatty acids or composed of amiphiphillic or hydrophobic peptides.

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Biosurfactant spontaneous release and function are often related to hydrocarbon uptake therefore they are predominantly synthesized by hydrocarbon degrading microorganisms .Some biosurfactants have been produced by an water soluble compounds such as glucose, sucrose, glycerol, or ethanol [3,4,5]. These compounds also have antibiotic properties which may serve to disrupt membrane of microorganisms competing for food.

Microbial surfactants can also be divided into two major classes according to their molecular-mass. The low molecular-mass biosurfactants include glycolipids such as rhamnolipids and sophorolipids, or lipopeptides like surfactin and polymyxin, has a function in lowering the surface and interfacial tensions. Whereas the high molecular-mass biosurfactants such as lipoproteins, lipopolysaccharides and amphipathic polysaccharides are more effective at stabilizing oil-in-water emulsions. Biosurfactants are classified mainly by their chemical composition and microbial origin. The major classes of biosurfactant include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants and particulate surfactants [6].

A biosurfactant must have the ability to improve water loss, which can wet the solid surfaces. Some of the biosurfactants also has the ability to act as an emulsifier. Unfortunately, many of the emulsifiers that were characterized were found to be polymeric, with minimal ability to lower surface tension (7).

Many biosurfactants and their surface activities are not affected by environmental conditions such as temperature and pH. McInerney reported that lichenysin from *B. licheniformis* JF-2 was not affected by temperature (upto 50°C), pH (4.5–9.0) and by NaCl and Ca concentrations up to 50 and 25 g/l respectively. A lipopeptide from *B. subtilis* LB5a was stable after autoclaving (121°C/20 min) and after 6 months at –18°C; the surface activity did not change from pH 5 to 11 and NaCl concentrations up to 20%.[8]

Biosurfactants may stabilize (emulsifiers) or destabilize (de-emulsifiers) the emulsion. High molecular- mass biosurfactants are in general better emulsifiers than low-molecular-mass biosurfactants. Sophorolipids from *T. bombicola* have been shown to reduce surface and interfacial tension, but are not good emulsifiers. By contrast, liposan does not reduce surface tension, but has been used successfully to emulsify edible oils. Polymeric surfactants offer additional advantages because they coat droplets of oil, thereby forming stable emulsions. This property is especially useful for making oil/water emulsions for cosmetics and food.

Biosurfactants (Microbial Surface Active Agents) have become recently an important product of biotechnology for industrial and medical applications. There as on for their popularity, as high value microbial products, is primarily in their specific action, low toxicity, relative ease of preparation and widespread applicability.

Recently, Multi-biotech (a subsidiary of Geodyne Technology) has commercialized biosurfactants for enhanced oil recovery applications. *Bacillus licheniformis* JF-2, an isolate from oilfield injection water which, in addition to producing the most effective biosurfactants, has other characteristics like being anaerobic, halotolerant, and thermo-tolerant, produces biosurfactants that are potentially useful for *in situ* microbially enhanced oil recovery. As such, the viscosity of heavy crude oil can be greatly reduced when it is treated with this biosurfactant. In addition, the use of a biosurfactant for desludging of a crude oil storage tank for Kuwait Oil Co., has been reported .So the present study was aimed to isolate biosurfactant producing bacteria from petrol bunk sample and screening for extracellular biosurfactant production.[9]

Sample collection

The sample were collected from car shed and petrol contaminated soil from petrol bunk, Adayar, Chennai. The samples were transferred in a sterile container and it was brought to the laboratory for further processing

Isolation of bacteria

The collected sample was serially diluted up to 10^{-9} dilutions using sterile saline as a blank and the diluted samples were plated in to the sterile SNB agar using spread plate method. The SNB plates were incubated at 37º C for 24 hours. The isolated colonies were further purified by streak plate method using sterile media plates. The pure cultures were inoculated into sterile nutrient agar slants for further uses.

Screening for the biosurfactant production

Hemolytic activity

The hemolytic assay was performed using blood agar. The blood agar plates were prepared by the mixing 5ml of fresh human or horse blood to 100ml of NA, after solidification bacterial cultures were streaked on blood agar media and incubated at 28ºC for 48-72 hrs .Observation was made for α, β, γ haemolysis .Hemolytic activity was correlated with the production of biosurfactant [10].

Blue agar plate (BAP) method

Anionic biosurfactant, specifically rhamnolipids were detected by this technique .Mineral salts agar medium supplemented with carbon sources (2%) and cetyltrimethyl ammonium bromide (CTAB: 0.5 mg/ml) and methylene blue (MB:0.2 mg/ml) were prepared. A dark blue halo zone around the culture was considered a positive for biosurfactant production.

Surface tension measurement

Drop collapsing test

Two microliters of mineral oil was added to each well of a 96-well microtiter plate lid (Nunc, Roskilde, Denmark). The lid was equilibrated for 1h at room temperature, and then 5 µl of the cultural supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant producing cultures giving flat drops were scored as positive. Those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production [11].

Emulsification test (E_{24})

Several colonies of a pure culture were suspended in test tubes containing 2ml 0f MSS after 48h incubation , 2ml hydrocarbon (oil) was added to each tube and mixture vortexed at high

speed for 1 min ,they were allowed to stand for 24h. The emulsion index (E_{24}) is the height of the emulsion layer (cm) divided by total height (cm), multiplied by 100[12,13].

$$\text{Emulsification index } (E_{24}) = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times 100$$

Biosurfactant production

Preparation of inoculums for bacteria

The inoculum for further production of biosurfactant and other studies was prepared using Luria broth (LB).The pure culture was inoculated at 37 ºC in a rotary shaker for overnight. The fresh over night culture was used as an inoculum for production of biosurfactant. The cells were removed by centrifugation at 10000 rpm at 10°C for 40 minutes

Acid precipitation

The Biosurfactant in the supernatant will be precipitated out by acidification with HCL to pH 2 .The proteins and lipids and was recovered by centrifugation at 12,000 rpm ,4ºC for 20 minutes .Washed three times with acid water (pH 2.0 HCL) and the precipitate was dissolved in alkaline water (pH 8.0 NaOH)

Biosurfactant Assay

Bacterial crude culture preparation

Aliquots of 10ml of the culture suspension from production medium was taken and centrifuged at 8000 rpm for 40 minutes and cell free extract was subjected to biosurfactant assay. This extract was stored at 4ºC for future analysis.

Hemolytic assay

Blood agar plates were prepared and after solidification 5 wells around 10mm diameters were cut out aseptically with the help of cork borer. Among 5 wells 4 wells named A, B, C and D was filled with culture filtrate of different concentration (10µl, 25µl, 50µl and100µl). In the well named E was filled with 100µl of distilled water as control. After incubating at 37ºC for overnight. Hemolytic activity was detected for the occurrence of a define clear zone around the colony.

Blue agar plate assay

Blue agar plates were prepared and allowed to solidification 2 wells around 10mm diameters were cut out aseptically with the help of cork borer. Among 2 wells one was filled with cultural filtrate of 100µl concentration and other with distilled water with control. After incubating at 72 hours biosurfactant activity was detected for the occurrence of a dark blue halo zone around the colony.

Chemical assay

Acid precipitated samples, dissolved in alkaline water were used in assay.

Orcinol method

The orcinol assay was used for direct assessment of the amount of glycolipids in the sample. An extracellular glycolipids concentration was evaluated in triplicate by measuring the concentration of rhamnose. To 100µl of each sample was mixed with the 900µl of 0.19% orcinol was added. After heating for 30 min at 80ºC the samples were cooled at room temperature and the OD at 421nm was measured. Control was prepared with distilled water. The rhamnolipid concentrations were calculated from a standard curves prepared with L-rhamnose and expressed as rhamnose equivalents

Hydrophobicity test

To determine the cell surface hydrophobicity a variation of the two phase method was used. Two microliters of a stationary -phase culture were washed once with 2 ml PUM buffer (22.2 g dipotassium hydrogen orthophosphate,7.26 g potassium dihydrogen orthophosphate, 1.8g urea, 0.2 g magnesium sulphate, 1 l distilled water) and resuspended in 2 ml of this buffer to an optical density (OD) of about 0.3(OD1) at 405 nm . 1 ml of kerosene was added and incubated at room temperature for 10 min. The contents of the test tubes were vortexed at high speed for 120 seconds. After 15 minutes of equillibriation, the optical density of the lower -water phase was measured (OD2).The degree of hydrophobicity was calculated using following formulae:

Degree of hydrophobicity =100 -100 (OD2/OD1)

Thin layer chromatography

A 40µl volume of the partially purified biosurfactant in the methanol was spotted onto the TLC plates (silica gel 60 Aº, 20 by 20 cm, 250 -µm thickness; whatman, Clifton, NJ) and developed in chloroform –methanol-5 M ammonium hydroxide (80:25:4,vol/vol/vol). For detection of peptides ,the plates were air dried, sprayed with ninhydrin, and heated at 110ºC for development. For detection of lipids, the plates were air dried, sparyed with bromothymol blue (0.1% in 10% aqueous ethanol). Migration distances of sample spots relative to the mobile phase (Rf values) were calculated.

Parameter optimization studies

Effect of incubation time on biosurfactant production

Around 500ml of sterile production medium was prepared and 5% bacterial inoculums was added aseptically. The inoculated medium was incubated at 37ºC temperature with shaking around 150 rpm .After incubation ,around 20ml of culture was aseptically drowned periodically at 12 hours intervals upto 48 hours. The culture filtrate was examined for the total biosurfactant activity.

Effect of temperature on biosurfactant production

100ml of sterile production medium was prepared in different conical flask with pH 6.7 and inoculated with 5% inoculum. Each flask was incubated at various temperature such as 28ºC, 32ºC, 30ºC, 42ºC, and 47ºC for 48 hours. The biosurfactant activity was estimated.

Effect of pH biosurfactant production

100ml of sterile production media was prepared in different conical flasks and each flask was adjusted to different pH such as 6, 6.5, 7, 7.5 and 8 using 0.1N NaOH and 0.1N HCL. After which was inoculated with 5% inoculum. The flasks were incubated at 37ºC for 48 hours. The biosurfactant activity was estimated.

Effect of carbon sources on biosurfactant production

100ml of sterile production medium was prepared in different conical flasks. Each flask was amended with different carbon sources such as n-hexadecane, mannitol, lactose, maltose and arabinose. The flasks were inoculated with 5% inoculum and incubated at 37ºC for 48 hours .The cultural filtrate was collected and biosurfactant activity was determined.

Effect of different glucose concentration on biosurfactant production

100ml of sterile production medium was prepared in different conical flasks. Each flask was amended with different concentration of glucose such as 0, 3, 5, 10, 20 mM. The flasks contain sterile medium were inoculated with 5% inoculums and incubated at 37ºC for 48 hours. The culture filtrate was collected and biosurfactant activity was determined.

Application of biosurfactant

Growth inhibition of Bacillus subtilis

Lawn cultures of fresh over night cultures of *Bacillus subtilis* was made on NA plates. 2 wells around 10mm diameters was cut out aseptically with the help of cork borer. Among 2 wells one was filled with cultural filtrate of 100µl concentration and other with distilled water as control, and observed for the formation of zone of inhibition by incubating at 37°C for 24 hrs.

Precoating of agar plates with biosurfactant

Ten microliter volumes of increasing concentrations of biosurfactant or 40µl of the purified biosurfactant were spotted onto NA plates and air dried for 2 hrs. Ten microliters of an overnight culture of the wild-type strains was spotted onto the biosurfactant-coated area, and plates were incubated at 32°C for 40 hrs.

Agarose gel electrophoresis

DNA fragments were separated using standard Agarose Gel Electrophoresis technique

PCR amplification

The PCR amplification of 16S ribosomal RNA gene was carried out based on the methodology of (in Thermocycler (PTC – 100 TM Programmable Thermal Controller, USA)**.[14]**

Agarose Electrophoresis

Electrophoresis was performed in a horizontal sub-marine apparatus (Medox, India). Agarose (2%) was melted with TE buffer and 2 µl of ethidium bromide was at the concentration of 4 mg/ml. TE buffer was used as the tank buffer and electrophoresis was carried out for 30 minutes at constant voltage. The gel was visualized under UV transilluminator and photographed.

Gene sequencing

The gene sequencing was carried out using Beckman Coulter CEQ 8000 auto analyzer. (The original sequence was done by Delhi university south campus). The amplified products were cleaned up using QIA Quick (Qiagen) Spin column. The cycle sequencing was carried out using DTCS quick start Dye terminator kit (Beckman Coulter). The removal unbound dye and nucleotide from cycle sequenced product was carried out using DyeEx spin columns (Qiagen). The purified samples were sequenced by Beckman Coulter CEQ8000 sequencer.

S rRNA Sequence analysis

BLAST algorithm detects local as well as global alignments, regions of similarity may provide important clues to the function of uncharacterized nucleotides and proteins.

Phylogenetic tree analysis

Phylogenetic tree analysis is the technique of methodically demonstrating an evolutionary relationship between species. This method is useful to determine whether group of genes are related through a process of divergent evolution from a common ancestor or the result of convergent evolution. In the present study a phylogenetic tree was constructed using BLAST tree tool.

RESULTS

Sample collection

In this study, the bacterial strains was isolated from petrol contaminated petrol bunk soil, (Adayar, Chennai) From the soil samples around 17 bacterial strains were isolated .But later during screening it was found that only 8 bacterial strains having the ability to produce biosurfactant. Among 8 bacterial strains which showed positive for all four screening was chosen for further study.

Screening for biosurfactant

Haemolytic Activity

The isolated bacterial strains were tested for hemolytic activity on the blood agar plates shows the maximum zone of haemolysis clearance indicates the presence of biosurfactant. (Figure-1)

Blue Agar Plate

The isolated bacterial strains were tested for dark blue halo zone. Among the isolates, 3 strains showed dark blue halo zone around the colony. Among the three positive isolates. colony no: 8 showed higher activity on CTAB plate and it is taken for further screening.
(Figure-2)

Surface tension measurement

Drop Collapse Test

In drop collapse test**,** if the drop gets spread and collapsed it is considered positive result for drop collapse test, where if the drop remained beaded it is considered as negative. The isolate which showed positive results in blood agar and blue agar medium was subjected to drop collapse test, showed positive result**.** (Figure-3)

Emulsification Index

Screening of microbial isolates performed in order to check the abilities of crude oil emulsifying bacteria .Among 17 strains, 12 strains had 60 % emulsification ability. The strains which showed better positive results both in blood agar and CTAB agar screened for emulsification index and it showed emulsification index of surfactant 62.5% in the medium supplemented with glucose. (Figure-4)

Fig 1 Blue agar method

Fig 2 Hemolytic activity

Fig 3 Drop collapse test

Fig 4 Emulsificatiuon index

Fig 5 Blue agar plate assay

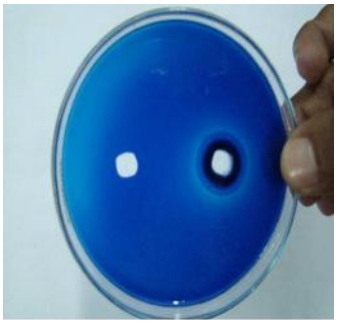


Fig 6 Haemolytic plate assay

$$\text{Emulsification index (E(24)} = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times 100$$

$$E(24) = \frac{2.5}{4} \times 100$$

$$= 62.5\%$$

Plate assay

Haemolytic activity

Haemolytic activity was showed by the formation of haemolytic zone around the wells loaded with crude filtrate of production medium which indicates the presence of biosurfactant producing bacteria. Increasing concentration of biosurfactant showed haemolytic zones of increased diameter. (Figure-5)

Blue agar medium

The well containing crude filtrate of production medium formed dark blue halo zones around the well. Whereas no zone was found around well containing distilled water as control. (Figure-6)

Parameter optimization

Effect of incubation time on biosurfactant production

The growth study of the organisms is essential for the production of biosurfactant because most of the extracellular biosurfactant are produced during log phase of the organisms. Generally, during growth study, the biomass of the cells will be estimated. The culture was withdrawn and checked up for biosurfactant activity. The bacterial cultures were with-drawn every six hours once.

Table 1 Effect of incubation time on growth

S.NO	Incubation period	Biosurfactant activity g/L
1	6	4.36
2	12	5.88
3	18	8.33
4	24	9.96
5	30	11.38
6	36	12.25
7	42	10.42
8	48	6.25

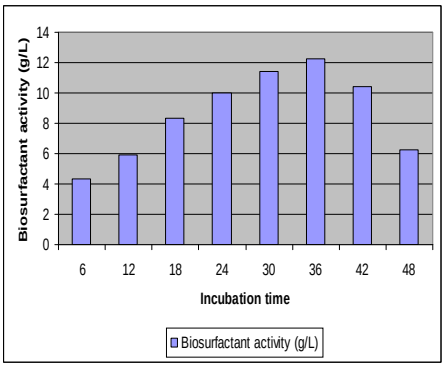


Fig 7 Effect of incubation time on growth

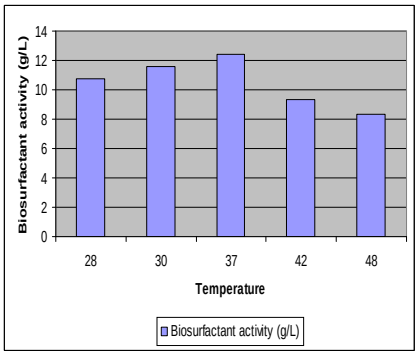


Fig 8 Effect of temperature on growth

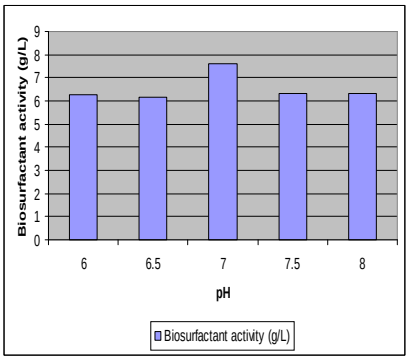


Fig 9 Effect of pH on growth

The results revealed that there is gradual increasing of production has occurred from 24th hours to 48th hours and higher production has occurred at 36th hours(12.25 g/L) followed by 11.38g/L of biosurfactant at 30th hour. (Table-1) (Figure-7). These shows that bacterial isolates should have maintained its log phase from around 24th hour to 36th hour.

Effect of temperature on biosurfactant production

In order to determine the effect of the incubation temperature for the better biosurfactant production, different incubation temperatures were maintained for production process. Based on the readings observed it was found that like mesophilic organisms, the higher Biosurfactant activity was found (12.45g/L) at 37ºC (Table-2) (Figure-8). These indicate that the optimum temperatures for better production of bacterial isolates are 37ºC .

Table 2 Effect of temperature on growth

S.NO	Temperature (° C)	Biosurfactant activity g/L
1	28	10.75
2	30	11.57
3	37	12.45
4	42	9.30
5	48	8.33

Effect of pH on biosurfactant production

Next to the temperature, pH is the important parameter which determines the growth of the organism and biosurfactant production .Generally most of the bacteria require medium pH for its growth. The study results showed that the optimum pH around 7.0 (7.62 g/L) followed by 7.5 and 8 (6.31g/L and 6.32 g/L) is better for bacterial isolate (Table-3) (Figure-9).

Table 3 Effect of pH on growth

S.NO	pH	Biosurfactant activity(g/L)
1	6	6.25
2	6.5	6.15
3	7	7.62
4	7.5	6.32
5	8	6.31

Effect of carbon source on biosurfactant production

Different carbon sources were screened for maximum production of Polyhydroxybutyrate for the selected isolate. As it is seen from Table- 4, except for starch, the rest of the carbon sources gave satisfactory production of biosurfactant. We can see the higher production of many other Biosurfactant due to amending of glucose.

But in this study, Glycerol (554.5mg/L) was found to be a right carbon source for bacterial strain for higher production of Biosurfactant. (Table-4) (Figure-10).

Table 4 Effect of carbon source on growth

S.NO	Carbon source(2ml/100ml)	Biosurfactant activity(mg/L)
1	Sunflower oil	505.6
2	Mannitol	451.8
3	Glycerol	554.5
4	Soyabean oil	298.6
5	Starch	157.5

Effect of initial glucose concentration on biosurfactant production

In this study, growth of bacterial isolates was carried out in batch culture system. Bacteria were grown in basal medium, supplemented with various initial concentration of glucose which acts as a substrate for biosurfactant production. The higher amount of biosurfactant was obtained in the medium amended with 3mM (5.83mg/L) of glucose followed by 5Mm and 10Mm (Table-5) (Figure-11).

Table 5 Effect of initial glucose concentration on growth

S.NO	Initial glucose concentration	Biosurfactant activity(mg/L)
1	0mM	1.62
2	3mM	5.82
3	5mM	5.75
4	10mM	4.56
5	20mM	3.57

Hydrophobicity test

The cells grown in the medium supplemented with glucose showed high degree of hydrophobicity of about 82.6. Hence they posse's hydrophobic groups in posses their repeating units. It increasing in cell hydrophobicity from 37 ± 3.8% in the logarithmic growth to 69.4±2.7% was observed in the beginning of the stationary phase in the case when washed cells were used as inoculum. This may facilitate cell adhesion and access to the substrate, as suggested by the subsequent maximal surfactant secretion. In the case when the whole broth was used as inoculum cell hydrophobicity did not changes significantly during growth (from 58 ± 1.7% to 67.3 ± 2.4 %, respectively).

Aminoacid analysis

Thin layer chromatography

The thin layer chromatography showed colored spots which showed the presence of corresponding aminoacids

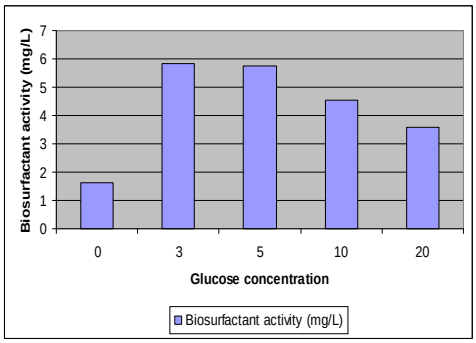


Fig 10 Effect of initial glucose concentration on growth

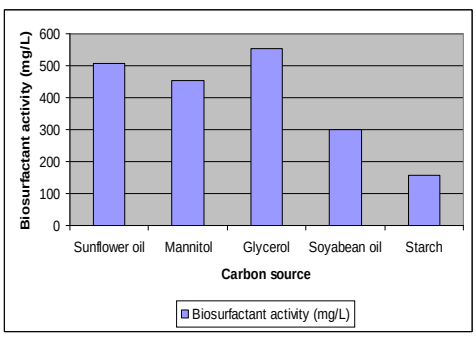


Fig 11 Effect of carbon source on growth

(Glu, Asp, Val, Leu, Ile) in the purified biosurfactant fraction when calculated the Rf value of 0.83 and from the colored spots when sprayed with ninhydrin. (Figure-12)

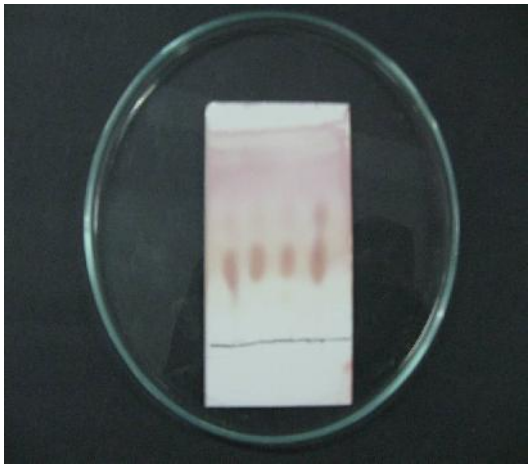


Fig 12 Thin layer chromatography

Applications

Growth inhibition of Bacillus subtilis

We tested whether the biosurfactant had growth inhibition activity for Bacillus subtilis. Clear zone of growth inhibition with diameter 26mm was obtained around the well containing 100µl of extracted Biosurfactant. Whereas no zone was found around the well containing distilled water as control.

Precoating of agar plates with biosurfactant

While testing the biosurfactant had growth inhibition activity against different species of bacteria. Among 7 bacteria tested 5 gram positive bacteria showed clear zone of inhibition around the coated Biosurfactant. While 2 gram negative bacteria did not showed any growth of inhibition around the coated Biosurfactant.

Isolation of genomic DNA

The 24 hour old bacterial culture of ***Bacillus licheniformis*** BIOS PTK1 grown in Luria Bertani medium at 37 °C under aerobic conditions at 100 rpm were collected and isolated the respective genomic DNAs was isolated. The isolated genomic DNA of the ***Bacillus licheniformis*** BIOS PTK1 was visualized under UV light after agarose gel separation. (Figure-13)

PCR amplification of 16S rRNA gene

Polymerase chain reaction was performed in Thermocycler (PTC – 100 TM Programmable Thermal Controller, USA) to produce multi-copies of the specified DNA. The PCR reaction was allowed for 30 cycles for amplification of 16S rRNA gene. Then the PCR product was run on 2 % agarose gel electrophoresis along with 100 bp DNA ladder mix and visualized under UV light. (Figure-14).

Sequence analysis of PCR-amplified product

The nucleotide sequence of PCR products of both forward and reverse primer sequences of the *Bacillus licheniformis* BIOS PTK1 16S rRNA gene were sequenced~ 682 bp sequences of *Bacillus licheniformis* BIOS PTK1 16S rRNA was observed. (Fig-15).

Molecular identification of Bacillus licheniformis BIOS PTK1

The potential Biosurfactant producing bacteria, *Bacillus licheniformis* BIOS PTK1 was ascertained its systematic position based on 16S rRNA sequence analysis and with the aid of computational programme, BLAST homology analysis was also carried out to compare with other 16S rRNA sequences available in the GenBank of NCBI. It revealed that the sequence of *Bacillus licheniformis* BIOS PTK1(Fig-16) (Report-1,2). Evolutionary relationship of *Bacillus licheniformis* BIOS PTK1 to be clearly showed by phylogenetic tree.(Fig-17).

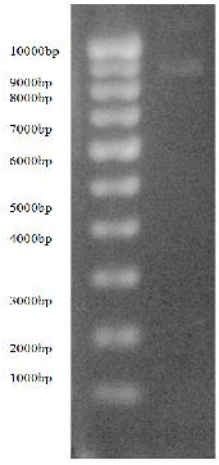


Fig 13 Genomic DNA

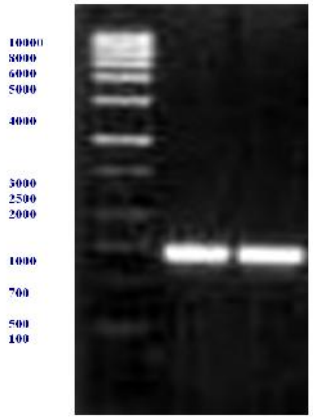


Fig 14 PCR amplified DNA

GGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCG
ACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAA
GCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGC

Fig 15 16S ribosomal RNA gene, partial sequence (682 bps) for *Bacillus licheniformis* strain BIOS PTK1

Fig 16 Sequences producing significant alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU201863.1	Bacillus licheniformis strain Gzn-9-1 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU458873.1	Uncultured Bacillus sp. clone T0552 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU458871.1	Uncultured Bacillus sp. clone T0501 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU323372.1	Bacillus licheniformis strain HS12 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU323368.1	Bacillus licheniformis strain HS7 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU391534.1	Bacillus licheniformis strain Na1 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
AB525389.1	Bacillus licheniformis gene for 16S ribosomal RNA, partial sequence	1260	1260	100%	0.0	100%
GU250455.1	Bacillus licheniformis strain BFE 5370 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU191917.1	Bacillus licheniformis strain SB 3131 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU191906.1	Bacillus licheniformis strain SB 3181 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU191905.1	Bacillus licheniformis strain SB 3180 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
FJ493055.1	Bacillus subtilis strain T-2 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
FJ493045.1	Bacillus licheniformis strain 2J-1 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU137298.1	Bacillus licheniformis strain Hsw-12 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU132507.1	Bacillus sp. JJM-1 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU086446.1	Bacillus licheniformis strain G7A 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU086433.1	Bacillus licheniformis strain N34 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GU086425.1	Bacillus licheniformis strain N22 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GQ918258.1	Bacillus licheniformis strain JP4 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GQ867231.1	Bacillus sp. BRAZ10 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
GQ867228.1	Bacillus sp. BRAZ2A 16S ribosomal RNA gene, partial sequence	1260	1260	100%	0.0	100%
AB523720.1	Bacillus licheniformis gene for 16S rRNA, partial sequence, strain: AN-sB	1260	1260	100%	0.0	100%
AB523719.1	Bacillus licheniformis gene for 16S rRNA, partial sequence, strain: AN-sS23	1260	1260	100%	0.0	100%
AB523718.1						

Job Title: BIOS PTK1

Tax BLAST Report

Lineage Report

Bacteria [bacteria]
. Bacillus [firmicutes]
. . Bacillus subtilis group [firmicutes]
. . . Bacillus licheniformis ---------- 1260 78 hits [firmicutes] Bacillus licheniformis strain Gzn-9-1 16S ribosomal RNA gen
. . . Bacillus subtilis ..... 1260 1 hit [firmicutes] Bacillus subtilis strain T-2 16S ribosomal RNA gene, partia
. . uncultured Bacillus sp. ---------- 1260 3 hits [firmicutes] Uncultured Bacillus sp. clone T0552 16S ribosomal RNA gene,
. . Bacillus sp. JJM-1 ..... 1260 1 hit [firmicutes] Bacillus sp. JJM-1 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. BRAZ10 ..... 1260 1 hit [firmicutes] Bacillus sp. BRAZ10 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. BRAZ2A ..... 1260 1 hit [firmicutes] Bacillus sp. BRAZ2A 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. enrichment culture clone S29 . 1260 1 hit [firmicutes] Bacillus sp. enrichment culture clone S29 16S ribosomal RNA
. . Bacillus sp. BCCS 057 ..... 1260 1 hit [firmicutes] Bacillus sp. BCCS 057 16S ribosomal RNA gene, partial seque
. . Bacillus sp. PS-99 ..... 1260 1 hit [firmicutes] Bacillus sp. PS-99 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. PS-39 ..... 1260 1 hit [firmicutes] Bacillus sp. PS-39 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. 24KZ ..... 1260 1 hit [firmicutes] Bacillus sp. 24KZ 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. SFK10 ..... 1260 1 hit [firmicutes] Bacillus sp. SFK10 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. gy12 ..... 1260 1 hit [firmicutes] Bacillus sp. gy12 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. L240 ..... 1260 1 hit [firmicutes] Bacillus sp. L240 partial 16S rRNA gene, isolate L240
. . Bacillus sp. L164 ..... 1260 1 hit [firmicutes] Bacillus sp. L164 partial 16S rRNA gene, isolate L164
. . Bacillus sp. L157 ..... 1260 1 hit [firmicutes] Bacillus sp. L157 partial 16S rRNA gene, isolate L157
. . Bacillus sp. sc_36 ..... 1260 1 hit [firmicutes] Bacillus sp. sc_36 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. cp-h68 ..... 1260 1 hit [firmicutes] Bacillus sp. cp-h68 16S ribosomal RNA gene, partial sequence
. . Bacillus sp. Mm1(2010) ..... 1256 1 hit [firmicutes] Bacillus sp. Mm1(2010) strain Mm1 16S ribosomal RNA gene, p
. rumen bacterium 37-H_48B ---------- 1260 1 hit [bacteria] Rumen bacterium 37-H_48B 16S ribosomal RNA gene, partial se
. rumen bacterium 37-H_43 ..... 1260 1 hit [bacteria] Rumen bacterium 37-H_43 16S ribosomal RNA gene, partial seq

Organism Report

Bacillus licheniformis [firmicutes] taxid 1402
gb|GU201863.1| Bacillus licheniformis strain Gzn-9-1 16S r... 1260 0.0
gb|GU323372.1| Bacillus licheniformis strain HS12 16S ribo... 1260 0.0
gb|GU323368.1| Bacillus licheniformis strain HS7 16S ribos... 1260 0.0
gb|GU391534.1| Bacillus licheniformis strain Na1 16S ribos... 1260 0.0
dbj|AB525389.1| Bacillus licheniformis gene for 16S riboso... 1260 0.0
gb|GU250455.1| Bacillus licheniformis strain BFE 5370 16S ... 1260 0.0
gb|GU191917.1| Bacillus licheniformis strain SB 3131 16S r... 1260 0.0
gb|GU191906.1| Bacillus licheniformis strain SB 3181 16S r... 1260 0.0
gb|GU191905.1| Bacillus licheniformis strain SB 3180 16S r... 1260 0.0
gb|FJ493045.1| Bacillus licheniformis strain 2J-1 16S ribo... 1260 0.0
gb|GU137298.1| Bacillus licheniformis strain Hsw-12 16S ri... 1260 0.0
gb|GU086446.1| Bacillus licheniformis strain G7A 16S ribos... 1260 0.0
gb|GU086433.1| Bacillus licheniformis strain N34 16S ribos... 1260 0.0
gb|GU086425.1| Bacillus licheniformis strain N22 16S ribos... 1260 0.0
gb|GQ918258.1| Bacillus licheniformis strain JP4 16S ribos... 1260 0.0
dbj|AB523720.1| Bacillus licheniformis gene for 16S rRNA, ... 1260 0.0
dbj|AB523719.1| Bacillus licheniformis gene for 16S rRNA, ... 1260 0.0
dbj|AB523718.1| Bacillus licheniformis gene for 16S rRNA, ... 1260 0.0
dbj|AB523715.1| Bacillus licheniformis gene for 16S rRNA, ... 1260 0.0
dbj|AB523714.1| Bacillus licheniformis gene for 16S rRNA, ... 1260 0.0
dbj|AB523713.1| Bacillus licheniformis gene for 16S rRNA, ... 1260 0.0
dbj|AB523712.1| Bacillus licheniformis gene for 16S rRNA, ... 1260 0.0
dbj|AB523711.1| Bacillus licheniformis gene for 16S rRNA, ... 1260 0.0
dbj|AB523710.1| Bacillus licheniformis gene for 16S rRNA, ... 1260 0.0
gb|GQ478407.1| Bacillus licheniformis strain 140-2 16S rib... 1260 0.0
gb|GQ478406.1| Bacillus licheniformis strain 137-12 16S ri... 1260 0.0
gb|GQ470399.1| Bacillus licheniformis strain DQgbc4 16S ri... 1260 0.0
gb|GQ503331.1| Bacillus licheniformis strain CrK21 16S rib... 1260 0.0

gb|GQ503324.1| Bacillus licheniformis strain AsK03 16S rib... 1260 0.0
gb|FJ915147.1| Bacillus licheniformis strain nju-1411-1 16... 1260 0.0
gb|GQ375247.1| Bacillus licheniformis strain CICC 10338 16... 1260 0.0
gb|GQ375245.1| Bacillus licheniformis strain CICC 10336 16... 1260 0.0
gb|GQ375244.1| Bacillus licheniformis strain CICC 10335 16... 1260 0.0
gb|GQ375243.1| Bacillus licheniformis strain CICC 10334 16... 1260 0.0
gb|GQ375235.1| Bacillus licheniformis strain CICC 10181 16... 1260 0.0

uncultured Bacillus sp. [firmicutes] taxid 83428
gb|GU458873.1| Uncultured Bacillus sp. clone T0552 16S rib... 1260 0.0
gb|GU458871.1| Uncultured Bacillus sp. clone T0501 16S rib... 1260 0.0
gb|EU371573.1| Uncultured Bacillus sp. clone CBIOS-09 16S ... 1260 0.0

Bacillus subtilis [firmicutes] taxid 1423
gb|FJ493055.1| Bacillus subtilis strain T-2 16S ribosomal ... 1260 0.0

Bacillus sp. JJM-1 [firmicutes] taxid 689850
gb|GU132507.1| Bacillus sp. JJM-1 16S ribosomal RNA gene, ... 1260 0.0

Bacillus sp. BRAZ10 [firmicutes] taxid 679145
gb|GQ867231.1| Bacillus sp. BRAZ10 16S ribosomal RNA gene,... 1260 0.0

Bacillus sp. BRAZ2A [firmicutes] taxid 679146
gb|GQ867228.1| Bacillus sp. BRAZ2A 16S ribosomal RNA gene,... 1260 0.0

rumen bacterium 37-H_48B [bacteria] taxid 672994
gb|GQ461838.1| Rumen bacterium 37-H_48B 16S ribosomal RNA ... 1260 0.0

rumen bacterium 37-H_43 [bacteria] taxid 672993
gb|GQ461837.1| Rumen bacterium 37-H_43 16S ribosomal RNA g... 1260 0.0

Bacillus sp. enrichment culture clone S29 [firmicutes] taxid 660044
gb|GQ288417.1| Bacillus sp. enrichment culture clone S29 1... 1260 0.0

Bacillus sp. BCCS 057 [firmicutes] taxid 664433
gb|GQ352448.1| Bacillus sp. BCCS 057 16S ribosomal RNA gen... 1260 0.0

Bacillus sp. PS-99 [firmicutes] taxid 613155
gb|FJ489842.1| Bacillus sp. PS-99 16S ribosomal RNA gene, ... 1260 0.0

Bacillus sp. PS-39 [firmicutes] taxid 613153
gb|FJ489838.1| Bacillus sp. PS-39 16S ribosomal RNA gene, ... 1260 0.0

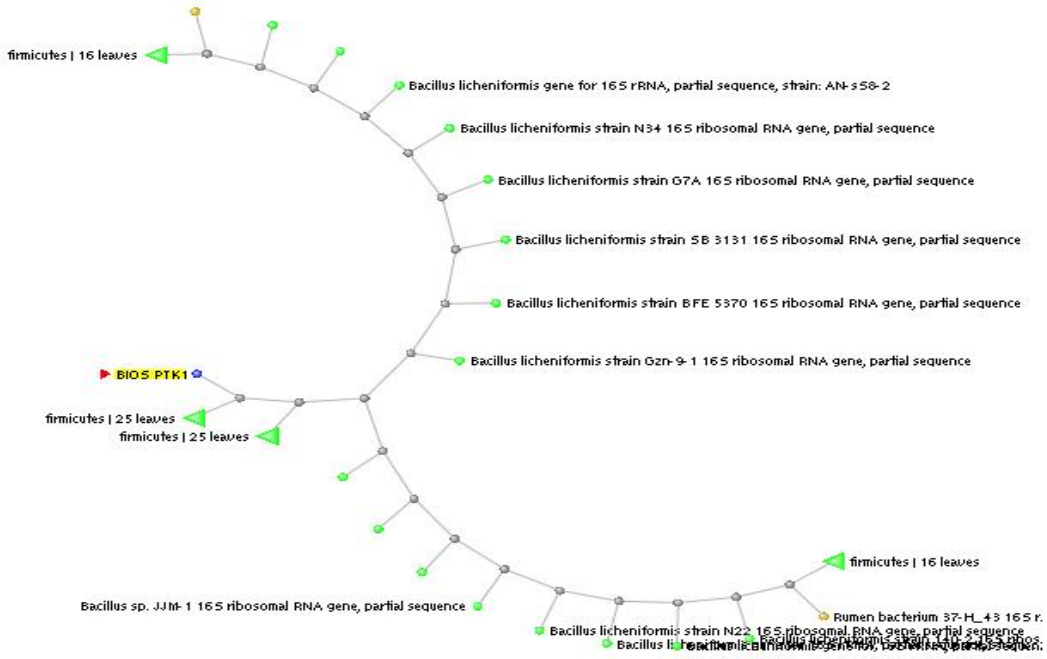
Bacillus sp. 24KZ [firmicutes] taxid 592981
gb|FJ615520.1| Bacillus sp. 24KZ 16S ribosomal RNA gene, p... 1260 0.0

Bacillus sp. SFK10 [firmicutes] taxid 588982
gb|FJ594463.1| Bacillus sp. SFK10 16S ribosomal RNA gene, ... 1260 0.0

Bacillus sp. gy12 [firmicutes] taxid 587699

Taxonomy Report
Bacteria ..... 100 hits 21 orgs [root; cellular organisms]
. Bacillus ..... 98 hits 19 orgs [Firmicutes; Bacilli; Bacillales; Bacillaceae]
. . Bacillus subtilis group ..... 79 hits 2 orgs
. . . Bacillus licheniformis ..... 78 hits 1 orgs
. . . Bacillus subtilis ..... 1 hits 1 orgs
. . environmental samples ..... 4 hits 2 orgs
. . . uncultured Bacillus sp. ..... 3 hits 1 orgs
. . . Bacillus sp. enrichment culture clone S29 . 1 hits 1 orgs
. . Bacillus sp. JJM-1 ..... 1 hits 1 orgs
. . Bacillus sp. BRAZ10 ..... 1 hits 1 orgs
. . Bacillus sp. BRAZ2A ..... 1 hits 1 orgs
. . Bacillus sp. BCCS 057 ..... 1 hits 1 orgs
. . Bacillus sp. PS-99 ..... 1 hits 1 orgs
. . Bacillus sp. PS-39 ..... 1 hits 1 orgs
. . Bacillus sp. 24KZ ..... 1 hits 1 orgs
. . Bacillus sp. SFK10 ..... 1 hits 1 orgs
. . Bacillus sp. gy12 ..... 1 hits 1 orgs
. . Bacillus sp. L240 ..... 1 hits 1 orgs
. . Bacillus sp. L164 ..... 1 hits 1 orgs
. . Bacillus sp. L157 ..... 1 hits 1 orgs
. . Bacillus sp. sc_36 ..... 1 hits 1 orgs
. . Bacillus sp. cp-h68 ..... 1 hits 1 orgs
. . Bacillus sp. Mm1(2010) ..... 1 hits 1 orgs
. unclassified Bacteria (miscellaneous) ..... 2 hits 2 orgs [unclassified Bacteria]
. . rumen bacterium 37-H_48B ..... 1 hits 1 orgs
. . rumen bacterium 37-H_43 ..... 1 hits 1 orgs

Fig 17 BLAST tree result for *Bacillus licheniformis* strain BIOS PTK1



results in blood agar and blue agar medium was subjected to drop collapse test, showed positive result.

DISCUSSION

Naturally occurring so many microorganisms are having ability to produce the various enzymes and compounds. Indeed, it is boon of god because nowadays most of this compounds are industrially important and human welfare. Biosurfactant is one of the important compound involved in bioremediation, which can be produced by various microorganisms.

In this study, the bacterial strains was isolated from petrol contaminated petrol bunk soil, (Adayar, Chennai) was collected because most of the petrol contaminated sites., surface tension was reduced by native microbes that are growing over the soil. In such a way, it is fact that microbes which are isolated from the petrol contaminated soil, having the ability to produce surface active compound, biosurfactant. From the soil samples around 17 bacterial strains were isolated. But later during screening it was found that only 8 bacterial strains having the ability to produce biosurfactant. Among 8 bacterial strains which showed positive for all four screening was chosen for further study.

All of the isolated strains were tested for hemolytic activity. Among the isolates, 8 strains showed hemolytic activity in blood agar medium. Selected strains were used for further screening**.**[15] All of the isolated strains were tested for dark blue halo zone formation on CTAB/MB plates. Among the isolates, 3 strains showed dark blue halo zone around the colony. Among the three positive isolates., colony no: 8 showed higher biosurfactant activity. The isolated strains formed halo zone which indicated the production of extracellular glycolipids by *Bacillus licheniformis* and for extracellular rhamnolipid by *Renibacterium salmoninarium* and *Pseudomonas aeruginosa* was studied.[16] If the drop gets spread and collapsed it is considered positive result for drop collapse test, where if the drop remained beaded it is considered as negative. The isolate which showed positive

Similarly the medium supplemented with corn oil showed positive result for the drop collapse test for surface tension [17]

Screening of microbial isolates performed in order to check the abilities of crude oil emulsifying bacteria .Among 17 strains, 12 strains had 60 % emulsification ability. The strains which showed better positive results both in blood agar and CTAB agar screened for emulsification index and it showed emulsification index of surfactant 62.5% in the medium supplemented with glucose. The other studies showed the emulsification index of the surfactant was greater in the medium supplemented with corn oil by *Bacillus subtilis* [18]

The growth study of the organisms is essential for the production of biosurfactant because most of the extracellular biosurfactant are produced during log phase of the organisms. Generally, during growth study, the biomass of the cells will be estimated. The culture was withdrawn and checked up for biosurfactant activity. The bacterial cultures were with-drawn every six hours once .The results revealed that there is gradual increasing of production has occurred from 24^{th} hours to 48^{th} hours and higher production has occurred at 36^{th} hours(12.25 g/L) followed by 11.38g/L of biosurfactant at 30^{th} hour. (Table .1).

These shows that bacterial isolates should have maintained its log phase from around 24^{th} hour to 36^{th} hour. Besides, it is believed that the higher production of biosurfactant has occurred in extreme log phase because even though the log phase was maintained between around 24^{th} to 36^{th} hours, the followed drop of production has indicated that the organism has indicated that the organism should have entered into the stationary phase of growth. This variation of log phase timing is based on the nutrient present in the medium and the culture condition of the organism. The environmental parameters also influencing the maintenance time of the bacteria. Interestingly

some of the gram- negative bacteria also show the same kind of growth time like *Bacillus* species. [19].

The environmental parameters are showing great influence in the growth of the organisms and the production of Biosurfactant. The main parameters like Temperature, pH, are very essential parameters of the production. To optimize the optimum temperature for the better production, Production were made in various temperatures. It was found that like mesophilic organisms, the higher biosurfactant activity was found (12.45g/L) at 37ºC (Table.2). These indicate that the optimum temperatures for better production of bacterial isolates are 37ºC .The temperature requirement of the organism is based on the nature of organisms. Many *Bacillus* spp. needs 32-37ºC for better production of Biosurfactant. [20].

Next to the temperature, pH is the important parameter which determines the growth of the organism and biosurfactant production .Generally most of the bacteria require medium pH for its growth. The study results showed that the optimum pH around 7.0 (7.62 g/L) followed by 7.5 and 8 (6.31g/L and 6.32 g/L) is better for bacterial isolate (Table.3). Like temperature, different organisms needs different pH ranges for its biosurfactant production. Most of the *Bacillus* spp. has produced high amount of biosurfactant between pH 7-9. [21]

The carbohydrates are soul energy source for most of the heterotrophic organisms .These shows great influence on the production of may biosurfactant. The production will be carried out by medium with Glycerol as a substrate and as a carbon source for better growth and production .We can see the higher production of many other Biosurfactant due to amending of glucose. But in this study, Glycerol (554.5mg/L) was found to be a right carbon source for bacterial strain for higher production of Biosurfactant. (Table.4). As for as Biosurfactant production concern, the production will be low in the medium amended with starch.[22]

In this study, growth of bacterial isolates was carried out in batch culture system. Bacteria were grown in basal medium, supplemented with various initial concentration of glucose which acts as a substrate for biosurfactant production. The higher amount of biosurfactant was obtained in the medium amended with 3mM (5.83mg/L) of glucose followed by 5Mm AND 10Mm (Table.5). [23]

The cells grown in the medium supplemented with glucose showed high degree of hydrophobicity of about 82.6. Hence they posses hydrophobic groups in posses their repeating units [24]

The thin layer chromatography showed coloured spots which showed the presence of corresponding aminoacids (Glu, Asp, Val, Leu, Ile) in the purified biosurfactant fraction when calculated the Rf value of 0.83 and from the coloured spots when sprayed with ninhydrin. Similarly the two glycolipids from the new isolated strain *Renibacterium salmoninarium* 27BN having Rf values 0.80 and 0.42 produces both types of rhamnolipids that are usually secreted by Pseudomonas spp. and biosurfactant from *B.cereus* have Rf value of 0.69 [25]

We tested whether the biosurfactant had growth inhibition activity for *Bacillus subtilis.* Clear zone of growth inhibition with diameter 26mm was obtained around the well containing 100µl of extracted Biosurfactant the well. Similarly clear growth inhibition zone with a diameter of 36mm was obtained when the concentrated culture supernatant of *P.putida* on top of the agar plate with freshly grown *B.subtilis* [26]

We tested whether the biosurfactant had growth inhibition activity against different species of bacteria. Among 7 bacteria tested 5 gram positive bacteria showed clear zone of inhibition around the coated Biosurfactant. While 2 gram negative bacteria did not showed any growth of inhibition around the coated biosurfactant.

Total community DNA was efficiently extracted at small-scale level using direct lysis with hot sodium dodecyl sulphate (SDS), glass bead beating and finally subjecting the sandy soil to liquid nitrogen freeze-thaw cycles. To amplify V3 region of bacterial 16S rRNA gene, universal conserved primers were used. Second round polymerase chain reaction (PCR) was attempted to increase product concentration and to minimize the effect of inhibitory substances. To enhance the detection sensitivity of the denaturing gradient gel electrophoresis (DGGE), the effect of change in template DNA concentration was studied. The separation of bands were greatly enhanced in the fingerprints obtained after the second round of PCR representing low abundant species which were not differentiated at single optimized concentration of DNA. [14]

Summary

The biosurfactant producing bacterial strain isolated from petrol bunk sample (ADYAR, Chennai). The isolated bacterial strain was identified as *Bacillus licheniformis*. In the production optimization studies, the bacterial strain needs temperature around 37°C, PH 7,glycerol as carbon source, incubation period of 36 hrs and initial glucose concentration of 3mM as optimum parameter for better production of biosurfactant. The biosurfactant produced by above organism was acid precipitated and extracted as partially purified one using ethyl acetate extraction technique. The extracted partially purified biosurfactant characterized through TLC and the presence of corresponding amino-acid (Glu, Asp, Val, Leu, Ile) was detected whose having Rf value of 0.83.When studied with application, biosurfactant inhibited the growth of *Bacillus subtilis* thus forming clear zone of lysis around the well. Moreover., it seemed to having antibacterial effect against 5 gram positive bacteria and no effect against 2 gram negative bacteria tested. The systematic position of *Bacillus licheniformis* BIOS PTK1 was determined based on 16S rRNA sequence. BLAST homology analysis revealed that the sequence of *Bacillus licheniformis* BIOS PTK1 showed 100% sequence identity with more than 60 *Bacillus licheniformis.*

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15. Banat IM 1995. Biosurfactant production and possible uses in Microbial Enhanced Oil Recovery and Oil Pollution Remediation: review. *Biores Tech*, 51: 1-12.
16. Siegmund Lang, Wagner. 2002. Biological amphiphiles žmicrobial biosurfactants. *Current Opinion in Colloid & Interface Science* 72:12-20.
17. Adria A. Bodour. 2004. Structure and Characterization of Flavolipids, a Novel Class of Biosurfactants Produced by *Flavobacterium* sp. Strain MTN11. *Applied and Env. Biotech*. 70:114-120
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