Journal of kerbala university , vol. 10 no.3 scientific . 2012

Isolation and characterization of two thermotolerant Kerosene degrading bacteria from oily contaminated soil

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Abstract

Seven bacterial isolates were isolated from soils contaminated with used diesel engine oils which had been used at a high temperature. Two isolates were gram negative bacteria and the other five were gram positive. All of these bacteria were tested for growth in Bushnell & Haas broth (BH broth) with kerosene as a carbon source at various temperatures (25˚ – 48˚C). One isolate grew well and gave significant growth at (37˚ – 48˚C) , another isolate grew well at (37˚ - 42˚C), the other five isolates lost viability after the first day of incubation at all temperature conditions tested. The two isolates which could grow in BH broth with kerosene were classified as *Sphingomonas spiritivorum* and *Pseudomonas chlororaphis*.

الخلاصة

تم عزل سبع عزلات بكتيريه من التربه الملوثه بزيوت محركات الديزل والتي استخدمت بدرجات حراره عاليه . كانت اثنتان منها سالبه لصبغه غرام والخمسه الاخرى موجبه. اختبرت جميع العزلات على قدرتها من حيث النمو في وسط الاملاح المعدنيه السائل(BH) الحاوي على الكيروسين كمصدر للكاربون بدرجات حراره مختلفه (25 – 48 م˚) , من هذه العزلات نمت واحده بشكل جيد بدرجات الحراره (37- 48 م˚) , وعزله اخرى اعطت نموا جيدا بدرجات الحراره (37 - 42 م˚) , اما العزلات الخمسه الاخرى فقد فقدت قدرتها على النموبعد اليوم الاول من الحضانه بجميع درجات الحراره المذكوره. العزلتان اللتان اظهرتا القدره على النمو في وسط الاملاح المعدنيه (BH) مع الكيروسين شخصتا بكونهما *Sphingomonas spiritivorum* و *Pseudomonas chlororaphis*

Introduction

Large numbers of bacterial species are able to oxidize a wide range of hydrocarbons including saturated or unsaturated aliphatic compounds, aromatic , naphthenic series and other hydrocarbon mixtures . According to Makut and Ishaya (1) the most prevalent bacterial hydrocarbon degraders in decreasing order belong to the genera *Pseudomonas, Achromobacter, Flavobacterium, Nocardia, Arthrobacter* and others which are able to degrade hydrocarbons including *Vibrio, Bacillus, Micrococus, Acinetobacter, Aeromonas* and *Alcaligenes* . Bacteria are the most active agents in petroleum degradation and they work as primary degraders, several bacteria are even known to feed exclusively on hydrocarbons (2). Inakollu *et al*.,(3) listed 25 genera of hydrocarbon degrading bacteria and 25 genera of hydrocarbon degrading fungi which were isolated from marine environment. Asimilar compilation by Mancera *et al*.,(4) included 22 genera of bacteria and 31 genera of fungi. Rosenberg (5) commented that the first priority of bacteria during hydrocarbon utilization is to gain energy and they may attack hydrocarbons in three different manners; (1) conversion of hydrocarbon to other similar and simple compounds (2) convert it to the carbon skeletons that compose the cell and (3) complete oxidation to CO_2 and water, and in all cases the rate of hydrocarbon oxidation depends on aeration, temperature, chain length, branching of chain and saturation or unsaturation. Fuel handling systems are very complex and at many points some form of microbial contamination, from variety of sources, is inevitable, microorganisms get into contact with oil products by various ways including ventilation and pumping systems. Generally it is very difficult to prevent microbial contamination of these products, because it is practically impossible to maintain sterile conditions during their transportation and storage, those

microorganisms capable of utilizing hydrocarbons as a sole source of carbon and energy occur practically everywhere; in air, water, and soil (6). Although the ability of bacteria to attack hydrocarbons has been known for many years, only recently has the importance of their influence on petroleum products quality been recognized, this may influence many properties of them including their color, emulsion stability, odor, octane rating, filterability, water tolerance and corrosive action on metals (7). In Iraq, there are a limited information on microbial degradation of hydrocarbons; the object of this work was to study the cultivable hydrocarbon bacterial strains isolated from polluted soils and their course of growth by determining their optimal recovery temperatures using the kerosene as a sole source for their carbon and energy and whether they can utilize it at a high temperature.

Material and methods

1- Isolation of oil degrading bacteria;

Bacteria were isolated from severely contaminated soils with used diesel and engine oils near shops of automobile engine changing oils in Hilla , by mixing 10 gr. of soil with 100 ml of Bushnell-Hass (BH) medium in 250 ml flasks and incubated at 37˚C in orbital shaker incubator at 170 rpm for two days, then 1ml of the suspension was taken for decimal serial dilutions up to 10^{-6}, aliquots of 0.1 ml were seeded on BH agar, which were covered with 100 microliter of kerosene , the plates were incubated at 37˚C for two days, the kerosene degrading individual different isolated colonies were subjected for re-streaking on another plates of the same media and conditions until purity (8).

2- Selection of kerosene assimilating strains at fixed different temperatures;

bacterial suspensions were prepared from the seven different strains which had been isolated . By picking a single colony of the isolate and suspending it in a 10 ml of BH medium, 1 ml of this bacterial inoculum (1 OD_{600} equivalent) was transferred to 100 ml BH medium containing 20 ml kerosene which was sterilized previously by filtration through sterile millipore membrane filter of 0.45 micron in diameter. Flasks were incubated at 25˚C, 30˚C 37˚C, 42˚C and 48˚C at 170 rpm in an incubator shaker, a control devoid of the bacterial isolate was prepared for each set of experiments. All experiments were performed in duplicate, the initial bacterial numbers and the subsequent bacterial growth in the aqueous phase were checked daily up to seven days by the plate dilution frequency technique using plates of Tryptose blood agar base (TBAB) (9) .

3- Identification, characterization and standardization of the isolates;

Two isolates which showed the ability to assimilate kerosene as a sole source of carbon and energy were identified to their species level using conventional microbiological and biochemical procedures. The tests were carried out according to Bergey's manual of systematic bacteriology (10) and confirmed by 16S rRNA sequencing (11), while the other isolates were identified to their genus only. Before usage in subsequent works, cells were standardized to the McFarland nephelometer standard (0.5) (12). In all cases, 1%(v/v) of standardized inoculum was used according to the volume of medium used.

4- Growth of *S. spiritivorum* and *P. chlororaphis* in various ratios of kerosene;

Aliquots of 1ml, 5ml and 10 ml of bacterial suspensions in BH broth were added to 100ml kerosene in conical flasks giving ratios v/v of 1:100, 5:100 and 10:100, flasks containing *S. spiritivorum* were incubated at 48˚C and that for *P. chlororaphis* were incubated at 42˚C, initial viable bacterial populations were determined and then the growth was estimated daily for one week using the plate dilution frequency technique for bacterial number count.

Results;

1- Isolation and identification of the isolates

The results of identification are shown in table 1. All the performed tests were incubated at the best recovery temperature of each bacterium, 42˚C for *P. Chlororaphis*, 48˚C for *S. spiritivorum* and 37˚C for the reminders.

2- Determination of the optimum recovery temperature of the isolates

Out of the seven isolates, only five isolates showed an approximate optimum recovery temperature at 37˚C, while the other two bacteria (*P. chlororaphis* and *S. spiritivorum*) had an optimum recovery temperature at 42˚C and 48˚C respectively. The ability of each isolate to grow at different temperatures and the optimum temperature for maximum growth with the initial and final cell population numbers are shown in table 2, *S. spiritivorum* can grow in a range of 25˚ - 48˚ C with the highest viable number of colonies on Tryptose blood agar base (TBAB) at 48˚C. Five isolates, (*Nocardia sp., Rhodococcuc sp., Arthrobacter sp., Corynebacter sp., and Micrococcus sp.)* failed to grow at 42˚C and 48˚C but gave the highest viable number of colonies at 37˚C, while *P. chlororaphis* gave the highest viable number of colonies at 42˚C, and its range of growth was between 25˚ – 42˚ C.

3- Growth in BH broth and kerosene at different temperatures;

Growth results of these experiments are shown in table 3, the isolates of *Nocardia sp., Rhodococcuc sp., Arthrobacter sp. Corynebacter. sp.,* and *Micrococcus sp.* gave visible growth on TBAB after incubation in BH broth with kerosene after the first day of incubation at 37˚C and were unable to grow at 42˚C and 48˚C, whiles the isolates of *S. spiritivorum* and *P. chlororaphis* grow very well and gave significant growth yields, *S. spiritivorum* reached its maximum growth yields from the second day of incubation at 48˚C and gave a higher growth yield at 42˚C than at 37˚C.

The optimum growth temperature for *P. chlororaphis* in BH broth with kerosene was 42˚C and it gave its maximum viable growth yield after 3 days incubation and produced a higher growth yield at 42˚C than at 37˚C.

4- Growth of *S. spiritivorum* and *P. chlororaphis* at different ratios of kerosene;

Maximum growth yield occurred after 2 days of incubation at 48˚C for *S. spiritivorum* and yields were almost similar at each ratios 1:100, 5:100 and 10:100, while at 42˚C maximum growth yields were obtained after 3 days for *P. chlororaphis* as shown in table 4, both isolates gave similar results in that they were unaffected by changes in the aqueous: non-aqueous phase ratio, although there was a very slight increase in numbers as the ratio increased, however, *S. spiritivorum* had a relatively higher yield than *P. chlororaphis* .

Discussion;

Seven bacterial spp. were isolated from fuel contaminated soil, only two of them (*S. spiritivorum* and *P. chlororaphis*) were thermotolerant and were able to utilize kerosene as a sole source of carbon and energy. Kerosene usually includes $C_{10} - C_{16}$ aliphatic HCs and antifreezing additives which can possess a biostatic properties, but it has been shown that some microorganisms are capable of degrading antifreeze agents added to kerosene (13). Most thermophiles known are moderate and show an upper temperature border of growth between 50˚ and 70˚ C, thermophiles, predominantly bacilli, possess a substantial potential for the conversion of environmental pollutants (14,15). Microbial action is directly influenced by several biotic and abiotic parameters, among these; special attention must be given to type and concentration of oil, type of environment that has been contaminated, prevalent of climatic conditions, pH, Nitrogen and phosphorous sources, microelements, as well as temperature which plays an important role by directly affecting the chemistry of the pollutants and also affecting the physiology and diversity of the microbial flora, therefore a higher reaction rates due to smaller boundary layers are expected at elevated temperatures (16). There are many ways to determine the optimum temperature of bacteria such as estimation of total yield of cells, the rate of respiration or fermentation, lag time , CO_2 measured in a bioreactor exhaust gas and spore production, however, the most common meaning of optimum temperature for bacteria is that temperature at which the specific growth rate is maximal (7,17) , Chung *et al*.,(18) shown that at higher or lower temperatures the growth rate is lower, and also commented that the rate of protein synthesis and rate of metabolic processes will decreased as the temperature is decreased below the optimum temperature for growth. The apparent viability of a

suspension of bacteria may vary according to the temperature at which the growth medium is incubated. The initial experiment established that the greatest recovery occurred at different temperatures according to each isolate, consequently, to determine the approximate temperature for optimum growth of the isolates. The growth yields were measured at different temperature range , five of the isolates had their optimum temperature at 37˚C and the other two (*P. Chlororaphis* and *S. spiritivorum*) had their optimum temperature at 42˚C and 48˚C respectively, it can thus be seen that the highest recovery of viable cells occur at these temperatures. Aetierlas (19) reported that different temperatures produced different growth yields when grown in kerosene and BH broth, moreover different media gave different yields and this was due to the different carbon source utilized in each case (20). All isolates were tested for their ability to assimilate kerosene with BH broth, only *S. spiritivorum* and *P. chlororaphis* are capable of assimilating it at temperatures between 37˚- 48˚C and 37˚- 42˚C respectively, (21) showed that some bacteria not capable of utilizing hydrocarbons could grow in fuel on metabolic byproducts of hydrocarbon-utilizing bacteria, this agrees with the statement of Alexander (22) that the acclimation of a microbial community to one substrate frequently results in the simultaneous acclimation to some, but not all structurally related molecules , also, individual microbial species have the ability to act on several structurally similar substrates, and therefore more easily act on their analogues after the first addition. Asia *et al*., (23) showed from their experiments that some of the bacteria isolated from the sludge present in the fuel tanks could grow in kerosene and mineral salts medium, while other isolates could not, and they stated that bacteria did so because they were capable of utilizing kerosene as their sole carbon source. In this work bacterial isolates of *S. spiritivorum* and *P. chlororaphis* were capable of utilizing kerosene, these species possess catabolic enzymes for hydrocarbons, and more importantly they have an immense capacity for adaptive change (24), it is believed that this adaptive capacity is promoted by their inherent patterns of regulation, which allows for the coincidental induction of different catabolic pathways resulting in novel patterns of biodegradation and this conforms to the high degradative ability and ubiquity associated with these bacterial types as it concerns biodegradation of both soil and water environments polluted with petroleum or its products (25) , all other bacteria tested, although isolated from hydrocarbon contaminated soil, failed to utilize kerosene, these bacteria probably lacked the hydrocarbon utilizing enzymes necessary for initial oxidation , however, they could grow in systems where hydrocarbon utilizing bacteria were present to carry out the initial oxidation, these bacteria are capable of utilizing the oxygenated metabolic by-product produced by the hydrocarbon utilizing bacteria (26) . Brooijmans *et al*.,(27) found that only three out of thirty three bacteria isolated from fuel tanks were fuel utilizer, the remainder was divided into doubtful fuel utilizers, fuel survivors and chance contaminants. the fuel survivors and the doubtful fuel utilizers could survive in mixed cultures of isolates but were not able to survive as pure cultures, these organisms utilize metabolic by-products produced by primary hydrocarbon oxidation by the fuel utilizers, however, others stated that to assume growth of microorganism in the presence of petroleum product as a “priori” evidence for hydrocarbon utilization, is not valid (28,29).

Table 1; Analytical profile index used in identification of the isolated strains.

Strains / Characters	*Nocardia sp.*	*Rhodococcuc sp.*	*Arthrobacter sp.*	*Corynbacter sp.*	*Micrococcus sp.*	*S. Spiritivorum*	*P. Chlororaphis*
Gram stain	+	+	+	+	+	--	--
Glucose fermentation	+	--	--	--	--	+	+
Urease production	+	+	+	+	+	+	--
Gelatin	+	--	--	--	--	--	--
Oxidase	+	+	+	+	+	+	+
Arabinose	+	--	--	--	--	--	+
Mannose	+	--	--	--	--	--	--
Maltose	+	--	--	--	--	--	--
Citrate	+	--	+	+	+	+	+
Ribose	+	--	+	+	+		
Galactose	+	--	--	--	+		
Sorbitol	+	--	+	+	+		
Rhamnose	+	+	--	--	--		
Dulcitol	+	--	--	--	--		
Inositol	+	+	+	+	--		
Melibiose	+	--	--	--	--		
Trehalose	+	--	--	--	+		
Glycogen	+	--	--	--	--		
Beta galactosidase	+	--	+	+	+	+	--
N-acetyl-glucosamine assimilation	+	--	+	+	+	+	--
Capronate assimilation	--	--	--	--	--	--	+
Adipate assimilation	+	--	+	+	+	+	--
Phenyacetate assimilation	+	--	+	+	+	+	--
B-Methyl-Xyloside	+	--	--	--	--		
α-Methyl-D-mannoside	+	--	--	--	--		
β-gentiobiose	+	--	--	--	--		
2Keto gluconate	+	--	--	--	+		
5Keto gluconate	+	--	--	--	--		

Note; + ; positive result , _ ; negative result , Blank ; test not performed

Table 2; Optimum recovery temperatures of the isolates with their initial and final population numbers on TBAB.

Isolates	Incubation temperatures (C˚)	Initial population (Log.no./m)l	Optimum temp. for maximum growth	Final population (log.no./ml)
S. Spiritivorum	25	2.880		2.716
	30	2.880		3.518
	37	2.880	48˚C	4.633
	42	2.880		5.963
	48	2.886		7.908
Nocardia sp.	25	2.869		3.908
	30	2.869		4.623
	37	2.869	37˚C	7.113
	42	2.875		Zero
	48	2.875		Zero
Rhodococcuc sp.	25	2.579		3.913
	30	2.579		4.903
	37	2.591	37˚C	7.732
	42	2.579		Zero
	48	2.591		Zero
Arthrobacter sp.	25	2.623		3.857
	30	2.623		4.653
	37	2.623	37˚C	7.079
	42	2.623		Zero
	48	2.612		Zero
Corynebact. Sp	25	2.612		3.505
	30	2.612		4.707
	37	2.623	37˚C	7.079
	42	2.612		Zero
	48	2.612		Zero
Micrococcus sp	25	2.732		3.740
	30	2.732		4.568
	37	2.732	37˚C	7.531
	42	2.732		Zero
	48	2.740		Zero
P. Chlororaphis	25	2.544		2.716
	30	2.544		3.579
	37	2.544	42˚C	4.707
	42	2.556		7.913
	48	2.556		Zero

Table 3; Growth of the bacterial isolates in BH broth with kerosene as a sole source of carbon and energy at different temperatures.

Incubation temperatures (C˚)	Incubation time (days)	*S. Spiritivorum*	*Nocardia sp.*	*Rhodoc occuc sp.*	*Arthrob acter sp.*	*Coryne bact sp.*	*Micro coccus sp.*	*P. chloro raphis*
25		+	+	+	+	+	+	+
30		+	+	+	+	+	+	+
37	1	+	+	+	+	+	+	+
42		+	--	--	--	--	--	++
48		++	--	--	--	--	--	--
25		+	--	--	--	--	--	+
30		+	--	--	--	--	--	+
37	2	+	--	--	--	--	--	+
42		++	--	--	--	--	--	++
48		+++	--	--	--	--	--	--
25		+	--	--	--	--	--	+
30		+	--	--	--	--	--	+
37	3	+	--	--	--	--	--	++
42		++	--	--	--	--	--	+++
48		+++	--	--	--	--	--	--
25		+	--	--	--	--	--	+
30		+	--	--	--	--	--	+
37	4	+	--	--	--	--	--	++
42		++	--	--	--	--	--	+++
48		+++	--	--	--	--	--	--
25		+	--	--	--	--	--	+
30		+	--	--	--	--	--	+
37	5	+	--	--	--	--	--	++
42		++	--	--	--	--	--	+++
48		+++	--	--	--	--	--	--
25		+	--	--	--	--	--	+
30		+	--	--	--	--	--	+
37	6	++	--	--	--	--	--	++
42		+++	--	--	--	--	--	+++
48		+++	--	--	--	--	--	--
25		+	--	--	--	--	--	+
30		+	--	--	--	--	--	+
37	7	++	--	--	--	--	--	++
42		+++	--	--	--	--	--	+++
48		+++	--	--	--	--	--	--

-- ; no growth, + ; slight growth , ++ ; moderate growth, +++ ; abundant growth.

Table 4; Growth of *S. Spiritivorum* and *P. Chlororaphis* in BH broth with different ratios of kerosene.

Isolate	incubation Temp. C°	Kerosene ratio	Incubation time for max. growth(day)	Initial population (log.no./ml)	Maximum yield (log. no./ml)
S. Spiritivorum	48 48 48	1: 100 5: 100 10:100	2 2 2	4.491 4.491 4.505	8.755 8.770 8.778
P. Chlororaphis	42 42 42	1: 100 5: 100 10:100	3 3 3	4.518 4.531 4.505	8.556 8.579 8.579

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