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Comparative Analysis of Biosurfactant Production Assays by Five Indigenous Oil Sludge Bacteria

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ABSTRACT

Although there are several screening assays available for screening and evaluating the presence of biosurfactants but many of these show variable results under the same conditions and for the same organism. Most commonly applied screening assays including oil spreading technique (OST), tilted glass slide assay (TGS), drop collapse assay, emulsification index E24 and emulsification assay were used to evaluate production of biosurfactants in five biochemically identified strains isolated form oil contaminated sites. All strains used were known producers of biosurfactants but showed variable results for different tests. Four of five strains produced similar results for OST, TGS and emulsification assay. Drop collapse assay produced negative results when small amounts of biosurfactants were present. So it was concluded that a single primary screening test alone cannot identify an organism's ability to produce biosurfactants and these tests should be used in combinations to get a reliable picture.

Key Words: Biosurfactants, screening assays, Oil Spreading Technique, Tilted Glass Slide Test, Emulsification index E24, Emulsification assays.

INTRODUCTION

Microbial flora of oil sludges produce surface active agents to lower oil and water as well as oil and soil interfacial tension that makes oil available for consumption or solubilization. Such chemical agents are called biosurfactants or bioemulsifiers, depending on their interaction with the hydrocarbon moiety and physiochemical properties (Uzoigwe *et al.*, 2015). Hydrocarbonclastic bacteria produce biosurfactants and therefore this can be considered as a survival mechanism in oil-polluted environment (Pacwa-Płociniczak *et al.*, 2011).

Oil-polluted areas have always been a grave concern in regard to the environmental pollution, and thus microbial activity to produce biosurfactants holds an important area for research (Colwell *et al.*, 1977; Atlas, 1981). Biosurfactants with their wide diversity and substrate specificity have functionality in the areas of biodegradability and inactivation/sequestering (Makkar & Cameotra, 2002). These properties account for their desirability in agriculture, food, textiles, cosmetics, petrochemicals etc (Haferburg *et al.*, 1986; Georgiou *et al.*, 1992; Prince, 1993). In addition, biosurfactants are helpful in microbial enhanced oil recovery (MEOR) (Brown *et al.*, 1985).

To harness the capability of microbes in biodegradation or rehabilitation of oil polluted areas; various assays have been developed. Initial testing of microbial production of biosurfactants is done by any one of the most commonly performed assays including oil spreading technique(OST) (Morikawa *et al.*, 2000), emulsification index E24 (Cooper & Goldenberg, 1987), emulsification assay (Patil & Chopade, 2001), tilted glass slide (TGS) assay (Persson & Molin, 1987) and drop collapse test (Bodour & Miller-Maier, 1998).

MATERIALS AND METHODS

Selection of Bacterial Strains: Soil samples from different oil contaminated areas of Lahore, Pakistan were collected. Five strains, namely G, H, J, K and L, of different phenotypic and morphological characteristics were selected and isolated on L-agar culture media. These isolates were then biochemically identified according to the identification schemes of Bergey's Manual (Garrity G. *et al.*, 2006).

Tests for biosurfactants: Primary screening tests were checked for the efficacy and accuracy in determination of biosurfactants, using coconut oil, in five bacterial strains i.e; G, H, J, K and L. which are known producers of biosurfactants at different levels (Bento *et al.*, 2005) (Hamed *et al.*, 2012). Each test was performed in duplicates and mean results were noted.

Oil spreading technique (OST) The assay was performed according to the technique of Morikawa *et al.,* 2000. Briefly, a Petri plate was filled

Author's Contribution: A.A., The research project was initiated and planned; **H.M.**, **S.N.CH.,** Protocols optimized; **W.F.**, Project was supervised; **A.A., H.M.,** Research methodology was conducted and troubleshot (where applicable).

with 20 ml of distilled water and on it 8 µl of crude oil was placed forming a uniform oil layer. 6 µl of bacterial culture was added to the center of plate; on top of the oil layer, and clear zones were observed after 30 seconds. Quantity of biosurfactant formation per cm2 of oil displaced was defined as one BS (biosurfactant) unit (Thaniyavarn *et al.*, 2003).

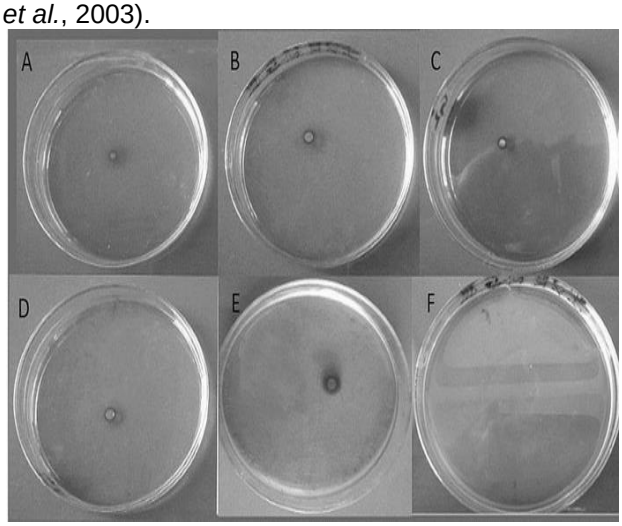

Fig., 1: Oil spreading technique, A: Strain G, B: Strain K, C: Strain L, D: Strain H, E:Strain J, F: Negative control (oil+water)

Drop Collapse assay Bodour & Miller-Maier (Bodour & Miller-Maier, 1998) drop collapse assay developed from Drop collapse assay by Jain et al (Jain *et al.*, 1991), was used. Glass slides were rinsed using hot water, treated with ethanol and dried after washing with distilled water. These were then coated with oil and equilibrated to form a thin oil coating. 5 µl of bacterial culture supernatant was placed onto the glass slide and results were noted as a positive if the drop collapsed from its beaded shape or a negative if it remained in its initial form.

Tilted glass slide test: A colony of each strain was mixed with normal saline (0.9% NaCl) at an end of the glass slide which was cleared of any oil or possible surfactants by carefully washing with ethanol or heat dried. The slide was tilted and the drop was observed for collapsing or any kind of similar changes like dipping down or flowing down of the drop (Persson & Molin, 1987).

Emulsification index E24: Equal volumes of (2ml) bacterial culture and oil were mixed and vortexed at high speed for 2 minutes. These were then left to stand for 24 hours for ensuring a stable emulsified layer of oil. E24 index was calculated by dividing the height of emulsification "he" by the total height "ht" and multiplied by 100 to get percentage emulsification (Cooper & Goldenberg, 1987). Clear distilled water was used as a negative control.

E24 = (he/ ht) x 100

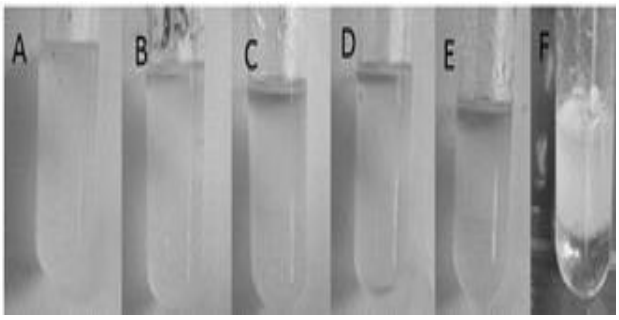

Fig., 2: Tubes are showing the Emulsification index test (E24) using coconut oil. A: Strain H, B: Strain J, C: Strain K, D: Strain G, E: Strain L, F: Negative control.

Emulsification assay: Twenty four hours fresh cultures grown under optimum conditions were centrifuged at 10,000 rpm for 15 minutes to ensure settling of cells. Three ml of each culture was collected and mixed with 0.5 ml of the test-oil. The mixture was vortexed for 2 minutes and incubated at 37 °C for 1 hour to allow separation of aqueous and oil phase. After that oil layer was removed and absorbance was measured for the aqueous phase at 400nm for each strain using uninoculated sterile growth medium as a blank. One emulsification unit (emulsification activity per ml [EU ml–1]) is equal to absorbance of 0.01 at 400nm multiplied by the dilution factor (in the case of diluted emulsifier preparation) (Patil & Chopade, 2001).

RESULTS AND DISCUSSION

Selected isolates were identified as *Aeromonas spp*. (Strain G), *Pseudomonas aeruginosa* (Strain H), *Escherichia coli* (Strain J), *Bacillus pumilis* (Strain K) and *Staphylococcus spp* (Strain L). All five biochemically identified strains were checked for biosurfactant production and activity by five screening assays. These screening assays were selected for their simple technique and rapid identification of results. Oil displacement assay utilizes the ability of biosurfactant to change the oil-water interface angle. The diameter of the zone was considered directly related to the activity of biosurfactant (Morikawa *et al.*, 1993). Using *E.coli* as a test strain highest activity was observed (Fig., 1) with a diameter of 0.6 cm and biosurfactant unit of 0.28. Previously reported studies have also shown that *E.coli* is one of strongest candidates for production of biosurfactants (Pruthi & Cameotra,

1997). *Aeromonas* was the weakest producer with diameter of 0.3cm and biosurfactant unit of 0.07.

Drop collapse and tilted glass slide assays also showed *E.coli* as the strongest while *Aeromonas spp*. as the weakest producer. Drop collapse assay was considered a bit non-specific as only positivity and negativity could be observed but the level to which biosurfactant is being produced was difficult to evaluate. Furthermore *Staphylococcus spp*. showed negative results in tilted glass slide assay but was positive in all others, this also questioned reliability of tilted glass assay as the sole technique for evaluating biosurfactant production (Table I).

Table I: Comparative analysis of screening techniques (+ positive, ++ strong positive, +++very strongly positive, - negative)

S train	OST	DC	TGS	Emulsificatin E_{24}	Emulsification Assay ($EUml^{-1}$)
Aeromonas spp.	0.07	-	+	40	79.6
P. aeruginosa	0.195	+	++	47.5	103.5
Bacillus pumilis	0.195	+	++	40	112.4
Escherichia coli	0.28	+	+++	42.5	123.8
Staphylococcus spp.	0.125	+	_	37.5	105.7

OST: Oil spreading technique; DC: Drop collapse assay; TGS: Tilted glass slide assay While using E24 it was observed that *Pseudomonas auregenosa* showed best results with E24 of 47.5%, *E.coli* with 42.5% was the second strongest while *Staphylococcus* showed the least ratio.

Fig., 2 shows the amount of biosurfactant produced for each strain and it is one of the first screening methods applied for the detection of biosurfactants (Willumsen & Karlson, 1996) (Christova *et al.*, 2004) (Bento *et al.*, 2005).

The results are summarized in table I facilitating comparative analysis of the tests that were performed and their consequent efficacy. The tests appear to be consistent for *Pseudomonas aeruginosa, Bacillus pumilis* and *Escherichia coli*, as they show good activity in TSG and drop collapse assay. This is further supported by their emulsification capacities in E24 and emulsification assay, but with the exception of *Pseudomonas* as it showed poor emulsification per ml capacity, even lower than *Staphylococcus spp*. The tests were variable in the case of these two strains as well in the case of *Staphylococcus spp*. Negative for TSG, lowest activity in OST after *Aeromonas spp*., lowest emulsification capacity, it appeared positive for drop collapse and showed emulsification per ml slightly higher than *Pseudomonas aeruginosa*. Similarly, in the case of *Aeromonas spp*., it has lowest results in OST and emulsification per ml, negative for drop collapse as well, but positive for TSG. Its emulsification paralleled with that of *Bacillus pumilis*.

Our results showed that *E.coli* a predominant strain for biosurfactant activity as it was consistent with highest activity in OST, TGS and EA, except in E24 essay, where *P. aeruginosa* was dominant. So non-pathogenic strains of *E.coli* are good source of biosurfactant productivity and they can be used for different purposes (Banat *et al.*, 2010) (Segura *et al.*, 2014; Thies *et al.*, 2014). *Pseudomonas aeruginosa* and *Bacillus pumilis* were the second most biosurfactant producing strains. One showing greater activity in E24 while other shows the greater activity in EA simultaneously. Hence, *Pseudomonas aeruginosa* and *Bacillus pumilis* also proved to be a good source of biosurfactants and these results are consistent with (Priya & Usharani, 2009) (El-Sheshtawy & Doheim, 2014). In our results *Aeromonas spp* and *Staphylococcus spp* showed the least activity for biosurfactant production. *Aeromonas spp* showing the negative results for TGS assay (Ilori *et al.*, 2005). In other tests like OST, E24 and EA, they also showed the least activity, possibly because of their small size and gram negative nature.

Our results also show that TGS and drop collapse assays can be used as nonspecific initial screening assays. Their results are less reliable. Compared to these E24 and EA are more specific and can be used with efficacy and much reliability for testing biosurfactant production. It should be noted that none of these tests measured amounts of biosurfactants in specific culture so these test could only be applied as initial screening assays.

Hence, bioemulsification is not consistant for the biosurfactant's activity on different substrates, rather only points to the presence of surfactants (Płaza *et al.*, 2006) (Segura *et al.*, 2014).

CONCLUSION

In the present study some primary screening tests, which are most frequently used for

the analysis of biosurfactants, were applied for those strains which are known to produce biosurfactants, to check the reliability of these tests. Variability suggested that only one test is not sufficient to determine presence or absence of biosurfactants. Therefore, it is recommended to use a combination of the most reliable tests for screening purposes.

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