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## Cloning and expression of PHB biodegradable biopolymer under different regulation elements in recombinant *E.coli*

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### ABSTRACT

In order to produce Polyhydroxybutyrate (PHB) polymer, as a biodegradable plastic, several expression cassettes for PHB operon was constructed under different regulatory systems. Poly (3)-hydroxyalkanoates (PHAs) are a group of biodegradable macromolecules that have material properties such as polypropylene. Polyhydroxybutyrate (PHB) was the first PHA to be discovered. It is considered as a valuable bio-plastic because of its biodegradability. PHB is an applicable material in industry and medical. In this study three genes of PHB operon from *Alcaligenes eutrophus* H16 were isolated using PCR method. The resulted 4kb PCR product that contained three genes initially was cloned in pJET1.2 vector and recombinant *E. coli* colonies were analyzed by enzymatic digestion and nested PCR. Subsequently PHB operon was sub-cloned in pET28a expression vector using a compatible end enzymatic digestion and placed under the control of T7 promoter. PHB was expressed in recombinant bacteria both in IPTG induction condition and without it. Production of PHB was confirmed by gas chromatography along with standard. Additionally PHB operon was cloned under *psbA* plastid promoter as polycistronic with neo gene in a designed plastid vector. This recombinant vector, pFNP, can be used for constitutive expression of PHB in bacteria and plant plastids. Additionally PHB operon was cloned under *groE* heat shock promoter and pFNPI plasmid was constructed. After transformation of these plasmids to *E. coli*, extraction of PHB was performed and caused to PHB polymer production for the first time from recombinant *E. coli* in Iran. Production of PHB from natural producers is expensive, so alternative hosts such as recombinant bacteria and transgenic plant would be valuable for the production of PHB.

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### KEYWORDS

Bioplastic;  
Cloning;  
Polyhydroxybutyrate (PHB);  
Recombinant *E. coli*.

### INTRODUCTION

Plastic wastes have led concerns for environment<sup>[4,6,23]</sup>. Conventional plastics take many decades

to be decomposed in nature and some toxin agent was produced during the process of their degradation. For this reason the use of plastics that can be readily eliminated from our environment is valuable. Poly (3)-

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hydroxyalkanoates (PHAs) are a group of biodegradable biopolymers that are synthesized by several microorganisms<sup>[1, 7, 10, 12, 24]</sup>. Polyhydroxybutyrate (PHB) was the first discovered Polyhydroxyalkanoate. Petroleum-derived plastics take several decades to degrade but PHAs can be completely bio-degraded within a year by a variety of microorganisms. The results of this biodegradation are carbon dioxide and water, which return to the environment<sup>[22]</sup>. The chemical structure and properties of PHAs was reviewed by<sup>[22]</sup>.

Long time for grow and difficulty of polymer extraction from natural PHB producers were caused that these microorganisms not considered to be suitable for industrial production of the biopolymer.

*E. coli* is considered as an appropriate host for generating higher yields of the biopolymer because of its fast growth and the ease with which it can be lysed<sup>[14]</sup>. This bacterium does not naturally produce PHA. Fast growth, high cell density, ability to use several inexpensive carbon sources and easy purification are the advantages of employing recombinant *E. coli* for the production of PHA<sup>[9]</sup>. The polycistronic *phb* operon encodes three enzymes for production of polyhydroxybutyrate<sup>[16]</sup>. At first<sup>[20]</sup> introduced *pha* genes into *E. coli*. Under optimal conditions was found that Recombinant *E. coli* accumulate PHB up to 85% of the cell dwt.<sup>[13, 25]</sup>. Culture condition is a key factor for molecular mass of the PHB produced in *E. coli* cells. In higher glucose concentration (20 g/l), 37 °C and pH 6.0, cells produced PHB with highest molecular mass value (20 MDa)<sup>[22]</sup>.

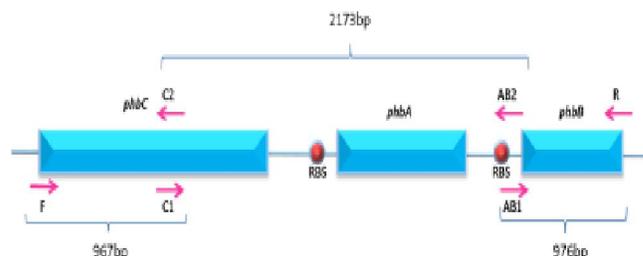
In this study the genes that involved in PHB synthesis from *Ralstonia eutropha* were isolated and cloned under the control of different genetically regulation elements and PHB was produced and extracted successfully from recombinant *E. coli*.

## MATERIALS AND METHODS

### Isolation and cloning of PHB operon

The strain of *Alcaligenes eutrophos* (*ralstonia eutropha*) was received from Iranian Research Organization for Science and Technology (IROST) (PTCC.irost.org) and cultured in LB and incubated in 28 °C for 48 hours. Genomic DNA was extracted from

this bacteria using Qiagen kit. A set of specific primers was designed for the 3 genes of PHB operon according to sequences from NCBI by Oligotech and Vector NTI software. In addition to designing a primers pair for isolation of three genes of entire PHB operon as a 4078bp, two another primer pairs were also designed for amplification of operon internal (Figure 1).



**Figure 1 : Schematic view of the landing sites of designed primers in PHB operon.**

PCR for amplification of whole PHB operon was performed using TLA DNA Polymerase (BioNer). The expected 4078bp band was appeared after electrophoresis on agarose gel and purified using High pure PCR product purification kit (Roche Company). Then this fragment was initially cloned in pJET1.2 plasmid (Fermentas). Ligation reaction was transformed into competent cells that was prepared from *E. coli*, XLI-Blue strain and selected on LB medium containing 100mg/l Ampicillin as selectable marker. Plasmid was extracted from appeared colonies and analyzed using PCR and enzymatic digestion reactions for detection of recombinant bacteria harboring *phbC*, *phbA* and *phbB* genes in pJET vector.

### Cloning of PHB genes in expression vector

After bioinformatics analysis for PHB operon cloning by preservation of correct ORF and expression cassette, this operon was isolated using *XhoI* and *HindIII* from pJET-PHB plasmid and cloned in *SalI* and *HindIII* digested pET28a expression vector. Recombinant *E. coli* XLI-Blue colonies were detected on LB medium containing 50mg/l Kanamycin and plasmids from positive recombinant colonies was transformed into BL21 bacterial strain for expression.

### PHB expression from pET-PHB in recombinant bacteria

BL21 containing recombinant pET-PHB vector was cultured in 10ml LB broth medium containing 50mg/

1 kanamycin and incubated overnight in 37 °C shaker incubator. Five ml of grown bacteria was diluted in 250ml broth medium in 500ml Erlenmeyer containing 50mg/l kanamycin and 2% glucose and incubated as above condition. Until  $OD_{600nm}$  received to 0.6, IPTG was added and incubated in 30 °C.

### Cloning of PHB genes in plastid vectors

PHB was isolated from pJET-PHB plasmid by *XbaI* and *XhoI* enzymes and after purification from agarose gel, was cloned into pNGi plasmid that was constructed by authors previously. pNGi plasmid was digested by *NheI* and *XhoI* enzymes and PHB operon was replaced to *gfp* gene in this vector. PHB genes were derived by *groE* heat shock promoter. Recombinant bacteria were recognized after transformation on LB medium containing kanamycin and Ampicillin. The resulted plasmid called pNPi was analyzed by PCR and digestion and after verification cloned in the center of a fragment that was isolated from plant plastid genome and replaced in an intergenic region of plastid flanking region (FR). PHB cassette was inserted in two direction related to plastid FR region and two plastid vector for PHB was obtained that called, pFNPi(+) and pFNPi(-).

Additionally for construction of a new cassette for PHB under plastid promoter, pNPi vector was partially digested using *XhoI* and *XbaI*. Ten bands were appeared after partial digestion that 8444bp fragment was isolated and purified from them. This fragment that was formed after *psbA* terminator and *groE* promoter removing was treated by klenow enzyme for blunt end formation. Re-circularization leads to expression of PHB operon as polycistronic with neo gene under common strong *psbA* plastid promoter. Resulting vector called pNP, after verification by molecular analysis was re-cloned into FR plastid region and pFNP recombinant plasmid was obtained.

All cloning procedures were carried out using standard methods described in<sup>[18]</sup>.

### Gas chromatography analysis

Biomass was analyzed for its PHB content according to<sup>[3]</sup> method that involves hydrolysis and subsequent methanolysis of lyophilized PHB containing biomass followed by gas chromatography analysis of the 3-hydroxybutyric acid methyl ester (Me-3HB) produced.

PHB was produced by *E. coli* BL21 containing pET28-PHB and XLI-Blue containing pFNP and pFNPi plasmids. Broth cultures including grown bacteria were centrifuged and the precipitated biomass was washed with distilled water to remove residual culture medium, frozen and then lyophilized prior to methanolysis of PHB for subsequent GC analysis.

Bacterial biomass containing PHB was lyophilized and treated with a 2mL MeOH/H<sub>2</sub>SO<sub>4</sub> (3% v/v) solution in 150mm×20mm screw-cap Kimax tubes. Chloroform (2 mL) was added to the resulting mixture. The tubes were sealed tightly and heated in shaker water bath at 100 °C for 4 hours. Pure PHB reference material was also treated with acidified methanol under conditions similar to the ones described for the Bacterial biomass. Me-3HB was quantified by a gas chromatograph (Varian 4000 Gas Chromatograph-Mass Spectrometer).

### PHB extraction

PHB extraction from bacterial biomass was performed based on the solubility of PHA in chloroform and insolubility in methanol<sup>[8]</sup>. PHA from chloroform solvent was recovered by solvent evaporation and precipitation by addition of methanol.

## RESULTS

### Isolation and cloning of PHB operon

Entire PHB operon was isolated as a complete three gene fragment about 4kb (Figure 2) after PCR by PHB-F and PHB-R primers and cloned in pJET vector. The accuracy of PHB isolation initially was confirmed using Nested PCR by appearance of expected bands amplified using internal primers. PHB-F and PHB-C2 primers were amplified 976bp fragment from *phbC* gene. PCR using PHB-C1 and PHB-AB2 primers also amplified 2173bp fragment from *phbA* gene and a part of *phbC* gene. PCR performance using PHB-C1 and phb-AB2 primer was appeared 976bp band from *phbB* gene (Figure 3).

Insertion of PHB operon was possible in two directions. Because of importance of genes direction for their further cloning under promoter and terminator regulatory elements, detection of recombinant plasmids har-

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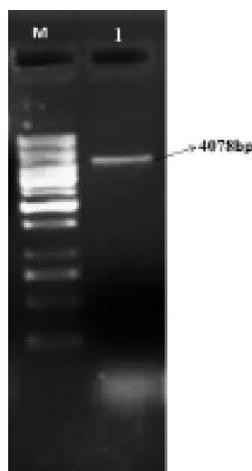


Figure 2 : Amplification of PHB operon using PCR. Expected 4078bp band from PHB operon that amplified using PHB-F and PHB-R primer was appeared. M.DNA Marker ladder (1Kb Ladder, Fermentas).

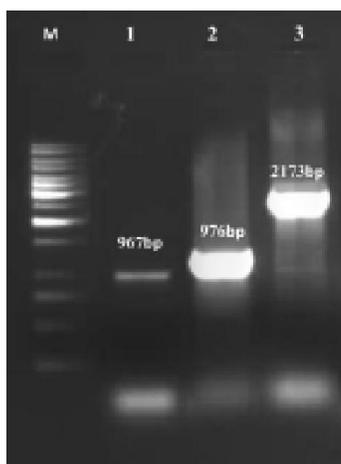


Figure 3 : Nested PCR reaction for confirmation of PHB operon isolation. M.DNA Marker ladder (1Kb Ladder, Fermentas); 1. Amplified fragment using PHB-F and PHB-C2 primers; 2. Amplified fragment using PHB-R and PHB-AB1 primers; 3. Amplified fragment using PHB-C1 and PHB-AB2 primers.

boring PHB operon in correct direction was performed using digestion reaction. Finally recombinant pJET-PHB plasmid was detected by appearance of 4103bp and 2949bp resulted from *Xho*I and *Xba*I double digestion and further proved by resulted 4109bp and 2943bp bands from *Eco*RV and *Hind*III digestion (Figure 4).

### Cloning and expression of polyhydroxybutyrate, gas chromatography and extraction

Recombinant colonies were recognized after plasmid extraction and digestion using *Eco*RI and *Xho*I (Figure 5). Appearance of 5335bp and 4378bp bands

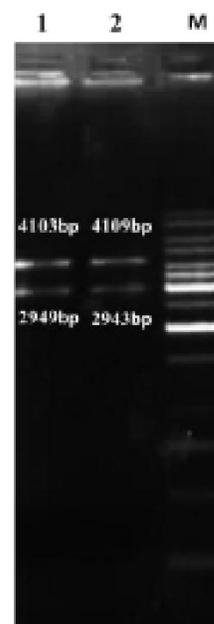


Figure 4 : Recognition of pJET-FR recombinant plasmid harboring PHB operon in correct direction using enzymatic digestion. M.DNA Marker ladder (1Kb Ladder, Fermentas); 1. Using *Xba*I and *Xho*I; 2. Using *Eco*RV and *Hind*III.

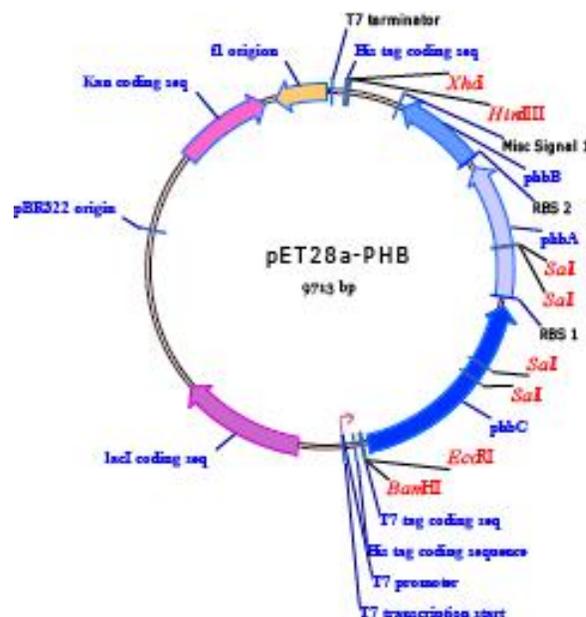


Figure 5 : Schematic view of pET28a-PHB recombinant plasmid

confirmed the accuracy of pET-PHB plasmid (Figure 6). This recombinant plasmid was transformed to BL21 for production of PHB. Finally gas chromatography by appearance of strong curve for PHB in recombinant *E. coli* (pic number 5 in Figure 7) was clearly indicated the accuracy of PHB gene isolation and cloning. PHB extraction resulted plastic form of PHB successfully

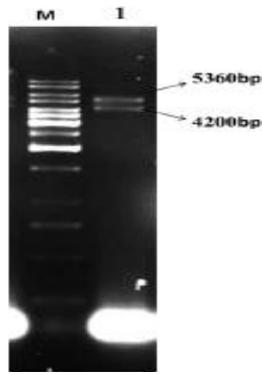


Figure 6 : Enzymatic digestion of recombinant pET28a-PHB using *EcoRI* and *XhoI*. M.DNA Marker ladder (1Kb Ladder, fermentas)

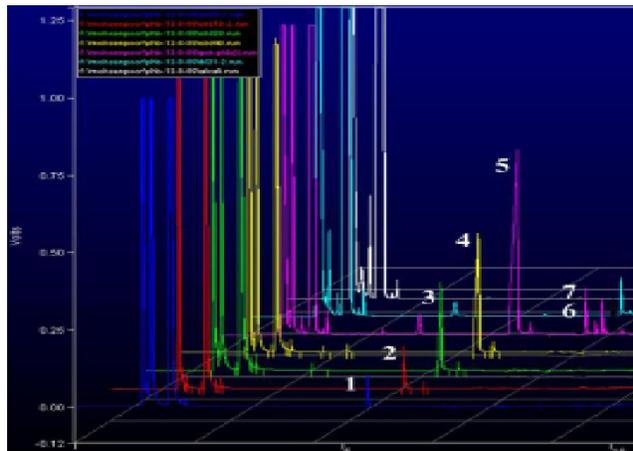


Figure 7 . Gas chromatography results for recombinant bacteria harboring pET28a-PHB along with standards PHB and control untransformed bacteria. 1, 2, 3 and 4. Standard PHB in content 5, 10, 20 and 40 mg respectively. 5. Recombinant *E. coli* BL21 harboring pET28a-PHB. 6. Blank *E. coli* BL21. 7. Blank *E. coli* XII-Blue.

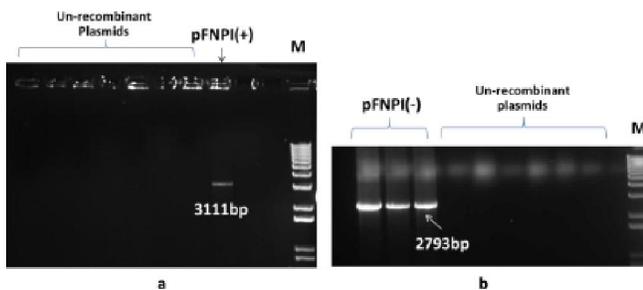


Figure 8 : PCR for recognition of pFNPI(+) recombinant plasmids. PCR using PHB-C2 and FRI-F primer was led to recognition of a recombinant pFNPI plasmid. Appearance of 4111bp band indicated that PHB cassette was cloned in the same direction of FR fragment. PCR using PHB-C2 and FRI-R primer was led to recognition of 3 recombinant pFNPI(-) plasmids. Appearance of 2793bp band indicated that PHB cassette was cloned in opposed direction of FR fragment. M. DNA Marker ladder (1Kb Plus Ladder, Invitrogen)

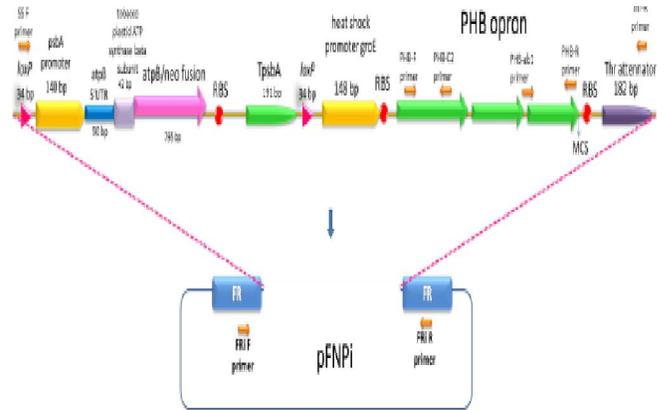


Figure 9 : Schematic view of pFNPI(+) recombinant plasmid. In the pFNPI(-) plasmid, the upper inserted fragment is reverse complement.

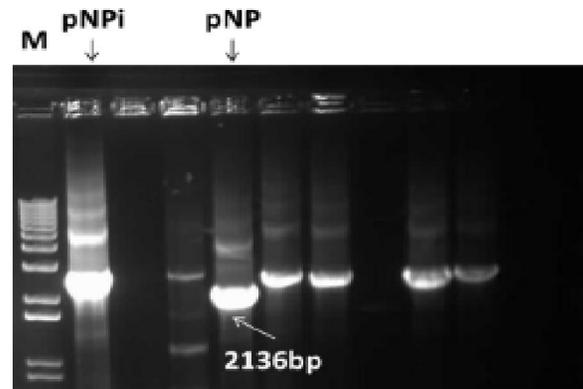


Figure 10 : PCR analysis for recognition of pNP polycistronic recombinant plasmid using SS-F and PHB-C2 primers. One of the plasmids appeared 2136bp band related to pNP. Other plasmids indicated band in parallel pNPI, so are not polycistronic. (pNP after confirmation was used for construction of pFNP) M.DNA Marker ladder (1Kb Plus Ladder, Invitrogen)

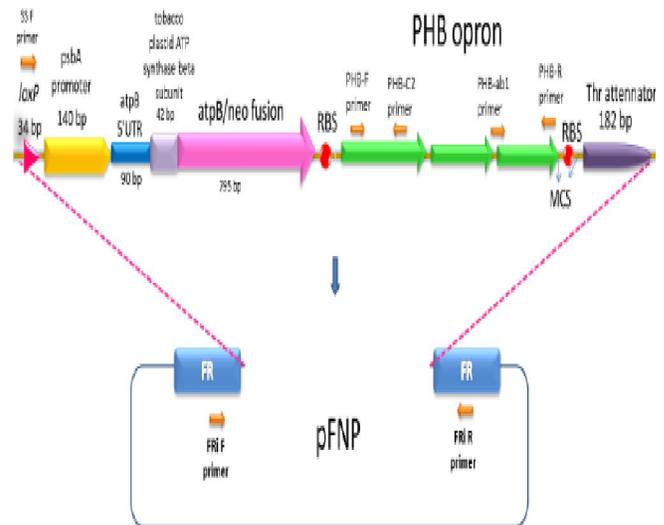


Figure 11 : Schematic view of pFNP recombinant plasmid



**Figure 12 : Biodegradable plastic production after isolation, cloning and expression of Polyhydroxybutyrate genes in recombinant *E. coli*.**

(Figure 12).

### Cloning of PHB in plastid vector

The accuracy of recombinant plasmids pFNPI(+), pFNPI(-) and pFNP were confirmed by PCR (Figure 8, 9, 10, 11). These plasmids can be used for PHB production in recombinant *E. coli* without need to IPTG for PHB induction. Additionally these plasmids can be used as plastid constructs for plant plastid engineering in order to PHB expression.

## DISCUSSION

Fossil fuels are a key factor for industrial processes and for the production of structural materials. The economy of most countries is still very oil dependent. If we can substitute a renewable source for carbon based structural materials would be valuable. Polyhydroxybutyrates because of no toxic effects in the host and certain advantages over petroleum-derived plastic are a suitable candidate for current plastics<sup>[22]</sup>. In recent years, a combination of genetic engineering and molecular microbiology techniques has been applied to enhance PHA production in microorganisms. *R. eutropha* is a natural producer that is well adapted to PHA accumulation in cells.

PHA producers are not suitable for industrial production of the biopolymer because most natural producers take a long time to grow during fermentation and extraction of polymers from their cells is difficult<sup>[22]</sup>. *E. coli* is considered as an appropriate host for generating higher yields of the biopolymer<sup>[14]</sup>.

Some parameters have been adjusted to enhance PHB production including increased carbon supply, changes in fermentation temperature, changes in the number of plasmid copies and choice of bacterial strains<sup>[15, 22]</sup>.

In this study the genes that involved in PHB synthesis from *Ralstonia eutropha* were isolated and cloned under different genetically regulation elements and PHB was produced and extracted successfully from recombinant *E. coli*. Expression PHB in pET28 vector required IPTG for PHB expression. Bacteria harboring pET-PHB was analyzed in two manners with and without adding IPTG to bacterial culture medium and indicated PHB was still produced even in absent of IPTG but in low concentration. However construction of pFNP vector that expresses PHB as constitutively under strong plastid promoter can be a suitable replacement for IPTG inducible T7 promoter in pET vectors for production of PHB. Additionally unlike pET vectors that required specific bacterial host (BL21), pNP and pFNP vectors are expressed easily in any current *E. coli* host such as XLI-Blue and DH5 $\alpha$ .

pNPi and pFNPI vectors that PHB was cloned under *groE* promoter in them, require a heat shock at 42 °C for PHB expression. In this study produced PHB from these vectors was not significant. The reason of this problem may be relying on the fact that PHB should be produced after expression of 3 genes that each of them should be cods a functional enzyme. These 3 enzymes to have effective on acetylc-CoA substrate and PHB was produced after processing. When heat shock is applied, just these 3 enzymes can be produced and their influences on substrate are related to the stability of these three enzymes in bacterial cells. If one of 3 PHB producing enzyme was inactivate, PHB would not be produced. In the case of pET vector, IPTG presents in culture medium during incubation and induced PHB expression, but in the case of pNPi and pFNPI, heat shock cannot be applied for a long time during incubation. However using of pNPi and pFNPI vector requires optimizing of culture condition for PHB expression. pNP and pFNP don't need to any inducing agent or condition, and can be easily used for PHB production.

However PHA production in bacteria and yeast

requires growth under sterile condition in a costly fermentation process with an external energy source such as electricity. In contrast, PHA production in plant systems is considerably less expensive because the system only relies on water, soil nutrients, atmospheric CO<sub>2</sub> and sunlight. In addition, a plant production system is much more environmentally friendly. Plants use photosynthetically fixed CO<sub>2</sub> and water to generate the bioplastic, which after disposal is degraded back to CO<sub>2</sub> and water. Synthesis of PHAs in crops is also an excellent way of increasing the value of the crops<sup>[17, 21]</sup>. In this study we constructed 2 kind of plastid vector, pFNPI and pFNP, each one in two different model. These PHB constructs have potential to triggered PHB cassette to plastid genome and can be used for plant plastid transformation using gene gun in order to PHB expression in high level in plastid genomes. Chloroplast genetic engineering offers a number of unique advantages, including a high-level of transgene expression, multi-gene engineering in a single transformation event, transgene containment via maternal inheritance, lack of gene silencing, position and pleiotropic effects, and undesirable foreign DNA<sup>[5]</sup>. By using pFNP and pFNPI recombinant plasmid for plastid transformation, PHB can be produced as constitutive and inducible respectively. Induction of PHB from pFNPI would be possible by transformation of a hybrid sigma factor into plant nuclear that targeted to plastid by signal peptide. The kind of sigma factor promoter would determine PHB expression induction.

Expression of several genes along with optimization of PHA synthesis in the host is a need for bioplastic production. In this study we produced PHB bioplastic from recombinant *E. coli* for the first time in Iran. Future effort was needed in order to improving the properties of resulting bioplastic such as modification of monomers or production of co-polymer. However Although excellent progress has been made in recombinant hosts, the barriers to obtaining high quantities of PHA at low cost still remain to be solved. The commercially viable production of PHA in crops, however, appears to be a realistic goal for the future.

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