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# Studies on the Role of Bacteria in Self Purification of the River Tamirabarani

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**Abstract:** Tamirabarani – A perennial river in South India satisfy the water demand of more than three southern districts in Tamil Nadu. The quality of the river is degraded by diverse pollutants including oil contaminants. Bio surfactants are amphiphillic compounds produced by microbes used for degrading oils. Investigating the ability of surfactant production in microbes help us to understand its self-purification nature. The total viable count in the water sample collected from the river at Kokkirakulam, Tirunelveli was found to be $5.5\times10^4$ CFU/ml. Initial screening methods indicated us that 40 % of the total bacterial isolates were found to be bio surfactant producers. Bacterial identification studies conducted on the positive surfactant producers through basic microbiological and biochemical tests indicated the presence of *Pseudomonas aeruginosa* and *Bacillus subtilis*. The efficacy of bio surfactant produced by the two bacterial isolates was checked against commonly used oils such as coconut oil, gingili oil and palm oil and also against glycerol by three methods such as oil displacement method, emulsification index and drop collapse method. Results of the methods employed revealed positive action of the bio surfactant against all the tested samples but more activity was observed on gingili oil followed by coconut oil. Effect of physicochemical parameters such as pH, temperature and carbon and nitrogen source was also investigated on the growth of bio surfactant producing bacteria. Comparative analysis between *P. aeruginosa* and *B. subtilis* declared more production of bio surfactant in the initial organism. Thin Layer Chromatography studies exposed a variation in the chemistry of surface active molecules. The biochemistry of the surface active molecules were found to be glycolipid in *Pseudomonas* sp and lipopeptide in *Bacillus* sp. Antimicrobial studies of the surface active molecules indicated the presence of bio controlling potential against human bacterial pathogens. The current study helped us to understand the role of microbes in pollution management of the river on its own.

**Key words:** Tamirabarnai River · Bio Surfactant · *Pseudomonas aeruginosa* · *Bacillus subtilis* · Submerged Fermentation

## INTRODUCTION

Water is an important natural resource of earth and plays a vital role in our life. Surface water and groundwater are the major sources of water.

The surface water qualities of major river basins are contaminated by the municipal and industrial discharges [1]. River Tamirabarani is well known for its historical and cultural values is not an exception from quality degradation by pollution. This perennial river satisfy the water demand of domestic, agricultural and industries in three districts in south Tamil Nadu. Plenty of research works have been carried out on determining the physico and chemical aspects in the water of this river [2-5]. Literature survey revealed, no work has been done explaining the bio surfactant producing microbe's distribution in the river so far.

**Bio Surfactants Are Biological Amphipathic:** Compounds produced by various bacteria, fungi and moulds [6-7]. They are capable of reducing surface and interfacial tension and forming micro emulsion where hydrocarbon can be solubilized in water or where water can solubilize in hydrocarbons [8]. Even though surfactants of microbial origin find applications in different sectors such as cosmetics, food, pharma industries etc. and its role in oil pollution management is something amazing [9-12].

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Oil in different forms enter into the river by human activities and domestic and industrial wastes.

Distribution of surfactant producing microbes shall help the river in tackling oil pollution and could be one of the tools of self-purification. The present work was designed to assess the self-purification ability of the river by studying bio surfactant producing microbes.

## MATERIALS AND METHODS

**About the River:** Water samples for the current study was collected from the Tamirabarani River Basin of Tirunelveli District, Tamil Nadu. It lies between Latitude 08° 8'N and 09°23'N and Longitude 77° 09' E and 77° 54'E. The location of the Study area is shown in Fig.1 and Index Map of Tamirabarani River Basin is shown in Fig.2. The major rainy season is from October to middle of January. The average annual rainfall prevails over the study area is 815mm. The total length of River is 120km of which 75km runs in Tirunelveli district. It is fed both by monsoons and by its tributaries. The area of the river basin is 5942km2. The relative humidity in general, during the year, is between 55 and 65 percent. Physico graphically, the area represents flat topography with gentle slope. It flows roughly east and enters the Gulf of Manner of the Bay of Bengal near Palayakayal. The wastewater from the industries and municipal areas are discharged and drained into Tamirabarani River [1].

**Study Area and Sampling Sites:** Water sample for the presence study was collected in 10 liter water cane from the River Tamirabarani at Kokkirakulam, Tirunelveli District. Sampling point was selected because of the prominence of oil pollution. At the point of sampling, color, odour and temperature were noted and brought to the laboratory for physicochemical and biological analysis.

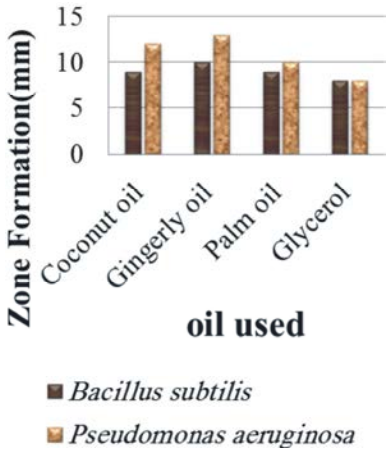


Fig 1: Oil Displacement Method *Bacillus Subtilis* and *Pseudomonas Aeruginosa*

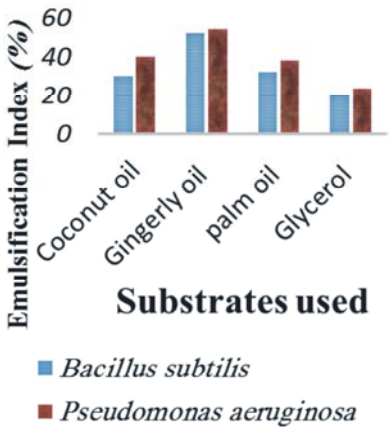


Fig 2: E24 Value of *Bacillus Subtilis* and *Pseudomonas Aeruginosa*

**Physicochemical Study:** Determinations like pH, electrical conductivity and turbidity were carried out in-situ using digital meters. The chemical parameters like dissolved oxygen, nitrate and phosphate were analyzed with the adaptation of standard methodologies [13 APHA].

**Enumeration of Total Heterotrophic Bacterial Population (THBP):** Water sample was serially diluted and spread plated on nutrient agar medium and incubated under aerobic conditions at 37°C for 24 hours for determining the THBP. Well isolated bacterial strains were picked up and stored in nutrient agar slants for further studies.

**Screening of Bio Surfactant Production:** Bacteria selected were tested for its ability to produce bio surfactant by four methods as mentioned below.

**Blood Hemolysis:** A loop full of inoculum was taken and streaked on the blood agar plate and incubated at 37°C for 48-72 hours. The bio surfactant positive colonies were selected based on the presence of a clear zone around the colonies and were recorded.

**Oil Displacement Test:** It is a method used to determine the diameter of the clear zone, which occurs after adding surfactant-containing supernatant collected by spinning the 24 hours culture at 10000 rpm for 10 minutes on an oil-water interphase. The diameter evaluation allows the surface tension reduction efficiency of a given bio

surfactant. In this test, 15 ml distilled water was added to a petri dish of 90 mm diameter. 100 ìl of test oil was added to the water surface, followed by the addition of 20 ìl of cell culture supernatant on to the oil surface. The diameter and the clear halo visualized under visible light was measured after 30 seconds [14].

**Drop Collapse Method:** This test was performed according to Youssef *et al.* [15] and Ghayyomi Jazeh *et al.* [16] with a slight modification. The test was carried out using 96 well microtiter-plates containing 100 ìl. Mineral oil which was equilibrated for an hour at room temperature. About 10 ìl of the culture was added to the surface of the oil. After 1 min, the shape of the drop on the surface of the oil was observed. The results were Interpreted as (+) to (+++) corresponding partial to Complete spreading on the oil surface. Those culture that gave rounded drops were scored as negative (–) indicating the lack of bio surfactant production.

**Emulsification Capacity ($E_{24}$):** Emulsification capacity of the bio surfactant towards three oils i.e., coconut, gingili and palm oil was checked following [17]. A mixture of 2 ml oil and 1 ml cell free extract obtained after the centrifugation of the cultures were taken in a test tube and homogenized by vortexing for 2 min. The emulsion activity was investigated after 24 hours and the $E_{24}$ was calculated by the following formula.

$E_{24}$ = total height of the emulsified layer/total height of the liquid layer

**Identification of The Strain:** The positive bio surfactant producing bacterial strains (TS3 &STS4) were identified following the Begay's manual of determinative bacteriology [18] and the results were tabulated.

**Selection of Suitable Carbon Source for the Production of Bio Surfactant:** The effect of 2% carbon sources like glucose, molasses and whey was studied on the production of bio surfactant.

**Selection of Suitable Inorganic Nitrogen Source for the Production of Bio Surfactant:** The effect of 2% nitrogen sources like ammonium sulphate, sodium nitrate, ammonium chloride and ammonium nitrate was studied on the production of bio surfactant [19].

**Extraction of Bio Surfactant:** The culture broth was centrifuged at 12,000 rpm for 15 mints to remove the cells as well as debris and the supernatant was filtered through 0.2µm filter. Filtered supernatant was used for the extraction of bio surfactants. Extraction was performed by acid precipitation followed by liquid-liquid extraction. The cell free supernatant was acidified with equal volume of concentrated HCl to attain a pH 2.0 and extracted with an equal volume of solvents such as ethyl acetate, diethyl ether and acetone. The resultant aliquot was concentrated to dryness in a rotary vacuum evaporator and tested for the emulsification activity [20].

**Determination of Bio surfactant Dry Weight:** The dry weight of the bio surfactant was calculated by the following formula.

Dry weight of the bio surfactant = Wt of dried plate – Wt of empty plates.

**Characterization of Bio Surfactant Using Thin Layer Chromatography:** The preliminary characterization of the bio surfactant was carried out by TLC method as proposed by earlier worker [21].

**Determination of Antimicrobial Activity of Bio Surfactant:** The antibacterial effect of bio surfactant at 30 µl concentration loaded in the wells of Mueller Hinton agar plates previously swabbed with potent human bacterial pathogens including Gram positive (*Bacillus* sp *and Staphylococcus aureus )* and Gram negative bacteria (*E.coli, Klebsiella, Pseudomonas aeruginosa* and *Salmonella* ). The plates were incubated at 37°C for 24 hours. After incubation the results were observed [22].

## RESULTS AND DISCUSSION

Water is the elixir of the life. The quality of rivers is reflected on the quality of life and development in that region. Tamirabarani river is the back bone of the south most districts of Tamilnadu. The physicochemical properties of the water sample collected from this river were presented in table 1 helping to understand that the water qualities are found within the limits of BIS [23]. Water quality properties in the rive have been documented by many researchers [1-5]. Presence of water qualities with in the admissible limits revealed the self-purification ability of the river beyond the occurrence of various forms of pollution.

Self -purification is a natural process occurring in water bodies due to physical, chemical and biological means. Natural aquatic micro flora could aid the river in self - purification. THBP in the water collected from the

Table 1: Biochemical Tests

| S.no | Tests | Results Produced by the Organisms | |
|---|---|---|---|
| | | TS3 | TS4 |
| 1 | Gram staining | G+ve rods | G-ve rods |
| 2 | Motility | Motile | Motile |
| 3 | Spore staining | Spore former | Non spore former |
| 4 | Oxidase | Positive | Positive |
| 5 | Catalase | Positive | Positive |
| 6 | Indole | Negative | Negative |
| 7 | Methyl red | Negative | Negative |
| 8 | Voges proskauer | Positive | Negative |
| 9 | Citrate | Positive | Positive |
| 10 | TSI | k/k | k/k |
| 11 | Casein Hydrolysis | Positive | - |
| 12 | Gelatin Hydrolysis | Positive | - |
| 13 | Starch Hydrolysis | Positive | - |

Table 2: Analysis Biosurfactant Using Thin Layer Chromatography

| Nature of Chemicals | Nature of Spot | | Rf Value | Organisms |
|---|---|---|---|---|
| | Anthrone | Ninhydrine | | |
| Lipo peptides | Red spot | No spot | 0.49 | *B. subtilis*(TS3) |
| Glycolipids | No spot | Yellow spot | 0.68 | *P.aeruginosa*(TS4) |

selected sampling site was found to be $5.5\times10^4$ CFU/ml. THBP which also involves bio surfactant producing microorganisms.

Bio surfactant are surface active molecules produced by microorganisms such as bacteria, yeast and filamentous fungi [24]. The role of bio surfactant has been considered important in oil pollution management by various workers [25, 26]. Among the various pollutants degrading the water qualities in the river, oil pollution is a common and more frequently occurring one from its starting points and especially when the rivers enter into towns and cities like Tirunelveli.

Bio surfactant producing microorganisms are naturally present in the oil contaminated water. Oil contaminated environment contain large amount of hydrocarbons. Microorganisms' exhibit emulsifying activity producing bio surfactants often mineralizing them (Or) converting them into harm less products. They are more active and less toxic than chemical surfactants which are difficult to remove or degrade from the environment. They can be used in handling industrial emulsions. Control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated water [27].

Detection of bio surfactant producing bacteria was tested by four methods such as blood hemolysis, emulsification index, drop collapse test and oil displacement method as prescribed by Padma priya *et al*. [28]. Investigation of bio surfactant producing bacteria in the water sample revealed its distribution at 40% level.

Following Bergey's manual, bio surfactant producing organisms were identified as *P. aeruginosa* (TS3) and *B. subtilis* (TS4) 21 (Table-1).The four different methods employed in the current work could also be used to evaluate the efficiency of produced bio surfactants.

Numerous experiments have been carried out with *P. aeruginosa* and *B. subtilis* for bio surfactant production [21, 29, 33]. Fig (2-4), indicating us the degrading effect of bio surfactants on different oils and glycerol. All the oils selected were degraded by the bio surfactants, irrespective of the source of production. Maximum derivative effect was noted on gingili oil followed by coconut oil. Gingili oil pollution in the river Tamirabarani is a common one due to human activities. It is used for taking oil bath by many following coconut oil.

Bio surfactant producing microorganisms are predominantly distributed in oil spilled contaminated regions [30]. The ability of bio surfactant production is influenced by various physical and chemical factors like temperature, pH, carbon source and nitrogen source etc., [31, 32]. The effect of various factors could be understood on bio surfactant production by screening their effect over the growth of microbes at different points. Optimization of different parameters like pH, temperature,

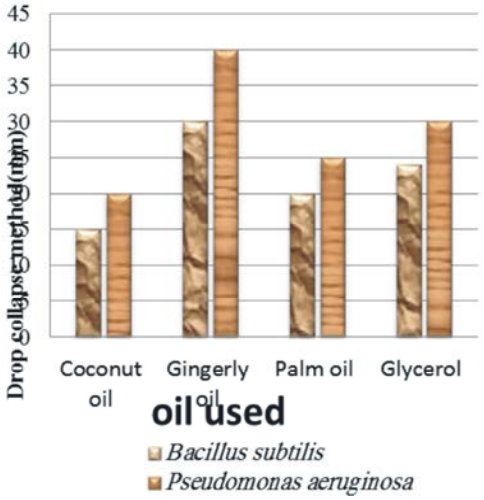


Fig 3: Drop Collapse Method of *Bacillus Subtilis* and *Pseudomonas Aeruginosa*

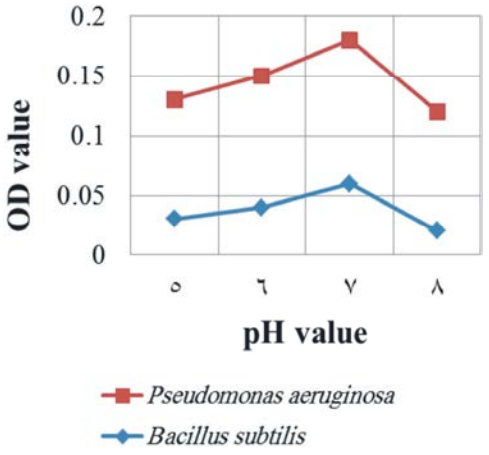


Fig 4: Effect of Ph on the Growth of Biosurfactant Producing Bacteria

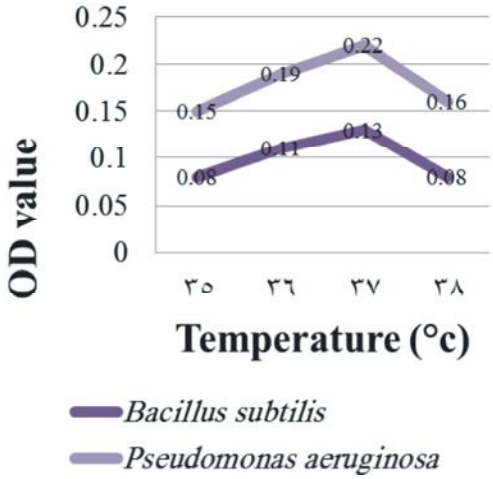


Fig 5: Effect of Temperature on the Growth of Biosurfactant Producing Bacteria

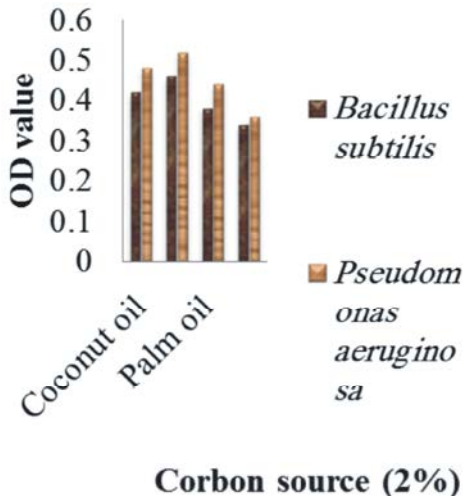


Fig 6: Effect of Different Carbon Source

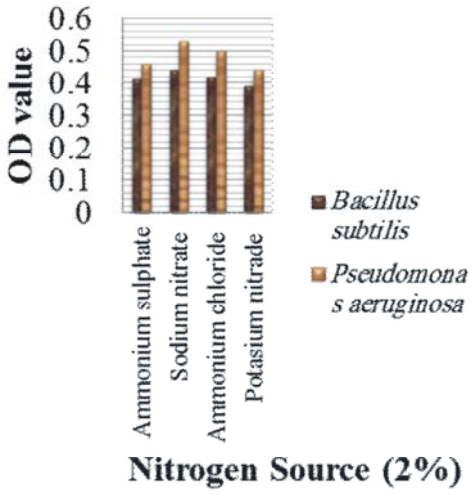


Fig 7: Effect of Different Nitrogen Source

carbon source and nitrogen source have been done [33]. Fig 5&6 explained the effect of selected physicochemical parameters on the growth of the two bacteria. From the figures, it was very clear that *Pseudomonas* sp was found to show a superior growth in all the parameters in the tested range than *Bacillus* sp. Highest growth was noted at the 37 °C and pH 7 in both organisms. Ainon Hamzah *et al*. [19] has reported 37°C is the optimum temperature for bio surfactant producing *Pseudomonas* sp but reported a variation in the pH for the same organism as 9. A fall of growth was noted beyond this point. The impact of studied physical parameters has also been reported [34].

Carbon and nitrogen are the two important elements influencing the production of any useful product of microbial origin by supporting their growth. Among the four carbon sources tested (Fig 7), highest supportive role

was noted from gingili oil followed by coconut oil. Least effect was noted from glycerol. Ainon Hamah *et al.* [19] documented the effectiveness of five different carbon sources including glycerol and palm oil, which were used in the current study. More growth and production of bio surfactant were noted with glycerol than palm oil. But in the current study, the effect of palm oil was found to be better than glycerol. The different in the activity of the organism over the utilization / degradation may be due to the difference in the biochemistry of bio surfactant produced [35, 36].

Nitrogen is a limiting factor. It is available for organism in organic or inorganic forms. Screening of the effect of inorganic nitrogen sources such as *P.aeruginosa* and *B. subtilis* revealed that the positive effect was in the following order: Sodium nitrate>ammonium chloride>ammonium sulphate>potassium nitrate (Fig). The same trend was noted on both the organism checked Fig 8 Impact of organic [37] and inorganic [24] nitrogen sources has also been tested on *P.aeruginosa* and *B.subtilis*.

Most microbial surfactant is complex molecules comprising different structure that includes lipo peptides, glycolipids, polysaccharides- protein complex, fatty acid and phospholipids [38]. The biochemical characterization of bio surfactant produced by the 2 different bacteria using TLC revealed that they are chemically different. (Table-3). Swapna Patil *et al*. [21] has reported the production rhamnolipids bio surfactant in *P. aeruginosa f23* isolated from oil contaminated sample by using TLC.

The same chemistry in bio surfactant has also been observed from *P. aeruginosa* by Praveesh *et al*. [39]. While comparing with the previous works, it is understood that the chemistry of bio surfactant produced by *P. aeruginosa* isolated from Tamirabarani River is different. Oil degrading efficiency of biochemically different bio surfactants shall be studied in the future.

River waters contain not only beneficial microbes, but also various pathogens affecting different hosts. Bio surfactants can benefit the aquatic environment dual process such as bioremediation and bio control. Antimicrobial role of bio surfactant produced by *P.aeruginosa* and *B. subtilis* organism was recorded against *Escherichia coli*, *Salmonella*, *Staphylococcus aureus* and *Bacillus cereus* organisms Kishore *et al*. [40]. Screening of antibacterial effect against *E. coli, Salmonella* sp, *S.aureus* and *B. cereus* indicated the presence of inhibitory activity against all the pathogens tested. More activity was noted against *E.coli* followed by *S. aureus*. It was applicable for the surface active components produce by both bacteria tested.

## CONCLUSION

From the findings of the current study, it is concluded that even though, the river Tamirabarani is polluted by various means, the pollution level is found to be manageable. It is because of the self-purification ability acquired by the river in different ways. One of the factors responsible for pollution management is its micro flora. The bio surfactant producing bacteria like *Bacillus* sp helping the river to tackle oil pollution to some extent and acting as a bioremediation agent. The finding insists the need to safeguard the river from pollution for the sustainability.

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