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A COMPARATIVE STUDY ON THE BIOSURFACTANT PRODUCING BACTERIA FROM OIL CONTAMINATED WATER

Permod Kumar[1], Monika Malik[2], Raj Singh[1], Anju Rani[1] and Amit Kumar[2]

Author's Affiliation: [1]Department of Botany, K.V. Faculty of Science Swami Vivekanand Subharti University, Meerut. [2]Department of Biotechnology, K.V. Faculty of Science Swami Vivekanand Subharti University, Meerut.

Corresponding Author: Permod Kumar
Department of Botany,
K.V. Faculty of Science,
Swami Vivekanand Subharti University,
Meerut, Uttar Pradesh 250005.
E-mail: pkkalhra@gmail.com
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Abstract

Bio surfactants are a structurally diverse group of surface- active molecules synthesized by microorganisms. It's have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility and the higher substrate selectivity. Oil spreading technique, blood hemolysis test, foaming activity, and emulsification activity were performed for the screening of the biosurfactant producing bacteria, and found positive oil spreading technique in bacterial strain *Bacillus subtilis* and *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* has high emulsifying activity and it was 29.62 % where as in *Bacillus subtilis* 20.68 %. *Bacillus subtilis* and *Pseudomonas aeruginosa* showed blood hemolysis test and it was observed that foaming stability in *Pseudomonas aeruginosa* is higher than *Bacillus subtilis*. *Pseudomonas aeruginosa* showed gram negative and *Bacillus subtilis* showed gram negative and they are in rod and circular shape respectively *Bacillus subtilis* and *Pseudomonas aeruginosa* both the organism have higher growth rate at pH 7 and *Pseudomonas aeruginosa* shows more growth than Bacillus**.** *Pseudomonas aeruginosa* shows higher growth rate as compare to *Bacillus subtilis* at 37^0C and the *Pseudomonas aeruginosa* shows better growth rate than *Bacillus subtilis* at 37°C.

Keywords: Pollution, Biosurfactant, Bacteria, oil spreading Techniques

INTRODUCTION:

Now a days, increased use of petroleum hydrocarbons and oils as main energy sources has increased the risk of leakage of these products while transportation, leading to soil and water contamination; which is not beneficial for human beings, plants, animals and microbes. Components of crude oil are of environmental concern due to their noxious belongings, so there should be an efficient method to remove these hazardous compounds thoroughly to clean and to save the environment.

Microbial Enhanced Oil Recovery (MEOR) has several advantages over chemical methods, including environmental compatibility; biosurfactant, biopolymers, and biomass are amongst the key players in MEOR. Biosurfactants are surface active agents can be produced by microorganism such as yeasts, bacteria and fungi (Mulligan *et al.,* 2004) etc. Biosurfactants have several advantages including low toxicity, environmental friendly, high biodegradability, low irritancy, higher foaming, high selectivity and specific activity at extreme temperature, pH, salinity and compatibility with human skin (Maker and Cameotra 1998; Mahalingam and Sampath 2014). They possess a wide range of industrial application such as health care, oil & food processing industries, pharmaceuticals and a potential place in bioremediation. Hence this present study is focussed on the isolation and chacterization of biosurfactant producing bacterial strains from oil contaminated water.

Various microorganisms are able to produce biosurfactant eg. *Pseudomonas aerogenosa, Bacillus subtilis, Brevibacteriumbrevis, Bacillus licheniformis. Candida bombicola, Candida apicola*, *Arthrobacter* species, *Acinetobacter* species but this biosurfactant producing ability get affected by carbon, nitrogen source used in medium, salinity, temperature, pH, pressure etc (Rahman *et. al.,* 2002).The aim of recent study is isolation and screening of biosurfactant producing bacteria from oil contaminated water samples.

MATERIAL AND METHODS

Isolation and biosurfactant producing techniques of Bacteria: Oil (Kerosene) contaminated Water samples were collected from different sampling sites of Abbott pharmaceuticals limited, Baddi Himachal Pradesh. 1ml of oil contaminated water sample were inoculated in 100 ml of Mineral Salt Medium (MSM) with 3ml kerosene oil added to the conical flask having capacity of 250 ml, as the carbon sources and then it was incubated for 72 hours at 30°C temperature. After then 1ml of incubated culture was streaked on the petriplates. The samples then were serially diluted up to 10^{-6} dilution. 1 ml of 10-6 time dilution was transferred to nutrient agar for spread culture. The plate was inverted and incubated at 37° C, for 72 hours. The isolated colonies were obtained in pure cultures and tested for their biosurfactant production by the following techniques:

Oil Spreading Technique: 10 µl of crude oil of kerosene was added to the surface of 40 ml of distilled water in a petridish to form a thin oil layer. Then, 10 µl of culture or culture supernatant was gently placed on the centre of the oil layer. The diameter of the clearing zone on the oil surface would be visualized under visible light and measured after 30seconds, which correlates to the surfactant activity.

Emulsification Assay: 2 ml of hydrocarbon *i.e.* kerosene oil was taken in a test tube to which 1ml of cell free supernatant obtained after centrifugation of the culture, was added and was vortexed for 2 minutes to ensure homogenous mixing of both the liquids.The emulsification activity was observed after 24 hours and it was calculated by using the following formula (Copper and Goldenberg, 1987):

$E_{24} = h_{emulsion} / h_{total} \times 100\%$

Blood Hemolysis Test: Isolated colony was taken and streaked on blood agar plate and incubated for 72 hours under 37°C temperature. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carello, *et al.,* 1996).

Foaming Activity: Isolated strains were grown biomass separately in 250 mL flasks, each containing 100 ml of nutrient broth medium. The flasks were incubated at 37°C on a shaker incubator (200 rpm) for 72 h.

Screening testing of biosurfactant bacteria:
Some physical and biochemical screenings of isolated bacteria are as following:

Physical Screening:
Colony Morphology: The shape, size, elevation, margin and color of the colony were observed in the culture plates with Luria Bertani Agar (LB Agar) used as the nutrient medium.

Biochemical Screening:
Catalase Test: Spread the bacteria on an agar plate and incubate the plate over night (18-24 h) under appropriate conditions. Collect bacteria from one colony with a sterile inoculating loop (of plastic or platinum) and apply the bacteria on a microscope slide by adding one drop of H_2O_2 (3%) to the bacteria and observe the suspension.

Citrate Test: A single bacterial colony from fresh culture was inoculated on a sodium citrate medium and a pH indicator such as bromothymol blue for 48 hrs and maintains temperature 37°C and then observed the color.

Urease Test: One pure single colony from fresh culture was inoculated in urease broth for 48 hrs and maintains temperature 37°C and then observed the color.

Methyl Red Test: Culture was inoculated in methyl red vogesproskauer (MR-VP) broth for 3 days at temperature of 37°C after the incubation added 2-3 drops of Methyl red and observed the color change that take place in few seconds.

Nitrate Test: Incubated the bacterial strain in nitrate broth at the optimal temperature 37°C for 48 hours further added few drops of nitrate reagent and see the reaction within the minute or less.

Indole Test: 24 hours pure culture was inoculated in tryptone broth. It was incubated for 24 hours at 37°C and then removed 2ml media and added in to the empty sterile test tube and added few drops of Kovac's reagent and agitate the tube for few minutes.

Study of Growth Kinetics
Effect of pH: In 100ml Erlenmeyer flasks containing 50ml mineral salt medium were adjusted at different pH values from 6, 7, 8. 0.5 ml of overnight culture was inoculated and incubated for 144 hours at 37°C on shaker incubator and reading was taken at regular interval of 12 hours at absorbance 600 nm. After removing the cells by centrifugation, supernatant was used for estimation of surfactant concentration by UV-VIS spectrophotometer measured at 600 nm.

Effect of Temperatures: In 100ml Erlenmeyer flasks containing 50ml sterile mineral salt medium were inoculated with overnight cultures and incubated at different temperature ranges from 35°C, 37°C, and 39°C for one week and further studied as in (a) and measured at 620 nm.

RESULTS AND DISCUSSION

A total of seven bacterial species were isolated from oil contaminated water as per details mentioned in table 1.1. All the isolated species were identified and are listed as under (with frequencies). Broad taxonomic arrangement is based upon that suggested by Bizet *et al.,* 1997.

Table 1: Frequency of Isolates Bacterial Species

S. No.	Isolates Bacterial Species	Frequency (%)
1.	*Bacillus subtilis*	27.72
2.	*Enterococcus faecalis*	18.80
3.	*Escherichia coli*	4.55
4.	*Micrococcus luteus*	8.20
5.	*Pseudomonas aeruginosa*	29.73
6.	*Salmonela sp.*	6.80
7.	*Staphylococcus aureus*	4.60

The high incident rate or frequency of *Bacillus subtilis* and *Pseudomonas aeruginosa*in oil contaminated waste water when compared to other organisms isolated, all the isolated strains species screened for the confirmation of biosurfactant producing bacteria in which above two species (*Bacillus subtilis* and *Pseudomonas aeruginosa*) showed good rate of frequency.

Biosurfactant Screening Tests:

In oil spreading technique both species show positively but *Bacillus subtilis* show higher zone formation of 3.2cm, on the other hand similarly our study focused. *Pseudomonas aeruginosa* shows 2.8 cm. Further study to ascertain the potential of both *Bacillus subtilis & pseudomonas aeruginosa* confirmed that they are products of surface active agents as shows table 1.1.This is in accordance to Jaysree *et al.,* 2011; Suganya *et al.,* 2013.

In emulsification, the highest E24 value was observed in *Pseudomonas aeruginosa* are 29.62 in kerosene oil. Similarly the highest E24 value was observed by Suganya *et al.,* 2013. So the high surfactant activity or be best surfactant activity are shown here (Table 1.2)

Table 2: Biosurfactant Screening Tests

Isolated bacterial strain	Oil Spreading Test		Emulsification Test			Foaming Activity	Blood Haemolysis Test
	Diameter of clear zone (cm)	Interpretation	Emulsifier layer (cm)	Total liquid layer (cm)	E24 (%)		
Bacillus subtilis	3.2	+ ve	0.6	2.9	20.68	+ ve	+ ve
Pseudomons aeruginosa	2.8	+ ve	0.8	2.7	29.62	+ ve	+ ve

In foaming activity, isolated bacterial strains were grown separately in 250ml flasks, each containing 100 ml of nutrient broth medium. The flasks were incubated at 30°C on a shaker incubator (200 rpm) for 72 h.

In blood hemolysis test, its test shows in preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic condition (Schulz *et al.,* 1991; Suganya, 2013). From our results both the isolates will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies (Table 1.2).

Kiran *et al.* (2009) recommended single screening method, unsuitable for identifying all types of biosurfactants and more than one screening method should be included during primary screening to identify potential biosurfactant producers. In the present study, therefore oil spreading technique, emulsification assay, foaming test and blood haemolysis test measurement were used to screen the biosurfactant producer. Strain *Bacillus subtilis* and

Pseudomonas aeruginosa. Showed positive results in all of the four screening methods used. Thus, we confirm that this bacterium can produce biosurfactants with positive responses.

Biochemical Charecterization

The characterization different bio-chemical tests were performed to identify the species of strains. For the identification and characterization of isolate bacterial strains.

Table 3: Result of Biochemical Test

Biochemical test	**Result**	
	Bacillus subtilis	***Pseudomonas aeruginosa***
Catalase test	+	+
Citrate test	+	+
Indole test	-	-
Methyl red test	+	-
Nitrate test	-	+
Urease test	-	-

Most researchers have used maximum two to three screening methods for characterization of bacterial isolates. It is suggested that a single method is not suitable to identify all types of bacteria (Yousef *et al.,* 2004). The biochemical and physiological features of *Pseudomonas aeruginosa* and *Bacillus subtilis* enabled only preliminary identification (Pacwa-Plociniczak *et al.,* 2010; Tong *et al.,* 2011). From our result both the isolates identification was confirmed by combining similar results of biochemical and physiological characteristics.

Growth Study

Growth study (freshly inoculated strains) was studied at different pH and temperature to find out the optimal condition for the growth of isolates bacterial strains. The readings were plotted against time from 0 hour to 144 hour and reading was taken on interval of 12 hours.

Effect of pH: The pH ranges from 6, 7 and 8 and were used for the effect of pH for the growth of *Bacillus subtilis* and *Pseudomonas aeruginosa*. Both the organisms have higher growth rate at pH 7 and PS shows more growth than BS (Fig. 1).

Effect of Temperature: The temperature ranges from 35°C, 37°C and 40°C for the effect at 37°C BS and PS shows higher growth rate and the PS shows better growth rate than BS at 37°C (Fig. 2).

Effect of different Ph from 6, 6.5, 7, 7.5, 8 and 8.5 and different temperature 35^0C to 40^0C. Both the organism have higher growth rate on pH7 and 37^0C (Suganya *et al.,* 2013). Both *Bacillus subtilis* & *Pseudomonas aeruginosa* confirmed in our results, both the bacterial strains were show similar result.

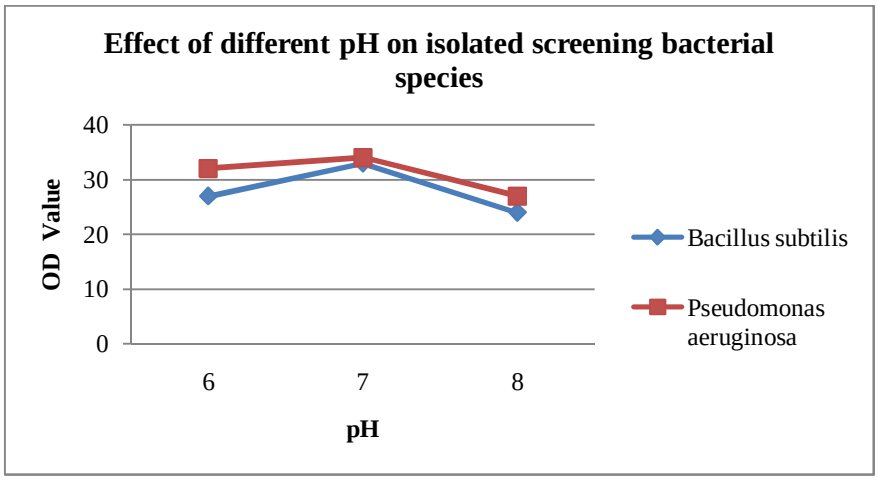
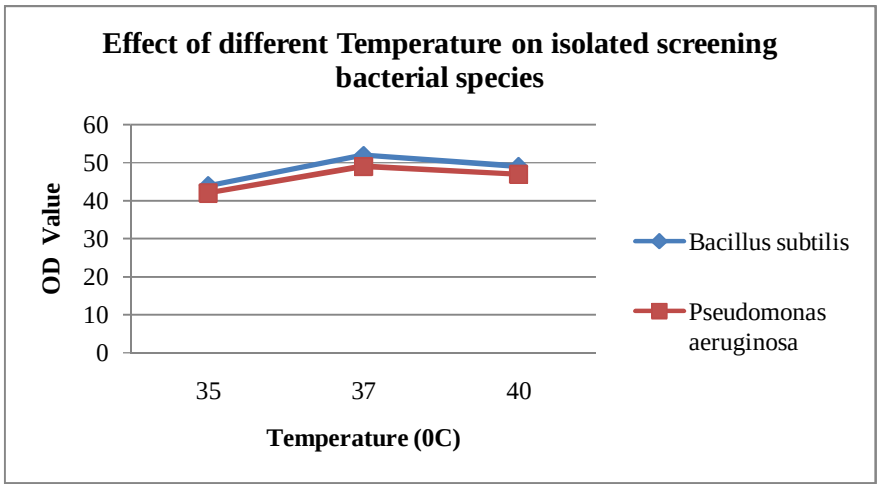


Fig.1: Effect of different pH on isolated screening bacterial species



2: Effect of different temperature on isolated screening bacterial species

CONCLUSION

This study can conclude the ability of *Bacillus spp*. isolated from oil contaminated water to grow on the enriched media with the hydrocarbon as a sole source of energy with high potential capacity of oil degradation and biosurfactant production.

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