Int. J. Chem. Sci.: 10(3), 2012, 1687-1697
ISSN 0972-768X
www.sadgurupublications.com

COMPARATIVE STUDY OF BIOSURFACTANT BY USING *BACILLUS LICHENIFORMIS* AND *TRICHODERMA VIRIDE* FROM PAPER WASTE CONTAMINATED SOIL

N. UMA MAHESWARI* and L. FIRTHOWS PARVEEN

P. G. and Research Department of Microbiology, Sengamala Thayaar Educational Trust Women's College, Sundarakkottai, MANNARGUDI – 614001, Thiruvarur (Dt), (T.N.) INDIA

ABSTRACT

This study deals with the production of biosurfactant producing organisms, which were isolated from paper waste contaminated soil from the paper industry at Boothalore, Thanjavur (Dt), Tamilnadu. From that sample, organisms were isolated by serial dilution agar plating method. The colonies were identified as *Bacillus licheniformis* and *Trichoderma viride* by morphological and biochemical characteristics. Biosurfactant producing organisms were scanned by using oil spreading technique and blood haemolysis test. Organisms were inoculated into the Mineral salt broth with diesel, petrol, kerosene and vegetable oil as a carbon source for biosurfactant production. Biosurfactant was extracted by acid precipitation method. Biosurfactant was analyzed by using different pH, temperature, carbon and nitrogen sources. Emulsification activity of biosurfactant was analyzed. The extracted biosurfactant was characterized by using thin layer chromatography. This study concludes that production of biosurfactants using the isolated organisms is the simplest and cheapest method for the surfactant production.

Key words: Biosurfactant, *Bacillus licheniformis, Trichoderma viride,* Emulsification, Thin layer chromatography.

INTRODUCTION

Biosurfactant are surface active organic compound synthesized by many micro-organism during their growth that cells for the utilization of hydrocarbon compounds[1]. The term "biosurfactant" refers to any compound obtained from microorganism which has striking influence on interfaces, further it brings down the interfacial tension between the two liquids[2].

*Author for correspondence; E-mail: umasamy2004@yahoo.co.in

Biosurfactant producing microorganisms are naturally present in the paper waste contaminated soil. This environment contain large amount of complex chemical structure i.e. aliphatic and aromatic hydrocarbons. Microorganisms exhibit emulsifying activity by producing biosurfactants and utilize the hydrocarbons as substrate of ten mineralizing them or converting them into harmless products[3].

Biosurfactant is known that these secondary metabolites can enhance nutrient transport across membrane, action various host-microbes interaction and provide biocidal and fungicidal protection to the producing organism[4]. Bacterial product with surface active characteristics (biosurfactants) constitutes a class of product with a large potential for industrial and biomedical application. Biosurfactants are usually biodegradable, possess a greater specificity and display a large diversity[5].

Surface active agents are needed for a large number of diverse applications[6]. Emulsion stabilization is a very common requirement for food products cosmetics and other products. Surfactants are also useful as soap and detergents both for cleaning applications and for petrochemical purposes such as enhanced oil recovery and oil spill cleanup. Surface wetting and solid dispersal are important for preparations of coal slurries for pipelining. Colloid preparations are necessary for paints and related products penetration of rates of inks and dyes are important for the pulp paper and textile industries. Foam stabilization is also necessary for five extinguishers and food industry.

Some microorganisms and microbial surfactants exhibit demulsifying activity. De-emulsification is important for the removal of water from emulsions prior to crude oil processing. In addition, biosurfactants are effective in the release of bitumen from tar sands.

Test organisms

Bacillus licheniformis is the gram positive red shaped bacterium. It is commonly found in the soil. Recently studies have also shown that is found on bird feathers especially chefs and back plumage. It has the ability to produce many surface active lipopeptides[7]. *Trichoderma viride* is a common fungi present is the soil. It has the ability to produces surface active biomolecules[8].

EXPERIMENTAL

Soil sample was collected from the paper industry at Boothalore, Thanjavur (Dt), Tamilnadu. The isolated colonies were identified by cultural, morphological and biochemical characteristics.

Screening for biosurfactant production[9]

The isolated colonies were tested for the biosurfactant production by two methods.

(i) Oil spreading technique.

(ii) Blood haemolysis test.

Oil spreading technique[10]

30 mL of distilled water was taken in the petriplate and 1 mL of coconut oil was added to the center of the plate. 20 mL of the culture supernatant was added to the centre of the plate. The biosurfactant producing organisms can displace the oil and spread in the water.

Blood haemolysis test[11]

The fresh single colony form the isolated culture was taken and inoculated into blood agar plates. The plates were incubated for 48-72 hrs at 37^{o}C then the plates were observed for the presence of clear zone around the colonies. The clear zone indicates the presence of biosurfactant producing organisms.

Biosurfactant production[12]

Bacillus licheniformis and *Trichoderma viride* culture were inoculated on the mineral salt broth containing 2% of oil (vegetable oil, kerosene, petrol and diesel) and it was incubated at an optimized condition for 24 to 48 hrs in a shaker operating at 120 rpm/min. After incubation the broth was screened for the production of biosurfactant.

Extraction of biosurfactant[12]

Biosurfactant produced by *Bacillus licheniformis* and *Trichoderma viride* were extracted by acid precipitate methods. After incubation the bacterial cells were removed by centrifugation at 5000 rpm 4^{o}C for 20 minutes. The surfactant was taken and pH of the supernatant was adjusted to using HCl. Equal volume of choloroform methanol (2 : 1) was added and mixed well. It was left overnight for evaporation. White colored sediment was obtained as a result of the production of biosurfactant.

Dry weight of biosurfactant[13]

Sterile petriplate was taken and the weight of the plate was measured. Now the sediment was poured on the plates. They were placed on the hot air oven for drying at 100^{o}C for 30 minutes. After drying the plates were weighed. The dry weight of the biosurfactant was calculated by the following formula.

Dry weight of biosurfactant = weight of the plate after drying - weight of the empty plate.

Physical parameters

Effect of temperature

100 mL of nutrient broth and potato dextrose broth were prepared 1% of inoculum was added. Then the broth was incubated at different temperature at 25^{o}C, 30^{o}C, 37^{o}C, 42^{o}C, for 48-72 hrs.

Effect of pH

100 mL of nutrient broth and potato dextrose broth were prepared and separated into different conical flasks each flasks were adjusted to different pH such as 6.5, 7, 7.5, 8 and 8.5.

After Sterilization, 1% inoculum was added into different flasks containing medium. Then the flasks were incubated for 48-72 hrs.

Chemical parameters

Effect of carbon sources

100 mL of nutrient broth and potato dextrose broth was prepared and separated into different conical flasks. 1% of inoculum was added. Carbon sources like glucose, starch, galactose were added. Then the broth was incubated for 24-72 hrs.

Effect of nitrogen sources

100 mL of nutrient broth and potato dextrose broth was prepared and separated into different conical flasks. 1% of inoculum was added. Nitrogen sources like urea, NaCl, $NaNO_3$ was added. Then the broth was incubated for 24-72 hrs.

Estimation of emulsification activity[13]

Partially purified biosurfactant (5 mg) was dissolved in 5 mL of Tris buffer (pH 8.0) in 30 mL test tubes. Hydrocarbons like waste motor lubricant oil, crude oil, peanut oil diesel, kerosene, naphthalene, anthracene and xylene were tested for emulsification activity. 5 mg of hydrocarbon was added to the above biosurfactant solution and shaken well for 20 min and the mixture was allowed to stand for 20 min. The optical density of the emulsified mixture was measured at 610 nm and the results were expressed as D_{610}.

Analytical method[13]

Thin layer chromatography

Preliminary characterization of the biosurfactant was done by TLC method. A portion of the crude biosurfactant was separated on a silica gel plate using chloroform: Methanol water (10 : 10 : 0.5 v/v/v) as developing solvent system with different color developing reagent. Ninhydrin reagent was sprayed to detect lipopeptide biosurfactant as red spot, produced by *Bacillus licheniformis* and *Trichoderma viride.*

R_f value is calculated by using following formula

$$R_f = \frac{\text{Distance moved by analyze from origin}}{\text{Distance moved by solvent front from origin}}$$

Statistical analysis was made.

RESULTS AND DISCUSSION

Biosurfactant or bioemulsifiers play a key role in emulsifying hydrocarbons. Biosurfactant and bioemulsifiers are thought to be very suitable alternatives to chemical surfactants due to their properties like eco friendly, less or no toxicity, biodegradability, high specificity, selectivity at temperature, pH, salinity and synthesis from cheaper renewable substrates. The present study was carried out to determine the production of biosurfactants by using the organism isolated from paper waste contaminated soil.

Isolation and identification of organism

Serial dilution plating method used for the isolation of colonies. These colonies were identified by gram's staining, biochemical tests and lacto phenol cotton blue mounting of fungi. Gram positive motile rods were observed in gram's staining and motility test. The results of biochemical tests showed positive results for indole, methyl red, vogesproskauer, citrate and oxidase test and negative results for urease and catalase. With all the above result, the isolated bacterial colony was compared with Bergey's manual of systemic Bacteriology and confirmed as *Bacillus licheniformi*s. Spores and filamentous hyphae of *Trichoderma viride* was observed by lactophenol cotton blue mounting of fungi.

Screening of biosurfactant producing organism

The isolated colonies for their biosurfactant production ability by two method.

(i) Oil spreading technique

In oil spreading technique, *B. licheniformis* and *T. viride* showed a zone of displacement in the oil. The biosurfactant production organisms can only be able to displace the oil. The result of this technique revealed that the maximum zone of displacement was noted in diesel (52 mm, 49 mm) for *B. licheniformis* and *T. viride*, respectively, when compared with other oils. (Table 1).

Table 1: Zone displacement of oil spreading technique by *Bacillus licheniformis* and *Trichoderma viride*

S. No.	Sample	*Bacillus licheniformis* (mm)	*T. viride* (mm)
1	Vegetable oil	43	42
2	Kerosene	45	46
3	Petrol	49	47
4	Diesel	52	49

i) Blood haemolysis test

On Blood agar plates, *Bacillus licheniformis* showed the beta – haemolytic activity.

Production of biosurfactants

Biosurfactant production in mineral salt broth with diesel, petrol, vegetable oil and kerosene as carbon sources showed a colloidal thin white layer formation on the surface of the broth.

Extraction of biosurfactants

The biosurfactants was extracted by acid precipitation method with chloroform; methanol solvent. White sediment was retained when the mixture was placed in the rotor.

Dry weight of biosurfactants

The dry weight of the biosurfactants was measured and estimated. The result revealed that the maximum dry weight was in Diesel (1.4 ± 0.89 g), (1.2 ± 0.86 g) for *B. licheniformis* and *T. viride*, respectively when compared with other oils (Table 2).

Table 2: Dry weight of biosurfactants for *Bacillus licheniformis* and *Trichoderma viride*

S. No.	Source	*Bacillus licheniformis*			*Trichoderma viride*		
		Plate weight (g)	After dry (g)	Dry weight (g)	Plate weight (g)	After dry (g)	Dry weight (g)
1	Vegetable oil	46.3±1.42	45.31±2.53	0.21±0.04	42.3±2.12	44.12±2.41	0.31±0.14
2	Kerosene	45.6±3.64	44.3±2.21	0.38±0.07	43.1±3.24	42.68±2.17	0.71±0.23
3	Petrol	45.1±2.68	46.2±1.54	0.4±0.34	44.2±1.61	43.27±1.81	0.62±0.34
4	Diesel	47.2±1.78	46.4±3.4	1.4±0.89	45.5±1.57	45.89±1.92	1.3±0.96

Values are represented as mean ± standard deviation

Physical parameters

Effect of pH

Biosurfactant was analyzed in various pH ranges such as 6.5, 7, 7.5, 8 and 8.5. The finding results were presented (Table 3)**.**

Effect of temperature

Biosurfactant was analyzed in various temperature ranges such as 25^{o}C, 30^{o}C, 37^{o}C, 42^{o}C. The finding results were presented (Table 3).

Table 3: Effect of pH and temperature on biosurfactant production

pH	pH				Temp. (^{o}C)	Temperature			
	B. licheniformis		*T. viride*			*B. licheniformis*		*T. viride*	
	OD Value	g/100 mL	OD Value	g/100 mL		OD Value	g/100 mL	OD Value	g/100 mL
6.5	0.51	0.43	0.43	0.33	25	0.36	0.25	0.32	0.21
7.0	0.53	0.48	0.49	0.39	30	0.35	0.27	0.31	0.23
7.5	0.55	0.49	0.48	0.38	37	0.35	0.24	0.38	0.30
8.0	0.40	0.36	0.36	0.37	42	0.21	0.16	0.26	0.14
8.5	0.42	0.39	0.32	0.30					

Chemical parameters

Effect of carbon sources

Biosurfactant was analyzed in various carbon sources such as glucose, starch, galactose. The finding results were presented (Table 4).

Effect of nitrogen sources

Biosurfactant was analyzed in various nitrogen sources such as urea, NaCl, $NaNO_3$. The finding results were presented (Table 4).

Table 4: Effect of carbon and nitrogen sources on biosurfactant production

Sources	Carbon sources				Sources	Nitrogen sources			
	B. licheniformis		***T.viride***			***B. licheniformis***		***T.viride***	
	OD Value	**g/100 mL**	**OD Value**	**g/100 mL**		**OD Value**	**g/100 mL**	**OD Value**	**g/100 mL**
Glucose	0.31	0.25	0.31	0.24	Urea	0.36	0.27	0.54	0.43
Starch	0.43	0.36	0.45	0.31	NaCl	0.48	0.36	0.35	0.21
Galactose	0.56	0.47	0.33	0.27	$NaNO_3$	0.57	0.48	0.42	0.36

Estimation of emulsification activity

Emulsification activity of biosurfactant was analyzed by using various hydrocarbon such as vegetable oil, petrol, diesel, kerosene, xylene. The finding results were presented (Table 5).

Table 5: Emulsification activity on biosurfactant production.

S. No.	Sources	***B. licheniformis***		***T. viride***	
		OD value	**g/100 mL**	**OD value**	**g/100 mL**
1	Vegetable oil	0.6	0.53	0.4	0.42
2	Petrol	0.13	0.12	0.9	0.81
3	Diesel	0.9	0.86	0.6	0.52
4	Kerosene	0.4	0.38	0.3	0.26
5	Xylene	0.11	0.17	0.9	0.82

Characterization of biosurfactants

The biosurfactant production was characterized by using TLC plates. On TLC plate, the biosurfactant produced by *B. licheniformis* and *T. viride* were detected as red spots. Larger red spot on TLC plate was observed for diesel. The R_f values for the biosurfactants production for *B. licheniformis* in diesel was (0.56 mm) which was higher than the vegetable oil (0.46 mm), kerosene (0.53 mm) and petrol (0.54 mm) and R_f values for *T. viride* in diesel was (0.58 mm) which was higher than the vegetable oil (0.44 mm), kerosene (0.52 mm) and petrol (0.53 mm).

From this study, *Bacillus licheniformis* was able to produce the biosurfactant by using pH (7.5), temperature (30^oC), carbon source (galactose), nitrogen source ($NaNO_3$). *Trichoderma viride* was able to produce the biosurfactant by using pH (7.0), temperature (37^oC), carbon source (starch), nitrogen source (urea). Among this study *Bacillus licheniformis* having highest ability to produce the biosurfactant than the *Trichoderma viride*.

In our study correlated with the biosurfactant isolated from *P. aeruginosa* and Triton x-100 showed maximum emulsification activity against waste motor lubricant oil. Biosurfactant producing organisms showed beta hemolytic activity on blood agar plate. In our study similar to the findings of rhamnolipid produced from *Pseudomonas aeruginosa* in TLC plate[14].

Our study is similar to the *P. aeruginosa* used for biosurfactant production by using whey waste within 48 h of incubation the yield of biosurfactant obtained was 0.92 g/L[15]. Potato substrates were evaluated as a carbon source for surfactant production by *Bacillus subtilis* ATCCC 21332[16].

In our study correlated with the lipopeptide were obtained as rhamnolipid i.e, a glycolipid while sprayed at ninhydrine reagent on the TLC plate[17]. Biosurfactants was to be more effective than chemically synthesized surfactants. It was used for the removal of lead from the water and it was easily biodegraded in environment. This study should be progressed in future in such a way to find more applications of biosurfactants in bioremediation. Microbially produced compounds are easily degraded suited for environmental applications such as bioremediation and dispersion of oil spills[18].

Our study reports similar to the potential applications of biosurfactants in pollution and environmental control are microbial enhanced oil recovery. Hydrocarbon degradation in soil environment and hexachlorocyclohexane degradation, heavy metal removal from contaminated soil and hydrocarbon in aquatic environment[19].

CONCLUSION

Soil sample was collected from paper waste contaminated soil from the paper industry at Boothalore, Thanjavur (Dt), Tamilnadu. From that sample, organisms were isolated by serial dilution agar plating method. The colonies were identified by morphological and biochemical characteristics. Biosurfactant producing organisms were scanned by using oil spreading technique and blood haemolysis test. Organisms were inoculated into the Mineral salt broth with diesel, petrol, kerosene and vegetable oil as a carbon source for biosurfactant production. The extracted biosurfactant was characterized by using Thin Layer Chromatography. This study concludes that *Bacillus licheniformis* have the highest ability to produce the biosurfactant than *Trichoderma viride*. Production of biosurfactants using the isolated organisms is the simplest and cheapest way than the surfactant production. They are safe to environment, low toxicity and low cost than synthetic surfactants. These biosurfactant are easily degradable and well suited for the environmental applications such as bioremediation and oil spills.

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Revised : 09.04.2012 *Accepted : 11.04.2012*