

## RESEARCH ARTICLE

# Purification and kinetics of the PHB depolymerase of *Microbacterium paraoxydans* RZS6 isolated from a dumping yard

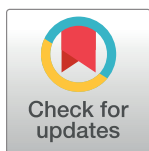
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**Citation:** Sayyed RZ, Wani SJ, Alyousef AA, Alqasim A, Syed A, El-Enshasy HA (2019) Purification and kinetics of the PHB depolymerase of *Microbacterium paraoxydans* RZS6 isolated from a dumping yard. PLoS ONE 14(6): e0212324. <https://doi.org/10.1371/journal.pone.0212324>

**Editor:** Pradeep Kumar, North Eastern Regional Institute of Science and Technology, INDIA

**Received:** January 30, 2019

**Accepted:** June 1, 2019

**Published:** June 18, 2019

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**Data Availability Statement:** All relevant data are within the manuscript.

**Funding:** The work in the present paper was funded by Deanship of Scientific Research at King Saud University through research group No (RG-1440-053) and MOE, UTM, RMC, Malaysia through HICOE, Grant No. R5130000.7846.4J262.

**Competing interests:** The authors have declared that no competing interests exist.

## Abstract

Poly-β-hydroxybutyrate (PHB) depolymerase is known to decompose PHB, biodegradable polymers and therefore has great commercial significance in the bioplastic sector. However, reports on PHB depolymerases from isolates obtained from plastic-contaminated sites that reflect the potential of the source organism is scarce. In this study, we evaluated the production of extracellular PHB depolymerase from *Microbacterium paraoxydans* RZS6 isolated from the plastic-contaminated site in the municipal area of Shahada, Maharashtra, India, for the first time. The isolate was identified using 16S rRNA gene sequencing, gas chromatographic analysis of fatty acid methyl esters (GC-FAME), and BIOLOG method. Ithydrolyzed PHB on minimal salt medium (MSM) containing PHB as the only source of carbon. The isolate produced PHB depolymerase at 45°C during 48 h of incubation. The enzyme was purified most efficiently using octyl-sepharose CL-4B column, with the highest purification yield of 6.675 Umg<sup>-1</sup>mL<sup>-1</sup>. The activity of the enzyme was enhanced in the presence of Ca<sup>2+</sup> and Mg<sup>2+</sup> ions but inhibited by Fe<sup>2+</sup> (1 mM) ions and mercaptoethanol (1000 rpm). The nzyme kinetic analysis revealed that the enzyme was a metalloenzyme; requiring Mg<sup>2+</sup> ions, that showed optimum enzyme activity at 30°C (mesophilic) and under neutrophilic (pH 7) conditions. Scale-up from the shake-flask level to a laboratory-scale bioreactor further enhanced the enzyme yield by 0.809 U mL<sup>-1</sup>. The molecular weight of the enzyme (40 kDa), as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, closely resembled the PHB depolymerase of *Aureobacterium saperae*. Our findings highlighted the applicability of *M. paraoxydans* as a producer of extracellular PHB depolymerase having potential of degrading PHB under diverse conditions.

## Introduction

Poly- $\beta$ -hydroxy alkananoate (PHA) and poly- $\beta$ -hydroxybutyrate (PHB) are stored as food and energy reserve in bacteria under the carbon-rich environment and are catabolized during nutrient stress conditions under the influence of PHB depolymerase [1–3]. PHB is a biocompatible, thermoplastic, nontoxic, completely biodegradable molecule exhibiting the properties of synthetic plastics. Moreover, it is easily degraded by PHB depolymerases and hence is an eco-friendly alternative to recalcitrant synthetic plastics [4–6]. PHB is degraded under natural conditions by the actions of PHB depolymerases produced by a wide variety of microorganisms [7–8]. Although PHB has commercial applications, identification of potent PHB degraders from relevant habitats and their evaluation for PHB depolymerase production, purification, and enzyme kinetic and scale-up must be carried out. Reports on bacterial PHB depolymerases isolated from plastic-contaminated sites, which reflect the true biodegradation potential of the enzyme, are scarce. Organisms isolated from plastic-contaminated sites that are capable of degrading PHB may serve as potential sources of efficient PHB depolymerases. Accordingly, in this study, we aimed to isolate PHB-degrading bacteria from plastic-rich dumping yards. We describe the isolation and polyphasic identification of a PHB depolymerase-producing *Microbacterium paraoxydans* RZS6 isolated from plastic-contaminated sites, production of PHB depolymerase, purification, characterization, and enzyme kinetics and scale-up of the identified PHB depolymerase.

## Materials and methods

### Chemicals and glassware

All the chemicals used in this study were of analytical research grade. PHB was purchased from Sigma-Aldrich (Germany); all other chemicals were purchased from Hi-Media Laboratories (Mumbai, India). The glassware was cleaned using 6N HCl and  $K_2Cr_2O_7$ , rinsed with double-distilled water and dried in hot air oven.

### Isolation and screening of PHB depolymerase-producing bacteria

Sample collection, isolation, and screening of the PHB depolymerase-producing bacteria were performed as described by Wani et al. [4]. *M. paraoxydans* RZS6 was isolated from plastic-contaminated site located at latitude 21° 30' 47.09" N and longitude 74° 28' 40.47" E. This sampling site was purposefully chosen due to the higher probability of finding microflora that would be metabolically very active in biopolymer degradation.

### Selection of potent isolate

Isolate producing the zone of PHB hydrolysis were grown on MSM containing different concentrations of PHB (0.1–0.4%) at 30°C for 10 days [9]. The degradation of PHB was detected by observing the time profile of the growth of the isolate, and by observing the formation of the zone of PHB clearance surrounding the colonies. The level of PHB degradation was measured from the diameter of the zone of PHB hydrolysis.

### Temperature profile of the potent isolate

In order to assess the thermostability of the PHB depolymerase, the isolate was subjected to PHB degradation assays for a period of 10 days at 28°C, 37°C, and 45°C in MSM containing different concentrations (0.1%, 0.2%, 0.3%, and 0.4%) of PHB. The influence of temperature on PHB degradation was assessed by measuring the zone of PHB hydrolysis on each plate.

## Polyphasic identification of the isolate

Isolates showing the highest potential for PHB degradation on the PHB-agar were considered potent PHB depolymerase producers and were subjected to polyphasic identification.

**Preliminary identification.** Colonies of PHB-degrading isolate on nutrient agar (NA) medium were characterized using the Gram-staining method, morphological characteristic, and taxonomic characterization by biochemical kits (Hi-Media, Mumbai, India). The isolate was identified according to Bergey's Manual of Determinative Bacteriology [10].

**16S rRNA gene sequencing.** Sequencing of 16S rRNA genes of the isolate RZS6 was performed as per the method of Gangurde et al. [11]. DNA of the isolate was extracted according to the methods of Sambrook and Russel [12] using HiPurA Plant Genomic DNA Miniprep purification spin kit. Amplification of the 16S rRNA genes was performed using the following primers [13].

27f (5'-AGAGTTTGATCCTGGCTCAG-3') and

1492r (3'-ACGGCTACCTTGTTACGACTT-5').

The amplified sequences were analyzed by using gapped BLASTn (<http://www.ncbi.nlm.nih.gov>) search algorithm and the phylogenetic analysis was performed by evolutionary distance and maximum likelihood with 1,000 bootstrap replicates using the neighbor-joining method in Clustal W software [14]. The evolutionary distances were computed using the neighbor-joining method and the Kimura 2-parameter model. Phylogenetic trees were constructed according to the methods described by Tamura and Kumar [15]. The 16S rRNA gene sequences of the isolate were submitted to GenBank.

**Fatty acid methyl ester (FAME) analysis.** Fatty acids from whole cells of the isolate RZS6 were derivatized to methyl esters and analyzed by gas chromatography using the Sherlock Microbial Identification System (MIDI, Inc., Newark, DE, USA) [16]. Identification and quantification of fatty acids was carried out by comparing the retention time and peak area of the samples with those of standard fatty acids. Differences in the fatty acid profiles were computed using the Sherlock bacterial fatty acid ITSA1 aerobe reference library [17].

**Phenotypic fingerprinting.** Phenotypic fingerprinting of isolate RZS6 was carried out using a GEN III MicroPlate test panel with 95 carbon source utilization assays and a Microbial Identification System, 1998 (Biolog Inc., CA, USA) with Micro Log version 4.2 database software (Biolog Microstation System; Biolog Inc.) [18].

## Production and activity assay of PHB depolymerase

**Growth kinetics and PHB depolymerase activity.** Growth curve experiments were performed to evaluate the ability of the isolate to mineralize the substrate (PHB), as described by Maria and Zauscher [19]. The growth rate of *M. paraoxydans* RZS6 in PHB MSM was monitored over time at 620 nm in the presence of PHB as a substrate by withdrawing sample after every 12 h. The PHB depolymerase activity of the isolate was estimated as described by Papanophytou et al. [7].

**Production of PHB depolymerase.** The production of PHB depolymerase was evaluated under shake-flask conditions by separately growing *M. paraoxydans* RZS6 at 30°C and 120 rpm for 4 days in MSM containing PHB (0.15% w/v) [20].

**PHB depolymerase assay.** After incubation, the MSM broth culture was centrifuged at 5000g for 15 min., and the PHB depolymerase activity of the supernatant was assayed as described by Papanophytou et al. [7]. For this purpose, *M. paraoxydans* RZS6 ( $5 \times 10^6$  cells mL<sup>-1</sup>) was grown in two reaction mixtures, each consisting of 50 mM Tris-HCl buffer (pH 7.0), 150 µg mL<sup>-1</sup> PHB (prepared by sonication at 20 kHz for 15 min), and 0.5 mL of 2 mM CaCl<sub>2</sub> at 30°C for 10 min. The PHB depolymerase activity was assayed as a decrease in the

PHB turbidity at 650 nm [7]. One unit of PHB depolymerase activity was defined as the quantity of enzyme required to cause a 0.1 unit decrease in absorbance at 650 nm per min.

## Purification of PHB depolymerase

Purification of PHB depolymerase from cell-free supernatant was carried out by using three approaches as given below.

**Ammonium sulfate precipitation.** The crude PHB depolymerase in the supernatant was precipitated by the gradual addition of increasing concentrations (10–70% w/v) of ammonium salt. The precipitate so obtained was dialyzed overnight [8], followed by estimation of enzyme activity as per Papanephoytu et al. [7] and the protein concentration as per Lowry et al. method [21].

**Solvent purification method.** The culture supernatant of the isolate was centrifuged at 5,000 g for 15 min, residues were dissolved in a pre-chilled 1:1 (v/v) mixture of acetone and ethanol and kept in a water bath at 50 °C to allow the evaporation of the solvent. The pellet left over after solvent evaporation was dissolved in Tris-HCl buffer (pH 7), and the protein content and enzyme activity were assayed as described earlier [21,7].

**Column chromatography.** The culture supernatant of *M. paraoxydans* RZS6 was loaded on to octyl-sepharose CL-4B column charged with glycine-NaOH buffer (pH 9.0) and eluted using a 0–50% gradient of ethanol [22]. The fractions were collected and subjected to the estimation of protein content [21] and enzyme activity [7].

## Determination of molecular weight

The molecular weight of the purified PHB depolymerase of the isolate was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with standard molecular weight markers (GeNei™, Bangalore, India) such as phosphorylase B (82.2 kDa), bovine serum albumin (64.2 kDa), egg albumin (48.8 kDa), carbonic anhydrase (37.1 kDa), trypsin inhibitor (25.9 kDa), lysozyme (19.4 kDa), lysozyme (14.8 kDa), and lysozyme (6.0 kDa). Separated bands were stained for 4–6 h under shaking with 0.025% Coomassie brilliant blue G prepared in 40% (v/v) methanol and 7% (v/v) acetic acid followed by destaining with acetic acid: methanol (7:5) and 2X loading buffer (10 mL) and bands were observed under UV transilluminator. The protein concentration of the purified band was measured using bovine serum albumin as a standard [21].

## Enzyme kinetics

**Effects of temperature on enzyme activity and determination of the thermostability of the enzyme.** To determine the temperature optima and sensitivity of the PHB depolymerase, log culture of *M. paraoxydans* RZS6 ( $5 \times 10^6$  cells mL<sup>-1</sup>) was grown in the reaction mixture at a different temperature ranging from 5 to 70 °C for 10 min, and the enzyme activity was then measured as described above [7].

**Effects of pH on enzyme activity and determination of the pH stability of the enzyme.** The effect of pH on enzyme activity and the pH stability of the enzyme were determined in reaction mixtures having varying pH values in the range of 2 to 13. For this purpose log culture of *M. paraoxydans* RZS6 ( $5 \times 10^6$  cells mL<sup>-1</sup>) was separately grown in reaction mixtures having different pH ranging from 2 to 13 at 30 °C for 10 min, and the enzyme activity was then measured as described above [7].

**Effects of metal ions on the enzyme.** In order to ascertain the metal requirement and type of metal required for the activity of the PHB depolymerase, *M. paraoxydans* RZS6 was separately grown in various reaction mixtures, each containing one type of metal ion, e.g.,

$\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}_2^+$ ,  $\text{Hg}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Fe}^{2+}$  (1 mM), at 30°C for 10 min. Enzyme activity was then measured as described earlier [7].

**Effects of solvents and chemicals on the enzyme.** In order to determine the effects of solvents, such as methanol (10%, v/v), ethanol (10%, v/v), acetone (10%, v/v), mercaptoethanol (1%, v/v), tween-20 (1%, v/v), tween-80 (1%, v/v), ethylene diamine tetraacetic acid (EDTA; 1 mM), NaCl (1 mM), KCl (1 mM), and  $\text{NaNO}_3$  (1 mM), on the activity of the PHB depolymerase. The solvents and chemicals were added individually into each reaction mixture, followed by inoculation with the isolate RZS6, incubation at 30°C for 10 min, and measurement of enzyme activity [7].

### Scale up of the optimized process to a laboratory-scale bioreactor

In order to evaluate the performance of the organism in the bioreactor and to confirm the validity of the optimized shake-flask studies, the process was scaled-up to a fully-automated bioreactor of 5-L capacity (Model LF-5; Murhopye Scientific Co., Mysore, India). The bioreactor was sterilized, along with the above-optimized medium (working volume 3 L), at 121°C for 20 min; cooled, then inoculated with 3% (v/v) inoculum of *M. paraoxydans* RZS6 and incubated at 30°C for 48 h at 80 rpm. The samples were withdrawn after 12 h and subjected to estimation of protein concentrations [21] and enzyme activity [7].

### Statistical analysis

All the experiments were performed in triplicate and the mean of three replicates was considered. Each mean value was subjected to Student's *t*-test and values of  $P \leq 0.05$  were taken as statistically significant [23].

## Results and discussion

### Isolation and screening of PHB depolymerase-producing bacteria

In total, 39 isolates were obtained from the respective plastic-contaminated sites; among these, seven isolates grew well and produced varying degrees of PHB hydrolysis on MSM containing PHB as the only carbon source. The isolate RZS6 produced the largest zone (27.9 mm) of PHB hydrolysis on MSM containing 0.4% (w/v) of PHB and was therefore selected as the best PHB depolymerase producer. The hydrolysis of PHB reflected the ability of the isolate to produce PHB depolymerase.

Although several bacteria are known to secrete PHB depolymerase which degrades PHB, our findings demonstrated, for the first time, the production of PHB depolymerase from *M. paraoxydans* RZS6. The PHB depolymerase produced by isolate RZS6 obtained from the plastic-contaminated environment may be relevant for applications in plastic/bioplastic degradation. Mergaert et al. [24] have isolated 295 strains that degraded PHB and P (3HB-co-3HV) copolymer on MSM amended with PHB as the only carbon source. Elbanna et al. [25] reported *Schlegelella thermodepolymerans* and *Pseudomonas indica* K2 as PHA degraders on MSM. Additionally, Gangurde et al. [3] also reported PHB biodegradation by soil micro-flora on MSM containing PHB.

**Selection of potent isolate.** PHB-degrading isolate exhibited a range of PHB biodegradation abilities in MSM containing varying concentrations of PHB. Degradation of PHB was dependent on the amount of PHB in the medium. The degradation was significantly influenced by the concentration of the PHB in the medium. Maximum biodegradation was recorded at 0.2% concentration of PHB. However, among all PHB degrading isolates, RZS 6 showed significant degradation of PHB. In the case of other isolates, the zone diameter of PHB

hydrolysis was comparatively smaller ranging from 0.9 to 2.9 mm. Variation in the degradation profile of PHB by different isolates suggested differences in their metabolic states [26]. Augusta et al. [27] have reported that the diffusion rate of an enzyme in plate assay differs with respect to the isolate and the amount of enzyme activity and rate of PHB degradation is influenced by environmental conditions. Kim et al. [28] also reported similar observations in the case of *Aspergillus* sp. strain NA-25.

### Temperature profile of the potent isolate

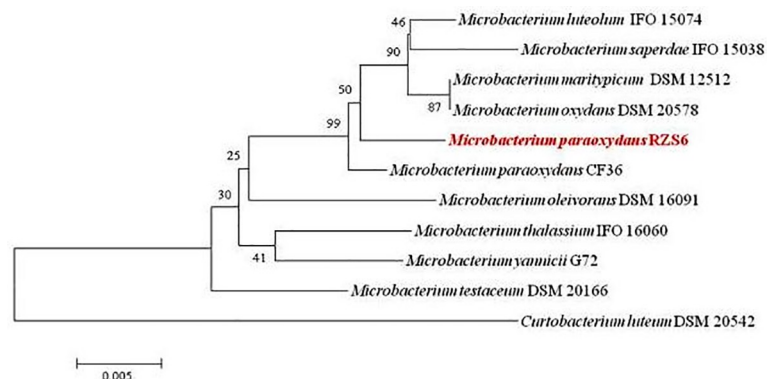
The isolate RZS6 exhibited optimum PHB degradation at 30°C with 0.2% PHB as a substrate. Importantly, the temperature profile of PHB degradation is dependent on the activity of the enzyme and therefore changes with the producing organism. Certain PHB depolymerases function in the mesophilic range of temperature, whereas others are thermotolerant or thermophilic in nature [6]. Wang et al. [29] have reported similar observations on a poly-depolymerase (3-hydroxybutyrate-co-3-hydroxy valerate) from *Acidovorax* sp. HB01.

### Polyphasic identification of potent PHB depolymerase producers

**Preliminary identification.** The phenotypic characteristics of the potent PHB-degrading isolate RZS6 were similar to those of *Microbacterium* sp.

**16S rRNA gene sequencing.** A BLAST search of the 16S rRNA gene sequences based on 1486 bp of the PHB depolymerase producing isolate with the 16S rRNA gene sequences of the NCBI GenBank database revealed the highest similarity and homology (99.2%) of the isolate RZS6 with *M. paraoxydans* (Fig 1). The phylogenetic tree based on 16S rRNA gene sequences of an isolate with the representative strains of its respective genus formed two distinct groups: Group I included the genus *Microbacterium* while Group II contained the strain *Curtobacterium leuteum* DSM 20542. Thus, based on phylogenetic analysis we identified the isolate RZS6 was identified as *M. paraoxydans*, The 16S rRNA gene sequence of the isolate was submitted to NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) under the name *M. paraoxydans* RZS6 with accession No. KP862607.

**Whole-cell FAME analysis.** The fatty acid profile of isolate RZS6 demonstrated the presence of characteristic fatty acids of *M. barkeri* (*Microbacterium barkeri*, *Corynebacterium*; similarity index: 0.859) and *M. chocolateum* (similarity index: 0.602) [17].



**Fig 1. Phylogenetic analysis of *M. paraoxydans* RZS6 based on 16s rRNA gene sequence drawn using the neighbor joining method (MEGA 5.0 software) with evolutionary distances computed using Kimura's two-parameter method showing the relationship of PHB depolymerase producing bacteria with the validly published sequences of related genera.**

<https://doi.org/10.1371/journal.pone.0212324.g001>

**Table 1. Identification of bacteria on the basis of 16S rRNA sequencing, GC FAME analysis, and BIOLOG profile.**

Strain	16s rRNA sequencing	Base Pair	Identity (%)	GC FAME analysis	SI	BIOLOG identification	SI
RZS 6	<i>Microbacterium paraoxydans</i>	1486	99.2	<i>Microbacterium barkeri</i> ( <i>Aureobacterium</i> , <i>Corynebacterium</i> )	0.859	<i>Microbacterium paraoxydans</i>	0.48

Phylogenetic analysis was carried out using the neighbor joining method. Evolutionary distances were computed using Kimura's two-parameter method.

FaAME analysis was carried out by Sherlock Microbial Identification System and comparison with of bacterial fatty acid with standard ITSA1 aerobic reference library.

Phenotypic fingerprinting was carried out using a GEN III Micro Plate Microbial Identification System with Micro Log version 4.2 database software

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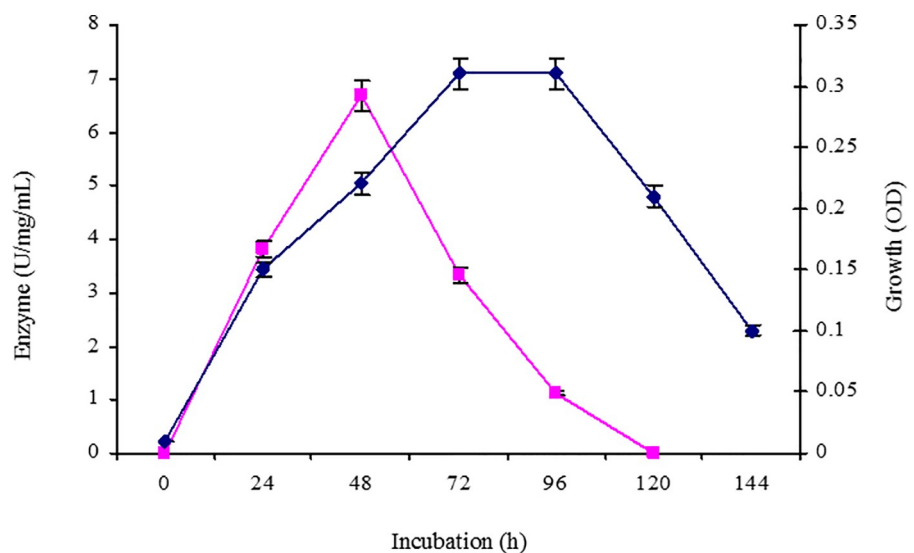
**BIOLOG identification.** The pattern of carbon source utilization assays for the isolate RZS6 demonstrated a maximum similarity index of 0.48 with *M. paraoxydans*.

Based on the preliminary characteristics, 16S rRNA gene sequencing, gas chromatographic analysis of FAMES, and BIOLOG profiles, the isolate RZS6 was identified as *M. paraoxydans*. Based on 16s rRNA gene sequencing, GC-FAME analysis BIOLOG characteristics, the isolate RZS6 was identified as *Microbacterium paraoxydans* (Table 1).

## Production and assay of PHB depolymerase

**Growth kinetics and PHB depolymerase activity.** Isolate RZS6 grew well in MSS containing PHB. The isolate showed faster growth, produced PHB depolymerase during the log phase of growth, and exhibited an optimum enzyme activity of  $6.675 \text{ U mg}^{-1} \text{ mL}^{-1}$ , obtained at 48 h. The activity gradually decreased from the beginning of the stationary phase (72 h) and was completely absent during the decline phase (96–120 h) of growth (Fig 2).

**Production of PHB depolymerase from the isolate.** *M. paraoxydans* RZS6 produced copious amounts of PHB depolymerase in MSM. After 48 h (log phase) of incubation, RZS6 produced 6.675 U of PHB depolymerase with  $0.247 \text{ mg mL}^{-1}$  protein content in 2 days at  $30^\circ \text{C}$ . Gowda and Srividya [30] reported the production of 4 U of extracellular PHB depolymerase with a protein content of  $0.05 \text{ mg mL}^{-1}$  from *Penicillium expansum*. We report higher yields of PHB depolymerase.



**Fig 2. Growth kinetics and PHB depolymerase activity of *M. paraoxydans* RZS6 in MSM containing PHB as the only source of carbon, growth rate and enzyme activity were monitored over time at 620 and 50 nm respectively by withdrawing the sample after every 12 h.**

<https://doi.org/10.1371/journal.pone.0212324.g002>

## Purification of the PHB depolymerase

Having confirmed the presence of a PHB depolymerase in the cell-free supernatant of the MSM broth culture of *M. paraoxydans* RZS6, the supernatant was subjected to purification using three approaches, as described below.

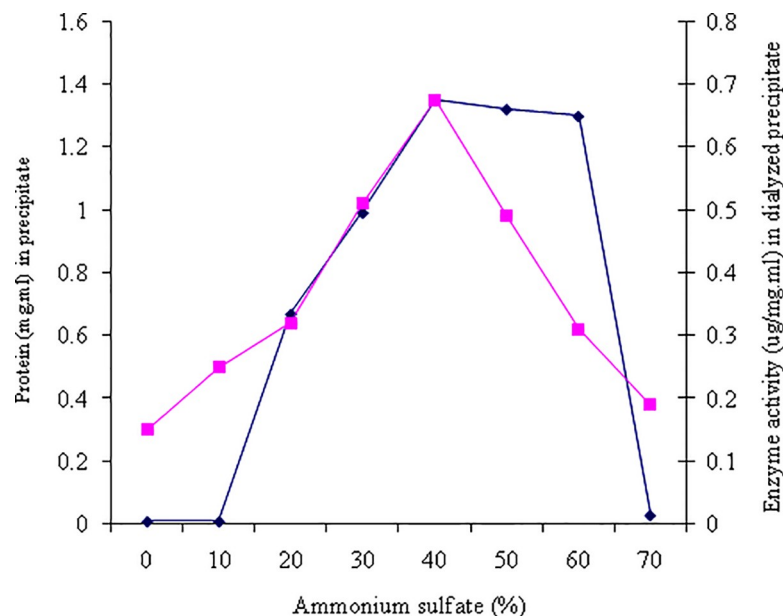
**Ammonium sulfate precipitation.** The maximum protein precipitation in the culture supernatant was obtained at 40% (v/w) concentration of ammonium sulfate. The protein concentrations, and enzyme activities in the dialyzed precipitate of *M. paraoxydans* RZS6 were  $1.350 \text{ mgmL}^{-1}$ , and  $6.675 \text{ U}$ , respectively (Fig 3). Zhou et al. [31] also reported the precipitation of PHB depolymerase from *Escherichia coli* and *Penicillium* sp. DS9701-D2 using 70% and 75% ammonium sulfate. Shivakumar et al. [32] have reported efficient precipitation of PHB depolymerase from *Penicillium citrinum* S2 using 80% ammonium sulfate.

**Solvent purification method.** Solvent mixture of acetone and ethanol (1:1) adversely affected PHB depolymerase activity, leaving behind only 45.66% enzyme activity. This significant loss in enzyme activity may be related to the precipitation of proteins (enzymes) by acetone and ethanol. Thus, the solvent purification method proved to be inefficient.

**Column chromatography.** Out of the five fractions, fraction 1, 2 and 5 did not show any enzyme activity, fractions 4 was less in enzyme activity ( $1.013 \text{ U}$ ) and protein content ( $0.062 \text{ mgmL}^{-1}$ ), fraction 3 exhibited the maximum enzyme activity of  $7.703 \text{ U}$ , with  $0.247 \text{ mgmL}^{-1}$  protein content. Other fractions. Thus, column chromatography using an octyl-sepharose CL-4B column resulted in the most efficient purification. Purification of PHB depolymerase from various organisms has been carried out using Sephadex columns [33–34]. Wang et al. [29], DEAE-Sepharose column and using column chromatography [6–8].

## Determination of the molecular weight of the purified enzyme

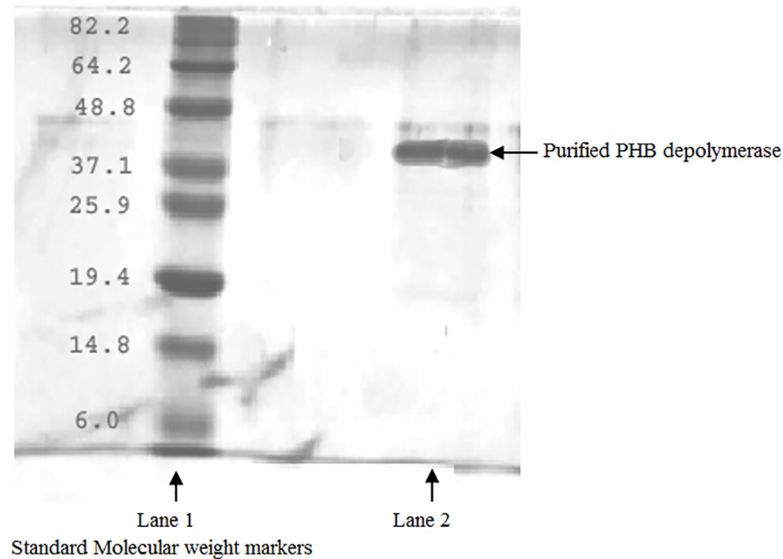
The purified protein fraction from *M. paraoxydans* RZS6 yielded single protein bands corresponding to the molecular weight of approximately 40 kDa (Fig 4). Sadocco et al. [35] also



**Fig 3. Ammonium sulfate purification of PHB depolymerase of *M. paraoxydans* RZS6.** The enzyme was precipitated with increasing concentrations from 10–70% (w/v) of ammonium salt and the enzyme activity and the protein were measured for each precipitation.

<https://doi.org/10.1371/journal.pone.0212324.g003>





**Fig 4. SDS-PAGE for determination of molecular weight of PHB depolymerase of *M. paraoxydans* RZS6 by comparing it with standard molecular weight markers as shown in Lane 1 and separated band of PHB depolymerase shown in Lane 2. Separated bands were stained with Coomassie brilliant blue G and destained with acetic acid:methanol (7:5) and observed under UV transilluminator.**

<https://doi.org/10.1371/journal.pone.0212324.g004>

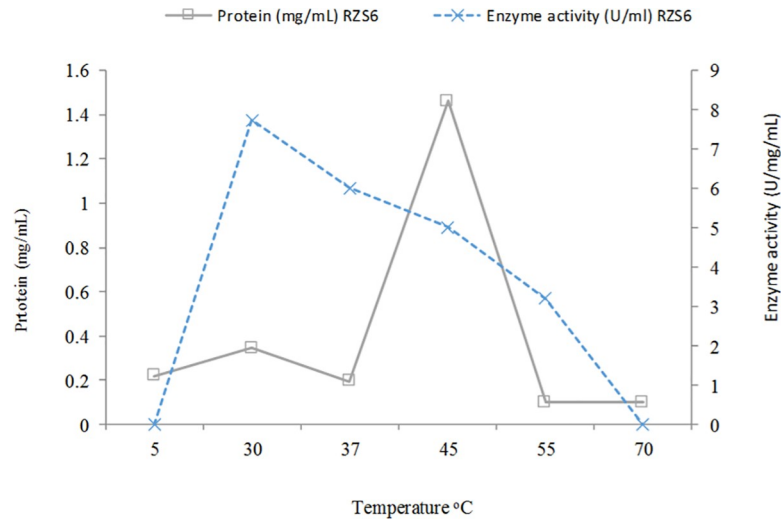
reported a PHB depolymerase of 42.7 kDa from *Aureobacterium saperdae*. Calabia and Tokiwa [36] identified a PHB depolymerase of 41 kDa from *Streptomyces* sp. MG 41.

## Enzyme kinetics

**Effects of temperature and determination of the thermostability of the enzyme.** The PHB depolymerase of RZS6 showed an optimum enzyme activity of 6.657 U with 0.247 mgmL<sup>-1</sup> protein content at 30°C, indicating the mesophilic nature of the enzyme. The enzyme activity decreased with as the temperature increase, and the enzyme was completely inactivated at 70°C (Fig 5). The decrease in the enzyme activity with the increase in temperature reflected the thermolabile nature of the enzyme. Wang et al. [29] and Gowda and Shivakumar [30] reported thermostable PHB depolymerases in *Pseudomonas mendocina* DSWY0601 and *Penicillium expansum*, respectively. Calabia and Tokiwa [36] reported optimum PHB depolymerase activity in *Streptomyces* sp. MG at 50°C.

**Effects of pH on enzyme activity and determination of the pH stability of the enzyme.** The optimum activity of the PHB depolymerase of *M. paraoxydans* RZS6 was obtained at pH (Fig 6), indicating the neutrophilic nature of the enzyme. The enzymes from isolate RZS6 remained stable at an acidic pH (4.0–6.5). Sadocco et al. [35] and Jeong [37] reported an optimum pH for PHB depolymerase from *A. saperdae* in the range of 7.0 to 9.0. The pH sensitivity of the PHB depolymerases of *Penicillium expansum* and *Pseudomonas mendocina* DSWY0601 has been reported to be between 6.0 and 9.0 respectively [29–30]. Soam et al. [6] reported pH 7.0 as the optimum pH for enzyme production in *Bacillus mycoides*. Calabia and Tokiwa [36] reported that the optimum PHB depolymerase activity of *Streptomyces* sp. MG was in the pH range from 6.5 to 8.5.

**Effects of metal ions on enzyme activity.** The presence of Mg<sup>2+</sup> ions significantly enhanced PHB depolymerases however, Fe<sup>2+</sup> ions negatively affected the enzyme activity, leaving behind only 22.26% activity. The other metal ions had a negligible effect on the enzyme activity (Table 2). The increase in PHB depolymerase activity in the presence of Mg<sup>2+</sup> ions was

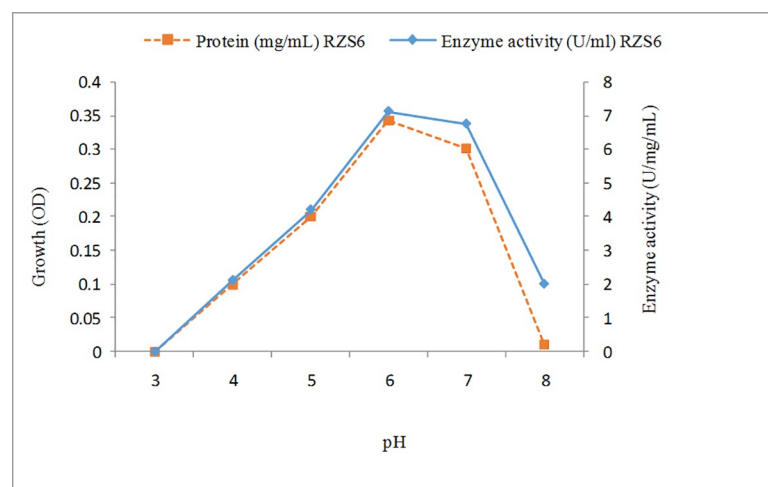


**Fig 5. Effects of temperature and determination of thermostability of PHB depolymerase of *M. paraoxydans* RZS6.** Enzyme activity was measured by separately incubating the enzyme in the reaction mixture at various temperature ranging from 5 to 70 °C for 10 min.

<https://doi.org/10.1371/journal.pone.0212324.g005>

attributed to the enzyme activator nature of these metal ions. Wang et al. [29] also reported a positive effect of Mg<sup>2+</sup> and Ca<sup>2+</sup> ions on the PHB depolymerase of *Pseudomonas mendocina* DSWY0601.

**Effects of different chemicals on enzyme activity.** Mercaptoethanol caused the maximum inhibition (85%) of enzyme activity. NaCl enhanced the activity of the PHB depolymerase (Table 2). The loss of enzyme activity in the presence of chemicals and solvents occur due to denaturation and proteolysis of the enzyme. Papanephytous et al. [7] have also reported mercaptoethanol as a strong inhibitor of PHB depolymerase in *Thermus thermophilus* HB8. Wang et al. [29] have also reported the negative effects of ethanol, acetone, tween, and other chemicals on the PHB depolymerase of *Pseudomonas mendocina* DSWY0601.



**Fig 6. Effects of pH and determination of pH stability of PHB depolymerase of *M. paraoxydans* RZS6.** Enzyme activity was measured by separately incubating the enzyme for 10 min in the reaction mixture of various pH ranging from 2–13.

<https://doi.org/10.1371/journal.pone.0212324.g006>

Table 2. Effects of metal ions and chemicals on the PHB depolymerase of *M. paraoxydans* RZS6.

Metal ion (1 mM)	Enzyme activity (U/mL <sup>-1</sup> )	Protein content (mg/mL <sup>-1</sup> )	Specific activity (U/mg <sup>-1</sup> mL <sup>-1</sup> )	% activity
No metal ions	0.146 (0.010)	0.247 (0.013)	0.6210 (0.022)	59.10 (0.119)
Mg <sup>2+</sup>	0.194 (0.100)	0.247 (0.013)	0.7854 (0.031)	78.54 (0.172)
Ca <sup>2+</sup>	0.192 (0.095)	0.247 (0.013)	0.7773 (0.030)	77.73 (0.161)
Mn <sup>2+</sup>	0.129 (0.081)	0.247 (0.013)	0.5627 (0.017)	56.27 (0.149)
Cu <sup>2+</sup>	0.099 (0.072)	0.247 (0.013)	0.4008 (0.016)	40.08 (0.132)
Co <sup>2+</sup>	0.101 (0.075)	0.247 (0.013)	0.4089 (0.016)	40.89 (0.136)
Hg <sup>2+</sup>	0.097 (0.086)	0.247 (0.013)	0.3927 (0.012)	39.27 (0.123)
Zn <sup>2+</sup>	0.094 (0.080)	0.247 (0.013)	0.3805 (0.012)	38.05 (0.125)
Fe <sup>2+</sup>	0.055 (0.050)	0.247 (0.013)	0.2226 (0.113)	22.26 (0.016)
<b>Chemicals</b>				
No chemical	0.134 (0.011)	0.219 (0.013)	0.6118 (0.019)	61.18 (0.014)
NaCl	0.154 (0.019)	0.219 (0.013)	0.7196 (0.022)	71.96 (0.022)
NaNO <sub>3</sub>	0.148 (0.100)	0.219 (0.013)	0.6757 (0.031)	67.57 (0.031)
Methanol	0.145 (0.092)	0.219 (0.013)	0.6621 (0.019)	66.21 (0.019)
KCl	0.138 (0.073)	0.219 (0.013)	0.6301 (0.018)	63.01 (0.018)
Ethanol	0.112 (0.074)	0.219 (0.013)	0.5114 (0.014)	51.14 (0.014)
Tween-80	0.11 (0.088)	0.219 (0.013)	0.5022 (0.009)	50.22 (0.009)
Tween-20	0.103 (0.069)	0.219 (0.013)	0.4703 (0.008)	47.03 (0.008)
Acetone	0.091 (0.086)	0.219 (0.013)	0.4155 (0.007)	41.55 (0.007)
EDTA	0.046 (0.079)	0.219 (0.013)	0.2100 (0.005)	21.00 (0.005)
Mercaptoethanol	0.023 (0.051)	0.219 (0.013)	0.1050 (0.003)	10.50 (0.003)

Values were taken to be statistically significant at  $P \leq 0.05$

Values are the mean of three replicates

Values in parenthesis are the values generated by comparing control with the test by the Students *t*-test

<https://doi.org/10.1371/journal.pone.0212324.t002>

### Scale up of the optimized process to a laboratory-scale bioreactor

Scale-up of the parameters optimized at the shake-flask level to the laboratory-scale bioreactor level enhanced PHB depolymerase yield from 7.703 to 8.512 U and protein content from 0.247 to 0.297 mgmL<sup>-1</sup>. An increase of 0.809 UmL<sup>-1</sup> in enzyme yield and 0.50 mgmL<sup>-1</sup> in protein content was obtained.

### Conclusion

The occurrence of PHB-degrading bacteria in plastic-contaminated sites and their growth in the presence of PHB on MSM containing PHB as the only source of carbon indicated their ability to degrade bioplastic (PHB). The growth and maximum enzyme activity of isolate at mesophilic temperature indicated the ability of an organism to survive well under environmental conditions of dumping yard. The highest purification yield obtained by using octyl-sepharose CL-4B column reflects the binding and adsorption specificity of the enzyme to sepharose. Enhanced activity of enzyme in the presence of metal ions such as Ca<sup>2+</sup> and Mg<sup>2+</sup> indicated the role of these metal ions as activators of the enzyme and metalloenzyme nature of the PHB depolymerase. Thus, PHB depolymerase-producing bacteria obtained from plastic-contaminated environments are expected to be important catalyst for applications in the biodegradation of bioplastics. However, further studies on the growth of *Microbacterium paraoxydans* and performance of PHB depolymerase under natural conditions shall help in this direction.

## Acknowledgments

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University, Saudi Arabia and MOE and UTM-RMC, HICOE, Malaysia for funding this research through the Research Group Project No. RG-1440-053 and R. J130000.7846.4J262.

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## References

1. Sayyed RZ and Chincholkar SB, Production of Poly- $\beta$ -hydroxy butyrate (PHB) from *Alcaligenes faecalis*, *Indian J Microbiol*, 2004;269–272.
2. Sayyed RZ, Gangurde NS, Chincholkar SB, Hypochlorite digestion method for efficient recovery of PHB from *A. faecalis*, *Indian J Microbiol*, 2009; 49: 230–232.
3. Gangurde NS, Patil YP, Jain R, Sayyed RZ, Poly- $\beta$ -hydroxybutyrate biodegradation by mixed culture population vis-à-vis single culture population under varying environmental conditions: a new approach, *Indian J Exp Biol*, 2017; 55: 311–320.
4. Wani SJ, Shaikh SS, Tabassum B, Thakur A, Gulati A, *Stenotrophomonas* sp. RZS7, a novel PHB degrader isolated from plastic contaminated soil in Shahada, Maharashtra, Western India, *3 Biotech*, 2016; 6: 179. <https://doi.org/10.1007/s13205-016-0477-8> PMID: 28330251
5. Wani SJ, Sayyed RZ, Production, efficient recovery and partial characterization of biodegradable polymer produced by soil *Streptomyces*, *Indian J Biotechnol*, 2016; 15: 127–129.
6. Soam A, Singh A, Singh R, Shahi S, Optimization of culture conditions for biopolymer producing *Bacillus mycoides* (wss2) bacteria from sewage, *Curr Discov*, 2012; 1: 27–32.
7. Papanephoytu CP, Pantazaki AA Kyriakidis DA, An extracellular polyhydroxybutyrate depolymerase in *thermosthermophilus hb8*. *Appl Microbiol Biotechnol*, 2009; 83: 659–668. <https://doi.org/10.1007/s00253-008-1842-2> PMID: 19214501
8. Hsu KJ, Tseng M, Don TM, Yang MK, Biodegradation of poly( $\beta$ -hydroxybutyrate) by a novel isolate of *Streptomyces bangladeshensis* 77T–4. *Bot Stud*, 2012; 53: 307–313.
9. Pushpita S, Parama S, Paul AK, Degradation of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-co-3-hydroxy valerate) by some soil *Aspergillus* spp. *J Poly Env* 2006; 14: 257–263.
10. Brenner DJ, Krieg NR, Staley JT, *Bergey's Manual of Systemic Bacteriology*. Second ed. 2005; Vol. 2. Springer, USA.
11. Gangurde NS, Sayyed RZ, Shashi K, Gulati A, Development of eco-friendly bioplastic like PHB from distillery effluent microorganisms, *Env Sci Poll Res*, 2013, 20: 488–497.
12. Sambrook J, Russel DW, *Molecular Cloning: A Laboratory Manual*. Third ed. Cold Spring Harbor Laboratory Press, New York, USA, 2001, pp 543–549.
13. Padiyar V, Adam KA, Badri NN, Patole M, Shouche YS, *Aeromonas culicicola* sp. nov., from the midgut of *Culexquinque fasciatus*, *Int J Syst Evol Microbiol*, 2002, 52: 1723–1728. <https://doi.org/10.1099/00207713-52-5-1723> PMID: 12361279

14. Thompson JD, Toby JG, Frederic P, François J, Desmond GH, The CLUSTAL × windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, *Nucleic Acids Res*, 1997; 25: 4876–4882. <https://doi.org/10.1093/nar/25.24.4876> PMID: 9396791
15. Tamura K, Nei M, Kumar S, Prospects for inferring very large phylogenies by using the neighbor-joining method, *Proc Natl Acad Sci*, 2004; 101:11030–11035. <https://doi.org/10.1073/pnas.0404206101> PMID: 15258291
16. Gulati A, Rahi P, Vyas P, Characterization of phosphate solubilizing fluorescent *Pseudomonads* from the rhizosphere of sea buckthorn growing in the cold desert of Himalayas, *Curr Microbiol*, 2008; 56: 73–79 <https://doi.org/10.1007/s00284-007-9042-3> PMID: 17909886
17. Sasser M, Bacterial identification by gas chromatographic analysis of fatty acid methyl esters (GC-FAME), 2006, Technical Note # 101, MIDI, Inc, Newark, DE, USA.
18. Shaikh SS, Patel PR, Patel SS, Nikam SD, Rane TU, Sayyed RZ, Production of biocontrol traits by banana field fluorescent *Pseudomonads* and comparison with chemical fungicide, *Indian J Exp Biol*, 2014, 52: 917–920.
19. Maria CG, Zauscher F, Biodegradation of two commercial herbicides (Gamaxone and Matancha) by the bacteria *Pseudomonas putida*. *Env Biotechnol*, 2002; 5: 182–195.
20. Han JS, Kim MN, Purification and characterization of extracellular poly(3-hydroxybutyrate) depolymerase from *Penicillium simplicissimum* LAR13, *The J Microbiol*, 2002; 40: 20–25.
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, Protein measurement with the Folin phenol reagent, *J Biol Chem*, 1951; 193: 265–275. PMID: 14907713
22. Kim DY, Yun JH, Kim HW, Bae KS, Rhee YH, Purification and characterization of poly (3-hydroxybutyrate) depolymerase from a fungal isolate, *Emericellopsis minima* W2, *J Microbiol*, 2002; 40: 129–133.
23. Parker RE, Continuous distribution: tests of significance. In: Parker RE (ed) *Introductory statistics for biology*, 2nd Edn; 1979; Cambridge University Press, London, pp 18–42.
24. Mergaert J, Webb A, Anderson C, Wouters A, Swings J, Microbial degradation of poly (3-hydroxybutyrate) and poly (3-F hydroxybutyrate-co-3-hydroxybutyrate) in compost, *J Environ Polym Degrad*, 1993; 2: 177–183.
25. Elbanna K, Lutke-Eversloh T, Jendrosseck D, Luftmann H, Steinbuechel A, Studies on the biodegradability of polythioester copolymers and homopolymers by polyhydroxyalkanoate (PHA) degrading bacteria and PHA depolymerases, *Arch Microbiol*, 2004; 182: 212–25. <https://doi.org/10.1007/s00203-004-0715-z> PMID: 15340783
26. Minna H, Aliphatic polyesters: abiotic and biotic degradation and degradation products, *Adv in Poly Sci*, 2002; 157: 114–134.
27. Augusta J, Muller RJ, Widdecke J, A rapid evaluation plate-test for the biodegradability of plastics, *Appl Microbial Biotechnol*, 1993; 39: 673–678.
28. Kim HJ, Nam JS, Bae KS, Rhee YH, Characterization of an extracellular medium-chain length poly (3-hydroxyalkanoate) depolymerase from *Streptomyces* sp. KJ-72. *Antony van Leeuwenhoek*, 2003; 83: 183–189.
29. Wang Y, Li F, Wang Z, Liu D, Xia H, Liu L, and Chen S, Purification and properties of an extracellular poly hydroxybutyrate depolymerase from *Pseudomonas mendocina* DSWY0601, *Chem Res Chinese Univ*, 2012; 28: 459–464.
30. Gowda USV, Shivakumar S, Poly (-β-hydroxybutyrate) (PHB) depolymerase PHAZPen from *Penicillium expansum*: purification, characterization and kinetic studies, *3Biotech*, 2015; 5: 901–909.
31. Zhou H, Wang Z, Chen S, Liu D, Xia H, Purification and characterization of extracellular poly (β-hydroxybutyrate) depolymerase from *Penicillium* sp. DS9701-D2, *Poly Plas Technol Engin*, 2008; 48: 58–63.
32. Shivakumar S, Jagadish SJ, Zatakia H, Dutta J, Purification, characterization and kinetic studies of a novel poly (β) hydroxybutyrate (PHB) depolymerase PhaZ Pen from *Penicillium citrinum* S2, *Appl Biochem Biotechnol* 2011; 164: 1225–1236. <https://doi.org/10.1007/s12010-011-9208-0> PMID: 21369777
33. Shah AA, Hasan F, Hameed A, Ahmed S, Isolation and characterization of poly (3-hydroxybutyrate-co-3-hydroxy valerate) degrading actinomycetes and purification of PHBV depolymerase from newly isolated *Streptoverticillium kashmirensis* AF1, *Ann Microbiol* 2007; 57: 583–588.
34. Garcia-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. <https://doi.org/10.1371/journal.pone.0071699> PMID: 23951224
35. Sadocco PS, Nocerino E, Dubini-Paglia A, Seres EG, Characterization of a poly (3-hydroxybutyrate) depolymerase from *Aureobacterium sperdae*: Active site and kinetics of hydrolysis studies, *J Env Polym Degrad*, 1997; 5: 57–65.

36. Calabia BP, Tokiwa Y, A novel PHB depolymerase from a thermophilic *Streptomyces* sp, *Biotechnol Lett*, 2006; 28: 383–388. <https://doi.org/10.1007/s10529-005-6063-5> PMID: 16614903
37. Jeong SJ, Purification and properties of a novel poly (3-hydroxybutyrate) depolymerase from *Pseudomonas* sp. GM101. Ph.D. Thesis, 1996, Gyeongsang National University.