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# INTERNATIONAL JOURNAL OF CURRENT RESEARCH IN BIOLOGY AND MEDICINE

ISSN: 2455-944X

[www.darshanpublishers.com](http://www.darshanpublishers.com)

DOI:10.22192/ijcrbm

Volume 1, Issue 8 - 2016

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**Original Research Article**DOI: <http://dx.doi.org/10.22192/ijcrbm.2016.01.08.004>

## Isolation and Characterization of Biosurfactant Producing *Pseudomonas* sp isolated from Porto Novo Coastal Region – Cuddalore

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### Abstract

Biosurfactants are amphiphilic compounds produced by microorganisms as secondary metabolite. Biosurfactants are microbially produced surface active agents and occur in nature as chemical entities such as glycolipids, phospholipids and lipopeptides. These molecules have attracted considerable scientific attention due to lower toxicity, higher biodegradability. The present study deals with the screening, production and characterization of a biosurfactant by *Pseudomonas* sp (PNB 34 and PNB 51) from the sediment samples of Porto Novo coastal region, Cuddalore, Tamil Nadu. Thus, the nature of the biosurfactants produced from the isolates in our study was a rhamnolipid - type. They had a good oil displacement ( 2.2), haemolytic and emulsifying properties (52.6) The obtained results suggests that the marine isolates has got the great industrial importance.

**Keywords:** Biosurfactant, *Pseudomonas* sp, Glycolipid.

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### Introduction

Biosurfactants are amphiphilic compounds that reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids or of a fluid and solid, and consequently, increase the surface areas of insoluble compounds leading to increased mobility, bioavailability and subsequent biodegradation and emulsification (Ron and Rosernberg, 2001; Vasileva-Tonkova and Gesheva, 2007). The biological function of this surface-active compound is related to hydrocarbon uptake, where spontaneous release occurs with hydrocarbons as substrates (Guerra-Santos *et al.*, 1984; Makkar and Cameotra, 1998; Singh *et al.*, 2006).

Biosurfactants are categorized by their chemical composition and microbial origin. One of the prevalent class is glycolipids constituting mono-, di-, tri- saccharides produced by *Rhodococcus erythropolis* used in oil spill cleanup operation (Peng *et al.*, 2007). Sophorolipids are another class of biosurfactant produced by *Candida bombicola* having environmental application (Daverey and Pakshirajan, 2010). Rhamnolipid produced by *Pseudomonas aeruginosa* too have application in bioremediation of oil contaminated sites (Chen *et al.*, 2007).

Biosurfactants are surface-active, degradable organic compounds produced by microorganisms when grown on water immiscible substrates. They help to reduce surface and interfacial tension (Pacheco *et al.*, 2010) thus forming stable water-oil emulsions which is important for maximum oil extraction. Biosurfactant solubility and activity may be affected by salt concentration and pH with effective pH ranging between 4 and 10 (Al-Bahry *et al.*, 2013). The objectives of the present investigation aims at isolation of an interesting isolate from the sampling site followed by production intended to the specific metabolite, further biochemical and application studies of the partially purified biosurfactant of the promising isolate. Isolation of biosurfactant producing bacteria from sediment samples of Porto Novo coastal sites, Cuddalore District, Tamil Nadu

## Materials and Methods

### Isolation of bacterial colonies:

5g of sediment sample was inoculated in 100 ml of Mineral Salt Medium (MSM) with 3ml crude oil added to the conical flask having capacity of 250 ml, as the carbon sources and then it was incubated for 72 hours at 30°C temperature. After then 1ml of incubated culture was streaked on the petriplates. The samples then were serially diluted up to 10<sup>-6</sup> dilution. 1 ml of 10<sup>-6</sup> time dilution was transferred to nutrient agar for spread culture. The plate was inverted and incubated at 30° C, for 72 hours. After incubation, morphologically five distinct colonies were selected for further studies and identified the species by Bergey's manual of Systematic Bacteriology (Palleroni *et al.*, 1984).

### Screening of Biosurfactant producing bacteria:

The isolated colonies were taken and tested for the confirmation of biosurfactant bacteria that comprises following test:

#### Oil spreading technique:

In oil spreading assay for oil displacement activity of surfactants was done as per the method described by Morikawa *et al.* (1993). The principle of this method was based on the ability of the biosurfactant to alter the contact angle at the oil-water interface. The surface pressure of the biosurfactant displaced the oil. In this method 10µl of crude oil was added to the surface of 20 ml distilled water in a petri dish, oil form a thin layer then 10µl cultured supernatant gently placed on

the center of oil layer. If the oil displaces and clear zone forms then it shows the presence of biosurfactant. The displaced diameter is measured after 30 second. This is also known as oil displacement activity. Measured area is express in BS unit, known as biosurfactant unit. One biosurfactant unit (BS unit) was defined as the amount of surfactant forming 1 cm<sup>2</sup> of oil displaced area.

#### Emulsification activity:

Emulsification activity was calculated by emulsification index known as E<sub>24</sub>. Emulsification assay was carried by adding 1ml crude oil in 1ml cell free supernatant which was obtained after the centrifugation, and then it was vortexed for 5 minutes confirm regular mixing of both the liquids. The emulsification activity was observed after 24 hours and it was calculated by using the formula:

$$E_{24} = \text{Total height of the emulsion layer/height of the aqueous layer} * 100$$

#### 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 37°C on a shaker incubator (200 rpm) for 72 h. Foam activity is detected as duration of foam stability, foam height and foam shape in the graduated cylinder.

#### Haemolytic activity

The haemolytic activity of the biosurfactant was evaluated on blood agar plates (Carillo *et al.*, 1996). Blood agar plates were prepared by adding blood from a voluntary human donor into sterile blood agar base medium and by allowing the plates to solidify under aseptic environment of a laminar air flow. After solidification of agar plates, wells were made using well cutter and 50 µl of the cell free supernatant was poured into the wells. Further, the plates were incubated under normal atmospheric conditions at 37°C overnight. After overnight incubation, the plates were checked for the zone of haemolysis.

#### Oil displacement test

The oil spreading test was done by adding 20 ml of distilled water to a Petri dish with a diameter of 8 cm in which 15 µl of crude oil was dropped to form a thin oil layer on the surface of the water and then 10 µl of a centrifuged supernatant was added onto the surface of

the oil (Youssef *et al.*, 2004). The diameter of the clear zone was observed under light and measured.

### Rhamnose test

The presence of carbohydrate groups in the biosurfactant molecule was assayed by rhamnose test (Dubois *et al.*, 1956). A volume of 0.5ml of surfactant was mixed with 0.5 ml of 5% phenol and 2.5ml of sulfuric acid and incubated for 15min before measuring absorbance at 490 nm.

## Results and Discussion

### Screening of Bacterial Isolates.

Five isolates were isolated from the marine sediment samples of Porto Novo coastal region, Cuddalore. The morphologically distinct isolates were identified as *Pseudomonas* sp by morphological, physiological

properties in accordance with Bergy's Manual of Determinative Bacteriology also screened for the confirmation of biosurfactant producing bacteria in which two isolates (PNB 34 and PNB 51) showed good result and these two were taken for the further study. Willumsen and Karlson, (1997) isolated biosurfactant producing bacteria from soil which was contaminated with polyaromatic hydrocarbons (PAHs). They used PAH-amended liquid minimal medium for enrichment culture.

### Oil Spreading Technique:

Supernatant of isolated strains were added to the plate containing kerosene oil. It was added to the center of oil layer. The isolates PNB 34 and PNB 51 displaced the oil and showing a clear zone. The results were noted down. Bacterial isolates PNB 34 and PNB 51 was chosen for the further study.

**Table.1 Result of oil spreading technique.**

Bacterial isolates	Diameter of clear zone (in cm)	Interpretation
PNB 34	2.2	Positive
PNB 51	1.9	Positive
PNB 42	0	Negative
PNB 45	No result	Negative
PNB 56	0	Negative

### Emulsification assay:

Marine bacteria were examined for emulsification activity (EA) and emulsification stability (ES) of wide variety of hydrocarbons and vegetable oils. Similar study was conducted by Aparna *et al.*, (2011) reported maximum emulsification activity of *P. aeruginosa* at 72h (80%). Priya and Usharani (2009) reported E24

40% at 24 h. 72% were also reported Sneha *et al.*, (2012). The isolates showed positive result were tested for their abilities emulsify crude oil and in this study crude was take for the study of emulsification assay. Test was done by the adding 1ml of crude oil and 1 ml of supernatant and kept overnight. After then results were noted down.

**Table.2 E<sub>24</sub> index of bacterial isolates**

Bacterial isolates	Emulsified layer(cm)	Total (cm)	liquid layer	E24 (%)
PNB 34	1.00		1.8	52.6
PNB 51	0.8		1.9	42.1

### Foaming Activity:

Total disappearance of the foam was observed at 1hr 18min. Abouseoud *et al.*, (2007) reported total disappearance of the foam was detected after 2h. Gujar and Hamde (2012) also reported total disappearance of the foam was detected after 2h. Emulsification activity gave indication on the presence of biosurfactant. Higher emulsification index indicated a higher

emulsification activity of the tested biosurfactant. Palm oil was the best substrates for biosurfactant having higher emulsification activity followed by soyabean, olive and mustard oil and least by coconut oil at 0h, 24h, 48h, 72h respectively. Isolates 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.

**Haemolytic activity:**

The present study revealed the  $\alpha$ -hemolytic pattern on blood agar for screening biosurfactant activity of *Pseudomonas* sp. Similar study was conducted by Anandraj and Thivakaran, (2010) in which *Pseudomonas* was screened for biosurfactant producing activity on blood agar medium that showed an alpha hemolytic pattern. Blood haemolysis assay is used for preliminary screening of microorganism for the ability to produce biosurfactants. Therefore, those microorganisms which shows positive blood haemolysis are considered as potential biosurfactant producers. The approach to the screening method is valid because biosurfactants would cause lysis of erythrocytes. The assay also predicts about the surface activity of biosurfactant producing microorganisms. In

comparison to the present study similar result with culture supernatant of *Pseudomonas aeruginosa* was observed alpha haemolytic activity by Satpute *et al.* (2008); Nicholls *et al.* (2000); Anandraj and Thivakaran (2010); Samanta *et al.* (2012) and Sneha *et al.* (2012) who used blood haemolysis test for screening of biosurfactant producing organisms..

**Rhamnose test:**

The optical density increase with increasing the concentration of supernatant which confirmed that the rhamnose test was positive and separated biosurfactant could be of glycolipid type. (Table: 3). Gujar and Hamde (2012) also reported rhamnose test positive indicating biosurfactant could be of rhamnolipid type. Abouseoud *et al.* (2007) also reported rhamnose test was positive which indicates that the separated biosurfactant was of glycolipid type.

**Table: 3 Quantitative estimation of carbohydrate by rhamnose test (PNB1 & PNB2)**

S.No	PNB 34		PNB 51	
	Concentration (ml)	Optical Density (490nm)	Concentration (ml)	Optical Density (490nm)
1	Blank	0	Blank	0
2	0.2	0.70	0.2	0.75
3	0.4	0.85	0.4	0.89
4	0.6	0.98	0.6	1.02
5	0.8	1.52	0.8	1.54
6	1.0	1.70	1.0	1.64
7	1.2	1.82	1.2	1.75

**Conclusion**

In this era of green technology biosurfactant have led considerable interest for present and future application. In this study, biosurfactant produced from *Pseudomonas* sp was chemically characterized as glycolipid mainly consisting of lipid and carbohydrate. The result from the study reports that even from the cheapest carbon source (crude oil) at very less concentration of 2% a good biosurfactant can be produced. This has opened up a practically significant and commercially viable biotechnological approach to produce varieties of biosurfactants having huge industrial application.

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**How to cite this article:**

Bhuvaneswari. M, Sivagurunathan. P, Uma. C. (2016). Isolation and Characterization of Biosurfactant Producing *Pseudomonas* sp isolated from Porto Novo Coastal Region – Cuddalore. *Int. J. Curr. Res. Biol. Med.* 1(8): 25-29. **DOI:** <http://dx.doi.org/10.22192/ijcrbm.2016.01.08.004>