BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Comparative study on the production of poly(3-hydroxybutyrate) by thermophilic *Chelatococcus daeguensis* TAD1: a good candidate for large-scale production

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Abstract In spite of numerous advantages on operating fermentation at elevated temperatures, very few thermophilic bacteria with polyhydroxyalkanoates (PHAs)-accumulating ability have yet been found in contrast to the tremendous mesophiles with the same ability. In this study, a thermophilic poly(3-hydroxybutyrate) (PHB)-accumulating bacteria (*Chelatococcus daeguensis* TAD1), isolated from the biofilm of a biotrickling filter used for NOx removal, was extensively investigated and compared to other PHB-accumulating bacteria. The results demonstrate that *C. daeguensis* TAD1 is a growth-associated PHB-accumulating bacterium without obvious nutrient limitation, which was capable of accumulating PHB up to 83.6 % of cell dry weight (CDW, *w/w*) within just 24 h at 45 °C from glucose. Surprisingly, the PHB production of *C. daeguensis* TAD1 exhibited strong tolerance to high heat stress as well as nitrogen loads compared to that of other PHB-accumulating bacterium, while the optimal PHB amount (3.44 ± 0.3 g l^{-1}) occurred at 50 °C and C/N=30 (molar) with glucose as the sole carbon source. In addition, *C. daeguensis* TAD1 could effectively utilize various cheap substrates (starch or glycerol) for PHB production without prehydrolyzed, particularly the glycerol, exhibiting the highest product yield ($Y_{P/S}$, 0.26 g PHB per gram substrate used) as well as PHB content (80.4 % of CDW, *w/w*) compared to other carbon sources. Consequently, *C. daeguensis* TAD1 is a viable candidate for large-scale production of PHB via utilizing starch or glycerol as the raw materials.

Keywords *Chelatococcus daeguensis* TAD1 ·
Growth-associated PHB-accumulating · Thermophilic ·
Starch · Glycerol

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Introduction

Due to the finite reserves of fossil resources, many efforts have been conducted to achieve identical alternatives not only for crude oil but also for the petroleum-based materials. Polyhydroxyalkanoates (PHAs), which were first reported in *Bacillus megaterium* several decades ago (Lemoigne 1926), have attracted great interest as suitable alternatives to petrochemical-based plastics because of their similar material properties to various thermoplastics and elastomers (Rehm 2010). Moreover, PHAs are aliphatic bacterial polyesters intracellularly accumulated by many bacteria and some archaea, and can serve as carbon and energy sources under unfavorable conditions (Akiyama et al. 2011; Rodgers and Wu 2010). This means that PHAs are biocompatible and could be completely degraded to carbon dioxide and water through natural microbiological mineralization (Bhatt et al. 2008; Tokiwa and Calabia 2004). Depending on the carbon chain length of their

monomers, PHAs are commonly classified into three major categories: short-chain-length PHAs (scl PHAs), commonly consisting of C3–C5 hydroxyl fatty acids such as poly(3-hydroxybutyrate) (PHB), which are produced by several bacteria like *Ralstonia eutropha* (Riedel et al. 2012); medium-chain-length PHAs (mcl PHAs) with C6–C14 hydroxyl fatty acids, such as poly-3-hydroxyhexanoate, which are synthesized in many *Pseudomonas* species (Guo et al. 2011); and copolymers of scl and mcl PHAs containing the above scl and mcl monomers (Chee et al. 2012).

Although many PHAs have been found, only four of them have been produced on a large scale for commercial exploitation, including PHB, poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P3HB4HB), and poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) (Chen 2010). Among them, PHB is the best-studied PHAs with a broad application in the field of green packaging, biomedical devices, and tissue engineering materials (Chen and Wu 2005). So far, there are two prevalent cultivation modes for PHB large-scale production. The more frequently used mode is realized by a complex two-stage cultivation process, constituted of cell growth to a high cell density firstly and then PHB accumulation with nutrient limitation in the next stage. The representative microorganism for this mode is *R. eutropha* (Lee 1996). In the other cultivation mode, meanwhile, PHB accumulation is along with the cell growth in a just one-stage process without obvious nutrient limitation, and the production cost could decrease conspicuously compared to that of first mode. *Alcaligenes latus* is a well-known growth-associated PHB-accumulating microorganism used in this mode (Yamane et al. 1996).

Generally, PHB production contains several steps, including fermentation, separation of biomass from the broth, biomass drying, PHB extraction, PHA drying, and packaging (Chen 2010). However, the production cost of PHB (about 4 US$/kg) is more than four folds higher than that of petrochemical plastics (<1 US$/kg), which significantly hinder its successful commercialization (Waltz 2008). Many efforts have been dedicated to reducing the production cost by developing superior microorganisms capable of utilizing cheap substrates (Cerrone et al. 2013; Sudesh et al. 2011; Wang et al. 2012) and also by applying more efficient fermentation strategies (Albuquerque et al. 2011; Passanha et al. 2013; Venkata Mohan and Venkateswar Reddy 2013) and economical recovery processes (Ibrahim and Steinbüchel 2009; Mohammadi et al. 2012; Madkour et al. 2013).

Recently, the study on the PHA accumulation in thermophilic bacteria has attracted growing interest because of their numerous advantages on operating fermentation at elevated temperatures, which is of benefit for reducing the production cost of PHA. For example, the diffusion rates and solubility of chemicals increase at elevated temperatures, while the risk of contamination decreases significantly because of fewer microbial organisms can survive at elevated temperatures (Holst et al. 1997). In spite of these advantages, few PHB-accumulating thermophiles have yet been found. The biosynthesis of PHAs in thermophilic bacteria was firstly reported in *Thermus thermophilus* HB8, which is capable of accumulating PHAs to approximately 35 or 40 % at a optimal temperature of 70 °C utilizing sodium gluconate or sodium octanoate as sole carbon sources (Pantazaki et al. 2003). However, the optimal temperatures of this strain are too high for industrial fermentation. *Caldimonas taiwanensis*, a thermophile isolated from a hot spring in southern Taiwan, could accumulate PHB at 55 °C from gluconate, glucose, glycerol, and starch under nitrogen-limited conditions with a short time to obtain peak PHB content (14 h) (Sheu et al. 2009). Meanwhile, this strain did not grow or accumulate PHAs from fatty acids as the sole carbon source. In addition, some PHAs-accumulating thermophilic bacteria have been isolated from the aerobic organic waste treatment plant and characterized to produce PHB from glucose or glycerol at 50 °C (Ibrahim et al. 2010).

However, there are still growing demands for exploring more appropriate sources for PHA-accumulating thermophiles due to the fact that extremely fewer thermophilic microorganisms with PHA-accumulating ability have yet been found in contrast to the tremendous mesophiles with this ability (Budde et al. 2011; Chen 2010; Chen et al. 2001; Delamarre and Batt 2006). In our group, a biotrickling filter had been set up and used for the removal of nitrogen oxides from a coal-fired power plant with elevated temperatures (around 50 °C) and sufficient carbon sources (glucose) (Jiang et al. 2009; Liang et al. 2012; Yang et al. 2012), which are the key parameters of suitable sources for PHA-accumulating thermophiles. In this study, we succeeded in isolating a thermophilic PHA-accumulating bacteria (*Chelatococcus daeguensis* TAD1) from the biofilm of this biotrickling filter. This study thus characterizes the PHB accumulation of *C. daeguensis* TAD1 at elevated temperatures. After that, further investigation refer to the influence of temperature, molar ratio of carbon to nitrogen (C/N), and carbon source on PHB accumulation were conducted to obtain the optimal PHB amount by *C. daeguensis* TAD1. Furthermore, the obtained results were compared to those of other thermophilic/mesophilic PHB-accumulating bacteria to verify whether this strain is a viable candidate for large-scale production of PHB.

Materials and methods

Characteristics of *C. daeguensis* TAD1

The novel thermophilic aerobic denitrifying bacterium *C. daeguensis* TAD1 investigated in this study was isolated

from the biofilm of a biotrickling filter in our group, which have been used for the removal of nitrogen oxides from a coal-fired power plant (Liang et al. 2012; Yang et al. 2012). *C. daeguensis* TAD1 had been deposited in China General Microbiological Culture Collection Center (CGMCC no. 5226). The 16S ribosomal DNA gene sequence of *C. daeguensis* TAD1 has been submitted to the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank databases under accession no. HM000004, which showed a high similarity (99 %) to that of *C. daeguensis* K106 (Liang et al. 2012). Therefore, the detailed characteristics of *C. daeguensis* TAD1 have been compared to that of *C. daeguensis* K106 as well as other *Chelatococcus* sp.

Observation of the cytoplasmic PHB inclusions in *C. daeguensis* TAD1

The presence of cytoplasmic PHB inclusions was investigated by staining the biopolymer with Nile red [0.25 mg dissolved in 1 ml dimethyl sulfoxide (DMSO)] and observing the cells under a fluorescence microscope (Ibrahim et al. 2010). The observations were carried out via a Nikon ECLIPSE 90i equipped with a Nikon JAPAN Plan Apo VC 100×/1.40 oil immersion Plan-Apochromat objective lens. The images were initially collected by a Nikon DIGITAL CAMAERA DXM 1200C, and then processed through an image software named as Nikon NIS-Elements License. Nile red fluorescence was excited using a Nikon INTENSILIGHT C-HGFI high-pressure mercury arc lamp, and the recording of fluorescence images were performed by using emission band-pass filters at EX 510–560 nm/DM 575 nm/BA 590 nm for Nile red.

Growth medium and cultivation of *C. daeguensis* TAD1

The growth media and their corresponding composition are given as follows: Luria-Bertani (LB) medium (grams per liter): tryptone, 10.0; yeast extract, 5.0; NaCl, 5.0. Mineral salt medium (MSM) (grams per liter) (Ibrahim and Steinbüchel 2010b): $Na_2HPO_4 \cdot 12H_2O$, 9.0; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.2; NH_4Cl, 1.0; $CaCl_2 \cdot 2H_2O$, 0.02; 1 ml of trace elements solution. Trace elements solution (grams per liter): EDTA, 50.0; $FeCl_3$, 8.3; $ZnCl_2$, 0.84; $CuCl_2 \cdot 2H_2O$, 0.13; $CoCl_2 \cdot 6H_2O$, 0.1; $MnCl_2 \cdot 6H_2O$, 0.016; H_3BO_3, 0.1.

TAD1 was first pre-cultured in 50 ml LB medium for 12 h at 45 °C and 180 rpm. 5 ml of this seed medium was inoculated to 100 ml MSM medium with glucose (20 g l^{-1}) as sole carbon source in 300-ml flask, and then grown for 36 h at 45 °C and 180 rpm. The pH of the medium was adjusted to 7.3 before sterilization. These cells were harvested by centrifugation for 20 min at 1,200×*g* and 4 °C, washed with distilled water twice, and then frozen and lyophilized. Cell

concentration, defined as cell dry weight (CDW) per liter of culture broth, was determined by weighing the lyophilized cells.

Optimization of the PHB amount from *C. daeguensis* TAD1

Influence of the temperature on PHB accumulation

The influences of the temperature on the PHB accumulation of TAD1 were determined by testing at various temperatures (35, 45, 50, 55, and 60 °C) in MSM medium. Other culture conditions were the same as described in the "Growth medium and cultivation of *C. daeguensis* TAD1."

Influence of the molar ratio of carbon to nitrogen (C/N) on PHB accumulation

The influences of various molar ratios of carbon to nitrogen (C/N=5, 10, 20, 30 and 60) on the PHB accumulation of TAD1 were investigated at MSM medium via adjusting the concentrations of NH_4Cl, with the coincident concentration of glucose. The other culture conditions were as described in "Growth medium and cultivation of *C. daeguensis* TAD1" except for the temperature optimized at "Influence of the temperature on PHB accumulation."

Influence of the carbon source on PHB accumulation

The ability of TAD1 to utilize various carbon sources for PHB accumulation was investigated in MSM at 45 °C. Based on their structures, carbon sources were divided into sodium salts of fatty acid, consisting of sodium acetate (C2), sodium propionate (C3), sodium succinate (C4), sodium citrate (C6), and sodium caprylate (C8); carbohydrates, containing glucose (monosaccharide), sucrose (disaccharide), and starch (polysaccharide); and glycerol, a biodiesel fuel (BDF) by-product. The culture conditions were as described in "Growth medium and cultivation of *C. daeguensis* TAD1" with the optimized temperature and C/N above.

Analytical methods

Lyophilized cells were treated with acetone, dried, and then stirred in 50 volumes of chloroform for 48 h at 30 °C. After filtration, the extracted PHB was concentrated by partially evaporating the solvent, and five volumes of cold methanol were used for precipitation. The precipitated PHB was filtered and dried at 37 °C for 24 h (Ibrahim and Steinbüchel 2010b). The PHB was then repurified by the same procedure.

To determine the PHB content of the cells and the composition, samples were subjected to methanolysis in the presence of 15 % (*v*/*v*) sulfuric acid. The resulting 3-hydroxybutyric acid methyl esters were analyzed by gas chromatography

(Rodgers and Wu 2010). PHB homopolymer (Sigma) was used as the standard for calibration. The content % (w/w) was defined as a percentage of PHB from CDW. The residual cell dry weight (RCDW) standing for cell growth was calculated by as the total CDW minus the mass of PHB.

The concentrations of ammonium in cell-free supernatants were determined by employing a gas-sensitive type 152303000 ammonium electrode (Mettler Toledo GmbH, Greifensee, Switzerland) (Ibrahim and Steinbüchel 2010b). The analysis of glucose, glycerol, and organic acids was monitored via high-performance liquid chromatography (Shimadzu, LC-20A, Japan) using a JADE-PAK™ OA column (300×7.8 mm, 10 μm) with a UV detector (Shimadzu, SPD-20AV) at 210 nm. Aliquots of 10 μl were injected and eluted with 2.5 mM sulfuric acid in double-distilled water at a flow rate of 0.5 ml/min and operation temperature of 60 °C.

Results

Characteristics of *C. daeguensis* TAD1

Table 1 shows the characteristics of *C. daeguensis* TAD1, which were compared to those of *C. daeguensis* K106[T] (a typical model of *C. daeguensis*) (Yoon et al. 2008), *Chelatococcus* sp. MW10, and *Chelatococcus* sp. MW11 (two newly isolated PHA-accumulating thermophilic bacteria

from waste treatment) (Ibrahim et al. 2010). Strain TAD1 is a Gram-negative, rod-shaped bacterium. Nitrate reduction, indole production, as well as citrate utilization was positive, while both urease and phenylalanine tests were negative. Heterotrophic growth was observed at pH 6.0~9.5 and 20~52 °C, whereas optimal growth occurred at 37~50 °C. Strain TAD1 is capable of utilizing both glucose and glycerol to accumulate PHB (a short-chain-length PHA), and the PHB inclusions stained with Nile red have been observed by fluorescence microscopy (Fig. 1). The PHB was tested by Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR) spectroscopy, and thermal analysis, and the results were shown in Fig. S1, Fig. S2, Fig. S3, respectively.

Although all the four bacteria investigated belong to the genus of *Chelatococcus*, the characteristics of strain TAD1 are significantly more comparable to that of *C. daeguensis* K106[T] than those of *Chelatococcus* sp. MW10 and *Chelatococcus* sp. MW11. However, no PHB accumulation has been reported by *C. daeguensis* K106[T] before, and its optimum growth temperatures (30~37 °C) are considerable lower than that of strain TAD1. In addition to the extremely high similarity (99 %) between the 16S rRNA sequences of strain TAD1 and K106[T], it can be inferred that strain TAD1 is a new isolate of *C. daeguensis*. Although *Chelatococcus* sp. MW10 and MW11 could also produce PHB (Ibrahim et al. 2010), these two isolates may form a separate species rather than belong to *C. daeguensis* due to the differential characteristics in contrast
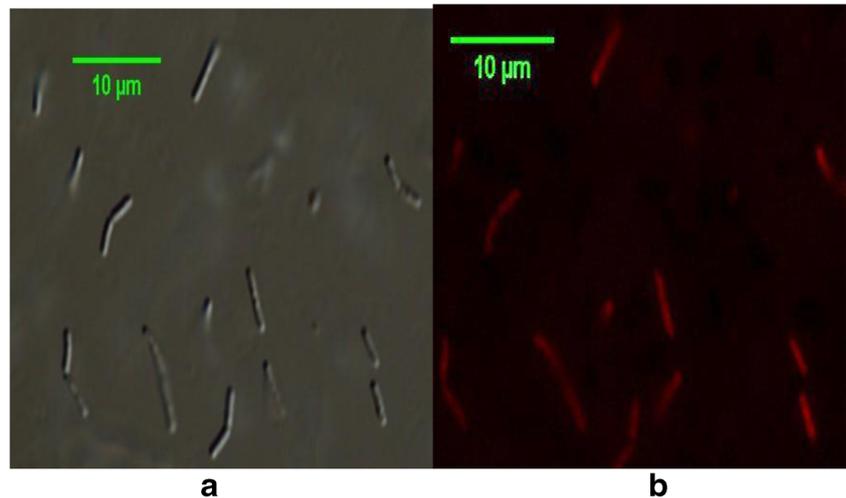
Table 1 The characteristics of *C. daeguensis* TAD1, *C. daeguensis* K106[T], *Chelatococcus* sp. MW10, and *Chelatococcus* sp. MW11

Test	*Chelatococcus daeguensis* TAD1	*Chelatococcus daeguensis* K106[T]	*Chelatococcus* sp. MW10	*Chelatococcus* sp. MW11
Morphological test				
Gram's reaction	−	−	−	−
Colony morphological	Circular	Circular	Circular	Circular
Colony color	Light yellow	Moderate yellow	Bright cream	White dull
Cell shape	Rod	Rod	Rod	Rod
Biochemical test				
Nitrate reduction	+	+	−	−
Urease test	−	−	+	+
Indole production	+	+	−	−
Citrate utilization	+	+	−	−
Phenylalanine	−	−	+	+
Physiological test				
Growth temperature (°C)	20~52	20~51	37~50	30~55
Optimal temperature (°C)	37~50	30~37	41~50	37~50
Growth pH	6.0~9.5	5.5~10.0	ND[a]	ND[a]
PHB production				
Glucose	+	ND	+	−
Glycerol	+	ND	−	+

+ positive reaction, − negative reaction, *ND* not detected

[a] The initial pH of both *Chelatococcus* sp. MW10 and MW11 for the PHB production is set as 7.3 (Ibrahim et al. 2010)

Fig. 1 Fluorescence microscopy of Nile red stained inclusions inside *C. daeguensis* TAD1. The cells were cultivated in mineral salts medium containing 20 g l^{-1} glucose at 45 °C and 180 rpm for 36 h. Images were observed by **a** phase contrast light microscopy and **b** fluorescence microscopy of Nile red stained cells. The *scale bars* indicate a length of 10 µm

to those of *C. daeguensis* K106^{T} as well as strain TAD1. For example, the nitrate reduction tests of *Chelatococcus* sp. MW10 and MW11 are negative, whereas that of *C. daeguensis* K106^{T} as well as strain TAD1 are positive. Consequently, to the best of our knowledge, this is the first report on PHB accumulation by thermophilic *C. daeguensis*.

Time course of growth and PHB accumulation by *C. daeguensis* TAD1

To investigate the characteristics of PHB accumulation by *C. daeguensis* TAD1, the alteration of CDW, PHB content (*w/w*), RCDW, carbon source (glucose), and nitrogen source (NH$_4$Cl) along with the growth of *C. daeguensis* TAD1 during MSM medium have been determined and illustrated in Fig. 2.

According to the variation of RCDW, the growth of *C. daeguensis* TAD1 was divided into two phases, including logarithmic phase and stationary phase. During the logarithmic phase, the RCDW as well as the PHB content simultaneously increased with the cultivation time. The highest RCDW (0.93 g l^{-1}) occurred at 28 h, while the PHB content reached its maximum value (83.6 % of CDW, *w/w*) at 24 h. Although all the glucose added in the medium used up at the same time, the cells still kept on growing until 28 h, at which the maximum PHB amount and cell density were 2.93 and 3.86 g l^{-1}, respectively. These results are in well accordance with those of growth-associated PHB-accumulating microorganisms (Ibrahim and Steinbüchel 2010b; Yezza et al. 2006), which could be able to obtain high cell densities for producing PHB in a simple one-step fermentation process without nutrient limitation (Gahlawat and Srivastava 2013; Ibrahim and Steinbüchel 2010a).

Although glucose was not detect in the stationary phase, the RCDW values were almost identical and remained in the range of 0.71 to 0.96 g l^{-1}. Even if nutrient starvation was prolonged up to 120 h, a portion of *C. daeguensis* TAD1 cells still survive, and the concentration of RCDW was 0.422 g l^{-1}.

Meanwhile, the PHB content (62.6 %) as well as CDW (2.01 g l^{-1}) exhibited a significant decrease after 60 h cultivation, which is attributed to the fact that the intracellular PHB of *C. daeguensis* TAD1 could be utilized as carbon and energy
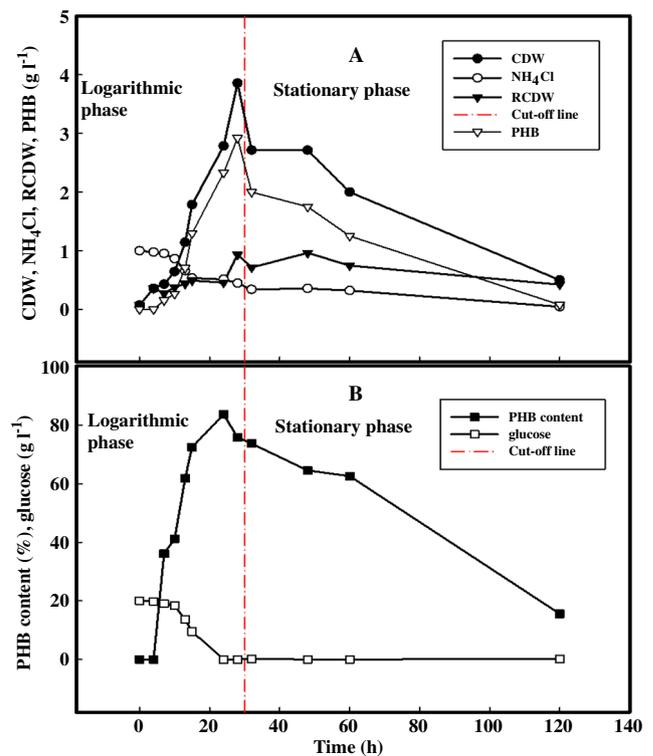

Fig. 2 Time course of the growth and PHB accumulation by *C. daeguensis* TAD1. According to the variation of RCDW, the growth of *C. daeguensis* TAD1 was divided into two phases by the cut-off line (*vertical dashed lines*, including logarithmic phase and stationary phase. **a** The concentrations of cell dry weight (*CDW*) (*closed circle*), NH$_4$Cl (*opened circle*), residual cell dry weight (*RCDW*) (*closed triangle*), and PHB (*opened triangle*); **b** PHB content (%, *w/w*) (*closed square*) and the concentrations of glucose (*opened square*). TAD1 was inoculated in a 300-ml flask containing 100 ml mineral salts medium with 20 g l^{-1} glucose. Cultures were incubated at 45 °C and 180 rpm

source for cell growth in the absence of extracellular carbon source (Jendrossek and Handrick 2002).

Influence of the temperature on PHB accumulation

Based on the time curse of growth and PHB accumulation by *C. daeguensis* TAD1 obtained above, we determined that the harvest time for *C. daeguensis* TAD1 cells was 28 h during the following experiments, when the highest PHB amount occurred. The influence of temperature on the growth and PHB accumulation of *C. daeguensis* TAD1 was investigated, and the results were shown in Fig. 3.

The CDW as well as PHB content were initially in positive proportion to the temperature and exhibited the maximum values at 50 °C, which were 4.20 g l^{-1} and 78.3 % (w/w). After that, the CDW and PHB decreased with rising temperature, but still exhibited considerable values even at temperatures up to 60 °C (1.50 g l^{-1} and 45.3 %). Although there was no observation on the growth at 65 °C (data not shown), *C. daeguensis* TAD1 still exhibit stronger tolerance to heat stress compared to other PHA-accumulating bacteria (Ibrahim et al. 2010; Satoh et al. 2011).

Influence of the molar ratio of carbon to nitrogen (C/N) on PHB accumulation

Based on the investigation of the influence of the temperature on PHB accumulation by *C. daeguensis* TAD1, we determined that the optimal culture temperature during the following experiments was set as 50 °C. In order to investigate the influence of nitrogen source on PHB accumulation, as well as to gain optimal C/N (molar) for growth and PHB production, we grew *C. daeguensis* TAD1 in MSM medium in the presence of various C/N (molar). The results were illustrated in Fig. 4.

The CDW was originally in positive proportion to the C/N (molar), and exhibited the maximum values at C/N=30, which was 4.50 g l^{-1}. After that, the CDW (2.96 g l^{-1}) decreased sharply while the C/N was up to 60. However, the PHB accumulation of *C. daeguensis* TAD1 increased sharply with the elevated C/N (molar), and the maximum PHB content (81.3 % of CDW, w/w) was obtained at C/N=60, indicating that the nitrogen limitation could significantly enhance the PHB accumulation. Meanwhile, even though the C/N was as low as 5, *C. daeguensis* TAD1 still exhibited considerable PHB content (55.7 % of CDW, w/w), showing strong tolerance to high concentrations of nitrogen. Consequently, the optimal C/N (molar) for PHB production by *C. daeguensis* TAD1 was 30, and the corresponding PHB amount was 3.44 g l^{-1} (Fig. 4).

Production of PHB from various carbon sources

In order to develop the most suitable carbon source for PHB production by *C. daeguensis* TAD1, we investigated the ability of *C. daeguensis* TAD1 to utilize various carbon sources (sodium salts of fatty acid, carbohydrates, and glycerol) for PHB accumulation in MSM at 50 °C. Table 2 shows the final pH, CDW, PHB content, PHB amount as well as the product yield ($Y_{P/S}$, gram PHB per gram substrate used) after 28 h cultivation of *C. daeguensis* TAD1 on a variety of carbon sources.

With regard to the sodium salts of fatty acid, most of them could support the growth and PHB accumulation of *C. daeguensis* TAD1 except for the sodium propionate as well as sodium caprylate. The sodium succinate exhibited the highest CDW (3.09±0.3 g l^{-1}) and PHB amount (1.98±
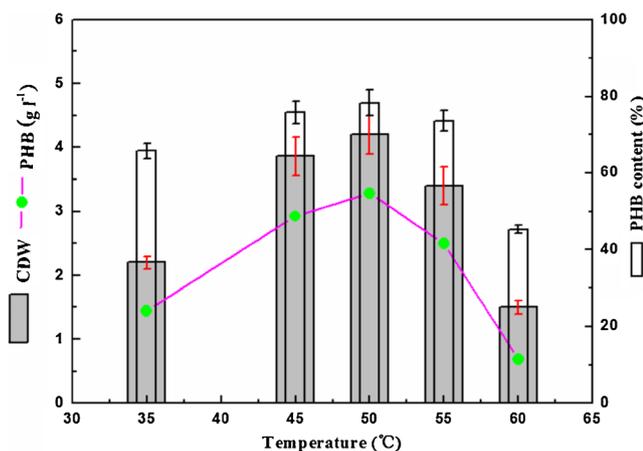

Fig. 3 Effects of incubation temperature on the growth and PHB accumulation of *C. daeguensis* TAD1. Cultures were inoculated in 300-ml flasks containing 100 ml mineral salts medium with 20 g l^{-1} glucose at pH 7.3 and were incubated for 28 h at 180 rpm at different temperatures. Values are means ± SD (*error bars*) for three replicates
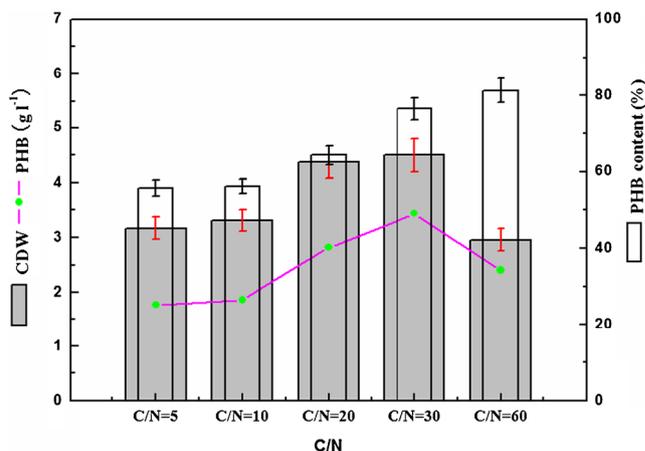

Fig. 4 PHB accumulation of *C. daeguensis* TAD1 under various molar ratios of carbon to nitrogen (C/N) with 20 g l^{-1} glucose as the sole carbon source at 50 °C. Cultures were inoculated in 300-ml flasks containing 100 ml mineral salts medium for 28 h at 180 rpm. Values are means ± SD (*error bars*) for three replicates

Table 2 The growth and PHB accumulation of *C. daeguensis* TAD1 utilizing various carbon sources

Carbon sources[a]	Final pH	CDW (g l^{-1})	PHB content (%)	PHB amount (g l^{-1})	Product yield ($Y_{P/S}$)[b] (g g^{-1})
Sodium acetate	9.26±0.11	1.71±0.1	33.0±1.4	0.57±0.1	0.02±0.00
Sodium propionate	9.13±0.09	2.35±0.2	<1	<0.01	0.00±0.00
Sodium succinate	9.14±0.08	3.09±0.3	64.4±1.8	1.98±0.2	0.11±0.01
Sodium citrate	9.12±0.09	2.11±0.2	22.1±1.5	0.47±0.1	0.03±0.00
Sodium caprylate	7.30±0.05	0	0	0	0.00±0.00
Glucose	5.37±0.05	4.50±0.3	76.5±2.9	3.44±0.3	0.17±0.02
Sucrose	5.06±0.03	2.40±0.2	4.6±0.2	0.11±0.1	0.01±0.00
Starch	6.86±0.07	2.35±0.2	47.9±1.5	1.13±0.1	0.06±0.00
Glycerol	7.67±0.04	2.47±0.2	80.8±2.5	2.00±0.2	0.26±0.03

[a] The bacteria is cultivated at 50 °C for 28 h, with the coincident molar ratio of carbon to nitrogen (C/N=30)

[b] $Y_{P/S}$, gram PHB per gram substrate used

0.2 g l^{-1}) as well as product yield (0.11±0.01 g g^{-1}), about 1.5–1.8, 3.5–4.2, and 3.6–5.5 folds higher than that of sodium acetate and sodium citrate, respectively. Meanwhile, the sodium propionate could support the growth of *C. daeguensis* TAD1 (CDW=2.35±0.2 g l^{-1}) but no PHB accumulation, whereas the sodium caprylate even did not support the cell growth.

For the carbohydrates, the glucose exhibited the highest PHB amount (3.44±0.3 g l^{-1}) and product yield (0.17±0.02 g g^{-1}), which were significantly higher than of the sucrose and starch. Previous studies (Ibrahim and Steinbüchel 2010b; Ibrahim et al. 2010; Sheu et al. 2009) have indicated that fewer bacteria are able to utilize starch as sole carbon source without hydrolysis to produce PHB. However, in this study, although the PHB content of *C. daeguensis* TAD1 (47.9±1.5 %) is comparable to that of *Bacillus cereus* CRF06 previously reported (Halami 2008) (45.5–48 %), the PHB amount (1.13±0.1 g l^{-1}) of *C. daeguensis* TAD1 is approximately 2.5 folds higher than that of *Bacillus cereus* CRF06 (Halami 2008). In addition to the considerable product yield (0.06±0.00 g g^{-1}), it can be inferred that using inexpensive starch as a carbon source for *C. daeguensis* TAD1 could be a feasibility to lower the production cost of PHB.

The PHB amount (2.00±0.2 g l^{-1}) of glycerol by *C. daeguensis* TAD1 is about 58.1 % of that of glucose and comparable to that of *Chelatococcus* sp. reported by Ibrahim et al. (2010). However, the product yield of glycerol (0.26±0.03 g g^{-1}) is 1.5 folds higher than that of glucose, and the PHB content is slightly higher than that of glucose. This is attributed to the fact that almost all the glucose added in the medium used up at 28 h (Fig. 2), meanwhile, more than half glycerol still existed in the medium. These results are also in well accordance with those of other PHB-accumulating microorganisms (Ibrahim and Steinbüchel 2010a, b; Ibrahim et al. 2010), indicating that glycerol would be more suitable for PHB accumulation rather than the growth of *C. daeguensis*

TAD1 in contrast to that of glucose. Nowadays, glycerol could be tremendously produced by the biodiesel industry as a by-product (Solaiman et al. 2006). As a result, glycerol may be a cheap alternative to the conventional fermentation substrates for large-scale PHB production by *C. daeguensis* TAD1.

Discussion

The study of PHA accumulation in thermophilic bacteria is an interesting approach aiming at exploring more and more of such microorganisms for new PHA synthesis and degradation enzymes, and above all reducing the production cost of PHA (Steinbüchel and Hein 2001). Although several kinds of thermophilic PHA-accumulating microorganisms have been found in hot springs (Pantazaki et al. 2003; Sheu et al. 2009) or aerobic organic waste treatment plants (Ibrahim et al. 2010), the amount of them is still extremely lower than that of PHA-accumulating mesophiles (Budde et al. 2011; Chen 2010; Chen et al. 2001; Delamarre and Batt 2006). In this study, we succeeded in isolating a thermophilic PHA-accumulating bacteria (strain TAD1) from a biotrickling filter used for the removal of nitrogen oxides from a coal-fired power plant (Jiang et al. 2009; Liang et al. 2012; Yang et al. 2012). As a result, this biotrickling filter would be a new appropriate source for PHA-accumulating thermophiles, and further studies need to be conducted on screening more thermophilic PHA-accumulating microorganisms from this filter.

Based on the characteristics and 16S rRNA sequences of strain TAD1 (Table 1), the strain TAD1 isolated was affiliated as *C. daeguensis* TAD1 (Liang et al. 2012). *C. daeguensis* TAD1 is testified to be capable of utilizing both glucose and glycerol to accumulate PHB (a short-chain-length PHA) via the fluorescence microscopy of the PHB inclusions stained with Nile red (Fig. 1), while no PHB accumulation has been reported by other strains of *C. daeguensis* before. Although *Chelatococcus* sp. MW10 and

Chelatococcus sp. MW11 could also produce PHB at elevated temperatures (Ibrahim et al. 2010), these two isolates may form a separate species rather than belong to *C. daeguensis* due to their differential characteristics in contrast to those of *C. daeguensis* K106[T] as well as strain TAD1. Consequently, to the best of our knowledge, this is the first report on PHB accumulation by thermophilic *C. daeguensis*.

As shown in Fig. 2, the thermophilic *C. daeguensis* TAD1 grows and accumulates PHB rapidly at elevated temperatures and is capable of accumulating PHB up to 83.6 % of CDW (*w/w*) within just 24 h at 45 °C. This fermentation time to obtain the highest PHB content is significantly shorter than that of other high-performance mesophilic PHB-accumulating bacteria, and merely contributes 25 % for that of *Zobellella denitrificans* MW1 (around 96 h) (Ibrahim and Steinbüchel 2010b), 33.3–50 % for that of *R. eutropha* (48–72 h) (Budde et al. 2011; Yang et al. 2010), and 60 % for that of *A. latus* (around 40 h) (Wang and Lee 1997). Yet, the PHB-accumulating content of *C. daeguensis* TAD1 (83.6 %) is significantly identical to that of these high-performance PHB producers, whose PHB content are in the range of 80 to 90 % (Budde et al. 2011; Ibrahim and Steinbüchel 2010b; Wang and Lee 1997; Yang et al. 2010).

Moreover, the PHB accumulation of *C. daeguensis* TAD1 is along with cell growth during logarithmic phase, indicating that *C. daeguensis* TAD1 is a growth-associated PHB-accumulating bacteria without obvious nutrient limitation. Previous studies (Ibrahim and Steinbüchel 2009, 2010a, b) have confirmed that growth-associated PHB-accumulating bacteria would be possible to obtain high cell densities accompanied by high polymer contents in a single-stage fermentation process without nutrient limitation. As a result, *C. daeguensis* TAD1 has good potentials as a suitable candidate for low-cost industrial production of PHB.

After that, further investigation refers to the influence of temperature, molar ratio of carbon to nitrogen (C/N), and carbon source on PHB accumulation to obtain the optimal PHB amount by *C. daeguensis* TAD1. The growth and PHB production of *C. daeguensis* TAD1 exhibited strong tolerance to a broad range of temperatures (35–60 °C), and the suitable temperatures for PHB production were in the range from 45 to 55 °C. In many warm countries, summer temperatures are frequently >40 °C and the temperature of fermentation vessel would be over 40 °C in the absence of a cooling system. Therefore, fermentation performed by this thermophilic microorganism could be an energy-efficient process because few cooling efforts are necessary. In addition to the influence of external temperature increases, fermentation broths could also self-heat up as a result of cell's exothermic metabolic reactions during high cell density (HCD) growth (Ibrahim and Steinbüchel 2010a). Thus, the running cost for maintaining suitable temperatures for microbial growth and PHB production would be cut down to some extent.

The fermentation temperature for PHB production was optimized from 45 to 50 °C with glucose as sole carbon source, where the PHB amount was slightly increased from 2.92 to 3.29 g l^{−1}. The PHB accumulation by *C. daeguensis* TAD1 occurred even if the temperature is up to 60 °C (Fig. 3), indicating that the PHB biosynthesis enzymes of this strain should exhibit stronger thermostability than those in previous reports (Ibrahim et al. 2010; Satoh et al. 2011). In addition, the temperature of 60 °C is also beyond the limit of growth temperature for *C. daeguensis* TAD1 via physiological test (Table 1), which is in the range of 20–52 °C. This improvement to the thermostability of *C. daeguensis* TAD1 is attributed to the fact that PHB accumulation by microorganisms would significantly strengthen their resistance to the high environment stress (Ayub et al. 2004), and that is why *C. daeguensis* TAD1 could thrive in the biotrickling filter investigated with fluctuating temperatures from 40 to 60 °C.

Previous studies (Lenz and Marchessault 2005; Meng et al. 2012) indicated that the nutrient limitation could activate a metabolic pathway, which shunts acetyl units from the tricarboxylic acid (TCA) cycle into the synthesis of PHB during whether or not a growth-associated PHB-accumulating microorganism was used. This can explain why the nitrogen limitation could dramatically enhance the PHB accumulation by the growth- associated PHB-accumulating *C. daeguensis* TAD1, and the maximum PHB content (81.3 % of CDW, *w/w*) was obtained at the lowest nitrogen concentration (C/N= 60, molar) (Fig 4), which is similar with that of other growth-associated PHB-accumulating bacterium (Ibrahim and Steinbüchel 2010b; Yezza et al. 2006). Surprisingly, *C. daeguensis* TAD1 exhibited strong tolerance to high nitrogen loads (molar C/N=5, i.e., 6 g l^{−1}) compared to other thermophilic PHB-accumulating bacterium (Ibrahim et al. 2010; Satoh et al. 2011; Sheu et al. 2009), which further confirm its ability for high cell density production of PHB in a single-stage fermentation process without nutrient limitation.

The cost of carbon source accounts for almost half of the total production cost of large-scale PHB production, so it is critical to utilize cheaper carbon sources for PHB production so as to enhance the economic viability of PHB as a cheap alternative to the petroleum-based plastics (Chen 2010; Koller et al. 2010). In this study, although glucose exhibited the highest PHB amount (3.44±0.3 g l^{−1}) and product yield (0.17±0.02 g g^{−1}), it was not a viable raw material for large-scale PHB production as a result of its rising prices caused by the increased demand for bioethanol production (Lee et al. 2013). Therefore, further investigations were conducted on the PHB production from various carbon sources in order to develop the most suitable carbon source for large-scale PHB production by *C. daeguensis* TAD1. As shown in Table 2, *C. daeguensis* TAD1 was able to utilize the sodium salts of scl fatty acid (C2–C6) for synthesizing PHB except for the

sodium propionate, from which PHV instead of PHB was synthesized. The results indicated that the strain possesses the scl-PHA biosynthesis pathway (Suriyamongkol et al. 2007), which exists in the wide range of bacteria such as *R. eutropha* (Riedel et al. 2012). However, neither PHB accumulation nor cell growth was observed in the cultivation of *C. daeguensis* TAD1 on sodium caprylate, indicating that sodium caprylate can strongly suppress the growth of *C. daeguensis* TAD1. Yet, further work still needs to confirm whether *C. daeguensis* TAD1 possesses the mcl PHA biosynthesis pathway through beta-oxidation metabolic pathway (Guo et al. 2011).

Starch is one of the most abundant and cheap carbon sources in the world. However, fewer bacteria could directly utilize starch for growth without pre-hydrolyzed into glucose or oligosaccharides, which would increase the production cost of PHB (Quillaguaman et al. 2010). In this study, *C. daeguensis* TAD1 is obviously capable of accumulating PHB amounting to 47.9 % (*w/w*) of CDW from starch without pre-hydrolyzed, supposing that this strain possesses intrinsic starch-degrading enzymes that can directly hydrolyze starch into a bacteria-usable carbon source (such as glucose) for PHB production (Sheu et al. 2009). Despite the relatively low product yield (0.06 g g^{-1}) compared to that of glucose (0.17), the starch could still be a cheap carbon source for PHB production by *C. daeguensis* TAD1.

Nowadays, the price of glycerol drops rapidly as a result of significant quantities of glycerol are produced by the biodiesel industry as a by-product (Solaiman et al. 2006). Interestingly, *C. daeguensis* TAD1 is able to effectively utilize glycerol as sole carbon source for PHB production, and exhibits considerable high product yield (0.26 g g^{-1}) as well as PHB content (80.4 % of CDW, *w/w*) compared to those of glucose (Table 2). Consequently, glycerol is the most suitable candidate for large-scale PHB production by *C. daeguensis* TAD1, and further investigations would be dedicated to the PHB production through the batch or fed-batch cultivation of *C. daeguensis* TAD1 with glycerol as the major carbon source.

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