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Screening of Rhamnolipid Producing Bacteria from Oil Contaminated Soil Sample

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ABSTRACT

Biosurfactant are amphiphilic compounds containing both hydrophobic and hydrophilic moieties. Biosurfactant reduces surface and interfacial tension by accumulating between two phases. They are produced by variety of microorganism's viz. Bacteria, fungi and yeast as secondary metabolites. Due to low toxicity, biodegradability and eco-friendly nature they are commonly used in several sectors. In the present research study, biosurfactant producing isolates were isolated from oil contaminated soil sample from different locations of Washim city area. Further the isolates were screened for biosurfactant production. Primary and secondary screening method was employed for screening test. Total 07 isolates were obtained and identified on the basis of cultural, morphological and biochemical characteristics. Out of the 07 isolates 04 isolates showed positive results in primary screening and further used for secondary screening. In secondary screening it is found that, RL1 demonstrated the highest biosurfactant-producing ability. Hence it is concluded that oil contaminated soil could be a good source for the isolation of biosurfactant producing bacteria. This finding could have significant implications for environmental and industrial applications, including bioremediation and oil spill control; highlight the ecological benefits of biosurfactant.

Keywords – Biosurfactant, Rhamnolipid, amphiphilic

INTRODUCTION

Biosurfactant are secondary metabolites synthesized by variety of bacteria, yeast and filamentous fungi. Biosurfactant are amphiphilic in nature contain both hydrophobic and hydrophilic moieties. These extracellular and amphiphilic compounds were discovered in the 1960s through the fermentation of hydrocarbon. Biosurfactant have much more advantages as compared to synthetic surfactant. Due to low toxicity, biodegradable nature, high selectivity, low critical micellar concentration and stability in drastic condition of temperature, pH and salinity biosurfactant received a lot of attention and used for various application in several sectors.

Biosurfactants are commonly grouped on the basis of biochemical nature or the producer species. With regard to structure, these compounds are classified into mainly five groups (Rahman and Gakpe, 2008). Biosurfactant are broadly classified as low molecular weight Lipopeptides, Glycolipids and high molecular weight Lipopolysaccharides, protein. A large diversity of micro-organism are capable to produce biosurfactant with different molecular structure.

Microorganisms synthesized various classes of biosurfactant such as phospholipid, glycolipids, fatty acids, and lipopeptides (Cooper 1986; Cooper *et al*., 1980; Velikonja and Kosaric 1993). Some biosurfactant synthesized by microorganisms produced on water soluble substrate such as glucose, ethanol and glycerol but majority of known biosurfactant is produced on water insoluble hydrocarbons (Haferburg *et al*., 2005). Sophorolipds were the first microbiological biosurfactant introduced in the market. Up to now, the best studied groups of biosurfactant are phospholipids and glycolipid compounds (Sandoval *et al.,* 1999). Currently rhamnolipid have the great potential for becoming the next generation biosurfactant (Muller *et.al*., 2012).Several studies reported that biosurfactant have antimicrobial activity against bacteria, fungi and viruses. Due to wide broad spectrum antimicrobial activity biosurfactant can be used as a new alternative to synthetic chemical drug (Rahman *et al*., *2002*)

Rhamnolipid produce by pseudomonas aeruginosa have been investigated widely.(Abdel-Mawgoud *et al.,* 2010; Chrzanowski *et al.*, 2012; Henkel *et al.,* 2012; Nitschke *et al.,*2011). Several strains of Pseudomonas aeruginosa synthesized various glycolipid type biosurfactants. Currently rhamnolipid have the great potential for becoming the next generation biosurfactant (Muller *et al*., 2012). Rhamnolipid are mainly produced by Psudomonas aeurogenosa (Edwards and Hayashi, 1965). They are formed by monosaccharide and disaccharides of rhamnose linked by glycolic bond to a beta hydroxy fatty acid molecule (Kumar *et al.,* 2021; Patowary *et al*.,2016). They are generally used in oil recovery processes and in agriculture sector to improve soil quality and to control plant pathogen.

Structure and yield of biosurfactant determine on the type of fermenter, nutrients, pH, base and temperatures utilized (Mulligan, 2005). Rhamnolipids have various potential industrial and environmental applications including the production of superior chemicals, the characterization of surfaces and surface coatings and supplements (Maier. R.M. *et al.,* 2000). They can be efficiently used in manipulation industrial emulsions, biodegradation and detoxification of industrial emissions, control of oil spills, and in bioremediation of contaminated soil (liang, M.W *et al.,* 2008)

Characteristics of biosurfactants depend not only on the producer's organisms but also on the growth conditions. So, it will be essential to evaluate various obtainable strains for their biosurfactant potential, the suitable nutrients, and the cultural conditions required to succeed high efficiency.

Hence the present study focus on screening of biosurfactant producing bacteria from oil contaminated soil sample.

MATERIAL AND METHOD

Collection of soil sample:

100 gram of oil contaminated soil sample was collected using sterile spatulas and transferred in a plastic polythene bags, labelled and transported to microbiology reserch laboratory, R.A. College Washim. The soil sample was further used for the isolation of Bacterial isolates.

Enrichment of soil sample

Enrichment was carried out by suspending 10 gm soil sample in 100 ml sterilized nutrient broth at 37º C for 24 hrs. The enriched sample was further used for the selective enrichment in 100 ml modified mineral salt medium.

Isolation and Identification of Bacterial isolates

The isolation of Bacterial strain from selective enriched sample was carried out. After 24 hrs. devloped colonies were screened for colony character. The isolated colonies were identified on the basis of cultural, biochemical and morphological characters.

Screening of biosurfactant producing organisms:

1. Primary screening:

Isolation of biosurfactant producing bacterial isolates was carried out. After inoculation the flask were incubated on rotary shaker at 30^0 c temperature and 250 rpm for 5 days. After incubation a drop from each flask was placed on mineral salt CTAB methylene blue agar for the detection of rhamnolipid production.

The culture showing positive result were used for secondary screening

2. Secondary screening:

The secondary screening of rhamnolipid producing bacterial strain was carried out as per the procedure suggested by Moussa *et al* , 2014.

2.1 Hemolytic activity:

Pure culture of bacterial isolates were streaked on the freshly prepared blood agar plates and incubated at 37°C for 48-72 h. 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 γ-hemolysis when there was no change in the medium surrounding the colony (Carrillo *et al*., 1996)

\2.2.Emulsification activity (EA):

Emulsification activity is measured by as per the procedure suggested by Cooper and goldenberg,1987. Emulsification index was determined by mixing 2 ml supernatant and 2 ml kerosene for 2 min and height of emulsion was measured by 24 hrs. The equation used to calculate the Emulsification index is as follows (Nitschke *et al*., 2010, Viramontes-Ramos *et al*., 2010,Cooper and Goldenberg,1987).

$$E = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100$$

2.3. Surface Tension (ST):

The surface tension of the culture media was determined by using surface tensiomat model 21 tensiometer by the Nouy ring method. The surface tension of pure water was determined for the calibration of the instrument. The criteria for the selection of biosurfactant producing bacterial isolates was the Emulsification and reduction of the surface tension of the medium below 40 dyne/cm.

2.4. Oil-spreading method (OSM):

Oil spreading technique was done as per the procedure suggested by (Morikawa *et al*., 1993). 10 microlitre crude oil was added on the surface of 40 ml of distilled water in a Petri plate to form a thin layer. After that 10 microlitre of culture supernatant of screened organism was placed on the centre of the oil layer.

2.5. Drop collapse test:

The drop Collapse test was performed according to method described by Bodour and miller- Maier (1998). Drop Collapse test was done by inoculating bacterial isolates in the enrichment medium and incubated for 5 days at 37 c and 150 rpm. After incubation culture broth was centrifuged at 10,000 rpm for 15 min. 96 well microtitre plate lids was used for drop Collapse test. The microtitre plate were washed with hot water and rinsed with ethanol. The microtitre plate lid were again rinsed with distilled water and dried. The microtitre plate was coated with oil. Soyabean oil was used for this test to ensure uniform oil coating plate were equilibrated for 24 hrs. A 5 microlitre of sample was combined with 2 microlitre of methylene blue and then using micropippete applied it onto the centre of oil drops.

RESULT AND DISCUSSION

Isolation and identification of bacterial isolates

The present study was carried out for the selective isolation of rhamnolipid Producing bacterial strains. Isolation of bacterial strains was done using oil contaminated soil sample. Total seven bacterial isolates were obtained. The purified isolates were labelled as RL1 to RL7 and subjected for Conventional identification and compared with standard literature.

Table 1. Morphological and biochemical characterization of Bacterial isolates

Test	Bacterial isolates						
	RL1	RL2	RL3	RL4	RL5	RL6	RL7
Size (µm)	0.6 -2	0.6-1.5	0.8 -2	0.7- 2	0.5-2	0.7-2	0.7-2.2
Gram stain	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Indol	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-
Voges Proskaur	-	-	-	-	-	-	-
Citrate Utilization	+	+	+	+	+	+	+

Among the all isolated strains, Pseudomonas aeruginosa is identified on the basis of cultural and biochemical tests.

Screening of surfactant producing bacterial strains

The pure cultures of Pseudomonas species was inoculated separately in the sterilized modified mineral salt medium containing test tubes as suggested by Dubey and Juwarkar (2001) for the isolation of rhamnolipid producing bacterial strains. After incubating on rotator shaker at 250 rpm and 30°C temperatures for 5 days, a drop from each tube was spotted on Mineral salt CTAB methylene blue agar to detect biosurfactant production (Seigmud–Wagner 1991). The plates were incubated at 45°C for 24 hrs. After incubation, the plates were observed for the formation of a dark blue halo around the drop of culture (Tahzibi et al., 2004).It is observed that out of 7 isolates, total 4 isolates shows positive results.

Table 2. Primary screening for rhamnolipid producing bacteria strain

Sr.No	Isolates	Appearance of dark blue halo on CTAB
1	RL1	+ ve
2	RL2	+ ve
3	RL3	-ve
4	RL4	-ve
5	RL5	+ ve
6	RL6	-ve
7	RL7	+ve

Secondary screening for efficient rhamnolipid producing bacterial strain.

Secondary screening for efficient rhamnolipid producing Pseudomonas Strains. The secondary screening was done by six different highly recommended Screening methods recommended by Sekhon et al., (2011) viz. haemolytic assay, oil Spreading assay, drop collapse method, emulsification assay, Surface tension Reduction and Phenol sulphuric acid test.

The results of secondary screening methods are as follows:

Table 2.1 Emulsification activity:

Table 2.1 and figure 1.1 shows the results on Emulsification activity of Primary screened isolates. It is found that high emulsification activity (42%) was given by RL 1 strain followed by RL7 (33%). RL 2 showed 31% emulsification Activity followed by RL5 (23%).

Sr. No	Isolates	Emulsification index (%E24)
1	RL1	42
2	RL2	31
3	RL5	23
4	RL7	33

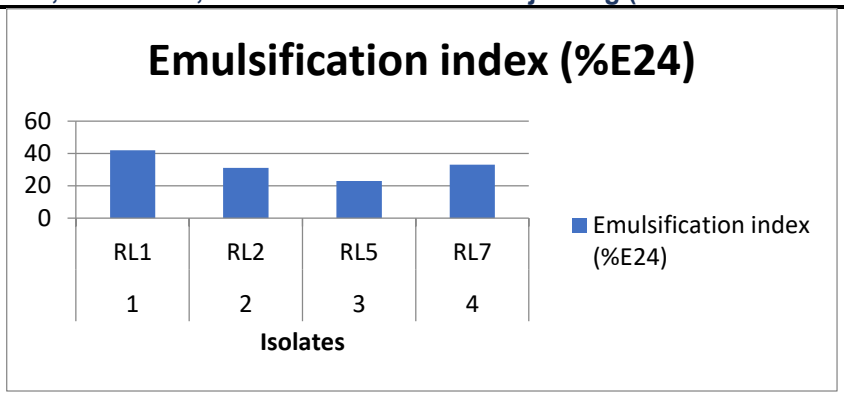

Table 2.2 Oil spreading technique

Table 2.2 and figure 1.2 shows the results of oil Spreading test. The primary screened isolates were subjected for oil spreading technique. It is found that out of 04 strains, total 03 strains showed positive results for this test. The Maximum clearance zone was given by RL 7 (2.5cm.) followed by RL2 (1.8cm.) and RL5 (1.3cm.) respectively.

Sr .no	Isolates	Diameter of zone of clearance (cm)
1	RL1	2.5
2	RL2	1.8
3	RL5	1.3
4	RL7	0

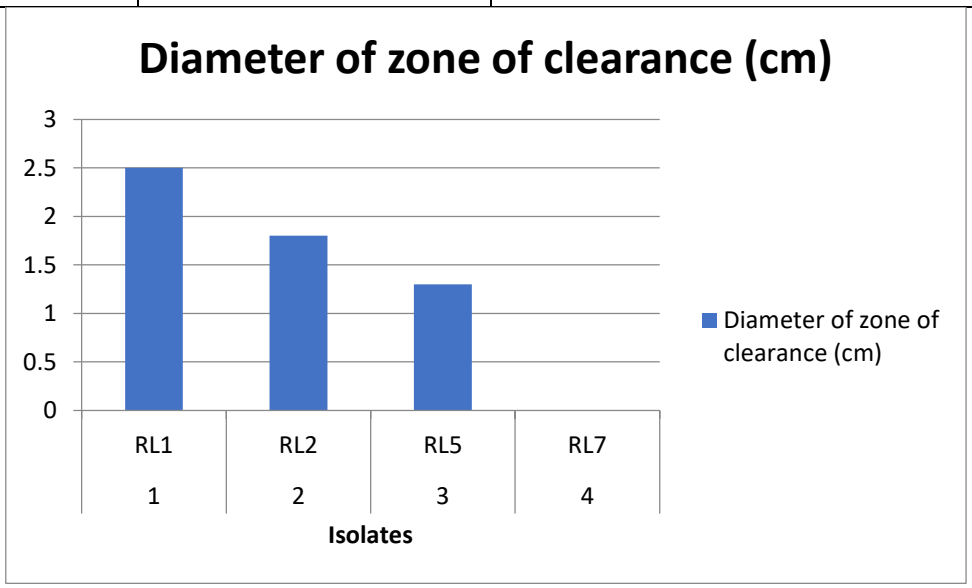

Table 2.3 Surface tension reduction

Table 2.3 and figure 1.3 shows the results on Surface Tension Reduction test of Primary screened Pseudomonas isolates. It is found that RL1 showed highest Surface tension reduction followed by RL5, RL 7 and RL 2**.**

Sr .no	Isolates	Surface tension (mN m-1)
1	RL1	27
2	RL2	19
3	RL5	23
4	RL7	21

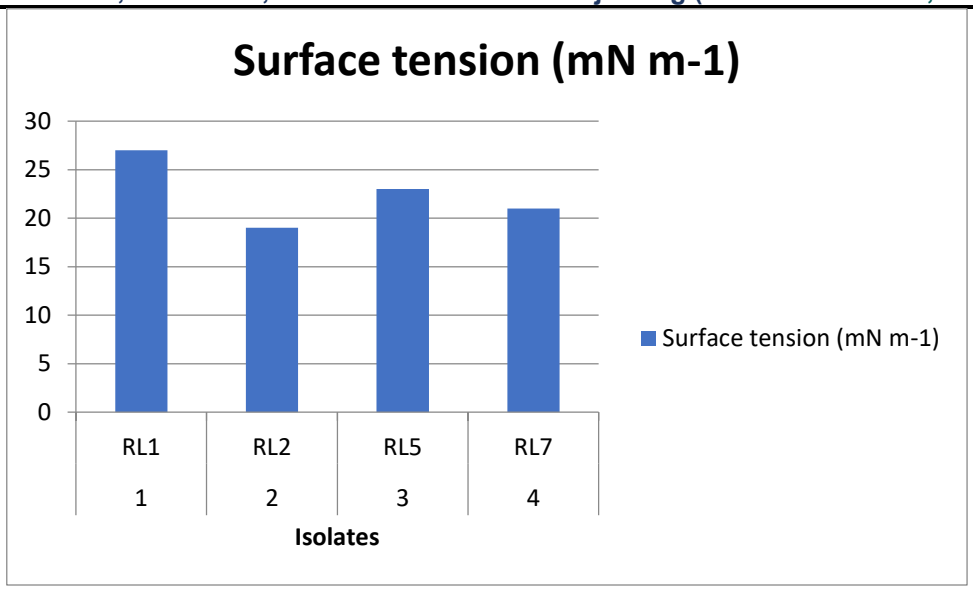

Table 2.4 Blood haemolysis test

The strains showing positive primary screening were used for blood Haemolysis test. It is found that Out of 04 primary screened isolates, 03 isolates showed B-haemolytic pattern on blood agar.

Sr .no	Isolates	Haemolytic activity
1	RL1	+ve
2	RL2	+ve
3	RL5	+ve
4	RL7	-ve

Table 2.5 Drop collapse test

During the drop collapse test it is found that all the 4 isolates showed positive results.

Sr .no	Isolates	Drop collapse test
1	RL1	+ve
2	RL2	+ve
3	RL5	+ve
4	RL7	+ve

CONCLUSION

The present study successfully isolates and screened bacteria from oil mill soil samples, identifying potential biosurfactant producers. The focus on multiple screening methods enhances the reliability of the results. Among the isolated strains, RL1 demonstrated the highest biosurfactant-producing ability. This finding could have significant implications for industrial and environmental applications.

SUGGESTIONS

This research study provides valuable insights; further research is suggested to delve into the specific properties and applications of the biosurfactants produced by the identified strains. Additionally, exploring optimization strategies for enhanced biosurfactant production could be a fruitful avenue for future investigations.

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