Research Article

Poly- β -hydroxybutyrate (PHB) Depolymerase from *Fusarium solani* Thom

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Fusarium solani Thom produced maximum PHB depolymerase by 48 h when grown in BHM containing 0.2%, w/v PHB, pH 8.0 at 25°C. Statistical optimization studies using Plackett Burman design of PHB depolymerase production yielded maximum PHB depolymerase activity after 2 days as against 4 days in the unoptimized conditions with a 2-fold increase in activity. Partial purification of the extracellular poly- β -hydroxybutyrate (PHB) depolymerase PHAZ_{Fus} from *F. solani* Thom by two steps using ammonium sulphate (80% saturation) and affinity chromatography using concanavalin-A yielded 162.3-fold purity and 63% recovery of protein. The enzyme composed of a single polypeptide chain of 85 KDa, as determined by SDS-PAGE. The enzyme stained positive for glycoprotein by PAS staining. Optimum enzyme activity was detected at pH 7.0 and 55°C. The enzyme was stable at pH 7.0 and 55°C for 24 h with a residual activity of almost 85%. Paper chromatography revealed β -hydroxybutyrate monomer as the major end product of PHB hydrolysis. Complete inhibition of the enzyme by 1 mM HgCl₂ (100%) indicated the importance of essential disulfide bonds (cystine residues) for enzyme activity or probably for maintaining the native enzyme structure.

1. Introduction

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

It has been reported that PHB-degrading bacteria are distributed widely in the natural environment, and several extracellular PHB depolymerases with differing biochemical properties have been isolated from various bacterial origins [2, 3]. However, the ability to degrade SCL-PHA is not restricted to bacteria, and some filamentous fungi also play an important role in the extracellular degradation of SCL-PHAs

such as PHB and its copolyesters with 3-hydroxyvalerate [4]. Many reports have been published on the fungal degradation of SCL-PHAs in the environment, however reports on the properties of the PHB depolymerases from fungi are relatively rare. Only few fungal PHB depolymerases have been purified and partially characterized to date [5–12]. Therefore, the biochemical properties of fungal PHB depolymerases are not well documented in comparison to those of bacterial PHB depolymerases.

The ever-increasing human demand for materials leads to a concomitant increase in waste production. The fact that soil serves as major landfill, it also means that the new generation of biosynthetic plastics will probably have to be degraded there as well. The fungal biomass in soils generally exceeds the bacterial biomass and thus it is likely that fungi may play a considerable role in degrading polyesters, just as they predominantly perform the decomposition of organic matter in the soil ecosystem. However, in contrast to bacterial polyester degradation, which has been extensively investigated, the microbiological and environmental aspects

of fungal degradation of polyesters are unclear [13]. Many PHA-degrading fungi have been identified [4, 14]. Soil-containing environments are the habitats from which the largest numbers of fungal PHB degraders are found [15]. To our knowledge there are only a few reports on the biochemical characteristics of fungal PHB depolymerases and no report so far on statistical optimization of medium components for production of any fungal PHB depolymerase.

Optimizing the parameters by statistical method reduces the time and expense; otherwise if required, conventional one-factor-at-a-time approach is used. Several statistical methods are available for optimization experiments [16]. Plackett and Burman's statistical method is one of such approaches involving a two-level fractional factorial saturated design that uses only $k + 1$ treatment combinations to estimate the main effects of k factors independently, assuming that all interactions are negligible [17]. Saturated designs are used in the early stages of experimentation to screen out unimportant factors from among a large number of possible factors. In full factorial designs, the number of factors increases exponentially leading to an unmanageable number of experiments [18]. Hence, fractional factorial design like Plackett-Burman becomes a method of choice for initial screening of medium components.

The present paper describes the production, purification process and some of the properties of the PHB depolymerase obtained from a fungal isolate, *Fusarium solani* Thom. The exceptional properties of this enzyme are emphasized and compared with those of other fungal depolymerases.

2. Experimental

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

2.2. Isolation of a SCL-PHA-Degrading Fungus. A SCL-PHA-degrading filamentous fungus from a wastewater sample was isolated by pure culturing a colony with high depolymerase activity among fungi grown on a mineral salt agar medium [7] containing PHB as the sole carbon source.

2.3. Optimization of the Medium Using Statistically Based Plackett-Burman Experimental Design. The Plackett-Burman experimental design, a fractional factorial design [17, 19] was used in this research to reflect the relative importance of various environmental factors on PHB degradation and PHB depolymerase activity in liquid cultures.

2.4. Production of the PHB Depolymerase from the Isolate. The isolate was cultivated in mineral salts agar medium at 30°C for 3-4 days as described earlier [12]. The culture supernatant was filtered using Whatmann filter paper no. 1 and used as enzyme source.

2.5. Protein Estimation. Protein concentrations were measured by Bradford's method using bovine serum albumin as the standard [20].

2.6. Enzyme Assay. The PHB depolymerase assay was carried out by adding the enzyme to 100 mM sodium citrate buffer (pH 6.0) containing 300 µg of PHB at 55°C for 1 h [11].

2.7. Purification of PHB Depolymerase. The extracellular PHB depolymerase of the fungus was purified from the culture filtrate by $(\text{NH}_4)_2\text{SO}_4$ precipitation (80% saturation), followed by affinity chromatography using concanavalin-A [12]. The fractions collected were assayed for enzyme activity and protein content by the method mentioned above.

2.8. Molecular Weight Determination. Electrophoresis (10%, w/v sodium dodecyl sulfate polyacrylamide gel electrophoresis) was carried out to measure the molecular weight according to Yu [19]. Phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.5 kDa) were used as standard molecular weight markers. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250.

2.9. Glycoprotein Staining. Carbohydrate staining of glycoprotein in SDS-PAGE gel was carried out with fuchsin sulfate after periodate oxidation [21].

2.10. Effects of Temperature and pH on the Enzyme Activity. To find out the effect of temperature on PHB depolymerase activity, enzyme activity was measured at different temperatures in the range of 25°C to 60°C at optimum pH. Temperature stability was measured by incubating the enzyme in standard reaction solution without PHB for 90 min at 25~60°C, followed by enzyme assay. The optimum pH for enzyme activity was determined by measuring the activity in each of the buffers containing PHB and enzyme. To examine the pH stability of the enzyme, different buffers containing enzyme without substrate were incubated at 25°C for 2 h, and then controlled to have the optimum pH in order to measure the residual activity of the enzyme [12].

2.11. Determination of K_m and V_{max} . A PHB stock solution (3 mg/mL) was used to prepare varying PHB (substrate) concentrations from 200–2000 µg/mL, making up the volume to 2 mL with buffer (optimal assay pH). Using the data, a double reciprocal plot (Lineweaver-Burke plot) was plotted and the value of K_m was determined [12].

2.12. Substrate Specificity. To examine the ability of the PHB depolymerase to degrade other polymers, PCL and copolymer of poly-β-hydroxybutyrate (5%) were used. The standard assay under optimal conditions was performed for the enzyme extract using P (HB-co-HV) (5%), PCL (5%) as the substrate instead of PHB. The absorbance was read at 660 nm against a blank [12].

2.13. Esterase Activity. Esterase activity was assayed in 2 mL of 100 mM sodium citrate buffer, pH 6.0, using *p*-nitrophenyl alkanoates-*para*-nitrophenyl acetate (PNPA),

TABLE 1: Degree of positive or negative effect of independent variables on *F. solani* Thom PHB depolymerase according to the levels in the Plackett-Burman experimental design.

Variable	Symbol	(-) Level	(0) Level	(+) Level	Main effect	T value	Significance level
Time (h)	a	48	72	96	0.6667	-3.89	NS
pH	b	4	6	8	0.1422	-0.853	NS
Temperature (°C)	c	25	30	35	-0.5687	4.32	S
Inoculum size (% v/v)	d	2	4	6	0.0688	-0.397	NS
PHB (g/L)	e	1	2	3	0	0	NS
Glucose (g/L)	f	5	0	10	0	0	S
Volume/flask (mL)	g	20	50	100	-0.2447	1.89	S
Nitrogen (g/L)	h	0.5	1.0	1.5	-0.02	3.761	S
KH ₂ PO ₄ (g/L)	i	0.1	0.7	1.3	-0.0512	0.57	NS

Bold type indicates positive effect.

Significant (S); nonsignificant (NS). $t_{0.1} = 1.875$; $t_{0.05} = 2.365$.

para-nitrophenyl butyrate (PNPB), and *para*-nitrophenyl decanoate (PNPD) incubated at 37°C for 10 min. The reaction mixtures contained 50 μ L of a 10 mM solution of the substrates in ethanol and 10 μ L of the enzyme solution [12]. Polycaprolactone (PCL; MW_{avg}, 42,500) and other substrates were purchased from the Aldrich Chemical Co. Suspensions of PCL were prepared as previously described [22].

2.14. Effect of Inhibitors. The inhibitory effect of various chemical reagents on enzyme activity was measured as follows: the reaction mixture (1.97 mL) containing 1 mL of the enzyme solution, reagent, and 100 mM sodium citrate buffer (pH 6.0) was initially pre-incubated for 1 h at 37°C, with the enzymatic reaction subsequently started by adding 2 mL of the PHB substrate. All the inhibitors were purchased from Sigma (St. Louis, MO, USA) [22].

2.15. Identification of PHB Hydrolysis End Products. One milligram of PHB was incubated in 1 mL 100 mM sodium citrate buffer (pH 6.0) at 50°C in the presence of 10 μ g purified PHB depolymerase for 4–6 h. After incubation, the reaction was stopped with 0.1 N HCl, reaction mixture acidified to pH 4.0, and the aqueous end product was repeatedly extracted with equal volumes of diethyl ether in presence of 1 N NaCl in a separating funnel. The ether extract was collected and air-dried. The concentrated extract was detected for the presence of aqueous PHB hydrolysis products by paper chromatography using 2° Butanol: H₂O: 28% NH₄OH in the ratio 1,000:200:3 and Bromocresol purple (0.1%, w/v in distilled water) as developer. The monomer of complete PHB hydrolysis- β hydroxybutyrate (Sigma) was spotted as reference. The paper was observed for yellow spots against purple background.

3. Results and Discussion

3.1. Microorganism: Identification and Characterization. Fungal species identified as *Fusarium solani* Thom (Fungal identification service, Agarkar Research Institute, Pune) based on morphotaxonomy, was used in this work.

FIGURE 1: Elucidation of fermentation factors affecting PHB depolymerase activity of *F. solani* Thom.

3.2. Optimization of PHB Depolymerase Production by Plackett-Burman Design. The independent variables examined in Plackett-Burman design and their levels are presented in Table 1 [23].

The design was applied with different fermentation conditions. The main effect of each variable upon PHB degradation and the production of PHB depolymerase enzyme was estimated and presented in Figure 1.

Within the range of the tested levels of variables, pH, time of incubation, and inoculum size affected activity at high levels (Figure 1). On the other hand, other variables showed effects on PHB depolymerase activity at low levels. Different concentrations of PHB did not show any effect on the enzyme activity in the range tested. Presence of glucose showed repressive effect on the enzyme activity suggesting the catabolic repression effect of glucose on PHB depolymerase production. Of all the factors tested, time of incubation and temperature showed profound effect on PHB depolymerase activity at high and low levels, respectively, indicating the importance of these two factors in production of the enzyme.

According to the data obtained from the Plackett-Burman experimental results, KH₂PO₄ and NH₄Cl should be added in the lowest concentration and the medium predicted to be near optimum should be of the following composition (g/L): PHB, 2; MgSO₄·7H₂O, 0.2; NH₄Cl, 1; KH₂PO₄, 0.1; CaCl₂, 0.02; FeCl₃, 0.05. Fungal spores were inoculated with 6% (v/v) inoculum of 5-day-old culture in a total culture volume

FIGURE 2: Plate showing PHB clearance by *F. solani* Thom.

FIGURE 3: Time course production of PHB depolymerase by *F. solani* Thom.

of 25 mL BHM and incubated at pH 8.0 and 25°C. Under optimized culture conditions, maximum PHB depolymerase activity was achieved after 2 days as against 4 days in the unoptimized conditions with a 2-fold increase in activity (Figures 2 and 3).

In order to optimize enzyme production, the Plackett-Burman design was applied, which has been demonstrated as an efficient approach to screen for medium components and/or factors affecting PHB degradation. Increasing pH level, proved to enhance polymer hydrolysis. On the contrary, increasing NH_4Cl and KH_2PO_4 level negatively affected enzyme activity. PHB concentration at the tested levels did not show profound influence on the enzyme activity. Supplementation of glucose negatively affected PHB depolymerase production by this isolate suggesting catabolite repression by glucose. Our results are in agreement to all other known PHB bacterial depolymerases, which are repressed in the presence of a soluble carbon source. In accordance with our results are those reported for *Pseudomonas lemoignei*, [24, 25] which produced polymerase maximally during growth on succinate. This result is in good agreement with a previous investigation which demonstrated the importance of carbon sources in the growth medium for enzyme production as the rate of polymer degradation was influenced by the degree and availability of secondary carbon and by the initial carbon

in the liquid medium. In contrast to this observation, the PHB depolymerase of *Nocardioopsis aegyptia* was found to be constitutive [23].

3.3. Purification of PHB Depolymerase. Purification of the enzyme by two steps using ammonium sulphate precipitation (80% saturation) and affinity chromatography using concanavalin-A yielded 162.31-fold purity with 63% recovery of the protein (Table 2). PHB depolymerases from different systems have been purified using different multistep methods. According to the literature, the yield of PHB depolymerase after purification was 66% from *P. funiculosus*; 27% from *A. faecalis*; 42% from *Pseudomonas* sp.; 66% from *A. fumigatus*; 73.5% from *Aureobacterium saperdae*; 61% from *P. simplicissimum* and 86.11% from *A. fumigatus* Pdf1. The increases of the enzyme activity were 4.5-fold for *P. funiculosus*; 1.5-fold for *A. faecalis*; and 5.67-fold for *Pseudomonas* sp.; 2.4-fold in *P. simplicissimum* and 33.56-fold in *A. fumigatus* Pdf1 [5, 7, 9, 11, 26–28].

3.4. Molecular Weight Determination. The purity and homogeneity of the enzyme was confirmed by (Figure 4(a), Lanes 2 and 3) protein staining. On performing activity staining on 2.5% (w/v) PHB plates, a zone of clearance was observed with different concentrations of the pure enzyme (Figure 4(b)). The molecular weight of *F. solani* PHB depolymerase determined here is in agreement to that of the PHB depolymerase obtained from *P. funiculosus*; *P. pinophilum*; *P. simplicissimum* and *A. fumigatus* Pdf1 and *P. citrinum* [5–7, 9, 12]; bacterial PHB depolymerase [24, 26–28] all of which showed a single polypeptide of different molecular weights. The PHB depolymerase of *A. faecalis* AE122 and *P. citrinum* S2 are the only depolymerases reported with unusually high apparent M_r of 96 KDa and 240 KDa [12, 27].

3.5. Glycoprotein Staining. On subjecting the PHB depolymerase of *F. solani* to glycoprotein staining by PAS method after SDS PAGE (Figure 5) revealed glycosylation. Carbohydrates were not detected in the PHB depolymerase either from *A. fumigatus* or from *A. saperdae* [11, 28]. However, the bacterial PHB depolymerase from *Pseudomonas lemoignei* [24]; and fungal PHB depolymerase from *P. funiculosus* [5]; *P. simplicissimum* [9]; *A. fumigatus* Pdf1 [7], and *P. citrinum* S2 [12] were glycosylated. The composition and function of the carbohydrate moiety of the glycosylated PHB depolymerase and other proteins studied so far are unknown. The cellulases of *Trichoderma reesei* and *Cellulomonas fimi* are known to be glycosylated in the proline/threonine rich region and an extracellular glucoamylase from *Aspergillus niger* is also glycosylated in the C-terminal region of the protein. A functional role of the carbohydrate moiety in binding the substrate of cellulases has been proposed. However, glycosylation is not essential for activity but may prove the resistance of the exoenzyme to elevated temperature and/or hydrolytic cleavage by proteases of competing microorganisms [29].

3.6. Effects of Temperature and pH on the Enzyme Activity. The optimum pH of the PHB depolymerase from fungi

TABLE 2: Purification of PHB depolymerase of *F. solani* Thom.

Purification step	Enzyme activity (U/mL)	Total enzyme activity (U)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Activity recovery (%)
Crude enzyme (1000 mL)	5.0	5000	0.260	160	18.75	1.0	100
NH ₄ SO ₄ ppt, dialysed (9.0 mL)	410	3690	2.5	28.8	128.125	6.83	73.8
Affinity chromatography (9 mL)	350	3150	0.115	1.035	3043.4	162.31	63

FIGURE 4: (a) SDS denatured PAGE of standard marker (Lane 1), purified PHB depolymerase by ammonium sulphate precipitation (Lane 2), and affinity chromatography (Lane 3). (b) Activity staining of PHB depolymerase of *F. solani* Thom on PHB plate.

[24, 26–28] is in the range of 7.5~9.5 depending on the strain. The PHB depolymerase enzyme of *F. solani* was most active at pH 7.0 as shown in Figure 6. pH stability of *F. solani* PHB depolymerase at pH 7.0, 55°C showed stability over a period of 24 h with a residual activity of almost 85% (Figure 7). The PHB depolymerase of *A. saporidae* [28] was completely inactivated at pH 1.5 in 2 hours and that of *P. lilacinus* [10]

maintained the activity only in the range of pH 4.0 to 6.0. Accordingly, it was concluded that the PHB depolymerase of *F. solani* was very stable under neutral conditions.

The enzyme exhibited the highest activity at 55°C and was stable at 55°C for 1 h with 84% residual activity (Figures 8 and 9), indicating that the enzyme was thermally as stable as other PHB depolymerases reported [5, 11, 27, 30]. The PHB

FIGURE 5: Glycoprotein staining of PHB depolymerase by PAS method. Standard molecular weight marker (Coomassie stained) (Lane 1); purified PHB depolymerase by ammonium sulphate precipitation (Lane 2), and affinity chromatography (Lane 3) after PAS method.

FIGURE 6: pH optima of *F. solani* PHB depolymerase.

FIGURE 7: pH stability of *F. solani* PHB depolymerase.

FIGURE 8: Temperature optima of *F. solani* PHB depolymerase.

FIGURE 9: Temperature stability of *F. solani* PHB depolymerase.

depolymerase produced by PHB decomposing organisms has high activity and stability at high temperatures.

3.7. Determination of K_m and V_{max} . The K_m values were determined by plotting a double reciprocal plot (Figure 10). The K_m and V_{max} values are 100 $\mu\text{g/mL}$ and 50 $\mu\text{g/min}$, respectively.

3.8. Effect of Inhibitors. Inhibitors are indicative of the various functional groups present in the active site of an enzyme. Effect of inhibitors on the activity of the enzyme was investigated in order to identify the active sites in the PHB depolymerase of *F. solani* (Figure 11). The activity of *F. solani* PHB depolymerase seemed to be unaffected by *N*-*p*-tosyl-*L*-lysinechloromethyl ketone (TLCK), which should transform the histidine groups. The PHB depolymerases of *P. funiculosum* and *A. saperdae* also exhibited 100% activity in the presence of 10 mM TLCK [5, 28]. Complete inhibition of the *F. solani* enzyme by 1 mM HgCl_2 (100%), indicates the importance of essential disulfide bonds (cystine residues) for enzyme activity or probably for maintaining the native enzyme structure. Partial inhibition of the enzyme by PMSF (10 mM), DAN (10 mM) and *N*-ethylmaleimide (10 mM), indicating less importance of serine residue, carboxyl group, tyrosine, and sulfhydryl groups in the active site of the enzyme respectively [28]. In contrast, *P. simplissimum* LAR 13 PHB depolymerase was not affected by

FIGURE 10: Determination of K_m and V_{max} of *F. solani* PHB depolymerase by double reciprocal plot.

FIGURE 11: Effect of inhibitors on *F. solani* PHB depolymerase.

N-acetylimidazole. Phenylmethylsulfonyl fluoride (PMSF) is known as an inhibitor of serine residues [5, 24, 30]. The PHB depolymerase of *A. saperae* was partially inactivated by 10 mM PMSF [28], and that of *P. lemoignei* [24] and *Agrobacterium* sp. [31] was completely inhibited by 1 mM PMSF. However, the PHB depolymerase of *P. simplicissimum* LAR 13 showed 58% activity in the presence of 10 mM PMSF [9]. The inhibitor studies clearly shows distinct nature of *F. solani* PHB depolymerase active site as compared to the PHB depolymerases reported till date.

3.9. Substrate Specificity. Of the PNP-alkanoates tested, PNP-decanoate and copolymer P(HB-co-HV) were hydrolyzed efficiently by *F. solani* PHB depolymerase and the esterase activity for the substrate was measured at 180 units, respectively (Table 3), indicating the true depolymerase nature of the enzyme. The enzyme also showed appreciable activity with other PNP-alkanoates like PNP-acetate (PNPA) and PNP-butyrate (PNPB) which is in agreement with the PHB depolymerase of *E. minima* [8] and *A. fumigatus* Pdf1 [7]. Absence of activity with PCL indicates that the enzyme is not a cutinase [32].

3.10. Identification of PHB Hydrolysis End-Products. Identification of the aqueous end-products of PHB

TABLE 3: Substrate specificities of *F. solani* PHB depolymerase on different polymers.

Substrates	Activity (Units/mL/min)
PNPA ^a	0.6
PNPB ^b	3.2
PNPD ^c	180
PCL ^d	0
P(HB-Co-HV) ^e	180

^aPolynitrophenyl acetate; ^bpolynitrophenyl butyrate; ^cpolynitrophenyl decanoate; ^dpolycaprolactone; ^epolymer of polyhydroxybutyrate and hydroxyvalerate.

FIGURE 12: Identification of PHB hydrolysis end products by PHB depolymerase of *F. solani*. Lane 1— β -hydroxybutyrate standard, Lane 2—end-product of PHB depolymerase reaction.

depolymerase reaction by paper chromatography revealed β -hydroxybutyrate monomer as major end product of PHB hydrolysis (Figure 12). Though high-resolution analysis or HPLC/LC-MS analysis is required to confirm the above observations, this preliminary observation by paper chromatography indicated mainly monomers as the aqueous end-product of PHB hydrolysis by *Penicillium* sp. S2 PHB depolymerase as in the case of *Comamonas* sp [33] and *P. picketti* [30], as against the depolymerase of *A. faecalis* T1, *A. faecalis* AE122, and *P. lemoignei* which hydrolyze PHB mainly to the dimeric and trimeric ester of hydroxybutyrate [8, 10, 24, 27].

4. Conclusion

Biodegradable plastics, such as PHB, continue to make progress in both the commercial and scientific fields. However, their use as a replacement for conventional plastics in a wide range of applications has been hindered by their brittleness, low mechanical strength, and high production cost. Improvements in the fermentation technology and genetically modified strains are being developed for

the economical and efficient production of PHB [34, 35]. Many fine chemicals produced by chemical processes can also be prepared using microbial fermentation [36]. Our isolate, *Penicillium expansum*, is a good candidate for the production of pure D-3-hydroxybutyric acid, which is the main component of the degradation products obtained by enzymatic degradation of PHB [1]. A pure monomer of PHB, D-3-hydroxybutyric acid, is also an important precursor of 4-acetoxazetidinone, which is used in making carbapenem antibiotics [37]. Intensive studies on this newfound strain as a useful microorganism for industrial application are necessary.

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