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SCREENING OF BIO SURFACTANT PRODUCING BACTERIA AND THEIR ABILITY TO PRODUCE INDUSTRIAL IMPORTANT ENZYMES

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Abstract: Biosurfactants are amphiphilic compounds having an unique properties that allows their use and which are natural replacement for chemically synthesized surfactant. Micro-organisms produced these biosurfactants on their surface or may also secret extracellularly and it contains both hydrophilic and hydrophobic moieties which reduces the surface and interfacial tension of the surface and interface respectively. In the present study a screening was done to check the ability of microorganisms to produce biosurfactant from various petroleum sites. Out of 40 soil samples 20 bacterial species were isolated. Based on morphology and biochemical characterization the bacterial species were identified and only 9 species were having the ability to produce bio surfactants. These bacterial species were also screened for their ability to produce different hydrolytic enzymes like amylase, lipase and proteases using standard methods. This study has opened new avenues for biosurfactant studies and their application in bioremediation of contaminated sites as well as in industrial purpose. Further work on purification and large scale production using cheap raw substrate is needed to explore these bacteria.

Keywords- Biosurfactants, hydrocarbons, surface tension, functional properties

1. Introduction

Currently the whole globe is concern with a serious issue of environmental pollution caused by hydrocarbon contamination, chemical solvents, heavy metals and industrial waste. They cause a severe hazards to living organisms including human being and leads to economic loss as they remain persistently in the environment including soil and water (Ismail et al., 2013). To some extends these toxic compounds are naturally degraded by indigenous microorganisms through the process of biodegradation (Hassanshahian et al., 2012). A biotechnological approach has been applied to identify such pollutant degrading microorganisms from different environment and used to remove these contaminants effectively (Kumari et al., 2012). Remediation of such polluted sites using microorganisms is eco-friendly, cost effective and most efficient than other methods (Ismail et al., 2013).

Some microorganisms including bacteria, algae and fungi have ability to adopt adverse environmental condition and produce industrially important extracellular compounds like biosurfactant. Biosurfactant are surface active compounds synthesized by specific groups of microorganisms using different substrates like simple sugars, oils and hydrocarbons. Surface active compounds have ability to reduce surface and interface tension amongst liquid and solid substances (Das and Mukherjee, 2005). Various uses of biosurfactant have been reported in food industry, oil industry, cleaning purpose, pharmaceutical industry and bioremediation of oil contaminated sites. Compared to chemical surfactants, biosurfactant have potential advantages, i.e., they are eco-friendly, easily degradable, active in any extreme conditions like high salinity/temperature regions and can be produced using cheap organic sources, which facilitates commercialization (Makkar et al., 2011; Freitas de Oliveira et al., 2013). An efficient strategy of co cultivation of native microbes to increase the contaminant removal in short time was reported by (Zhang et al., 2012). Several studies on biosurfactant production by *Achromobacter, Acinetobacter, Arthrobacter, Pseudomonas, Halomonas, Myroides,*

Corynebacteria, Bacillus and Alteromonas sp. has been extensively carried out, but still the cost effective production of biosurfactant is not achieved from the cheap raw substrate (Satpute et al., 2010).

Apart from biosurfactant production bacteria also has ability to produce some industrially important hydrolytic enzymes like amylase lipase protease, L-asparginase, Dnase etc. which can be helpful in industrial processes. Enzymes acts as biocatalyst and hydrolyse the substrate to produce various products like dextrins and small polymer units (Natasa et al., 2011; Mohammed and Mastan, 2013). Microbial amylase has potential application in detergent industry and has great significance in biotechnological studies. In present study soil isolates from petroleum contaminated sites were investigated for biosurfactant production using different oil and were further analysed for their ability to produce hydrolytic enzymes like amylase, lipase and proteases which will be further used for industrial use.

2. Materials and Methods:

2.1 Collection of sample

A soil sample contaminated with petroleum products were collected from different automobile workshops and garages by using standard protocol and were transferred to sterile zip lock bag with proper identification mark and brought to the laboratory for further study.

2.2 Enrichment and isolation of bacteria: 1g of soil sample was inoculated in the 250mL Erlenmeyer's flask containing 100mL mineral salt medium as suggested by (Tambekar and Gadakh, 2012). The flasks were incubated at 37^0C with 200 rpm for 7days. After incubation inoculation was done on freshly prepared Nutrient agar plate and well isolated and morphologically distinct colonies were selected.

2.3 Screening for biosurfactant producing bacteria

A basic biochemical and morphological characterization of selected bacterial strains were carried out and were further screen out for potential to produce biosurfactant using different methods:

2.3.1 Surface tension measurement

A significant method for detection of biosurfactant production is reduction in surface tension. After 5 days of incubation broth were centrifuged at 8000rpm for 20 min for cell removal and supernatant was processed for measurement of reduction in surface tension using Traube's stalagmometer as suggested by Morikawa et al., (1993). γ (surface tension) = F (γ_o/m_o) x m where γ_0 is the surface tension of water, m_o is the weight of water and m is the weight of sample.

2.3.2 Oil displacement test

Oil displacement test was performed in petri dish containing 20ml distilled water and 20µl of oil. A 10µl culture supernatant was carefully placed on the Centre of oil layer and diameter of clear zone of oil layer was measured as suggested by Morikawa et al., (1993). A tween 80 sample was considered as positive control.

2.3.3 Drop collapse test

A micro titre plate coated with oil was taken and 20µL of culture supernatant was added to the well. After 1min, the collapsing of drops was examined visually as suggested by Satpute et al., (2010).

2.3.4. Emulsification activity: Emulsifying activity was measured by adding 6 mL of oil to 4 mL of cell free culture supernatant and vortexes vigorously for 2 min and after 24 h formed emulsion was measured.). Emulsification activity is a ratio of height of emulsion layer / total height of layer X 100 (Cooper and Goldenberg, 1987).

3. Enzymes assay

3.1 Assay of Amylase: a fresh culture of selected isolates were point inoculated on starch agar plate and incubated at 37^0C for 24h, after incubation the plates were flooded with 1% iodine solution and observed for clear zone of hydrolysis around the colony, a clear zone indicates starch hydrolysis and considered positive amylase test (Adeoyo, 2020).

3.1 Assay of Protease

The bacterial isolates were point inoculated on the freshly prepared casein agar plate and incubated for 24h at 37^0C. A clear zone around the colony indicates positive result for protease activity (Hemke et al., 2015).

3.2 Assay of Lipase

Freshly prepared egg yolk agar medium was point inoculated with bacterial isolates and was incubated for 24h at 37^0C and zone of lipid hydrolysis was recorded after incubation (Hemke et al., 2015).

4. Biochemical characterization of isolates

Selected isolates were examined morphologically and biochemically characterized using Hi media kit. Growth pattern of, bacterial isolate was investigated using different growth condition like pH (5, 6, 7, 8, 9) temperature range between 4 – 50^0C and salt concentration from 1% to 5%. These isolates were identified according to Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986).

4. Result and discussion:

Hydrocarbon contaminated sites are generally treated with chemically derived surface active agents, which exerts an additional toxic effect to the environment. A special attention was given towards eco-friendly, nontoxic and biodegradable product extracted from microorganisms (Bicca et al., 1999). In present investigation, about 40 soil samples collected from hydrocarbon contaminated site and morphologically distinct 20 bacterial isolates were selected and were further investigated for biosurfactant production using standard methods as discuss earlier. Out of 20 isolates only 9 isolates shows biosurfactant producing property (Table No. 1). These selected bacterial isolates were cultivated with same type of medium and investigated for surface tension reduction and oil displacement test. Similar type of cultivation work was carried out by Parthipan et al., (2017).

Table No. 1: Biosurfactant production property shown by the isolates

Isolate	Surface tension	Oil displacement Test (mm)	Drop collapse test	Emulsification Index (%)
P1	28.1±0.20	35	+	22.2
P2	66.03±0.07	0	-	6.7
P3	60.72±0.17	5	-	5
P4	29.62±0.25	40	+	11.5
P5	50.35±0.13	5	-	8.3
P6	45.85±0.20	25	+	9.7
P7	31.6±0.36	55	+	16.4
P8	27.85±0.16	60	+	30
P9	28.22±0.06	70	+	23.3

±= standard deviation;

Result showed that a highest reduction 27.85 mN/m was reported for isolate P8, followed by 28.1 mN/m, 28.22 mN/m and 29.62 mN/m for isolate P1, P9 and P4 respectively. Vijaya et al., (2013), reported a reduction in surface tension upto 30 mN/m while working on biosurfactant production from *Pseudomonas* and *Bacillus* using coconut oil as substrate. Reduction in surface tension reported for isolate P2 and P3 are negligible and were also gives drop collapse test negative and minimum activity for emulsion formation. Similar type of result were reported by Partovi et al., (2013), while studying biosurfactant production from *Pseudomonas aeruginosa* using soybean oil refinery waste as substrate. An oil displacement activity about 70 mm was reported for isolate P9 followed by 60 mm and 55 mm for isolate P8 and P7 respectively. Isolate P2 was found negative for oil displacement test and drop collapse test.

While highest emulsification activity of 30% was reported for isolate P8 followed by P9 (23.3%) and for P2 (22.2%) respectively. Our study results are much less than, the study reported by Obayori et al., (2009), while working on *Pseudomonas sp,* they reported 80% oil emulsion activity by isolates.

Result of biochemical characterization showed that, most of isolates were aerobic Gm-ve short rod with sluggishly or fast motile and non spore former. All the isolates were reported indole negative, methyl red negative and VP test negative, while able to utilize citrate except isolate P5. These isolates grows normally at pH range 6 to 9, while shows ability to survive at 2% salt concentration. All the isolates show hemolysis property except isolate P5 and P6 and grow normally at temperature range between 20-42^0C (Table

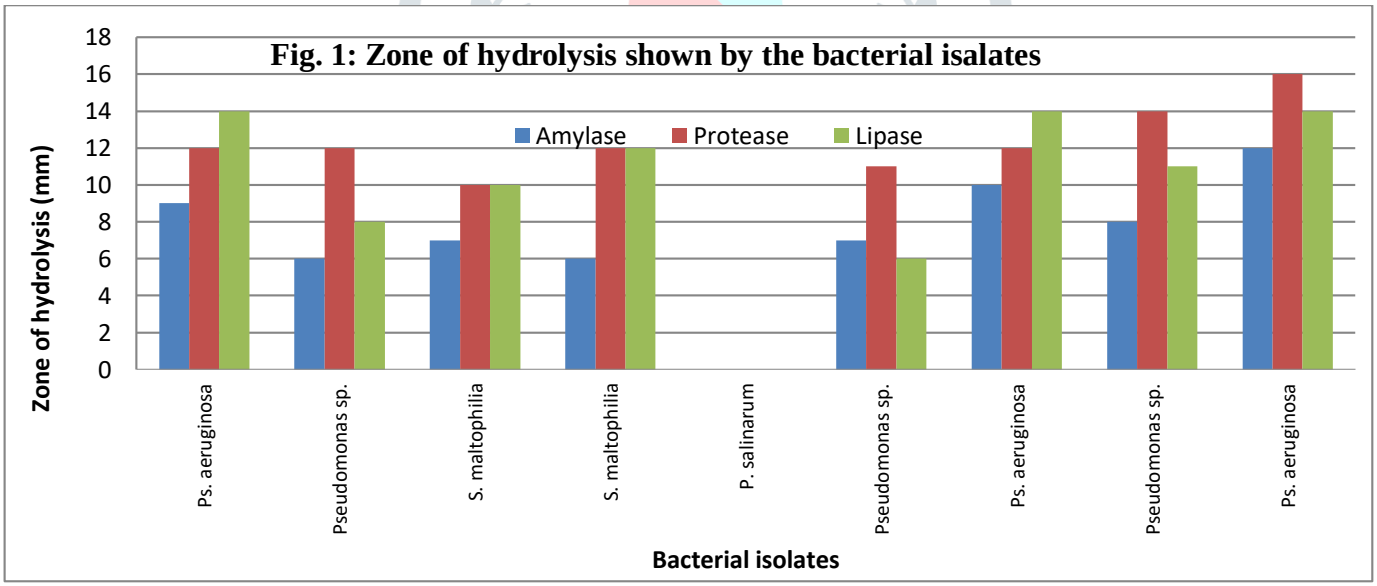
2).

Table 2: Morphological and biochemical characteristics of selected biosurfactant producing isolates

Bacterial isolates	Gram Staining	Shape	Motility	Spore	Catalase	Oxidase	Indole	Methyl red	Growth at 2% salt	Citrate utilization	Nitrate reduction	β-haemolysis	Name of Isolates
P1	-	SR	+	-	+	+	-	-	+	+	+	+	*Ps. aeruginosa*
P2	-	SR	+	-	+	-	-	-	+	+	-	+	*Pseudomonas* sp.
P3	-	SR	+	-	+	-	-	-	+	+	-	+	*S. maltophilia*
P4	-	SR	+	-	+	-	-	-	+	+	-	+	*S. maltophilia*
P5	+	CB	-	-	+	-	-	-	+	-	+	-	*P. salinarum*
P6	-	SR	+	-	+	-	-	-	+	+	-	-	*Pseudomonas* sp.
P7	-	SR	+	-	+	+	-	-	+	+	+	+	*Ps. aeruginosa*
P8	-	SR	+	-	+	+	-	-	+	+	+	+	*Pseudomonas* sp.
P9	-	SR	+	-	+	+	-	-	+	+	+	+	*Ps. aeruginosa*

+=Positive;-=Negative;SR= Short rod; CB= Cocco bacilli

Enzyme activity of biosurfactant producing isolates: biosurfactant producing bacteria also produce some industrially important hydrolytic enzymes. A fresh culture of bacterial isolates was inoculated on respective medium and was observed for zone of hydrolysis. Adeoyo, (2020) has studies the hydrolytic amylase producing bacteria *Bacillus* species isolated from agricultural soil, which was reported as biosurfactant producer. Apart from amylase and protease isolates also shows activity for lipid hydrolysis. According to Colla et al., (2010) for the metabolism of water insoluble substrate microorganisms produces lipase and biosurfactant.



Result was showed that highest zone (12 mm) of starch hydrolysis was recorded with isolate P9, while isolate P5 identified as *P. salinarum* was found unable to hydrolyse starch, casein and lipid respectively. Zone of casein hydrolysis was recorded 16mm with isolate *Ps. aeruginosa* (P9). Similarly highest zone of lipid hydrolysis (14mm) was recorded with isolate P1, P7 and P9 and all three were identified as *Ps. aeruginosa*. Bacterial isolates with biosurfactant and lipase activity was also reported by Rahayu et al., (2019), while recording a keratinolytic bacterial isolates. Amylase can be derived from different sources like plant, animal and microorganisms.

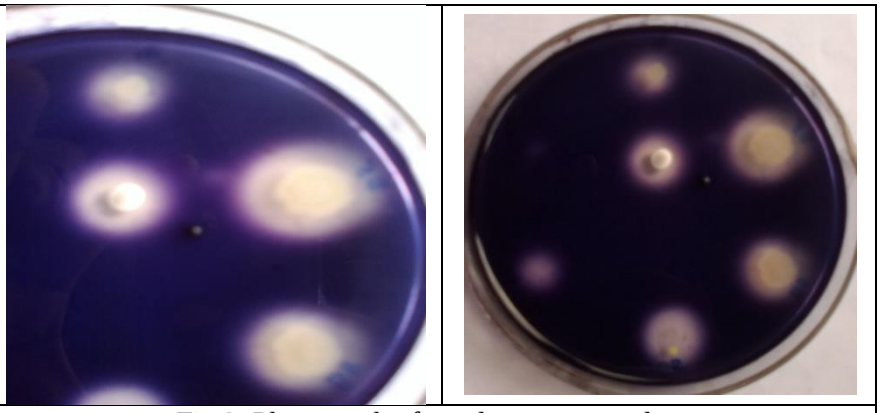


Fig 2: Photograph of amylase enzyme plate

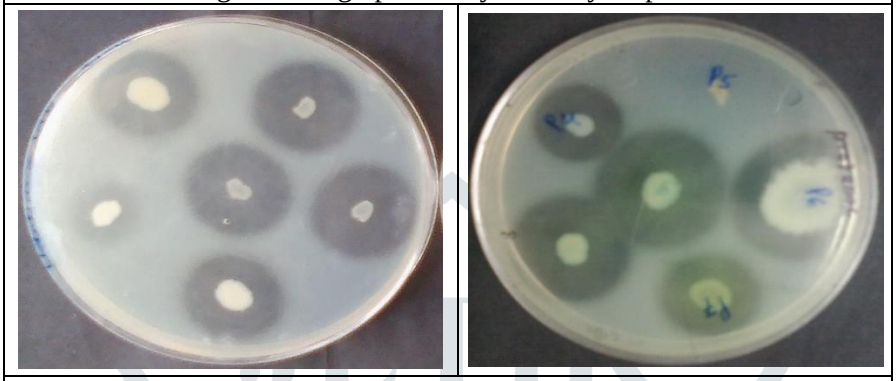


Fig. 3: Photograph of enzyme lipase and protease plate

Microbial production of amylases are most effective process as it is cost effective, can be produced from cheap raw substrate and be modified to obtain enzymes of interest (Ashwini et al., 2011). In present investigation all the isolates shows the ability to produce amylase enzyme when grown on starch agar plate. Amylase producing *Pseudomonas aeruginosa* was isolated from garden soil by Raju and divakar (2013). They reported four isolates having ability to hydrolyse starch. Further they studied the effect of surfactant and nitrogen source, carbon source, temperature on amylase production.

5 Conclusion

The report of present investigation suggest that microorganisms isolated from the different sites having ability to produce biosurfactant by metabolizing hydrophobic components present in medium, apart from that the isolates are also capable to produce different hydrolytic enzymes which enlighten the importance of these bacteria for application in bioremediation of contaminated sites as well as in industrial purpose. Further work on purification and large scale production using cheap raw substrate is needed to explore these bacteria.

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