

Cloning of acetyl-CoA acetyltransferase gene from *Halomonas elongata* BK-AG18 and *in silico* analysis of its gene product

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ABSTRACT Polyhydroxybutyrate (PHB) is a biodegradable polymer that can be used as a substitute for petrochemical plastics. Bacteria accumulate PHB in their cells as carbon and energy reserves because of unbalanced growth conditions. This study aimed to amplify *phbA* from the chromosomal DNA of *Halomonas elongata* BK-AG18, a PHB-producing bacterium that was previously isolated from the Bledug Kuwu mud crater of Central Java, Indonesia. The obtained *phbA* amplicon was 1176 bp. This fragment was cloned into a pGEM-T Easy cloning vector and used to transform *Escherichia coli* TOP10. The recombinant colonies were selected using blue-white screening, confirmed by size screening, reconfirmed by re-PCR, and sequenced. When putative *phbA* sequences were aligned with *H. elongata* DSM2581 chromosome using BLASTN, this sequence showed 99% identity. The deduced amino acid sequences of this clone showed 100% identity to PhbA of *H. elongata* DSM2581, suggesting that the obtained cloned fragment is a *phbA* gene. The 3D structure predicted by I-TASSER showed that PhbA of *H. elongata* BK-AG18 had a high similarity to the acetyl CoA acetyltransferase structure of *Ralstonia eutropha* H16. PhbA of *H. elongata* BK-AG18 possesses three catalytic residues, namely Cys88, His348, and Cys378.

KEYWORDS acetyl-CoA acetyltransferase; *Halomonas elongata* BK-AG18; polyhydroxybutyrate

1. Introduction

Plastic materials are widely used for various human activities. This massive plastic usage has caused serious environmental problems because of their non-biodegradability (Singh 2014). Common plastics are manufactured from non-renewable fossil fuels and their increasing demand will speed up fossil fuel depletion (Colwill et al. 2012). Therefore, the alternative and more environmentally friendly plastics are needed. Polyhydroxyalkanoates (PHAs) are biodegradable polymers that can be used as plastics, synthesized and accumulated by bacteria as intracellular carbon storage granules in response to nutrient limitations (Dietrich et al. 2013). Polyhydroxybutyrate (PHB) is the most common type of PHA that accumulating in bacteria and archaeal cells (Muangsuwan et al. 2015).

The biosynthetic pathway of PHB in bacteria includes three reactions catalyzed by three different enzymes, namely acetyl-CoA acetyltransferase, beta-ketoacyl-ACP reductase, and PHB polymerase. The acetyl-CoA acetyltransferase, encoded by *phbA* gene, catalyzes the condensation of acetyl-CoA molecules to produce acetoacetyl-CoA molecule. Beta-ketoacyl-ACP reductase, encoded by *phbB* gene, reduces acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA. On the other hand, PHB poly-

merase, encoded by *phbC* gene, polymerizes the (R)-3-hydroxybutyryl-CoA monomers to form PHB (Jari et al. 2015). *Halomonas elongata* BK-AG18 isolated from Bledug Kuwu, Central Java, Indonesia was known to produce detectable PHB (Hertadi et al. 2017). In this study, the *phbA* gene was isolated from this strain and cloned into a pGEM-T Easy vector in *Escherichia coli* TOP10.

2. Materials and methods

2.1. Bacterial strain, plasmid, and chemicals

H. elongata BK-AG18 local strain was used as a source of chromosomal DNA. *E. coli* TOP10 was used as the host strain for gene cloning. These bacteria were obtained from the Biochemistry Laboratory of the Institut Teknologi Bandung. The pGEM-T Easy (Promega) was used as cloning vector. Oligonucleotide primers were ordered from Macrogen, South Korea. KAPA Taq Ready Mix PCR Kit, T4 DNA ligase and DNA ladder were purchased from Promega. Sodium chloride from Amresco and yeast extract, tryptone, bacto agar from Deben Diagnostics Ltd. were used as Luria-Bertani medium's ingredients. Ampicillin and tetracyclin from Bioline were used as antibiotics to screen a recombinant clone. Isopropyl-

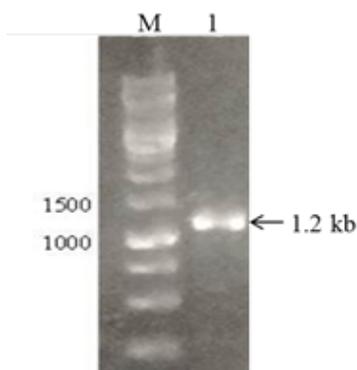


FIGURE 1 Electropherogram of PCR result. M: Generuler 1 kb DNA Ladder, 1: *phbA* amplicon. The obtained amplicon size was approximately 1.2kb which is similar to the size of the *phbA* gene from *H. elongata* DSM2581 from GenBank.

β -D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indoyl- β -D-galactoside from Promega were also used to screen positive recombinant clones.

2.2. Primer design

The *phbA* primers were designed based on *phbA* full nucleotide sequences from *H. elongata* DSM2581 in GenBank database by the authors. Some forward and reverse primer candidates were designed and the parameters of % GC, T_m , the absence of hairpin, dimer, and loop structure were analyzed using Oligo Analyzer. Fast PCR was performed to see the mispriming probability.

2.3. Culturing condition, genomic DNA isolation and amplification of *phbA* gene

H. elongata BK-AG18 was grown in a modified high glucose medium (HM) as described by Hertadi et al. (2017). Chromosomal DNA of *H. elongata* BK-AG18 was isolated using Klijn method (Klijn et al. 1991). Amplification of *phbA* gene was carried out by PCR by the following primers: *phbAF*(5'-ATGTCAGAAGTGATCGTC-3') as forward primer and *phbAR* (5'-TCAGCGCTCGATGGCCAG-3') as re-

verse primer. PCR were conducted on an automatic thermal cycler (BIORAD) for one cycle predenaturation at 95°C for 4 min, 34 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 90 s. The reaction was completed by final extension at 72°C for 5 min. The *phbA* amplicon was confirmed by electrophoresis on 1% (w/v) agarose gel.

2.4. Cloning and recombinant clone selection

The amplicon of *phbA* gene was ligated into pGEM-T Easy using T4 DNA ligase and transformed into competent *E.coli* TOP10 cells using heat shock method (Sambrook and Russell 2001). The transformants were blue-white selected on Luria Bertani solid medium supplemented with 100 μ g/ml ampicillin, 20 μ g/ml X-gal, and 0.1 M IPTG. The recombinant plasmids were confirmed by size screening and re-PCR. Recombinant plasmids DNA were isolated from the transformants with Geneaid High Speed Plasmid Mini Kit (Geneaid Biotech Ltd., Taiwan).

2.5. Sequencing of *phbA* gene

Determination of *phbA* nucleotide sequences was performed by 1stBASE, Malaysia with dye terminator dideoxy Sanger Method using universal primer of T7 and SP6 promoter.

2.6. Catalytic residues and three dimensional structure prediction of PhbA

PhbA amino acid sequence was deduced from *phbA* gene sequences. Prediction of PhbA catalytic residues was performed using Sequence Annotated by Structure (SAS) and Catalytic Site Atlas (CSA). On the other hand, the prediction of PhbA 3D structure was conducted using I-TASSER.

3. Results and discussion

3.1. Chromosome isolation and gene amplification

The chromosome of *H. elongata* BK-AG18 was successfully isolated. Using this chromosome and the designed

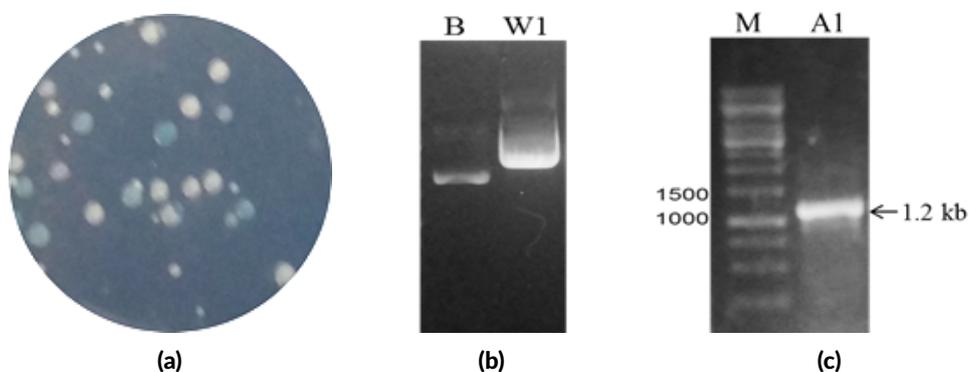


FIGURE 2 Selection of recombinant clones. (a) Blue-white screening of *E.coli* TOP10 transformants, (b) Size screening of recombinant clones, B: plasmid from blue colony, W1: recombinant plasmid from white colony. (c) re-PCR of *phbA* gene in pGEM-*phbA*, M: Generuler 1 kb DNA Ladder, A1: *phbA* amplicon from W1.

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10      20      30      40      50      60      70      80      90
ATGTCCAGATGGTGTATGTCCTCCGCGCCAGCCACCGCCCTCGCGCGCTCTCCGCGCGATTCGCCAGAGCCATCGGCGCCAGCAGTCA
HSEVIVIAARRRTAVGAFGGGSLAAGIIPASDLAGAHVZ
100
TCAGGACATTCGCGCCAGCCGGGTGGCCGCGACAGTGTATGAGTCTGCTCCGCGCAGGTCTGACAGCCGCGCCAGGATCGCGCCG
KDLIAGTGVAAADQVDEVLLVLLGQVLTAGTGTQGNPAA
200
CCAGGCTCCTCAGGCGGAGCTCCGCGCGCCGATCCGCGCCATGACCATCAACAGGCTCGCGTTCGCGCTCAAGGCTCGACTCGCCAG
QASIRKAGLPEAVPAPARTINRVCVSGSLKALHLATQ
300
GGATTCGCGCGCGGCTCGCGCGGAGGAGGATGCTCGCGCCATGCTCGCGCCATGCTCGCGCTCGCGCGGATCGCGCGGAGGAG
AIRCGDAELIILAGGQENHSLSPHVLPNRSRNDQRH
400
TGGGCGATCGAGGCGCATGCGCTCATGTCGCGCGCGCTGTGGATGCTTCAGCGATCCAGCGATCGCGCGGAGGAGGAGGAGGAGG
SDMRAIDSMVHDGLMDAFNDVYHMGITAEENLAAK
500
GTACGGCTACCCCGCGGCGCATGGACGATTCGCGCGCGCTCCGCGCGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
YGITRQKQDPTAGNASHALNDGAAVAVVHMCSDAEKAAEL
600
GGCATCGACATTCGCGCGCGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
GIDIPQRKQDPTAGNASHALNDGAAVAVVHMCSDAEKAAEL
700
CCTTCAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
EKEDQSDVTAQNAASHALNDGAAVAVVHMCSDAEKAAEL
800
CGGCTCCTCAGGCGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
GLTIPLEAASDVAARASDSDKAAASIREGRKQIV
900
GAAGCGCGCGGCGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
ERAGMSLDELDELLEANEAFAPAAQAALSVYKLELDWDT
1000
CCGACAGGCTCAAGCTCAAGCGCGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
DKVNVNNGGAAIALGHPFIGAASGCRILVTLVHERIA
1100
CCGAGGCGCCAGGCGGCTCGCGCGCGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
RDAHKKGLLATLCTGGGGGQVLAATER*
    
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FIGURE 3 The nucleotide sequences of *phbA* gene and its deduced amino acid sequences.

primers, the performed PCR reaction has produced a *phbA* gene as shown by a single band of the amplicon on agarose gel electrophoresis (Figure 1).

3.2. Cloning and recombinant clone selection

The 1.2 kb obtained amplicon was inserted into the pGEM-T Easy vector and transformed into *E.coli* TOP10. The transformants will survive in LB-ampicillin medium, and the recombinant clones will appear as white colonies. On the other hand, the transformants with plasmid without insert will appear as blue colonies (Figure 2a). The positive recombinant clones were confirmed by size screening (Figure 2b) and re-PCR (Figure 2c). As presented in Figure 2b and 2c, the recombinant plasmid has larger size compared to the non-recombinant plasmid and the re-PCR produces 1.2 kb fragment as expected.

3.3. Sequencing of *phbA* gene in pGEM-*phbA*

The sequence of 1176 bp *phbA* gene and its deduced amino acids is shown in Figure 3. The BLASTN analysis indicated that this *phbA* gene showed 99% identity with the whole genome of *H. elongata* DSM2581 (Figure 4a). The BLASTP analysis on the deduced amino acid of this gene, PhbA, showed 100% identity with acetyl-CoA acetyltrans-

ferase from *H. elongata* DSM2581 (Figure 4b).

Literature studies stated that PhbA super family contains three catalytic residues: Cys89, His348, and Cys378 as shown in *Zooglea ramigera* (Modis and Wierenga 2000).The PhbA-deduced amino acids from *H. elongata* BK-AG18 also has Cys88, His348, and Cys378, confirming that the isolated gene is *phbA*. The PhbA 3D structure predicted by I-TASSER showed 68.72% identity to acetyl-CoA acetyltransferase structure from *Ralstonia eutropha* H16 (RePhbA). The modelling 3D structure of PhbA predicted by I-TASSER with its catalytic residues can be seen in Figure 5.

In RePhbA, Cys89 has a function as covalent catalyst, His349 as general base, while Cys378 was a second nucleophile (Kim and Kim 2014). The superposition showed that Cys88, His348 and Cys379 residues in our PhbA structure have the same positions with Cys89, His349 and Cys378 from RePhbA.

4. Conclusions

The designed primers had successfully amplified *phbA* gene that code acetyl-CoA acetyltransferase from *H. elongata* BK-AG18 chromosomal DNA. This *phbA* gene showed 99% identity to the whole genome of *H. elongata* DSM2581. The deduced amino acid sequences of this gene showed 100% identity with acetyl-CoA acetyltransferase of *H. elongata* DSM2581.The three amino acid on PhbA catalytic sites are Cys88, His348, and Cys378 which is similar to the PhbA catalytic sites of *Zooglea ramigera*, confirming that the *phbA* gene isolated is coding for PhbA protein superfamily.

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| Description | Query cover | E value | Ident | Accession |
|--|-------------|---------|-------|------------|
| Halomonas elongata DSM 2581, complete genome | 100% | 0.0 | 99% | FN869568.2 |
| Halolamina sediminis strain Hb3, complete genome | 99% | 0.0 | 88% | CP018139.1 |
| Halomonas sp. 1513, complete genome | 99% | 0.0 | 85% | CP019326.1 |
| Halomonas chromatireducens strain AGD-8-3, complete genome | 98% | 0.0 | 85% | CP014226.1 |
| Cobetia marina strain JCM 21022, complete genome | 99% | 0.0 | 83% | CP017114.1 |
| Halomonas sp. R57-5 genome assembly HalomonasR57-5, chromosome : 1 | 99% | 0.0 | 81% | LN813019.1 |
| Halomonas sp. KO116, complete genome | 98% | 0.0 | 81% | CP011052.1 |

(a)

| Description | Query cover | E value | Ident | Accession |
|--|-------------|---------|-------|----------------|
| acetyl-CoA acetyltransferase [Halomonas elongata DSM 2581] | 100% | 0.0 | 100% | CBV44015.1 |
| MULTISPECIES: acetyl-CoA acetyltransferase [Halomonas] | 100% | 0.0 | 98% | WP_064702380.1 |
| acetyl-CoA acetyltransferase [Halomonas sinaiensis] | 100% | 0.0 | 98% | WP_064700538.1 |
| acetyl-CoA acetyltransferase [Halomonas smymensis] | 100% | 0.0 | 91% | WP_016853970.1 |
| acetyl-CoA acetyltransferase [Halomonas shengliensis] | 100% | 0.0 | 92% | SDQ45355.1 |
| acetyl-CoA acetyltransferase [Halomonas sp. PRN3] | 100% | 0.0 | 91% | WP_023005010.1 |
| acetyl-CoA acetyltransferase [Halomonas sp. HG01] | 100% | 0.0 | 90% | WP_046079367.1 |
| acetyl-CoA acetyltransferase [Halomonas salina] | 100% | 0.0 | 90% | WP_035592997.1 |

(b)

FIGURE 4 (a) The BLASTN result of the *phbA* gene and (b) the BLASTP result of PhbA.

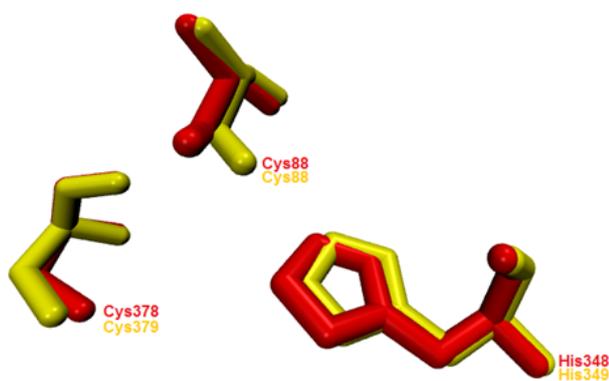


FIGURE 5 Prediction of PhbA 3D structure using I-TASSER. Residues in red color are PhbA and yellow are RePhbA.

Authors' contributions

RH designed the study. Molecular cloning strategy was led by ER. NPY carried out the laboratory work. ER, RH, NPY analyzed the data and prepared the manuscript. All authors read and approved the final version of manuscript.

Competing interests

The authors declare no competing interest.

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