

# Role of *Leifsonia* sp. in degradation of Paraffin

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**ABSTRACT:** *Leifsonia* sp. was isolated by enrichment culture technique from paraffinic crude oil of oil wells. The degradation potential of paraffin wax and crude oil by *Leifsonia* sp. was confirmed by Gas chromatography. Effect of various factors on paraffin degrading ability of *Leifsonia* sp. was evaluated by DCPIP dye (2, 6-dichlorophenol indophenol) reduction method. *Leifsonia* sp. showed maximum dye reduction as an indicator of paraffin degradation at 37 °C, pH 7, 5% inoculum concentration, 1% paraffin substrate concentration, 2% crude oil concentration and 1% NaCl concentration. *Leifsonia* degraded eicosane more when compared to other hydrocarbons and utilized ammonium chloride more than other nitrogen sources.

**KEYWORDS:** Paraffin, Crude oil, *Leifsonia*, Gas chromatography, DCPIP dye

## I. INTRODUCTION

Paraffins are high molecular weight alkane hydrocarbons derived from petroleum compound. Paraffin wax is generally considered to be composed of alkanes of carbon chain length C16 (hexadecane) to C18 (octadecane) and above [1-2]. Paraffinic wax deposition causes, the oil companies to spend millions of dollar per year as removal costs and the cost of lost production [3]. Physical and chemical methods are in use, but they have certain disadvantages. Mechanical scraping causes losses due to production downtime. Chemicals and solvents, besides being hazardous, only partially dissolve or disperse paraffin and they are deposited elsewhere in the tubing. Moreover, these remedial options are expensive.

A wide variety of microbes are able to use long chains hydrocarbons as their sole source of carbon and energy. In the laboratory, aerobic degrading bacteria from soil showed a clear tendency to liquid paraffin and can emulsify liquid paraffin [4]. Several studies have identified organisms which are able to decompose paraffin. The main goal of the present study is to evaluate the role of *Leifsonia* sp. in degradation of paraffin.

## II. MATERIALS AND METHODS

### 2.1. Materials:

All chemicals and reagents used for the study were of analytical reagent grade. The chemicals used for media preparation was purchased from Hi Media (Mumbai, India) and the paraffinic wax (Melting point 58–60°C) used in the study was obtained from Merck India Ltd. (Mumbai, India).

### 2.2. Methods:

#### 2.2.1. Sample Collection:

Paraffinic crude oil samples were collected from different oil wells of an oil field in Limbodara village, Gandhinagar of Gujarat state of Western India. Crude oil samples contained asphaltenes, resins and wax in the range 0.4 – 2.4 %, 7.28 - 10.58 %, 15.06 - 21.45%, respectively.

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## 2.2.2. Enrichment and isolation of paraffin wax degrading bacteria:

The paraffin wax degrading bacteria was isolated by enrichment culture technique from paraffinic crude oil samples. For enrichment, 5 ml paraffinic crude oil sample was inoculated into 100 ml of minimal salts medium (MSM) [5] and incubated at 37°C on a rotary shaker (150 rpm) for 7 days. After three cycles of enrichment, 0.1 ml of enriched medium was plated on to MSM agar plates with paraffinic wax (0.1% w/v) as the carbon source. Bacterial colonies were formed and purified on the MSM agar plates with paraffinic wax.

## 2.2.3. Screening of paraffin wax degrading bacteria:

Screening of potential paraffin wax degrading bacteria was carried out by modified Hanson *et al.*, (1993) and Bidoia *et al.*, (2010) method using DCPIP (0.01%) as redox indicator[6- 7]. Inoculum was prepared by transferring cultures into LB medium for 24 h at 37°C at 120 rpm. Screening of potential paraffin-wax degrading bacteria was carried out in 100 ml Erlenmeyer flasks containing 22 ml MSM, 2 ml DCPIP (0.01 %) dye, 0.05 g of paraffinic wax as the sole carbon source and 1 ml culture inoculum. The samples were incubated on a rotary shaker (120 rpm) at 37°C for 6 days. The absorbance was measured at 600 nm at regular time interval of 24 hours till 6 days. The percentage decolorization was measured by comparing with the control. Control was prepared by using the same method as mentioned above i.e. without any inoculum. Percentage decolorization of DCPIP was measured by using the following formula

$$\% \text{ dye reduction} = \frac{\text{Control} - \text{test}}{\text{Control}} \times 100$$

The bacterial strains, which showed maximum decolorization of DCPIP in the quickest time, were chosen as the best paraffin wax degraders.

## 2.2.4. Characterization and Identification of the selected paraffin-wax degrading bacterial strain:

The efficient paraffin-wax degrading bacterial strain was grown on Luria Brentani agar at 37°C and characterized by morphological studies. The strain was also characterized by Gram's staining and biochemical tests. The identification of the bacteria was carried out by 16s rRNA technique at GSBTM (Gujarat State Biotechnological mission).

## 2.2.5. Environmental and physiological factors affecting paraffin wax degradation:

Effect of various factors on paraffin degrading ability of selected isolate was monitored in 100 ml Erlenmeyer flasks (in triplicate) containing 22 ml MSM, 2 ml DCPIP (0.01 %) dye, 0.05 g of paraffinic wax as the sole carbon source and 1 ml culture inoculums. The samples were incubated on a rotary shaker (120 rpm) at 37°C for 6 days. The absorbance of all the assays was measured at 600 nm at regular time interval of 24 h till 6 days. The parameters that studied were included temperatures (30, 37, 45°C), pH values (5, 6, 7, 8, 9, 10), carbon sources (paraffin, crude oil, eicosane, heneicosane, octacosane, triacontane (1% v/v or 1% w/v)), nitrogen sources (ammonium chloride, ammonium nitrate, potassium nitrate, peptone, tryptone (1% w/v)), inoculum concentration (1, 2, 3, 5, 10 % v/v), substrate (paraffin wax) concentration (0.01, 0.05, 0.1, 0.5, 1.0 % w/v), crude oil concentration (1, 2, 3, 5, 10 % v/v) and NaCl concentration (1, 2, 3, 4, 5, 10 % w/v).

## 2.2.6. Gas chromatographical analysis of paraffin wax and crude oil degrading ability of selected organism.

Paraffin wax degradation potential of the selected isolate was measured by Gas chromatographic method. For this 250 ml Erlenmeyer flasks containing 95 ml MSM, 1 g NH<sub>4</sub>Cl, 1 g of paraffinic wax as the sole carbon source and 5 % of inoculum of the selected strain samples were incubated on a rotary shaker (150 rpm) at 37°C for 10 days. Uninoculated control was maintained to monitor abiotic loss of paraffin-wax. The residual undegraded paraffin-wax was extracted thrice with equal volumes of hexane [8]. For quantitative analysis, 1 µl of the paraffinic wax (dissolved in 10 ml hexane) was analyzed by GC (Perkin Elmer, USA; Auto-system XL Model with flame ionization detectors (FID)) on a silica based column using the method described by Mishra *et al.* [9]. The profile of the paraffinic fraction, extracted from inoculated flasks, was compared with that obtained from uninoculated control flask. The above protocol was used for Gas chromatographical analysis of crude oil degrading ability of selected isolate (instead of paraffin, crude oil (2%) was used).

## 2.2.7. Statistical Analysis:

To compare the effect of various treatments employed in the study, the experimental data were analyzed statistically using the ANOVA technique. P<0.05 was considered to be significant.

### III. RESULTS AND DISCUSSION

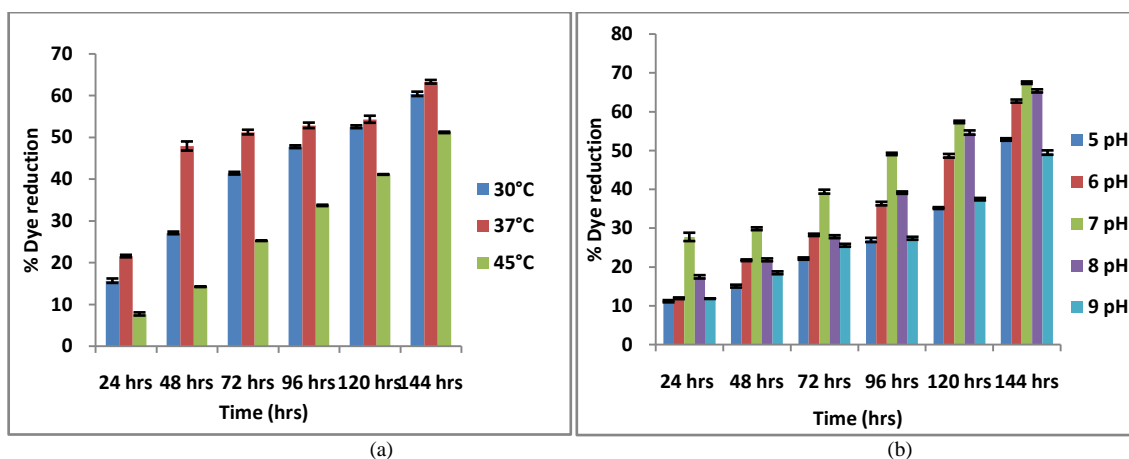
Microbes able to grow on hydrocarbons have shown the ability to accumulate the paraffinic substrate intra cellularly in inclusion bodies. The passage of hydrocarbon through the cells is a crucial step for the catabolism of these chemicals. It has been shown that it can even be limiting for very long chains (up to 36 carbons). To be able to catabolise the long-chain hydrocarbons, a microorganism must have the ability to produce the degrading enzymes. Hydrocarbon degradation processes by microorganisms involve redox reaction, in which the initial attack on the alkanes is carried out by monooxygenase enzyme present in the microbes. The monooxygenase incorporated oxygen to alkanes, and electrons are transferred to electron acceptors, such as hydrogen. Reducing condition can be detected using redox indicators. Generally, it is observed that oxidized forms are coloured and reduced forms are colourless. Due to this fact, colour change can be monitored with a spectrophotometer [10]. By incorporating an electron acceptor such as DCPIP to the culture medium, it is possible to ascertain the ability of the microorganism to utilize hydrocarbon substrate by observing the colour change of DCPIP from blue (oxidized) to colourless (reduced), which is monitored at 600 nm wavelength. A peak in the absorbance was observed at 600 nm; as reported by Yoshida *et al.*, 2001 [11]. Moreover, the colour of the DCPIP indicator in the presence of light is reasonably stable [12].

#### 3.1. Isolation and Identification of paraffin wax degrading organism:

The organism which showed maximum dye reduction was selected. The selected strain was a gram-positive rod and non-motile. It was able to grow at temperatures from 25 to 40 °C and could utilize sucrose, glucose, mannitol and trehalose. It was a catalase positive and citrate, urease, nitrate reductase, malonate, ONPG and oxidase negative. Colonies of strain were smooth, circular and yellowish white. The organism was identified as *Leifsonia* sp. by 16s rRNA sequencing and the sequence was submitted in NCBI with an accession number KJ210576.

#### 3.2. Environmental and physiological factors affecting paraffin wax degradation:

Effect of various factors like temperature, pH, carbon sources, nitrogen sources, inoculum concentration, paraffin wax concentration, crude oil concentration and NaCl concentration on the paraffin degrading ability of *Leifsonia* sp. was assessed. The results are represented in Fig. 1 and Fig. 2. *Leifsonia* culture showed a maximum of 63.28% dye reduction at 37 °C on 144<sup>th</sup> hours when compared to other temperatures, a maximum of 67.43% dye reduction was observed at 7 pH on 144<sup>th</sup> hours. A maximum of 69.72 % dye reduction was observed in the medium containing eicosane when compared to other carbon sources and a maximum of 82.34 % dye reduction was observed in the medium containing ammonium chloride when compared to other nitrogen sources. A maximum of 79.05 % dye reduction was observed in the medium containing 5% inoculum concentration. A maximum of 79.30 % dye reduction was observed in the medium containing 1% substrate (paraffin wax) concentration and a maximum of 78.70 % dye reduction was observed in the medium containing 2% crude oil concentration. A maximum of 77.43 % dye reduction was observed in the medium containing 1% NaCl concentration.



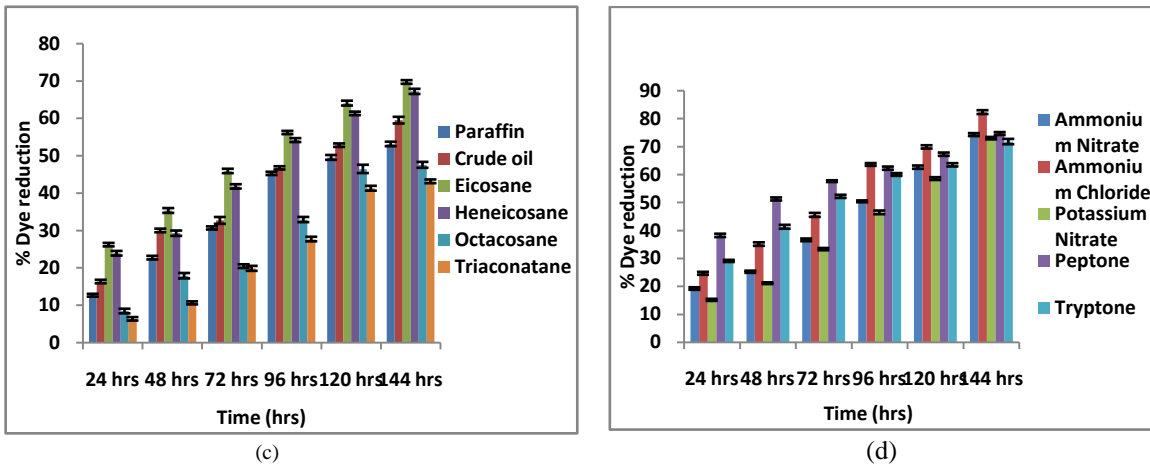


Fig. 1. Effect of different temperature (a), pH (b), carbon sources (c) and nitrogen sources (d) on percentage dye reduction as an indicator of paraffin degradation ability of *Leifsonia* sp.

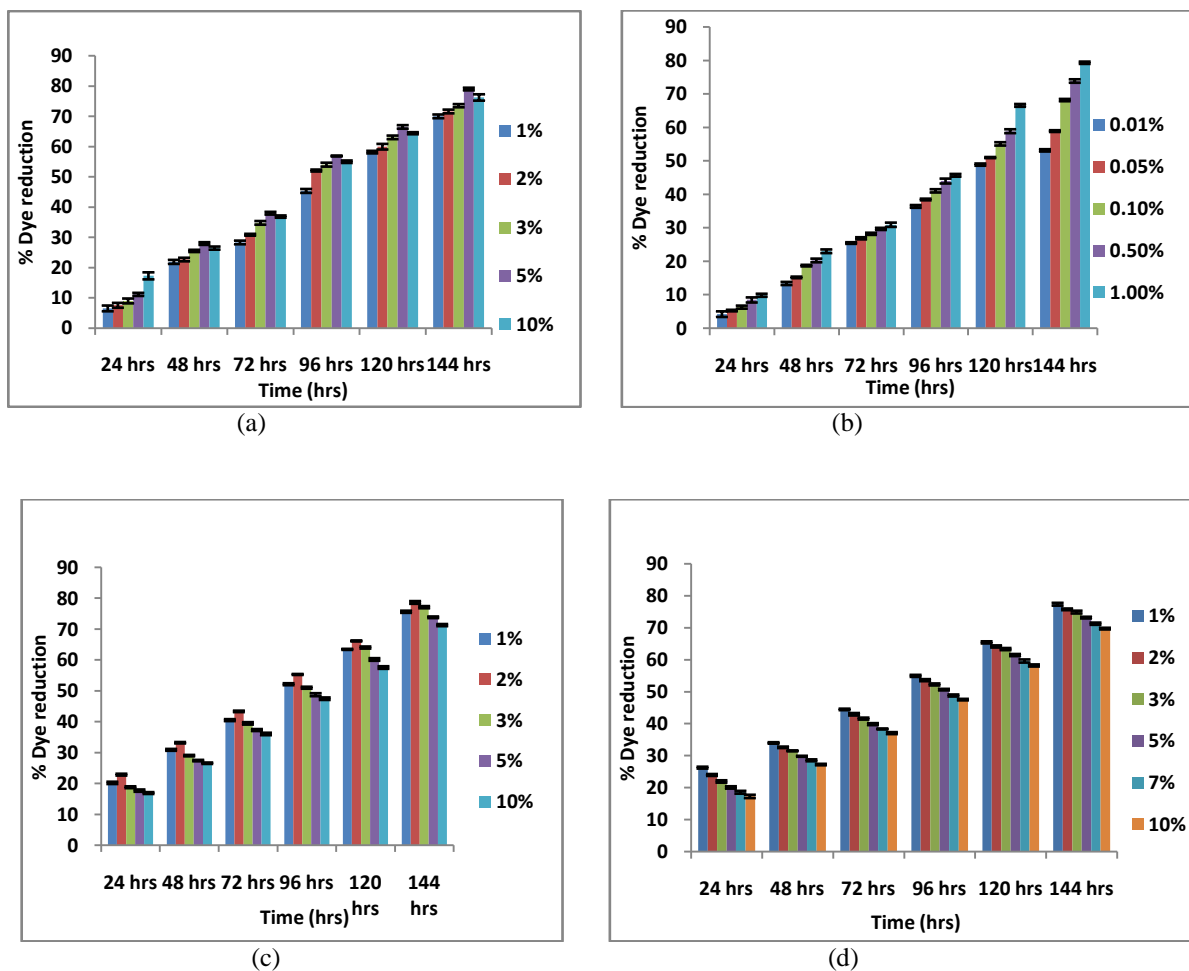


Fig. 2. Effect of different inoculums conc. (a), paraffin substrate conc. (b), crude oil conc. (c) and NaCl conc. (d) on percentage dye reduction as an indicator of paraffin degradation ability of *Leifsonia* sp.

### 3.3. Gas chromatographical analysis of paraffin and crude oil degrading ability of *Leifsonia* sp.:

Paraffin wax degradation potential of the *Leifsonia* sp. was monitored by Gas chromatographic method. Gas chromatogram of paraffin wax shows various paraffin components from heneicosane (C21) to pentatriacontane (C35). Gas chromatogram of paraffin wax treated with *Leifsonia* sp. shows 30 % degradation of this paraffinic wax fraction after incubation of 10 days. Gas chromatogram of paraffinic crude oil shows various paraffin components with a carbon chain length C5 to C29 (Fig.6). Gas chromatogram of paraffinic crude oil treated with *Leifsonia* sp. shows 40% degradation of this paraffinic crude oil fraction after incubation of 10 days. The results are represented in Fig. 3 and Fig. 4.

### 3.4. Statistical analysis:

Values are mean  $\pm$ S.D n=2. In all the treatments there was significant difference between control and treated group at 144 hr (P<0.05).

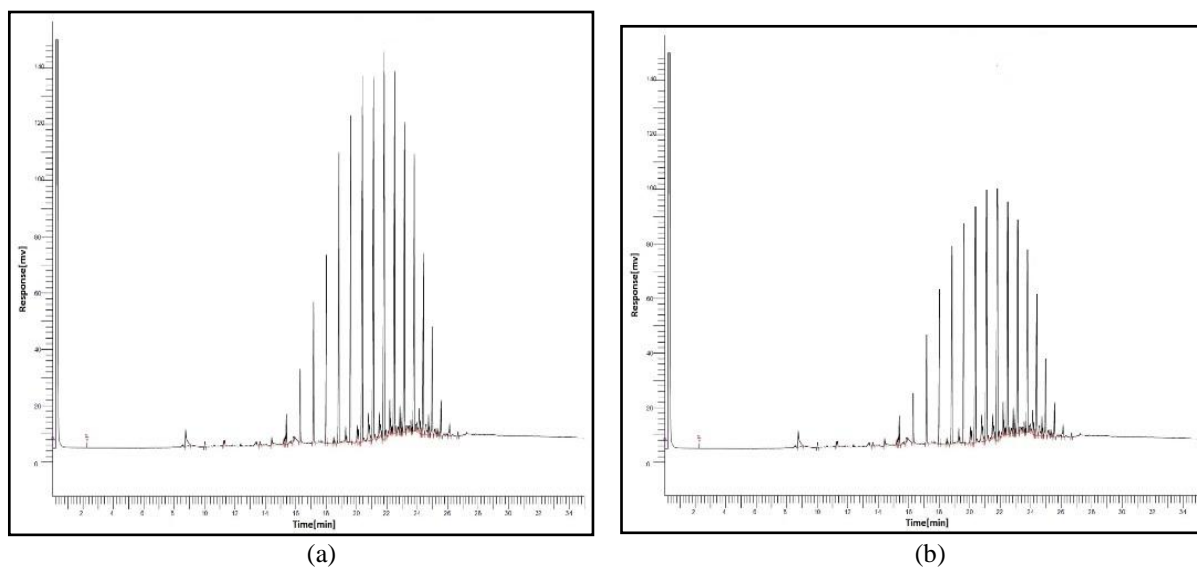


Fig.3. Gas chromatograph of standard paraffin wax (a) and paraffin wax treated with *Leifsonia* sp.

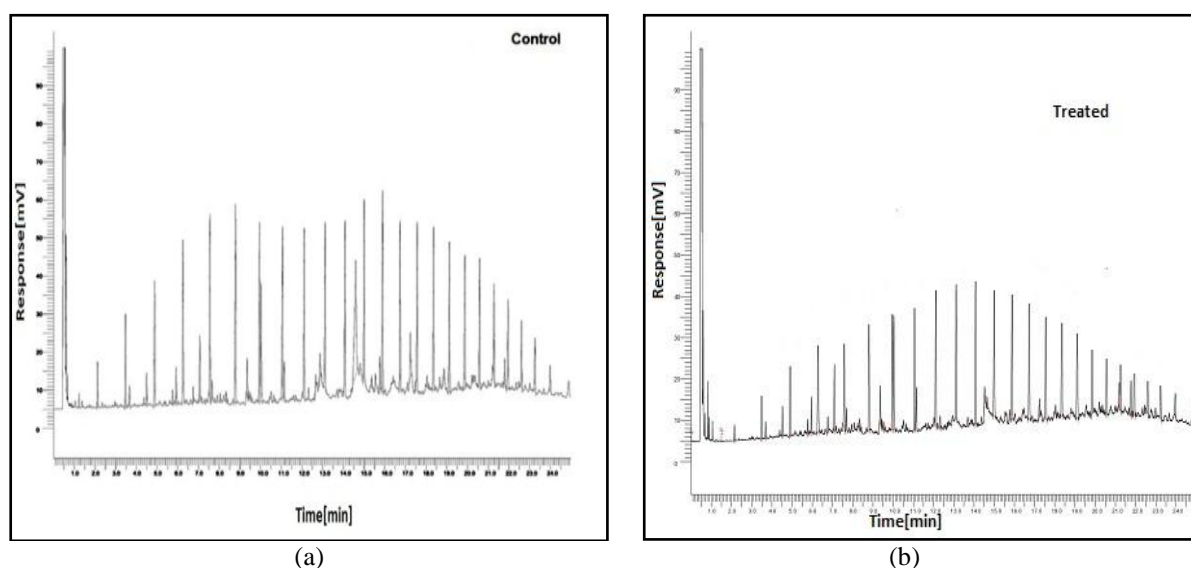


Fig.4.. Gas chromatograph of standard paraffinic crude oil (a) and paraffinic crude oil treated with *Leifsonia* sp.

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## III. CONCLUSION

The role of *Leifsonia* sp. in paraffin degradation was studied. The *Leifsonia* was isolated by enrichment culture technique and identified by Gram staining, Biochemical test and 16s rRNA technique. *Leifsonia* sp. showed maximum dye reduction as an indicator of paraffin degradation at 37 °C, pH 7, 5% inoculum concentration, 1% paraffin substrate concentration, 2% crude oil concentration and 1% NaCl concentration. *Leifsonia* sp. degraded eicosane more when compared to other hydrocarbons and utilized ammonium chloride more than other nitrogen sources. The gas chromatographical analysis showed 30% and 40% degradation of paraffin wax and paraffinic crude oil fraction respectively.

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