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Production of Biosurfactant from Fungi and Yeast by Using Oil Mill Waste

E.SherylChrisentia&Dr.PriyaIyer
PG & Research Department of Biotechnnology,
Women's Christian College, Chennai, Tamil Nadu, India
(An Autonomous Institution Affiliated to the University of Madras)

Abstract
One yeast and three fungal species were isolated from oil mill waste and was identified as Millerozymafarinosa, Aspergillusflavus, Aspergillusniger and Aspergillusfumigatus. The yeast species was isolated on Mannitol yeast agar and the fungal species on Sabouraud dextrose agar. The biosurfactant was produced through submerged fermentation in minimal salt media broth supplemented with 2%dextrose and the biosurfactant screened using various methods. The biosurfactant was characterized. Millerozymafarinosa yielded maximum positive results and the biosurfactant produced by it was purified using dialysis membrane and thenanalysed using Thin layer chromatography and Fourier transform infrared spectroscopy. The optimum growth conditions were standardized using the factors of pH, temperature, carbon and nitrogen source. It was measured using the drop weight quantitative test. The yeast species was given for Internal Transcribed Spacer sequencing and was identified as Millerozymafarinosa. The biosurfactant produced is a glycerophospholipid which was then used to bio-remediate crude oil and in the production of an efficient handwash.

Keywords: *Glycerophospholipid, Yeast, TLC,FTIR*

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I. Introduction

Biosurfactants are amphiphillic compounds produced extracellularly by filamentous fungi, yeast and bacteria.(2) They are surface active compounds that are biodegradable, non-toxic and eco-friendly.(1). They also have a good temperature and pH tolerance.They help in reducing surface and interfacial tension by accumulating between two interfaces. They are also able to from emulsions, break emulsions and are used as anti-adhesive agents.(3)

II. Materials and Methods

2.1.Isolation and Identification of Organisms

Coconut, sesame and groundnut pomace was collected from two different shops in Chennai,India and was added to nutrient agar, nutrient broth and on Minimal salt media plates(supplemented with 2%dextrose).(2) The organisms were identified using simple staining and Lactophenol cotton blue staining method.

The fungal organisms were identified and grown on Sabouraud Dextrose Agar. The yeast species was isolated on Mannitol yeast agar. All the species were grown in minimal salt media broth supplemented with 2% dextrose with shaking. Solid state fermentation was done using 5g of the pomace and a salt solution of 0.5g of Ammonium chloride, 0.9g of Sodium chloride, 0.1g of Magnesium sulphatehepatahydrate dissolved in 100ml of distilled water to moisten the pomace.(17)

2.20. Screening of Biosurfactant

2.21. CTAB Test

To test the presence of anionic surfactants, 100µl of biosurfactant produced was added to wells in agar plates. The agar plate constitutes 0.5g of glucose, 0.25 g of peptone, 0.025g of beef extract, 0.02 g of CTAB,0.0001 g of methylene blue, 0.013g of yeast extract and 2.5-3% agar. The pH was adjusted to 7.2. (18)

2.22. Emulsification Assay

2ml of kerosene was added to cell-free supernatant and vortexed for 2 minutes. It was then incubated for 24 hours. The emulsion index was calculated as:

E_{24}= Height of emulsion x 100%
Height of total liquid

E_{24} correlates with the surfactant concentration and indicates the presence of biosurfactant.

Other tests like oil spreading assay, microplate assay, penetration assay and oil collapse/drop collapse test was also done.(18)

2.30. Characterisationof Biosurfactant

2.31. Rhamnose Test

0.5ml of the cell free supernatant is taken and 0.5ml of 5%phenol solution is added to it. 2.5ml of sulphuric acid is then added and it is incubated for 15minutes. The readings were then taken at 490nmusing a UV spectrophotometer.(13)

2.32. Rhamnolipid Quantification using Methylene blue method

Dry extract was dissolved in 4ml of chloroform and then mixed with 400µl of freshly prepared methylene blue solution. It was then mixed by vortexing for 5 minutes. It was then incubated at room temperature for 15minutes to allow colour formation. 1ml of the lower chloroform layer was then takenin a centrifuge tube and vortexed for 20seconds with 500µl of 0.2N HCl. It was then centrifuged at 100g for 1 minute. It was incubated at room temperature for 10 minutes. 200µl of the upper acidic phase was taken and the reading was taken at 638nm. (14)

2.33. Phosphate Test

10 drops of 6M HNO_3 was added to 2ml of cell free supernatant and heated to 70°C. 5% ammonium molybdate is added drop by drop and was observed. (10)

Molisch's test for carbohydrates and biuret test was also done to all the samples.(10)

2.4. Extraction of Biosurfactant

The broth was centrifuged at 5000rpm for 20minutes at 4°C using a REMI coolingcentrifuge. The crude biosurfactant was extracted using 1:1 ethyl acetate: cell free supernatant ratio in a separating funnel at room temperature. The ethyl acetate phase was collected and evaporated at room temperature, to obtain crude product. (11)

2.50. Analysis of Biosurfactant

2.51. Thin Layer Chromatography

Partial purification and identification of the crude biosurfactant was done using TLC plates impregnated with sample. The solvent system was chloroform: methanol: acetic acid: water in the ratio 25:15:4:2. It was then dried and sprayed with α-naphthol dissolved in concentrated sulphuric acid. It was then placed in the hot air oven at 110°C for 5minutes.

2.52. Fourier Transform Infrared Spectroscopy

The pure biosurfactant was obtained in hexane and FTIR was done using the Bruker Alpha-T ATR-FTIR Spectrometer. The crystal was cleaned using isopropanol. The background was measured and the sample was placed on the ATR crystal. The infrared beam reflected of the surface and was detected using a suitabledetector.

2.6. Purification of Biosurfactant

The cell free supernatant was extracted using hexane in 1:1 ratio. It was then purified using dialysis membrane and placed in double distilled water at 20°C. The dialysis membrane was changed once.

2.70. Standardisation for Optimum Growth using Drop Weight Quantitative Test

It is done using the cell free supernatant. A pre-weighed beaker is placed beneath a burette filled with the supernatant. The supernatant was released slowly drop by drop till 20 drops were collected. The weight of the 20 drops along with the beaker was then measured.

The mass of 1 drop = (Beaker + sample weight) – Empty beaker weight/number ofdrops. Surface tension (T) = $Mg/\pi r \times 10^{-3} \times nm^{-1}$ (Where, M – Mass of one drop, g – Gravity, r – Radius of glasstube)Surface activity = (surface tension of un-inoculated medium) – (surface tension of supernatant) (8)

2.71. Standardisation of pH for Optimum Growth

The pH was adjusted to 3 by addition of concentrated HCL in flask 1. The pH was adjusted to 7 in flask 2. The pH was adjusted to 9 by addition of NaOH. The culture was inoculated and incubated at room temperature in a shaker. The best growth conditions were observed using drop weight quantitative method.**2.72. Standardisation of Temperature for Optimum Growth**

The culture was inoculated and incubated at the following temperatures of 4°C, 10°C, 30°C and 40°C. The best growth conditions were observed using drop weight quantitativemethod.**2.73.Standardisationof Carbon source for Optimum Growth**

Carbon source standardization was done using glucose, sucrose, fructose and maltose. The culture was inoculated and incubated at room temperature in a shaker. The best growth conditions were observed using drop weight quantitative method.

2.74. Standardisation of Nitrogen Source for Optimum Growth

Nitrogen source standardization was done using peptone and ammonium nitrate. The culture was inoculated and incubated at room temperature in a shaker. The best growth conditions were observed using drop weight quantitative method.

2.8. Sequencing Studies

The yeast species was sent for Internal Transcribed Spacer sequencing (ITS) method done by BT Scientific Services, Tamil Nadu.

- DNA extraction- Yeast extract peptone dextrose agar (YEPD) was prepared by adding 1.63g of media in 25ml of distilled water. The yeast species was grown on it. Two loops of the culture were collected and lysed using preheated CTAB. It was incubated at 65°C for 30minutes. The suspension was cooled and equal amount of phenol:chloroform(1:1) was added. It is mixed well by vortexing. The suspension is centrifuged at 7000rpm for 10minutes at room temperature. The aqueous layer is transferred to another eppendorf and the phenol:chloroform extraction is repeated twice. Ice cold isopropanol is added to it and mixed well.It is then centrifuged at 10,000rpm for 10 minutes. The pellet is collected and air dried. The pellet is re-suspended in Tris-EDTAbuffer.
- Amplification and Sequencing- Amplification was performed in a PCR machine in a volume of 20 µlcontaining 3 mM$MgCl_2$, 0.5 µM ofeach specific primer, 2 µlof SYBR green master mix, and 2 µlof eluted DNA. Taq DNA polymerase (Genei) was also used. Cycling parameters includedan initial denaturation for 10 min at 95°C and 50 cycles of 1 sec at 95°C, 5 sec at 53°C, and 40 sec at72°C.
- The amplified products were checked on 1% agarose gel.The amplified product was purified using polyethylene glycol. Equal volume of 1.6M NaCl containing 13%(W/V) Polyethylene glycol 10,000 was added. It is mixed well and incubated for 30minutes on ice. The pure DNA is then recovered by centrifugation at 12,000rpm for 3 minutes at room temperature. The DNA is extracted using Tris-EDTAbuffer.
- The purified DNA was sequenced using ABI 3730XL with BDT ver 3.1 chemistry.

2.90. Application Studies

2.91. Bioremediation of Crude oil

3 conical flasks with minimal media were prepared. 2ml of crude oil was added to them under sterile conditions. In flask 2, 10ml of extracted crude biosurfactant was added. In flask 3, 1g of soil collected from a mechanic shed was added. It was incubated for 5days in ashaker.

Paper chromatography was done using solvent system as chloroform: acetic acid (1:1). The paper was dried completely and then placed in an iodine chamber.

10ml of extracted crude biosurfactant was added to flask 3 with the soil. It was then incubated for 5 days. Paper chromatography was done and once the paper dried completely, it was placed in an iodine chamber.

2.92. Hand Wash Formulation

The ethyl acetate extracted biosurfactant was dried. It was then heated for 2-3 minutes. 5ml of potassium hydroxide dissolved in water was added and stirred continuously. Once it starts boiling, it's removed from the heat and 20%boric acid solution is added to neutralize thecaustic action of potassium hydroxide. Sodium bicarbonate was added to increase the foaming ability. Colour, fragrance and glycerol are added in appropriate quantities. It's allowed to cool for an hour to let the mixture thicken.

III. Results and Discussion

3.1.Isolation and Identification

Growth was observed in nutrient agar, nutrient broth, minimal salt media plates and minimal salt media broths. Solid state fermentation also yielded growth of the fungus and yeast species.(1)

Table 1: Colony Characteristics of Fungi and Yeast species

CHARACTERISTICS	*Aspergillusfumigatus*	*Aspergillusniger*	*Aspergillusflavus*	Yeast species
Surface colour	Green	Black	Greyish green	Light cream coloured
Margins	Entire	Entire	Entire	Entire
Reverse side	Yellow	Without colour	Yellowish	Creamish
Elevations	Umbonate	Umbonate	Umbonate	Convex
Spore	Oval and multinucleate	Oval and multinucleate	Oval and uninucleate	-
Arrangement	Septate	Septate	Septate	-

On simple staining and Lactophenol cotton blue staining, the fungal species were identified as *Aspergillusniger, Aspergillusflavus*and *Aspergillusfumigatus.* The cream coloured growth was identified as yeast.
3.2. Screening of Biosurfactant 3.21.CTAB Test Negative results were obtained indicating the absence ofextracellular glycolipids and other anionic surfactants.(18)**3.22. Emulsification Assay** All the samples responded positively for this assay.

TABLE 2: The emulsion index of the various organisms

SPECIES	CALCULATION	EMULSION INDEX
Yeast spp	(0.9/1.8)x100	50.0 %
Aspergillusfumigatus	(0.8/1.8)x100	44.4%
Aspergillusflavus	(0.5/1.8)x100	27.7%
Aspergillusniger	(0.4/1.8)x100	22.2%

All the samples responded positively for the oil spreading assayby displacing the oil and creating a clear zone. The biosurfactant from yeast yielded larger clearancezones.(36) All the samples responded positively for the oil collapse/drop collaspse assay and this was observed by collapsing of the drops. (7) All the samples responded positively for the microplate assay. All the samples responded positively for this assay by changing from red colour to cloudy white. (18)

3.3. Characterisation of Biosurfactant
3.31. Rhamnose Test
The fungal species gave positive results for this test. The absorbance was measured at 490nm.

TABLE 3: Rhamnose test-Absorbance values of the organisms at 490nm

SPECIES	ABSORBANCE AT 490nm
Yeast spp	0.79
Aspergillusfumigatus	0.84
Aspergillusflavus	0.89
Aspergillusniger	0.83

3.32. Rhamnolipid Quantification using Methylene blue method
The chloroform phase developed a blue colourin the *Aspergillusflavus*samples. This was added to HCL and phase separation was done. The upper acidic phase was taken and absorbance measured at 638nm**.** It is a quantification method. The amount of biosurfactant in the *Aspergillusflavus*broth sample is approximately 12µg/ml. The amount of biosurfactant in the *Aspergillusflavus*coconut sample (solid state fermentation) is approximately 25-50µg/ml.

TABLE 4: Rhamnolipid quantification of different samples and species

SAMPLE	ABSORBANCE AT 638nm
BROTH SAMPLES	
Yeast species	0.001
Aspergillusfumigatus	0.001
Aspergillusflavus	**0.17**
Aspergillusniger	0.001
COCONUT SAMPLES-SOLID STATE FERMENTATION	
Yeast species	0.001
Aspergillusfumigatus	0.001
Aspergillusflavus	**0.264**
Aspergillusniger	0.001
SESAME SAMPLES-SOLID STATE FERMENTATION	
Yeast species	0.001
Aspergillusfumigatus	0.001
Aspergillusflavus	0.001

Aspergillusniger	0.001

3.33. Phosphate Test

Yeast species gave positive result by producing a yellow colour and a fine yellow precipitate which slowly formed. It indicates the presence of phospholipidbiosurfactant.(10)

*Aspergillusflavus*and the *Aspergillusfumigatus*gave positive results for Molisch'stest.All samples gave a negative result for the Biuret test indicating the absence of lipopeptide.

3.4. Analysis of Biosurfactant

3.41. Thin Layer Chromatography

The spots appeared and darkened after placing it in the hot air oven indicating the presence of carbohydrates. (33)

3.42. Fourier Transform Infrared Spectroscopy

- The PO_2 group's presence is indicated by a small peak at around $980cm^{-1}$.
- The P=O vibration is between 1250 and $1300cm^{-1}$in the referred paper and in comparison, there is a slight peak in the same region indicating the presence of phosphate bond.
- The C=O bond is indicated by the peak around $1700cm^{-1.}$
- A peak is observed between 1020 and $1282cm^{-1}$ indicating the presence ofC-O-C bonds(Ether).

Therefore, the compound is aglycerophospholipid.(19)

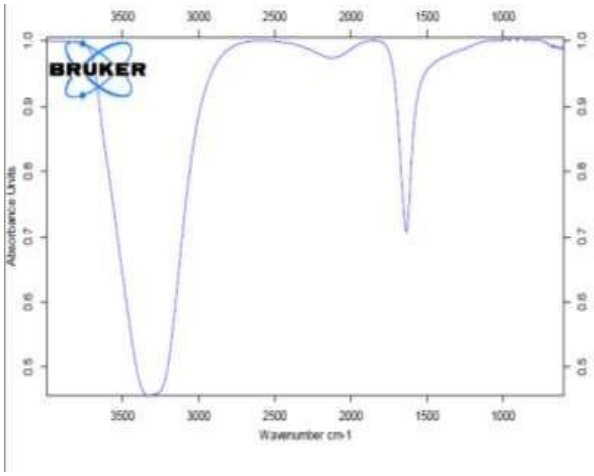

FIGURE 1: FTIR graph of absorbance against wavenumber.

3.50.Standardisation for Optimum Growth using Drop Weight Quantitative Test

3.51.Standardisation of pH for Optimum Growth The optimum pH was identified as pH 9 for yeast and Aspergillus*fumigatus*. The *Aspergillusflavus*and *Aspergillusniger*species grew best at pH 3. (14)

3.52.Standardisation of Temperature for Optimum Growth

The optimum temperature for yeast species growth was identified as 40 ºC. The optimum temperature for *Aspergillusfumigatus*growth was identified as 30 ºC. The optimum temperature for *Aspergillusflavus*growth was identified as 30 ºC. *Aspergillusniger*grew at 30 ºC. (14)

It was also observed that better results and faster growth was obtained when the culture was incubated in a shaker.

3.53.Standardisation of Carbon Source for Optimum Growth The optimum carbon source for yeast species growth was found to be sucrose. The optimum carbon source for *Aspergillusfumigatus'* growth was found to be glucose, sucrose and mannitol.

The optimum carbon source for *Aspergillusflavus*growth was found to be glucose and sucrose. The optimum carbon source for *Aspergillusniger*growth was found to be fructose. (14)

3.54.Standardisation of Nitrogen Source for Optimum Growth

The optimum nitrogen source for yeast species growth was found to be peptone. The optimum nitrogen source for *Aspergillusfumigatus'* growth was found to be peptone and ammonium nitrate. The optimum nitrogen source *Aspergillusflavus*growth was found to be peptone. The optimum nitrogen source for *Aspergillusniger*growth was found to be ammonium nitrate. (14)

3.6. Sequencing Studies

The species was identified as *Millerozymafarinosa*using the ITS sequencing method.

Classification
Division: Ascomycota
Subdivision: Saccharomycotina
Class: Saccharomycetes
Subclass: Saccharomycetidae
Order: Saccharomycetales
Family: Debaryomycetaceae
Genus: Millerozyma

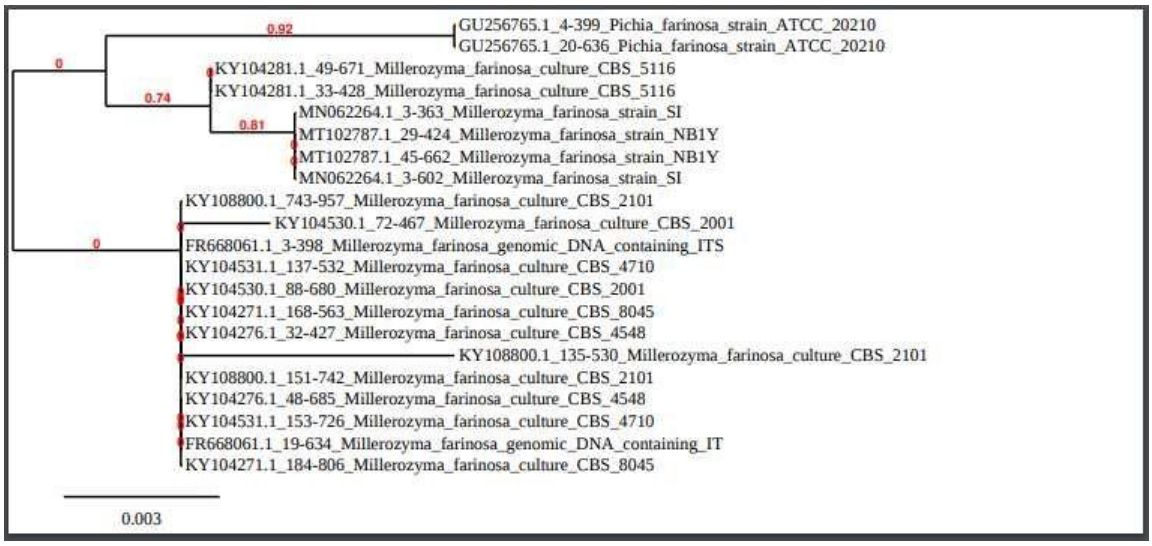

FIGURE 2: Phylogenetic tree of *Millerozymafarinosa*

3.7. Application Studies

3.71. Bioremediation of Crude Oil

The paper chromatography from flask 1 indicated the presence of crude oil. In flask 2, it was observed that the biosurfactant was able to break the crude oil but not very efficiently. In flask 3, it was observed that there was a slight break down of oil.
Paper chromatography was done after the addition of biosurfactant to flask 3 and after a five day incubation period indicated that the crude oil broke down completely.
(a) (b)

FIGURE 3: (a) Paper chromatograhy done after 5 day incubation;**(b)** Paper chromatography done after addition of biosurfactant to flask 3

This study shows that biosurfactant with the organisms capable of oil degradation were successful in bio-remedying the crude oil contamination. This shows that it can be used in large scale bioremediation activities.(4)

3.72. Hand Wash Formulation

It formed an efficient soap and produced mild foam.(5)

IV. Conclusion

The entire production of biosurfactants was relied upon submerged fermentation. Further work can be done on using solid state fermentation, which is a greener bioprocess by using cheap agricultural waste and

spent crude oil as the solid state. (Kiran.et.al.,2015) This would be a preferred method as there is lesser water, raw materials and agricultural waste as substrates requirement. It also has a lower energy requirement. It also produced a higher quantity of biosurfactants. Genetic analysis can also be done for the isolated organisms.

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