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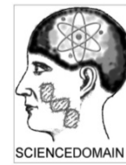

Investigation the Consumption of Aromatic Hydrocarbons by *Anoxybacillus rupeinsis* Ir3 (JQ912241) using FTIR and HPLC Analyses

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MHAJ and AMAF designed the study and wrote the protocol. Author MSM wrote the first draft of the manuscript and managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To confirm the ability of *Anoxybacillus rupiensis* strain Ir3 (JQ912241) to utilize the aromatic compounds using FTIR and HPLC analyses.
Study Design: Experimental study.
Place and Duration of Study: Department of Biotechnology, College of Science, Al-Nahrain University. Baghdad, Iraq, between December 2012 and April 2013.
Methodology: *Anoxybacillus rupiensis* strain Ir3 (JQ912241), a newly thermophilic bacterium, isolated from hydrocarbon contaminated soil in Iraq, was used. Analytical experiments include HPLC (High performance liquid chromatography) and FTIR (Fourier transform infrared) were used to determine the ability of this strain to utilize the aromatic compounds.
Results: The quantitative analysis (HPLC) indicated that this bacterium showed as much as 99.62% consumption of carbazole, 99.4% of ρ-nitrophenole, 97.73% of nitrobenzene and 98.89% of naphthalene. Qualitative analysis of FTIR spectra showed that *A. rupiensis* strain Ir3 (JQ912241) has the ability to convert carbazole to anthranilic acid, indicating the presence of the *meta* cleavage

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enzyme, this also confirmed by using 2, 3-dihydroxybiphenyl through converting the colony color on Luria-Bertani (LB) and minimal agar plates to brown.

Conclusion: The good ability of *A. rupiensis* strain Ir3 to utilize the studied aromatic hydrocarbons make it a good candidate as biocatalist. Its ability to convert carbazole to anthranilic acid and to oxidize catechol to 2-hydroxymuconic semialdehyde is through *the meta cleavage enzyme*.

Keywords: Thermophilic bacteri; Anoxybacillus; biodegradatio; nitroaromatic; PAH.

1. INTRODUCTION

Anoxybacillus (A means without; Oxy is shortened from oxygenium, oxygen; bacillus is a small rod; *Anoxybacillus* is a small rod living without oxygen). Cells are rod-shaped and straight, often ranged in pairs or chains, with rounded ends. Gram positive, endospores are round and resistant to heat and freezing. Spores are located at the end of the cell. There is not more than one spore per cell [1]. The description of this genus was corrected to be aerotolerant anaerobes or facultative anaerobes.

Inan et al. reported that *Anoxybacillus* species are widely distributed and readily isolated from geothermal heated environments, with a continually increasing industry interest in their thermostable gene product. Therefore, isolating the new strains of this novel bacterial genus is not a taxonomical concern, but also a necessity in order to exploit its biotechnological potential completely [2].

Anoxybacillus rupiensis sp. Nov. was isolated from hydrocarbon-contaminated soil in Iraq [3], and it is an efficient strain for utilizing aromatic compounds and the identity of the carbazole-degrading culture. It was characterized using biochemical tests, microscopic observation, and a determination of its 16S rDNA gene. The culture is a facultative Gram positive or (to gram variable) long rod that form, medium sized, smooth, round colonies with cream color, regular and complete margins on LB agar plate. Cell of this strain is appeared as motile, strictly aerobic, thermophile. Most cells occur in exponential growth phase singly or in chain. Terminal endospores are observed. Obligate thermophilic growing between 40 and 70℃ (optimum 55-65℃) and in the pH range from 5.0-9.0 (optimum 7.0), indole is not produced, the voges-proskauer reaction is negative, catalase and oxidase reaction are positive and methyl red test is negative. The 16S rRNA gene sequence of this bacterium compared with a database of NCBI with BLAST program has 1500 bp and 97% similarity to *Anoxybacillsus rupiensis* (HQ 696615.1).These data indicated that this carbazole-degrading bacterium can be identified to the genus and species level as *Anoxybacillus rupiensis* (JQ 912241) [4].

Sites contaminated by xenobiotics need urgent remedial solutions. The search for which has revealed a diverse range of bacteria that can utilize these xenobiotics as substrates, often mineralizing them or converting them into harmless products, and in the process helping to clean up the environment [5].

Aromatic compounds can be defined as organic molecules that containing one or more aromatic rings, specifically benzene rings. Different aromatic compounds co-exist as complex mixtures in petroleum refining and distillation sites [6,7]. Aromatic hydrocarbons enter the global environment through human activities such as crude oil spillage, fossil fuel combustion and gasoline leakage as well as natural inputs like forest fire smoke and natural petroleum seepage. These hydrocarbons comprise simple aromatics like benzene and toluene as well as polycyclic aromatic hydrocarbons (PAHs) from naphthalene to pyrenes, as well as myriad alkyl-substituted isomers. Annually, large inputs of such compounds affect both aerobic and anaerobic environments such as aquifers, surface freshwater bodies, soils, and terrestrial and marine sediments [8]. This article aimed to trace the consumption of aromatic hydrocarbons by *Anoxybacillus rupeinsis* Ir3 (JQ912241) using FTIR and HPLC analyses.

2. MATERIALS AND METHODS

2.1 Bacterial Strain

The bacterium used in this study (*Anoxybacillus rupeinsis* Ir3 (JQ912241)) is a novel strain able to utilize aromatic compounds. It was isolated from hydrocarbon-contaminated soil [3] and obtained from the Department of Biotechnology at Al-Nahrain University.

2.2 Tracing the bacterial consumption of aromatic compounds

Chemically defined media [9] (100 ml in 250 ml flask) containing 1 mM of the different aromatic compounds (naphthalene, carbazole, ρ-nitrophenol and nitrobenzene) were inoculated with a fresh culture of efficient bacterial isolate *A. rupiensis* strain Ir3 (JQ912241) and incubated at 55℃ for 7 days with shaking at 150 rpm. After incubation, centrifugation (13000 rpm) for 10 min at 4℃. The resulting cell-free supernatant was analyzed by HPLC for tracing the consumption of the aromatic compounds.

2.2.1 HPLC analysis

Cell- free culture supernatant was analyzed to trace the utilization of different aromatic compounds, naphthalene, the N-containing groups, namely nitro aromatics, and N-heterocyclic compounds by using HPLC system. 10 µl of supernatant were injected into a C18 column (5 µM, 4.6*250 mm, supelcosil Lc-2010AHT) and the following conditions were followed:

The solvent system used (60% acetonitrile in H_2O) was run at a flow rate of 1 ml/min., for UV detection of naphthalene, carbazole, ρ-nitrophenol and nitrobenzene, the UV detector was adjusted to 295 nm. Under these conditions, the observed retention time for authentic samples of naphthalene, carbazole, ρ-nitrophenol and nitrobenzene were 3.5 min, 3 min, 3 min, and 3 min, respectively.

2.2.2 FTIR analysis

One hundred milliliter of CDM in 250 ml Erlenmeyer flasks, supplemented with crude oil (sterilized by tyndallization) 0%, 1%, 2%, 5%, 10%, 20% and 30% (v/v). All the flasks were inoculated with 1% of fresh culture of efficient bacterial isolate *A. rupiensis* strain Ir3 (JQ912241), and the inoculated flasks, as well as uninoculated controls were incubated in a shaker incubator (150 rpm) at 55℃ for 30 days. Growth was monitored daily by plate count on LB agar medium.

2.2.2.1 Extraction procedure

Each inoculated flask and uninoculated controls were extracted twice by using separating funnel with 50 ml of Diethyl ether. The extracts were combined, dried over (1-2) gm of Na_2SO_4, and filtered through (Whitman no.1) filter paper. The extracts were kept in the refrigerator until the time of analysis with Fourier Transform Infrared Spectrophotometer (prestige 21). Polystyrene was used as the standard material for the calibration of the instrument [10].

FTIR analysis was used to follow the changes in the functional groups.

2.3 Meta- Cleavage Enzyme Test

The *Meta* cleavage enzyme activity was examined by monitoring the conversion of 2, 3-dihydroxybiphenyl to 2-hydroxy-6-Oxo-6-phenyl-hexa-2, 4-dienoic acid. *A. rupiensis* strain Ir3 (JQ912241) was grown on LB agar plates (containing 1mM carbazole) and CDM agar plates and sprayed with 2, 3-dihydroxylphenyl-acetone solution. A change in colony color from yellow to brown indicating *meta*-cleavage activity was sought [11].

3. RESULTS AND DISCUSSION

3.1 Tracing the *Anoxybacillus rupiensis* Strain Ir3 (JQ912241) Consumption of Pure Aromatic Compounds

The consumption of 1 mM carbazol, ρ-nitrophenole, nitrobenzene as the sole carbon and nitrogen source and naphthalene as the sole carbon source was traced by HPLC in cell-free supernatants of cultures. The *A. rupiensis* strain Ir3 (JQ 912241) showed clear growth with the four aromatic compounds.

The HPLC analysis indicated that carbazole showed as much as 99.62% consumption depending on the area of the peaks eluted at 3 min as shown in Figs. 1a, b. ρ-nitrophenol showed as much as 99.4 % consumption depending on the area of the peaks eluted at 3 min as shown in Figs. 2a, b whereas nitrobenzene showed as much as 97.73% consumption depending on the peaks eluted at 3 min as shown in Figs. 3a, b, and naphthalene showed as much as 98.89% consumption depending on the peaks eluted at 3.5 min as shown in Figs. 4a, b.

It was considered the potential of *A. rupiensis* strain Ir3 (JQ912241) for degradation of carbazol, ρ-nitrophenol, nitrobenzene, and naphthalene. Carbazole and other nitrogen compounds are oxidized to nitrogen oxides throughout petroleum combustion and causes

acid rain. During petroleum upgrading, carbazole, as other nitrogen- containing compounds, reduces the efficiency of the hydrodesulfurization (HDS) process of diesel feed stocks because of the deactivation of the catalyst. Therefore, the elimination of nitrogen containing molecules from petroleum distillates enhances the quality of the final fuels [12,13].

Several methods to eliminate nitrogen from fuels have been devised and the most widely used is the hydrodenitrogenation (HDN) at high temperature and pressure, carried out simultaneously with HDS. An alternative to HDN for carbazole elimination is the bio-treatment of diesel feed stocks. Some bacterial strains have been reported to be able to metabolize carbazole [14,15] and the most studied strain is *Pseudomonas resinovorans* CA10 [16]. This strain carries the carbazole degrading enzyme coded in a gene cluster (car) that resides in a large plasmid, pCAR1 [11].

3.2 FTIR Analysis

Anoxybacillus rupiensis strain Ir3 (JQ912241) was grown in 100 ml CDM supplemented with crude oil (0%, 1%, 2%, 5%, 10%, 20%, and 30%), in addition to a negative control (crude oil only). The bacterium showed high resistance and still life until the time of analysis and there were great differences among the control and the test flasks appeared through the FTIR analysis (data are not shown).

It was also possible to verify alteration in superficial film oil, which indicates possible bio-degradation process. Some observed alteration suggests the capability of bioemulsifer production by this strain through the formation of oil drop coalescence. In another word, during the incubation, the crude oil morphology went through three obvious changes. In the first, the crude oil either appeared as a large stretch covering the upper part of the aqueous medium or attached to the glass wall. Later, the large stretch or attachment of petroleum turned gradually into disperses oil drops. Immediately after that, the oil drops disappeared even without shaking. This phenomenon is closely related to the emulsification.

3.2.1 Utilization of carbazole in oil mixture

Since *A. rupiensis* strain Ir3 (JQ912241) degrades carbazole in experiments employing model compounds, it was of interest to determine if similar results could be obtained in experiments using petroleum where a complex of chemicals is present and exposure to petroleum could be potentially damaging to biocatalysts. To test the ability of *A. rupiensis* strain Ir3 (JQ912241) to degrade carbazole (nitrogen compound) in a complex oil mixture, the FTIR analyses (Figs. 5a, b) were conducted with the same flasks that is containing 30% crude oil and negative control.

The characteristic absorption bands (KBr disc cm^{-1}): at 3549, 3475 and 3417 correspond O-H stretching of anthranilic acid and catechol which were produced from the CAR degradation. The spectrum also showed bands at 2954, 2924 and 2854, which attributed to C-H aliphatic stretching from the different hydrocarbons in crude oil. The three bands are as follows: bands at 1620 due to C=C stretching for benzene ring, bands at 1458, 1377 due to C-H aliphatic banding, while the band at 1118 could be attributed to C-O stretching of the phenol group.

The results of FTIR analysis of biotreated crude oil sample illustrate clearly that *A. rupiensis* strain Ir3 is capable of removing carbazole from crude oil.

The cleavage of the aromatic ring is a key reaction in the oxidation of aromatic compounds. One of the most frequently encountered key dihydroxy aromatic pieces before the occurrence of ring cleavage is catechol. These results suggest that *A. rupiensis* strain Ir3 has genes encoding a catechol catabolic enzyme.

3.3 Meta- Cleavage Enzyme Test

A. rupiensis strain Ir3 (JQ912241) colonies grown on LB (containing 1 mM carbazole) and CDM agar plates were examined for the presence of *meta*-cleavage enzyme activity for monitoring the conversion of 2, 3-dihydroxybiphenyl to brown metabolite, 2-hydroxy-6-Oxo-6-phenylhexa-2, 4-dienoic acid (HOPDA) (oxidation products of the aromatic substrate). *A. rupiensis* strain Ir3 (JQ912241) may produce HOPDA on these media, and this may also suggest a constitutive expression of the carbazole degradation trait. Hamzah and Al-Baharna showed that *P. cepacia* ATCC29351 possesses the genetic capacity for enzyme of both *ortho* and *meta* cleavage pathway of benzoate degradation, also Cunha et al. found that *Bacillus* strains contain the genetic capacity for enzymes involved in cleavage pathways of aromatic degradation [17,18].

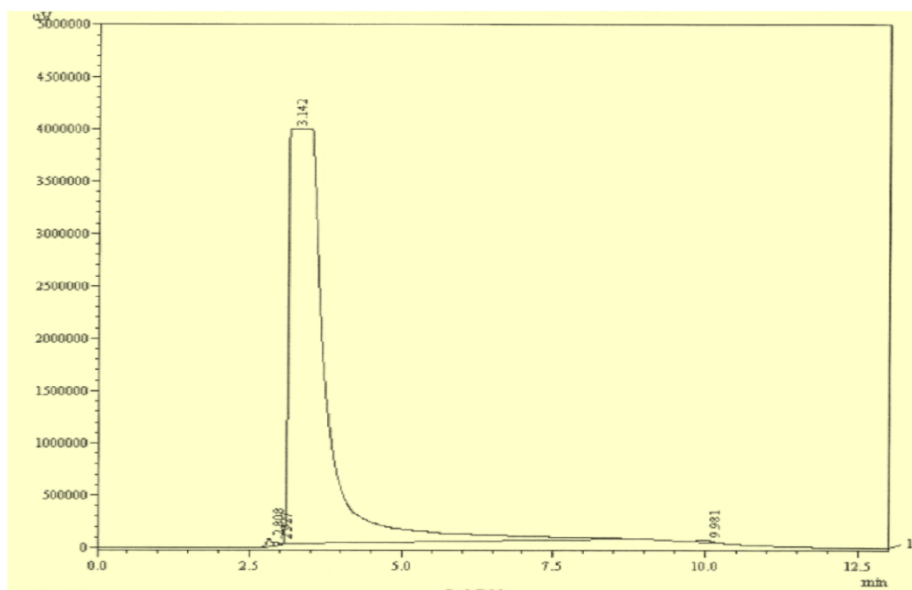

Fig. (1-a). HPLC chromatogram showing retention time (3 min) for authentic sample of carbazole. Absorbance was followed at 295 nm

Fig. (1-b). Tracing the consumption of carbazole by HPLC in cell-free supernatant of bacterial culture

Hence, and to the researcher's best knowledge, this is the first study on the ability of this genus to mineralized carbazole. Moreover, it is noteworthy that although all of the known carbazole-utilizing bacteria are Gram-negative bacteria, strain Ir3 was identified as a Gram-positive *A. rupiensis*, in addition to its ability to degrade a variety of aromatic compounds, including ρ-nitrophenol, nitrobenzene (nitroaromatic compounds) and naphthalene (polyaromatic hydrocarbon). Such results are similar to those of Inoue et al. who found that a Gram positive *Nocardioides* sp strain IC177 was capable of mineralizing carbazole [15]. *Nocardioides* species have previously been shown to degrade a variety of aromatic compounds, including 2, 4, 6-trinitrophenol, 2, 4, 5- trichlorophenxyacetic acid, ρ-nitrophenol, phenanthrene, and dibenofuran. Their results also indicated that the degradation pathway for carbazole to anthranilic acid in the strain IC117 was similar to that of the Gram-negative *Pseudomonas resinovoran* CA10.

The results suggest that the degradation pathway for CAR to anthranilic acid in this strain may be similar to strain CA10. The capability of bacterial strain to degrade CAR to anthranilic acid is one of the most important characteristics since anthranilic acid is easily degradable which regarded as harmless substance. The anthranilic acid is assimilated for the tryptophan biosynthesis pathway by various organisms [19]. Zaki found that the typical pathway for metabolizing an aromatic compound like phenol is to dihydroxylate the benzene ring to form a catechol derivative followed by open the ring through ortho and meta oxidation [20]. Also Omokoko et al. indicated that *Geobacillus sterothermophilus* is able to utilize phenol as a sole carbon source. A DNA fragment encoding a phenol hydroxylase catalyzing the first step in the *meta*-pathway has been isolated previously. Catechol is either oxidized in a reaction catalyzed by catechol-1, 2-oxygenase which is described as an *ortho* pathway, or is oxidized in a reaction catalyzed by catechol-2, 3-dioxygenase (C23O) the *meta* pathway to 2-hydroxymuconic semialdehyde. The final products of both the pathways are molecules that can enter the tricarboxylic acid cycle [21].

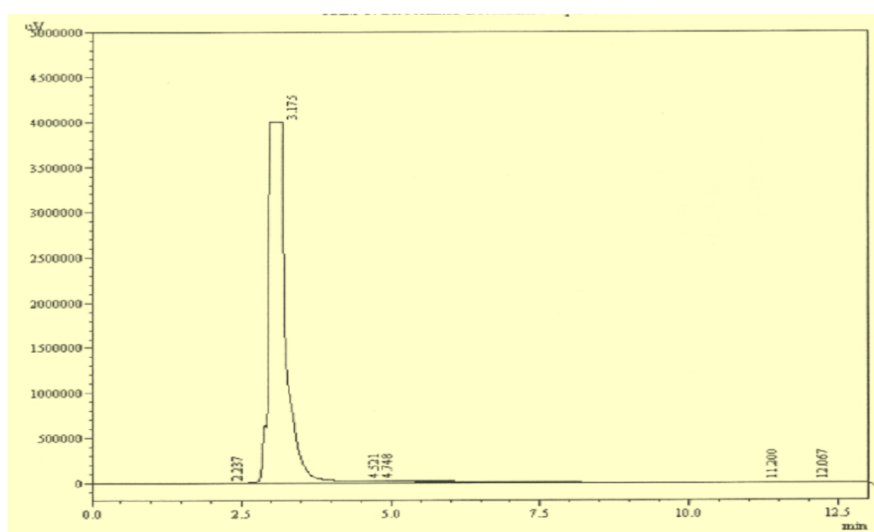

Fig. (2-a). HPLC chromatogram showing retention time (3 min) for authentic sample of ρ-nitrophenol. Absorbance was followed at 295 nm

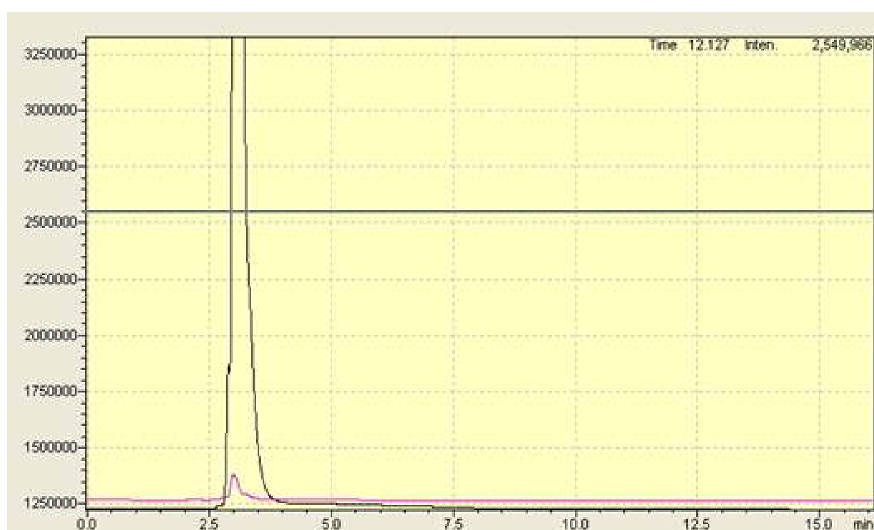

Fig. (2-b). Tracing the consumption of ρ-nitrophenol by HPLC in cell-free supernatant of bacterial culture

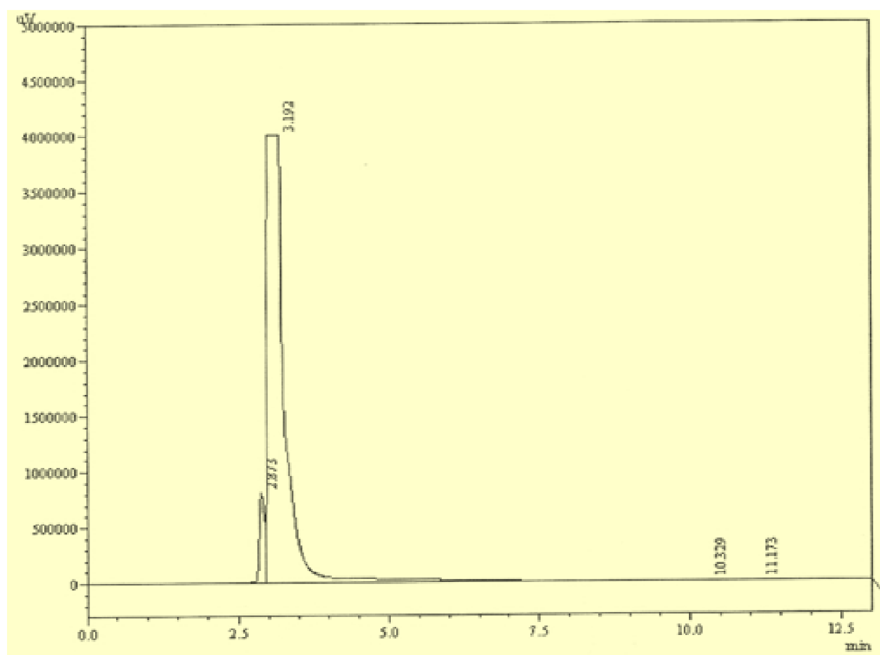

Fig. (3-a). HPLC chromatogram showing retention time (3 min) for authentic sample of nitrobenzene. Absorbance was followed at 295 nm

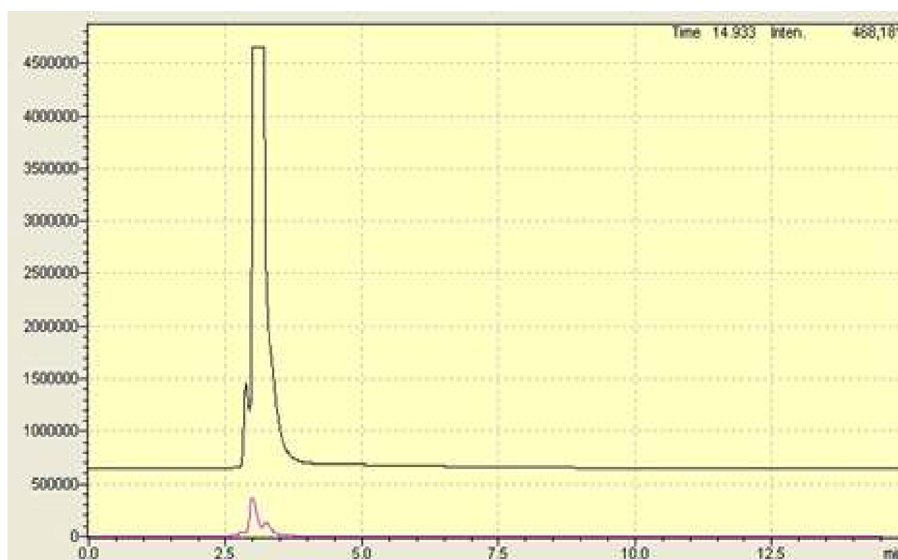

Fig. (3-b). Tracing the consumption of nitrobenzene by HPLC in cell-free supernatant of bacterial culture

Leon and Manoj reported that the heavy crudes (bitumen) are extremely viscous and contain high concentrations of asphaltene, resins, nitrogen and sulfur containing heteroaromatics and several metals, particularly nickel and vanadium. These properties of heavy crude oil present serious operational problems in heavy oil production and downstream processing. There are vast deposits of heavy crude oils in many parts of the world. In fact, these reserves are estimated at more than seven times the known remaining reserves of conventional crude oils. It has been proven that reserves of conventional crude oil are being depleted, thus there is a

growing interest in the utilization of these vast resources of unconventional oils to produce refined fuels and petrochemicals by upgrading [22]. Presently, the methods used for reducing viscosity and up-gradations are costly, less selective and environmentally reactive. Biological processing of heavy crudes may provide an eco-friendly alternative or complementary process with less severe process conditions and higher selectivity to specific reactions to upgrade heavy crude oil.

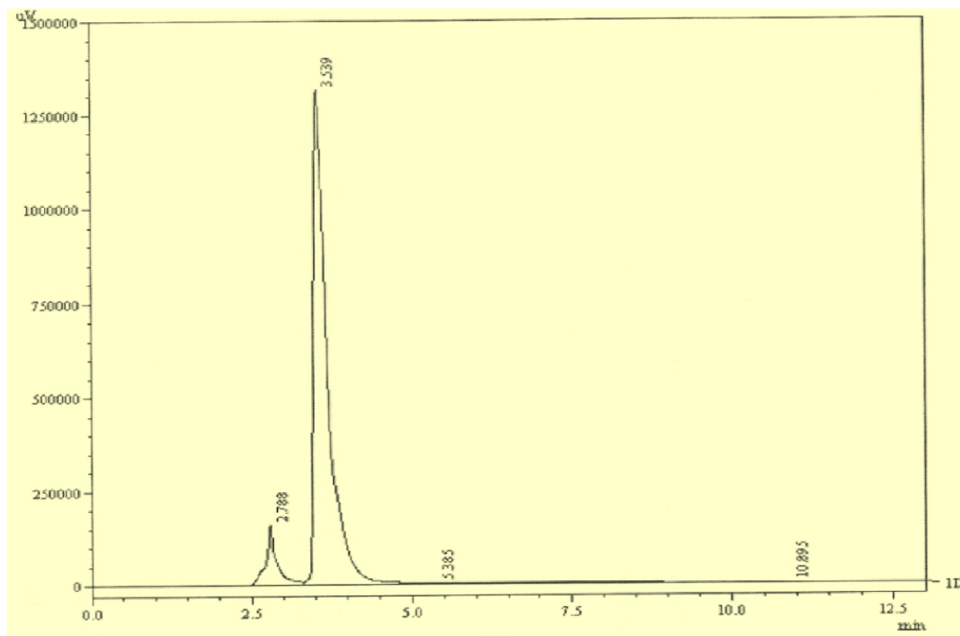

Fig. (4-a). HPLC chromatogram showing retention time (3.5 min) for authentic sample of naphthalene. Absorbance was followed at 295 nm

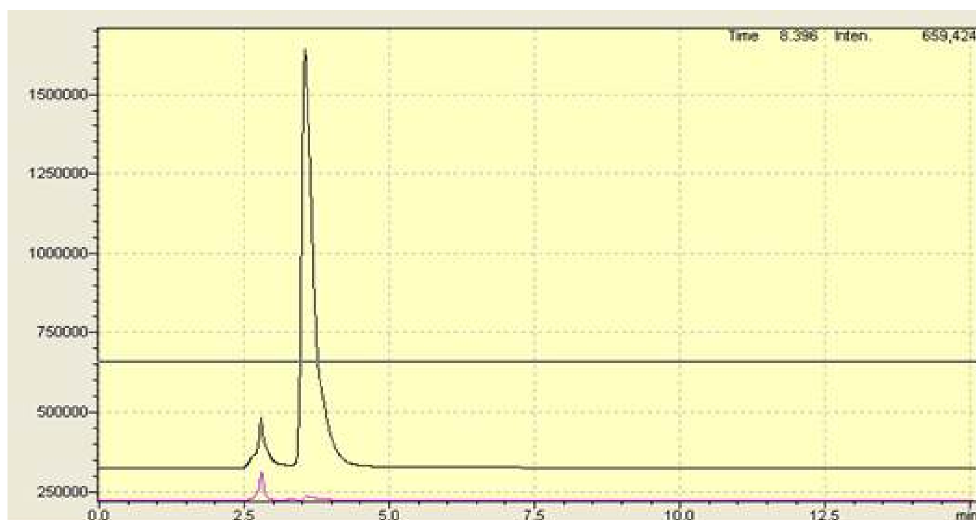

Fig. (4-b). Tracing the consumption of naphthalene by HPLC in cell-free supernatant of bacterial culture

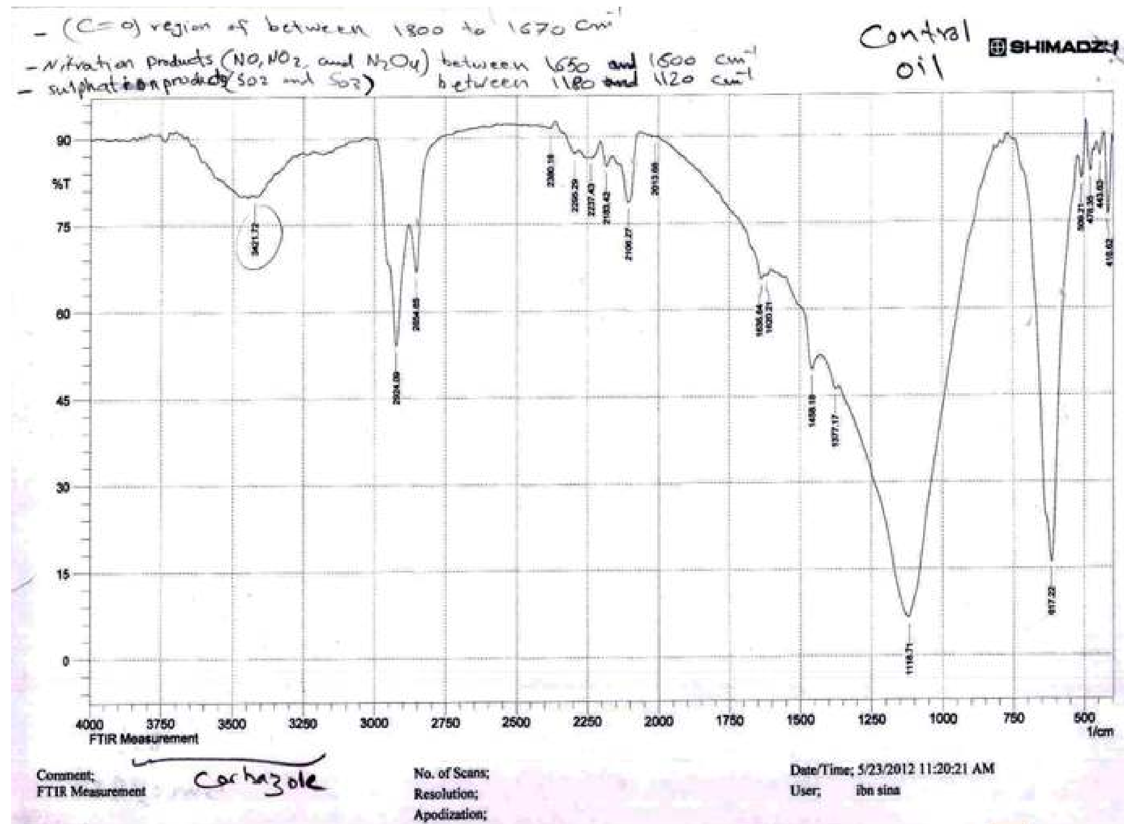

Fig. (5-a). FTIR spectrum for crude oil (control)

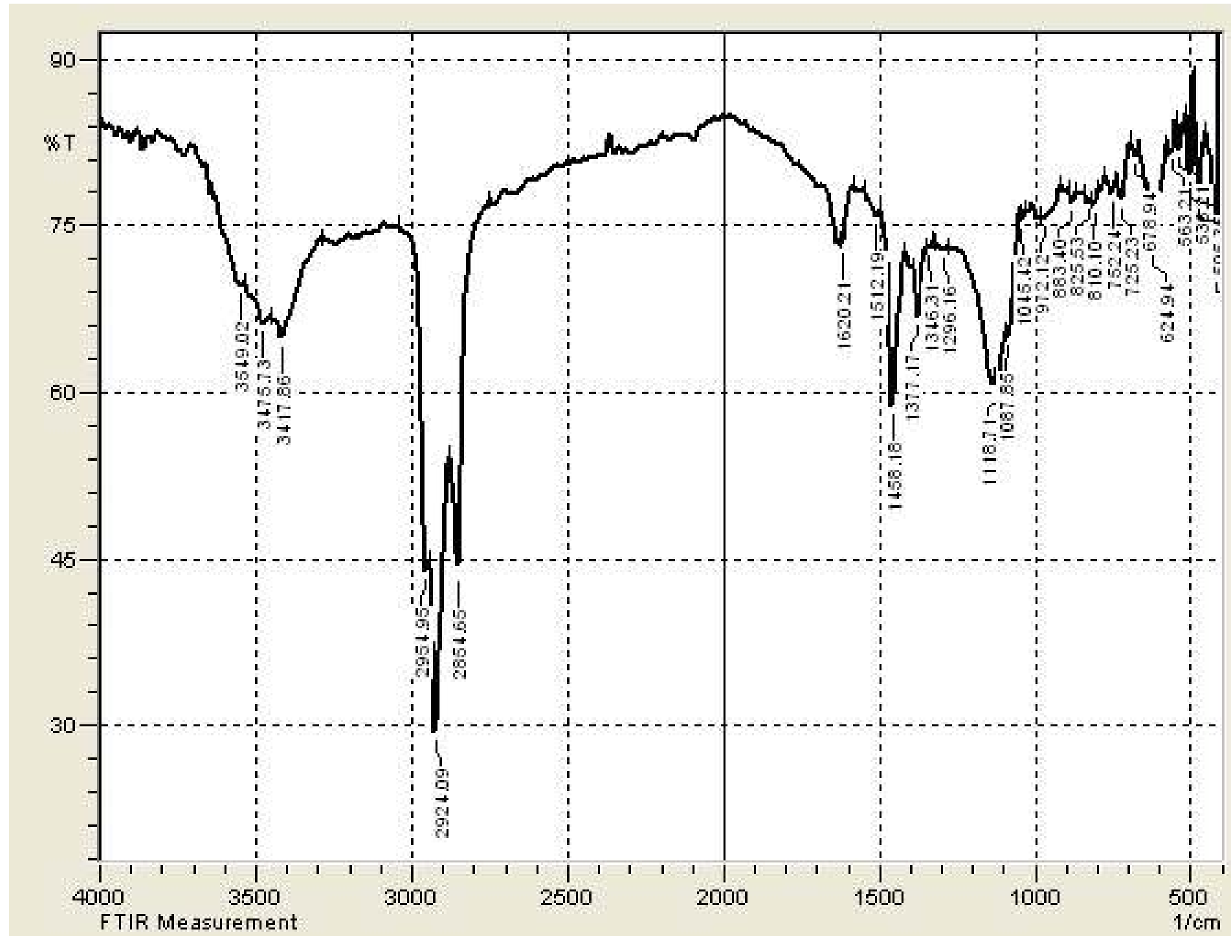

Fig. (5-b). FTIR spectrum for 30% crude oil in presence of *A. rupiensis* strain Ir3 (JQ912241), at 55C°, (150 rpm) shaking for 30 days

4. CONCLUSIONS

A. rupiensis strain Ir3 could be used as alternative to hydrodenitrogenation (HDN) for nitroaromatic compounds elimination (bio-treatment) of crude oil and its derivatives. The bio-treatment of crude oil illustrated that *A. rupiensis* strain Ir3 is capable of removing carbazole from crude oil. Its ability to convert carbazole to anthranilic acid is through the *meta* cleavage enzyme.

This bacterium might possess the genetic capacity for a catechol catabolic enzyme. Catechol is oxidized in a reaction catalyzed by catechol-2, 3-dioxygenase (C23O), the *meta* pathway to 2-hydroxymuconic semialdehyde.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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