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Biodegradation of Poly(β-hydroxybutyrate) by a novel isolate of *Streptomyces bangladeshensis* 77T-4

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ABSTRACT. A thermophilic *Streptomyces bangladeshensis* isolate designated 77T-4 that can degrade poly(β-hydroxybutyrate) (PHB) was isolated from a soil sample in Taiwan. Isolate 77T-4 grew well in urea fructose oatmeal medium and produced a clear zone around the colony when grown on an agar plate containing emulsified PHB, indicating the presence of an extracellular PHB depolymerases. A PHB-degrading enzyme was purified to homogeneity from the culture supernatant. The molecular weight of the PHB-degrading enzyme was estimated to be approximately 40 kDa. A small portion of the N-terminal sequence (Ala-Val-Pro-Leu-Thr-Arg) of the purified enzyme was determined and found to share a significant homology with that of the PHB depolymerase of *Streptomyces* sp. MG.

Keywords: PHB-degraders; PHB depolymerases; *Streptomyces bangladeshensis*; Thermophilic actinomycetes.

INTRODUCTION

Polyhydroxyalkanotes (PHAs) are insoluble polyesters of hydroxyalkanoic acids. They are synthesized and deposited as inclusion bodies in many microorganisms for carbon and energy reserves. Because PHAs are thermostable, biodegradable, and biocompatible, they can be used as osteosynthetic materials, bone plates, or surgical sutures (Lenz and Marchessault, 2005). Among the various PHA polymers, poly-(β)-hydroxybutyrate (PHB), a homopolymer of β-hydroxybutyrate, is the most well characterized polymer. Other aliphatic polyesters such as poly(propiolactone) (PPL), poly(ε-caprolactone) (PCL), poly(L-lactide) (PLA), poly(ethylene succinate) (PES), poly(butylene succinate) (PBS), and poly(ester carbonate) (PEC) also have properties of conventional plastics and are being developed for use as biodegradable substitutes for non-degradable plastics (Tokiwa and Calabia, 2004).

Degradation of PHAs can take place in natural environments such as soil, water, and compost by microorganisms (Jendrossek et al., 1996). Many kinds of PHA-degrading bacteria have been isolated. Most of them belong to genera *Bacillus, Pseudomonas, Comamonas, Alcaligenes*, and *Streptomyces* (Tokiwa et al., 2009), while PHA-degrading fungi are distributed among at least 95 genera (Jendrossek et al., 1996). Most PHA-degrading microorganisms, especially PHB degraders, are present in mesophilic environments. Very few of them can degrade PHB at a temperature higher than 45°C. Since composting at high temperature is more effective in recycling biodegradable plastics (Tokiwa et al., 2009), identification of thermophilic PHB-degraders is essential.

We have previously isolated 341 polyester-degrading thermophilic actinomycetes from different environments in Taiwan (Tseng et al., 2007). Some of them degrade either PHB, PCL, or PES; others can degrade two or all three of these polyesters. *Streptomyces bangladeshensis* 77T-4, isolated from soil in this study, has the highest growth and PHB hydrolysis rates. To be able to use it in an industrial scale, we characterized the organism and purified its PHB-degrading enzyme.

MATERIALS AND METHODS

Screening and identification of PHB-degrading thermophilic actinomycetes

To isolate thermophilic actinomycetes, soil samples were plated on modified HV agar plates (humic acid, 1.0 g; KCl, 1.7 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; Na_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; cycloheximide, 50 mg; 0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxine-HCl, inositol, Ca-pantothenate, p-aminobenzoic acid, and 0.25 mg of biotin; agar, 18.0 g; distilled water 1 L, pH 7.2) and incubated at 45-50°C for 7-10 days as described previously (Tseng et al., 2007). The powdery colonies with branched hyphae were picked, streaked on oatmeal agar plates (oatmeal, 20 g; 1 mg each of $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot H_2O$ and $ZnSO_4 \cdot 7H_2O$; agar, 18 g; in

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1L of distilled water, pH 7.2), and incubated at 45°C for 3-7 days for identification.

The PHB-degradation ability of the isolates was visualized by the presence of a clear zone around the colonies on an emulsified PHB agar plate (Tseng et al., 2007). The plate was made by dissolving one gram of PHB powder in 50 ml of methylene chloride and then emulsifying the mixture in 50 ml of basal medium which contained (per liter) the following: yeast extract, 0.1g; $FeSO_4 \cdot 7H_2O$, 10 mg; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1 g; $CaCl_2 \cdot 2H_2O$, 20 mg; NaCl, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg; $NaWO_4 \cdot 2H_2O$, 0.5 mg; $MnSO_4 \cdot H_2O$, 0.6 mg; and dishwashing liquid, 1 mL (Poas, Nice Co., Taiwan). Methylene chloride was then evaporated in a fume hood.

Isolate 77T-4 was identified based on its microscopic characteristics, cell wall compositions (*meso*- and LL-diaminopimelic acid isomer, A_2pm), and nucleotide sequences of the 16S rRNA gene. Morphological examinations of 77T-4 were performed with a scanning electron microscope (S-4700, Hitachi, Tokyo) as described previously (Itoh, 1989). Cultural characteristics were assessed by using 7-day cultures grown at 45°C on yeast extract-malt extract agar (ISP 2 medium), oatmeal agar (ISP 3 medium), inorganic salts-starch agar (ISP 4 medium), and glycerol-asparagine agar (ISP 5 medium) that were developed by the International *Streptomyces* Project (ISP) (Shirling, 1966). Color designations of substrate mycelium and aerial mass were performed according to the ISCC-NBS Color Name Charts. Organisms for chemotaxonomic and molecular studies were grown in yeast-glucose broth (yeast extract 1.0%, glucose 1.0%, w/v) for 7 days at 45°C. Isomers of diaminopimelic acid (A_2pm) and sugars in whole-cell lysates were determined by the method of Hasegawa et al. (1983).

Total DNA was extracted from 7-day cultured cells using the QIAGEN® Genomic DNA Kit. The 16S rRNA gene was PCR-amplified according to the method of Nakajima et al. (1999), and the amplified product was sequenced directly on an ABI automatic DNA sequencer (Model 3730) using the BigDye Terminator V3.1 kit (Applied Biosystems). The resulting 16S rDNA sequence was identified by comparing it with all the sequences in the EzTaxon server (Chun et al., 2007).

Growth conditions for *S. bangladeshensis* 77T-4

To investigate growth requirements, 77T-4 cells were grown in Nutrient Broth (NB), Luria Bertani (LB), 1% Oatmeal, and UF medium which contained the following: 15 g of fructose, 10 g of peptone, 10 g of yeast extract, 2 g of urea, 20 mg of $CaCl_2 \cdot 2H_2O$, 200 mg of $MgSO_4 \cdot 7H_2O$ in one liter of distilled water. Determination of the optimal growth temperatures of 77T-4 was performed by growing it in the UF medium. For PHB-degrading enzyme production, 77T-4 cells were grown at 45°C in UF medium for 14 h to stationary phase and then in basal medium supplemented with 0.1% (w/v) PHB at 45°C for various lengths of time.

PHB-degrading enzyme assay

To examine the extracellular PHB-degrading enzyme, culture supernatants were assessed for their ability to decrease the turbidity of emulsified PHB. The assay was performed by incubating 3 ml overnight liquid culture of 77T-4 cells with 1 ml of basal medium containing 0.1% PHB at 45°C for 12 h and then examined spectrophotometrically at 650 nm. To further evaluate PHB-degrading activity, weight loss of a PHB film was determined after being incubated with a 77T-4 culture. Five vacuum dried thin PHB films (size: 20×20 mm; initial weight:0.3-0.4 g) were placed in a flask containing 100 ml overnight culture of 77T-4 and incubated at 45°C for 18 h in a rotary shaker. The films were periodically removed, washed with distilled water, dried under a vacuum, and weighed. The surface morphology of the enzymatically degraded PHB films was examined under a scanning electron microscope.

Purification of PHB depolymerase

The extracellular PHB depolymerase was produced by growing 77T-4 cells in a basal medium containing PHB films at 45°C for 12 h with rotary shaking. After removing the cells by centrifugation at 12,000 ×*g* for 20 min at 4°C, the crude enzyme in the supernatant was precipitated by adding solid ammonium sulfate with continuous stirring at 4°C for 1 h and harvested by centrifugation. The precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.2) and then dialyzed against four volumes of the buffer. Protein concentration was determined by the Lowry method using bovine serum albumin as the standard.

The PHB-degrading activity of the isolated enzyme was detected by zymography. After electrophoresis of the proteins on a native polyacrylamide gel, the gel was placed on another polyacrylamide gel containing 0.1% PHB and incubated at 45°C for 30 min. The protein bands with PHB-degrading activity were detected as clear zones. The gel was then stained with Coomassie brilliant blue R-250 to determine the size of the protein with PHB-degrading activity.

RESULTS

Isolation and identification of a PHB-degrading *S. bangladeshensis* 77T-4

To isolate thermophilic actinomycetes, 1 g of soil from various locations in Taiwan was added to 9 mL of sterilized distilled water. The sample was 10-fold serially diluted, and 0.1 mL of each dilution was spread onto a modified HV agar plate. After 7-10 days of incubation at 45-50°C, powdery irregular colonies with branched hyphae were observed in most of the cultures. These bacterial colonies were picked, streaked on oatmeal agar, and assessed for PHB degradation. Thirty-three isolates were found to form a clear zone around each of the colonies on PHB-containing basal medium plates. Six of these colonies also formed clear zones on PCL- or PES-containing agar plates. The isolate designated 77T-4 had the highest PHB-degrading

activity among all the isolates; it could also degrade PCL.

Isolate 77T-4 produced fluffy whitish round-shaped colonies on oatmeal agar plates. Peptidoglycan analyses showed that LL-DAP is the major component of the cell wall. Glucose and xylose were also found, suggesting that 77T-4 has a chemotype IC cell wall. 77T-4 cells grew well and produced spores on ISP media 2, 3, and 4, but not on ISP medium 5. The aerial mass was yellowish white to light gray, and the substrate mycelia were pale yellow to yellowish white. Mature spore chains with smooth surface were long, containing 7-50 spores per chain. No soluble pigment was detected. Based on these observations, isolate 77T-4 was assigned to the *Streptomyces* genus (Lechevalier and Lechevalier, 1970). With the Eztaxon program, the 16S rRNA sequences of 77T-4 were aligned with those of the members of the genus *Streptomyces* and found to be 99.9% homologous to that of *S. bangladeshensis*. Therefore, isolate 77T-4 was identified as *S. bangladeshensis*.

Growth and PHB degrading activities of *S. bangladeshensis* 77T-4

To determine the growth requirements, 77T-4 was grown in various culture media. Significant growth ($O.D_{600}$=1.8) was observed 12 h after incubation in all media, except the oatmeal medium. The growth in the UF medium reached the peak ($O.D_{600}$=2.0 and $O.D_{600}$=1.8, respectively) after 12 h of incubation at 45 and 50°C (Figure 1A). A slower growth rate was seen when the incubation temperature was changed to 37 or 28°C, in which the cell density reached approximately $O.D_{600}$=2.0 and $O.D_{600}$=1.7 after 14 h and 24 h of incubation, respectively. 77T-4 cells did not grow well at 55°C. These results suggest that the optimal growth temperature for 77T-4 is 45°C.

To investigate the PHB degradation activity of 77T-4, an aliquot of the culture was plated on an agar plate with basal medium containing emulsified PHB. After incubating the plates at various temperatures, the diameters of the clear zones were measured at different time points. A clear zone of 1.2 mm in diameter was observed after incubating at 45°C for 1 day. The diameter of the clear zone was increased to 4.2 mm after 2 days of incubation, reached 5.5 mm after 4 days, and stayed at this level for the remaining period of the study up to 6 days. When the incubation temperature was raised to 50°C, a smaller clear zone of approximately 0.5 mm in diameter was observed after 1 day of culture and then gradually increased to 5 mm over time during the 6-day period of incubation. At 37°C, a clear zone of 0.7 mm was observed at the 2-day time point and peaked at 4 mm after 6 days of culture. Very tiny clear zones were produced at 28°C, and no clear zones were seen at 55°C. These results (Figure 1B) indicated that 77T-4 grew well on basal medium containing PHB and produced PHB-degrading enzyme optimally at 45°C.

Induction in the production of the PHB depolymerase by PHB was then investigated. 77T-4 cells were grown in UF medium for 12 h at 45°C and then switched to basal

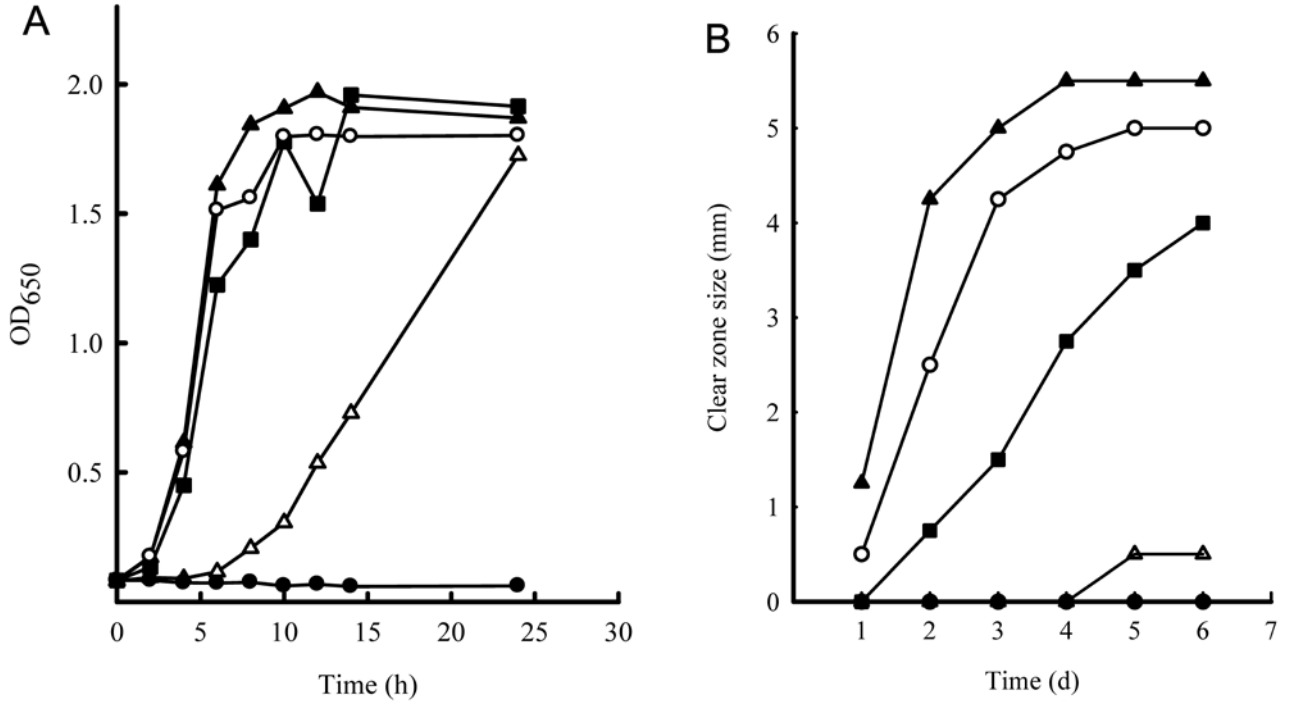

Figure 1. Effect of temperature on the growth and PHB degradation of *S. bangladeshensis* strain 77T-4. (A) Growth curve of 77T-4 cultured in UF medium at 28°C (—△—), 37°C (—▲—), 45°C (—■—), 50°C (—○—), or 55°C (—●—); (B) Time course of PHB degradation as indicated by diameters (mm) of the clear zones on agar plates containing PHB at 28°C (—△—), 37°C (—■—), 45°C (—▲—), 50°C (—○—), or 55°C (—●—).

medium containing different concentrations of PHB. The PHB hydrolytic activity of the culture supernatant was assayed every 2 h for 12 h. Figure 2 shows a time-dependent decrease in the turbidity of PHB at 45°C. The hydrolysis of PHB by 77T-4 was minimal when it was grown in UF or basal medium, but was greatly increased when it was grown in basal medium containing 0.025% PHB. When the PHB concentration was increased to 0.05 or 0.1%, a significant decrease in PHB degradation was observed. This result suggests that the optimal PHB concentration for induction of the PHB depolymerase is 0.025%.

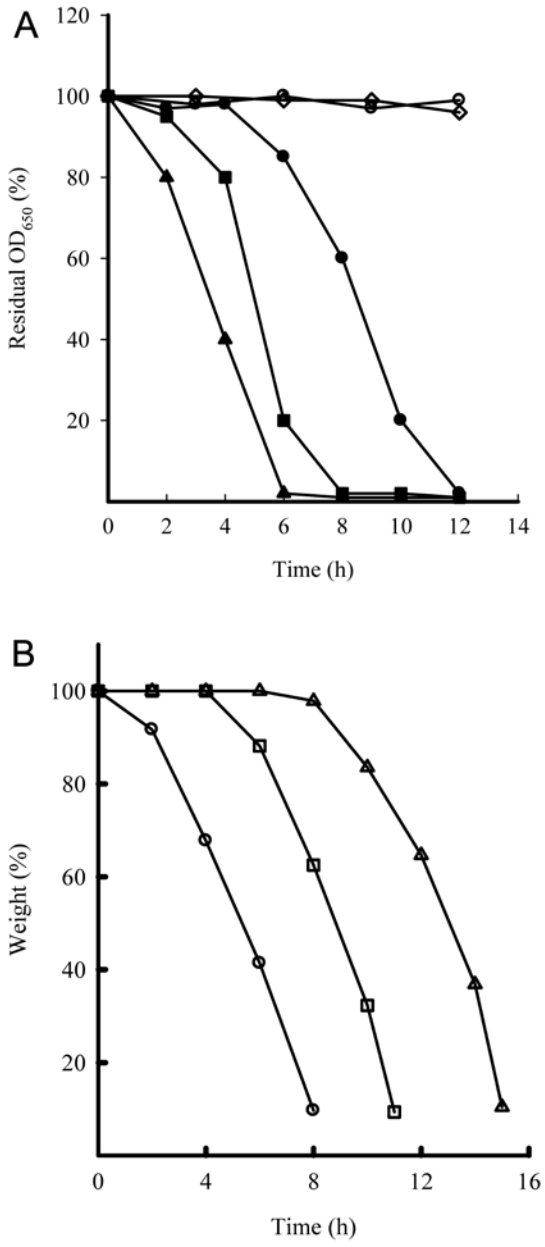

Figure 2. Degradation of PHB by *S. bangladeshensis* strain 77T-4. (A) 77T-4 cells were cultured at 45°C in basal medium containing 0.025% (—▲—), 0.05% (—■—), or 0.1% (—●—) emulsified PHB as the sole carbon source. The PHB depolymerase activity in the culture supernatant was assayed every 2 h for 12 h; (B) PHB films were incubated with 77T-4 culture in basal medium (—△—) without PHB, with 0.025% PHB powder (—□—), or with 0.025% emulsified PHB (—○—). Degradation of the PHB films was assessed by mass loss every 2 h for 16 h. Data are means of five independent experiments.

Degradation of PHB films by *S. bangladeshensis* 77T-4

Isolate 77T-4 was then assayed for degradation of PHB films at 45°C in basal medium. The PHB films were produced by dissolving PHB powder in chloroform, followed by casting it in a mold, and finally evaporating the solvent. The surface morphology of the PHB films was examined by scanning electron microscopy. The films were porous with pore sizes ranging from 3 to 5 μm (Figure 3A). After incubation with 77T-4 cultures at 45°C, the surface porosity of the films was increased, and the pores became larger and deeper, indicating substantial degradation of the PHB films (Figure 3B).

Figure 4 shows that a significant weight loss of the PHB films was observed after 8 h incubation with 77T-4

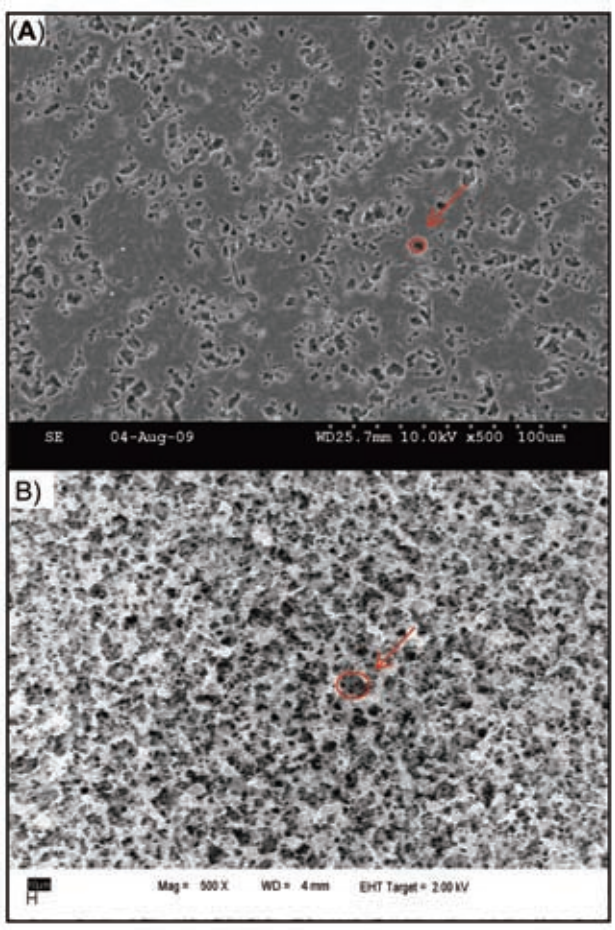

Figure 3. SEM pictures of solvent-casted PHB films. (A) The surface morphology of the PHB film before incubation with *S. bangladeshensis* 77T-4 cultures at 45°C with pore sizes ranging from 3 to 10 μm; (B) The surface morphology of the PHB film after incubation with *S. bangladeshensis* 77T-4 cultures at 45°C for 14 h with pore sizes increased to 30 to 40 μm.

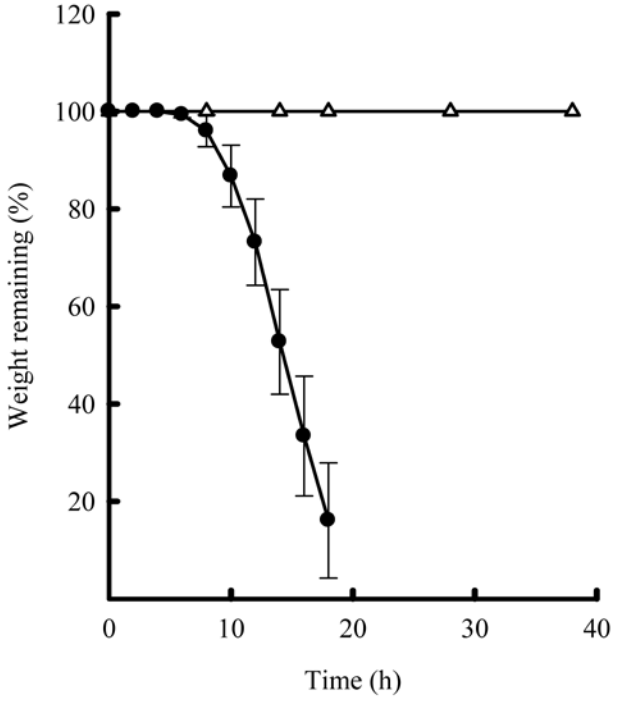

Figure 4. Degradation of PHB films by *S. bangladeshensis* strain 77T-4. PHB films were incubated with *S. bangladeshensis* 77T-4 culture (—●—) in basal medium at 45°C. The films were assessed for mass loss every 2 h for 20 h. PHB films incubated with basal medium without *S. bangladeshensis* 77T-4 (—△—) at 45°C were examined in an identical manner to serve as controls.

culture at 45°C, and a 50% weight loss of the film was observed after 14 h of incubation. At the 18 h time point, a 95% loss in weight was observed. These results indicated that the enzyme secreted by 77T-4 was highly active in degrading PHB.

Purification of the PHB depolymerase

The PHB degradation enzyme of 77T-4 was then isolated. 77T-4 cells were grown under optimal conditions. The proteins in the cell-free culture supernatant were precipitated with increasing amounts of ammonium sulfate. Maximum protein precipitation was achieved at 60% ammonium sulfate. Multiple protein bands were found (Figure 5A) when the precipitated proteins were analyzed by a 10% native PAGE. After performing a zymography by overlaying the native gel on a 10% polyacrylamide gel containing PHB, a single band of clear zone due to PHB hydrolysis was seen (Figure 5B). The protein with the active PHB depolymerase activity was eluted from the native gel and electrophoresed on another 10% SDS polyacrylamide gel. A single protein band with a molecular mass of approximately 40 kDa was observed (Figure 5C, lane 2). To identify the protein, the N-terminal amino acid sequence of this purified PHB depolymerase was determined and found to be Ala-Val-Thr-Leu-Thr-Arg. A similarity search of the DDJB nucleic acid sequence database indicated that this sequence is similar to that of the PHB depolymerase of *Streptomyces* strain MG (Ala-Val-Pro-Leu-Thr-Arg-Val-Thr-Gly-Phe).

DISCUSSION

PHB, a promising compound for making biodegradable plastics, has been investigated for its degradation in many terrestrial and aquatic environments (Jendrossek and Handrick, 2002). Many different PHB-degrading bacteria and fungi including members of *Bacillus*, *Streptomyces, Aspergillus, Penicillium*, *Acidovoraxs*, and *Variovorax* have been isolated from soil (Mergaert et al., 1993). Because of their ability to degrade extracellular PHB, these microorganisms have the potential to become useful for industrial applications. In this study, we isolated a PHB-degrading bacterium designated 77T-4 from soil and identified it as *Streptomyces bangladeshensis*. A strain designated AAB-4 found in a soil sample from Natore, Bangladesh was the first *Streptomyces bangladeshensis* isolated (Al-Bari et al., 2005). Strain AAB-4 was found to produce bis-(2-ethylhexyl) pathalate which has both antibacterial and antifungal activities (Al-Bari et al., 2005). Our isolate 77T-4 exhibits a high level of 16S rRNA gene sequence similarity to strain AAB-4. However, it is unknown whether AAB-4 is the same as 77T-4 and whether it can also degrade PHB.

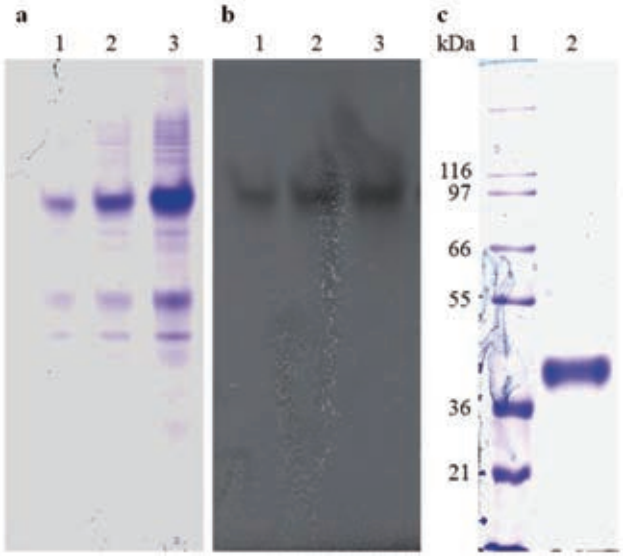

Figure 5. PAGE analysis of ammonium sulfate fractionated protein of *S. bangladeshensis* 77T-4. Electrophoresis was carried out on a 10% native polyacrylamide gel which was then stained with Coomassie brilliant blue R-250 (a). The PHB depolymerase activity was detected by zymography by overlaying the native gel on a 10% polyacrylamide gel containing PHB (b). Lane 1, 5 μg; lane 2, 15 μg; lane 3, 30 μg of purified proteins. The protein in the band with PHB depolymerase activity was isolated and electrophoresed in a 10% SDS-polyacrylamide gel which was then stained with Coomassie brilliant blue R-250 (c). Lane 1, molecular mass marker; lane 2, 20 μg of purified protein.

Isolate 77T-4 formed clear zones on PHB-containing agar plates and degraded PHB films at 45-50°C within a day (Figure 1B), indicating that 77T-4 is a fast-growing thermophile with potent PHB degradation ability. Various *Streptomyces* strains have been used to produce a wide variety of antibiotics and other secondary metabolites (Manteca et al., 2008). Interestingly, the antibiotic production activity of *Streptomyces* is correlated with its ability to sporulate (Kendrick and Ensign, 1983). Therefore, enormous efforts have been devoted to develop methods to induce rapid sporulation in submerged *Streptomyces* cultures (Manteca et al., 2008; Novella et al., 1992). It is unknown whether sporulation also regulates PHB degradation in 77T-4. The expression of PHB-degrading enzyme in 77T-4 cells occurred in the presence of PHB (Figure 5), indicating that the synthesis of its PHB depolymerase is inducible. This induction was maximal when 0.025% of PHB was present in the culture medium (Figure 2). However, the synthesis of the PHB-degrading enzyme was diminished when higher concentrations of PHB were present (Figure 2), suggesting that the production of the enzyme is tightly regulated.

The purified PHB depolymerase from 77T-4 cells has a molecular mass of approximately 40 kDa (Figure 5) which is in the size range of all bacterial and fungal PHB depolymerases. However, the N-terminal amino acid sequence (AVTLTR) of the 77T-4 PHB depolymerase is different from those of the known PHB depolymerases except that of *Streptomyces* strain MG (Calabia and Tokiwa, 2006). PHB depolymerase from the thermophilic *Streptomyces* sp. MG was reported to be a novel type of PHB depolymerase with respect to substrate specificity, thermostability, amino acid sequence, and yields of the end products (Calabia and Tokiwa, 2006).

The PHB depolymerase with high activity and stability at high temperatures would be useful in waste disposal (Lenz and Marchessault, 2005; Mergaert et al., 1993). The 77T-4 PHB depolymerase exhibited the highest activity at 45°C and was stable at 50°C (Figure 1A), indicating that the enzyme is thermally stable similar to other PHB depolymerase (Mergaert et al., 1993; Calabia and Tokiwa, 2004; Calabia and Tokiwa, 2006; Tseng et al., 2007). Several thermal stable PHB depolymerases have been characterized (Tokiwa and Calabia, 2004). However, little information is available on the PHB depolymerase from *Streptomyces* (Klingbeil et al., 1996; Kim et al., 2003). Further studies on the 77T-4 PHB depolymerase gene will provide novel knowledge of the *Streptomyces* PHB depolymerase.

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放線菌 *Streptomyces bangladeshensis* 77T-4 分解聚烴基丁酯

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Streptomyces bangladeshensis 77T-4 是自台灣苗栗通霄海岸土壤所分離之能分解聚烴基丁酯（poly-β-hydroxybutyrate 簡稱 PHB）的放線菌，此菌於 Nutrient Broth (NB) 及 Urea Fructose (UF) 培養基中，於 37-50°C 溫度下都能生長，但最適之生長溫度為 45°C，屬於中高溫菌。當培養 14 小時菌數可達最高峰，分解 PHB 的能力也最強，於菌落之四周會形成明顯的透明環；去除菌體之上清液只需 6 小時就可將 PHB 乳化液完全分解，顯示 77T-4 的快速分解 PHB 是胞外分解酶作用的結果。以 PHB 材料製成的 PHB 薄膜與 77T-4 菌液一同培養，只需 16-18 小時就完全被分解，於掃描式電子顯微鏡下可觀察到許多凹陷孔洞的形成，表示 77T-4 菌液確能分解由 PHB 所製得的成品。將菌液利用硫酸銨沉澱分離出分子量約為 40 kDa 的蛋白，具有明顯的 PHB 分解能力，為一種 PHB 分解酶。將此蛋白以質譜儀進行身份鑑定，並將其 N 端序列 (Ala-Val-Pro-Leu-Thr-Arg) 訂出，發現與一般之 PHB 分解酶不同，但與 *Streptomyces* sp MG 之 PHB 分解酶較為類似，推測 77T-4 所產生的為一不同的 PHB 分解酶。

關鍵詞：聚烴基丁酯 PHB；PHB- 分解酶；高溫放線菌 *Streptomyces bangladeshensis*。