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ISOLATION, EXTRACTION, PURIFICATION AND APPLICATIONS OF BIOSURFACTANT PRODUCING BACTERIA

H. Sahana[1], Harshini R.[1], Lakshmi Badiger[1], Dr. Siddalingeshwara K. G.[2*] and Prakruthi[2]

[1]The Oxford College of Science, Bangalore.
[2]Scientific & Industrial Research Centre, Bangalore.

***Corresponding Author: Dr. Siddalingeshwara K. G.**

Scientific & Industrial Research Centre, Bangalore.

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ABSTRACT

Biosurfactants are surface active compounds produced by a wide range of microorganisms. They have different chemical structures like – lipopeptides, glycolipids, neutral lipids and fatty acids. The objective of this project was to perform screening of biosurfactant producing microorganisms from different samples like soil and water from different places were used, Soil samples from garage, garden, forest and water samples from sewage treatment plant were used in the study. The isolates were cultured in nutrient broth with tween 80 and their extracts were evaluated according to the drop collapse, oil dispersion, emulsification assay. This present study deals with the isolation, screening, production and purification of bio surfactant produced from bacteria and fungi and its application in biofilm assay, antimicrobial property and oil degradation in water.

KEYWORDS: Bio surfactant, Oil displacement, Emulsification, drop collapse, antimicrobial properties, oil degradation, anti-biofilm activity.

1. INTRODUCTION

Bio surfactants are chemicals produced by microorganisms that have clearly defined hydrophilic and hydrophobic groups. They are found in nature in bacteria, yeasts, and fungi, particularly in bacteria that grow on a water-impermeable substrate and use it as a food source. Surface-active compounds produced by a wide variety of microorganisms are known as bio surfactants. They have both hydrophobic and hydrophilic domains and can reduce growth medium surface tensionand inter facial tension. Biosurfactants comeina variety of chemical structures, including lip opeptides, glycolipids, neutral lipids, and fatty acids. They are biodegradable, non-toxic bio-molecules that emulsify hydrophobic compounds well.

They can form stable emulsions. The low water solubility of these compounds restricts the iravailability to microorganisms. Microbe-secreted surfactants improve the bio availability of such hydrophobic compounds for bioremediation. Asaresult, biosurfactant-enhanced pollutant solubility has potential applications that is bioremediation. Biosurfactants are useful in a variety of industrial processes and are also critical to the microbes in the environment. Adhesion, emulsification, bioavailability, desorption, and defence strategy are all important considerations (Anuraj Nayarisseri*et al*., 2018).

Oil pollution is a continuous phenomenon, especially in oil - producing countries. Oil pollution despite the progress of recent years will remain a considerable problem. A promising method that can improve bioremediation effectiveness of hydrocarbon contaminated environments is the use of biosurfactants. Biosurfactants over the years have found a greatmany uses in industry, for example, (1) oil recovery, (2) oil spill clean-up, (3) textiles, (4) pharmaceuticals, (5) cosmetics. Due to their special qualities, biosurfactants can be used in avariety of industrial processes in place of chemically synthesized surfactants (Ade sumiardi*etal*., 2018).

The most isolated and the bes studied groups of biosurfactants are mainly glycolipids and phospholipids in nature. Nevertheless, rhamnolipids are glycolipid compounds produced by *Pseudomonas* sp. that can reduce water surface tension and emulsify oil (Babu *etal*. 1996). Biosurfactants are environmentally friendly and havepotentialindustrial and environmental applications.

The present study is to perform screening of biosurfactant producing bacteria from different samples and their applications. This study deals with the is olation, screening, production and purification of biosurfactant produced from bacteria, including applications inanti-biofilm activity, antimicrobial activity

and oil removal.

2. MATERIALSANDMETHODS

1. ISOLATIONOFMICROORGANISMSFROMSOILANDWATER

A. COLLECTIONOFSAMPLES

Three soil samples from Garage, garden and forest and two water sample from Sewage treatment plant water and inland water were collected from different places in and around industrial area, Bangalore. The soil samples were collected in plastic pouches and water samples were collected in screw cap bottle and brought to laboratory for analysis.

B. ISOLATIONOFTHE BACTERIA BY SPREAD PLATE METHOD

The collected soil and water samples were suspended in Minimal Salt Medium (MSM) broth containing g/lt, Sodium nitrate – 15gm, potassium chloride – 1.1gm, sodium chloride – 1.1g, ferrous sulphate – 0.00028gm, potassium di hydrogen ortho phosphate – 3.4gm, di potassium hydrogen phosphate – 4.4gm, magnesium sulphate – 0.5gm, yeast extract – 0.5gm, pH: 7.0 and incubated for 48 hrs at 35◦C after incubation period MSM broth was subjected for serial dilution, 10^{-5} and 10^{-6}dilutionsamples were inoculated onto nutrient agar medium by spread plate technique and the plates were incubated at 35^0C for 24hours. The bacterial isolates obtained were sub-cultured regularly on nutrient agar and storedat 4^0C for further work. (Anuraj et al).

2. SCREENING FOR BIOSURFACTANT PRODUCING MICROORGANISMS

Bacteriali solates obtained from the samples were subjected to screening to check bacterial biosurfactantability. Screening methods like oil-spread assay, drop-collapse assay, Emulsification assay and CTAB assay were performed.

Extraction of crude biosurfactant: The bacterial isolates were inoculated on to nutrient broth containing g/lt of peptone – 5g, yeast extract – 3g, sodium chloride – 3g, and incubated at 35◦C for 24 hours, after the incubation period the bacterial broth culture was centrifuged at 5000rpm for 10minutes, later the cell free supernatant (Crude Biosurfactant) was collected and used in performing screening test.

1.1 DROPCOLLAPSE TEST

The wells of a polystyrene 96 well microtiter plate (Tarsons) were coated with 2 µL of tween80, coconut oil, Diesel, Refined oil and left to dry for 24 h at 25°C. Filtered cell-free supernatant (5 µL) was transferred to the centre of the oil coated well. The results were recorded after 1–2 min and considered positive for bio surfactant production when the oil drop was flat. Those that gave rounded drops were negative, an indication of the absence of bio surfactant production (Anuraj et al).

1.2 OIL DISPLACEMENT TEST

The oil spread assay was performed in petridishes (Borosil) containing 20µL ofoil, oils such as Tween 80, coconut oil, refined oil and diesel was used were carefully layered over 20 mL of distilled water. A drop (approx10 µL) of filtered supernatant was carefully pipetted onto the centre of the oil layer. The diameter of the clearzone on the surface of the oil layer was measured and compared to the negative controls (AdeSumiardi *et al*.).

1.3 CTABAGARASSAY

The CTAB agar plate assay is a semi-quantitative assay for the detection of extra cellularglycolipids or other anionic surfactants. It was developed by Siegmund and Wagner. The microbes of interest are cultivated on amineral saltsagar plate containing the cationic surfactant cetyl trim ethyl ammonium bromide (CTAB) and the basic dye methylene blue. CTAB agar plates were prepared by adding 0.15 g of Cetyl trimethyl ammonium bromide. Three wells (5mm) were made in the CTAB plates, and approximately 20 µL of cell-freesuspensionwas loaded insideeach well. Plates were incubated at 37°C for 2-3days.

Anionic surfactants are secreted by the microbes, form a blue halos which is in solubleion pair with cetyltrimethyl ammoniumbromide andmethyleneblue. Thus, productive coloniesaresurrounded, by bluezone of clearance. The diameter of the halo was measured and compared with positive and negativecontrols (Mamta Rani*et al*., 2020).

EMULSIFICATION TEST

Equal volume of cell free suspension and oil was taken in a test tube (Borosil). Tween 80 andrefined oil was tested for emulsification activity. Emulsification capacity was evaluated byadding 2.0 mL of oil in a test tube containing 2.0 mL of cell free crude biosurfactant suspension, the suspension was mixedvigorously for 2 min and then the suspension was allow edtosettle to visualize emulsification layer. Measurementswereper for medafter 0 minand 24 hours at room temperature. The emulsification index E24h was calculated by the ratio between emulsion layer height after24 hours and total column height. The stability was determined considering the columnemulsionheightafterE-0, E-24respectively (Mamta Rani *et al*., 2020)

2. Identification of Biosurfactant producing Bacterial Strain

Biosurfactant producing bacterial isolate obtained was subjected to perform the biochemical tests like gram's staining, catalase test, oxidase test, indole test, methyl red test, vogues – proskauer test, citrate utilization test, nitrate reductase test, carbohydrate fermentation test (Glucose, lactose).

3. OPTIMIZATIONOFCULTURECONDITIONSFORTHEPRODUCTIONOFBIOSURFACTANT

Optimization of culture conditions like, pH, temperature

and inoculum size was designed to increase biosurfactant production.

a) OPTIMIZATIONOFpH

Production medium containing g/lt of 5gm – peptone, 3gm – sodium chloride, 3gm – yeast extract was prepared, and the pH of the medium was varied from pH 5.0 to pH 9.0 in each set of fermentation media and the positive isolate was inoculated and incubated at 35◦C for 24 hours. After the incubation period biosurfactants was extracted from each production medium using chloroform and methanol. (Anuraj Nayarisseri *et al*., 2018).

b) OPTIMIZATIONOFTEMPERATURE

To determine optimum temperature for biosurfactant production, production media as described above was prepared with optimum pH, inoculated with positive biosurfactant strain and incubated at different incubation temperatures ranging from 25◦C to 40◦C with 5◦C variation for 24 hours, after completion of incubation period the biosurfactant produced was extracted from each production media using solvents.(Anuraj Nayarisseri *et al*., 2018).

c) OPTIMIZATIONOFINOCULUMSIZE

To know the optimum size of inoculum required for the production of biosurfactant, the inoculum size of the inoculate was varied ranging from 0.25ml to 1.25ml with 0.25ml variation. The production media was inoculated with respective inoculum size and incubated for 24hrs at 35°C, after incubation period biosurfactants produced were extracted using solvent extraction. (Anuraj Nayarisseri *et al*., 2018).

4. PRODUCTION AND EXTRACTION OF BIOSURFACTANT

Production media containing g/lt of peptone – 5gm, sodium chloride – 3gm, yeast extract – 3gm with optimum pH was prepared and inoculated with positive bio surfactant producing strain, incubated for 24 hours at optimum temperature. After completion of incubation period, the production medium with culture was centrifuged at 5000rpm for 15minutes to get cell free crude biosurfactant. The crude biosurfactant obtained was used to extract pure biosurfactant.

Extraction of Biosurfactant: Crude biosurfactant obtained was pre-treated by acidifying to pH 2.0 and refrigerated for 1hour to precipitate the biosurfactants, after incubation the biosurfactant was treated with chloroform and methanol in the ratio 2: 1 and mixed vigorously, later the biosurfactant with solvent mixture was collected in a pre- weighed evaporating dish and the dish was kept on boiling water bath to extract the biosurfactant, after complete evaporation biosurfactant formed was dark – honey colour in appearance, the weight of evaporating dish with biosurfactant was taken and the percentage of biosurfactant was calculated.(Anuraj Nayarisseri *etal*., 2018).

5. APPLICATION

1. Evaluation of Antibacterial Property

Biosurfactant extracted was checked for its antibacterial property against strains of *E.coi, Staphylococcus aureus, Pseudomonas*. Nutrient agar containing g/lt, Peptone – 5gm, yeast extract – 3gm, sodium Chloride – 3gm, Agar – 18gm, pH :7.0 was used in the study, the sterilized nutrient agar slants were inoculated with test organisms and spread evenly all over the plate using sterile swabs, later wells were created and wells were loaded with pure biosurfactant and solvent, later the culture plates were incubated at 35°C for 24 hours, after 24 hours the plates were observed for zone of inhibition. (Mayank Garg, 2018).

2. Anti- Biofilm Activity

Biosurfactant ability against biofilm forming bacteria was studied. Nutrient agar was inoculated with biofilm forming bacteria and spread evenly all over the plate, later well was created, and the well was loaded with biosurfactant and incubated at 35°C for 24 hours, after incubation period the plates were observed for zone of inhibition. (Karthik Sambanthamoorthy*et al*., 2014).

3. Application in oil removal from waste water

Role of biosurfactant in removing oil content was studied in the present study, three varieties of waste waters were used in the study. Oil & grease present in the sample before treatment and after treating with biosurfactant was determined by using hexane solvent and the percentage of oil & grease removed was calculated. (APHA 23rd Edition, Christina et al).

4. RESULT AND DISCUSSION

4.1 Isolation of bacteria

Soil samples and water samples collected were aseptically serially diluted, 10^{-5} and 10^{-6} dilutions were used in the study, and the samples were inoculated onto nutrient agar by spread plate technique, and incubated at 35°C for 24 hours. After incubation 07 isolated isolates were selected and sub cultured on nutrient agar for further work and preserved under 4°C. The isolates obtained were designated as HLS01, HLS02.....HLS07.

4.2 Screening for Biosurfactant producing bacteria

Isolates obtained HLS 01 to HLS 07 were subjected to screening to check for the presence of biosurfactant. Screening tests like oil displacement test, CTAB assay, drop collapse test, Emulsification test were performed for all the isolates as described as described above. Out of HlS01 to HLS07 isolates, HLS05 strain showed positive results with increased biosurfactant value in all screening tests performed. Results of test isolates specific to each screening tests are tabulated in table 4.2.1.

Table 4.2.1: Results of HLS01 to HLS07 towards screening test performed.

Test	Test Material	HLS01	HLS02	HLS03	HLS04	HLS05	HLS06	HLS07
Drop Collapse Test	Tween 80	+ve	-ve	+ve	-ve	+ve	-ve	+ve
	Coconut Oil	+ve	-ve	+ve	-ve	+ve	-ve	+ve
	Diesel	+ve	+ve	+ve	-ve	+ve	+ve	+ve
	Refined Oil	+ve	-ve	+ve	-ve	+ve	+ve	+ve
Oil Displacement Test in cm	Tween 80	1.1	-ve	1.0	-ve	1.0	-ve	0.9
	Coconut Oil	1.2	-ve	0.5	-ve	1.0	-ve	1.3
	Diesel	1.5	-ve	1.5	-ve	2.3	-ve	1.7
	Refined Oil	0.5	-ve	0.4	-ve	0.4	-ve	0.5
Emulsification Test in %	Tween 80	36	4.5	27	4.5	40	4.5	40
	Coconut Oil	31	4.5	31	4.5	40	4.5	45
	Diesel	40	4.5	31	4.5	45	4.5	45
	Refined Oil	31	4.5	40	4.5	45	4.5	36
CTAB Assay Zone of clearance in cm	CTAB	1.5	-ve	-ve	-ve	1.7	-ve	0.4

4.3 Identification of Biosurfactant Producing Strain

HLS05 strain which showed better positive results were selected for the further study, hence HLS05 was tested for biochemical tests, HLS05 showed gram negative reaction with rods shaped bacteria for gram's staining, HLS 05 strain showed negative results for indole test, methyl red test, voges-proskauer test, hydrogen sulphide test, and carbohydrate fermentation test using sucrose, lactose, glucose, where as positive results were observed for citrate test, catalase test, oxidase test, nitrate test and carbohydrate (mannitol) fermentation test. From the biochemical test results the HLS05 strain was identified to be *Pseudomonas sps*, hence HLS05 is designated asHLS05 *Pseudomonas sps*in further work. Results of biochemical tests are listed in table 4.3.1.

Table 4.3.1: Results of strain HLS05 towards biochemical tests.

Sl.No	Biochemical Results	Results
1	Gram's staining	Negative
2	Shape	Rods
3	Catalse Test	Positive
4	Oxidase Test	Positive
5	Indole Test	Negative
6	Methy;l Red Test	Negative
7	Voges – Proskauer Test	Negative
8	Citrate utilization Test	Positive
9	Nitrate Reductase test	Positive
10	Carbohydrate fermentation test	
	Glucose	Negative
2.	Sucrose	Negative
3.	Lactose	Negative
	Mannitol	Positive

4.4 Optimization of Culture conditions for the production of biosurfactants

Optimization of culture conditions like pH, temperature and inoculum size were studied to increase the biosurfactants production yield, from the study optimum pH was found to be pH 5.0, Optimum temperature was 25°C and optimum inoculum size was 1ml for the BS production.

4.5 Production and Extraction of biosurfactants

Production media with optimized pH 5.0 was inoculated with HLS05 *Pseudomonassps* strain and incubated at 25◦C for 24hours, after completion of incubation period, biosurfactant produced was extracted using chloroform methanol solvent extraction, 1% of biosurfactant yield was obtained using HLS05 *Pseudomonassps* strain with optimized culture conditions at the end of 24hours.

4.6 Application

Application of biosurfactant produced by HLS05Pseudomonas sps was checked towards anti – microbial property, anti – biofilm property and oil removal in wastewater. Biosurfactant exhibited anti-microbial property against *E. coli*, *S.aureus* and *Bacillus sps* with 17mm, 30mm and 20mm zone of inhibition respectively, whereas 30mm zone of inhibition was exhibited by biosurfactant towards biofilm forming bacteria.

From the study it was found that oil & grease content present in wastewater was reduced in all the three wastewater samples tested, results of the work are tabulated in table 4.6.1.

Table 4.6.1: represents the reduction in oil & grease present in wastewater after treating with biosurfactant produced by HLS05 *Pseudomonas sps.*

Sample	Oil & grease mg/lt Before Treatment	Oil & grease mg/lt After treating with BS
Sample 1	120	40
Sample 2	40	40
Sample 3	60	30

5. CONCLUSION

Biosurfactants are surface active compounds produced by a wide range of microorganisms. A few microorganisms have been stated to produce several classes of biosurfactants such as glycolipids, lipopeptides, phospholipids, neutral-lipids or fatty acids

and polymeric biosurfactants. Few of the *Pseudomonassp.* Isfoundto producegroup of biosurfactants called Rhamnolipids. In the present study isolation of organisms was done using soil and water samples. 07 isolateswere obtained where tested for biosurfactant production by performing screening tests like oil displacement test, drop collapse test, emulsification test and CTAB assay, out of 07 isolates, 04 isolates exhibited BS property. Out of 04 positive isolates HLS05 strain showed higher efficiency, hence HLS05 strain was selected for the further work and identified as *Pseduomonassps* from the biochemical tests, Culture conditions required to produce biosurfactants from HLS05 *Pseudomonas sps* was optimized and production was carried, later the purified biosurfactant was applied in the application studies. Application studies proved that biosurfactant produced from HLS 05 *Pseudomonas sps* is effective in anti-microbial property, anti – biofilm property and efficient in reducing oil & grease content in contaminated source.

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