Original Article

Optimization of the expression of phaC2 encoding poly (3-hydroxyalkanoate) synthase from *Pseudomonas aeruginosa* PTCC1310 in Fad B deleted *Escherichia coli*

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Abstract

Background: Poly3-hydroxyalkanoates (PHAs) are potential candidates for the industrial production of biodegradable plastics. Therefore, in the present study, expression and activity of one of the enzymes involved in the PHA synthesis, phaC2 (isolated from *Pseudomonas aeruginosa* PTCC1310), were investigated in Fad B deleted *Escherichia coli*.

Materials and Methods: The inserts obtained from recombinant pTZ57R plasmids were ligated into the pGEX-5x-1 expression vector and then transformed into Fad B deleted *E. coli* cells using the heat shock method. This protein was then expressed using isopropyl beta-d-thiogalactoside (IPTG) as an inducer. By changing expression conditions such as IPTG and glucose concentration, time and temperature of incubation with IPTG, the expression conditions were optimized.

Results: The optimum condition for the expression of this enzyme was: 1.5 mM IPTG, 1 mM glucose, incubated at 37°C for 2 hours.

Conclusion: We obtained functional expression of the phaC2 gene and investigated various conditions that could influence the expression of protein to optimize production of PHA synthase enzymes. This would allow us to study PHA production in large quantities.

Key Words: Fad B deleted *E. coli,* IPTG, phaC2, polyhydroxy alkanoate, protein expression

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Received: 01.06.2013, **Accepted:** 09.12.2014

INTRODUCTION

Biodegradable polymers offer a great solution to the environmental hazards caused by conventional plastics.[1-4] One of these biodegradable plastics are biopolymers called poly-3-hydroxyalkanoates (PHAs) which behave similar to the synthetic plastics.[1-5] PHAs are a family of intracellular polyesters produced by a variety of microorganisms under stressful conditions such as sulfur or nitrogen limitation and after returning to normal conditions, cells would disintegrate these polymers.[1]

There are three groups of PHAs: Short chain length (SCL) PHAs, containing 3 to 5 carbon atoms in monomer units, medium chain length (MCL) PHAs containing 6 to 14 carbon atoms, and scl-co-mcl a

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.178790

How to cite this article: Abedi D, Moazen F, Akbari V, Mirzaalian F, Sadeghi HM. Optimization of the expression of phaC2 encoding poly (3-hydroxyalkanoate) synthase from *Pseudomonas aeruginosa* PTCC1310 in Fad B deleted *Escherichia coli*. Adv Biomed Res 2016;5:50.

repeat-unit monomers containing 3 to 14 carbon atoms.[1,6] Most *Pseudomonase* strains are able to accumulate mcl-PHA and they have long generation time, ease of lysis, good understanding of their biochemical properties and genetics. Therefore, it would be advantageous to obtain large quantities of PHAs using recombinant *Escherichia coli*.[7-9]

PHA synthase are the most important enzymes involved in PHA biosynthesis. Four major classes have been recognized for PHA synthases. The class two PHA synthase has been found only in mcl-PHA-producing *Pseudomonade* strains. This system contains two PHA synthase genes, phaC1 and phaC2, which are separated by a phaZ gene (encoding for PHA depolymerase).[10]

In our laboratory, several isolates of *Pseudomonas aeruginosa* obtained from Iranian resources were examined for the presence of PHA synthase genes. One of those species (PTCC1310) contained these genes and thus used for further studies.

In previous studies the phaC2 gene was amplified from *P. aeruginosa* PTCC1310 by PCR, cloned and then expressed in *E. coli* BL21 (DE3). The results revealed that for increasing the yield of PHA production, mutant strains of *E. coli* that could block the oxidation of fatty acids and accumulate greater levels of PHA should be used. Therefore, the aim of the present study were to express the phaC2 gene in Fad B deleted *E. coli* (a mutant *E. coli* which blocks the oxidation of fatty acids and leads to more expression) and optimize the conditions for expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used were Fad B deleted *E. coli* and *P. aeruginosa* PTCC 1310. Fad B deleted *E. coli* was grown at 37°C in Luria-Bertani medium (LB). *Pseudomonas aeruginosa* was grown in minimal salt medium (MSM) containing 1.5% glucose. When needed, ampicillin, kanamycin and isopropyl-β-D thiogalactopyranoside (IPTG) were added to the medium. Fad B deleted *E. coli* is resistant to kanamycin so the plates were supplemented with this antibiotic.

Construction of the expression plasmids

pTZ57R plasmid containing the PHA synthase gene (phaC2) from *P. aeruginosa* PTCC1310 was used for this study.[11] At first, this plasmid was digested with restriction enzymes (*Xho*I for 45 min in 37°C and then *Eco*RI added for 30 min at 37°C). After digestion with *Eco*RI and *Xho*I restriction enzymes, the obtained phaC2 inserts were ligated into the pGEX-5x-1 expression vector. For this digestion, the first *XhoI* enzyme was added for 45 min at 37°C and after that *Eco*RI enzyme was added for 30 min at 37°C. This recombinant vector was transformed into competent Fad B deleted *E. coli* cells using the $CaCl_2$ method.[12] We used fast digest enzymes in this study.

Expression of phaC2 genes

For the expression of PHA synthase, a positive clone was inoculated into 5 ml of LB medium (100 μg/ml amp). After an overnight culture, bacterial growth (1% v/v) was added to 50 ml of fresh LB medium in 250 ml flasks and incubated at 37°C until OD 600 nm of 0.4–0.6 was reached. By adding IPTG to a final concentration of 1 mM, the expression of phaC2 was induced.

Optimization of conditions for phaC2 gene expression

For optimization of the enzyme expression, IPTG concentration at four levels (0.5, 1, 1.5, 2 mM), four different times of incubation with IPTG (1, 2, 3 and 4 hrs), the temperature of incubation with IPTG at three levels (25, 30, 37°C) and glucose concentration at three levels (0.5, 1, 2 mM) were examined. Expressed protein samples were analyzed on a 12% SDS-PAGE gel.

Detection of PHA accumulation

The accumulation of PHA in bacteria was detected with Nile-red.[13] Recombinant pGEX as well as pGEX and *P. aeruginosa* (as the positive control) were investigated for PHA production. *E. coli* cells were spread on an LB plate supplemented with sodium-gluconate (w/v 0.5%), Nile-red (0.05 μg/ml), IPTG (0.1 mM) and ampicillin (100 μg/ml). *P. aeruginosa* was grown on MSM supplemented with sodium-gluconate (0.5% w/v), glucose (1.5% w/v) and Nile-red (0.05 μg/ml). The plates were incubated at 37°C for 24 hours. After that, plates which contained rpGEX revealed an orange color under UV light at 302 nm.

RESULTS

The phaC2 gene was ligated to the pTZ57R vector and for confirming the presence of the insert, double digestion with *Eco*R1 and *Xho*1 restriction enzymes was performed. Gel electrophoresis of this digested products revealed that the expected band in 1770 bp was present. Subsequently, the phaC2 sequence was digested out and ligated into pGEX-5x-1 expression vector and the clones containing this insert were identified using PCR [Figure 1]. rpGEX also was digested with *Xho*I and *Eco*RI and the correct band was revealed by gel electrophoresis (1770 bp), so the phaC2 gene was correctly ligated to the vector [Figure 2].

The functional expression of PHA synthase was assessed by Nile-red. The PHA enzyme was expressed by adding IPTG. As demonstrated in Figure 3, both *P. aeruginosa* and *E. coli* containing rpGEX revealed an orange color under the UV light at 302 nm. So Nile-red plate assay revealed functional expression of PHA enzymes by recombinant Fad B deleted *E. coli*.

The effects of incubation time and temperature, IPTG concentration and glucose concentration were analyzed by SDS-PAGE which revealed that the target 84-KD protein was expressed as is demonstrated in Figures 4 and 5. The maximum production of PHA synthase enzymes occurred when 1.5 mM IPTG and 1 mM glucose were used at 37°C for 2 hours.

DISCUSSION

Escherichia coli strains have been widely used for the expression of recombinant proteins. Fad B deleted *E. coli* cells have been the host organism of choice for the expression of certain enzymes because of their ability to block the oxidation of fatty acids and accumulate greater levels of PHA.[14]

In many studies, optimization of conditions for the expression of recombinant proteins has been performed. Wen and his coworkers investigated the effect of temperature and concentration of IPTG on the expression of PET-ILC-HIL-2-MGM.They found that maximum protein expression occurred when 0.3 mM IPTG was used at 42°C.[15] In another study, Qun *et al*. expressed two PHAC genes in *E. coli* and the effect of IPTG concentration on PHA monomer composition was evaluated.[16] In the present study we investigated four factors: The concentration of IPTG, temperature, glucose concentration and time

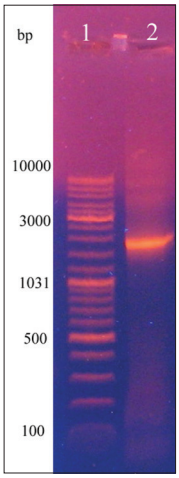

Figure 1: Colony PCR of rpGEX. Presence of 1770 bp band indicated that this colony contained the recombinant plasmid (lane 2). Lane1: molecular weight marker

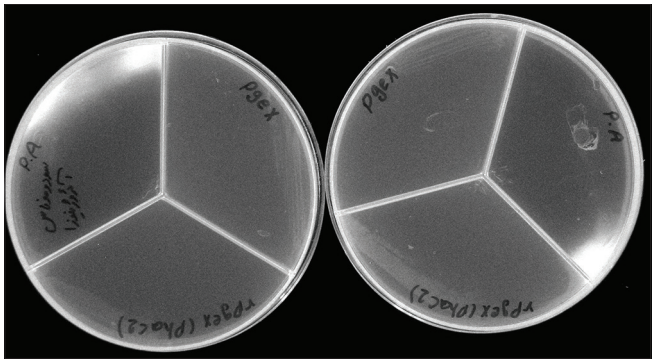

Figure 3: *P. aeruginosa* and *E. coli* containing rpGEX revealed an orange color under UV light

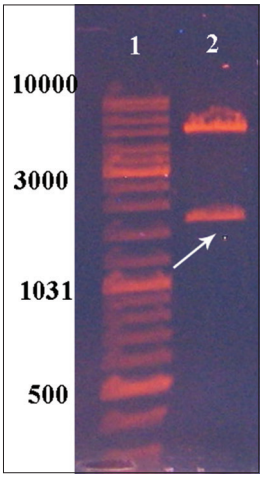

Figure 2: rpGEX digestion with *Xho*I and *Eco*RI, the insert was detected as 1770 bp band (lane 2). Lane 1: molecular weight marker

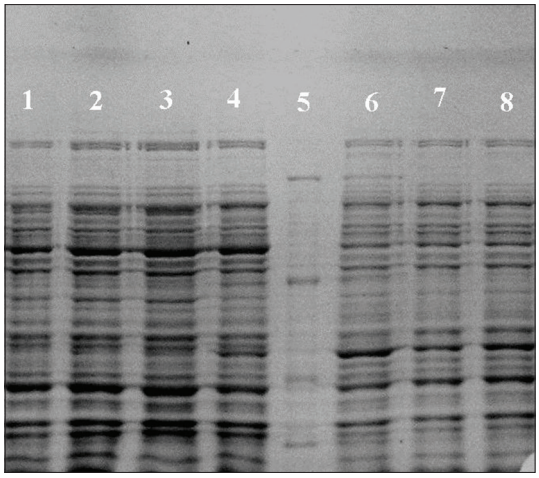

Figure 4: Lane 1 is rpGEX with 0.5% glucose, lane 2 is rpGEX with 1% glucose, lane 3 is rpGEX with 2% glucose. Recombinant protein expression in these experiments was induced with 1.5% IPTG. Lane 4 is uninduced rpGEX, lane 5 is protein molecular weight marker, lane 6 is rpGEX induced at 25°C, lane 7 is rpGEX induced at 30°C, lane 8 is rpGEX induced at 37°C. Recombinant protein expression in these experiments was induced by 1.5% IPTG. As it is demonstrated, lanes 3 and 8 have sharper bands in comparison to others

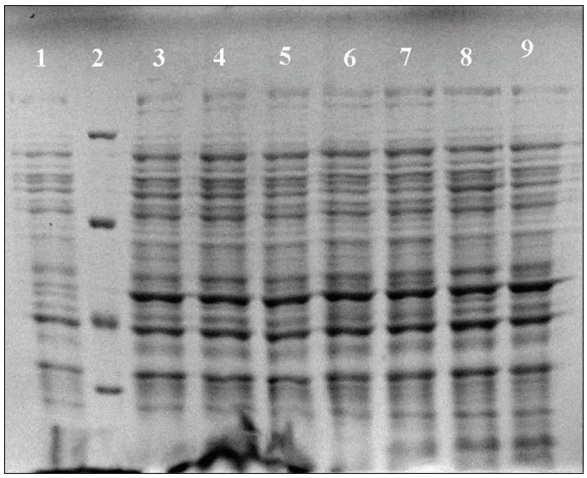

Figure 5: Lane 1 is uninduced rpGEX, lane 2 is protein molecular weight marker, lane 3 is rpGEX induced with 2 % IPTG for 2 hrs, lane 4 is rpGEX induced with 1% IPTG for 2 hrs, lane 5 is rpGEX induced with 1% IPTG for 3 hrs, lane 6 is rpGEX induced with 1% IPTG for 4 hrs ,lane 7 is rpGEX induced with 1.5% IPTG for 1 hrs, lane 8 is rpGEX induced with 1.5% IPTG for 2 hrs, lane 9 is rpGEX induced with 1.5% IPTG for 3 hours. As it is demonstrated in the figure lane 8 has a sharper band as compared to others

of induction of the target protein. Our results show that the optimum conditions for the expression of the phaC2 gene are 1.5 mM IPTG and 1 mg glucose for 2 hrs at 37°C. In comparison with other studies, optimum incubation time for PHA synthase enzyme expression was shorter and IPTG concentration was greater than in those studies.

In a study conducted by Abedi *et al*., phaC1 and phaC2 genes were expressed in *E. coli* BL21(DE3).[17] Perivious studies showed that caltivating *E. coli* with fatty acids as sole carbon source resulting in accumulation higher amounts of PHA.[18] In the present study, we used a mutant strains of *E. coli* (Fad B deleted) in order to block the oxidation of fatty acids and accumulate greater levels of PHA. In agreement with our results, the functional expression of phaC1 in *E. coli* fad and fab mutants were reported.[19]

In summary, we obtained functional expression of the phaC2 gene and investigated various conditions that could influence the expression of protein to optimize production of PHA synthase enzymes. This would allow us to study PHA production in large quantities.

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Source of Support: This work was supported by Isfahan University of Medical Sciences (grant No 391170)., **Conflict of Interest:** None declared.