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# STUDY ON ALIPHATIC HYDROCARBON DEGRADATION BY TWO BACTERIAL STRAINS ISOLATED FROM CRUDE OIL CONTAMINATED SOIL OF UPPER ASSAM

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Abstract

*In Assam, release of petroleum contaminants to the ecosystem started with the beginning of oil exploration activities in various oilfields. Currently many large areas are present in Assam which is contaminated with petroleum and diverse petroleum constituents. Crude oil is a composite mixture of hundreds of hydrocarbons. Out of hundreds of different hydrocarbon molecules, mainly aliphatic hydrocarbons (also known as paraffins) from C1 to C40 straight chain, C6–C8 branched-chain, cyclohexanes, aromatics compounds (Stafford et al., 1982) are present in crude oil. An investigation was conducted to study the efficiency of two bacterial strains Cellulosimicrobium sp. Strain 26ML and Bravibacillus laterosporus strain 3SG in degradation of aliphatic hydrocarbons viz n-heptane, n-dodecane and n-hexadecane (purity 99%). Comparative study on ability of growth of the two strains was evaluated in LMM (Liquid Mineral Media) supplemented with 200ppm (v/v) of the respective hydrocarbons using CFU method,UV spectrophotometer, estimating cell biomass and GC analysis up to a period of 240h.*

Keywords :*Aliphatic hydrocarbon, heptanes, dodecane, hexadecane*

Short Profile

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## INTRODUCTION :

The most important classes of organic pollutants in the environment are crude oil constituents (Wolf gang et al, 2008). The different constituents of crude oil are aliphatic, alicyclic and polyaromatic hydrocarbon as well as oxygen, nitrogen and sulphur-containing substances. Aliphatic and aromatic hydrocarbon can act as source of carbon and energy for the growth of soil microorganisms. Aliphatic hydrocarbons are non- straight-chain and branched alkanes, as well as cycloalkanes . The proportion of aliphatic fractions in a crude oil normally decreases with increasing molecular weight fractions, thus the aliphatic hydrocarbons generally are the lightest fraction of the crude oil. Among the different chain types of aliphatic hydrocarbon, aliphatic hydrocarbons with increasing chain le gth- of C12 and above are almost water insoluble. Short –chain alkanes (less than C9) are toxic to many microorganisms, but they get evaporated rapidly from hydrocarbon polluted site. Mid-length (C14-C20) alkanes are non-polar and virtually water insoluble. Such alkanes have low aqueous solubilities; for example, hexadecane has a water solubility of 0.9 µgl-1 and is in a liquid state at room temperature. Therefore mid length aliphatic contaminants are not readily volatilized or leached from soils. As these alkanes are less soluble in water hence they have a better tendency to bind with soil like aromatic hydrocarbons. Microorganisms such as bacteria, fungi, yeast

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and microalgae can degrade hydrocarbon (Bundy et al., 2004). However, bacteria are mainly responsible in degradation of aliphatic hydrocarbon in contaminated ecosystem. Many heterotrophic bacteria are able to utilize hydrocarbons as a source of carbon and energy, and most ecosystems support indigenous communities of microbes that are capable of significant hydrocarbon degradation (Nichols et al., 1996). The most commonly used heterotrophic soil bacteria are Pseudomonas and Arthrobacter. They are capable of breaking down hydrocarbons through various metabolic pathways (Morelli et al., 2005) and breakdown of the hydrocarbon compounds depends on solubility and concentration of hydrocarbons. At low concentrations of hydrocarbon, all fractions are to be expected of being attacked by bacteria. However, at high concentrations, only those fractions most susceptible to degradation will be broken down. Also the concentration of contaminants will affect the number of organisms present.

Ko and Lebeault (1999) reported that Pseudomonas aeruginosa and Rhodococcus equi were able to degrade alkanes with chain length from C7 to C28.Pseudomonas aeroginosa can degrade alkanes of different chain length (C6-C11 or C12-C16). Many researchers were involved in the studies of biodegradation potential of different bacterial strains in presence of alkanes with different chain lengths. Gill and Ratledge (1972) reported a variety of effects of aliphatic hydrocarbons like n-alkanes, n-alkenes, n-alkan-1-ols and alkyl bromides on yeast. Cobet and Guard(1973) examined the effect of pentane,0.2% decane, hexadecane, Pristane, bicyclohexyl, 2,3,6-trimethylnaphthalene, anthracene soluble fraction of Chevron bunker fuel on 221 bacterial isolates from a fuel impacted beach area. Haines and Alexander (1974) studied on microbial degradation of 14 high molecular weight alkanes such as n-heptane, n-octane, n-dodecane, n-hexadecane,n-octadecane , eicosane, docosane etc. Sakai et al. (1994) reported that very long chain alkanes (up to C44) are degradable by Acinatobacter.

This paper describes the comparative study on the growth rates of two bacterial strains Cellulosimicrobium sp. 26ML and Bravibacillus laterosporus 3SG in LMM containing n-heptane (200ppm v/v), n-dodecane (200ppm v/v)and n-hexadecane (200ppm v/v) as sole source of carbon and energy for bacterial growth.

## Material and methods

The n-heptane, n-dodecan and n-hexadecane (purity99%) used in this study were purchased from Sigma-Aldrich. Bacteriological media and chemical were purchased from Himedia Laboratory Pvt. Ltd. India. All the chemicals and solvents were of analytical grade.

Table1: Morphological, biochemical and molecular characterization of the two isolates

**Morphological characteristics of bacterial colony**

| in | Pigmentation | Form | Consistency | Margin | Elevation | Shape |
|---|---|---|---|---|---|---|
| L | White, waxy | Filiform | Translucent | Irregular | Flat | Rod |
| | Smooth,Dirty white, medium turns green | Filiform | Translucent | Entire | Convex | Rod |

**Biochemical characteristics**

| | $NO_3$ reduction | Indole production | MR | VP | Citrate utilization | Gelatin | Starch hydrolysis | TSI | Catalase |
|---|---|---|---|---|---|---|---|---|---|
| L | + | - | - | - | + | + | - | - | + |
| | + | - | - | - | + | + | - | - | + |

**Molecular identification of the bacterial strains**

| | Identified by 16s sequencing | Accession no |
|---|---|---|
| L | *Cellulosimicrobium sp.* | KF732709 |
| | *Brevibacillus laterosporus* | KF732710 |

Table 2: Growth of Cellulosimicrobium sp. 26ML and Bravibacillus laterosporus 3SG in LMM supplemented with aliphatic hydrocarbon

| Strain | Time of observation | Aliphatic hydrocarbon (200ppm v/v) | | |
|---|---|---|---|---|
| | | n-heptane | n-dodecane | n-hexadecane |
| 26ML | 24 | + | + | + |
| | 48 | + | + | + |
| | 72 | ++ | ++ | ++ |
| 3SG | 24 | + | + | + |
| | 48 | + | + | + |
| | 72 | ++ | ++ | ++ |

N.B: +: Growth
++: Good growth

Table 3: Counts (log10CFU/ml) of 26ML and 3SG in LMM supplemented with different aliphatic hydrocarbons

| Time (h) | Aliphatic hydrocarbon | | | | | |
|---|---|---|---|---|---|---|
| | n-heptane | | n-dodecane | | n-hexadecane | |
| | 26ML | 3SG | 26ML | 3SG | 26ML | 3SG |
| 0 | 7.17±0.2 | 7.17±0.2 | 7.17±0.2 | 7.17±0.2 | 7.17±0.2 | 7.17±0.2 |
| 12 | 7.3±0.6c | 7.3±1.0c | 7.3±0.9c | 7.65±0.2a | 7.39±1.0 | 7.47±0.4b |
| 24 | 7.9±0.5b | 7.77±0.1b | 8.04±0.3a | 8.07±0.8a | 8.14±1.4a | 7.95±0.5b |
| 48 | 8.14±0.2a | 8.07±1.9b | 8.27±0.8a | 8.34±1.0a | 8.4±0.4a | 8.27±0.5a |
| 72 | 8.71±2.4b | 8.66±2.0c | 8.84±0.7a | 8.86±2.5a | 8.9±0.3a | 8.78±0.6b |
| 96 | 8.91±0.8b | 8.85±1.2c | 9.0±2.5a | 9.03±1.9a | 9.08±1.9a | 8.92±0.4b |
| 120 | 9.09±1.2b | 9.05±2.9b | 9.4±0.5a | 9.07±0.5b | 9.6±0.8a | 9.06±0.9b |
| 144 | 15.0±0.3d | 14.9±0.3e* | 15.19±2.8b* | 15.06±2.8c* | 15.34±0.3a* | 15.0±1.2d* |
| 168 | 15.69±0.4a* | 14.6±1.7c | 15.03±0.2b | 14.95±0.3c | 15.23±3.0a | 14.84±1.7c |
| 192 | 8.31±1.2b | 8.2±0.6b | 8.6±2.0a | 8.81±1.4a | 8.79±0.4a | 8.63±0.4a |
| 216 | 8.04±0.9c | 7.9±1.3c | 8.39±0.3a | 8.49±0.5a | 8.48±1.2a | 8.25±0.2b |
| 240 | 8.08±1.0c | 7.6±0.4e | 8.06±2.0c | 8.04±2.0d | 8.16±0.8b | 8.95±0.5a |

Data are mean of three individual values ; ±1.0= SE of observed values; SE followed by similar letters are not significantly different from each other within the column ($P < 0.05$) according to DMRT.

Table 4: Cell biomass production of 26ML and 3SG in LMM supplemented with different aliphatic hydrocarbons

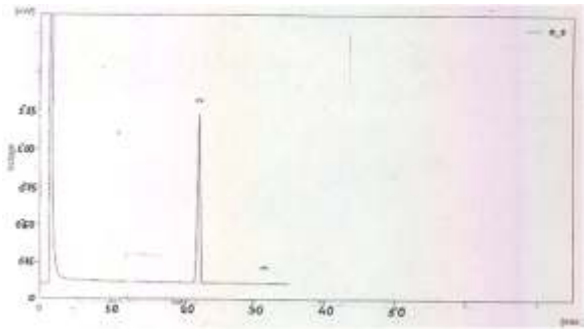
| Time(h) | n-heptane | | n-dodecane | | n-hexadecane | |
|---|---|---|---|---|---|---|
| | 26ML | 3SG | 26ML | 3SG | 26ML | 3SG |
| | Biomass g/L | | | | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 0.2±0.03e | 0.27±0.04e | 0.39±0.02d | 1.96±0.01a** | 0.9±0.04b | 0.66±0.09c |
| 48 | 0.6±0.02c | 0.4±0.02c | 1.07±0.1b | 2.07±0.03b** | 3.9±0.1a** | 1.75±0.2b |
| 72 | 0.9±0.07c | 0.5±0.1d | 1.1±0.02c | 2.4±1.0b** | 4.0±0.09a** | 1.9±0.4c |
| 96 | 1.06±0.02c** | 0.6±0.06d | 1.24±0.08c | 2.76±0.01b** | 4.95±0.2a** | 2.0±0.2b |
| 120 | 1.2±0.8d** | 0. 66±0.04d | 1.5±0.9d | 3.0±0.9b** | 5.2±0.04a** | 1.9±0.09c |
| 144 | 2. 0±0.1d** | 0. 8±0.09d | 2.9±0.02c | 4.69±0.02b** | 6.95±0.02a** | 2.3±0.01c |
| 168 | 1.7±0.6c** | 1.3±0.02d | 1.5±0.03cd | 4.08±0.03b** | 6.5±0.02a** | 1.0±1.0e |
| 192 | 1.3±0.4c** | 1.09±0.09d | 1.5±0.02c | 3.64±0.02b** | 6.14±0.01a** | 0.8±0.01d |
| 216 | 0.8±0.01c | 0.7±0.02c | 0.9±0.08c | 2.0±0.05b** | 4.5±1.3a** | 0.5±0.003d |
| 240 | 0.8±0.03c | 0.7±0.0c4 | 0.6±0.02c | 1.7±0.008b | 2.0±0.3a** | 0.4±0.03d |

Data are mean of three individual values ; ±1.0= SE of observed values; SE followed by similar letters are not significantly different from each other within each row (P < 0.05) according to DMRT. **= statistically significant at p<0.01 by paired comparison between biomass production of strain 26ML and 3SG.

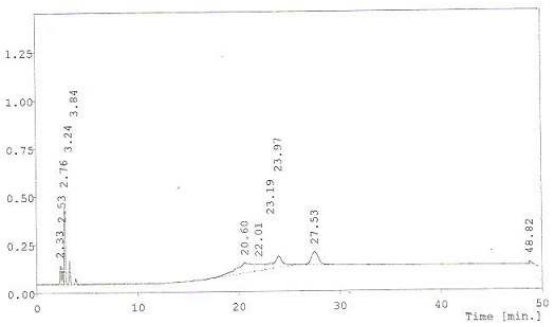
Table5: pH of 26ML and 3SG in LMM supplemented with different aliphatic hydrocarbons

| Time(h) | n-heptane | | n-dodecane | | n-hexadecane | |
|---|---|---|---|---|---|---|
| | 26ML | 3SG | 26ML | 3SG | 26ML | 3SG |
| 0 | 8.0±0.1b | 8.0±1.4b | 8.2±0.04a | 8.2±0.01a | 8.2±0.2a | 8.2±0.2a |
| 24 | 8.0±0.4d | 8.2±0.4b | 8.2±0.05b | 8.1±0.4 | 8.1±0.2c | 8.3±0.2a |
| 48 | 8.05±0.3b | 8.1±0.7a | 8.1±0.3a | 8.0±0.9b | 8.0±0.3b | 8.1±0.4a |
| 72 | 7.9±0.08b | 7.8±0.2c | 8.09±1.5a | 7.9±1b | 7.8±0.4c | 8.0±0.6a |
| 96 | 7.0±0.2e | 7.4±1.2b | 7.3±1.2c | 7.5±1.0a | 7.4±1.4b | 7.2±0.3d |
| 120 | 6.6±1.2c | 6.8±1.7b | 7.0±0.3a | 6.6±0. | 6.8±0.8b | 7.0±0.3a |
| 144 | 6.2±0.4b | 6.0±0.4c | 6.5±0.4a | 6.1±1.4c | 6.0±1.7c | 6.5±1.0a |
| 168 | 6.2±1.2b | 5.9±0.6d | 6.4±1.4a | 6.1±0.2c | 6.0±0.3cd | 6.5±1.2a |
| 192 | 6.1±0.9a | 5.8±2.0b | 6.4±0.09a | 6.0±0.5b | 5.8±1.2b | 6.2±0.4a |
| 216 | 6.0±0.3b | 5.8±0.9c | 6.2±0.02a | 5.9±0.4b | 5.5±0.4d | 6.0±0.3b |
| 240 | 5.7±0.2b | 5.8±0.2b | 6.2±0.03a | 5.6±0.4bc | 5.5±0.3c | 6.0±0.5a |

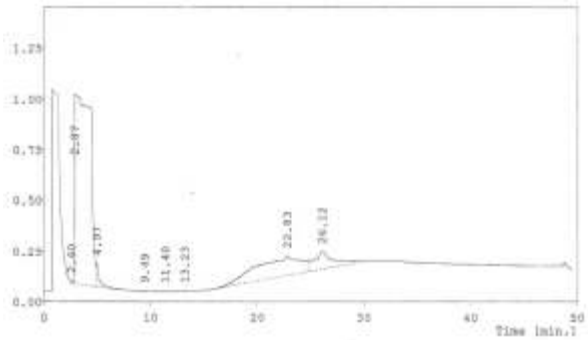
Data are mean of three individual values ; ±1.0= SE of observed values; SE followed by similar letters are not significantly different from each other within each row ($P < 0.05$) according to DMRT



(A)

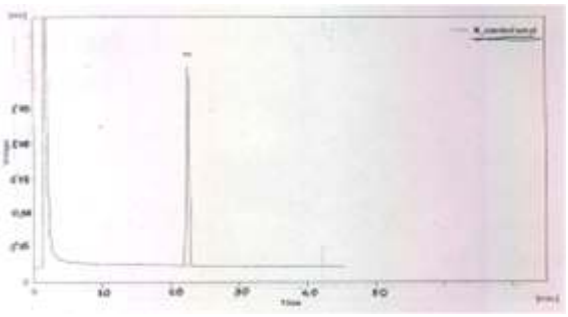



(B)

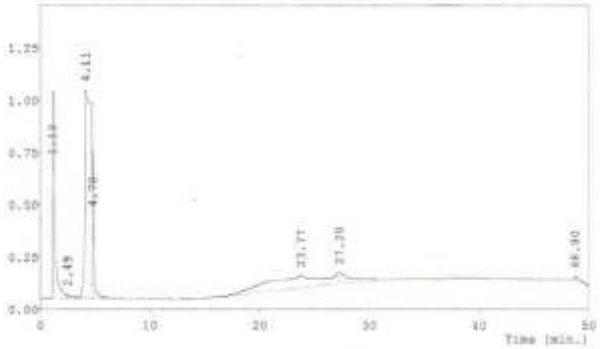



(c)

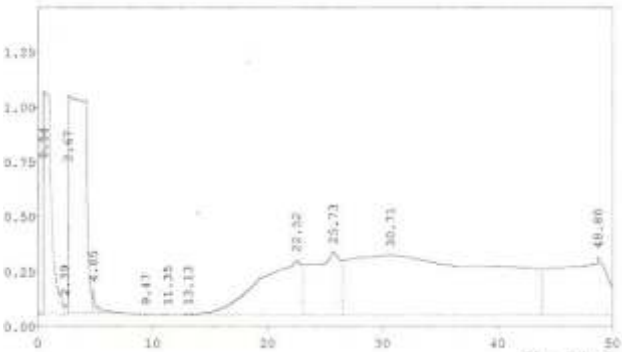
Fig 1: The GC profile for n-Heptane (A) without inoculum (B) with 26ML (C) with 3SG after 144 h of incubation period.



(D)

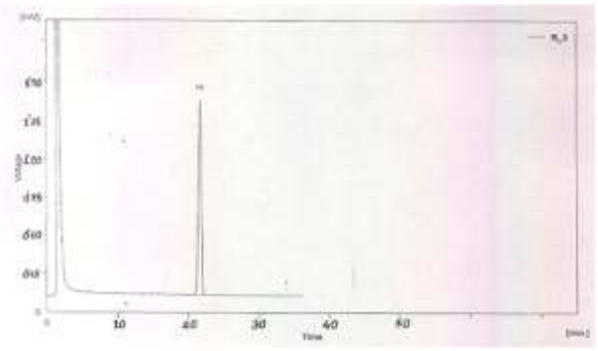



(E)

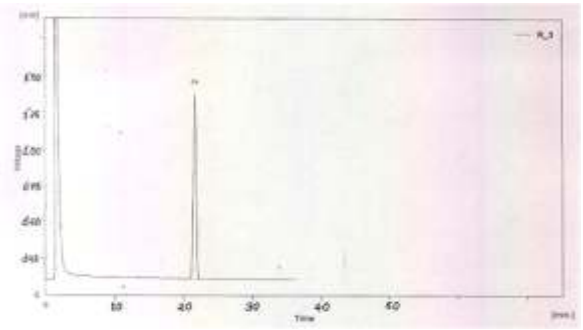



(F)

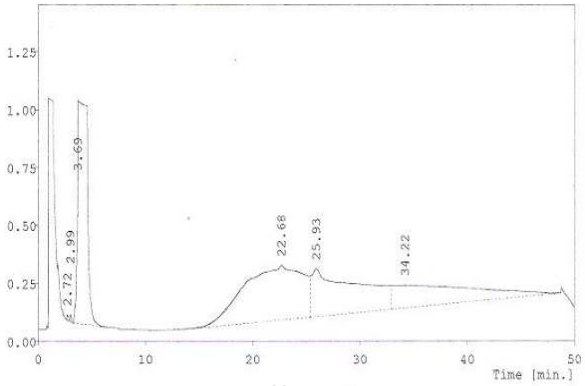
Fig 2: The GC profile for n-dodecane (A) without inoculum (B) with 26ML (C) with 3SG after 144 h of incubation period.



(G)



(H)




(I)

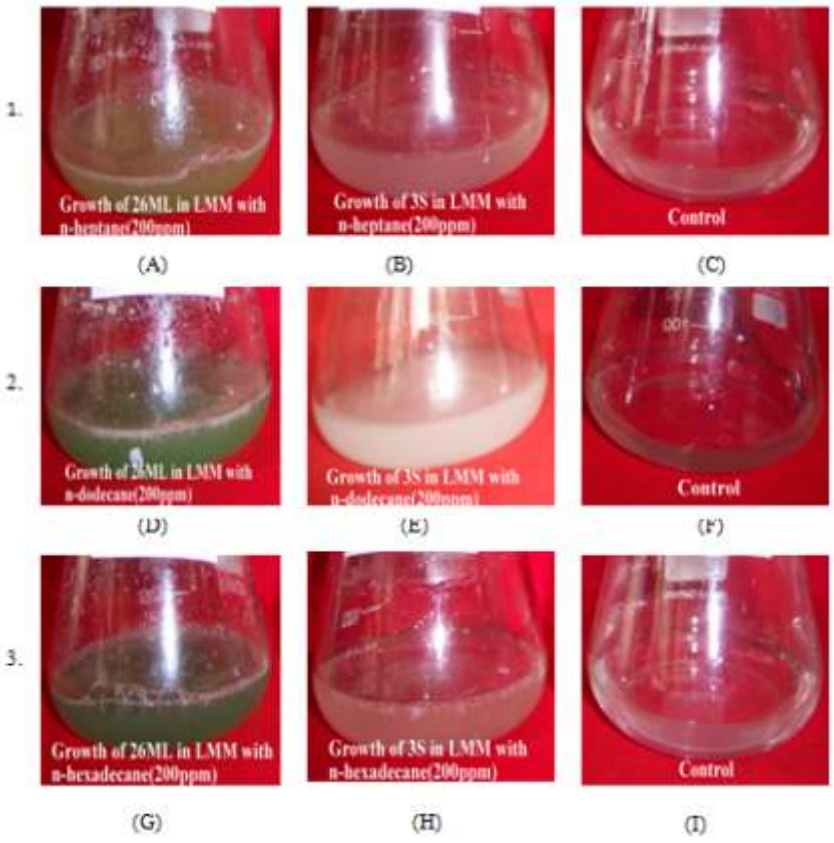



Plate 1: Growth of 26ML and 3SG in LMM supplemented with different aliphatic hydrocarbons after 144 h of incubation. (1) n-heptane inoculated with (A) 26ML (B) 3SG (C) Control; (2) n-dodecane inoculated with (D) 26ML (E) 3SG (F) Control ; (3) n-hexadecane inoculated with (G) 26ML (H) 3SG (I) Control.

### Microorganisms and growth conditions

3SG and 26ML were isolated from crude oil contaminated soil of Gelaky and Lakowa oilfield of Assam.The cultures were maintained in mineral slants supplemented with hexadecane. LMM was supplemented with individual hydrocarbons to achieve final concentrations of 200ppm (v/v) aliphatic hydrocarbon and 200ppm aromatic hydrocarbon. For this, LMM saturated with, n-heptane, n-hexadecane and n-dodecane individually were prepared by adding 1ml of each hydrocarbon in conical flask containing 50ml LMM followed by filtration and autoclaving. The stock solution (200ppm) of naphthalene, anthracene and fluoronnnthene were prepared in (Dimethyl sulfoxide (DMSO) and was sterilized by Millipore micro syringe filter assembly (0.45 µm pore size). LMM was prepared by dissolving NaNO3-4 g/L, Na2HPO4-3.67g/L, KH2PO4-1.75g/L, MgSO4.7H2O-0.2g/L, CaCl2.2H2O-0.05g/L, FeSO4-0.001g/L, Trace element-1% in distilled water and the final pH of the medium was adjusted to pH 7 and autoclaved at 121ºC for 20 minutes. Both the cultures were stored at 40C and used as inoculums in the experiment.

### Utilization/ bacterial degradation of aliphatic and aromatic hydrocarbon

Bacterial growth associated with aliphatic hydrocarbon was verified by demonstrating cfu method and cell biomass estimation. Degradation of hydrocarbon in the culture broth was determined using gas chromatographic analysis. Cultures were analyzed for the increase in bacterial cell number at various hours (0, 24, 48, 72, 96,120,144,168,192,216 and 240 h). For this 100 µl of each culture were taken out and diluted serially up to 10-1 to 10-10. After that 100 µl of each serially diluted culture were spread over MMA (Mineral media agar) and incubated for 24h at 28ºC. Log10 CFU/ml was estimated for each culture after 24 h of incubation.

### Assessment of hydrocarbon degradation in term of cell biomass production and GC analysis

After 240h of cultivation, each hydrocarbon remained in media was extracted from the total volume of each flask by shaking three times with 10 ml tolune. The mixture was centrifuged at 4000 rpm for 5 min. The organic phase was transferred to a fresh tube .The remaining cells were washed with a mixture of acetone and petroleum ether (3:1) thrice to remove the associated hydrocarbon and air dried to obtain dry biomass (g/L). The extent of different hydrocarbon biodegradation was checked by quantifying left over hydrocarbons in the biodegraded extracts by gas Chromatographic

## DISCUSSION

### Isolation and characterization of isolates

The two bacterial strains 3SG and 26ML were isolated from crude oil contaminated soil of Gelakey and Lakowa drillsite, upper Assam with a previous history of crude oil contamination of 25.5% and 45 % respectively (Saikia et al,2009).The identification and characterization of the two strains were done according to Bergey's Manual of Systematic Bacteriology. Morphologically the strain 26ML is a rod shaped, gram negative bacterium with green pigmentation and 3SG is a coccus, gram positive bacterium without pigmentation colour. DNA sequence analysis of PCR-amplified 16S rRNA as compared to those of reference sequences from NCBI GenBank database confirmed that the strains 26ML belongs to the genera Cellulosimicrobium (GeneBank accession no. KF732709) and the strain 3SG was identified as Brevibacillus laterosporus (GeneBank accession no.KF732708).

### Viability of 26ML and 3SG in the presence of aliphatic hydrocarbons.

The ability of the two isolates 26ML and 3SG in degradation of aliphatic hydrocarbon compounds was studied (Table2). Total viablecell count in LMM supplemented with the respective hydrocarbon fractions was assessed in terms of CFU of 26ML and 3SGobserved at different time intervals (0, 12, 24, 48, 72, 96,120,44,168, 216 and 240 h) . As shown in table 16, strain 26ML attained highest growth in LMM supplemented with n-hexadecane (200ppm v/v) which was indicated by highest 15.34log10 CFU/mland showed comparatively less growth in LMM containing n-heptanes which was indicated by 15.0log10CFU/ml in 144h. After 12 h of lag phase, log10 CFU/ml of 26ML on n-hexadecane increases exponentially with increase in incubation time up to

144 h and then it decreases. Initially, 7.17log10 CFU/ml ( 1.5×107 CFU/ml) of 26ML was added to the n-hexadecane medium, which almost remains constant (7.54log10CFU/ml) for 12h, started increasing linearly after 24h (8.14log10CFU/ml) attaining maximum 15.34log10 CFU/ml(10×1014) on 144 h of incubation showing approx 2×107 fold increase in the cell number. Thereafter a decrease in cell number of 8.16log10CFU/ml (14.5×107) was recorded after 240 h of incubation. In contrast, there is an increase in cell population of 3SG in LMM containing n-dodecane than in n-hexadecane on 144h of incubation. The initial cell population of 3SG was 7.17log10CFU/ml(1.5×107) in n-dodecane which simultaneously attained 8.07log10CFU/ml (12×107) after 24 h, subsequently reached up to 15.06 log10 CFU/ml (11.5×1014) at 144 h of incubation and followed by decreasing cell number h (8.04log10 CFU/ml) on 240 .While in n-heptanes and n-hexadecane, cell number of 3SG reached up to 14.9log10CFU/ml and 15.0log10CFU/ml respectively. Concentration of cell biomass estimated in 26ML and 3SGis given in table 4. Cell biomass of 26ML grown in LMM containing n-hexadecane (200 ppm v/v) showed luxuriant growth with biomass formation of 6.95gL-1 of in 144 h of cultivation. While, 26ML produced only 1.70 gL-1 and 1.80 gL-1 cell biomass in LMM containing n-heptanes (200 ppm v/v) and n-dodecane (200 ppm v/v) respectively. On the other hand, 3SG showed comparatively fine growth in n-dodecane with biomass formation of 4.69 gL-1 than in n-hexadecane and n-heptane in 144 h of incubation.

## Evaluation of bacterial growth in terms of pH change

pH of each culture each medium was checked at 0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240h (Table 5) .The initial pH of the media inoculated with 26ML and 3SG individually in n- heptanes, n-dodecane, n-hexadecane was in the range of 8.0 - 8.2. In case of 26ML inoculation pH was slowly reduced to 5.7, 5.8 and 6.2 within 240h in LMM containing n-heptanes, n-dodecane, n-hexadecane respectively. In case of 3SG, in 240h the pH turned down to 5.8. , 5.6 and 6.0 respectively.

## Verification of degradation of aliphatic hydrocarbon by 26ML and 3SG

The degradation ability of the n-heptanes, n-dodecane and n-hexadecanewas studied using gas chromatography(G C) analysis of biodegradation products of the respective aliphatic hydrocarbons (Fig1, 2 and 3). G C profile for n-heptanes, n-dodecane, n-hexadecane and their metabolites after 144 h of incubation of 26MLand 3SG in LMM was compared with that of negative control (without inoculums). The chromatogram of n-heptanes degradation by 26ML and 3SG showed 56.64 % and 19.49 % degradation along with ten and six additional peaks respectively which indicates formation of different metabolites during the degradation of n-heptanes. While the gas chromatographic profile for n-hexadecane in LMM inoculated with 26ML in 144 h was compared with the biodegradation products of n-dodecane. The chromatogram showed only trace amount of n-hexadecane and ten additional peaks could be detected by G C indicating greater utilization of n-hexadecane by 26ML in LMM indicating higher percentage of n-hexadecane degradation by 26ML (99%). But GC profile for 26ML in degradation of n-dodecane showed only 62% degradation. While cultivation of 3SG in LMM containing n-dodecane on 144 h of incubation showed greater utilization of n-dodecane as shown by the increase in bacterial cell number with a concomitant loss of dodecane which was detected 96% by G C analysis.There are several reports on biodegradation of different hydrocarbon compounds by a number of bacteria (Wongsaet al.2004; Mashreghi and Marialigeti, 2005; Mahammad et al.2007;Kim et al., 2009). Most of the studies have established that the gram negative bacteria, most of them belong to the genusPeudomonas are common in degradation of aliphatic compounds. Mahammad et al. (2007) worked on a strain WatG which can degrade aliphatic hydrocarbon with chain length uptoC31. Bardietal. (2000) worked on bacterial degradation of n-dodecane with addition of β-cyclodextrin. During the present study it has been observed that both the strain showed increasing number of viable cells in LMM containing various hydrocarbons within 144h of incubation which is directly correlated with the ability of Cellulosimicrobiumsp26ML and Bravibacilluslaterosporus strain 3SG to utilize different hydrocarbons as the sole source of carbon and energy which leads to high increase in cell number and starts decreasing afterwards possibly due to the exhaustion of hydrocarbons. Further,

between the two isolates i.eCellulosimicrobium sp. strain 26ML and Bravibacilluslaterosporus strain 3SG inoculated in LMM containing different aliphatic hydrocarbons, strain 26ML showed best growth in hexadecane than the two other aliphatic hydrocarbons.In contrasts,a few studies are available which described low biodegradation of n-hexadecane (Watts and Stanton, 1999;Huesemannet al., 2003, 2004).

## CONCLUSION

Growth study ofCellulosimicrobium sp. strain 26ML andBravibacilluslaterosporus strain 3SGwas evaluate4d in Liquid mineral medium containing various aliphatic hydrocarbon compounds for period of 240 h in laboratory. The result demonstrated thatCellulosimicrobium sp. strain 26ML attained highest growth in LMM containing n-hexadecane than the other two hydrocarbons. While,Bravibacilluslaterosporus strain 3SGshowd highest activity for dodecane degradation indicating that these two isolates would be useful for the bioremediation strategy for soil polluted by petroleum aliphatic hydrocarbon.

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