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Isolation of Biosurfactant Producing Bacteria from Oil Contaminate Site

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Abstract

Naturally produced surface active compounds of biological origin are called biosurfactant. The biosurfactant producing bacteria were isolated from different oil contaminated site. Seven oil contaminated soil samples were collected from local garage located in various areas of Ahmedabad and Ghandhinagar. Total 19 morphologically distinct colonies were obtained on Mineral salt medium (MSM) supplemented with 2% engine oil. All the isolates were grown as shake flask culture and were further screened for biosurfactant production using different tests such as drop collapse test, oil displacement test, emulsification activity, hemolytic activity and blue agar plate method. By these tests, 6 bacterial isolates viz. KP1, KP2, KP8, KP12, KP13 and KP16were screened for biosurfactant production. Among these isolates, Isolate KP8 was further evaluated for reduction ofsurface tension by drop collapse test, oil displacement test and emulsification activity.Effect of various carbon sources like glucose, glycerol, paraffin oil, kerosene and peanut oil was checked on biosurfactant production. Isolate KP8 gave the best results to reduce surface tension when grown in MSM supplemented with 1% paraffin oil.

Key words:Biosurfactants, Emulsification index, Drop collapse, Oil displacement.

Introduction

Surfactants are found to enhance degradation and recovery of crude oil. Surfactants and emulsifiers which may be synthetic or of biological origin are inseparable components of daily life (Das andChandran, 2011). Surfactants are amphiphilic molecules possessing wide variety of surface activity. As they are of both hydrophilic and hydrophobic in nature they reduce surface and interfacial tensions by accumulating at the interface by formation of micelle between two immisible fluids like oil and water and facilitate hydrocarbon uptake and emulsification (Thavasi, Sharma, Jayalakshmi, 2011). This molecules have ability to aggregate between the interfaces of fluids with different

polarity i.e., water and oil and therefore reducing interfacial tension between them (Banat, 1995). Depending upon their charge they are classified anionic, cationic and non-ionic.Biosurfactantis a substance that tends to reduce surface tension produce by different microorganisms (Vingeshwaram et al., 2016). Microorganisms have attracted much attention because of advantageous characteristics such as a structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity and lower CMC. These properties have led to several biosurfactantapplication in the food, cosmetic and pharmaceutical industries (Xiao-Xia et al., 2003; Thanomsub et al., 2004).

Unlike the chemically synthesized surfactants that are categorized on the basis of on the type of the polar group present, biosurfactants are in general classified chiefly by their chemical composition and microbial origin. Rosenberg and Ron(1999) suggested thatbiosurfactants could be devided into low molecular mass molecules and high molecule mass molecules. The main classes of low mass surfactants are lipopeptides, glycolipids and phospholipids, whereas large mass surfactants include polymeric and particulate surfactants. The majority biosurfactants are anionic or neutral and the hydrophobic moiety is based on long chain fatty acids or fatty acid derivatives, whereas the hydrophilic moiety can be a carbohydrate, phosphate, amino acid or cyclic peptide. Thus, based on their chemical composition, the biosurfactants produced by microorganisms are of many types such as glycolipids, lipopolysaccharides, oligosaccharides, and lipopeptides(Rosenberg and Ron, 1999).

Biosurfactantshas received considerable attention in the field of environmental remediation processes such as bioremediation, soil washing, and soil flushing. Biosurfactants influence this processes because of their efficacy as dispersion and remediation agents and their environmentally friendly characteristics such as low toxicity and high biodegradability. Although biosurfactants exhibit such important advantages, they have not yet been employed extensively in industry because of relatively high production costs (Satpute et al., 2010). Moreover, they can be used as moistening agents, dispersing agents, emulsifiers, foaming agents, beneficial food elements and detergents in many industrial regions such as organic chemicals, metallurgy, mining, petroleum, petrochemicals, biological control and management and many others(Banat et al., 2000; Perfumo et al., 2010; Vedaraman and Venkatesh, 2011).Biosurfactant can raise the surface area of hydrophobic resources, such as pesticides in water and soil surroundings, thus increases in water solubility. Hence, the existence of surfactants might increases microbial degradation of pollutants. The utilization of biosurfactants for degradation of pesticides in soil and water environment has gained significance recently. The identification and characterization of biosurfactant produced by a variety of microorganisms have been broadly reviewed (Lin, 1996; Desai, 1987; Parkinson, 1985).

Materials and Methods

Sample collection

Seven oil contaminated soil samples were collected from garage located in various areas of Ahmedabad and Gandhinagar district, Gujarat, India.

Enrichment and isolation of Bacteria

One gram of each soil sample was inoculated into 50 ml of mineral salt medium(Tahzibi et al., 2004) containing (g/l); 15g $NaNO_3$, 1.1g NaCl, 1.1g KCl, 0.00028g $FeSO_4.7H_2O$, 4.4g K_2HPO_4, 3.4g KH_2PO_4, 0.5g $MgSO_4.7H_2O$incubated at room temperature on shaker at 120 rpm for 4-5 days.After enrichment, the samples were serially diluted till 10^{-5} in sterile distilled water and spreaded 0.5ml sample onto MSM plate containing 2% engine oil. After incubation, different bacterial isolates were selected on the basis of different colony morphology on MSM. All the different bacterial isolates were preserved on mineral salt agar slants at 4°c. The selected isolates were screened for the production of biosurfactants.

Screening for Biosurfactant production

Cultural medium and Bacterial growth: Bacteria were grown aerobically in 250 ml Erlenmeyer Flask with 100ml of MSM supplemented with 2% v/v engine oil. Flasks containing sterilized MSM were inoculated with loopfull of bacterial culture grown on MSM plates and then flasks were incubated on shaker at room temperature, 120 rpm for 4-5 days. After incubation, 10ml culture broth from each flask was centrifuged at 12000rpm for 10 minutes and the supernatant collected in sterile test tubes. This cell free supernatant was used for drop collapse test, oil displacement test, emulsification activity, hemolytic activity, blue agar plate method. All the screening experiments were performed in triplicates and the mean value were presented as results.

Drop collapse test: 10µl of culture supernatant was placed carefully on an oil coated glass slide and observed after 1 min. If the drop of supernatant collapsed and spread on the oil coated surface, it indicates positive test for biosurfactant. This test simultaneously carried out on distilled water and 1% Sodium DodecyleSulphate as negative control and positive control respectively (Jain et al., 1991).

Oil Displacement test: 20ml of distilled water added to the petri plate followed by addition of 40µl of engine oil to the surface of water. 20µl of cell free supernatant was then added to the oil surface (Morikawa et al., 2000). If biosurfactant is present in the cell free supernatant, the oil will be displaced with an oil free clearing zone and diameter of this clear zone indicates surfactant activity, also called oil displacement activity. A negative control was maintained with distilled water, in which no oil displacement or clear zone was observed and SDS was used for positive control.

Emulsification Activity: Biosurfactant activity was measured by adding 2ml of engine oil and 2ml of cell free supernatant. The mixture was vortexed at high speed for 2 min. The height of emulsion was measured by taking the layer formed in between aqueous and engine oil layer (Cooper and Goldenberg, 1987). Measurement was taken after 24 hrs. The emulsification activity was determined using the following formula.

Emulsification activity (%) = Height of emulsion layer ×100/Total height

Emulsion by the isolates were compare to those form by distilled water as control.

Hemolytic Activity: Pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 37°C for 48-72 hrs. Results were recorded based on the type of

clear zone observed i.e., α-hemolysis when the colony was surrounded by greenish zone, β-hemolysis when the colony was surrounded by a clear white zone and Ò-hemolysis when there was no change in the medium surrounding the colony(carrillo et al., 1996).

Blue Agar Plate Method: Mineral salt agar medium supplemented with glucose as carbon source(2%) and cetyltrimethyl ammonium bromide(CTAB 0.5mg/ml) and methylene blue (MB 0.2mg/ml) were used for the detection of anionic biosurfactant(Satpute et al., 2008). 30µl of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer (4mm). The plate was then incubated at 37°C for 48-72 hrs. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production.

Effect ofCarbon Sources on Biosurfactant production: Mineral salt medium supplemented with various sources like glucose(w/v),glycerol, peanut oil, paraffin oil and kerosene at three different concentrations 1%, 2% and 3%(v/v) inoculated with biosurfactant producing isolate and incubated at room temperature, 120 rpm for 4-5 days to check the activity of biosurfactant producing isolate in various sources.

Results and Discussion

Total 19 bacterial isolates were isolated from different oil contaminated soil samples by serial dilution technique. Out of 19 isolates, 11 strains were gram positive and 08 gram negative. In present study out of 19 isolates, 06 were further screenedas positive for biosurfactant production by different tests like oil displacement test, drop collapse test, emulsification activity, etc. Among this 06 isolates, isolate KP1, KP2, KP8, KP12, KP16 were gram positive and KP13 was gram negative. Conoly characteristics of these six bacterial isolates are described in the Table 1.Ghayyomi, et al (2012) isolated biosurfactant producing bacteria from petroleum contaminated soil and they obtained 160 strains were able to producebiosurfactant.

Name	Size	Shape	Margin	Elevation	Texture	Consistency	Opacity	Pigment
KP1	Medium	Irregular	Uneven	Raised	Rough	Dry	Opaque	Golden-yellow
KP2	Medium	Round	Entire	Convex	Smooth	Mucoid	Opaque	Off-white
KP8	Small	Round	Even	Convex	Smooth	Moist	Opaque	Pinkish-orange
KP12	Small	Round	Uneven	Raised	Rough	Dry	Opaque	Golden-yellow
KP13	Small	Round	Even	Convex	Smooth	Moist	Opaque	Yellow
KP16	Small	Round	Even	Convex	Smooth	Mucoid	Opaque	Nil

Table: 1 Colony characteristics on MSM

Selected six bacterial isolates were grown in MSM with 2% engine oil and incubated at room temperature, 120 rpm for 5 days. Samples were withdrawn on 4th, 5th and 6th days of incubation and further tests were carried out from the supernatant. And the results are documented in Table2.

Drop collapse test(DCT): Drop collapse method is a sensitive and easy to perform method which requires small volume of culture broth or biosurfactant solution to test the surfactant property. Among the 19 strain screened, 06 strains are positive for drop collapse test. Isolate KP2 & KP13, isolate KP12, isolate KP1 and isolate KP8& KP16 showed positive test, moderate activity, good activity and very good activity respectively for drop collapse test.Suthar et al., (2017) isolated 09 bacterial isolates from oil contaminated site and reported 04 isolates shown positive results.

Oil displacement test(ODT): Oil displacement test results were in corroboration with drop collapse test results. Strains found with positive drop collapse results were positive for oil displacement test also. These results confirmed the presence and absence of surface active compound in the cell free culture broth. The area of oil displacement in oil displacement test is directly proportional to the concentration of the biosurfactant in solution (Morikawa et al., 2000). As found in Drop collapse test, all 06 isolates were positive for the oil displacement test and showed same results as DCT.Thavasi et al.,(2011) isolated 105 bacterial strains from which 82 strains were positive for oil displacrement test. The advantage of DCT and ODT is very easy and less sample volume 10-20µl is required to check the drop collapse and oil displacement test, which helps in repeating the measurements. Also it can be performed without an aid of specialized instruments.

Emulsification activity: Emulsification activity is an indirect method used to screen biosurfactant production. It was assumed that if the cell free culture broth used in this method contains biosurfactant then it will emulsify the hydrophobic substrate present in the test solution. In this study, engine oil was used as the hydrophobic substrate. Isolate KP8 showed highest emulsification of 58%. Remaining isolates KP12, KP16, KP1, KP13 and KP2 showed 46%, 42%, 34%, 30% and 19% emulsification activity, respectively. Cheng et al.(2008) has studied the emulsion property of bacterial biosurfactant and they isolated three unknown biosurfactant producing bacteria and their emulsification activity was compared with the two artificial surfactant, SDS and Triton X-100.

Hemolytic test: Among 06 bacterial strains tested, isolate KP2, KP8 and KP16 strains were positive for hemolysis and isolate KP16 indicates α-hemolysis and KP2, KP8 indicates β-hemolysis and other remaining three isolates KP1, KP12, KP13 indicates Ò-hemolysis. It has been previously reported that blood agar lysis test gives high percentage of either false negative or false positive results. The reason for false result could be virulence factor for poor diffusion of biosurfactant in blood agar plate. Another drawback of hemolytic test is that hydrophobic substrate can not be included as sole carbon source in this test.Thavasi et al.,(2011) isolated 105 bacterial strain from which 101 strains were positive for hemolysis and *Ps. aeruginosa* showed the maximum hemolytic activity.

Blue Agar Plate method: Blue agar plate method was reported as a new, rapid, easy, systematic, non-tedious, non-laborious method for biosurfactant detection which could be transferred to liquid culture conditions. Positive results were noted as dark blue halo zone around the well on a light

blue plate background. Out of 06 isolates, isolates KP8& KP1 showed dark blue halo zone, 04 isolates did not show dark blue halo zone indicative of anionic biosurfactant production.Saravanan and Vijayakumar,(2012) isolated 243 bacterial isolates from oil contaminated soil from which only 10 isolates showed dark blue halo zone around colonies. The disadvantage of this method is that CTAB is harmful and inhibits the growth of some microbes and CTAB could be replaced by another cationic surfactant.

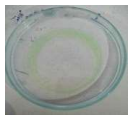
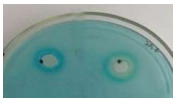
Name	Drop collapse test	Oil displacement test	Emulsification Activity	Hemolytic activity	Blue agar plate
KP1	++	++	34%	?	+
KP2	+	++	19%	B	-
KP8	++++	++++	58%	B	+
KP12	+++	++	46%	?	-
KP13	+	+	30%	?	-
KP16		++	42%		-
Figure	 +++			 α	

Table: 2 (Results of screening tests) where(++++)=best result, (+++)=good result, (++)=average result, (+)=weak result

Five screening methods mainly (Drop collapse test, Oil displacement test, Emulsification activity, Hemolytic activity and Blue agar plate) were employed for biosurfactant and assessed for their suitability for these purpose. It was found that Drop collapse and Oil displacement tests are reliable methods to screen large number of samples for biosurfactant production because these methods require only 10-20 µl of biosurfactant solution and have direct correlation with each other as reported with in this study and in other earlier studies. Results from this study shows that the order of suitable methods to screen biosurfactant production is drop collapse test = oil displacement test > emulsification activity > hemolytic test = blue agar plate. KP8 was selected as a potential biosurfactant producing isolate with MSM supplemented with 1% paraffin oil from 19 isolates obtained from oil contaminated sites and used for screening purpose in the study.

Effect of various carbon sources: Various bacterial isolates gave different results with various sources depending on their ability to degrade hydrophilic and hydrophobic substrates to produce biosurfactant. Isolate KP1, KP8, and KP12 showed best biosurfactant activity with MSM

supplemented with glycerol, paraffine oil and kerosene, respectively. Isolate KP16 showed best activity with MSM supplemented with both paraffine oil and engine oil

Various sources	KP1	KP8	KP12	KP16
Glucose	+	++	-	-
Glycerol	+++	+	+	+
Peanut oil	++	+	-	++
Paraffin oil	+	+++	++	+++
Kerosene	-	++	+++	++
Engine oil	+	++	+	+++

Table: 3 (Result of various sources) where(+++)=best result, (++)=average result, (+)= weak result, (-)=negative result

Wen-Jie Xia et al (2011), in that research they were isolated three biosurfactant producing bacteria from reservoir formation water, *B. subtilis, Ps. aeruginosa*and *R. erythropolis,* by using this three bacteria three biosurfactant was extracted and studied using crude oil as a carbon source.Suthar et al.,(2017) studied biosurfactant production from *Ps. stutzeri* by using waste fried oil as a carbon source.Varjani and Upasani,(2014) studied biosurfactant production with crude oil, glucose and glycerol and showed best biosurfactant activity with 1% glucose by *Ps.aeruginosa* and selected as a potential biosurfactant production.

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