Characterization of Biosurfactant Producing Bacteria Isolated From Petroleum Contaminated Sites

Gadakh PV[1] & Fuse SA[2] & PM Deshmukh[3]
[1]Assistant Professor, Department of Microbiology,Art, Science and Commerce College, Badnapur, Dist. Jalna (MH)
[2]Assistant Professor, Department of Chemistry, Art, Science and Commerce College, Badnapur, Dist. Jalna (MH)
[3]Research Scholar, Department of Microbiology, Shri Shivaji Science College, Amravati, Dist. Amravati (MH)

Received: September 02, 2018 Accepted: October 18, 2018

ABSTRACT *Petroleum derived hydrocarbons acts as a persistent contaminant of the soil and aquatic environment. Recent year's biosurfactant plays a vital role to solve this issue. Petroleum contaminated sites may harbor the microorganisms capable of degrading the hydrocarbons by producing extracellular products. Present study focused on to isolate and enriches the biosurfactant producing bacteria from such sites. So far petroleum contaminated soil samples were collected and processed to screen out the biosurfactant producing bacteria using surface tension measurement, drop collapse test, oil emulsion test, blood hemolysis etc. Out of 30 soil samples 18 different bacteria were isolated of which one Gram +ve bacterium was selected and processed further using different waste oil as source of carbon. Optimization study of the produced biosurfactant was done and GC-MS analysis of extracted and purified biosurfactant was done. The isolate shows an excellent biosurfactant activity when vegetable oil was used as substrate, while the moderate activity was recorded when waste engine oil was used as substrate. On the basis of the results of biochemical and 16S rRNA sequencing the isolate was identified as Aneurinibacillusmigulanus (G1) and further submitted to gene bank with accession number JQ337949. Result of study reveals that the isolate is able to produce biosurfactant efficiently from waste oil and will probably be used as alternative to chemical surfactant and suggest possible use in remediation of polluted sites.*

Keywords: *Biosurfactant, 16S rRNA sequencing, GC-MS analysis, Aneurinibacillusmigulanus*

Introduction:

The pollution caused by the crude petroleum oil and its derivative becoming a major issue of concern as they are most pervasive and persistent environmental pollutant (Okoh and Trejo-Hernandez, 2006).The profuseness of petroleum in any petroleum producing locality arises both as a blessing and a curse, because unfortunately most of the crude oil drilling sites and storage facilities are based at thenearby of human settlement (Patowary*et al.,*2017). During the process of oil exploration, collection and transportation from drilling sites, accidental leakage of crude oils results in the wide-range of contamination of adjacent agricultural fields and water bodies. These pollutants also have a major threat to ecosystem and biota through transfer of toxic organic materials into the food chain (Reddy *et al.,* 2011; Wang *et al.,* 2015). Such polluted sites are generally clean up by the chemically synthesized surface active compounds apart from this chemically derived surface active compounds are used widely in almost every sector of industries (Henkel *et al.,* 2012).To overcome these detrimental hydrocarbon pollutants and the expansion of environmental carefulness make attention for development of a remediation technology essential for cleaning up of polluted sites. A serious consideration of biological surfactants recognized as one of the most promising alternative to existing chemically derived products as they are effective, ecofriendly and inexpensive technologies (Bento *et al.,* 2005).

Interest in biosurfactant increased in recent years as a high value microbial product for application in biotechnology, industrial and medical field (Nitschke and Costa, 2007; Makkar*et al.,* 2011). They have different application as emulsifiers, dispersing agent, foaming agent, food stabilizer, detergency in organic chemicals, pharmaceuticals, cosmetics, mining, petroleum and many others as reported by (Banat *et al.,* 2000; Perfumo*et al.,* 2006; Vedaraman and Venkatesh, 2011). In spite of these applications commercial and large scale production of biosurfactant was failed due to low yield, high production cost and tedious recovery process. Possible economic biosurfactant production using agricultural products like sugars, molasses, plant oils, whey, distillery waste,animal fat were reported by (Makkar*et al.,* 2011).Various biosurfactant producing microorganisms like bacteria, filamentous fungi and yeast have been reported

previously. The bacterium genus *Bacillus* is ubiquitous in nature and not well reported for biosurfactant production. The automobile workshop and servicing centers also play an important role in the mixing of petroleum products to the surrounding environment including soil and biota. Present study focused on biosurfactant production using soil bacteria were isolated from garages and automobile workshop to check the adaptability and ability to utilize and degrade the petroleum product which acts as a source of soil and water contamination.

Materials and Methods

Collection of soil samples: In present study petroleum contaminated soil samples were collected from contaminated sites such as automobile work shop, garages and petrol pump of different areas of Amravati city. From each site about 10 gram oil mixed soil was collected in a sterile zip lockbag. The samples were labeled properly according to site and date of collection.All samples were transported to the laboratory in sterile polyethylene containers. The soil samples were serially diluted up to 10^{-3} of soil samples in 0.85% sterile saline and were preserved for further processing (Bordoloi and Konwar, 2008).

Enrichment and Isolation of Bacteria: From each serially diluted soil samples 1ml of soil sample was transferred to the sterile 250ml Erlenmeyerconical flask containing 100ml pre-sterile mineral salt medium having composition:2.5g/l of $NaNO_3$, 3.0g/l of KH_2PO_4, 7.0g/l of K_2HPO_4, 0.01g/l of $CaCl_2$, 0.5g/l of $MgSO_4.7H_2O$ and trace element solution containing 0.116g/l of $FeSO_4$.$7H_2O$, 0.232g/l of H_3BO_3, 0.41g/l of $CoCl_2.6H_2O$, 0.008g/l of $CuSO_4$.$5H_2O$, 0.008g/l of $MnSO_4$ .H_2O, 0.022g/l of $[NH_4]$ $6Mo7O_{24}$, 0.174g/l of $ZnSO_4$ with 2% used engine oil (v/v) as source of carbon was added to the medium. The trace element solution was added after the sterilization of production media, prior to inoculation the trace element solution was filtered through 0.2µm membrane filter.The inoculated flasks were incubated for 7 days in shaking incubator at 37^0C at 100rpm. Theflasks were continuously observed for the growth of bacteria.Direct isolation of bacteria was done by inoculating 1ml of sample on the surface of Nutrient agar medium. The plates were incubated at 37^0C for 24hr. Pure cultures with different morphological properties were selected and picked by repetitive streaking and stored in nutrient agar slants at 4^0C (Elazzazy*et al.,* 2015).

Screening Assay for Potential Biosurfactant Producing Strains: The isolated bacterial strains were further screened for their ability to produce biosurfactant using qualitative test namely drop collapse test, oil displacement test, blood hemolysis test and quantitatively using emulsification activity and measuring decrease in surface tension of cell free broth by Du Nouy ring method (Elazzazy*et al*., 2015). All the experiments were done in triplicates. Among the selected strain which shows highest surface tension reduction value was selected for further study accordingly (Youssef*et al*., 2004).

Fig. 1: Haemolysis test

Fig. 2: Crude biosurfactant

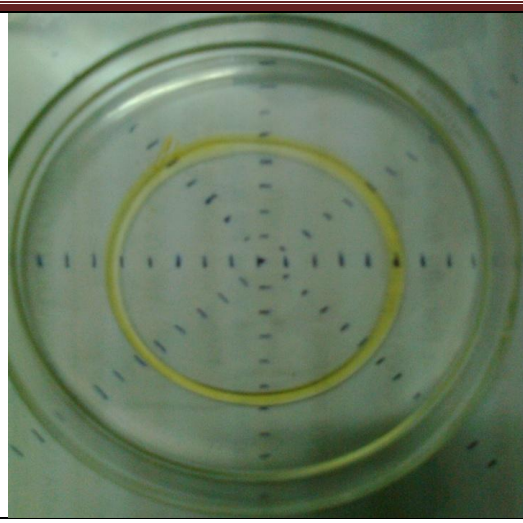


Fig.3: Oil Displacement test

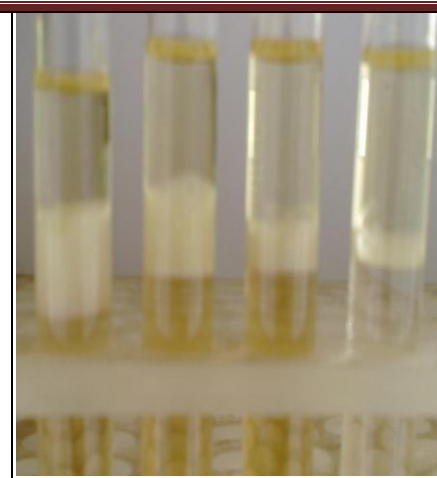


Fig. 4: Emulsification test

Biochemical andMolecular Identification of Bacteria: The qualitatively and quantitatively active bacterial strains were further selected and characterized biochemically using distinct biochemical test and identificationwas done by molecular method depending on phylogenetic gene approximation.PCR amplification was performed with 16S rRNAEu-bacterial primer (NCCS, Pune) 16F 27 (5'CCAGAATTGATC MTGGCTCAG-3') and 16R 1525 (5'TTCTGCAGT CTA GAAGGA GGTGWTCCAGCC-3'). The obtained nucleotide sequences from 16S rRNA gene sequencing were BLAST analyzed using NCBI. The nucleotide sequences were aligned in CLUSTAL X and phylogenetic tree was constructed using MEGA 4.0 software using neighbor joining method and assessed with 1000 bootstrap replication (Tamura *et al.*, 2007).

Biosurfactant Production and Optimization of Media Components: The mineral salt medium having composition as described above was used throughout the study. 1ml overnight culture of selected bacteria was aseptically inoculated in the flask containing 100 ml media and 2% respective oil as carbon source. The flasks were incubated at 37^{0}C for 7 days on shaking incubator for proper mixing of oil in the medium. After incubation cell free supernatant was obtained and further experiments were performed (Elazzazy*et al*., 2015). A series of experiments were performed to obtained higher productivity of biosurfactant. To optimize the media components and cultivation condition one variable was changed by keeping all other variable constant. The optimization of factors like pH, temperature and NaCl concentration was done by following the method suggested by Elazzazy*et al*., (2015). The pH range of the medium was adjusted from 2 - 10 pH by adjusting the pH before inoculation while the temperature range was set from 10 - 60^{0}C. The effect of salt was also evaluated by changing the salt concentration from 1% - 5% (v/v). Same time different vegetable oil like fried soybean oil, linseed oil, castor oil, coconut oil and mustard oil was added in the medium as well as diesel, engine oil, brake oil, kerosene andparaffin were also used to evaluate the maximum biosurfactant production and production cost (Abouseoud*et al*., 2008a)

Time course for biosurfactant production: Time required for the biosurfactant production was determined by inoculating the mineral salt medium with standard inoculum and were incubated for desired time at 100rpm. During the incubation period sample from the flask was collected at various time interval and surface tension and emulsification assay of cell free broth was determined (Abouseoud*et al*., 2008a)

Biosurfactant recovery: The cell free broth obtained by centrifugation was used to recover the biosurfactant. ThepH of the cell free broth was adjusted to 2.0 using 6N HCl and the flask were kept at 4^{0}C overnight. The biosurfactant precipitated in the form of pellets were thus collected by centrifugation at 8000 rpm for 15 min at 20^{0}C and dissolved in distilled water. Then the pH adjusted to 8.0 with 1N NaOH, and the extract was lyophilized(Abouseoud*et al*., 2008a). The extracted and purified biosurfactant was further analyzed by the GC-MS.

Result and Discussions:

Isolation and primary screening: In present investigation 30 petroleum mixed soil samples were collected from different garages and petrol pumps of Amravati city. From these soil samples isolation was done by continuous screening and enrichment of the flask containing mineral salt media, 1gm soil sample and 2% (v/v) oil as source of carbon. On the basis of morphological characteristics about 18 different colonies were

selected and were further studied for their potential to produce biosurfactant. Most of study over biosurfactant production suggests the predominance of gram -ve bacteria Bicca*et al*., (1999), similar results were recorded in present investigation. Out of 18 isolates 83% bacteria were reported gram -ve while 13% were gram +ve. All reported isolates were aerobic gives positive catalase test and varies with different biochemical and fermentation ability. Out of them one isolates which was gram +ve and shows primary biosurfactant production ability was further selected for the biosurfactant production irrespective of other test. The major differentiating biochemical characteristics were given in table 1.

Table 1 : Biochemical Characteristics of biosurfactant producing bacteria

Gram reaction	+	pH 10	-
Shape	LR	Catalase	+
Arrangement	S	Oxidase	+
Motility	+	Methyl red	-
Spore formation	+	Indole	-
4°C	-	Citrate utilization	-
42°C	+	VogesProskauer	-
30°C	+	Nitrate reduction	+
40°C	+	Urea hydrolysis	-
50°C	+	β-haemolysis	+
1% NaCl	+	Glucose	-
2% NaCl	+	Lactose	-
3% NaCl	+	Mannitol	-
4% NaCl	-	Arabinose	-
pH 6	+	Trehalose	-
pH 7	+	Xylose	-
pH 8	+	Rhamnose	-
pH 9	+	Sorbitol	-

Selection of oil source:production economy is the bottleneck of most of biotechnological process. It is estimated that cost of raw materials for biosurfactant production account for 10 to 30% of the total production costs in most biotechnological processes. Use of cheap and agro-based raw materials as substrate for biosurfactant production to reduce the production cost is one of extensively studied alternative (Makkar and Cameotra, 2002).Differentcheap raw materials including plant-derived oils, oil wastes, starchy substances, lactic whey and distillery wastes were previously reported to support biosurfactant production (Mukherjee *et al*., 2006). Present study was also focused on use of cheap and used substrate for the production of biosurfactant including different vegetable and hydrocarbon oil. Primary screened isolate was further tested using different oils as source of carbon and nitrogen in minimal media.

Result of the study reveals that the isolate shows the highest surface tension reduction up to 30.61±0.3 when coconut oil was used as substrate, it also shows moderate oil displacement activity while strong emulsification activity 25±02 after 24hr incubation. Hassan *et al.,* (2014) also reported the biosurfactant production and strong oil spreading and emulsification activity using coconut oil as substrate. Vijaya*et al.,* (2013) also studied the biosurfactant production from *Bacillus* species, there results showed that the highest reduction in surface tension was recorded as 47.61mN/m.

Table 2: Biosurfactant production using different cheap raw substrate

Different oils	Surface Tension (mN/m)	% reduction in surface tension	Oil Displacement Test (mm)	Drop Collapse Test	Emulsification Index (E24)	% emulsification
Mustard oil	52.27 ±0.5	73.2	+++	++	18.33 ± 0.1	30.6
Soybean oil	44.23 ± 0.4	61.9	+++	+	9.84 ± 0.1	16.1

Linseed oil	56.84 ± 0.6	79.6	+	-	33.33 ± 0.2	55.6
Coconut oil	30.61 ± 0.3	42.9	+++	++	25 ± 02	41.7
Castor oil	61.62 ± 0.4	86.3	+	-	21.67 ± 0.1	36.1
Diesel	61.21 ± 0.4	85.7	+	-	3.23 ± 0.2	5.2
Engine oil	36.13 ± 0.5	50.6	+	+	16.5 ± 0.3	25.4
Brake oil	54.28 ± 0.3	76.0	++	+	10 ± 0.3	16.7
Kerosene	41.93 ± 0.5	58.7	+++	++	4.92 ± 0.1	8.1
Paraffin	54.58 ± 0.3	76.4	++	+	0 ± 0.3	0.0
± = means SD of values of three independent experiments with three replicates each +++ = strong activity; ++ = moderate activity; + = low activity; - = no activity						

Jaysree*et al.,*(2011) also studied the biosurfactant production from *Bacillus* they reported the emulsification index of 15 % and 20%, while our study reports emulsion activity about 16% after 24hr when engine oil was used as substrate.Our isolate also showed the reduction in surface tension up to36.13±0.5 when waste engine oil was used as substrate and also showed the drop collapse test and emulsification activity. Mellor *et al.,* (2011) also reported ability of microbial biosurfactant using canola oil, olive oil, sunflower oil, vegetable oil and paraffin oil. Our isolate also showed the biosurfactant activity when vegetable oil and different hydrocarbon oils were used as substrate.

Optimization of biosurfactant activity:for optimization study environmental factors like pH, temperature and salt concentrationwere taken under consideration the reduction in surface tension and emulsification activity was recorded after each 24 hr. results of the study reveals that the isolate showed a highest biosurfactant activity including surface tension reduction and emulsification formation when used engine oil was added as substrate at normal temp i.e. 37^{0}C and at pH 7 with 1% salt concentration. With increase or decrease in the temperature and pH it affects the biosurfactant activity of the isolate (Fig. 5,6,7). Different reports suggest that the optimum condition for biosurfactant production varies with organism to organism Abouseoud*et al.,* (2008) studied the activity of *Pseudomonas* strain, which showed that maximum value of reduction in surface tension at temperature 37^{0}C, pH 12 and salt 20% while Khopade*et al.,* (2012) reported the stable biosurfactant production from bacterialstrain, at 100^{0}C temperature and remains stable at alkaline pH and at 3% salt concentration.

Fig. 5: Effect of pH on biosurfactant activity of *An. migulanus* (G1)

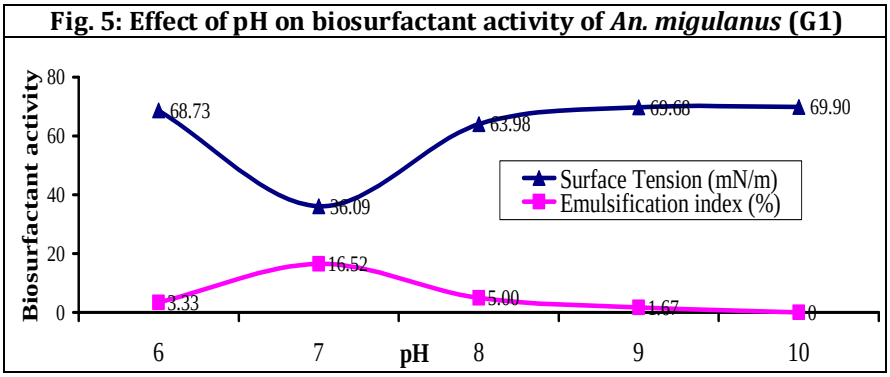


Fig. 6: Effect of temperature on biosurfactant activity of *An. migulanus* (G1)

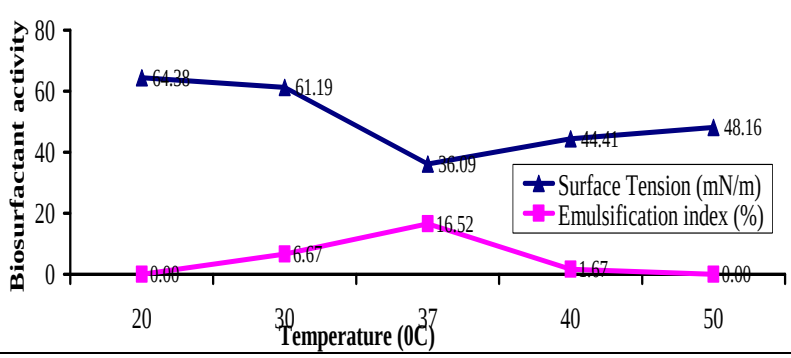
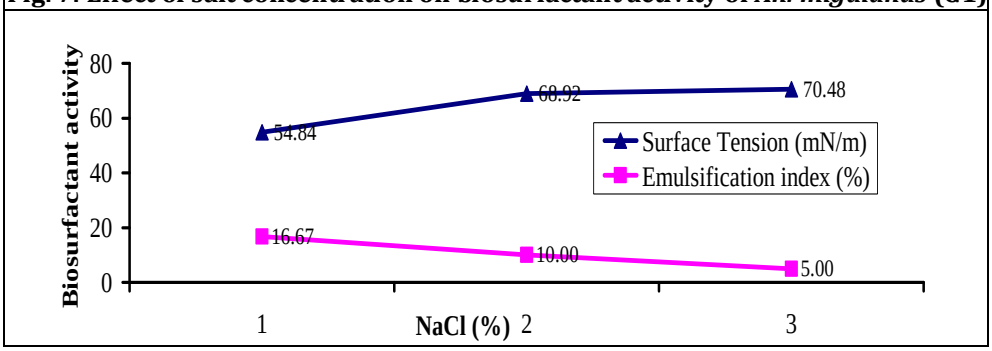
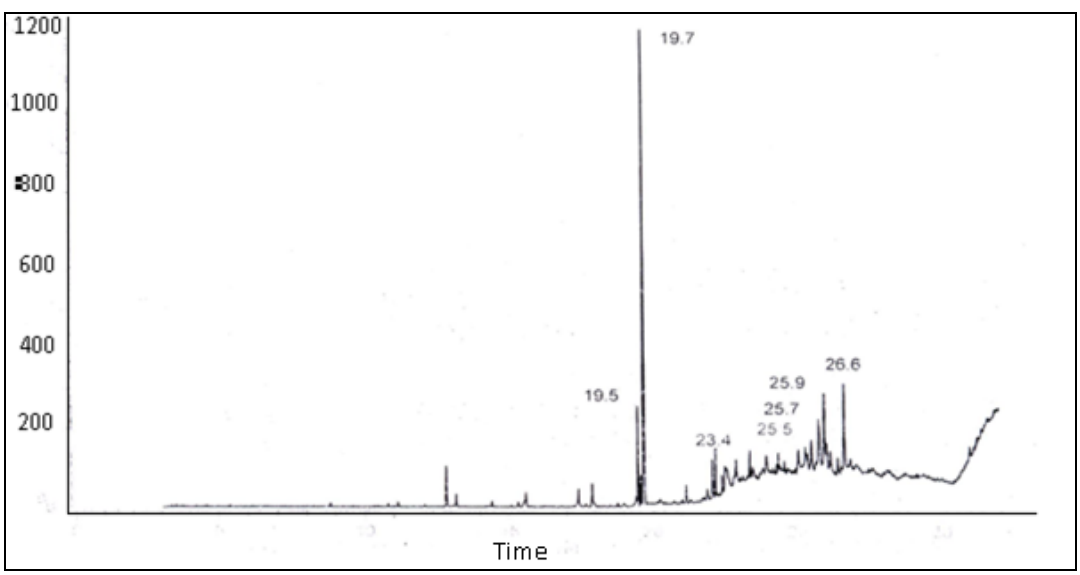


Fig. 7: Effect of salt concentration on biosurfactant activity of *An. migulanus* (G1)



Biosurfactant stability study and GC-MS analysis: stability study of the extracted biosurfactant was done with different pH, salt and temperature range. Results of the study reveal that no change in activity of biosurfactant was reported. The purified crude biosurfactant was further analyzed with gas chromatography-mass spectroscopy technique toget the clear information regarding substances present in the biosurfactant and their structure.



Result of the Gas chromatography and mass chromatography revels that the highest peak was recordedat retention time 19.7 and the product was Dibutylpthalate. Kiran*et al.,* (2010) while studied biosurfactant production from bacteria *B. aureum* MAS13 isolated from marine environment also reported similar report of mass spectra. The Dibutyl phthalate and hexadecane reported in mass spectra were having minor quantity in the produced biosurfactantwhile the different peak were recorded at different time interval from 23.4 to 26.6 depending upon the molecular weight of the molecules present in the produced biosurfactant. Table 3 showing the different molecules detected with their molecular weight and formula.

Table 3: Predicted compounds and major fractions of GC-MS data of *A.migulanus* (G1)

No.	Rt	Compound	Formula	Mol. Wt. (g/mol)	Rel. Per. (%)
1	19.7	Dibutylpthalate	$C_{16}H_{22}O_4$	278.34	3.74
2	22.0	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294.47	9.30
3	22.1	9-Octadecenoic acid (z)-methyl ester	$C_{19}H_{36}O_2$	296.48	43.25
4	8.8	Hexadecane	$C_{16}H_{34}$	226.44	2.84
5	13.2	Hexadecane	$C_{16}H_{34}$	226.44	5.71
6	19.6	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	10.56
Rt=Retention time, Mol. Wt.=Molecuolar weight, Rel. per.=Relative percent					

Phylogenetic analysis: The obtained 16S rRNA sequences of bacterial culture from NCCS, Pune was analyzed phylogenetically using MEGA 4.0 software package. The phylogenic position of bacteria indicated that the bacterial strain was related to phylum Firmicutes and is belongs to genera *Aneurinibacillus.*The phylogenetic tree was constructed using phylogenetic neighbors identified by Ribosomal Database Project (RDP-II) by neighbor-joining (NJ) algorithm (Saitou and Nei, 1987, Tamura *et al.*, 2007). A distance matrix was determined using Kimura's, (1980) model for 16S rRNA gene and bootstrap analysis was used to evaluate phylogenetic tree stability among the clades according to a consensus tree based on 1,000 replicates for each. According to the 16S rRNA gene sequences, the strain G1 showed a high level of similarity with the type strain of genus*Aneurinibacillus* and a substantial degree of relatedness to references 16S rRNA sequences of genus*Aneurinibacillus* in the database.

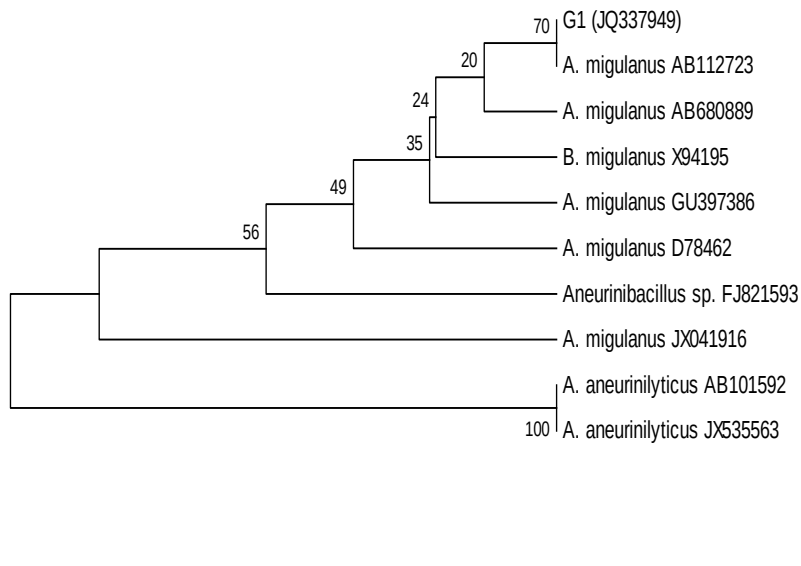


Fig. 8: Phylogenetic tree based on 16S rRNA gene comparisons of the biosurfactant producer, tree was constructed by neighbor-joining method.

The strain G1 from present study showed high value of similarity (70%) with isolate *An. migulanus* (AB112723) which was previously reported as *Brevibacillusbrevis* and reclassified as *An. migulanus* by Goto*et al*., (2004).

Conclusion: On the basis of the above production and characterization study showed that use of waste fried oil and used engine oil can also be act as alternative source of cheap substrate for biosurfactant production. Also study reveals that possible use of organism *Aneurinibacillusmigulanus* in the remediation study as it is able to utilize and produce biosurfactant from waste oils including vegetable and hydrocarbon oil. Though the produced biosurfactant showed the activity at normal growth condition of organisms possibly can be used in pharmaceutical, cosmetic industry and further for bioremediation of polluted sites from such kind of oils and need more exploration further.

Acknowledgement: Author thankful to the Department of Microbiology, SGBAU, Amravati for providing infrastructure and all help needed during work for smooth conduction of work and also thankful to Late. MN Kale fellowship for financial assistance.

References:

1. Abouseoud M, Maachi R, Amrane A, Boudergua S, Nabi A. (2008a). Evaluation of different carbon and nitrogen sources in production of biosurfactant by Pseudomonas fluorescens. Desalination. 223: 143–151.
2. Banat IM, Makkar RS, Cameotra SS. (2000). Potential commercial applications of microbial surfactants. ApplMicrobiolBiotechnol. 53:495-508.
3. Bento FM, Camargo FA, Okeke BC, Frankenberger WT. (2005). Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour Technol. 96 :1049–1055.
4. Bicca CF, Fleck LC, Ayub MAZ. (1999). Production of biosurfactant by hydrocarbon degrading Rhodococcus rubber and Rhodococcuserythropolis. Braz J Microbiol. 30:231-236.

5. Bordoloi NK, Konwar BK. (2008). Microbial surfactant-enhanced mineral oil recovery under laboratory conditions. Colloids Surf B: Biointerfaces. 63 (1):73–82.
6. Elzzazy AM, Abdelmoneim TS, Almaghrabi OA. (2015). Isolation and characterization of biosurfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia. Saudi J Biol Sci. 22 : 466-475.
7. Goto K, Fujita R, Kato Y, Asahara M,Yokota A. (2004). Reclassification of Brevibacillusbrevisstrains NCIMB 13288 and DSM 6472 (NRRL NRS-887) as Aneurinibacillusdanicussp. nov. and Brevibacilluslimnophilussp. nov. Int J SystEvolMicrobiol. 54(2):419-427.
8. Hassan M, Essam T, Yassin AS, Salama A. (2014). Screening of Bio- Surfactant Production Ability among Organic Pollutants Degrading Isolates Collected From Egyptian Environment. J MicrobBiochem Technol. 6:195-201. doi:10.4172/1948-5948.1000143
9. Henkel M, Mu¨ller MM, Ku¨gler JH, Lovaglio RB, Contiero J, Syldatk C.(2012). Rhamnolipids as biosurfactants from renewable resources: concepts for next-generation rhamnolipid production. Process Biochem.47(8) :1207–1226.
10. Jaysree RC, Basu S, Singh PP, Ghosal T, Patra PA, Keerthi Y, Rajendran N. (2011). Isolation of biosurfactant producing bacteria from environmental samples. Pharmacologyonline. 3:1427-1433.
11. Khopade A, Ren B, Liu XY, Mahadik K, Zhang L, Kokare C. (2012). Production and characterization of biosurfactant from marine Streptomyces species B3. J Colloid and Inter Sci. 367: 311-318.
12. Kimura M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 16 (2):111-120.
13. Kiran GS, Thomas TA, Selvin J, Sabarathnam B, Lipton AP. (2010). Optimization and characterization of a new lipopeptide biosurfactant produced by marine Brevibacteriumaureum MSA13 in solid state culture. Biores Technol. 101:2389-2396.
14. Makkar R, Cameotra S. (2002). An update on the use of unconventional substrates for biosurfactant production and their new applications. ApplMicrobiolBiotechnol. 58 (4):428-434.
15. Makkar SR, Swaranjit SC, Ibrahim MB. (2011). Advances in utilization of renewable substrates for biosurfactant production. AMB Express. 1(1):5.
16. Mellor GE, Bentley JA, Dykes GA. (2011). Evidence for a role of biosurfactants produced by Pseudomonas fluorescens in the spoilage of fresh aerobically stored chicken meat. Food Microbio. 28 (5):1101-1104.
17. Mukherjee S, Das P, Sen R. (2006). Towards commercial production of microbial surfactants. Trends Biotechnol. 24: 509-515.
18. Nitschke M, Costa SG. (2007). Biosurfactants in food industry. Trends Food Sci Technol. 18: 252–259.
19. Okoh AI, Trejo-Hernandez MR. (2006). Remediation of petroleum hydrocarbon polluted systems: exploiting the bioremediation strategies. Afr J Biotechnol. 5: 2520–2525. 10.4314/ajb.v5i25.56067
20. Patowary K, Patowary R, Kalita MC, Deka S. (2017). Characterization of Biosurfactant Produced during Degradation of Hydrocarbons Using Crude Oil As Sole Source of Carbon. Front Microbiol.8:279. doi: 10.3389/fmicb.2017.00279
21. Perfumo A, Banat IM, Canganella F, Marchant R. (2006).Rhamnolipid production by a novel thermophilic hydrocarbon-degrading Pseudomonas aeruginosa AP02-1.ApplMicrobiolBiotechnol. 72:132-138.
22. Reddy CM, Arey JS, Seewald JS, Sylva SP, Lemkau KL, Nelson RK, et al. (2011). Composition and fate of gas and oil released to the water column during the Deepwater Horizon oil spill. Proc Nat AcadSci U.S.A. 109: 20229–20234.
23. Saitou N, Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Bio Evol.4 (4):406-425.
24. Tamura K, Dudley J, Nei M, Kumar SM. (2007). Molecular evolutionary genetics analysis (MEGA) software version 4.0. MolBiolEvol. 24 (8): 1596–1605.
25. Vedaraman N, Venkates N. (2011). Production of surfactin by Bacillus subtilisMTCC 2423 from waste frying oils. Braz J Chem Eng. 28(2):175-180.
26. Vijaya B, Jayalakshmi NR, Manjunath K. (2013). Enumeration of biosurfactant producing microorganisms from oil contaminated soil in and around Bangalore (India). Int J Curr Sci. 5:86-94.
27. Wang M, Wang C, Hu X, Zhang H, He S, Lv S. (2015). Distributions and sources of petroleum, aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) in surface sediments from Bohai Bay and its adjacent river. China Mar pollut Bull. 90: 88–94. doi: 10.1016/j.marpolbul.2014.11.017
28. Youssef NH, Duncana KE, Naglea DP, Savagea KN, Knappb RM, McInerney MJ. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. J Microbiol Methods. 56: 339–347.