Research Article

Kulsoom Bano, Mohammed Kuddus, Mohd Rehan Zaheer and Roohi*

A novel, thermotolerant, extracellular PHB depolymerase producer *Paenibacillus alvei* PHB28 for bioremediation of biodegradable plastics

Biyobozunur plastiklerin biyoremediasyonu için yeni bir ısıya toleranslı ekstraselüler PHB depolimeraz üreticisi *Paenibacillus alvei* PHB28

<https://doi.org/10.1515/tjb-2018-0207>

Received May 25, 2018; accepted September 4, 2018; previously published online December 5, 2018

Abstract

Background: Poly-β-hydroxybutyrate (PHB) is the most important and versatile class of biodegradable polymers used successfully in the medical, agricultural and industrial field. Idea is to find the novel isolate for degradation of biodegradable plastics that can enhance the bioremediation.

Materials and methods: Thirty-one PHB and PHB depolymerase enzyme producing isolates out of 80 mesophilic bacteria from Lucknow region were further screened for PHB degradation capability by secreting extracellular PHB depolymerase enzyme in minimal salt media supplemented with PHB (0.15%). Various biodegradable plastic films were tested by soil burial method for weight loss determination.

Result: 37.3% weight loss has been observed in PHB films when buried under the soil for 45 days in the presence of a novel PHB degrader identified as *Paenibacillus alvei* PHB28 by 16S rRNA sequencing (GenBank accession number KX886342). These Gram-negative, spore-forming, thermotolerant bacteria produce maximum PHB depolymerase

(5.03 U/mL) at 45°C, pH 8.0, with 0.15% substrate concentration when incubated for 96 h with starch (0.1%) and yeast extract (0.01%) as an additional nutrient supplements.

Conclusion: To the best of our knowledge this is the first report of PHB depolymerase production by *P. alvei* PHB28 which may contribute successfully to combat plastic pollution and to sustain the green environment.

Keywords: Biodegradation; Poly-β-hydroxybutyrate; PHB depolymerase; Optimization; Bioremediation.

Öz

Amaç: Poli-β-Hidroksibutirat (PHB), tıbbi, tarımsal ve endüstriyel alanda başarıyla kullanılan, biyolojik olarak çözünebilen polimerlerin en önemli ve çok çeşitliliğe sahip sınıfıdır. Amaç, biyobozunur plastiklerin bozunması için biyoremediasyonu arttıracak yeni bir izolat bulmaktır.

Gereç ve Yöntem: Lucknow bölgesinden 80 mezofilik bakteriden elde edilen otuz bir PHB ve PHB depolimeraz enzimi üreten izolat, PHB (%0.15) ile desteklenmiş minimal tuz ortamında hücre dışına PHB depolimeraz enzimi salgılayarak PHB'yi bozundurma kabiliyeti açısından tarandı. Çeşitli biyobozunur plastik filmler, kilo kaybı tespiti için toprağa gömme metodu ile test edildi.

Bulgular: PHB filmleri 16S rRNA dizilemesine göre *Paenibacillus alvei* PHB28 olarak tanımlanan yeni bir PHB indirgeyici varlığında (GenBank erişim numarası KX886342) toprağın altında 45 gün süreyle gömüldüğünde %37.3 ağırlık kaybı gözlemlenmiştir. Bu Gram-negatif, spor oluşturan, termotolerant bakteriler, 45°C'de pH 8.0'de, % 0.15 substrat konsantrasyonu ile, ek besin takviyesi olarak nişasta (% 0.1) ve maya ekstraktı (%96) ile inkübe edildiğinde maksimum PHB depolimeraz (5.03 U/mL) üretmektedir.

*Corresponding author: Dr. Roohi, Protein Research Laboratory, Department of Bioengineering, Integral University, Lucknow, India, Phone: 09450358872, Fax: +91 5222890809, e-mail: roohi0607@gmail.com. <https://orcid.org/0000-0003-4296-6105>

Kulsoom Bano: Protein Research Laboratory, Department of Bioengineering, Integral University, Lucknow, India

Mohammed Kuddus: Department of Biochemistry, University of Hail, Hail, Kingdom of Saudi Arabia

Mohd Rehan Zaheer: Department of Chemistry, Gagan College of Management and Technology, Aligarh, U.P., India

Sonuç: Bildiğimiz kadarıyla bu, plastik kirliliğe karşı mücadeleye ve yeşil çevreyi sürdürmeye başarıyla katkıda bulunabilecek olan PHB depolimerazın *Paenibacillus alvei* PHB28 tarafından üretimine dair ilk rapordur.

Anahtar Kelimeler: Biyodegradasyon; Poli- β -Hidroksibütirat; PHB depolimerase; Optimizasyon; Biyoremediasyon.

Introduction

The intractable non-biodegradable synthetic waste [1] have led to the replacement of petrochemical-derived polyester with the production of biodegradable and eco-friendly plastic polymers. Biodegradable but water-insoluble polyesters such as poly-hydroxyalkanoate (PHA) can be originated by living microorganisms with the supplement of organic carbon source in rich amounts as an energy reservoir and intracellular storage granule. Among numerous PHA components, poly- β -hydroxybutyrate (PHB) have been investigated with a high degree of interest for commercial applications due to their unusual properties. These insoluble, semi-crystalline, denatured granules are degraded by extracellular PHB depolymerase enzymes [2].

PHB is fragmented into monomer in nutrient-limiting condition by the effect of PHB depolymerase [3]. PHB degrading microorganisms have been isolated earlier from diverse ecological aspects such as soil, water, hot spring, sea water, sewage sludge and waste effluent and agro-waste [3–8]. PHB degrading activity of PHB depolymerase has been recently reported from *Streptomyces ascomycinicus*, *Stenotrophomonas* sp. RZS7, *Streptomyces* sp. MG and *Paucimonas lemoignei* [2, 9–11]. Despite this fact, degradation of biopolymers by the microorganisms in the environment is initiated by the surface erosion and/or adsorption on the polymer surface. The rate of biodegradation is highly influenced by several changing environmental factors such as temperature, pH, moisture, alkalinity, availability of carbon and nitrogen source and heavy metal ions within the soil.

The purpose of the present study was to screen and isolate novel potential PHB depolymerase producing bacteria from waste, polluted soil that is able to perform efficient biodegradation of biodegradable plastics. The study was further extended for its production optimization input that can meet the industrial demand for enzyme utilization.

Materials and methods

Isolation of poly β -hydroxybutyrate(PHB) producing bacteria

Collection of sample

Samples were collected from different plastic polluted enriched ecological niches of Lucknow region (India) viz potato storage soil, river water, and river soil, sewage sludge, wastewater, decomposing vegetable soil. The samples were collected from 2 to 4 cm depth of the soil with a sterile spatula in sterile poly-bags while the water samples were collected in falcon tubes. The collected samples were stored at -20°C . The soil and water samples were serially diluted in sterile distilled water, followed by plating on nutrient agar medium with 1% glucose. The plates were then incubated at $35 \pm 2^{\circ}\text{C}$ for 2–7 days and the colonies with distinct morphological features were selected for further studies. For rapid detection and isolation of PHB producing bacteria, 0.02% alcohol solution of Sudan black B staining viable colony method was used [12].

Source of poly β -hydroxybutyrate (PHB) polymer

The PHB powder used in this study was purchased from Sigma-Aldrich Chemicals, Germany. The molecular weight of PHB was 470 KDa. All the experiments were performed using PHB powder.

Preparation of PHB film

PHB film was prepared by dissolving 0.3 g of PHB powder in 30 mL of chloroform with magnetic stirring for 20 min and fancied into a film by pouring into clean sterilized glass petriplate of 10 cm diameter and then chloroform was vaporized slowly. The complete plastination resulted in the formation of polymer films and allowed to stand for a further 24 h until their weights had established in the air [13].

Soil burial method for isolation of PHB degrading bacteria

The weight of each experimental piece of polymer films Polypropylene (P1), Polyethylene (P2, P3), Polystyrene (P4) PHB (P5), before and after treatment is calculated

from the change in the weight for each plastic film. To screen positive strains, aliphatic PHB polyester films were buried in soil for different incubation time period (15 days, 30 days and 45 days) at ambient temperature (35–37°C). The polymer film of about 5.0×6.0 cm with an initial weight of 0.1 g was buried in pots containing about 150 g of garden soil at room temperature amended with a mineral solution to maintain the availability of mineral salts and the moisture content was maintained around 60%. The films were hanged with thread for easy follow up.

After different incubation periods, the pre-weighed films were removed from the soil medium, brushed softly, washed with distilled water several times to clean off the soil particles then dried at room temperature [13];

$$\text{Degradation (\%)} = [(W_1 - W_2) / W_1] 100$$

W_1 = initial weight of films, W_2 = final weight of buried films.

The polymer film showing maximum weight loss after doing this experiment was further analyzed by scanning electron microscopy (SEM).

The surface morphology of soil buried film via scanning electron microscopy (SEM)

The surface properties of soil buried film were investigated via SEM. Polymer film, which had been in the soil for the different incubation period were primarily fixed in 4% (v/v) glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2. Following fixation in glutaraldehyde, the polymer was post-fixed in 20 g/L aqueous osmium tetroxide and examined under SEM after dried to critical point [14].

Screening of PHB depolymerase producing bacteria

Extracellular degradation of PHB on solid medium by PHB depolymerase

The extracellular degradation of PHB by isolated strain on solid surface medium was done with clear zone method [15]. The minimal salt media (MSM) of the following composition was prepared in g/L: NaCl-10, NH_4Cl -1, CaCl_2 -0.015, yeast extract-0.1, K_2HPO_4 -1.6, KH_2PO_4 -0.2, MgSO_4 -0.123 supplemented with 0.15% PHB as carbon source and 6 mL of a trace element solution prepared as per the following concentration per liter (H_3PO_4 -1.96 mg, FeSO_4 -56 mg, ZnSO_4 -29 mg, MnSO_4 -22 mg, CuSO_4 -2.5 mg,

$\text{Co(NO}_3)_2$ -3 mg, H_3BO_3 -6 mg) [16]. PHB suspension was prepared by sonicating PHB powder in the mineral salt medium for 15 min in an ultrasonic water bath [17]. The solid plate was prepared with the addition of 2.0% agar in the PHB suspension [18]. The plate was incubated for 96–144 h to screen out the extracellular PHB degrading bacteria from the PHB producing positive isolates by the formation of the clear zone.

Identification of bacterial isolate

The morphological identification of isolated bacterium was done by growing on MSM. The various morphological, biochemical and physiological characteristics of the isolate was determined by using Bergey's Manual of Determinative Bacteriology [19]. The identity was further confirmed by 16S rRNA sequencing.

Phylogenetic analysis by 16S rRNA sequencing

The 16S rRNA sequence analysis of isolate was carried out from Eurofins Genomics India Pvt. Ltd, Bangalore, India. Amplification was performed by using the forward primer sequence 5' AGAGTTTGATCMTGGCTCAG 3'. The amplified sequence was analyzed with the nBLAST run and the evolutionary tree was further computed by using neighbor-joining method with Molecular Evolutionary Genetics Analysis software version 7.0.21 (www.ncbi.nlm.nih.gov).

PHB depolymerase enzyme assay

For the determination of PHB depolymerase activity of the crude or purified enzyme, a stable suspension of PHB in 50 mM Tris-HCl buffer solution, pH 7.0 was prepared by sonication at 20 kHz for 10 min [20]. The assay was performed by the modified method of Jendrossek et al. [18]. The standard reaction mixture contained 0.9 mL of 50 mM Tris-HCl buffer (pH 8.0) suspended with PHB powder (150 µg/mL). The reaction was started by the addition of the crude enzyme (0.1 mL) and was incubated for 20 min at 30°C. The activity was arrested by the addition of 0.1N HCl solution to the reaction mixture. The enzyme activity was measured as a decrease in PHB turbidity by spectrophotometer at 650 nm. One unit of PHB depolymerase activity was defined as the amount of enzyme that was required to decrease the OD_{650} by 0.001 mL/min [9].

Optimization of PHB depolymerase production from *P. alvei* strain PHB 28

Effect of the incubation period and growth kinetics

The optimum incubation period for PHB depolymerase enzyme production by *P. alvei* strain PHB 28 was determined by inoculating the MS medium (0.1 mL of inoculated growth medium with CFU of $2 \times 10^7/\text{mL}$) for the different time interval (24–192 h) at 120 rpm. The enzyme activity was assayed after every 24 h of incubation for the next consecutive 8 days by standard assay procedure.

Effect of incubation temperature and pH

The temperature optima of the production medium were measured at different incubation temperatures such as 15°, 25°, 35°, 45° and 55°C for 96 h. The pH optima of the production medium were obtained by incubating the inoculated PHB containing MS media with different pH range (4.0–12.0) on a rotatory shaker at optimum growth temperature for the optimum incubation period. The activity was measured as per the standard assay procedure.

Effect of carbon/nitrogen sources on enzyme activity

To optimize the effect of carbon and nitrogen sources on enzyme production, optimized media was supplied with different carbon sources by replacing PHB as glucose, fructose, lactose, mannitol, starch and sucrose with 0.1% concentration in optimized parameters. For effect of inorganic (ammonium sulfate, ammonium nitrate, ammonium chloride) and organic nitrogen sources (urea, peptone and tryptone), enzyme production was optimized at 0.01% concentration in MS medium by replacing yeast extract (in control). The activity was measured as per the standard assay procedure.

Effect of substrate concentration on enzyme activity

Different concentration of PHB substrate was incorporated in the optimized medium ranging from 0.05 to 0.25% for continuous 192 h to optimize enzyme production from *P. alvei* strain PHB28. The activity was measured as per the standard assay procedure in all pre-optimized conditions.

Effect of heavy metal ions on PHB depolymerase production by *P. alvei* PHB28

To study the effect of heavy metal ions on the PHB depolymerase production from *P. alvei* PHB28, various metal ions (Mg^{+2} , Zn^{+2} , Cu^{2+} , Hg^{2+} , Co^{2+} , Fe^{+3} , K^{+}) in their chloride form were assayed in the concentration of 10 mM for 96 h of incubation. The activity was measured as per the standard assay procedure in all pre-optimized conditions.

Results and discussion

PHB producing microbes

Among the soil samples collected from different locations, maximum PHB producing bacteria were observed in river soil and wastewater (Table 1). Total 80 bacterial isolates were screened for natural production of PHB. On the basis of black-blue coloration, when stained with Sudan black B (preliminary screening agent for lipophilic compounds), 26 positive isolates with distinct morphology on nutrient agar were randomly selected for having the capability of extracellular degradation on PHB enriched minimal salt medium. Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black (Figure 1). As reported by Getachew and Woldesenbet [21] a potent PHB producer *Bacillus* sp., was isolated at 37°C from municipal sewage [22]. Hungund et al. [23] has reported the isolation of PHB producing *Paenibacillus durus*

Table 1: Screening of PHB producing microbes in the different soil sample.

S. no.	Sample location site	CFU/mL	No. of bacteria isolated	No. of positive isolates	PHB producing bacteria (%)
1	Potato storage soil	1.3×10^9	12	4	33
2	River water	1.8×10^9	15	5	30
3	River soil	2.0×10^9	20	5	40
4	Sewage sludge	1.4×10^9	10	3	30
5	Waste water	2.4×10^9	8	2	40
6	Field soil	1.5×10^9	15	7	20

Bold values shows the best values which were taken to explain the result.

Figure 1: Positive bacterial colonies showing intracellular PHB granules (bluish-black) stained with Sudan black B dye.

BV-1 from oil mil soil. Only one report is available showing PHB synthesis from *P. alvei* [24], while here for the first time *P. alvei* PHB28 is reported as PHB depolymerase producing strain possessing PHB synthesizing ability as well.

Weight loss determination of the soil buried plastic films

The weight of the test piece before and after treatment is calculated from the change in the weight for each plastic film (Table 2). The weight loss degradability for PHB (P5) film was found to be maximum (37.3%) at the end of treatment.

Surface morphology of soil buried film by SEM

The incubation of PHB film in the soil for degradation at different time period was microscopically observed

using SEM. In the first 15 days of degradation under soil, the surface was observed to be smooth with no pits and grooves (Figure 2B) as compared to the control (Figure 2A) and then progressively became rough with the markedly larger grooves (Figure 2C,D) as the exposure of the film to the soil for biodegradation was increased (~45 days) [6, 9].

Wen and Lu [25] also reported microbial degradation of PHB films buried in soil was enhanced with surface erosion process and 15% degradation was achieved with exposure of soil for 60 days when observed under SEM.

Screening of PHB depolymerase producing bacteria

From the soil burial treatment method, five potent PHB producing microbes were isolated by applying the same process of Sudan black B staining. Amongst 31 positive PHB producers (26 obtained from different locations and five from soil buried polymer film), the varying degree of PHB degradation was achieved with the colonies growing on solid plates with PHB as the sole source of carbon.

Primary screening was done on the basis of zone diameter on PHB solid plate after the incubation period of 7 days at $35 \pm 2^\circ\text{C}$, from which five strains were selected. Secondary screening was determined on the basis of PHB depolymerase enzyme activity of isolates (Table 3) in minimal medium under standard enzyme assay procedure, from which one potential isolate was further analyzed [9].

Characterization of bacterial isolate

Five potential isolates were characterized by morphological, biochemical and physiological observation according to the Bergey's Manual of Determinative Bacteriology (Table 4). Based on maximum PHB depolymerase enzyme production, PHB28 was chosen for further study.

Table 2: Weight loss (%) of plastic film buried in soil for an incubation period of 45 days.

Type of polymer film	Weight of film (g)		Weight loss degradability (%)
	Before treatment	After treatment	
P1(Polypropylene)	0.312 ± 0.001	0.304 ± 0.000	12.56
P2 (Polyethylene)	0.305 ± 0.001	0.298 ± 0.003	18.3
P3 (Polyethylene)	0.214 ± 0.005	0.195 ± 0.001	19.4
P4 (Polystyrene)	0.133 ± 0.002	0.130 ± 0.002	14.6
P5 (Polyhydroxybutyrate)	0.284 ± 0.001	0.242 ± 0.002	37.3

Bold values shows the best values which were taken to explain the result.

Figure 2: Scanning electron micrographs of the PHB films.
(A) Control and soil buried, (B) after 15 days, (C) after 30 days and (D) after 45 days.

Table 3: PHB depolymerase production after 72 h of incubation.

S. no.	Culture no.	Source of isolation	Renaming	Diameter of hydrolysis zone (mm)	Enzyme assay (U/mL)
1.	2	Waste water	PHB2	1.5	2.982
2.	18	Waste water	PHB18	0.5	0.220
3.	26	Sewage sludge	PHB26	0.6	0.882
4.	28	Potato soil	PHB28	2.0	4.01
5.	67	Potato soil	PHB67	0.25	0.280

Bold values shows the best values which were taken to explain the result.

Identification of bacterial strain

DNA sequence alignment

Partial homologous 16S rRNA sequences were analyzed using BLAST algorithm search tool in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). BLAST showed that the isolate PHB 28 which is of 614 bp has maximum homology identity (99%) with *P. alvei* strain FJAT-46015 (KY038690) with nucleotide base count of Adenine:163, Guanine:187, Cytisine:152, Thymine:131 through direct submission at NCBI-BLAST with an assigned GenBank accession number KX886342. Phylogenetic relationship and molecular evolution of the identified isolate was inferred by Neighbor-Joining analysis as presented in Figure 3.

Optimization of PHB depolymerase production by *Paenibacillus alvei* strain PHB28

Effect of incubation period

The conversion of PHB (0.15% in the turbid medium) into its monomer/oligomer under the suitable growth conditions was found to be maximum (3.39 U/mL) with 96 h of incubation as shown in Figure 4A.

Effect of incubation temperature

PHB depolymerase production from *P. alvei* strain PHB28 was optimized in the temperature range of

Table 4: Biochemical characterization of PHB depolymerase producing positive isolate.

Characteristics	PHB2	PHB18	PHB26	PHB28	PHB67
Configuration	Entire	Flat	Undulate	Entire	Entire
Shape	Irregular	Irregular	Irregular	Circular	Circular
Surface	Smooth	Mucoid	Rough	Smooth	Mucoid
Pigment color	White	Orange	Greenish	Light green	Yellowish
Colony size (diameter in mm)	3–4	2–3	2–4	1–2	1–2
Gram's staining	+	+	–	–	+
Spore formation	–	–	+	+	+
Biochemical tests					
Indole production	–	–	–	+	+
Catalase test	–	+	+	+	+
Citrate utilization	–	–	+	–	–
Starch hydrolysis	–	–	–	+	–
H ₂ S production	–	–	–	–	–
Voges-praskeur	–	–	–	+	–
Methyl red	–	–	–	–	–
Acid production from					
Glucose	–	+	+	+	–
Lactose	–	–	–	+	–
Fructose	–	–	–	+	–
Mannitol	–	–	+	–	–
Physiological tests					
Growth at temp.	25–40°C	35–40°C	25–40°C	30–45°C	25–35°C
Growth at NaCl (%)	2–8	2–8	4–8	2–8	2–6
Growth at pH	6–7	6–9	7–9	6–8	6–8
Aerobic/anaerobic condition	Anaerobic	Aerobic	Facultative anaerobic	Strictly aerobic	Aerobic
Agitation required for growth	+	+	+	+	+

Figure 3: Phylogenetic tree showing the homology of strain PHB 28 with *P. alvei*.

15°C–65°C with 96 h of incubation period. Maximum production of PHB depolymerase was achieved at 45°C (3.92 U/mL) after which a sharp decline (1.03 U/mL) in the

enzyme production was obtained with the rise in temperature (Figure 4B). Temperature optima results suggest that PHB28 is a thermotolerant strain. Aly et al. [26] found

Figure 4: Optimization of PHB depolymerase production by *Paenibacillus alvei* strain PHB28. (A) Effect of incubation time (B) Effect of incubation temperature (C) Effect of pH.

similar results where also 45°C is the most suitable temperature for PHB depolymerase enzyme production from *Streptomyces lydicus* MM10. A few numbers of thermotolerant ($\geq 40^\circ\text{C}$) PHB degrading microbial strain (*Aspergillus fumigatus*) has been reported [27].

Effect of pH

Among different pH range (4.0–12.0), maximum degrading activity was obtained at pH 8.0 (4.76 U/mL) as shown in Figure 4C. Loss of degrading activity was observed by a sharp decline in the pH range of (10.0–12.0) as shown in the graph. Lodhi et al. [27] have reported the maximum PHB depolymerase production at pH 7.0 from *A. fumigatus*.

Effect of carbon sources

Among additional carbon sources, maximum hydrolytic activity was observed in the presence of Starch (4.88 U/mL) after 96 h of incubation at 45°C, pH 8.0 as shown in Figure 5A, while in the presence of Sucrose, Mannitol, Fructose, Lactose and Glucose, the PHB depolymerase activity was 1.36, 1.28, 1.08, 0.85 and 2.98 U/mL, respectively.

Effect of nitrogen sources

Among various organic and inorganic nitrogen sources, highest activity was exhibited with yeast extract (5.03 U/

mL) followed by peptone (3.23 U/mL) under the optimized condition of temperature (45°C) and pH (8.0) for 96 h of the incubation as shown in Figure 5B. Supplementation of ammonium sulfate (2.69 U/mL) and ammonium nitrate (1.62 U/mL), was found not significant for enzyme production whereas urea proved to be a potent inhibitor for PHB depolymerase production as activity goes down to just 0.55 U/mL as compared to control (3.16 U/mL) having PHB as a carbon source. On the contrary of our result, Vignesswari et al. [28] have reported that urea was found best for enzyme production from *Acidovorax* sp. DP5.

Effect of substrate concentration

It was observed that the activity of an enzyme is in direct proportion with the concentration of PHB, thereby, best production (3.97 U/mL) was achieved at 0.15% of PHB in minimal media (Figure 5C). A higher or lower concentration is markedly inhibiting or reducing the activity of PHB depolymerase. However, Aly et al. [26] observed the maximum production of PHB depolymerase at 0.3% after 24 h of incubation with *S. lydicus* MM10. Similar to our results, maximum production of PHB depolymerase was reported by with 0.1% of substrate concentration from *Thermus thermophilus* HB8 after 24 h of incubation [29].

Wani et al. [9] have reported the optimum yield of PHB depolymerase from *Stenotrophomonas* sp. RZS7 with five days of incubation period at 37°C and at pH 6.0. The two PHB depolymerase from *Pseudomonas mendocina* PHAase

Figure 5: Optimization of PHB depolymerase production by *Paenibacillus alvei* strain PHB28.

(A) Effect of carbon sources, (B) effect of nitrogen sources, (C) effect of substrate concentration and (D) effect of metal ions.

I and PHAase II have been reported previously [30]. Several thermophilic bacterial strains have been isolated for PHB depolymerase production. The presence of carbon sources other than PHB also effects or inhibits the PHB depolymerase production [6, 26]. The optimum production of PHB depolymerase was achieved with lactose while in the current study, the best PHB depolymerase activity was observed with starch in addition to PHB.

Effect of metal ions

The effect of monovalent (K^+), divalent (Mg^{+2} , Zn^{+2} , Cu^{+2} , Hg^{+2} , Co^{+2}) and trivalent (Fe^{+3}) heavy metal ions on PHB depolymerase production was observed at 10 mM concentration under the optimized parameters of 45°C, pH 8.0, 0.1% starch, 0.01% yeast extract in enzyme production media for 96 h. The enhanced activity was observed (Figure 5D) only with Co^{+2} (4.30 U/mL) as compared to control (4.03 U/mL) while Mg^{+2} (3.84 U/mL) had no significant change over the activity while rest of the metal ions found as an inhibitor for production of the enzyme like Zn^{+2} , Fe^{+3} and Hg^{+2} showed 3.13 U/mL, 0.77 U/mL and 0.88 U/mL activity, respectively. In support of our results, Bhatt et al. [31] also found the enhanced activity of PHB depolymerase with Mg^{+2} , Ca^{+2} and Co^{+2} in a concentration of 5 mM while Hg^{+2} was proved as a potent inhibitor where activity goes down to zero. However, 85% and

81% of enzyme inactivation were observed with Fe^{+3} and Cu^{+2} . Thus it can be summarized that PHB depolymerase (PHB degrading activity) producing isolate is thermostable (45°C), and an increased activity was observed in the presence of starch remarks the additional requirement of the carbon source to enhance the activity of PHB depolymerase. Further, it will be significant to characterize the amino acid sequences essential for the functioning of PHB depolymerase enzyme. Up to best of our knowledge, this is the first report of PHB degradation and PHB depolymerase production simultaneously from *P. alvei* PHB28 strain.

Acknowledgment: Authors are grateful to the Department of Science and Technology (DST-SERB) for providing financial support and Integral University for providing the necessary infrastructure and manuscript communication number IU/R&D/2017-MCN000217.

Conflict of interest: All authors declare that they have no conflict of interest.

Declarations of interest: None

References

1. Barnes DK, Galgani F, Thompson RC, Barlaz M. Accumulation and fragmentation of plastic debris in global environments. *Philos Trans R Soc Lond B Biol Sci* 2009;364:1985–98.

2. García-Hidalgo J, Hormigo D, Arroyo M, de la Mata I. Novel extracellular PHB depolymerase from *Streptomyces ascomycinicus*: PHB copolymers degradation in acidic conditions. PLoS One 2013;8:e71699.
3. Kim HJ, Nam JS, Bae KS, Rhee YH. Characterization of an extracellular medium-chain-length poly (3-hydroxyalkanoate) depolymerase from *Streptomyces* sp. KJ-72. Antonie van Leeuwenhoek 2003;83:183–9.
4. Tokiwa Y, Calabia BP. Review degradation of microbial polyesters. Biotechnol Lett 2004;26:1181–9.
5. Takaku H, Kimoto A, Kodaira S, Nashimoto M, Takagi M. Isolation of a Gram-positive poly (3-hydroxybutyrate)(PHB)-degrading bacterium from compost, and cloning and characterization of a gene encoding PHB depolymerase of *Bacillus megaterium* N-18-25-9. FEMS Microbiol Lett 2006;264:152–9.
6. Shah A, Hasan F, Hameed A, Ahmed S. A novel poly (3-hydroxybutyrate)-degrading *Streptovorticillium kashmirensis* AF1 isolated from soil and purification of PHB-depolymerase. Acta Biol Hung 2008;59:489–99.
7. Song JH, Murphy RJ, Narayan R, Davies GB. Biodegradable and compostable alternatives to conventional plastics. Philos Trans R Soc Lond B Biol Sci 2009;364:2127–39.
8. Nielsen C, Rahman A, Rehman AU, Walsh MK, Miller CD. Food waste conversion to microbial polyhydroxyalkanoates. Microb Biotechnol 2017;10:1338–52.
9. Wani SJ, Shaikh SS, Tabassum B, Thakur R, Gulati A, Sayyed RZ. *Stenotrophomonas* sp. RZS 7, a novel PHB degrader isolated from plastic contaminated soil in Shahada, Maharashtra, Western India. 3 Biotech 2016;6:179.
10. Calabia BP, Tokiwa Y. A novel PHB depolymerase from a thermophilic *Streptomyces* sp. Biotechnol Lett 2006;28:383–8.
11. Jendrossek D, Hermawan S, Subedi B, Papageorgiou AC. Biochemical analysis and structure determination of *Paucimonas lemoignei* poly- (3-hydroxybutyrate PHB) depolymerase PhaZ7 mutants reveal the PHB binding site and details of substrate–enzyme interactions. Mol Microbiol 2013;90:649–64.
12. Bhuwal AK, Singh G, Aggarwal NK, Goyal V, Yadav A. Isolation and screening of polyhydroxyalkanoates producing bacteria from pulp, paper, and cardboard industry wastes. Int J Biomater 2013;2013:1–10.
13. Gouda MK, Swellam AE, Omar SH. Biodegradation of synthetic polyesters (BTA and PCL) with natural flora in soil burial and pure cultures under ambient temperature. Res J Environ Earth Sci 2012;4:325–33.
14. Altaee N, El-Hiti GA, Fahdil A, Sudesh K, Yousif E. Biodegradation of different formulations of polyhydroxybutyrate films in soil. SpringerPlus 2016;5:762.
15. Wang Z, Gao J, Li L, Jiang H. Purification and characterization of an extracellular poly (3-hydroxybutyrate-co-3-hydroxyvalerate) depolymerase from *Acidovorax* sp. HB01. World J Microbiol Biotechnol 2012;28:2395–402.
16. Pantazaki AA, Tambaka MG, Langlois V, Guerin P, Kyriakidis DA. Polyhydroxyalkanoate (PHA) biosynthesis in *Thermus thermophilus*: purification and biochemical properties of PHA synthase. Mol Cell Biochem 2003;254:173–83.
17. Nadhman A, Hasan F, Shah AA. Production and characterization of poly (3-hydroxybutyrate) depolymerases from *Aspergillus* sp. isolated from soil that could degrade poly (3-hydroxybutyrate). IJB 2015;7:25–8.
18. Jendrossek D, Knoke I, Habibian RB, Steinbüchel A, Schlegel HG. Degradation of poly (3-hydroxybutyrate), PHB, by bacteria and purification of a novel PHB depolymerase from *Comamonas* sp. J Environ Polym Degrad 1993;1:53–63.
19. Hoseinabadi A, Rasooli I, Taran M. Isolation and identification of poly β -hydroxybutyrate over-producing bacteria and optimization of production medium. Jundishapur J Microbiol 2015;8:1–10.
20. Ghanem NB, Mabrouk ME, Sabry SA, El-Badan DE. Degradation of polyesters by a novel marine *Nocardiopsis aegyptia* sp. nov.: application of Plackett-Burman experimental design for the improvement of PHB depolymerase activity. J Gen Appl Microbiol 2005;51:151–8.
21. Getachew A, Woldesenbet F. Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. BMC Res Notes 2016;9:509.
22. Reddy SV, Thirumala M, Mahmood SK. Production of PHB and P (3HB-co-3HV) biopolymers by *Bacillus megaterium* strain OU303A isolated from municipal sewage sludge. World J Microbiol Biotechnol 2009;25:391–7.
23. Hungund B, Shyama VS, Patwardhan P, Saleh AM. Production of polyhydroxy alkanoate from *Paenibacillus durus* BV-1 isolated from oil mill soil. J Microb Biochem Technol 2013;5:013–7.
24. Boyandin AN, Prudnikova SV, Filipenko ML, Khrapov EA, Vasil'ev AD, Volova TG. Biodegradation of polyhydroxyalkanoates by soil microbial communities of different structures and detection of PHA degrading microorganisms. Appl Biochem Microbiol 2012;48:28–36.
25. Wen X, Lu X. Microbial degradation of poly-(3-hydroxybutyrate-co-4-hydroxybutyrate) in soil. J Polym Environ 2012;20:381–7.
26. Aly MM, Tork S, Qari HA, Al-Seeni MN. Poly-hydroxy butyrate depolymerase from *Streptomyces lydicus* MM10, Isolated from Wastewater Sample. IJAB 2015;17:891–900.
27. Lodhi A, Hasan F, Abdul Z, Hameed A, Faisal S, Shah A. Optimization of culture conditions for the production of poly 3-hydroxybutyrate depolymerase from newly isolated *Aspergillus fumigatus* from soil. Pak J Bot 2011;432:1361–2.
28. Vigneswari S, Lee TS, Bhubalan K, Amirul AA. Extracellular polyhydroxyalkanoate depolymerase by *Acidovorax* sp. DP5. Enzyme Res 2015;2015:1–8.
29. Papaneophytou CP, Pantazaki AA, Kyriakidis DA. An extracellular polyhydroxybutyrate depolymerase in *Thermus thermophilus* HB8. Appl Microbiol Biotechnol 2009;83:659–68.
30. Mao H, Jiang H, Su T, Wang Z. Purification and characterization of two extracellular polyhydroxyalkanoate depolymerases from *Pseudomonas mendocina*. Biotechnol Lett 2013;35:1919–24.
31. Bhatt R, Patel KC, Trivedi U. Purification and properties of extracellular poly (3-hydroxybutyrate) depolymerase produced by *Aspergillus fumigatus* 202. J Polym Environ 2010;18:141–7.