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Purification, Characterization and Kinetic Studies of a Novel Poly(β) Hydroxybutyrate (PHB) Depolymerase $PhaZ_{Pen}$ from *Penicillium citrinum* S2

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Abstract A fungal isolate, identified as *Penicillium citrinum* S2, produced ≈1 U/mL of PHB depolymerase by 72 h when grown in BHM containing 0.2%, *w/v* PHB, pH 6.0 at 30 °C. Partial purification of an extracellular poly(-β-)hydroxybutyrate (PHB) depolymerase $PhaZ_{Pen}$ from *P. citrinum* S2 by two steps using ammonium sulphate (80% saturation) and affinity chromatography using concanavalin A yielded 16.18-fold purity and 21.53% recovery of protein. The enzyme was composed of three polypeptide chains of 66, 43 and 20 kDa, respectively, as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. All the three bands stained positive for glycoprotein by PAS staining. Optimum enzyme activity was detected at pH 6.0 and 50 °C. The enzyme was stable between pH 4.0 and 7.0 at 50 °C, 2 h. β-hydroxybutyrate monomer was detected as the major end product of PHB hydrolysis. The enzyme also showed distinct behaviour towards different inhibitors tested, which suggests the role of serine, serine residue, carboxyl group, tyrosine and sulfhydryl groups in its active site.

Keywords PHB depolymerase · *P. citrinum* · Purification · Characterization · Kinetic studies

Introduction

Polyhydroxyalkanoates, PHAs, are versatile biopolyesters synthesized by numerous bacterial strains as intracellular storage compounds of carbon and energy. These polyesters can be divided primarily into two classes of short-chain-length (SCL) PHAs and medium-chain-length (MCL) PHAs, according to the carbon-chain-length of constituents: SCL-PHAs consist of (*R*)-hydroxyalkanoates of C3–C5, and MCL-PHAs are comprised of (*R*)-hydroxyalkanoates of C6–C14. Poly(3-hydroxybutyrate) (PHB), a representative SCL-PHA, is a promising material for use as a renewable and biodegradable plastic [1, 2].

It has been reported that PHB-degrading bacteria are distributed widely in the natural environment, and several extracellular PHB depolymerases with differing biochemical

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properties have been isolated from various bacterial origins [3, 4]. However, the ability to degrade SCL-PHA is not restricted to bacteria, and some filamentous fungi also play an important role in the extracellular degradation of SCL-PHAs such as PHB and its copolyesters with 3-hydroxyvalerate [5]. Many reports have been published on the fungal degradation of SCL-PHAs in the environment; however, reports on the properties of the PHB depolymerases from fungi are relatively rare. Only few PHB depolymerases viz. from *Penicillium funiculosum* ($PhaZ_{Pfu}$) [6], *Penicillium pinophilum* ($PhaZ_{Ppi}$) [7], *Penicillium simplicissimum* LAR13 [8], *Aspergillus fumigatus* ($PhaZ_{Afu}$) [9, 10], *Emericellopsis minima* W2 [11] and *Paecilomyces lilacinus* ($PhaZ_{Pli}$) [12] have been purified and partially characterized to date. Therefore, the biochemical properties of fungal PHB depolymerases are not well documented in comparison to those of bacterial PHB depolymerases.

The used PHB is normally buried under the ground and can be decomposed by the extracellular PHB depolymerase secreted from the soil microbes. The present paper describes the purification process and some of the properties of the PHB depolymerase obtained from a fungal isolate, *Penicillium citrinum* S2. The exceptional properties of this enzyme are emphasized and compared with those of other fungal depolymerases.

Materials and Methods

PHB

PHB was obtained as a kind gift from Biomer Inc., Germany. The molecular weight of PHB was 470,000 g/mol. All experiments were performed using PHB powder.

Isolation of a SCL-PHA-Degrading Fungus

A SCL-PHA-degrading filamentous fungus from a wastewater sample was isolated by pure culturing a colony with high depolymerase activity among the fungi grown on a mineral salt agar medium [10] containing PHB as the sole carbon source.

Production of the PHB Depolymerase from the Isolate

The isolate was cultivated in mineral salts agar medium at 30 °C for 3–4 days. Spore suspension was inoculated into mineral PHB media (0.2% PHB, 0.7 g K2HPO4, 0.7 g KH2PO4, 0.7 g MgSO4, 1.0 g NH4Cl, 1.0 g NaNO3, 5 mg NaCl, 2 mg FeSO4, 7 mg ZnSO4 in 1 L of D.W.) and cultivated at 30±2 °C for 3 days. The culture supernatant was filtered using Whatman filter paper no. 1 and used as crude enzyme source.

Protein Estimation

Protein concentrations were measured by Bradford's method [13] using bovine serum albumin as the standard.

Enzyme Assay

The PHB depolymerase assay was carried out by adding the enzyme to 100 mM sodium citrate buffer (pH 6.0) containing 200 μg of PHB at 50 °C for 1 h. The activity was arrested by adding 0.5 ml of 1 N HCl to the 3-ml enzyme substrate system. The decrease in the

turbidity was measured at 600 nm using a colorimeter. One unit of PHB depolymerase is defined as the amount of enzyme required to decrease the A600 nm by 1.0 per hour [14].

Purification of PHB Depolymerase

The extracellular PHB depolymerase of the fungus was purified from the culture filtrate by $(NH_4)_2SO_4$ precipitation (80% saturation), followed by affinity chromatography using concanavalin-A. The fractions collected were assayed for enzyme activity and protein content by the methods mentioned above.

Modified In Situ Gel Activity Staining

The proteins of the crude culture supernatant and the purified PHB depolymerase were resolved in native polyacrylamide gel electrophoresis (PAGE; 5%, *w*/*v*) containing PHB (2.5%, *w*/*v*) as described earlier [14] and subjected to activity staining by incubating the gel in Tris–HCl buffer (100 mM, pH 8.0) to observe for PHB clearance at the position of the enzyme in the gel.

Native PAGE

The molecular mass of native protein was determined on 8% (*v*/*v*) native PAGE using the following native molecular weight standards: catalase (240 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), trypsin soyabean inhibitor (20.1 kDa) and lactoglobulin (18.4 kDa). After electrophoresis, proteins were visualized by staining with Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid.

Molecular Weight Determination

Electrophoresis (10% sodium dodecyl sulphate (SDS)-PAGE) was carried out to measure the molecular weight according to Laemmli (1970). Phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.5 kDa) were used as standard molecular weight markers. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. The protein concentration was measured with the Bio-Rad protein assay [13] using bovine serum albumin as the standard protein.

Glycoprotein Staining

Carbohydrate staining of glycoprotein in SDS-PAGE gel was carried out with fuchsin-sulphate after periodate oxidation according to Zacharius et al. [15].

Identification of End Products of PHB Hydrolysis by Paper Chromatography

One milligramme of PHB was incubated in 1 ml 100 mM sodium citrate buffer (pH 6.0) at 50 °C in the presence of 10 μg purified PHB depolymerase for 4–6 h. After incubation, the reaction was stopped with 0.1 N HCl, reaction mixture acidified to pH 4.0 and the aqueous end product was repeatedly extracted with equal volumes of diethyl ether in presence of 1 N NaCl in a separating funnel. The ether extract was collected and air-dried. The concentrated extract was detected for the presence of aqueous PHB hydrolysis products by

paper chromatography using 2° Butanol:H_2O:28% NH_4OH in the ratio 1,000:200:3 and Bromocresol purple (0.1%, *w*/*v* in distilled water) as developer. The monomer of complete PHB hydrolysis-β hydroxybutyrate (Sigma) was spotted as reference. The paper was observed for yellow spots against purple background.

Effects of Temperature and pH on the Enzyme Activity

To find out the effect of temperature on PHB depolymerase activity, enzyme activity was measured at different temperatures in the range of 25 °C to 60 °C at optimum pH. The ability of the enzyme to withstand heat was measured by incubating the enzyme in standard reaction solution without PHB for 90 min at 25~60 °C. The effect of pH on enzyme activity was determined by using buffers of pH 3.0~4.0 (0.1 M sodium acetate), pH 5.0~6.0 (0.1 M sodium citrate) and pH 7.0~8.0 (0.1 M phosphate). The optimum pH for enzyme activity was determined by measuring the activity in each of the buffers containing PHB and enzyme. To examine the pH stability of the enzyme, buffers containing enzyme without substrate were incubated at 25 °C for 2 h, and then controlled to have the optimum pH in order to measure the residual activity of the enzyme.

Determination of K_m and V_{max}

A PHB stock solution (3 mg/ml) was used to prepare varying PHB (substrate) concentrations from 200 to 2,000 μg/ml, making up the volume to 2 ml with buffer (optimal assay pH). Four such sets of varying concentration of substrate were prepared, one for each range and the blank. One millilitre of enzyme extract was added to each of the tubes of their respective set. The tubes were incubated at 50°C for 1 h. The reaction was stopped by adding 0.5 ml of 1 N HCl, and the absorbance was read at 600 nm against a blank for each tube. Using the data, a double reciprocal plot (Lineweaver–Burke plot) was plotted, and the value of K_m and V_{max} was determined.

Effect of Metal Ions

To study the effect of metal ions on PHB depolymerase activity, 0.08 ml of each of the metal ion solutions (1 mM) was mixed with the enzyme-buffer system (4 ml) with an appropriate blank and incubated at 50 °C (optimum assay temperature) for 1 h. The reaction was stopped by adding 0.5 ml of 1 N HCl, and the absorbance was read at 600 nm. The metal ions used were calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$), ferric chloride ($FeCl_2$), cobalt chloride ($CoCl_2$), cadmium nitrate [$Cd(NO_3)_2$], zinc sulphate ($ZnSO_4$) and manganese acetate [$Mn(CH_3COO)_2$].

Substrate Specificity

To examine the ability of the purified PHB depolymerase of *P. citrinum* S2 to degrade other polymers, *para*-nitro phenyl butyrate (PNPB; 10 mM) and copolymer of poly-β-hyroxybutyrate and polyhydroxyvalerate [P(HB-co-HV)] (5%) were used. The standard assay under optimal conditions was performed with the purified enzyme extract using P (HB-co-HV; 5%) as the substrate instead of PHB. The absorbance was read at 600 nm against a blank. Esterase activity was assayed in 2 ml of 100 mM sodium citrate buffer, pH 6.0, using *p*-nitrophenylbutyrate (PNPB) incubated at 37 °C for 10 min. The reaction mixture contained 50 μl of a 10-mM solution of PNPB in ethanol and 1 ml of the enzyme

solution. One unit of esterase activity was defined as the amount of protein required to produce 1 μmol of PNP from PNPB per hour.

Effect of Inhibitors

The inhibitory effect of various chemical reagents on enzyme activity was measured as follows: the reaction mixture (1.97 ml) containing 1 ml of the enzyme solution, reagent and 100 mM sodium citrate buffer (pH 6.0) was initially pre-incubated for 1 h at 37 °C, with the enzymatic reaction subsequently started by adding 2 ml of the PHB substrate.

Results and Discussion

The fungal isolate, S2, showed good growth at 30 °C when cultivated on Sabouraud's dextrose agar (SDA) for 3 days. Colonies were blue gray, often producing yellow droplets on the surface and yellow in reverse. Conidiophores were bi-verticillate, having only metulae and flask-shaped sterigmata. The metulae were not compressed to each other with space between them. Conidia were globose and usually smooth. Based on these characteristics, S2 was identified as *P. citrinum* Thom (Fungal Identification service, Agharkar Research Institute, Pune) by morphotaxonomy (Fig. 1a, b).

Production of enzyme depends on the growth period of microorganisms [7, 9]. *P. funiculosum* [6] and *Alcaligenes faecalis* [16] cultured at 30 °C produced the most PHB depolymerase in the stationary phase. In this study, *P. citrinum* S2 produced maximum PHB depolymerase (~1 U/mL) by 72 h when grown in BHM containing 0.2%, *w*/*v* PHB, pH 6.0 at 30 °C (Fig. 2). There are limited reports on the ability of different organisms to utilize PHB. *P. funiculosum* and *Eupenicillium* sp. have also been shown to take almost 3–4 days to degrade the same amount of PHB [17].

Production of the $PhaZ_{Pen}$ was significantly inhibited in the presence of glucose, an easily utilizable carbon source (data not shown), indicating that the synthesis of the PHB depolymerase is inhibited by catabolite repression. On the other hand, it has been reported that the PHB depolymerase from *A. faecalis* T1 can be produced to a significant level when this organism is grown with glucose as the sole carbon source [18].

Purification of the enzyme by two steps using ammonium sulphate precipitation (80% saturation) and affinity chromatography using concanavalin A yielded 16.18-fold purity with 21.53% recovery of the protein (Table 1). PHB depolymerases from different systems have been purified using different multi-step methods. According to the literature, the yield of PHB depolymerase after purification was 66% from *P. funiculosum* [6], 27% from *A. faecalis* [19], 42% from *Pseudomonas* sp. [20], 66% from *A. fumigatus* [9], 73.5% from *Aureobacterium saperdae* [21], 61% from *P. simplicissimum* and 86.11% from *A. fumigatus* Pdf1 [8, 10]. The purification fold was found to be 4.5 for *P. funiculosum* [6], 1.5 for *A. faecalis* [19], 5.67 for *Pseudomonas* sp. [20], 2.4 in *P. simplicissimum* [8] and 33.56 in *A. fumigatus* Pdf1 [10].

The purity and homogeneity of the enzyme were confirmed by activity (Fig. 3a, lanes 2, 3 and 4) as well as protein staining (Fig. 3b, lanes 3 and 4). On performing activity staining on 8% native PAGE incorporated with 2.5% (*w*/*v*) PHB, a single band of clearance was observed by the active fractions of both the crude and the partially purified enzyme (Fig. 4a, lanes 1, 3 and 4, respectively), suggesting the presence of a single PHB depolymerase in this strain. SDS-PAGE (10%) of the purified PHB depolymerase from *Penicillium* sp. S2 revealed three polypeptides with a molecular weight of approximately

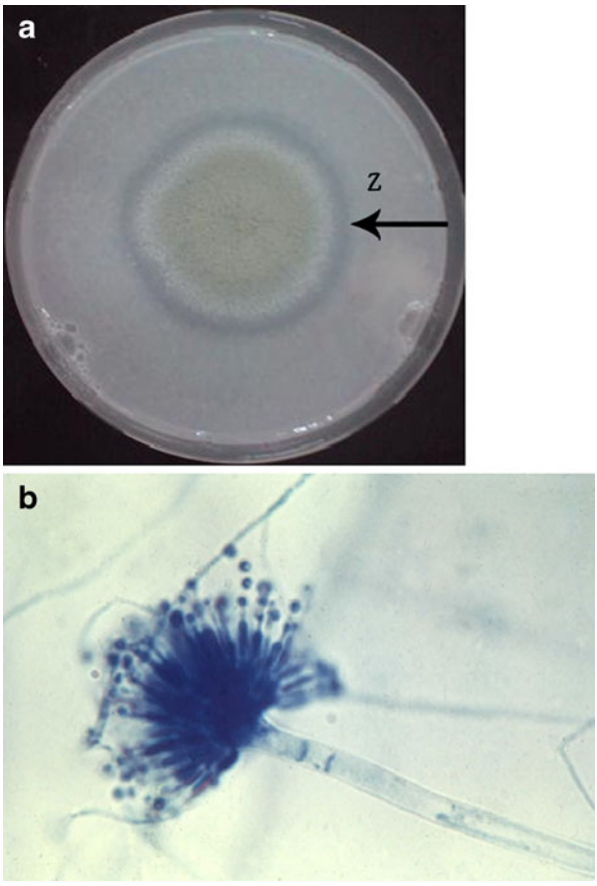

Fig 1 **a** *P. citrinum* (stained with lactophenol cotton blue). **b** PHB clearance by *P. citrinum* S2; "*Z*" indicates zone of clearance

66, 43 and 20 kDa, respectively. The molecular weight of the native enzyme on 8% native PAGE revealed a molecular weight of approximately 240–250 kDa, which suggests that the protein is comprised of three homodimers. The molecular weight of *P. citrinum* PHB depolymerase determined here is in contrast to that of the PHB depolymerase obtained from

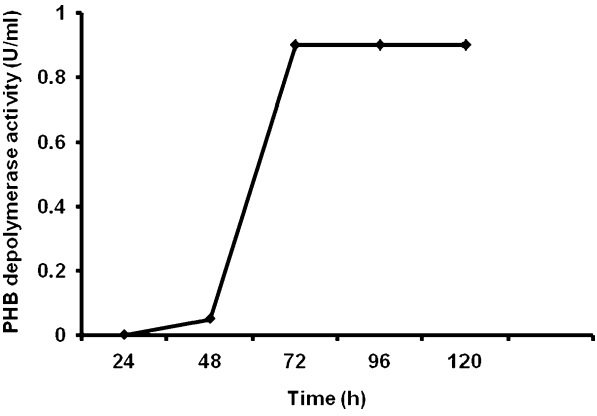

Fig. 2 Time course production of PHB depolymerise by *P. citrinum* S2

Table 1 Purification of PHB depolymerase of *P. citrinum*

Purification step	Enzyme activity (U/ml)	Total enzyme activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Activity recovery (%)
Crude enzyme (900 ml)	1.3	1,170	0.0075	6.75	173	1.0	100
NH_4SO_4 ppt, dialysed (7.5 ml)	138	1,035	0.125	0.94	1,101	6.36	88.46
Affinity chromatography (7 ml)	36	252	0.0125	0.09	2,800	16.18	21.53

P. funiculosum [6], *P. pinophilum* [7], *P. simplicissimum* [8], *A. fumigatus* Pdf1 [10], and bacterial PHB depolymerase [9, 12, 19–22], all of which showed a single polypeptide of different molecular weights. Only the poly(3-hydroxyoctanoate) (PHO) depolymerase of *Pseudomonas fluorescens* GK13 is composed of two identical polypeptides (M_r, 26,000) [23]. The PHB depolymerase of *A. faecalis* AE122 is the only depolymerase reported with unusually high apparent M_r of 96 [24].

Subjecting the PHB depolymerase of *P. citrinum* to glycoprotein staining by PAS method after SDS-PAGE (Fig. 3b, lanes 5 and 6) revealed glycosylation of all the three bands. Carbohydrates were not detected in the PHB depolymerase either from *A. fumigatus* or from *A. saperdae* [9, 21]. However, the fungal PHB depolymerase from *P. funiculosum* [6], *P. simplicissimum* [8] and *A. fumigatus* Pdf1 [10] were glycosylated.

The optimum pH of the PHB depolymerase from bacteria was in the range of 7.5~9.5 depending on the strain [19–22]. The enzyme was most active at pH 6.0 as shown in Fig. 5. pH stability of S2 PHB depolymerase at varying pH over a period of 2 h at 50 °C showed the enzyme to be stable between pH 4 and 7 and completely inactivated at pH 8 (Fig. 5). The PHB depolymerase of *A. saperdae* [21] was completely inactivated at pH 1.5 in 2 h and that of *P. lilacinus* [12] maintains the activity only in the range of pH 4.0 to 6.0. Accordingly, it was concluded that the PHB depolymerase of *P. citrinum* S2 was very stable under acidic conditions and maintains activity with variations of pH.

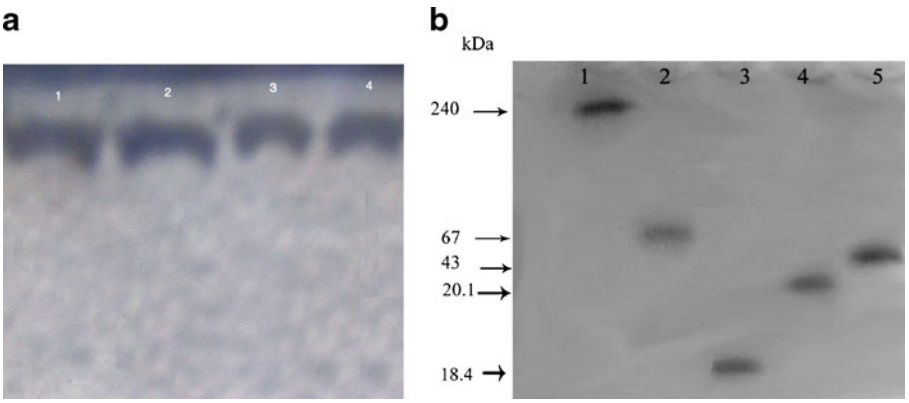

Fig. 3 **a** Activity staining of crude (*lane 1*) and purified PHB depolymerase by NH_4SO_4 precipitation (*lane 2*), affinity column (*lane 3*) and preparative gel (*lane 4*). **b** Molecular weight calibration of native PHB depolymerase by native PAGE using standard molecular weight markers: *lane 1*, catalase (240 kDa); *lane 2*, bovine serum albumin (67 kDa); *lane 3*, lactoglobulin (18.4 kDa); *lane 4*, trypsin soyabean inhibitor (20.1 kDa); *lane 5*, ovalbumin (43 kDa)

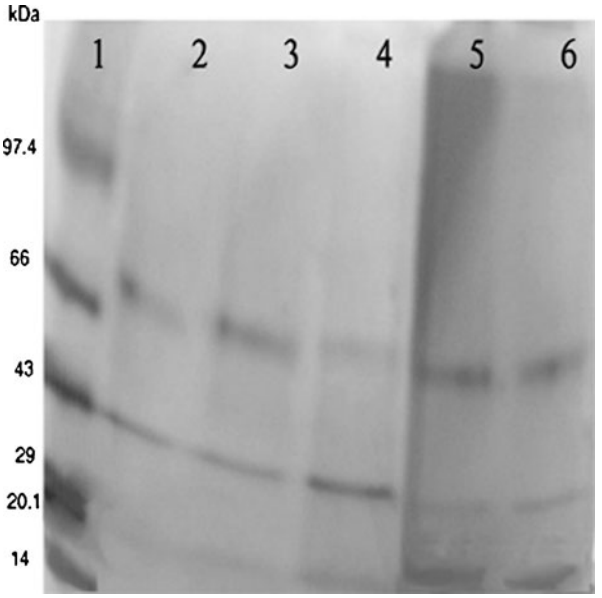

Fig. 4 SDS-denatured PAGE of purified PHB depolymerase of S2 by NH_4SO_4 precipitation. *Lane 1*, molecular standard containing phosphorylase b (MW, 97.4 kDa), BSA (MW, 66 kDa), egg albumin (MW, 45 kDa), carbonic anhydrase (MW, 31 kDa), trypsin inhibitor (MW, 21.5 kDa), lysozyme (MW, 14.5 kDa); *lane 2*, affinity column purified enzyme; *lanes 3 and 4*, preparative gel purified enzyme and glycoprotein-PAS method (*lanes 5 and 6*)

The enzyme exhibited the highest activity at 50 °C and was stable at 50 °C (Figs. 6 and 7), indicating that the enzyme was thermally as stable as other PHB depolymerases reported [6, 8, 9, 19, 25]. Mergaert et al. [26] reported that PHB showed a high degradation rate in summer in their PHB decomposition experiments and explained that such a result was due to high activation of the PHB decomposing organisms. The PHB depolymerase produced by PHB decomposing organisms has high activity and stability at high temperatures.

The K_m values were determined by plotting a double reciprocal plot. The K_m and V_{max} values are 1,250 μg/ml and 12.5 μg/min, respectively.

The activity of S2 PHB depolymerase remained unaltered in the presence of metal ions (Fig. 8). Nojima et al. [25] observed that the activity of the PHB depolymerase from

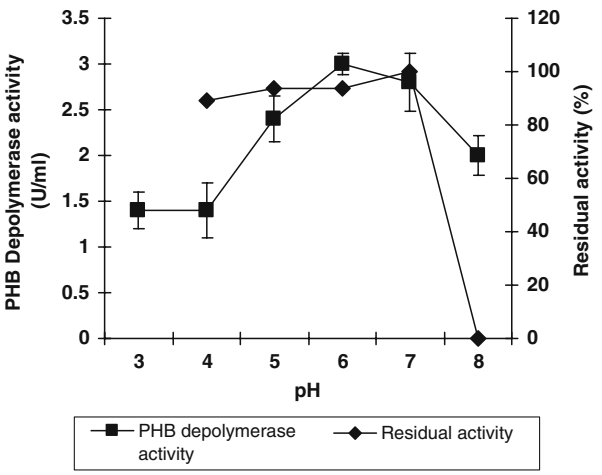

Fig. 5 Effect of pH on PHB depolymerase activity and stability from *P. citrinum* S2

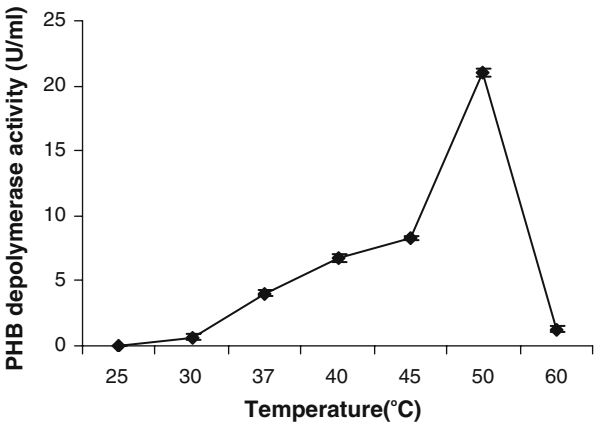

Fig. 6 Effect of temperature on PHB depolymerase activity from *P. citrinum* S2

Agrobacterium sp. was nearly independent of Fe^{2+} as well as Co^{2+}, Mg^{2+}, Mn^{2+} and Cu^{2+}. However, the PHB depolymerase from *P. lilacinus* [12] was 80% deactivated by Cu^{2+}, Zn^{2+} and Mn^{2+}, and 88% by Fe^{2+} at the same concentration. The PHB depolymerase of *Agrobacterium* sp. [25], *P. pinophilum* [7] and *P. simplicissimum* LAR13 [8] are relatively stable in the presence of ions.

Inhibitors are indicative of the various functional groups present in the active site of an enzyme. Effect of inhibitors on the activity of the enzyme was investigated in order to identify the active sites in the PHB depolymerase of *P. citrinum* S2 (Fig. 9). The activity of S2 PHB depolymerase seemed to be unaffected by *N*-*p*-tosyl-L-lysinechloromethyl ketone (TLCK), which should transform the histidine groups. The PHB depolymerases of *P. funiculosum* [6] and *A. saperdae* [21] also exhibited 100% activity in the presence of 10 mM TLCK. Iodoacetate partially inhibited the enzyme activity, suggesting the importance of reduced sulphur groups, heavy metal ions or Ca^{2+} ions. S2 depolymerase activity was reduced by ~20% in presence of 1 mM of DTT (94%), BME (37%) and 1 mM $HgCl_2$ (87%), thus indicating the importance of essential disulfide bonds (cystine residues) for enzyme activity or probably for maintaining the native enzyme structure. Complete

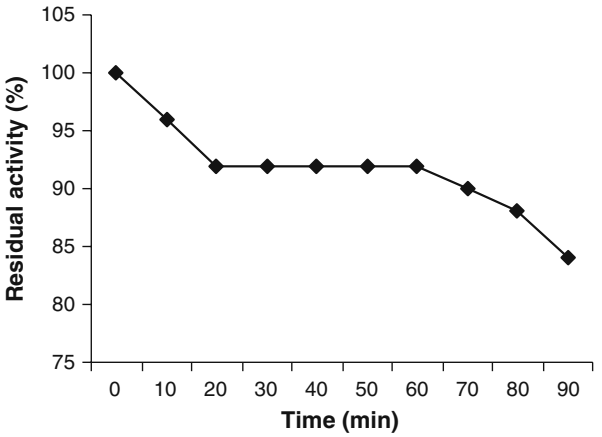

Fig. 7 Temperature stability of PHB depolymerase activity from *P. citrinum* S2

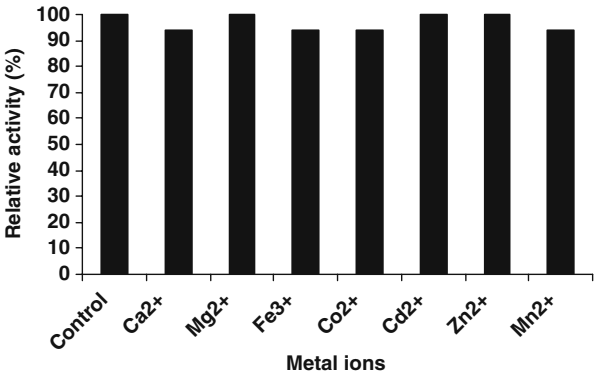

Fig. 8 Effect of metal ions on PHB depolymerase activity from *P. citrinum* S2

inhibition of the enzyme by PMSF (10 mM), DAN (25 mM), *N*-acetylimidazole (10 mM) and *N*-ethylmaleimide (10 mM) indicates the importance of serine residue, carboxyl group, tyrosine and sulfhydryl groups in the active site of the enzyme, respectively [21, 27] (Fig. 8). In contrast, *P. simplicissimum* LAR 13 PHB depolymerase was not affected by *N*-acetylimidazole. Phenylmethylsulfonyl fluoride (PMSF) is known as an inhibitor of serine residues [6, 22, 24]. The PHB depolymerase of *A. saperdae* [21] was partially inactivated by 10 mM PMSF, and that of *Pseudomonas lemoignei* [22] and *Agrobacterium* sp. [25] was completely inhibited by 1 mM PMSF. However, the PHB depolymerase of *P. simplicissimum* LAR 13 showed 58% activity in the presence of 10 mM PMSF [21]. The inhibitor studies clearly show distinct nature of *Penicillium citrinum* S2 PHB depolymerase active site as compared to the PHB depolymerases reported till date.

Of the PNP-alkanoates tested, PNP-butyrate and copolymer P (HB-co-HV) were hydrolyzed efficiently by $PhaZ_{Pen}$, and the esterase activity for the substrate was measured at 1.6 and 27 U, respectively. PNP-acetate and PNP-butyrate were hydrolyzed efficiently by the PHB depolymerase of *E. minima* [11] and *A. fumigatus* Pdf1 [10].

Identification of the aqueous end products of PHB depolymerase reaction by paper chromatography revealed β-hydroxybutyrate monomer as the major end product of PHB

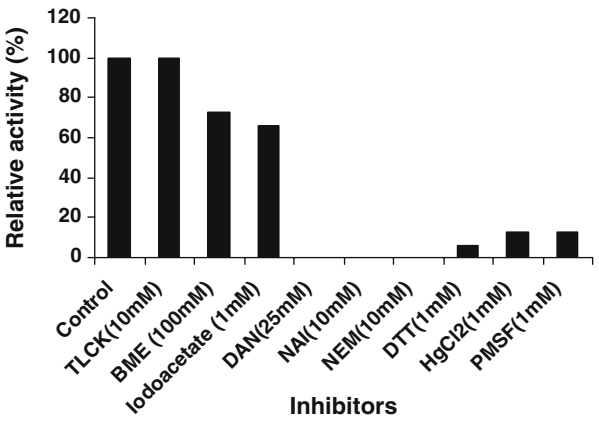

Fig. 9 Relative activity of the PHB depolymerase from *P. citrinum* S2 in the presence of various inhibitors

hydrolysis (Fig. 10). Though high resolution analysis or HPLC/LC-MS analysis is required to confirm the above observations, this preliminary observation by paper chromatography indicated mainly monomers as the aqueous end product of PHB hydrolysis by *Penicillium* sp. S2 PHB depolymerase as in the case of *Comamonas* sp. [28] and *Pseudomonas picketti* [24], as against the depolymerase of *A. faecalis* T1, *A. faecalis* AE122 and *P. lemoignei* which hydrolyze PHB mainly to the dimeric and trimeric ester of hydroxybutyrate [11, 12, 19, 24].

Conclusion and Future Prospects

PHB depolymerase of *Penicillium* sp. S2 ($PhaZ_{Pen}$) is distinct to other eukaryotic depolymerases in its M_r, glycosylation and similar to other fungal depolymerases in terms of pH and temperature optima on activity. Production of 3HB monomer from PHB with $PhaZ_{Pen}$ as the main hydrolysis product is also comparable to that of the 3HB monomer produced by $PhaZ_{Afu}$ [9] and *A. fumigatus* Pdf1 [10]. The enzyme also shows distinct behaviour towards different inhibitors tested, which suggests the role of serine, serine residue, carboxyl group, tyrosine and sulfhydryl groups in its active site. Present results suggest that PHB depolymerase of *Penicillium* sp. S2 ($PhaZ_{Pen}$) is an enzyme with distinct characteristics, different from those of other eukaryotic PHB depolymerases reported to date.

Biodegradable plastics, such as PHB, continue to make progress in both the commercial and scientific fields. However, their use as a replacement for conventional plastics in a wide range of applications has been hindered by their brittleness, low mechanical strength and high production cost. Improvements in the fermentation technology and genetically modified strains are being developed for the economical and efficient production of PHB [29, 30]. Many fine chemicals produced by chemical processes can also be prepared using microbial fermentation [31]. Our isolate, *P. citrinum* S2, is a good candidate for the production of pure D-3-hydroxybutyric acid, which is the main component of the degradation products obtained by enzymatic degradation of PHB [32]. A pure monomer of PHB, D-3-hydroxybutyric acid, is also an important precursor of 4-acetoxyazetidinone, which is used in making carbapenem antibiotics [33]. Intensive studies on this newfound strain as a useful microorganism for industrial application are necessary.

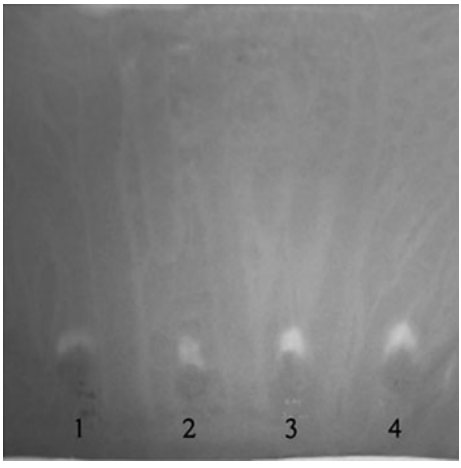

Fig. 10 Identification of PHB hydrolysis end products by PHB depolymerase of *P. citrinum* S2. *Lane 1*, β-hydroxybutyrate standard; *lanes 2–4*, end product of PHB depolymerase reaction

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