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RESEARCH ARTICLE OPEN ACCESS

Cloning and Expression of *Pseudomonas aeruginosa AlkB* Gene in *E. coli*

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Abstract

Pre identified hydrocarbons degrading bacteria were used in this study, specific primer was conducted to amplification of *AlkB* gene, approximately 1206bp band size of this gene for Pseudomonas aeruginosa was detected and proofed by sequence and alignment analysis with NCBI database. The *AlkB* gene was inserted in PET-21a(+) plasmid vector as expression vector, then transformed in BL21(DE3) competent *E. coli* and confirmed by colony PCR technique using the T7 promoter and T7 terminator primers. The expression of the inserted gene was checked by determined the concentration of *AlkB* protein for multiple periods by Bradford assay method and the SDS-polyacrylamide gel electrophoresis method was revealed band of ~46 KD molecular weight of the concerned protein. The gene amplification and cloning strategy was lay out before the practical part of the study by SnapGene software, this study was conducted to introduce cloned bacteria which facilitate the first step (key step) of alkane's biodegradation and propose an appropriate strategy to construct genetically engineered microorganisms with multiple recombinant plasmid for enhance the degradation of the aliphatic fraction of hydrocarbon

Keywords: Cloning, *AlkB* gene, *Pseudomonas aeruginosa*

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INTRODUCTION

Iraq is one of the main petroleum oil producers. As a result of this elevated production, the probability of accidents such as the spillage of oil and environmental pollution became obvious, particularly concerns regarding possible pollution of soil and subterranean water sources.

Hydrocarbons in different forms are one of the most important pollution sources of the environment, it is causing critical damages to the human being and the ecosystem, the most promising of these is bioremediation. The process of organisms usage or their enzyme systems to provide an effective alternative is called bioremediation[1]. The proportion of alkanes in crude oil is estimated about 20-50%. also, it is produced by living organisms as structural elements, waste, part of protection mechanisms or as a chemoattractants[2]. A lot of plasmids include genes which express of necessary enzymes for the important steps to bioremediation, for example enzymes essential in the biodegradation of naphthalene, toluene, octane etc. have been consider as plasmid encoded[3]. The techniques of molecular biology can be used to cut portions of DNA involves genes responsible for particular biodegradation pathways and insert it in plasmids. The recombinant plasmid can then be hosted into unique organisms to produce genetically engineered microorganisms (GEM) with new biodegradation abilities[4].

The bacteria responsible for the degradation of the alkanes are widespread in the environment[5]. Bacteria genetically contained the *Alk* enzymes, which is involved in the in the metabolic steps of alkanes degradation, alkanes are the major compounds present in petroleum and its related components[6]. *Alk* enzyme system includes alkanes monooxygenases which is encoded by the *AlkB* genes, these genes are involved in the first hydroxylation step of the aerobic metabolism for aliphatic compounds, with the participation of the rubredoxin and rubredoxin reductase co-factors. The action of these enzymes is transport the electrons required in the *AlkB* function[7], Some studies depend on *AlkB* gene as a bioindicator for the diversity of alkanes-degrading bacteria in the environment[8] . In *Pseudomonas*, the responsible system for the first hydroxylation step of n-alkanes is known as alkane hydroxylase (monooxygenase) system[9].

Molecular cloning allows combination of genes from different organisms for multiple purposes; the process depends on the following four main steps[10]:

1- Selection and isolation of a target gene to be cloned.

2- Selection the carrier (vector) of our target DNA sequence.

3- Ligation of the target to the vector followed by genetic transformation-vector set in to a host cell.

4- Selection host cell having the recombinant DNA.

The gene amplification and cloning strategy were lay out before the practical part of the study by SnapGene software [11] ,this study was conducted to facilitate the first step (key step) of alkanes biodegradation and propose an appropriate strategy to construct genetically engineered microorganisms with multiple recombinant plasmid for enhance the degradation of aliphatic fraction of hydrocarbon.

MATERIALS AND METHODS

Hydrocarbons degrading bacteria and Molecular identification

In this study, pre-diagnosed hydrocarbons degrading bacterial strains were used, bacterial identification was done using 16S rRNA. The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using a specific set of primer 27F 5'AGAGTTTGATCCTGGCTCAG'3 1492R 5'GGTTACCTTGTTACGACTT'3[12] and characterized by alignment with NCBI data bank[13].

Amplification of *AlkB* gene

The polymerase chain reaction (PCR) was performed to amplify the *AlkB* of the isolated strains as shown in (Table 1) using the primes set supported with restriction sites of *Eco*RI in the forward primer plus (CAG) as flanking region and *Hind*III in the reverse primer plus (GAC) as flanking region, (Table 2) shows the PCR program details, the annealing temperature was fixed by gradient PCR.

Construction protocol of the recombinant (PET-21a (+)-*AlkB*/BL21 (DE3) *E. coli*)

The amplification of *alkB* gene was performed using the primers supported with the RE sites of *Eco*RI (G↓AATTC) and *Hind*III (A↓AGCTT)

(Bioneer, Korea) as shown in (Table 1). QIAGEN gel extraction Kit was used to purify the amplification product of PCR, and the PET-21a(+) (Novagen) plasmid was used as a vector. PCR products and plasmids were restricted with the same two endonucleases; *Eco*RI and *Hind* III at 37°C, and then T4 DNA ligase enzymes (Bioneer, Korea) were used in ligation process following the standard protocol from Bioneer company. In brief, the vector and the insert were mixed at a ratio of 1:5. The mixture was incubated at 16°C for 10min After heat inactivation at 65°C for 10 min, the reaction was chilled on ice[14]. The recombinant plasmid (PET-21a(+)-*AlkB*) was transformed to BL21(DE3) competent *E. coli* (Biolabs, New England) using heat shook method, briefly, 5µl of the ligation products and 50µl of competent DH5α cells were mixed and incubated for 30 min on ice, and subsequently heat shocked at 42°C for 10 sec and then placed back on ice. SOC medium (950µl) was added, and the transformed cells were incubated at 37°C for 60 min with agitation. After incubation, cells were pelleted and resuspended in 100µl SOC medium, which was then spread on LB agar plates containing ampicillin (100µg/ml). The plates were incubated overnight at 37°C 15, and then the PCR colony strategy was performed to recognise the recombinant bacterial cell using the T7 promoter and T7 terminator primers (Novagen) to amplify AlkB gene (Table 3).

Expression of the *AlkB* gene in PET-21a(+)-*AlkB*/ BL21(DE3) *E. coli*

According to the modified procedure of the article[17], IPTG (100µl/100ml) was used as an inducer and added when PET-21a(+)-*AlkB*/BL21(DE3) was at the logarithmic phase (OD600,0.4) in the LB broth medium with ampicillin(10µg ml^{-1}). The experiment was carried out in three replicates [2 flasks (experiment and control) contains 100 ml for each replicate] using shaker incubator (250 rpm, 37°C), the harvest process of the cells for each replicates were conducted at different times (2, 4 and 6 hours), the cell paste was pelleted by centrifugation (12000g, 10 min.) then kept at -20°C till next experiment. The precipitated cells were suspended in sterile ddH_2O and boiled for 5min to disrupt the cells. Bradford assay method was used to determine the concentration of *AlkB* protein in the supernatant [18].

SDS-polyacrylamide gel electrophoresis

IPTG was used as inducer for different periods (4h, 6h, 8h) as well as the control (without inducer) was used too. About 120 V as operation voltage for 10% resolving gels and 80V applied to 5% stacking gels was used to perform SDS-polyacrylamide gel electrophoresis[17,19]. In each lane 15µl of protein was loaded. Coomassie Brilliant Blue G-250 was used to stain the gel.

RESULTS AND DISCUSSION

Detection of the *AlkB* gene (amplification of the AlkB gene)

The oil degrading bacteria have been conducted to amplification using *AlkB* primer, the 1218 bp-band size for *Alkb* gene of *Pseudomonas aeruginosa* genomic DNA was detected on the agarose gel as shown in Fig. 1, there are two goals from the amplification process using these types of primers (table 2); the 1st goal was the detection of the gene while the 2nd one was to addition of *Eco*RI and *Hind* III restriction sites to the ends of gene for subsequent cloning protocol. Concerned gene sequence with terminal restriction sites was confirmed by the NCBI gene bank.

Construction protocol of PET-21a(+)-*AlkB* plasmid vector

In this study, the 1206 bps *AlkB* gene was inserted into the MCS of the PET-21a(+) plasmid. Fig. 4 shows the recombinant plasmid map and complete protocol designed by SnapGene software which downloaded from the official website[11]. The pET plasmid vectors are the most effective

Table 1. Primers set for amplification of *AlkB* gene supported with restriction sites of *Eco*RI and (CAG) as flanking region in the 5 end of the forward primer and *Hind*III and (GAC) as flanking region in the 5 end of reverse one

AlkB gene primers	Sequence	GC content (%)	PCR product (bp)	Reference
Forward	5'- cagGAATTCatgcttgagaaacacagagttc -3'	41%	1218	Current study
Reverse	5'- gacAAGCTTctacgatgctaccgcagagg -3'	60%		

system build up for the cloning and expression of recombinant proteins in *E. coli*. Target genes are inserted in pET plasmids and controlled by strong bacteriophage T7 transcription and (optionally) translation signals[20]. The name of the new recombinant plasmid vector was PET-21a(+)-*AlkB.*

The recombinant plasmid vector PET-21a(+)-*AlkB* was transformed into competent *E. coli* BL21(DE3) ,the ampicillin(10µg/ml) was used to select the positive transformants because this plasmid have Ampicillin resistance gene (amp.R) which enable only *E. coli* harboring PET-21a(+) to grow in the medium.

Table 2. PCR amplification program of *AlkB* gene

Stage	Temp.	Time	Cycles
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	1 min	35
Annealing	50°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	7 min	1

Colony PCR technique was used to confirm the positive transformants colony harboring the PET-21a(+)-*AlkB* vectors, moreover the forward T7 promoter primer and reverse T7 terminator primer was used in PCR procedure to confirm that coding region of this gene is also under the transcriptional and translational control of the TEF1 promoter and terminator. The amplified segment was about 1452bp which is equal to that of the predicated fragment as shown in Fig. 2, the PET-21a(+) plasmid includes *AlkB* gene was called PET-21a(+)-*AlkB* vectors.

The band size (1452bp) and the analysis of the amplified gene sequencing result by using NCBI BLAST database proved the colony to be the *AlkB* cloned recombinant *E. coli*, the constructed bacteria was called PET-21a(+)-*AlkB*/BL21(DE3) *E. coli*.

Fig. 3 shows two bands in the lane 2 as a result for digestion of the PET-21a(+)-*AlkB* with *Eco*RI/*Hind*III restriction enzymes ,the lower band was equal to the *AlkB* gene size and the upper

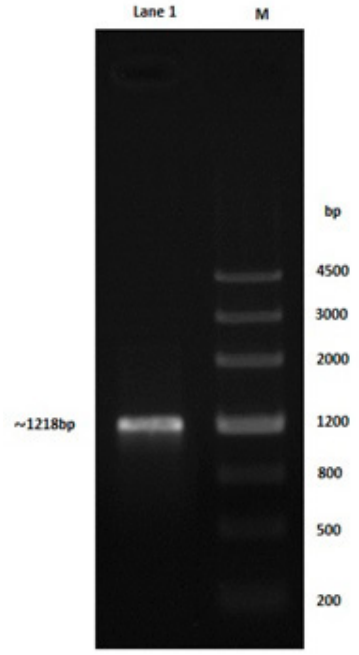

Fig. 1. Right lane; 4500bp ladder, left lane; ~1218 bp *AlkB* gene amplified with primers supported with *Eco*RI and *Hind*III restriction sites

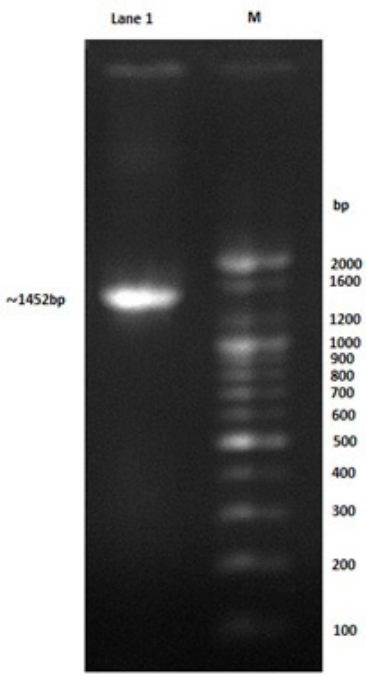

Fig. 2. Gel electrophoresis of colony PCR product using forward T7 promoter primer and reverse T7 terminator primer: 100bp marker, lane1: 1452bp amplified fragment

band was equal to the PET-21a(+) size while the lane 1 show about four bands as a result of the gel electrophoresis of the native uncut PET-21a(+) plasmid ,these bands related to supercoiled, relaxed, nicked respectively and that agree with[21].

Expression of *AlkB* gene in PET-21a(+)-*AlkB*/ BL21(DE3) *E. coli*

Bradford assay demonstrate that the crude protein was produced in each period of experiment (2h,4h,6h,) using IPTG as inducer (Fig. 5). protein concentration increased gradually with the time, but highest concentrations were detected at induction, so that there are significant differences in total protein production between the induce PET-21a(+)-*AlkB*/BL21(DE3) *E. coli* and the non-induced bacteria due to the high levels of *AlkB* protein produced as a result for IPTG induction which then confirmed by SDS page.

SDS-polyacrylamide gel electrophoresis

Approximately 46-48 KD of *AlkB* protein bands on the polyacrylamide gel were appear in all the times of the experiment using IPTG as inducer, the band size was closely related to the calculated value from the sequence of amino acids and agreed with[22]. The results shows, there is no significant differences between the different induction periods of IPTG (Fig. 6), which indicates that the minimum time of induction applied in the study was sufficient for bacteria to induce and to produce the recombinant protein, as well as the continuation of protein production with induction time indicates that the protein produced was not have toxic affection to the recombinant cells.

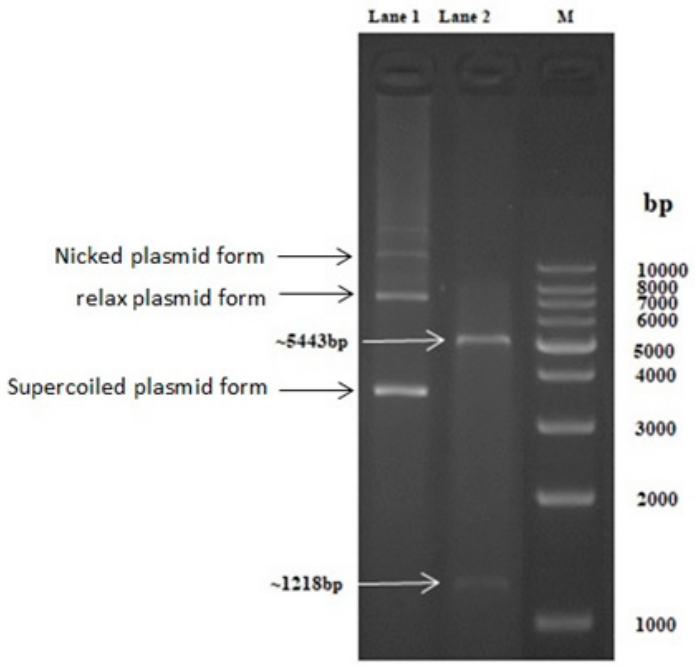

Fig. 3. Lane 1: Native PET-21a(+) plasmid, lane 2: linear PET-21a(+)-*AlkB* digested with *Eco*RI/*Hind*III, lane 3:1kb DNA ladder

Table 3. T7 promoter and T7 terminator primers for PCR colony to detection of *AlkB* gene inserted in BL21 (DE3) competent *E. coli*

T7 primers set	Sequence	GC content (%)	PCR product (bp)	Reference
Forward T7 Promoter	5-TAATACGACTCACTATAGGG-3	40%	1452	16
Reverse T7 Terminator	5-GCTAGTTATTGCTCAGCGG-3	52%		

In conclusion, using of competent *E. coli* (BL21(DE3) and PET21a(+) system for expression of alkane hydroxylase-1 (*ALKB1*) suggestion a feasible and appropriate strategy for a wide conception of the catalytic mechanism of this interesting non-heme oxygenases. The newly synthesized alcohols don't convert to subsequent aldehyde and fatty acid because the absence of alcohol oxidation enzymes in *E. coli*, therefore the downstream enzymes of the native strains is necessary to the function of recombinant *alkB1*.

The availability of different hydroxylases and catalyst enzymes (rubredoxin and rubredoxin reductase) will lead to enhancement of decontamination systems for more friendly environment studies which focus on bioremediation field. Moreover, there are many applications of biotransformation of common starting materials into fine chemical production related to this enzyme system.

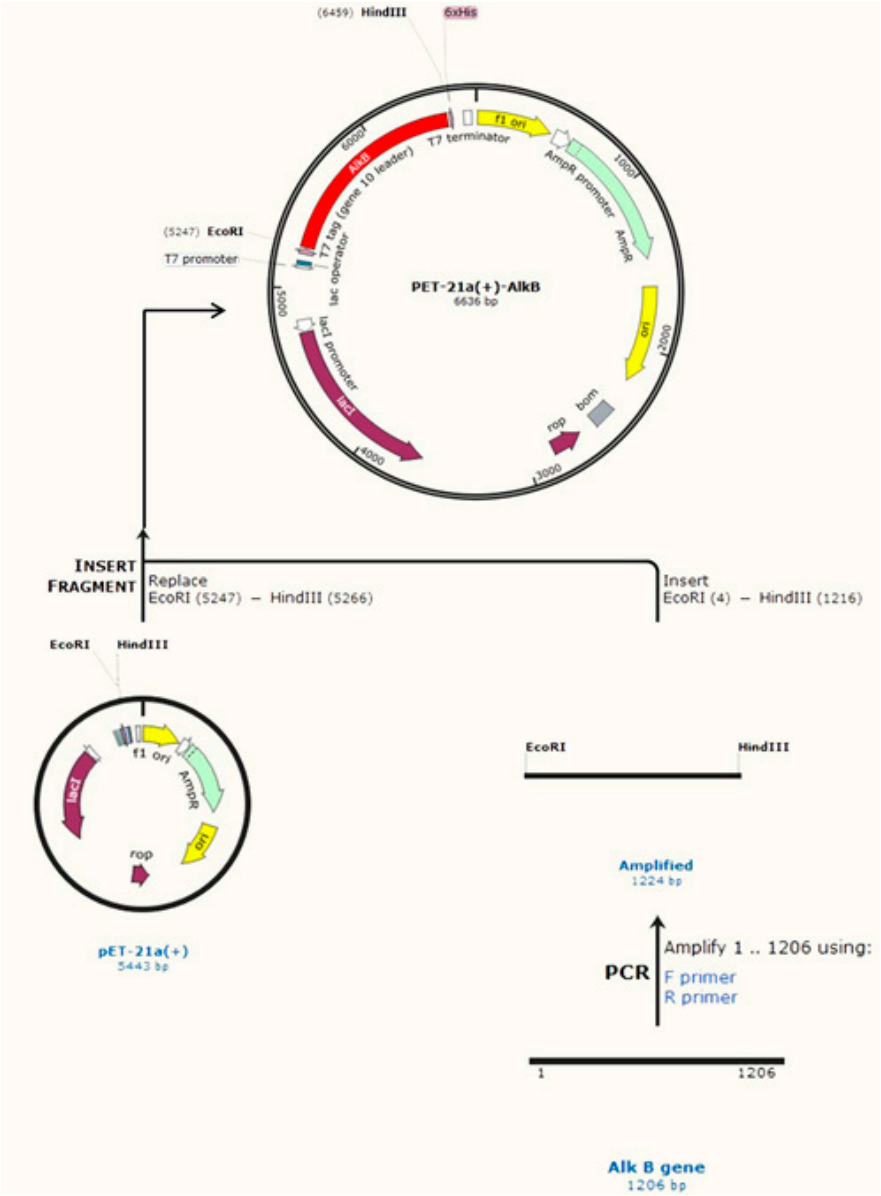

Fig. 4. Construction protocol of PET-21a(+)-AlkB plasmid vector

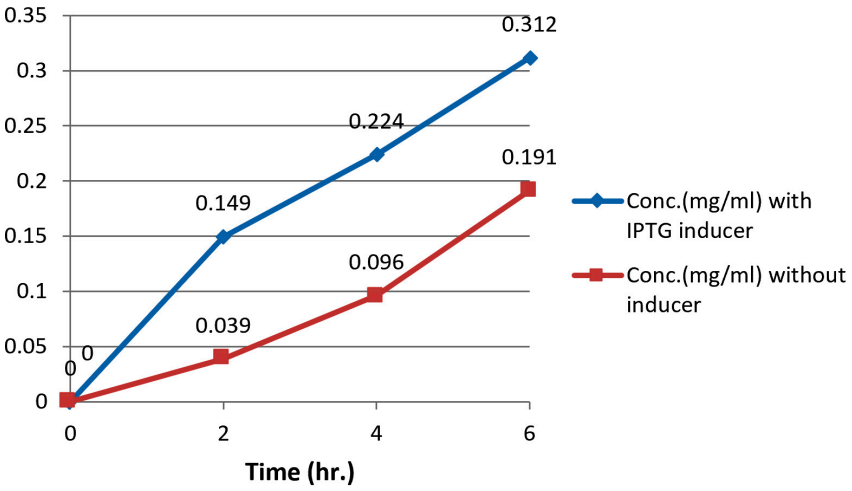

Fig. 5. Protein conc. at (2,4,6) hr. using IPTG as inducer

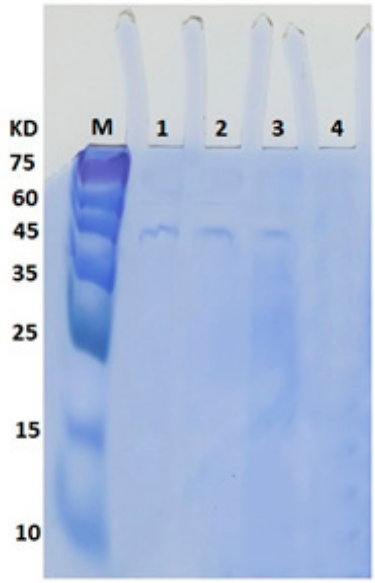

Fig. 6. Protein gel of *AlkB* with different induction times of IPTG. M: protein marker, 1: IPTG(4h),2: IPTG(6h), 3:IPTG(8h), 4:without inducer.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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None.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript

ETHICS STATEMENT

Not applicable.

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