Revelation of the ability of *Burkholderia* sp. USM (JCM 15050) PHA synthase to polymerize 4-hydroxybutyrate monomer

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**Abstract**

The nutrition-versatility of *Burkholderia* sp. strain USM (JCM 15050) has initiated the studies on the use of this bacterium for polyhydroxyalkanoate (PHA) production. To date, the *Burkholderia* sp. has been reported to synthesize 3-hydroxybutyrate, 3-hydroxyvalerate and 3-hydroxy-4-methylvalerate monomers. In this study, the PHA biosynthetic genes of this strain were successfully cloned and characterized. The PHA biosynthetic cluster of this strain consisted of a PHA synthase (*phaC*), β-ketothiolase (*phaA*), acetoacetyl-CoA reductase (*phaB*) and PHA synthesis regulator (*phaR*). The translated products of these genes revealed identities to corresponding proteins of *Burkholderia vietnamiensis* (99–100 %) and *Cupriavidus necator* H16 (63–89%). Heterologous expression of *phaCBs* conferred PHA synthesis to the PHA-negative *Cupriavidus necator* PHB¯4, confirming that *phaCBs* encoded functionally active protein. PHA synthase activity measurements revealed that the crude extracts of *C*. necator PHB¯4 transformant showed higher synthase activity (243 U/g) compared to that of wild-types *Burkholderia* sp. (151 U/g) and *C. necator* H16 (180 U/g). Interestingly, the transformant *C*. necator PHB¯4 harbouring *Burkholderia* sp. PHA synthase gene accumulated poly(3-hydroxybutyrate-co-4-hydroxybutyrate) with 4-hydroxybutyrate monomer as high as up to 87 mol% from sodium 4-hydroxybutyrate. The wild type *Burkholderia* sp. did not have the ability to produce this copolymer.

**Keywords:** Biopolymer, Polyhydroxyalkanoate, PHA synthase, PHA operon, *Burkholderia* sp.

**Introduction**

Since the introduction of phenoformaldehyde plastic in 1909 by Leo Hendrik Baekeland, petrochemical plastics have developed into a major industry and an indispensable commodity for modern life (Meikle, 1995). It is estimated that more than 100 million tonnes of plastics are produced yearly. Most of these plastics end up after their useful life as discarded waste and some are disposed into the marine environment which pose a threat to the aquatic wildlife. In recent years, the widespread and increasing use of petrochemical plastics has raised concerns about the adverse impact of these recalcitrant plastics on the environment. Hence, biobased and biodegradable polymers are gaining widespread interest and acceptance as an alternative to some synthetic plastics. Polyhydroxyalkanoate (PHA), a storage and reserve compound accumulated naturally in the cytoplasm of numerous bacteria, is being considered as one of the most attractive and promising biodegradable thermoplastics for various industrial and biomedical applications (Sudesh and Iwata, 2008).

Poly(3-hydroxybutyrate) [P(3HB)], the most common type of PHA, has mechanical properties such as Young's modulus and tensile strength similar to polypropylene. Nevertheless, P(3HB) is a stiffer and more brittle plastic material compared to polypropylene (Tsuge, 2002). Many other types of PHA with improved mechanical properties are synthesized by incorporating co-monomers such as 3-hydroxyvalerate, 3-hydroxyhexanoate and 4-hydroxybutyrate. The underlying challenge for the commercialization of PHA is the higher production cost compared to petrochemical plastics. To achieve the commercial application and wide use of PHA, efforts are directed on effectively lowering the production cost of PHA. Much research has been focused on reducing the production cost by strain...
development, developing more efficient fermentation and recovery processes and using inexpensive carbon sources. At the same time, more PHA applications are being developed, including the high value applications such as medical and pharmaceutical field at which cost of production is not the main concern (Chen, 2009).

Structural analyses in recent years revealed that different types of granule-associated proteins are located on the surface of PHA granules. The proteins include PHA synthases, intracellular PHA depolymerases, phasins, PHA synthesis regulator proteins and etc. (Pötter and Steinbüchel, 2006; Rehm, 2006; Jendrossek, 2009). PHA synthases are the most important enzymes involved in PHA biosynthesis. They can be grouped into four classes synthases are active towards short-chain length (R)-hydroxyacyl-CoA consisting of three to five carbon atoms (Pötter and Steinbüchel, 2006). Class II synthases are active towards medium-chain length (R)-3-hydroxyacyl-CoA that contain six to fourteen carbon atoms and are represented by Pseudomonas aeruginosa. Class I and II PHA synthases comprise enzymes consisting of only one type of subunit (PhaC) with molecular masses (Mw) between 61 and 68 kDa. Class III PHA synthases, represented by the Allochromatium vinosum PHA synthases, comprise two subunits: PhaC and PhaE. Class IV PHA synthases, represented by the enzyme of Bacillus megaterium, consist of two different types of subunits (PhaC and PhaR). Both class III and IV PHA synthases prefer short-chain length (R)-hydroxyacyl-CoA. (Rehm, 2003).

Burkholderia sp. USM (JCM 15050) was isolated from oil-polluted wastewater and has been known to utilize various carbon sources e.g. sugars, organic acids and triglycerides for PHA production (Chee et al., 2010; Lau et al., 2010). The focus of this research was the PHA biosynthetic genes cloned from this bacterium. Here, we reported for the first time that the heterologous expression of Burkholderia sp. PHA synthase in C. necator PHB-4 lead to the accumulation of poly(3-hydroxybutyrate-co-4-hydroxybutyrate), P(3HB-co-4HB) with high 4-hydroxybutyrate (4HB) composition. Although the accumulation of PHA containing 4HB monomer was not observed in wild-type Burkholderia sp. culture, the PHA synthase of Burkholderia sp. was able to polymerize the 4HB monomer.

Materials and methods
Bacterial strains, plasmids and media
All bacterial strains and plasmids used in this study are listed in Table 1. C. necator PHB-4 was cultivated in nutrient rich (NR) medium containing meat extract (10 g/L), peptone (10 g/L) and yeast extract (2 g/L) at 30°C. Escherichia coli strains were grown at 37°C in Lysogeny Broth (LB) medium consisting of the following components (per L): 10 g casein enzyme hydrolysate, 5 g yeast extract and 10 g NaCl. For maintenance of plasmid, kanamycin (50 μg/mL) or ampicilin (100 μg/mL) were added.

Culture conditions for the synthesis of PHA
To determine the functional expression of the cloned PHA biosynthetic genes in vivo, PHA biosynthesis was carried out with transformant C. necator. Both one-stage cultivation and two-stage cultivation were carried out according to the method described previously (Lau et al., 2010; Lau et al., 2011). For inoculum preparation, transformant C. necator was grown in 50 mL of NR at 30°C, 200 rpm. 3% (v/v) of the total volume of the seed culture (OD600 nm = 4.5) were inoculated into nitrogen-limiting mineral salts medium (MM). The MM contained (per liter): 3.32 g NaN2HPO4, 2.80 g KH2PO4, 0.50 g NH4Cl, 0.25 g MgSO4·7H2O and 1 mL trace elements solution (Doi et al., 1995). The trace element solution consisted of 0.22 g CoCl2·6H2O, 9.7 g FeCl3, 7.8 g CaCl2, 0.12 g NiCl2·6H2O, 0.11 g CrCl3·6H2O, 0.16 g CuSO4·5H2O in one liter 0.1 N HCl (Kahar et al., 2004). Different carbon sources (crude palm kernel oil, jatropha oil, fruitose, 4-methylvaleric acid, sodium propionate, sodium valerate, γ-butyrolactone and sodium 4-hydroxybutyrate) were tested for their ability to promote PHA synthesis in transformant C. necator. The cultures were harvested by centrifugation (6000 g, 7 min and 4°C) after 48 h of incubation at 200 rpm, 30°C. Cell pellets were washed with hexane to get rid of excess oil-based carbon sources before being washed with distilled water. Cells grown in non-palm oil based carbon sources were washed only with distilled water.

For two-stage cultivation, 3% (v/v) of the inoculum from transformant C. necator culture was transferred into fresh NR broth which was then incubated for additional 24 h. The culture was centrifuged aseptically (6000 g, 7 min and 4°C) and washed with sterile distilled water before being transferred into nitrogen-free MM. The cultures were incubated at 30°C, 200 rpm for 48 h before being harvested.

Analytical procedures
The PHA content and composition were analyzed by gas chromatography (GC) spectrometry. Approximately 25 mg of lyophilized cells were subjected to methanolytic acid in methanol. The resulting hydroxyacyl methyl esters were assayed according to the method of Braunegg and coworkers. (Braunegg et al., 1978).
DNA manipulation

The isolation of genomic DNA from *Burkholderia* sp., plasmids DNA isolation, agarose gel electrophoresis and transformation of *E. coli* were performed following standard procedures (Sambrook et al., 1989). Restriction endonucleases, all other DNA-manipulating enzymes and kits were used as recommended by the manufacturers (Promega, USA).

Cloning of PHA biosynthetic genes from *Burkholderia* sp

The putative PHA synthase (phaC<sub>Bs</sub>), β-ketothiolase (phaA<sub> Bs</sub>), acetocetyl-CoA reductase (phaB<sub> Bs</sub>) and PHA synthesis regulator (phaR<sub> Bs</sub>) genes were amplified from the chromosomal DNA of *Burkholderia* sp. using primer pairs I, II and III respectively (Table 2). The resulting polymerase chain reaction (PCR) products were cloned into pGEM-T vector (Promega, USA) and sent for sequencing at 1st BASE Laboratory (Malaysia). Sequence comparisons and alignments were performed with the Basic Local Alignment Search tool (BLAST, National Center for Biotechnology Information) and ClustalW Multiple Sequence Alignment program (Thompson et al., 1994). The potential promoter regions recognized by Sigma factor D (σ<sup>D</sup>) were predicted using prediction of bacterial promoters (BPROM) provided by Softberry Inc. (http://www.softberry.com).

Construction of plasmids

To construct a plasmid for expression of phaC<sub> Bs</sub> in *C. necator* PHB<sup>−4</sup>, PCR was performed with primer pairs IV to obtain the gene fragment containing the putative coding region, ribosome binding site and promoter. This PCR product was digested with *Bam*HI- and *Hind*III- and inserted in frame into *Bam*HI- and *Sac*I- restricted pBBR1MCS-2. The phaC<sub> Bs</sub> was also cloned in reverse orientation to *lac* promoter in pBBR1MCS-2 to confirm the expression under its native promoter. The plasmid for expression was constructed by ligation of phaC<sub> Bs</sub>.

### Table 1 Bacterial strains and plasmids used in this study

| Strain or plasmid         | Relevant characteristics                                                                                 | Source or reference |
|---------------------------|----------------------------------------------------------------------------------------------------------|---------------------|
| **Strains**               |                                                                                                          |                     |
| *E. coli* JM109           | recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB)/F<sup>[</sup>traD36, proAB<sup>+</sup>, lacI<sup>+</sup>, lacZD<sup>−</sup>]<sup>[</sup>15]<sup>] [89]<sup>2</sup> | Promega             |
| *E. coli* S17–1           | relA<sup>+</sup>, ΔlacI<sup>−</sup>, (q<sup>80</sup>lacZ<sup>−</sup>Δ<sup>−</sup>15), thi<sup>+</sup>, proA<sup>+</sup>, hsdR17, hsdM<sup>+</sup>, recA<sup>+</sup>, RP4-tra function | Simon et al., 1983   |
| *C. necator* PHB<sup>−4</sup> | PHA-negative mutant of wild-type H16                                                                  | Schlegel et al., 1970 |
| *Burkholderia* sp. USM (JCM 15050) | Wild type                                                                                           | Chee et al., 2010   |
| **Plasmids**              |                                                                                                          |                     |
| pGEM-T                    | Ap<sup>+</sup>, lacZ, cloning vector                                                                    | Promega             |
| pBBR1MCS-2<sup>−</sup>    | Km<sup>+</sup>, lacPO<sup>−</sup>, mob<sup>+</sup>, broad host range                                    | Kovach et al., 1995 |
| pBBR1MCS-2 phaC<sub>b</sub> <sup>−</sup> | pBBR1MCS-2 derivative harbouring *Bam*HI-*Hind*III phaC<sub>b</sub> from *Burkholderia* sp. with putative promoter | This study          |
| pBBR1MCS-2 phaC<sub>b</sub> <sup>−</sup> | pBBR1MCS-2 derivative harbouring *Bam*HI-*Eco*I phaC<sub>b</sub> from *Burkholderia* sp. with putative promoter | This study          |

### Table 2 List of primers used in this study<sup>a</sup>

| Primer pairs | Primers’ sequences                                      | Target gene            |
|--------------|----------------------------------------------------------|------------------------|
| I            | F: GCGAGTCCACGTTTTATTTATGGT                              | *Burkholderia* sp. phaC gene |
|              | R: TGGGCCCTCAGTATTTGTC                                   |                        |
| II           | F: AAGAGAAGGCGACGCTAGGCTGGTC                             | *Burkholderia* sp. phaA gene |
|              | R: TTGAAAGCGCTGCGATCGGTTG                                |                        |
| III          | F: GGGCGAGCACCATTTACCTGACGGCGC                           | *Burkholderia* sp. phaB and phaR genes |
|              | R: GCGACGAGCTTTAATCTTTCG                                 |                        |
| IV           | F: GCGAGGATTCGAAAATGTTTTATGGTT                           | *Burkholderia* sp. phaC gene |
|              | R: ATGAGGCTACAGGCTCGATATTCC                              |                        |
| V            | F: GCGAGGATTCGAAAATGTTTTATGGTT                           | *Burkholderia* sp. phaC gene |
|              | R: ATGAATTCATAGGCTCCCGATATTCC                             |                        |

<sup>a</sup> All primers were synthesized by 1st BASE Laboratory (Malaysia). Restriction enzymes digestion sites were underlined.
amplified with primer pairs V into pBBR1MCS-2 at BamHI and EcoRI sites. Conjugation of C. necator PHB’4 with E. coli S17-1 harbouring broad-host-range plasmids were performed as described by Friedrich et al., 1981.

**PHA synthase activity assay**

Wild-type Burkholderia sp. and transformant C. necator harbouring pBBR1MCS-2 pphaCBsBH were cultivated for 24 h under conditions as described in the materials and methods section. Cells were harvested and resuspended in 20 mM Tris-HCl (pH 8). Cell extracts were obtained by disruption using sonication (two cycles, 7 min each) with a TOMY UD-200 sonicator and centrifugation at 13700 x g for 10 min at 4°C. Activities of PHA synthase were determined spectrophotometrically by monitoring the release of CoA at 412 nm (30°C). The standard assay contained 40 mM potassium phosphate buffer (pH 7.5), 2 mM 3HB-CoA, 10 mM 5,5’-dithio-bis(2-nitrobenzoic acid) and 35 to 40 μg of protein from soluble protein fraction (Bhubalan et al. 2011). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1.0 μmol CoA/min using a molar absorption coefficient of 13,600 M⁻¹ cm⁻¹. The protein concentration was assayed as described by Bradford using bovine serum albumin as standard.

**Results**

**Identification and cloning of PHA biosynthetic genes from Burkholderia sp. USM (JCM 15050)**

In order to determine the nucleotide sequence of PHA locus of Burkholderia sp. USM (JCM15050), primers for amplification were designed based on the annotated phaC, phaA, phaB and phaR obtained from genomic DNA sequencing of Burkholderia vietnamiensis, Burkholderia thailandensis, Burkholderia ambifaria, Burkholderia glumae, Burkholderia mallei, Burkholderia multivorans and Burkholderia cenocepacia. The nucleotide sequence obtained were deposited with GenBank (accession no. JN022533, JN835296-JN835297, JQ936592). The deduced amino acid sequence of pphaCBs exhibited great similarity to PhaC of B. vietnamiensis (99% identity, accession no. YP_001119555.1), Pseudomonas putida (73% identity, accession no. BAB96552.1) and Cupriavidus necator (63% identity, accession no. YP_725940.1). The putative Shine-Dalgarno consensus sequences (TAAGG) were found 10 base pairs upstream of the putative start codon for pphaCBs. The putative −35 region (TTTACA) and −10 region (TGATAAAA) found were similar to the corresponding sequences of E. coli σ70 consensus promoter sequence. The product of pphaCBs is a protein composed of 625 amino acids with a calculated molecular mass of 68.25 kDa. Although the nucleotide sequence of pphaC from B. vietnamiensis was available following complete genomic DNA sequencing, functional characterization of the gene has not been reported. Comparison of the deduced amino acids sequence of phaC from Burkholderia sp. with homologous phaC genes from other bacteria was done by multiple alignment (Figure 1). The deduced amino acids sequence of pphaCBs showed relatively high identity to other PHA synthase and the putative lipase box that has been found conserved in all the PHA synthase was also identified. The deduced amino acid sequences of putative pphaR were similar to those β-ketothiolase from B. vietnamiensis (99% identity, accession no. YP_001119555.1), P. putida (90% identity, accession no. BAB96553.1) and C. necator (87% identity, accession no. YP_725941.1). The putative pphaB exhibited significant identity to acetocacetyl-CoA reductase from B. vietnamiensis (99% identity, accession no. YP_001119555.1), P. putida (92% identity, accession no. BAB96554.1) and C. necator (89% identity, accession no. YP_725942.1). A putative PHA synthesis regulator is encoded by pphaR and it is located downstream of pphaB. The deduced amino acid sequence of pphaR exhibited high identity to B. vietnamiensis (100% identity, accession no. YP_001119555.1) and C. necator (83% identity, accession no. YP_725943.1). As Burkholderia sp. 16 S rDNA gene (accession no. FJ667272.1) exhibited 92% identity to the corresponding gene of C. necator (accession no. CP000090.1), it is not surprising to find that the translated products of Burkholderia sp. PHA biosynthetic genes revealed identities to corresponding proteins of C. necator (63–89%).

**PHA accumulation in transformant C. necator PHB’4**

The functionality of the cloned Burkholderia sp. USM (JCM 15050) PHA synthase gene was investigated by heterologous expression of the gene in the PHA-negative C. necator host. Plasmid pBBR1MCS-2 pphaCBsBH containing pphaCBs and its putative promoter collinear to and downstream of the lac promoter was constructed. The expression was confirmed by complementation of the mutant host deficiency in PHA synthesis and the PHA-producing ability of the transformant strains were examined (Table 3). Interestingly, the heterologous expression of pphaCBs in C. necator PHB’4 has conferred the ability to synthesize P(3HB-co-4HB) copolymer with high 4HB composition in the mutant host strain. The 4HB composition reached 87 mol% in cultures supplemented with sodium 4-hydroxybutyrate in two-stage cultivation. In contrast, wild type Burkholderia sp. has limited ability to utilize 4HB-related carbon sources for growth and PHA production (results not shown). The highest PHA content of 66 wt% was obtained in transformant C. necator harbouring pphaCBs cultivated on crude palm kernel oil (CPKO) in one-stage.
cultivation. Although a low fraction of 3-hydroxy-4-
methylvalerate (3H4MV) monomer was synthesized by
the transformant strain culture from 4-methylvaleric
acid, it is in agreement with previous studies on the
ability of *Burkholderia* sp. PhaC to polymerize 3H4MV
monomer (Lau et al., 2010). 3-hydroxyvalerate (3HV)
monomer was also incorporated into the polymer in the
presence of precursor carbon sources e.g. sodium

![Putative lipase box](image)

**Figure 1** Multiple alignment of the partial deduced amino sequences of *phaC* of *Burkholderia* sp. USM (JCM 15050) with corresponding
*phaC* sequence from *Burkholderia vietnamensis* G4 (Genbank accession no. YP_001119557.1), *Burkholderia* sp. DSMZ9242 (GenBank accession no.
AAF23364.1), *Cupriavidus necator* H16 (GenBank accession no. YP725940.1), *Chromobacterium* sp. USM2 (Genbank accession no. ADL70203.1) and
*Delftia acidovorans* (Genbank accession no. BAA3155.1).

### Table 3 Effect of different carbon sources on the biosynthesis of PHA by transformant *Cupriavidus necator* PHB^4 harboring the *PHA synthase gene* of *Burkholderia* sp.

| Carbon sources | Dry Cell Weight^b (g/L) | PHA content^c (wt %) | PHA composition^d (mol %) |
|----------------|-------------------------|-----------------------|---------------------------|
|                |                         |                       | 3HB  | 3HV  | 3H4MV | 4HB  |
| One-stage cultivation |                         |                       |     |     |       |      |
| pBBR1MCS-2 *phaC*, BE |                         |                       |     |     |       |      |
| CPKO | 2.1 ± 0.4 | 64 ± 9 | 100  | 0   | 0   | 0    |
| pBBR1MCS-2 *phaC*, BH |                         |                       |     |     |       |      |
| CPKO | 2.4 ± 0.1 | 66 ± 2 | 100  | 0   | 0   | 0    |
| Jatropha oil | 2.3 ± 0.3 | 53 ± 1 | 100  | 0   | 0   | 0    |
| Fructose | 2.5 ± 0.1 | 61 ± 1 | 100  | 0   | 0   | 0    |
| Fructose+ 4MV | 1.6 ± 0.2 | 40 ± 8 | 97   | 0   | 3   | 0    |
| Two-stage cultivation |                         |                       |     |     |       |      |
| Sodium propionate | 2.7 ± 0.1 | 27 ± 3 | 70   | 30  | 0   | 0    |
| Sodium valerate | 3.2 ± 0.1 | 35 ± 1 | 60   | 40  | 0   | 0    |
| γ-butyrolactone | 2.3 ± 0.1 | 29 ± 1 | 69   | 0   | 0   | 31   |
| sodium 4-hydroxybutyrate | 2.0 ± 0.1 | 14 ± 2 | 13   | 0   | 0   | 87   |

CPKO, crude palm kernel oil; 4MV, 4-methylvaleric acid; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate 3H4MV, 3-hydroxy-4-methylvalerate; 4HB, 4-
hydroxybutyrate.

^aCells were cultivated for 48 h, at 30 °C, 200 rpm in MM medium containing the indicated carbon sources (0.2 M carbon concentration: CPKO, jatropha oil,
fructose, sodium propionate, sodium valerate, γ-butyrolactone, sodium 4-hydroxybutyrate and 0.02 M carbon concentration: 4-methylvaleric acid).

^bDry cell weight after freeze-drying.

^cPHA composition of the freeze-dried cells was determined by gas chromatography.
valerate and sodium propionate. To examine whether the expression of phaC<sub>B</sub>, was affected by the lac promoter, pBRR1MCS-2 phaC<sub>B</sub>B<sub>E</sub> was constructed by inserting phaC<sub>B</sub> in reverse orientation to lac promoter. Transformant C. <i>necator</i> PHB<sup>4</sup> harbouring pBRR1MCS-2 phaC<sub>B</sub>B<sub>E</sub> showed similar cell growth and PHA accumulation to strain harbouring pBRR1MCS-2 phaC<sub>B</sub>BH<sub>E</sub>. These results suggest that the <i>Burkholderia</i> sp. PHA synthase gene was constitutively expressed in <i>C. necator</i> PHB<sup>4</sup> from the native promoter.

The ability of transformant <i>C. necator</i> PHB<sup>4</sup> harbouring <i>Burkholderia</i> sp. PHA synthase gene to incorporate 4HB monomer into the PHA produced was investigated. To study the effect of 4HB-related carbon sources efficiency and concentration on 4HB composition of PHA synthesized, shake-flask cultures of transformant <i>C. necator</i> were cultivated on medium with different concentrations of γ-butyrolactone or sodium 4-hydroxybutyrate in two-stage cultivation. The dry cell weight, PHA content and 4HB composition showed slight increase with the increase of sodium 4-hydroxybutyrate concentration from 0.1 to 0.2 M carbon concentration for transformant <i>C. necator</i> cultures (Table 4). A maximum of 87 mol% 4HB composition was produced by the transformant strain cultivated on medium supplemented with 0.2 M carbon concentration sodium 4-hydroxybutyrate. The increase of sodium 4-hydroxybutyrate concentration from 0.2 to 0.4 M carbon concentration resulted in the decrease of 4HB composition. On the other hand, increased mol fractions of 4HB monomers in PHA produced were achieved by increasing γ-butyrolactone concentrations.

**Assay of PHA synthase activity**

In order to determine and compare the expression of <i>Burkholderia</i> sp. PHA synthase gene in <i>C. necator</i> PHB<sup>4</sup> and its native host, crude extracts of transformant <i>C. necator</i> and wild-type <i>Burkholderia</i> sp. were subjected to PHA synthase assay (Table 5). The crude lysate from <i>Burkholderia</i> sp. exhibited specific activity of 151 U/mg, which was similar and comparable with that from <i>C. necator</i> H16 results published previously (180 U/mg) (Schubert et al., 1988). <i>C. necator</i> PHB<sup>4</sup> harbouring <i>Burkholderia</i> sp. PHA synthase exerted slightly enhanced in vivo PHA synthase activity compared to wild-type <i>Burkholderia</i> sp.

**Discussions**

Bacteria belonging to the genus <i>Burkholderia</i> was first used in 1989 for the production of P(3HB) homopolymer from fructose (Ramsay et al., 1989). They are one of the most nutritionally versatile microorganisms that are capable of utilizing a wide range of carbon sources. In this study, the PHA biosynthetic genes from a locally isolated <i>Burkholderia</i> sp. strain USM (JCM 15050) were successfully amplified. The PHA biosynthetic genes of <i>Burkholderia</i> sp. consisted of a PHA synthase (phaC), β-ketothiolase (phaA), acetoadetyl-CoA reductase (phaB) and PHA synthase regulator (phaR). The phaC, phaA and phaB seems to be organized in an operon and the structural organization of these genes is closely related to other bacteria harbouring type I PHA synthase e.g. <i>C. necator</i> and <i>Alcaligenes latus</i> (Choi et al., 1998; Rehm and Steinbüchel, 2002) (Figure 2). Although the PHA synthase and other PHA biosynthetic genes are often found clustered in the bacterial genomes, there are some exceptions to these observations. In the genomes of <i>Caulobacter crescentus</i>, <i>Paracoccus denitrificans</i>, <i>Methylotracerium extorquens</i> and <i>Aeromonas caviae</i>, the genes related to PHA biosynthesis are not directly linked to the PHA synthase (Rehm and Steinbüchel, 2002).

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**Table 4 Effect of different 4HB-related carbon sources concentrations on the biosynthesis of PHA by transformant Cupriavidus necator PHB<sup>4</sup> harboring the PHA synthase gene of Burkholderia sp.**

| Carbon sources<sup>a</sup> | Carbon concentration (M) | Dry Cell Weight<sup>b</sup> (g/L) | PHA content<sup>c</sup> (wt %) | PHA composition<sup>d</sup> (mol %) |
|---------------------------|--------------------------|----------------------------------|-------------------------------|-----------------------------------|
|                           |                          |                                  |                               | 3HB                              |
| γ-butyrolactone           | 0.1                      | 1.9±0.1                          | 10±1                          | 78                                |
|                           |                          |                                  |                               | 4HB                              |
|                           |                          |                                  |                               | 22                                |
| γ-butyrolactone           | 0.2                      | 2.3±0.1                          | 29±1                          | 69                                |
|                           |                          |                                  |                               | 31                                |
| γ-butyrolactone           | 0.3                      | 2.4±0.2                          | 31±3                          | 68                                |
|                           |                          |                                  |                               | 32                                |
| γ-butyrolactone           | 0.4                      | 2.2±0.1                          | 24±1                          | 59                                |
| sodium 4-hydroxybutyrate  | 0.1                      | 1.5±0.1                          | Trace                         | 28                                |
|                           |                          |                                  |                               | 72                                |
| sodium 4-hydroxybutyrate  | 0.2                      | 2.0±0.1                          | 14±2                          | 13                                |
|                           |                          |                                  |                               | 87                                |
| sodium 4-hydroxybutyrate  | 0.3                      | 1.9±0.1                          | 8±1                           | 40                                |
|                           |                          |                                  |                               | 60                                |
| sodium 4-hydroxybutyrate  | 0.4                      | 2.0±0.1                          | 11±2                          | 46                                |
|                           |                          |                                  |                               | 54                                |

<sup>a</sup>Cells were cultivated for 48 h, at 30 °C, 200 rpm in MM medium containing different concentrations of 4HB-related carbon sources at two-stage cultivation.

<sup>b</sup>Dry cell weight after freeze-drying.

<sup>c</sup>PHA content of the freeze-dried cells was determined by gas chromatography.

<sup>d</sup>PHA composition of the freeze-dried cells was determined by gas chromatography.

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Table 5 Analysis of PHA synthase activity in wild-type
Burkholderia sp. and transformant C. necator

| Strain                        | PHA synthase sp act (U/mg of protein) | References                  |
|-------------------------------|--------------------------------------|-----------------------------|
| Burkholderia sp. USM (JCM 15050) | 151                                  | This study                  |
| C. necator PHB’4 (pBBR1MCS-2 phaC, phaB) | 243                                  | This study                  |
| C. necator H16                | 180                                  | Schubert et al., 1988       |

* One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1.0 μmol CoA/min.

bacteria e.g. *P. denitrificans* contains other genes related to PHA synthesis (phasin, *phaP* and *phaR*) map close to the PHA locus. Both *C. necator* and *Burkholderia* sp. PHA locus also possess a putative *phaR* immediately downstream of *phaB*. The *phaR* encodes a protein with putative regulatory function in PHA metabolism (York et al. 2002; Stubbe and Tian, 2003; Pötter and Steinbüchel, 2006).

The putative translated product of *Burkholderia* sp. PHA biosynthetic genes exhibited high homologies to the respective *B. vietnamiensis* and *C. necator* genes. As the most important enzyme involved in PHA biosynthesis, PHA synthase of *Burkholderia* sp. belongs to Class I that is active on short chain-length (*R*)-hydroxyacyl-CoA with three to five carbon atoms (Rehm and Steinbüchel, 2002; Rehm, 2007). Site-specific mutagenesis analysis of *C. necator* PHA synthase had shown that the conserved cystein-319, aspartate-480 and histidine-508 of the class I synthase are required for enzyme activity. These amino acid residues are conserved in all class I synthases and it was suggested that they are involved in covalent catalysis. In addition, another conserved residue, tryptophan-398 was suggested to function in PHA synthase subunit dimerization by the provision of a hydrophobic surface (Gerngross et al., 1994; Jia et al., 2001). In recent years, PHA synthases with improved substrate specificity, enzyme activity and stability were engineered by various approaches e.g. random mutagenesis, gene shuffling and recombination (Nomura and Taguchi, 2007). The introduction of a mutant PHA synthase (Ser325Thr/Gln481Lys) from *Pseudomonas* sp. 61-

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**Figure 2** Molecular organizations of PHA biosynthetic genes in *Burkholderia* sp. USM (JCM 15050) and other bacteria containing type I PHA synthase (Choi et al., 1998; Kolibachuk et al., 1999; Rehm & Steinbüchel, 2002; Sudesh et al., 1998).
3 and lactate (LA) monomer supplying enzymes into E. coli had enabled the production of LA-based polyesters in a biological system (Shozui et al., 2010). The same mutant *Pseudomonas* sp. PHA synthase was expressed in *E. coli* for the synthesis of glycolate-based polyesters containing medium-chain-length 3-hydroxyalkanoates (Matsumoto et al., 2011).

The types and compositions of PHA that are produced by the biological system depend on the PHA synthase substrate specificity, the carbon sources supplied and the metabolic pathways that are functioning in the cell (Sudesh and Doi, 2005). PHA-negative mutant PHB-4 of *C. necator* is a desirable host for PHA production due to the strain ability to achieve stable high cell density fermentation and produce high PHA content from simple, inexpensive substrates (Taguchi et al., 2003). Heterologous expression of *Burkholderia* sp. PHA synthase gene in PHA-negative mutant of *C. necator* enabled the synthesis of P(3HB-co-4HB) containing 87 mol% of the 4HB fraction. In contrast, wild-type *Burkholderia* sp. had reduced ability in utilizing 4HB-related carbon sources for growth and PHA production. It is for the first time that the *Burkholderia* sp. PHA synthase was shown to polymerize 4HB monomer. It is well documented that *C. necator* was equipped with the metabolic pathway to supply 4HB-CoA substrate even though only small amount of P(4HB) was accumulated (Nakamura et al., 2001). The expression of *Burkholderia* sp. PHA synthase in a host that are capable of producing 4-hydroxybutyryl-CoA as substrate for the PHA synthase allowed the polymerization of 4HB monomer. These results support previous findings which suggested that the monomer supplying pathways operating in the cells are an important factor determining the provision of substrate for the polymerization by the PHA synthase (Sudesh et al., 1998). In addition, the synthesis of P(3HB-co-4HB) with high 4HB composition by transformant *C. necator* suggested the preference of *Burkholderia* sp. PHA synthase for 4-hydroxybutyryl-CoA.

In this study, the PHA biosynthetic gene cluster *Burkholderia* sp. USM (JCM 15050) which consisted of a *phaC*, *phaA*, *phaB* and *phaR* were successfully cloned. The heterologous expression of *Burkholderia* sp. PHA synthase gene in *C. necator* PHB-4 showed that the PHA synthase was capable of polymerizing 4HB monomer. The biosynthesis results also suggested *Burkholderia* sp. PHA synthase preference for 4HB-CoA as PHA with high 4HB composition (87 mol%) was synthesized by transformant *C. necator* harbouring the synthase. PHA synthase activity assay of wild-type *Burkholderia* sp. and transformant *C. necator* indicated that these two strains exhibited activities that are comparable and in a similar range with *C. necator* H16. More efforts to analyze the substrate specificity of PHA synthase in various hosts need to be done in order to understand fully the potential of the synthase.

**Competing interests**
The authors declare that they have no competing interests.

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**References**
Bhupalan K, Chua J-A, Zhou F, Brigham CJ, Taguchi S, Sinkey AJ, Rha C, Sudesh K (2011) Characterization of the highly active polyhydroxyalkanoate synthase of *Chromobacterium* sp. strain USM2. Appl Environ Microbiol 77:2926–2933
Braungg G, Sonnleitner B, Lafferty RM (1978) A rapid gas chromatographic method for the determination of poly-3-hydroxybutyric acid in microbial biomass. Eur J Appl Microbiol 6:29–37
Chee J-Y, Tan Y, Samian M-R, Sudesh K (2010) Isolation and characterization of a *Burkholderia* sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty Acids and glycerols. J Polym Environ 18:584–592
Chen G-Q (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Rev 38:2434–2446
Choi J-H, Lee SY, Han K (1998) Cloning of the *Alcaligenes latus* polyhydroxyalkanoate biosynthesis genes and use of these genes for enhanced production of poly(3-hydroxybutyrate) in *Escherichia coli*. Appl Environ Microbiol 64:4897–4903
Doi Y, Kitamura S, Abe H (1995) Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). Macromol 28:4822–4828
Friedrich B, Hogrefe C, Schlegel HG (1981) Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J Bacteriol 147:198–205
Gerngross TU, Snell KD, Peoples OP, Sinskey AJ, Cruhail E, Masamune S, Stubbe J (1994) Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus* evidence for a required posttranslational modification for catalytic activity. Biochemistry 33:9311–9320
Jendrossek D (2009) Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). J Bacteriol 191:3195–3202
Jia Y, Yuan W, Wodzinska J, Park C, Sinkey AJ, Stubbe J (2001) Mechanistic studies on class I polyhydroxybutyrate (PHB) synthase from *Rhizobium* etoraphus class I and III synthases share a similar catalytic mechanism. Biochemistry 40:1011–1019
Kahar P, Tsuge T, Taguchi K, Doi Y (2004) High yield production of polyhydroxyalkanoates from soybean oil by *Rhizobium* etoraphus and its recombinant strain. Polym Degrad Stabil 83:79–86
Kolbchak D, Miller A, Dennis D (1999) Cloning, molecular analysis and expression of the polyhydroxyalkanoic acid synthase (phaG) gene from *Chromobacterium violaceum*. Appl Environ Microbiol 65:3561–3565
Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM II, Peterson KM (1995) Four new derivatives of the broad-host-range cloning vector pBR322MCs, carrying different antibiotic-resistance cassettes. Gene 166:175–176
Lau N-S, Chee J-Y, Tsuge T, Sudesh K (2010) Biosynthesis and mobilization of a novel polyhydroxyalkanoate containing 3-hydroxy-4-methylvalerate monomer produced by *Burkholderia* sp. USM (JCM15050). Bioreourc Technol 101:7916–7923
Lau N-S, Tsuge T, Sudesh K (2011) Formation of new polyhydroxyalkanoate containing 3-hydroxy-4-methylvalerate monomer in *Burkholderia* sp. Appl Microbiol Biotechnol 89:1599–1609
Matsumoto K, Ishiyama A, Sakai K, Shiba T, Taguchi S (2011) Biosynthesis of glycolate-based polyesters containing medium-chain-length 3-hydroxyalkanoates in recombinant *Escherichia coli* expressing engineered polyhydroxyalkanoate synthase. J Biotechnol 156:214–217
Meikle JL (1995) American plastic: a cultural history. Meikle Rutgers University Press, New Brunswick, N.J
Nakamura S, Doi Y, Scandola M (1992) Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-4-hydroxybutyrate). Macromolecules 25:4237–4241

Nomura CT, Taguchi S (2007) PHA synthase engineering toward superbiocatalysts for custom-made biopolymers. Appl Microbiol Biotechnol 73:969–979

Pötter M, Steinbüchel A (2006) Biogenesis and structure of polyhydroxyalkanoate granules. In: Shively JM (ed) Inclusions in prokaryotes. Springer-Verlag, Berlin, pp 110–129

Ramsay BA, Ramsay JA, Cooper DG (1989) Production of poly-β-hydroxyalkanoic acid by Pseudomonas cepacia. Appl Microbiol Biotechnol 55:584–589

Rehm BHA (2003) Polyester synthases: natural catalysts for plastics. Biochem J 376:15–33

Rehm BHA (2006) Genetics and biochemistry of polyhydroxyalkanoate granule self-assembly: the key role of polyester synthases. Biotechnol Lett 28:207–213

Rehm BHA (2007) Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. Curr Iss Mol Biol 941–62

Rehm BHA, Steinbüchel A (2002) PHA synthases: the key enzymes of PHA synthesis. In: Doi Y, Steinbüchel A (eds) Biopolymers. Wiley, VCH, Weinheim, pp 173–215

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Schlegel HG, Lafferty R, Krauss I (1970) The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch Microbiol 70:283–294

Schubert P, Steinbüchel A, Schlegel HG (1988) Cloning of the Alcaligenes eutrophus genes for synthesis of poly-β-hydroxybutyric acid (PHB) and synthesis of PHB in Escherichia coli. J Bacteriol 170:5837–5847

Shozui F, Matsumoto K, Nakai T, Yamada M, Taguchi S (2010) Biosynthesis of novel terpolymers poly(lactate-co-3-hydroxybutyrate-co-3-hydroxyvalerate)s in lactate-overproducing mutant Escherichia coli JW0885 by feeding propionate as a precursor of 3-hydroxyvalerate. Appl Microbiol Biotechnol 85:949–954

Simon R, Priefert U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering. Transposon mutagenesis in Gram negative bacteria. Bio/Technology 1:784–791

Stubbe J, Tian J (2003) Polyhydroxyalkanoate (PHA) homeostasis: the role of the PHA synthase. Nat Prod Repo 20:445–457

Sudesh K, Doi Y (2005) Polyhydroxyalkanoates. In: Bastioli C (ed) Handbook of biodegradable polymers. Rapra Technology Limited, United Kingdom, pp 219–241

Sudesh K, Kivata T (2008) Sustainability of biobased and biodegradable plastics. CLEAN-Soil, Air, Water 36:433–442

Sudesh K, Fukui T, Doi Y (1998) Genetic analysis of Comamonas acidovorans polyhydroxyalkanoate synthase and factors affecting the incorporation of 4-hydroxybutyrate monomer. Appl Environ Microbiol 64:3437–3443

Taguchi S, Nakamura H, Kichise T, Tsuge T, Yamato I, Doi Y (2003) Production of polyhydroxyalkanoate (PHA) from renewable carbon sources in recombinant Ralstonia eutropha using mutants of original PHA synthase. Biochem Eng J 16:107–113

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res 22:4567–4680

Tsuge T (2002) Metabolic improvements and use of inexpensive carbon sources in microbial production of polyhydroxyalkanoates. J Biosci Bioeng 94:579–584

York GM, Stubbe J, Sinskey AJ (2002) The Ralstonia eutropha PhaP protein couples synthesis of the PhaP phasin to the presence of polyhydroxybutyrate in cells and promotes polyhydroxybutyrate production. J Bacteriol 184:59–66