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Original Article

Recovery and Molecular characterization of poly-β-hydoxybutyrate polymer by *Bacillus cereus* isolate P83

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ABSTRACT

Production of polyhydroxyalkanoates by *Bacillus cereus* isolate P83 was carried out in a 14L laboratory fermentor using mineral salt medium containing 0.7% corn oil and 0.1 g/L ammonium chloride as carbon and nitrogen sources, respectively at 28°C, 200 rpm, 2 vvm and 7.2 pH. The maximum level of polyhydroxyalkanoates (53% per dry weight) was achieved after 24 hours of incubation. Different methods (Digestion by sodium hypochlorite, dispersion of chloroform and hypochlorite, mechanical dispersion and chloroform extraction) were used for polymer recovery. According to the obtained results, the highest molecular weight of polymer ($2.15*10^4$ g/mole) and lowest polydispersity (1.15) were achieved using chloroform extraction method. The results of the characterization techniques, IR and 1H-NMR spectroscopy, showed polyhydroxybutyrate identity. Analysis of PHA synthase and PhaR subunit PCR products indicated class IV PHA synthase with enzymatic and specific activity (measured by DTNB assay) of 195U and 1500U/mg, respectively. In conclusion, *Bacillus cereus* isolate P83 is a candidate for commercial production of PHB polymer with high growth rate and promising polyhydroxybutyrate production level. The produced polymer is characterized by comparable molecular weight of polymer with low polydispersity value.

Keywords: PHA synthase- Polyhydroxyalkanaotes- *Bacillus cereus* - Swiss model- chloroform extraction

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INTRODUCTION

Polyhydroxyalkanoates (PHA) biopolymers have picked up considerable biotechnological interest in the last few years. They are biocompatible and biodegradable biopolymers produced by bacteria under nutrient limitation with excess carbon source in the medium [1,2]. They are good alternatives for synthetic plastics inspite of their high production cost [3,4]. The ways addressed to decrease the cost are using inexpensive carbon sources, efficient recovery method, studying the PHA biosynthesis and mechanism of its formation inside the bacterial cells [2,5]. The carbon source contributes to 80% of the final cost of PHA so simple carbon sources as sugars and plant oils are usually used [6]. Different recovery methods affect the quality of the produced polymer in addition to its molecular weight so for each new strain recovery methods are investigated [7].

Poly-β-hydroxybutyrate (PHB) is the first member in the PHA family and the most characterized one [8] . PHB biosynthetic pathway is initiated by condensation of two molecules of acetyl-CoA by β-ketothiolase to acetoacetyl-CoA, which is reduced by acetoacetyl-CoA reductase to 3-hydroxybutyryl-CoA which will then be polymerized into PHB by PHA synthase [9]. The concentration and molecular weight of PHB polymer is largely depended on the activity of the rate limiting enzyme in the biosynthetic pathway, PHA synthase enzyme [5]. PHA synthases are divided into four classes depending on their substrate specificity and composition of their subunits [2]. Class I and class II PHA synthases consist of one subunit (PhaC) while class III and IV have extra smaller subunit; PhaE and PhaR, respectively [10].

Regarding PHA formation inside the bacterial cell, they are formed as discrete granules intracellulary without any alteration in osmotic state or general fitness of the cell [11]. Two models were proposed for PHA accumulation. The budding model assumes initiation of polymer formation in the cytoplasmic membrane via membrane-attached PHA synthase [9]. The micelle model, the second model, proposes the availability of PHA synthase in the cytoplasm then it starts PHA polymerization once the cell has provided enough substrate molecules [9].

In this study, we used *Bacillus cereus* isolate P83, previously isolated and identified as a good PHA producer [12], for the polymer production at large scale level using 14 L fermentor. Different recovery methods were investigated to select the suitable method for polymer downstream processing. Moreover, molecular chrachterization and enzymological analysis of PHA synthase enzyme were done and its consequences on intracellular PHA formation were addressed.

MATERIALS AND METHODS

Fermentation: For PHB production, a 20 h culture of *Bacillus cereus* isolate P383 in Luria bertani (LB) broth was used for inoculation of 14L fermentor (CelliGen 310 glass fermentor, new Brunswick Scientific, Edison, NJ, USA). The main production medium, named as mineral salt medium, composed of 7 ml corn oil, 0.2 g ammonium chloride, 10 g sodium chloride, 10 g disodium hydrogen phosphate and 1.5 g potassium dihydrogen phosphate per liter, adjusted to initial pH 7.2. The working volume was 4L with applied conditions of 2 vvm aeration (8 SLPM), 200 rpm agitation, 28°C temperature and 100% oxygen saturation. Samples were drawn to measure PHB concentration [13] biomass [12] and PHB percentage per dry weight. pH and dissolved oxygen percentage were monitored during fermentation process.

Recovery methods of PHB biopolymer

About 2 mg pellet dry bacterial weight was used for all the studied methods of recovery. For all recovery methods, PHB concentration, molecular mass and polydispersity index were measured [14].

a. **Recovery using sodium hypochlorite (5%):** The bacterial pellet was mixed with sodium hypochlorite solution in shaking incubator at 37°C for 1 hour at 160 rpm. PHB granules were separated by centrifugation at 12,000 rpm for 10 min, then rinsed with 1ml distilled water, ethanol and acetone, successively. Extraction of PHB granules was carried out using hot chloroform and evaporating the chloroform extract to obtain the dried PHB residue [15].

b. **Recovery using dispersion of sodium hypochlorite and chloroform:** The bacterial pellet was treated with a dispersion containing 1 ml of chloroform and 1 ml of sodium hypochlorite solution. The mixture was left for 1, 2 and 3 hours at 30°C with shaking and then centrifuged at 6,000 rpm for 10 min forming three separate phases; the upper phase was hypochlorite solution, the middle phase contained non-PHB cell materials and undisrupted cells, and the bottom phase was chloroform-containing PHB. For recovering the PHB, the chloroform layer was aspirated and PHB was precipitated using cold methanol: water (At ratio7:3) [15].

c. **Recovery using mechanical disruption:** At the end of fermentation, the bacterial pellet was

dissolved in lysis buffer (0.3 g Tris –HCL pH 7.4, 0.36 g Potassium chloride, 0.046 g Magnesium chloride and 0.0146g EDTA in 100 ml distilled water) [16]. The disruption of the cells using an ultrasonic processor for PHB liberation was investigated for total time of 5-15 min in an ice bath where sonication period was 30 s followed by a 5-s cooling period. PHB extraction was done by adding 1 ml chloroform to the disrupted cells at 30 °C and left overnight in shaking incubator. After centrifugation at 6000 rpm for 10 min, the chloroform layer was aspirated and the dissolved PHB was then precipitated via the addition of cold methanol :water (At ratio7:3) [14].

d. **Recovery using chloroform extraction:** About 2 ml chloroform were added to the bacterial pellet and incubated for 24-72 hours at 30°C in shaking incubator. At the end of incubation, one ml distilled water was added, vortexed and the chloroform layer containing PHB was then aspirated and the polymer was separated as described before [17].

Characterization of the recovered biopolymer

a. Transmission electron microscope: At the end of fermentation process, the bacterial pellets were obtained by centrifugation at 10,000 rpm for 10 min. Then the steps required for examination by transmission electron microscope were carried out according to Tian et al (2005) [18].

b. Fourier transform infrared spectroscopy (FT-IR): The PHB extracted from the tested organisms was dissolved in chloroform and casted on KBr pellets. The spectra for extracted PHB were analyzed and compared against the spectra of commercial PHB (Sigma-Aldrich Ltd., UK). It was done within spectral range, 4000-400 cm $^{-1}$ using Fourier transform spectrometer [19].

c. Nuclear magnetic resonance (NMR analysis): H1 NMR was performed to verify the composition of PHB produced. The extracted PHB polymer was dissolved in deuterated chloroform where tetramethylsilane (TMS) was used as an internal standard. The H1 NMR produced spectrum was compared against the spectra of commercial PHB (Sigma-Aldrich Ltd., UK) [19].

PHA synthase enzyme assay: PHA synthase activity was measured spectrophotometrically by monitoring the release of CoA at 410 nm using Ellman's reagent,5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) [20]

The crude lysate preparation: *Bacillus cereus* isolate P83 was cultured in 83M medium (0.7% corn oil, 2mg/L ammonium chloride, 10g/L disodium hydrogen phosphate dodecahydrate, 1.5g/L potassium dihydrogen phosphate and 10g/L sodium chloride) for 48 hours of incubation, then centrifugation of the cells were carried out at 6000 rpm for 10 min and suspended in 3 ml lysis buffer (50 Mm potassium dihydrogen phosphate (pH 7) containing 5% glycerol) [21]. Release of the enzyme was done by 3 min total time sonication (30s sonication followed by 30s cooling) [22], then it was centrifuged at 2800xg at 4°C for 15 min to inactivate NADH oxidase.

DTNB assay: The reaction starts by adding the crude lysate to the reaction mixture containing 1.0 mM HBCoA (Hydroxybutyryl-coA lithium salt) and 143 mM potassuim hydrogen phosphate (pH 7.0). Aliquots (40 µL) were removed at timed intervals and stopped by the addition of 100 µL of 5% trichloroacetic acid. The protein was precipitated by centrifugation for 10 min, and an aliquot (125µL) of the supernatant was added to 675 µL of 500 mM potassuim hydrogen phosphate (pH 7.5). DTNB [l0 µL of a 10 mM stock solution in 500 mM potassuim hydrogen phosphate (pH 7.5)] was added to this mixture and incubated for 2 min at room temperature [20]. The absorbance was measured at 410 nm. One U of enzyme is defined as the amount which produced 1µM product per minute under the assay conditions employed [23]. Protein concentration of the bacterial crude lysate was measured by the method of Lowry [24].

Molecular characterization of PHA synthase enzyme

Chromosomal DNA manipulations: Extraction of the DNA was done from fresh culture of *Bacillus cereus* isolate P83 (16 h) using GeneJET Genomic DNA Purification Kit according to manufacturer specifications. DNA yield was verified by agarose gel electrophoresis to ensure efficient DNA extraction.

Primer design: For designing the PHA synthase primers, the nucleotide sequences of PHA synthase class IV of different *Bacillus* species and its R-subunit were collected from NCBI GenBank. For designing *B. cereus* PHA synthase primers, the same procedures were carried out as described before. The conserved regions of the amino acid alignment data at the beginning and the end of the protein sequences were analyzed using pDRAW32 program (http://www.acasoft.dk/acaclone/download/install.htm) and the melting temperatures (Tm) were calculated. The confirmations of the designed primer specify was carried out using Primer-BLAST on the NCBI database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers are listed in table 1.

Polymerase chain reaction (PCR): The total reaction mixture for PCR was 50µl containing 25 µl master mix (Red Taq Ready mix, Sigma Aldrich), 1 µl (20 pmole/µl) of each of forward (P_f) and reverse (P_r) primers, 1µl of template chromosomal DNA. The thermal cycle program, run on NYX TECHNIK ATC 40I thermocycler, USA is listed in table 2. Detection of the amplified genes was done by agarose gel electrophoresis.

Sequencing of the amplified PHA synthase gene: GeneJET^{TM} PCR Purification kit, (Fermentas) was used for purification of the PCR products of PHA synthase enzymes at Sigma Scientific Services Company, Egypt. Then, nucleotides Sequencing was done at GATC Company, through Sigma Scientific Services Company, Egypt, by the use of ABI 3730xl DNA Sequencer. Afterwards, the forward and reverse obtained sequences were assembled into final consensus sequences using Staden package program version 3 (http://staden.sourceforge.net/) [25]. Then, detection of the open reading frames (ORFs) in the final consensus using Frameplot (http://www0.nih.go.jp/~jun/cgi-bin/frameplot.pl) [26]. Conserved domain analysis was conducted using Basic Local Alignment Search Tool (NCBI): http://www.ncbi.nlm.nih.gov/Structure/index.shtml [27].

Prediction of the tertiary structure of the PHA synthase enzyme: The putative tertiary structures of the sequenced enzymes were predicted using Swiss-Model software (http://swissmodel.expasy.org/) [28]. The structural class of the tertiary structure of the obtained gene product was performed using Modular Approach to Structural class prediction (MODAS, http://biomine.ece.ualberta.ca/MODAS/).

RESULTS

PHA production on 14L fermentor: After applying the shake flask optimum conditions (200 rpm agitation, 28 °C temperature and initial pH 7.2) on 14 L fermentor, *Bacillus cereus* isolate P83 produced about 53% PHA production after 24 hours of incubation (Figure 1). The dissolved oxygen percentage reached 38% after 8 hours then increased gradually to 90% at the end of the fermentation process.

Different recovery methods: For downstream processing of PHA, different methods of recovery were tested. As displayed in figure 2a, treatment of bacterial pellet with dispersion of both sodium hypochlorite and chloroform for 2 hours was enough to liberate highest amount of polymer. Regarding recovery by sonication, 10 min total time sonication released the largest amount of polymer (Figure 2b), while 72 hours incubation with chloroform was the best with no significant difference from 24 hours incubation (Figure 2c).Taken together (Figure 3), the highest polymer concentration and molecular weight was recovered by the chloroform extraction method for 24 hours.

Characterization of the produced polymer

Transmission electron microscope: Figure 4 showed electron dense PHA granules in the center of the bacterial cell.

FT-IR spectroscopy: The spectrum for extracted PHA was compared against the spectrum of commercial PHB (Sigma-Aldrich Ltd., UK). The FTIR spectra for the extracted PHB showed almost identical peak positioning when compared to the spectrum obtained from a standard PHB (Figure 5).The most prominent peak for PHB is the ester carbonyl band at 1720-1740 cm-1. The signals seen at 1000–1500 cm $^{-1}$ range are due to the bendings of CH2 and CH3,–C–O– and –C–C– groups. In-between 1280 and 1050 cm $^{-1}$,signals are due to valence antisymmetric and symmetric vibration of C–O–C .The peak at 344.5 cm $^{-1}$ is correlated to O-H bending.

1H-NMR spectroscopy: The 1H-NMR spectrum as shown in figure 6 revealed the presence of signals characteristic of PHB. The signals are illustrated in the 1H-NMR spectrum as chemical shifts from tetramethylsilane (TMS) which appeared at zero ppm. The PHB spectrum included the doublet at 1.3 ppm attributed to the methyl group (CH3) coupled to one proton; and the doublet of the quadruplet at 2.57 ppm attributed to the methylene group (CH2) adjacent to an asymmetric carbon atom bearing a single proton and the multiplet at 5.28 ppm attributed to the (CH) group. The 1H-NMR spectra of PHB were compared to 1H-NMR spectrum of a PHB standard, it showed identical positioning of signals for PHB standard except for signals appeared at 3.8-4.5 ppm which refer to the terminal esterification of glycerol to PHB through the primary hydroxyls (C1 or C3 positions of glycerol).

PHA synthase activity assay: *Bacillus cereus* isolate P83 showed a 195 U activity of PHA synthase enzyme and specific activity of 1500 U/mg. As shown in figure 7, the maximum activity was observed after 6 min.

Molecular characterization of PHA synthase enzyme: The results of the PCR and agarose gel electrophoresis displayed the size of the target gene

as expected 1.08 Kb and R-subunit at 0.3 Kb (Figure 8).

The nucleotide sequence of the PCR product was analyzed and submitted to the GenBank database under the following accession code (KX358865). Clustal Omega software was used for aligning the PHA synthase enzyme sequence with other homologous proteins sequences (Figure 9). The alignment results showed conserved lipase box (G-X-[S/C]-X-G) and cysteine (C) residue.

The protein sequence of *Bacillus cereus* isolate P83 PHA synthase enzyme exhibited class III domain and alpha beta hydrolase super family as deduced by Blast Conserved Domain Analysis. MODAS server predicted the structural class of PHA synthase to be multi domain (α and β) with 59.12% probability and 1.7% probability membrane protein.

Prediction of the putative tertiary structure of the PHA synthase was carried out using Swiss model and it showed 25.09% similarity to *Ralstonia eutropha* polyhydroxyalkanoate synthase class I C-terminal domain (Figure 10). The central beta sheets were surrounded by alpha helix, a common feature of α/β hydrolase fold. The conserved cysteine residue was found at the junction between β sheet and α helix in the so-called nucleophile elbow, another characteristic feature of the α/β hydrolase super family (Figure 10). In addition, the Swiss model mentioned 17% similarity between the obtained PHA synthase sequence of *Bacillus cereus* isolate P83 and lipase enzyme.

DISCUSSION

The high cost of PHA biopolymer production is a barrier against its commercialization. For overcoming such barrier, certain strategies can be adapted as strain development, more efficient fermentation, effective recovery process, inexpensive carbon substrate and deep understanding of PHA formation inside the cells. In this study, many of these strategies were tracked for competent usage of *Bacillus cereus* isolate P83 as a PHB commercial producer. The carbon source used was corn oil which is an economic renewable source supporting PHB biosynthesis. Cuellar and his coworkers (2011) reported that plant oils are cheap carbon sources containing mixture of useful fatty acids required for PHA accumulation [6]. By using corn oil as carbon source, *Bacillus cereus* isolate P83 produced up to 53% PHA per dry weight after 24 hours of incubation on large scale level using 14L lab fermentor. Tsuge (2016) reported that most bacteria accumulate from 30-50% PHA per dry weight, moreover Aarthi and Ramana (2011) worked on *B. mycoides* producing 57% per dry weight but after 48 hours of incubation [29,30]. Chaijamrus and Udpuay (2008) stated that *B. megateruim* produced 32% per dry weight [31].

The PHA recovery process contributes for 70-80% of PHA production cost [7] and affects the quality of the produced polymer so different methods of polymer recovery were tested. The obtained results revealed that extraction by chloroform produced the highest concentration of polymer with high molecular weight and low polydisperisty index. As reported previously, chloroform extraction is mostly acknowledged for high purity of the recovered polymer and protection of polymer chains against degradation [7,15].

PHA characterization was carried out using FT-IR and 1H-NMR spectroscopy. After interpretation of both charts in comparison to PHB standard chart, PHB polymer identity was confirmed. The only intriguing signals were found in 1H-NMR charts referring to glycerol peaks which may act as PHB chain terminator. The glycerol may be produced in the medium as a metabolic product of corn oil [33]. Ashby and his coworkers (2011) pointed that chain termination of PHB can be done using glycerol which was used in the fermentation process as the main carbon source [32].

Biosynthesis of Poly-β-hydroxybutyrate polymer is an interesting biological phenomenon in microbial physiology. It is carried out using three enzymes (3-ketothiolase, acetoacetyl reductase and PHB synthase) [34,35]. The rate limiting enzyme is PHA synthase which catalyze the conversion of (*R*)-3-hydroxybutyryl-CoA to PHB with the concomitant release of CoA. Four classes for PHA synthase enzyme are well-known in literature [5,21,22]. Since PHA synthase enzyme directly affects the PHB polymer production, molecular weight and polydisperisity, molecular characterization and enzymatic analysis of the respective enzyme offers an opportunity for more efficient bioplastics industry. The PHA synthase activity was measured by discontinuous assay using Ellman reagent to prevent the inhibitory effect of Ellman reagent on thiol groups of PHA synthase [9]. The results of this assay displayed interesting features of *Bacillus cereus isolate* P83 PHA synthase enzyme. It showed high activity of 195 U and specific activity 1500 U/mg. The specific activity of PHA synthase enzyme in *Aeromonas punctata* wild-type and mutant strains cell extracts was in the range of 118 – 768 U/g [36]. On the contrary, the PHA synthases of *Pseudomonas* sp. 61-3 harboring class II PHA synthase had activity of 50 U/g towards the

polymerization of 3HB-CoA [30]. Bhubalan et al (2011) reported specific activity of 2462U/g for recombinant PhaC of *Chromobacteruim* USM2 expressed in *Escherichia coli* [22].

The PHA synthase class IV composes of two subunits: PHA synthase class III and R- subunit. This class is capable of polymerizing short chain monomer units and it is widespread in *Bacillus* species [37]. In the present study, PHA synthase class IV identity was confirmed in *Bacillus cereus* isolate P83 due to presence of its two components: PHA synthase class III domain and R-subunit. The conserved domain analysis confirmed the presence of class III PHA synthase domain in the obtained sequence and PCR with agarose gel electrophoresis displayed the R-subunit at its expected size (0.3 Kb). In addition, Swiss model software predicted the putative tertiary structure of the PHA synthase protein of the respective isolate showing 25.09% similarity to *Ralstonia eutropha* polyhydroxyalkanoate synthase class I C-terminal domain. This emphasized the class IV identity because the class IV PHA synthase protein sequences generally exhibits amino acid sequence similarity of 21–28% to class I and II PHA synthases [10]. Moreover, the isolated PHA synthase protein sequence showed 17% similarity to lipase enzyme by Swiss model software. Jia and his coworkers (2000) reported a similarity between PHA synthases and lipases in both amino acids alignment and mechanism of action [28]. Bacterial lipases are interfacial catalysts as PHA synthases, both of them function on water-oil interface. The PHA synthase enzymes are attached to the surfaces of the insoluble granules to continue polymerization process [28]. Moreover, both lipases and PHA synthase belong to the α/β-hydrolase superfamily of proteins which comprises many enzymes with marked variation in substrate specificity, including: thioesterases , hydrolases , and cholesterol esterases [10].

Researchers postulated two models for PHB granules formation; budding and micelle models [9]. To know which model is used for PHA formation in *Bacillus cereus* isolate P83, molecular characterization of PHA synthase enzyme was done. The MODAS structural class prediction of the putative tertiary structure of the isolated PHA synthase enzyme excluded the probability of being membrane protein; instead, it showed 59% probability multidomain protein and 1.75% membrane protein. In addition, Swiss model estimated the presence of the conserved catalytic cysteine residue in the known nucleophilic elbow. This elbow is a known feature of α/β hydrolase superfamily [38]. Kim et al (2017) reported the presence of a series of conserved hydrophobic residues among PHA synthases class I, III and IV, the reason behind same substrate specificities, to act as a tunnel for entrance of substrate to the cysteine residue example valine at 321 and illeucine at 246 [5]. This tunnel along with the PHA synthase similarity with lipase enzyme and MODAS structural class prediction supported the cytoplasmic nature of PHA synthase which in turn advocates the micelle model for PHA formation in *Bacillus cereus* isolate P83.

In conclusion, *Bacillus cereus* isolate P83 is a good candidate for commercial production of PHB polymer for biomedical practice due to promising production level, high molecular weight and low polydisperisty. Efficient PHB recovery process was achieved by using chloroform extraction technique. Class IV PHA synthase is responsible for PHB production in the respective isolate by micelle model.

Table 1: list of primers sequences, annealing temperature (T_a) and expected PCR size

Gene	Primer	Primer sequence	Expected product size (Kb)	Ta (°C)
PHA-synthase (*Bacillus*)	P_f	5'ATGACTACATTCGTAACGGAATGGGAAAAGC-3'	1.08	54
	P_r	5'TTAATTAGAACGCTCTTCAAGCCAATTTCCAATC-3'		
R-subunit	P_f :	5'ATGGGCAGTGTTCTAGATTTG-3'	0.3	51
	P_r	5'TCATTTTTTATTTTCTGGCTTATTCGTAG-3'		

Table 2: Conditions of PCR cycles (Thermocycler input data)

Steps	Temp (°C)	Duration	No. of cycles
Initial denaturation	95	2 min	1
Subsequent denaturation	95	45 sec	35
Annealing	55	45 sec	
Extension	72	1 min	
Final extension	72	5 min	1
Hold	4		

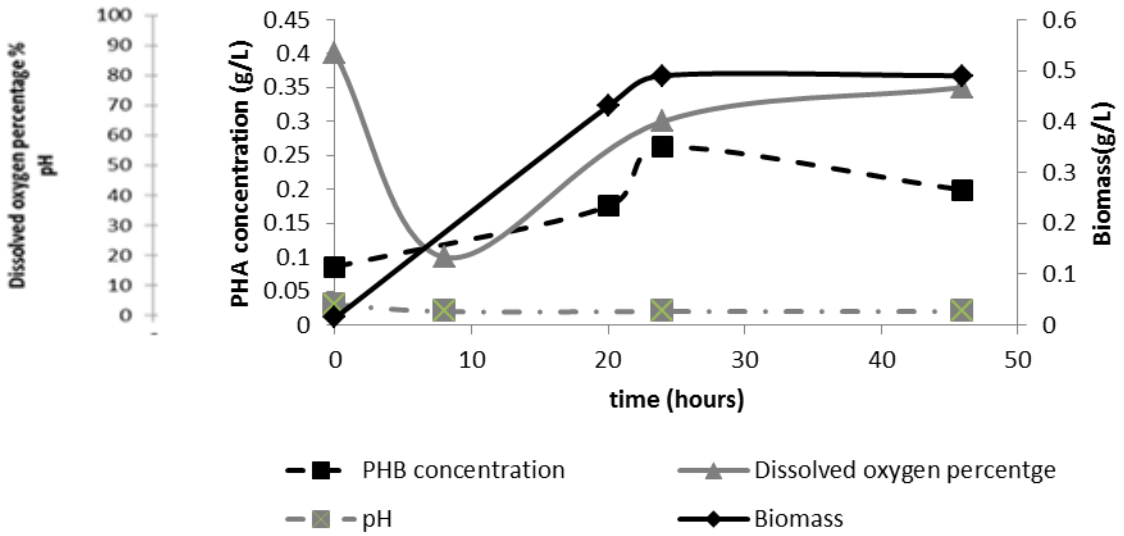

Figure 1: Time course of PHA production, biomass, pH and dissolved oxygen percentage by *Bacillus cereus* isolate P83 on 14 L fermentor

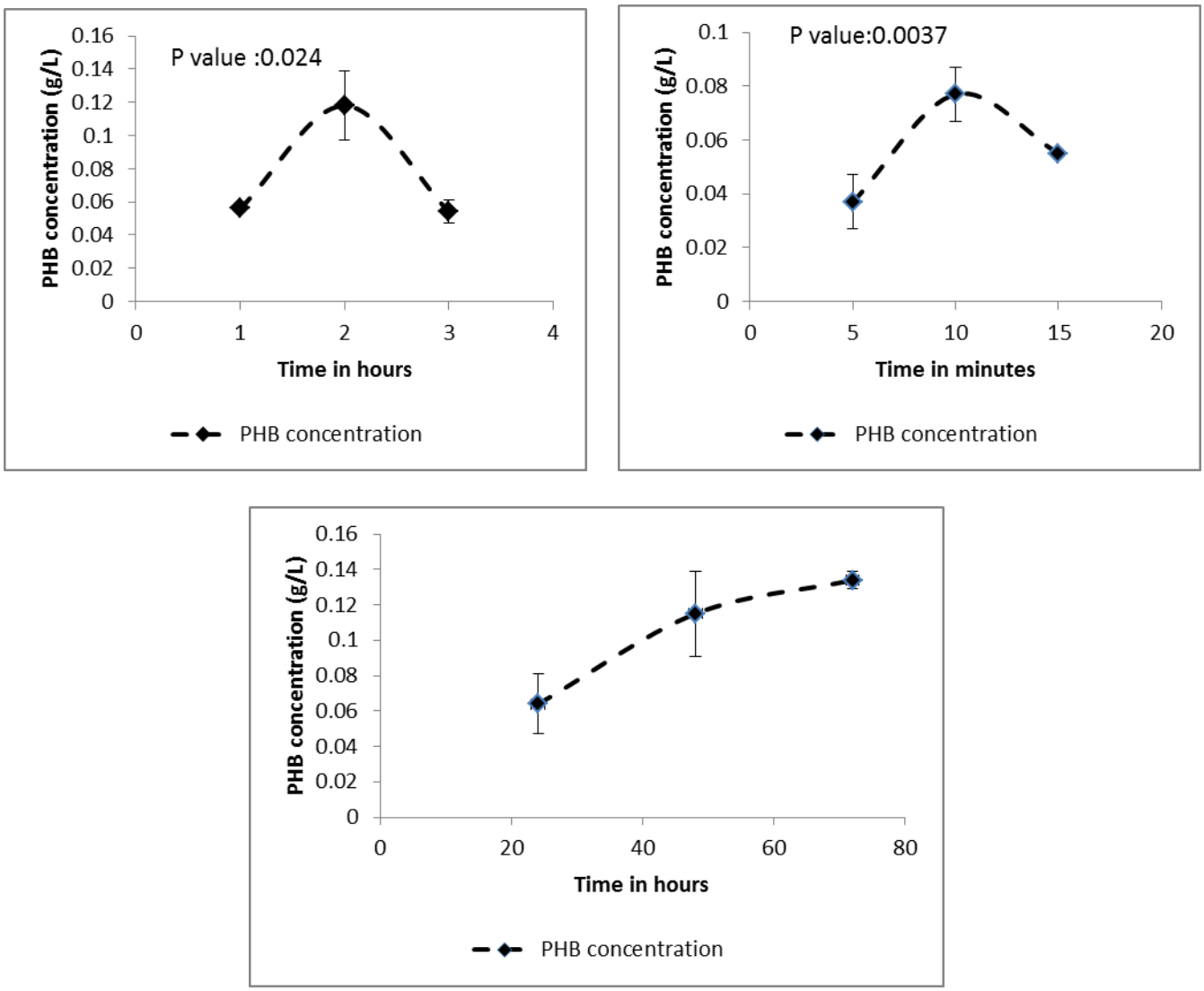

Figure 2: Effect of recovery methods with different treatment times, dispersion (A)- sonication (B)- chloroform extraction (C) on PHA concentration of *B. cereus* isolate P83

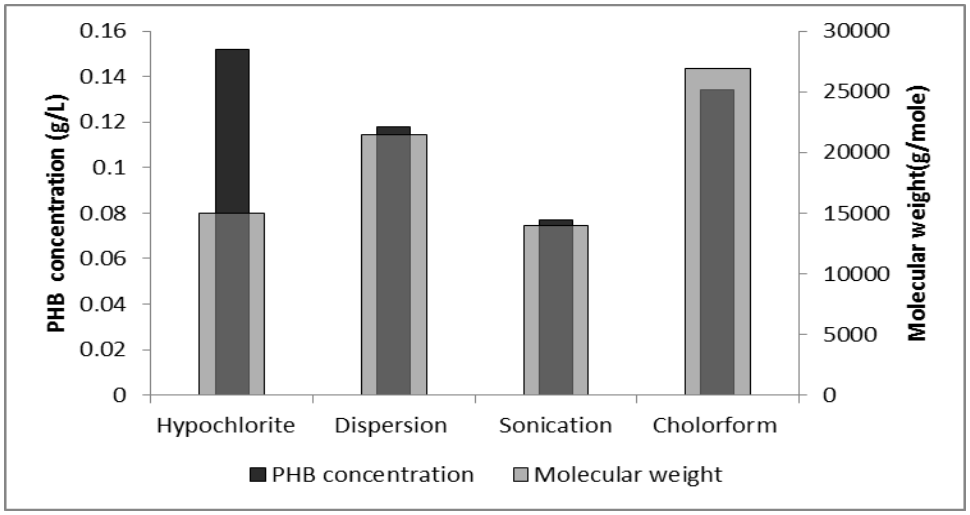

Figure 3: Effect of different recovery methods on molecular weight and concentration of PHA produced by *B. cereus* isolate P83

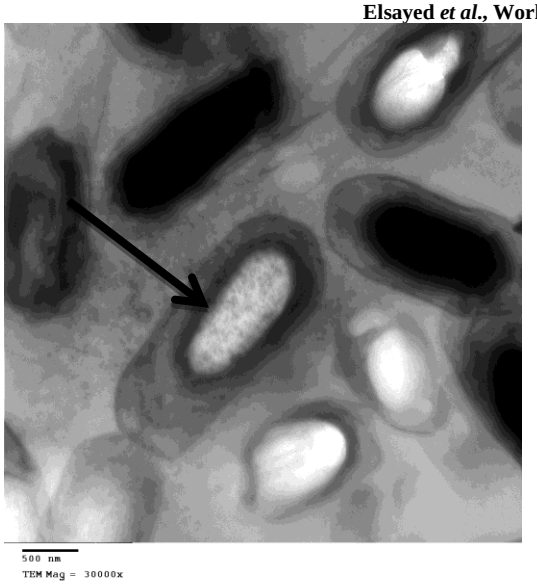

Figure 4: Electron micrographs of *B. cereus* isolate P83. Arrow showed PHB granules inside the cells.

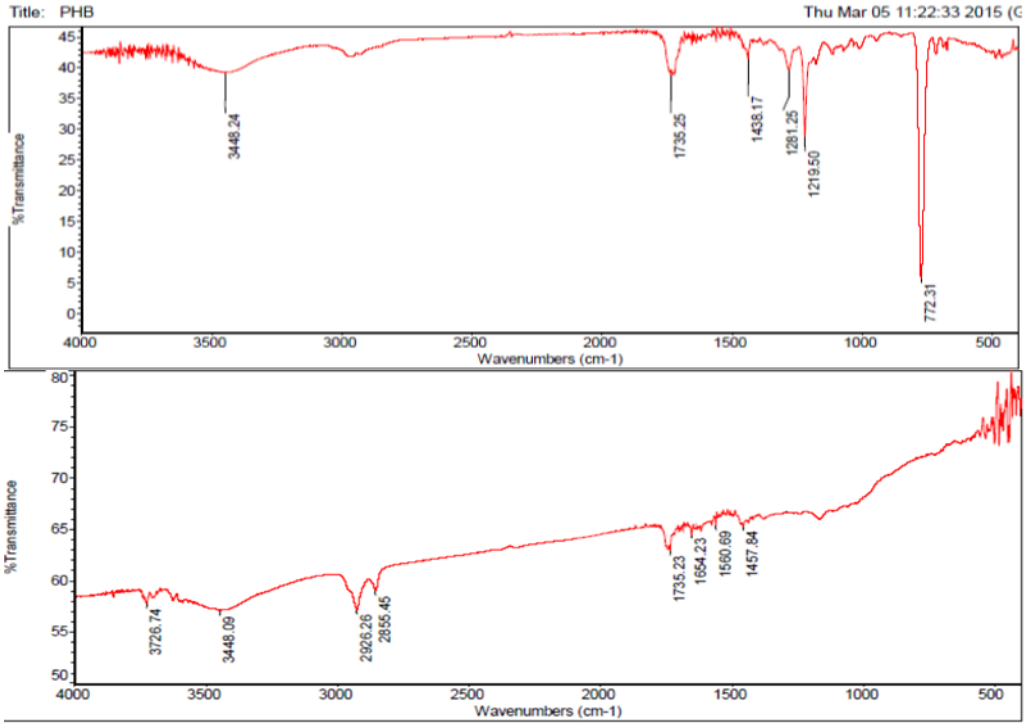

Figure 5: FTIR spectrum of PHB standard (A), PHB biosynthesized from, *B. cereus* isolate P83 (B)

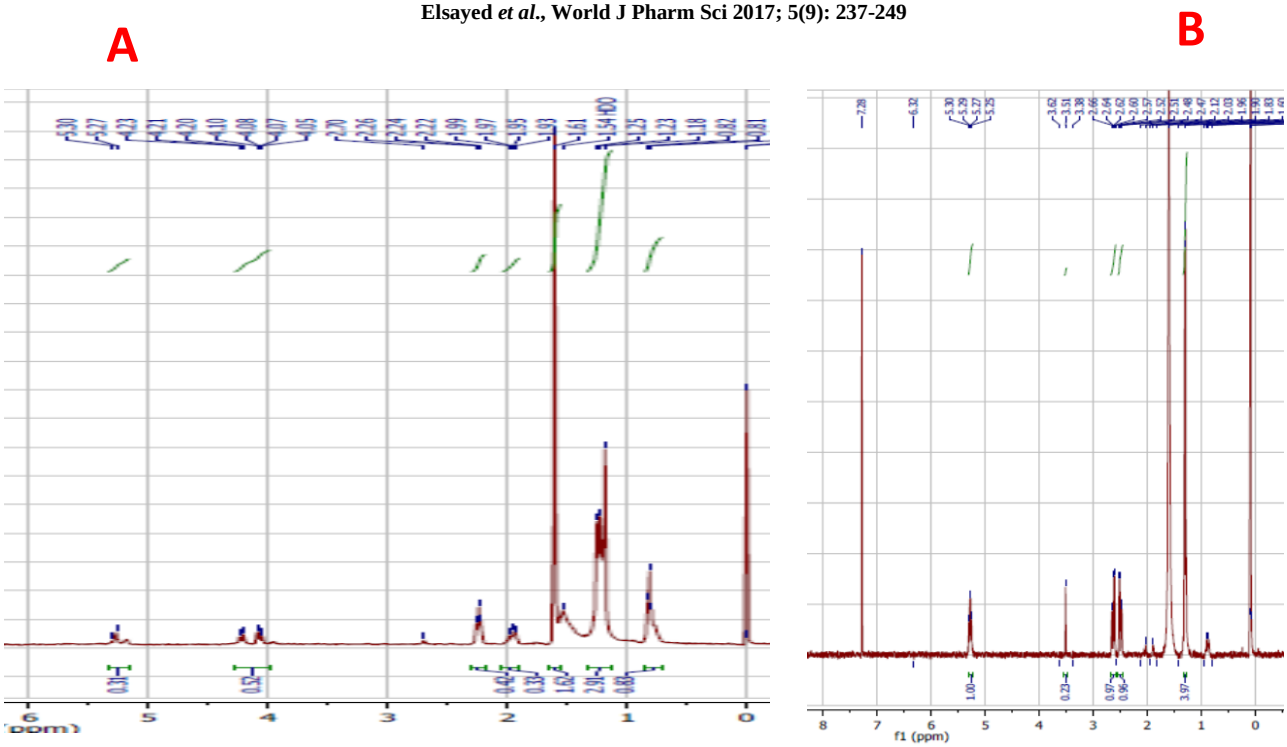

Figure 6: 1H-NMR spectra of PHB synthesized from *B. cereus* isolate P83 (A) and Standard PHB (B), The marked peaks are as follows: TMS standard (1), CH3group (2), CH2 (3), CH (4) , glycerol peaks (5).

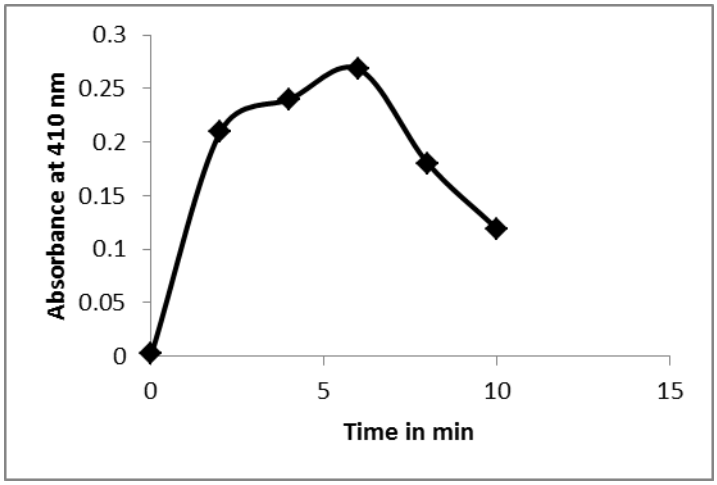

Figure 7: Time course of CoA release by PHA synthase displayed as absorbance at 410 nm from 1mM 3-hydroxybutyryl–CoA. CoA release was monitored in a discontinuous assay using DTNB.

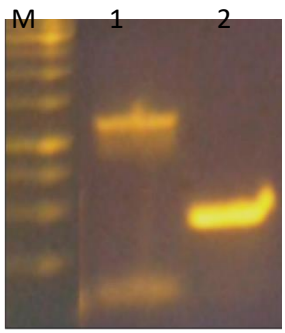

Figure 8: Gel electrophoresis showing amplified PHA synthase (1.08 Kb). Lane M: DNA ladder (2 kb) ,Lane 1: PHA synthase of *Bacillus cereus* isolate P83 and Lane 3: R-subunit of PHA synthase (0.3 bp). Molecular sizes mentioned in the figure is bP (base pair).

Figure 9: Alignment of the amino acids of PHA synthase of *B. cereus* isolate P83 (Query) with other homologous proteins in GenBank database using Clustal omega. It was aligned with EEL29879.1 (PHA synthase PhaC of *B. cereus* Rock1-15), EEK90425.1 (PHA synthase PhaC of *B. cereus* m1550), WP_071724047.1 (class III poly(R)-hydroxyalkanoic acid synthase subunit PhaC of *B.* sp. N24), WP_000206337.1 class III poly(R)-hydroxyalkanoic acid synthase subunit PhaC of *B. cereus*), ABF29870.1 (PhaC of *B. cereus*). Conserved cysteine is boxed. Lipase box in conserved sequences are shaded in grey.

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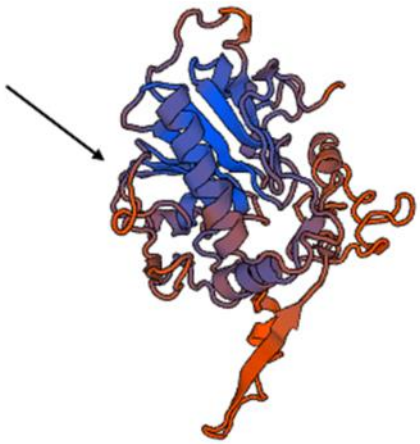

Figure 10: The putative tertiary structure of PHA synthase enzyme in cartoon diagram of *Bacillus cereus* isolate P83 as predicted by Swiss model software. The arrow pointing to the cysteine residue

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