Shahid Bahonar University of Kerman
Agricultural Biotechnology Journal
Iranian Biotechnology Society
Print ISSN: 2228-6705 Online ISSN: 2228-6500

Exploring the role of Alk, PAH and CYP153 genes in removing of bitumen contaminants

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Abstract

Objective

Alkane hydroxylase is an enzyme involved in the first stage of alkane degradation the alkB gene is important for the biodegradation of bitumen because bitumen contains a lot of hydrocarbons such as alkanes, bacteria with the is alkane monooxygenase (AlkB) gene can break it down. The regulatory mechanisms can be intricate and species-specific; there are enzymes encoded by the PAH gene that initiate the breakdown of polycyclic aromatic hydrocarbons (PAHs); and PAHs are dangerous pollutants that can be detected in water and soil. An alkane hydroxylase, specifically a cytochrome P450 enzyme, is encoded by the cytochrome P450 Class I P450 (CYP153) gene. this enzyme is essential for the biodegradation of hydrocarbons, which includes bitumen. Enzymes belonging to the cytochrome P450 family play an important role in the metabolism of many different compounds, including those that are foreign to the body. One of bitumen's main components, alkanes, can be oxidized by the enzyme CYP153. Thus, the aim of this study was to explore the role of Alk, PAH and CYP153 genes in removing of bitumen contaminants.

Materials and Methods

Genomic DNA was extracted using the standard DNA extraction Kit. The quality and quantity of extracted DNA were determined using nanodrop device. The specific primers were used to amplify AlkB, PAHs and Cyp153 genes. Visualization of the amplified fragments was performed using a transilluminator under ultraviolet light and photographed.

Results

Extracted DNA had good quality and quantity (10ng/µL). Alkane monooxygenase (alkB), PAH, and CYP153 are three key enzyme-encoding genes that play an essential role in the mineralization of aliphatic and PAH chemicals, respectively. The presence of these three genes (alkB, PAH, and CYP153) was detected based on PCR amplification and visualized on agarose gel. Frequency of bitumen utilization genes was different. It was the highest for CYP152 gene and the lowest for AlkB gene.

Conclusions

The results highlight the potential for bioremediation applications, especially in bitumen-contaminated areas of employing native bacteria such as *Pseudomonas aeruginosa* to validate these bacteria's effectiveness in practical settings and create scalable bioremediation techniques for reducing hydrocarbon contamination field research is necessary.

Keywords: Bitumen, DNA, enzyme, PCR amplification

Paper Type: Research Paper.

Citation: Hassouni DM, Mohammed MM (2024) Exploring the role of Alk, PAH and CYP153 genes in removing of bitumen contaminants. *Agricultural Biotechnology Journal* 16 (4), 377-390.

Agricultural Biotechnology Journal 16 (4), 377-390. DOI: 10.22103/jab.2024.24493.1634

Received: October 09, 2024. Received in revised form: December 19, 2024.

Accepted: December 20, 2024. Published online: December 30, 2024.

Publisher: Faculty of Agriculture and Technology Institute of Plant Production, Shahid Bahonar University of Kerman-Iranian Biotechnology Society.

Introduction

As asphalt is constantly exposed to environmental factors including sunlight, precipitation and temperature it undergoes changes in both its mechanical characteristics and chemical makeup these alterations may lead to issues including potholes, cracks and loosening which would ultimately shorten the pavement's lifespan and performance (Wu et al. 2024). Because asphalt is impermeable, less water can percolate through it, leading to higher runoff volumes. On the plus

side, asphalt helps keep soil from washing away nutrients and pollutants, but it also adds potentially harmful substances like PAHs and aromatic hydrocarbons, which can linger in the soil and stunt plant and animal life (Norin 2004). Another research examined the efficacy of bioremediation in removing asphalt-contaminated soil and discovered that it successfully decreased the concentration of asphalt-derived polycyclic aromatic hydrocarbons (PAHs) (Pathak et al. 2022). Since native bacterial strains have been found to be significantly more effective in hydrocarbon biodegradability than introduced strains outcompeting, biotic and/or abiotic interacting elements may be the cause of this therefore, the effectiveness of petroleum hydrocarbon cleaning in a given area is highly dependent on the isolation of new bacteria that are adapted to the local conditions (Wu et al. 2013). A technique for cleaning up PAH-contaminated soil (Li et al. 2020; Zhang et al. 2021). Due to its great efficiency, affordability and sustainability, the bio-augment remediation strategy is regarded as the ideal choice (Ghosal et al. 2016). Environmentally speaking, it is of the utmost importance to have a better understanding of biodegradation, the process by which organic contaminants are converted or mineralized by local microorganisms (Das and Chandran 2011). Compared to other remediation methods, microbiological applications are more cost-effective because they use microorganisms to degrade organic materials and remove hydrocarbon toxins like petroleum from the environment (Das and Chandran 2011). According to Wang et al. (2016), genes that degrade polycyclic aromatic hydrocarbons (PAHs) in soil are helpful biomarkers for determining how well bacterial populations can break down PAHs. Reductase, ferredoxin and terminal oxygenase subunits are frequently found in dioxygenase, a multicomponent enzyme (Ghosal et al. 2016). The two categories of dioxygenases are those that hydroxylate rings (RHDs) and those that cleave rings (RCDs) (Chikere and Fenibo 2018). The cleavage of C12O, which codes for catechol 1, 2-dioxygenase during the biodegradation of PAHs is linked to the last aromatic ring in the PAH degradation pathway the quantity and expression of these genes are critical (Liao et al. 2021). One of the non-heme diiron enzymes catalyzing the hydroxylation of alkanes is alkane monooxygenase (AlkB). This enzyme is usually existed in alkanotrophic organisms. These organisms use alkanes to receive energy and carbon for living. Two-electron reduction of AlkB diferric active site activate this enzyme through facilitating of binding, activation, and cleavage of molecular oxygen for introducing into an inert C-H bond (Williams et al. 2022). Currently, the enzymes involved in aerobic alkane activation can be clustered in: soluble diiron methane monooxygenases (sMMO) and membrane-bound copper-containing methane monooxygenases (pMMO) (van Beilen and Funhoff 2005), cytochrome P450 Class I P450 (CYP153) and cytochrome P450 Class II P450 (CYP52, CYP2E and CYP4B) (Maier et al. 2001), integral membrane di-iron alkane hydroxylases (AlkB) (van Beilen and Funhoff 2005), flavin-binding

monooxygenases (AlmA), firstly found in *Acinetobacter* strain DSM 17874 (Throne-Holst et al., 2007), or long chain alkane monooxygenases (LadA), initially found in *Geobacillus thermodenitrificans* NG80-2 (Feng et al. 2007) superfamilies. Moreover, biochemical tests are incapable of distinguishing different types of parasites (Ahsani et al. 2010; Mohammadabadi et al. 2004). PCR is the most modern practical technology in diagnosing infectious diseases, different close bacterial strains and detection of specific genes and compared with classical techniques, it has been shown to be more rapid, with results obtained in a few hours, and also more reliable (Mohammadabadi et al. 2011; Khabiri et al. 2023). PCR allows a faster bacterial identification directly from clinical samples (Shahdadnejad et al. 2016; Mohammadabadi et al. 2024). Thus, the aim of this study was to explore the role of Alk, PAH and CYP153 genes in removing of bitumen contaminants.

Materials and Methods

DNA extraction: Genomic DNA was extracted using the standard DNA extraction Kit performing below steps. The microcentrifuge tube was filled with bacterial cells for one minute and the sample was centrifuged at 14000–16000 rpm. Then, 20 µL of proteinase K and 180 µL of GT buffer were added to the sample. After that the sample was transferred to the GD column and 200 µL of GB buffer and 200 µL of 100% ethanol were added. Centrifugation was done and the GD column was filled with 400 µL of W1 buffer. Then 600 µL of washing buffer was used to repeat the washing process. After adding 50 µL of elution buffer and letting the sample remain at room temperature for at least three minutes, the eluted DNA was kept at -20°C until it was needed. The quality and quantity of extracted DNA were determined using nanodrop device. For all samples, the DNA concentration was almost 10ng/µL.

Polymerase chain reaction: Primers forward 5'-CCTGCTCCCGATCCTCGA-3' and reverse 5-'TCGTACCGCCCCGCTGTCCAG-3' for amplification of AlkB gene (Temitayo et al. 2019), forward 5'-TGCGGCGGGTGTNAAYGGNAT-3' and reverse 5-' CCTGAGGAATCTCGGACATYTSTGCCCARAA-3' for amplification of PAHs gene (Liang et al. 2019), and forward 5'- GATCCGCTCGCGTGTC-3' and reverse 5-' GGGAGTGAGGCGAACCA-3' for amplification of Cyp153 gene (Ivanova et al. 2014) was used to amplify the target genes.

The PCR reaction consisted of 2.5 ng of DNA, each oligonucleotide at 0.25 mM, 1X reaction buffer, 3.12 mM MgCl2, 0.125 mM dNTPs each, 1.25 U of DNA polymerase in a final volume of 20 µL. Amplification was performed with initial denaturation at 94°C for 3 minutes, followed by 25 cycles at 94°C for 45 seconds, oligonucleotide annealing at 59°C for 45 seconds, extension

Agricultural Biotechnology Journal; Print ISSN: 2228-6705, Electronic ISSN: 2228-6500

at 72°C for 2 minutes and final extension at 72°C for 5 minutes. A 2 µL aliquot of the PCR were stained and subjected to electrophoresis in a 1.6% agarose gel, using M100 DNA Ladder as a molecular weight marker. Visualization of the amplified fragments was performed using a transilluminator under ultraviolet light and photographed.

Results and discussion

Extracted DNA had good quality and quantity (10ng/µL). Alkane monooxygenase (alkB), PAH, and CYP153 are three key enzyme-encoding genes that play an essential role in the mineralization of aliphatic and PAH chemicals, respectively. We used the catabolic genes as a tool to detect bacterial strain-specific activities. Alkane hydroxylase an enzyme involved in the first stage of alkane degradation the alkB gene is important for the biodegradation of bitumen because bitumen contains a lot of hydrocarbons such as alkanes, bacteria with the alkB gene can break it down. Genomic DNA from the selected strains served as templates for the AlkB gene amplification. One of the many functions of the multipurpose bacterium *Pseudomonas aeruginosa* is to degrade hydrocarbons. The alkB gene of this bacterium may play a role in degrading alkylated compounds found in certain environments, but enzymatic genes play a much larger role, according to other bacteria. Bitumen biodegradation relies on the alkB gene, which encodes the enzyme alkane hydroxylase, which is involved in the initial phase of alkane degradation (Temitayo et al. 2019). Because of its high hydrocarbon content, bitumen can be degraded by bacteria that have the alkB gene. Through the use of this gene, bacteria are able to catalyze the conversion of alkanes to alcohols. From there, fatty acids and other intermediates can be produced through downstream metabolic pathways. Bacteria can biodegrade bitumen by using its hydrocarbons as a carbon and energy source through these metabolic activities. Bioremediation relies on bacteria carrying alkB genes to break down complicated hydrocarbon molecules into simpler, more biodegradable compounds. These bacteria are essential in environments polluted with hydrocarbons, including soil that has been contaminated with bitumen. The existence of alkB in bacterial communities suggests that these microbes may be able to break down aliphatic hydrocarbons, a key component of bitumen. This might lead to a decrease in bitumen pollution levels or even its bioremediation. The presence of three genes (alkB, PAH, and CYP153) was detected based on PCR amplification. The existence of these genes was confirmed, and the results are shown in the Figures 1, 2, and 3.

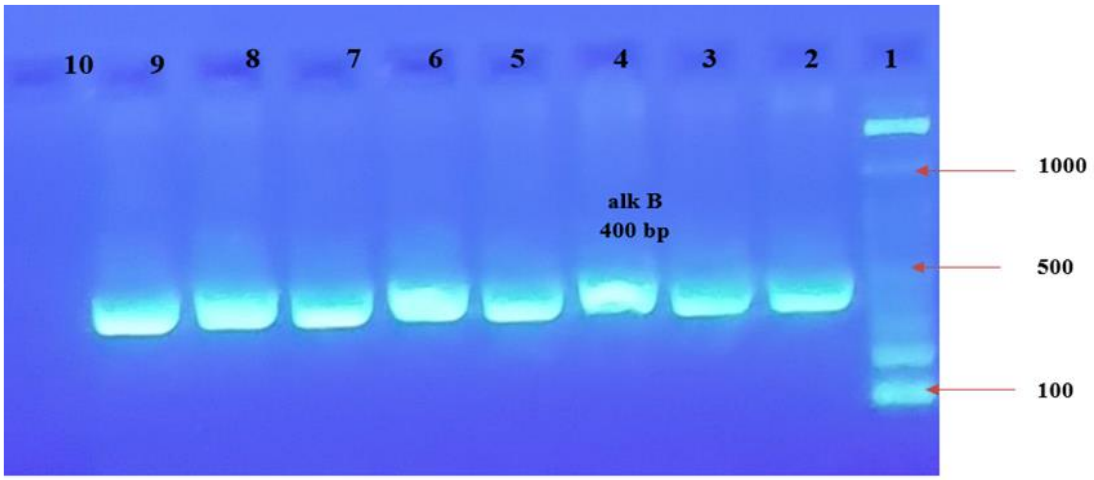

Figure 1. The electrophoresis of PCR products for the alkane monooxygenase (AlkB) gene on 1.6% agarose gel. Lane 1 is M100 DNA ladder

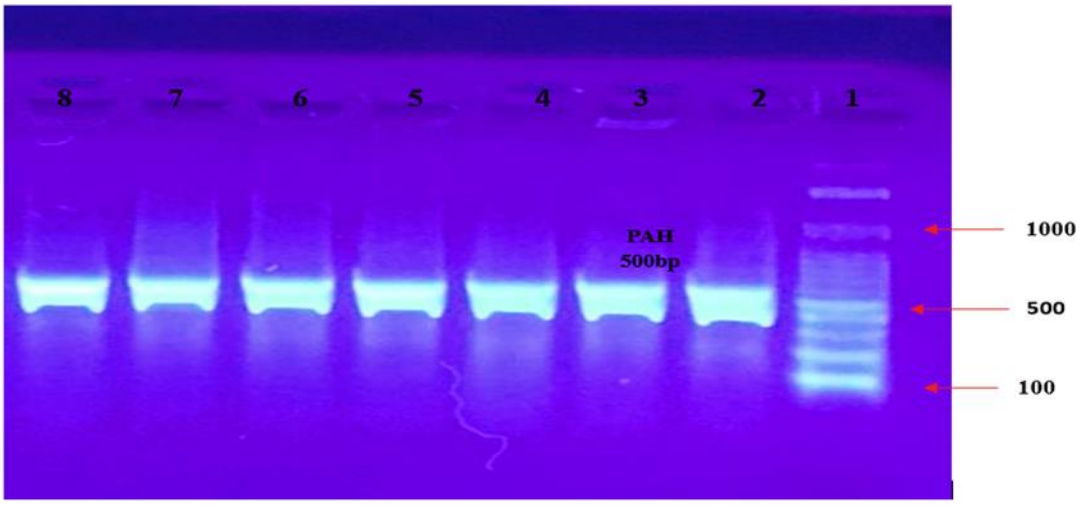

Figure 2. The electrophoresis of PCR products for the PAH gene on 1.6% agarose gel. Lane 1 is M100 DNA ladder

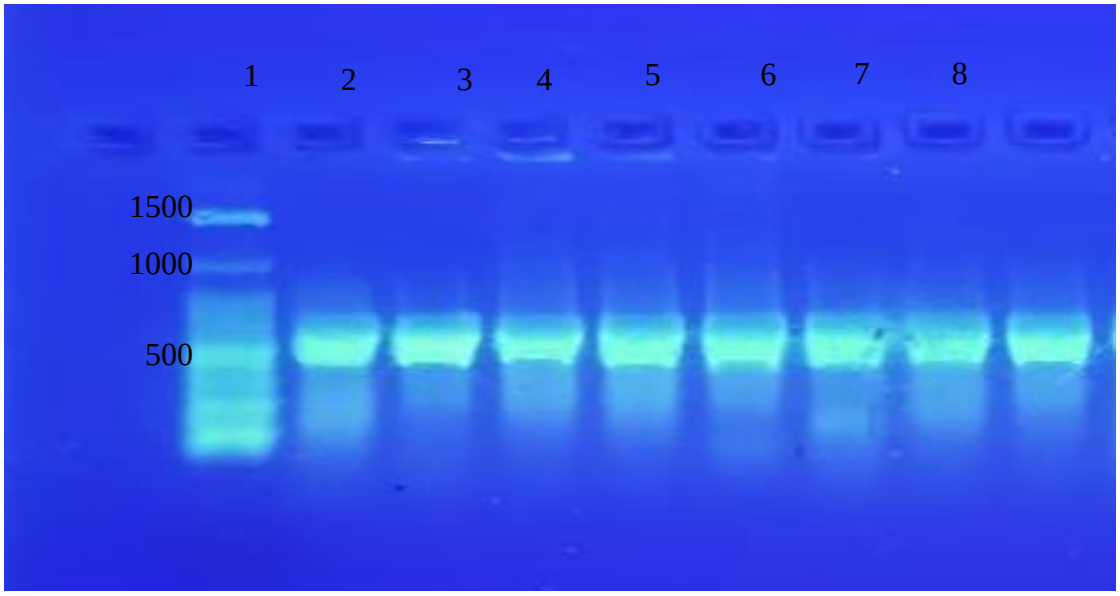

Figure 3. The electrophoresis of PCR products for the CYP153 gene on 1.6% agarose gel. Lane 1 is M100 DNA ladder

Frequency of bitumen utilization genes is shown in figure 4.

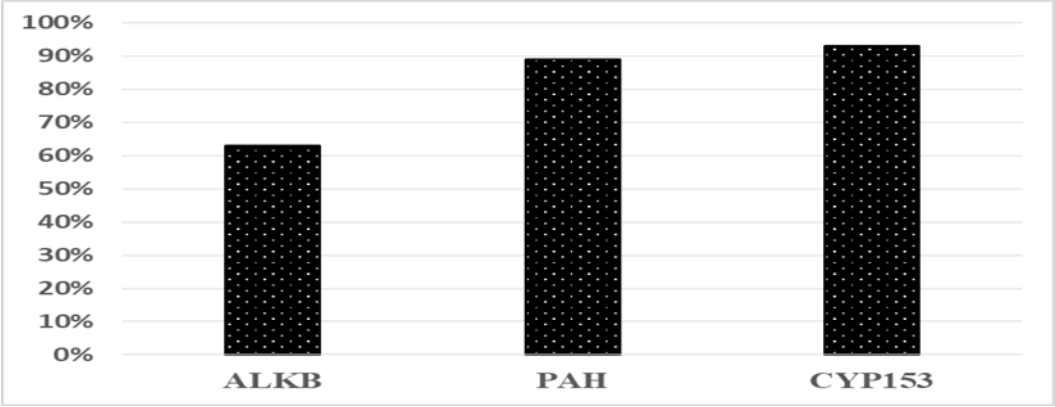

Figure 4. Bitumen utilization genes frequency

All bacterial isolates produced positive findings when the AlkB gene universal primer pair was used. A 400 bp amplification product was also detected in *Pseudomonas aeruginosa.* This gene most likely codes for an enzyme involved in DNA repair alkylated DNA damage so it can be repaired by AlkB. The two catabolic genes that encode the enzymes involved in the pathways leading to the degradation of alkanes were discovered in the purified DNA by PCR screening as noted by Lima et al. (2020). The discovery of the alkane hydroxylase gene in bacterial isolates led to the earlier findings of other researchers indicating that the presence of certain enzymes in

the bacteria is necessary for them to break down hydrocarbons. Because of their high enzymatic capacity, microbial communities can degrade complex hydrocarbons like aliphatics and polyaromatics. Additionally, several bacteria that consume hydrocarbons have functional genes that facilitate hydrocarbon degradation (Eze et al. 2021). Control of PAH gene expression the presence of PAHs typically causes the expression of PAH genes, this indicates that only when the bacteria come into contact with PAHs in their surroundings do the genes become active. With controlling PAH gene expression typically, PAHs are able to induce the expression of PAH genes. This indicates that PAHs are the sole environmental cues needed to activate the genes in bacteria. The regulatory mechanisms can be intricate and species-specific; there are enzymes encoded by the PAH gene that initiate the breakdown of PAHs; and PAHs are dangerous pollutants that can be detected in water and soil. Since crude oil and oil spills frequently contain significant amounts of polycyclic aromatic hydrocarbons like naphthalene, alkylnaphthalenes, phenanthrene and alkyl phenanthrenes, the possibility of the consortium for the remediation and reclamation of petroleum-contaminated soils is indicated by the presence of putative genes encoding PAH dioxygenases in the consortium's metagenome (Ahmed and George 2004; Eze and George 2020). An alkane hydroxylase, specifically a cytochrome P450 enzyme, is encoded by the CYP153 gene. According to Rojo and Wackett (2000), this enzyme is essential for the biodegradation of hydrocarbons, which includes bitumen. Enzymes belonging to the cytochrome P450 family play an important role in the metabolism of many different compounds, including those that are foreign to the body. One of bitumen's main components, alkanes, can be oxidized by the enzyme CYP153.

In a study, a biosurfactant was found to significantly enhance the desorption of tetracyclic PAHs from soil and to enhance their biodegradation by Pseudomonas alkaligenes strain PA-10. This was likely due to a combination of solubility and increased biomass, as biosurfactants can also be used as a carbon source (Hickey et al. 2007). Thus, microorganisms that overproduce biosurfactants may play an important role in the hydrocarbon degradation process.

In other study Temitayo et al. (2019) investigated the polyaromatic hydrocarbon degradation potentials of some bacteria: *Campylobacter hominis, Bacillus cereus, Dyadobacter koreensis, Pseudomonas aeruginosa* and *Micrococcus luteus* isolated from Agbabu bitumen sediments in Ondo State. Their results showed that these isolates degradated poly aromatic hydrocarbons (PAHs). Their consortium exhibited the highest PAH reduction (73%) while *C. hominis* had the least PAH reduction (56%). *Dyadobacter koreensis, P. aeruginosa, Micrococcus luteus and B. cereus,* displayed 66%, 60%, 59% and 58% PAH reduction respectively. The catabolic gene *nahH* gene was present in B. *cereus, D. koreensis, P. aeruginosa* and *M. luteus, alkB* gene was present in *B. cereus, C. hominis,* and *D. koreensis* while *CatA* was not detected in any of the isolates.

Their findings affirmed the hydrocarbon-degrading abilities and presence of catabolic genes in these bacteria, these make them potential tools in oil prospecting and cleaning up of hydrocarbon contaminated sites. Mirzaee (2022) studied six different magnetic granular and powder activate carbons (MPAC and MGAC) were synthesized to assess their affinity for USEPA priority polycyclic aromatic hydrocarbons (PAHs) in aqueous solutions and in a combined soil washing process. Two of the magnetised ACs (MPAC-Prec. and MGAC-CoPrec.) outperformed the others in removing PAHs from the aqueous phase, and therefore, were selected for removal of PAHs from aqueous phase and soil washing experiments, respectively. They used tween 80 surfactant in the last phase in conjunction with MGAC in soil washing process to enhance PAHs removal from a real contaminated soil. They concluded that the electron-donor and electron-acceptor interactions (π-π interactions) were the main adsorption mechanism formed between the PAHs molecules and MPAC adsorption sites, according to the FTIR test results. The obtained PAH removal efficiency was a function of PAH concentration and mass of the magnetic adsorbent in the samples. They also showed the highest PAH-removal efficiency from the aqueous solutions. Hosseini et al. (2022) assessed the isolation and identification of biodegradable *Mycobacterium* species from diverse Markazi province ecosystems. They demonstrated that the *M. porcinum* and *M. celeriflavum* have a significant capability to biodegrade the PAHs. Therefore, they recommended more investigations for separation and applicational use of the mycobacterium species for bioremediation of PAHs. Hesham et al. (2014) isolated strains capable of degrading low and high molecular weight PAHs. They obtained and identified a strain as *S. koreensis* according to morphological characteristics and 16S rRNA gene sequence analysis and its ability to degrade naphthalene, phenanthrene, anthracene, and pyrene. They also investigated the production of biosurfactants and increasing cell-surface hydrophobicity, the metabolites during the degradation process, and the genetics of catabolic genes in the isolated PAH-degrading bacterium.

Gonçalves et al. (2016) isolated microorganisms with degradation capacity of oil hydrocarbons (oil and diesel), evaluating the biodegradation rate and the presence of the *alkB* gene in soil samples. Four microorganisms isolated and identified as bacteria showed potential for hydrocarbon degradation, in addition to the presence of the alkB gene in their genomic DNA. They proposed that complementary studies, such as the identification of microorganisms and sequencing of amplified fragments of the alkB region, may corroborate the understanding of the degradation process. The study and use of microorganisms selected in natural soils and especially in areas already contaminated by hydrocarbons represent an important strategy for the bioremediation of these areas. For bioremediation to reflect satisfactory results, knowledge of the

principle and techniques used is essential. This helps in the correct use and selection of the microorganism according to the conditions of each area and each contaminant present.

Alkanmonooxygenase enzymes AlkB and Cyp153 are responsible for the aerobic degradation of *n*-alkanes of petroleum and petroleum products. To prove the usage of *n*-alkanes from oil and petroleum products by hydrocarbon-oxidizing bacteria isolated from aviation kerosene TS-1 and automobile gasoline AI-95, Shapiro et al. (2022) carried out the detection of the key genes *alkB*, *Alk1*, *Alk2*, *Alk3* and *Cyp153* encoding alkanmonooxygenases AlkB and Cyp153 (responsible for the oxidation of hydrocarbons with a certain chain length). They revealed the activity of the *alkB* gene in all strains of hydrocarbon-oxidizing bacteria isolated from TS-1 jet fuel and AI-95 gasoline by real-time PCR. They showed a significant quantitative difference in the activity of this gene in the isolated strains. For strains isolated from gasoline, the activity data correspond to physiological and biochemical data on bacterial growth in the presence of a model mixture of hydrocarbons and the efficiency of their degradation (Shapiro et al., 2021). They indicated the need to use a set of methods (a polyphase approach) for a comprehensive assessment of the ability of hydrocarbon-oxidizing bacteria strains to degrade petroleum hydrocarbons, including the usage of molecular (in particular, PCR) and physiological methods to analyze the distribution and homology of the specific studied gene in bacteria.

Conclusions: The results highlight the potential for bioremediation applications, especially in bitumen-contaminated areas of employing native bacteria such as *Pseudomonas aeruginosa* to validate these bacteria's effectiveness in practical settings and create scalable bioremediation techniques for reducing hydrocarbon contamination field research is necessary.

Acknowledgement: The authors thank all reviewers for their valuable comments in improving the quality of the article.

Conflict of Interest: There is no conflict of interest.

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مجله بیوتکنولوژی کشاورزی
شاپا چاپی: ۶۷۰۵-۲۲۲۸ شاپا الکترونیکی: ۶۵۰۰-۲۲۲۸

بررسی نقش ژن‌های Alk، PAH و CYP153 در حذف آلاینده‌های قیر

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تاریخ دریافت: ۱۴۰۳/۰۷/۱۸ تاریخ دریافت فایل اصلاح شده نهایی: ۱۴۰۳/۰۹/۲۹ تاریخ پذیرش: ۱۴۰۳/۰۹/۳۰

چکیده

هدف: آلکان هیدروکسیلاز آنزیمی است که در مرحله اول تجزیه آلکان نقش دارد و ژن alkB برای تجزیه زیستی قیر مهم است زیرا قیر حاوی مقدار زیادی هیدروکربن مانند آلکان است، باکتری‌هایی با ژن alkane monooxygenase (AlkB) می‌توانند آن را تجزیه کنند. مکانیسم‌های تنظیمی می‌توانند پیچیده و مختص گونه باشند. آنزیم‌هایی وجود دارند که توسط ژن PAH کدگذاری شده‌اند که تجزیه هیدروکربن‌های آروماتیک چند حلقه‌ای (PAHs) را آغاز می‌کنند و PAHها آلاینده های خطرناکی هستند که می توانند در آب و خاک شناسایی شوند. یک آلکان هیدروکسیلاز، به ویژه آنزیم سیتوکروم P450، توسط ژن سیتوکروم P450 کلاس I P450 (CYP153) کدگذاری می‌شود. این آنزیم برای تجزیه بیولوژیکی هیدروکربن ها، که شامل قیر است، ضروری است. آنزیم‌های متعلق به خانواده سیتوکروم P450 نقش مهمی در متابولیسم بسیاری از ترکیبات مختلف، از جمله آن‌هایی که برای بدن خارجی هستند، ایفا می‌کنند. یکی از اجزای اصلی قیر، آلکان ها، می توانند توسط آنزیم CYP153 اکسید شوند. بنابراین، هدف از این مطالعه بررسی نقش ژن‌هایAlk، PAH و CYP153 در حذف آلاینده‌های قیر بود.

مواد و روش‌ها: DNA ژنومی با استفاده از کیت استاندارد استخراج DNA استخراج شد. کیفیت و کمیت DNA استخراج شده با استفاده از دستگاه نانو دراپ تعیین شد. از پرایمرهای اختصاصی برای تکثیر ژن‌های AlkB، PAHs و Cyp153 استفاده شد. مشاهده قطعات تکثیر شده با استفاده از ترانس‌ایلمیناتور در زیر نور ماوراء بنفش انجام و عکسبرداری شد.

نتایج: DNA استخراج شده کیفیت و کمیت خوبی (10ng/µL) داشت. آلکان منواکسیژناز (alkB)، PAH و CYP153 سه ژن کلیدی کدکننده آنزیم هستند که به ترتیب نقش اساسی در کانی سازی مواد شیمیایی آلیفاتیک و PAH دارند. حضور این سه ژن (alkB، PAH و CYP153) بر اساس تکثیر PCR شناسایی و روی ژل آگارز مشاهده شد. فراوانی ژن‌های استفاده‌کننده از قیر متفاوت بود. بالاترین فراوانی برای ژن CYP152 و کمترین فراوانی برای ژن AlkB بود.

نتیجه‌گیری: نتایج پتانسیل کاربردهای زیست پالایی را به ویژه در مناطق آلوده به قیر با استفاده از باکتری‌های بومی مانند سودوموناس آئروژینوزا برای تایید اثربخشی این باکتری‌ها در محیط‌های عملی و ایجاد تکنیک‌های زیست پالایی مقیاس‌پذیر برای کاهش آلودگی هیدروکربنی نشان می‌دهد.

واژه‌های کلیدی: آنزیم، تکثیر توسط PCR، قیر، DNA

نوع مقاله: پژوهشی.

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Publisher: Faculty of Agriculture and Technology Institute of Plant Production, Shahid Bahonar University of Kerman-Iranian Biotechnology Society.