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Production and characterization of poly(3-hyroxybutyrate) depolymerases from *Aspergillus* sp. isolated from soil that could degrade poly(3-hydroxybutyrate)

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Key words: Poly(3-hydroxybutyrate), *Aspergillus* sp. strain NA-25, Poly(3-hydroxybutyrate) depolymerase, Purification, Characterization.

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Abstract

The degradation ability of *Aspergillus* sp. strain NA-25 was determined against poly (3-hydroxybutyrate) (PHB), since it was previously tested against co-polymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate). PHB depolymerase enzymes were produced from strain NA-25 at 45°C and pH 7.0 after 96h of incubation. The enzymes were purified to homogeneity through column chromatography using Sephadex G-75 gel, as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Four bands of different molecular sizes, approximately 48, 75, 80 and 85 kDa, were found which revealed that strain NA-25 was producing 4 types of PHB depolymerases. The enzymes utilized *p*-nitrophenyl caproate (C_6) as preferred substrate among various *p*-NP-acyl esters, indicating that these are a type of esterases, therefore, designated as *A*est and numbered as *A*est-1–4, respectively. The enzymes were stable at wide range of temperature (37–60°C) and pH (6–9). All the PHB depolymerases were stable in the presence of different metal ions except enzyme *A*est-4. Ethylenediamine teteracetic acid (EDTA), β-mercaptoethanol and sodium dodecyl sulphate (SDS) inhibited the activity of all depolymerases. PHB depolymerases were related to the serine group of hydrolases, as indicated by the inhibitory effect of phenylmethyl sulphonyl fluoride (PMSF) against all the depolymerases. The enzymes from *Aspergillus* sp. strain NA-25 could degrade aliphatic polyesters; therefore, it might be applied for bioremediation in the polyesters-contaminated environments.

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Introduction

Poly (3-hydroxybutyrate) (PHB) is a universal carbanosome that is synthesized by the microorganisms when carbon compounds are present in excess amount and also they are used in starve condition as an energy source by these microorganisms (Jendrossek, 2009). In the modern era, this carbanosome is used for a number of purposes e.g. in packaging, health, and agriculture. The main attraction lies in the complete degradation of this polymer in the natural environment and also having similar properties like that of the fossil fuel polymers (Kasuya *et al.*, 1998). The interest in degrading these polyhydroxyalkanoates (PHA) also lies in the production of chiral hydroxyalkanoates (Arroyo *et al.*, 2011). Extracellular PHA depolymerases are a type of hydrolases, which have attracted the attention of the researchers these days, because of their degradation ability against PHAs, especially against PHB and its co-polymer poly (3-hydroxybutyrate-co-3-hdyroxyvalerate) (PHBV) in the environment. PHB is decomposed by the soil microorganisms through extracellular PHB depolymerases, once it is buried under the ground (Shivakumar *et al.*, 2011). Thus, this enzyme also plays a vital role in the recycling of PHB, by degrading the PHB discarded materials to its oligomers and monomers and finally mineralizing to CO_2 and H_2O in the environment (Hiraishi *et al.*, 2009).

A number of microorganisms are capable of degrading PHB but the fungi possesses s high depolymerase production and activity, thus they are considered as appropriate candidates for the PHB degradation (Han *et al.*, 2002). Till date, all of the studied fungi carries only a single depolymerase enzyme for the degradation of PHB (Kim and Rhee, 2003). Furthermore, protease, lipase and esterase activities are also known to be associated with the degradation of certain polyesters by fungi and bacteria (Pathirana & Seal, 1984; Ruiz *et al.*, 1999; Rowe & Howard, 2002). This might be because of the presence of serine, histidine and aspartate residues in the active sites of PHB depolymerases (Jendrossek *et al.*, 1995). Serine is the part of lipase box such as Gly-aa-Ser-aa-Gly and it is found in all known hydrolases like esterases, lipases and serine proteases (Jaeger *et al.*, 1994). Some of the PHB depolymerases are also found to be serine esterases because they contain the lipase box (Schirmer and Jendrossek, 1994).

Owing to the aforementioned properties, the current study was designed to isolate, purify and characterize PHB depolymerases from *Aspergillus* sp. Strain NA-25. Interestingly, unlike other fungi, the Strain NA-25 was found to have four different sizes of PHB depolymerases. Importantly, the optimization of these enzymes was carried out, with special emphasis on temperature and pH. Despite an excellent depolymerase activity, the isolated enzymes were also assessed for any additional activities including esterases and substrate specificity. Further, the effect of different metal ions and enzyme inhibitors was also carried out on the purified enzymes. To the best of our knowledge, no fungus has been reported previously which could produce four different PHB depolymerases. The three of the depolymerases may be dimeric proteins because of large molecular weight (>70kDa).

Materials and methods

Materials

Poly (3-hydroxybutyrate) (PHB) was obtained in powder form from Sigma-Aldrich Chemicals, Germany. Strain NA-25 was selected on the basis of clear zone around its growth on PHB emulsified mineral salt agar plates. All the chemicals and reagents were obtained of highest commercial grade available.

PHB agar plates

PHB degradation ability of strain NA-25 was checked by inoculating on mineral salt agar supplemented with 0.2% (w/v) of PHB as a sole carbon source. PHB suspension was prepared by sonicating PHB powder in a flask, containing mineral salt medium (MSM) for 20 min in ultrasonic water bath (35 KHz, 285 W). 2% agar was added to the PHB suspension and poured into petri plates for the clear zone assay.

Identification of Fungal Strain NA-25

Strain NA-25 was identified by macroscopic (conidial and mycelial color) and microscopic (seriation, vesicle, conidia and ascospores) examination as well as through 18S rRNA gene sequencing. The DNA was extracted from *Aspergillus* sp. NA-25 and 18S rRNA gene was amplified from DNA using ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers. For PCR reaction, 1 µl of template DNA was mixed in 20 µl of PCR reaction solution, then 35 amplification cycles at 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s were made. DNA fragments were amplified about 500~800 bp. The unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore, USA). The purified PCR products of approximately about 500~800 bp were sequenced by using ITS1/ITS4 primers. Sequencing was performed by using Big Dye terminator cycle sequencing kit v.3.1 (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) from the Macrogen Company Limited, Korea.

Production of PHB Depolymerase

Spore suspension (2.6 x 10^5 cells/ml) of strain NA-25 was inoculated in 1000 ml of MSM with 0.2% of PHB as a sole carbon source. The flask was shifted into shaker incubator (200 rpm) for 96 h at 45°C. After incubation, the culture broth was centrifuged at 8,000×g for 10 minutes and the supernatant was collected for the enzyme purification.

PHB Depolymerase Assay

PHB depolymerase activity was determined by the method described by Kobayashi *et al.* (1999). PHB was taken in a concentration of 0.03% and was suspended in 50 mM Tris-HCl, pH 7.0. The suspension was autoclaved after sonicating for 1 hour at 90 duty cycles using a Branson sonifier (Branson Ultrasonic Cooperation, Danburry, CT, USA). Activity was measured as decrease in optical density (OD) after adding 0.1ml of culture supernatant to 0.9ml of substrate suspension and incubated for 24h at 30°C; using substrate buffer blank. One unit of activity is defined as the activity resulting in a decrease in OD at 650 nm per 24 hours (Kobayashi *et al.*, 1999).

Enzyme Purification

Ammonium Sulfate Precipitation

Approximately, 950 ml of supernatant was collected through above procedure. Finely grind ammonium sulfate $(NH_4)_2SO_4$ of protein precipitation grade was added to the supernatant with gentle stirring until the solution reached 60% saturation. The solution was centrifuged at 15,000×g for 10 min using Kokusan centrifuge Model H-251, Tokyo, Japan, and the supernatant was collected. More $(NH_4)_2SO_4$ was added to the supernatant until the solution reached 80% saturation. The solution was centrifuged, and precipitate was dissolved in 5 ml of 100 mM potassium phosphate buffer (pH 7.0). This product was designated as crude enzyme extract and stored at –80°C until use.

Column chromatography and Molecular weight determination

Crude enzyme extract was purified by size exclusion chromatography using column (10/50 mm) packed with Sephadex G-75. The crude protein was first dialyzed by protein dialysis kit to remove excess of salt. 2 ml of sample was loaded in the Sephadex G-75 column and eluted by using 100mM phosphate buffer at a flow rate of 2.0ml/min. A total of 25 fractions (3ml each) were collected. Fractions showing maximum enzyme activity were pooled and then lyophilized; the concentrate was re-suspended in the same buffer. The molecular mass of the purified PHB depolymerases was determined using SDS-PAGE with standard protein marker of 26–170 kDa (Fermentas, USA). SDS-PAGE was performed using 12% polyacrylamide gel by the method as described by Laemmli (1970).

Characterization of purified PHB depolymerase

Esterase activity

During purification process, the activity of PHB depolymerases was approximately calculated based

on the decrease in the turbidity of PHB. Esterase activity was determined by the modified method of Kay *et al.* (1993) using *p*-nitrophenyl acetate as a substrate and the absorbance was measured at 405 nm. One unit was defined as the amount of enzyme required to liberate 1 µM of *p*-nitrophenol per min. The *p*-nitrophenol produced indicates the presence of esterase (U/ml) in the medium. The protein concentration was determined using the method described by Lowry *et al.* (1951).

Substrate specificity

The substrate specificity towards various *p*-NP acyl esters such as, acetate (C_2), butyrate (C_4), caproate (C_6), caprylate (C_8), caprate (C_{10}), palmitate (C_{16}) and stearate (C_{18}) was determined by the method of Eggert *et al.*, (2002). Alternatively, gum arabic and deoxycholate were omitted from the reaction mixture, and the reaction was performed with 100 mM potassium phosphate buffer (pH 7.0) at 37°C.

Effect of temperature and pH on enzyme activity

The effect of temperature on enzyme activity was determined by pre-incubating the enzymes samples in 20mM phosphate buffer at different temperatures (25–80°C) for 1 h and residual activity was measured using the standard assay conditions. The effect of pH on enzyme activity was studied over a pH range of 3.0–9.0. The pH stability of the enzymes was determined by incubation with different buffer systems at 45°C for 1 h, and residual activity was measured using the standard assay conditions. The following buffer systems (20mM) were used: sodium acetate buffer, pH 3.0–5.0; potassium phosphate buffer, pH 6.0–7.0; Tris–HCl buffer, pH 8.0; glycine-NaOH buffer, pH 9.0.

Effect of metal ions and enzyme inhibitors on enzyme activity

The effects of various metal ions on enzyme activity were determined by assaying the residual activity after incubating the PHB depolymerases with 1mM metal ions for 1 h at 37°C. The chloride and sulphate salts of the metal ions tested were: Ca^{+2}, Mg^{+2}, Zn^{+2} and Co^{+2}. Effect of 1mM chemical reagents ethylenediamine tetra-acetic acid (EDTA), β–mercaptoethanol, sodium dodecyle sulphate (SDS) and phenylmethyl sulphonyl fluoride (PMSF) on activity was determined by incubating PHB depolymerases with these reagents for 1 h at 37°C and pH 7.0 and the residual activity was calculated.

Statistical analysis

All the experiments were repeated three times. Data is presented as mean, standard deviation and F-test was used for significance testing, and p value of <0.05 is considered to be statistically significant. The analysis was done on using Graphpad prsim 5.

Results and discussion

PHB Degrading Fungal Strain NA-25

Strain NA-25 was inoculated on PHB emulsified mineral salt agar plates. Zone of hydrolysis appeared after 48h of incubation at 45°C, with maximum size after 120h, it revealed that the strain NA-25 utilized PHB as carbon source. The formation of clear zones around bacterial growth is an indication that the polymer is hydrolyzed by the enzyme into water-soluble products (Nishida & Tokiwa, 1993).

Identification of Strain NA-25

On visual examination, fungal conidia were greyish turquoise or dull green; white mycelia; reverse side of growth plate was yellowish green; colonies become 40-70µm in diameter within 4 days, with no pigmentation, sclerotia, cleistothecia and colorless exhudates. As per microscopic examination, conidia were globosely to broadly ellipsoidal, smooth to finely roughened or spinose about 2-3mm in diameter; vesicle 15-30 µm in diameter; uniseriate; stipes greyish near the apices and smooth walled; hull cells and cleistochecial wall absent; ascopspores were purple, lenticular and 4.5-6µm in diameter.

According to the sequencing results at total of 967 bases of the 18S rRNA gene were used for identification. The nucleotide sequence reported in this paper can be obtained from the NCBI nucleotide sequence database under accession number HM807309. The 18S rRNA gene of strain NA-25 was

completely matched with that of *Aspergillus* sp. DX12 (type strain, GenBank accession No., GU726139.1.678).

Various microorganisms have been isolated and characterized from the environment with capability to degrade both PHB and PHBV, by many of the scientists. *Bacillus strains*, *Streptomyces*, *Acidovorax facilis* and *Variovorax paradoxus* (Mergaert *et al.*, 1993), *Nocardiopsis aegyptia* (Ghanem *et al.*, 2005), *Clostridium* sp. (Abou-Zeid *et al.*, 2001) and *Bacillus megaterium* strain AF3 respectively, from soil and sewage sludge (Shah *et al.*, 2007b). According to systematic screening of 45 soil fungi for degradation of PHAs, 6 potent *Aspergillus* isolates belonging to *Aspergillus flavus, A. oryzae, A. parasiticus*, and *A. racemosus* were selected (Sanyal *et al.*, 2006).

Table 1. Purification of PHB depolymerases from *Aspergillus* sp. strain NA-25.

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	450	879.00	632.00	0.72	1.00	100
Ammonium sulfate precipitation	15	83.50	319.73	3.83	5.32	51
Sephardi G-75						
Aest-1	3	5.09	180.42	35.45	49.24	28
Aest-2	4	8.41	201.01	24.00	33.33	32
Aest-3	3	10.66	300.90	28.23	39.21	47
Aest-4	2	7.88	260.00	33.00	45.83	41

Production and Purification of PHB depolymerases

In our study, we have used *Aspergillus* sp. NA-25 for the production of PHB depolymerase. PHB depolymerases were produced under optimized conditions and the crude protein was obtained by using increasing concentrations of ammonium sulfate upto 75% ($p<0.05$). Jendrossek *et al.* (1995) and Zhou *et al.* (2008) had also preceipitated out PHB depolymerase enzyme from recombinant *Escherichia coli*, and *Penicillium* sp. DS9701-D2 after using 70 and 75% of ammonium sulfate, respectively whereas 80% of ammonium sulphate in case of *Penicillium citrinum* S2 (Shivakumar *et al.*, 2011).

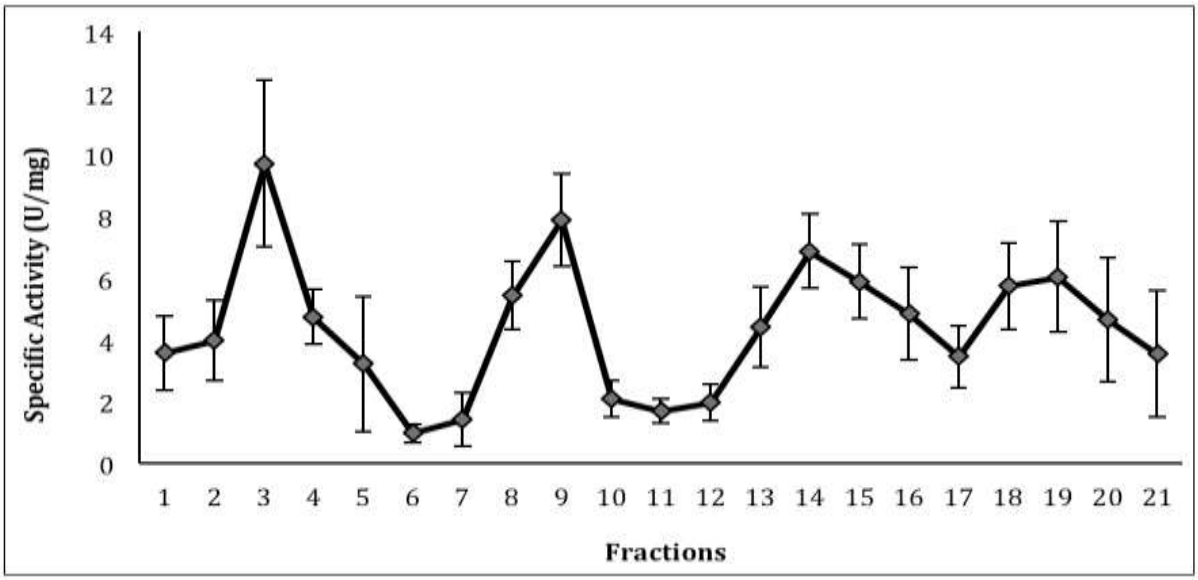

Fig. 1. Purification of PHB Depolymerases in crude extract by molecular exclusion chromatography on Sephadex G-75. Vo = 15 ml; Vt = 60 ml; Flow rate = 2ml/min; Fraction volume = 3ml; Eluent = phosphate buffer (pH 7.0).

PHB depolymerases have been purified through molecular exclusion chromatography using various types of gels such as Sephadex G-75–150. Shah *et al*., (2007a,b) used sephadex G-75 for the purification of PHBV depolymerases from *Bacillus* sp. AF3 and *Streptoverticillium kashmirense* AF1. Sephadex G-100 and Sephdex G-150 has also been reported for purification of PHB depolymerase from *Emericellopsis minima* W2 and *Streptomyces* sp. KJ-72 (Kim *et al*., 2003).

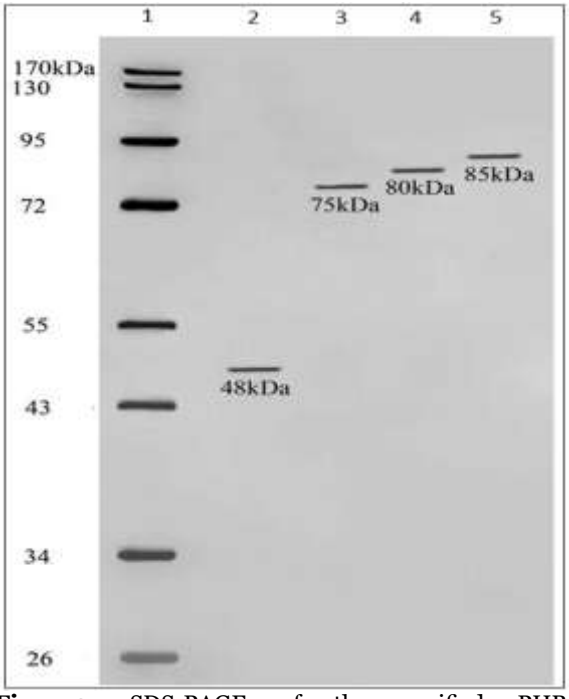

Fig. 2. SDS-PAGE of the purified PHB depolymerases. Lanes: 1, proteins molecular marker; 2, *A*est-1; 3, *A*est-2; 4, *A*est-3; 5, *A*est-4.

Specific activity of column fractions was calculated and plotted against activity; four proteins of different molecular sizes were eluted into fractions, as shown in figure 1. In Table 1, steps for purification of PHB depolymerases from *Aspergillus* sp. strain NA-25 has been summarized. The enzyme activity increased 49.24 fold after the purification, and the yield was 28%.

Uptill now, a number of PHB depolymerases have been purified from prokaryotes and eukaryotes, with variable molecular sizes such as 33.0-57.0 kDa, indicating that these are single polypeptide chain proteins (Kim & Rhee, 2003). In addition most of PHA degrading microorganisms are known to produce only one type of PHA depolymerase (Jendrossek, 1998), with the exception of *Pseudomonas lemoignei*, recombinant *Escherichia coli* and *Streptoverticillium kashmirense* AF1 have been reported to produce more than one type of PHA depolymerases (Jendrossek *et al*., 1995; Shah *et al*., 2007a). In our study, the *Aspergillus* sp. NA 25 has been found to produce four different types of PHB depolymerases with different molecular sizes i.e., 48 (Rf=0.52), 70 (Rf=0.22), 75 (Rf=0.20) and 80 (Rf=0.18) kDa, indicating that strain NA-25 produce both single and double polypeptide chain proteins (Fig. 2). To the best of our knowledge, high molecular sizes PHB have not been reported from any other fungal strain except strain NA-25.

Characterization of purified PHB depolymerases

Esterase activity

In our study, we found that the four PHB depolymerases have esterase activity as determined by the modified method of Kay *et al.* (1993). A number of PHA depolymerases have been reported with esterase activity. Kim *et al*., (2003) found that the medium chain length PHA depolymerases of *Streptomyces* sp. KJ-72 showed higher esterase activity then short chain length PHA depolymerases. In another study, the PHB depolymerase of a fungus *Emericellopsis minima* W2 and a bacterium *Thermus thermophilus* HB8 showed esterase activity (Papaneophytou *et al*., 2009). Similarly, it has recently been observed that PHA depolymerase from *Pseudomonas fluorescens* GK13 catalyzed ester-forming reactions in non-aqueous media by simply employing preparations of the lyophilized enzyme (Santos *et al*., 2013).

Substrate specificity

The substrate specificity towards various *p*-nitrophenyl acyl esters (C_2–C_{18}) was examined. The ester cleavage rate increased significantly as the number of carbon atoms increased from C_2 to C_6, but gradually started decreasing from C_8 to C_{18}, as shown in figure 7. The enzymes catalyzed an esterolytic activity against various *p*-nitrophenyl acyl esters, especially against short chain length esters, with

maximum activity against hexanoic acid ester. Lipases catalyze the hydrolysis of acylglycerols with acyl chain lengths of >10 carbon atoms, but esterases catalyze the hydrolysis of glycerolesters with acyl chain lengths of <10 carbon atoms (Rhee *et al.*, 2005). Esterases usually hydrolyze triglycerides bearing fatty acids shorter than C_6 (Bornscheuer, 2002). Therefore, our results suggested that these are a type of esterases. We tentatively designated the enzymes with *Aest*-1–4.

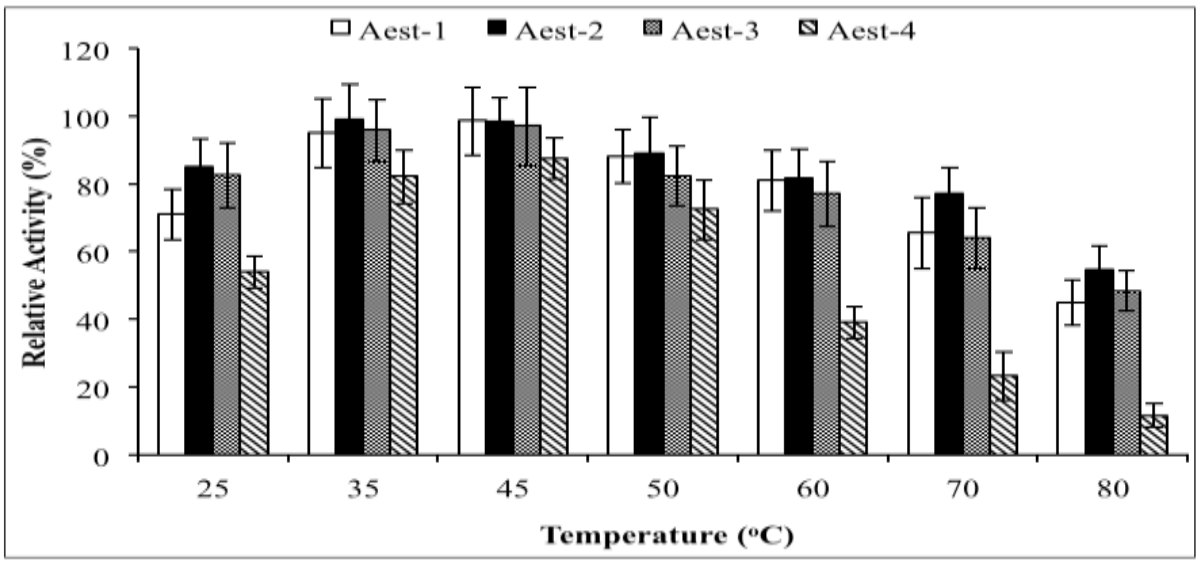

Fig. 3. Effect of temperature on activity of the purified PHB depolymerases. For determining the stability, the activity of the enzymes without any treatment was taken as 100%. Data are the average of three independent experiments.

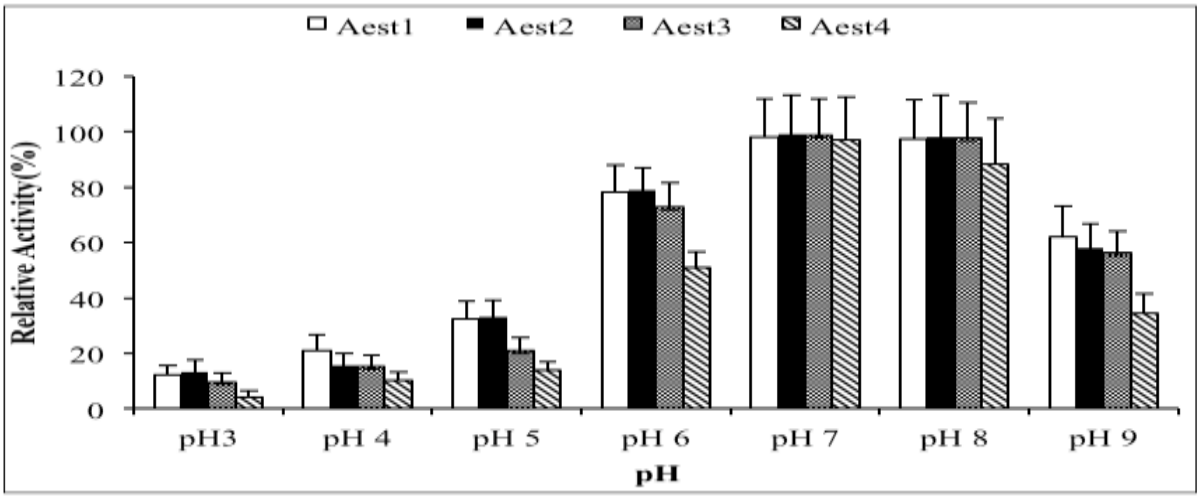

Fig. 4. Effect of pH on activity of the purified PHB depolymerases. For determining the stability, the activity of the enzymes without any treatment was taken as 100%. Data are the average of three independent experiments.

Effect of Temperature and pH on Purified PHB depolymerases activity

In the environment especially in the summers, the PHB degradation activity increases due to the high activity of PHB degrading microorganisms in the environment by releasing thermo-tolerant PHB deplymerases. In the current study, PHB depolymerases from strain NA-25 were stable at wide temperature range i.e., 50–80°C ($p<0.05$) with almost 80–100% ($p<0.01$) stability at 37–60°C (Fig. 3). PHBV depolymerase from *Acidovorax* sp. HB01 and *Streptomyces venezuelae* SO1 were found stable at 50°C and at neutral and alkaline pH (Wang *et al.*, 2012; Santos *et al.*, 2013). Enzymes from strain NA-

25 were stable at pH 6.0–9.0, with almost 100% ($p<0.01$) stability at pH 7.0–8.0 (Fig. 4). PHB depolymerase from *Penicillium citrinum* S2 was found 100% stable in between pH 4.0–7.0 and completely unstable at pH 8.0 (Shivakumar *et al.*, 2011). The PHB depolymerase of *Aspergillus saperdae* was completely inactivated at low pH (Calabia & Tokiwa, 2006) and but on the other hand *Penicillium lilacinus* maintained its stability at low pH (Oda *et al.*, 1997).

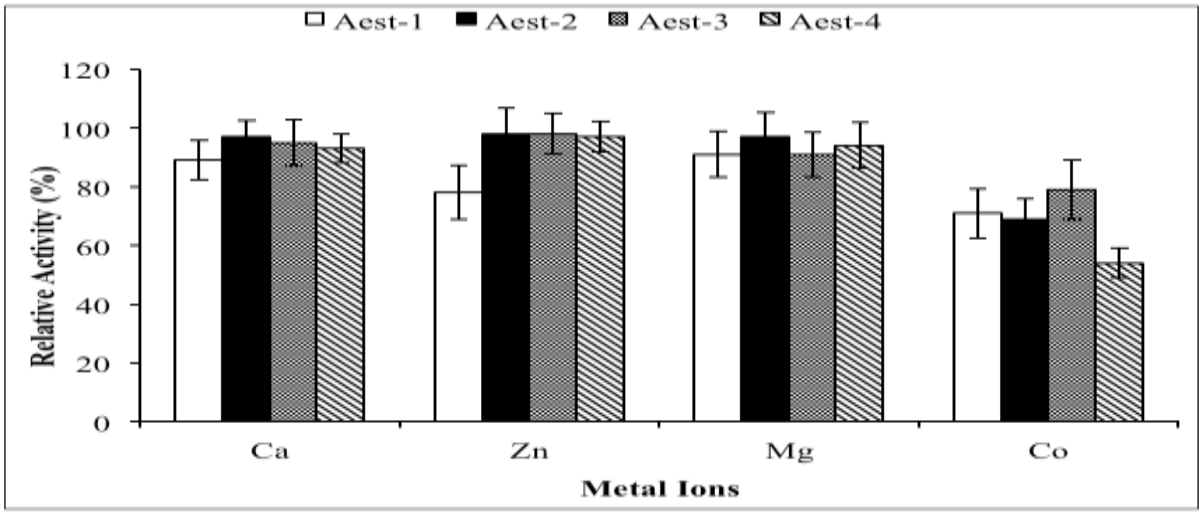

Fig. 5. Effect of metal ions on activity of purified PHB depolymerases. For determining the stability, the activity of the enzymes without any treatment was taken as 100%. Data are the average of three independent experiments.

Effect of metal ions on purified PHB depolymerases activity

The activity of PHB depolymerases from *Agrobacterium* sp. was not affected in the presence of metal ions (Nojima *et al.*, 1996). In our study, the PHB depolymerases from *Aspergillus* sp. NA-45 were more than 80–90% ($p<0.05$) stable in the presence of dianionic ions with the exception of *A*est-4, which was effected in the presence of metal ions (Fig. 5). PHB depolymerase from *Penicillium citrinum* S2 was also found stable in the presence of dianionic ions (Shivakumar *et al.*, 2011) whereas the PHBV depolymerase from *Acidovorax* sp. HB01 was inhibited by these ions (Wang *et al.*, 2012).

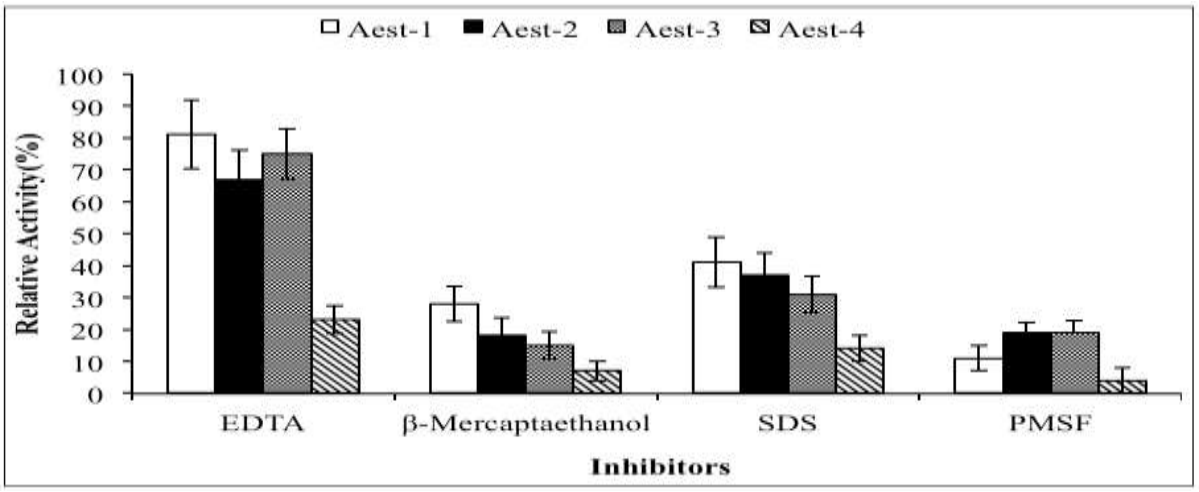

Fig. 6. Effect of inhibitors on activity of purified PHB depolymerases. For determining the stability, the activity of the enzymes without any treatment was taken as 100%. Data are the average of three independent experiments.

According to previous reports, the exact mechanism is not known but metal ions may modify protein structures. Changes in enzymes may be due to binding of metal ions to the specific sites of enzyme, which are generally formed by negatively charged amino acid side chains. If the metal ions bind to the amino acids far away from the catalytic site then there will be no or negligible effect on the activity of enzyme. But if the metal ions bind in the catalytic region then it may either enhances the enzyme activity acting as cofactor or decrease the activity due to resistance in substrate binding to the catalytic amino acids. In many cases the activity of PHB depolymerases is increased by the addition of Ca^{+2} ions in *Alcaligenes faecalis* AE122, *Pseudomonas lemoignei*, and *Pseudomonas stutzeri* (Kasuya *et al*., 1998) while in some cases the monovalent ions like K^{+} had inhibited the activity of PHB depolymerases (Sznajder & Jendrossek, 2011). These divalents also had negligible effect on the activity of *Penicillium simplicissimum* LAR13 (Han & Kim, 2002). But the other two divalents i.e. Zn^{+2} and Fe^{+2} had inhibitory effects on the activity of the PHB depolymerase from *Penicillium lilacinus*. Iron (Fe^{+2}) had inhibitory effect on purified PHB depolymerase from *Penicillium* sp DS9701-D2 (Zhou *et al*., 2008). Addition of Cu^{2+}, Co^{2+} and Zn^{2+} stimulated the PHB depolymearse activity whereas Mn^{2+} Fe^{2+}, Ni^{2+} and Ag^{+} caused inhibition (Calabia & Tokiwa, 2006). It has been suggested that the effect of metal ions could be attributed to a change in the solubility and in the catalytic properties of the enzyme itself (Kasuya *et al*., 1994).

Effect of inhibitors on PHB depolymerases activity

Effect of inhibitors on the activity of the enzyme was investigated in order to identify the active sites in the PHB depolymerase from *Aspergillus* sp. strain NA-25 (Fig. 6). Phenylmethylsulfonyl fluoride (PMSF) is known as an inhibitor of serine residues which has something to do with the activity of the PHB depolymerase (Sadocco *et al*., 1997; Nakayama *et al*., 1985; Nojima *et al*., 1996). PHB depolymerases from strain NA-25 were inhibited in the presence of PMSF indicating the presence of serine in their active sites. Enzymes *A*est-1–3 were more than 60% ($p<0.05$) stable in the presence of EDTA except *A*est-4 which indicates that it belongs to metalo-group of depolymerases. β-mercaptaethanol and SDS had inhibited the activity of all the enzymes. Papaneophytou *et al*., (Papaneophytou *et al*., 2009) has reported that EDTA and β-mercaptoethanol are strong inhibitors of extracellular PHB depolymerase from *Thermus thermophilus* HB8.

Hence it has been concluded from the results that PHB depolymerases of *Aspergillus* sp. strain NA-25 are enzymes with remarkable characteristics, different from those of the other eukaryotic PHB depolymerases reported till date. The p value less than 0.05 shows the linear relationship among all the experiments which indicates the significance of the data.

References

Abou-Zeid DM, Müller RJ, Deckwer WD. 2001. Degradation of natural and synthetic polyesters under anaerobic conditions. Journal of Biotechnology **86,** 113-126. http://dx.doi.org/10.1016/S0168-1656(00)00406-5

Arroyo M, Garcia-Hidalgo J, Villalon M, De Eugenio L, Hormigo D, Acebal C, García J, Prieto M, de la Mata I. 2011. Characterization of a novel immobilized biocatalyst obtained by matrix-assisted refolding of recombinant polyhydroxyoctanoate depolymerase from *Pseudomonas putida* KT2442 isolated from inclusion bodies. Journal of Industrial Microbiology and Biotechnology **38,** 1203-1209. http://dx.doi.org/10.1007/s10295-010-0898-z

Bornscheuer UT. 2002. Microbial carboxyl esterases: classification, properties and application in biocatalysis. FEMS Microbiology Review **26,** 73-81. http://dx.doi.org/10.1111/j.15746976.2002.tb00599.x

Calabia BP, Tokiwa Y. 2006. A novel PHB depolymerase from a thermophilic *Streptomyces* sp. Biotechnology Letters **28,** 383-388.

http://dx.doi.org/10.1007/s10529-005-6063-5.

Eggert T, van Pouderoyen G, Pencreac'h G, Douchet I, Verger R, Dijkstra BW, Jaeger KE. 2002. Biochemical properties and three-dimensional structures of two extracellular lipolytic enzymes from *Bacillus subtilis*. Colloids and Surfaces B: Biointerfaces **26,** 37-46.
http://dx.doi.org/10.1016/S0927-7765(02)00033-4

Ghanem NB, Mabrouk ME, Sabry SA, El-Badan DE. 2005. Degradation of polyesters by a novel marine *Nocardiopsis aegyptia* sp. nov.: application of Plackett-Burman experimental design for the improvement of PHB depolymerase activity. Journal of General and Applied Microbiology **51,** 151-158.
http://doi.org/10.2323/jgam.51.151

Han JS, Kim MN. 2002. Purification and characterization of extracellular poly (3-hydroxybutyrate) depolymerase from *Penicillium simplicissimum* LAR13. Journal of Microbiology **40,** 20-25.

Hiraishi T, Komiya N, Matsumoto N, Abe H, Fujita M, Maeda M. 2009. Degradation and adsorption characteristics of PHB depolymerase as revealed by kinetics of mutant enzymes with amino acid substitution in substrate-binding domain. Biomacromolecules **11,** 113-119.
http://dx.doi.org/10.1021/bm900967a

Jaeger K-E, Ransac S, Dijkstra BW, Colson C, van Heuvel M, Misset O. 1994. Bacterial lipases. FEMS Microbiology Review **15,** 29-63.
http://dx.doi.org/10.1111/j.15746976.1994.tb00121.x

Jendrossek D. 1998. Microbial degradation of polyesters: a review on extracellular poly (hydroxyalkanoic acid) depolymerases. Polymer Degradation and Stability **59,** 317-325.
http://dx.doi.org/10.1016/S0141-3910(97)00190-0

Jendrossek D. 2009. Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). Journal of Bacteriology **191,** 3195-3202.
http://dx.doi.org/10.1128/JB.01723-08

Jendrossek D, Frisse A, Behrends A, Andermann M, Kratzin HD, Stanislawski T, Schlegel HG. 1995. Biochemical and molecular characterization of the *Pseudomonas lemoignei* polyhydroxyalkanoate depolymerase system. Journal of Bacteriology **177,** 596-607.

Kasuya KI, Doi Y, Yao T. 1994. Enzymatic degradation of poly [(R)-3-hydroxybutyrate] by *Comamonas testosteroni* ATSU of soil bacterium. Polymer Degradation and Stability **45,** 379-386.
http://dx.doi.org/10.1016/0141-3910(94)90208-9

Kasuya K, Takagi K, Ishiwatari S, Yoshida Y, Doi Y. 1998. Biodegradabilities of various aliphatic polyesters in natural waters. Polymer Degradation and Stability **59,** 327-332.
http://dx.doi.org/10.1016/S0141-3910(97)00155-9

Kay M, McCabe R, Morton L. 1993. Chemical and physical changes occurring in polyester polyurethane during biodegradation. International Biodeterioration and Biodegradation **31,** 209-225.
http://dx.doi.org/10.1016/0964-8305(93)90006-N

Kim D, Rhee Y. 2003. Biodegradation of microbial and synthetic polyesters by fungi. Applied Microbiology and Biotechnology **61,** 300-308.

Kim HJ, Nam JS, Bae KS, Rhee YH. 2003. Characterization of an extracellular medium-chain-length poly(3-hydroxyalkanoate) depolymerase from *Streptomyces* sp. KJ-72. Antony van Leeuwenhoek **83,** 183-189.
http://dx.doi.org/10.1007/s00253-002-1205-3

Kobayashi T, Sugiyama A, Kawase Y, Saito T, Mergaert J, Swings J. 1999. Biochemical and genetic characterization of an extracellular poly (3-hydroxybutyrate) depolymerase from *Acidovorax* sp.

strain TP4. Journal of Environmental Polymer Degradation **7,** 9-18.
http://dx.doi.org/10.1023/A:1021885901119

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680-685.
http://dx.doi.org/10.1038/227680a0

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. Journal of Biology Chemistry **193,** 265-275.

Mergaert J, Webb A, Anderson C, Wouters A, Swings J. 1993. Microbial degradation of poly (3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in soils. Applied Envionmental Microbiology **59,** 3233-3238.

Nadhman A, Hasan F, Shah Z, Hameed A, Shah AA. 2012. Production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) depolymerase from *Aspergillus* sp. NA-25. Applied Biochemistry and Microbiology **48,** 482-487.
http://dx.doi.org/10.1134/S0003683812050080

Nakayama K, Saito T, Fukui T, Shirakura Y, Tomita K. 1985. Purification and properties of extracellular poly (3-hydroxybutyrate) depolymerases from *Pseudomonas lemoignei*. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology **827,** 63-72.
http://dx.doi.org/10.1016/0167-4838(85)90101-3

Nishida H, Tokiwa Y. 1993. Distribution of poly (β-hydroxybutyrate) and poly (ε-caprolactone) aerobic degrading microorganisms in different environments. Journal of Envionmental Polymer Degradation **1,** 227-233.
http://dx.doi.org/10.1007/BF01458031

Nojima S, Mineki S, Iida M. 1996. Purification and characterization of extracellular poly (3-hydroxybutyrate) depolymerases produced by *Agrobacterium* sp. K-03. Journal of Fermentation and Bioengineering **81,** 72-75.
http://dx.doi.org/10.1016/0922-338X(96)83124-2

Oda Y, Osaka H, Urakami T, Tonomura K. 1997. Purification and properties of poly (3-hydroxybutyrate) depolymerase from the fungus *Paecilomyces lilacinus* D218. Current Microbiology **34,** 230-232.
http://dx.doi.org/10.1007/s002849900174

Papaneophytou CP, Pantazaki AA, Kyriakidis DA. 2009. An extracellular polyhydroxybutyrate depolymerase in *Thermus thermophilus* HB8. Applied Microbiology and Biotechnology **83,** 659-668.
http://dx.doi.org/10.1007/s00253-008-1842-2

Pathirana R, Seal K. 1984. Studies on polyurethane deteriorating fungi. II: An examination of their enzyme activities. International Biodeterioration **20,** 229-235.

Rhee JK, Ahn DG, Kim YG, Oh JW. 2005. New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. Applied and Environmental Microbiology **71,** 817-825.

Rowe L, Howard GT. 2002. Growth of *Bacillus subtilis* on polyurethane and the purification and characterization of a polyurethanase-lipase enzyme. International Biodeterioration and Biodegradation **50,** 33-40.
http://dx.doi.org/10.1016/S0964-8305(02)00047-1

Ruiz C, Main T, Hilliard NP, Howard GT. 1999. Purification and characterization of two polyurethanase enzymes from *Pseudomonas chlororaphis*. International Biodeterioration and Biodegradation **43,** 43-47.
http://dx.doi.org/10.1016/S0964-8305(98)00067-5

Sadocco P, Nocerino S, Dubini-Paglia E, Seves A, Elegir G. 1997. Characterization of a poly (3-hydroxybutyrate) depolymerase from

Aureobacterium saperdae: Active site and kinetics of hydrolysis studies. Journal of Environmental Polymer Degradation **5,** 57-65.

Santos M, Gangoiti J, Keul H, Möller M, Serra JL, Llama MJ. 2013. Polyester hydrolytic and synthetic activity catalyzed by the medium-chain-length poly(3-hydroxyalkanoate) depolymerase from *Streptomyces venezuelae* SO1. Applied Microbiology and Biotechnology **97,** 211-222.
http://dx.doi.org/10.1007/s00253-012-4210-1

Sanyal P, Samaddar P, Paul A. 2006. Degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by some soil *Aspergillus* spp. Journal of Polymer and Environment **14,** 257-263.
http://dx.doi.org/10.1007/s10924-006-0022-7

Schirmer A, Jendrossek D. 1994. Molecular characterization of the extracellular poly (3-hydroxyoctanoic acid)[P (3HO)] depolymerase gene of *Pseudomonas fluorescens* GK13 and of its gene product. Journal of Bacteriology **176,** 7065-7073.

Shah AA, Hasan F, Hameed A, Ahmed S. 2007a. Isolation and characterisation of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) degrading actinomycetes and purification of PHBV depolymerase from newly isolated *Streptoverticillium kashmirense* AF1. Annals of Microbiology **57,** 583-588.
http://dx.doi.org/10.1007/BF03175359

Shah AA, Hasan F, Hameed A, Ahmed S. 2007b. Isolation and characterization of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) degrading bacteria and purification of PHBV depolymerase from newly isolated *Bacillus* sp. AF3. International Biodeterioration and Biodegradation **60,** 109-115.
http://dx.doi.org/10.1016/j.ibiod.2007.01.00.4

Shivakumar S, Jagadish SJ, Zatakia H, Dutta J. 2011. Purification, characterization and kinetic studies of a novel poly (β) hydroxybutyrate (PHB) depolymerase PhaZ Pen from *Penicillium citrinum* S2. Applied Biochemistry and Biotechnology **164,** 1225-1236.
http://dx.doi.org/10.1007/s12010-011-9208-0

Sznajder A, Jendrossek D. 2011. Biochemical characterization of a new type of intracellular PHB depolymerase from *Rhodospirillum rubrum* with high hydrolytic activity on native PHB granules. Applied Microbiology and Biotechnology **89,** 1487-1495.
http://dx.doi.org/10.1007/s00253-011-3096-7

Wang Z, Gao J, Li L, Jiang H. 2012. Purification and characterization of an extracellular poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) depolymerase from *Acidovorax* sp. HB01. World Journal of Microbiology and Biotechnology **28,** 2395-2402.
http://dx.doi.org/10.1007/s11274-012-1048-8.

Zhou H, Wang Z, Chen S, Liu D, Xia H. 2008. Purification and characterization of extracellular poly(β-hydroxybutyrate) depolymerase from *Penicillium* sp. DS9701-D2. Polymer-Plastics Technology Engineering **48,** 58-63.
http://dx.doi.org/10.1080/03602550802539627