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Cloning and Partial Sequencing of *phac1* and *phac2* Genes Encoding Poly (3-hydroxyalkanoate) Synthases from *Pseudomonas aeroginusa* PTCC 1310

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Abstract: Poly (hydroxy alkanoic acid) (PHA) polymers are synthesized by numerous microorganisms. These compounds have similar properties to synthetic plastics with excellent biodegradability. Here we report identification and cloning of $phaC_1$ and $phaC_2$ genes of type II PHA synthase gene from the Iranian isolate of *P. aeroginosa* PTCC 1310 bacterium. The sequences of both genes were isolated using PCR amplification with specific primers and cloned into pTZ57R cloning vectors as pTZPHAC1 and pTZPHAC2. The correct sequence of the cloned genes was confirmed by restriction digestion followed by partial sequencing. The vectors could be used for future sub-cloning and expression analysis purposes.

Key words: Cloning, poly (hydroxy alkanoic acid), *P. aeroginosa* PTCC 1310

INTRODUCTION

During the past years the use of petroleum based plastics has complicated the problem of disposal of solid wastes (Salehizade and Van Loosdrecht, 2004). In order to solve the problem, world-wide programs for efficient management of used-plastic materials such as recycling, source reduction and incineration have been started. Another solution to reduce plastic wastes is the use of biodegradable plastics. Among the different candidates for biodegradable plastics, the poly(hydroxy alkanoic acid) (PHA) polymers have been the focus of extensive research and commercial interest. This is mainly due to their similar properties to synthetic plastics and their potential use as biodegradable thermoplastics (Agus *et al*., 2006a; Steinbüchel, 2005; Braunegg *et al*., 1998).

PHAs are natural storage compounds, which are synthesized and intracellularly deposited by numerous microorganisms when in the presence of excessive carbon source, encounter nutritional deficiencies like lack of nitrogen, phosphorus, sulfur, oxygen or magnesium (Agus *et al*., 2006a; Solaiman and Ashby, 2005; Kessler and Witholt, 1999). PHAs can be classified into three groups depending on the number of carbon atoms in the monomer units, I) Short-Chain-Length (SCL) PHAs, which consist of 3-5 carbon atoms; ii) medium-chain-length, which consist of 6-14 carbon atoms; iii) scl-co-mcl PHA with repeat unit monomers containing 3 to 14 carbon atoms (Rehm, 2003; Solaiman and Ashby, 2005; Ramsay and Ramsay, 1999). PHA synthases (PHASs) are enzymes, which are responsible for the polymerization of hydroxyacyl-CoA monomers into the PHA polymers. Based on their structural properties and substrate specificity, these enzymes are classified into four classes I, II, III and IV. The gene clusters that code for the synthesis of each class of the PHAs have different genetic organizations (Agus *et al*., 2006b; Rehm, 2003; Sudesh *et al*., 2000). To date, more than 250 different microorganisms are known to synthesize and accumulate various PHAs (Salehizade and Van Loosdrecht, 2004).

Most *Pseudomonas* strains are able to accumulate polyhydroxyalkanoic acids as medium-chain-length PHAs. These strains belong to rRNA homology group I containing γ subdivision *Proteobacteria* and produce class II PHA synthases (Qi *et al*., 1997; Hein *et al*., 2002). The enzymes are encoded by type II *pha* loci, which consists of three genes namely $phaC_1$, $phaC_2$ and *phaZ*. The *phaZ* gene is located between $phaC_1$ and $phaC_2$ and encodes for PHA depolymerase (Hoffmann and Rehm, 2004; Solaiman, 2000; Zhang *et al*., 2001; Brandl *et al*., 1988).

Several isolates of *Pseudomonas aeroginosa* (e.g., PTCC 1310 and PTCC 1740) have been previously reported from Iranian resources. Molecular characterization of these isolates using a PCR-based identification method (Solaiman *et al*., 2000) demonstrated that only the PTCC 1310 contains type II *pha* synthase

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genes (Abedi *et al.*, 2004). To date there is no report on the cloning of type II PHA synthase gene from Iranian isolate, *Pseudomonas aeroginos* PTCC 1310. In this study $phaC_1$ and $phaC_2$ genes of type II pha synthase gene from *P. aeroginosa* PTCC 1310 were isolated and cloned into cloning vectors and the sequence of the genes was confirmed by partial sequencing. The vectors could be used for future sub-cloning and expression analysis purposes.

MATERIALS AND METHODS

Bacterial strains, plasmid and growth conditions: *Pseudomonas aeroginosa* PTCC 1310 was obtained from Persian type culture collection (PTCC), Tehran, IR Iran and was cultivated in nutrient broth at 30°C. *Escherichia coli* XL1 blue was used as competent cell and was cultivated at 37°C in Luria-Bertani (LB) (1% w/v tryptone; 0.5% w/v yeast extract; 0.5% w/v NaCl) medium. In order to maintain the stability of the plasmids ampicilline was added to the medium at a final concentration of 100 μg mL^{-1}. pTZ57R vector included in TA cloning system was purchased from Fermentas, Germany.

Genomic DNA extraction, PCR amplification and cloning: Genomic DNA from *Pseudomonas aeroginosa* PTCC 1310 was isolated using High Pure PCR template preparation kit (Roche, Germany) following the manufacturer's recommendations. The sequences of *phaC1* and *phaC2* genes were isolated by PCR amplification using specific primers. The primers were designed against the available sequences of *phaC1* and *phaC2* genes of *Pseudomonas aeroginosa* PAO1 (Accession number AE004919, AE004091) using *WDNASIS* 2.6 software (Hitachi Software Eng. Co, Ltd, Japan). Primer pairs for PCR amplification of *phaC1* (N-terminus: 5′-dGCC GAT GAG TCA GAA GAA C-3′; C-terminus: 5′-dCTT TTC ATC GTT CAT GCA C-3′) and *phaC2* (N-terminus: 5′-dGTC CAT GCG AGA AAA GCA G-3′; C-terminus: 5′-dATC TTT CAG CGT ATA TG-3′) genes were obtained form Fazapajoh Company (Tehran, IR Iran).

The PCR reaction cycles consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C and a final extension of 10 min at 72°C. The PCR reaction was performed in 50 μL volume containing 1 unit of Taq DNA polymerase (Biotools, Spain) and 20 pmol of each primer in a PCR reaction buffer provided with Taq DNA polymerase using Bio-Rad thermal cycler (Bio-Rad, USA).

The PCR products were analyzed using agarose gel electrophoresis. Then the DNA fragments were extracted using QIA Quick Gel extraction kit (Qiagen, Germany) and cloned into pTZ57R cloning vector of the T/A clone™ system (Fermentas, Germany). Plasmid preparation was performed using a standard alkaline lysis procedure as reported previously (Sambrook *et al.*, 2001).

RESULTS AND DISCUSSION

Genomic identification and PCR amplification of *phaC1* and *phaC2* genes: In this study *phaC1* and *phaC2* sequences of type II poly (hydroxyalkanoic acid) (PHA) synthase gene cluster of *Pseudomonas aeroginosa* were isolated and cloned. This cluster contains three genes namely *phaC1*, *phaZ* and *phaC2* genes. As shown in Fig. 1, both *phaC1* and *phaC2* sequences have approximately similar sizes. In order to isolate and amplify the *phaC1* and *phaC2* gene sequences, two pairs of primers were designed against the flanking sequences of the genes. The primers were used to amplify the genes from the genomic DNA, which was extracted from the Iranian isolate of *aeroginosa* PTCC1310. Since the genomic sequence of this Iranian isolate is not known yet, the genomic sequence of the known isolate of *Pseudomonas aeroginosa* PAO1, with accession number AE004919 and AE004091, was used as the template sequence for designing the primers. Following preparation of the primers, the PCR condition was optimized and the best annealing temperature was determined. As shown in Fig. 2, amplification of the PHA genomic cluster using both pair of primers, resulted in two approximately similar bands of about 1700 bp long (lane a and b). The size of the bands correlates with the size of the genomic length of the *phaC1* and *phaC2* genes (Fig. 1).

In order to confirm that the amplified fragments were indeed *phaC1* and *phaC2* gene sequences, a restriction mapping analysis for the genes based on the restriction map of the original genomic sequence of

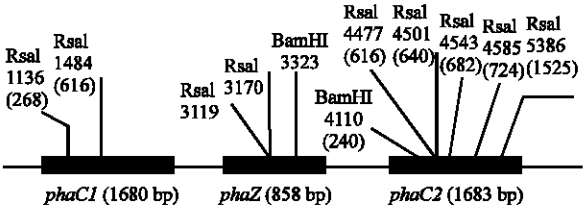

Fig. 1: A schematic representation of the organization of *P. aeroginosa pha* locus. The locus contains *phaC1*, PHA synthase 1 gene; *phaZ*, PHA depolymerase gene; *phaC2*, PHA synthase 2 gene. The length of the genes (base pair, bp), their genomic positions and some major restriction sites are also shown

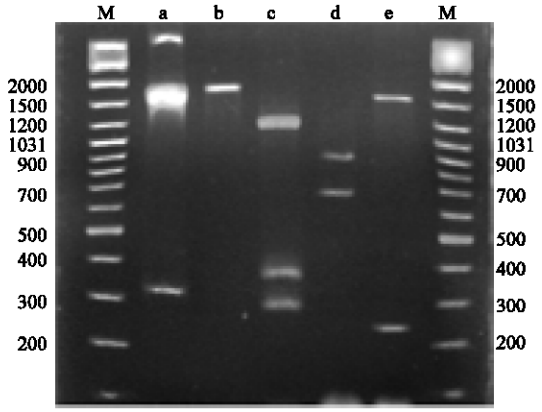

Fig. 2: PCR amplification and characterization of *phaC1* and *phaC2* gene sequences. The *phaC1* and *phaC2* gene sequences were PCR amplified, lanes a and b. The sequences were characterizes using *Rsa*I, lanes c and d. The sequence of *phaC2* gene was further analyzed using *Bam*HI. M represents the DNA size marker

Pseudomonas aeroginosa PAO1 was performed. One of the diagnostic restriction sites in both genes was *RsaI*. This enzyme recognizes two sites at nucleotides 1136 and 1484 in *phaC1* gene (It should be noted that the start codon in *phaC1* of *P. aeroginosa* PAO1 is located at nucleotide 868 in the given locus) and five sites at nucleotides 4477, 4501, 4543, 4585 and 5386 in *pahC2* gene (Fig. 1). Moreover, *Bam*HI recognizes one site at nucleotide 4101 in *phaC2*, but not in *phaC1* and used as a further diagnostic site (Fig. 1). As represented in Fig. 2, digestion of the *phaC1* PCR product with *RsaI* resulted in three bands of 268, 348 and 1064 bp (lane C). Digestion of *phaC2* PCR product with *RsaI* produced five fragments of about 24, 42, 152, 621 and 802 bp long (lane d). Note that only the latter two bands are seen on the figure presented. Moreover, digestion of *phaC2* PCR product with *Bam*HI produced two bands of about 245 and 1438 bp long (lane e). Therefore, based on the restriction mapping, it could be concluded that the amplified sequences were most likely belong to *phaC1* and *phaC2* genes.

Cloning of *phaC1* and *phaC2* genes sequences into pTZ57R vector: Following amplification and restriction mapping analysis of the sequences for *phaC1* and *phaC2* genes, the gene sequences were cloned into pTZ57R vector at the TA site. Figure 3 shows the map and multiple cloning site of pTZ57R cloning vector. In order to clone the *phaC1* and *phaC2* sequences, the PCR amplified fragments for both genes were extracted from agarose gel and ligated to the linearized pTZ57R plasmid. This cloning was performed with taking advantage of the overhang As at the 3' end of the PCR products, amplified using the Taq DNA polymerase enzyme. This could match the 5' overhang Ts in the linearized pTZ57R vector. Following ligation reaction, the *E. coli* XL1 blue bacterial competent cells were transformed by the recombinant plasmids containing *phaC1* and *phaC2* genes. The cells were cultured in the presence of ampiciline for selection and a number of the resulted colonies were picked and used in plasmid mini-preparations.

Following plasmid DNA preparation, restriction mapping analysis was performed to confirm the presence of the *phaC1* and *phaC2* DNA fragments. To quickly screen for the presence of the insert, the *Eco*RI site in the plasmid was used. This site is only present in the vector (Fig. 3), but not in the inserts and can be used as a diagnostic site to distinguish between the vectors with and without insert by size difference. The plasmids containing *phaC1* and *phaC2* genes were first digested with *Eco*RI and analyzed on agarose gel electrophoresis. As expected, a large band at about 4570 bp, which corresponds to the vector plus the insert, was observed for both plasmids. To further confirm the presence of the sequences for *phaC1* and *phaC2* genes and also to determine the orientation of the inserts in the pTZ57R plasmid, the recombinant plasmids were digested with different restriction endonucleases (Fig. 3). Plasmid containing *phaC1* gene (PTZPHAC1) was digested with *RsaI* endonuclease enzyme as a useful diagnostic enzyme. This enzyme recognizes two sites in *phaC1* and also two sites in pTZ57R plasmid (Fig. 3). Therefore, digestion of PTZPHAC1 plasmid in which the 5′ end of the inserts was near T7 promoter, could show bands with 348, 1066, 1074 and 2078 bp length (Fig. 4, lane a). Note that bands 1066 and 1074 appear at a similar position as single band.

Furthermore, plasmids extracted from bacterial colonies containing *phaC2* gene (PTZPHAC2) were digested with *Bam*HI restriction endonuclease that cuts the recombinant plasmid at two positions, one in the insert and the other in the plasmid (Fig. 3). Therefore, the PTZPHAC2 plasmids in which the 5′ end of the inserts was near T7 promoter could produce bands with about 4319 and 250 bp sizes. As shown in Figure 4, digestion of PTZPHAC2 plasmid with *Bam*HI gave two bands as expected (lane C). This not only confirmed the cloning of *phaC2* gene in pTZ57R plasmid, but demonstrated that the *phaC2* gene was cloned with right orientation in the plasmid.

The correct cloning of the *phaC1* and *phaC2* genes was further analyzed by sequencing the first half of the cloned sequence in PTZPHAC1 and PTZPHAC2 plasmids using general T7 sequencing primers. The primers were

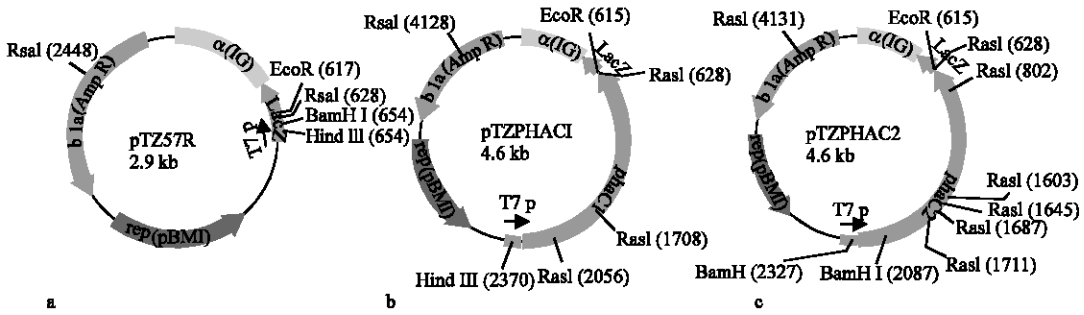

Fig. 3: Schematic representation of plasmids pTZ57R, pTZPHAC1 and pTZPHAC2. A brief restriction map for pTZ57R plasmid and the derived recombinant plasmids pTZPHAC1 and pTZPHAC2 are presented. The positions of *pha*C1 and *pha*C2 genes in the plasmids are shown

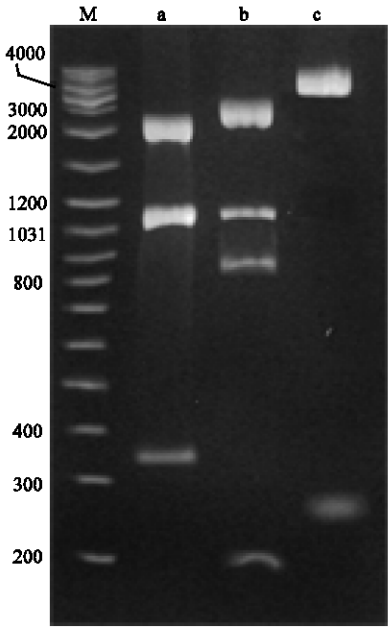

Fig. 4: Restriction digestion of pTZPHAC1 and pTZPHAC2 plasmids. The pTZPHAC1 and pTZPHAC2 plasmids were digested with *Rsa*I restriction endonuclease, lanes a and b, respectively. Lane c shows digestion of pTZPHAC2 plasmid with *Bam*HI. M represents the DNA size marker

complement to T7 promoter sequences of the pTZ57R plasmid, which was present at the upstream sequences of the *phaC1* and *phaC2* genes in both PTZPHAC1 and PTZPHAC2 plasmids (Fig. 3). The sequencing data were compared with the sequences for *phaC1* and *phaC2* genes in *P. aeroginosa* PAO1 using the BLAST program (NCBI, NIH). A diagram of the comparison of our sequence (Query) with the sequence of *phaC1* and *phaC2* from *P. aeroginosa* PAO1 in gene bank (Subject) is shown in Fig. 5A and 5B. Blast analysis revealed a high degree of similarity between the cloned genes and the genes of other *Pseudomonas* strains belonging to rRNA homology group I.

As shown in Fig. 5A, the data demonstrated that the first sequenced gene corresponded to *phaC1* gene and there was approximately a 99% similarity. In fact, the only difference was a C nucleotide in position 762 in the sequenced gene which is T in *P. aeroginosa* PAO1. This was a silent mutation which does not alter the coded amino acid (Serine).

We observed several base differences in *phaC2* sequence once compared with the corresponding sequence in the database (Fig. 5B). The first base difference was at site 90, in which a T nucleotide was located on cloned *phaC2* gene while a C nucleotide was located on *phaC2* gene of *P. aeroginosa* PAO1. This was a silent mutation not altering the coded amino acid (Aspartic acid). The second difference was at site 306, in which a G nucleotide was located on the cloned gene while an A nucleotide was located on *phaC2* gene of *P. aeroginosa* PAO1. This was also a silent mutation which did not change the coded amino acid (Lysine). The third difference was at site 430, in which a G nucleotide was located on *phaC2* cloned gene while a T nucleotide was located on *phaC2* gene of *P. aeroginosa* PAO1. This mutation altered the coded amino acid from Valine to Leucin.

It is not clear whether these differences are bona fide mutations or caused by Taq DNA polymerase because of its non-proof reading activity. Therefore, analysis of more clones and the repetition of the sequencing would clarify the nature of these nucleotide mismatches in the future experiments.

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Query  1     ATGAGTCAGAAGAACAATAACGAGCTTCCCAAGCAAGCCGCGGAAAACACGCTGAACCTG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  868   ATGAGTCAGAAGAACAATAACGAGCTTCCCAAGCAAGCCGCGGAAAACACGCTGAACCTG  927

Query  61    AATCCGGTGATCGGCATCCGGGGCAAGGACCTGCTCACCTCCGCGCGCATGGTCCTGCTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  928   AATCCGGTGATCGGCATCCGGGGCAAGGACCTGCTCACCTCCGCGCGCATGGTCCTGCTC  987

Query  121   CAGGCGGTGCGCCAGCCGCTGCACAGCGCCAGGCACGTGGCGCATTTCAGCCTGGAGCTG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  988   CAGGCGGTGCGCCAGCCGCTGCACAGCGCCAGGCACGTGGCGCATTTCAGCCTGGAGCTG  1047

Query  181   AAGAACGTCCTGCTCGGCCAGTCGGAGCTACGCCCAGGCGATGACGACCGACGCTTTTCC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1048  AAGAACGTCCTGCTCGGCCAGTCGGAGCTACGCCCAGGCGATGACGACCGACGCTTTTCC  1107

Query  241   GATCCGGCCTGGAGCCAGAATCCACTGTACAAGCGCTACATGCAGACCTACCTGGCCTGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1108  GATCCGGCCTGGAGCCAGAATCCACTGTACAAGCGCTACATGCAGACCTACCTGGCCTGG  1167

Query  301   CGCAAGGAGCTGCACAGCTGGATCAGCCACAGCGACCTGTCGCCGCAGGACATCAGTCGT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1168  CGCAAGGAGCTGCACAGCTGGATCAGCCACAGCGACCTGTCGCCGCAGGACATCAGTCGT  1227

Query  361   GGCCAGTTCGTCATCAACCTGCTGACCGAGGCGATGTCGCCGACCAACAGCCTGAGCAAC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1228  GGCCAGTTCGTCATCAACCTGCTGACCGAGGCGATGTCGCCGACCAACAGCCTGAGCAAC  1287

Query  421   CCGGCGGCGGTCAAGCGCTTCTTCGAGACCGGCGGCAAGAGCCTGCTGGACGGCCTCGGC  480
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Sbjct  1288  CCGGCGGCGGTCAAGCGCTTCTTCGAGACCGGCGGCAAGAGCCTGCTGGACGGCCTCGGC  1347

Query  481   CACCTGGCCAAGGACCTGGTGAACAACGGCGGGATGCCGAGCCAGGTGGACATGGACGCC  540
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Sbjct  1348  CACCTGGCCAAGGACCTGGTGAACAACGGCGGGATGCCGAGCCAGGTGGACATGGACGCC  1407

Query  541   TTCGAGGTGGGCAAGAACCTGGCCACCACCGAGGGCGCCGTGGTGTTCCGCAACGACGTG  600
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Sbjct  1408  TTCGAGGTGGGCAAGAACCTGGCCACCACCGAGGGCGCCGTGGTGTTCCGCAACGACGTG  1467

Query  601   CTGGAACTGATCCAGTACCGGCCGATCACCGAGTCGGTGCACGAACGCCCGCTGCTGGTG  660
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Sbjct  1468  CTGGAACTGATCCAGTACCGGCCGATCACCGAGTCGGTGCACGAACGCCCGCTGCTGGTG  1527

Query  661   GTGCCGCCGCAGATCAACAAGTTCTACGTCTTCGACCTGTCGCCGGACAAGAGCCTGGCG  720
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Sbjct  1528  GTGCCGCCGCAGATCAACAAGTTCTACGTCTTCGACCTGTCGCCGGACAAGAGCCTGGCG  1587

Query  721   CGCTTCTGCCTGCGCAACGGCGTGCAGACCTTCATCGTCAGCTGGCGCAACCCGACCAAG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1588  CGCTTCTGCCTGCGCAACGGCGTGCAGACCTTCATCGTCAGTTGGCGCAACCCGACCAAG  1647

Query  781   TCGCAGCGCGAATGGGGCCTGACCACCTATATC  813
             |||||||||||||||||||||||||||||||||
Sbjct  1648  TCGCAGCGCGAATGGGGCCTGACCACCTATATC  1680
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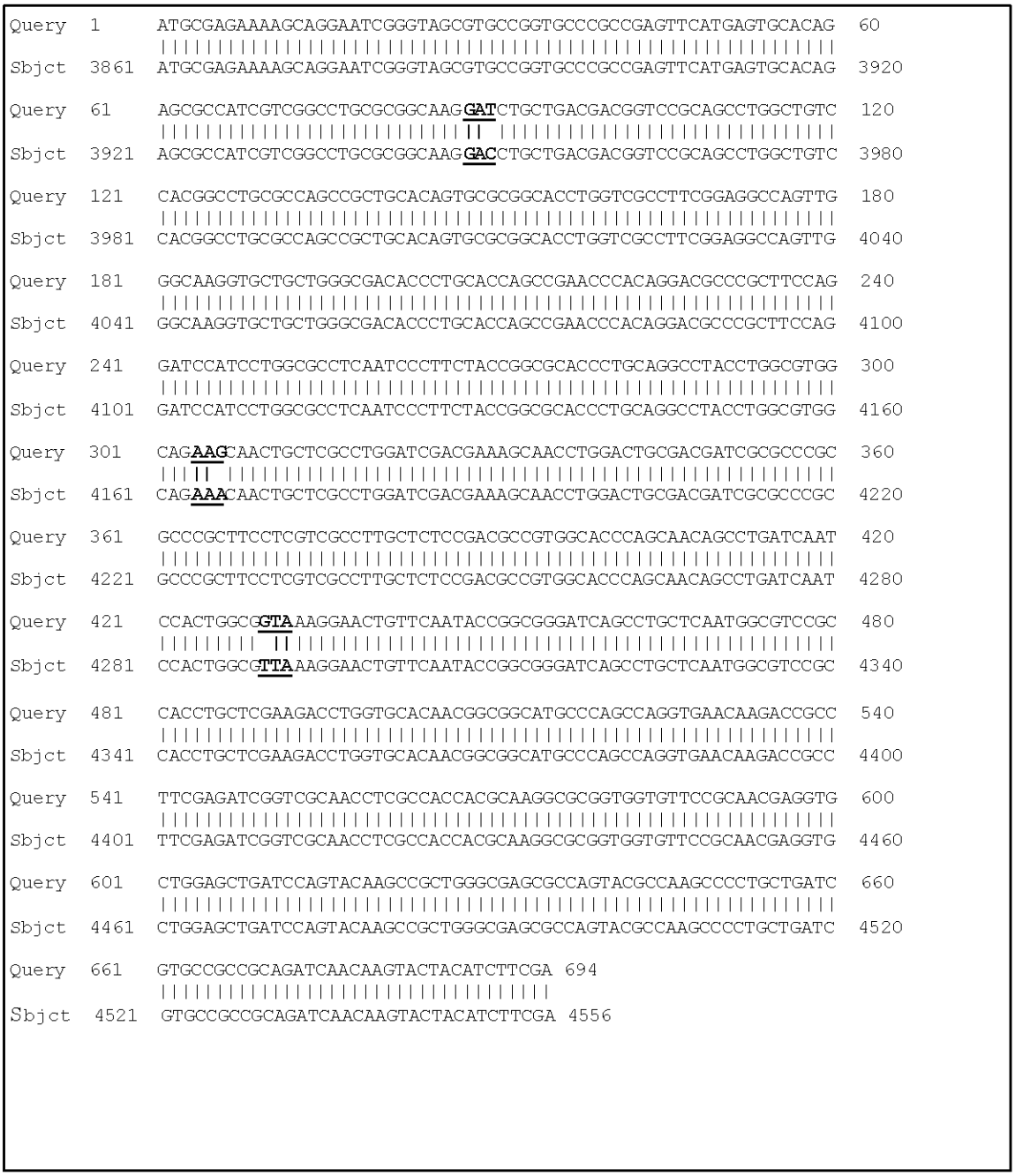
(A)

Fig. 5: Continued



(B)

Fig. 5: A BLAST search comparison on the sequence of *phaC1* and *phaC2* genes. The sequencing for (A) *phaC1* and (B) *phaC2* genes from the Iranian isolate of *P. aeroginosa* (PTCC1310) was compared with the sequences for *phaC1* and *phaC2* genes in *P. aeroginosa* PAO1 using the BLAST program (NCBI, NIH). A diagram of the comparison data for the sequences from *P. aeroginosa* (PTCC1310) (Query) with: A) the sequence of *phaC1* gene; and B) *phaC2* gene, from *P. aeroginosa* PAO1 in gene bank (Subject) are presented

CONCLUSION

In this study the *phaC1* and *phaC2* genes from an Iranian isolate of *P. aeroginosa* (PTCC1310) were successfully cloned into pTZ57R cloning vector. To our knowledge, this was the first time that these genes had been cloned form an Iranian isolate of *P. aeroginosa.* Restriction digestion of the recombinant plasmids primarily confirmed the cloned sequences. The preliminary sequencing which was performed in this study shows that the cloned sequences were indeed belong to *pha*C1 and *pha*C2 genes. Comparing the sequencing data with the genes of *P. aeroginosa* PAO1 revealed one nucleotide mismatch in cloned *phaC1* and three nucleotide mismatches in cloned *phaC2* gene. Clearly, these sequencing data could only be served for the purpose of confirming the correct cloning of *pha*C1 and *pha*C2 genes. It is, therefore, necessary that the cloned genes to be fully sequenced in both directions before using for any applications in the future.

The cloned sequences could be utilized in studies analyzing the expression and recombinant production of polyhydroxyalkanoate synthase, which has important industrial values in the production of biodegradable biopolymers.

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