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Degradation of Quinoline and Indole by Thermophilic Bacteria (*Anoxybacillusrupiensis* Ir2 (JQ912240))

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Abstract: *Anoxybacillus rupiensis* Ir2 (JQ912240), thermophilic bacteria capable of utilizing aromatic hydrocarbon especially N-compounds that form part of petroleum components.In an attempt to investigate the ability of this bacterium to degrade quinoline and indole, the strain Ir2 was grown on these N-compounds separately as a sole source of carbon and nitrogen. Results showed that strain Ir2 was able to degrade these compounds.Optimum conditions for degradation of quinoline and indole by strain Ir2 was investigated. It was found that these conditions are growing this bacterium in mineral salt medium (pH 7 for both quinoline and indole) containing (60mM) of quinoline and (90mM) of indole, and incubated with shaking (150rpm) at 55 °C for seven days. To confirm the ability of *A.rupiensis* Ir2 (JQ912240) to utilize the aromatic compounds(quinoline and indole), analytical experiments HPLC was used. Results indicated that thisbacterium showed as much as 99.55% consumption of quinoline and 96.11% of indole.PCR amplification with narG2-Respiratory nitrate reductase primer reveals the presence of that system in this bacterium.

Keywords: *Anoxybacillus rupiensis, microbial degradation, quinolin, indole*

1. INTRODUCTION

Numbers of *Anoxybacillus* spp. have been isolated from around the world since the first introduction of the genus in 2000 [1].There are 22 species and two subspecies of *Anoxybacillus* are described [2,3,4,5]. The cells of *Anoxybacillus* spp. are generally rod-shaped and straight or slightly curved, often present in pairs or short chains, and they form endospores. Interestingly, *Anoxybacillus* spp. can be either alkaliphilic or alkalitolerant, and most of them are able to grow well at neutral pH. *Anoxybacillus* spp. are moderately thermophilic [OGT(optimum growth temperatures) 50−62°C], with a slightly lower OGT than *Geobacillus* spp. (55−65°C). The *Anoxybacillus* spp. are either aerobes or facultative anaerobes. Among the *Anoxybacillus* spp., the genome of *A. flavithermus* WK1 (PRJNA59135) remains the only completely sequenced genome [6].

The closest genus to *Anoxybacillus* is *Geobacillus*, yet the genomes of the latter genus are larger and the cells grow at higher temperatures. Based on the genome annotation, the thermophily of *Anoxybacillus* is attributable to many features that stabilize proteins, DNA, and RNA. The presence of adaptive genes is sufficient for the cells to live in an alkaline environment with the presence of organic nitrogen and carbohydrates and to overcome the threats from UV radiation. In addition, for *Anoxybacillus* spp. to survive under extreme conditions, genetic exchange, especially uptake of genetic material via HGT (horizontal gene transfer), is important, this process can take place via transduction or transformation [7].

In this study, *Anoxybacillus rupiensis* sp. Nov. isolated from hydrocarbons contaminated soils in Iraq. It was efficient in utilizing aromatic compounds such as Carbazol, ρ-nitrophenol, nitrobenzene and naphthalene. It was investigated using biochemical tests, microscopic observation, and a determination of its 16S rDNA gene. The bacterium is a gram positive or (gram variable) long rod that formssmall sized, round colonies with cream color, obligated thermophilic growing between 40 and 70°C (optimum 55-65°C) and in a pH range from 5.0-9.0 (optimum 7.0). It is catalase positive, oxidase positive, and nitrate reductase positive. The 16S rDNA gene sequence of this bacterium was compared with database of NCBI and it has 99% similarity to *A. rupiensis* (HQ69661501) [8].

Aromatic compound can be defined as organic molecules that are among the most prevalent and persistent compounds in the environment, containing one or more aromatic rings, especially benzene

rings. Different aromatic compounds co-exist as complex mixtures in petroleum refining and distillation sites [9-10]. 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 impact both aerobic and anaerobic environments such as aquifers, surface fresh water bodies, soils, and terrestrial and marine sediments [11].There are three major categories: PAHs, heterocyclic, and substituted aromatics. Indole is an aromatic heterocyclic organic compound. It has a bicyclic structure, consisting of a six-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring. Indole is widely distributed in the natural environment and can be produced by a variety of bacteria [12]. Quinoline and its derivatives occur widely in coal tar, bone oil, oil shale and plant alkaloids and serve asintermediates and solvents in chemical industry. Quinolineand some of its derivatives were reported to be toxic, carcinogenic and mutagenic [13]. The widespread use of quinoline and its derivatives entails that these compounds, together with many other environmentalchemicals are distributed in the environment thuspolluting soil and water [14]. Degradation of quinolineby microbial process has attracted more and moreconcerns in recent years.Traditional biological treatment processes can destroya large fraction of biodegradable organic compoundsexisted in wastewater. Moreover, the biological treatmentcost is much lower than that of physical andchemical methods. However, many hazardous compoundsare poorly removed in conventional biologicalprocesses due to their toxicity, recalcitrance or inhibition [15].

The aim of the study is to characterize the ability of the thermophilic bacteria *Anoxybacillus rupiensis* Ir2 (JQ912240) to utilize some of the organonitrogen compounds that encountered in fossil fuels such as quinoline and indole.

2. METHODS

2.1. Microorganism

The strain was purified by successive streak transfers agar-plate medium. It was identified as *Anoxybacillus rupiensis* through PCR amplification of 16S rDNA using seven primers (fd1, fd2, fd3, fd4, rd1, rp1 and rp2) which represent primers for the PCR amplification of eubacterial 16S rDNA, and followedby sequencing. The nucleotide sequence data was compared with 16S rDNA sequences of other culture on BLAST of the National Center of Biotechnology Information database (NCBI database).

2.2. Medium

Luria-Bertani (LB) medium [17] and thechemical define media (CDM)[18], were used for the growth of the microorganism and degradationrespectively. Quinoline and indole were used as the sole source of carbon and nitrogen in all experiments.

2.3. Optimization of Indole and Quinoline Biodegradations

The experimentswere carried out through dispensing 100ml of (CDM) in (250 ml) Erlenmeyer flasks; inoculated with 1ml of mild- exponential phase of *Anoxybacillus rupiensis* Ir2. The flasks were incubated in a shakerincubator (150rpm) at 55°C. After incubation period, bacterial growth was determined by measuring the optical density at 600nm.

2.4. Effect of Indole and Quinoline Concentration

Quinoline and indole were added at different concentration (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100mM). PH was adjusted to 7.0, and then incubated in a shaker incubator (150rpm) at 55°C for seven days. Bacterial growth was measured as mentioned above.

2.5. Effect of Temperature

CDM (pH 7) supplemented with 60mM of quinoline and 90mM of indole and incubated in a shaker incubator (150rpm) at different temperatures (55°C, 60°C and 65°C) for seven days.

2.6. Effect of pH

CDM supplemented with 60mMquinoline and 90mM of indol were prepared at different pH values (6, 7, 8 and 9). The cultures were incubated in a shaker incubator (150rpm) at 55°C for seven days. Bacterial growth was measured as mentioned above.

2.7. HPLC (High Performance Liquid Chromatography) Analysis

Chemically defined media (100ml in 250 ml flask) containing (60mMquinoline and 90mM of indole) were inoculated with fresh culture of efficient bacterial isolate *A.rupiensis* strain Ir2 (JQ912240) and incubated at 55°C, pH 7, for 7 days shaking at 150 rpm. After incubation, centrifugation at 13000 rpm for 10min at 4°C. The resulting cell-free supernatant was analysed by HPLC (High Performance Liquid chromatography) for tracing the consumption of the aromatic compounds.

2.8. Extraction Procedure

The inoculated flasks and uninoculated control was extracted by using separating funnel inpresence of ethyl acetate as a solvent. One ml of culture supernatant was taken from cultures growing with (quinoline and indole) and extracted with 3ml of ethyl acetate. The ethyl acetate solvent was evaporated and the residue was dissolved in 1ml ethanol [19].

2.9. Analytical Method

The extracts were analysed to trace the utilization of quinoline and indole compounds by using HPLC system. Tenµl of supernatant were injected in C_{18} column (5 µM, 4.6*250mm, supelcosil Lc-2010AHT) and the following conditions were followed:

The solvent system used (60% acetonitrile in H_2O) was run at flow rate of 1ml/min. for UV detection of quinoline and indole, the UV detector was adjusted to 295nm. Under these conditions the observed retention time for authentic samples of quinoline and indole were 3min, and 2min, respectively.

2.10. Extraction of Genomic DNA with Wizardgenomic DNA Purification Kit

One ml of overnight culture was transferred to a1.5 ml centrifuge tube. Centrifugation at 13000rpm for 2 was used min to pellet the cell. Six hundredmicroliter of nuclei lysis solution was added andgently pipetted until the cells wasresuspended.The tube was incubated at 80° C for 5 min to lyse the cells, and then cooled to roomtemperature. RNase solution (3 µl) was addedto cell lysate.The tube was mixed by invertion2-5 times, incubated at 37° C for 15min, andcooled to room temperature. Twohundred microliter of protein precipitationsolution was added to the RNase - treated celllysate andvortexed vigorously. The sample incubated on ice for 5 min. and centrifuged at13000 rpm for 3 min. The supernatantcontaining the DNA was transferred to a clean1.5 ml microcentrifuge tube containing 600 µlof isopropanol,gently mixed by inversion untilthe thread like strands of DNA form a visiblemass, Centrifuged at 13000rpm for 2min. The supernatant was poured off and thetube was drained on a clean absorbent paper,600 µl of 70% ethanol was added, the tubewas gently inverted several times to wash the DNA pellet. The tube was centrifuged at13000rpm for 2 min;the ethanol was carefully aspiratedand one hundred microliter of DNArehydration solution was added to the tube and incubated at 65 °C for 1 hour.

2.11. PCR Amplification

PCR amplification was done by using the primer pair narG2 Forward (5´ CGAAGACGATCTCCACT CGCTAAC-´3) and narG2 Reverse (5´ATCTTCAAGCCAATCAACCTCCTC-´3). One microliter of DNAwas added to a 25µl PCR mixture containing 12.5µl PCR master mix solution, template DNA (genomic DNA) 0.5µl, primer (F: 10pmol/µl) 0.5 µl, primer (R: 10 pmol/µl) 0.5µl and 11µl H2O. The reaction was initially denatured at 95 ° C for 5 min. followed by 38 cycles of 94 °C for 45s, 55°C for 45s, and 72°C for 45s, followed by a final extension stepat 72°C for 10 minutes.

3. RESULTS AND DISCUSSION

Optimization of quinoline and indole biodegradation by *Anoxybacillus rupiensi*s Ir2 (JQ912240)

3.1. Effect of Quinoline and Indole Concentration

Different concentrations of quinoline and indole were used to grow of *Anoxybacillus rupiensi*s Ir2 (JQ912240) in order to determine the optimum concentration. Results in Figures (1) and (2) indicated that the optimum concentrations for growth were 60mMand 90mMof quinoline and indole respectively.

Crude oils contain organic nitrogen compounds; usually comprise not more than 2%, with 70 to 75% consisting of pyrrols, indoles and carbazole nonbasic compounds. The presence of these nitrogenous compounds in crude oil and oil´s refinery products is undesirable for two main reasons: the adverse

impacts on environment and human health due to emission of nitrogen oxides (NOx) as well as the economical impacts due to interfering with the processing of petroleum by poisoning of catalysts and contributing to corrosion [20].

The microorganism used in this studywas identified as *A. rupiensi*s according to16S rDNA results, it was a pure strain of aromatic-degrading microbe, which was isolated from the oil contaminated soils in Iraqwhich are chosen for isolation of thermophilic aromatic degrading bacteria since petroleum oil contains aromatic compounds that are toxic for most life forms [16].

3.2. Tracing the Anoxybacillus Rupiensis Strain Ir2 (JQ912240) Consumption of Pure Aromatic Compounds (Quinoline and Indole)

The consumption of 60mM quinoline and 90mM of indole as the sole of carbon and nitrogen source was traced by HPLC in cell-free supernatants of cultures. The *A. rupiensis* strain Ir2 (JQ 912240) showed clear growth with the two aromatic compounds.The HPLC analysis indicated that quinoline showed as much as 99.55% consumption depending on the area of peaks eluted at 3min (Figure 7 a, b). Indole showed as much as 96.11% consumption depending on the area of peaks eluted at 2min (Figure 8 a, b).

It was considered the potential of *A. rupiensis* strain Ir2 (JQ912240) for degradation of quinoline and indole. Crude oil is a heterogeneous mixture of organic molecules including all-hydrocarbon alkenes and aromatics, as well as sulfur and nitrogen–containing heteroaromatic compounds [21]. Many applications of crude oil are hindered by the presence of sulfur and nitrogen-containingcompounds [22, 23]. It is known that the chemical and physical petroleum refining processes are currently used to remove most of the nitrogen–containing organic compounds in crude oil [21, 24-26]. Nitrogen heterocyclic compounds can deactivate refining catalysts and can also contribute to chemical instability of refined petroleum products. Therefore, it is necessary to establish a method for removing these nitrogen compounds from crude oil for global environmental protection. To date, there has been an increasing interest in the use of microorganisms to treat heterocyclic nitrogenous compounds because such a bioprocess enables selective degradation and proceeds under milder conditions than the chemical and physical processes, which need high-temperature and high-pressure conditions [27-28].

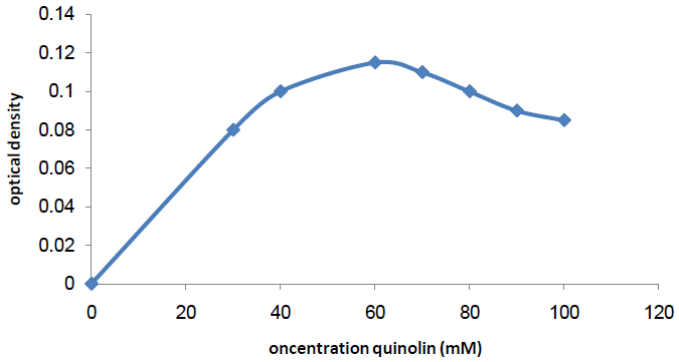

Fig1. *The effect of quioline concentration on Anoxybacillus rupiensis Ir2 (JQ912240) growth.*

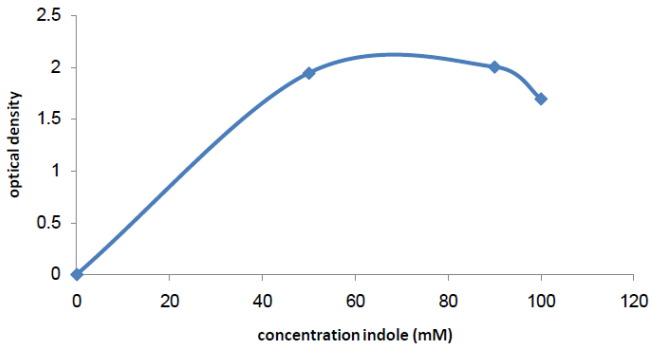

Fig2. *The effect of indole concentration on Anoxybacillus rupiensis Ir2 (JQ912240) growth.*

3.3. Effect of pH

CDM was prepared at different pH values (6,7and 8) in an attempt to determine the optimum pH required for growth of *A. rupiensis* Ir2 (JQ912240) on quinoline and indole.The obtained results as shown in Figures (3)and(4)elucidated that the optimum growth was occurred at pH7.0of both quinoline and indole.

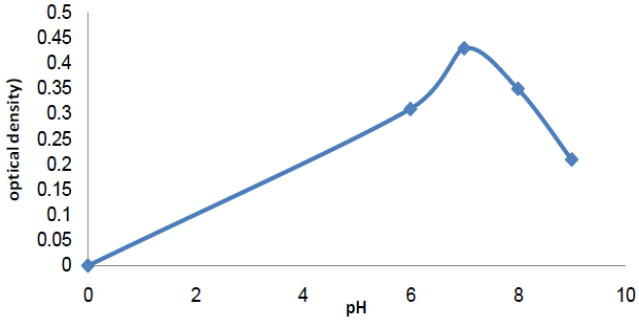

Fig3. *The effect of pH on Anoxybacillus rupiensis Ir2 (JQ912240) grew on 60mM of quinoline*

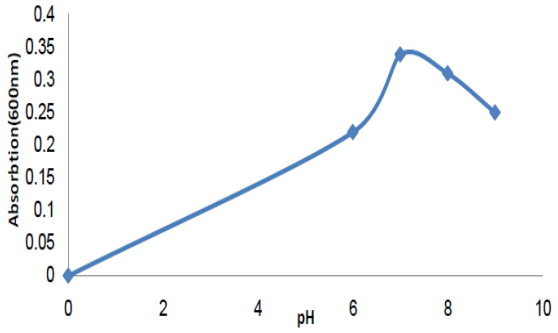

Fig4. *The effect of pH on Anoxybacillus rupiensis Ir2 (JQ1922240) grew on 90mM of indole*

3.4. Effect of temperature

Anoxybacillus rupiensis Ir2 (JQ912240) was grown and incubated at different temperatures (55, 60 and 65°C). Results shown in Figures (5) and (6) pointed out that the optimum temperature for growth in presence of quinoline and indole were 55°C for both.

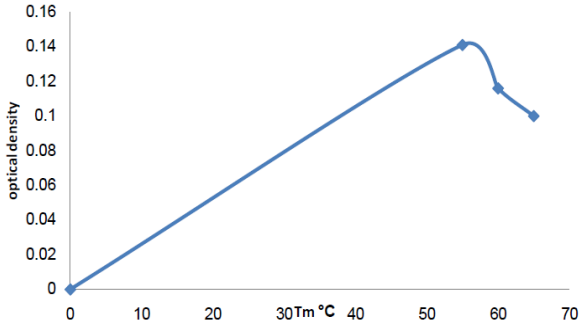

Fig5. *The effect of temperature on AnoxybacillusrupiensisIr2 (JQ192240) grew on 60mM of quinoline and pH 7.0*

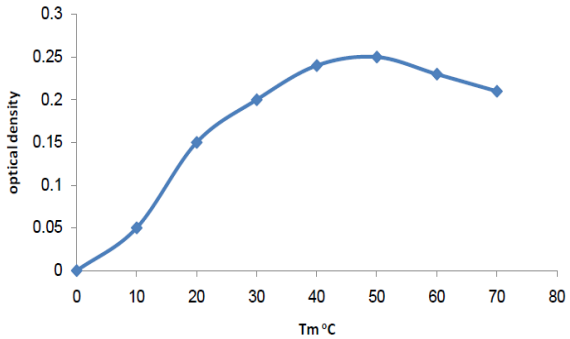

Fig6. *The effect of temperature on Anoxybacillus rupiensis Ir2 (JQ192240) grew on 90mM of indole and pH 7.0*

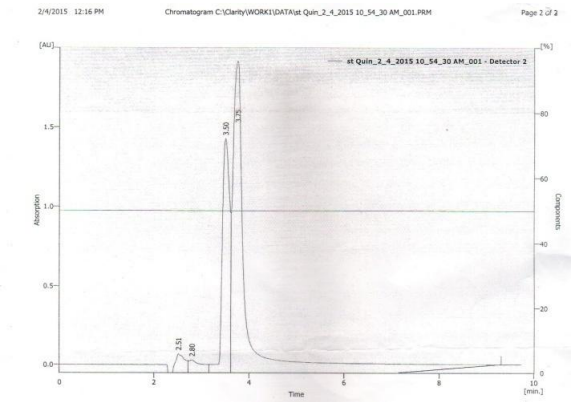

Fig7a. *HPLC chromatogram showing retention time (3min) for authentic sample of quinoline. Absorbance was followed at 295nm.*

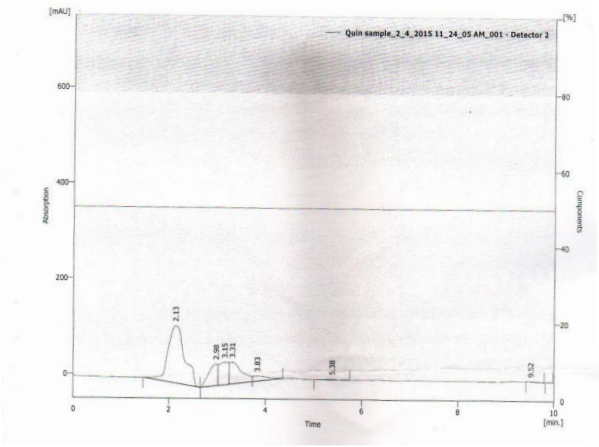

Fig7b. *Tracing the consumption of quinoline by HPLC in cell-free supernatant of bacterial culture.*

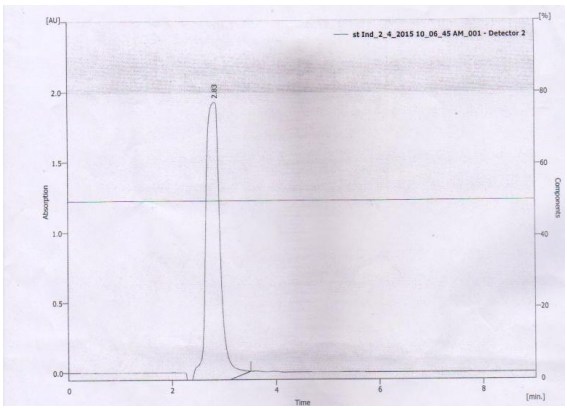

Fig8a. *HPLC chromatogram showing retention time (2min) for authentic sample of Indoel. Absorbance was followed at 295nm.*

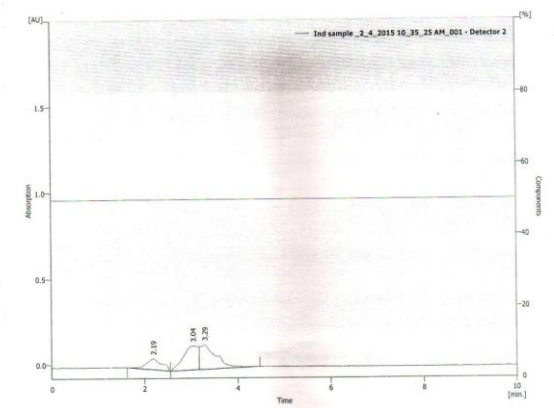

Fig8b. *Tracing the consumption of Indole by HPLC in cell-free supernatant of bacterial culture.*

3.5. PCR amplification

In this study molecular approaches have been used to investigate the presence and the expression of one the narG genes (narG2) in *A. rupiensis* strain Ir2 (JQ912240) which assessed with degenerated PCR primer. PCR amplification accrued with the narG primer on DNA extracted from the bacteria as show in (Figure 9).

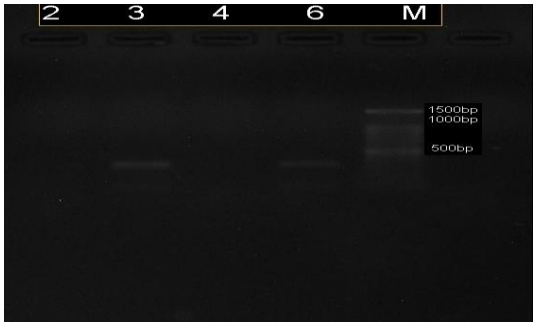

Fig9. Gel electrophoresis for PCR amplification of narG2 gene by using specific primer. Electrophoresis was performed on (1.5%) agarose gel and run with 5V/cm for 1 hr. M: DNA ladder 2, 3, 4 and 6): thermophilic bacterial strains. 3: represents the *A. rupiensis* strain Ir2 (JQ912240)

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