

## Research Article

# Genome Structure of *Bacillus cereus* tsu1 and Genes Involved in Cellulose Degradation and Poly-3-Hydroxybutyrate Synthesis

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In previous work, we reported on the isolation and genome sequence analysis of *Bacillus cereus* strain tsu1 NCBI accession number JPYN00000000. The 36 scaffolds in the assembled tsu1 genome were all aligned with *B. cereus* B4264 genome with variations. Genes encoding for xylanase and cellulase and the cluster of genes in the poly-3-hydroxybutyrate (PHB) biosynthesis pathway were identified in tsu1 genome. The PHB accumulation in *B. cereus* tsu1 was initially identified using Sudan Black staining and then confirmed using high-performance liquid chromatography. Physical properties of these PHB extracts, when analyzed with Raman spectra and Fourier transform infrared spectroscopy, were found to be comparable to the standard compound. The five PHB genes in tsu1 (*phaA*, *phaB*, *phaR*, *phaC*, and *phaP*) were cloned and expressed with TOPO cloning, and the recombinant proteins were validated using peptide mapping of in-gel trypsin digestion followed by mass spectrometry analysis. The recombinant *E. coli* BL21 (DE3) (over)expressing *phaC* was found to accumulate PHB particles. The cellulolytic activity of tsu1 was detected using carboxymethylcellulose (CMC) plate Congo red assay and the shift towards low-molecular size forms of CMC revealed by gel permeation chromatography in CMC liquid culture and the identification of a cellulase in the secreted proteome.

## 1. Introduction

Since 1960s, driven by public concerns about environmental pollution by petroleum-derived plastics [1–6] and the escalating crude oil price due to the depletion of fossil oil resources, bioplastics have attracted widespread attention, as eco-friendly, biodegradable, and sustainable alternatives [4, 7]. Among all the biodegradable plastics, the polyhydroxyalkanoates (PHAs) family has unique properties like insolubility in water, biocompatibility, oxygen permeability, and ultraviolet (UV) resistance [8]. Because of these advantageous characteristics, comprehensive applications have been discovered and developed using PHAs-derived materials for packaging plastics, medical materials, chiral monomer, and others [9, 10]. Also stable engineered industrial microbial strains have been developed overexpressing genes in PHAs

biosynthesis pathway with additional functions in regulating cellular metabolisms and stress resistance [11, 12]. The main member of the PHAs family is polyhydroxybutyrate (PHB). These polymers are accumulated intracellularly in PHB producing bacteria when cultured under carbon-excess and other nutrients-limited conditions [13].

A large number of microorganisms have been found to accumulate PHA as lipoidic storage materials in the cytosol [14–17]. These microorganisms are mainly divided into four classes (I, II, III, and IV) based on the type of PHA synthases, which are the key enzymes for PHA biosynthesis [18]. While a single subunit PhaC was found in class I (e.g., *Ralstonia eutropha*) and class II (e.g., *Pseudomonas aeruginosa*) synthases, two subunits, PhaE and PhaC, or PhaR and PhaC, were suggested to be used, respectively, in type III (e.g., *Allochromatium vinosum*) and type IV (e.g.,

*Bacillus megaterium*) synthases [19, 20]. Classes I, III, and IV synthases act on polymerase short-chain monomers (C3–C5) whereas class II synthase acts on medium-chain length (mcl) monomers (C6–C14). The most recently discovered class IV PHA synthase is only present in *Bacillus* sp. There is little information about the capacity of PHAs production and the substrate specificity of class IV PHA synthase.

The higher production cost compared to petroleum-derived plastics is the primary factor limiting practical application of these biodegradable polymer materials. The following two approaches have been taken to make the PHAs mass production economically feasible. Firstly, engineered *E. coli* strains producing higher yield of PHAs [21, 22] have been developed using recombinant DNA technology. The optimization of the intermediate substrates and fermentation conditions is the key step in utilizing this technology in large scale PHAs production. The second approach focuses on looking for cheaper raw materials. Carbon source for PHB production accounts for up to 50% of the total production costs. Agricultural byproducts like soybean cake [23], biogas methane [24], and palm oil [25] as inexpensive carbon feedstock exhibit a high potential to accelerate the commercialization of PHAs. Rapeseed (canola seed) oil is one of the preferred oil stocks for biodiesel production, partly because rapeseed produces more oil per unit of land area compared to other oil sources, such as soybeans [26]. Rapeseed cake is generated as a byproduct during the oil extraction process from rapeseed (canola seed), which will be tested in this study for its potential use as a substrate for PHB production. The physical properties of PHB products produced by the bacteria cultured in aqueous extracts of rapeseed cake were confirmed with Raman spectrum and Fourier transform infrared spectroscopy (FTIR).

Previously, we reported the genome sequence of *B. cereus* tsul [27]. This paper reports on the assembly of the genomic structure, and characterization of cellulolytic and PHB producing activities of this strain. PHB biosynthesis pathway genes were cloned and (over)expressed in *E. coli* BL21 (DE3) using TOPO cloning system. The recombinant bacterial clones were confirmed to accumulate PHB granules.

## 2. Materials and Methods

**2.1. Genomic Structure Analysis.** In previous research, draft genome of *Bacillus cereus* tsul was generated using next generation sequencing analysis [27]. To generate an alignment map of the assembled tsul scaffolds (GenBank: KN321896–KN321931) using MUMmer (version 3.0) on the galaxy working station (biou.psc.edu/galaxy) [28], the genome sequence of *B. cereus* B4264 (NCBI GenBank: CP001176.1) with the highest identity similarity clustered by phylogenetic COG (PCOGR) was downloaded from NCBI database and used as the reference (Figure S1 in Supplementary Material, available online at <https://doi.org/10.1155/2017/6192924>) [29]. Based on the alignment, 20 scaffolds of tsul were selected to construct a circular genomic map using DNAPlotter (version 10.2) [30, 31]. Localizations of annotated genes on these scaffolds and on the reconstructed circular map were described in Supplementary Table S1.

**2.2. Cloning and Expression of PHB Pathway Genes.** Putative genes encoding for enzymes in PHB biosynthesis pathways were identified in the annotated genome. Five genes were amplified from tsul genomic DNA using the polymerase chain reaction (PCR) with primers designed against the assembled gene sequences (see Supplementary Table S2). The PCR program was conducted as follows: after a hot start cycle of 94°C for 2 mins, there were 35 cycles of denaturation at 94°C for 30 s, annealing at a melting temperature ( $T_m$ ) for each individual primer, and extension at 72°C for 1 min, followed by a final cycle of 72°C for 10 mins. PCR products were separated on a 0.7% agarose gel. After staining with ethidium bromide, DNA fragments were isolated from the gel, purified using Qiagen Gel Extraction Kit (Cat. number 28704), and then cloned into TOPO pET101 vector (Invitrogen, CA). Plasmid with gene inserts was sent to GenHunter (624 Grassmere Park Drive, St 17, Nashville, TN 37211) for Sanger sequencing with primers (T7 forward and reverse) that flank the insertion site. Recombinant plasmids carrying full-length gene sequences were transformed into *E. coli* BL21 (DE3) cells and expression of recombinant proteins was induced by the addition of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) in LB broth to a final concentration of 0.5–0.8 mM. Cells were harvested after 4-hour induction by centrifugation. Cell pellets were frozen in liquid N<sub>2</sub> and stored at –20°C before conducting protein analyses.

For two-dimensional (2D) protein gel electrophoresis of the recombinant proteins, cell pellets were homogenized in 500  $\mu$ L dissolution buffer consisting of 7 M urea, 2 M thiourea, and 4% (3-((3-cholamidopropyl) dimethylammonio)-1-propylsulfonate) (CHAPS). After centrifugation at 16,000  $\times$ g, 4°C for 20 min, supernatants were collected. Protein concentration was assayed following the Bradford method using BSA as the standard protein (Bio-Rad). Supernatants containing 200  $\mu$ g protein samples were mixed with 5  $\mu$ M dithiothreitol (DTT), and 1.25  $\mu$ L IPG buffer (pH 3–10 NL, GE Healthcare). Upon bringing to a final volume of 250  $\mu$ L using DeStreak Rehydration Solution (GE), proteins were loaded onto 13 cm pH 3–10 NL Immobiline DryStrips (GE). After an overnight passive rehydration at room temperature, proteins were focused on an Ettan IPGphor II (Amersham Biosciences) until reaching 24,000 total voltage hours (VhT). Prior to second dimensional electrophoresis, IPG strips were reduced with 1% DTT and then neutralized in 2.5% iodoacetic acid (IAA). Both of these steps were performed in a buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS. The second dimensional separation was performed on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (1 mm in thickness) using a SE 600 Ruby Standard Vertical Unit (GE). Gels were run at a constant current (20 mA/strip after an initial run of 10 mA/strip for 30 min) until the bromophenol front reached the bottom of the gel and then stained with Colloidal blue staining kit (Invitrogen, LC6025). Gel images were captured by scanning on a Typhoon 9400 variable mode imager (GE). Recombinant proteins were localized to the spots on 2D gels according to their hypothetical isoelectric point and molecular weight. These protein spots and spots at the same

position on gels loaded with proteins without IPTG induction were picked followed by in-gel tryptic digestion [32].

Tryptic peptide samples were reconstituted in 15  $\mu\text{L}$  of 3% acetonitrile with 0.1% trifluoroacetic acid. Nano-liquid chromatography (LC) separation of tryptic peptides was performed using a nanoAcquity UPLC (Waters, Manchester), equipped with a Symmetry  $\text{C}_{18}$  5  $\mu\text{m}$ , 20 mm  $\times$  180  $\mu\text{m}$  trapping column and a bonded ethyl hybrid  $\text{C}_{18}$  1.7  $\mu\text{m}$ , 15 cm  $\times$  75  $\mu\text{m}$  analytical column (Waters). Mobile phase A consisted of water with 0.1% formic acid (FA) in water and mobile phase B acetonitrile with 0.1% FA. Samples, at 5  $\mu\text{L}$  injection volume, were transferred to the trapping column at a flow rate of 7  $\mu\text{L}/\text{min}$  100% mobile phase A for 5 min. Following desalting and concentrating, the trapping column was eluted to the analytical column equilibrated with 2% mobile phase B at 300 nL/min. The eluent from the analytical column was delivered to a Xevo G2 Q-TOF mass spectrometer (MS) via a nanolockspray ion source (Waters). Data dependent acquisition (DDA) mode was used to obtain one 0.25 s MS survey scan. MS survey scans were acquired from  $m/z$  300–1500, while product ion scans were acquired from  $m/z$  50–2000. All data were acquired using MassLynx 4.1 SCN 862 (Waters). ProteinLynx Global Server v.2.5 was used to convert raw spectral data files for each injection into a peak list (.pkl format). To identify the matching sequences, the peak list from each protein spot was compared to theoretical tryptic digestion fragments of recombinant proteins.

**2.3. Cellulolytic Activity Assay.** Bacteria were cultured on the double-layered carboxymethyl cellulose sodium salt- (CMC-Na-) containing plates of which the bottom M9 minimal salt (11%; wt/vol) medium was overlaid with soft-agar containing 1% (wt/vol) CMC-Na (Sigma, St. Louis, MO). A cellulolytic bacterial strain *Paenibacillus polymyxa* 25A2<sup>T</sup> [33–35] was obtained from the Bacillus Genetic Stock Center (Columbus, OH). In these assays, *P. polymyxa* 25A2<sup>T</sup> was used as the positive control for testing cellulase activity and *E. coli* was used as the negative control. After incubation at  $37 \pm 1^\circ\text{C}$  for 2 days, the CMC agar plates were stained with 0.1% Congo red solution following the method described previously [36, 37]. Plates were recorded for the formation of a clear zone around colonies, which indicates extracellular cellulolytic activity of the bacteria.

To further characterize the bacterial strain, a single colony of *B. cereus* tsul was inoculated into LB broth and incubated at  $37^\circ\text{C}$  under constant agitation at 200 rpm. Aliquots of an overnight culture of *B. cereus* tsul (1 mL) were inoculated into 10 mL M9 minimal salt medium supplemented with 1% (wt/vol) CMC-Na, and the control culture used the broth solution only. Cultures were continued under the same conditions. Two culture period treatments of 2 days and 6 days each with three replicates were conducted. At the end of each treatment period, bacterial cultures were centrifuged at  $16,000 \times g$  for 1 min to collect supernatants. For gel permeation chromatography (GPC) assays of cellulose degradation, 2 mL of the supernatant from each sample was filtered through a 0.22  $\mu\text{m}$  sterile filter (EMD Millipore, Massachusetts) and eluted into a clean 2 mL Eppendorf centrifuge

tube. Gel permeation chromatography (GPC) analyses were performed on a Varian Prostar chromatography system (Walnut Creek, CA) equipped with a Waters Ultrahydrogel 2000 column (Milford, WA). Each 800  $\mu\text{L}$  sample was diluted in 200  $\mu\text{L}$  GPC solution (25 mM Na-acetate : methanol; 9 : 1). For each sample, 20  $\mu\text{L}$  was injected each time. Analyses were performed at room temperature with a mobile phase consisting of 25 mM Na-acetate : methanol (9 : 1) at a flow rate of 0.75 mL/min and the UV/Vis absorbance was monitored at 210 nm using a photodiode array (PDA) detector. The collected data was used to compare the molecular weight changes between samples.

Supernatant collected for GPC analysis was mixed with acetone (1 : 3; v/v) followed by incubation overnight at  $-20^\circ\text{C}$  for secreted protein precipitation. After centrifugation at  $16,000 \times g$  for 1 min at  $4^\circ\text{C}$ , supernatant was removed. Protein pellets were air-dried, solubilized in a 1x Laemmli protein sample buffer (Biorad), and denatured by boiling for 5 min. Proteins were separated on a SDS-PAGE protein gel [27]. After staining with Coomassie Blue, protein bands were isolated from the gel and digested with trypsin [32], followed by liquid chromatography (LC)/mass spectrometry (MS) as described above. The generated peptides were searched against annotated protein database of *B. cereus* tsul (download from <https://www.ncbi.nlm.nih.gov/protein/?term=bacillus+cereus+tsul>).

#### 2.4. Poly-3-Hydroxybutyrate (PHB) Producing Activity Assay.

The intracellular accumulation of PHB in *B. cereus* tsul was determined using the Sudan Black B staining method [38]. Bacterial smears were prepared using a four-day culture in LB broth and stained in a 0.3% Sudan Black stain solution (w/v) in 60% ethanol for 10 min. After rinsing with water, bacterial cells were counter-stained with 0.25% safranin for 1 min. Stained bacterial cells were observed and photographed under a Nikon Eclipse E600 Pol microscope (Japan).

Rapeseed cake samples (25 g) were soaked in 1L water overnight under constant stirring. The supernatant was filtered through a 0.22  $\mu\text{m}$  filtration system (EMD Millipore), and the rapeseed cake substrate (RCS) was used for bacterial cultures without any supplements. The total protein concentration in RCS was quantified using Bradford protein assay method. Bacterial cultures were incubated overnight in RCS and harvested by centrifugation at  $3,220 \times g$ ,  $24^\circ\text{C}$ , for 10 min. Cell pellets were oven-dried at  $70^\circ\text{C}$  to a constant weight and lysed by dispersing in 6% sodium hyperchlorite. After incubation at  $37^\circ\text{C}$  for 1 h, cell pellets were washed in 5 mL alcohol and then in 5 mL acetone. PHB was extracted by bathing the pellet in chloroform at  $60^\circ\text{C}$  for 1 h [39–41]. Then, chloroform was evaporated to obtain PHB crystals. PHB extracts were digested in 1 mL concentrated sulfuric acid at  $100^\circ\text{C}$  for 30 min, chilled to room temperature, and then diluted in 0.001 N  $\text{H}_2\text{SO}_4$  to a final concentration of 0.8 mg/mL adipic acid, which are the 250x stock solutions.

The digested PHB-containing mixture was fractionated using high-performance liquid chromatography (HPLC) equipped with an Aminex HPX-87H ion-exclusion resin for organic acid analysis column (300 by 7.8 mm). The poly-(R)-3-hydroxybutyric acid (Sigma, MO) was used as the standard.

The crotonic acid formed from PHB acid digestion was detected by the absorbance peak at 210 nm [42] (Supplementary Table S4). Aliquots of PHB extracts were also analyzed on a spectrophotometer (SpectraMax M5, Molecular Devices, CA). The absorbance of the diluted sample (1:250) from acid digestion was measured at 235 nm. A standard curve was constructed using commercial PHB (Sigma) [43]. After confirmation of both methods giving nearly the same results, PHB content from bacterial batch culture was assayed using the spectrophotometric method (Supplementary Table S4).

**2.5. Physical Structural Property Analysis of *tsu1*-PHB.** An Xplora Raman spectrometer (LabRAM; HORIBA Jobin Yvon, NJ) was used with a NIR diode laser ( $\lambda = 785$  nm, power = 2.5 mW) as an excitation source. The instrument settings were 100  $\mu\text{m}$  confocal hole, 100  $\mu\text{m}$  wide entrance slit, 600 gr/mm grating, and Olympus SLM Plan N 10x objective lens. Samples were mounted on a computer-controlled, high-precision  $x$ - $y$  stage. An exposure time of 40 s and 5 accumulations were used to collect the spectra. Baseline fitting was performed using a LabSPEC 5 (HORIBA Jobin Yvon).

Samples were placed on the diamond crystal top plate of an attenuated total reflectance (ATR) accessory (Thermo Scientific Nicolet IS10, Thermo Scientific, Waltham, MA). Thumbscrew pressure was used to ensure that samples were in contact with the crystal. Data from sixteen scans were averaged over the spectral range of 4000 to 650  $\text{cm}^{-1}$ , with a resolution of 4  $\text{cm}^{-1}$ . Ambient air was used as the reference for the background spectrum before each sample. Between samples, the ATR crystal was cleaned using distilled water and dried. All spectra were recorded at room temperature. No data processing was performed on the raw spectra. The PHB standard (Sigma) was used as the reference for these analyses.

### 3. Results

**3.1. Characterization of Microbial Genome of *B. cereus tsu1*.** The 36 assembled scaffolds of *B. cereus tsu1* were each aligned to a distinct region of the genome from *B. cereus* B4262 [27]. Regions of scaffolds 4, 6, and 15 did not match the reference genome (Figure 1). The circular genome map of *B. cereus tsu1* (Figure 2) was constructed using the annotation of the assembled scaffolds in reference to *B. cereus* B4264 genome (see Table S1 in Supplementary Material).

**3.2. Characterization of the PHB Biosynthesis Pathways.** Six PHA synthesis related genes were located on scaffold 9. *phaR* (PHB synthase subunit), *phaB* (acetoacetyl-coA reductase), and *phaC* (PHB synthase subunit) are divergently transcribed as a tricistronic operon; *padR/phaQ* (transcription regulator), *phaP* (Phasin protein), and *phaJ* are transcribed in one direction. The *padR* gene is a PHB-responsive repressor controlling expression of *phaP* and *phaR*. Phasins are proteins that accumulate during PHA synthesis; they bind to PHA granules and promote further PHA synthesis. Gene *phaA* is located on a separate scaffold. The *phaJ* gene encodes for (R)-specific enoyl-CoA hydratase which is involved in fatty acid metabolism (Figure 3(a)). PHB granules were seen inside bacterial cells (Figure 3(b)). The five genes in PHB

synthesis pathway (*phaA*, *phaB*, *phaC*, *phaP*, and *phaR*) were cloned. The size and sequences of these genes are identical to the predicted gene sequences in the annotated genome sequences (Figure 3(c)). The recombinant proteins matched the predicted proteins in both molecular size and isoelectric points (pI value). Furthermore, mass spectrometry (MS) analysis of the tryptic digests of recombinant proteins showed that the peptide coverage ratio (identified/predicted) is 70%, 95%, 82%, 78%, and 100% for *PhaA*, *PhaB*, *PhaC*, *PhaR*, and *PhaP*, respectively (Figure 3(d); see Table S3 in the Supplementary Material). When the *phaC*-(over)expressing recombinant *E. coli* cells were cultured overnight under the IPTG induction condition, PHB granules were observed in Sudan Black stained cells (Figure 3(e)). These results confirmed that the PHB genes from *B. cereus tsu1* can drive biosynthesis of the polymer in recombinant *E. coli* clones.

**3.3. Characterization of Cellulolytic Pathways.** The conversion of cellulose into glucose consists of two steps. During the first step, beta-1,4 glucanase breaks the glucosidic linkage to cellobiose. Subsequently, this beta-1,4 glucosidic linkage of cellobiose is broken down by beta-glucosidase to produce glucose. Both endo-beta-glucanase and beta-glucosidase were found in the *tsu1* genome. One xylanase gene for the degradation of hemicellulose was identified in the genome (Figure 4(a)).

The Congo red test showed that the *B. cereus tsu1* colonies formed a clear distinct yellow halo, which is an indication of extracellular cellulase enzymes produced by the bacterium. The same reaction was observed in *Paenibacillus polymyxa*, but not in *E. coli* colonies (Figure 4(b)). Gel permeation chromatography (GPC), also known as size-exclusion chromatography, is often used to characterize the molar mass distribution of natural and synthetic polymers. Various incubation-period products from CMC-containing substrates after culturing *B. cereus tsu1* were compared. With longer treatment time (in 6-day sample), a lower intensity of high molecular weight component (between 11.5 and 12.5 min) was observed whereas a higher peak intensity of small molecules (between 13 and 14 min) emerged. The clear shift to longer retention times of the bacterial-treated products relative to the untreated products clearly demonstrates CMC degradation into smaller molecules in the bacterial-treated samples (Figure 4(c)).

The bacterial secreted proteins were separated into five major bands on SDS-PAGE gels (Supplementary Figure S2). Among all the proteins identified by searching the tryptic digestion peptides against the *B. cereus tsu1* protein dataset annotated by prokaryotic genome annotation pipeline, one endo-glucanase matching (GenBank ID: KGT43479.1) was identified (Table S5 in the Supplementary Material) [27].

Conclusively, the Congo red test, GPC analysis, and secreted proteome analysis all supported the extracellular cellulase activity of *B. cereus tsu1* which concurs with its genome structure.

**3.4. PHB Production Efficiency of *Bacillus cereus tsu1* on Rapeseed Cake Substrate (RCS).** In this study, bacterial cell cultures were grown in RCS without any additional materials.

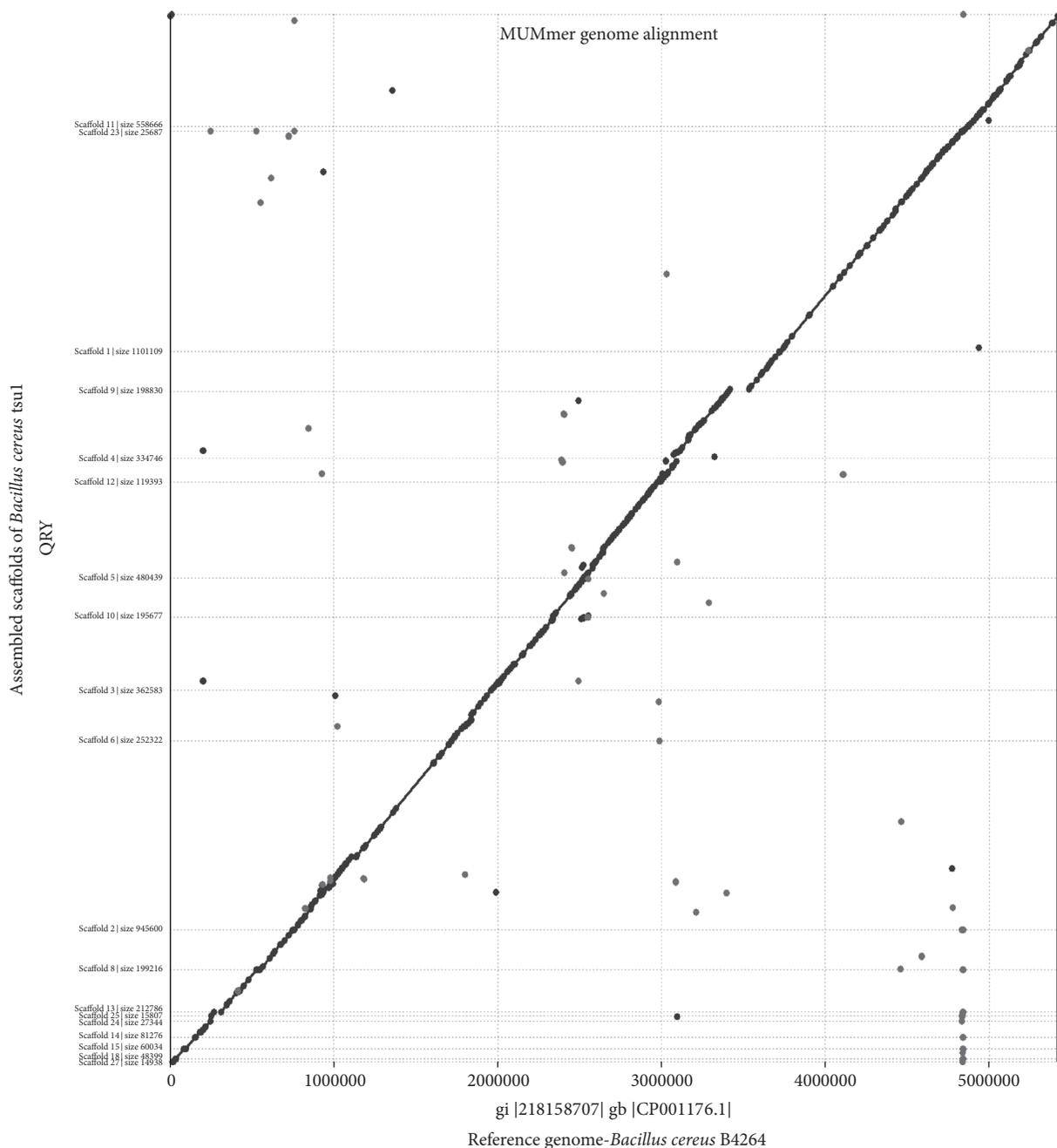


FIGURE 1: Alignment of assembled scaffolds from *Bacillus cereus* tsul with the reference genome of *B. cereus* B4262 using MUMmer on [biou.psc.edu/galaxy](http://biou.psc.edu/galaxy).

After overnight batch culture, the cell dry weight reached 540–575 mg/L, which produced a 13–14% PHB content per cell biomass (Table 1).

**3.5. Physical Structural Properties of PHB.** PHB extracts from tsul cultures in RCS were compared to poly-(R)-3-hydroxybutyric acid (Sigma, MO) as the standard. The spectroscopic data of Raman and FTIR were used to determine the physical properties of PHB polymers [8]. The Raman spectra from regions 300–2200  $\text{cm}^{-1}$  and from regions

2200–3000  $\text{cm}^{-1}$  showed that the tsul PHB and the standard were both crystalline. The presence of sharp and narrow peaks in the Raman spectra at the major positions 434 and 841  $\text{cm}^{-1}$  also indicated the crystallinity of the tsul PHB. However, peaks corresponding to C=O stretching and to  $\text{CH}_3$  stretching occur at 1731.2 and 2935.4  $\text{cm}^{-1}$ , respectively, showing that there were some amorphous regions in the tsul PHB extracts (see Table S6 in the Supplementary Material). Both Raman and FTIR spectra of tsul PHB extracts matched those from the standard (Figure 5). The mean square deviation is 3.7

TABLE 1: *Bacillus cereus* tsul cell propagation and PHB production on RCS substrate<sup>†</sup>.

Items	Batch 1	Batch 2	Average
Cell biomass (mg/L culture)	575.17 ± 56.02	540.89 ± 18.43	558.03 ± 28.58
PHB mixture extract (mg/L) <sup>a</sup>	139.44 ± 14.18	112.78 ± 12.54	126.11 ± 9.88
Pure PHB content (mg/L) <sup>b</sup>	74.66 ± 8.54	80.61 ± 10.32	77.64 ± 6.45
PHB content in cell biomass (%) <sup>c</sup>	13%	14.9%	13.95%
PHB conversion efficiency of rape seed cake (g/kg)	2.99	3.22	3.10

<sup>†</sup>*Bacillus cereus* tsul was batch cultured for 24 hours in one-liter bottle containing (25 g/L) aqueous extracts of RCS. Two independent experiments each containing six replicates were conducted; <sup>a</sup>PHB was extracted from dried bacterial cells. Six replicates were conducted; <sup>b</sup>PHB content in crude extracts was determined by spectrophotometry analysis; <sup>c</sup>PHB content measured using the spectrometric method is similar to the HPLC method (37).

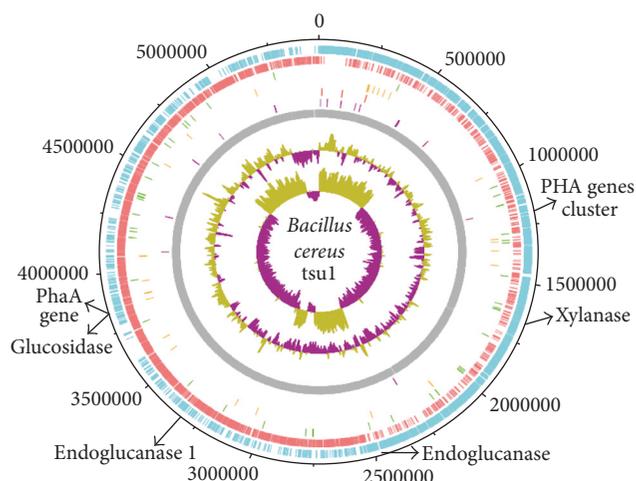


FIGURE 2: Circular genome map of *Bacillus cereus* tsul. The map was generated using DNAPlotter (version 10.2) based on annotation data of the assembled scaffolds of *B. cereus* tsul (NCBI accession KN321896-KN321931). The draft genome sequence is 5.81 Mb, and 5.4 Mb was aligned to create the circular genome map. Genes in PHB biosynthesis pathway *phaA*, *phaR*, *phaB*, *phaC*, *phaQ*, *phaP*, and *phaJ* and cellulase genes for endoglucanase, glucosidase, and hemicellulase gene xylanase were indicated on the genome map. Locations of genes on the assembled scaffolds and their linkage to the circular map were provided in Supplementary Table S1.

to  $7.1 \text{ cm}^{-1}$ , which is within the range of discrepancy between Raman and FTIR spectra of PHB reported in previous studies [44–46].

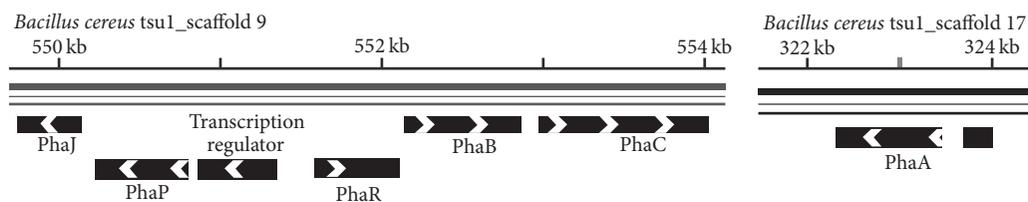
#### 4. Discussions

In this paper, we are reporting the characterization of *B. cereus* tsul, a bacterium that has the ability to produce PHB polymer and degrade cellulose. In order to understand the molecular basis for these biochemical activities and evaluate the biotechnological potential of this bacterial strain, we proceeded to analyze its genome structure and test its extracellular cellulase and PHB producing ability. Analysis of cellulose degradation activity using GPC and Congo red staining methods suggests that the bacteria can degrade cellulose into smaller molecular products, but the end-products of degradation were not identified [47–49]. Cellulose has been used as a low-cost substrate in PHAs fermentation

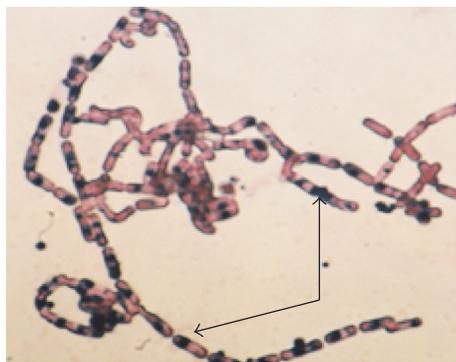
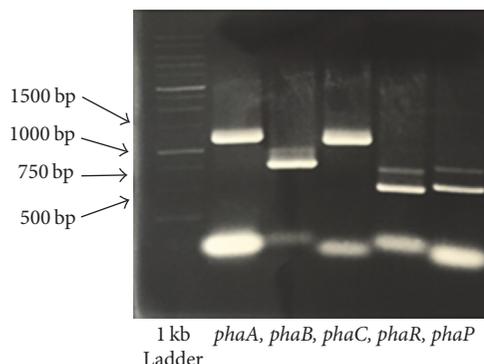
studies. Gao et al. reported that the successful transformation of cellulase and PHB synthesis pathway genes into *E. coli* resulted in the production of PHB directly from cellulose; however, the conversion efficiency and PHB yield were very low [50, 51]. In our study, the annotated *B. cereus* tsul genome contained cellulase (GenBank ID: KGT42715.1; KGT43479.1) and xylanase (GenBank ID: KGT44235.1) genes. A cellulase was also identified in secreted proteomes in CMC liquid culture. In addition, genes in each step of PHB biosynthesis pathway were identified in *B. cereus* tsul genome including several *phaA* genes and a gene cluster with six PHA genes: *phaR* (PHB synthase subunit), *phaB* (acetoacetyl-coA reductase), *phaC* (PHB synthase), and a *phaJ*, the downstream *phaP* (Phasin protein), and the *padR* (PhaQ transcription regulator) (see Table S7 in the Supplementary Material).

Based on the PHB gene cluster, *B. cereus* tsul should express class IV PHA synthase, which is composed of subunits PhaC and PhaR. PhaC is the key enzyme involved in the polymerization process; it determines the types of monomers (R-hydroxyacyl-CoAs) incorporated into the PHA polymer chain based on the enzyme's substrate specificity, as well as controlling PHA chain length and polydispersity. Previous researches consistently indicate that class IV synthases favor short-chain-length monomers such as 3-hydroxybutyrate (C4) and 3-hydroxyvalerate (C5) for polymerization, but it can also polymerize some unusual monomers as minor components [52–54]. There is an increasing interest in class IV PHA synthase, due to the possible alcoholysis activity as an inherent feature among these enzymes [55]. This alcoholysis reaction is useful not only for the regulation of PHA molecular weight but also for the modification of the PHA carboxy terminus, which can be manipulated to produce more promising PHA materials with more beneficial properties [56, 57]. The discovery of these genes has increased our understanding of the PHB synthesis pathway because they have a specific role that would affect the efficiency and the types of PHB polymers being synthesized. These genes found in *B. cereus* tsul genome related to PHB synthesis and cellulose degradation will contribute in building a library of information for constructing more efficient PHB fermenter hosts in future work.

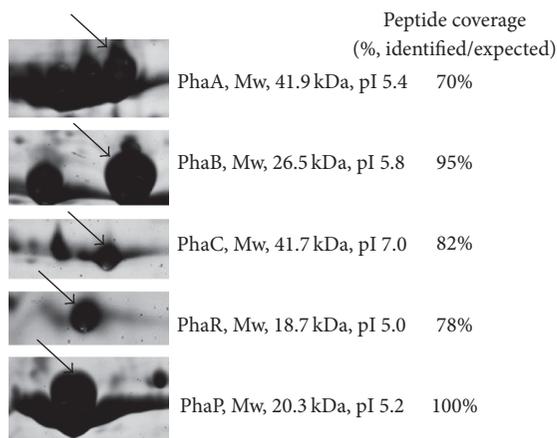
The high cost associated with the production of PHAs remains a major barrier for large scale use of bioplastics [58]. In order to produce PHAs economically, a reliable and economical supply of raw material is essential; meanwhile, new bacterial strains and genes need to be discovered in order



(a) Loci of genes in PHB biosynthesis pathways

(b) Accumulation of PHB granules (indicated by arrows) in *Bacillus cereus* tsu1

(c) PCR cloning of PHB genes



(d) Recombinant PHB proteins (indicated by arrows) on 2D gels and confirmation using peptide mapping

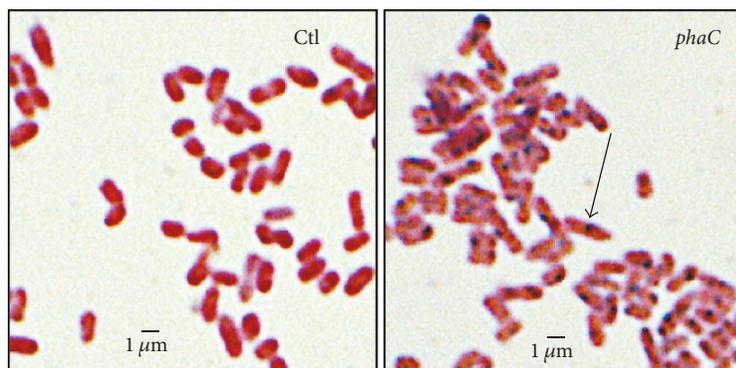
(e) *E. coli* with *phaC* genes displayed PHB producing activity

FIGURE 3: Characterization of genes in the poly-3-hydroxybutyrate (PHB) biosynthesis pathways in *Bacillus cereus* tsu1. (a) Genes in the PHB biosynthesis pathways: *phaR*, *phaB*, and *phaC* on the same operon, *phaJ*, *phaP*, and *phaQ* on reverse direction, and *phaA* located on a separate locus. (b) Sudan Black staining of *B. cereus* tsu1 showing accumulation of PHB granules in the bacterial cells. (c) The PHB gene fragments amplified from the genomic DNA of *Bacillus cereus* tsu1 using polymerase chain reaction (PCR) and separated on a 0.7% agarose gel. (d) Two-dimensional gel electrophoresis of recombinant proteins of *PhaA*, *PhaB*, *PhaC*, *PhaR*, and *PhaP*. The PCR amplified gene fragments showing identical sequence matches with the PHB synthesis genes were cloned into TOPO pET101 vector (Invitrogen, CA); recombinant proteins were expressed in DE3 cells. Recombinant proteins were separated on 2D gels. Protein spot with matching molecular size and isoelectric point (pI) of each predicted protein was picked from the gel. Protein identity was confirmed using the peptide fingerprinting (70–100% coverage with identical predicated peptides) using mass spectrometry analysis of tryptic digests of these proteins. (e) Sudan Black staining of the recombinant *E. coli* (over)expressing *phaC* gene showing intracellular PHB granules; *E. coli* transformed with the empty vector had no PHB accumulation. The arrow refers to the black spots inside of the bacterial cell, which is the PHB accumulation stained with Sudan Black.

to use a wider variety of substrates [59, 60]. The aqueous extract of rapeseed cake (RCS) can be a promising raw material for PHB production. In this study, PHB content was 13–14% per cell dry weight in overnight batch culture in RCS. Taking into consideration that no other nutrients were added

in RCS, the bacterial growth performance and PHB content are relatively lower than other well-developed nutrient substrates. Meanwhile, in this batch culture for PHB production, no pH buffer solution was added, which may limit the biomass production, and further limit the PHB production

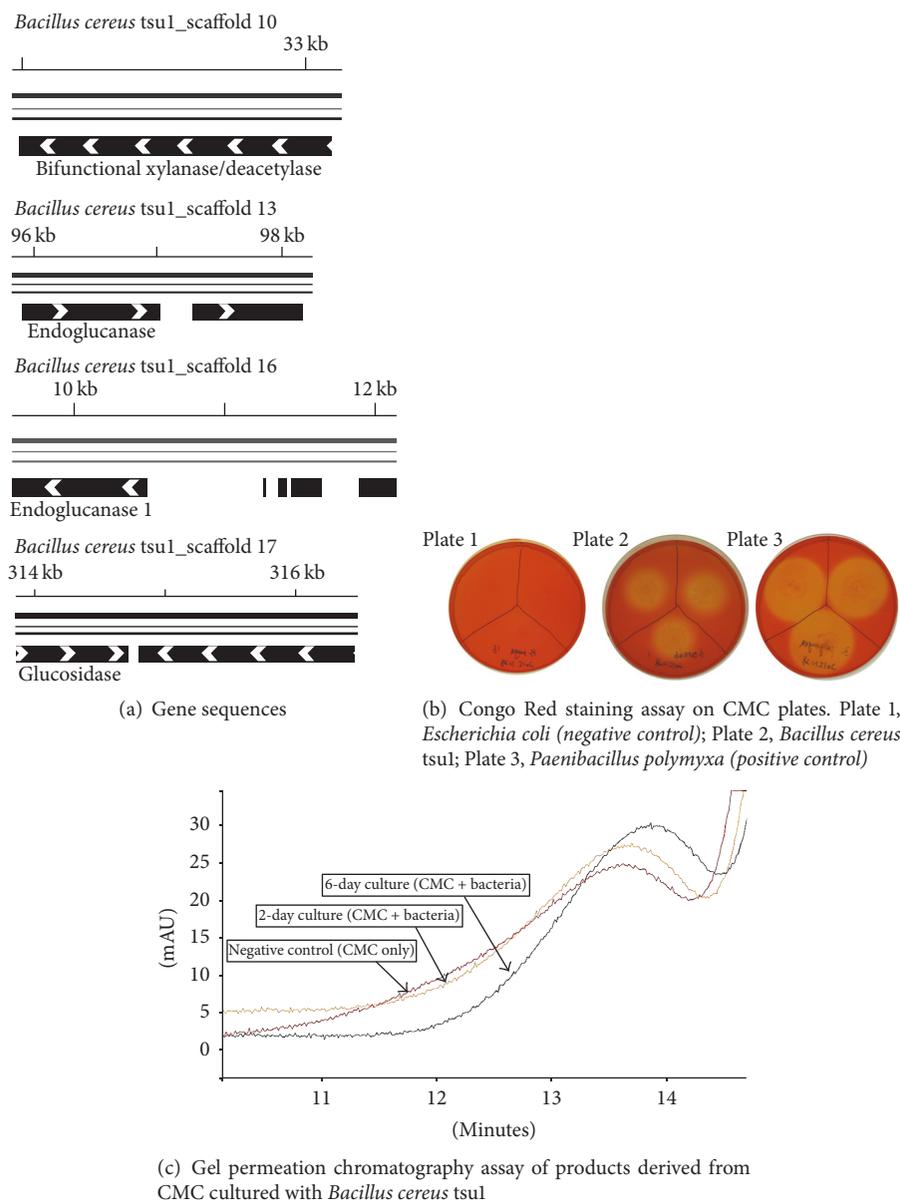
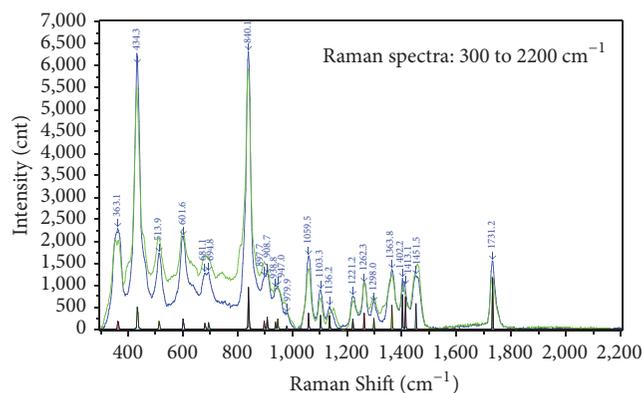


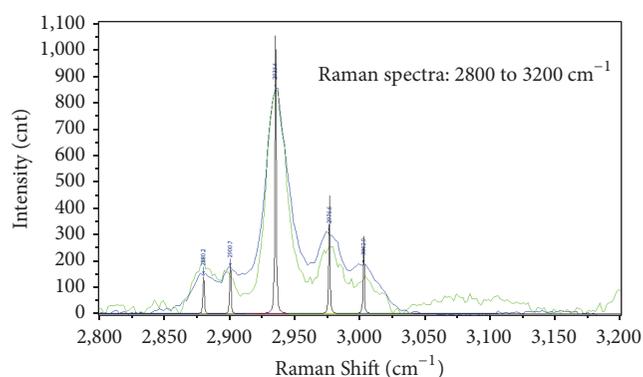
FIGURE 4: Characterization of cellulase gene and enzymatic activity of *Bacillus cereus* tsu1. (a) Cellulase genes annotated in the *Bacillus cereus* tsu1 genome. (b) Congo red plate assay of cellulase activity. Bacteria were cultured on the double-layered carboxymethylcellulose sodium salt- (CMC-Na-) containing plates of which the bottom M9 minimal salt (11% wt/vol) agar medium was overlaid with soft-agar containing 1% (wt/vol) CMC-Na (Sigma, St. Louis, MO). Plates from two-day incubation at 37°C were stained with 0.1% Congo red. The yellowish halo around the bacterial colony indicates degradation of CMC. Plate 1, *E. coli* showing no CMC degradation activity (negative control); Plate 2, *B. cereus* tsu1; Plate 3, *Paenibacillus polymyxa* 25A2<sup>T</sup> with CMC degradation activity (a positive control of cellulolytic bacterial strain from the Bacillus Genetic Stock Center, Columbus, OH). (c) Gel permeation assay of CMC derived products after incubation with *B. cereus* tsu1 for 2 days and 6 days. The right shifts of the peaks indicate that the CMC derived molecules after the digestion with *B. cereus* tsu1 were smaller in size and therefore they were eluted at a delayed time-frame than the original CMC. These results confirmed the extracellular cellulase activity of the bacterial strain.

[61]. RCS was estimated to have 8.98 g/L crude proteins, and it also contained all the essential amino acids for bacterial growth, and some  $\alpha$ - and  $\gamma$ -amino-butyric acid (AIB, GABA) (see Figure S3 in Supplementary Material). Some of these amino acids can potentially be converted into PHB through various alternative pathways (see Figure S4 in Supplementary Material). In a previous study [62], GABA was reported as an

alternative route of catabolism in *Saccharomyces cerevisiae*. It is involved in the conversion of GABA into succinate-semialdehyde (SSA) by 4-aminobutyrate aminotransferase; meanwhile, glutamate can be converted into GABA by glutamate decarboxylase. In 2007, Valappil et al. reported that *B. cereus* 14579 genome contained all the alternative pathway genes involved for the conversion of succinyl-CoA from

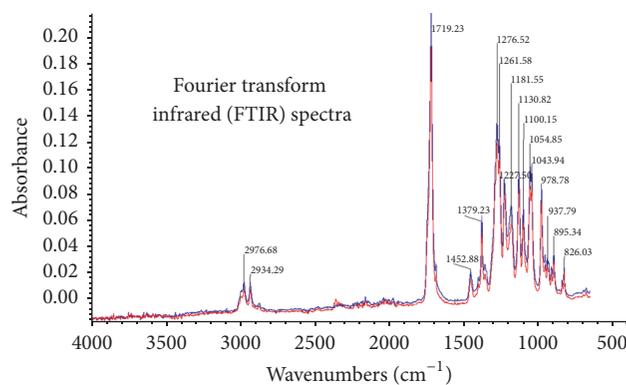


■ 3-22 PHB sample 300–2200 40-5 10x-auto baseline-peaks assigned  
 ■ 4-12 PHB standard-7 1-40-5  $\times 10$  300–2200-auto baseline



■ 3-22 PHB sample 2800–3200 40-5 10x-baseline-peaks assigned  
 ■ 4-12 PHB standard-6 1-40-5  $\times 10$  2800–3200-baseline

(a)



(b)

FIGURE 5: Physical properties of polyhydroxybutyrate PHB produced by *Bacillus cereus* tsul on rapeseed cake substrate (RCS). The PHB standard (Sigma) was used as the reference for these analyses. Raman spectra of PHB produced by *B. cereus* tsul and standard (a). FTIR spectra of PHB produced by *B. cereus* tsul and standard (b).

TCA cycle into P4HB/PH3B/P(3HB-co-4HB) [63]. P(3HB-co-4HB)-like copolymer was found in *B. cereus* SPV. In this alternative pathway, succinyl-CoA is first converted to SSA by SSA dehydrogenase followed by reduction of SSA into GABA by 4-hydroxybutyrate dehydrogenase. GABA is activated to 4-hydroxybutyryl-CoA by a Co-A transferase enzyme. The R-4-hydroxybutyryl-CoA and/or R-3-hydroxybutyryl-CoA are then to be polymerized to form P4HB/PH3B/P(3HB-co-4HB) by PHB synthase.

The (R)-specific enoyl-CoA hydratase/MaoC-like protein (PhaJ) is a monomer supplying enzyme from fatty acid  $\beta$ -oxidation. Tajima et al. demonstrated that PhaJ may make a channeling route from  $\beta$ -oxidation to PHA biosynthesis and PhaC synthases from *B. cereus* and its relatives have the ability to incorporate both scl and mcl PHAs [64]. This alternative metabolic pathway was confirmed [65] in a recombinant *E. coli* strain which utilizes two substrate-specific enoyl-CoA hydratases, R-hydratase (PhaJ) and S-hydratase (FadB). In these bacterial strains, the PhaJ works in coordination

with S-specific hydratases to provide (R)-3HB-CoA for PHA synthesis from crotonyl-CoA. In 2012, Cai reported that *B. cereus* may be another example harboring *phaJ* in the pha locus [66]. In *B. cereus* tsul, the *phaJ* gene encoding R-specific enoyl-CoA hydratase (GenBank ID: KGT44860.1) may function coordinately with an acyl-CoA dehydrogenase to form (R)-3HB-CoA via crotonyl-CoA intermediate which is derived from fatty acid  $\beta$ -oxidation (see Figure S4 in Supplementary Material). Genes in this pathway were found in the genome of *B. cereus* tsul. The production of PHB from RCS may support this alternative catabolism, which may shed new light on the PHB biosynthesis pathway and open up new opportunities for its industrial applications.

## Disclosure

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or

endorsement by the U.S. Department of Agriculture. The funders of the project had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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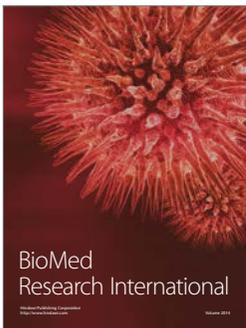
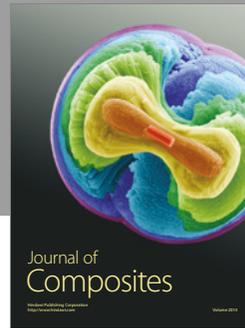
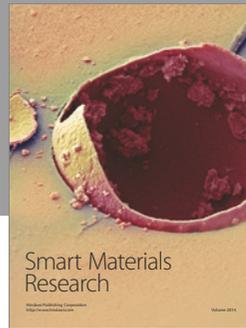
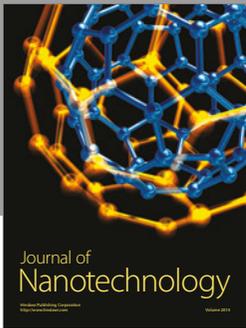
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