

Optimization of the Expression of Genes Encoding Poly (3-hydroxyalkanoate) Synthase from *Pseudomonas aeruginosa* PTCC 1310 in *Escherichia coli*

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Abstract

Over the years, the use of plastics has complicated the problem of disposal of solid wastes. One strategy to reduce plastic waste is the use of biodegradable plastics. A group of these plastics are polyhydroxyalkanoates (PHAs). To date more than 250 different microorganisms are known to synthesize and accumulate PHA. Most *Pseudomonas* strains are able to accumulate mcl-PHA. In previous studies, the *phaC1* and *phaC2* genes were identified in *Pseudomonas aeruginosa* (*P.aeruginosa*) PTCC 1310 and were cloned. The aim of this study was to express these genes and optimize the conditions for their expression. The inserts obtained from vectors pTZPHAC1 and pTZPHAC2 were subcloned into pET15b expression vector. After transformation of competent *Escherichia coli* (*E.coli*) BL21 (DE3) cells with recombinant plasmids, expression was induced using IPTG. By changing expression conditions such as IPTG concentration, time and temperature of incubation with IPTG, the expression conditions for these enzymes were optimized, and the obtained results were compared using proper statistical analysis. The PHA synthase genes were induced with IPTG and the expressed 62 kDa protein was observed and purified. By changing expression conditions, 1 mM IPTG, 37 °C and a 2 hr incubation provided the highest level of protein production in *E.coli* cells. These results suggest that induction condition of *PhaC* genes can influence expression of PHA synthase enzymes.

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Key words: *PhaC1*, *PhaC2*, Polyhydroxyalkanoate, Protein expression

Introduction

Poly (hydroxyalkanoates) (PHAs) are natural biopolymers produced by many microorganisms. These polymers are accumulated by cells as storage materials under stress conditions such as nitrogen limitation and excess supply of carbon sources⁽¹⁻³⁾.

PHAs have a wide range of applications because of their thermo-plasticity and degradability properties. Controlled drug release systems, scaffold materials in tissue engineering, absorbable surgical sutures, prosthesis and bioimplants are some of their uses in medicine⁽⁴⁾. Due to the growing needs for these biopolymers in industrial applications, there is great interest to produce them in large amounts through recombinant DNA technology.

Although many wild-type microorganisms are known to produce PHAs, natural PHA producers have a long generation time, are often hard to lyse, and contain pathways for PHA degradation. There are several advantages of using recombinant *E.coli* for the production of PHAs such as fast growth, ease of lysis, very good understanding of their genetics and biochemical properties, and broad substrate utilizing abilities⁽⁵⁻⁸⁾.

PHA synthases are the key enzymes of PHA biosynthesis. In our previous study, the PHA biosynthesis genes of *P.aeruginosa* PTCC 1310 (*phaC1* and *phaC2* genes) were cloned and sequenced⁽⁹⁾. The aim of this study was to express these genes and optimize the conditions for their expression in *E.coli*. The optimization of conditions

for *phaC* genes expression can serve as an important stage for establishing PHA production in *E.coli*.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used were *E.coli* BL(DE3) and *Pseudomonas citranolleise* (*P.citranolleise*) (DSMZ 11735). *E.coli* was grown at 37°C in Luria-Bertani medium (LB). *P.citranolleise* was grown in Minimal Salt Medium (MSM) containing 1.5% glucose. When needed, ampicillin and Isopropyl-b-D thiogalactopyranoside (IPTG) were added to the medium.

Construction of expression plasmids

Two plasmids, pTZPHAC1 and pTZPHAC2, which contained the PHA synthase genes (*phaC1* and *phaC2*) from *P. aeruginosa* PTCC 1310, were constructed from genomic DNA as described previously.⁽⁹⁾ These plasmids were extracted and digested with restriction enzymes. The *NdeI* and *BamHI* enzymes were used to digest pTZPHAC1 and for digestion of pTZPHAC2 *NdeI* was used (*BamHI* was not used because it digests the *phaC2* sequence). The obtained *phaC1* and *phaC2* inserts were ligated into pET15b (Novagen, USA) expression vector digested with *NdeI*, *BamHI* and *NdeI*, respectively. These recombinant vectors were transformed into competent *E.coli* BL21 (DE3) cells using the CaCl₂ method⁽¹⁰⁾.

Expression of *phaC* genes and purification of His-tagged enzymes

For the expression of PHA synthase, a positive cell clone was inoculated into 5 ml of LB medium (100 µg/ml Amp). An overnight culture was used to inoculate (1% v/v) 50 ml of fresh LB medium in 250 ml flasks and incubated at 37°C to an OD_{600nm} of 0.4-0.6. Then the expression of *phaC* genes was induced by the addition of IPTG to a final concentration of 1 mM. The expressed enzymes were purified with Ni-NTA His Bind Resin (Novagen, USA) as described by the manufacturer.

Briefly, 1 ml of each sample was collected and centrifuged after an appropriate growth period. The pellets were resuspended in 200 µl of buffer A and clarified by centrifugation at 15000 g for 10 min and the supernatant was taken for the next steps. Fifty µl of the 50% 1X Ni-NTA His Bind Resin was added to each sample and mixed gently for 30 min at room temperature. After centrifuga-

tion of samples, the supernatant was removed and the pellet was washed two times with 250 µl of buffer C. Finally, the His-tagged enzyme was eluted with buffer E and 20 µl of each sample was electrophoresed on a 12% polyacrylamide gel using SDS-PAGE.

Optimization of conditions for *phaC* genes expression

IPTG concentration, time and temperature of incubation with IPTG were optimized for *phaC* genes expression in *E.coli* BL21 (DE3) cells. For optimization of enzyme expression, IPTG concentration at four levels (0.5, 1, 1.5 and 2 mM), three different times of incubation with IPTG (1, 2 and 3 hrs) and the temperature of incubation with IPTG at three levels (25, 37 and 39°C) were examined. Each assay was repeated three times for pET-*phaC1* and pET-*phaC2* and one time for pET15b as the negative control (a total of 252 experiments). Expressed protein samples were analyzed on a 12% SDS-PAGE gel and the intensity of bands were quantified and compared using SPSS 10 version and differences at p<0.05 were considered as significant.

Detection of PHA accumulation

To confirm the expression of functional PHA synthase enzymes by *E.coli* BL21 (DE3) cells carrying the *phaC* genes, the accumulation of PHA in bacteria was detected with Nile red⁽¹¹⁾. The *E.coli* BL21 (DE3) cells carrying the *phaC* gene, *E.coli* BL21 (DE3) without the *phaC* gene (as the negative control) and *P.citranolleise* (as the positive control) were investigated for PHA production. *E.coli* BL21 (DE3) cells were streaked on an LB plate supplemented with sodium-gluconate (0.5% w/v), Nile red (0.05 µg/ml), IPTG (0.1 mM) and ampicillin (100 µg/ml). *P.citranolleise* 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 agar plates were incubated at 37°C for 24 hr.

Results

Expression and purification of *phaC* synthase

The PHA synthase enzymes were expressed by the addition of IPTG, then the cell suspension was centrifuged. The pellets were added to Ni-NTA His Bind resins. After purification, samples were analyzed by SDS-PAGE, which revealed that the target protein of 62 kD was expressed (Figure1).

Optimization of conditions for *phaC* gene expression

The effect of incubation temperature and IPTG

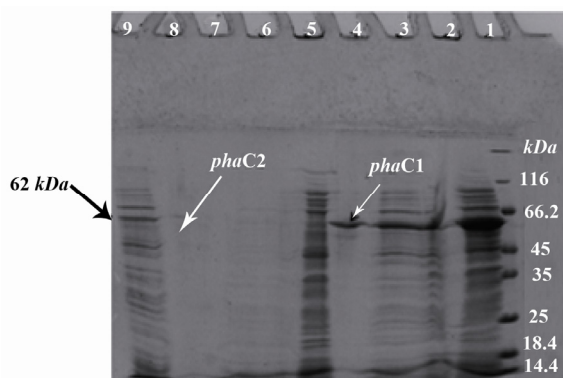


Figure 1. Electrophoresis of the expressed *phaC1* and *phaC2* proteins on 12% SDS-PAGE. 1) Protein marker; 2) Total protein from induced *E.coli* BL21(DE3) containing pET-*phaC1*; 3) Total protein from induced *E.coli* BL21 (DE3) containing pET-*phaC1* after incubation with Ni-NTA His Bind resin; 4) Purified his-tagged *phaC1* protein form Ni-NTA His Bind resin; 5) Total protein from induced *E.coli* BL21(DE3) containing pET15b without insert; 6) Total protein from induced *E.coli* BL21(DE3) containing pET15b without insert after incubation with Ni-NTA His Bind resin; 7) Purified sample from induced *E. coli* BL21 (DE3) containing pET15b without insert; 8: Purified his-tagged *phaC2* protein form Ni-NTA His Bind resin; 9) Total protein from induced *E.coli* BL21(DE3) containing pET-*phaC2* after incubation with Ni-NTA His Bind resin

concentration on SDS-PAGE band intensity of *phaC1* and *phaC2* were investigated and the results are shown in figure 2. The maximum production of the PHA synthase enzymes occurred when 1 mM IPTG was used at 37°C. The effects of incubation time and IPTG concentration on

SDS-PAGE band intensity of *phaC1* and *phaC2* were evaluated (Figure 3). Maximum production of PHA synthase enzyme was observed when 1 mM IPTG was used for 2 hr.

Detection of PHA accumulation

Nile red plate assay revealed functional expression of PHA synthase by recombinant *E.coli* BL21 (DE3) cells (Figure 4).

Discussion

Optimization of conditions for the expression of recombinant proteins has been performed in many studies. Bai et al evaluated the effect of concentration of IPTG and cell density on the level of expression of catalase⁽¹²⁾. In another study performed by Wen and his coworkers, the effect of temperature and concentration of IPTG on the expression of PET-ILC-HIL-2-MGM was investigated⁽¹³⁾. They found that maximum protein expression occurred when 0.3 mM IPTG was used at 42°C. Yang et al optimized four factors, including the initial cell density, concentration of IPTG, incubation temperature and induction time during the induction of the target proteins⁽¹⁴⁾. According to their results, the expression of superoxide dismutase was optimal when *E.coli* cell density was OD600_{nm}=0.6, IPTG concentration was 0.125 mM and incubation at 37°C was for four hours. In another research performed by Qun et al, two *phaC* genes from *P.putida* were expressed in *E.coli* and the effect of IPTG concentration on PHA content and PHA monomer composition was

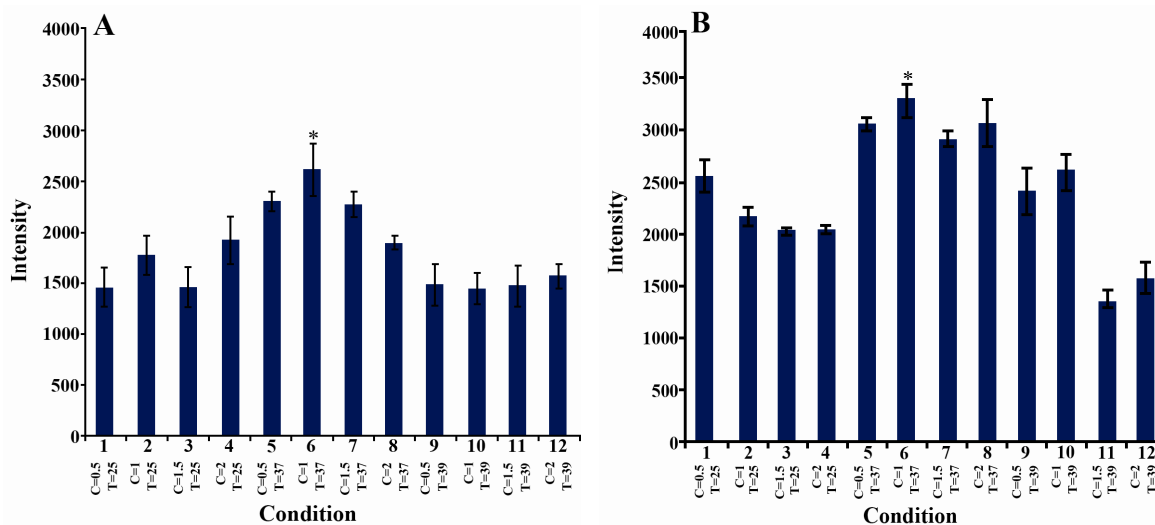


Figure 2. The effect of temperature of incubation with IPTG (°C) and IPTG concentration (mM) on SDS-PAGE band intensity of target protein: Error bars show standard errors of mean. C: IPTG concentration, T: temperature of incubation with IPTG. A) *phaC1*, the intensity of the band observed in lane 6 was significantly different from lanes 1, 3, 9,10, 11 and 12. B) *phaC2*, the intensity of the band observed in lane 6 was significantly different from lanes 1, 2, 3, 4, 9, 10, 11 and 12. (ANOVA, post hoc, n=3 and p<0.05)

Expression of PHA Synthase in *E.coli*

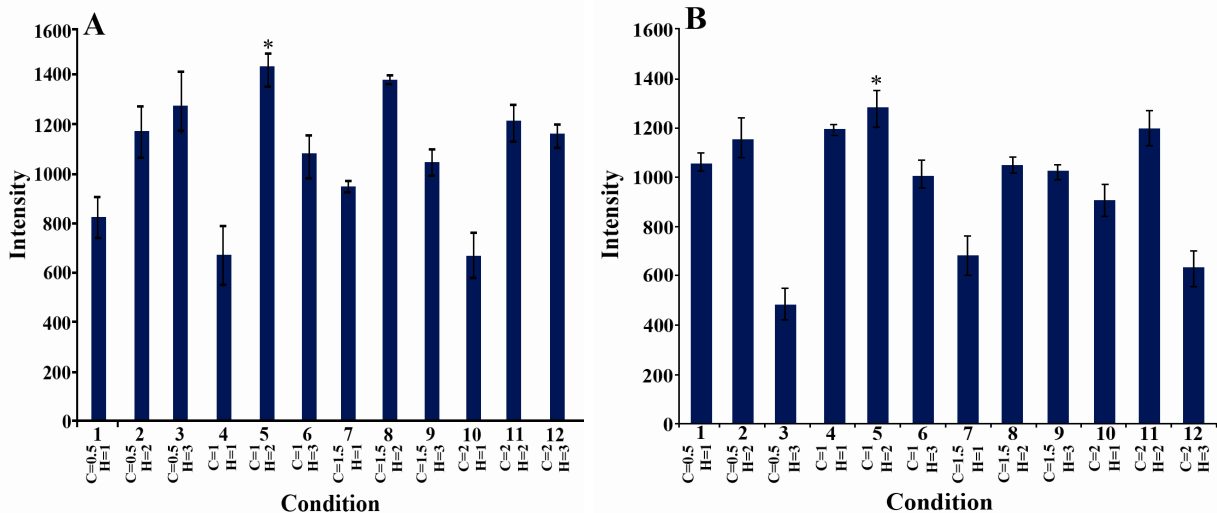


Figure 3. The effect of the time of incubation with IPTG (h) and IPTG concentration (mM) on SDS-PAGE band intensity of target protein: H: time of incubation with IPTG, C: IPTG concentration. A) *PhaC1*, the intensity of the band observed in lane 5 was significantly different from lanes 1, 4, 7 and 10. B) *PhaC2*, the intensity of the band observed in lane 5 was significantly different from lanes 3, 7, 10 and 12. (ANOVA, post hoc, n=3 and p<0.05)

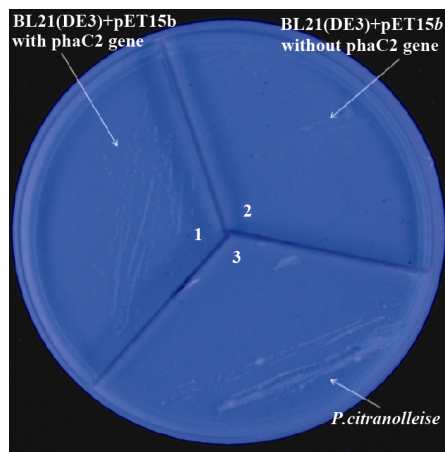


Figure 4. Expression of *phaC* gene as visualized by Nile Red plate assay. 1) *E.coli*BL21(DE3) carrying the *phaC2* gene, 2) *E.coli* BL21(DE3) cells without the *phaC2* gene (as a negative control) and 3) *P.citranolleise* (as a positive control)

evaluated ⁽¹⁵⁾.

In the present study we investigated three factors: the concentration of IPTG, temperature, and time of induction of the target proteins. Our results showed that the expression of *phaC1* and *phaC2* with 1 mM of IPTG for 2 hr at 37°C was the optimum condition for the expression of these genes. In comparison with other studies, optimum incubation time for PHA synthase enzyme expression was shorter and IPTG concentration was greater than those studies.

Using a Nile red plate assay, the accumulation of PHA in bacteria was detected which confirmed the expression of functional PHA synthase en-

zymes in *E.coli* BL21(DE3) cells. To increase the yield of PHA production, using mutant strains of *E.coli* that could block the oxidation of fatty acids and accumulate greater level of PHA is also recommended ⁽¹⁶⁾.

Conclusion

In this study, *PhaC* genes from an Iranian isolate, *P.aeruginosa*, were expressed in *E.coli* and their expression was optimized. According to our findings, the concentration of IPTG, incubation temperature and incubation time during the induction with IPTG can affect the expression of these proteins. Therefore, it is now possible to continue our studies in producing these enzymes in larger quantities for industrial production of biodegradable polymers.

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