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*RESEARCH ARTICLE*

# ISOLATION, SCREENING AND IDENTIFICATION OF BIOSURFACTANT PRODUCING FUNGAL STRAINS.

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## Abstract

Biosurfactants are the surface active compounds which are of microbial origin that are produced by different strains of bacteria fungi and actinomycetes. Soil samples were collected and 10 fungal colonies were isolated using pour plate method. Out of 10 fungal strains three were selected for further studies based on foaming activity. Preliminary and confirmatory tests were used for the identification of biosurfactant produced by three fungal strains. PS7 showed high biosurfactant production in emulsification index compared to other isolates. In this study morphology of fungal isolates were seen using trinocular microscope. Biosurfactants had different applications in the areas of oil recovery, cosmetics, agriculture, food processing.

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## Introduction:-

Biosurfactants are surface active amphiphilic molecules which are produced by a number of microorganisms such as bacteria, yeast and filamentous fungi **(G. Seghal Kiran et al., 2009)**. These biosurfactants possess both hydrophilic and hydrophobic moieties with different polarities such as hydrocarbons and water hence decrease the interfacial tension (**Karanth et al., 1997**). The production of biosurfactants by bacterial species is well known, relatively some fungi are known to produce biosurfactants. *Candida bombicola*, *Candida lipolytica*, *Candida ishiwadae*, *Candida batistae*, *Aspergillus ustus* are the well known fungi for the production of biosurfactants. The type of biosurfactant produced mainly by these strains are sophorolipids (**Gharima Bhardwaj et al., 2013**).

Biosurfactants are having a wide range of applications in the areas of bioremediation, food processing and pharmaceutical industries. (**Kamal preet kaur et al., 2017**). Biosurfactants are preferred over synthetic surfactants because of their low toxicity, biodegradable nature (**sammer M. Al-Hulu 2016**). These biosurfactants place an important role in the protection of environment and petrochemical industry. Biosurfactants are used in the bioremediation of petroleum hydrocarbons in ground water and soil and in degradation of hazardous compounds (**P.G. Carrillo et al., 1996**) .

These are used in oil recovery process in the oil industry. In cleaning of contaminated vessels and in transportation of heavy crude oil in pipe lines (**Ghurye et al., 1994**). These are used in the lipid solubilisation. The screening of biosurfactant producing microorganisms is generally carried out by

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monitoring parameters that estimate surface activity, such as surface tension, interfacial tension and ability to emulsify oils or hydrocarbons (**cooper and Zajic 1980**).

## Materials and Methods:-

**Collection, Isolation and Screening:-**

Samples were collected from the areas like oil mills in and around Guntur district of Andhra Pradesh. A total of five samples each of 50g weight were collected into sterile plastic bags and transported to lab under ambient conditions for the systematic screening of fungi and stored at 4ºC.

5g of soil sample in 50ml sterile water placed on rotary shaker for half an hour from the stock solution 1ml was used prepare the final volume of $10^{-1}$ to $10^{-8}$ by serial dilution method. Samples were inoculated on Sabouraud agar medium. To inhibit the growth of bacteria and actinomycetes the media was supplemented with rifampicin. The growth of fungi was confirmed by compact hair like structure and spore forming cells. The plates were incubated at 30 ºC for 3 to 4 days. After the incubation period colonies were identified and picked and further subcultured on to the Sabouraud Agar Slants and 10 fungal strains were isolated.

Isolated strains were grown separately in 250ml Erlenmeyer flask, each containing 100ml of sabouraud broth medium. The flasks are incubated at $30^{o}$c on a shaker incubator (200rpm) for 72hrs. After incubation out of 10strains 3 strains produced foam based on this observation we select PS7, PS3, PS9 for further studies

**Preliminary Tests For Biosurfactant Production:-**

**Phenol: $H_2SO_4$ Method:-**

To 1ml of supernatant, 1ml of 5% phenol was added. To this mixture, 2-5 ml of conc. $H2SO_4$ was added drop by drop, until orange color was developed. The development of orange color indicated the presence of glycolipids containing biosurfactant.

**Biuret Test:-**

This test was used to detect the lipopeptide containing bio-surfactant. 2ml of supernatant was heated at 700C, and then was mixed with 1ml of 1M NaOH solution. Drops of 1% $CuSO_4$ were added slowly until violet or pink ring was observed. Formation of violet or pink ring indicates the presence of lipopeptides containing biosurfactant.

**Phosphate Test:-**

Upto ten drops of 6M $HNO_3$ was added to 2ml of supernatant, and was heated at 70ºC. 5%(w/v) ammonium molybdate was added to this mixture, drop by drop, slowly until the formation of yellow color, and then the yellow precipitate. This indicates the presence of Phospholipids containing biosurfactant. **(Kalyani.A.L.T et al., 2014)**

**Confirmatory Test For Biosurfactant Production:-**

**Para film-M test:-**

One drop of Bromophenol blue indicator was added to 2 ml of cell-free supernatant. 10 µl of this sample was carefully placed like a drop on Para film-M with a micropipette. The shape of this drop on the surface was inspected after 1 min. Sodium lauryl sulphate and distilled water were used as positive and negative controls respectively. If the drop becomes flat, it indicates the presence of biosurfactant. If it remains in a dome shape, it indicates the absence of biosurfactant **(Youssef NH et al., 2004).**

**Emulsification Index:-**

Emulsification activity was measured by vortexing 1ml of culture supernatant grown in SAB at 30ºC for 24hrs. Further, 4ml of water and 6ml of petrol were shaken for 2mins to obtain maximum emulsification. After 48hrs emulsification index **(Ellaiah P et al.,2002)** was calculated by measurement of the height of the emulsion layer(a), divided by the total height(b), multiplied by 100 (EI=a/b*100). This assay was performed in same size glass test tubes.

**Oil spread method:-**
24hrs old inoculum grown in SAB was used. Petriplate was filled with 50ml of distilled water. On this water, 20 microliter of coconut oil was spreaded uniformly. Further 10 microliter of culture was added at different spots on coconut oil which was coated on the water surface. Then appearance of clear zone was the indication of biosurfactant production **(Morikawa M et al., 2000)**

**Drop collapse method:-**
A clean slide was taken. At the one end of the slide indicator mixed oil drop was added. Then five microliter cell free suspension of fungal culture was added to the oil drop. After 2min the drop was collapsed indicating the presence of biosurfactant in the cell free suspension.

**Morphology:-**
Freshly subcultured and fully sporulated slant of each isolate was taken 2ml spore suspension was prepared by inoculating a loopful of each isolate. Pour plate starch casein agar medium and was allowed to solidify and after pouring spore suspension over the plate it is incubated. After proper incubation, growth morphology was observed under trinocular microscope.

## Results and Discussion:-

**Isolation and Screening** From five soil samples used, 10 fungal colonies were identified and isolated and out of them 3 isolates PS3, PS7, PS9 showed foam during their growth so the three isolates were purified and selected for the further studies.

**Fig 1:-**Primary screening for isolation of fungi using sabouarud agar medium

**Foaming Activity:-**
Isolated strains were grown separately in 250 mL Erlenmeyer flasks, each containing 100 mL of nutrient broth medium. The flasks were incubated at 30°C on a shaker incubator (200 rpm) for 72 h.

**Fig 2:-**Foaming activity of both bacterial strain

**Preliminary Tests For Biosurfactant Production:-**
**Phenol: $H_2SO_4$ Method:-**
All the three isolates showed the appearance of Orange color indicating production of Glycolipids containing surfactants which is a positive result.

**Fig 3:**-Positive Phenol: $H_2SO_4$ test results of all the 3 isolates

**Biuret Test:-**

After the addition of NaOH and $CuSO_4$ to the fungal supernatant violet ring was observed in three isolates. This indicates that all three isolates have lipopeptide containing surfactants producing capability.

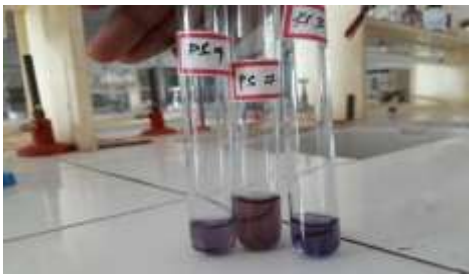

**Fig4:**-Positive Biuret Test results of all the 3 isolates

**Phosphate Test:-**

After the addition of 5% w/v ammonium molybdate solution drop by drop to the fungal supernatant containing 6M $HNO_3$ white precipitate was observed in PS7. This indicates the presence of phospholipid containing surfactant producing capability. PS3 and PS9 did not show any precipitate indicating the negative result.

**Fig 5:**-Positive Phosphate Test results of PS7 isolate

**Confirmatory Tests:-**

**Para film-M test:-**

These three isolates were then tested for biosurfactant production by para film-M test, where a flat drop was shown by PS7 taking sodium lauryl sulphate as positive control and distilled water as a negative control.

**Fig 6:**-Positive para film test results of PS7 isolate

**Emulsification Index:-**

High emulsification index was observed for PS7 and other fungal isolates were PS3 and PS9. The culture showing good emulsification activity was seen to be positive in atleast one of the above mentioned screening methods. The lowest emulsification activity was observed in PS9.

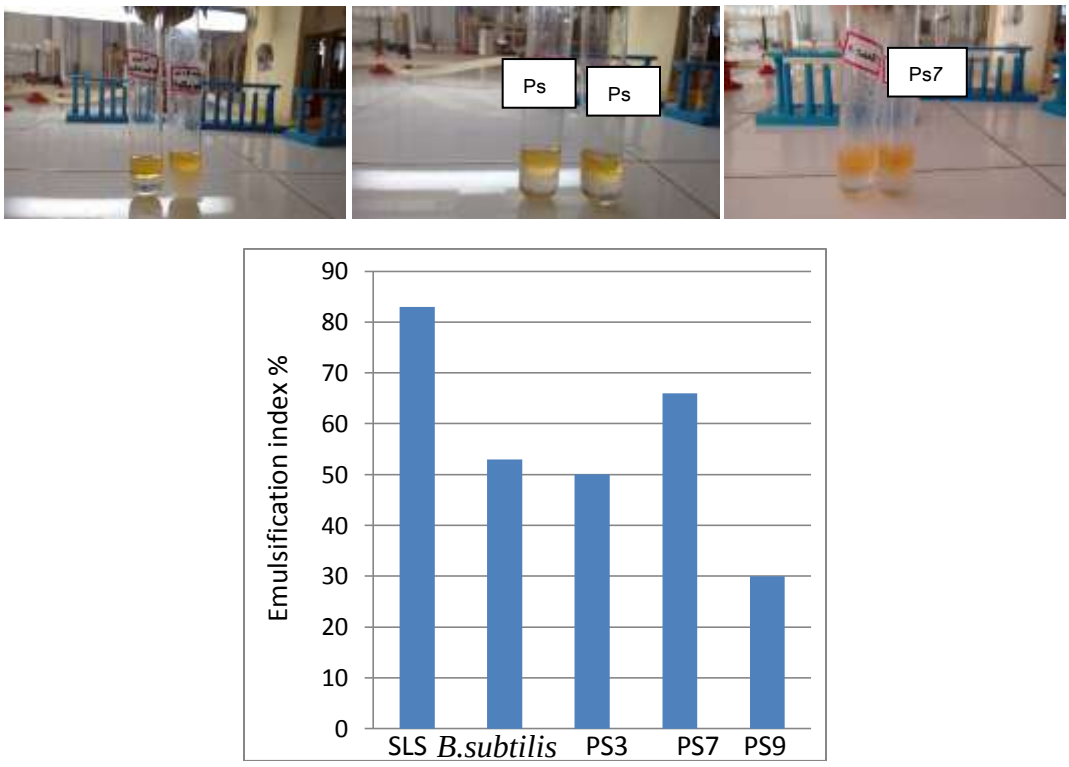


**Fig 7:-**Emulsification index results of fungal isolates

**Oil spread method:-**

The presence of biosurfactant activity of the supernatant of PS7 isolate was observed by the displacement of oil and formation of clear zone. This was compared with the positive control like SLS and *B.subtilis*

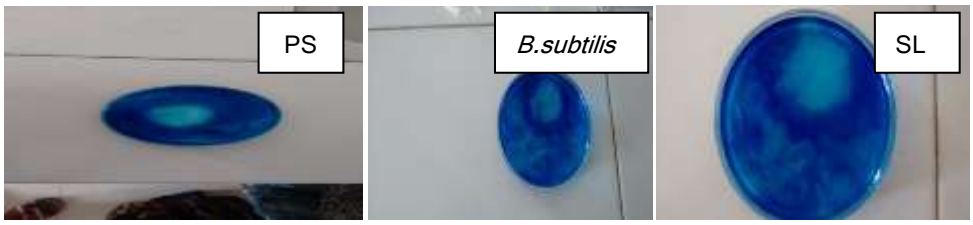


**Fig 8:-**Oil spread results of fungal isolate PS7

**Drop collapse method:-**

In presence of surfactant, the culture supernatant drop spreads over hydrophobic surface as the interfacial tension between the droplet and hydrophobic surface is reduced. In contrast the droplet remains beaded or rounded in the absence of surfactant.

**Fig9:-**Drop collapse results of fungal isolates

**Morphological Studies:-**

The morphology of isolated fungal colonies were observed under the Trinocular microscope

**Fig 10:-**Morphology of three fungal isolates PS3, PS7, PS9

## Conclusion:-

It was concluded that all three isolates have been isolated were screened and found to possess biosurfactant producing capabilities from good supporting medium. Biosurfactants are natural surface active agents produced by fungi these products are shown to be efficient in process of microbial enhanced oil recovery and bioremediation in hydrocarbon contaminated environments. They possess potential applications in agriculture, pharmaceutical and cosmetic industries. They are more advantageous than the synthetic derived ones when considering their biodegradability, low toxicity, lower CMC and better environmental compatibility

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