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Study on paraffin wax degrading ability of *Pseudomonas nitroreducens* isolated from oil wells of Gujarat, India

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ABSTRACT

Microbial remediation is an efficient method of removing paraffin wax from oil wells and pipelines and is found to be environment friendly and economical. In this study, a potential paraffin wax degrading bacterial strain was isolated from oil wells of Gujarat and was identified as *Pseudomonas nitroreducens* by 16S rRNA sequencing. *Pseudomonas nitroreducens* showed 100% utilization of heneicosane, 79.74% utilization of pentacosane and 72.50% utilization of triacontane, the major components of paraffin wax in 8 days at 37°C. *Pseudomonas nitroreducens* degraded 70% of paraffinic crude oil in 10 days at 37°C. The probable end products formed after degradation of eicosane and heneicosane by *Pseudomonas nitroreducens* were analysed by Mass Spectrum. The above results proving isolate as a potential candidate for degradation of paraffin wax in oil wells.

KEYWORDS

crude oil; degradation; gas chromatography; mass spectrum; paraffin wax

1. Introduction

Paraffins are high molecular weight alkane hydrocarbons commonly found in crude oil (Sood and Lal, 2008). Paraffins consist of a broad class of compounds that have the general formula C_nH_{2n+2} where "n" is the number of carbon atoms which are joined by single bonds. They consist of various forms and combinations of aliphatic hydrocarbons, aromatic hydrocarbons, asphaltenes, resins and naphthenes (Addison, 1984). They have relatively high melting points and cloud points. At high temperatures and pressure, paraffinic crude oil flows freely through oil wells and continuously carrying the oil. Below the cloud point (45°C), the paraffins begin to precipitate and form paraffin wax deposits on the inner walls of the oil wellbore tubulars. These deposits grow thicker and more complex and eventually results into a significant narrowing or complete blockage of the oil wellbore tubulars, reducing the crude oil flow through oil wells (Soni and Lal, 2009). The remediation and prevention methods for paraffin deposits are very costly. A billions of dollars are lost per year worldwide due to decreased production, well shut-in, failures of transport equipments and pipelines, replacements of pipelines and requirements of extra man power (Barker et al., 2001).

Paraffin deposition problems are generally controlled by techniques like hot water, mechanical scraping, chemicals and solvents. These techniques are costly and environmentally hazardous. Microbial remediation is an efficient method of removing paraffin wax from oil wells and pipelines and is found to environment friendly and economical. Microbes have the ability to utilize hydrocarbons and is widely distributed. Microorganisms use paraffinic hydrocarbons as their exclusive source of carbon and energy (Tan, 2011) and has been recognized for their ability to digest paraffin wax depositions from oil wells. Examples of paraffinic hydrocarbons degrading bacteria are *Acinetobacter, Pseudomonas, Phenylobacterium, Stenotrophomonas, Gluconobacter, Agrobacterium, Vibrio, Micrococcus, Aeromonas, Beijerinckia,*

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Flavobavterium, Nocardia, Corynebacterium, Spingomonas, Microbacterium, Paracoccus, Burkholderia and many more (Jacques et al., 2007). Use of microbes for the mitigation of paraffin deposition problems is a non hazardous and economically viable approach. The main objective of this study is to isolate microbes required for the mitigation of paraffin deposition problems in the surface flow lines of oil wells.

2. Materials and methods

2.1. Sample collection

Paraffinic crude oil samples were collected from different oil wells of an oil field in Limbodara village, Gandhinagar of Gujarat state, India. Crude oil samples contained asphaltenes, resins and wax in the range 0.4 – 2.4%, 7.28 – 10.58% and 15.06 – 21.45% respectively. The crude oil sample used for isolating paraffin degrading microorganisms had a density (15°C) of 861.3 kg/m^3, API of 32.69, viscosity (30°C) of 602 cps and pour point of 33°C.

2.2. Enrichment and Isolation of paraffin wax degrading microorganisms

The paraffin wax degrading microorganisms were isolated by enrichment culture technique from paraffinic crude oil samples. For enrichment, 5 ml paraffinic crude oil samples were inoculated into 100 ml of minimal salts medium (MSM) (Lal and Khanna, 1996) and incubated at 37°C on a rotary shaker (150 rpm) for 7 days. After three cycles of enrichment, 0.1 ml was plated on MSM agar plates with paraffinic wax (0.1% w/v) as the carbon source. Colonies with different morphological characters were purified by subculturing on to paraffin (0.1% w/v) containing MSM media.

2.3. Screening and Identification of efficient paraffin wax degrading microorganisms

Screening of efficient paraffin wax degrading microorganisms was carried out by using DCPIP dye (2,6-dichlorophenol indophenols) as redox indicator (Dolly and Lakshmi, 2016). The selected paraffin wax degrading microbial strain was grown on Luria Bertani agar at 37°C and identified by 16S rRNA gene sequencing at GSBTM (Gujarat State Bio-Technology Mission). The sequence was deposited in the NCBI (National Center for Biotechnology Information) database and was assigned the accession number KP208174.

2.4. Degradation of paraffin wax by the selected isolate

Degradation potential of paraffin wax by the selected isolate was monitored in 250 ml Erlenmeyer flasks (in triplicate) containing 100 ml MSM with 0.1% (w/v) of paraffinic wax as the sole carbon source and samples were incubated on a rotary shaker (150 rpm) at 37°C for 8 days. The selected isolate was grown overnight in Luria Bertani broth and 5% (v/v) inoculum (10^8 cells/ml) was used to inoculate MSM with paraffin wax. Uninoculated control was maintained to monitor abiotic loss of paraffin wax. The residual undegraded paraffin wax was extracted thrice with equal volumes of hexane. For quantitative analysis, 1 µl of the paraffinic wax (dissolved in 10 ml hexane) was analyzed by GC (Perkin Elmer, USA; Autosystem XL model with flame ionization detectors (FID)) on a silica based column (Mishra et al., 2001). The GC profile of the paraffinic fraction, extracted from inoculated flask, was compared with that of obtained from uninoculated control flask.

2.5. Degradation of paraffinic crude oil by the selected isolate

Degradation of paraffinic crude oil by the selected isolate was monitored in 250 ml Erlenmeyer flasks (in triplicate) containing 100 ml MSM with 2% (w/v) of paraffinic crude oil collected from problematic wells of Limbodara oil wells of Gujarat state, India. The medium was inoculated with 5% overnight grown culture (10^8 cells/ml) of selected paraffin wax degrading strain in LB medium and incubated in shaking

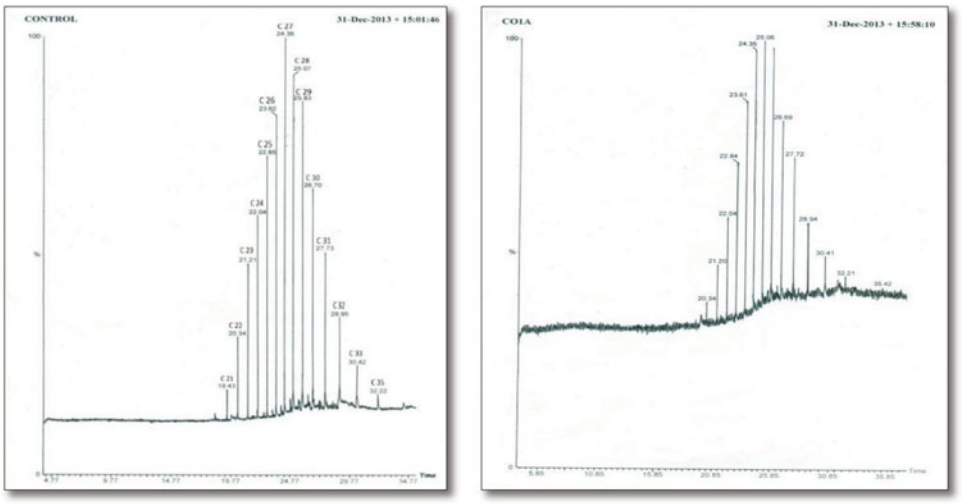

Figure 1. Degradation of different carbon fractions of the paraffin wax by *Pseudomonas nitroreducens* at 37°C in 8 days. (a) Gas Chromatogram of paraffin wax (control), (b) Gas Chromatogram of paraffin wax (residual) treated with *Pseudomonas nitroreducens*.

conditions (150 rpm) at 37°C for 10 days. At the end of the incubation period, the residual paraffinic crude oil was extracted thrice with equal volumes of hexane and analyzed by GC (Perkin Elmer, USA; Auto-system XL model, silica based column with flame ionization detectors (FID)).

2.6. Evaluation of the ability of selected isolate to degrade various alkanes present in paraffin wax

Degradation of alkanes (eicosane, heneicosane, octacosane, triacontane) that represent the paraffin wax was monitored in 100 ml Erlenmeyer flasks (in triplicate) containing 22 ml MSM, 2 ml DCPIP (0.01%) dye, 0.05 g of alkanes as the sole carbon source and 1 ml culture inoculum (10^8 cells/ml). The samples were incubated on a rotary shaker (120 rpm) at 37°C for 6 days. The absorbance were measured at 600 nm at regular time interval of 24 h till 6 days.

2.7. Evaluation of the degradation ability of the selected isolate to degrade paraffinic alkanes like eicosane and heneicosane using Mass Spectrophotometric method

The degradation of various paraffinic alkanes was studied by growing the selected isolate on alkanes like eicosane and heneicosane as the sole carbon source. The selected isolate was inoculated (5% (v/v)

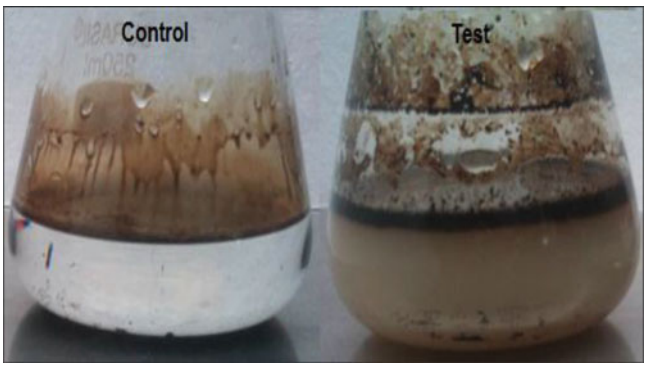

Figure 2. Dispersion of degraded crude oil due to degradation by *Pseudomonas nitroreducens* after incubation of 10 days at 37°C.

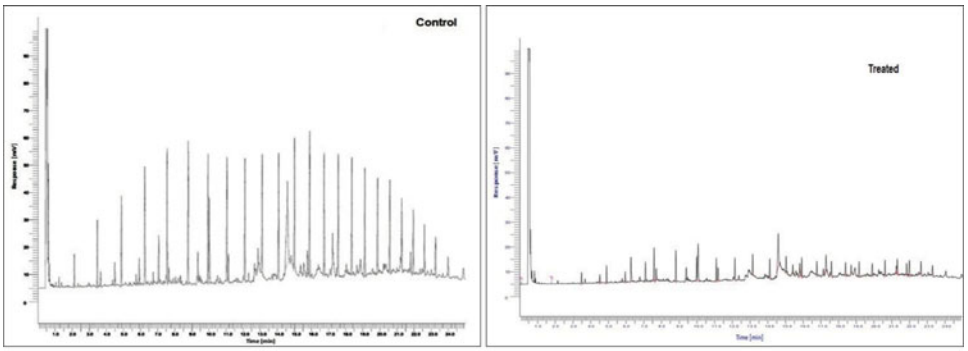

Figure 3. Degradation of different carbon fractions of the paraffinic crude oil by *Pseudomonas nitroreducens* at 37°C in 10 days. (a) Gas Chromatogram of paraffinic crude oil (control), (b) Gas Chromatogram of paraffinic crude oil (residual) treated with *Pseudomonas nitroreducens*.

-10^8cells/ml) in 250 ml Erlenmeyer flasks containing 100 ml MSM with 0.1% (w/v) of alkanes (eicosane and heneicosane). The inoculated flask along with a control (without inoculum) were incubated on a rotary shaker (150 rpm) at 37°C for 10 days. Then the degraded compounds were extracted by hexane using the liquid–liquid extraction method and analyzed by Electrospray Ionization Mass Spectrophotometer (Advion Expression CMS).

3. Results

3.1. Enrichment, Isolation, Screening and Identification of paraffin wax degrading microorganisms

By the enrichment culture technique from paraffinic crude oil samples, 12 morphologically distinct bacterial colonies were isolated. All these 12 isolates were screened for their efficiency of paraffin wax degradation (as a sole carbon source) using 2,6-dichlorophenol indophenol (DCPIP). Out of 12 isolates, the isolate which showed 90% dye reduction within 6 days was selected and identified as *Pseudomonas nitroreducens* by 16S rRNA gene sequencing.

3.2. Degradation of paraffin wax by the selected isolate

Degradation potential of paraffin wax by the selected isolate was measured by Gas Chromatographic method (Figure 1). Gas Chromatogram of paraffin wax (control) showed various paraffin components from heneicosane (C_{21}) to pentatriacontane (C_{35}). Gas Chromatogram of paraffin wax treated with selected isolate showed 100% utilization of heneicosane, 79.74% utilization of pentacosane and 72.50% utilization of triacontane, the major components of paraffin wax after incubation of 8 days.

3.3. Degradation of paraffinic crude oil by the selected isolate

Dispersion of degraded paraffinic crude oil was observed in conical flask inoculated with *Pseudomonas nitroreducens* indicating the ability to break down the paraffinic crude oil (Figure 2). Degradation efficiency of paraffin crude oil by the selected isolate was measured by Gas Chromatography (Figure 3). Gas Chromatogram of paraffinic crude oil (control) showed various paraffin components with a carbon chain length C_5 to C_{29}. Gas Chromatogram of paraffinic crude oil treated with selected isolate showed 70% degradation of this paraffinic crude oil fraction after incubation of 10 days. *Pseudomonas nitroreducens* was able to effectively degrade all carbon fractions.

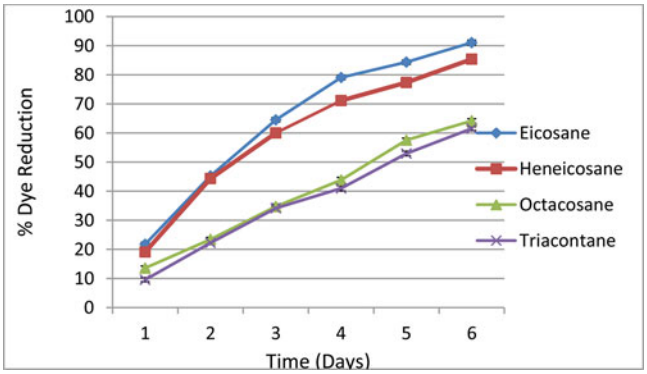

Figure 4. Degradation of different alkanes representing the paraffin wax by *Pseudomonas nitroreducens* at 37°C (Values are mean ± S.D. n = 3).

3.4. Degradation of alkanes representing the paraffin wax by the selected isolate

The selected isolate efficiently degraded alkanes (eicosane, heneicosane, octacosane and triacontane) that represent the paraffin wax but there was a difference in the rate of utilization of the four alkanes. The % dye reduction in eicosane, heneicosane, octacosane and triacontane containing MSM media was found to be 91%, 85%, 64% and 61% respectively. Octacosane and triacontane were degraded less by the organism as the % dye reduction was found to be less when compared to eicosane and heneicosane (Figure 4).

3.5. Evaluation of the degradation ability of the selected isolate to degrade paraffinic alkanes like eicosane and heneicosane using Mass Spectrophotometric method

Electrospray Ionization Mass Spectrum was carried out for the analysis of degraded products formed during the degradation of eicosane and heneicosane by *Pseudomonas nitroreducens* [Figure 5(a) and (b), 6(a) and (b)]. The compounds with highest peak percentage (%) obtained in the Mass Spectrums (ESI-MS) were identified based on m/z, molecular weight and molecular formula. The compounds with highest peak % (95.5% and 99%) obtained in the Mass Spectrums had a m/z of 104 and 180. The compound with m/z 104 was found to have molecular formula matching with 3- Ethoxy-1- propanol and 2-hydroxy-2-methylpropanoic acid. The compound with m/z 180 was found to have molecular formula matching with Heptane-1,3,4,5,7-pentol and 1,12- Tridecadiene respectively (Table 1 and 2). It can be concluded that either of these products were formed during the degradation.

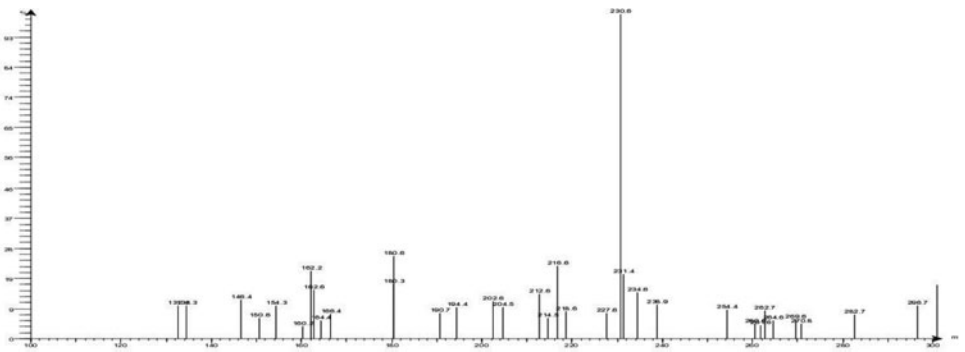

Figure 5 (a). Electrospray Ionization Mass Spectrum (ESI-MS) of eicosane.

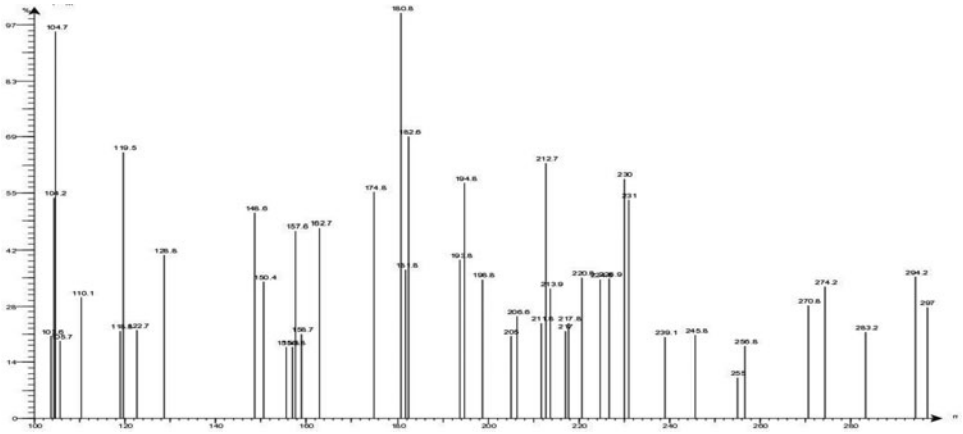

Figure 5 (b). Electrospray Ionization Mass Spectrum (ESI-MS) of degraded products of eicosane by *Pseudomonas nitroreducens*.

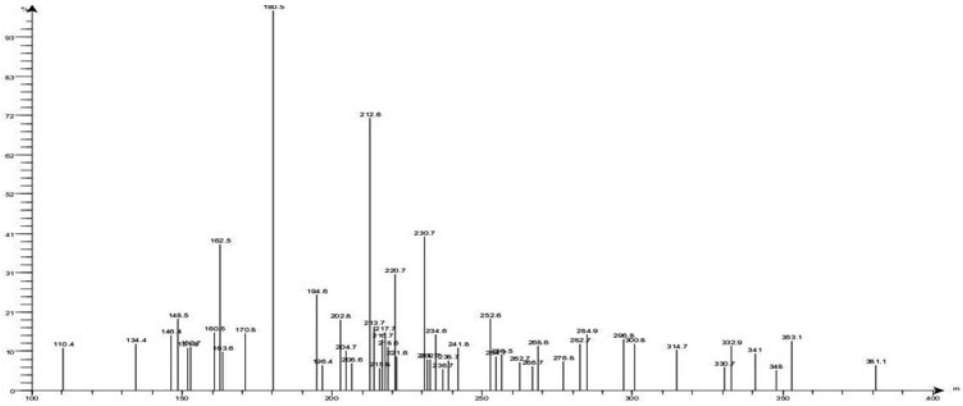

Figure 6 (a). Electrospray Ionization Mass Spectrum (ESI-MS) of heneicosane.

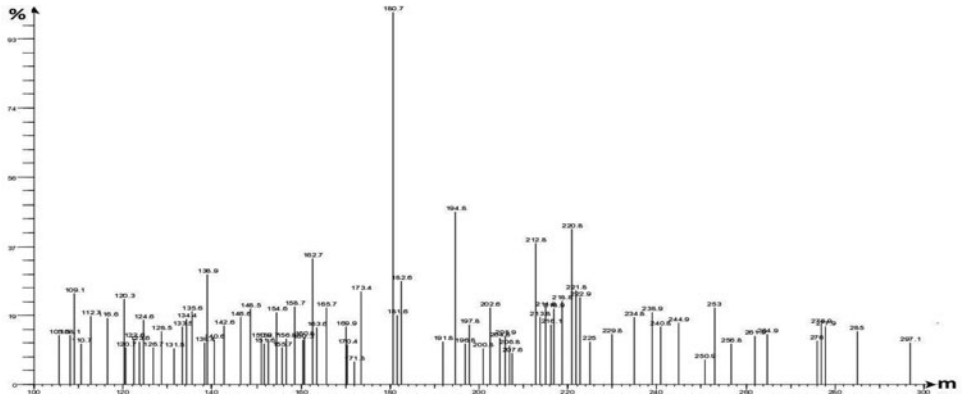

Figure 6 (b). Electrospray Ionization Mass Spectrum (ESI-MS) of degraded products of heneicosane by *Pseudomonas nitroreducens*.

4. Discussion and Conclusion

Many oil fields in India produce parafinic crude oil. Deposition of paraffin wax in the well bore region and flow lines is one of the most severe problems in such oil fields which adversely affects the well productivity. Paraffin deposition related problems in oil well tubings have crude oil containing paraffins of carbon chain length of C_{16} (hexadecane) and higher alkanes (Lazar et al., 1999). To address the problem

Table 1. End products formed* during the degradation of eicosane by *Pseudomonas nitroreducens*.

m/z	Molecular formula	Name of compound	Peak %
104	$C_5H_{12}O_2$	3- Ethoxy-1- propanol 1- Methoxy-2- butanol 1,5- Dihydroxypentane	95.5%
	$C_4H_8O_3$	2-hydroxy-2-methylpropanoic acid 2-Hydroxyisobutanoic acid 1,2- ethanediol	
180	$C_7H_{16}O_5$ $C_{13}H_{24}$	Heptane-1,3,4,5,7-pentol 1,12- Tridecadiene	99%

*probable products formed (any of the above compounds will be formed).

of paraffin deposition in oil well tubing and pipelines, paraffinic crude oil samples were collected from oil wells of Gujarat and were enriched using minimal salts medium for isolation of paraffin wax degrading microorganisms.

Screening for potential hydrocarbon utilizing bacteria by DCPIP assay has been reported (Mariano et al., 2008; Afuwale and Modi, 2012; Varjani et al., 2013). All the isolates were screened for their efficiency to utilize paraffin wax (as a sole carbon source) using DCPIP indicator. Color change of DCPIP was observed periodically at interval of 24 h till six days visually and optical density was measured. Based on percentage of DCPIP decolorization, the isolate which showed 90% dye reduction within 6 days was selected. All isolates were not able to decolorize DCPIP completely in six days. The selected isolate was identified as *Pseudomonas nitroreducens* by 16S rRNA sequencing. Das and Mukherjee (2006) isolated *Bacillus subtilis* and *Pseudomonas aeruginosa* strains from a petroleum-oil contaminated soil from North-East India were able to degrade crude petroleum-oil. Etoumi (2007) described the use of *Pseudomonas* for mitigation of wax precipitation in waxy crude oils.

The ability of degradation of paraffin wax by selected isolate was confirmed by Gas Chromatographical analysis. Gas Chromatogram of paraffin wax treated with *Pseudomonas nitroreducens* showed 100% utilization of heneicosane, 79.74% utilization of pentacosane and 72.50% utilization of triacontane, the major components of paraffin wax after incubation of 8 days. Koma et al. (2001) analyzed efficient degradation of C_{12}-C_{30} types of n-paraffins by *Acinetobacter* sp. ODDK71 using GC. Gas Chromatographical analysis revealed that *Pseudomonas nitroreducens* degraded 70% of paraffinic crude oil in 10 days. Sood and Lal (2008) showed that *Geobacillus kaustophilus* TERI NSM could degrade 60% of paraffinic fraction of the crude oil in 7 days and was also analyzed by GC. Tan (2011) reported that *Acinetobacter calcoaceticus* achieved enhancing degradation of crude oil from carbon chain length C_8 to C_{28} in 17 days.

Pseudomonas nitroreducens was inoculated in a media containing eicosane and heneicosane as only carbon source and the degraded products formed were analyzed by using ESI-MS. The compounds can be 3- Ethoxy-1- propanol and 2-hydroxy-2-methylpropanoic acid or Heptane 1,3,4,5,7-pentaol and 1,12, Tridecadiene. Koma et al. (2001) identified 1-hexadecanol and 1-hexadecanoic acid during hexadecane degradation by *Acinetobacter* sp. ODDK71 using GC-MS. Tan (2011) identified Octadecanal compound and 4-(2,2-Dimethyl-6 methylenecyclohexylidene)-3-methylbutane-2 from *Acinetobacter calcoaceticus* culture medium with crude oil on day 17 by Mass Spectroscopy.

Table 2. End products formed* during the degradation of heneicosane by *Pseudomonas nitroreducens*.

m/z	Molecular formula	Name of compound	Peak %
180	$C_7H_{16}O_5$ $C_{13}H_{24}$	Heptane-1,3,4,5,7-pentol 1,12- Tridecadiene	99%
194	$C_{13}H_{22}O$	3-(1-adamantyl)-1- propanol	45%

*probable products formed (any of the above compounds will be formed).

5. Conflict of interest

We declare that we have no conflict of interest.

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