SCREENING AND CHARACTERIZATION OF BIOSURFACTANT-PRODUCING BACTERIA ISOLATED FROM OIL-CONTAMINATED SOIL AND SLUDGE SAMPLES

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ABSTRACT: The present study was undertaken to isolate and characterize the hydrocarbon-degrading bacterial isolates from oil-contaminated soil and sludge samples. Totally 17 bacterial isolates were obtained from 35 oil contaminated soil (n=31) and sludge samples (n=4). Among them, six of were finally selected through various screening methods including blue agar plate, hemolysis, drop collapse and oil spreading test using crude oil as a carbon source. From these isolates, the single potential isolate was selected based on the emulsification activity and gravimetric analysis, which was further identified by 16s rRNA gene sequence. The 16S rRNA sequence alignment shows that the isolate 32 was closely related to *Pseudomonas stutzeri*. Results indicate that this isolate have the potential for future environmental friendly applications such as bioremediation.

Key words: Biosurfactant, *Pseudomonas stutzeri, blue agar*, emulsification index and gravimetric analysis.
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I. INTRODUCTION

In the current years, high ranges of polluting compounds are discharged into the atmosphere by many anthropogenic activities. Particularly, aromatic pollutants have caused the contamination of soil and water with negative impacts on environmental quality and healthy. Many polycyclic aromatic hydrocarbons (PAHs) are known to exhibit high toxicity and cancerous properties in humans and animals. Therefore, research on environmental contamination via PAH compounds have expanded over current years (Constantini *et al.*, 2009).
Among the various remediation methods, a number of researches interest in biological methodologies, because no side effect and that may help to reduce the risk of organic pollutants and effectively remediate polluted sites (Andreoni and Gianfreda, 2007). The use of microbial isolates for biodegradation of toxic organic compounds such as PAH is promoting excellent results in the clean-up of different environments including water, sludge and soil (Andrea *et al.*, 2012).
In recent years, increasing global awareness of the environment has resulted in much greater interest in biosurfactants compared to their chemical materials. It is due to unique properties of biosurfactants, including biodegradability, low toxicity, mild production conditions and acceptability of the environment, lower critical concentration of micelles, higher selectivity and better extreme-temperature activity (Odalys *et al.*, 2017). The natural properties of biosurfactant vary according to microorganisms such as bacteria, yeast and fungus. Biosurfactants have received great attention in the field of environmental remediation processes such as bioremediation, soil washing, and soil flushing. Biosurfactants influence these processes because of their effectiveness as dispersion and remediation agents and their ecologically friendly properties.
In this context, the aim of this study was to isolate and screen biosurfactant that produce microorganisms from sludge and oil contaminated soil samples.

II.MATERIALS AND METHODS

2.1Collection of sludge samples

Surface sediment of sludge sample and oil-contaminated soil were collected. The collected samples were pooled and transferred to pre-sterilized, labeled, plastic container and transported at 4 °C to the laboratory and maintained at 4 °C until analysis.

2.2 Isolation of bacterial isolates from oil contaminated soil

The serially diluted samples were spread on each of BHA plates overlaid with 100 µl of Hydrocarbon (crude oil) and were incubated at 25°C for 7 days (Pradnya *et al.*, 2014). After the incubation period, observed the colonies and subcultured into nutrient agar for further analysis.

2.3 Screening of biosurfactant producing isolates

All isolates were subjected to screening of biosurfactant production with various methods including blue agar plate, Blood hemolysis, Drop collapse, oil spreading test (Jayanth and Hemashenpagam, 2015). After completed these screening tests, potential isolates were subjected to Emulsification index (Pradnya *et al.*, 2014) and gravimetric method (Oloke *et al.*, 2005).

2.4 Characterization and Identification of isolates

All isolates were identified based on their cultural, morphological and physiological characteristics in accordance with the taxonomic scheme of Barrow and Feltham (1993) and reference to Holt *et al.*, (1992). The tests performed include Gram stain, spore stain, motility test, catalase test, oxidase, coagulase, urease, indole production, hydrogen sulphide production, nitrate reduction, methyl red, Voges-Proskauer, oxidative/fermentative test and utilization of carbon sources.

2.5 16 s rRNA sequence analysis

The16s rRNA genes were amplified using PCR with the universal primer FD1 and RP2. The sequence of FD1 and RP2 were 5'-AGAGTTTGATCTGGCTCAG-3'and5'AAGGAGGTCATCCAGCC3'respectively. A search of the GenBank Nucleotide library for a sequence similar to those determined was made by using BLAST (Altschul *et al*., 1990), through National Center for Biotechnology information (NCBI) internet site http://www.ncbi.nlmnih.gov/BLAST. Sequences with more than 98% identity with a Genbank sequence were considered to be of the same species as the highest score matching sequence on the public sequence databases.

III.RESULT AND DISCUSSION

Soil and water contamination with hydrocarbons caused extensive damage to the local system; this contamination is a disaster to plant life and animal. A green manner of remediation the oil-contaminated sites might be the employment of unique microorganisms, including microorganism, microalgae, and fungi. Bacteria are the most important microbes in this process because they break the dead materials into organic matter and nutrients (Marcelo *et al*., 2005).

In this present study, the ability of 17 isolates to grow on medium containing (crude oil), which eventually proves their ability for biosurfactant production any isolate which grow on BHA agar plates containing hydrocarbon. In this study, among the 17 isolates, 15 isolates were observed from soil samples and 2 of were from sludge samples. These isolates were carried out to further studies for the screening of biosurfactant producing isolates. The previous study of Marcelo (2005) and Wang (2008) were observed the hydrocarbon-degrading bacteria with different crude oil containing BH media. Out of the 17 isolates, 6 of were showed positive results in following screening test namely Blue agar, hemolysis, oil spreading test and drop collapse test. Meenal *et al*., (2016), devised blood agar lysis as a primary screening method for biosurfactant production. None of the studies within the literature reported non haemolytic property of biosurfactant. Although, hemolysis does not always mean biosurfactant production compounds other than biosurfactants may cause hemolysis.

Among the screening methods, oil spreading method was highly sensitive method than other methods; this phenomenon was agreed with a previous study of Youssef *et al*. (2004). In 2014, Pradnya *et al*., also reported that oil spreading test was suitable method for screening of biosurfactant producing isolates. The previous study of Satpute *et al*., (2008) were screened the biosurfactant producing isolates with more than one methods. In 2014, Rehman *et al*., also observed the biosurfactant producing isolates with the number of methods including oil displacement, drops collapse and blue agar methods. In recently, Odalys *et al*., (2017) were observed the biosurfactant producing isolates with the same screening methods. Jayathi and Hemashenpagam (2015) were obtained the biosurfactant producing bacterial isolates with using 6 types of screening procedure and using BTX as substrate.

From this current study, 6 isolates were showed positive results in all 4 screening methods, which were selected and subjected to emulsification and gravimetric analysis for confirmation of biosurfactant producing isolates. For emulsification index test found that 6 isolates could give emulsification index ranging from 28% to 38%. The isolate 32 showed 38% of emulsification index and followed by isolate 31 showed 34% of emulsification index. Furthermore, gravimetric analysis was accomplished to determine the degradation ranged of crude oil. Among the 6 isolates, isolate 32 was degraded around 25.2% of the crude oil by 7th day.

Overall, tests analysis indicated the positive response from isolate 32 as it acted as the best performer in most of the tests which was further identified by 16s rRNA gene. The potent biosurfactant producing isolate belonged to the genus *Pseudomonas*. The 16S rRNA sequence alignment shows that the strain 32 was closely related to *Pseudomonas stutzeri* (Fig1 and Table 1).

It is evident from this study that, hydrocarbon degrading organisms are ubiquitous in the environment, and they can be isolated from hydrocarbon polluted sites. The potent biosurfactant producer *Pseudomonas stutzeri* (isolate 32) was selected as it gave maximum biosurfactant yield and the isolate also showed strong emulsification ability. These properties suggest its potential application in environmental clean -up procedures.

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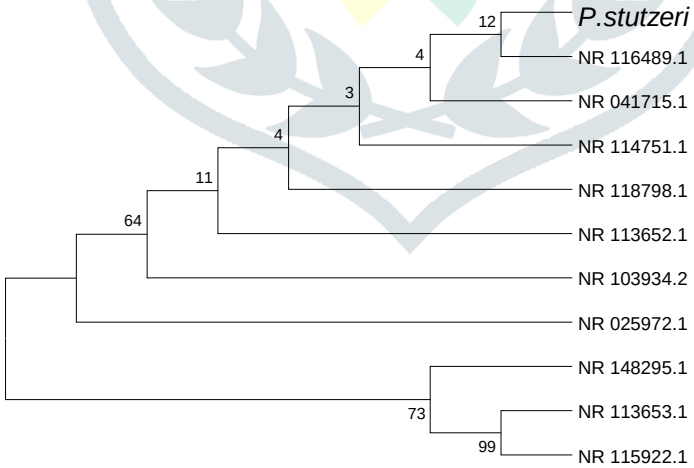

Fig. 1. Phylogenetic Tree

		1	2	3	4	5	6	7	8	9	10	11	
1	*Pseudomonas stutzeri*		0.002	0.002	0.002	0.002	0.002	0.002	0.004	0.007	0.008	0.008	1
2	NR_116489.1	0.002		0.000	0.000	0.000	0.000	0.000	0.003	0.006	0.007	0.007	2
3	NR_041715.1	0.002	0.000		0.000	0.000	0.000	0.000	0.003	0.006	0.007	0.007	3
4	NR_114751.1	0.002	0.000	0.000		0.000	0.000	0.000	0.003	0.006	0.007	0.007	4
5	NR_118798.1	0.002	0.000	0.000	0.000		0.000	0.000	0.003	0.006	0.007	0.007	5
6	NR_113652.1	0.002	0.000	0.000	0.000	0.000		0.000	0.003	0.006	0.007	0.007	6
7	NR_103934.2	0.002	0.000	0.000	0.000	0.000	0.000		0.003	0.006	0.007	0.007	7
8	NR_148295.1	0.007	0.005	0.005	0.005	0.005	0.005	0.005		0.006	0.006	0.006	8
9	NR_025972.1	0.018	0.015	0.015	0.015	0.015	0.015	0.015	0.015		0.009	0.009	9
10	NR_113653.1	0.020	0.017	0.017	0.017	0.017	0.017	0.017	0.012	0.028		0.000	10
11	NR_115922.1	0.020	0.017	0.017	0.017	0.017	0.017	0.017	0.012	0.028	0.000		11
		1	2	3	4	5	6	7	8	9	10	11	

Table 1. Distance Matrix: Estimates of Evolutionary Divergence between Sequences