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Naphthalene biodegradation by alkaliphilic *Pseudomonas aeruginosa* EGDS2

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Abstract

A naphthalene degrading bacterium was isolated from diesel contaminated soil in Damietta County, Egypt. It is identified as *Pseudomonas aeruginosa* strain EGDS2 based on classical and 16S rDNA sequence techniques. P. aeruginosa EGDS2 was highly able to degrade naphthalene in addition to benzene, toluene, catechol, and xylene. The normalize dioxygenases of P. aeruginosa EGDS2 responsible for naphthalene degradation was optimized for producing the maximum activity. The results revealed that the maximum dioxygenases activity was after 48 hours at pH 9.0 and 30oC. The best nitrogen source was sodium nitrate, and 7g/l of naphthalene was the optimum concentration for highest dioxygenases activity. The promising yield of dioxygenases of P. aeruginosa EGDS2 makes this isolate very useful for bioremediation of aromatic hydrocarbon pollutants.

Keywords: Naphthalene, dioxygenase, *Pseudomonas aeruginosa*, optimization.

Introduction

Biodegradation of aromatic compounds is a nature method to remove pollutants in the environments where it has been known for a long time that microorganisms can degrade pollutants in different environments under aerobic or anaerobic condition. Polycyclic aromatic hydrocarbons (PAHs) likes naphthalene, anthracene and phenanthrene are among largest industrial chemicals produced, with production figures of millions of tons per year. These compounds are used as fuels, which in nature is of great environmental concern due to their toxic, mutagenic, and carcinogenic properties.

Lower-molecular-weight PAHs, such as naphthalene and phenanthrene, are relatively easy to degrade, and able to be metabolized by a large number of different microorganism strains (Cerniglia, 1992). In soil environments, degradation of PAHs is strongly affected by the low bioavailability of the compounds, as they have only limited water solubility and tends to be sorbet strongly to particularly organic matter. Biodegradation using microorganisms is usually the preferred and major route of PAH removal from contaminated environments because of its

cost effectiveness and complete cleanup (Pothuluri and Cerniglia, 1994).

The first step of naphthalene degradation by bacterial metabolism, is the formation of the corresponding 1,2-dihydrodiol in the optically pure *cis*-(lR, 2s)-dihydroxy-1,2-dihydro naphthalene form. The cis-relative stereochemistry is characteristic of the bacterial degradation in contrast to the mammalian metabolism, which yields *trans* diols. This reaction in bacteria is carried out by normalized dioxygenases enzymes which also can oxidize indole to indigo dye (Ensely *et al.*, 1983) that can be easily monitored spectrophotometry.

Previous research on this issue has been reported the effectiveness of many bacterial strains with regard to aromatic degradation, and the most commonly bacteria are *Pseudomonas* sp. (Sorensen *et al*., 2010) like *P. putida* and *P. fluorescens* (Shim and Yang, 2002 and 2006; Shim *et al*. 2002 and 2005) and *P. aeruginosa* (Kim *et al*. 2003). *B. pumilus* was one of the consortia members which were able to degrade PAH present in the crops rhizosphere contaminated with field PAH (Ma *et al*. 2010).

There is a need for more information on microbial degradation of polycyclic aromatic hydrocarbons in Egypt. In this work, we report the isolation and identification of naphthalene degrading *Pseudomonas aeruginosa* from Egyptian contaminated soils with diesel. Also, optimization of normalized dioxygenases enzymes activity was considered.

Materials and methods

Isolation of naphthalene-metabolizing bacteria

10 g of diesel contamination soil samples around irrigation pumps (at Damietta, Egypt) were used as inoculum. Samples were placed into sterile polythene bag then immediately brought to the laboratory. Each sample was mixed well to insure sample homogeneity then added to 100 ml of sterile distilled water. Leave it for 15 min in shaker (200 rpm) at room temperature.

1 ml of soil suspension used as inoculum in modified mineral salt agar medium (Ortiz *et al.,* 2005) consisted of (g/l):0.6 KH_2PO_4; 2.4 K_2HPO_4; 1.5 $MgSO_4 \cdot 7H_2O$; 0.15 $CaSO_4 \cdot 2H_2O$; 0.03 $FeSO_4 \cdot 7H_2O$; 3.0 $(NH_4)_2SO_4$, pH 7 and modified by adding trace element 1ml per liter of (Beauchop and Elsden, 1960). In addition to naphthalene (2g/l), other hydrocarbon compounds (benzene, toluene, catechol, and xylene) were added separately to the previous medium were after autoclaving the pervious medium.

Identification of naphthalene degradation isolate

Classical Identification

The identification based on morphological, physiological and biochemical characters including standard method of Bergey's manual (Palleroni, 1984).

Molecular identification

This identification was based on the 16S rDNA sequencing. Genomic DNA was extracted by phenol/chloroform extraction followed by ethanol precipitation according to Ausubel *et al* (2003).

The extracted DNA was subjected to PCR amplification condition following Eden *et al*., (1991) using the 16S rDNA bacteria universal primers: 27F (5̀-AGAGTTTGATCMTGGCTCAG-3̀) and 1492R (5̀-GGTTACCTTGTTACGACTT-3̀). The PCR product was sequenced by an automated sequencer sequencing (Macrogen co. Korea) using the same pervious primers.

Blast (Altschul *et al*., 1990 and 1997) was performed to obtain the DNA sequence match with the best similarities with other related 16S rRNA genes on database. Pairwise and multiple DNA sequence alignment were carried out using CLUSTALW multiple sequence alignment programme version 1.82 (Thompson *et al*., 1994). Bootstrap neighbor joining tree was generated using MEGA version 4 (Tamura *et al*., 2007). The *Saccharomyces cerevisiae* was used as an out group strain.

Assay of normalized dioxygenases activity

Bacterial cells were cultured on previously described mineral salt broth medium

supplemented with naphthalene as the only carbon source and incubated at 30°C with shaking (200 rpm). After 48hr, 1ml of medium was harvested at 4°C and cells were washed with phosphate buffer (pH=7.2) by centrifugation (1 min at 14,000 rpm). The cells were resuspended in 1 ml menial salt broth medium without naphthalene. The sample was then immediately shaking in water bath at 30°C and the reaction was initiated by adding 5 µl of 100 mM indole in *N*,*N*-dimethlylformamide (DMF). The formation of indigo was measured spectrophotometrically at OD_{600} as a function of time where control contains all reaction components except indole. The enzymes activity was defined as the initial rate of indigo formation normalized to the protein content of the sample (Wooa *et al*., 1999). The protein concentration in each sample was determined by Lowry *et al*., (1951).

Optimization of normalized dioxygenases from P. aeruginosa EGDS2

Pseudomonas aeruginosa EGDS2 was grown on previously modified MSM supplement with naphthalene for incubation period varied from 24hrs to 120hrs.The naphthalene concentration were1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 gram per liter was applied. A range of pH 4-10 was studied. Also temperature 25, 30, 35, 40 and 45°C were conducted.

Effect of supplementary nitrogen source on dioxygenases activity by replacing ammonium sulfate in the MSM according to equivalence with ammonium chloride, ammonium nitrate, ammonium dihydrogen phosphate, sodium nitrite, sodium nitrate, beef extract, yeast extract, peptone and tryptone, thus were applied at 30°C and pH 9.0 for 48 hours.

All experiments were run in parallel triplicates and the results analysis in IBM SPSS statistics version 22

Results

Isolation and identification of naphthalene metabolizing bacterial isolate

The bacterial isolate that had the ability to grow and metabolizing naphthalene (2g/l) on modified mineral salt agar medium was identified according to Bergey's manual. Its morphological characteristics and biochemical reactions are listed in Table 1 indicated that the bacterial isolate could be identified as *Pseudomonas aeruginosa* strain EGDS2.

The 16S sequence alignment showed that the EGDS2 isolate was highly similar to the other strains of P. aeruginosa present in the data base. The highest similarity reached more than 99% with P. aeruginosa S3 (KX911719), P. aeruginosa FA-HZ1 (CP017353), P.aeruginosa L15 (KX832717), P. aeruginosa M1 (KX495190) and P. aeruginosa ATCC-15692 (CP017149) as indicated in the phylogenetic tree analysis (Figure 1).

Table 1. Morphological characteristics and biochemical reactions for identification of the P. aeruginosaEGDS2 isolate.

Morphological, physiological and Biochemical properties	**Reactivity**
Morphological characteristics	
Gram Stain	-ve
Shape	Rods
Spore formation	-ve
Physiological characteristics	
Air condition	Aerobic
Oxidase	+ve
Catalase	+ve
Indole	-ve
Voges-Proskauer	-ve
Citrate utilization	+ve
Casein Hydrolysis	+ve
Starch Hydrolysis	-ve
Gelatin Liquefaction	+ve
Nitrate reduction	+ve
H_2S production	-ve
Deamination of Phenylalanine	-ve
Urease activity	+ve
Utilization of sugars	
Glycerol	+ve
Arabinose	+ve
Xylose	+ve
Galactose	+ve
Lactose	-ve
Mannose	+ve
Fructose	+ve
Glucose	+ve

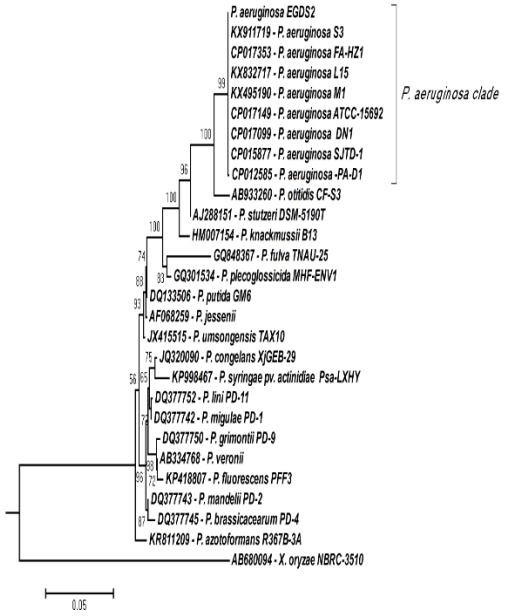


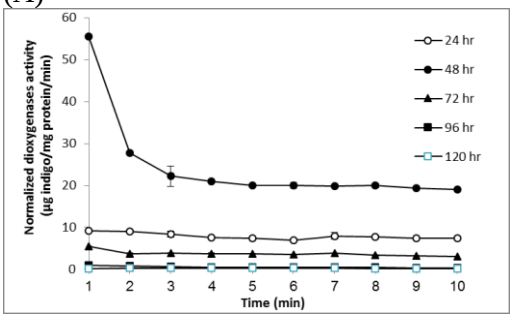
Figure 1. Phylogenetic tree analysis based on the 16S rDNA sequence alignment for P. aeruginosa EGDS2 with some other related and Pseudomonas species which possessed the best similarity. The 16S rDNA sequence of Xanthomonas oryza(AC:AB680094) was used as out-group to root the tree.

Optimization of normalized dioxygenases activity from P. aeruginosa EGDS2

Effect of incubation period

To ascertain the effects of cultivation time on the normalized dioxygenases enzymes activity, *P. aeruginosa* EGDS2 was cultivated for different periods. The results revealed that the activity of the dioxygenase of *P. aeruginosa* EGDS2 was increased progressively until the second day (48 hours) that reached the maximum activity (55.4905µg indigo/mg protein/min) at the first minute, and then it decreased dramatically to 27.7452 µg indigo/mg protein/min after two minutes. It then stabled to the average 20.16841 µg indigo/mg protein/min till ten minutes (Figure 2.A). On the other hand, the first 24 hours showed steady dioxygenases activity with approximate average 7.4140 µg indigo/mg protein/min. After 72 hours, it decreased to approximate average 3.3439 µg indigo/mg protein/min of enzymes activity, and then it lowered 0.5163 and 0.2434 µg indigo/mg protein/min at 96 and 120 hours, respectively (Figure 2.B).

(A)



(B)

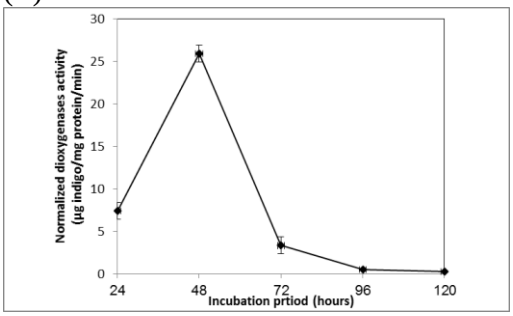
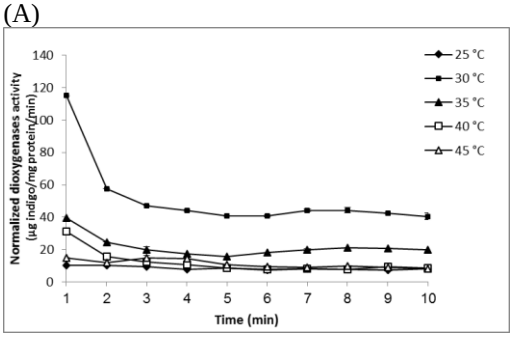


Figure 2. (A)The effect of incubation period on normalized dioxygenases enzymes activity from P. aeruginosa EGDS2, the activity was measured for ten minutes intervals. (B) The activity of normalized dioxygenases after 5 minutes for each incubation period.

Effect of temperature

The maximum activity of dioxygenases of P. aeruginosa EGDS2 was optimum at 30°C with enzymes activity 50.9494 µg indigo/mg protein/min. The highest value of normalized dioxygenases activity was recorded at the first minute (115.11µg indigo/mg protein/min) then decreased to 57.55µg indigo/mg protein/min in the 2nd minute (Figure 3.A). Total average of normalized dioxygenases activity was decreased to minimum at 40°C (11.8µg indigo/mg protein/min) and 45°C (5.08µg indigo/mg protein/min) (Figure 3.B).

(A)



(B)

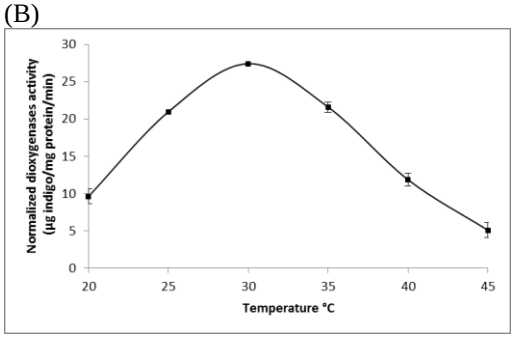
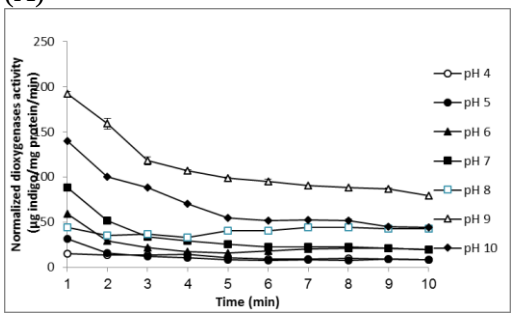


Figure 3. (A) The effect of temperature on normalized dioxygenases activity produced by P. aeruginosa EGDS2, the activity was measured for ten minutes intervals. (B) The activity of normalized dioxygenases after 5 minutes of different temperatures.

Effect of pH

The pH 9.0 was the optimum for dioxygenases activity (106.7601µg indigo/mg protein/min) followed by pH 10 (53.7µg indigo/mg protein/min). After one minute, the activity decreased gradually till 10 minutes (Figure 4.A). The minimal activity was recorded at pH 4 (2.09µg indigo/mg protein/min) and pH 5 (6.73µg indigo/mg protein/min) as represented in Figure 4.B.

(A)



(B)

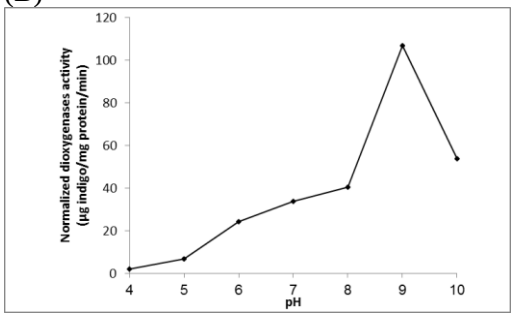
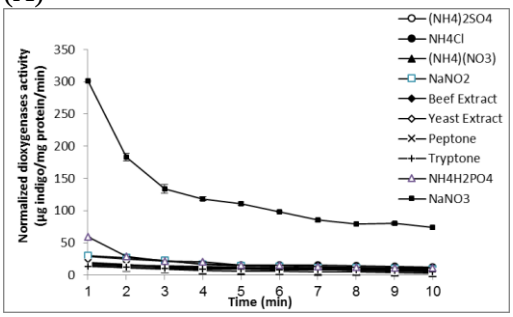


Figure 4. (A) The effect of pH on normalized dioxygenases activity produced by P. aeruginosa EGGS2, the activity was measured for ten minutes intervals. (B) The activity of normalized dioxygenases at 5 minutes for different pHs.

Effect of different nitrogen sources

Interestingly the best nitrogen source of dioxygenases activity of P. aeruginosa EGGS2 was NaNO3 giving the highest activity value (301µg indigo/mg protein/min) at the 1st minute, and then decreased to 189.9 µg indigo/mg protein/min and 133.8 µg indigo/mg protein/min after two and three minutes, respectively. Afterword, it reached 74.4µg indigo/mg protein/min after 10 minutes (Figure 5.A). The other nitrogen sources exhibited lower normalized dioxygenases activity compared with NaNO3 (Figure 5.B). The next lower activity was recorded using NH4H2PO4 at one minute (59µg indigo/mg protein/min), then decreased to 29.5µg indigo/mg protein/min after 2 minutes.

(A)



(B)

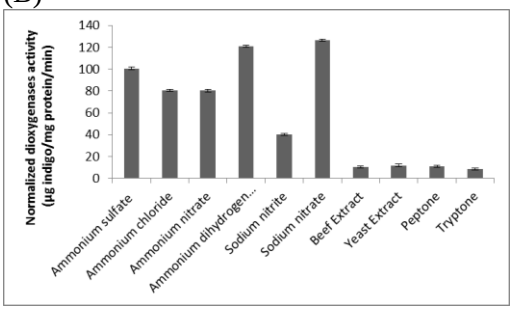


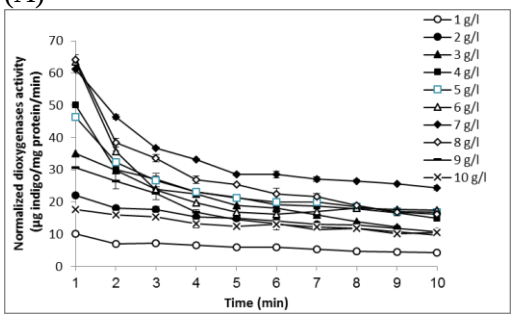
Figure 5. (A) The effect of different nitrogen sources on normalized dioxygenases activity produced by P. aeruginosa EGDS2, the activity was measured for ten minutes intervals. (B) The activity of normalized dioxygenases at 5 minutes for each nitrogen sources.

Effect of naphthalene concentration

Generally, the best naphthalene concentration for dioxygenases activities was at 7g/l followed by 8g/l. At the 1st minute, 8g/l recorded the highest enzymes activity (64.2µg indigo/mg protein/min) followed by 7g/l (61.2µg indigo/mg protein/min) (Figure 6.A). The enzymes activity then decreases to minimal at 1g/l

(10.22µg indigo/mg protein/min) and 10g/l (17.7µg indigo/mg protein/min) as showed in Figure 6.B.

(A)



(B)

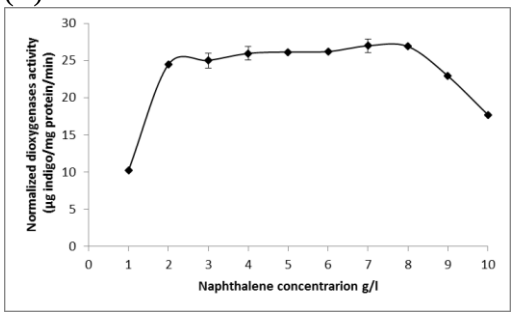


Figure 6. (A) The effect of different naphthalene concentrations on normalized dioxygenases activity produced by P. aeruginosa EGDS2, the activity was measured for ten minutes intervals. (B) The activity of normalized dioxygenases at 5 minutes for naphthalene concentration.

Discussion

Identification of naphthalene degradation bacterium was performed using the morphological and physiological properties in combination molecular tools of 16S rRNA gene sequencing. The bacterial isolate is a Gram negative rods, non-endospore forming, catalase and oxidase positive refers to be identified as *Pseudomonas aeruginosa* according to Bergey's Manual of Systematic Bacteriology (Palleroni, 1984). It was widely known that the genus *Pseudomonas* is a very diverse group of bacteria including many different species (Wagner *et al*., 2008), so this might need additional modern tools such as DNA sequencing for confirmation their identification. Consequently, the molecular identification based on 16S rDNA sequence alignment of the *P. aeruginosa* EGDS2 gave a high similarity, reached to 99%, with other *P. aeruginosa* strains present on the database. Also, the 16S rDNA sequencing based phylogenetic tree revealed that it is clustered in the same clade with the other *P. aeruginosa* strains and grouped away from the other species of the genus *Pseudomonas*. Furthermore, it is not totally related to the genus *Xanthomonas*.

Environmental factors such as temperatures pH and nutrient composition had been reported to have some important influences on an enzymes activity by bacterial organism. According to the above records, this investigation is made to enhance the maximum activity of normalized dioxygenases produced by the isolated *P. aeruginosa* EGDS2. The optimization of their cultural conditions such as cultivation period, optimum pH, temperature, the best nitrogen sources and optimal naphthalene concentrations, of the isolate were studied.

The maximum activity of normalized dioxygenases by *P. aeruginosa* EGDS2 was determined at 48 hours. Further increase in the incubation period beyond the optimum incubation time resulted in decrease in the enzymes activity. Our result was quite identical with optimal time line production of catechol 2,3-dioxygenase produced by *Pseudomonas* sp. strain ZJF08 (Zou *et al*., 2007). This probably because the enzymes might be expressed highly at the late log phase of the bacterial growth which in case of *Pseudomonas* sp. is at about 48 hours, then the activity is dramatically dropped when started the stationary phase.

The maximum dioxygenases activity of *P. aeruginosa* EGDS2 was observed at 30°C. In agreement with our result, Wojcieszynska *et al*., (2011) indicated that catechol 2,3-dioxygenase from *Stenotrophomonas maltophilia* KB2 possessed extremely high activity at 30°C. Most of normalized dioxygenases are produced at 35°C-40°C by many mesophilic bacteria. For example, catechol 1,2-dioxygenase from *P. putida* N6 and *B. cereus* was optimally active at 35°C (Guzik *et al*., 2011; Jayanthi and Hemashenpagam, 2015).

Most of natural environments usually have pH values 5.0 to 9.0. Consequently, this range is considered also optimal for biodegradation of hydrocarbon by microorganisms (Fatajeva *et al*., 2014). *Pseudomonas* sp. ZJF08 and *P. putida* N6

expressed catechol 2,3-dioxygenase and 1,2-dioxygenase, respectively, with optimal activity at pH 7.5 (Zou *et al*., 2007; Guzik *et al*., 2011), while *B. cereus* possessed optimally active catechol 1,2-dioxygenase at pH 7.0 (Jayanthi and Hemashenpagam, 2015). Degradation of BTEX compounds by alkaliphilic *Pseudomonads* species had been rarely reported (Hirota *et al.*, 2011). Catechol 2,3-dioxygenaseobtained from *Pseudomonas* sp. HB01 was maximally active at pH 10.5. The dioxygenase of *P. aeruginosa* EGDS2 strain was highly active at pH 9.0, which considered tending to be alkaliphilic enzyme.

The nitrogen source is one of the limiting factors for a bacterial growth and its enzymatic activities. $NaNO_3$ was the best nitrogen source for dioxygenases from the *P. aeruginosa* EGDS2 strain compared with the other tested sources. Nigam *et al*., (1998) reported that the maximum growth of *P. putida* UUC-1 and another two bacterial strains PNA-2 and PNC-2 were on medium supplemented with naphthalene and $(NH_4)_2SO_4$ rather than KNO_3 and NH_4Cl. On the other hand, NH_4Cl was the best nitrogen source for degrading benzene, toluene and xylene when *B. cereus* isolate was tested (Jayanthi and Hemashenpagam, 2015). Our strain (*P. aeruginosa* EGDS2) behaved differently as it utilized $NaNO_3$ as the best nitrogen source for degrading the naphthalene over the other tested nitrogen sources.

P. aeruginosa EGDS2 was able to utilize naphthalene perfectly, as its maximum dioxygenases activity was recorded at 7g/l naphthalene concentration. Though there was slight decline beyond 7g/l of naphthalene, the enzyme activity still fine. This decrease might be associated with metabolizing capacity of the isolate and toxicity of naphthalene. Aromatic hydrocarbon BTEX compounds (Benzene, Toluene, Ethylbenzene and Xylene) upon reaching certain concentrations can inhibit the microbial activity due to complex micro- and macro- level interactions (Jo *et al*., 2008). Naphthalene dioxygenase from *P. putida* G7 was improved before 56 ppm of naphthalene concentration, but decreased at 240 ppm and higher (Chang *et al*., 2015). Two bacterial strains PNA-2 and PNC-2 were compared with *P. putida* UUC-1as all gave maximum growth at 2g/l of naphthalene (Nigam *et al*., 1998). *P. putida* N6 strain degradedphenol completely up to concentrations of 10 mM, while hydroquinone and vanilic acid were degraded up to concentrations of 5 mM. On the other hand, The higher concentration above 9 mM, 11 mM, and 14 mM, for benzoic acid, protocatecuic acid, 4-hydroxybenzoic acid and, respectively, caused the growth inhibition of *P. putida* strain N6 (Guzik *et al*., 2011). Furthermore, *P. putida* strain F1 could not completely degrade Benzene at concentration above 380 mg/l and Toluene above 420 mg/l (Hamed *et al*., 2003).

In conclusion, The *P. aeruginosa* EGDS2 isolate exhibited highly derivative properties towards aromatic hydrocarbon including naphthalene. Its dioxygenases tends to be alkaliphilic and produced maximally after 48 hours at 30ºC. This isolate is a promising strain for bioremediation of multi aromatic hydrocarbon pollutants. Further studies would be considered for identification and characterization of the types of those dioxygenases including DNA sequencing for their genes.

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الملخص العربي

عنوان البحث: التحلل البيولوجي للنفثالين بواسطة *Pseudomonas aeruginosa* EGDS2 المحبة للقلوية

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تم عزل البكتريا المحللة للنفثالين من التربة الملوثة بالديزل من محافظة دمياط ، مصر . تم تعريف عزلة بكتيرية بالطرق الكلاسيكية والطرق الحديثة باستخدام تتابع 16 S rDNA علي أنها *Pseudomonas aeruginosa* EGDS2, وقد أظهرت تلك العزلة قدرة عالية علي تكسير النفثالين بالإضافة إلى البنزين و الطولوين و الكاتيكول و الزيلين .

تم دراسة الظروف المثلي لإنتاج أفضل نشاط للإنزيمات المسئولة عن عملية تكسير النفثالين المسماه normalize dioxygenases، وقد وضحت النتائج أن النشاط الأمثل لهذه الإنزيمات تم بعد 48 ساعة من فترة النمو و عند درجة 9 للأس الهيدروجينى، وكانت الحرارة المثلى هى 30 درجة سيليزية، و أفضل مصدر نيتروجيني استخدمته البكتريا كان نترات الصوديوم، أما التركيز الأمثل للنفثالين في الوسط الغذائي كان 7 جرام في اللتر.

أكدت النتائج أن العزلة *P. aeruginosa* EGDS2 تعتبر سلالة واعدة لإنتاج إنزيمات ذات نشاط عالى محللة للمواد الهيدوكربونية مما يؤهل استخدامها تباعا للتخلص من التلوث البيئي الناتج عن تلك المركبات.