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Cloning, Expression, and *In Silico* Analysis of Class IV Poly-(*R*)-3-hydroxybutyrate Genes from New Strain of *Bacillus thuringiensis* TH-01

Zuhdina Sabiqoh, Rukman Hertadi, Enny Ratnaningsih*

Biochemistry Research Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Bandung, Indonesia

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ABSTRACT

Poly-(*R*)-3-hydroxybutyrate (PHB) is a bioplastic derivative of polyhydroxyalkanoate (PHA) which can be synthesized by bacteria under certain growth conditions. Previous study has reported a new strain of *Bacillus thuringiensis* TH-01 isolated from thermite, which found to accumulate PHB. This research aimed to clone PHB biosynthesis genes from *B. thuringiensis* TH-01 and study its expression as well as predict the tertiary structure of the enzymes. The clone of *pha*A gene, which encodes PhaA, was obtained as 1182 bp. On the other hand, 2546 bp clone of *pha*RBC gene cluster was obtained to consist of 744 bp *pha*B, 1086 bp *pha*C, and 483 bp *pha*R, encoding respective PhaB, PhaC, and PhaR proteins. *In silico* analysis indicated that PhaA, PhaB, PhaC, and PhaR, revealed to have 393, 247, 361, and 160 amino acid, respectively. The predicted model of PhaA, PhaB, and PhaC showed dominant structure of α/β folding motif, while PhaR was dominated by a helix-loop-helix motif. The catalytic residues of PhaA were Cys88, His349, and Cys379, whereas the catalytic residues of PhaB were Ser142, Tyr155, and Lys159. These catalytic residues were identical to those residues obtained in other PHB biosynthetic enzymes reported elsewhere, confirming that our clones were of PHB biosynthetic genes.

1. Introduction

The development of human understanding in nature sustainability increases the need for the use of sustainable materials. Poly-(*R*)-3-hydroxybutyrate (PHB) as biodegradable thermoplastic is one of sustainable materials that is expected to be a substituent of conventional petroleum-based plastics (Sharma 2019). Various studies revealed that PHB possessed much wider application compared to the scope of petroleum-based plastics (Ray and Kalia 2017). These findings further increase the demand of PHB but seem to have encountered challenges in its production, particularly from bacteria (Markets and Markets 2019, Polyhydroxyalkanoate (PHA) market).

Recombinant DNA technology is considered as the most powerful tool to increase the production of various enzymes involved in biomaterials production, including PHB (El Rabey *et al.* 2017). One of the concerns in this approach was the selection of targeted genes sources. In this case, wild type bacteria that able to produce high amount of PHB are potential candidates to be used as PHB biosynthetic genes sources. Previous report had identified a new strain of *Bacillus thuringiensis* TH-01 that was accumulate 10.5% (w/w of dry cell) of PHB (Rizki *et al.* 2021). PHB production could be further improve by cloning the PHB biosynthetic genes from this strain into a particular expression vector regulated under a very strong promoter.

In bacteria, PHB biosynthesis begins with the uptake of carbon sources from the environment into the cells. These compounds were then converted into acetyl-CoA through glycolysis and followed by three stages of secondary metabolism involving three different enzymes, known as acetoacetyl-CoA acetyltransferase (PhaA) that catalyzes the condensation of two molecules of acetyl-CoA into acetoacetyl-CoA, acetoacetyl-CoA reductase (PhaB) that catalyzes the reduction of acetoacetyl-CoA to produce (*R*)-3-hydroxybutyryl-CoA, and PHA synthase that catalyzes the polymerization of (*R*)-3-hydroxybutyryl-CoA to poly-(*R*)-3-hydroxybutyrate (Tsuge *et al.* 2015). PHA synthase is classified into four types of proteins (Chek *et al.* 2017). Type I which is PhaC, type III that consists of PhaC and PhaE

* Corresponding Author
E-mail Address: ennyratnaningsih1958@gmail.com

subunits, and type IV that comprises of PhaC and PhaR catalyzes the polymerization of C3-C5 substrates. On the other hand, type II is a dimeric protein of PhaC1 and PhaC2 subunits, catalyzes the polymerization of C6-C14 substrates. *Bacillus* strains, including *B. thuringiensis* TH-01, was identified as having type IV PHA synthase, therefore PHB synthesis in this strain would involve PhaA, PhaB, PhaC, and PhaR (McCool and Cannon 2001), suggested that *B. thuringiensis* TH-01 possesses *pha*A, *pha*B, *pha*C, and *pha*R genes. This study aimed to clone these four PHB biosynthetic genes from *B. thuringiensis* TH-01 into pET-30a (+) and express them in *Escherichia coli* BL21(DE3) host.

2. Materials and Methods

2.1. Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study were listed in Table 1. *B. thuringiensis* TH-01 was the courtesy from Prof. Gomathi Velu (Departement of Agricultural Microbiology, Tamil Nadu Agricultural University, India), which was then kept and grown in Biochemistry Laboratory, Chemistry Department, Faculty of Mathematics and Natural Sciences ITB. In our previous study, the PHB production from this wild type bacterium has been confirmed and the PHB has been characterized (Rizky *et al.* 2021).

2.2. Chromosomal DNA Isolation

The chromosomal DNA of *B. thuringiensis* TH-01 was isolated using GeneaidTM DNA isolation kit (Geneaid Biotech Ltd.) according to the manufactural protocol. Chromosomal DNA isolation was performed from 5 ml of LB overnight fresh culture, lysed using lysozyme, RNA was degraded using RNaseA, protein was removed by protein removal buffer, and the DNA was precipitated using isopropanol. The obtained DNA pellet was air-dried and dissolved into 100 µl of DNA hydration buffer.

2.3. Primer Design

The primer used to amplify the *pha*A and *pha*R, *pha*B, *pha*C clustered as *pha*RBC genes were listed in Table 2. These primers were designed according to similar genes available in Genbank. Primers with restriction site addition to facilitate direct subcloning were also presented in this table.

2.4. PCR Amplification of *pha*A and *pha*RBC Genes

Amplification of *pha*A and *pha*RBC genes were conducted by PCR using chromosomal DNA of *B. thuringiensis* TH-01 as template and pair of primers as stated in section 2.3. Amplification was carried out using protocol of Go-Taq® Green Master Mix (Promega Corporation) with pre-denaturation at 95.0℃ for 2 minutes, denaturation at 95.0℃ for 30 seconds, annealing for 30 seconds at 56.5℃ for *pha*A and 50.3℃ for *pha*RBC. Considering the length of the amplified fragment, elongation at each cycle was performed at 72.0℃ for 1 minutes 11 seconds for *pha*A amplification and 2 minutes 40 seconds for *pha*RBC. In both cases, the PCR was conducted for 30 cycles and completed by final elongation at 72.0℃ for 5 minutes and renaturation at 12.0℃ for another 5 minutes.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains/plasmids	Relevant description	Source
*Bacillus thuringiens*is TH-01	A wild type bacterium used as *pha*A and *pha*RBC genes source	Courtesy of Prof. Gomathi Velu (Departement of Agricultural Microbiology, Tamil Nadu Agricultural University, India) which then cultured in Biochemistry Laboratory, Chemistry Department, FMIPA ITB
Eschericia coli TOP10	Host for pGEM-*Bt-pha*A and pGEM-*Bt-pha*RBC recombinant clones	Purchased from Invitrogen
Eschericia coli BL21(DE3)	Host for pET-*Bt-pha*A and pET-*Bt-pha*RBC recombinant clones expression	Purchased from Novagen
pGEM-T Easy	Cloning vector	Purchased from Promega
pET-30a(+)	Expression vector	Purchased from Novagen
pGEM-*Bt-pha*A	pGEM recombinant plasmid containing *pha*A gene from *B. thuringiensis* TH-01	Constructed in this study
pGEM-*Bt-pha*RBC	pGEM recombinant plasmid containing *pha*R, *pha*B, and *pha*C genes from *B. thuringiensis* TH-01	Constructed in this study
pET-*Bt-pha*ARBC	pET recombinant plasmid containing *pha*A, *pha*R, *pha*B, and *pha*C genes from *B. thuringiensis* TH-01	Constructed in this study

Table 2. Primers used to amplify *pha*A and *pha*RBC genes

Primer	Tm (℃)	% GC	Source
BtphaAF: 5'-ATGAGTAAA ACAGTTATTTTAAGTG-3'	50	24	Primers for amplification of phaA gene for cloning it into pGEM-T Easy
BtphaAR: 5'- TTAGTGG ACTTCAATCATCAC-3'	51	38	
BtphaRBCF: 5'-GTGATTG ATCAAAAATTCG-3'	46	32	Primers for amplification of phaRBC gene cluster for cloning it into pGEM-T Easy
BtphaRBCR: 5'-AAGCTTAT ATGCTCGTACCCCTTTTTC-3'	59	41	
BtphaAF1: 5'-AGATCTGAT GAGTAAAACAGTTATTTT AAGTG-3'	56/50	28/24	Primers for amplification of phaA gene with the addition of BglII site on forward primer and NcoI site on reverse primer for subcloning it into pET-30a(+)
BtphaAR1: 5'-CCATGGTTAGTGGACTTCAATCATCAC-3'	55/50	26/24	
BtphaRBCF1: 5'-CCATGGA AGGAGATGGTGATTGAT CAAAAATTCG-3'	46/63	32/41	Primers for amplification of phaRBC gene cluster with the addition of NcoI site on forward primer and XhoI site on reverse primer for subcloning it into pET-30a(+)
BtphaRBCR1: 5'-CTCGAGT TACTTAGAGCGCTCGT-3'	51/60	47/52	
BtphaRR_int: 5'-TCACTTTTTA TTTTCTGGCTTAT-3'	50	26	Walking primer 1 used to sequence phaRBC gene
BtphaBF_int: 5'-ATGGTTCAATT AAATGGCAAAG-3'	52	32	Walking primer 2 used to sequence phaRBC gene
BtphaBR_int:5'-TTACATATATA ATCCGCCGTTAATG-3'	53	32	Walking primer 3 used to sequence phaRBC gene
BtphaCF_int:5'-ATGACTACATT CGCAACAGAATGGG-3'	59	44	Walking primer 4 used to sequence phaRBC gene

Bolded F and R in primers' name indicated forward and reverse primers. Restriction sites of *Bgl*II (AGATCT), *Nco*I (CCATGG), and *Xho*I (CTCGAG) in the primers were underlined, added RBS and added start codon were bold

2.5. Cloning the *pha*A and *pha*RBC Genes into pGEM-T Easy Vector and its Characterization

Prior to cloning, the PCR products were purified through agarose gel electrophoresis. The amplicon band were excised out from the gel and then extracted according to the protocol of Promega. The pure *pha*A and *pha*RBC fragments were individually ligated into linearized pGEM-T Easy vector by taking advantage of TA cloning (Green and Sambrook 2012). Ligation mixture were used to transform *E. coli* TOP10 and the transformants were grown on LB medium supplemented with 10 µg/ml tetracycline to select *E. coli* TOP10, 100 µg/ml ampicillin to select the pGEM-T, and 20 µg/ml X-gal for blue-white selection with 0.1 mM IPTG induction. Transformants harbouring recombinant plasmids will appear as white colonies, whereas those that contain only pGEM-T Easy plasmids without any insert will remain blue. Confirmation of recombinant plasmids in white colonies were performed by isolating the plasmids using protocol of High-Speed Plasmid Mini Kit GeneaidTM (Geneaid Biotech Ltd.), analyzing its size by gel electrophoresis, reconfirmed by re-PCR utilizing suitable primers pairs, and then sequenced. Sequencing of *pha*A and *pha*RBC genes was carried out by Macrogen, Singapore, using the dye terminator dideoxy Sanger Method. Sequencing of *pha*A was carried out using the universal primer pair of T7 promoter and SP6 promoter, while sequencing of *pha*RBC was carried out by walking primer approach using four other primers presented in Table 1 as additional primers to universal primer. The confirmed clones were named as pGEM-*Bt-pha*A and pGEM-*Bt-pha*RBC.

2.6. Subcloning the *pha*A and *pha*RBC Genes into pET-30a(+) Expression Vector

For direct subcloning into expression vector, the *pha*A gene from pGEM-*Bt-pha*A and *pha*RBC gene from pGEM-*Bt-pha*RBC were re-amplified using pairs of primers with addition of restriction sites at both ends as stated in section 2.3. The obtained amplicon was again cloned into pGEM-T Easy to obtain pGEM-*Bt-pha*A1 and pGEM-*Bt-pha*RBC1 which were confirmed by sequencing. The pGEM-*Bt-pha*A1 was then double-digested using *Bgl*II and NcoI to release the *pha*A gene, whereas pGEM-*Bt-pha*RBC1 was double-digested using *Nco*I and *Xho*I to release the *pha*RBC gene. Each gene fragment was subsequently isolated from agarose gel following electrophoresis,

and the concentration were roughly determined using nanodrop instrument. These fragments were then ligated together with pET-30a(+) that already linearized using *Bgl*II and *Xho*I with 1:1:1 weight ratio. The obtained ligation mixture was used to transform *E. coli* TOP10 to obtain pET-*Bt-pha*ARBC which subsequently confirmed by restriction analysis, re-PCR, and sequencing. The confirmed pET-*Bt-pha*ARBC was then used to transform *E. coli* BL21(DE3) and selected on LB medium containing 30 µg/ml kanamycin. The plasmid in *E. coli* BL21(DE3) was again confirmed by re-PCR using BtphaAF1 and BtphaRBCR1 primers. The flowchart of plasmids construction in this study is presented in Figure 1.

2.7. Expression of the Recombinant *E. coli* BL21(DE3)/pET-*Bt-pha*ARBC

Expression of the recombinant pET-*Bt-pha*ARBC was studied in *E. coli* BL21(DE3) by analyzing the produced protein in SDS PAGE. Single colony of *E. coli* BL21(DE3)/pET-*Bt-pha*ARBC was inoculated into 5 ml LB medium containing 30 µg/ml kanamycin and incubated at 37°C with shaking at 150 rpm to reach OD_{600} nm ≈ 0.4. A 1 ml of this fresh culture was taken out as a control while the rest of the culture was induced using 1 mM IPTG followed by incubation at the same condition for another 2 hours. Subsequently, the culture sample before and after induction were harvested separately by centrifugation and lysed using 12.5% (w/v) trichloroacetic acid according to protocol by Rout Lab. The cell debris was removed by centrifugation and the crude extract was analyzed by SDS PAGE. The gel was stained using a commasie blue staining buffer and destained using 9:9:2 (v/v/v) of methanol:ddH_2O:acetic acid glacial. Analysis was performed by comparing the observed profile of protein bands before and after induction. Expression of *pha*ARBC genes were considered as the appearance of protein bands in PAGE which have the same size as PHA proteins from *B. cereus* mm7 (El Rabey *et al.* 2017).

2.8. *In Silico* Analysis of PhaA, PhaB, PhaC, and PhaR

The *in silico* analysis of PhaA, PhaB, PhaC, and PhaR proteins deduced from its nucleotide sequences were performed using multiple online services. The ProtParam program (Gasteiger *et al.* 2005) was used to analyze the secondary structure of proteins, I-TASSSER (Yang and Zhang 2015) and RaptorX

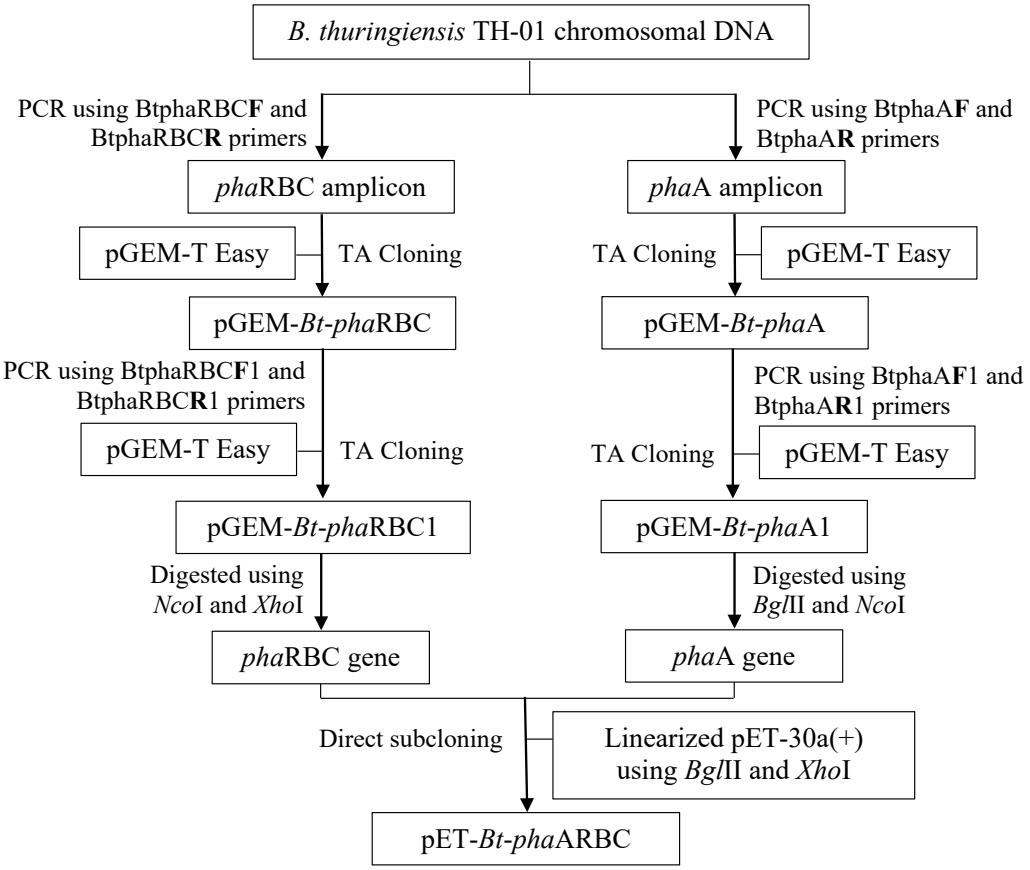

Figure 1. Flowchart of plasmids construction

(Wang *et al.* 2016) to predict tertiary structure of proteins, and Visual Molecular Dynamics (VMD) (Humphrey *et al.* 1996) to visualize the 3D proteins' structure.

3. Results

3.1. PCR Amplification of *pha*A and *pha*RBC Genes

The amplicon of *pha*A and *pha*RBC genes obtained by PCR, as well as the used chromosomal DNA template, were presented in Figure 2. The obtained amplicon using BtphaAF and BtphaAR was a clear single band of ~1 kb for *pha*A (Figure 2B). Similarly, a ~3 kb band was obtained for *pha*RBC fragment utilizing BtphaRBCF and BtphaRBCR primers (Figure 2C).

3.2. Cloning of *pha*A and *pha*RBC into pGEM-T Easy Vector

The purified fragments of *pha*A and *pha*RBC were separately ligated into pGEM-T Easy vector to generate pGEM-*Bt-pha*A and pGEM-*Bt-pha*RBC recombinant plasmids which were subsequently used to transform *E. coli* TOP10. Both recombinant plasmids were screened by electrophoresis, and the inserted fragment in pGEM-*Bt-pha*A and pGEM-*Bt-pha*RBC were sequenced, where the results of *pha*A and *pha*RBC sequencing were contigted using SnapGene™ and analyzed using BLAST technique separately (Boratyn *et al.* 2019) to identify each *pha*A, *pha*B, *pha*C, and *pha*R genes. The obtained sequences of *pha*A and *pha*RBC were represented in Figure 3A and B.

Sequence of *pha*A gene was presented as separate figure because in *Bacillus* species this *pha*A gene was

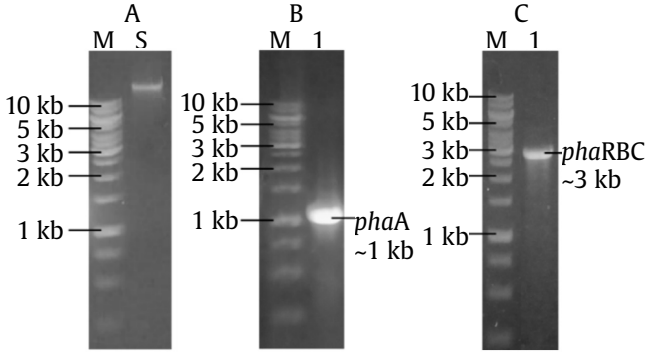

Figure 2. Electropherogram of the isolated chromosomal DNA from *B. thuringiensis* TH-01 and the amplicons of *pha*A and *pha*RBC genes. M = DNA 1 kb ladder marker (Thermo Fisher), (A) lane S = chromosomal DNA of *B. thuringiensis* TH-01, (B) lane 1 = amplicons of *pha*A, and (C) lane 1 = *pha*RBC genes

located far from PHA operon that contains *pha*RBC gene cluster (Tsuge *et al.* 2015). In our cloning, these four genes were unified as a single construct in pET plasmid named pET-*Bt-pha*ARBC explained in section 3.3.

3.3. Sub-cloning of *pha*A and *pha*RBC into pET-30a(+) Expression Vector

The sub-cloning of *pha*A and *pha*RBC into pET-30a(+) expression vector was first done by reamplifying both genes using modified primer pairs and re-cloning it into pGEM-T Easy to generate pGEM-*Bt-pha*A1 and pGEM-*Bt-pha*RBC1, followed by double digestion using suitable restriction enzymes stated in section 2.6. Figure 4 showed the electropherogram of double digested pGEM-*Bt-pha*A1 with *Bgl*II and *Nco*I (Figure 4A lane 1) and pGEM-*Bt-pha*RBC1 with *Nco*I and *Xho*I (Figure 4B lane 1), which resulted two sharp bands representing the pGEM-T Easy vector and the DNA fragments of ~1 kb for *pha*A and ~2.5 kb for *pha*RBC gene. For comparison, the linearized pET-30a(+) was shown in Figure 4C, and all undigested plasmids were presented in lane 2 respectively.

The obtained pET-*Bt-pha*ARBC in *E. coli* TOP10 was confirmed by restriction analysis as depicted in Figure 5. Restriction of pET-*Bt-pha*ARBC by *Bgl*II and *Xho*I resulted two DNA fragments with the size of ~4 kb that representing the *pha*ARBC and ~5.5 kb of the vector (Figure 5A). The pET-*Bt-pha*ARBC in *E. coli* BL21(DE3) was also confirmed by re-PCR and the obtained amplicon was shown in Figure 5B.

3.4. Analysis of PhaA, PhaB, PhaC, and PhaR Proteins

The SDS PAGE analysis on the crude enzyme extract from *E. coli* BL21(DE3)/pET-*Bt-pha*ARBC was shown in Figure 6. Lane 1 and 2 shows extract from *E. coli* BL21(DE3) before and after induction, whereas lane 3 and 4 were those from *E. coli* BL21(DE3)/pET-*Bt-pha*ARBC before and after induction, respectively. Size of bands detected in lane 4 were ~41 kD of PhaA, ~26.5 kD of PhaB, ~41.7 kD of PhaC, and ~18.5 kD of PhaR. As control, crude enzyme extract from *E. coli* BL21(DE3)/pET-*Bt-pha*A and *E. coli* BL21(DE3)/pET-*Bt-pha*RBC were shown in lane 5 and 6 respectively.

3.5. *In Silico* Analysis of PhaA, PhaB, PhaC, and PhaR Proteins

Amino acid sequences deduced from obtained DNA sequences of every genes were used in this analysis. *In silico* prediction of PhaA and PhaB structures using I-TASSER service are shown in Figure 7A and B. On the other hand, the structure of the best proposed PhaC and PhaR of *B. thuringiensis* TH-01 are

A

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atg agt aaa aca gtt att tta agt gct gca aga aca cca gtt gga aaa ttt gga gga tct tta aaa gat gta aaa gca aca gaa ctt gga ggg att  96
 M  S  K  T  V  I  L  S  A  A  R  T  P  V  G  K  F  G  G  S  L  K  D  V  K  A  T  E  L  G  G  I
gca att aaa gcg gcg ctt gaa aga gca aat gtt tct gct agt gat gtt gag gaa gtt ata ttc gga acg gtt att caa ggt ggg cag ggg caa att 192
 A  I  K  A  A  L  E  R  A  N  V  S  A  S  D  V  E  E  V  I  F  G  T  V  I  Q  G  G  Q  G  Q  I
cca tcg cgc caa gcg gcg agg gct gct gga atc cct tgg gaa gtg cag acg gaa aca gtg aat aaa gtt tgt gca tcg ggg ctt cgt gcg gtt acg 288
 P  S  R  Q  A  A  R  A  A  G  I  P  W  E  V  Q  T  E  T  V  N  K  V  C  A  S  G  L  R  A  V  T
tta gcg gat caa att att cgt act ggc gat caa tca ttg att gta gct ggt ggt atg gag tcg atg agt aac agt cct tac att tta cga gga gca 384
 L  A  D  Q  I  I  R  T  G  D  Q  S  L  I  V  A  G  G  M  E  S  M  S  N  S  P  Y  I  L  R  G  A
aga tgg gga tat aga atg ggc aac aac gaa gtc gtt gat tta aac gtt gct gac ggt tta aca tgc gca ttt tca ggt ata cac atg ggt gtt tat 480
 R  W  G  Y  R  M  G  N  N  E  V  V  D  L  N  V  A  D  G  L  T  C  A  F  S  G  I  H  M  G  V  Y
ggc gga gaa gtt gcg aag gaa gat gga att tct cgc gaa gcg caa gat gaa tgg gca tat cgt agc cac cag cgt gcg gtt tca gca cat aat gaa 576
 G  G  E  V  A  K  E  D  G  I  S  R  E  A  Q  D  E  W  A  Y  R  S  H  Q  R  A  V  S  A  H  N  E
gga cgt ttt gaa gag gaa atc gtg cca gta acg att ccg caa aga aaa ggc gat cct att gtc gtt gca aag gat gag gcg cca cgc gcg gat aca 672
 G  R  F  E  E  E  I  V  P  V  T  I  P  Q  R  K  G  D  P  I  V  V  A  K  D  E  A  P  R  A  D  T
aca att gaa aag tta gca aaa tta aaa cct gta ttt gat aaa aca gcg aca gtg aca gct ggt aat gca cca ggc tta aac gat ggt ggt gct gca 768
 T  I  E  K  L  A  K  L  K  P  V  F  D  K  T  A  T  V  T  A  G  N  A  P  G  L  N  D  G  G  A  A
ctt gta tta atg agc gaa gac aga gcg aag caa gaa gga aga aag ccg tta gcg aca att ttg gca cat aca gca att gca gtg gag tct aaa gat 864
 L  V  L  M  S  E  D  R  A  K  Q  E  G  R  K  P  L  A  T  I  L  A  H  T  A  I  A  V  E  S  K  D
ttc ccg aga acg cca ggt tat gca att aac gca ttg ctt gaa aaa aca ggc aag aca att gaa gac atc gat tta ttc gag att aat gaa gca ttt 960
 F  P  R  T  P  G  Y  A  I  N  A  L  L  E  K  T  G  K  T  I  E  D  I  D  L  F  E  I  N  E  A  F
gca gca gta gca att gca agt aca gaa atc gca gga att gac cca gaa aaa ttg aat gta aat ggc ggc gca gta gcg atg gga cat ccg att gga 1056
 A  A  V  A  I  A  S  T  E  I  A  G  I  D  P  E  K  L  N  V  N  G  G  A  V  A  M  G  H  P  I  G
gca agc gga gcg cgc att atc gtt aca cta atc cat gca ctt aag caa cgc ggc ggt gga att gga att gct tcg att tgt agt ggt ggc ggt caa 1152
 A  S  G  A  R  I  I  V  T  L  I  H  A  L  K  Q  R  G  G  G  I  G  I  A  S  I  C  S  G  G  G  Q
ggc gat gca gtg atg att gaa gtc cac taa                                                                                    1182
 G  D  A  V  M  I  E  V  H  -
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B

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gtg att gat caa aaa ttc gat cca cta caa gca tgg aaa aat gct tat gaa caa acc gaa aca ttt tgg gga aaa gcg ctc aat gaa aca att aaa  96
 V  I  D  Q  K  F  D  P  L  Q  A  W  K  N  A  Y  E  Q  T  E  T  F  W  G  K  A  L  N  E  T  I  K
aca gaa gaa tat tct gct tgg atg ggc agc gtt cta gat ttg aat ttg ttt tat caa aaa gca tta aat gat acg aca aaa aat tat tta gag cag 192
 T  E  E  Y  S  A  W  M  G  S  V  L  D  L  N  L  F  Y  Q  K  A  L  N  D  T  T  K  N  Y  L  E  Q
gta aat gtg cct acg aaa gag gat atc gct aga gtg gct acg ctt gtt att aac tta gaa aat aaa gtg gat aac att gag gag ttt cta gaa gat 288
 V  N  V  P  T  K  E  D  I  A  R  V  A  T  L  V  I  N  L  E  N  K  V  D  N  I  E  E  F  L  E  D
aag gta gag tca gta gga caa gct cct aca tta aag cgc gat gtt acg aaa gta aaa caa gat att cgc aca tta gaa acg aaa gtt gat caa att 384
 K  V  E  S  V  G  Q  A  P  T  L  K  R  D  V  T  K  V  K  Q  D  I  R  T  L  E  T  K  V  D  Q  I
tta gaa ttg cta gaa aag caa aat gca gta cta gca aaa cta caa gaa cct gta aaa gaa gaa gta aaa cct acg aat aag cca gaa aat aaa aag 480
 L  E  L  L  E  K  Q  N  A  V  L  A  K  L  Q  E  P  V  K  E  E  V  K  P  T  N  K  P  E  N  K  K
tga taa tag cgc ttg ttt ata aca tat gaa aga gaa agg gag aat ctc ttt tct atg tat aaa cag cga aac ata att ata tag gga ctc cat agt 576
 -
atg agt acc aaa atg att cat aat agt cgt tca caa att cat tta agg ggg aaa agaa atg gtt caa tta aat ggc aaa gta gca atc gta aca ggt 673
                                                                            M  V  Q  L  N  G  K  V  A  I  V  T  G
ggg gca aaa ggg att gga aaa gca atc aca gta gcg tta gca caa gag ggc gca aaa gtt gtt att aac tat aac agc agt aaa gaa gca gct gaa 769
 G  A  K  G  I  G  K  A  I  T  V  A  L  A  Q  E  G  A  K  V  V  I  N  Y  N  S  S  K  E  A  A  E
aac tta gta aat gaa cta gga aaa gaa gga cat gac gtt tat gca gtt caa gca gat gtt tct aaa gta gaa gac gca aac cga ctt gta gaa gaa 865
 N  L  V  N  E  L  G  K  E  G  H  D  V  Y  A  V  Q  A  D  V  S  K  V  E  D  A  N  R  L  V  E  E
gca gtg aat cat ttt ggt aaa gtt gat att ctt gtt aat aat gct ggt att aca aga gat cgt aca ttc aaa aag tta aat cgt gaa gat tgg gaa 961
 A  V  N  H  F  G  K  V  D  I  L  V  N  N  A  G  I  T  R  D  R  T  F  K  K  L  N  R  E  D  W  E
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 R  V  I  D  V  N  L  S  S  V  F  N  T  T  S  A  V  L  P  Y  I  T  E  A  E  E  G  R  I  I  S  I
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 Q  L  S  I  N  G  G  L  Y  M  -
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 M  L  K  P  I  T  N  F  V  G  P  Y  V  A  L  V  D  R  S  E  N  E  R  F  V  E  S  W  R  L  V  Q
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 V  I  R  G  Q  K  V  D  L  A  N  I  K  A  N  V  L  N  I  S  G  K  R  D  H  I  A  L  P  C  Q  V
gaa gcg ttg cta gat cat att tct agc aca gat aaa caa tat gta tgt tta cca acg gga cat atg tcg att gtt tac ggt gga aca gcg gta aaa 2498
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cag acg tat ccg acg att gga gac tgg ctt gac gag cgc tct aag taa                                                          2546
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C

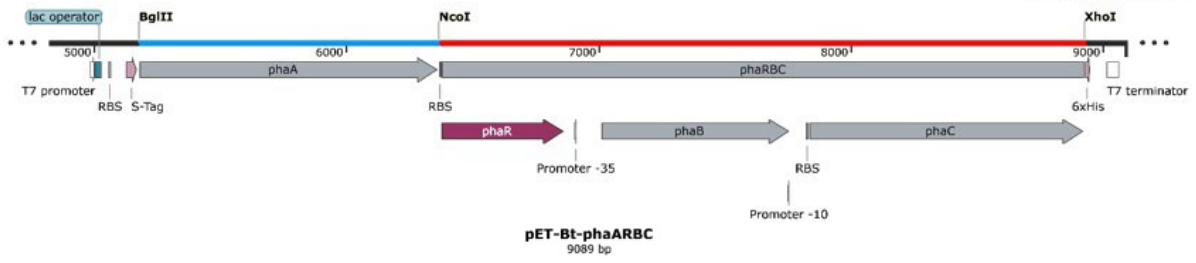

Figure 3. The nucleotide sequences of targeted genes. (A) *pha*A, (b) *pha*RBC, red = *pha*R gene, yellow = *pha*B gene, green = *pha*C gene, the putative promoter was marked as red underline and the RBS sequence were marked by blue underline, and (C) map of *pha*ARBC in the pET-*Bt*-*pha*ARBC recombinant clone created using SnapGene™

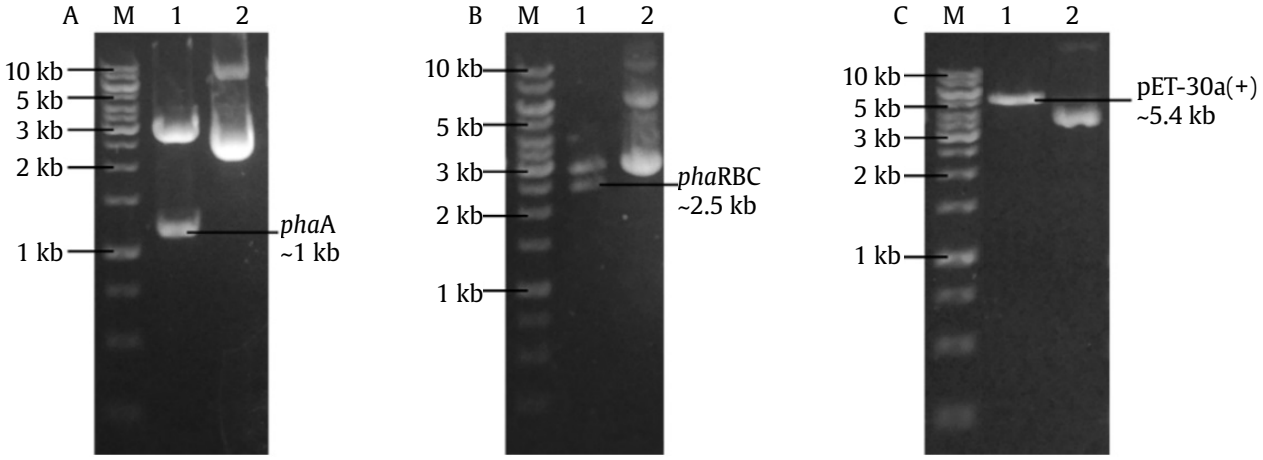

Figure 4. Electropherogram of double digested pGEM-T Easy clones and pET-30a(+) expression vector. M = DNA 1 kb ladder marker (Thermo Fisher) (a) lane 1 = digested pGEM-*Bt-pha*A1 with *Bgl*II and *Nco*I, lane 2 = circular pGEM-*Bt-pha*A1, (b) lane 1 = digested pGEM-*Bt-pha*RBC1 with *Nco*I and *Xho*I, lane 2 = circular pGEM-*Bt-pha*RBC1, (c) lane 1 = digested pET-30a(+) with *Bgl*II and *Xho*I, lane 2 = circular pET-30a(+)

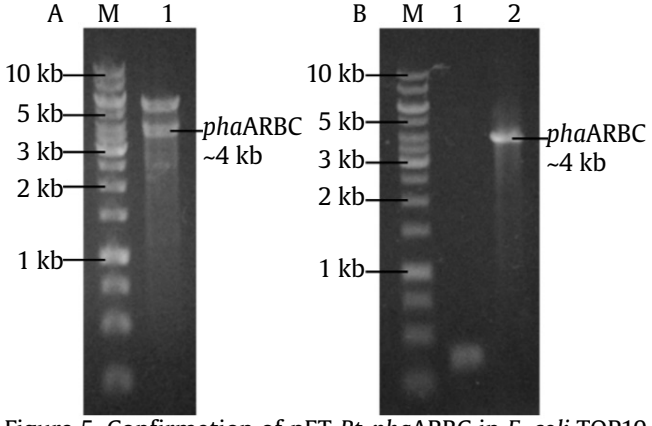

Figure 5. Confirmation of pET-*Bt-pha*ARBC in *E. coli* TOP10 by restriction analysis and in *E. coli* BL21(DE3) by re-PCR. M = DNA 1 kb ladder marker (Thermo Fisher), (a) lane 1 = pET-*Bt-pha*ARBC from *E. coli* TOP10 double digested by *Bgl*II and *Xho*I, (b) lane 1 = re-PCR product of empty pET-30a(+), lane 2 = re-PCR product of pET-*Bt-pha*ARBC from *E. coli* BL21(DE3). The primer used in this re-PCR were BtphaAF1 and BtphaRBCR1

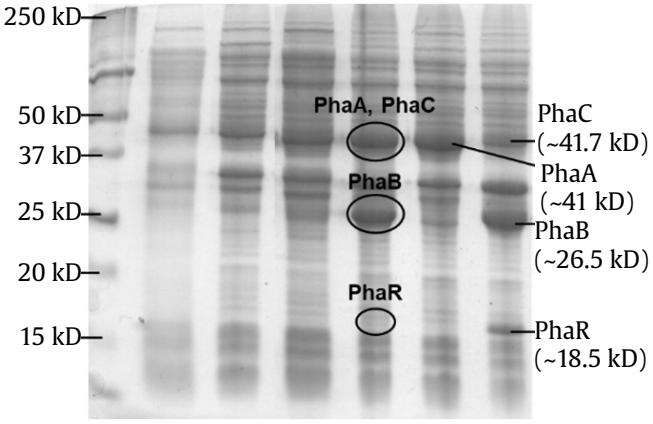

Figure 6. SDS PAGE electropherogram of crude extract from *E. coli* BL21(DE3). M = precision plus protein standards (BioRad), lane 1 = *E. coli* BL21(DE3) before induction, lane 2 = *E. coli* BL21(DE3) after induction, lane 3 = *E. coli* BL21(DE3)/pET-*Bt-pha*ARBC before induction, lane 4 = *E. coli* BL21(DE3)/pET-*Bt-pha*ARBC after induction, lane 5 = control of *E. coli* BL21(DE3)/pET-*Bt-pha*A, lane 6 = control of *E. coli* BL21(DE3)/pET-*Bt-pha*RBC

shown in Figure 7C and D. Amino acids in the boxes indicate catalytic residues of the predicted structures aligned to similar proteins available in Protein Data Bank, which was 4O99 for PhaA and 2UVD for PhaB. No similarities observed for PhaC and PhaR.

4. Discussion

In this study, pair of primers to amplify *pha*A gene for cloning into pGEM-T Easy was designed based on the nucleotide sequence of gene encoding acetyl-CoA acetyltransferase from *Bacillus thuringiensis* strain CTC (Accesion No. CP013274.1), whereas primer pair to amplify *pha*B, *pha*C, and *pha*R genes were based on PHA gene cluster named as *pha*RBC from *Bacillus thuringiensis* ATCC10792 (Accesion No. AY331151). To facilitate further subcloning into pET-30a(+) expression vector, these primer pairs were modified by adding restriction site at each 5' ends. The restriction sites selected for *pha*A were *Bgl*II and *Nco*I for the respective forward and reverse primer, whereas restriction sites added to the *pha*RBC primers were *Nco*I and *Xho*I. These restriction sites were chosen because they are absent in the targeted

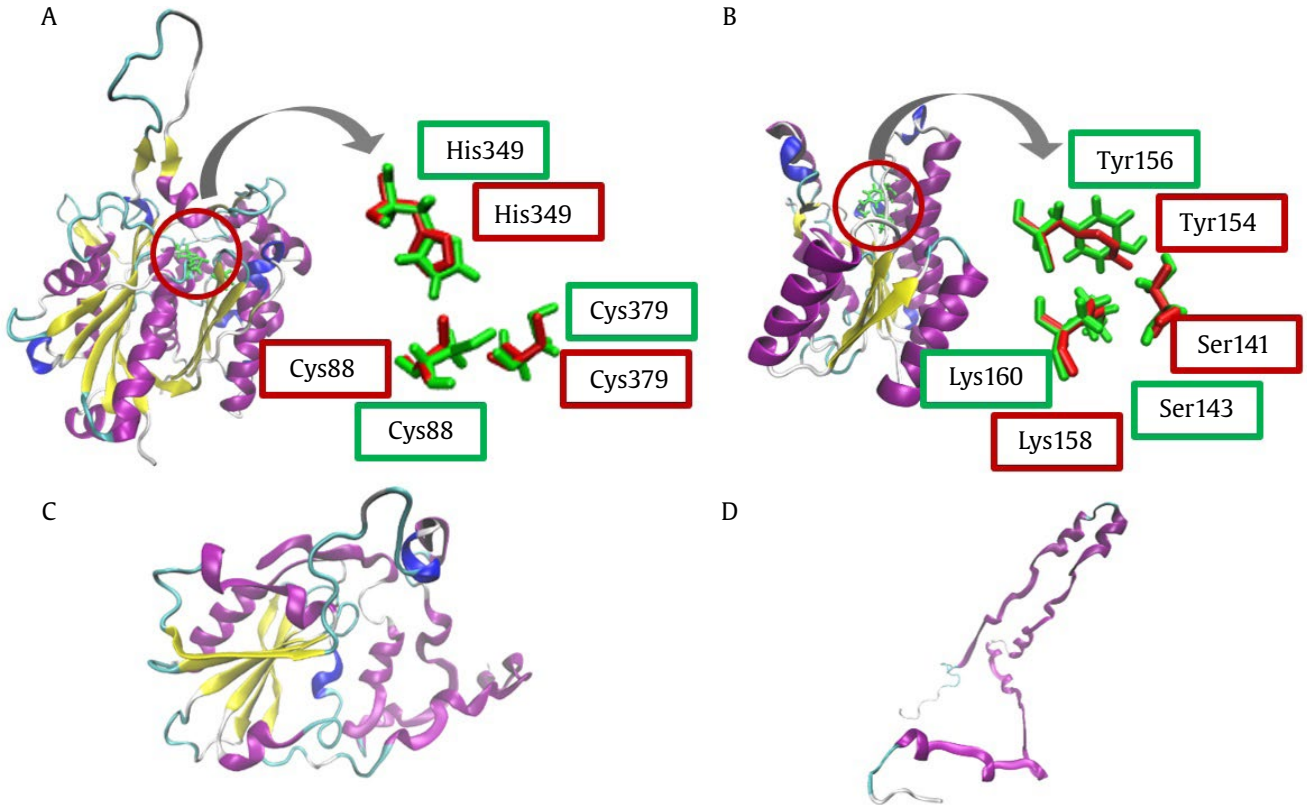

Figure 7. Predicted tertiary structure of proteins (A) PhaA and its structural alignment with 4O99, (B) PhaB and its structural alignment with 2UVD, (D) PhaC, and (D) PhaR

genes but exist in the multiple cloning site of pET-30a(+).

PCR amplification of *pha*A and *pha*RBC genes were conducted using the chromosomal DNA isolated from *B. thuringiensis* TH-01 as a template. As depicted in Figure 2A, the isolated chromosomal DNA of *B. thuringiensis* TH-01 was observed as a clean single band with no smear background above and below the concerned band, indicated that the isolated chromosome was intact and no proteins nor RNA contamination in the sample. Utilization of this chromosomal DNA as template in PCR results a clear single band with the amplicon size corresponds to targeted genes as shown in Figure 2B and C, indicating that the primers were properly anneal with no mispriming, results successful amplification of each targeted genes from *B. thuringiensis* TH-01.

The obtained clones were sequenced and the result were presented in Figure 3. BLASTX analysis of these sequences suggest successful cloning. As could be seen in Figure 3A, the obtained 1182 bp of *pha*A gene sequence shows 100% identity for gene encoding acetyl- CoA acetyltransferase from *Bacillus* (Accesion No. WP_141108629.1). On the other hand, the sequence of 2546 bp of *pha*RBC gene cluster consisted of 744 bp *pha*B gene, 1086 bp *pha*C gene, and 483 bp *pha*R gene (Figure 3B). The obtained *pha*B sequence in this cluster showed 99.60% identity for gene encoding acetoacetyl-CoA reductase from *Bacillus* (Accesion No. WP_000250412.1). The *pha*C sequence revealed 100% identity for gene encoding PhaC subunit of type III PHA synthase from *Bacillus* (Accesion No. WP_000206335.1), whereas the *pha*R sequence indicated 100% identity for gene encoding PhaR subunit of PHA synthase from *Bacillus thuringiensis* (Accesion No. WP_001997000.1). Our finding was similar to that obtained by other researcher who stated that the protein sequence of PhaC type IV PHA synthase was similar to PhaC of type III PHA synthase (McCool and Cannon 2001) excluding the PhaR, indicating that PhaC of both types are the same protein but with different subunit accessories (Pradani *et al.* 2020).

Further analysis of this obtained *pha*RBC gene sequence showed nucleotide sequence similarity with PHA gene cluster from *B. thuringiensis* R1 (Desetty *et al.* 2008). Remarkably, the *pha*B and *pha*C in this gene cluster in our strain own its specific promoters and RBS sequence upstream to the gene sequence, the same as those observed in *B. thuringiensis* R1 (Desetty *et al.* 2008). Other researchers stated that the presence of this own promoter and RBS sequence

allows independent expression of this gene cluster (McCool and Cannon 1998; El Rabey *et al.* 2017).

The double digestion of *pha*A and *pha*RBC genes in each pGEM-T Easy vector resulting two clear expected bands as indicated in Figure 4A and B. Afterwards, the successful construction of pET-*Bt-pha*ARBC were then confirmed by restriction analysis and re-PCR resulting clear DNA bands corresponding to that constructed ~4 kb phaARBC (Figure 5A and B). As expected, expression of *pha*A and *pha*RBC genes in *E. coli* BL21(DE3) results several bands of proteins with different sharpness as shown in Figure 6. The ~41 kD of PhaA and ~41.7 kD of PhaC were observed as an overlapped strong band, whereas the strong band of PhaB protein was observed as ~26.5 kD. However, the ~18.5 kD PhaR was observed as a faint band, indicating the low level of expression. This fact was similar to that previously reported (McCool and Cannon 2001), where expression of this PHA gene cluster, particularly PhaC and PhaR, which were expressed using its own native promoter, were not resulting in equimolar protein in the cell. Nevertheless, it could be stated that all targeted genes were successfully expressed in *E. coli* BL21(DE3). In *Bacillus megaterium,* the function of PhaR was stated as correlated to PhaC, hence small amount of PhaR still able to produce an active PHA synthase (McCool and Cannon 2001). The presence of these two proteins together with PhaA and PhaB were apparent to accumulate PHA in the recombinant cell (Desetty *et al.* 2008).

The bioinformatics analysis of PhaA, PhaB, PhaC, and PhaR reveals various protein characteristics. The results indicated that PhaA (acetyl-CoA acetyltransferase) of *B. thuringiensis* TH-01 consists of 393 amino acid residues with molecular mass of 41.05 kDa and a theoretical isoelectric pH of 5.63. On the other hand, PhaB (acetyl-CoA acetyl reductase) consists of 247 amino acid residues with a molecular mass of 26.5 kDa and a theoretical isoelectric pH of 5.84. *In-silico* prediction of PhaA and PhaB structures using I-TASSER service with a multiple threading alignment approaches resulted in tertiary structure with dominant α/β folding as shown in Figure 7A and B.

The PhaA from our strain were observed to be structurally close to PhaA from *Ralstonia eutropha* H16 (PDB ID 4O99; Kim and Kim 2014), whereas the PhaB revealed to be structurally close to PhaB from *Bacillus anthracis* (BA3989) (PDB ID 2UVD; Zaccai *et al.* 2008) with TM-scores 0.994 and 0.978 respectively. The PhaA was predicted to have Cys88, His349, and Cys379 catalytic residues, while the PhaB was predicted to have Ser143, Tyr156, and Lys160. This structural alignment analysis indicated that catalytic residues of PhaA and PhaB from *B. thuringiensis* TH-01 were conserved.

The PhaC and PhaR, which are subunits of type IV PHA synthase of *B. thuringiensis* TH-01, consist of 361 and 160 amino acid residues with molecular weights of 41.68 kDa and 18.48 kDa, respectively. Using the I-TASSER service, the PhaC was identified to have dominant folding of α/β (Figure 7C) with structural analog to PhaC of type I PHA synthase from *Cupriavidus necator* (PDB ID 5T6O; Wittenborn *et al.* 2016) with TM-scores 0.868. However, the PhaR of our type IV PHA synthase does not have any structural similarities to other type of known PHA synthase, since type IV PHA synthase complex has yet to be elucidated by crystallography (Zhang 2015). The absent of similar protein in PDB suggested us to predict PhaR tertiary structure using distance-based protein folding approach with RaptorX service. As shown in Figure 7D, the structure of the best proposed PhaR of *B. thuringiensis* TH-01 is dominated by helix-loop-helix motifs.

This research has been successfully combined the *pha*A and *pha*RBC genes of new strain *B. thuringiensis* TH-01 into pET-30a(+) expression vector in *E. coli* BL21(DE3) and the constructed clones has been successfully expressed. The results indicated that amino acid residues in the active site of each enzyme were evidently conserved.

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