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Research Article

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Utilization of Different carbon source for Biosurfactant Production by a Marine *Pseudomonas* sp PNB 34 from Cuddalore District.

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Abstract

Marine microorganisms produce extracellular or membrane associated surface-active compounds (biosurfactants). Surfactants are surface active compound that reduce the interfacial tension between two liquids, or that between a liquid and a solid. The specific surface active compounds produced by marine bacteria are able to utilize different carbon substrates for the production of an extracellular biosurfactant was evaluated. Bushnell Haas (BH) liquid medium was used as the enrichment medium with 1 %(v/v) crude as the sole carbon source to sole ate diesel degrading bacteria.. Among the several carbon substrates tested, production of the crude biosurfactant was found to be highest with sucrose.. The examination of biosurfactant production with different carbon sources revealed that it was produced maximum with 2% of sucrose . The results showed the *Pseudomonas* sp (PNB 34) could be effective for crude oil biodegradation Also the carbon substrate was found to affect biosurfactant production both in a qualitative and quantitative manner.

Keywords: Bushnell Haas, *Pseudomonas* sp, Biosurfactant, , Sucrose.

Introduction

"Biosurfactants" are microbially produced, structurally diverse group of surface active biochemical molecules. The huge diversity of biosurfactants makes them an interesting moiety for application in many areas including agriculture, health care, public health, food, waste utilization, and environmental pollution control, like degradation of hydrocarbons present(Al-Araji *et al*., 2007).

Biosurfactants often have interesting characteristics not possessed by petrochemical or oleochemical surfactants, like their application in "pharmaceuticals", "bioremediation", "food processing", nevertheless, for this to happen actually in an industrial scale, there needs to be the improved development of fermentation processes and downstream separation techniques for effective biosurfactant production (Al-Araji, 2007; Banat, 2010 Sharma, 2015).

Surfactants are organic compound that contain both hydrophobic (head part of the surfactant) and

hydrophilic (tail part of the surfactant) moieties. Thus surfactant contains both water insoluble i.e. water repellent groups as well as water soluble i.e. water loving group. Biosurfactants are also surface active compound like chemical surfactants but unlike the chemical surfactant, biosurfactant are synthesized by microbes like bacteria, fungi and yeast. Biosurfactants comprise the properties of dropping surface tension, stabilizing emulsions, promoting foaming and are usually non-toxic and biodegradable. Recently interest in biosurfactant has increased because of its diversity, flexibility in operation, and more ecofriendly then chemical surfactant (Abadi *et al*., 2009).

Efforts should be oriented toward the optimization of processes for the production of biosurfactants that result in high productivity on a commercial scale so that they can compete with synthetic surfactants in terms of cost. One of the strategies that has been suggested in the literature is the limitation of nutrients (Desai & Banat, 1997; Chayabutra, Wu & Ju, 2001), mainly nitrogen sources, as well as multivalent cations (Syldatk *et al*., 1985; Glick *et al*., 2010) and some anions (Mulligan, Mahmourides & Gibbs, 1989; Clarke, Ballot & Reid, 2010), as a condition necessary for stimulating the synthesis of rhamnolipids by P. aeruginosa. In addition to nutritional modifications, changes in physical factors such as temperature and pHcan also influence the synthesis of rhamnolipids by *P. aeruginosa* (Sousa *et al*., 2011; Jamal *et al*., 2014). The aim of this work was to improve the cultivation conditions for the production of biosurfactant by a marine bacterial isolate from Porto Novo coastal Region- Cuddalore. This study involved the investigation of the effect of a variation in the pH of the culture medium; the medium supplementation with recycled lubricant oil and peanut oil cake, Soybean oil cake, Oil recovery and estimation of emulsification activity of the biosurfactant.

Materials and Methods

Collection of sample

Isolation, screening and identification of biosurfactant producing marine bacteria from sea sediment samples were collected from Porto Novo coastal region – Cuddalore, Tamil Nadu, India. These samples were transported to the laboratory in 250 ml pre-sterilized bottles and stored at 4°C until further processing.

Isolation and screening of potential bacteria

The central portions of the samples were serially diluted using pre-sterilized seawater and spread plated on Bushnell Haas agar prepared with sea water and supplemented with 1% (v/v) crude oil. After 4 days incubation, morphologicaly distinct colonies were isolated, and sub cultured 4-5 times on Zobell marine agar plates to obtain axeniccultures and stored at 4°C for further studies (Morikawa *et al.*, 1993).

Characterization of biosurfactant-producing isolates

The selected biosurfactant-producing bacteria were characterized morphologically and biochemically. Further, identified to species level by following Bergey's Manual of Determinative Bacteriology (Buchanan *et al.*, 1974).

Screening of biosurfactant producing microorganism

Biosurfactants production is detected by various techniques.
Drop Collapsing technique
Hemolytic activity
CTAB Agar Plate method

Drop collapsing technique

The isolates were grown in BH medium with diesel as carbon source, incubated with shaking for 48 hours at 37ºC and 200 rpm. The glass slides used was rinsed with hot water, ethanol and distilled water, and dried. The slides were coated with diesel and equilibrated for24 hours to ensure a uniform oil coating. 1 µl of supernatant sample was then applied to the center of the oil drops using 10µl micropipette. The results were monitored visually after 1 hour. If the drop remained beaded, the result was scored as negative. If the drop collapsed, the result was scored as positive. (Jain *et al*. (1991) and modified by Bodour and Maier (1998)

Heamolysis test

Fresh colonies were prepared by streaking on nutrient agar and incubate at 37ºC for 24hrs.These fresh single colonies of culture are restreaked into blood agar plates and the plates were incubated at37ºC 48-72hrs.The bacterial colonies were then observed for the presence of clear zones around the colonies. These clear zones were used as qualitative method for biosurfactant production. (Carrillo *et al*., 1996).

CTAB Agar Plate method

The CTAB agar plate method is a semi-quantitative assay for the detection of extra cellular glycolipids

orother anionic surfactants. It was developed by Siegmund and Wagner. The microbes of interest are cultivated on a light blue mineral salts agar platecontaining the cationic surfactant cetyltrimethylammonium bromide and the basic dyemethylene blue. If anionic surfactants are secreted by the microbes growing on the plate, they form a darkblue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos.

Biosurfactant production on MSM supplemented with different carbon source in the form of oil

The *Pseudomonas aeruginosa* isolate was inoculated in Mineral salt liquid medium is used for production of biosurfactant. The pH was adjusted to 7 before autoclaving at 121ºC for 15minutes. After sterilization, 2 % of the different carbon sources such as glucose, fructose, xylose, maltose, sorbitol, mannitol and sucrose separatively were added. Then a bacterium was inoculated in to the mineral salt medium and it was placed on a reciprocal shaker at 100rpm at 37°C for 3 days.

Shake flask cultivation

Cultivation experiment was carried out in 500 ml shake flask. Cheap carbon source had to use, hence Glucose is used as a standard carbon source. The phosphate regulation was carried out by adapting minimal salt media (MSM) composition from Cameotra *et al*. (1998). Carbon to the nitrogen ratio i.e. C:N was maintained 20, by using $NaNO^3$ as a nitrogen source.

The optimization strategy was carried out by varying the percentage of carbon source used. 1%, 3% and 5% of carbon sources mentioned above were examined for the highest production of rhamnolipid. At the same time, pH of the media was varied using 0.1N HCl and 0.1N NaOH. Shake flask was operated at pH 5, pH 7 and pH 9 respectively after the Carbon source and its percentage optimisation.

The optimum carbon source, its concentration and optimum pH was calculated for highest rhamnolipid production. The other operating conditions, like temperature, agitation speed were properly maintained. The operating temperature was set 30°C and agitation speed was maintained 150 rpm in remi orbital shaker-incubator. During the cultivation period, biomass concentration, substrate concentration and rhamnolipid concentration were checked every after 24 hrs.

Influence of different carbon source

Different carbon sources were examined for the enhanced production of biosurfactant. Various carbon substrates such as glucose, fructose, maltose, sorbitol, Xylose ,manitol and sucrose with 2% (v/w) were individually examined in the basal mineral medium replacing glucose in the actual medium.

Results and Discussion

In this study, marine sediment samples were collected from Porto Novo coastal region- Cuddalore (Dt), Tamil Nadu, India. The examples were handled for the isolation of biosurfactant bacteria and among five colonies of bacteria were acquired on Bushnell Haas agar plate. Among them,only one bacteria produced biosurfactant. So examined the selected bacteria by morphologically unmistakable colony was separated and named as PNB 34 (Table 1).

Table: 1 Characterization of biosurfactant-producing isolate- PNB 34

S. No	Test	Result
1.	Grams staining	Gram negative, rod shape organisms
2.	Motility test	Motile rods
3.	Catalase test	Positive
4.	Oxidase test	Positive
5.	Indole	Negative
6.	Methyl red	Negative
7.	Voges proskauer	Negative
8.	Citrate utilization	Positive

Screening of Biosurfactant Producing Isolate PNB 34

10 µl cell suspension was placed on the polystyrene coated glass plate that was coated by immersion oil. If the cell suspension contains biosurfactant then the gave positive result and also that forming a clear zone around the colonies on blood agar plate, it positive result in CTAB Agar Plate method. According to the drop collapses or spread due to the reduction of hydrophobic surface and if there is no biosurfactant in the cell suspension then the drops remain stable as the polar water molecules are repelled from the hydrophobic surface. Stability of the drop depends on biosurfactant concentration. Only one isolate PNB 34 screening, the selected. *Pseudomonas* sp PNB 34 confirmed as biosurfactant producing bacteria (Table-2).

Table: 2 screening of Biosurfactant isolate- PNB 34

S. No	Screening techniques	Result
1.	Drop collapse test	Positive (Droplet collapse with the hydrocarbon)
2.	Haemolysis Test	Positive
3.	CTAB Agar Plate method	Positive

Effect of various carbon source

The enhanced production of biosurfactant is mainly dependent upon the selection of carbon sources to be used and in the present study seven different carbon sources were examined that supports growth and biosurfactant production. Among the tested carbon sources, sucrose showed the maximum biosurfactant concentration of 73.4% $E_{24.}$

The utilization of 7 carbon sources by *Pseudomonas* sp PNB 34 isolate was studied. Among the carbon sources, glucose, fructose, maltose, sorbitol, Xylose and sucrose were utilized by isolate PNB 34 where, mannitol was not utilized **(Table 3)**.

Table: 3 Utilization of carbon sources

S.No	Test	PNB 34
1	Glucose	+
2	Fructose	+
3	Xylose	+
4	Maltose	+
5.	Sorbitol	+
6	Mannitol	-
7	Sucrose	+

+: Presence of growth; -: Absence of growth

The examination of biosurfactant production with different carbon sources revealed that it was produced maximum with 2% of sucrose which is in good agreement with the findings of Das and Mukherjee, (2007) using *B. subtilis*. Different carbon sources showed excellent results that glucose and glycerol were effective carbon substrates for rhamnolipid production with 136.4 and 71.8 mg/L/h using *P. aeruginosa* (Wu *et al.,* 2008). . In this study, pH of the medium also plays an important role in the biosurfactant production, the highest yield of biosurfactant was observed at pH 7.0 , followed by pH 7.5 and the biosurfactant production decreased with increasing acidic conditions from pH 6.5 – 5.

It is possible that pH acts at a transcriptional level on the synthesis of one of the rhamnosyltransferases or on the synthesis of a specific glycolipid transporter channel, or even on the synthesis of L-rhamnose in *P. aeruginosa* (Olvera *et al*., 1999). *Escherichia coli* represents an example in which various enzymes and periplasmic proteins are expressed in a pH-dependent mechanism (Stancik *et al*., 2002). In any case, the possibility of obtaining a product with distinct percentages of rhamnolipid types synthesized by the appropriate choice of pH allows for different uses and applications for this natural formulation. Indeed, different rhamnolipid types or their mixtures present emulsifying activity, critical micellar concentration, hydrophobicity or bioavailabilities distinct from one another (Mata-Sandoval, 2001).

Conclusion

The isolated bacteria *Pseudomonas* sp PNB 34 showed Gram's negative, and they are in rod shape respectively. In addition to demonstrating the most appropriate pH for stimulating the production of biosurfactant by *P. aeruginosa* PNB 34, selective synthesis of different types of biosurfactant caused by certain pH ranges appeared as an unexpected and timely result. A two-fold increase in volumetric productivity was obtained using this strategy. The fed-batch experiment using the limitation of the carbon source was successful and could be employed along with an appropriate formulation of the cultivation conditions.

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