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Biodegradation of Naphthalene by *Staphylococcus pasteuri* RD2 Isolated from Oil Contaminated Soil

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A B S T R A C T

Naphthalene is a polycyclic aromatic hydrocarbon (PAHs) consisting of a pair of benzene ring and is considered as priority pollutants among 16 PAHs by US Environmental Protection Agency (USEPA, 1994, 2004) is highly toxic, mutagenic and carcinogenic. Due to its lipophilic property it enters the ecosystem and polluted the environment. A Bacterial strain isolated from oil polluted site of Guwahati Oil Refinery was identified as *Staphylococcus pasteuri* RD2, based on 16SrDNA sequences and phylogenetic analysis. The bacterial strain was able to degrade naphthalene in culture broth in to a number of its transformation products were monitored through FT-IR and GC-MS. Detection of degraded products in the culture broth such as1-cyclohexane-1-carboxylic, 1,2-cyclohexanedicarboxyldehyde, ethylhexyl salicylate, phthalic acid, acetic acid, oxalic acid, propanoic acid, 2-ethyl hexyl salicyclic acid and 1,2-cyclohexanedicarboxyldehyde suggested the degradation of naphthalene by bacterial strain *Staphylococcus pasteuri* RD2.

Introduction

Hydrocarbons are a group of organic compounds composed exclusively of carbon and hydrogen and are either monocyclic- or polycyclic aromatic in structure. Monocyclic aromatic hydrocarbons like benzene, ethylbenzene, toluene and xylene (BTEX) having a single benzene ring, are commonly found in gasoline and are highly volatile substances (Coates *et al.,* 2002). Polycyclic aromatic hydrocarbons (PAHs) contain two or more benzene rings and are relatively less aqueous soluble than monocyclic aromatic hydrocarbons. Aromatic hydrocarbons are considered as highly toxic, and carcinogenic to human health. These pollutants are released from combustion of fossil fuels and hydrocarbons that enter the ecosystem due to

their lipophilic property and polluted the environment. The aromatic hydrocarbons like Naphthalene, Benzene, toluene, ethylbenzene, and xylene are commonly found in crude petroleum and petroleum products are considered as one of the major causes of environmental pollution (Farhadian *et al.,* 2008).

Among the PAHs naphthalene is the simplest one and has been extensively used as a model for biodegradation study. Biodegradation of polycyclic aromatic compounds by aerobic and anaerobic microorganisms has been extensively studied and the biochemical degradation pathways have been elucidated by different groups of authors (Mahajan *et al.,*

1994, Annweiler *et al.,* 2002; Zhang and Young, 1997, Rainer *et al.,* 2004, Meckenstock *et al.,* 2004). Naphthalene consists of a fused pair of benzene rings with chemical formula $C_{10}H_8$. It is a white, volatile, crystalline solid with a characteristic strong odour which is detectable at concentration as low as 0.08 ppm by mass. Main source of naphthalene is coal tar and petroleum distillation. Naphthalene is one of the 16 PAHs classified as priority pollutant by US Environmental Protection Agency (USEPA, 1994, 2004). Naphthalene is toxigenic, mutagenic and carcinogenic which is among the most prevalent pollutants in the environment and as such for assuring human health, removal of naphthalene from the environment is very much important.

Microorganisms play a vital role in cleaning up the environment by degrading the environmental pollutants. Disintegration of the pollutants by bacteria is called biodegradation which is one of the effective ways in the context of removing environmental pollutants. Bioremediation is generally considered a safe and less expensive method for removal of hazardous contaminants and production of non-toxic bi-products (Ward *et al.,* 2003). Recently many authors have reported degradation of naphthalene using different microbial agents with several pathways and degradation metabolites. Mittal and Singh (2009) measured the microbial degradation value of Naphthalene through Gas Chromatography. Chen Lin *et al.,* (2010) worked on biodegradation of naphthalene by strain *Bacillus fusiformis* (BFN). Othman *et al.,* (2010) performed degradation of polycyclic aromatic hydrocarbon using *Micrococcus* sp. *Rhodococus* sp. *Corynebacterium* sp. and *Padiococcus* sp. isolated from municipal sludge. Kafilzadeh *et al.,* (2011) reported degradation of naphthalene by 5 bacterial species belonging to *Staphylococcus* sp.

Corynebacterium sp. *Pseudomonas* sp. *Bacillus* sp. and *Micrococcus* sp. Pawar *et al.,* (2013) characterized gram positive and gram negative bacteria belonging to genus *Micrococcus* sp. *Bacillus* sp. *Staphylococcus* sp. and *Pseudomonas* sp. isolated from marine and petroleum soil samples which showed degradation of naphthalene. B. Prajapati *et al.,* (2016) reported degradation of Naphthalene by *Bacillus subtilis* isolated from petroleum contaminated soil. Here, in our study we report degradation of naphthalene by bacterial strain identified as *Staphylococcus pasteuri* RD2 isolated from Guwahati Oil refinery able to utilize naphthalene as a sole source of carbon and energy. The degradation metabolites of naphthalene in bacterial culture were identified through FTIR and GC-MS spectroscopy.

Materials and Methods

Sample collection

Oil contaminated soil samples were collected in a sterilized polythene bag from Guwahati oil refinery, Assam, India. The soil sample was allowed to dry for two days.

Isolation, characterization and identification of microorganisms

Stock solution was prepared by mixing 1gm of the oil contaminated soil sample in 100ml of sterilized distilled water and then subjected to serial dilution from 10^{-2} to 10^{-9}. Plating was done in Nutrient agar media with pH 7.3 and then incubated at 37°C. After 7 days of incubation period, different colonies were isolated which were again transferred in nutrient agar plates supplemented with 100mg naphthalene with pH 7.3 for preliminary screening to isolate potent strain with naphthalene degrading ability. It was again incubated at 37°C for another 7 days.

The isolated strain was characterized through different biochemical tests according to Cappuccino and Sherman (1983) and Bergy's manual of systematic bacteriology (1984).

For identification of the isolated strain, DNA was isolated from the culture. Its quality was evaluated on 1.0% Agarose Gel. A single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 base pairs was observed when resolved on Agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genebank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs. Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7. Sequencing of the 16S rDNA was carried out by Eurofin Genomic, Bangalore, India.

The morphology of the isolate was observed by viewing under Scanning Electron microscope (SEM).

Growth determination of the isolate

To determine the growth of the isolate, different factors like temperature, pH, salinity and naphthalene concentration was taken under consideration. Experiments were performed to examine the effect of these factors on the growth of the isolate and degradation of naphthalene. The isolate was inoculated in nutrient broth and incubated at different temperatures of 25, 27, 30, 32, 35, 37 and 40°C. pH of the medium for the experiment was adjusted to 6, 6.3, 6.5, 6.8, 7, 7.3, 7.5 and 8 using 1M HCl and 1M NaOH. Salinity used was 0, 1, 4, 7, 10, 12 and 15/L. 1000 ppm of stock solution of naphthalene dissolved in n-hexane was prepared and from it 60 ppm, 80 ppm, 100 ppm, 150 ppm and 200 ppm naphthalene concentration was used for the experiment.

Naphthalene degradation experiment

For study of bacterial growth and biodegradation of naphthalene, 1ml of bacterial suspension inoculated in each of the two 250 ml conical flask, containing 100ml of nutrient broth supplemented with 60 ppm naphthalene and incubated at 37°C.

Bacterial growth and naphthalene degradation were monitored periodically at an interval of 24 hrs in UV-Vis spectrophotometer at 600nm and 310 nm respectively. Culture broth supplemented with naphthalene without inoculation of naphthalene was treated as control.

FTIR and GC analysis for naphthalene degradation

After 7 days of incubation, culture broths containing 60 ppm naphthalene were centrifuged separately at 15000 rpm for 15 minutes. The supernatant were extracted twice in 25ml n-hexane and filtered through Whatman No.42 filter paper. The filtrate was was dry under vacuum and used for FT-IR and G-C-MS analysis. For FT-IR analysis, a small amount of KBr was mixed with the dried sample and pellets were made to record the FT-IR spectra of the intermediate metabolites. 60 ppm of naphthalene without inoculation of bacteria was taken as a control in ATR mode for FTIR analysis.

For GC-MS analysis, dried sample was dissolved in 4ml n-hexane and transferred in a 5ml glass vile and then taken for GC-MS analysis (Fig. 6).

Results and Discussion

Isolation of the bacteria

A total number of 13 different colonies were isolated from the oil contaminated soil sample, labeled as R1 to R13. Among this only RD2 was recorded as having optimum growth in culture broth supplemented with 40 ppm, and 60 ppm naphthalene. Thus, RD3 was considered for identification and further experiments for naphthalene degradation in this study.

Characterization of isolated Naphthalene degrading strain

The biochemical and morphological study revealed that the bacterial strain RD2 was Gram positive, spherical and non-motile. It showed positive Catalase, Citrate and Urease test and was able to ferment Glucose, Lactose, Sucrose and Dextrose. It showed negative test for Methyl Red, Vogues Proskeur, Oxidase, Coagulase, Casein and Starch hydrolysis, Nitrate Reduction and Gelatin liquefication (Table 1).

Bacterial identification by 16S rDNA analysis

Amplification of the fragment of 16S rDNA was done by 27F and 1429R primers which resulted in the production of 15000 base pairs (Fig. 1a). From forward and reverse sequence data consensus sequence of 16S rDNA gene was generated which was used to carry out BLAST with the NCBI genebank database. First ten sequences were selected based on maximum identity score and aligned using multiple alignment software programs and the phylogenetic tree was constructed using MEGA 7 (Fig. 1b). From the analysis, the isolated strain has been identified as *Staphylococcus pasteuri* with 100% identity.

SEM studies

SEM studies revealed the topography and surface structure of the bacteria, *Staphylococcus pasteuri* RD2 (Fig. 2) from which the spherical structure of the bacteria was evident.

Factors affecting the growth of naphthalene degrading bacteria

To determine the growth of bacteria, it was incubated at different temperatures ranging from 25, 27, 30, 32, 35, 37, 40 and 45°C. Within 24 hrs of incubation the bacteria shows maximum growth at 37°C but after 40°C the growth of the bacteria decreases due to reduction in metabolic activity which shows that 37°C is more suitable for the growth of bacteria (Fig. 3a).

The pH of the growth of bacteria showed more effective on 7.3. With increase or decrease of pH greatly affects the growth of bacteria (Fig. 3b).

The optimum salinity for the growth of bacteria ranges from 0 to 4 mg/L of NaCl. With increase in salinity above 4, metabolic activity of the bacteria greatly reduces (Fig. 3c).

The effect of naphthalene concentration on the growth of bacteria showed that up to 80 ppm naphthalene, bacteria shows increase in growth (Fig. 3d) which suggests the naphthalene degradation and further increase in naphthalene concentration resulted in decrease of bacterial growth. Thus, 40 ppm and 60 ppm naphthalene concentration was considered for further experiment.

Table.1 Biochemical characteristics of naphthalene degrading strain

Biochemical test	Results
Catalase test	Positive
Citrate utilization test	Positive
Urease test	Positive
Glucose fermentation test	Positive
Lactose fermentation test	Positive
Sucrose fermentation test	Positive
Dextrose fermentation test	Positive
Methyl Red test	Negative
Vogues Proskeur test	Negative
Oxidase test	Negative
Coagulase test	Negative
Casein hydrolysis test	Negative
Starch hydrolysis test	Negative
Nitrate reduction test	Negative
Gelatin liquefication test	Negative

Fig.1 (a) gDNA 16S PCR amplicon ladder specification

Base pairs
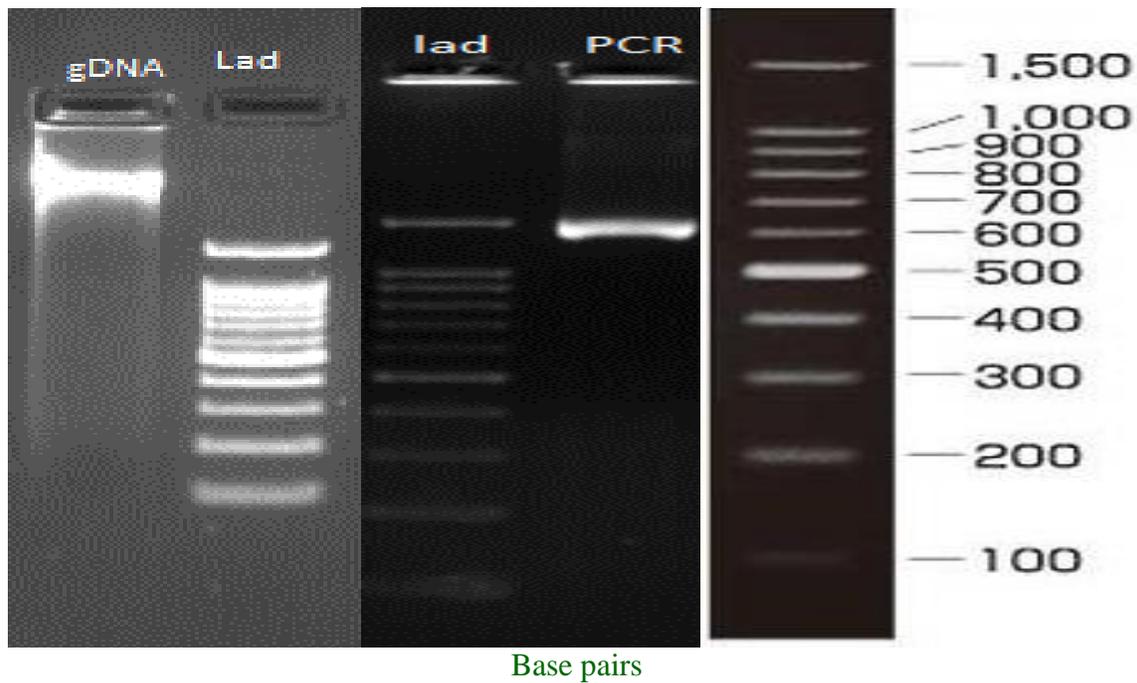
Fig.1 (b) Phylogenetic tree of *Staphylococcus pasteuri* strain labeled as RD2
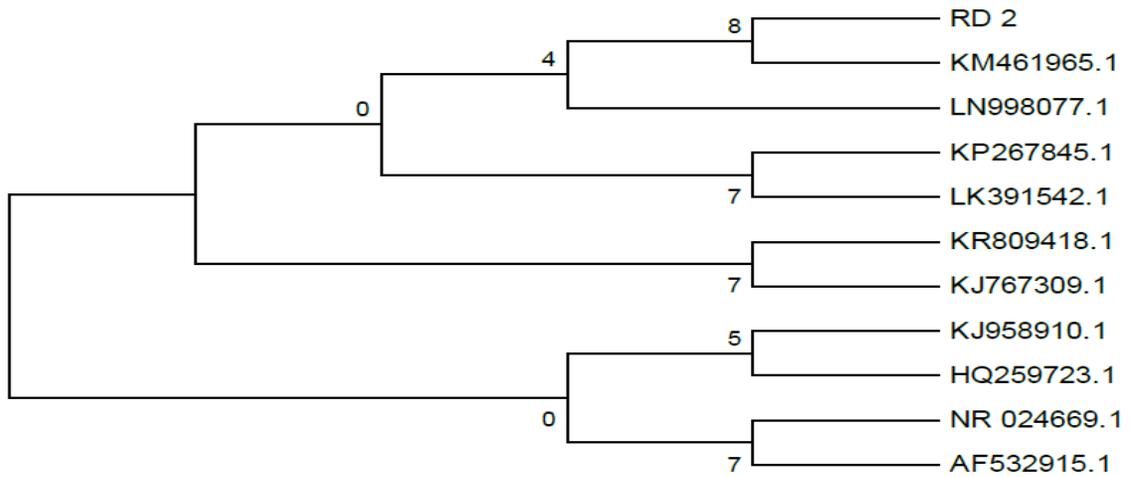
Fig.2 (a) and 2(b) SEM images of *Staphylococcus pasteuri* in colonies and chains


2(a)   2(b)
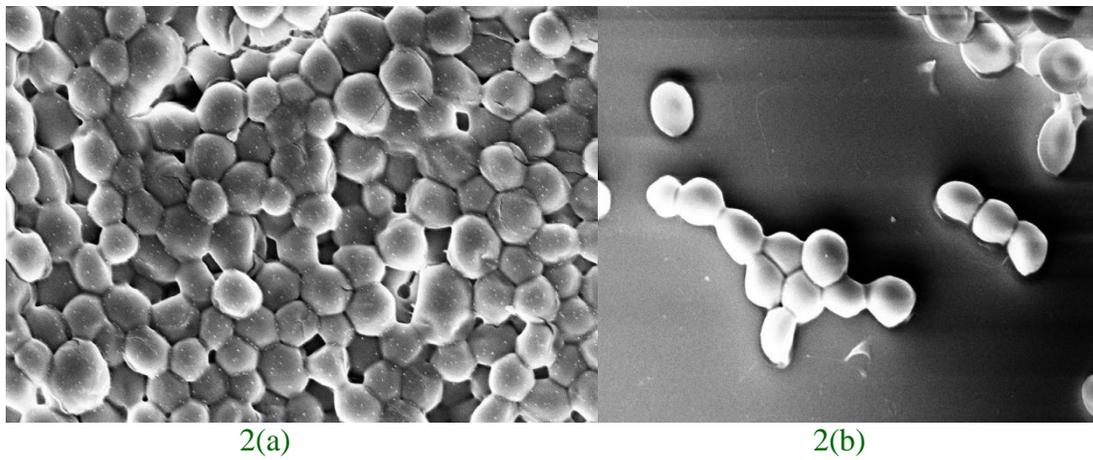
**Fig.3 (a)** Effect of temperature on bacterial growth; **(b)** Effect of pH on bacterial growth
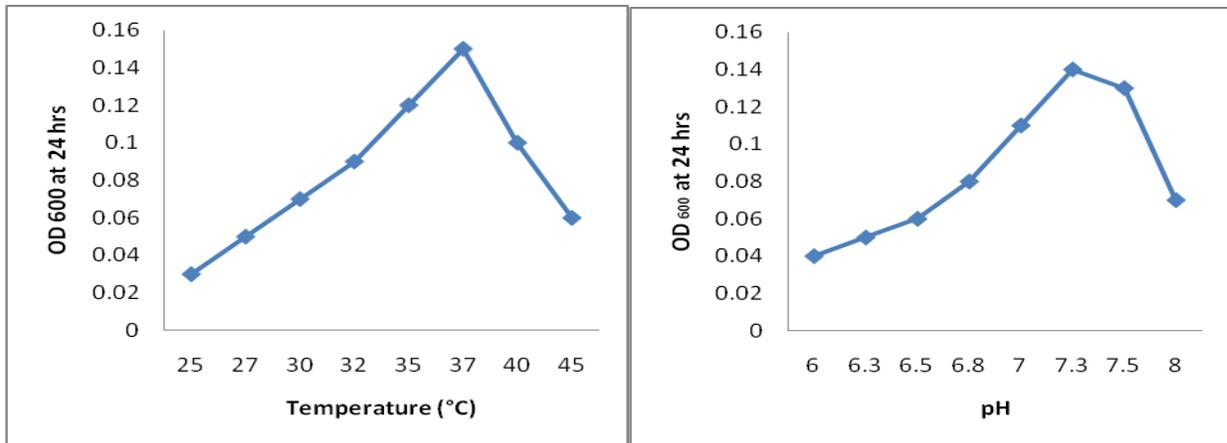
**Fig.3 (c)** Effect of salinity on bacterial growth; **(d)** Effect of different concentration of naphthalene on growth
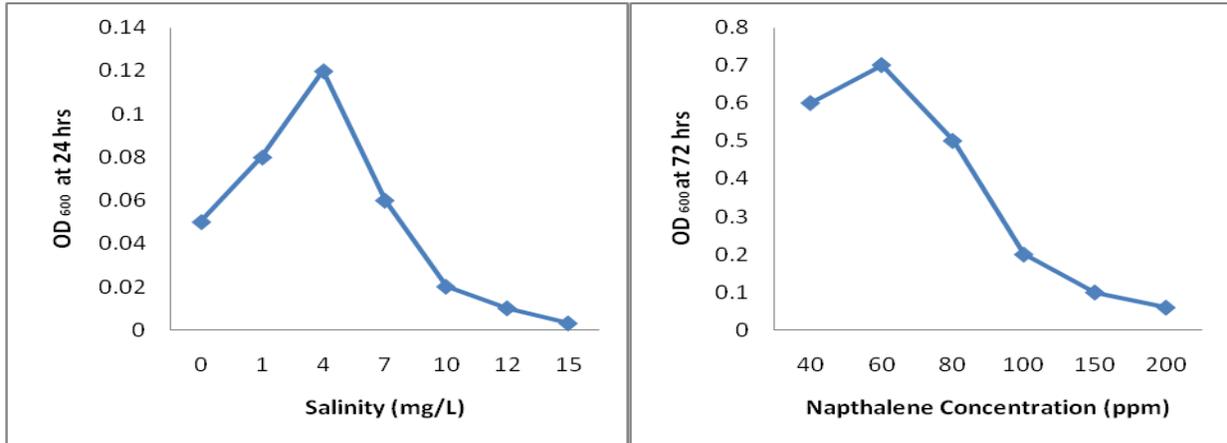
**Fig.4** Growth **(a)** of *Staphylococcus pasteuri* and naphthalene degradation **(b)** in 60 ppm naphthalene broth

4(a)   4(b)
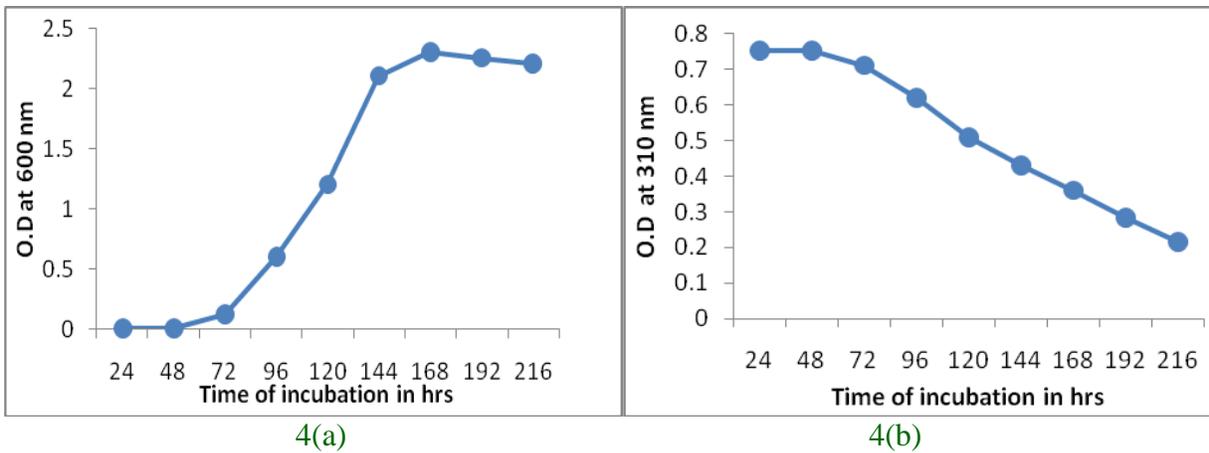
**Fig.5 (a)** FT-IR spectra of 60ppm naphthalene dissolved in hexane (control) taken in ATR mode
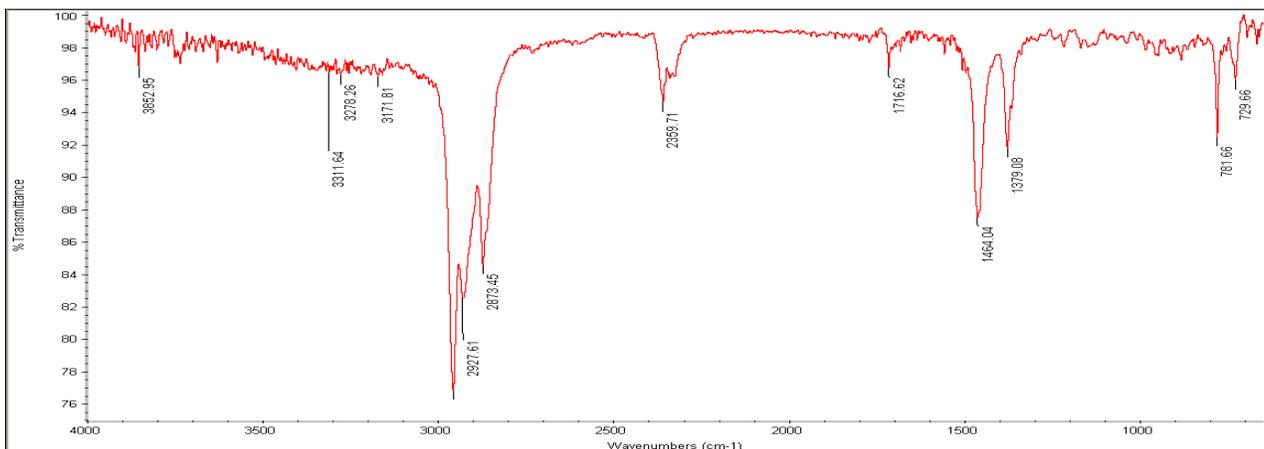
Fig.5 (c) FT-IR spectra of naphthalene treated with *Staphylococcus pasteuri* RD2
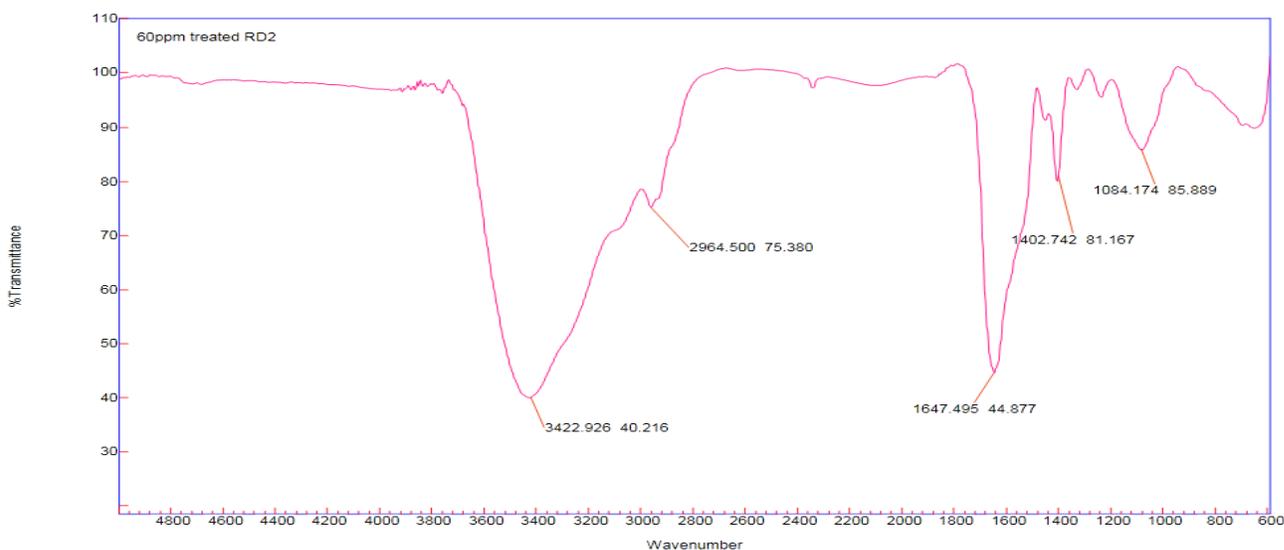
5(b)   5(c)

Fig.6 GC-MS of naphthalene degradation by *Staphylococcus pasteuri* RD2
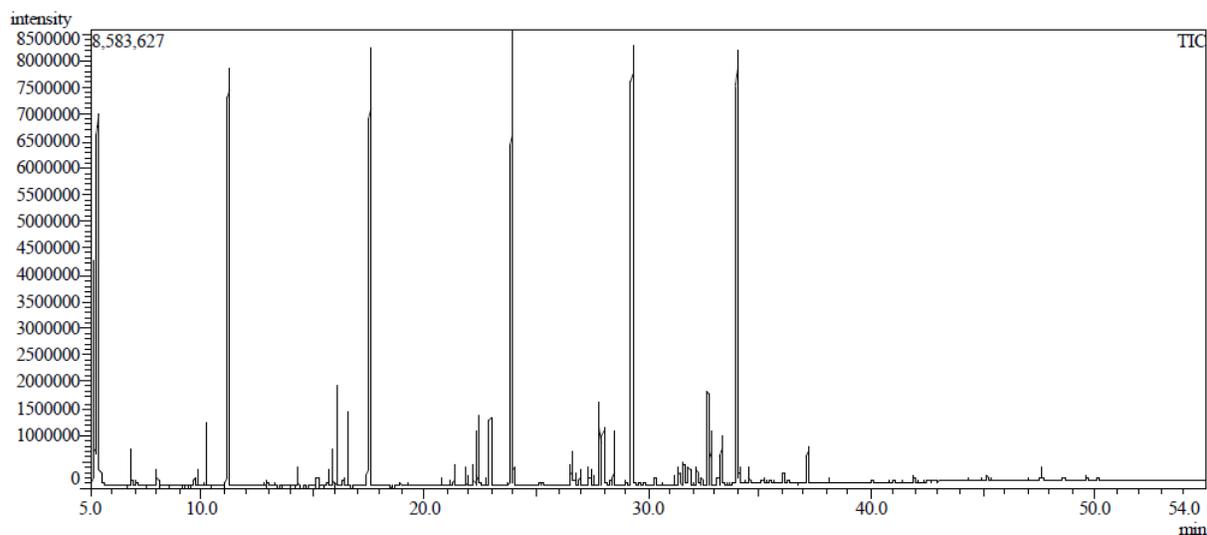
Degradation of naphthalene by *Staphylococcus pasteuri* strain

By utilizing naphthalene as the sole source of carbon and energy, the bacterial strain *Staphylococcus pasteuri* RD2 was able to grow on the culture media. The bacterial growth after lag phase (48 hs) increased exponentially indication of utilization of naphthalene as carbon source for cell growth (Fig. 4a). Residual naphthalene in the culture broth decreased (OD_{310}) gradually from 48th

hour indicated the metabolism of naphthalene in the culture broth by the bacterial strain (Fig. 4b). Thus there is a correlation between the cell growth and breakdown of naphthalene. The FT-IR spectra of the control, 60 ppm Naphthalene taken in ATR mode (Fig. 5a) show peaks at 3311, 3278, 2927, 2873, 1716, 1464 and 1379 cm^{-1} which corresponds to N-H stretch, O-H stretch, C-H stretch, C=O stretch, C=C stretch and CH_3 bending. The results of FT-IR spectra of hexane extract of culture broth supplemented

with 40ppm naphthalene (Fig. 5b) exhibited characteristic peaks at 3450and 2921 cm^{-1} indicating free O-H stretch, peak at 1647cm^{-1} suggesting NH$_2$ bending vibration and peaks at 1402, 1056 showing CH$_3$, CH$_2$ bending deformation and C-N stretching. Similarly, 60 ppm naphthalene culture broth (Fig. 5c) also showed characteristic peaks of free O-H stretch and CH$_2$, CH strong vibrations, NH$_2$ bending vibration, CH$_3$, CH$_2$ bending deformation and C-N stretch around 3422, 2964, 1647, 1402 and 1084cm^{-1} respectively. The absence of such peaks at these regions in the control after treatment indicates the degradation of naphthalene by *Staphylococcus pasteuri*.

Annweiler *et al.,* (2000) reported 2-carboxycinnamic acid, phthalic acid and benzoic acid as transformation products of naphthalene. They concluded phthalic acid as second transformation product of naphthalene with 2-carboxy functionality, arise from degradation of 2-carboxycinnamic acid, that arise through ortho-type cleavage of 1,2-dihydroxy naphthalene or meta-type of 2,3-dihydroxy naphthalene. In our study we have not detected 2-carboxycinnamic acid and benzoic acid but detected phthalic acid (RT 35.15 min.), may arise from further degradation of 2-carboxycinnamic acid. 2-carboxycinnamic acid was not detected in our study, probably it has been instantly transformed to phthalic acid before extraction (Doley*et al.,* 2017). R.U. Meckenstock *et al.,* (2004) detected 2-carboxycyclohexylacetic acid that was probably generated by cleavage of a C$_2$-fragment via beta-oxidation of other metabolite, a C$_{11}$H$_{16}$O$_4$-diacid. They reported that naphthalene, methyl naphthalene and tetraline degraded via a novel central degradation pathway with cyclohexanoic compounds as intermediate. In our study we have detected 1-Cyclohexane-1-Carboxylic acid (RT 23.35 min) as ring cleavage product along with acetic acid (RT 19.35 min), propanoic acid (RT 30.26) and oxalic acid (RT 30.26 min) as other metabolites of naphthalene. The enzyme system of microorganisms convert the aromatic compounds in to ortho or paradihydroxy phenol derivatives, followed by cleavage of the ring to aliphatic acids. However, a large number of microorganisms able to modify aromatic compound either by hydroxylation or elimination of substituent groups without causing ring fission (Evans 1963). Benzoate, salicylate etc are the common intermediates in degradative pathways of more complex aromatic compounds. These acids are converted to catechol by bacterial oxidases enzyme before ring fission. Ethylhexyl salicylate (RT 30.09 min.) and 1,2-Cyclohexanedicarboxyldehyde (RT 20.99 min.) are the another naphthalene degradative products detected in our study, reveals that the bacterial strain *Staphylococcus pasteuri* RB2 efficiently degrade naphthalene to its derivatives.

Acknowledgement

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