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ISOLATION, PURIFICATION AND SOME PROPERTIES OF AN EXTRACELLULAR PHBV DEPOLYMERASE FROM *STREPTOMYCES* SP. SSM 5670

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Abstract–*Streptomyces* sp. SSM 5670 with the ability to degrade a natural polymers, polyhydroxyalkanoates (PHAs), showed a high activity of the extracellular poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) depolymerase when it was grown in a basal medium containing PHBV as the sole carbon source. The extracellular PHBV depolymerase of the organism could be purified to electrophoretic homogeneity by ion exchange column chromatography and gel filtration approximately 10.5-fold. The recovery of its activity was 20.5%. The molecular mass of the PHBV depolymerase was estimated to be 24 kDa. The maximum activity was observed near pH 7.5 and 30°C, the activity was lost at temperatures above 45°C. This enzyme might be used to promote the degradation of PHBV products buried in soil, stabilized compost or other environments around room temperature.

INTRODUCTION

Under unbalanced growth conditions a variety of bacterial species are known to produce and accumulate polyhydroxyalkanoates (PHAs) as intracellular carbon and energy storage compounds (Sudesh *et al.*, 2000). Two of such polymers, poly(3-hydroxybutyrate) (PHB) as well as the copolymer 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), are of considerable interest because of a broad range of interesting properties, including biodegradability and biocompatibility, which most of the synthetic polymers do not have (Khanna and Srivastava, 2005). The copolymer PHBV exhibits better mechanical strength than PHB; this mechanical feature varies widely, depending on the monomer proportions, hence they have potential use for different applications as reported in a recent review (Khanna and Srivastava, 2005; Zinn *et al.*, 2001; Hazer and Steinbüchel, 2007).

The microbial degradation rate of the PHA appears to be two-fold dependent: on the microbial population and on the degradation ability of the PHA-degrading microorganisms. The later is widely distributed among diverse groups of microorganisms, including bacteria, actinomycetes, and fungi (Mergaert *et al.*, 1992). The first may be estimated from the population of aerobic and anaerobic PHA-degrading microorganisms isolated from various ecosystems such as soil, compost, aerobic and anaerobic sewage sludge, fresh and marine water, estuarine sediment or air (Jendrossek and Handrick, 2002). Particularly, the genus *Streptomyces* turned out to be a significant component of the microbial population in most soils. In fact, Mergaert *et al.* (1993) identified 68 strains of streptomycetes during degradation studies of PHB and PHBV in soil samples.

Degradation of extracellular PHAs by *Streptomyces* sp. and the use of its by-products as a source of carbon and energy is controlled by the secretion of specific extracellular PHAs depolymerase. Until now, more than 80 extracellular PHA depolymerases have been purified and characterized from various microorganisms but most of the isolated enzymes are specific to short-chain-length PHAs and mainly for PHB (Tokiwa and Calabia, 2004; Papaneophytou *et al.*, 2011). Most of the studies have focused on Gram-negative bacteria, however, fewer reports have addressed the isolation and characterization of PHA depolymerases from Gram-positive bacteria,

especially *Streptomyces* sp., despite their abundance in the soil (Klingbeil *et. al.*, 1996; Kim *et. al.*, 2003; Calabia and Tokiwa, 2006; Allen *et al.*, 2011).

Therefore, the goal of the present study was to isolate, purify and analyze the PHBV depolymerase of *Streptomyces* sp. SSM 5670, a mesophilic Gram-positive bacteria which degrades both PHB and PHBV, and to find out the optimum conditions for the enzymatic activities.

MATERIALS AND METHODS

Bacterial PHBV with 12% of hydroxyvalerate, was purchased from PHB Industrial SA, Brazil. PHBV was used as received: white granules with a weight average molecular weight 165 kg/mol. A mesophilic *Streptomyces* sp. SSM 5670, used throughout this study, was obtained from Culture collection SSM, AGRAL FAUBA, Argentina. This microorganism had been isolated from a soil sample as a PHBV degrader and maintained at 30±1°C on Actinomyces agar slants (Hermida *et al.*, 2009).

For the PHBV depolymerase production, *Streptomyces* sp. SSM 5670 was grown in 200 ml basal medium containing (g/l) KNO_3, 3.6; K_2HPO_4, 0.5; $Na_2HPO_4 \cdot 7H_2O$, 0.9; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.02; $CaCO_3$, 0.02; PHBV powder 1.0; and pH 7.0. The medium was inoculated with 5% (v/v) inoculum growing during 24 h at 30°C in Tryptone-Yeast extract broth (Shirling and Gottlieb, 1966). The culture was incubated under aerobic conditions in 500 ml Erlenmeyers flasks on a rotary shaker (125 rpm) at 30±1°C for 110 h. Following incubation, the culture broths were centrifuged (Refregerated Universal Centrifuge Z323K) at 13000 rpm for 10 min. The supernatant was collected and concentrated 40 fold by high pressure ultrafiltration (Stirred Ultrafiltration Cells Modes 8200 Device, Amicon) through a YM 10 regenerated cellulose membrane (Millipore). All the purification procedures were done at 4ºC. The concentrated supernatant was applied to a DEAE-Sephedex A-50 column (1.5 ´ 25 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with the same buffer using a linear gradient of NaCl (0-0.7 M) at a flow rate of 15 ml/h. The fractions with a high depolymerase activity were collected and concentrated by ultrafiltration. The enzyme was subsequently purified by size-exclusion chromatography using a Sephadex G-100 column (1.5 ´ 10 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with the same buffer and active fractions of 1 ml were combined and concentrated by ultrafiltration.

Protein concentrations were determined according to Bradford method using the protein assay reagent (BIO-Red Laboratories) with bovine serum albumin as standard (Bradford, 1996). The PHBV depolymerase activity of the enzyme was routinely assayed by measuring the turbidity decrease of the PHBV suspension at 650 nm with a Beckman DV®530 UV/Vis spectrophotometer. The reaction mixture (final A_{650} was 1.0±0.02) containing 0.1 ml of the PHBV suspension, 0.2 ml of the enzyme, and 50 mM of the Tris-HCl buffer (pH 7.5) in a total volume of 2 ml, was incubated at 30ºC for 30 min. One unit of depolymerase activity was defined as the amount of enzyme capable of decreasing A_{650} by 1 absorbance unit per min. The relative activity was expressed as a percentage of the maximum of the enzymatic activity under the assay conditions (Calabia and Tokiwa, 2006). The relative molecular mass (M_r) of the denatured enzyme was determined using a Mini-ProteanIII® system. A standard protocol was used for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel.

RESULTS AND DISCUSSION

As depicted in Figure 1, *Streptomyces* sp. SSM 5670 showed a high activity of the extracellular PHBV depolymerase when it was grown in a basal medium containing PHBV as the sole carbon source. The enzyme activity, that increased as the culture growth does, reached a maximum at the end of the exponential growth period (80 h).

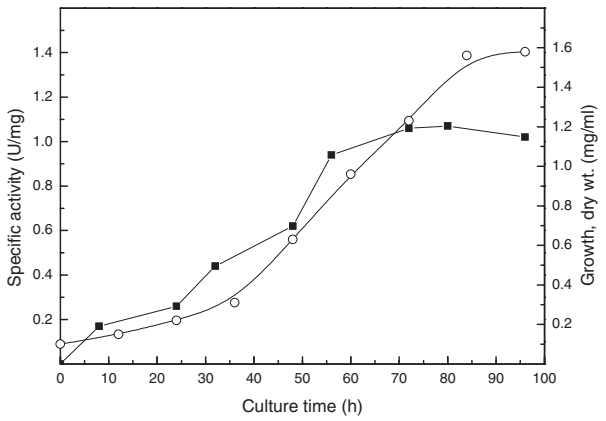

Fig. 1. Time course of PHBV depolymerase activity detection in the supernatant(%) and culture growth of *Streptomyces* sp. SSM 5670 (%).

The purification of the extracellular PHBV depolymerase from the culture broth was carried out by the ion exchange and gel filtration column chromatography. Generally, the PHAs depolymerases do not bind to anion exchangers such as DEAE but have strong affinity to hydrophobic materials. In contrast, PHBV depolymerase from *Streptomyces* sp. SSM 5670 was completely bound to a DEAE Sephadex A-50 resin; this feature confirmed previous studies by Kim *et al.* (2003) for the PHA-depolymerase of *Streptomyces* sp. KJ-72. A quantitative evaluation of the results observed for the consecutive purification steps is shown in Table 1. The enzyme was purified approximately 10.5-fold and the recovery of its activity was 20.5%. The homogeneity of the final enzyme preparation was ascertained using SDS-PAGE analysis, in which the purified depolymerase gave a single protein band as shown in Figure 2. The molecular weight of the purified enzyme was found to be approximately 24 kDa by SDS-PAGE (Fig. 2).

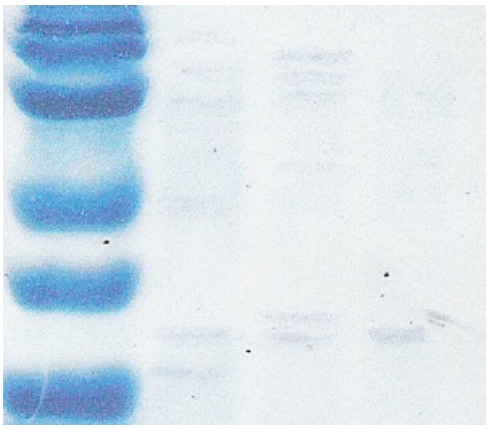

Fig. 2. SDS-PAGE of the purified PHBV depolymerase from *Streptomyces* sp. SSM 5670. Proteins were separated on a SDS-12% polyacrylamide gel and stained witch Coomassie Brillant Blue R-250. Lanes: 1, molecular weight marker (b-galactosidase, 116 kDa; phosphorylase b, 97 kDa; serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; lysozyme, 14 kDa); 2, concentrated culture supernatant; 3, concentrated DEAE Sephadex A-50 pool; 4, the purified PHBV depolymerase (arrow indicates location of protein).

Table 2 summarizes the sensitivity of the enzyme to various protein inhibitors. Addition of Mg^{2+} and Ca^{2+} had a negligible influence on the enzyme activity, whereas Mn^{2+}, Fe^{2+}, Ag^{+}, Zn^{2+} and Hg^{2+} caused inhibition. The relative inhibition depends on the metal ion. Nonionic detergents such as Tween 80, widely used as plasticizer in the manufacture of PHBV products, also reduced the enzymatic activity to 24%. His feature indicates that a hydrophobic region may be located near or at the active site of the enzyme. No inhibition of the enzymatic activity by the effect of sulfhydryl reagents such as sodium azide and ethylenediaminetetraacetic acid (EDTA) was observed.

Table 2. Effects of the metal ions and chemical reagents on the purified PHBV-depolymerase activity

Compounds (1mM)	Relative activity (%)
None	100
$CaCl_2$	102
$HgCl_2$	48
$FeCl_2$	16
$ZnCl_2$	18
$MgCl_2$	99
$MgSO_4$	98
$MnSO_4$	25
$AgNO_3$	77
EDTA	100
NaN_3	98
Acetic anhydride	83
Tween 80	24

The purified enzyme was optimally active at pH 7.5 (Fig. 3a). Following pre-incubation at 5°C for 8 h at different pH values, the enzymatic activity was fairly stable over a pH range from 6.5 to 7.5 (Fig. 3b). The optimum temperature of the enzyme was 30°C after 30 min of the enzyme reaction (Fig. 4a). The thermal stability of the enzyme was determined after incubating in 50 mM Tris-HCl buffer (pH 7.5) from 15 to 60 min at different temperatures; then the activity was assayed after 30 min enzyme reaction at 30°C. As shown in Figure 4b, the PHBV depolymerase was highly stable at 30°C. After 60 min incubation at 37°C, the purified enzyme still

Table 1. Purification steps of PHBV depolymease from *Streptomyces* sp. SSM 5670

Purification step	Total protein (mg)	Total activity (U)	Specific activity(U/mg)	Recovery (%)	Purification fold
Culture supernatant	300	340	1.1	100	1.0
DEAE Sephadex A-50	78	210.6	2.7	61.9	2.5
Sephadex G-100	6	69.6	11.6	20.5	10.5

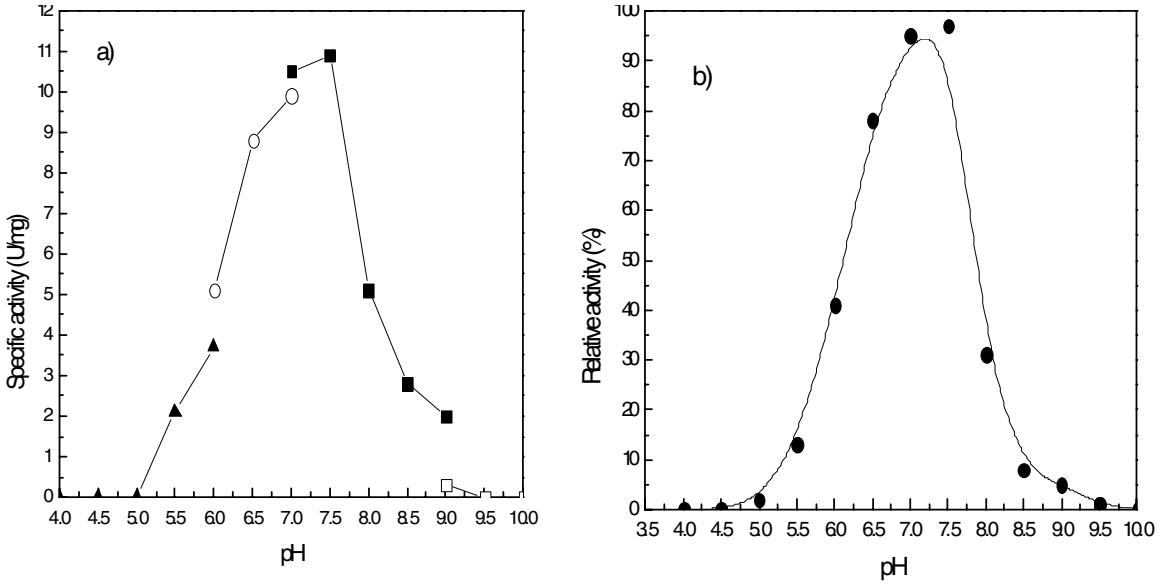

Fig. 3. Effect of pH on the specific (a) and relative (b) activity of the purified PHBV-depolymerase. Various buffers (0.1 M) were used: (%) citrate- phosphate buffer (pH 4.0-6.0); (%) phosphate buffer (pH 6.0-7.0); (%) Tris-HCl buffer (pH 7.0 – 9.0) and (%) 50 mM sodium borate buffer (pH 9.0-pH 10.0).

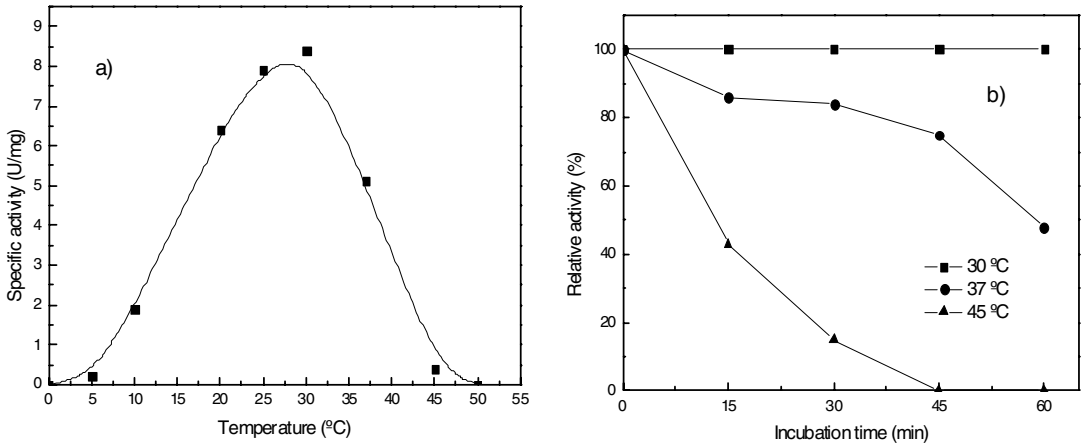

Fig. 4. Effect of the temperature on the specific (a) and relative activity (b) of purified PHBV-depolymerase.

retained about 50% activity and was almost completely inactivated after 45 min at 45°C. Previous work reported that the extracellular depolymerases from *Streptomyces exfoliatus* K10 (Klingbeil *et al.*, 2006) and *Streptomyces* sp. KJ72 (Kim *et al.*, 2003) showed relatively high stability below 40°C and were almost completely inactivated after 1 h at 60°C. Furthermore, the depolimerase isolated and purified from *Streptomyces* sp. MG (Calabia and Tokiwa, 2006) is highly stable at 50°C and loses 100% of the activity after incubation at 70°C for 60 min.

Therefore, the present study showed how the PHBV depolymerase could be purified from *Streptomyces* sp. SSM 5670. This enzyme has some different features from other *Streptomyces*. Particularly, it is thermolabile and maintains its activity over a narrow range of pH. Thus, this enzyme might be used to promote the degradation of PHBV products buried in soil, stabilized compost or other environments around room temperature.

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