Research Article

Production of Biosurfactant Using Cashew Nut Shell Liquid Renewable Source as Substrate

Satheesh. M [a], **R. Usha** [a] ***, **P. Kavitha kumari** [b], **V.P. Potty** [b]
[a] Department of Microbiology, Karpagam Academy of Higher Education, Coimbatore-21, Tamilnadu, India.
[b] CEPC Laboratory and Technical Division, The Cashew Export Promotion Council of India, Inda.
***Corresponding author's E-mail:** usha.anbu09@gmail.com

Received: 21-04-2018; **Revised:** 28-05-2018; **Accepted:** 23-06-2018.

ABSTRACT

The present study demonstrates the production and properties of a biosurfactant by Pseudomonas sp isolated from Cashew nut shell liquid contaminated soil. Biosurfactant production was confirmed by conventional screening methods including Emulsification Activity, Emulsification Power, Rhamnolipid Quantification, Drop Collapse method and Oil Spreading test. These studies revealed that the isolate produced Ramnolipid. Based on Indian standard and bacteria identification chart, the isolate was considered to be a Pseudomonas spp. The cashew nut shell liquid was the best carbon source for the production of biosurfactant. The rhamnose production was higher in the medium containing ammonium nitrate and lower in the medium containing ammonium chloride. The effect of agitation on the production of biosurfactant was also investigated. Considering the biosurfactant production by utilizing the cashew nut shell liquid which is one of the waste products from cashew industry, inexpensive and renewable sources as carbon source, This could be used as potential source for large scale production of biosurfactant.

Keywords: Biosurfactants, Pseudomonas spp, Cashew nut shell liquid, carbon source.

INTRODUCTION

Microbial surfactants are complex molecules comprising a wide variety of chemical structures such as glycolipids, lipopeptides, fatty acids, polysaccharide, protein complex, peptides and phospholipids. They are biodegradable non-toxic and eco friendly materials. The unique properties of biosurfactants allow their use and possible replacement of chemically synthesised surfactants in a number of industrial operations[1]. Biosurfactants reduce surface tension, Critical Micelle Concentration (CMC) and interfacial tension in aqueous solutions and hydrocarbon mixtures[2, 3].

In recent years, much attention has been directed toward biosurfactants owing to their advantages such as low toxicity, high biodegradability, better environmental compatibility, high foaming capability, higher selectivity, specific activity at extreme temperature, pH, salinity, and the ability to synthesize them from renewable food stocks. Recently, biosurfactants have been widely used in environmental protection, including EOR, oil spills control, biodegradation, and detoxification of oil contaminated industrial effluents and soil. Biosurfactants can also be used in bioremediation of soil and sand or in the cleanup of hydrocarbon contamination in groundwater [4]. Biosurfactants can improve the bioavailability of hydrocarbons to the microbial cells by increasing the area at the aqueous-hydrocarbons interface. This increase the rate of hydrocarbon dissolution and other utilisation by microorganisms[5]. Due to the features of high surface activity and bio-availability, biosurfactants produced by a variety of microorganism have been studied extensively in recent years[6]. The potential applications of biosurfactants include emulsification, phase separation, wetting, foaming and surface activity that can be exploited in food, oil, cosmetic, etc[7]. In the environmental sector microbial surfactants shows promising applications in bioremediation and waste treatment to remove hazardous material[8]. The objective of the present study was production and characterizes the biosurfactant from bacteria by using the cashew shell liquid. The biosurfactant production was investigated by giving the cashew nut shell liquid as carbon and by changing nitrogen sources in the production media.

MATERIALS AND METHODS

Isolation and identification of biosurfactant producing bacteria

Cashew nut shell liquid contaminated soil was collected from a cashewnut processing factory at kollam, Kerala for the study. Aseptically transported the sample to the Cashew Export Promotion Council Laboratory for further studies. The sample was serially diluted, pour plated and incubated at 37°C for 48 hrs. After incubation isolated colonies were selected based on the morphology and were streaked on to nutrient agar plates for purification. The slant culture was used for further studies. Isolated bacteria were identified by following bacterial identification chart [9].

Inoculum preparation and culture conditions

The isolated *Pseudomonas* sp was inoculated into 50ml nutrient broth and incubated at 37ºC for overnight. The overnight culture was used as inoculum for the biosurfactant production. Production of biosurfactant was

carried out in 250ml Erlenmeyer flasks containing 100ml of the mineral salt medium with pH 7.0. After sterlizing the media 1% cashewnut shell liquid (CNSL) was added as substrate and 1% of the overnight culture was inoculated in to the medium and incubated at 30ºC for a period 9 days in an incubator. From the biosurfactant production media 5ml of the culture was withdrawn periodically for bacterial growth measurement. It was monitored by measuring absorbance at a wavelength of 610 nm using spectrophotometer for biomass determination.

Identification of Biosurfactant Production

Emulsification Activity

The Emulsification activity of the culture sample was determined by adding the 0.5 ml of the sample supernatant with equal volume of hydrocarbon (Kerosene) and 4 ml of distilled water and mixing with a vortex mixer for 2 minutes. Hold stationary for 1 minute and examine for turbidity of stable emulsion layer.

Emlsification Power (E.24)

Emulsification Index of culture sample was determined by adding 2ml of a hydrocarbon (kerosene) to the same amount of sample supernatant and mixing with a vortex mixer for 2 minutes. The mixture was left to stand for 24 hrs. The E.24 index was calculated by using the equation [10].

$$\text{E.24 index} = \frac{\text{Hight of the emulsion layer}}{\text{Total Height}} \times 100$$

Rhamnolipid Quantification

The Quantification of the rhamnolipid expressed in rhamnose (mg/L) was measured in the cell free culture medium by a colorimetric determination of sugars with orcinol [11].

Orcinol assay

A modified orcinol method was used to assess the amount of glycolipids in the sample, from this method culture supernatant was obtained after removal of cells by centrifugation (8000 rpm, 15 minutes). The concentration of rhamnolipid was calculated by comparing the data with those of rhamnose standards between 0 and 175 ppm.

Drop Collapse method

For this method the servo engine oil was spreaded uniformly on the entire surface of the well cavity and equilibrated the plate for 1hr. Then 50µl aliquot of supernatant was delivered to the centre of the wells. The shapes of the drops were noted. If the drop spreaded and collapsed which indicates the positive for biosurfactant production(qualitative measurement)[12].

Oil spreading test

This was done by measuring the diameter of the clear zone occurred when a drop of a biosurfactant containing solution were placed on the oil -water surface. For this distilled water was added to petridish followed by addition of the crude oil to the surface of the water and 10µl of supernatant of culture. The diameters of clear zone of triplicate assays from the same sample were determined [13].

Factors Affecting The Biosurfactant Production

Effect of agitation on the production of biosurfactants

For studying the effect of agitation on biosurfactant production a set of flasks were kept in a rotary shaking incubator at 180 rpm at a temperature of 30°C for the same period of incubation.

Effect of nitrogen sources on the production of biosurfactant

The mineral salt medium was prepared by changing the Nitrogen sources. Four nitrogen sources were used viz $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl and $NaNO_3$. The MSM was prepared by adding 1g of the considered nitrogen sources . After sterilization 1% carbon source (CNSL) was added as substrate. Inoculate the medium with 1% of overnight culture of the isolated *Pseudomonas* Spp. This was incubated at 30ºC for a period of 9 days in a rotary shaking incubator (180 rpm). Periodically barterial growth measurement and rhamnolipid estimation was done.

RESULTS AND DISCUSSION

The bacteria were isolated from the cashewnut shell liquid contaminated soil. The serial dilution of the cashew nut shell liquid contaminated soil in nutrient agar plate showed several colonies after incubation. From that eight different colonies were identified as *Enterobacterioceae, Arthobacter* and *Pseudomonas* sp based on the bacterial identification chart (Table.1).

Table 1: Identification of Colonies formed on nutrient agar plates

S.No	Colony morphology	Bacteria identified
1	Small yellow colour mucoid colony	Enterobacterioceae
2	Small white mucoid colony	Enterobacterioceae
3	Small cream coloured mucoid colony	Enterobacterioceae
4	Transparent subsurface colony	Arthrobacter
5	Slightly transparent colony	Arthrobacter
6	Small white coloured irregular colony	*Pseudomonas*
7	Pinpoint cream coloured colony	Enterobacterioceae
8	Yellow colour with dry cater colony	Enterobacterioceae

The *Pseudomonas* sp. was identified based on the bacterial identification chart and in the Indian standard (IS: 13428 Annex D) which shown that the organism was Gram negative, rod shaped bacteria. Penicillin sensitivity was negative, cytochrom oxidase positive, oxidative metabolism in H&L medium (Table 2).

Table 2: Identification of *Pseudomonas* sp as per bacteria identification chart

S.No	Test	Result
1	Gram staining	Negative
2	Morphology	Rod
3	Penicillin sensitivity	Resistant
4	Cytochrome oxidase test	Positive
5	Reaction in H & L medium	Non fermentative (oxidative)

Table 3: Identification of *Pseudomonas* sp. As per Indian standard (IS: 13428 Annex D)

S.No	Test	Result
1	Growth on Asparagines praline broth	Turbidity and florescence under UV.
2	Growth on skim milk agar	Greenish yellow Colour colony with cleaning around the growth and florescence
3	Cytochorome oxidase test	Positive
4	Catalase test	Positive
5	Gelatin liquefaction	Positive

The strain showed turbidity and florescence under UV light Asparagines praline broth and greenish yellow colony with clearing around the growth and florescence on skim milk agar under UV (Fig.1). Organism showed positive for catalase test and gelatin liquefaction. Gelatin tubes were liquid after freezing at 4˚C (Table 3). Based on the result isolates were identified as *Pseudomonas* sp.

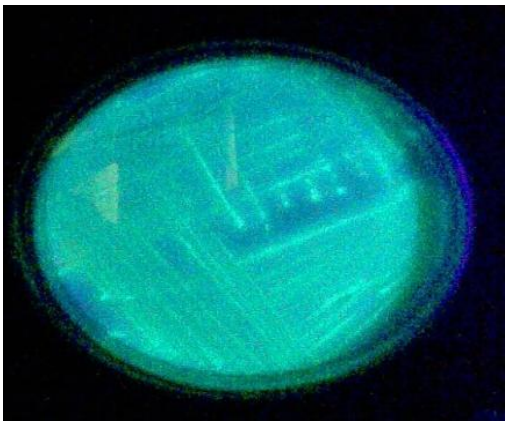

Figure 1: *Pseudomonas* on skim milk agar

Figure 2: Cashew nut shell liquid UV spectrophotometer

The biosurfactant production was carried out with the isolated *Pseudomonas* sp using Cashew nut shell liquid as carbon source (Fig. 2). The carbon source is a major parameter in production of biosurfactants. The carbon source generally used can be divided in to three categories: carbohydrates, hydrocarbons and vegetable oils. In this study the biosurfactants produced by the isolated *Pseudomonas* species was examined in the presence of 1% cashew nut shell liquid. For identifying the growth and biosurfactant production Growth OD (610nm), Emulsification activity, Emulsification power (E24), Rhamnolipid Quantification by Orcinol assay, Drop collapse method and Oil spreading test were performed. The influence of agitation and nitrogen sources for biosurfactant production were also monitored.

The result shown that the isolated *Pseudomonas* spp utilized the CNSL and produced rhamnolipid type biosurfactant. While looking the media, CNSL was displaced in to the media as globules after incubation, there was no change observed in control, i.e. the added CNSL was floating on the MSM medium.

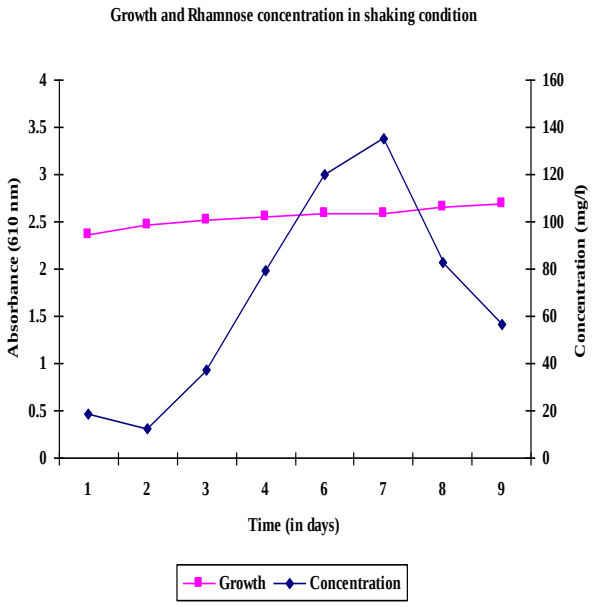

Figure 3: Influence of agitation on the growth, rhamnose production

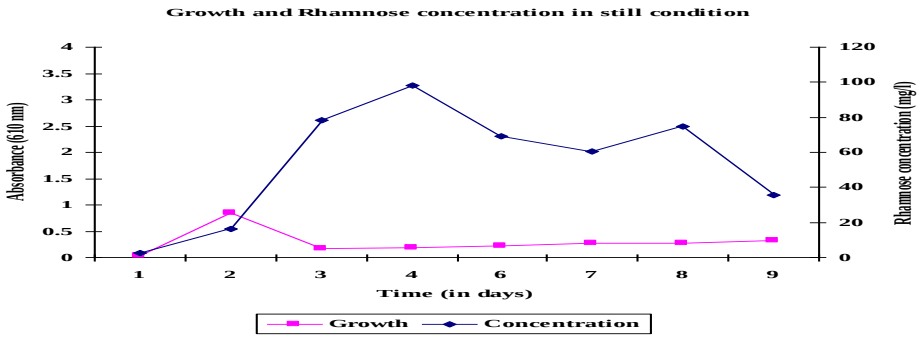

Figure 4: Growth of *Pseudomonas* spp. and rhamnose concentration in still condition

The *Pseudomonas* spp. produced rhamnolipid type biosurfactant in both still and shaking condition. The present result showed that the production range was higher in the case of shaking than still condition. In both cases the growth was increased up to ninth day of incubation period. The highest production of rhamnose in the shaking condition was (135mg/l) in the sixth day of incubation period (Fig. 3). In still condition the highest production was (98mg/l) was observed in the fourth day of incubation period (Fig.4). Rhamnolipid formation by *Pseudomonas* spp occurs under limiting concentrations of the late exponential and stationary phase of growth [14].

Table 4: Influence of agitation on the emulsification index, oil spreading activity, Emulsification and Drop collapsing activity

Time days)	Still				Shaking			
	Emulsification index(E24)	OSA (diameter)	EA*	DC**	Emulsification index(E24)	OSA (diameter)	EA	DC
1	-	0.5	_	_	-	0.3	_	_
2	-	0.9	_	_	-	0.5	_	_
3	33.3	3.4	_	_	8.3	4.0	+	+
4	41.6	5.4	+	+	33.3	4.6	+	+
6	33.3	3.2	+	+	45.8	4.9	+	+
7	25	3.0	+	+	50	5.2	+	+
8	33.3	3.1	+	+	37.5	5.0	+	+
9	8.3	2.0	+	+	20.8	4.3	+	+

EA: Emulsification, DC: Drop collapsing activity**,** OSA: Oil spreading activity

The emulsion layer was formed in both still and shaking condition, but the highest activity was shown in the case of the media kept under shaking condition. The emulsification index (E24) was calculated and tabulated (Table. 4). The result showed that the agitation has an influence in E24 activity. While comparing the still and shaking condition the highest activity was in shaking (50%) and (41.6%) in still condition. The drop collapsing activity was shown by the culture after 48 hrs of incubation both in still and shaking condition. The shaking condition had the highest oil displacement activity than still condition.

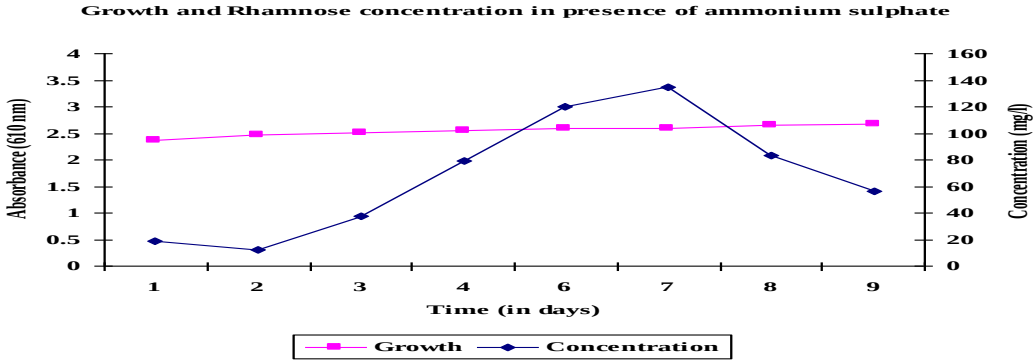

Figure 5: Influence of ammonium sulphate on growth and rhamnose concentration

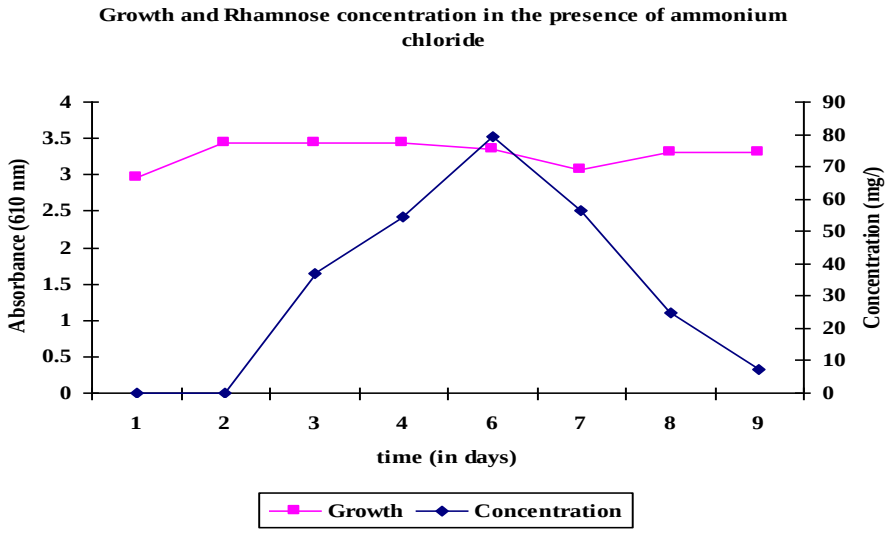

Figure 6: Influence of ammonium chloride on growth and rhamnose concentration

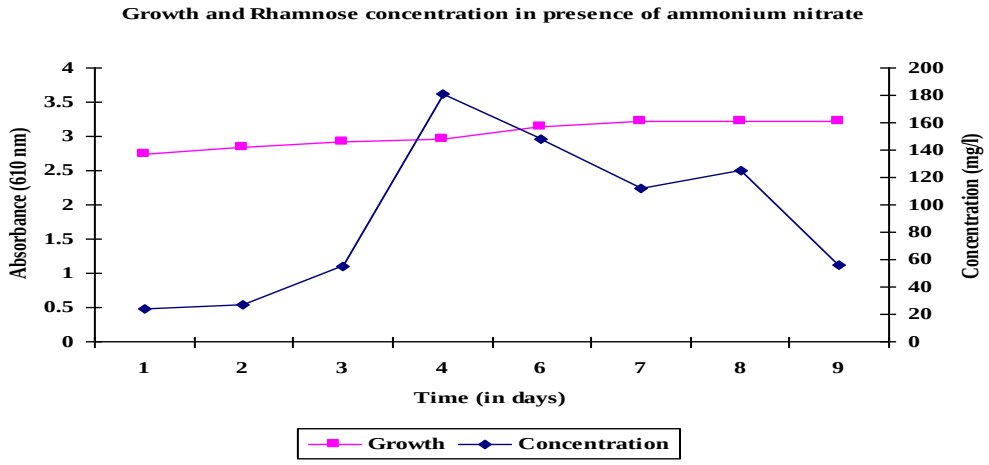

Figure 7: Influence of ammonium nitrate on growth and rhamnose concentration

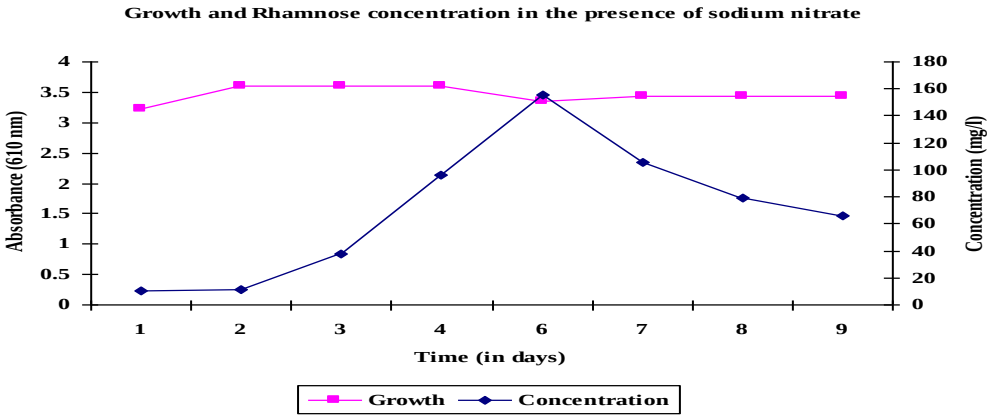

Figure 8: Influence of sodium nitrate on growth and rhamnose concentration

To evaluate the most appropriate nitrogen sources for the production of rhamnolipids by *Pseudomonas* spp the following nitrogen sources were used namely $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl and $NaNO_3$. In this the ammonium nitrate containing media gave the highest productivity of rhamnose (181mg/l) noted in the fourth day of incubation period (Fig.7). The less production was noted in the MSM media with NH_4Cl (79.50mg/l) in the fifth day of incubation period (Fig.7). In the case of $NaNO_3$ the highest production (155.50 mg/l) was reached in the fifth day of incubation period (Fig.8) and in NH_4SO_4 (135mg/l) in the seventh day (Fig.5). The *Pseudomonas* spp. utilized the nitrogen in the form of ammonia and nitrates, but the highest production was in the case of nitrates [15].

Table 5: Influence of nitrogen sources on the Emulsification index (E24), Oil spreading activity, Emulsification and Drop collapsing activity

Time (days)	$(NH4)_2SO_4$				NH_4NO_3				NH_4Cl				$NaNO_3$			
	E24	OSA	EA	DC	E24	OSA	EA	DC	E24	OSA	EA	DC	E24	OSA	EA	DC
1	-	0.3	_	_	-	0.5	_	_	-	0.5	_	_	-	0.5	_	_
2	-	0.5	_	_	-	1.2	_	_	-	1.3	_	_	-	1.0	_	_
3	8.3	4.0	+	+	20.8	2.4	+	+	8.3	2.1	+	+	8.3	2.2	+	+
4	33.3	4.6	+	+	58.3	6.7	+	+	20.8	3.8	+	+	41.6	4.8	+	+
6	45.8	4.9	+	+	54.16	6.1	+	+	20.8	4.2	+	+	54.16	6.3	+	+
7	50	5.2	+	+	45.8	4.9	+	+	20.8	3.4	+	+	45.8	5.4	+	+
8	37.5	5.0	+	+	50	5.3	+	+	-	1.8	+	+	33.3	3.6	+	+
9	20.8	4.3	+	+	20.8	2.6	+	+	-	0.8	-	-	29.16	3.1	+	+

E24-Emulsification index; **OSA-**Oil spreading activity; **EA-**Emulsification activity; **DC-Dropcollapsing activity**

The emulsification activity was showed by the cultures after 48 hrs of incubation period. In the positive result emulsion layer was formed after vortexing the mixture with kerosene and distilled water. The NH_4NO_3 showed the highest activity of emulsion formation, the less activity was in the case of NH_4Cl as nitrogen source (Table.5).

Figure 9a: Emulsion layer

Figure 9b: Well cavity plate

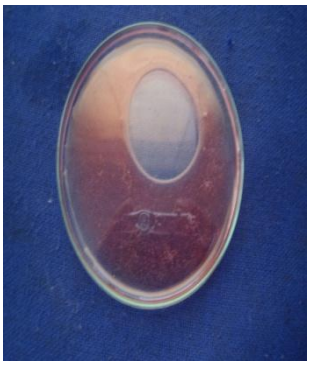

Figure 9c: Oil displacement

C - Drop collapse control; P1&P2 – *Pseudomonas*; S - Surfactant

The emulsification index (E24) was calculated and tabulated (Table 5). The result showed that the nitrogen sources have an influence in E24 activity. In the nitrogen sources the highest activity (58.3%) by NH_4NO_3. In the case of NH_4CL (20.8%) and $NaNO_3$ (54.16%), which in agreement with other studies reported in the literature[16]. In the nitrogen sources NH_4NO_3 showed the highest drop collapsing activity. The strain exhibiting emulsifying activity showed the positive result with drop collapsing test. The similar results shown in the reports of Maneerat and Phetrong (2007) and other research reports[17, 18].

The oil displacement showed by the culture supernatant that the oil was spreaded on to the sites of the Petri dish while adding the sample supernatant to the oil surface (Fig 9c). The oil displacement activity was varying according to the nitrogen sources, in that NH_4NO_3 showed the highest activity. The less activity was noted in the case of NH_4Cl as nitrogen source. The condition exhibiting positive result for emulsifying activity showed the oil displacement activity [19].

Pseudomonas spp. was able to use CNSL as carbon source and nitrogen source such as ammonia or nitrate. However in order to obtain high concentration of rhamnolipids it is necessary to have restrained conditions of this macronutrients and the physical factors such as agitation. Our studies showed that agitation was good for the production of rhamnolipid and the nitrate is more effective nitrogen source in the production of rhamnolipid than ammonia. Those results were agreement with the studies reported in the literature[20].

CONCLUSION

Cashew oil shell liquid can be used as substrate for production of specific metabolites and general bacterial growth. Based on the experimental result, the isolated *Pseudomonas* species utilized the Cashew oil shell liquid as carbon source and produced the rhamnolipid biosurfactants. Therefore, it is feasible to use relatively inexpensive and renewable sources for industrial production.

Acknowledgments: The authors would like to acknowledge the management, Karpagam University for their encouragement and support for this research work. Two of the authors thankful to the cashew export promotion council of India, Kollam for providing facilities.

REFERENCES

1. Kosaric N, Biosurfactants in industry, *Pure Appl Cherm*, 64, 1992, 1731-1737.
2. Rahman KSM , Banat I M, Thahira J, Thayumanavan T & Laksmanaperumalsamy P, Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhaminolipid biosurfactant. *Bioresour. Technol*, 81, 2002, 25-32.
3. Banat I M, Characterization of biosurfactants and their use in pollution removal – State of the Art. (Review), *Acta Biotechnol*, 15 (1995) 251- 267.
4. Urum K & Pekdemir T, Evaluation of biosurfactants for crude oil contaminated soil washing, *Chemosph*, 57, 2004, 1139-50.
5. Gerson D F, The biophysics of microbial surfactants: growth on insoluble substrates, *In: Surfactant Science Seri, Biosurfact,* 48, 1993, 269–286.
6. Van Hamme J D, Singh A & Ward O P, Physiological aspects, Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology.*Biotechnol. Adv,* 24, 2006, 604.
7. Makkar R S, & Cameotra S S, An update on the use of unconventional substrates for biosurfactant production and their new applications. *Applied Microbiol Biotechnol*, 58, 2002, 428-434.
8. Mulligan, C N, Environmental applications for biosurfactants. *Environ. Pollut*, 133, 2005, 183-198.
9. Surendran P K & Gopakumar K, Scheme for identification of bacterial cultures, *Fish tech*, 18, 1981, 133- 141.
10. Tabatabaee A, Mazaheri M A, Noohi A A & Sajadian V A, Isolation of Biosurfactant Producing Bacteria from Oil Reservoirs, *Iran J Environ Health Sci Eng*, 2, 2005, 6-12.
11. Chandrasekaran E V & Bemiller J N , Constituent analysis of glycosaminoglycans. *In Meth in Carbohy Chem*. (Academic Press Inc, New york) 1980, 89–96.
12. Youssef N, Duncan K, Nagle D P, Savage K N, Knapp R M & McInerney M J, Comparison of methods to detect biosurfactant production by diverse microorganism, *J Microbiol Meth,* 56, 2004, 339-347.
13. Rodrigues L, Banat I M, Teixeira J, Oliveira R, Biosurfactants: potential applications in medicin, *J Antimicrobial Chemo*, 57, 2006, 609- 618.
14. Ochsener U A, Reiser J, Fiechter A & Witholt B , Production of *P. aeruginosa* rahamnolipid biosurfactants in heterologous hosts, *Appl Environ Microbiol*, 61, 1995, 3503-3506.
15. Rashedi H., Jamshidi E , Assadi M M & Bonakdarpour B, Biosurfactant production with glucose as a carbon source, *Chem. Biochem. Eng*, 20, 2006, 99- 106.
16. Abouseoud M, Maachi R, Amrane A , Boudergua S & Nabi A, Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens,* Desalination, 223, 2008, 143- 151.
17. Maneerat S & Phetrong K, Isolation of biosurfactant-producing marine bacteria and characteristics of selected biosurfactant, *J. Sci. Technol*, 29, 2007, 781-791.
18. Mulligan C N , Cooper D G and Neufeld R J, Selection of microbes producing Biosurfactants in media without hydrocarbons, *J. Ferment. Technol*, 62, 1989, 311- 314.
19. Lin S C, Lin K G, Lo C C & Lin Y M, Enhanced biosurfactant production by a *Bacillus lichemniformis* mutant. *Enzyme Microb Technol*, 23, 1998, 267-273.
20. Rashedi H, Assadi M, Jamshidi M, Esmaeil, Bonakdarpour, Babak. Optimization of the Production of Biosurfactant by *Psuedomonas aeruginosa* HR Isolated from an Iranian Southern Oil Well, *Iran. J Chem Chem Eng,* 25 2006, 25 - 30.

Source of Support: Nil, **Conflict of Interest:** None.