GENETIC IMPROVEMENT OF SOME BACTERIAL ISOLATES IN UTILIZATION OF HYDROCARBON COMPOUNDS

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Abstract

Six bacterial isolates belonging to the genus ***Pseudomonas*** were selected for their high ability to utilize crude oil. These isolates showed a diverge ability for utilization of different hydrocarbon compounds and 2 isolates (***P. aeruginosa*** RB19 and ***Pseudomonas*** sp. RB29) were able to utilize all the tested compounds except tetrahydrofuran, sodium toluate and *trans*–1, 2–diphenylethylene. Conjugation between ***P. aeruginosa*** RB19 and ***P. aeruginosa*** RB27 was made in an attempt to obtain bacterial isolates, capable to utilize a wide range of hydrocarbon compounds. The conjugation was successful and a number of transconjugants were obtained. All transconjugants were able to utilize sodium toluate, and some of these transconjugants were able to utilize salicylic acid. This indicated that ***P. aeruginosa*** RB27 was the recipient bacterium which received pSR202 plasmid (responsible for salicylic acid and benzoate utilization) from ***P. aeruginosa*** RB19.

تحسين وراثي لبعض العزلات البكتيرية في استهلاك المركبات الهايدروكاربونية

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الخلاصة

أنتخبت ستة عزلات بكتيرية تعود لجنس ***Pseudomonas*** وذلك لقابليتها الجيدة على استهلاك النفط الخام. اظهرت هذه العزلات قابليات مختلفة على استهلاك عدد من المركبات الهيدروكاربونية المختلفة، وتمكنت عزلتين منها (***Pseudomonas aeruginosa*** RB19 و***Pseudomonas*** sp. RB29) من استهلاك كل المركبات المختبرة باستثناء الـ *trans*-1, 2-diphenylethylene, tetrahydrofuran, sodium toluate. أجريت عملية اقتران بين العزلتين ***P. aeruginosa*** RB19 و***P. aeruginosa*** RB27 في محاولة للحصول على عزلات بكتيرية قادرة على استهلاك مدى واسع من المركبات الهيدروكاربونية. كانت عملية الأقتران ناجحـة وتم الحصول على عدد من المقترنات. وتبين ان جميع المقترنات كانت قادرة على استغلال sodium toluate، كما ان بعض المقترنات كانت قادرة على استغلال salicylic acid، مما يشير الى ان بكتريا ***P. aeruginosa*** RB27 كانت هي البكتريا المستلمة وان بعض خلايا هذه البكتريا أكتسبت البلازميد pSR202 (المسؤول عن استغلال salicylic acid وbenzoate) من بكتريا ***P. aeruginosa*** RB19.

Introduction

Crude petroleum is a mixture of several complex hydrocarbons. Hydrocarbons can be classified generally into three major groups including: aliphatic, alicyclic and aromatic hydrocarbons, upon their chemical structure [1].

Large amounts of crude petroleum ranging from 1.9×10^6 to 11.4×10^6 tones reaches the environment through the accident of oil transport tankers, ballast water, the offshore oil production, illegal industries and refineries wastes discharge [2].

Cleaning-up of such pollutants by physiochemical means like dispersion, sinking, collecting and the use of mechanical recovery are expensive and labor intensive and often leads to air pollution [3]. A better way would be to use biological degradation and/or bioemulsifiers, such a process is usually cheap and environmentally friendly and entails no major technical hurdles [4].

The biological method has taken two directions one involves the provision of suitable conditions like temperature, pH, and addition of nutrients. The other direction depends upon the genetic development of specific microbial strains especially the bacteria present in polluted area [5, 6, 7].

It has been noted that the bacteria ***Pseudomonas*** was the most important of hydrocarbon–degrading bacteria. ***Pseudomands*** spp. exhibit a wide range of metabolic activity against a wide range of hydrocarbons [8] like monoterminal oxidation systems for aliphatic hydrocarbons [1], besides the catabolic pathways that cleave and oxidize aromatic hydrocarbons through *ortho*, *meta* and *para* pathways [9].

This bacterium is considered as a natural host for a wide array of plasmids ex: plasmid for antibiotic resistance, heavy metal resistance, ultra–violet resistance and degradative plasmids [10], which represent a group of naturally occurring plasmids, that are responsible for the petroleum hydrocarbons and synthetic compounds degradation [11].

Degradative plasmids are generally transmitssible or conjugative plasmids, with high molecular weights (may reach 500 Kb) enough for loading the biodegradation and conjugation genes. These plasmids are member of Incompatibility P family of plasmids [12, 13]. Horizontal gene transfer, is a genetic mechanism by which micro-organism can acquire novel metabolic capabilities [7].

Conjugation is considered as a suitable mean for hydrocarbon degrading plasmids transfer, because most of these plasmids are conjugative especially those found in Pseudomonas, and as a result, several Pseudomonas strains have been constructed for harboring different aliphatic and aromatic hydrocarbon degradative plasmids [5, 4].

So this research was aimed to investigate the ability of some bacterial isolates to utilize various hydrocarbon compounds and attempt to improve strain(s), that capable to utilize a wide range of hydrocarbon compounds.

Materials and Methods

Bacteria

Pseudomonas sp. RB7, RB29, RB31, ***P.aeruginosa*** RB19, RB7 and ***P.fluorescens*** RB16, were isolated in previous study [14].

Culture Media

LB (Luria-Bertani) Broth and agar [15].

Where used for the growth of bacterial isolates.

Mineral Salts Medium MSM [16].

Where used for the detection of bacterial utilization for different types of pure hydrocarbon compounds as a sole source of carbon and energy.

Utilization of Pure Hydrocarbon Compounds

The liquid hydrocarbons were sterilized by filtration (Millipore filter 0.45µm). The solid hydrocarbons as naphthalene, naptholbenzene and salicylic acid were dissolved in diethyl ether to prepare stock solutions and sterilized by filtration. Other solid hydrocarbons as sodium benzoate and sodium toluate were dissolved in distilled water to prepare stock solutions, sterilized by filtration, while anthracene and *trans*-1, 2-diphenyl ethylene were sterilized by autoclaving at 121^{o}C for 15 min. Hydrocarbons were added to MSM in concentration of 0.2%.

Flasks were inoculated with mid exponential phase culture of bacterial isolates after being washed and resuspended in MSM. Cultures then incubated with shaking (180rpm) at 37°C for 7 days. The growth was determined by measuring the optical density at 600nm wavelength.

Bacterial Conjugation

Conjugation was performed between ***Pseudomonas aeruginosa*** RB19 (Neo^r and Sm^s), and ***Pseudomonas aeruginosa*** RB27 (Neo^s and Sm^r), according to the procedure described by Stuart – Keil *et al.*, [6].

Samples were taken from the conjugation mixture and diluted appropriately, and then 0.1ml samples from proper dilution was spread on selective medium (LB agar containing 50µg/ml Neomycin and 50µg/ml Streptomycin), that allowed the growth of transconjugants, but not recipient and donor colonies. Both donor and recipient controls were plated onto the same selective medium to account for spontaneous Neomycin and Streptomycin resistance and/or experimental errors. The growing colonies were picked using sterile toothpicks and plated onto the same selective medium (as a master plate) and tested for their ability to utilize hydrocarbon compounds as described previously.

Results and Discussion

The ability of six ***Pseudomonas*** isolates to utilize pure hydrocarbons as a sole source of carbon and energy were described in the Table (1).

Table 1: GROWTH OF BACTERIAL ISOLATES ON HYDROCARBON COMPOUNDS AT 37°C FOR 7 DAYS.

Compound	*P. aeruginosa* RB19	*P. fluorescens* RB16	*P.* sp. RB7	*P. aeruginosa* RB31	*P.* sp. RB29	*P. aeruginosa* RB27
Octane	+++	++	++	++	+++	++
n–Decane	++	+	++	+	++	-
Hexadecane	+++	-	-	-	++	+
Sodium benzoate	+++	+++	+++	+++	+++	+++
Sodium toluate	-	-	++	+++	-	++
Salicylic acid	+++	-	++	-	++	-
Xylene	+	-	-	-	+	-
Naphthalene	++	-	-	-	++	-
Naphtholbenzene	++	-	-	++	++	+
Anthracene	++	-	-	-	+	-
Cyclohexane	++	-	-	-	++	++
Phenol	+	-	-	-	+	-
Tetrahydrofuran	-	-	-	-	-	-
trans–1,2–diphenyl ethylene	-	-	-	-	-	-

(-) No growth, (+) Slight growth ($O.D_{600}$= 0.2–0.4), (++) Moderate growth ($O.D_{600}$= 0.4–0.6), (+++) Good growth ($O.D_{600}$= 0.6–0.8).

Results indicated that all of these isolates were able to utilize octane (small chain n–alkane) and benzoate (single ring aromatic compound). Their ability to utilize benzoate was the highest in contrast with the utilization of other hydrocarbons. None of these isolates were capable to utilize *trans*–1, 2–diphenylethylene and tetrahydrofuran.

Pseudomonas aeruginosa RB19 and ***Pseudomonas*** RB29 were capable to utilize all of the hydrocarbon compounds except toluate (single ring aromatic hydrocarbon), *trans*-1, 2-diphenyl ethylene and tetrahydrofuran. Other isolates showed a diverse ability to utilize hydrocarbon compounds as a sole source of carbon and energy.

Results indicated that, generally, most of these isolates showed a good utilization of aliphatic hydrocarbons, because of their simple compositions, less toxicity and easy to utilize [1]. Also, all of these isolates showed a high ability to utilize benzoate, since benzoate is the simple in structure aromatic hydrocarbon that could be degraded by bacteria and utilized by different metabolic pathways [16].

Only ***Pseudomonas aeruginosa*** RB19 and ***Pseudomonas*** RB29 were able to utilize polynuclear aromatic hydrocarbons, but failed to grow on trans–1,2–diphenyl ethylene. It was known that the ability of microorganisms to utilize hydrocarbon compounds decreased with the increase of complexity of these hydrocarbons [17, 18].

According to the results obtained Table (1), only 3 isolates (***P. aeruginosa*** RB19, ***Pseudomonas aeruginosa*** RB27 and ***Pseudomonas*** RB29) were able to utilize cyclohexane (alicyclic compound). Alicyclic compounds were characterized by their high toxicity toward microbial cells and their complex structure, therefore the biodegradation of alicyclic compounds as a sole source of carbon and energy needs synergistic cooperation of two or more microbial species [19].

The failure of these isolates to utilize tetrahydrofuran as a sole source of carbon and energy may be attributed to its antimicrobial activity [20].

The chemical structures of hydrocarbon compounds not only have an extensive effect on their utilization by different bacterial isolates, but also the genetic diversity of these isolates affect the biodegradability of hydrocarbon compounds. As well as, some bacteria may contain different genetic elements like plasmids or transposons, harboring biodegradative genes that might participate in diverging the utilization of hydrocarbons [8, 4, 21].

Conjugation Experiment:

Conjugation was performed between ***P. aeruginosa*** RB19 and ***P. aeruginosa*** RB27 in order to obtain transconjugants with a better metabolic capabilities.

It was known that ***P. aeruginosa*** RB19 was the most efficient isolate for hydrocarbon utilization and contained two plasmids, pSR101 (confer resistance to Neomycin, Carbenicillin and Trimethoprim), and pSR202 (carry genes responsible for utilization of sodium benzoate and salicylic acid) [22, 21].

Results indicated that a successful conjugation between RB19 and RB27 occurred, and transconjugants (Neor and Smr) were obtained on selective media.

A number of transconjugants (20 colonies) were selected and tested for their ability to utilize hydrocarbon compounds that were utilized originally by ***P. aeruginosa*** RB19 and ***P. aeruginosa*** RB27, as a sole source of carbon and energy.

Results (Table 2) showed that all (20) transconjugants were able to utilize sodium toluate in presence of Neomycin and Streptomycin, and five from them (PT8, PT6, PT12, PT13 and PT14) were able to utilize salicylic acid in addition to sodium toluate. None of these transconjugants were able to utilize n–decane, xylene, phenol, anthracene and naphthalene.

From these results it could be concluded that all the transconjugants were ***P. aeruginosa*** RB27 (recipient) received genetic elements from ***P. aeruginosa*** RB19 (donor), because all the strains were capable of utilizing sodium toluate just like ***P. aeruginosa*** RB27, and only five from these transconjugants were capable of utilizing salicylic acid (received genetic elements from ***P. aeruginosa*** RB19).

Not all transconjugants gained the ability to utilize salicylic acid but all of them gained the ability of being Neomycin resistant because selection was based on the antibiotic resistant traits. ***P. aeruginosa*** RB19 contain small plasmid pSR101 which carry genes responsible for Neomycin resistance [21] may have transferred with self transmissible plasmid or by conduction [23, 7].

The reason that only five transconjugants showed the ability to utilize salicylic acid may be attributed to the transfer of the self transmissible plasmid pSR202 that carry genes responsible for salicylic acid utilization [21].

This plasmid was transferred to ***P. aeruginosa*** RB27 and efficiently expressed in the new host. The plasmid pSR202 was successfully transferred by conjugation to ***E. coli*** MM294 and was able to show its expression in the new host [21].

Conjugation between different ***Pseudomonas*** spp. in order to improve their ability of utilizing a wide range of hydrocarbon compounds had been attempted previously and recently. It was found that SAL–plasmid in ***P. putida*** AC36 is a self–transmissible plasmid and was trans-

ferred to ***P. putida*** AC536, and its ability to utilize salicylate was confirmed [24]. TOL–plasmid pWWO of ***P. putida*** was transferred to ***P. fluorescens*** via conjugation; the transconjugants obtained were gained the ability to utilize xylene and toluene [9].

Table 2: Hydrocarbons Utilization of Transconjugant Strains Resulted from Conjugation between *P. aeruginosa* RB19 and *P. aeruginosa* RB27, Growing in Mineral Salts Medium Containing Neo and Sm at 37°C for 7 Days.

	n–Decane	Sodium toluate	Salicylic acid	Xylene	Naphthalene	Anthracene	Phenol
***P. aeruginosa* RB19**	++	-	+++	+	++	++	+
***P. aeruginosa* RB27**	-	++	-	-	-	-	-
Trasconjgant Strains							
PT1	-	++	-	-	-	-	-
PT2	-	++	-	-	-	-	-
PT3	-	++	-	-	-	-	-
PT4	-	++	-	-	-	-	-
PT5	-	++	-	-	-	-	-
PT6	-	++	+++	-	-	-	-
PT7	-	++	-	-	-	-	-
PT8	-	++	+++	-	-	-	-
PT9	-	++	-	-	-	-	-
PT10	-	++	-	-	-	-	-
PT11	-	++	-	-	-	-	-
PT12	-	++	+++	-	-	-	-
PT13	-	++	+++	-	-	-	-
PT14	-	++	+++	-	-	-	-
PT15	-	++	-	-	-	-	-
PT16	-	++	-	-	-	-	-
PT17	-	++	-	-	-	-	-
PT18	-	++	-	-	-	-	-
PT19	-	++	-	-	-	-	-
PT20	-	++	-	-	-	-	-

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