

# Isolation, cloning and sequencing of poly 3-hydroxybutyrate synthesis genes from local strain of *Bacillus cereus* MM7 and expressing them in *E. coli*

## Abstract

Poly 3-hydroxybutyrate (*PHB*) is the most known degradable biopolymers, produced by some genera of bacteria under unfavorable growth conditions. Isolation, cloning, and expression of *PHB* genes from *PHB* producing *Bacillus cereus* was achieved. A highest Poly 3-hydroxybutyrate producer bacterium was selected and identified as *Bacillus cereus* MM7, based on morphological and physiological characters in addition to 16S rRNA gene sequences. The four genes of *Bacillus cereus* MM7 that involved in Poly 3-hydroxybutyrate production were successfully amplified using specific primers which were designed depending on the sequence alignment of the known *PHA* biosynthetic genes from *Bacillus cereus*, cloned in *E. coli* DH5 $\alpha$  and completely sequenced. Moreover, the expression of the four genes responsible for *PHA* biosynthetic in *E. coli* XL1-Blue and *E. coli* MJ 109 was assayed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the current study *Bacillus cereus* was able to synthesize *PHB*. *PHB* genes were isolated, cloned and expressed in *E. coli* DH5 $\alpha$ . The *PHB* encoding proteins were analyzed in SDS-PAGE and the *PHB* corresponding protein bands were analyzed using mass spectrometry to nominate their amino acid sequence.

**Keywords:** bioplastic; bacillus, *PHB*, gene, cloning, expression, Polyacrylamid

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## Introduction

Poly 3-hydroxybutyrate (*PHB*) is a polyhydroxyalkanoate (*PHA*) which is a polymer belonging to the polyesters class that are of interest as bio-derived and biodegradable plastics produced by some genera of bacteria under unfavorable conditions.<sup>1</sup> *PHA* can be accumulated by microorganisms in the environment naturally or *PHA* can be produced artificially by microorganisms if they are fed with specific chemicals in the laboratory which are not the normal substrates for cell growth and survival.<sup>2</sup> It is the only bioplastics completely synthesized by over 30% of soil bacteria.<sup>3</sup> *PHA* synthases are divided into four groups i.e. I- II- III and IV, based on the enzymes substrate specificities and the enzymes subunit composition.<sup>4</sup> The three classes I (found in *Ralstonia eutropha*), III (found in *Allochromatium vinosum*), and IV (found in *Bacillus megaterium*) *PHA* synthases have substrate specificities toward short-chain-length monomers (C3-C5), whereas class II (found in *Pseudomonas aeruginosa*) *PHA* synthase utilizes medium-chain-length monomers (C6-C14).<sup>5</sup> Excess carbon sources with limitation of nitrogen and phosphate were used to produce *PHB* in different kinds of bacteria.<sup>6,7</sup> Furthermore, Ghate et al.<sup>8</sup> isolated different *Bacillus* species for the production of polyhydroxybutyrate (*PHB*) from soil samples. Sangkharak et al.<sup>9</sup> isolated twenty five *Bacillus* sp. strains that were able to accumulate polyhydroxyalkanoates (*PHAs*). Davis et al.<sup>10</sup> used new pathway employing genes for b-ketothiolase and NADPH dependent acetoacetyl CoA reductase (*PHAA* and *PHAB*) isolated from *Bacillus* sp. 256 and genes for *PHA* synthase and (R)-specific enoyl CoA hydratase (*PHAC1* and *PHAJ1*) cloned from *P. aeruginosa* for the production of *PHB* in recombinant *E. coli*. *PHA*

biosynthetic genes from different species were cloned for mass production of *PHAs* for commercial use.<sup>6,11-13</sup> Tomizawa et al.<sup>14</sup> studied the expression of *PHA* synthases from *Bacillus megaterium* NBRC15308 (*PHARCBm*), *B. cereus* YB-4 (*PHARCYB4*) and hybrids (*PHARBmCYB4* and *PHARYB4CBm*) in *Escherichia coli* JM109 to characterize the molecular weight of the synthesized poly (3-hydroxybutyrate) [P (3HB)]. The aim of the current study was the selection and identification of the most active *PHB* producing bacteria, isolating the *PHB* genes, cloning and sequencing them and assaying the expression of *PHB* genes in *Escherichia coli*. The *PHB* encoding proteins were analyzed in SDS-PAGE and the *PHB* corresponding protein bands were analyzed using mass spectrometry to nominate their amino acid sequence.

## Materials and Methods

A highly *PHB* producing isolate was isolated in our lab from sea water sample collected from North Beach of Jeddah, Saudi Arabia and identified as *Bacillus cereus* MM7 based on morphological and physiological characters in addition to 16S rRNA gene sequences.<sup>15</sup> This bacterium was used as a source for *PHB* genes.

### Pre culture

The pre culture was prepared in 250ml Erlenmeyer flasks containing 50ml of nutrient broth.<sup>16</sup> Each flask was inoculated with a loop of the tested cultures and incubated in a 130 rpm speed rotary shaker at 30°C for 24h.

## Isolation of genomic DNA

The DNA of *PHB* producing strain was extracted using Nucleo Spin Tissue Kit (Macheri Nagal, Germany) according to the manufacturer's instruction.

## Colony PCR

For colony PCR amplification, bacterial colonies were picked using a sterile tooth pick and dipped into 10µl of water plus 10µl PCR reaction mixture consisting of 0.4µl of forward and 0.4µl reverse primers, 0.5µl dNTPs, 2 µl PCR buffer, 0.6µl MgCl<sub>2</sub>, 0.3µl Taq DNA polymerase and 5.8 µl. Amplification reaction was performed using MWG-Biotech Primus 96 thermocycler, Galileo Equipos S.L, Spain) under the following reaction conditions; initial denaturation at 95°C for 5min, followed by 25 cycles denaturation at 94°C for 1m, annealing at 55°C for 1m and elongation at 74°C for 3min and final elongation at 72°C for 10min.

## Recombinant *E. coli* medium

The medium used for *E. coli* growth was LB medium (5g yeast extract, 10g tryptone, 10g NaCl per liter of deionized water). For *PHB* production, it was supplemented with 20g/L glucose in a 500ml shaking flask containing 100ml medium at 37°C for 24h. Sterilized *ampicillin* was added to the *E. coli* culture medium to a final concentration of 100µg/ml.

## PCR amplification of the *PHA* genes

The isolated DNA from *B. cereus* MM7 strain was used as a template for PCR amplification of *PHA* biosynthesis genes which contain (*PHAA*, *PHAR*, *PHAB* and *PHAC*). The oligonucleotide primers for PCR amplification are listed in Table 1. The primer of *PHAA* gene was designed based on the sequence alignment of the known *PHA* synthases from the *B. cereus* group (organisms *B. cereus*, accession number EU239690),<sup>10</sup> while the oligonucleotide primers for PCR amplification of *PHAR*, *PHAB* and *PHAC* were designed in this study based on the sequence alignment of the known *PHA* synthases from *B. cereus*. (Accession number AB525763).<sup>17</sup>

## PCR amplification protocol for *PHAA*, *PHAB*, *PHAC* and *PHAR*

PCR reactions were achieved using genomic DNA as a template in 25µl PCR reaction mixture consisted of 2.5 µl PCR buffer, 0.5µl of each forward and reverse primers, 0.5µl dNTPs, 0.5µl polymex enzymes, 1µl DNA (containing 50-100 ng DNA) and 19.5µl water. PCR reaction condition was as follows: under the following reaction conditions; initial denaturation at 95°C for 5 min, followed by 25 cycles denaturation at 94°C for 1m, annealing at 55°C for 1m and

elongation at 74°C for 3 min and final elongation at 72°C for 10min. The PCR product was excised using clean scalper under UV light, purified using NucleoSpin® Gel and PCR Clean-up kit (Macheri Nagal, Germany) and then sequenced (at DNA sequencing unit at Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany). The PCR product was resolved on 0.8% or 1.2% agarose gel (w/v agarose in 1X TBE) buffer depending on the size of DNA and the experiment. The running buffer was 1X TBE buffer. The gel was stained with 0.1µg ml ethidium bromide, visualized under UV light, and then photographed using gel documentation system.

## Ligation of *PHAA* PCR product to pJET-PHARBC

The ligation was performed according to the instruction of ligation protocol supplied with T4 DNA ligase (New England Biolabs).

## Bacterial transformation of *DH5α*, *E. coli* XLI-blue and *E. coli* JM109

Heat-shock transformation was performed in *DH5α*, *E. coli* XLI-Blue and *E. coli* JM109 according to the instruction of Lamitina Lab Protocols.

## Cloning of *PHA* synthase genes from the strain *Bacillus cereus* MM 7

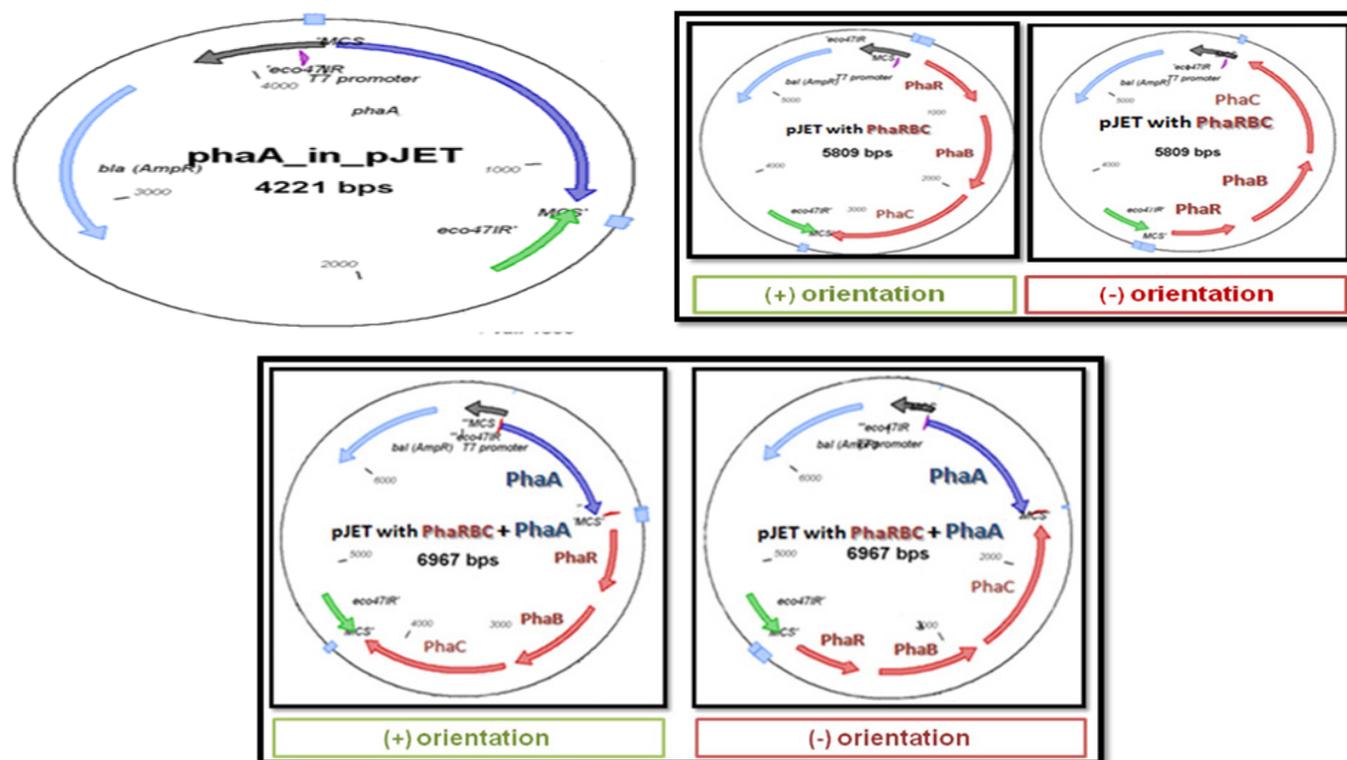
All *E. coli* strains, plasmid and primers are listed in Table 1. The purified PCR product was ligated to the pJET1.2/blunt cloning vector (Clone JET PCR Cloning Kit, Fermentas, Thermo Fisher Scientific Inc) according to the instructions of the supplier to get three kinds of plasmids; pJET-*PHAA* (Figure 1A), pJET-*PHARBC* (Figure 1B) and pJET-*PHAARBC* (Figure 1C). After plasmid was extracted using miniprep Kit (Nucleo Spin Plasmid kit, Macheri Nagal, Germany). All the three types of plasmids were transformed separately into *E. coli* *DH5α* using heat shock transformation protocol according to Lamitina Lab protocols. Afterwards, the plasmid was extracted and the concentration of plasmid was measured and digested with XhoI (Biolabs, England) to check the size after cutting with restriction enzyme and check if there is other site can be cut. Oligonucleotide primers containing NotI and XhoI restriction sites sequences were designed by Biolabs for PCR amplification of *PHAA* gene in pJET +*PHAA*. After that, the PCR product of *PHAA* and (+) pJET +*PHARBC* were digested with (NotI and XhoI), and then *PHARBC* and *PHAA* were ligated to each other and to the pJET vector according to the ligation protocol of T4 DNA ligase protocol (M0202), (New England Biolabs) to get (+) pJET +*PHAARBC* operon (Figure 1C). The construct was transformed into *E. coli* *DH5α*, *E. coli* XLI-Blue and *E. coli* MJ 109. The (+) pJET +*PHAARBC* operon was sequenced at (Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany).

**Table 1** *E. coli* strains, plasmid and primers used in this study

Strains/ Plasmid / Primers	Relevant Description	Source or Reference
<i>E. coli</i> <i>DH5α</i>	Cloning strain	Biolab.
<i>E. coli</i> XLI-Blue	Expression strain	promega
<i>E. coli</i> JM109	Expression strain	promega

Table continued...

Strains/ Plasmid / Primers	Relevant Description	Source or Reference
pJET1.2/blunt cloning vector	linearized with Eco32I (EcoRV) (GenBank/EMBL Accession number EF694056), and has the highest efficiency cloning of PCR products	Thermo Scientific CloneJET PCR Cloning Kit.
Fw pJET1.2 5'-d(CGACTCACTATAGGGAGAGCGGC)-3'	pJET1.2 primers	From Thermo Scientific Fermentas™ pJET™ 1.2
Rv pJET1.2 5'-d(AAGAACATCGATTTTCCATGGCAG)-3'		
Fw (1) PHAA 5-TACGTAACATAACGAGGGGAG-3	Amplification of PHAA gene of Bacillus cereus for cloning it into pJET	Designed at
Rv (1) PHAA 5-GCTTCCAACGCCCTCCTGTTCTAAACG-3		Fraunhofer
Fw (2) PHAA 5-CTAATAACGAGGGGAGATATCGTTC-3		Institute,
Rv (2) PHAA 5-GTCGTTTCACCCCTTCCTGTGAGGC-3		Aachen
Fw (1) PHARBC operon 5-AAATATAGACTTGAAGTTTTATTTCAG-3	Amplification of PHARBC genes of Bacillus cereus for cloning it into pJET	At
Rv (1) PHARBC operon 5-AACATAGGCTAGTTGGATTTTTTAT-3		Fraunhofer
Fw (2) PHARBC operon 5-GACTTGAAGTTTTATTTCAGAATATTAG-3		Institute,
Rv (2) PHARBC operon 5-CATAAAAAAATCCAACATAACATAGGC-3		Aachen
Fw PHARBC operon: 5- GCGCTTGTTTATAACATATGAAAG-3	Amplification to detection of B gene of Bacillus cereus	Designed at Fraunhofer
Rv PHARBC operon: 5-CTATCTCCTTTTTGGTCAATTC-3		Institute, Aachen
Fw PHAA 5-ATTAATAATGCGGCCGCCATGAGAGAAGCTGTCATTG-3	Amplified PHAA gene for subcloning it into pJET-PHARBC(+)	Designed at Fraunhofer
Rv PHAA 5-ATTAATAATCTCGAGTTATAGTAATTCAAATACTCCCG-3		Institute, Aachen



**Figure 1A:** pJET -PhaA plasmid.

**Figure 1B:** The (+) and (-) pJET -PhaRBC operon plasmid.

**Figure 1C:** The (+) and (-) pJET -PhaARBC operon plasmid.

### Determination of the (+) orientation for pJET +PHARBC operon and (+) pJET+PHAARBC using HindIII restriction enzyme

The PHARBC operon was then digested with HindIII to determine the orientation, if it is (+) or (-) before ligation and also to determine the orientation for PHAARBC if it is (+) or (-). The samples were resolved on agarose gel, and then estimated the bands size to know the kind of orientation.

### Determination of the (+) orientation for pJET +PHARBC operon and (+) pJET+PHAARBC by colony PCR

To determine the orientation of pJET +PHARBC operon, if it is (+) or (-), colony PCR amplification was achieved using forward B gene detection primer and forward pJET1.2 primer (which must give no band in the gel with only (+) orientation for pJET +PHARBC operon). Also, it was done using forward B gene detection primer and the reverse pJET1.2 primer (which must give band in the gel with only (+) orientation for pJET +PHARBC operon). After detecting the positive colony was incubated in 5ml LB medium and the plasmid was isolated and its concentration was measured using Nano drop spectrophotometer. PCR was run using the same primers that was used in colony PCR to assure results. On the other hand, to check the orientation for pJET +PHAARBC if it is (+) or (-) after ligation, forward pJET1.2 primer and reverse PHAA gene primer were used for amplification (It must give band in the gel with (+) orientation). Then the positive colony was inoculated in 5ml LB medium and the plasmid was isolated and its concentration was measured using Nano drop spectrophotometer. PCR amplification was achieved using the

same primers listed in Table 1 and the same PCR reaction conditions mentioned above.

### PHB genes expression

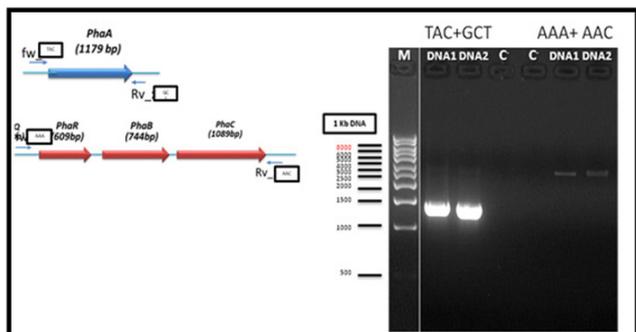
The gene expression of PHB genes was achieved according to McCool et al.<sup>18</sup> After getting the (+) pJET - PHAARBC operon construct, it was transformed to *E. coli* DH5a, *E. coli* XL1-Blue and *E. coli* MJ 109, and then grown overnight. The transformed strains were grown at 37°C in Luria Bertani medium alone and also in Luria Bertani medium supplemented with glucose. The crude extracts of *E. coli* strains were prepared after 7h and 24h of incubated by sonication. Then, spun for 5min at 11000rpm. The crude extracts of *E. coli* strains were precipitated and soluble proteins (supernatant) were separated from insoluble proteins (pellet). Then, proteins were loaded on SDS-PAGE 12% (w/v) polyacrylamide gel and run at 35mA for 1hour. All the expected protein bands of PHB genes expression (PHAA 41,13 KD: PHAB 26.54KD: PHAC 41.69KD: PHAR 18.5KD) were excised from the SDS-gel and analyzed using mass spectrometry (Mascot software. Matrix science) at the proteins analyses unit in Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany. The amino acid sequence of all PHB genes was compared to that present in the databases using BLAST search to find the most homologous sequences to them.

## Results

### PCR amplification of the PHB genes

The genomic DNA of PHA producing bacteria (*Bacillus cereus* MM7) was used as a template for PCR amplification of the PHA biosynthesis genes which consists of PHAA, PHAR, PHAB and

**PHAC.** Two of the oligonucleotide primers for PCR amplification of *PHAA* gene gave positive result with clear bands in the gel while the other two primers did not give bands. Also, the oligonucleotide primers for PCR amplification of *PHAR*, *PHAB* and *PHAC* operon gave positive result with clear bands in the gel, whereas the other two primers did not give any band. PCR reaction resulted in a successful amplification of an approximately 900bp (for partial fragment) and distinct 1.8kb (for full-length gene) from the tested bacteria as shown in Figure 2.



**Figure 2** Amplification of *PHAA* and *PHARBC* genes from *Bacillus cereus* MM7. M, 1 Kb DNA ladder. Lane 2 and 3, Amplification of *B. cereus PHAA* region using primers TAC+GCT. Lane 6 and 7, Amplification of *PHARBC* operon from *B. cereus* using primers AAA+ AAC. Line 4+5 (C-), PCR negative control, PCR mix without *B. cereus* genomic DNA. DNA1, DNA2, duplicate preparations of genomic DNA.

### Cloning of PHA synthesis genes from the strain *Bacillus cereus* MM7

After PCR product purification to *PHAA* and *PHAR*, *PHAB*, *PHAC* operon and cloning them into the pJET1.2/blunt cloning vector, three recombinant plasmids (pJET-*PHAA*, pJET-*PHARBC* operon and pJET-control) were obtained. These plasmids were successfully transformed separately into *E. coli* DH5 $\alpha$  by using heat shock transformation. Then, again the plasmid was extracted and digested with *Xho*I to check the size after cutting with restriction enzyme and check if there is another sites can be cut and the results showed that the size of pJET-*PHAA* (4221pb) and for pJET-*PHARBC* operon (5809pb) were correct as expected and it was also appeared that only one restriction site in the plasmid was there, which mean that all the four genes *PHAA* and *PHAR*, *PHAB*, *PHAC* operon were successfully cloned in two pJET1.2/blunt cloning vectors (Figure 3). Then, the *PHAA* gene in pJET +*PHAA* was amplified and digested with restriction enzymes *Not*I and *Xho*I before running the digestion product in the gel to check the size of *PHAA* gene (1179pb) (Figure 4). The (+) pJET +*PHARBC* operon with *Not*I and *Xho*I was digested and successfully ligated to get (+) pJET +*PHAARBC* operon which transformed to *E. coli* DH5 $\alpha$ , *E. coli* XL1-Blue and *E. coli* MJ 109.

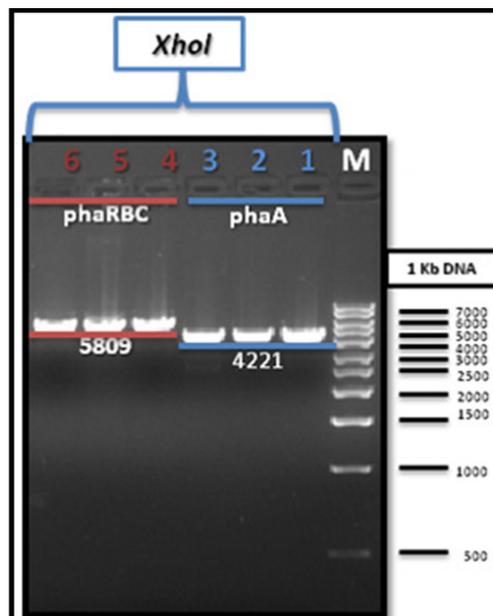
### Detecting if the pJET+*PHAA* was successfully ligated to pJET+*PHARBC* operon

The forward primer pJET1.2 and the reverse primer *PHAA* were used to detect the orientation of pJET +*PHAARBC*, whether it is (+) or (-). The PCR result showed a band which means that pJET +*PHAARBC* was (+) orientation (Figure 5).

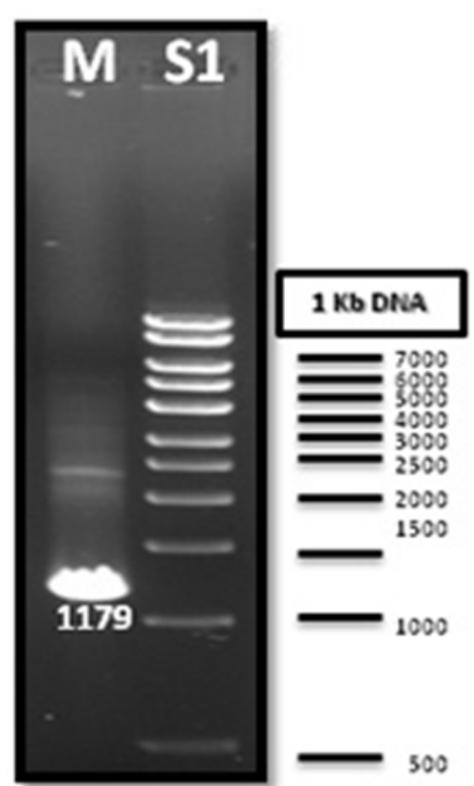
### The sequence data of *PHAA*

The sequence data of *PHAA* was 1186 bp as shown in the Supplementary Figure 1. The BLAST search of this sequence showed

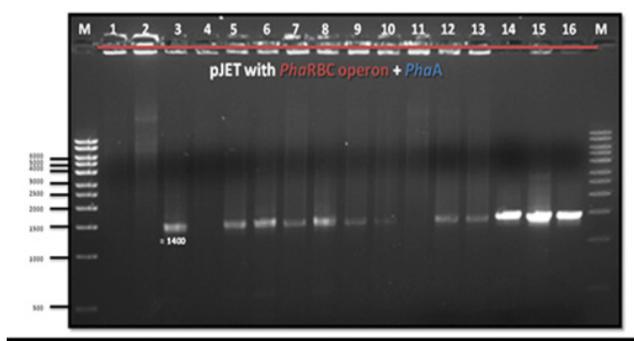
99% homology to the sequence of acetyl-CoA acetyltransferase from *Bacillus thuringiensis* BMB171, accession No. (NC\_014171.1),<sup>19</sup> and *Bacillus cereus* ATCC 14579, accession No.: NC\_004722.1.<sup>20</sup>



**Figure 3:** Determination of the size of *phaA* and *phaRBC* by digestion with *Xho*I restriction enzyme. M: 1 Kb DNA ladder. Lane 1, Lane 2 and Lane 3 are *phaA* plasmid from different colonies. One expected fragment 4221 bp. Lane 3, lane 4 and lane 5 are *phaRBC* operon plasmid from different colonies. One expected fragment 5809 bp.



**Figure 4:** Amplification of *phaA* gene, *phaA* gene was sub cloned into (+) pJET-*phaRBC* operon and the amplification product was cut by *Not*I and *Xho*I enzymes.



**Figure 5** Detecting of the correct ligation of PHAA with pJET - PHARBC by colony PCR. In this experiment, the inserted gene PHAA was tested if it is correctly ligated to pJET -PHARBC operon using colony PCR (using fw vec. + rv PHAA). Many colonies were amplified which means that the inserted gene PHAA is ligated correctly with pJET -PHARBC operon.

### The sequence data of PHAB

The sequence data of PHAB fragment is 745bp as shown in the [Supplementary Figure 2](#) and the BLAST search showed 98% homology to acetoacetyl-CoA reductase of *Bacillus thuringiensis serovar finitimus* YBT-020 NC\_017200.1<sup>21</sup> and *Bacillus thuringiensis serovar finitimus* YBT-020 NC\_017200.1<sup>21</sup> and *Bacillus cereus* FRI-35, accession No.: NC\_018491.1.

### The sequence data of PHAC

The sequence data of PHAC is 1086bp as given in the [Supplementary Figure 3](#) and the BLAST search showed 98% homology to poly(R)-hydroxyalkanoic acid synthase subunit PHAC from *Bacillus thuringiensis serovar finitimus* YBT-020, accession No. NC\_017200.1.<sup>21</sup>

### The sequence data of PHAR

The sequence data of PHAR is 484bp as given in [Supplementary Figure 4](#) and also aligned sequence ata of PHAR using BALAST search, showed 99% homology to PHA synthase subunit PHAR; regulator of PHAP of *Bacillus cereus* ATCC 10987, accession No.: NC\_003909.8.<sup>22</sup>

### The complete sequence of the PHA operon

The complete sequence of the PHA operon is 4279 bp as shown in [Supplementary Figure 5](#). The BLAST search gave 98% homology to PHAR protein acetoacetyl-CoA reductase of *Bacillus cereus* ATCC 14579, accession No. NC\_004722.1.<sup>20</sup>

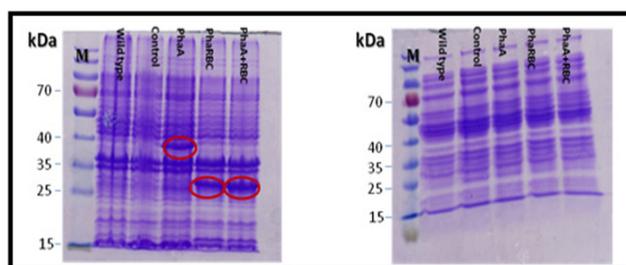
### PHA synthase gene expression

All the expected protein bands in the SDS-gel (PHAA 41,13 KD: PHAB 26.54KD: PHAC 41.69KD: PHAR 18.5KD ) were cut and the proteins were analyzed using mass spectrometry (Mascot software, Matrix science) at the proteins analyses unit in Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany. The result confirmed the presence of PHAA, PHAR and PHAB, which means that PHAA, PHAR and PHAB are expressed. But there was no clear protein band in the gel for PHAC which means that PHAC is probably expressed with very little amounts or not expressed. Also, the current results showed that using the proteins pellet better than soluble proteins in SDS-PAGE gel for expression analysis (Figure 6). Moreover, the proteins were expressed clearly when using medium not supplemented with glucose better than using medium supplemented

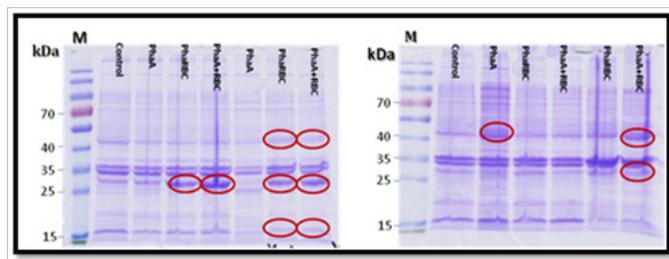
with glucose (Figure 7). The proteins bands were clearer when the strains were incubated for 24h than 7h as shown in Figure 8.

### PHA expression

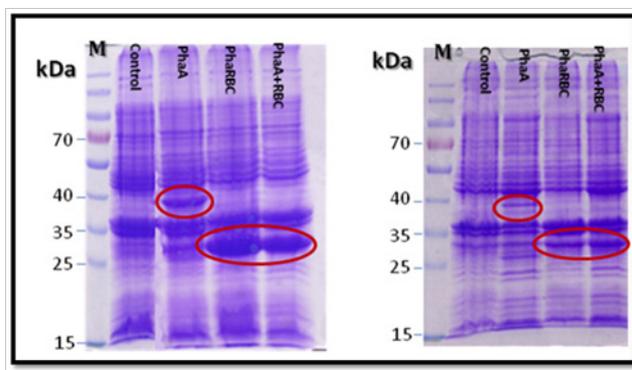
The expression of PHA genes in *E. coli* (transformed with PHA genes), showed the production of three protein subunits, PHAA, PHAB, PHAR and the absence of PHAC protein subunit. The MS analysis of the three proteins showed that PHA was 390 amino acids as shown in the [supplementary materials Figure 6](#), PHAB was 247 amino acids ([Supplementary Figure 7](#)) and the PHR was 176 amino acids ([Supplementary Figure 8](#)). On the other hand, the BLASTP search of the amino acid sequence for PHAA subunit. The PHAA amino acid sequence showed 100% homology to acetyl-CoA acetyltransferase ATCC 14579 of *Bacillus cereus* Accession No.: NP\_834674.1.<sup>20</sup>



**Figure 6** A. M, Protein marker. Lane 1, Wild type of *E. coli* JM109. Lane 2 control. Lane3 PHAA Lane 4 PHARBC. Lane 5, All PHAARBC. Using Pellet 1.5 ml. B. M, Protein marker. Lane 1, Wild type of *E. coli* JM109. Lane 2 control. Lane3 PHAA Lane4 PHARBC. Lane 5, All PHAARBC.



**Figure 7** A. M, Protein marker. Lane 1, control of *E. coli* JM109. Lane2 PHAA, Lane 3 PHARBC. Lane 4, All PHAARBC. Using pellet 1.5ml (LB with glucose). Lane5 PHAA, lane 6 PHARBC. Lane 7, PHAARBC. Loaded without glucose. B. M, Protein marker. Lane 1, control of *E. coli* XLI- Blue. Lane2 PHAA, lane 3 PHARBC. Lane 4, All PHAARBC. Using pellet 1.5ml. (LB with glucose). Lane5 PHABCR lane 6. PHAARBC. Loading was done without glucose.



**Figure 8** A. M, Protein marker. Lane 1, control of *E. coli* JM109. Lane2 PHAA, Lane 3 PHARBC. Lane 4, All PHAARBC. Using Pellet 1.5ml. 24h (15µl). B. M, Protein marker. Lane 1, control of *E. coli* JM109. Lane2 PHAA, lane 3 PHARBC. Lane 4, All PHAARBC, using pellet 1.5ml. 7h (15µl).

## Discussion

Four classes of PHA synthase have been classified based on subunit composition of enzymes and their substrate specificities.<sup>23</sup> *Bacillus* strains belong to class IV synthase that comprises PHAC and its subunit PHAR.<sup>24</sup> The most important genes that involves in PHB production in *Bacillus* strains were PHAA and PHAR, PHAB, PHAC.<sup>3,23</sup> On the other hand, Valappil et al.<sup>25</sup> and Tomizawa et al.<sup>14</sup> proved that in *Bacillus* genus, two proteins, PHAC, the normal PHA synthase enzyme, and PHAR have been proven to be essential for the PHA synthase activity. The PHA synthases was exist in two forms; an active form in PHA-accumulating cells and an inactive form in non-accumulating cells, where PHAC is more susceptible to degradation in cell.<sup>18</sup> All PHA genes; PHAA, PHAR, PHAB, PHAC were amplified and assembled together, and then cloned in *E. coli*. The results showed that PHAA sequence (1186bp) gave the maximum homology of 99% to acetyl-CoA acetyltransferase of *Bacillus thuringiensis* BMB171, accession No.: NC\_014171.1 and *Bacillus cereus* ATCC 14579, accession No.: NC\_004722.1.<sup>20</sup> In addition, the sequence data of PHAB fragment was found 745 bp and the BLAST search gave a maximum homology of 98% to many *Bacilli* strains sequence e.g. acetoacetyl-CoA reductase of *Bacillus thuringiensis* serovar finitimus YBT-020 NC\_017200.1<sup>21</sup> and *Bacillus cereus* FRI-35, accession No.: NC\_018491.1. Moreover, the sequence data of PHAC was found 1086bp and the BLAST search of PHAC sequence showed 98% homology to poly(R)-hydroxyalkanoic acid synthase subunit PHAC from *Bacillus thuringiensis* serovar finitimus YBT-020, accession No. NC\_017200.1.<sup>21</sup> Also, the sequence data of PHAR was 484bp and the BLAST search of homologous sequence to PHAR showed 99% homology to PHA synthase subunit PHAR; regulator of PHAP of *Bacillus cereus* ATCC 10987, accession No.: NC\_003909.<sup>8,22</sup> and *Bacillus thuringiensis* serovar finitimus YBT-020, accession No.: NC\_017200.1.

Finally, the complete sequence of the PHA operon resulted from PCR amplification and sequencing using the specific primers was 4279 bp and the BLAST search gave 98% homology to PHAR proteinacetoacetyl-CoA reductase of *Bacillus cereus* ATCC 14579, accession No. NC\_004722.1.<sup>20</sup> This result is consistent with many studies in different bacteria such as; *Rhodospirillum rubrum*,<sup>26</sup> *Bacillus megaterium*,<sup>18</sup> *Bacillus cereus* SPV<sup>25</sup> and *Bacillus thuringiensis* R1.<sup>4</sup> It is worthy to mention that in this study, it is the first time to isolate and clone all the four genes; PHAA and PHAR, PHAB, PHAC responsible for PHB production from the same bacterial isolate “*Bacillus cereus* MM7”, in contrast with the previous studies which used only two or three genes of PHB biosynthetic genes and cloned them in plasmid that carry the remaining PHB biosynthetic gene.<sup>4</sup> The four PHA genes were cloned and expressed in *E. coli* XL1-Blue and *E. coli* MJ 109 and the resulting proteins were analyzed using SDS-PAGE.<sup>18</sup> The results of polyacrylamide gel electrophoresis and proteins mass spectrometry data (Mascot software. Matrix science) confirmed that the PHAA (41,13 KD), PHAR (18.5KD) and PHAB (26.54KD) were expressed very well and the their bands in the gel were also clear, but there was no clear protein band in the gel for PHAC (41.69KD) which means that PHAC is probably expressed with very little amounts or not expressed. The MS analysis was performed for the corresponding protein bands for PHB genes according to Slater et al.,<sup>27</sup> Valappil et al.<sup>25</sup> and Zhu et al.<sup>13</sup>

The current results showed that using the proteins pellet better than soluble proteins in SDS-PAGE gel to analyze the expression, this may be due to the elongation of PHA chain and phasing proteins or the specific proteins that are associated with the produced PHA

granules or budding of a vesicle with a phospholipid layer and leading to granule formation.<sup>2</sup> Many other studies used whole-cell extracts for determination of one protein or more of PHA synthesis proteins activity.<sup>10,18,28</sup> In contrast, Zhu et al.<sup>13</sup> used soluble protein to detect PHB expression proteins (PHBA, PHBB and PHBC) which were all expressed at high levels. Moreover, in the current study, the PHB encoding proteins were expressed clearly when using a medium not supplemented with glucose better than using glucose. This result agrees with Davis et al.<sup>10</sup> who used LB media without glucose and noted that PHAC1, PHAJ1, PHAA and PHAB proteins were expressed very clear. In the current study, the amino acid sequence analysis of the three proteins using MS showed that PHAA was 390 Amino acids with 100% homology to acetyl-CoA acetyltransferase ATCC 14579 of *Bacillus cereus* Accession No.: NP\_834674.1.<sup>20</sup> PHAB was 247 amino acids and it showed 100% homology with acetoacetyl-CoA reductase (*Bacillus cereus* ATCC 14579) accession No.: NP\_831099.1.<sup>20</sup> PHAR was 176 amino acids and it showed 89% similarity to hypothetical protein [*Bacillus cereus*], accession No.: WP\_002195298.1. Similar results were obtained in many previous studies which sequenced and analyzed the amino acids of PHB proteins in *Rhodospirillum rubrum*,<sup>26</sup> *Pseudomonas* sp.<sup>29,30</sup> *Bacillus* sp.<sup>4,18,25</sup> In conclusion, *Bacillus cereus* MM7 isolate was able to synthesize Poly 3-hydroxybutyrate. The PHB genes were isolated, cloned, sequenced and expressed in *E. coli* DH5 $\alpha$ . The PHB encoding proteins were analyzed in SDS-PAGE and the PHB corresponding protein bands were analyzed using mass spectrometry to nominate their amino acid sequence.

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## Conflict of interest

Author declares that there is no conflict of interest.

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