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Pyrene degradation of biofilm-forming *Paracoccus* sp. DG25 isolated from oil polluted samples collected in petroleum storage Duc Giang, Hanoi

Khả năng phân hủy pyrene của chủng Paracoccus sp. DG25 phân lập từ các mẫu nhiễm dầu lấy tại kho xăng Đức Giang, Hà Nội

Research article

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In this study, a well biofilm-forming bacterial strain was isolated from oil contaminated water and sediment samples collected in petroleum storage Duc Giang, Hanoi. It was identified as *Paracoccus* sp. DG25 and registered in the GenBank database with the accession numbers KJ608354. Several biophysical and bio-chemical conditions for the biofilm formation of the strain were estimated such as pH, temperature, carbon sources and nitrogen sources. As the results the biofilm forming capacity was highest at pH 7, 37 ^oC, on maltose and supplemented with KNO_3. Using these optimal conditions, the formed biofilm degraded 76.07 % of pyrene after 7 day-incubation, with the initial concentration of 300 ppm by high-performance liquid chromatography (HPLC) analysis. To our knowledge, there is rare publication on pyrene degradation by biofilm-forming bacteria. Therefore, the obtained results show that biofilm formed the strain *Paracoccus* sp. DG25 may considerably increase the degrading efficiency of pyrene and may lead to a new approach to treat polycyclic aromatic hydrocarbons containing in petroleum oil contaminated water in Vietnam.

Trong nghiên cứu này, từ các mẫu đất và nước nhiễm dầu lấy tại kho xăng Đức Giang, Hà Nội, chúng tôi đã phân lập được chủng vi khuẩn có khả năng tạo màng sinh học tốt. Chủng vi khuẩn này đã được phân loại và định tên là Paracoccus *sp. DG25 với số đăng ký trên ngân hàng Gen là KJ608354. Chúng tôi cũng đã nghiên cứu một số điều kiện hóa lý ảnh hưởng tới khả năng hình thành màng sinh học như pH, nhiệt độ, nguồn Carbon và nguồn Nitơ. Kết quả cho thấy, chủng DG25 có khả năng tạo màng tốt nhất ở các điều kiện pH 7, 37 ^oC, nguồn Carbon là maltose và nguồn Nitơ là KNO_3. Sử dụng các điều kiện tối ưu này để tạo màng và đánh giá khả năng phân hủy pyrene của màng tạo thành. Bằng phương pháp sắc ký lỏng cao áp, chúng tôi đã đánh giá được hàm lượng pyrene bị phân hủy sau 7 ngày nuôi tĩnh bởi màng sinh học của chủng DG25 lên tới 76,07 % với nồng độ ban đầu là 300 ppm. Cho tới nay, chưa có nhiều công bố về hiệu quả phân hủy pyrene của các chủng vi khuẩn tạo màng sinh học. Do vậy, kết quả đạt được này mở ra khả năng sử dụng màng tạo thành bởi chủng DG25 để nâng cao hiệu quả phân hủy pyren và có thể mở ra phương pháp mới nhằm xử lý các hợp chất hydrocarbon thơm có trong nước ô nhiễm dầu ở Việt Nam.*

Keywords: biodegradation, biofilm, *Paracoccus* sp., pyrene

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1. Introduction

Pyrene is one of the polycyclic aromatic hydrocarbons (PAHs) that have been considered to be priority pollutants by the United States Environmental Protection Agency (US EPA) (Yan *et al*., 2004). Pyrene possessing four benzene rings is a by-product of gasification processes and other incomplete combustion processes. Due to its chemical structure is highly recalcitrant and resistant to microbial degradation (Seo *et al*., 2009). The microbial PAHs-degradation is an effective strategy to remove pollutants from the environment by bioremediation. A number of microorganisms including algae, heterotrophic bacteria, cyanobacteria and fungi have been reported to play a role in PAH degradation (Kim *et al*., 2005; Seo *et al*., 2009; Klankeo *et al*., 2009). However few of them demonstrated that biofilm-forming microorganisms effectively degrade pollutants at the contaminated sites (Moslemy *et al*., 2002; Mrozik and Piotrowska-Seget, 2010). Biofilms are communities of microorganisms formed on solid faces or interfaces (Morikawa, 2006). It has recently been demonstrated that microorganisms exist prevalently as biofilms and gather high tolerance to physical, chemical, and biological stresses because in biofilms, cells exhibit specific gene expression (Gorbushina and Broughton, 2009). Therefore, forming biofilms is considered a natural strategy of microorganisms to survive and maintain a favorable niche in stressful environments (Morikawa, 2006). The purpose of the present study was to investigate the effects of culture conditions (pH, temperature, carbon and nitrogen sources) on biofilms formation of a strain *Paracoccus* sp. DG25 isolated from oil contaminated water and sediment samples collected in petroleum storage Duc Giang, Hanoi and high performance of biofilms to degrade pyrene.

2. Materials and Methods

2.1 Materials

Oil polluted soil and water samples from petroleum storage Duc Giang, Hanoi were collected in to special equipments. The samples were stored at 4 ^oC for further steps.

2.2 Methods

2.2.1 Enrichment and isolation of pyrene degrading bacteria

For enrichment, 5 ml of water or 1 g of sediment samples were inoculated into 50 ml Gost medium (Na_2HPO_4-0.7g, KH_2PO_4-0.3g, KNO_3-3g, NaCl-5g, $MgSO_4$-0.4g and distilled water-1 liter at pH 7.0-7.2). The medium was supplemented with 50 ppm of pyrene, shaked at 30°C and 180 rpm for 5 days. After three cycles of enrichment, 100 µl of the incubation were cultured on Gost agar plates supplemented with pyrene. The different bacterial colonies were purified on separated Gost agar plates supplemented with 50 ppm of pyrene. The pure colony which could grow well on pyrene was routinely subcultured in Gost and stored with 35% of glycerol at -80°C.

2.2.2 Screening biofilm-forming bacteria

Based on biofilm – estimation method published by Morikawa *et al*. (2006): An overnight culture was diluted to OD_{600} = 0.3 and inoculated (1 %) into 300 µl of LB liquid medium in a 1.5 ml micro-centrifuge tube (TC131615, Nippon Genetics, Tokyo, Japan). The tube was kept standing at 30 °C for 3 days. The pellicles and the medium were removed from the tube, which was gently rinsed with distilled water and filled with 500 µl of 1 % CV solution. After 25 min, the CV solution was removed and the tube was washed with distilled water. The CV attached to the biofilm was dissolved in 400 µl of DMSO and quantified by measuring its absorbance at 570 nm. All the data are average of triplicate experiments.

2.2.3 Identification of the bacterial strain forming biofilm by cell morphological and molecular biology methods

Morphological methods

Cells of the best biofilm formation were observed under S-4800 Scanning Electron Microscope – SEM (Hitachi, Japan).

Molecular biology methods

The total genomic deoxyribonucleic acid (DNA) of this strain was isolated and purified by using the methods as described by Zhou *et al*. (1996). The universal primers for amplification of 16S rRNA region contain forward primer 27f (5′-GAGTTTGATCCTGGCTCAG-3) and 1527r (5′-AGAAAGGAGGTGATCCAGCC-3′). The PCR reaction (25 µl) mix contained 0.5 µM of each primer, 10 µM deoxynucleotides, 1.5 mM $MgCl_2$, 1 x buffer, 1 unit of *Taq* polymerase (Fermentas) and annealed at 52°C for 2 min. PCR product was separated by agarose gel electrophoresis and recovered by using Accuprep PCR purification kits (Bioneer). The sequence was identified under sequencing ABI Systems (USA). After that, the sequence was analyzed by the software's such as Bioedit, Clustal X and Mega4.

2.2.4 Effect of some conditions to biofilm formation

The values of pH from 6 to 11; temperature (25, 30, 37, 40, 45 and 50°C); carbon sources (glucose, saccharose, mantose and lactose) and nitrogen sources (yeast extract, KNO_3, peptone and $NaNO_3$) were used to estimate optimal conditions to biofilm formation.

The selected strain was cultured to form biofilm as described in 2.2.2 with different conditions of cultivation (pH, temperature, carbon and nitrogen sources). Then, after 24h, 48h and 72h, the biofilms were quantified by measuring its absorbance at 570 nm. The highest results were selected for further experiments. All the data are average of triplicate experiments

2.2.5 Analysis of pyrene by high performance liquid chromatography (HPLC)

The selected bacterium was cultured at the optimal conditions to form biofilm in LB medium. The biofilms were washed twice with distilled water. The biofilms were then incubated into Gost medium supplemented with 300 ppm pyrene. The incubation afterthat was extracted to analyze by HPLC to determine the amount of degraded pyrene by the biofilms. It was performed on a Hewlett-Packard (Bad Homburg, Germany) HPLC apparatus 1100 M equipped with a quaternary pump system, a diode array detector 1100 M series I, and an HP Chemstation. The separation was achieved with a Spesiorb ODS C18 end-capped (5-µm) column (Merck, Darmstadt, Germany). Elution profile is characterized by an initial solvent composition of 30 % water- 70 % acetonitril (0.1 %), reaching 100 % acetonitril after 30 min at a flow rate of 0.5 ml min^{-1}, column temperature is 25 ^{o}C.

3. Results and discussion

3.1. Enrichment and isolation of pyrene degrading bacteria

After three times of enrichment, samples were diluted and plated on Gost medium supplemented with 50 ppm of pyrene. These plates were incubated at 30°C and eight bacterial strains having different morphologies were collected. After seven days (168 hours) of culturing on Gost liquid medium supplemented with 50 ppm of pyrene, the growth capacity of these bacteria was showed in Figure 1.

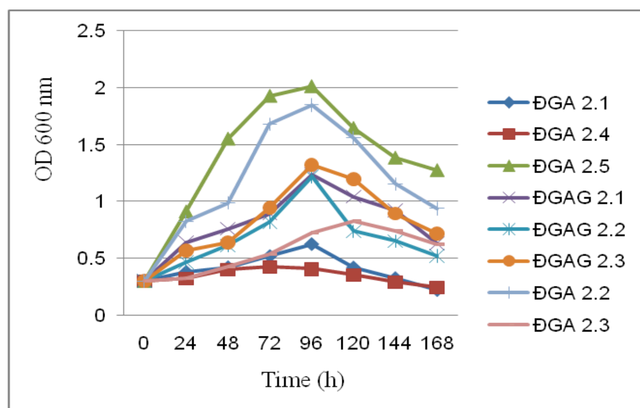
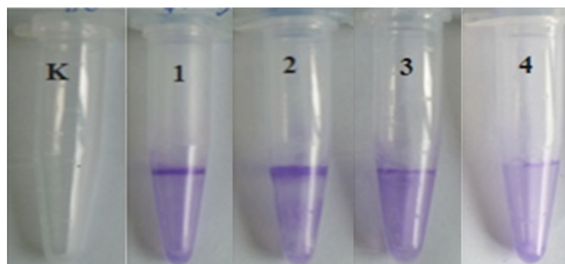


Figure 1. Growth capacity of isolated bacteria on pyrene



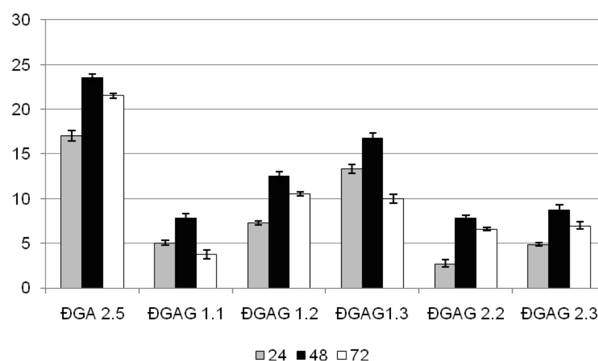


Figure 2. Biofilm formation of isolated bacterial strains (1, ĐGA 2.2; 2, ĐGA 2.5; 3, ĐGAG 2.1 and 4, ĐGAG 2.3)

The results indicated that the four bacterial strains including ĐGA 2.2, ĐGA 2.5, ĐGAG 2.1 and ĐGAG 2.3 have highly pyrene utilazation ability. Therefore, these microorganisms were selected for biofilm formation test.

3.2 Screening bacterial strains forming biofilm

The four bacterial strains were rinsed with cristal violet and the results showed in Figure 2. Of which, the strain ĐGA 2.5 was shown as the highest biofilm-forming and pyrene-utilizing strain, thereby, the ĐGA 2.5 was chosen for further study.

3.3 Identification of the bacterial strain by cell morphological and molecular biology methods

The strain ĐGA 2.5 was negative Gram with milk color, curve, wet and colony diameter was 1.5-2 mm. Under SEM with magnification of 30,000 X, the cells of ĐGA 2.5 have short - rod shape, (0.6-1.1) µm x (0.4-0.6) µm size (Figure 3).

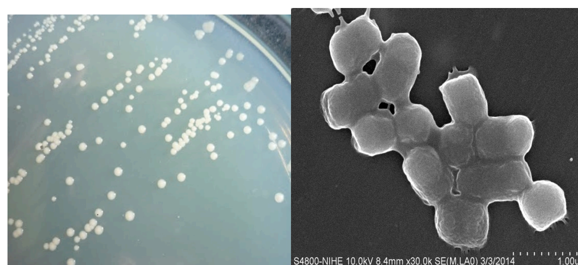


Figure 3. Morphology of ĐGA 2.5 colonies and cells

Then, the ĐGA 2.5 cells were identified by 16S rRNA analysis. Comparing with gene sequences of other bacterial strains in NCBI, gene sequence of ĐGA 2.5 strain was similar from 96 to 99% with *Paracoccus* genus. And, BQN11 strain was named *Paracoccus* sp. DG25. This sequence was registered at NCBI with KJ608354 accession. A phylogenic tree was built basing on similarity sequences in NCBI, using clustal X and Mega4 software (Figure 4).

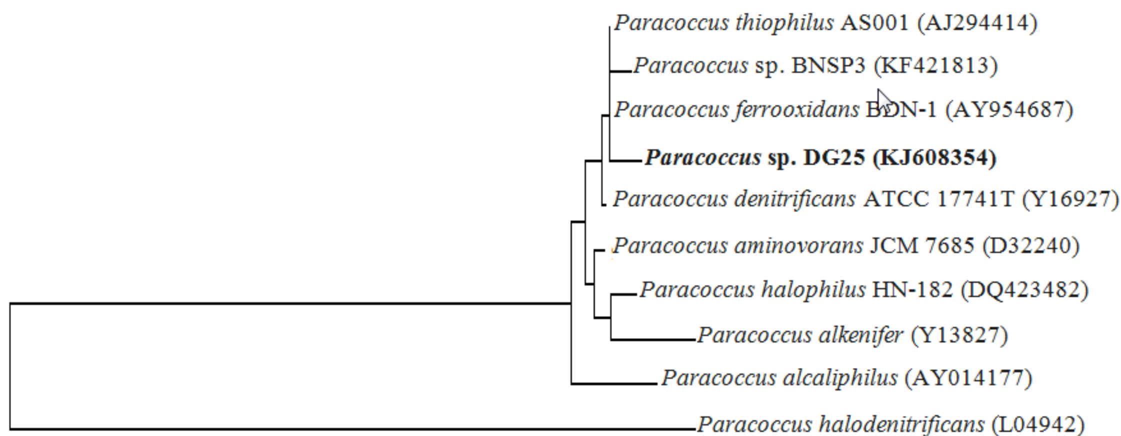


Figure 4. Phylogenic tree of ĐGA 2.5 gene 16S rRNA sequence

3.4 Effect of some experimental conditions on biofilm formation

The effects of incubation temperature, pH, carbon and nitrogen sources on the biofilm formation of *Paracoccus* sp. DG25 strain are shown in Fig 5. The highest biofilm formation of DG25 strain was observed in culture at 37 ^{o}C, followed by those at 39, 40 and 25 ^{o}C. The initial pH of the growth media ranged from 4.0 to 9.0. After 3 d incubation, the biofilms well formed in the cultures at pH 6 to pH 9. The highest formation occurred in the cultures in which the initial pH was 7.0. Similarly, matose was shown as the best carbon source for biofilm formation and KNO_3 was the optimal nitrogen source. According to Nghiem Ngoc Minh (2012), the *Paracoccus* sp. BTL4 isolated from wastewater of Tu Liem industrial zone grew best at 37°C, pH from 6 to 8. However, the BTL4 was investigated as suspended type, meanwhile the DG25 strain was examined as biofilm type. *Paracoccus* sp. are common in the environment and have been reported to be able to utilize and/or degrade many variety of xenobiotics when given as plantonik type (Teng *et al*., 2010).To the best of our knowledge, few studies have studied the biofilms formation of *Paracoccus* sp. to apply in aromatic hydrocarbon treatment. Therefore, these optimal conditions were used for further investigation.

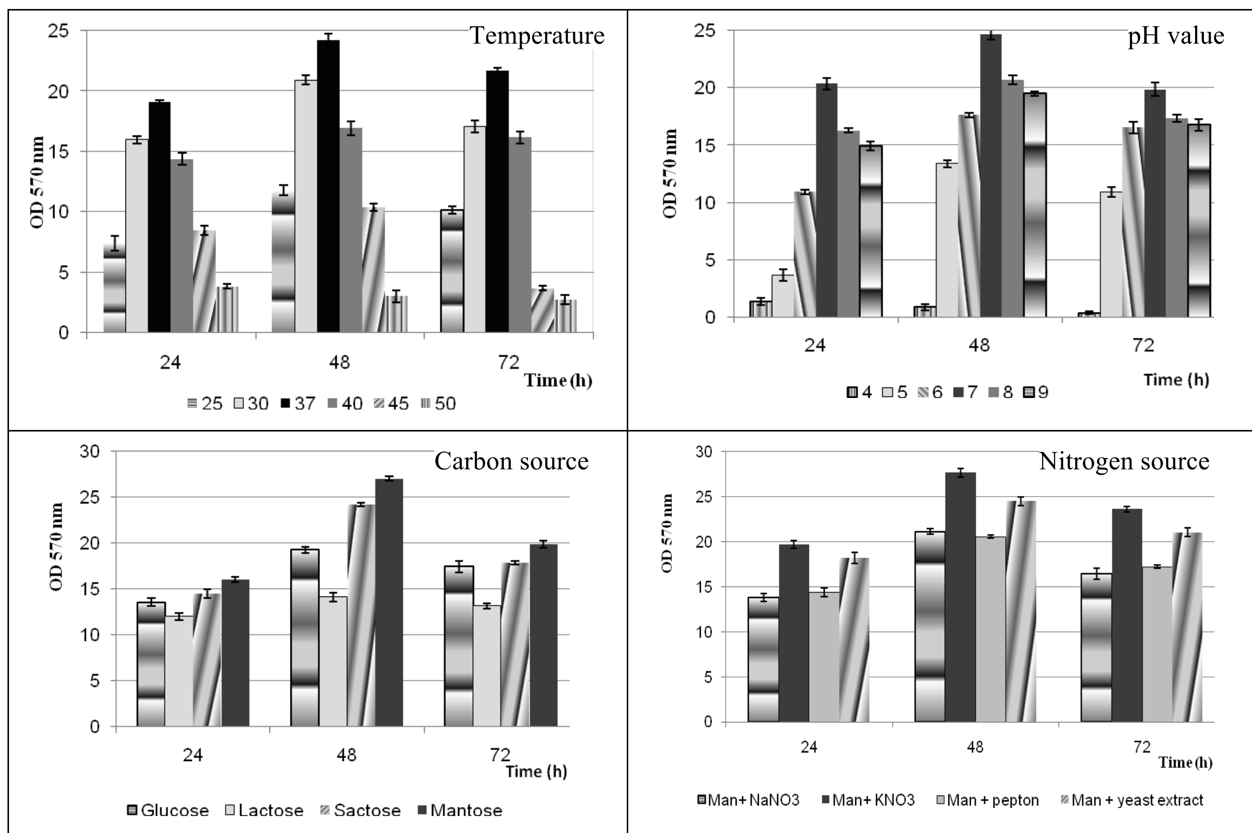


Figure 5. Effect of some experimental conditions on biofilm formation

3.5 Degradation of pyrene by *Paracoccus* sp. DA 2.5

The pyrene degradation was estimated by HPLC analysis and the results were shown in Fig 6. Depending on the peak high, the percentage of degraded pyrene was 76.07 % after 7 day incubation with the initial concentration of 300 ppm.

There are many publications on PAH degradation by *Paracoccus* sp. In 2005, Guo *et al* reported on *Paracoccus* (SPNT) isolated from mangrove sediments were capable to degrade mixed PAHs (phenanthrene + fluoranthene + Pyrene). However, pyrene was hardly biodegraded (<20% degradation) then other compounds. In 2010, Teng *et al*. published PAH degradation of *Paracocus aminovorans* HPD-2which could degrade 36 % amount of PAHs containing 3 rings and also degraded 26 % amount of PAHs containing 5 rings after 14 day incubation (Teng *et al*., 2010). Nghiem Ngoc Minh *et al*. (2012) showed that the strain *Paracocus*. sp. BTL4 degraded 25.5 % of pyrene after 7 day incubation with the initial concentration of 100 ppm. In comparison with these results, the biofilm formed by *Paracoccus* sp. DG25 degraded higher than these other *Paracoccus* sp. This result suggested the capacity of application biofilm formed by *Paracoccus* sp. DG25 in pyrene and PAH polluted soil and water samples. Moreover, to enhance this ability, using biofilm formed by mixture of species was published (Le Thi Nhi Cong *et al*., 2014). It is explained that the ability of a biofilm to tolerate environmental stresses could be used to increase biodegradation.

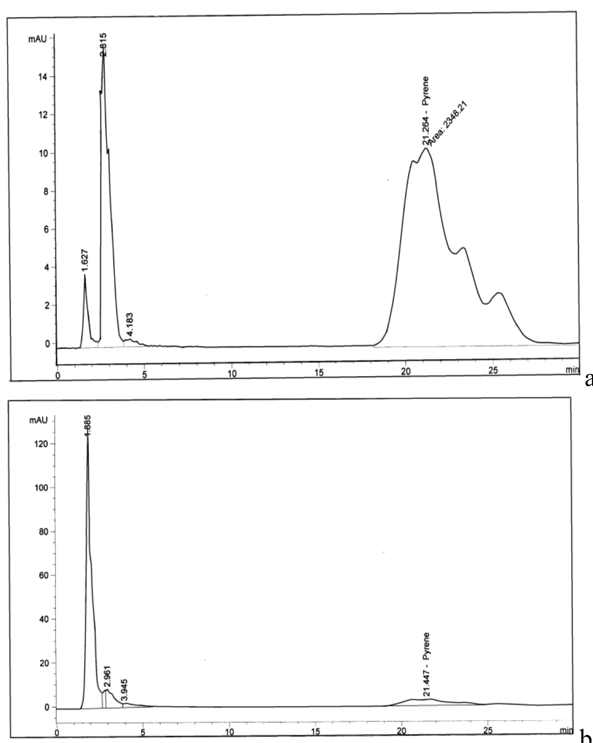


Figure 6. Chromatograpy of pyrene degradation by DG25 after 7 day incubation. a, control; b, experiment

Once these cells are firmly bound, the activity of the community is dependent on the metabolism and growth of each member species under local surface conditions. Thus, the *Paracoccus* sp. DG25 is considered to increase the diversity of biofilm forming microorganism to treat PAH polluted wastewater.

4. Conclusion

From oil contaminated water and sediment samples collected in petroleum storage Duc Giang, Hanoi we have isolated a biofilm forming *Paracoccus* sp. DG25 strain with the accession numbers KJ608354 in the GenBank database. The optimal biofilm formation was at pH 7, 37°C, on maltose and supplemented with KNO_3. Using these optimal conditions, the formed biofilm degraded 76.07 % of pyrene after 7 day-incubation with the initial concentration of 300 ppm. These results may lead to a new approach to treat polycyclic aromatic hydrocarbons containing in petroleum oil contaminated water in Vietnam.

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