# Assessment of orange peel for biosurfactant production by *Rhodococcus pyridinivores* isolated from marine ecosystem and its potential to degrade napthalene

**Thanki Neha[1], Bhavsar Nirav[1], Raol Gopal[2], Vaidya Yati[1] and Surati Viral[1*]**
1 Shri Alpesh N. Patel Post Graduate Institute of Science and Research, Anand, Gujarat, INDIA
2 Shri RP Arts, Shri KB Commerce and Smt. BCJ Science College, Khambhat, Gujarat, INDIA
*viral4host@gmail.com

***Abstract***
*A Gram-positive bacterium was isolated from a marine soil sample collected from Porbandar, Gujarat, India and identified as Rhodococcus pyridinivorans (OK036732) using 16S rRNA sequencing. The present study evaluates the ability of R. pyridinivorans to utilize various natural waste carbon sources for biosurfactant production and hydrocarbon biodegradation.*

*Among the tested substrates, orange peel proved to be the most effective, yielding 1.25 g/L of biosurfactant with an emulsification activity of 69.62% against the tested hydrocarbon. Biodegradation studies indicate that the biosurfactant produced by R. pyridinivorans holds significant promise as an environmental friendly and biodegradable alternative.*

**Keywords:** *Rhodococcus pyridinivores*, Biosurfactant, Hydrocarbon, Emulsification.

## Introduction

In recent decades, the environmental damage caused by petroleum and petrochemical products has gained significant attention. Researchers have identified various microorganisms capable of breaking down polycyclic aromatic hydrocarbons (PAHs) and have conducted extensive studies on their mechanisms[16]. However, the limited accessibility of these hydrophobic pollutants to microorganisms often hampers the bioremediation of PAH-contaminated soils[18]. The increasing use of cars and machinery has led to widespread use of lubricating oils, resulting in heightened environmental pollution from spent motor oils such as diesel and jet fuel spills[1].

The improper disposal of this waste poses serious environmental challenges due to the presence of heavy PAHs and metals in old motor oil which elevate the risk of chronic health issues, including cancer and mutagenicity[6,15].

Biosurfactants, which are microbial surface-active agents, consist of both hydrophobic and hydrophilic components. These molecules interact with surfaces of varying polarities and reduce surface and interfacial tension, enhancing hydrocarbon uptake and emulsification or dispersion. By increasing the contact area at the aqueous–hydrocarbon interface, biosurfactants improve the bioavailability of hydrocarbons to microbial cells, accelerating hydrocarbon dissolution and their subsequent microbial utilization[21,24,25].

Traditionally, the production of biosurfactants has depended on expensive carbon sources; however, the cost of biosurfactant production can be reduced using natural raw materials. Agro-industrial wastes, which are rich in lipids and carbohydrates, present a viable alternative carbon source for biosurfactant synthesis[17]. Current research mainly concentrates on the potential of individual biosurfactant-producing bacteria for remediation.

## Material and Methods

**Isolation and Identification of Biosurfactant producing bacteria:** 100 g of marine soil were collected from a depth of 0–5 cm in the coastal area of Porbandar, Gujarat, India (21.2046° N, 77.4977° E). One gram of the sediment sample was added to 50 ml of Bushnell Haas (BH) broth in a 250 ml Erlenmeyer flask along with 2% glycerol as the carbon source to promote bacterial growth. The flasks were incubated at 30 °C and 150 rpm for five days. Subsequently, one milliliter of the culture broth was transferred to 50 milliliters of fresh BH broth in a 250-milliliter flask and the incubation process was repeated under the same conditions. This enrichment procedure was carried out three times.

After enrichment, one milliliter of the cultures was diluted in sterile 0.85% saline solution and plated on nutrient agar to isolate the microorganisms. Morphologically distinct colonies were re-isolated by transferring them to fresh nutrient agar plates three times to ensure pure cultures. These pure cultures were then subjected to Gram staining and selected for biochemical characterization and biosurfactant screening. The pure isolates were preserved on nutrient agar slants at -20 °C for future use[4].

**Molecular characterization of Biosurfactant producing bacterial isolate:** The sequencing analysis of the 16S *r*RNA was conducted by the National Centre for Microbial Resource in Pune, Maharashtra, India. The sequences obtained from DNA extraction were blasted against the GenBank database using the BLAST network service[5]. Further analysis included multiple sequence alignment with ClustalW followed by construction of the consensus neighbor-joining phylogenetic tree using the Molecular Evolutionary Genetics Analysis (MEGA) software [27].

**Screening of potential biosurfactant producer:** The screening of potential biosurfactant-producing bacteria was

conducted through several methods such as hemolytic assays, drop collapsing tests, oil displacement tests and lipase activity assessments[12,14,30]. These techniques were utilized to evaluate the bacteria's capability to produce biosurfactants by examining their interactions with oils and their surface-active characteristics.

**Selection of optimum natural waste substrates for biosurfactant production:** To evaluate the optimum natural waste substrates as carbon sources for biosurfactant production, orange peels, banana peels and potato peels were used instead of glycerol in a BH medium at a concentration of 2%. These waste substrates were dried and ground into powder before use. The experiments took place in 250-mL Erlenmeyer flasks containing 50 mL of medium, inoculated with 2% (v/v) of bacterial inoculum and incubated at 30 °C for 120 hours. After fermentation, the broth was centrifuged at 10,000 rpm at 4 °C for 10 minutes to separate the supernatant for further analysis.

**Biosurfactant production by using orange peel as carbon source:** The effects of incubation time, temperature and substrate concentration on biosurfactant production were optimized. In each experiment, 50 mL of medium in a 250-mL flask was inoculated with 2% (v/v) of bacterial inoculum and incubated at 35 °C. To examine the impact of incubation time, the medium was incubated for periods ranging from 1 to 5 days, using an orange peel concentration of 2%. The biosurfactant yield was measured at 24-hour intervals and the minimum incubation period for maximum production was determined to be the optimal incubation time. Furthermore, biosurfactant production was assessed at different temperatures (25, 30, 35, 40 and 45 °C) to find the optimal temperature. The concentration of orange peel was also optimized by testing concentrations from 1% to 5%.

**Extraction of Biosurfactant:** After fermentation, the cell pellet was separated from the culture broth by centrifuging at 10,000 rpm for 10 minutes. To precipitate the lipids and proteins, 6 N HCl was added to the culture supernatant until a final pH of 2.0 was reached and the mixture was stored at 4 °C overnight. The biosurfactants that precipitated, were extracted by adding an equal volume of ethyl acetate to the supernatant in a separating funnel and shaking it vigorously. The organic phase was then collected and concentrated using rotary vacuum evaporation. Finally, the extracted biosurfactant was lyophilized and weighed to determine the yield.

**Study of growth and biosurfactant production:** A minimal salt medium culture (MSM) of 250 mL conical flasks, each with a working volume of 50 mL, was used to study the growth and development of biosurfactants in various carbon substrates. Samples of fermented broth were taken every two hours to measure biomass, biosurfactant concentration and emulsification activity. Biomass was calculated as dry cell weight[26]. The emulsification activity was determined by adding equal volumes of hydrocarbon and culture supernatant, vortexing for 2 minutes and incubating at room temperature for 24 hours[2].

**TLC analysis for crude biosurfactant:** Crude biosurfactants obtained from *Rhodococcus pyridinivorans* were characterized through thin-layer chromatographic (TLC) analysis. 100 µL of each sample was spotted on a preparative TLC plate of 20 cm x 20 cm pre-coated with silica gel 60 and developed in a solvent system of chloroform-methanol-acetic acid (65:15:2, v/v/v). TLC plates were sprayed with orcinol-$H_2SO_4$ reagent and heated at $100^0$C for 5 min[19].

**Biodegradation of PAH compounds (naphthalene) and crude oil:** Bacteria grow in batch culture under conditions that allow the use of two individual sterile 250 mL flasks, each containing a volume of 50 mL minimal salt medium (MSM) and with a carbon source by a polycyclic aromatic hydrocarbon (PAH). The first flask has naphthalene (100 mg) while the second flask contains crude oil (500 µL), both supplemented with a 2% overnight broth culture. The two flasks were incubated at a temperature of 150 rpm and 37 °C for 10 days. At the intervals given within the 10-day incubation period, 1 mL of culture was drawn from the batch for measurement of its optical density (OD) at 600 nm. The same broth was used for estimating the biodegradation end product, salicylic acid and analyzing crude oil degradation.

**Analysis of naphthalene intermediate:** The concentrations of hydroxylated aromatic metabolites from naphthalene degradation were determined according to Box[7]. Because salicylic acid was expected to be the primary metabolite produced from naphthalene degradation, the sodium salicylate was used for preparation of standard curves. Concentrations of the hydroxylated metabolic intermediates were subsequently expressed in terms of salicylic acid equivalents in units of mg/mL.

**Bacterial adhesion to hydrocarbons (BATH assay):** The cell hydrophobicity of the *R. pyridinivorans* with crude oil was measured using the Bath assay[23]. Hydrophobicity expressed as the percentage of cell adherence to crude oil was calculated as follows:

$$\text{Percentage of bacterial adherence (\%)} = (1 - (OD_{\text{aqueous phase}} / OD_{\text{original}})) \times 100$$

**Statistical analysis:** All the biosurfactant production experiments were repeated in triplicate and the results were factored as the average of the three determinations. All measures were below 5% statistical deviation.

## Results and Discussion

**Isolation, screening and identification of biosurfactant-producing microorganism:** A Gram-positive bacterium with cocci morphology was isolated from coastal area of Porbandar, Gujarat, India. The sequence of the 16S *r*RNA of the isolated bacteria demonstrated a high degree of similarity

(99.86 %) with *Rhodococcus pyrinivorans* (Figure 1). The 16S *r*RNA sequences of strain *R. pyrinivorans* were determined and were submitted to Gen-Bank database with accession numbers OK036732.

In the present study, the bacterial isolates were first screened with primary screening method for determining hemolytic activity. *R. pyrinivorans* showed good hemolytic activity. Blood agar lysis has been used to quantify surfactin[20,23] and rhamnolipids[11] and has been used to screen biosurfactant production finding an association between hemolytic activity and surfactant production. The use of blood agar lysis as a primary method to screen biosurfactant production was recommended[10,31]. None of the studies reported in the literature mention the possibility of biosurfactant production without a haemolytic activity[11,20,31]. However, in some cases, hemolytic assay excluded many good biosurfactant producers[14].

The supernatant from *R. pyrinivorans* has shown good activity of 30 mm in diameter oil displacement activity. The presence of biosurfactant was determined by oil displacement and clearing zone formation. The diameter of this clearing zone on the oil surface correlates to surfactant activity and hence satisfies oil displacement activity. Youssef et al[30] demonstrated that the oil spreading technique was very reliable method to detect biosurfactant production by diversified microorganisms.

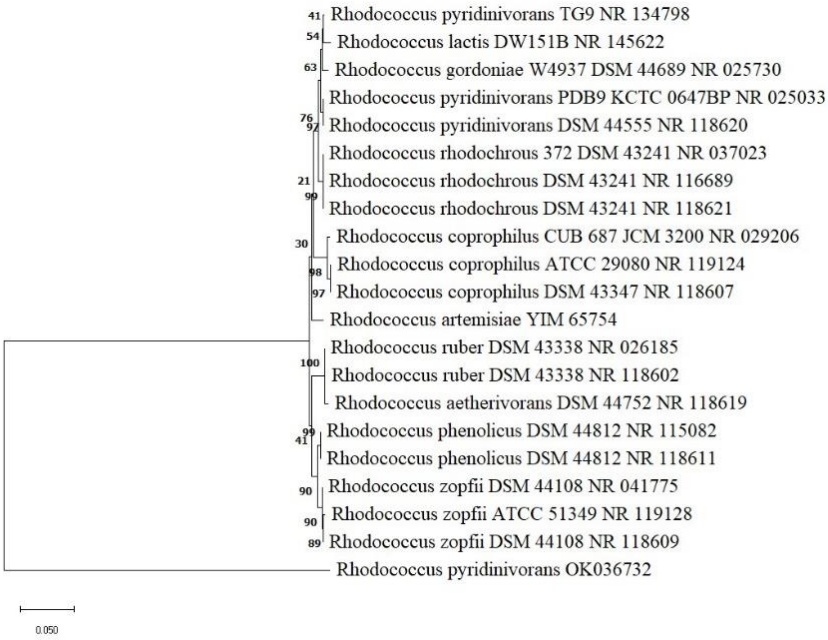


**Figure 1: Phylogenetic tree of the strain *Rhodococcus pyridinovorans* and closest NCBI (BLASTn) strain based on the 16s *r*RNA gene sequences (neighbour joining tree method). The scale bar indicates 0.050 nucleotide substitutions per nucleotide position. The numbers at node show the bootstrap values.**

**Figure 2: Liplytic activity of marine soil isolate *R. pyridinivorans***

In the lipase screening activity, *R. pyrinivorans* showed positive lipolytic activity (Figure 2). Inoculation of the bacterial isolates on tributyrin agar plate produced a clear zone which indicates production of the enzyme i.e. lipase. These results confirmed that the bacterial isolates were potential producers of surface-active molecules. According to Kokare et al[13], lipase acts on water–oil surfaces and therefore it was suggested that actinomycetes showed the presence of lipases and are able to produce bioemulsifiers.

**Evaluation of biosurfactant production by *R. pyrinivorans*:** Biosurfactant production from *R. pyrinivorans* with different natural waste substrates as carbon sources was studied using a mineral salt medium. The composition (g/L) of mineral salt medium prepared in distilled water was as follows: $NH_4NO_3$ (1.0), $KH_2PO_4$ (1.0), $K_2HPO_4$ (1.0), $MgSO_4$ $7H_2O$ (0.2), $CaCl_2$ $2H_2O$ (0.2) and $FeCl_3$ $6H_2O$ (0.05). Among the three substrates tested, the highest percentage of emulsification and biosurfactant yield was obtained on using orange peels used as carbon source and the lowest emulsification index was found on banana peels (Table 1). The biosurfactant concentration of 1.063 g/L was recorded and the emulsification activity of 67.69 % was measured. Based on these studies, the orange peel was used for further studies as a carbon source to enhance the biosurfactant production.

The growth and biosurfactant production by *R. pyrinivorans* were monitored in mineral salts medium at different time intervals. *R. pyrinivorans* actively produced biosurfactants in the exponential growth phase and it was completed within 72 h. Then onwards it decreased gradually in orange peel containing media (Figure 3). Maximum emulsification activity and biosurfactant yield of 67.69 % and 1.063 g/L were obtained at 96 h of incubation respectively (Figure 3). The biosurfactant production significantly increased with the increase in the orange peel concentration to obtain the maximum yield of 1.25 g/L and emulsification activity of 69.62% when 4 % orange peel is used as a carbon source (Table 2). The optimum temperature for maximum biosurfactant yield was found at 35 °C and there onwards the biosurfactant production decreased with increase in temperature (Table 3).

**Table 1**
**Biosurfactant produce by *R. pyrinivorans* with different natural waste products.**

| Substrates | Biosurfactant concentration (g/L)* | Emulsification activity (%)* |
|---|---|---|
| Orange peels | 1.063 ± 0.051 | 67.69 ± 0.231 |
| Potato peels | 0.909 ± 0.007 | 56.04 ± 1.066 |
| Banana peels | 0.853 ± 0.068 | 53.46 ± 2.713 |

*Mean ± SD, no = 3

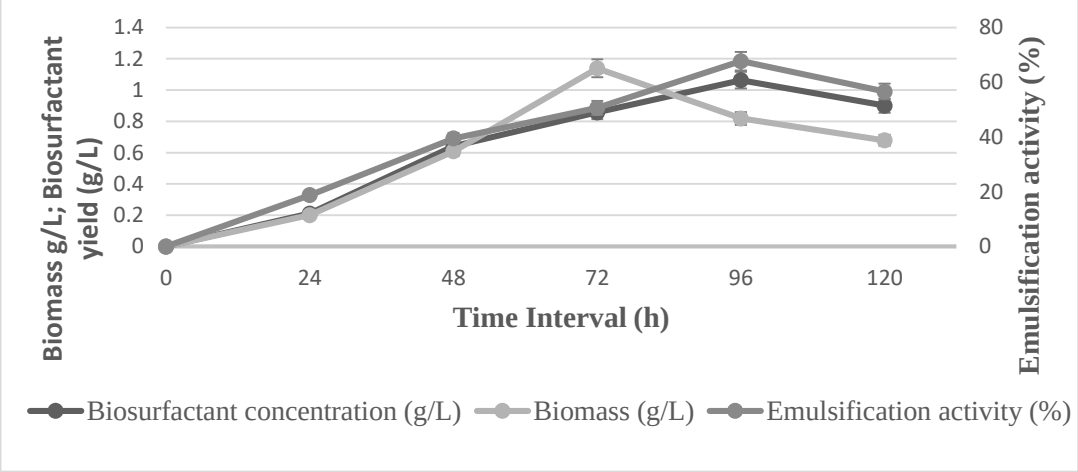


**Figure 3: Effect of incubation time on biomass, Biosurfactant yield and emulsification activity**

**Table 2**
**Effect of different orange peel concentration on Emulsification activity and biosurfactant production by *R. pyridinivorans***

| Orange peel concentration (%) | Emulsification index (%) | Biosurfactant yield (g/L) |
|---|---|---|
| 1 | 56.62 ± 0.561 | 0.67 ± 0.015 |
| 2 | 64.77 ± 0.623 | 0.89 ± 0.065 |
| 3 | 65.49 ± 0.426 | 0.97 ± 0.021 |
| 4 | 69.62 ± 0.223 | 1.25 ± 0.092 |
| 5 | 67.30 ± 0.233 | 1.06 ± 0.046 |

**Table 3**
**Effect of incubation temperature concentration on Emulsification activity and biosurfactant production by *R. pyridinivorans***

| Temperature (°C) | Emulsification index (%) | Biosurfactant yield (g/L) |
|---|---|---|
| 25 | 51.95 ± 1.030 | 0.60 ± 0.153 |
| 30 | 63.27 ± 0.553 | 1.04 ± 0.071 |
| 35 | 71.98 ± 0.825 | 1.17 ± 0.093 |
| 40 | 62.69 ± 0.237 | 0.80 ± 0.026 |
| 45 | 19.95 ± 1.070 | 0.14 ± 0.305 |

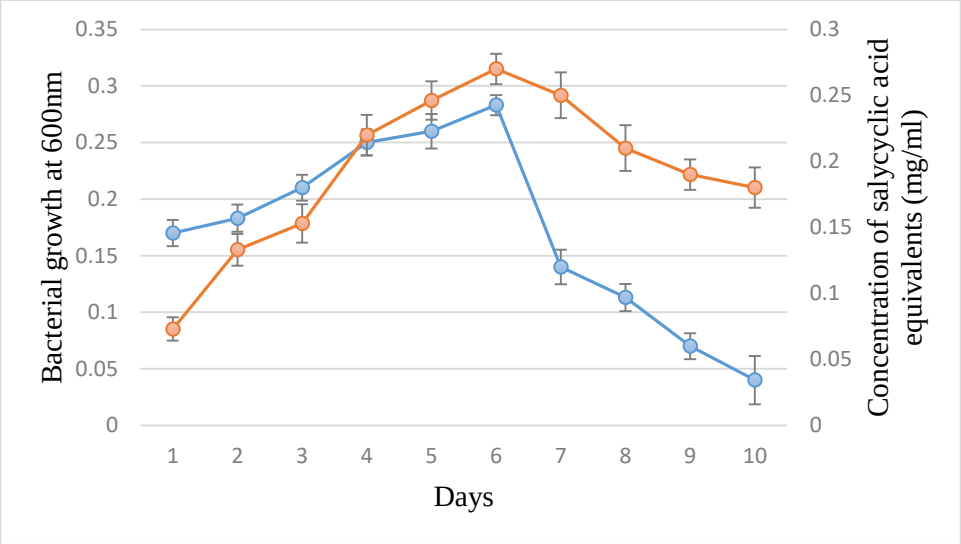


**Figure 4: Biodegradation of naphthalene by *R. pyrinivornas***

**TLC analysis of biosurfactant from *R. pyrinivorans*:** The crude biosurfactant obtained after solvent extraction from the culture supernatant of *R. pyrinivorans* was further purified by different chromatographic techniques. Crude extract of the biosurfactant was recovered from the culture supernatant of bacterial isolate by ethyl acetate extraction process. After acid precipitation, crude biosurfactant was examined and separated using thin layer chromatography (TLC). Single spot was observed at an Rf value 0.56 and suggested presence of glycolipid content in biosurfactant. There was no color development observed for amino acid.

**Biodegradation studies of naphthalene:** In this study, the potent bacterial isolate *R. pyrinivorans* was used for the naphthalene degradation studies. The bacterial degradation of naphthalene was quantified indirectly by the determination of naphthalene polar intermediates i.e. salicylic acid. Accumulation of the major metabolite salicylic acid started slowly by day 1 onwards and it reached maximum at 6th day of incubation for *R. pyrinivorans* and later it decreased (Figure 4). In the earlier studies, the production of biosurfactants by microorganisms can reduce the surface tension of the broth and subsequently can increase the bioavailability of the naphthalene to the bacteria.

In general, the mechanism of aerobic degradation of naphthalene involves the incorporation of molecular oxygen into one of the aromatic rings by naphthalene dioxygenase, leading to the formation of cis-1,2-Naphthalene dihydrodiol. The later undergoes a number of further degradative steps and finally gets metabolized to carbon dioxide through salicylic acid[26]. Naphthalene biodegradation is the best studied PAH because it has simple structure and is most soluble in nature. Naphthalene-degrading microorganisms are relatively easy to isolate. Recently Dasari et al[8] reported that *P. aeruginosa* degraded the naphthalene in presence of biosurfactant on 6th day of incubation.

The maximum percentage of bacterial adherence of 38.25 % was reported by *R. pyrinivorans* with crude oil. The Bath assay was confirmed by visualization of cells adhered to crude oil and it was confirmed by the affinity of cells towards crude oil facilitated by producing biosurfactant[28]. The hydrocarbon degraders normally produce surfactants which adhere effectively to hydrophobic substrate[3]. Pruthi and Cameotra[22] showed that the ability of bacteria to adhere to hydrocarbons is a characteristic feature of biosurfactant producing microbes. According to Thavasi et al[28] the bacterial isolates with high hydrophobicity are likely to be more efficient degraders. Franzetti et al[9] stated that the cell hydrophobicity is also an indication of biosurfactant production.

## Conclusion

A Gram-positive bacterium was isolated from coastal area of Porbandar, Gujarat, India and it was identified as *R. pyrinivorans* with an accession number OK036732. The

orange peel substrate was utilized by *R. pyrinivorans* as a very effective carbon source and biosurfactant production. From the biodegradation studies, it was concluded that the biosurfactant produced by *R. pyrinivorans* further can add to its value as an ecofriendly and biodegradable product.

## References

1. Abioye O.P., Agamuthu P. and Abdul Aziz A.R., Biodegradation of used motor oil in soil using organic waste amendments, *Biotechnology Research International*, **2012**, 587041 **(2012)**

2. Akbari E., Beheshti-Maal K., Rasekh B., Emami-Karvani Z. and Omidi M., Isolation and identification of current biosurfactant-producing *Microbacterium maritypicum* ABR5 as a candidate for oily sludge recovery, *Journal of Surfactants and Detergents*, **23**, 137-144 **(2020)**

3. Aliyu A., Usman M.D., Mohammed N., Ashafa M.A., Saidu M., Umar A., Salim C.A., Saifullah G., Shafi'u A., Usman M. and Ukwaja V.C., Identification and screening of biosurfactant producing bacteria from mechanic workshops soil in Gusau Metropolis, Nigeria, *UMYU Scientifica*, **2**, 46-52 **(2023)**

4. Arutchelvi J. and Doble M., Characterization of glycolipid biosurfactant from *Pseudomonas aeruginosa* CPCL isolated from petroleum-contaminated soil, *Letter of Applied Microbiology*, **51**, 75-82 **(2010)**

5. Blast N., Nucleotide BLAST, Basic Local Alignment Search Tool **(2020)**

6. Boonchan S., Britz M.L. and Stanley G.A., Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures, *Applied Environmental Microbiology*, **66**,1007-1019 **(2000)**

7. Box J.D., Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters, *Water Research*, **17**, 511-525 **(1983)**

8. Dasari S., Venkata Subbaiah K.C., Wudayagiri R. and Valluru L., Biosurfactant-mediated biodegradation of polycyclic aromatic hydrocarbons-naphthalene, *Bioremediation Journal*, **8**, 258-265 **(2014)**

9. Franzetti A., Caredda P., La Colla P., Pintus M., Tamburini E., Papacchini M. and Bestetti G., Cultural factors affecting biosurfactant production by Gordonia sp. BS29, *International Biodeterioration & Biodegradation*, **63**, 943-947 **(2009)**

10. Gaur D., Jain P.K., Sisodia Y.S. and Bajpai V., Estimation of extracellular lipase enzyme produced by thermophilic bacillus sp. isolated from arid and semi-arid region of Rajasthan, India, Nature Proceedings **(2012)**

11. Johnson M.K. and Boese-Marrazzo D., Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa*, *Infection and Immunity*, **29**, 1028-1033 **(1980)**

12. Kiran G.S., Hema T.A., Gandhimathi R., Selvin J., Thomas A., Rajeetha Ravji T. and Natarajaseenivasan K., Optimization and production of a biosurfactant from the sponge-associated marine fungus *Aspergillus ustus* MSF3, *Colloids Surfaces B: Biointerfaces*, **73**, 250-256 **(2009)**

13. Kokare C.R., Kadam S.S., Mahadik K.R. and Chopade B.A., Studies on bioemulsifier production from marine *Streptomyces* sp. S1, *Indian Journal of Biotechnology*, **6**, 78-84 **(2007)**

14. Kolapo Kehinde T., Biosurfactant producing abilities of some bacteria isolated from bitumen contaminated soils, *Open Journal of Environmental Biology*, **6**, 17352 **(2021)**

15. Lee L.S., Mats H., Delflno J.J., Suresh P. and Raot C., Partitioning of polycyclic aromatic hydrocarbons from diesel fuel into water, *Environmental Science Technology*, **26**, 2104-2110 **(1992)**

16. Li J.L. and Chen B.H., Surfactant-mediated biodegradation of polycyclic aromatic hydrocarbons, *Materials*, **2**, 76-94 **(2009)**

17. Makkar R. and Cameotra S., An update on the use of unconventional substrates for biosurfactant production and their new applications, *Applied Microbiology and Biotechnology*, **58**, 428-434 **(2002)**

18. Mihelcic J.R., Lueking D.R., Mitzell R.J. and Stapleton J.M., Bioavailability of sorbed- and separate-phase chemicals, *Biodegradation*, **4**, 141-153 **(1993)**

19. Monteiro S.A., Sassaki G.L., de Souza L.M., Meira J.A., de Araújo J.M., Mitchell D.A., Ramos L.P. and Krieger N., Molecular and structural characterization of the biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614, *Chemistry and Physics of Lipids*, **147**, 1-13 **(2007)**

20. Morán A.C., Martínez M.A. and Siñeriz F., Quantification of surfactin in culture supernatants by hemolytic activity, *Biotechnology Letter*, **24**, 177-180 **(2002)**

21. Nirmala K. and Veerendra Kumar B., Evaluation of Antiproliferative and Cytotoxic potential of organic solvent fractions of potential plant sources against Human colorectal carcinoma cell lines, *Res. J. Biotech.*, **19(5)**, 22-30 **(2024)**

22. Pruthi V. and Cameotra S.S., Rapid identification of biosurfactant-producing bacterial strains using a cell surface hydrophobicity technique, *Biotechnology Techniques*, **11**, 671-674 **(1997)**

23. Rosenberg M., Microbial adhesion to hydrocarbons: Twenty-five years of doing MATH, *FEMS Microbiology Letters*, **262**, 129-134 **(2006)**

24. Satpute S.K., Banpurkar A.G., Dhakephalkar P.K., Banat I.M. and Chopade B.A., Methods for investigating biosurfactants and bioemulsifiers: A review, *Critical Reviews in Biotechnology*, **30**, 127-144 **(2010)**

25. Singh A., Van Hamme J.D. and Ward O.P., Surfactants in microbiology and biotechnology: Part 2. Application aspects, *Biotechnology Advances*, **25**, 99-121 **(2007)**

26. Suwansukho P., Rukachisirikul V., Kawai F. and H-Kittikun A., Production and applications of biosurfactant from *Bacillus subtilis* MUV4, *Songklanakarin Journal of Science and Technology*, **30**, 87-93 **(2008)**

27. Tamura K., Dudley J., Nei M. and Kumar S., MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0, *Molecular Biology and Evolution*, **24**, 1596-1599 **(2007)**

28. Thavasi R., Jayalakshmi S., Balasubramanian T. and Banat I.M., Production and characterization of a glycolipid biosurfactant from *Bacillus megaterium* using economically cheaper sources, *World Journal of Microbiology and Biotechnology*, **24**, 917-925 **(2008)**

29. Walton J.L. and Buchan A., Evidence for novel polycyclic aromatic hydrocarbon degradation pathways in culturable marine isolates, *Microbiology Spectrum*, **12**, 03409-23 **(2024)**

30. Youssef N.H., Duncan K.E., Nagle D.P., Savage K.N., Knapp R.M. and McInerney M.J., Comparison of methods to detect biosurfactant production by diverse microorganisms, *Journal of Microbiological Methods*, **56**, 339-347 **(2004)**

31. Zobaer M., Ali F., Anwar Md.N., Bappi M.S.H., Bakar T.B. and Hossain T.J., Isolation of Biosurfactant Producing Bacteria from Oil-Spilled Soil and Characterization of Their Secreted Biosurfactants in Pathogen-Inhibition and Oil-Emulsification, *International Journal of Pharmaceutical Sciences and Research*, **15**, 1174-1183 **(2024)**.

(Received 03rd February 2025, accepted 17th March 2025)