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Molecular identification and characterization of *Pseudomonas stutzeri* strain showing potent degradation activity towards pyrene

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Abstract. Pyrene is a Polyaromatic hydrocarbon ubiquitously found in soils. Bioremediation of this lethal and toxic compound is of great importance due to its toxicity toward humans. It is imperative to identify and isolate bacterial species that readily degrade pyrene present in the soil. In this study soil samples were collected from different regions in the Madinah region and studied for the presence of bacterial species that readily degrade pyrene. Isolates were thoroughly studied for the degradation of Pyrene using Spectrophotometer, 2,6-Dichlorophenolindophenol Assay, and dry weight method. About 18 bacterial isolates were isolated from soil samples of Madinah. Among them, only P12 show high degradation activity. Furthermore, the potent strains were Identified using 16s rRNA gene sequencing as *Pseudomonas stutzeri*. Results show that *Pseudomonas stutzeri* change color from blue to white in the presence of DCPIP dye compare to the control one. The optical density of this strain was also high at 600 nm wavelength compare to the other strains. High Pressure Liquid Chromatography analysis shows that one of the selected sample P12 shows 100 % degradative activity compare to the control one. The dry weight also confirms its degradative activity towards pyrene. Our study concludes that these strains can be used to clean the environment.

Keywords: Pyrene, Polyaromatic hydrocarbons, Biodegradation, *Pseudomonas*

Introduction

Polyaromatic hydrocarbons (PAHs) are abundantly found in the environment, it contaminates the environment primarily by anthropogenic activities including fossil oil burning, gasification and liquefaction of coal, accidental spill and seepage of petrochemicals (Guerin and Jones, 1988; Juhasz and Naidu, 2000). PAHs are made up of fused aromatic rings (two or more) in different arrangements. According to the number of aromatic rings these compounds are classified as low molecular (LMW) and high molecular weight compounds (HMW). Comparatively, LMW aromatic compounds are less stable regarded as volatile, soluble and easy to break down while HMW compounds are stable and hydrophobic in nature. Hence, due to strong attachment to oil particles, HMW aromatic hydrocarbons are therefore not easily degraded by microorganisms. PAHs shows great risks towards human health on long term exposure

as they are highly toxic, mutagenic, tertragenic and carcinogenic properties (Abd-Elsalam et al., 2009; Abou-Arab et al., 2010). Volatilization, oxidation by light, oxidation by chemical, bioaccumulation and adsorption to soil particles probably the fate of PAHs in the environment. But, degradation of PAHs contaminants is assumed to be environment friendly and acceptable strategy to solve the problem in the soil (Gibson et al., 1975). PAHs unavailability to bacteria due to its high solubility and adsorption to particles may be the major problem in biodegradation soil (Thomas et al., 1986; Volkering et al., 1998). Many research documents are available regarding bacterial degradation of PAHs (Cerniglia, 1992; Wilson and Jones, 1993), but the bacteria were isolated from the oil sludge contaminated sites mainly, which probably have got more efficiency to degrade due to selective pressure as compared to uncontaminated site. Nevertheless, reports regarding bacterial degradation of PAHs in uncontaminated soil are also available (Juhasz and Naidu, 2000; Kanaly et al., 2000). Various genera *Pseudomonas*, *Alcaligenes*, *Mycobacterium*, *Rhodococcus* of bacteria have been known for degradation of PAHs (Cerniglia, 1992; Mueller et al., 1996). PAH mineralization capacity of mycobacterium isolates in two different sites one is contaminated the other one uncontaminated soil Lease et al. (2011). In the research study it was found that among HMW PAHs, pyrene is difficult to degrade due to its high hydrophobicity therefore the cellular uptake was restricted by microbes (Seo et al., 2009).

Pyrene degradation was first reported as a lonely carbon source of energy by *Rhodococcus* sp. strain UWI (Walter et al., 1991). *Mycobacterium* sp. strain KMS, showed pyrene degradation involve formation of catabolic enzymes and metabolites during degradation process (Liang et al. 2006). More pyrene degradation was found by khan et al. (2009) by in which the catabolic genes nahAc and pdo 1 responsible for PAH degradation was identified through rhizospheric bacteria than non-rhizospheric bacteria. Involvement of two ring hydroxylating dioxygenases and pyrene induced proteins in *Mycobacterium* sp. was recognized by Krivobok et al. (2003). Besides, an Enteric Bacterium *Leclercia adecarboxylata* PS4040 was reported by Sarma et al. (2010) for pyrene degradation. A novel strain Proteus vulgaris 4Bi for degradation of pyrene more efficiently along with the production of non-toxic and non-stable metabolites in nature (Ceyhan 2012).

Moreover, bioremediation of pyrene is an eco-friendly and cost effective technique for contaminated soil and water. Different approaches were performed involving various microorganisms' isolation used for pyrene degradation. Nevertheless, these approaches were limited for LMW PAHs and less effective against HMW PAHs (Pyrene). The facts of some findings may be a future clove for the advancement of pyrene degradation by microbes at polluted locations. The aim of our study was to isolate a potent bacterium that has the ability to degrade pyrene which is a toxic Polyaromatic hydrocarbon.

Material and Methods

Soil Sampling

The soil sample used in this study was collected from fifteen different oil contaminated sites of Madinah region Kingdom of Saudi Arabia. The list of the soil samples is shown in Table 1. pH of the soil was monitored before collection of soil samples. The samples were immediately transfer to the ice box and then save in refrigerator. Latter on one gram of soil sample was added to BHS medium broth containing 1% pyrene.

Table 1. List of samples along with their location

S. No	Sample id	Location
1	P12	24.4491309,39.5916872 Madinah
2	P11a	24.4487329,39.5914122 Madinah
3	P10a	24.3763372,39.4747129 Madinah
4	P4a2	24.4558907,39.4653767 Madinah
5	P15a2	24.4803043,39.656965 Madinah
6	P4a1	24.4558907,39.4653767 Madinah
7	P7p	24.3732491,39.4581344 Madinah
8	P9a	24.3754613,39.4731897 Madinah
9	P3ap	24.5826375,39.6769229 Madinah
10	P9b	24.3754613,39.4731897 Madinah
11	P5a2	24.4526987,39.4634692 Madinah
12	P16p	24.5487025,39.6215736 Madinah
13	P1ap	23.1401931,40.5509347 Safinah
14	P4a	24.4558907,39.4653767 Madinah
15	P13b	24.4477094,39.5903678 Madinah
16	P16a	24.5487025,39.6215736 Madinah
17	P1b	23.1401931,40.5509347 Safinah
18	P1ap	23.1401931,40.5509347 Safinah
19	P20p2	24.5866504,39.673842 Madinah
20	P18b	24.584012,39.675255 Madinah
21	P15	24.4803043,39.656965 Madinah
22	P10b	24.3763372,39.4747129 Madinah
23	P8a1	24.371981,39.4689554 Madinah
24	P1a	23.1401931,40.5509347 Safinah
25	P20a	24.5866504,39.673842 Madinah
26	P21a	24.587492,39.6733615 Madinah
27	P30p	24.584475,39.6723545 Madinah
28	P20p	24.5866504,39.673842 Madinah
29	P8p	24.371981,39.4689554 Madinah
30	P17b	24.5473568,39.623398 Madinah
31	P11a	24.4487329,39.5914122 Madinah
32	P2ap	24.4071249,39.4960288 Madinah

Isolation

Exactly 1 g of the each collected sample was added to 100 mL freshly prepared Bushnell Haas Media (BHM) and placed at 35 °C with continuous stirring at 180 rpm for one week. After that the culture containing microorganisms was spread on nutrient Agar. Colonies grown on the plates were aseptically taken with a sterile loop and inoculated into 5mL of LB broth and incubated at 35 °C and stirred at 180 rpm for 24 hours. Then these isolates were used for further characterization whether it can grow in the presence of pyrene.

Characterization

Selection and Screening of pyrene-degrading Bacteria

For screening and selection Pyrene was used throughout the study. Bacteria isolates was cultured in BHS medium containing Pyrene as sole carbon source. Optical density was observed at 600 nm, after incubation with 1 percent of Pyrene, in a shaker incubator at 200 rpm.

DCPIP Assay

Using sterile loops colonies was taken aseptically and incubated in 5 ml Luria broth at 35 ºC and 180 rpm till OD observed at 660nm is over 1.0. Latter, 1 ml of sample was taken and centrifuged for 5 min at 4000xg. After centrifugation the supernatant was discarded and pellet was washed with saline solution. Optical density was adjusted to an OD of 1.0 at 660 nm. Regents & media used were autoclaved and 750 ml of Bushnell Haas Media, 50 mL of Ferric chloride hexahydrate ($FeCl_3$-$6H_2O$) solution (150 mg/ml) and 50 ml of 2,6-Dichlorophenolindophenol solution (37.5 mg/ml) were taken into a test tube along with 80 ml of cell suspension. 5 ml of pyrene was added to same test tube for incubation of 48 h at 30 ºC and adjust shaking to 100 rpm. After 48 h, each tube was observed for presence or absence of Pyrene depending on the color. Tubes that are colorless would affirm the degradation process by microbes whereas tubes that are blue would indicate that the microbes failed to degrade Pyrene present in the tube, suggesting absence of hydrocarbon degrading microbes (Quinn et al., 2011).

Spectrophotometric analysis

To assess pyrene degradation, selected bacteria was supplemented with 1g/liter of pyrene and incubated under same conditions as mentioned above. After 24 h of incubation absorbance was be observed at 600 nm using spectrophotometer in comparison to blank. Degradation of pyrene was measured using linear standard curve and quantified by using the following formula:

$D = C_1 - C_2 / C_1 \times 100\%$

D: % degradation;

C_1: initial PAH concentration;

C_2: residual concentration

Weight Method for pyrene

Weight method was used to know the degradation of pyrene in the presence of bacteria. For this bacteria were grown for two weeks in the presence of pyrene. Then after two weeks of culture, Pyrene was extracted using Dichloromethane as a solvent.

Calculating Dry weight

Use the following steps to calculate the dry weight of pyrene extracted from culture broth: First Weigh the empty container selected to hold the pyrene and record the weight. Then Place the extracted pyrene in the container. After that Weigh and record the container and pyrene weight. Finally Subtract the weight of the container from the total weight (Step 3) to determine the weight of the pyrene.

Container weight = A gm
Container and sample weight after drying = B gm
Dry sample weight = g (Calculation: A gm – B gm = C gm)

HPLC Analysis of the selected sample

Sample preparation for HPLC analysis

Pyrene was extracted from culture medium using dichloromethane (DCM) (1:9). The sample was then transfer to 200 microliter Dichloromethane in amber glass vial and stored at −20 °C before analysis by HPLC. For HPLC sample preparation the tip of the syringe was dip in the extracted sample from culture medium. Then the tip was dip in the glass vial

containing 200 microliter of DCM. Same procedure was done for sample preparation of control sample. The HPLC analysis was done to know the Pyrene percent degradation for the selected bacteria.

Identification

DNA isolation

Whole genomic DNA was extracted according to manufacturer's instructions. GeneJET Genomic DNA Purification Kit (Thermo Scientific) was used for genomic DNA extraction. In short DNA was enzymatically released from cells. Columns were prepared and the DNA was washed via centrifugation. Latter the DNA was eluted with elution buffer, centrifuged and collected into a collection tube.

PCR Amplification

DNA was isolated from the selected sample using GeneJET genomic extraction kit. DNA was isolated and 16S rDNA was amplified using Forward primer sequence (5-AGAGTTTGATCCTGGCTCAG-3) and Reverse primer sequence (5-GGCTACCTTGTTACGACTT-3) primers. Following reaction conditions was used; An initial incubation for 8 min at 94 °C. Then 25 cycles of 94 °C for 20 seconds, followed by annealing at 54 °C for 20 second, and extension at 72 °C for one minute. Finally, extension for 5 min at 72 °C. PCR product was run on agarose gel and amplified product corresponding to the size of bacterial rDNA was purified and sequenced. The Phylogenetic trees were constructed using MEGA software version 10.0.5.

Results

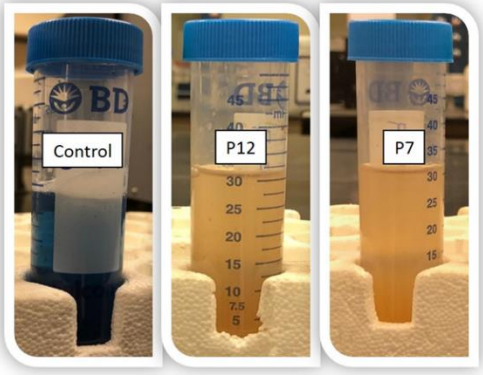
Isolation

Bacteria were isolated from different area of Makkah region that has the ability to grow in the presence of pyrene. Most of the bacteria did not grow because they don't have the ability to use this large molecule. The strains which grow in this stress environment was used for qualitative and quantitative analysis.

Characterization

DCPIP Assay

Strain P12 and P7 was grown in the presence of pyrene at 35 °C for two weeks. The



color of the control did not change, but the tube containing bacteria change its color from blue to white as shown in Figure 1.

Figure 1. show DCPIP assay for the selected samples and their control.

OD using spectrophotometer

All of the bacterial isolates were grown in the presence of 1 % Pyrene. Few of the bacteria grow in the presence Pyrene. Based on the optical density, P12 was selected for further studies as shown in Table 2.

Table 2. Spectrophometric analysis of selected samples.

S. No	Sample id	Optical density (600 nm)
1	P12	0.91
2	P11a	0.495
3	P10a	0.492
4	P4a2	0.467
5	P15a2	0.475
6	P4a1	0.456
7	P7p	0.445
8	P9a	0.441
9	P3ap	0.440
10	P9b	0.430
11	P5a2	0.430
12	P16p	0.426
13	P1ap	0.418
14	P4a	0.418
15	P13b	0.386
16	P16a	0.385
17	P1b	0.369
18	P1ap	0.355
19	P20p2	0.355
20	P18b	0.350
21	P15	0.349
22	P10b	0.347
23	P8a1	0.345
24	P1a	0.336
25	P20a	0.335
26	P21a	0.332
27	P30p	0.331

28	P20p	0.330
29	P8p	0.320
30	P17b	0.319
31	P11a	0.318
32	P2ap	0.315

Sample	**(Weight)**	**Day one Control (Weight)**	**Two weeks Treated (Weight)**
P12	Test tube	Test tube+ pyrene	Test tube+ pyrene+ bacteria
	7.911	8.915	8.001
	7.890	8.891	7.830
	7.903	8.904	7.800
Average	7.901	8.806	7.877
Degradation (gm)			0.92

Dry Weight Method

Bacteria strain was grown for two weeks at 35 °C in the presence of Pyrene. Then Pyrene was extracted from the culture medium using Dichloromethane (DCM). Empty test Tube of control was weighted and then extracted Pyrene was transfer. Then tube was weight after the Pyrene was dried. Similar technique was used for the treated sample. Results show that the weight of the Pyrene decreases significantly as show in Figure 2 and Table 3.

Table 3. Shows the degradation analysis of selected strain.

Figure 2. Show the degradation analysis of selected strain.

HPLC Analysis

The extracted Pyrene from control and treated samples were furthermore subjected to qualitative analysis. The result of the control was shown in Figure 3A and Table 4. Also result of the treated sample is shown in Figure 3B and Table 5

Peak	Ret. Time	Area		
1	2.855	2056		
2	3.028	1493		
3	3.552	1341		
4			4.460	1174
Total				6064

Figure 3. Show HPLC analysis of control and treated sample having media, Pyrene and P12. a). Control b). Treated

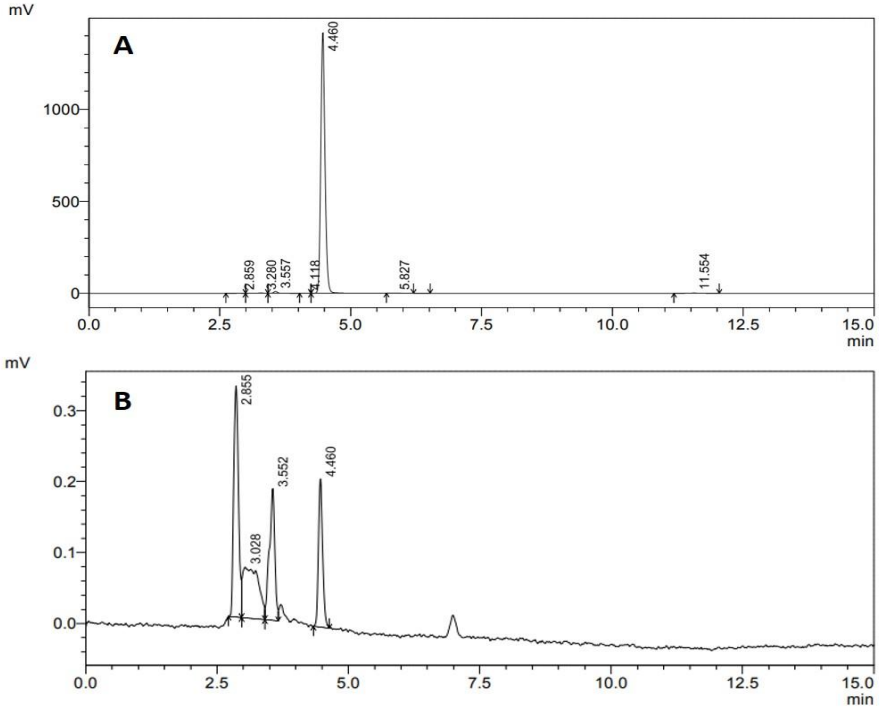


Table 4. HPLC Profile of Control sample

Peak	Ret. Time	Area		
1	2.855	2056		
2	3.028	1493		
3	3.552	1341		
4			4.460	1174
Total				6064

Table 5. HPLC Profile of Treated sample.

Identification

Molecular Analysis

16s r RNA gene sequencing

These strain was identified using 16s rRNA gene sequencing as *Pseudomonas stutzeri*. The sequence was submitted to genbank for accession number. The phylogenetic tree of these strains were constructed using MEGA version 4 (Tamura et al., 2007; Tamura et al., 2004). Results show that *Pseudomonas stutzeri* strain YA 8805 (MK648317) 16s rRNA sequence laid in the same cluster with many of *Pseudomonas* strains from genbank, reflecting high percentage of similarity with these strains as shown in Figure 4.

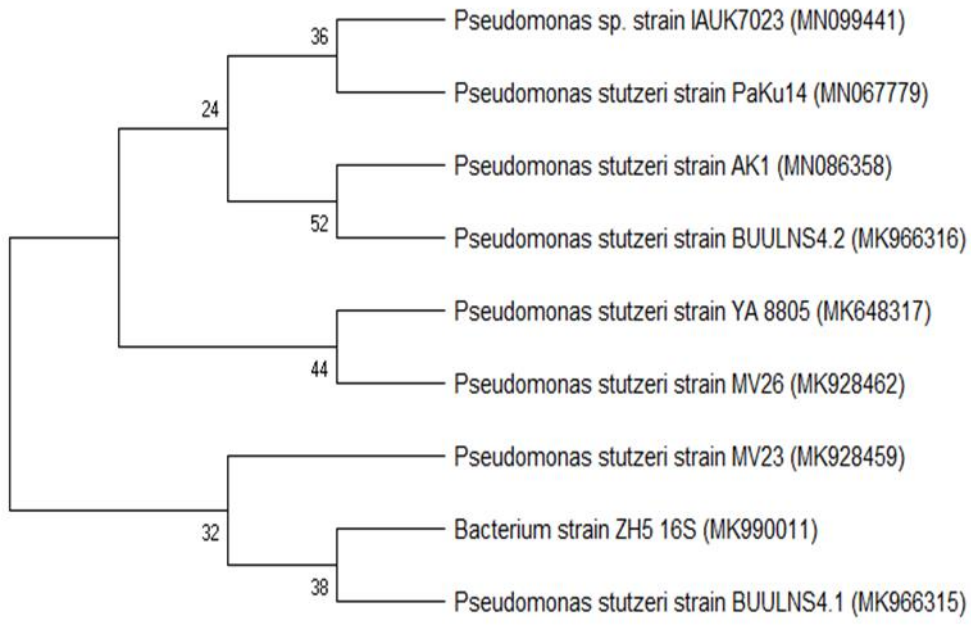


Figure 4. Phylogenetic tree of *Pseudomonas stutzeri* along with the other closely related species.

Discussion

Environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) are widespread due to increased anthropogenic activities such as, coal liquefaction, fossil fuel combustion, gasification processes and unwarranted spillage of hydrocarbons (Guerin and Jones., 1988: Juhasz and Naidu., 2000). Pyrene is one of the polyromantic hydrocarbon responsible for polluting our environment. Recently, interest in using bioremediation techniques via microbes has been gaining moment due to its cost-effectiveness, efficacy and environment friendly nature (Margesin and Schinner., 1998). In this regard, numerous bacterial species including *Pseudomonas*, *Alcaligenes*, *Mycobacterium*, *Rhodococcus*, *Cornybacterium*, *Diaphorobacter*, *Pseudoxanthomonas*, *Bacillus vallismortis* have been utilized in biodegradation of pyrene (Kafilzadeh et al., 2012: Klankeo et al., 2009: Ling et al., 2011). During our study eighteen strains were isolated from different location of Madinah region based on that they can grow in the presence of pyrene as sole carbon source. P12 and P7 were further selected based on its ability to degrade in the presence of DCPIP (2,6-Dichlorophenolindophenol), dry weight and spectrophotometer.

About eighteen bacteria were isolated from different areas of Madinah region. These strains were maintained at 35 ℃ for 24 hours. Out of these the potent bacteria, which was designated as P12 was molecularly identified using 16s rRNA primer as *Pseudomonas stutzeri* (MK648317). The phylogenetic tree of this strain was constructed against the other *pseudomonas* species retrieve from NCBI website.

Biodegradation of crude oil was evaluated by introducing an electron acceptor, such as

DCPIP to the culture medium. The color of the medium from DCPIP was observed and evaluated to be positive that revealed the degrading capability of the bacterial strains to degrade crude oil by changing the color from blue (oxidized) to colorless (reduced). Isolate P12 was grown in the presence of pyrene along with DCPIP. After one-week control color was blue showing no activity takes place. But in case of our sample color changes from blue to white showing its degradation ability.

For the dry method the sample was grown for two weeks in the presence of pyrene. The extracted control and treated samples were weighted. After weight the results show that about 90 % of pyrene is degraded by our selected strain (P12). This method gives us the idea that our strain has the ability to degrade pyrene. Similar method was used to weight the polyromantic hydrocarbons from different samples.

Samples were grown in the presence of pyrene 1 gm/l. Isolate P12 has 0.91 O. D at 600 nm wave length. The O.D of this strain was compare to the other isolates. Based on this reason P12 was consider the best degrader of pyrene. Furthermore, the sample was given for the qualitative analysis of pyrene degradation. Similar studies show that the spectrophotometer is used for the qualitative analysis of polyaromatic hydrocarbons (Christopher et al., 2011; Ping et al., 2017).

After treated sample with P12 for two weeks at 35 °C. the extracted pyrene was used for the qualitative analysis (Spectrophotometer and Dry Method) as well as quantitative analysis (HPLC analysis). The control retention time for pyrene was 4.46, with peak area of 8097431 and the treated one has a peak area of 1174. This shows that it degraded almost 100 % of pyrene in the treated sample (P12). Similar results also show that bacteria have the ability to degrade different types of polyromantic hydrocarbons. Similar studies have done in order to quantify the degradative ability of microorganism's (Abd et al., 2014; Joanna et al., 2004).

Conclusion

Pyrene a high molecular weight compound found in soil has gain importance due to its toxicity towards human and animals. During our study we isolate bacteria from Madinah region that has the ability to degrade more than 90 % of Pyrene (Polyaromatic hydrocarbon). This potent bacterium was identified as *Pseudomonas stutzeri* (MK648317). So it is concluded that these indigenous strains can be used to clean our environment from this toxic compound.

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التحديد الجزيئي والتوصيف لسلالة Pseudomonas stutzeri يظهر نشاط تحول قوي تجاه البيرين

هاني محمد علي

قسم العلوم البيولوجية

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مستخلص. البيرين هو هيدروكربون متعدد العطريات موجود في كل مكان بالتربة. المعالجة البيولوجية لهذا المركب القاتل والسام لها أهمية كبيرة بسبب سميتها تجاه البشر. من الضروري تحديد وعزل الأنواع البكتيرية التي تحلل البيرين الموجود في التربة بسهولة. في هذه الدراسة تم جمع عينات من التربة من مناطق مختلفة في منطقة المدينة المنورة وتمت دراستها لوجود أنواع بكتيرية تعمل على تحلل البيرين بسهولة. تمت دراسة العزلات بدقة لتحلل البيرين باستخدام مقياس الطيف الضوئي، ومقايسة ٢،٦–ثنائي كلوروفينولليندوفينول، وطريقة الوزن الجاف. تم عزل حوالي ١٨ عزلة بكتيرية من عينات تربة المدينة المنورة. من بينها، يظهر P12 فقط نشاط تحلل عالي. علاوة على ذلك، تم التعرف على السلالات القوية باستخدام تسلسل جينات الرنا الريباسي s١٦ مثل Pseudomonas stutzeri. أظهرت النتائج أن Pseudomonas stutzeri يتغير لونها من الأزرق إلى الأبيض في وجود صبغة DCPIP مقارنة مع صبغة التحكم. وكانت الكثافة البصرية لهذه السلالة عالية أيضًا عند الطول الموجي ٦٠٠ نانومتر مقارنة بالسلالات الأخرى. يوضح التحليل الكروماتوجرافي السائل عالي الضغط أن إحدى العينات المختارة P12 تظهر نشاطًا تحلليًا بنسبة ١٠٠٪ مقارنة بعينة التحكم. كما يؤكد الوزن الجاف نشاطه التحللي تجاه البيرين. وخلصت دراستنا إلى أنه يمكن استخدام هذه السلالات لتنظيف البيئة.