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Original Article

Cloning *phb*CABoperon of *Alcaligeneseutrophus* H16 into Escherichia coli DH5α to manufacture Poly 3-Hydroxybutyrate using molasses as carbon source

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ABSTRACT

In this study,a 4985 bp DNA fragment including the whole phbCAB operon from Alcaligeneseutrophus H16 was clonedinto Escherichia coli DH5α. This fragment contains three genes phbC, phbA, phbB encoding three enzymes P(3HB) polymerase, β-ketoacyl CoA thiolase and acetoacetyl-CoA dehydrogenase, respectively, which participate in PHB biosynthesis. This recombinant E. coli DH5α was performed batch fermentation in molasses pretreated with acid, 2.5g/l cell dried weight (CDW) and 22.5% PHB accumulation were obtained. This is the basis for future studies of optimization of PHB biosynthesis by recombinant bacteria using molasses ascarbon source.

1. Introduction

PHB (Poly 3-Hydroxybutyrate) is a biodegradable polymer which can be alternatives for traditional petroleum plastics[1]. Three enzymes participated in PHB biosynthesis. The first one is ketothiolase which condensates acyl-coA into alkanoacyl-coA.The second one, reductase, reduces alkanoalkyl-coA to hydroxyacyl-coA.AndPHBsynthase, the third one, polymerizes hydroxyacyl-coA into PHB products. In PHB synthesizing bacteria such as Alcaligenes latusandAlcaligenes eutrophus, these enzymes are coded by phbA, phbB andphbC genes, respectively, which occur in the same operon named phbCAB and are located by the order of phbC – phbA – phbB[2]. PHB production by natural organisms has several limitations, for example the PHB producers can hydrolyzed the accumulated polymer for their own uses. Moreover, these organisms themselves are hard to be disintegrated, cause significant difficulties and low efficiency in PHB recovery. Therefore, it is more beneficial to use recombinant Escherichia colias an alternative; the recombinant E. coli genome can be further refined to increase growth rate and utilize inexpensive nutrient sources[3].

The three A. eutrophusgenesphbA, phbBandphbChave been identified and clone intoE. colifor a long time[4,5,6]. The bacterium has no internal PHB hydrolysis enzyme, can achieve optimal cell concentration after24 – 48 h of cultivation and PHB accumulation up to 80 – 90 % of CDW,is able to utilize various kinds of substrate including molasses[7]hence it is an effective alternative for PHB manufacture.

In this study, the whole 4985bpphbCABoperon was amplified and then cloned intopCR®2.1 expression vector harboring bla (beta-lactamase) selecting gene and transferred into E. coli DH5α to evaluate PHB biosynthesis using molasses as carbon sources.

2. MATERIALS AND METHODS

2.1 Amplification of targeted genes

Genomic DNA of A. eutrophus NBRC 102504 (supplied by NBRC gen museum, Japan in freeze-dried form) was extracted following the instruction of Doyle and Doyle (1987)[8]. The 4985bp DNA fragment consisted of the whole 3851bpphbCAB operon, the 842bp upstream region harboring transcription regulation factors and the 292bp downstream region (also named as phbCAB-4985fragment) which could be amplified by forward and reverse primers harboring restriction sitesSmaIandHpaI, respectively. The primers (phbCAB-H16-F:CCCGGGCAAGTACCTTGCCGACATCand phbCAB-H16-R: GTTAACTGCAGCCTCGCCCCCGC) were designed by the authors of this study. Binding of the two SmaI andHpaI restriction enzymes will facilitate the extraction of targeted DNA from the vector in further studies.

Amplification of DNA was performed via MasterAmp™ Extra-Long PCR(MHF 9220, Epicenter)thermal cycler. The 5kb phbCAB

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operon underwent 3 minutes at 98°C, then 30 cycles of 15 seconds at 98°C, then 1 minute at 56°C, 5 minutes at 72°C, finally 10 minutes at 65°C. The reaction mixuture was then kept overnight at 4°C.

2.2 Construction of recombinant vector

PCR products of *phb*CAB operon pCR®2.1 vector (Invitrogen) were ligated by T4 DNA ligase enzyme at 16°C for 16 hours follow the instruction of the supply enterprise (15224-041, Invitrogen, U.S.).

2.3 Transfering gene into bacteria and assessment of gene incorporation

Transfering vector into competent cells was performed using CaCl2method[9]. To guarantee the successful of ligation targeted DNA into vector and vector transferinto targeted cell, eight intermediate primers scattered in vector and phbCAB operon were used to directly sequence with the bacterial DNA mould. Name, sequence and binding site of the primers are presented in Table 1.

Table 1: Name, sequence, binding sites of sequencing primers for phbCAB-4985 fragment

Name	Sequence (5' – 3')	Binding site
phb-CAB-H16-F1	GGCCCACCCGTGAAGGTGAGCC	On the vector, 100 bp apart from *phb*CAB-4985 insertion
phbCAB-H16-F2	GCGCGTGCGTTGCAAGGCAACAATGG	539th position of *phb*CAB-4985 fragment
phbCAB-H16-F3	CACCAACCTCCCATATCGCTTCG	1161st position of *phb*CAB-4985 fragment
phbCAB-H16-F4	CCAGGACAAGATCAACGTGCTC	1770th position of *phb*CAB-4985 fragment
phbCAB-H16-F5	GCAGCCACTGGACTAACGATGCGCTGCC	2404th position of *phb*CAB-4985 fragment
phbCAB-H16-F6	GCACGTGCTGCCGGGCTCGCGCGAT	3062nd position of *phb*CAB-4985 fragment
phbCAB-H16-F7	GACACCTCCAAGGTCAATGTGAACG	3696th position of *phb*CAB-4985 fragment
phbCAB-H16-F8	GCGACGTGGTGTTCCGCAAGATGACC	4231st position of *phb*CAB-4985 fragment

2.4 Cultivation of recombinant cells

Cells of recombinantE. coli DH5α harboring vector which consisted ofphbCAB-4985fragment (abbreviated as E. coli DH5α phbCAB-4985) were cultivated in LB medium supplemented by 100 µg/mL ampicillin at 37°C and 150rpm of shaking until optical density achieved maximum level. Centrifuged the cultivation liquid at 5000 rpm for 10 minutes and harvested the cell residue.

2.5 Identification of Poly 3-Hydroxybutyrate producing cells usingSudan Black B and Nile Blue A stains

Harvested E. coli DH5α phbCAB-4985 cells after cultivation and inoculated (using streaking technique) into screening medium (LB supplemented by ampicillin). Incubated the cells overnight until bacterial colonies became visible. Air-dried the cell samples and stained with 3% Sudan Black B solution (Sigma) for 10 minutes, then washed and air-dried the products. Stained samples were stain again with 5% Safranin solution (Sigma) for 10 seconds, then washed by distilled water, dried and observed. The samples were also put on microscopic slides and stained similarly to be observed via oil immersed lens (X100)[10].

RecombinantE. coli DH5α cells were also stained with Nile Blue A to identify PHB granules since the granules exhibits orange fluorescence under 460nm wavelength[11]. Spread the cells on microscopic slides then stained with1% Nile blue A at 55°C for 10 minutes. Washed the slides with distilled water and then with 8% acetic acid for 1 minute. The slides were washed again with distilled water and dried by blotting paper. For observation, covered the stains by microscopic slides and observed under fluorescence microscope at 460nm wavelength[12].

2.6 Acid treatment of molasses

Molasses underwent pre-treatment with concentrated sulfuric acid as described by Liuet al. (1998)[13]. The treated molasses was analyzed at Center of Quality Measurement No. 3 for the assessment of basic properties includingtotal amount of carbohydrates measured in glucose, proteins, Na, Ca, K, P, S, and Pb.

2.7 Cultivation of recombinant E. coli phbCAB-4985 in molasses medium for PHB

RecombinantE. coli DH5α phbCAB-4985 was cultivated in 150ml treated molasses medium (40g/l) contained in conical flask and supplemented with mineral nutrients at 37°C and 150rpm of shaking. Supplemental mineral nutrients consisted of (per liter) 4g (NH4)2SO_4, 13.3g KH_2PO_4, 1.2g $MgSO_4.7H_2O$, 1.7g citric acid and 1ml of micronutrients. The micronutrient solution contained (per liter) 10g $FeSO_4.7H_2O$, 2.25g $ZnSO_4.7H_2O$, 1g $CuSO_4.5H_2O$, 0.5g $MnSO_4.4H_2O$, 2g CaCl2.$2H_2O$, 0.23g $Na_2B_4O_7.10H_2O$, 0.1g (NH4-)$_6M_{o7O24}$,10 mL HCl 35 %[13].

Cultivated RecombinantE. coli DH5α for 24 – 48 hours, then centrifuged at 5000rpm for 10 minutes. Harvested fresh cell mass was treated with distilled water then air-dried at 90oCfor 24 hours.

2.8 Extraction of PHB from cell dried weight (CDW)

Treated CDW with 30% NaClO and chloroform (at 1:1 ratio). The mixture was stirred and incubated at3^{0}C for 90 minutes, then was centrifuged at 4000 rpm and 30^{o}C for 15 minutes. Harvested the chloroform phase and treated with methanol solution (7 methanol : 3 H_2O), the amount of methanol was equal to 10% of used chloroform.Air-dried the samples in 48 hours for evaporation of excessed products. The remaining white powder was PHB[14].

2.9 Quantification of PHB by optical density (OD)

PHBwas quantified via brown-color crotonic acid after hydrolyzed by hot and concentratedH_2SO_4at 235 nm wavelength; then constructed a directrix using obtained data. Mixed 100 mgPHB into 10 mL hot concentrated H_2SO_4,and then boiled in water bath for 10 minutes so that PHB transformed into 10mg/ml crotonic acid. Dilated the solution with concentrated H_2SO_4at different concentrations and measured the absorption spectrum at 235 nm. Constructed a linear correlation directrix between OD235and PHB concentration[15].

2.10Data processing

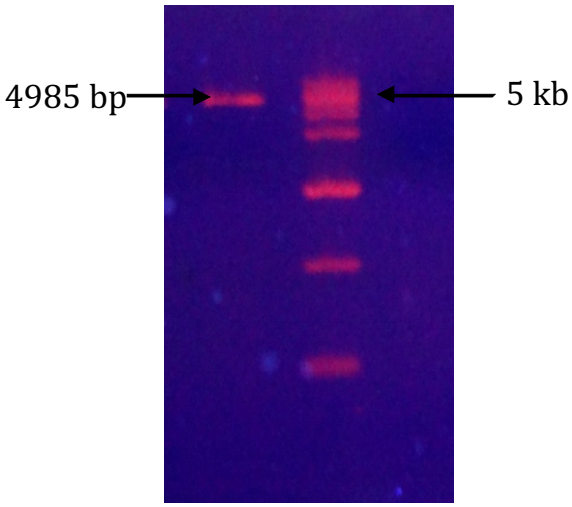
Used Microsoft Excel 2010 and Minitab 17.0software to processed the dataand calculate means value and standard error

3. RESULTS

3.1 Amplification of 4985pb phbCABoperon

The 4985bpA. eutrophus (AM260479.1) genome sequence from position 1.556.511 to 1.561.495harbors the whole 3852bpphbCABoperon and the 842 bp upstream regions containing transcription regulation factor and 292bp downstream region which was chosen to be amplified. PCR results shown an approximately 5kb product identified on agarose gel(Figure 1).

Figure 1:PCR product of phbCAB-4985fragment (left lane)



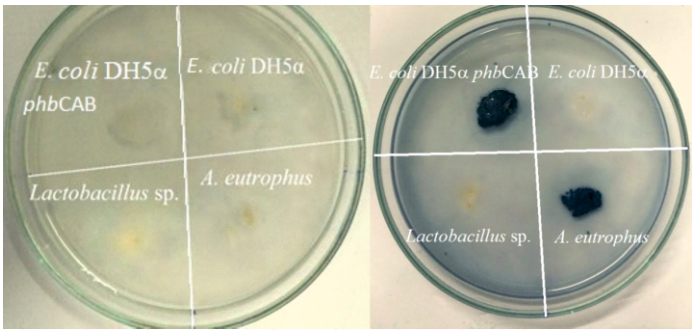
ThephbCABoperon was amplified using the two primers harboring two restriction sites could recognize the SmaIandHpaIenzymes, which can facilitate the extraction of targeted DNA from and the cloning of DNA product into vector in further studies.

TheE. coli DH5α phbCAB-4985 cells were cultivated in LB medium supplemented withampicillin for 24 hours, then were harvested for checking the presence of phbCAB-4985 region by sequencing. Sequencing primers was designed to be 600 – 700 bp apart from each other for the obtained sequences to achieve high reliability, overlap the other ones and cover the whole 5000bp of phbCAB-4985 fragment and the upper and lower ends of the vector. All the eight primers generated satisfying products with the length of 681, 764, 770, 768, 697, 812, 803 and 746 bp, respectively. Results of checking via BLAST showed 100% similarity to A. eutrophusphbCAB region available onGenBank. The products of first and eight primers (phbCAB-H16-F1 and phbCAB-H16-F8, respectively) contained a 32bp sector at upstream region and a 54 bp sector at downstream region, which matched the upper and lower ends of the vector, respectively. All these proved the successful cloning of 4985bpphbCABregion into pCR®2.1 vector.

3.2 Identification of PHB in E. coli DH5α using Sudan Black B and Nile Blue A stains

Besides sequencing phbCAB-4985 region to verify the success of recombination, observation of PHB granules in recombinant cells was also performed using Sudan Black B and Nile Blue A stains for assessment of transcription and PHB biosynthesis in recombinant E. coli DH5α.A. eutrophusand wild-type E. coli DH5α were used as positive and negative controls, respectively. PHB granules in Sudan Black B stained recombinant E. coliwere observed via optical microscope. Lipid inclusion bodies were stained by Sudan Black B and obtained grayish-blue colour, whilethe cytoplasm obtained dark blue colour(Figure 2).

Figure 2: E. coli DH5α phbCAB-4985 colonies before and after stained with Sudan Black



Furthermore, also to verify the presence of PHB, 1ml recombinant bacteria was stained with Nile blue A and observed via fluorescence microscope at 460nm wavelength and 60X magnification. The stain exhibited bright orange colourwhich proved the presence of PHB granules in bacterial cells(Figure 3).

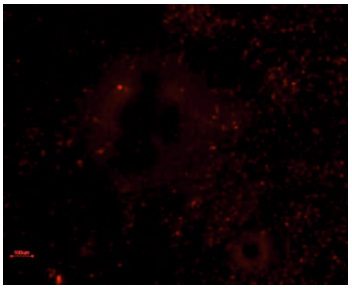


Figure 3: PHB granules in E. coli DH5α phbCAB-4985 cells stained with Nile blue A

These results proved successful expression of 4985bpphbCABfragment in E. coli DH5α cells.

3.3 Evaluation of CDW and PHB accumulation of recombinant E. coli DH5α harboring phbCAB-4985 using molasses as substrate E. coli DH5α phbCAB-4985 was grown in a cultivation system using 8 conical flasks, each contained 150 ml molasses medium.CDW yields amounted to 2.5g/l with 22.5% (w/w) PHB (Figure 4), only equal to 40 – 50% of the ones obtained by Liuet al. (1998)[13]. Nonetheless, this study only aimed to verify the growth and PHB accumulation of E. coli DH5α phbCAB-4985 in molasses medium. Optimization of PHB biosynthesis will be performed in further experiments to achieve similar yields to other studies[13].

Figure 4: PHBPowder from E. coli DH5α phbCAB-4985 cultivated in molasses medium

4DISCUSSIONS

Previous studies already detailed the PHB biosynthesis and accumulation of A. eutrophus, however utilization of this bacterium meet with several limitations.Therefore, many studies tried to develop recombinant alternatives to overcome the obstacles caused by natural bacteria. Various reports used PHB genes fromA. eutrophusandA. latusin recombinant E. colifor PHB production[4,5,6,13,16,17]. The A. eutrophus3851bp operon harboringphbC, phbAandphbBgenes was identified and cloned using the technique of identification of restriction fragments. These genes – as a result – consist of predetermined restriction sites at both ends and are contained in plasmids for convenience in sharing amongst research groups. However, junior research groups like many in Vietnam may find it harder to access the genetic materials of the other ones.

Amongst various approaches in genetic engineering, long range PCRtechnique[18] enables rapid harvest of large DNA fragment (up to 40kb) with high accuracy and acceptable cost. This study correspondingly selected this approach instead of classical one performed by Parket al. (1995a, 1995b and 1995c)[4,5,6] to amplify and obtain the whole DNA fragment harboring phbCABoperon before cloning into E. coli DH5 α for PHB synthesis.

Concurrent with this study, Jariet al. (2015)[19]published a similar work using the same aforementioned approach. Yet Jariet al. (2015) only amplified the 3851bp DNAofphbCABoperon before cloning into expression vector. This study instead used a 4985bp one including phbCAB operon and regulation fragments at upstream and downstream to guarantee the successful expression of the operon in vector. Moreover, DNA sequencing was implemented to assess the success of inserted fragment used in place of traditional double digestmethod[4].

There have been attempts to reduce production cost by means of bioprocess design or modification of the metabolic pathway[3]. This study aimed to set the foundation or further researches in utilization of molasses as an inexpensive and economical substrate for PHB biosynthesis using recombinant E. coli DH5α harboringphbCAB operon.

Acid-treated molasses obtained in Ho Chi Minh Citycontained 14.4 – 18.5g/100gcarbohydrates measured in glucose, 2g/100g of protein. The mineral nutrients content was relatively high, about 137 – 164 mg/kg (P), 1530 – 1658 mg/kg (Na),4314 – 4375 mg/kg (Ca) and 5731 – 5946 mg/kg (K). On the contrary, there was only 0.1g/100g S and no Pbdetected. Therefore, molasses content of 40g/l (corresponding with 5.76– 7.2 g/l of carbohydrates measured in glucose)was chosen to cultivate E. coli DH5α phbCAB-4985for preliminary evaluation of PHB accumulation. CDW yield (2.5g/l) and PHB accumulation (22.5% of CDW) were consistent with previous reports[13].

This study demonstrated functional activities of recombinant E. coli DH5α harboring phbCABoperon via cell growth and PHB accumulation. PHB accumulation was still low (only equal to 30% of the previous experiments) yet it provedthe direction of the studyand served as basis for further optimization of PHB biosynthesis – the stage which Jariet al. (2015) haven't reached yet.

5. CONCLUSIONS

4985bp DNA fragments harboring phbCAB operon was isolated, amplified and cloned into pCR®2.1 vector. The recombinant vector was successfully transferred into E. coli DH5α cells for PHB biosynthesis using acid-treated molasses. Growth and PHB accumulationof E. coliwere evaluated. Although CDW yields and PHB accumulation were still low (~ 20 %) compared with other published studies, it can served as basis for further studiescentered on optimization of fermentation conditions to implement this process at industrial scale.

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REFERENCES

1. Amass W, Amass A, Tighe B. A review of biodegradable polymers: uses, current developments in the synthesis and characterization of biodegradable polyesters, blends of biodegradable polymers and recent advances in biodegradation studies. PolymInt. 1998;47(2):89-144.
2. Fidler S, Dennis D. Polyhydroxyalkanoate production in recombinant Escherichia coli. FEMS Microbiol Rev. 1992; 9(2-4):231-235.
3. Chee J-Y, Yoga S-S, Lau N-S, Ling S-C, Abed RM, Sudesh K. Bacterially produced polyhydroxyalkanoate (PHA): converting renewable resources into bioplastics. CurrResTechnolEduc Top ApplMicrobiolBiotechnol.2010; 2:1395-1404.
4. Park H-C, Lim K-J, Park J-S, Lee Y-H, Huh T-L. High frequency transformation of Alcaligeneseutrophus producing poly-β-hydroxybutyric acid by electroporation.Biotechnol Tech. 1995a; 9(1):31-34.

5. Park H-C, Park J-S, Lee Y-H, Huh T-L. Manipulation of the genes for poly-β-hydroxybutyric acid synthesis in Alcaligeneseutrophus.BiotechnolLett. 1995b; 17(7):729-734

6. Park J-S, Park H-C, Huh T-L, Lee Y-H. Production of poly-β-hydroxybutyrate by Alcaligeneseutrophustransformantsharbouring cloned phbCAB genes. BiotechnolLett. 1995c; 17(7):735-740.

7. Liu Q, Ouyang S-P, Chung A, Wu Q, Chen G-Q.Microbial production of R-3-hydroxybutyric acid by recombinant E. coli harboring genes of phbA, phbB, and tesB.ApplMicrobiolBiotechnol.2007; 76(4):811-818.

8. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue.Phytochem Bull1987; 19:11-15.

9. Green MR, Sambrook J. Molecular cloning: a laboratory manual. 4th ed. Cold Spring Harbor, New York, Cold Spring Harbor Lab Press,2012.

10. Hartman T. The use of Sudan Black B as a bacterial fat stain. Stain Technol. 1940;15(1):23-28.

11. Tokiwa Y, Ugwu CU. Biotechnological production of (R)-3-hydroxybutyric acid monomer.JBiotechnol.2007; 132(3):264-272.

12. Ostle AG, Holt J. Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. ApplEnviron Microbiol. 1982; 44(1):238-241.

13. Liu F, Li W, Ridgway D, Gu T, Shen Z. Production of poly-β-hydroxybutyrate on molasses by recombinant Escherichia coli. BiotechnolLett.1998; 20(4):345-348.

14. El-Sayed AA, Abdel Hafez A, Abdelhady M, Khodair T. Production of polyhydroxybutyrate (PHB) using batch and two-stage batch culture strategies. Aust J Basic Appl Sci. 2009;3(2):617-627.

15. Law JH, Slepecky RA. Assay of poly-β-hydroxybutyric acid. JBacteriol.1961; 82(1):33-36.

16. Chien C-C, Hong C-C, Soo P-C, Wei Y-H, Chen S-Y, Cheng M-L, Sun Y-M. Functional expression of phaCAB genes from Cupriavidustaiwanensis strain 184 in Escherichia coli for polyhydroxybutyrate production. ApplBiochemBiotechnol.2010; 162(8):2355-2364.

17. Choi J-I, Lee SY, Han K. Cloning of the Alcaligeneslatuspolyhydroxyalkanoate biosynthesis genes and use of these genes for enhanced production of poly (3-hydroxybutyrate) in Escherichia coli. Appl EnvironMicrobiol. 1998;64(12):4897-4903.

18. Wilton S. Long-range PCR.eLS. 2002.

19. Jari M, Khatami SR, Galehdari H, Shafiei M. Cloning and Expression of Poly 3-Hydroxybutyrate Operon Into Escherichia coli. JundishapurJ Microbiol.2015; 8(2).

20. Gorenflo V, Schmack G, Vogel R, Steinbüchel A. Development of a process for the biotechnological large-scale production of 4-hydroxyvalerate-containing polyesters and characterization of their physical and mechanical properties. Biomacromolecules.2001; 2(1):45-57.

21. Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, Eitinger T, Ewering C, Pötter M, Schwartz E. Genome sequence of the bioplastic-producing "Knallgas" bacterium Ralstoniaeutropha H16. Nature Biotechnol. 2006;24(10):1257-1262.