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Purification and Characterization of Poly(3-hydroxybutyrate) Depolymerase from a Fungal Isolate, *Emericellopsis minima* W2

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The fungus, *Emericellopsis minima* W2, capable of degrading poly(3-hydroxybutyrate) (PHB) was isolated from a waste water sample. Production of the PHB depolymerase from *E. minima* W2 ($PhaZ_{Emi}$) was significantly repressed in the presence of glucose. $PhaZ_{Emi}$ was purified by column chromatography on Octyl-Sepharose CL-4B and Sephadex G-100. The molecular mass of the $PhaZ_{Emi}$, which consisted of a single polypeptide chain, was estimated to be 48.0 kDa by SDS-PAGE and its pI value was 4.4. The maximum activity of the $PhaZ_{Emi}$ was observed at pH 9.0 and 55°C. It was significantly inactivated by 1 mM dithiothreitol, 2 mM diisopropyl fluorophosphate, 0.1 mM Tween 80, and 0.1 mM Triton X-100, but insensitive to phenylmethylsulfonyl fluoride and *N*-ethylmaleimide. The $PhaZ_{Emi}$ efficiently hydrolyzed PHB and its copolyester with 30 mol% 3-hydroxyvalerate, but did not act on poly(3-hydroxyoctanoate). It also hydrolyzed *p*-nitrophenylacetate and *p*-nitrophenylbutyrate but hardly affected the longer-chain forms. The main hydrolysis product of PHB was identified as a dimer of 3-hydroxybutyrate.

Key words: *Emericellopsis minima,* PHB depolymerase, poly(3-hydroxyalkanoate), poly(3-hydroxybutyrate)

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 C_3-C_5 and MCL-PHAs are comprised of (*R*)-hydroxyalkanoates of C_6-C_{14}. Poly(3-hydroxybutyrate) (PHB), a representative SCL-PHA, is a promising material for use as a renewable and biodegradable plastic (Lee, 1996; Madison and Huisman, 1999).

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 (Jendrossek *et al.*, 1996; Jendrossek, 2001). 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 (Matavulj and Molitoris, 1992). 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 four PHB depolymerases from *Penicillium funiculosum* ($PhaZ_{Pfu}$) (Brucato and Wong, 1991), *Penicillium pinophilum* ($PhaZ_{Ppi}$) (Han *et al.*, 1998), *Aspergillus fumigatus* ($PhaZ_{Afu}$) (Scherer, 1996), and *Paecilomyces lilacinus* ($PhaZ_{Pli}$) (Oda *et al.*, 1997) have been purified and partially characterized to date. Therefore, the biochemical properties of fungal PHB depolymerases are not well documented in comparison to those of bacterial PHB depolymerases.

The present paper describes the purification process and some of the properties of the PHB deploymerase obtained from a fungal isolate, W2. The exceptional properties of this enzyme are emphasized and compared with those of other fungal depolymerases.

Materials and Methods

Preparation of polyesters and latex suspensions

PHB (MWavg, 350,000) and its copolymer with 30 mol% 3-hydroxyvalerate (PHBV, MWavg, 320,000) were produced from *Ralstonia eutropha* (Chung *et al.*, 2001) and *Alcaligenes* sp. SH-69 (Yoon *et al.*, 1995), respectively.

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PHB and PHBV granules were isolated from the cells as described in our previous paper (Kim *et al.*, 2002) and latex suspensions were made by ultrasonically dispersing the polymer granules in distilled water. Poly(3-hydroxyoctanoate) (PHO; MWavg, 50,000) was synthesized by *Pseudomonas putida* KCTC 2407 from octanoate (Kim *et al.*, 2002). Polycaprolactone [PCL; MWavg, 42,500] was purchased from the Aldrich Chemical Co. Suspensions of PHO and PCL were prepared as previously described (Jaeger *et al.*, 1995).

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 (Kim *et al.*, 2000) containing PHB or PHBV as the sole carbon source.

Production of the PHB depolymerase from the isolate

The strain W2 was cultured by shaking at 200 rpm for 3 days at 30°C using three 500 ml Erlenmeyer flasks, each containing 100 ml of potato dextrose broth, and then transferred to a fermentor containing a mineral salt medium supplemented with 0.2% (wt/v) PHB granules. The fermentation experiments were conducted in a 5 L jar fermentor with a working volume of 3 L for 3 days. The temperature and pH were automatically controlled at optimal values of 30°C and 8.0, respectively. The airflow rate and stirring speed were 0.5 vvm and 250 rpm, respectively.

Purification of the PHB depolymerase from the isolate

The culture supernatant was first applied onto an Octyl-Sepharose CL-4B column pre-equilibrated with 50 mM glycine-NaOH buffer, pH 9.0, and eluted with a gradient of 0 to 50% ethanol. The fractions with high depolymerase activity were collected, concentrated by ultrafiltration using a PM10, and the enzyme was subsequently purified by gel permeation chromatography (GPC) using Sephadex G-100.

Enzyme assays

PHB depolymerase activity was routinely assayed by measuring the turbidity decrease at 650 nm of PHB suspension. The reaction mixture (O.D. 1 ± 0.02) containing 150 μl of PHB suspension, 50 μl (1.5 U) of the enzyme, and 50 mM of glycine-NaOH buffer (pH 9.0) in a total volume of 2 ml, was incubated at 37°C for 30 min. One unit of depolymerase activity was defined as the decrease of the value of A_{650} by 1 unit per hour. Esterase activity was assayed in 2 ml of 50 mM potassium phosphate buffer, pH 7.5, using various *p*-nitrophenylalkanoates (PNP-alkanoates). The reaction mixtures contained 30 μl of a 10 mM solution of the respective PNP-alkanoates in ethanol and 50 μl (1.5 U) of the enzyme solution. One unit of esterase activity was defined as the amount of protein required to produce 1 μmol of PNP from PNP-alkanoate per hour. The inhibitory effect of various chemical reagents on enzyme activity was measured as follows: the reaction mixture (1.97 ml) containing 50 μl (1.5 U) of the enzyme solution, reagent, and 50 mM potassium phosphate buffer (pH 7.5) was initially pre-incubated for 1 h at 37°C, with the enzymatic reaction subsequently started by adding 30 μl of a 10 mM solution of PNP-acetate.

Analysis

The quaternary structure of the native enzyme was determined by GPC using Sephacryl S-200 (Kim *et al.*, 2002). The relative molecular mass (M_r) of the denatured enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 11% gel according to the method described by Laemmli (1976). Isoelectric focusing was performed using PhastGel slabs (pH 3 to 9). Protein concentrations were measured by Bradford's method (1976) using bovine serum albumin as the standard. Identification of the hydrolysis products was performed by electron impact gas chromatography/mass spectrometry (EI GC/MS) according to the method described by Kim *et al.* (2002).

Results and Discussion

The fungal isolate, W2, showed good growth at 25°C when cultivated on potato dextrose agar (PDA), malt extract agar (MEA), and oat meal agar (OMA) for 10 days and the colony color turned from pink to blackish-brown with age on PDA and OMA. It formed sexual structures on PDA and OMA, but not on MEA, when grown at 25°C for 1 month. The isolate had brown ascomata of a cleistothecium type, 50-90 μm in diameter, and its globose asci contained 8 ascospores. Specifically, the ascospores ($5.1\text{-}7.6 \times 3.3\text{-}4.1$ μm in size) were pale brown, ellipsoidal with two pointed ends and surrounded by hyaline wings. These morphological characteristics of the strain W2 were nearly identical to those of type strain *E. minima* Stolk 1955. From these results, the isolate was identified as *E. minima* and deposited in the Korean Collection for Type Cultures under code no. *E. minima* KCTC 26195.

When *E. minima* W2 was grown on a mineral salt medium containing 2% glucose, it showed a maximal growth rate at pH 8.0 and its growth was approximately 80% of the maximal value even at pH 8.5. The organism secreted a PHB depolymerase ($PhaZ_{Emi}$) into the culture broth when it was grown with either PHB or PHBV, although the $PhaZ_{Emi}$ in the culture supernatant did not show any hydrolyzing activity toward PHO and PCL as determined by a turbidity decrease test. In contrast, strain

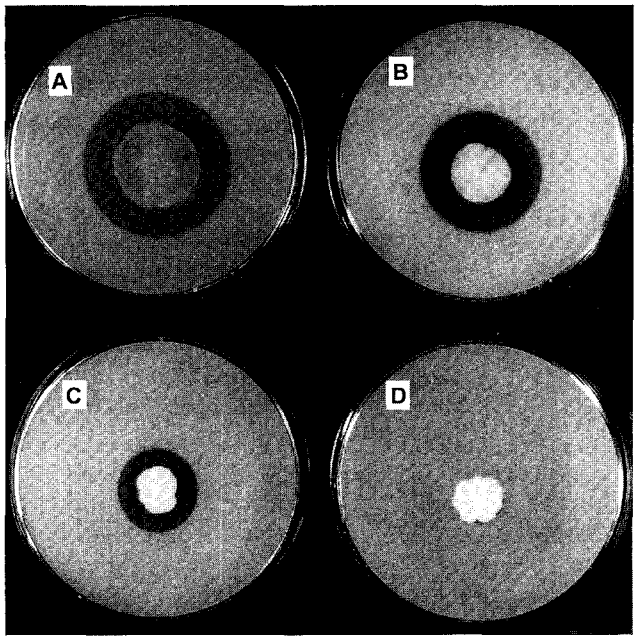

Fig. 1. Production of the PHB depolymerase by *E. minima* W2 grown on a mineral salt medium containing 0.2% PHB granules in the presence of 0% (A), 0.1% (B), 0.5% (C), and 1.0% (D) glucose for 10 days.

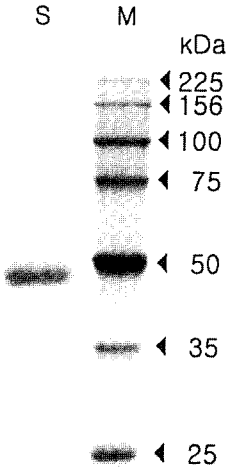

Fig. 2. SDS-PAGE of the purified PHB depolymerase from *E. minima* W2. Proteins were separated on a SDS-11% polyacrylamide gel and revealed by Coomassie brilliant blue R-250 staining. The numbers on the right show the molecular masses (kDa) of the standard proteins. Lanes: M, molecular mass standards; S, the purified PHB depolymerase (4 μg).

W2 efficiently produced the PCL depolymerase without PHB depolymerase activity in the presence of PCL. From these results, it is believed that production of the two different hydrolases from *E. minima* W2 is dependent on the kind of substrate. Production of the $PhaZ_{Emi}$ was significantly inhibited in the presence of glucose, an easily utilizable carbon source (Fig. 1). The gradual decrease in the clear zone size for the colonies with increasing concentrations of glucose tends to indicate that the synthesis of the PHB depolymerase is inhibited by catabolite repression. On the other hand, it has been reported that the PHB depolymerase from *Alcaligenes faecalis* T1 can be produced to a significant level when this organism is grown with glucose as the sole carbon source (Zhang *et al.*, 1992).

$PhaZ_{Emi}$ was strongly bound to hydrophobic materials in the column and was eluted by a gradient of approximately 30% ethanol. For further purification the proteins obtained from hydrophobic interaction chromatography were repurified by GPC using Sephadex G-100. A quantitative evaluation of the results obtained from the consecutive purification steps is listed in Table 1. The enzyme was purified 20-fold with an overall yield of 51.1%.

The molecular mass (M_r) of the native $PhaZ_{Emi}$ was estimated to be 50.0 kDa by GPC and the M_r of the denatured $PhaZ_{Emi}$ was evaluated to be 48.0 kDa by SDS-PAGE (Fig. 2). The present results indicate that the enzyme is one single polypeptide chain. The pI value of $PhaZ_{Emi}$ was determined to be 4.4 by isoelectric focusing and its maximum activity was obtained at pH 9.0 and 55°C, indicating that it is an alkalophilic enzyme, relatively distinct from other eukaryotic PHB depolymerases. It has been demonstrated that the PHB depolymerases from *A. faecalis* AE122 (Kita *et al.*, 1995) and *P. funiculosum* (Brucato and Wong, 1991) have pI values in the acidic pH range. However, the majority of bacterial PHB depolymerases and $PhaZ_{Afu}$ that have been purified and characterized to date are hydrolases with neutral or alkaline pI values (Jendrossek *et al.*, 1996). The purified $PhaZ_{Emi}$ was completely inhibited by nonionic detergents such as 0.1% Tween 80 and 0.1% Triton X-100, indicating that a hydrophobic region may be located near or at the active site. It is noteworthy that $PhaZ_{Emi}$ activity was markedly decreased to below 10% of its original activity by dithiothreitol (DTT) at a concentration of 1 mM, similar to other bac-

Table 1. Purification of the PHB depolymerase from *E. minima* W2

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Supernatant	12.92	1850	143	100.0	1.0
Octyl-Sepharose CL-4B	1.19	1378	1158	74.5	8.1
Sephadex G-100	0.33	946	2866	51.1	20.0

terial and fungal PHB depolymerases. This finding strongly suggests that disulfide bridges in the active site of PHB depolymerases play an essential role in enzyme activity. On the other hand, $PhaZ_{Emi}$ was insensitive to 10 mM *N*-ethylmaleimide (NEM) and sodium azide, which is a sulfhydryl reagent. The significant decrease of $PhaZ_{Emi}$ activity to below 15% of its original activity by 2 mM of diisopropyl fluorophosphate (DFP) indicated that the enzyme is an esterase belonging to the serine hydrolase family. However, due to its compact architecture, $PhaZ_{Emi}$ was not susceptible to phenylmethylsulfonyl fluoride (PMSF), another serine residue-directed inhibitor, which probably makes the active serine fairly inaccessible to PMSF with its bulky aromatic moiety, as demonstrated by Schirmer *et al.* (1995). Of the PNP-alkanoates tested, PNP-acetate and PNP-butyrate were hydrolyzed efficiently by $PhaZ_{Emi}$ and in these cases the esterase activity for each substrate was measured at 4.5 U and 2.3 U, respectively. However, the esterase activities of the $PhaZ_{Emi}$ toward various PNP-alkanoates containing even numbers of carbon atoms (between six and sixteen) in the carbon chain, which are substrates for esterase and/or lipase, were significantly low or not detected. This result was highly comparable to that of Oda *et al.* (1997), in which $PhaZ_{Pli}$ revealed the highest esterase activity for PNP-octanoate.

The composition and relative amounts of hydrolysis products of PHB by $PhaZ_{Emi}$ are shown in Fig. 3. The GC chromatogram (Fig. 3a) shows that a dimer of 3HB is the main hydrolysis product after the enzymatic hydrolysis of PHB for 12 h, as determined by EI GC/ MS analysis. The EI mass spectra of Fig. 3b and Fig. 3c clearly indicated that the two distinct peaks on the GC chromatogram contained ion fragments with *m/z* values of 43, 60, 71, and 89, which are characteristic ones produced from the 3HB monomer and ion fragments with *m/z* values of 43, 87, and 128, and 175, which are characteristic ones produced from 3HB dimer. In this study, the relative ratio of dimer to monomer that was produced by PHB degradation was nearly constant, regardless of the enzymatic reaction time.

Table 2 provides some of the biochemical properties of $PhaZ_{Emi}$ and other fungal PHB depolymerases. The $PhaZ_{Emi}$

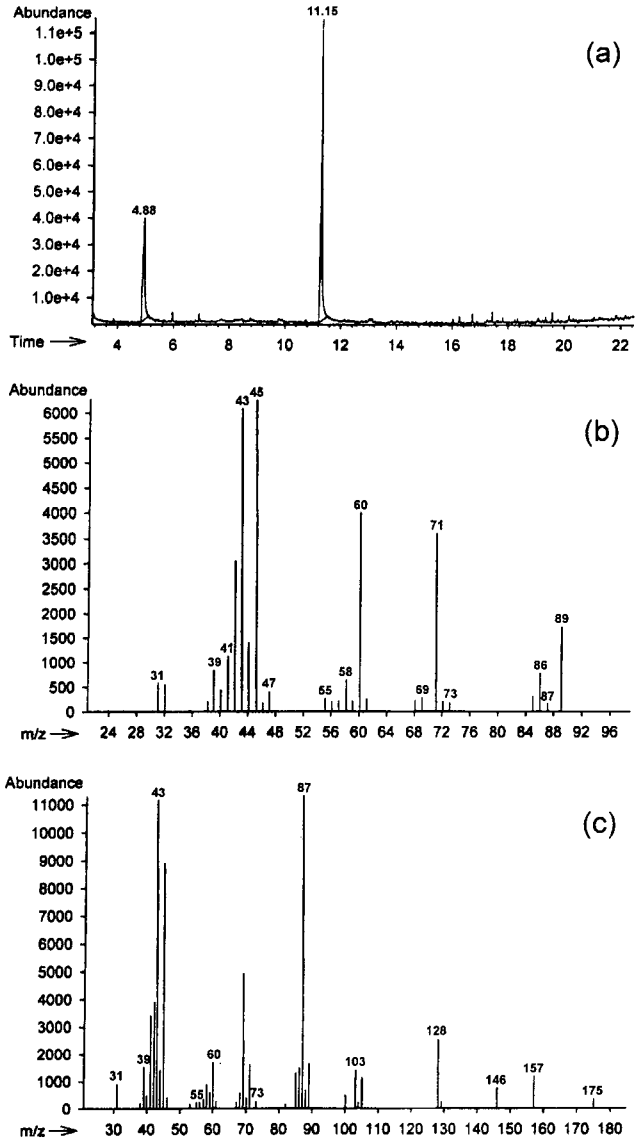

Fig. 3. GC chromatogram of hydrolysis products formed after the enzymatic degradation of PHB for 12 h in the standard assay mixture (a) and EI mass spectra of 3HB monomer [RT: 4.88] (b) and 3HB-*co*-3HB dimer [RT: 11.15] (c).

Table 2. Biochemical properties of various fungal PHB depolymerases

Characteristics	$PhaZ_{Emi}$	$PhaZ_{Pfu}$	$PhaZ_{Ppi}$	$PhaZ_{Pli}$	$PhaZ_{Afu}$
Quaternary structure	monomer	monomer	monomer	ND [a]	monomer
MW (kDa)	48.0	37.0	35.0	48.0	57.0
pI	4.4	5.8	ND	ND	7.2
Optimum pH	9.0	6.0	6.0	7.0	8.0
Optimum temp. (°C)	55	ND	50	45	70
Sensitivity to					
sodium azide	-	ND	ND	ND	ND
DTT	+	+	ND	+	+
PMSF	-	-	ND	+	+
DFP	+	+	ND	ND	ND

Table 2. Continued

Characteristics	$PhaZ_{Emi}$	$PhaZ_{Pfu}$	$PhaZ_{Ppi}$	$PhaZ_{Pli}$	$PhaZ_{Afu}$
NEM	-	-	ND	+	ND
Triton X-100	+	+	ND	ND	+
Tween 80	+	+	ND	+	+
Main hydrolysis product of PHB	dimer	ND	ND	ND	monomer
Reference	This study	Brucato and Wong, 1991	Han *et al.*, 1998	Oda *et al.*, 1997	Scherer, 1996

[a]Not determined.
DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; NEM, *N*-ethylmaleimide.

is similar to other eukaryotic depolymerases in its quaternary structure and susceptibility to DTT and nonionic detergents such as Tween 80 and Triton X-100, while its M_r, p*I* value, and pH and temperature optima on activity differ from those of the PHB depolymerases from *Penicillium* and *Aspergillus* species. Notably, the M_r of $PhaZ_{Pli}$ (Oda *et al.*, 1997) is identical with that of $PhaZ_{Emi}$ although its pH and temperature optima on activity and sensitivity to PMSF and NEM differ greatly from those of $PhaZ_{Emi}$. The production of the 3HB dimer from PHB with $PhaZ_{Emi}$ as the main hydrolysis product is also very comparable to that of the 3HB monomer produced by $PhaZ_{Afu}$ (Scherer, 1996). The present results suggest that $PhaZ_{Emi}$ is an enzyme with distinct characteristics, different from those of the other eukaryotic PHB depolymerases reported to date.

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References

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

Brucato, C.L. and S.S. Wong. 1991. Extracellular poly(3-hydroxybutyrate) depolymerase from *Penicillium funiculosum*: general characteristics and active site studies. *Arch. Biochem. Biophys.* 290, 497-502.

Chung, S.H., G.G. Choi, H.W. Kim, and Y.H. Rhee. 2001. Effect of levulinic acid on the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Ralstonia eutropha* KHB-8862. *J. Microbiol.* 39, 79-82.

Han, J.S., Y.J. Son, C.S. Chang, and M.N. Kim. 1998. Purification and properties of extracellular poly(3-hydroxybutyrate) depolymerase produced by *Penicillium pinophilum*. *J. Microbiol.* 36, 67-73.

Jaeger, K.E., A. Steinbüchel, and D. Jendrossek. 1995. Substrate specificities of bacterial polyhydroxyalkanoate depolymerases and lipases: bacterial lipases hydrolyze poly(ω-hydroxyalkanoates). *Appl. Environ. Microbiol.* 61, 3113-3118.

Jendrossek, D., A. Schirmer, and H.G. Schlegel. 1996. Biodegradation of polyhydroxyalkanoic acids. *Appl. Microbiol. Biotechnol.* 46, 451-463.

Jendrossek, D. 2001. Microbial degradation of polyesters. *Adv. Biochem. Eng. Biotechnol.* 71, 293-325.

Kim, D.Y., Y.B. Kim, and Y.H. Rhee. 2000. Evaluation of various carbon substrates for the biosynthesis of polyhydroxyalkanoates bearing functional groups by *Pseudomonas putida*. *Int. J. Biol. Macromol.* 28, 23-29.

Kim, D.Y., J.S. Nam, and Y.H. Rhee. 2002. Characterization of an extracellular medium-chain-length poly(3-hydroxyalkanoate) depolymerase from *Pseudomonas alcaligenes* LB19. *Biomacromolecules* 3, 291-296.

Kita, K., K. Ishimaru, M. Teraoka, H. Yanase, and N. Kato. 1995. Properties of poly(3-hydroxybutyrate) depolymerase from a marine bacterium, *Alcaligenes faecalis* AE122. *Appl. Environ. Microbiol.* 61, 1727-1730.

Laemmli, U.K. 1976. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lee, S.Y. 1996. Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.* 49, 1-14.

Madison, L.L. and G.W. Huisman. 1999. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiol. Mol. Biol. Rev.* 63, 21-53.

Matavulj, M. and H.P. Molitoris. 1992. Fungal degradation of polyhydroxyalkanoates and a semiquantitative assay for screening their degradation by terrestrial fungi. *FEMS Microbiol. Rev.* 103, 323-332.

Oda, Y., H. Osaka, T. Urakami, and K. Tonomura. 1997. Purification and properties of poly(3-hydroxybutyrate) depolymerase from the fungus *Paecilomyces lilacinus* D218. *Curr. Microbiol.* 34, 230-232.

Scherer, T.M. 1996. Biological and enzymatic mechanisms of polyester biodegradation by fungi. Ph.D. Thesis, University of Massachusetts, Amherst.

Schirmer, A., C. Matz, and D. Jendrossek. 1995. Substrate specificities of poly(hydroxyalkanoate)-degrading bacteria and active site studies on the extracellular poly(3-hydroxyoctanoic acid) depolymerase of *Pseudomonas fluorescens* GK13. *Can. J. Microbiol.* 41(Suppl. 1), 170-179.

Yoon, J.S., J.Y. Kim, and Y.H. Rhee. 1995. Effects of amino acid addition on molar fraction of 3-hydroxyvalerate in copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate synthesized by *Alcaligenes* sp. SH-69. *J. Ferment. Bioeng.* 80, 350-354.

Zhang, J., T. Saito, A. Ichikawa, and T. Fukui. 1992. Secretion of poly(3-hydroxybutyrate) depolymerase by *Alcaligenes faecalis* T1. *Chem. Pharm. Bull.* 40, 713-717.