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PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*CO*-3-HYDROXYVALERATE) DEPOLYMERASE FROM *Aspergillus* sp. NA-25

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Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) degrading thermophilic fungus was isolated from soil sample collected from waste disposal site, Islamabad, Pakistan. It was able to grow efficiently on a medium containing PHBV as a sole source of carbon and has been identified as *Aspergillus* sp. NA-25 by 18S rRNA. Using 9% of inoculum maximum production of PHBV depolymerase was observed at 45°C, pH 7.0 in the presence of 0.2% lactose as an additional carbon source. PHBV depolymerase was purified by precipitation with 80% ammonium sulfate and gel filtration chromatography on Sephadex G-75. The four enzyme forms obtained after gel filtration were analyzed on SDS-PAGE and their molecular weights (36, 68, 72 and 90 kDa) were determined. They were characterized on the basis of effect of different temperatures, pH, metal ions and different reagents on the PHBV activity and stability. It is obvious that the fungal strain *Aspergillus* sp. NA-25 is capable of degrading PHBV with the help of different types of depolymerases.

Polyhydroxyalkanoates (PHAs) may form complex subcellular structures referred to as "carbonosomes", produced by microorganisms due to excess of carbon source [1]. They have gained much attention as they are a better replacement of fossil fuel polymers. These polymers have similar material properties to that of polyolens [2]. Microorganisms in isolated form or in consortia can degrade them easily [3]. The fascinating feature of these polymers is their complete degradation to carbon dioxide and water under aerobic conditions and into methane under anaerobic conditions. Natural PHA degraders of various bacterial species from soil and water include genera i.e. *Pseudomonas, Alcaligenes, Comamonas, Streptomyces, Ilyobacter* [4, 5], as well as fungi belonging to *Ascomycetes, Basidiomycetes, Deuteromycetes, Mastigiomycetes, Myxomycetes* [3].

At present, approximately 150 different constituents of PHAs have been identified as homopolymers or as copolymers [6]. The most important are polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV). PHBV is a copolymer of PHB, which contain 3-hydroxyvalerate monomers in the side chain. Because of the better mechanical performance of PHBV, it is used in making various products like films, compost bags, disposable food service ware and moulded products [7]. The PHBV molecules are large enough to be transported directly across the cell wall and, therefore, nature has provided bacterial and fungal species with the ability to degrade PHBV extracellularly. This ability is realized in the form of extracellular depolymerases, which convert these large polymers to its respective monomers i.e. 3-hydroxybutyrate and 3-hydroxyvalerate [8].

Among the microorganisms, fungi are considered to be appropriate candidates for the PHA degradation because they show fast surface growth rate and high depolymerase activity. Fungi belonging to *Aspergillus* species contribute considerably to PHA breakdown. Many thermotolerant strains of *Aspergillus fumigatus* that are capable of degrading PHB and PHBV at high temperatures (above 40°C) have been isolated from soil samples [9] and compost [10]. A number of researchers have isolated different strains of *A. fumigatus*, degrading PHA as a carbon source [10–13].

Many extracellular PHA depolymerases from prokaryotic and eukaryotic microorganisms have been purified and characterized. Scherer with coworkers [11] reported that the extracellular PHB depolymerase of *Aspergillus fumigatus* M2A isolated from compost had a molecular weight of 57 kDa whose activity was maximum at pH 8.0 and 70°C. It was revealed [14] that the extracellular PHB depolymerase of *Paecilomyces lilacinus* D218 isolated from soil had a molecular weight of 48 kDa and its optimum activation conditions were pH 7.0 and 45°C. Han and Kim [15] reported that the extracellular PHB depolymerase of *Penicillium simplicissimum* had a molecular weight of 36 kDa and activity at 45°C at pH 5.0. In another study, Shah et al. [16] purified two types of extracellular PHBV depolymerases from *Streptoverticillium kashmirense* AF1, isolated from municipal sewage sludge. They had molecular weights of 35 and 45 kDa and maximum activity at 45°C and at pH 7.0 and 8.0. PHA depolymerases purified from recombinant *Escherichia coli* appeared as 4 bands on SDS-PAGE with molecular masses 44, 46, 49, and 65 kDa. *Pseudomonas lemoignei* was found to have five PHB depolymerases [17].

The aim of the study was to isolate and identify PHBV degrading fungi from soil, and to purify and characterize the degradative enzymes.

MATERIALS AND METHODS

Material. Poly-3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid (PHBV) containing 5% 3-hydroxyvaleric acid (3-HV), was obtained in powder form from Sigma-Aldrich (Germany).

Sample collection. Soil samples were collected from waste disposal sites, Islamabad, Pakistan, and were used to screen for PHBV utilizing microorganisms.

PHBV-agar plates. PHBV-degrading microorganisms were isolated from soil, by inoculating on mineral salt agar [17] supplemented with 0.2% (w/v) of PHBV as a sole source of carbon source. PHBV suspension was prepared by sonicating PHBV 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 PHBV suspension and poured into petri plates for the clear-zone assay.

Isolation of PHBV degrading microorganisms. The PHBV degrading microorganisms were isolated through enrichment technique. The soil sample (1.0 g) was added to Erlenmeyer flask containing 100 ml of MSM containing 0.2% PHBV (pH 7.0) as the sole carbon source and incubated at 30°C with shaking (150 rpm/min). MSM contained (g/l): K_2HPO_4 – 0.5; KH_2PO_4 – 0.04; NaCl – 0.1; $CaCl_2 \cdot H_2O$ – 0.002; $MgSO_4 \cdot 7H_2O$ – 0.02; $(NH)_2SO_4$ – 0.2 and $FeSO_4 \cdot \cdot 7H_2O$ – 0.001. After a week, 0.5 ml of this culture broth was transferred into a fresh MSM supplemented with PHBV. This step for re-culturing into fresh medium was repeated 5 times. At the end of the experiment, the culture was spread on PHBV emulsified mineral salt agar plates for single-colony isolation.

Identification of PHBV degrading microorganism. A fungal strain NA-25 was isolated on the basis of clear zone around its growth on PHBV emulsified mineral salt agar plates. It was identified by macroscopic (conidial and mycelial color) and microscopic (seriation, vesicle, conidia and ascospores) examination and also through 18S rRNA sequencing.

18S rRNA sequencing procedure. 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) at the Macrogen (Korea).

Preparation of spore suspension. Spore suspension was prepared by adding autoclaved 0.9% NaCl solution directly on to sporulating fungal colonies grown on Petri plates, and then spore suspension was collected under aseptic conditions in screw caped tubes. The spores were counted as 2.3×10^5 cells/ml, with the help of hemocytometer (424027 – BD BBL™ Hemacytometer Cover Glass, USA).

Optimization of various factors for the production of PHBV depolymerase. PHBV depolymerase production was observed at different temperatures (30, 37, 45, 50, 55°C), pH ranges (3.0–9.0), substrate concentrations (0.1–0.5% w/v), 1% carbon sources (glucose, fructose, lactose, sucrose) and size of inoculum (1, 3, 5, 7, 9, 11%). For these experiments, PHBV was emulsified in MSM; the suspension was prepared by sonicating the mixture for 30 min at 90 duty cycles using a Branson sonifier (Branson Ultrasonic Cooperation, USA).

PHBV depolymerase assay. PHBV depolymerase activity was determined by the method described by Kobayashi et al. [18]. PHBV was taken in a concentration of 0.03% in 50 mM Tris-HCl, pH 8.0. This suspension was sonicated for 30 min in Branson sonifier (Branson Ultrasonic Cooperation, USA). After centrifugation at 10000 g for 10 min, 0.1 ml of culture supernatant was added to 0.9 and the mixture was incubated for 24 h at 30°C. Activity was measured as the decrease in turbidity of PHBV suspension at OD_{650} against blanks (substrate + buffer). One unit of activity is defined as the activity resulting in a decrease in OD at 650 nm per 24 h.

Protein estimation. The protein determination was done by using the method described by Lowry et al.

Purification of PHBV depolymerase. The fungal strain was grown in liquid MSM containing PHBV as a carbon source under optimized culture conditions at 30°C and 150 rpm for 2 days.

Ammonium sulfate precipitation. The culture was centrifuged at 10,000 g for 30 min at 4°C. To precipitate the protein, cell free extract was subjected to increasing concentrations (10–80%) of ammonium sulfate. The material was sedimented at 10,000 g, for 20 min at 0°C. The pellet was dissolved in minimum amount of 0.1 M phosphate buffer, pH 7.0, and kept at –20°C.

Gel permeation chromatography (*Sephadex G-75*). About 2 ml of crude enzyme was loaded in the Sephadex G-75 column and eluted by using the same phosphate buffer. Fractions showing maximum enzyme activity were pooled, lyophilized and used for SDS-

PAGE analysis on a 12% polyacrylamide gel according to the method of Laemmli [19]. The samples were run along with the standard protein markers of 26–170 kDa (Fermentas, USA). The gel was stained with silver staining kit (Bio-Rad, USA) and was visualized on gel documentation system (Bio-Rad, USA).

Characterization of purified PHBV depolymerases. The purified enzymes were characterized on the basis of effect of temperature, pH, metal ions and inhibitors on enzyme activity and stability.

Effect of temperature on the activity of purified PHBV depolymerases. The residual PHBV depolymerase activity was determined after incubating the purified enzyme for 1 h at 25, 35, 45, 55, 60 and 70°C at pH 7.0. The percentage stability was also calculated.

Effect of pH on the activity of purified PHBV depolymerases. The PHBV depolymerase activity was measured by incubating the purified enzyme forms in the presence of buffers of various pH (4.0–9.0) for 1 h at 37°C and residual enzyme activity was calculated. Buffer solutions (20 mM) of different pH values were used: acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–7.0), Tris- HCl (pH 8.0) and glycine- NaOH buffer (pH 9.0).

Effect of metal ions on the activity of purified PHBV depolymerases. The effects of various metal ions on the activity of the purified PHBV depolymerase were examined by assaying the remaining activity after incubating the enzymes with 1 mM metal ions (chloride and sulphate salts of the metal ions included Na^+, Ca^{2+}, Mg^{2+}, Co^{2+}, and Zi^{2+}) for 1 h at 37°C at pH 7.0.

Effect of reagents influencing enzyme activity. Effect of 1 mM inhibitors (EDTA, β-mercaptoethanol, SDS) was determined after incubation with the PHBV depolymerase for 1 h at 37°C at pH 7.0 and determining the residual activities.

RESULTS AND DISCUSSION

Isolation and identification of PHBV degrading microorganism. A fungal strain was isolated from waste disposal site by enrichment and plate assay techniques. The sequencing result of microorganism named strain NA-25 showed a total of 967 bp nucleotides of 18S rRNA. The resultant sequence was matched with those in the NCBI GeneBank and Ribosomal Database Project (RDP). The blast analysis of the 18S rRNA sequence revealed that the strain NA-25 belongs to genus *Aspergillus* having 100% similarities with *Aspergillus* sp. DX12 with accession number GU726139.1.678. The polymer-mineral salt agar plates were used to determine the PHBV degradation by this fungus. The results showed that *Aspergillus* sp. NA-25 was capable of degrading PHBV as a carbon source by producing clear zones of hydrolysis. *Aspergillus* species are considered as predominant PHA-degraders [20]. 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 [21]. Recently, Shah et al. [16] isolated an *Actinomycetes* strain i.e. *Streptoverticillium kashmirense* AF1, from sewage sludge capable of degrading PHBV and Ghanem *et al.* [22] isolated PHBV and PHB degrading *Nocardiopsis aegyptia* from marine seashore environment.

Production of PHBV depolymerase by *Aspergillus* sp. NA-25. Different factors (temperature, pH, substrate concentration, different carbon sources and inoculum size) influence the production of PHBV depolymerases. These environmental factors had a drastic effect on the degradation of polymer [23]. The maximum degradation of PHBV by *Aspergillus sp.* NA-25 was found at temperature 45°C (6.70 U/mg). Thermotolerant *Aspergillus* sp. ST-01, *Streptomyces* MG and *Bacillus* TT96 isolated from soil were able to degrade polycaprolactone (PCL), PHB and poly(butylene succinate-co-adipate) at 50°C after 144 h of incubation [24]. Many thermotolerant *A. fumigatus* strains that are capable of degrading PHB and PHBV at high temperatures (above 40°C) have been isolated from soil samples [12] and compost [10]. Phithakrotchanakoon et al. [25] isolated 5 bacterial species, degrading PHA's at high temperature.

The influxes of H^+ and OH^- ions have a drastic impact on the production of degradation enzymes. *Aspergillus* NA-25 was capable of degrading PHBV at vast range of pH but the maximum degradation occured at neutral pH (7.22 U/mg). Phithakrotchanakoon et al. [25] isolated a *Streptomyces* sp. BCC23167 showing degradation of several biopolymers including PHA copolymers, PCL and polybutylene succinate at neutral pH. A fungus *Paecilomyces lilacinus* F4-5 showed maximum enzyme production at pH 7.0 for PHB and PHBV depolymerases [26].

The substrate concentration plays an important role because it suppresses the activity of enzyme [27]. *Aspergillus* sp. NA-25 had maximum depolymerase production in the presence of 0.2% of PHBV (6.39 U/mg). Elbanna et al. [28] used 0.2% PHB for the production of PHB depolymerase from *Schlegelella thermodepolymerans*. Increase in concentration of PHBV decreased the production of depolymerases. The decrease in enzyme production at higher concentration of polymer in growth medium seems to be due to saturation of extracellular depolymerase by the substrate in the immediate vicinity of the microbial growth. PHA degrading activity is known to be regulated by simple carbon substrates. We have found lactose as the best carbon source, which enhanced enzyme production (8.62 U/mg). Regulation of PHA degrading activity generally acts through a catabolite repression mechanism [25] and lactose can participate in this process. According to Manna and Paul [27], degradation of PHB by bacteria isolated from soil and sewage sludge, was affected significantly when the PHB-containing medium was supplemented with easily consumable carbon sources.

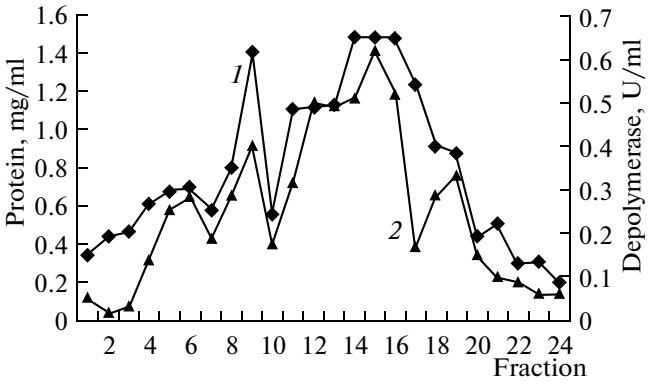

Fig. 1. Purification of PHBV depolymerase from *Aspergillus* sp. NA-25 by gel permeation chromatography on Sephadex G-75. *1* – protein; *2* – enzyme activity.

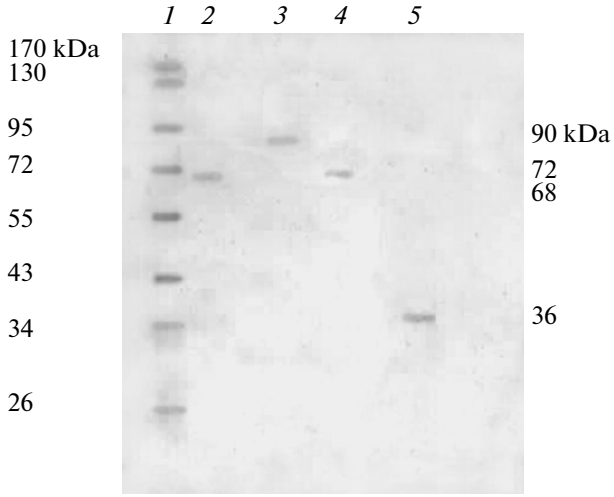

Fig. 2. SDS-PAGE of the puried PHBV depolymerase (E) from *Aspergillus* sp. NA-25. *1* – molecular mass standards; *2* – E1; *3* – E2; *4* – E3; *5* – E4.

The utilization of the nutrients is largely dependent on the population of microorganisms. To ensure a high production of enzyme in the limited volume of medium, the inoculum size should be controlled [29]. Maximum production of PHBV depolymerase from *Aspergillus* sp. NA-25 (13.18 U/mg) was observed in the presence of 9% of inoculum. In these conditions the incubation time decreased from 120 h (5% inoculum) to 48 h.

Purification of PHBV depolymerase from *Aspergillus* sp. NA-25. *Precipitation of PHBV depolymerase.* To purify PHBV depolymerase from *Aspergillus* sp. NA-25 precipitation by 80% of ammonium sulphate was made. Shah *et al.* [16, 30] had used the same procedure for the purification of PHBV depolymerases from *Streptoverticillium kashmirense* AF1 [16] and *Bacillus* sp. AF3 [30].

Gel permeation chromatography. It is obvious that 4 peaks of PHBV depolymerase activity obtained by gel filtration represent different forms of the enzyme (Fig. 1). Sephadex G-75 was also used for PHBV depolymerase purification and one peak was obtained from *Bacillus* sp. AF3 [35] but two peaks from *Streptoverticillium kashmirense* AF1 [16]. Sephadex G-100 was used for purification of PHB depolymerase from fungus *Emericellopsis minima* W2 [9]. Kim et al. [31] used Sephadex G-150 for PHA depolymerase purification from *Streptomyces* sp. KJ-72.

SDS-PAGE. The purified forms of the enzyme were further analyzed by SDS-PAGE showing 4 bands with molecular weights of 68, 90, 72 and 36 kDa, respectively (Fig. 2). It can be assumed that 4 forms of PHBV depolymerase from *Aspergillus* sp. NA-25 are encoded by 4 different genes, which can be further sequenced. Scherer et al. [11] purified PHB depolymerase from *A. fumigatus* M2A having molecular mass 57 kDa. PHBV depolymerase isolated from a fungus *Paecilomyces lilacinus* F4-5 [26] had molecular weight 45 kDa. PHB depolymerases purified from a fungus *Penicillium simplicissimum* LAR13 [15] and thermophilic bacterium *Thermus thermophilus* HB8 [37] had molecular weights 36 kDa and 42 kDa, respectively. Shah et al. [16] purified two PHBV depolymerases i.e. 35 and 45 kDa, from *Streptoverticillium kashmirense* AF1. The *Pseudomonas lemoignei* enzyme had at least 5 forms, which differ slightly in their biochemical properties [17].

Characterization of purified forms of PHBV depolymerases. *Effect of temperature on the activity of the purified PHBV depolymerase forms.* After incubation at 70°C for 1 h the residual activity of the two enzyme forms from *Aspergillus* sp. NA-25, E2 and E1, was 99.16% and 86%, respectively (Fig. 3a). PHB depolymerase, isolated from *T. thermophilus* HB8 showed 75% of stability at 60°C and 50% at 70°C after 4 h of incubation [32]. Scherer et al. [11] isolated a PHB depolymerase from *A. fumigatus* M2A, showing maximal stability at 70°C. The PHB depolymerase of *Penicillium simplicissimum* LAR13 remained stable at 45°C for 0.5 h [15]. The PHB depolymerases from *Comamonas acidovorans* YM1609 [33] and *Paecilomyces lilacinus* F4-5 [26] were stable for 30 min at temperature below 37 °C and 40°C, respectively.

Effect of pH on the activity of purified PHBV depolymerase forms. The residual activity of all enzyme forms from *Aspergillus* sp. NA-25 was 100% at pH 7.0 while decreased of it above or below neutral pH (Fig. 3b). The PHA depolymerase of *Pseudomonas alcaligenes* showed over 60% of its maximum activity in the pH range 7.0 to 9.5 [34]. The purified enzyme of *T. thermophilus* HB8 showed stability at pH 8.0 [32]. In another work, the depolymerase from *Pseudomonas indica* K2 showed activity at a wide range of pH (6.0–10.5) but the maximal stability was found at pH 8.2 [28].

Effect of metal ions on activity of PHBV depolymerase forms. Different types of metal ions were added at a concentration of 1.0 mM to incubation mixture

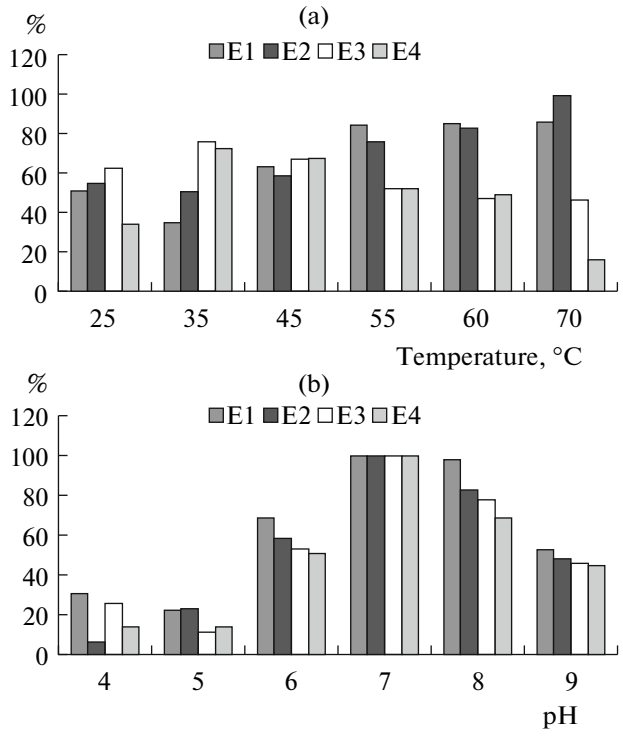

Fig. 3. Effect of temperature (a) and pH (b) on the activity of the purified PHBV depolymerase from *Aspergillus* sp. NA-25.

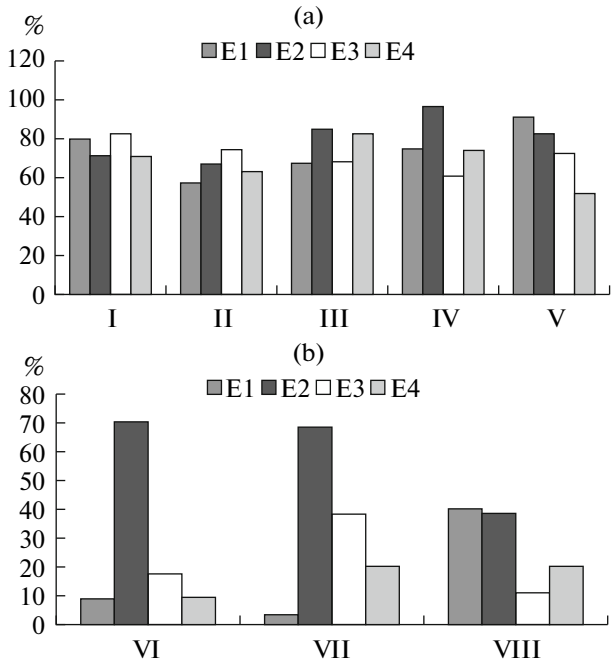

Fig. 4. Effect of metal ions (a) and reagents (b) on the activity of the purified PHBV depolymerase (E) from *Aspergillus* sp. NA-25. *1–4* – E1–E4; I – Na^{+}, II – Ca^{+2}; III – Zn^{+2}, IV – Mg^{+2}, V – Co^{+2}, VI – EDTA, VII – 2-mercaptoethanol, VIII – SDS.

and the activities of all the purified enzyme forms were detected. Their residual activities were less in the presence of metal ions except E1 and E2 which showed 91% and 97% of residual activity in the presence of Co^{2+} and Mg^{2+}, respectively (Fig. 4a). Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+} had a negligible effect on the activity of *P. simplicissimum* LAR13 [19]. Kobayashi et al. [18] observed that the activity of the PHB depolymerase from *Agrobacterium* sp. and was nearly independent of Co^{2+}, Mg^{2+}. The PHB depolymerase from *P. lilacinus* was 80% deactivated by Zn^{2+}, Mn^{2+} and Cu^{2+} [14]. Na^{+} and K^{+} and Mg^{2+} caused inhibitory effect on the PHB depolymerase of *T. thermophilus* HB8, when they were used in high concentration i.e. 5 and 10 mM [37]. 5 mM Zn^{2+} and 5 mM Mg^{2+} had also inhibitory effect on the PHB depolymerase of *Penicillium* sp. DS9701-D2 [35].

Effect of reagents influencing the activity of purified PHBV depolymerase. In our study, all enzyme forms were strongly inhibited by reagents such as EDTA, SDS and 2-mercaptoethanol except form E2, which was partially inhibited by EDTA and 2-mercaptoethanol (Fig. 4b). The PHB depolymerase of *Schlegelella thermodepolymerans* was also partially inhibited by EDTA. The recombinant PHB depolymerases of *Escherichia coli* were totally inhibited, when the enzymes were pre-incubated for 5 min in 3 mM EDTA [17]. The extracellular PHB depolymerase of *T. thermophilus* HB8 was greatly inhibited by EDTA and 2-mercaptoethanol [32]. These facts indicate that this enzyme belongs to the serine hydrolase family like other PHA depolymerases because the enzyme requires some metal ions and disulfide bonds, which play an essential role in enzyme activity [17].

The present study shows that the *Aspergillus* NA-25 has the ability to produce the extracellular PHBV depolymerase that degrades PHBV. The enzyme forms produced by the *Aspergillus* NA-25 are robust with the ability to perform at a wide range of temperature, pH and in the presence of different metal ions and protein inhibitors. The application of specific plastic degrading enzymes is highly advantageous where pure monomers can be recovered from the mixed plastic wastes without distinction. The results imply that the PHA can be applied to enzymatic monomer recycling using specific microbial enzymes.

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REFERENCES

1. Jendrossek, D., *J. Bacteriol.,* 2009, vol. 191, pp. 3195–3202.

2. Reddy, C.S., Ghai, R., Rashmi, and Kalia, V.C., *Bioresour. Technol.,* 2003, vol. 87, pp. 137–146.
3. Jendrossek, D. and Handrick, R., *Ann. Rev. Microbiol.*, 2002, vol. 56, pp. 403–432.
4. Müller, R.J., Kleeberg, I., and Deckwer, W.D., *J. Biotechnol.*, 2001, vol. 86, pp. 87–95.
5. Sudesh, K., Abe, H., and Doi, Y., *Prog. Polym. Sci.*, 2000, vol. 25, pp. 1503–1555.
6. Steinbüchel, A. and Lutke-Eversloh, T., *Biochem. Eng. J.*, 2003, vol. 16, pp. 81–96.
7. Khanna, S. and Srivastava, A.K., *Process Biochem.* 2005, vol. 40, pp. 607–619.
8. Luzier, W.D., *Proc. Natl. Acad. Sci. USA,* 1992, vol. 89, pp. 839–42.
9. Kim, D.Y., Yun, J.H., Kim, H.W., Bae, K.S., and Rhee, Y.H., *J. Microbiol.*, 2002, vol. 40, pp. 129–133.
10. Mergaert, J., Wouters, A., and Swings, J., *Syst. Appl. Microbiol.*, 1994, vol. 17, pp. 601–612.
11. Scherer, T.M., Fuller, R.C., Lenz, R.W., and Goodwin, S., *Polym. Degrad. Stab.*, 1999, vol. 64, pp. 267–275.
12. Kim, M.N., Lee, A.R., Yoon, J.S., and Chin, I.J., *Europ. Polym J.,* 2000, vol. 36, pp. 1677–1685.
13. Iyer, S., Shah, R., Sharma, A., Jendrossek, D., and Desai, A., *J. Polym. Environ.*, 2000, vol. 8, pp. 197–203.
14. Oda, Y., Osaka, H., Urakami, T., and Tonomura, K., *Curr. Microbiol.,* 1997, vol. 34, pp. 230–232.
15. *Han, J.* and Kim, M., *J. Microbiol.*, 2002, vol. 1, pp. 20–25.
16. Shah, A.A., Hasan, F., Hameed, A., and Safia, A., *Ann. Microbiol.,* 2007, vol. 57, no. 4, pp. 583–588.
17. Jendrossek, D., Frisse, A., Beherends, A., Andermann, M., Kratzin, H.D., Stanislawskit, T., and Schlegel, H.G., *J. Bacteriol.,* 1995, vol. 177, pp. 596-607.
18. Kobayashi, T., Sugiyama, A., Kawase, Y., Saito, T., Mergaert, J., and Swings, J., *J. Environ. Polym. Degrad.*, 1999, vol. 7, pp. 9–17.
19. Laemmli, U.K., *Nature* 1970, vol. 227, pp. 680–685.
20. Matavulj, N. and Molitoris, H.P., *FEMS Microbiol. Rev.*, 1992, vol. 103, pp. 323–332.
21. Puspita, S., Parama, S., and Paul, A.K. *J. Polymer. Environ.*, 2006, vol. 14, pp. 257–263.
22. Ghanem, N.B., Mabrouk, M.E., Sabry, S.A., and El-Baden, D.E., *J. Gen. Appl. Microbiol.,* 2005, vol. 51, pp. 151–158.
23. Abou-Zeid, D.M., Müller, R.J., and Deckwer, W.D., *J. Biotechnol.,* 2001, vol. 86, pp. 113–126.
24. *Tokiwa, Y.* and Calabia, B.P., *Biotechnol. Lett.*, 2004, vol. 26, pp. 1181–1189.
25. Phithakrotchanakoon, C., Rudeekit, Y., Tanapongpipat, S., Leejakpai, T., Aiba, S., Noda, I., and Champreda, V., *Biologia,* 2009, vol. 64, pp. 246–251.
26. Sang, B.I., Lee, W.L., Hori, K., and Unno, H., *World J. Microbiol. Biotechnol.*, 2006, vol. 22, pp. 51–57.
27. Manna, A. and Paul, A.K., *Biodegradation*, 2000, vol. 11, pp. 323–329.
28. Elbanna, K., Lütke-Eversloh, T., Jendrossek, D., Luftmann, H., and Steinbüchel, A., *Arch. Microbiol.*, 2004, vol.182, pp. 212–224.
29. Abusham, R.A., Raja, N.Z., Rahman, R.A., Abu, B.S., and Mahiran, B., *Microb. Cell Fact.,* 2009, vol. 8, pp. 20–28.
30. Shah, A.A., Hasan, F., Hameed, A., and Safia, A., *Int. Biodeterior. Biodegrad.* 2007, vol. 60, pp. 109–115.
31. Kim, D.Y. and Rhee, Y.H., *Appl. Microbiol. Biotechnol.,* 2003, vol. 61, pp. 300–308.
32. Papaneophytou, C.P., Pantazaki, A.A., and Dimitrios, A., *Appl. Microbiol. Biotechnol.*, 2009, vol. 83, pp. 659–668.
33. Kasuya, K., Inoue, Y., Tanaka, T., Akehata, T., Iwata, T., Fukui, T., and Doi, Y., *Appl. Environ. Microbiol.,* 1997, vol. 63, pp. 4844–4852.
34. Kim, D.Y., Kim, H.C., Kim, S.Y., and Rhee, Y.H., *J. Microbiol.* 2005, vol. 43, pp. 285–294.
35. Zhou, H., Wang, Z., Chen, S., Liu, D., and Xia, H., *Polym. Plast Technol. Eng.,* 2009, vol. 48, pp. 58–63.