

Original Research Article

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Diversity and Distribution of Biosurfactant Producing Plant Growth Promoting Bacteria (PGPB) from Hydrocarbon Contaminated Soils of Upper Assam

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ABSTRACT

Bioremediation is a highly potential approach to restore and rehabilitate oil polluted soils having many recalcitrant polyaromatic hydrocarbons (PAHs) such as dibenzothiophene (DBT), benzothiophene (BT), etc by use of microorganisms. In the present study biosurfactant producing bacterial diversity and distribution for bioremediation of oil contaminated soils of upper Assam for the growth of Muga host plant Som (*Persea bombycina*) were studied. Crude oil contaminated soil samples were collected from Lakwa, Gelakey, and Nazira regions of upper Assam and heterotrophic bacteria were isolated. Various screening methods for biosurfactant production as well as plant growth promoting properties were characterized for the bacterial isolates. Results of the drop collapse assay indicated the presence of biosurfactant producing bacteria. Emulsification index (EI) of 40 bacterial isolates revealed that L3, L5, L6, N2, N9, N13, N14, G1, G3, G7, G8, G9, G10, G11 isolates had higher biosurfactant production. EI values of these isolates were significantly high (> 35%) indicating potentiality higher biosurfactant production. Blue agar CTAB method was done to characterize the presence of anionic biosurfactants. Screening of PGPR activity was checked by starch hydrolysis, phosphate solubilization and extracellular enzyme screening such as catalase, lipase and cellulose. Among all the isolates, N6, N14, G1, G3, G7, G8, G9 showed good plant growth promoting characters along with biosurfactant producing ability. Heterotrophic biosurfactant producing bacterial diversity has been deciphered by 16S rRNA gene sequencing. Results indicated that abundance of *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Enterococcus* sp., *Klebsiella* sp. and *Serratia* sp. in hydrocarbon contaminated soils.

Keywords

Bioremediation, Crude oil polluted soils, Bacterial diversity, Biosurfactant producing bacteria, Plant growth promoting bacteria

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Introduction

With increase in industrialization, not only air, but soil is also getting contaminated by

various toxic pollutants such as sulfur polycyclic aromatic hydrocarbons (PASHs) (released by oil and energy industries which are recalcitrant in nature (Kuppusamy *et al.*,

2017). This ultimately have a negative impact on soil beneficial microbiota and subsequently reduced agricultural yield including production of sericulture (Ghosal *et al.*, 2016, Prasad *et al.*, 2011; Subrahmanyam *et al.*, 2016; Thavamani *et al.*, 2012). Increased production of industrial waste, traffic pollution, and household garbage has led to migration of many sulfur polycyclic aromatic hydrocarbons (PASHs) and other pollutants into soils through dry and wet atmospheric deposition. PAHs tend to accumulate in the soils as they are sparingly soluble, readily absorbable by soil particles, and difficult to be degraded Tang *et al.*, 2005; Wang *et al.*, 2007). The most important sink for PAHs (polycyclic aromatic hydrocarbons) in the environment are soils.

By physical, chemical, and other biological methods soil which gets accidentally contaminated with petroleum hydrocarbons can be remediated (Bharali *et al.*, 2011). To our knowledge most of the hydrocarbons are biodegradable; nevertheless their rates of biodegradation in the environment are limited due to their hydrophobicity or less accessibility to microbes and low aqueous solubility (Santos *et al.*, 2016). Use of biosurfactant is one of the approaches to enhance biodegradation of crude oil contamination (Cubitto *et al.*, 2004) which could increase the solubility of hydrophobic substrates like oils in aqueous medium and enhances the bioavailability of the hydrophobic substrates leading to higher oil degradation.

Biosurfactants are biological surfactants that are amphiphilic containing both hydrophilic and hydrophobic moieties in nature and are produced by microorganisms. By accumulating at the interface between two immiscible fluids such as oil and water biosurfactants reduce surface and interfacial tensions (Nitsehke and Costa, 2007) and

potentially increase their bioavailability for use as carbon and energy sources (Mulligan, 2009). The microorganisms survive in the aqueous phase by producing biosurfactant which effectively adsorb, emulsify and disperse or solubilize the oil or hydrophobic substrate (Satpute *et al.*, 2010). The partially purified biosurfactant is known to exhibit antimicrobial activity by inhibiting the growth of several bacterial and fungal species. The biosurfactants with antimicrobial properties has an enormous potential to combat diseases caused by antibiotics-resistant microbes (Ilori *et al.*, 2008).

The aim of improving nutrients availability for plants by using microorganisms is an important practice and necessary for agriculture (Freitas *et al.*, 2007). Plant growth promoting bacteria (PGPB) are recognized both as efficient soil microbes and bio-fertilizers to enhance the growth of several crops and control soil borne pathogens. They can affect plant growth by different direct and indirect mechanisms (Heydarian *et al.*, 2016). Various mechanisms that are active due to use of PGPRS are (1) increase in nitrogen fixation and mineral nutrient solubilization (2) repression of pathogens which are soilborne by production of hydrogen cyanide, siderophores, antibiotics, and/or competition for nutrients; (3) improving plant stress tolerance to drought, salinity, and metal toxicity; and (4) production of phytohormones such as indole-3-acetic acid (IAA) and ACC deaminase (Subrahmanyam *et al.*, 2018). Reports are present that suggest significant increase in growth and yield of agronomically important crops in response to inoculation with PGPB. PGPB strains producing rhamnolipids could be a better measure for reclamation of petrol contaminated sites (Youssef *et al.*, 2004).). It is anticipated that indigenous bacteria existing in hydrocarbon contaminated soils areas usually acquire natural resistance against toxic pollutants

present in their environment. The resistant PGPB ability to promote plant growth in contaminated environment makes them the preferred choice for the bioremediation and sustainable agriculture technologies (Subrahmanyam *et al.*, 2018). Therefore, the present study was aimed to isolate and characterize biosurfactant producing PGPB for enhanced production of Muga silkworm (*Antheraea assamensis* Helfer) host plants (*Persea bombycina*) in oil polluted soils of Upper Assam. Mitigating the phytotoxic effects of crude oil in Muga silkworm host plant fields would be beneficial for sustainable sericulture and restoration of soil health.

Materials and Methods

Soil sampling and Isolation of heterotrophic bacterial isolates

Hydrocarbon contaminated soil of Muga host plants were collected in triplicates from three regions of Sivsagar district; Lakwa (27.0136° N, 94.8572° E), Nazira (26.9007° N, 94.7226° E), Gelakey (26.7959° N, 94.6915° E) from where isolation of heterotrophic bacterial isolates were done in Luria-Bertani medium 2.5 g l⁻¹ supplemented with bacteriological agar 2 g l⁻¹. Pure bacterial isolates were obtained from mixed bacterial cultures by streak plate method after an incubation period of 2 days at 30± 5°C.

Screening for biosurfactant producing bacteria

Drop collapse test

Drop collapse assay has been done in order to check the capability of the isolates to produce biosurfactant. A film was xylene was taken on a petriplate where pure broth cultures of bacterial isolates were dropped with the help of a dropper, and water was taken as control (Irorere, 2017).

Emulsification test

Quantitative estimation for biosurfactant production was done by this method. Several colonies of a pure culture were suspended in test tubes containing 2ml of LB broth after 48 h incubation, 2ml xylene was added to each tube and mixture vortexed at high speed for 1min, they were leaved to stand for 24 h. The emulsification index is given as percentage of height of emulsified layer (cm) divided by total height of the liquid column (cm) following Saruboo's method (Saravanan and Vijaykumar 2012).

Blue agar CTAB plate assay

Blue agar CTAB plate assay was done to check the anionic property of the biosurfactant produced by the bacterial isolates. This was done by making wells of about 4 mm by cork borer at equal distances where bacterial broth was incubated.

The medium used for detection anionic biosurfactants was Bushnell-Hass agar 23.27 l⁻¹ supplemented with 2% glucose, cetyltrimethyl ammonium bromide (CTAB) 0.5 mgml⁻¹, methylene blue 0.2 mgml⁻¹. Appearance of a dark blue halo around the spot indicates the anionic property of the biosurfactant (Satpute *et al.*, 2008).

Screening of PGPR activity of different isolates

Phosphate solubilization

A loop full of fresh bacterial cultures will be streaked on the centre of agar plates modified with Pikovskaya agar with insoluble tricalcium phosphate (TCP) and incubated for 120 h at 30±2°C (Bhattacharyya and Jha, 2012). The presence of halo zone around the bacterial colonies indicates positive for phosphate solubilization ability.

Catalase activity

Bacterial cultures were grown in nutrient agar medium for 48 h at 32°C. 48 hr old bacterial colonies were added with 2-3 drops of hydrogen peroxide (3%) on a clean glass slide and mixed using a sterile tooth pick. The evolution of oxygen as effervescence is an indicative of catalase activity (Joseph *et al.*, 2012).

Lipase activity

Bacterial isolates were inoculated in nutrient agar medium amended with egg yolk. After 48 hours of incubation plates were flooded with saturated solution of CuSO₄ and kept for 15-20 minutes. Greenish blue color zones around the colonies are an indicative of lipase activity (Kumar *et al.*, 2012; Geetha *et al.*, 2014).

Starch hydrolysis test

Bacterial isolates were inoculated on starch agar plates for a period of 2 days incubation at 32°C. After 2 days plates were flooded with iodine solution for a minute. Clear zone indicates production of extracellular amylase or starch hydrolysis (Geetha *et al.*, 2014).

Biochemical tests

Biochemical testing of the isolates were done on the basis of the protocol mentioned in KB002 HiAssorted™ Biochemical Test Kit (for gram-negative), and KB013 HiBacillus™ Identification Kit (for gram-positive) made of HiMedia Laboratories Pvt. Ltd, Mumbai-400086, India. (Email: techhelp@himedialabs.com.)

Molecular identification of biosurfactant producing bacterial isolates

Genomic DNA was extracted from 5 ml of each of the grown screened bacterial isolates

have following Kate Wilson genomic DNA extraction protocol (Wilson, 2001). The size of the extracted DNA was checked on a 0.8 % agarose gel and the purity of DNA was evaluated by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA) Subsamples of extracted DNA were kept at -20 °C for further quantitative and qualitative analysis (Subrahmanyam *et al.*, 2014). Bacterial 16S rRNA genes suitable for diversity profiling were amplified using universal eubacterial primer sets 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Eurofins Genomics, Bangalore, India). The PCR was performed in 50 µl volumes containing 25 µl of DreamTaq Green PCR Master Mix (2X) (ThermoFisher Scientific, Mumbai, India), 1 µl (0.1-1.0 µ mol) of each primer and 1 µl (10pg) of template DNA. Briefly, a touchdown thermal cycle strategy consisting of 30 cycles (30 s at 94 °C, annealing for 1 min at temperature at 55 °C and extension at 72 °C for 1 min then a final extension for 7 min at 72 °C were performed in Applied Biosystems Veriti™ 96-Well Fast Thermal Cycler (ThermoFisher Scientific, Mumbai, India). The PCR products were resolved in 1% agarose gels, stained with ethidium bromide (5 ng ml⁻¹) and analyzed using a gel documentation system (Gel Doc™ XR+, BioRad, USA).

16S rRNA sequence analysis

Purified PCR amplicons of the biosurfactant producing bacterial isolates were sent for 16S rRNA gene sequencing to Eurofins Genomics, Bangalore, India. The obtained sequences were subjected to BLAST analysis in the NCBI GenBank (www.ncbi.nlm.nih.gov) database, and sequence chromatograms were manually edited with DNASTar and DNAMAN version 6.0. Phylogenetic analysis was conducted using MEGA X. (Kumar *et al.*, 2018)

NCBI accession numbers

16S rRNA gene sequences obtained in this study were submitted to NCBI GenBank (www.ncbi.nlm.nih.gov) under the accession numbers MH909822 to MH909836.

Results and Discussion

A total of 40 pure heterotrophic bacterial colonies were obtained from hydrocarbon contaminated soils of Lakwa, Gelakey, Nazira regions (Sivasagar district) of upper Assam. 10 heterotrophic bacterial isolates were isolated from Lakwa region which were named as L1 to L10; 15 from Nazira, N1 to N15; and 15 from Gelakey, G1 to G15. All the bacterial isolates were screened for biosurfactant producing capacity and PGPR activity.

Screening of biosurfactant production was done by three methods; drop collapse assay, emulsification index and blue CTAB agar method. Drop collapse assay indicated the presence of biosurfactants. Drops of that bacterial culture broth capable of producing biosurfactants on xylene film appeared flat, and spread out with uneven boundaries whereas water drop taken as control appeared more round and intact. Almost all the isolates showed some amount of biosurfactant produced. Therefore a more reliable test i.e. emulsification index was done for quantitative analysis of the biosurfactant produced by the bacterial isolates.

Results of emulsification index method showed that 15 bacterial isolates were capable of producing biosurfactants in good quantity. Isolate number L3, L5, L6, N2, N6, N9, N13, N14, G1, G3, G7, G8, G9, G10, G11 showed quantifiable amount (>35%) of biosurfactants produced (Fig. 1A, 1B, 1C). Results also showed that isolates obtained from Gelakey region showed a very high amount of

biosurfactant produced where the value of emulsification index EI was > 50% (Fig. 1C). EI value of G1 58.48 %, G3 55.45 %, G7 54.54%, G8 61.51%, G9 62.42% and these values are comparable to EI values standard bacterial strains reported earlier, for eg. *Bacillus subtilis* MTCC 2422 showed an EI value of 75%; *Bacillus subtilis* MTCC 2423 showed EI value of 78% (Satpute *et al.*, 2008).

Blue CTAB agar method was done to check the chemical nature of the biosurfactant i.e. whether the biosurfactant produced was anionic or cationic in nature. A dark blue color halo around the bacterial colony indicated anionic nature of biosurfactant produced. Out of the 15 biosurfactant producing isolates only two of them, N2 and N6 produced biosurfactant that were anionic in nature. From the above results it can be inferred that isolate G1, G3, G7, G8, G9 as potential biosurfactant producing bacterial isolates. Since, biosurfactants are biological agents that reduce the interfacial tension between two immiscible liquid; the property of releasing biosurfactants by these bacterial isolates will make enzymes, hormones or chemicals released by these bacteria to be at an accessible state or reach for the Muga host plants Som. Hence, after screening of biosurfactants produced, these bacterial isolates were again screened for PGPR activity and biochemical test.

Different PGPB screening that were done were starch hydrolysis test, catalase test, phosphate solubilising test, and lipase activity (Table 1). Positive starch hydrolysis test showed a clear zone around the bacterial colony as indication of enzyme amylase being produced which is responsible for breakdown of amylase. Isolates N6, N14, G1, G7, G8, G8 and G9 showed positive starch hydrolysis test. Interestingly all the 15 bacterial isolates tested for phosphate solubilisation showed negative results.

Table.1 Characterization of plant growth promoting activities of biosurfactant producing bacterial isolates obtained from crude oil contaminated soils

Isolates	Starch hydrolysis	Catalase activity	Lipase activity	Phosphate solubilizing ability
L3	-	++	-	-
L5	-	+++	-	-
L6	-	+	-	-
N2	-	+	+	-
N6	+++	++	-	-
N9	-	+	-	-
N13	-	+	-	-
N14	++	+	-	-
G1	+	++	+	-
G3	-	+	-	-
G7	+++	+	-	-
G8	+	++	+	-
G9	+	++	+	-
G10	-	+	+	-
G11	-	+	-	-

Table.2 Biochemical tests of the biosurfactant producing bacterial isolates (Gram negative) isolated from Lakwa, Nazira and Gelakey region of upper Assam using in KB002 HiAssorted™ Biochemical Test Kit (HiMedia Laboratories Pvt. Ltd, Mumbai-400086, India)

S.no	Tests	G1	G8	G9	N2	N13
1	Citrate utilization	+	+	+	-	-
2	Lysine utilization	+	+	+	-	-
3	Ornithine utilization	+	+	+	-	-
4	Urease	+	+	+	-	-
5	Phenylalanine Deamination	+	+	+	-	-
6	Nitrate Reduction	-	-	-	-	-
7	H2S production	-	-	-	-	-
8	Glucose	-	-	-	+	+
9	Adonitol	-	-	-	+	+
10	Lactose	-	-	-	+	+
11	Arabinose	+	+	+	+	+
12	Sorbitol	-	-	-	+	+

Table.3 Biochemical tests of the biosurfactant producing bacterial isolates (gram positive) isolated from Lakwa, Nazira and Gelakey region of upper Assam using KB013 HiBacillus™ Identification Kit (HiMedia Laboratories Pvt. Ltd, Mumbai-400086, India)

Sl.no	Tests	N6	N9	L3	L5	L6	G3	G7	G11
1	Malonate	-	-	-	-	-	+	+	-
2	Voges Proskaver	-	-	-	-	-	-	-	-
3	Citrate	-	-	-	-	-	+	-	-
4	ONPG	-	-	-	-	-	-	-	-
5	Nitrate Reduction	+	+	-	-	-	+	+	-
6	Arginine	+	-	-	-	-	-	-	-
7	Sucrose	-	+	+	+	+	+	-	+
8	Mannitol	-	+	-	+	+	-	-	+
9	Glucose	-	+	-	+	+	+	+	+
10	Arabinose	+	+	-	+	+	+	-	+
11	Trehalose	-	+	-	+	+	+	-	+

Fig.1 Emulsification index (EI) of the biosurfactant producing bacterial isolates obtained from (A) Lakwa, (B) Nazira and (C) Gelakey region of upper Assam.

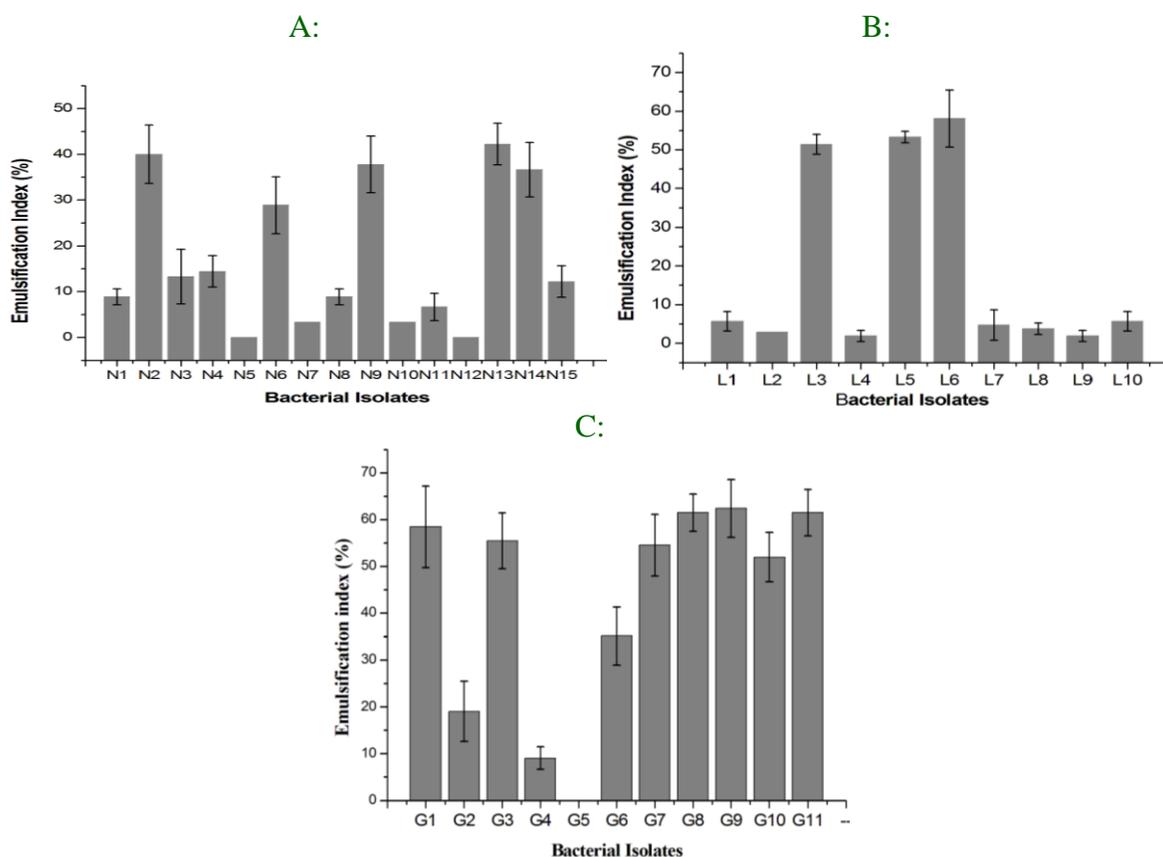
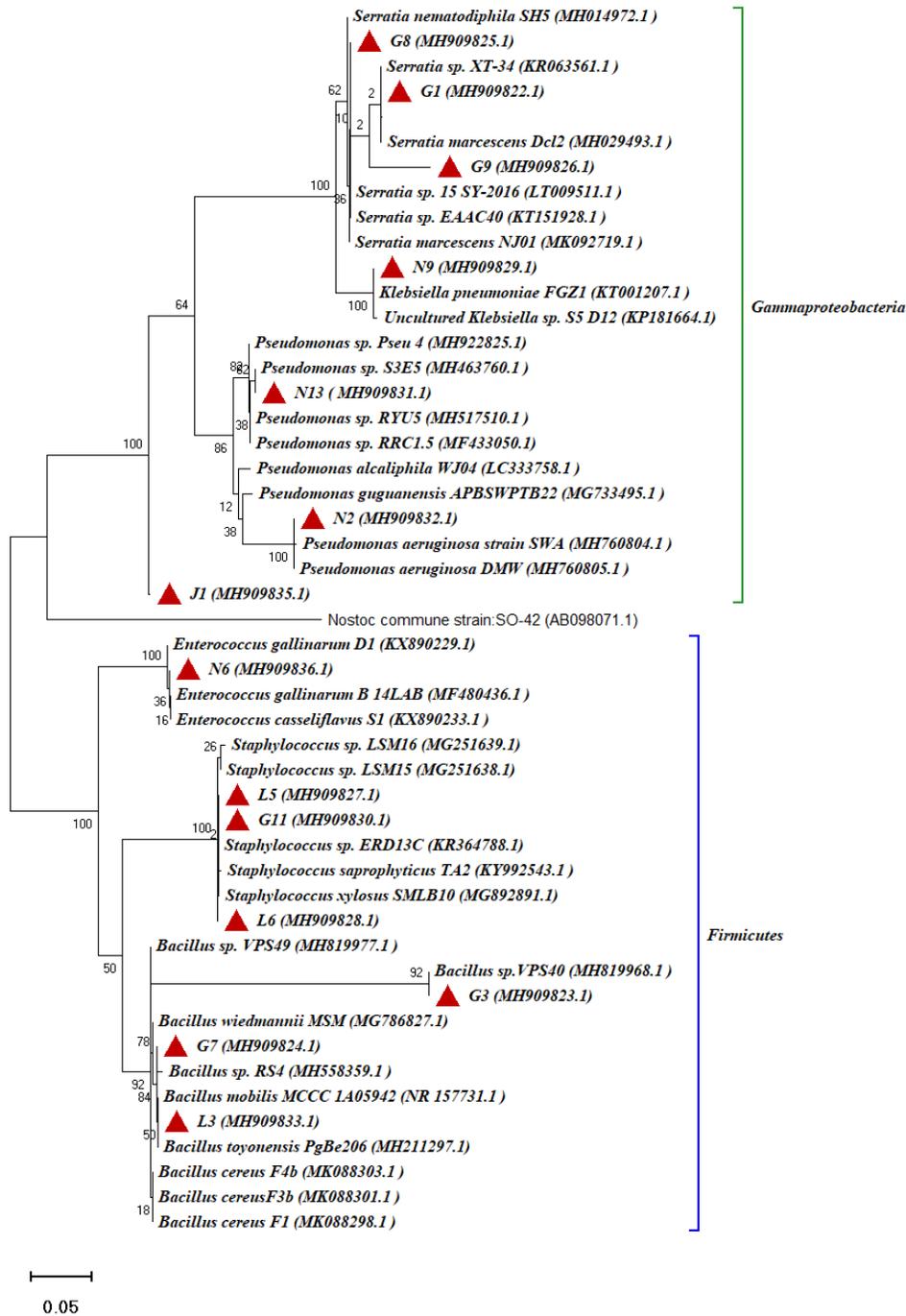


Fig.2 Identification and phylogenetic relation of biosurfactant producing bacterial isolates obtained from crude oil polluted soils. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood (-9170.94) is shown. Bootstrap values of trees in which the associated taxa clustered together is shown next to the branch points. Biosurfactant producing bacterial isolates are shown with triangles on phylogenetic tree. NCBI accession numbers for all the isolates are shown in parenthesis



Positive lipase activity showed dark greenish blue color halo around the bacterial isolate indicating the production of the enzyme lipase which is responsible for breakdown of fats. N2, G1, G8, G9 and G10 showed positive results for lipase activity. Catalase activity was checked; production of effervescence indicated positive catalase activity. All 15 bacterial isolates showed positive catalase activity. These results that isolate G1, G8, G9 showed good PGPR activity (Table 1)

Biochemical characterization of the all 15 biosurfactant bacterial isolates was also done and it was found that isolate G1, G3, G7, G8, G9 found to show positive results in almost all the tests performed by biochemical test kit (Table 2 and 3).

This could be indicative that these bacterial isolates have a potentiality in utilization of wide variety of carbon compounds for their cellular growth. These bacterial isolates shall be used as suitable candidates for remediation of soil pollution as the isolated showed to metabolize diverse carbon sources (Table 2 and 3).

Molecular identification of the biosurfactant producing bacterial isolates were done with 16S rRNA gene amplification and sequencing. Results showed that isolates found were L3 as *Bacillus sp.*, L5 as *Staphylococcus sp.*, L6 as *Staphylococcus sp.*; N2 as *Pseudomonas sp.*; N6 as *Enterococcus sp.*; N9 as *Klebsiella sp.*; N13 as *Pseudomonas sp.*; G1, as *Serratia sp.*; G3 as *Bacillus sp.*; G7 as *Bacillus sp.*; G8 as *Serratia sp.*; G9 as *Serratia sp.*; G11 as *Staphylococcus sp.* (Fig. 2). The bacterial isolates were identified till genus level and it was found that majority of the bacterial isolates were from the genus *Bacillus sp.*; *Pseudomonas sp.*, and *Serratia sp.*, *Klebsiella sp.* under phyla *Firmicutes* and class *Gammaproteobacteria* (Fig. 2).

The 16S rRNA gene sequences of the bacterial isolates were submitted in NCBI and the accession numbers of the bacterial isolates were obtained (MH909822 to MH909836). These bacteria populations may have a great potential for the bioremediation of toxic chemicals. Indigenous microorganisms as potential bioremediators for environments contaminated with heavy metals were reported in earlier study (Subrahmanyam *et al.*, 2016; Keshri *et al.*, 2014). In the present study abundance of bacteria under phyla "*Firmicutes*" and class "*Gammaproteobacteria*" noticed in crude oil polluted soils (Fig. 2). These results are in accordance with previous findings where class *Betaproteobacteria* and *Gammaproteobacteria* were detected in polluted soils (Subrahmanyam *et al.*, 2016; Thavamani *et al.*, 2012). These phyla are frequently observed in soils contaminated with persistent organic pollutants, hydrocarbons and heavy metals in many previous studies (Popp *et al.*, 2006; Thavamani *et al.*, 2012, Vivas *et al.*, 2008). In earlier studies it was found that bacteria from phyla *Firmicutes*, *Actinobacteria* and *Acidobacteria* found to be the abundant lineages pointing their adaptability to pollution stresses. The phylogenetic classification of bacterial isolates obtained from hydrocarbon contaminated soils is consistent with *in situ* analyses of bacterial community structure in other studies (Subrahmanyam *et al.*, 2016; Keshri *et al.*, 2014).

Serratia sp. G1, *Bacillus sp.* G7, *Serratia sp.* G9 and *Enterococcus sp.* N6 showed good biosurfactant producing capability as well as PGPR activity. Hence, these bacterial isolates could be used to improve soil health which in turn might help muga host plants to grow better in soils contaminated with hydrocarbons. Oil industries have been carrying out oil exploration, production and

refining activities for decades in upper Assam (Sivasagar District, Dibrugarh District, Golaghat District and Digboi District etc.). In these areas, soils of Muga rearing fields are polluted during oil drilling and refining operations and subsequently pose toxic effect on metabolism and growth of host plants mainly Som, *Persea bombycina* a primary food plant of Muga silkworm, *Antheraea assamensis* Helfer. In general, bacteria will be able to degrade oil pollutants by excreting extra cellular enzymes and thereby increasing soil quality. Thus the bacterial isolated obtained in this study will be explored for improving host plant "Som" growth, production, and palatability of leaves for productivity of Muga culture especially in Muga rearing fields polluted by oil contaminates in upper Assam region.

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