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RESEARCH ARTICLE

Isolation and Characterization of a New Thermophilic, Carbazole Degrading Bacterium (*Anoxybacillus rupiensis* Strain Ir3 (JQ912241)

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Abstract

The main aim of this study was to isolate and identify thermophilic bacteria capable of utilizing aromatic hydrocarbon especially N-compounds that form part of petroleum components. A new aerobic Gram-variable long rod-shaped, thermophilic bacterium was isolated. The isolate cannot be distinguished by ordinary morphological, physiological and biochemical tests; therefore, it was subjected to molecular identification through PCR amplification of 16S rDNA by using 7 primers (fd1, fd2, fd3, fd4, rd1, rp1 and rp2) which represent primers for the PCR amplification of eubacterial 16S rDNA, and followed by 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). According to the results of molecular identification, the isolate characterized as *Anoxybacillus rupiensis* strain (Ir3), and deposited in the National Genebank database under the accession number, (JQ912241). Optimum conditions for growth of *A. rupiensis* strain Ir3 (JQ912241) were determined. Results showed that growing in LB medium (pH 7) containing 0.5 to 1% of NaCl, and incubated with shaking (150 rpm) at 55 -65◦C for 24h. It was also found that this bacterium was able to withstand 80◦C for 90 min. The optimum conditions for growth of *A. rupiensis* strain Ir3 (JQ912241) in minimal medium (CDM) were adjusted, the pH to 7, incubated at 55-65◦C and the bacterial growth was increased with carbazole concentration increased. This means that bacterial growth with CAR as an N-source was concentration-dependent

Introduction

The genus Anoxybacillus is separate from the genus Bacillus, and the type species is *A. pushchinoensis* DSM 12423^T [1]. [1] first described the type species of the genus as an obligate anaerobe. Later, [2] corrected the description of the species *A. pushchinoensis* from 'obligate anaerobe' to 'aerotolerant anaerobe' and also changed the description of the genus Anoxybacillus from one comprising obligate anaerobes to facultative anaerobes to one comprising aerotolerant anaerobes or facultative anaerobes. [3] mentioned that the genus Anoxybacillus contained 16 species: *A. pushchinoensis* [1], *A. gonensis* [4], *A. amylolyticus* [5], *A. ayderensis* [6], *A. bogrovensis* [7], *A. contaminans* [8]. *A. eryuanesis* [9], *A. flavithermus* [1], *A. kamchatkensis* [10]. *A. kestanbolensis* [6], *A. mongoliensis* [11], *A. rupiensis* [12], *A. salavtliensis* [13], *A. tengchongensis* [9], *A. thermarum* [14], and *A. voinovskiensis* [15]. [16] Reported that Anoxybacillus species are widely distributed and readily isolated from geothermal heated environments, with a continually increasing industrial interest for 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.

The thermophiles are defined as organisms capable of living at high temperature. These organisms do not only survive but might even thrive in boiling water [17]. As of their cellular components, proteins are inherently more stable to heat than those of conventional organisms called "mesophiles". This thermal stability is not due to any specific characteristic but resulted of a consequence of various changes which contribute to the whole stability of the protein in additive manner [18]. The ability of thermophilic bacteria to grow at high temperature and to produce stable extracellular enzymes was attributed to the probability of increasing their enzyme excoriation and activity by means of genetic manipulation. Therefore, these microorganisms were the first candidates for massive enzyme production for industrial applications [19].

Carbazole, is an environmental pollutant, it is a heteroaromatic compound containing nitrogen found in coal–tar creosote. Because, bioremediation has proven to be an effective technique for cleaning sites polluted by persistent compounds such as carbazole, carbazole-degrading bacteria namely *Pseudomonas resinovorans* strain CA10 [20, 21] Novosphingobium sp. strain KA1 [22], Janthinobacterium sp. strain J3 [23], *Pseudomonas stutzeri* strain OM1[24], Sphingomonas sp. strain CB3 [25], and *Nocardioides aromaticivorans* strain IC177 [26] have been isolated and studied. Bacteria degrade carbazole by conversion to 2-hydroxypenta- 2, 4-dienoate and anthranilate via dioxygenation by carbazole 1,9 a-dioxygenase (CarA), aromatic ring cleavage by the meta-cleavage enzyme (CarB), and hydrolytic cleavage by the meta-cleavage compound hydrolase (CarC). Next, 2-hydroxypenta-2, 4-dienoate is converted to TCA cycle intermediates by 2-hydroxypenta-2, 4-dienoate hydratase (CarD), 4-hydroxy-2-oxovalerate aldolase (CarE), and acetaldehyde dehydrogenase (CarF). Anthranilate is also converted to TCA cycle intermediates via dioxygenation by anthranilate 1, 2-dioxygenase (AntABC), spontaneous deamination, decarboxylation, and further degradation [27].

According to those mentioned above, the aim of .present work was isolating and identifying thermophilic bacteria capable of utilizing aromatic hydrocarbon.

Materials and methods:

Bacterial isolation

Samples of soils contaminated with hydrocarbon compounds were collected. The soil samples (200g) were collected randomly from the top soil layer (15cm in depth). Soil samples were transferred to the laboratory using sterile plastic bags to isolate thermophilic aromatic hydrocarbons degrading bacteria. The microbial selection procedures were performed in minimal medium (CDM) [28], constituted of minerals and the crude oil as sole source of carbon, nitrogen and energy. This experiment was designed to enrich indigenous bacterial communities growing at (60 ◦C and 75 ◦C). This was followed by isolation and purification of pure cultures capable of utilizing individual aromatic compounds, naphthalene (polycyclic aromatic hydrocarbon), carbazole (N-heterocyclic aromatic compound) and ρ-nitrophenol, nitrobenzene (nitroaromatic compounds).

One hundred milliter of chemical defined medium (CDM) were dispensed in the (250 ml) Erlenmeyer flasks, supplemented with one milliter of crude oil (sterilized by tyndallization) as sole source of carbon, nitrogen, and energy. Then one percent (w/v) of soil samples were added to the flasks and incubated at both temperatures (60 ◦C and 75 ◦C) on a rotary shaker (150 rpm). After 3 days of incubation, each flask was supplemented again with crude oil. After 7 days incubation, samples 0.1 ml of appropriate dilution were spread on LB (Luria- Bertani) agar plates [29], incubated at both temperatures for 24hrs. A single colony was picked with a sterile loop to prepare a pure subculture in a fresh LB agar plates by streaking. The purity of the selected colonies was checked by microscopic examination.

Utilization of pure aromatic compounds

Thermophilic bacterial isolate was grown on the respective compounds as the sole source of carbon and energy for naphthalene and as carbon, nitrogen, and energy source for carbazole, ρ-nitrophenol and nitrobenzene to study its degradation ability. Aliquot of 50 ml of chemically defined medium (CDM) distributed into 100 ml Erlenmeyer flasks. The flasks were sterilized by autoclaving at 121◦C for 15 min, 1mM of aromatic compounds (sterilized by filtration) were added.

All the flasks were inoculated with 1% of fresh culture (18hrs), and incubated under shaking (150 rpm) at 55◦C for 2 days. Cell density was determined by measuring the optical density at 600nm.

Characterization and identification of the isolate

Morphological, physiological and biochemical tests were performed to identify the thermophilic bacterial isolates [30].

Molecular identification

The genomic DNA was extracted from isolated bacterium (Wizard genomic DNA purification kits solutions, www.promega.com, USA), and the amplification of the 16S rDNA was performed through PCR technique. The purified PCR products were sequenced by Macro gene Inc., (Seoul, Korea) using ABI, 3310 automated sequencing system. The16S rDNA sequence of the isolate was aligned with the 16S rRNA gene sequences of the genus Anoxybacillus obtained from the Ribosomal RNA from NCBI Genebank (www.ncbi.nlm.nih.gov/BLAST). The phylogenetic tree was constructed using the program MEGA5.

Optimization of growth conditions of *A. rupiensis* strain Ir3 (JQ912241)

Some factors (pH medium, incubator temperature and concentration of sodium chloride) were studied to determine the optimum conditions. Optimization experiments were carried out through dispensing of 50 ml Luria-Bertani (LB) medium in 250 ml Erlenmeyer flasks, inoculated with 1% of 18hrs bacterial cultures. Then Flasks were incubated in shaker incubator (150 rpm). Cell density was determined by measuring the optical density at 600nm.

Studying the death curve (Thermal death time) of *A. rupiensis* strain Ir3 (JQ912241)

Using thermal death time method, *A. rupiensis* strain Ir3 (JQ912241) grew in LB broth was subjected to 80 ◦C using water bath for ninety minutes. A sample of culture was taken at time 0**,** 15, 30, 45, 60, 75, and 90 minutes. Aliquot of 0.1ml of bacterial culture was quadrant streaked on LB agar plates and then incubated at 55◦C for 24hrs.

Growth of *A. rupiensis* strain Ir3 (JQ912241) at different carbazole concentrations

The experiments were carried out in Erlnmeyer flasks (250ml) containing 100 ml of CDM with glucose (1%) and different carbazole concentrations, 0.2, 1, 5, and 10 mM, in addition to control flask that contain NH_4Cl as a source of nitrogen. The flasks inoculated by 1ml of 18hrs growth culture, then incubated at 55◦C in shaker incubator (150rpm). Cell density was determined by measuring the optical density at 600nm.

Results and Discussion

Isolation and identification of thermophilic, carbazole degrading bacteria

Out of 48 isolates, 10 isolates were showed thermophilic character in addition to their ability to utilize the crude oil as a sole source of carbon, nitrogen, and energy.

Thermophilic bacterial isolates were tested for their ability to utilize individual aromatic compounds. The results indicated that the most efficient bacterial isolate was 4A as shown in Fig. (1), which gave the best result in its ability to utilize all the aromatic compounds.

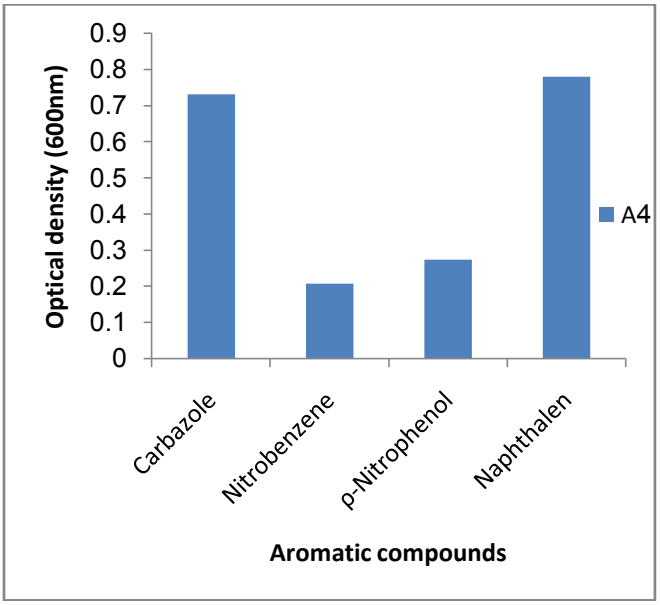

Fig. (1): **Growth density (600nm) of the thermophilic bacterial isolate on aromatic compounds after 2 days incubation at 55◦C**

Molecular identification

The isolate cannot be distinguished by ordinary morphological, physiological and biochemical tests; therefore, it was subjected to molecular identification**.** In order to amplify 16S rDNA for thermophilic isolate, genomic DNA of the isolate 4Awas extracted to provide a PCR template for the amplification as shown in Fig. (2).

The identity and purity of the thermophilic bacterial isolate (4A) was carried out at Hamdi Mango Center for Scientific Reserch, Jordan and was checked by amplification and sequencing of eubacterial 16S rDNA gene.

Primers (fD1, fD2, fD3, fD4, rD1, rP1 and rP2) for amplification of eubacterial 16S rDNA gene were used (Integrated DNA Technologies, http//www:idtdna.com), which are universal primers that bind at the conserved 5ó and 3ó ends of 16S rDNA of eubacteria, 1500 bp PCR fragments were obtained as shown in Fig. (3) and this indicates that the 4A isolate was affiliated to eubacteria. Partial sequence around the obtained PCR fragments with forward and reverse primers was performed and compared with 16S rDNA nucleotide sequences present in gene bank, using the standard basic local alignment search tool (BLAST). The results of this BLAST search showed the highest percentage (97%) of sequence similarity with both 5ó and 3ó ends of the same gene from Anoxybacillus sp (HQ696615.1).

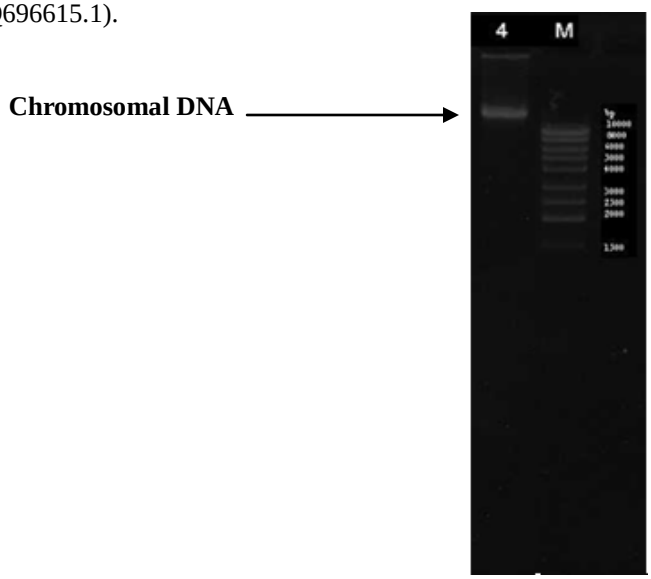

Fig. (2): Gel electrophoresis for genomic DNA of bacterial isolate. Electrophoresis was performed on (1%) agarose gel and run with 5V/cm for 1.5 hrs. 4:4A. M: molecular marker 10kb (mass ruler DNA ladder, high range).

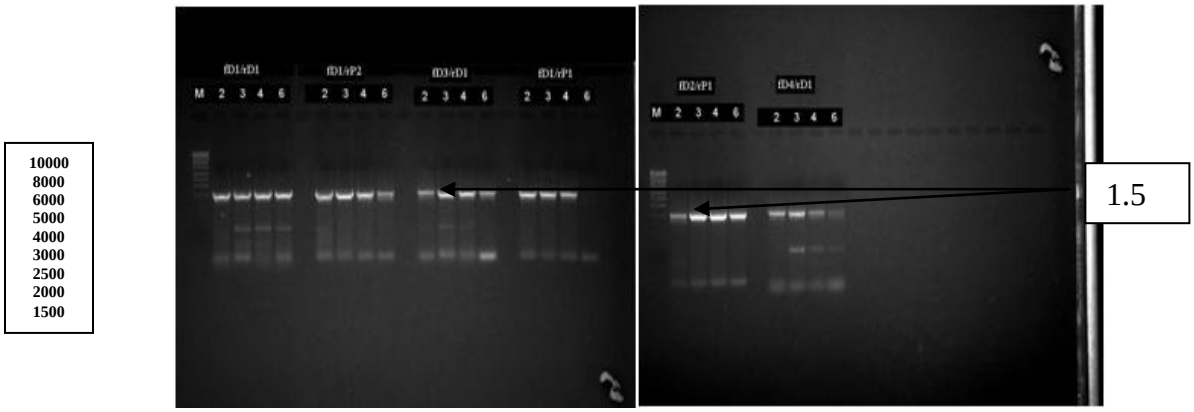

Fig. (3): Gel electrophoresis for amplification of 16S rDNA gene using eubacterial specific primers fD1, fD2, fD3, fD4, rD1, rP1 and rP2. Electrophoresis was performed on (1.5%) agarose gel and run with 5V/cm for 1hrs. M: Mass Ruler High range. DNA ladder, 4: 4A(Ir3) .

DNA sequencing and phylogenetic analysis revealed that the isolate which obtained from oil contaminated soil in Iraq showed 97% similarity with the sequence within the gene bank. The closet phylogenetic neighbors according to the 16S rRNA sequence data for the isolate 4A, was A. rupiensis as shown in Fig. (4), the phylogenetic tree was constructed using the program MEGA5. According to the results of molecular identification, the isolate characterized as *Anoxybacillus rupiensis* strain (Ir3), and were deposited in the National Genebank database under the accession number, (JQ912241).

The identification based on 16S rDNA gene sequencing has a higher accuracy than conventional testing [31]. Techniques such as the polymerase chain reaction (PCR) and 16S rRNA sequencing have made it possible to specifically detect and identify microorganisms with high level of precision [32].

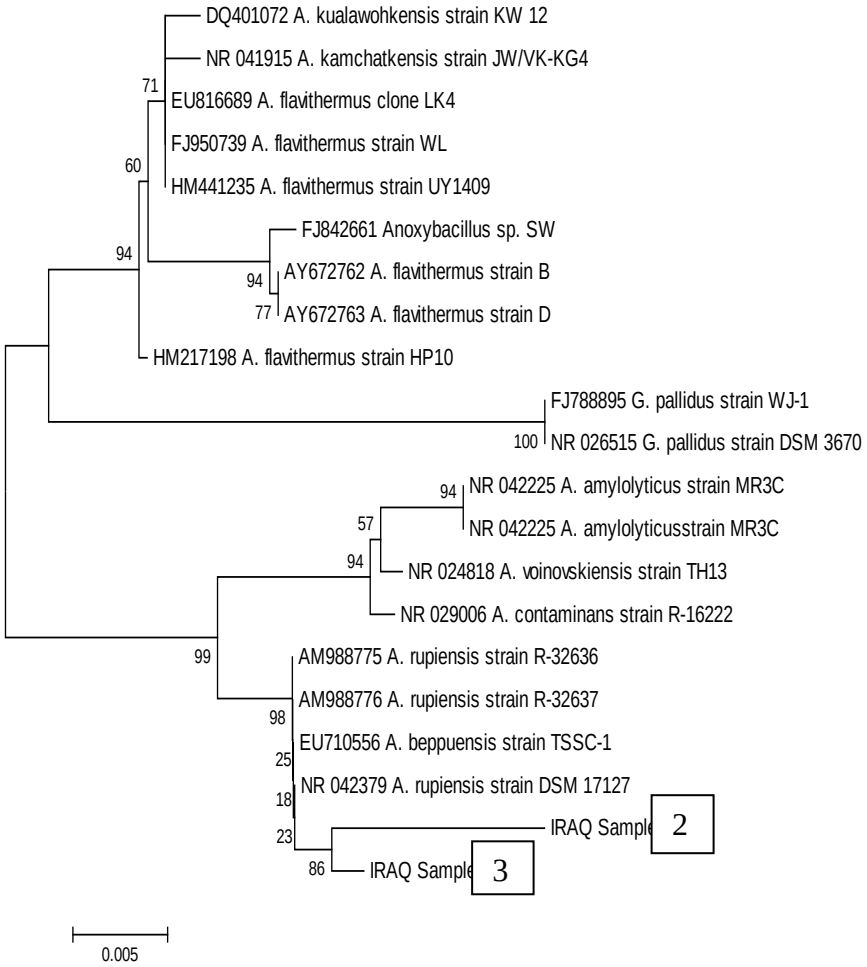

Fig. (4) Phylogenetic tree showing the position of *Anoxybacillus rupiensis* strain Ir3, other representative of the genus Anoxybacillus. Based on a comparison of 16S rDNAsequence.

Optimization of growth conditions of *A. rupiensis* strain Ir3 (JQ912241):

Determination of the optimum pH

The optimum growth pH of *A. rupiensis* strain Ir3 (JQ912241) was studied in the range between 5-9 and at optimum temperature, 55◦C. The result showed that this strain could grow well between pH 5-9, which indicated that *A. rupiensis* strain Ir3 (JQ912241) was a neutrophilic bacterium, and the optimum peak for growth was at pH 7 as shown in Fig. (5). Like some other members of the Anoxybacillus genus, *A. gonensis* CTISari, *A. kestanbolensis* AC26, *A. voinovskiensis* B9.3 [16] and *A. flavithermus* DSM 2641^T [1] and the optimum peak for growth was at pH 7 which resemble to *A. contaminans* DSM15866T [8] and *Geobacillus tepidamans* GS5-97 [12]. Bioremediation of

oil contamination can only be accomplished by using indigenous bacteria capable of degrading petroleum compounds.

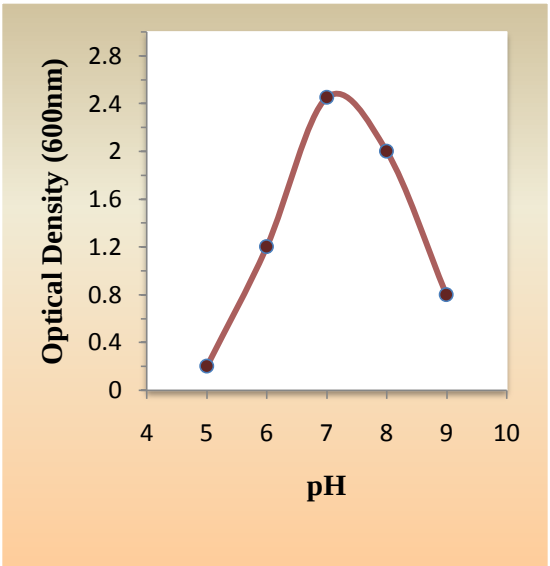

Fig. (5): Effect of pH on the growth of *A. rupiensis* strain Ir3 (JQ912241), at 55◦C in a shaker incubator (150 rpm) for 24hrs.

Determination of the optimum temperature

The optimum growth temperature of *A. rupiensis* strain Ir3 (JQ912241) was studied between 35- 70°C in LB broth medium. The optimum temperature for growth was 55-65◦C as shown in the Fig. (6).

A. rupiensis strain Ir3 (JQ912241) grows best in different temperatures. One plate was incubated at either: 35, 40, 45, 50, 55, 60, 65 and 70°C. *A. rupiensis* strain Ir3 (JQ912241) grows at 40 °C to 70 °C; therefore it is thermophilic. Thermophilic bacilli grow best at temperatures between 45 and 70°C. and the first research about the characterization of thermophilic bacteria, which were forming aerobic spores and able to grow at 70°C was done by [33]. And this strain may have good potential for application in microbial oil recovery.

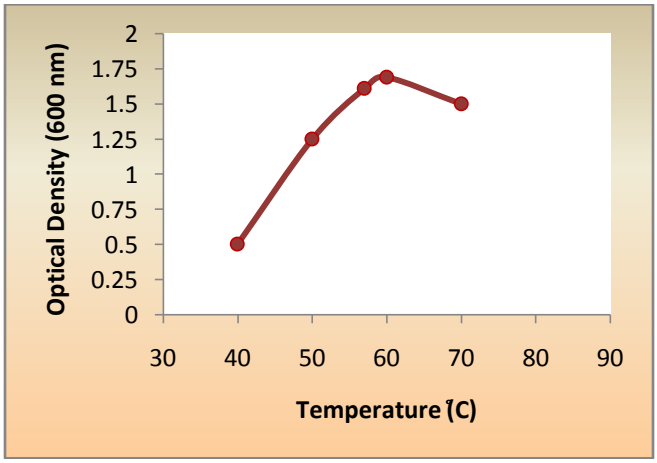

Fig. (6): Effect of temperature on the growth of *A. rupiensis* strain Ir3 (JQ912241), at pH 7, in a shaker incubator (150 rpm) for 24hrs.

A. rupiensis strain Ir3 (JQ912241) grew in wide ranges of temperatures and pH. It was similar to other Anoxybacillus spp. that also grow in the wide ranges of temperatures and pH such as *A. rupiensis* DSM 17956 sp. nov., which grow at pH 5.5- 8.5 and 35 -67 °C [12], *A. amylolyticus* sp. nov., which grow at pH 5.0- 6.5 and 45-65◦C [5], *A. flavithermus* comb. nov., which grow at pH 5.5- 9.5 and 30- 72 ◦C [1], and *A. contaminans* strain JT-12, which grow at pH 5.0- 10.0 and 45-75◦C [34]. Also the results indicated that Iraqi soils are a rich source of many thermophilic bacteria which could be a good source of interested enzymes from the industrial point of view and

further studies are recommended on these thermal soils including study of microbial biodiversity and the biotechnological potent of the isolated strain.

Growth of *A. rupiensis* strain Ir3 (JQ912241) at different sodium chloride concentrations

The result showed that the growth occurred at 0.5% to 4% of sodium chloride concentration. The optimum concentration for growth was 0.5% to 1% as shown in Fig. (7). Growth of *A. rupiensis* strain Ir3 (JQ912241) was inhibited gradually in the presence of NaCl concentration above 4%. It was similar to other Anoxybacillus spp. such as *A. kestanbolensis* strain K4^T, *A. ayderensis*, and *A. pushchinensis* as show in Table (1).

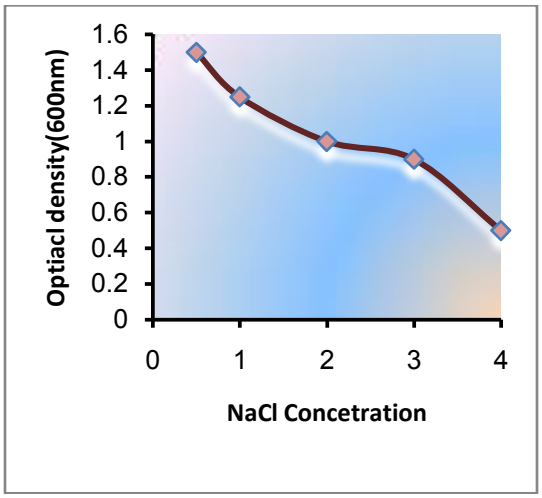

Fig. (7): Growth of *A. rupiensis* strain Ir3 (JQ912241) at different concentrations of NaCl, at pH 7, 55°C in a shaker incubator (150rpm) for 24hrs.

Studying the thermal death curve for *A. rupiensis* strain Ir3 (JQ912241)

The thermal death curve of strain *A. rupiensis* strain Ir3 (JQ912241) after exposure to 80 degree Celsius in water bath for ninety minutes was investigated. The result is showed in Fig. (8).

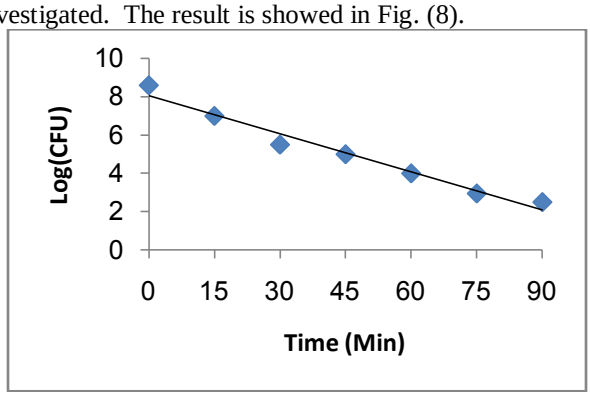

Fig. (8): Thermal death curve for *A. rupiensis* strain Ir3 (JQ912241) at (80°C).

Common practices to determine how temperature affects microorganisms are thermal death time (TDT), which is the shortest time it takes to kill specimens, so temperature is kept constant. Most organisms have an optimal temperature ranges for their best growth.

After analyzing the results, *A. rupiensis* strain Ir3 (JQ912241) withstands 80°C for at least 90 minutes. No growth was evident over 90 minutes. *A. rupiensis* Ir3 (JQ912241) could claim to be a thermophilic bacterium, as it grew well at 45 to 75◦C but could not grow at 37◦ C.

Since petroleum is often produced, transported, and processed at elevated temperatures, thermophilic microorganisms/ enzymes that can function at temperatures ranging from 60 to 100°C may be the most appropriates microorganisms to examine for biorefining applications. Performing biorefining processes at higher temperatures is compatible with existing industry practices and may also result in higher catalytic rates. The reduced viscosity of petroleum at higher temperatures will allow lower processing costs. Microorganisms which function at thermophilic temperatures can also function at mesophilic / ambient temperatures; whereas, mesophilic microorganisms almost

never function at thermophilic temperatures. Therefore, the isolation of thermophilic cultures capable of selectively removing organic nitrogen from petroleum should result in a highly flexible biorefining process that can be used at a wide range of temperatures. Thermophilic microorganisms have not been well studied and no systematic examination of thermophilic cultures for possible use in biorefining has been reported. Moreover studies seek to identify or develop cultures, thermophilic or otherwise, for the removal of nitrogen or metals from petroleum are rare [35].

Growth of *A. rupiensis* strain Ir3 (JQ912241) at different carbazole concentrations

Anoxybacillus rupiensis strain Ir3 (JQ912241) is a representative of carbazole-utilizing Gram-positive bacterium, which utilize CAR as a sole nitrogen and energy source, was tested at 0.2, 1, 5, and 10 mM in the presence of glucose as carbon source. The obtained result revealed higher bacterial growth at 10 mM than 0.2 mM, i.e. the observed bacterial growth with CAR as an N-source was concentration-dependents shown in Fig. (9). An attempt was made to study the biodenitrogenation ability of *A. rupiensis* strain Ir3 (JQ912241**).** The results showed better growth of the bacterium in the minimal medium in presence of carbazole as a nitrogen source and glucose as a carbon source than in presence of carbazole as the only source N and C, and also better than using NH_4CL (N-source) and glucose (C- source)**.** This indicated that *A. rupiensis* strain Ir3 (JQ912241) might be utilizing organonitrogen compound as a nitrogen source only via the specific cleavage of C-N bond (denitrogenation of carbazole) (data not show).

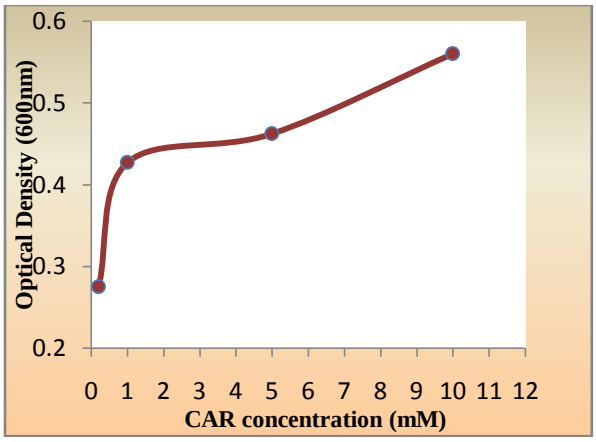

Fig. (9): Effect of carbazole concentration on the growth of *A. rupiensis* strain Ir3 (JQ912241), at pH 7, 55°C **in a shaker incubator (150 rpm) for 24hrs.**

To the researcher's best knowledge, this is the first study showing that the *A. rupiensis* belong to CAR-degrading bacteria. Various terrestrial bacteria belonging to Pseudomonas and Sphingomonas show CAR- degrading ability [36, 37, 38, 25, 22 23 and 26].

Characterization and identification of *Anoxybacillus rupiensis* Ir3 (JQ912241).

A. rupiensis strain Ir3 (JQ912241) was isolated from hydrocarbon contaminated soils in Iraq; it was the most efficient isolate for utilizing aromatic compounds and the identity of the carbazole-degrading culture. It was investigated using biochemical tests, microscopic observation, and a determination of its 16S rDNA gene. The culture is a Gram positive or (gram variable) long rod that form medium sized, smooth, round colonies with cream color, regular and complete margins on LB agar plate as shown in Fig. (10). 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°C optimum 55-65°C and in 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 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), with comparison to other species in that genus as shown in Table (1).

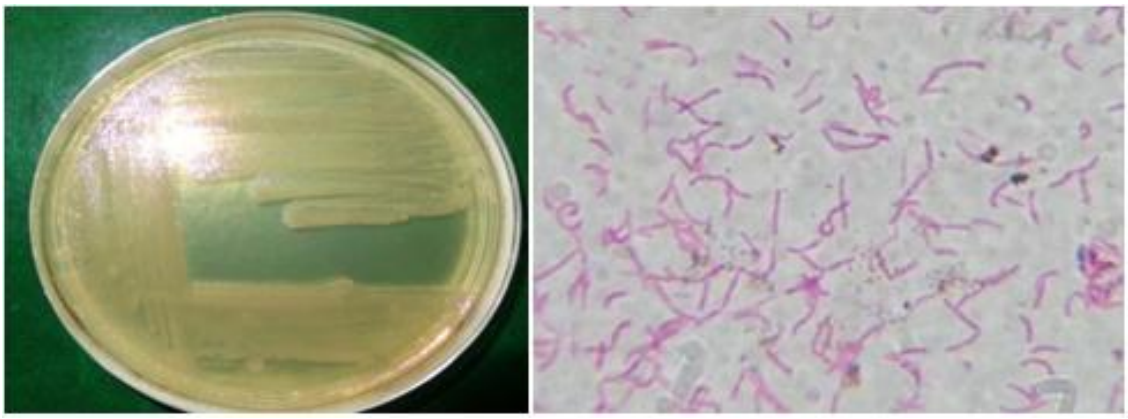

Fig. (10): The macroscopic and microscopic appearance for *Anoxybacillus rupiensis* Ir3 (JQ912241).

Table (1): Comparison of the phenotypic and biochemical characteristics of *Anoxybacillus rupiensis* Ir3 (JQ912241) and other Anoxybacillus species.

characterization		Anoxybacillus species						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Colony color		Creamy	White	Creamy	Creamy	Creamy	Creamy	yellow
Growth conditions		Aerobic	Anaerobic	Facultative	Facultative	Facultative	Facultative	Facultative
Temperature range (◦C)		40-70	37-66	40-70	30-70	40-70	45-65	37-69
Optimum temperature		55-65	62	55-60	50	50-55	61	60
pH range		5-9	8-10.5	6-10	6-11	6-10.5	5-6.5	5.5-9.5
Optimum pH		7	9.5-9.7	7.5-8	7.5-8.5	7.5-8.5	5.6	8-9
Tolerance to NaCl (w/v)%		4	3	4	2.5	4	<3	4.5
Tetracycline hydrochloride		-	ND	-	-	-	-	ND
Ampicillin		-	+	-	-	-	-	ND
Oxidase		+	ND	+	+	+	-	+
Catalase		+	-	+	+	+	+	+
Motility		+	-	+	+	+	+	+
Nitrate reduction		+	+	+	+	+	-	+
Utilization of	**Starch**	+	+	+	+	+	+	+
	Gelatin	-	-	+	+	-	+	+
	Glucose	+	+	+	+	+	-	+

1. *A. rupiensis* Ir3 (JQ912241), 2.*A. pushchinensis*, 3. *A. gonensis*, 4. *.A. aydernsis*, 5. *A. kestanbolensis*,
6. *A. amylolyticus*, 7. *A.salavatliensis*,
+: positive, - : negative, ND: not determined.

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