Original article

Cloning of poly (3-Hydroxybutyrate) synthesis genes from *Azotobacter vinelandii* into *Escherichia coli*

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Abstract
The *Azotobacter vinelandii* is an aerobic, nitrogen-fixing bacterium with the ability to accumulate polyesters such as poly (3-hydroxybutyrate), a particular group of the polyhydroxyalkanoates (pha), when will be cultivated on several carbon sources, including sucrose. Although the capacity of *Azotobacter* strains to accumulate polyesters is well known, and the genes responsible for their synthesis have recently been identified. However, high production costs have hindered the use of polyhydroxyalkanoates as bioplastics, since their final price is considerably high. The phb gene clusters including phbBAC genes can alternatively be cloned and expressed in the *Escherichia coli* with the goal to produce high cell density cultivation. We amplified separately the phbB, phbA and phbC genes from extracted *Azotobacter* genome with the designed primers and could clone the fragments in the cloning vector pCR 2.1- TOPO with the subsequent transformation of *E. coli* strain DH5α.

Keywords: *Azotobacter vinelandii*, Recombinant *Escherichia coli*, phb gene cluster, poly (3-hydroxybutyrate)

Introduction

A group of polyesters such as polyhydroxyalkanoates (PHA) are produced by a large number of bacteria. The accumulated polymers are forming intracellular granules that act in bacteria as an answer to the environmental stress and nutrient imbalance [1]. Today, there are great interests in the field of nanobiotechnology to produce such nanopolymers in a large amount, because of their thermo-plasticity and degradability properties and further as a renewable carbon source [2]. The *Azotobacter* is for instance an aerobic, nitrogen-fixing bacterium with the ability to accumulate poly (3-hydroxybutyrate) (PHB), a special form of the polyhydroxyalkanoates, when it will be cultivated on several carbon sources, including sucrose [3].

The capacity of *Azotobacter* strains to accumulate PHA is well known [4,5] and the genes responsible for their synthesis have recently been identified [1]. However, the expression of these genes in the *Azotobacter* is usually under strong regulation and consequently very low [6]. In contrast, because of failure of phb gene cluster, the *E. coli* is biologically not able to produce PHB. But cloning of the three genes and transformation into the *Escherichia coli* would yield under optimized conditions appropriate amount of gene products which

are responsible for building of such biopolymers [6].

PHAs are a group of polyesters produced by a large number of bacteria with variable properties according to their monomer compositions [7]. PHB is the best-known PHA and has been studied most often as a model product in the development of fermentation strategies [8,9]. In the majority of PHB-accumulating species, it is synthesized in three sequential enzymatic steps: a 3-ketothiolase condenses two acetyl-coenzyme A [CoA] moieties to form acetoacetyl-CoA; a NADPH-dependent acetoacetyl-CoA reductase catalyzes the stereoselective reduction of acetoacetyl-CoA to D-[−]-3-hydroxybutyryl-CoA; and a PHB synthase links the 3-hydroxybutyryl-CoA monomers to the growing PHB chain by an ester bond [10].

A copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate was commercialized in the 1980s, but high production costs have hindered the use of PHAs as bioplastics, since their final price is considerably higher than that of petrochemical-based synthetic plastic materials [7]. Additionally, environmental pollution has renewed interest in the development of PHAs, which are fully biodegraded by microorganisms present in most environments [11]. In addition, these polymers can be produced from different renewable carbon sources [11]. Therefore, our aim here was the amplification and cloning of the *phb* gene cluster of *Azotobacter vinelandii* to the *E. coli* strain DH5α, the first important step to produce biopolymers.

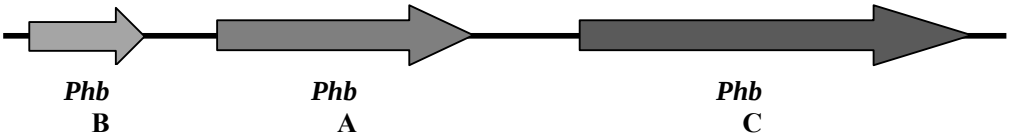

Fig. 1: Organization of the *phb* gene cluster in the *Azotobacter* genome containing *phbB* encoding acetoactyl coA reductase, *phbA* for ketotiolase and *phbC* for PHA depolymerase

Materials and Methods

Culturing condition

Dried culture of the bacterium *A. vinelandii* was purchased from German collection for microorganisms (DSMZ, GmbH). *A. vinelandii* was cultured on the suitable culture medium. Ingredients of culture medium were glucose 5.00g, mannitol 5g, $CaCl_2$ x $2H_2O$ 0.1g, $MgSO_4$ x $7H_2O$ 0.1g, Na_2MoO_4 x $2H_2O$ 5mg, K_2HPO_4 0.90g, KH_2PO_4 0.1g, $FeSO_4$ x $7H_2O$ 0.01g, $CaCO_3$ 5g, agar 15g and distilled water 950 ml. Glucose and mannitol were separately sterilized (in 50ml H_2O) and added to the medium after autoclaving (all ingredients ware obtained from Sigma company, USA). Final pH in prepared medium was 7.3.

Separate amplification of the genes phbA, phbB and phbC

Genomic DNA of *A. vinelandii* was isolated by a method adapted from Sambrook *et al.* [12]. Three fragments with the length 1179 bp for phbA gene, 744 bp for phbB gene and 1704 bp for phbC gene have been obtained by PCR using forward and reverse primers as is listed in the table 1. The primers were designed by Oligo7 software from the reference sequences of the phb gene cluster in the 'National Centre of Biotechnology Information (NCBI) website.

The PCR reaction mix contained 25 pmol of each primer, 50 to 100ng bacterial DNA, 0.2 mM of each deoxynucleoside triphosphate (fermentase), two mM $MgCl_2$ (fermentase), three units of Taq DNA polymerase (house recombinant Taq) and 13µl PCR buffer in a final volume of 25µl. The cycling parameters were an initial denaturation at 95°C for five minutes, followed by 30 cycles of denaturation at

95°C for one minute, annealing at 55-57°C (Table 1) for one minute, and extension at 72°C for two minutes. The final cycle was followed by a long extension at 72°C for 10min.

Table 1: Summary of information about the primers that were used in this report

Name	Sequence	Tm	Product length
PhbA-F	5-ATGAAAGAGGTTGTAATCGTCGCT-3	57	1179 bp
PhbA-R	5-TCAACGCTCCACTGCGAG-3		
PhbB-F	5-ATGAGCAATCAACGAATTGCA-3	55	744 bp
PhbB-R	5-TCATTGCATGTTCAGACCGC-3		
PhbC-F	5-ATGGATCAAGCCCCCTCTTT-3	55	1704 bp
PhbC-R	5-TCAGCCTTTCACGTAACGG-3		

Insertion of PCR products into the cloning vector

The vector pCR 2.1-TOPO was purchased from Invitrogen (Invitrogen, Iran). The produced fragments by PCR were purified from a one percent agarose gel by PCR product purification kit (Qiagen, Iran) according to the manufacturer's instruction and ligated with the linear TA-vector.

The Taq DNA polymerase has an adenine-Transferase activity, so that almost all of the PCR products contain an adenine-overhang at the 3'-site, which can be used for the cloning into the vectors such pCR-2.1-TOPO that are having in linear form a thymine-overhang at both ends. *E. coli* strain DH5α (Invitrogen, Iran) was transformed by the three constructs and plated at 37ºC on LB agar plates supplemented, when required, with 50 mg/ml ampicillin (Sigma, USA) *E. coli* colonies were selected and plasmid DNA was purified.

Bacterial transformation

Transformation of the *E. coli* strain DH5α was performed by the $CaCl_2$ method as described by Sambrook *et al*. [12]. Regarding the concentration, the relation of the vector to the insert was chosen as 1:3 for optimal transformation efficiency.

DNA plasmid extraction

Large amount of endotoxin-free plasmid DNA were obtained by plasmid extraction miniprep Kit (Qiagen, Iran) according to the manufacturer's instruction. Plasmid concentration and DNA contamination were checked by agarose gel.

Restriction enzymes

To prove the successful cloning of PCR products, plasmid DNA was extracted from some bacterial clones and the plasmid was digested with EcoRI (fermentase) overnight at 37°C and the resulted fragments from digestion reactions were loaded on the one percent agarose gel.

Results and discussion

After transformation of the *E. coli* with the pCR-2.1-TOPO vector including appropriate inserts, a number of the single colons were selected from the plates containing LB-ampicillin, from which the plasmid DNA was extracted. The extracted plasmids were digested with the restriction enzyme EcoRI, which did cut the cloned fragments (inserts) in the full length. We note that the EcoRI has no restriction site for the inserts. This step was achieved to prove the correct insertion of the PCR products. Additionally, we used the extracted plasmids as template for the subsequent amplification of phb genes by the specific primer pairs that has been used before (Table 1). The three PCR products with the length of 1704 bp, 1179 bp

and 744 bp was generated as has been shown in figure 2.

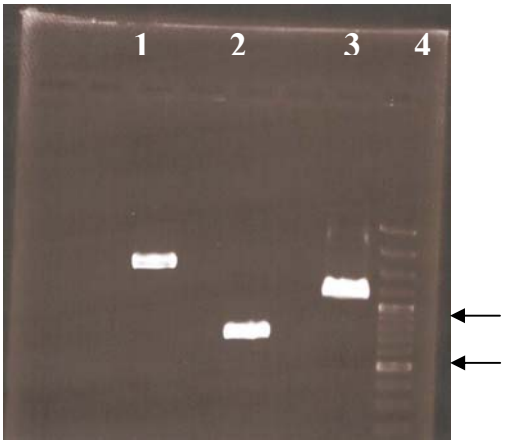

Fig. 2: Extracted plasmids that were obtained from bacterial clones were used as template to detect positive clones containing appropriate inserts. 1) bacterial clone with 1) the 1704 bp insert (Phb C); 2) the 744 bp insert (Phb B); 3) 1179 bp insert (Phb A); 4) One kb DNA size marker (Fermentase).

We have successfully cloned the three phb genes in the pCR 2.1- TOPO vector, which is designed for direct cloning of PCR products. This vector contains in linear form the 5'-T-overhang ends that can be joined with the 3'-A-overhangs of PCR products. The vectors have been subsequently transformed into the *E. coli* strain DH5α. Nevertheless, the PHB production costs can be reduced by using recombinant *E. coli* [2,11]. *E. coli* is a suitable host as a heterologous expression background for foreign genes that can be easily manipulated and improved by means of recombinant DNA methodologies.

In addition, high-cell-density cultivation strategies for numerous *E. coli* strains are well established [13,14]. *E. coli* cells that accumulate large amounts of PHB become fragile, facilitating the isolation and purification of the biopolymer, and the bacterium does not express PHA-degrading enzymes [9]. However, the present work is the basis step of designing recombinant *E. coli* that is containing the three genes responsible for production of biopolymers in the *E. coli*.

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