

Molecular Identification of *Pseudomonas* Strains with Polyethylene Degradation Ability from Soil and Cloning of *alkB* Gene

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Abstract

Today, high-density plastics such as polyethylene are recognized as one of the major pollutants of the environment by the Global Environment Organization. This study has been designed to isolate bacterial strains of *Pseudomonas* with polyethylene degradation power and to clone the *alk-B* gene to be used in the future to expedite the biological degradation of plastic waste. To isolate the polyethylene degrading bacteria, two culture methods were used, direct culture and pre-enriched medium. Bacteria were cultured in MSM medium, and the best strains that could decrease the plastic weight were selected for PCR of the *alk-B* gene. After phylogenetic analysis of PCR sequences and TA cloning, the *alk-B* gene was introduced into the *E. coli* XL1-Blue by PTG19-T vector. After confirming the presence of the gene in the cloning vector by colony PCR, the samples with recombinant plasmids were sequenced. The percentage of polyethylene degradation by *Pseudomonas* strains was 7.2% at most and 4.5% on average. All the polyethylene-degrading strains were isolated and all had the *alk-B* gene. Also, the data showed that with TA cloning can colon *alk-B* in the vector. The data of the present study show that by optimizing the polyethylene-degrading bacteria isolated from the soil could greatly accelerate the process of biodegradation of polyethylene. It seems by developing genetic methods based on the genomes of bacteria, especially the strain of *Pseudomonas aeruginosa*, it is possible to devise methods by which all types of polyethylene to be degraded at a shorter time.

Keywords: Polyethylene, *Pseudomonas aeruginosa*, *alk-B* gene, gene cloning, alkane hydroxylase

INTRODUCTION

Today, synthetic polymers, commonly named plastics, play a very important role in the production of various industries. The unique properties of plastics and their alloys make them a very suitable alternative for metals, wood, and glasses in various applications. On the other hand, the vast applications and the increasing production of these materials and their insolubility have caused major environmental problems. Plastics are among the most widespread pollutants of the environment by the Global Environment Organization ^[1-3].

About 75% of the synthetic plastics used in the world consist of polyethylene, polypropylene, polystyrene, and PVC. Therefore, the environmental problems today are mainly due to the use of these types of plastics. Polyethylene is used intensively in most industries, including automotive, home appliances, packaging, health, and medicine, so that the packaging industry has the highest share in the use of this synthetic polymer, and consequently in contamination of the environment ^[4].

Large molecules within the polymeric structure of polyethylene prevent the natural decomposition of these materials. On the other hand, it has been proved that

biological methods are one of the most important and least costly procedures for eliminating biological contamination. Hence, in recent years, there has been a lot of research about the function of bacteria in the decomposition of non-recyclable and environmental pollutants. Most bacteria that are capable of decomposing such substances are commonly called extremophiles. Some of these bacteria have been found in environments where no carbon source other than polyethylene has been found. Therefore, it is concluded that

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due to the occurrence of different mutations and natural selection, the population of bacteria with polyethylene degrading enzymes would be more than the others [5, 6].

The decomposition of various plastics in natural conditions, depending on their type and structure, lasts between 100 to 700 years and more. Biological methods are among the most important procedures for eliminating biological contamination, especially in the field of chemical pollution. On the other hand, the study of the ability of bacteria to decompose non-recyclable and pollutant materials has been one of the most important studies in recent years [6, 7].

Studies in different parts of the world have shown that among the genus *Pseudomonas*, two species of *Pseudomonas putida* and *Pseudomonas aeruginosa* have the highest degree of decomposition of polyethylene plastics in a month. The results show that out of 169 isolates identified from 64 soil samples, *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* had the highest LDPE degradation [7, 8]. On the other hand, various studies have shown that the *alk-B* gene by alkane hydroxylase production can break down alkane and polyethylene chains [8-10].

Objectives: This study has been designed to isolate bacterial strains of *Pseudomonas* with polyethylene degradation power, and to clone the *alk-B* gene to be used in the future to expedite the biological degradation of plastic waste.

MATERIALS AND METHODS:

In this study, samples from soil and deposited plastics from 10 units of the disposal of waste material in Tehran and Kerman municipality were collected from October 2017 to January 2018. Samples were collected with sterile spatulas from surface layers and depths of 15 and 20 cm, especially those areas where the moisture content of the soil was preserved. The samples were sent to the laboratory in a cold condition and kept at 2 to 5 °C until use.

Culturing and isolating polyethylene degrading bacteria:

To isolate the polyethylene degrading bacteria, two methods of direct culture and pre-enriched culture were used. In direct culture, for each sample, 10 g of soil was added to 90 ml of Ringer solution. After 10 minutes on the shaker at 150 rpm, 10^{-1} to 10^{-5} dilutions were made from the suspension, and then one ml of each dilution was cultured on the surface of Petri dishes containing trypticase soy agar (TSA) and cetrimide agar and then kept 10 days at 30 °C. In the pre-enrichment culture method, 5 g of soil was added to 95 ml of Malachite Green Broth medium and incubated at 37 °C for 48 hours. When turbidity was observed in the medium, the contents were cultured linearly (streak method) on the solid surface of cetrimide agar and then grown at 37 °C for 24-48 hr. To purify target bacteria by these two methods, oxidase solution was added on top of the medium and those colonies that stained purple for less than 10 seconds was quickly removed

by a needle and grown on the solid TSA medium and then cultured at 30 °C for 24-48 °C and then observed in terms of growth and purity. Gram-negative and oxidase-positive bacteria suspected to be *pseudomonas* were kept in a slant TSA medium at 2-5 °C for short-term storage and long-term storage, they were kept at -20 °C in tubes containing an equal ratio of glycerol and liquid TSB medium, as described by Careaga *et al.* [11].

Screening of polyethylene-degrading strains

After preparing the polyethylene granules (2 x 2 cm), they were placed in xylol and in 100 °C water bath for 15 minutes to be dissolved, then ethanol was added and the content dried at 60 °C oven. For initial screening, 0.1% of the weight of the polyethylene powder was added to the minimal salt medium (MSM) and sterilized By autoclaving then poured into the Petri dishes. Then, strains isolated from the soil and control strains were spot cultured and were incubated at 30 °C for 7 days and the presence of halo around the colony was investigated. Finally, *Pseudomonas* strains isolated from the soil and control *Pseudomonas* strain, in terms of percentage of plastic weight loss, were compared and the best strain was selected for molecular analyzes. The following formula was used to check the percentage of plastic weight loss [12].

$$\frac{\text{Primary Weight} - \text{Lost Weight}}{\text{Initial Weight}} \times 100$$

Genomic studies

Out of 60 isolated strains of *Pseudomonas*, two strains were selected for molecular analyses based on the highest percentage of polyethylene degradation. The DNA extraction was performed by a special kit for gram-negative bacteria (from Iranian Genetic Center Reserves). Specific primers of the *alkB* gene include F5'-TCGAGCACATCCGCGGCCACCA-3 and R5'-CCGTAGTGCTCGACGTAGTT-3 and the product had a length of 330 bp derived from reference 14. After the blast in the National Center for Biotechnology Information (NCBI) site, it was ordered by Aryan Teb Company (Iran, Tehran). The PCR reaction (20 µL) was performed 30 cycles as follow: 30 seconds at 94 °C for initial denaturation, 30 seconds at 61 °C annealing, and 45 seconds at 72 °C for an extension. PCR products were electrophoresed on 1% agarose in the presence of negative control (Blank) and positive control (330 bp *alkB* gene) and then were sequenced and analyzed by Bio-Edit software. The forward and reverse sequences were analyzed by DNA Baser assembler software and one sequence was aligned from '5 to 3'. The amplified sequences were compared with the isolates registered in NCBI and each sequence was searched separately in the GenBank.

Phylogenetic analysis:

The *alkB* homology was examined by aligning the gene sequences with those of the representative members of the selected genera with the CLUSTAL W program, and then the phylogenetic trees were mapped from the neighbor-joining method using the MEGA7 programs. The bootstrap was

selected based on 1000 replications for the MEGA7 program. Excel was used to plot the histogram based on the nucleotide distances.

Cloning *alkB* gene:

After confirming the sequences from the purified bacteria, cloning of the gene was performed by TA cloning using a specific kit (Sinaclon, Iran) as follow: 2 µl of PTG19-T vector, 1 µl attachment buffer (X10), 1.5 µL purified piece, 0.6 U T4 DNA ligase in final volume of 10 µl were mixed. The cloning reaction was carried out according to the manufacturer's instructions. The insertion of the product into the susceptible cells of the *XL1-Blue* strain of *E. coli* was performed using the heat shock method. After adding 800 µl of the LB medium without antibiotics and incubating at 37 °C for one hour, 200 µl of transformed bacteria were spread on Petri dish containing 40 µg/ml ampicillin, X-Gal and IPTG and then incubated at 37 °C for 14 hours. To select the colonies with the transferred gene, screening of white and blue colonies was performed, and the white colonies were isolated and the recombinant plasmid was extracted. The PCR of the encoding region of the *alkB* gene together with the recombinant PTG19-T vector were sent for sequencing (Fanavaran Gene Co. Iran, Tehran). The sequences were analyzed using the DNAMAN software and the sequence available at GenBank as reported by Bai *et al.*

RESULTS:

The rate of polyethylene decomposition in the culture medium

The data show that in all the culture media (except control medium), all types of polyethylene in the culture had less weights than the start of the experiment (Table 1). The data also show that the average percentage of polyethylene breakdown by *Pseudomonas* strains was 4.5%.

Duplication and sequencing of the *alkB* gene

Electrophoresis of the PCR products of the *alkB* gene encoding region on the 1% agarose gel showed that the specific fragments with a length of 330 bp were well replicated (**Fig. 1**). The presence of a specific band in the columns of samples indicates the specificity of primers designed to connect to the *alkB* gene encoding region. The presence of specific components and the absence of bands in the negative control column showed the accuracy of the reaction, therefore, the PCR products were sequenced.

Phylogenetic analysis results based on DNA sequences

The phylogenetic tree represents the relationship between the isolated 16SrRNA sequences and the reference sequences in GenBank. The bar indicates sequence divergence and the numbers in the branching node represent the bootstrap value (%). *Pseudoxanthomas* was used as outgroup. Two unknown samples of the present study, S10, S18, were identified as *Pseudomonas aeruginosa* (**Fig. 2**).

TA cloning results and sequences of the *alkB* gene

The results of the electrophoresis of reproductive cells of the target gene are depicted in the strains mentioned in **Fig. 3**. After cloning, the obtained products were sequenced and the sequences were verified to confirm amplification and clone of the *alkB* gene.

DISCUSSION:

Studies have shown that in an environment with a very low concentration of nutrients or carbon sources, during randomized mutations and based on natural selection, some bacteria could have the ability to secrete enzymes that can lead to the breakdown of polyethylene into ethylene. Therefore, bacteria that use polyethylene as a carbon source are more dominant. Many scientists have tried to use this feature to break down plastic wastes. In this report, we aimed to accelerate the biological degradation of plastic waste by identification and isolation of *Pseudomonas* strains with degradation power of polyethylene components and to clone the *alkB* gene ^[10].

Our data show that the percentage of polyethylene degradation by *Pseudomonas* strains was 7.2% at most and 4.5% on average. Also, all of the purified bacteria had an *alkB* gene.

Hussein *et al.* (2015) by isolating, screening and identifying the LDPE decomposing bacteria from the contaminated soil with plastic wastes, demonstrated that out of 169 isolated from 64 soil samples, *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* had the highest LDPE biodegradation ^[8]. In another study, Nanda *et al.* (2010) showed that among polyethylene degrading strains such as *Bacillus*, *Pseudomonas*, and *Rhodococcus*, the *Pseudomonas* strains have the highest ability to decompose polyethylene ^[9]. Singh *et al.* (2016) have measured the amount of polyethylene degradation by measuring the amount of lost weight in 40 days. Out of 15 bacteria isolated from different parts of soils, only 3 strains of *Staphylococcus*, *Pseudomonas*, and *Bacillus* had the highest ability to decompose polyethylene ^[13]. Our results correspond with the studies by Singh, Nanda, and Hussein, however, none of them cloned the *alkB* gene.

We showed that at most 7.2% of polyethylene weight was degraded by *Pseudomonas* species, but Nanda *et al.* ^[9], showed that after 3 weeks of incubation, the highest percentage of polyethylene decomposition by *Pseudomonas* was equal to 40%. Vatseldutt *et al.* showed that the percentage of polyethylene degradation by *Pseudomonas* was about 11% ^[11]. Our results also in agreement with Roy *et al.* that the highest polyethylene degradation was 8.4% by *Bacillus cereus* ^[12]. The difference in the percentage of polyethylene degradation can be attributed to the different strains of isolated bacteria and also to the expression of different polyethylene degrading genes.

Van Beilen *et al.* cloned the *alkB* gene of *P. putida* strains in *E. coli* that were isolated from different environments such as oil-contaminated soil and groundwater. The results show that the alkane hydroxylase gene of these bacteria could be expressed in the *E. coli* host [6]. These data are in agreement with the results obtained in the present study.

Also, Hara *et al.* cloned the *alk-B1* and *alk-B2* genes of *P. putida* bacteria in *Alcanivorax borkumensis* and subsequently destroyed these two genes in the original strain and studied the growth characteristics of distorted mutants [14]. The results indicated that the alkane hydroxylase is responsible for the decomposition of alkanes. This study confirmed that by expression of alkane hydroxylase gene using cloning in the host can determine its role in the decomposition of alkanes and their products, such as polyethylene.

These data are in agreement with the results obtained in the present study.

CONCLUSION:

Our results indicate that by using microorganisms isolated from soil, the process of decomposition of plastics is significantly accelerated. Thus, by promoting genetic methods, based on the genome of bacteria, especially the strain of *Pseudomonas aeruginosa*, all types of polyethylene that are used commonly could be degraded at much less time

than the normal situation. Also, by cloning the *alkB* gene in *E. coli* *XL1 Blue* host, we have succeeded to increase the expression of the desired gene, and as a result the production of alkane hydroxylase enzyme, which accelerates the process of plastics decomposition.

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Table 1. The numerical values of polyethylene weight changes in the medium

Culture Medium	Polyethylene weight after bacterial growth	Lost weight	% lost weight
1	0.168	0.007	4.1
2	0.180	0.007	3.9
3	0.159	0.006	3.6
4	0.179	0.004	2.2
5	0.180	0.013	7.2
6	0.175	0.008	4.5
7	0.174	0.009	5.1
8	0.168	0.009	5.3
9 (control)	0.164	0.000	0.0

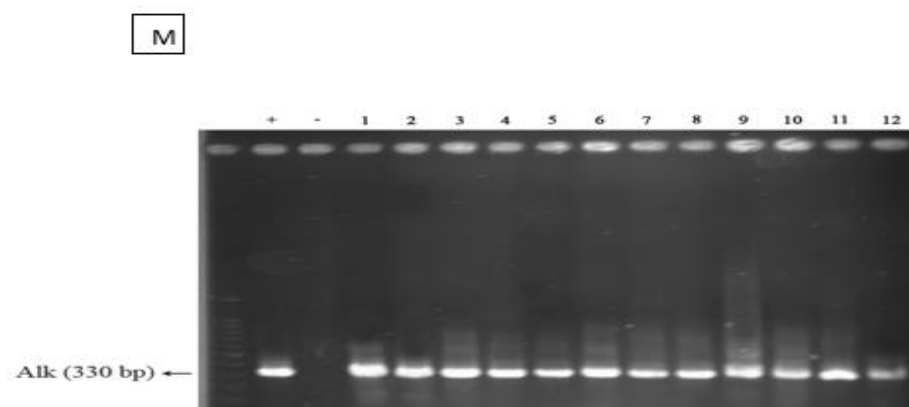


Figure 1: Frequency of the *alkB* gene in *Pseudomonas* strains. From the left, lane M: marker 100bp, +: positive control, -: negative control and lanes 1-12: PCR products of the *alkB* genes of *Pseudomonas* strains separated from the soil.

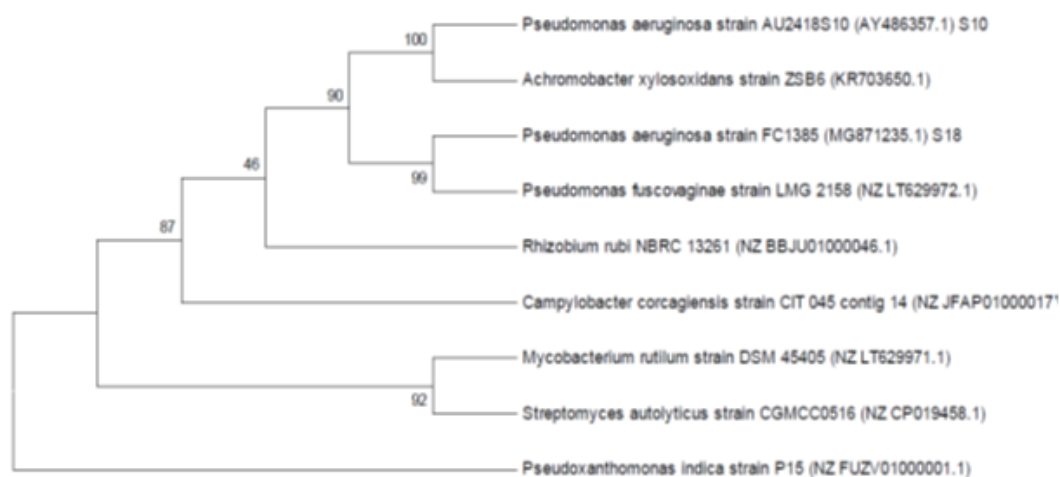


Figure 2: Phylogenetic tree of *Pseudomonas aeruginosa* based on 16S rRNA gene sequence analysis.

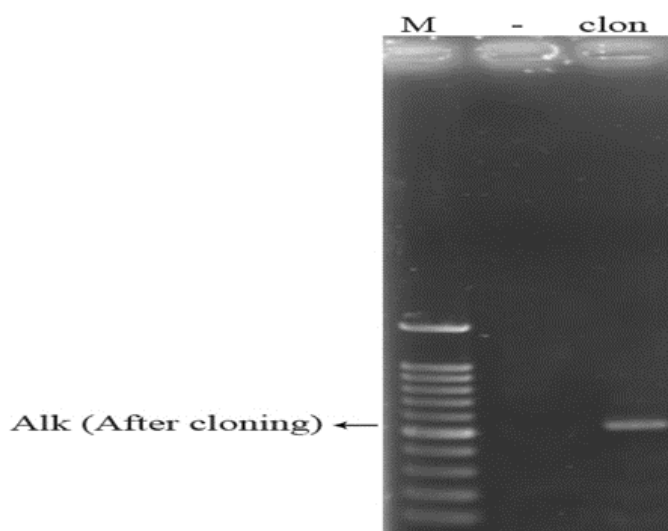


Figure 3: Electrophoresis of PCR Products. The *alkB* gene was amplified in *XL1-Blue E. coli*. PCR amplification of the *alkB* gene (After cloning 550 bp).

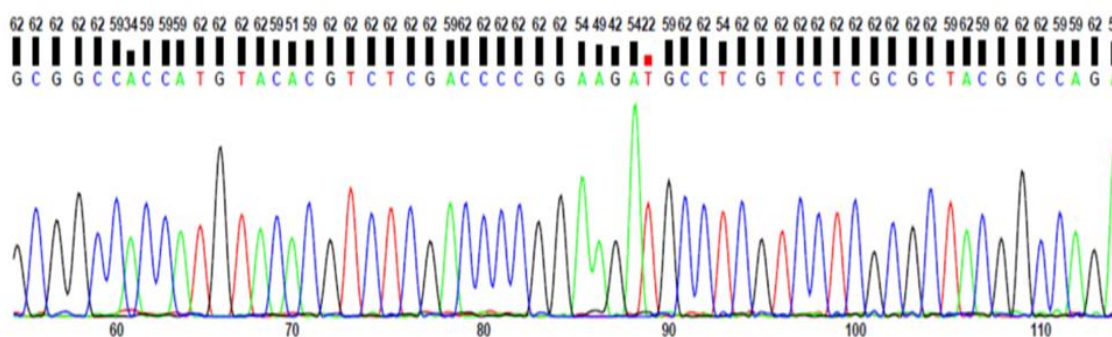


Figure 4: Part of nucleotide sequences of the *alkB* gene from *Pseudomonas aeruginosa*.

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