Appl Microbiol Biotechnol (2013) 97:7679–7688
DOI 10.1007/s00253-012-4595-x

Affinity purification and characterization of a biodegradable plastic-degrading enzyme from a yeast isolated from the larval midgut of a stag beetle, *Aegus laevicollis*

Ken Suzuki · Hironori Sakamoto · Yukiko Shinozaki · Jun Tabata · Takashi Watanabe · Atsushi Mochizuki · Motoo Koitabashi · Takeshi Fujii · Seiya Tsushima · Hiroko K. Kitamoto

Received: 4 September 2012 / Revised: 9 November 2012 / Accepted: 13 November 2012 / Published online: 8 December 2012

Abstract Two yeast strains, which have the ability to degrade biodegradable plastic films, were isolated from the larval midgut of a stag beetle, *Aegus laevicollis*. Both of them are most closely related to *Cryptococcus magnus* and could degrade biodegradable plastic (BP) films made of poly(butylene succinate) (PBS) and poly(butylene succinate-*co*-adipate) (PBSA) effectively. A BP-degrading enzyme was purified from the culture broth of one of the isolated strains employing a newly developed affinity purification method based on the binding action of the enzyme to the substrate (emulsified PBSA) and its subsequent degradative action toward the substrate. Partial amino acid sequences of this enzyme suggested that it belongs to the cutinase family, and thus, the enzyme was named CmCut1. It has a molecular mass of 21 kDa and a degradative activity for emulsified PBSA which was significantly enhanced by the simultaneous presence of Ca^{2+} or Mg^{2+} at a concentration of about 2.5 mM. Its optimal pH was 7.5, and the optimal temperature was 40 °C. It showed a broad substrate specificity for *p*-nitrophenyl (pNP)-fatty acid esters ranging from pNP-acetate (C2) to pNP-stearate (C18) and films of PBSA, PBS, poly(ε-caprolactone), and poly(lactic acid).

Keywords Biodegradable plastics · *Aegus laevicollis* · Midgut · *Cryptococcus* · Biodegradable plastic-degrading enzyme · Cutinase · Lipase

Introduction

Driven by a growing demand for sustainable solutions to the problem of huge volume of plastic wastes, the commercial market of biodegradable plastics (BPs) has been expanding. Consequently, the European market for BPs is growing at an annual rate of roughly 20 %. The global production capacity of BPs is 56,500 tonnes in 2010 and is estimated to increase to 143,500 tonnes by 2015 (Lange 2011). Products that show vast growth rates include bags, catering products, agricultural mulching films, and food/beverage packaging materials.

Each year after harvest, farmers have to gather and dispose used nondegradable plastic agricultural materials for recycle as industrial wastes. Unlike typical plastics, BPs can be degraded by microorganisms in the natural environment, thus users do not have to recover used BP materials from the fields. Because of these beneficial characteristics, i.e., environment friendly and labor saving, a wide range of agricultural BP products (e.g., mulch film, landfill cover, vine nets, and pots) for various uses are already sold in the market. However, controlling the degradation speed of the BP products is difficult because of its considerable dependence on environmental factors, such as temperature, humidity, as well as the activities of soil microorganisms. It is essential for useful BP products to have characteristics that strike a balance between physical strength and biodegradability suitable for their intended uses (Gross and Kalra 2002; Kyrikou and Briassoulis 2007). To encourage the further spread and use of BP products, it would be necessary to solve these problems.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-012-4595-x) contains supplementary material, which is available to authorized users.

K. Suzuki · H. Sakamoto · Y. Shinozaki · J. Tabata · T. Watanabe · A. Mochizuki · M. Koitabashi · T. Fujii · S. Tsushima · H. K. Kitamoto (✉)
National Institute for Agro-Environmental Sciences (NIAES), 3-1-3 Kannondai,
Tsukuba, Ibaraki 305-8604, Japan
e-mail: kitamoto@affrc.go.jp

Microorganisms or their enzymes with strong degrading ability can accelerate the degradation speed of BPs (Acero et al. 2011; Chatterjee et al. 2010; Kasuya et al. 2009; Maeda et al. 2005; Masaki et al. 2005; Ronkvist et al. 2009; Uchida et al. 2000). Their contribution will make BPs more user friendly and would make possible the replacement of various plastic products with BPs. For this purpose, cutinase-like BP film degrading enzymes, e.g., CLE from a yeast *Cryptococcus* sp. S-2 (Masaki et al. 2005) and CutL1 from a fungus, *Aspergillus oryzae*, (Maeda et al. 2005), were isolated and characterized. We previously reported that phyllosphere is one of the sources of yeast strains of *Pseudozyma* spp. and *Cryptococcus* spp. with strong biodegradable plastic-degrading ability (Kitamoto et al. 2011). A novel, wide-spectrum enzyme, which could degrade various BPs, had been isolated from *Pseudozyma antarctica*, identified and characterized (Shinozaki et al. 2012a, b).

Insects are organisms which have evolved most diversely on earth and have adapted themselves to various extreme habitats and foods. Gut-inhabiting microorganisms have been deeply involved in the diversification of insects. It is well known that the gut-inhabiting microorganisms play important roles in digesting food to provide nutrients needed by the host (Breznak and Brune 1994; Douglas 1998). Recently, vast numbers of unknown microorganisms were discovered in the gut of beetles, suggesting that the gut of insects serves as a good habitat for diverse species of microorganisms (Park et al. 2007; Suh et al. 2005). Some of the larvae of stag beetles grow by exclusively eating decayed woods which contain macromolecular components such as cellulose, lignin, and hemicellulose (Araya 1993; Tanahashi et al. 2010). Intestinal microorganisms of such larvae would be associated with the digestion activity of such macromolecular components, which are expected to have various chemical structures. It can be reasonably expected that microorganisms which can degrade the BPs exist in the beetle's gut.

The objectives of this study are (1) to isolate and identify BP-degrading microorganisms from the gut of stag beetle larvae, (2) to produce BP-degrading enzymes from the isolated strain with the strongest BP-degrading ability, (3) to purify the enzyme with a newly developed purification method using the BP-affinity binding activity and BP-degradation characteristics of the enzyme, and (4) to characterize the purified enzyme.

Materials and methods

Preparation of the homogenate from organs of stag beetles

Larvae of *Aegus laevicollis* were collected from brown-rotted woods in May 2007 at Miyake-jima-island, Japan. Larvae are placed in a plastic container with decayed wood pieces of Fagaceae and maintained at 23 °C and 16-h light/8-h dark daily photoperiodic cycles. Within a week after collection, midguts, hindguts, and integuments of the larvae of stag beetles were separated using a sterile needle in a clean bench. Each tissue sample (midgut, hindgut, and integument) isolated from each larva was separately homogenized in 500 μl of sterilized 10 mM sodium phosphate buffer (pH 7.0). Each homogenate sample was used as a seed stock of microorganisms for isolation.

Strains and media

Yeast strains of BPD1A and BPD2A were isolated and evaluated in this study. These strains were stocked at −80 °C. Strain BPD1A was deposited in the NIAS Genebank in the National Institute of Agrobiological Sciences, Japan (accession number, MAFF 306841). Yeast type strains of *Cryptococcus magnus* JCM 9038 (CBS 140), *Filobasidium floriforme* JCM 10631 (CBS 6241), and *P. antarctica* JCM 10317 were supplied from Japan Collection of Microorganisms of the Riken Bio-resource Center, Wako, Japan.

For isolation and evaluation of the microorganisms which degrade emulsified poly(butylene succinate-*co*-adipate) (PBSA) from larvae of stag beetles, two kinds of selective media composed of double layers (10 ml of bottom layer and 3 ml of upper layer) were used. For the bottom layer, 10 ml each of NA medium (Bacto Nutrient agar (Becton Dickinson & Company, Sparks, MD)) or fungal minimum (FMM) agar medium (Kitamoto et al. 2011) with 1.5 % of agarose were used. Over the solidified bottom layer, 3 ml of upper layer containing 1 % emulsified PBSA (Bionolle EM-301, weight-average molecular weight—M_w, $1.2–1.5\times10^5$ supplied by Showa Denko K.K., Tokyo, Japan), 1 % soy bean oil, and 1.5 % agarose was poured and solidified. These selective media were named NA-PBSA-oil medium and FMM-PBSA-oil-agar medium, respectively. For screening, the microorganisms present in the gut homogenate, the following antibiotics were added on the surface of the selective media: cycloheximide (final 50 μg ml^{-1}) to NA-PBSA-oil medium and chloramphenicol (final 40 μg ml^{-1}) to FMM-PBSA-oil-agar medium. For the production of biodegradable plastic-degrading enzyme, a basal medium composed of 1 % yeast extract, 1 % KH_2PO_4, 0.1 % $MgSO_4{\cdot}7H_2O$, and 1 % soy bean oil (Kamini et al. 2000) was used.

Isolation of biodegradable plastic-degrading microorganisms

From each gut homogenate sample, 100 μl were diluted with 10 mM sodium phosphate buffer (pH 7.0) by 10^3 times and 50 μl each of the diluted gut homogenate was spread

onto the selective media (NA-PBSA-oil medium and FMM-PBSA-oil-agar medium) containing antibiotics as described above. The plates were incubated at room temperature (at around 25 °C). A single colony appearing at the centre of the clarified emulsified PBSA on the plate within 1 week was selected and isolated as a strain with the ability to degrade emulsified PBSA and stocked in 10 % glycerol at −80 °C.

Sequence analysis of rDNA of BP-degrading microorganisms

To identify the selected yeast-like strains, the nucleotide sequences of the rDNA of each strain were determined and compared with those in the GenBank by using the Blast search with nucleotide sequence database. For PCR amplification, genomic DNA was extracted from cells and the gene sequences of the D1/D2 regions of 18S rRNA gene were identified as described in Kitamoto et al. (2011). By using the universal fungal primers for ITS1 (5′-gtcgtaacaaggtttccgtaggtg-3′), ITS2 (5′-gctgcgttcttcatcgatgc-3′), and ITS4 (5′-tcctccgcttattgatatgc-3′), the sequences of ITS1-5.8S-ITS2 and ITS1-5.8S-ITS4 were also analyzed with the same procedure and listed the accession numbers at the end of "Materials and methods."

D1/D2, ITS1-5.8S-ITS2, and ITS1-5.8S-ITS4 regions of the *C. magnus*-type strain CBS 140 (JCM 9038; GenBank accession No. AF18185 and AF190008) and those of *F. floriforme*-type strain CBS 6241 (JCM 10631; accession No. AF075498 and AF190007) were used for the identification of isolated strains.

Evaluation of biodegradable plastic-degrading ability of selected yeast strains

To evaluate their solid polymer-degrading activity, an assay for PBS (Bionolle 3001G; M_w, 2.0–2.5×10^5 and thickness, 20 μm) and PBSA (Bionolle 1001G; M_w 2.0–2.5×10^5 and thickness, 20 μm; 2×2 cm) mulch film degradation activity by isolated microorganisms was performed on agarose plates as described previously (Kitamoto et al. 2011), except that in this study, the selective conditions used were NA-PBSA-oil medium at 25 °C and FMM-PBSA-oil-agar medium at 28 °C. In the assay, the extent of degradation of film was estimated by the luminance of the 2×2-cm area of residual film (three films at the same time) as described previously (Kitamoto et al. 2011). The type strain of *P. antarctica* JCM 10317, previously reported to have strong BP-degrading ability (Kitamoto et al. 2011), was used for comparison.

Production of BP-degrading enzyme in liquid culture

Yeast strain BPD1A was incubated at 30 °C for 3 days on FMM agar plate. After incubation, colonies were collected and suspended in 450 μl of 10 mM sodium phosphate buffer (pH 7.0). The cell suspension (100 μl) was inoculated into 500-ml Erlenmeyer flasks containing 150 ml of the basal medium, which were then shaken at 30 °C on a rotary shaker at 170 rpm for 4 days.

Assay for emulsified PBSA-degrading activity

Emulsified PBSA-degrading enzyme activity was measured by spectrophotometry as a decrease in turbidity of the emulsified PBSA solution (Uchida et al. 2000; Masaki et al. 2005; Maeda et al. 2005). To ensure uniform quality, commercially available emulsified PBSA (Bionolle EM-301) was used as described previously (Kitamoto et al. 2011; Shinozaki et al. 2012a; Koitabashi et al. 2012) with some modifications, as follows: 15 μl of 4 % (*w*/*v*) emulsified PBSA was diluted with 2 ml of 25 mM HEPES-NaOH buffer (pH 7.4) containing 2.5 mM $CaCl_2$ in a glass test tube (11.5 mm inner diameter; 100 mm height) and was held at 30 °C for about 10 min in the water bath with reciprocal agitation (120 rpm). Immediately after adding the appropriate amount of enzyme solution (up to 10 μl), the tube was briefly vortexed and then returned to the water bath. Subsequently, the optical density (OD) of the reaction mixture at 660 nm was measured at appropriate intervals. After subtracting the OD of the control containing no enzyme, the activity was evaluated. One unit (U) of PBSA-degradation enzyme activity was defined as the activity that can decrease the OD by 1 (with a 1-cm light path at 660 nm) in 1-ml reaction solution at 30 °C in 1 min.

Purification of BP-degrading enzyme

The yeast cells were separated from the medium by centrifugation. The considerably turbid supernatant was clarified by adding $CaCl_2$ to a final concentration of 10 mM and then left overnight at room temperature. The resulting unidentified sediment was removed by filter paper (VCF-01, Hario, Tokyo, Japan) and subsequently by 0.2 μm pore-sized membrane filter (568 Filter unit, Nalgene, NY). The collected filtrate, ca. 250 ml, was concentrated to approximately 40 ml with Stirred Ultrafiltration Cell (Model 8200, Millipore, Billerica, MA) using a regenerated cellulose ultrafiltration membrane (PLGC06210, Millipore), and then the solvent was exchanged to 25 mM HEPES-NaOH buffer (pH 7.4) by gradually adding the buffer (total 200 ml) simultaneously with continuous concentration. The exchanged buffer solution (crude enzyme solution) was finally concentrated to 2.4 ml (ca. 104-fold concentration).

A part of the crude enzyme solution (100 μl), 200 μl of 25 mM HEPES-NaOH buffer (pH 7.4) and 4 mg of the emulsified PBSA were mixed well in a micro-glass tube (8-mm inner diameter; 50-mm height) and kept for 15 min at 15 °C on the water bath. Then the tube was centrifuged at

5,000×*g* for 3 min, and the supernatant (PBSA unadsorbed fraction) was removed. The precipitate was washed thoroughly by repeating the procedure of re-suspension (with 300 μl of 15 °C of 25 mM HEPES-NaOH buffer (pH 7.4)), centrifugation (at 5,000×*g* for 3 min) and supernatant removal for five times. Finally, the precipitate was suspended in 300 μl of 0.5 M HEPES-NaOH buffer (pH 7.4) containing 2.5 mM $CaCl_2$. The suspension was then transferred into a microdialysis unit (EasySep MD-003, TOMY SEIKO, Tokyo, Japan) and was dialyzed against 250 ml of 25 mM HEPES-NaOH buffer (pH 7.4) containing 2.5 mM $CaCl_2$ at room temperature overnight. The emulsified PBSA was degraded during dialysis and the suspension became clear. Finally, 400 μl of dialyzed solution (purified enzyme solution) was recovered. The purity and the molecular weight of the enzyme thus obtained were checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using Tris-tricine buffer system (e-PAGEL E-T15S and EzRunT, ATTO, Tokyo, Japan). The protein bands were visualized by silver staining (Silver Staining Kit Protein, GE Healthcare Bio-Sciences, Uppsala, Sweden). Protein concentrations were determined by the Quick Start Bradford Dye Reagent (Bio-Rad, Hercules, CA) according to the manufacturer's instructions with bovine gamma globulin as the standard. Purification steps for the enzyme are shown in Table 1.

Partial amino acid sequence analysis of the purified BP-degrading enzyme

A single CBB-stained protein spot was subjected to peptide sequencing by the method described by Shevchenko et al. (1996). A single CBB-stained band, representing the purified enzyme separated by SDS-PAGE, was excised from the gel and was digested by trypsin. The resulting peptide fragments were separated by HPLC and the amino acid sequences of three fragments were determined by Edman degradation on a PPSQ-23A gas-phase protein sequencer (Shimadzu, Kyoto, Japan).

Characterization of the BP-degrading enzyme

To determine the effect of Ca^{2+} and Mg^{2+} on BP-degrading activity, the purified enzyme was assayed in the presence of various concentrations of $CaCl_2$ or $MgCl_2$ in 25 mM HEPES-NaOH buffer (pH 7.4) at 30 °C. To determine the optimal pH, BP-degrading activity of the purified enzyme was assayed in 25 mM MES-NaOH buffer at pH from 5.48 to 6.60, 25 mM HEPES-NaOH buffer at pH from 6.91 to 8.01, or 25 mM TAPS-NaOH buffer at pH from 7.80 to 9.04, at 30 °C. MES, HEPES, and TAPS were purchased from Dojindo (Kumamoto, Japan). The Na^+ concentrations of all buffers were equalized to 20 mM with 1 M NaCl. All buffers contained 2.5 mM $CaCl_2$. The optimum temperature of the enzyme was evaluated by measuring its activity in 25 mM HEPES-NaOH buffer (pH 7.4) containing 2.5 mM $CaCl_2$ at each temperature (from 15 to 60 °C).

Esterase activity was assayed as described previously (Kitamoto et al. 2011) using 0.73 μg ml^{-1} of the enzyme, 25 mM HEPES-NaOH buffer (pH 7.4) containing 2.5 mM $CaCl_2$ as a reaction buffer and 200 μM *p*-nitrophenyl (pNP) ester (pNP-acetate, pNP-butyrate, pNP-valerate, pNP-octanoate, pNP-decanoate, pNP-dodecanoate, pNP-palmitate, or pNP-stearate; Sigma-Aldrich, St. Louis, MO) as substrate. One unit of esterase activity was defined as the release of 1 μmol of *p*-nitrophenol/min.

Lipase activity was measured using the Lipase Kit S (DS Pharma Biochemical, Osaka, Japan) with several modifications. All reagents used here with the exception of HEPES buffer were provided with the kit and prepared according to the manufacturer's instructions. Purified enzyme (172 ng) was preincubated in 160 μl of 25 mM HEPES-NaOH buffer (pH 7.4) containing 0.5 mM phenylmethylsulfonyl fluoride as an esterase inhibitor at 30 °C for 5 min. After the preincubation, 20 μl of substrate solution (20 mM 2,3-mercapto-1-propanol tributyrate–20 mM SDS in ethanol) was added and allowed to react for 20 min at 30 °C. Then, the reaction was stopped by adding 400 μl of the Reaction terminator solution provided with the kit (composition not provided by manufacturer). Twenty microliters of buffer solution (composition not provided by manufacturer) followed by 20 μl of Chromogenic reagent (0.25 mM 5,5′-dithiobis(2-nitrobenzoic acid)) was added to the reaction solution, and the absorbance at 412 nm (with a 1-cm light path) was measured within 1 h. After subtracting the absorbance of the control containing no enzyme from that of the test sample, lipase activity was calculated as micromoles of

Table 1 Purification of PBSA-degrading enzyme from BPD1A culture medium

Fraction	Total protein[a] (mg)	Total activity[a] (U)	Recovery (%)	Specific activity (U/mg)	Purification fold
Concentrated culture filtrate (crude enzyme solution)	0.30	0.95	100	3.20	1
PBSA-unadsorbed fraction	0.23	0.084	8.8	0.36	0.11
Purified enzyme solution	0.014	0.61	64.6	44.4	13.9

[a] These values are of per 100 μl concentrated culture filtrate

liberated SH groups per milligram of enzyme per minute, as described in the manufacturer's instructions. As a comparison, the activity of porcine pancreatic lipase (Type VI-S, Sigma-Aldrich) was also measured.

Protease activity in 25 mM HEPES-NaOH buffer (pH 7.4) was measured using the Pierce Fluorescent Protease Assay Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. After subtracting the change in relative fluorescent units (ΔRFU) of the control containing no enzyme from that of the test sample, protease activity was calculated as ΔRFU per microgram of enzyme per minute As a comparison, the activity of tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (provided with the kit) was also measured.

All assays in this section were repeated 3 times, and the activities were expressed as the average±standard deviation (SD). For lipase and protease activity assays, activities in buffers containing 2.5 mM $CaCl_2$ or $MgCl_2$ were also measured.

Evaluation of the enzymatic degradation of BP films

The BPs used in the degradation experiments were as follows: PBSA (Bionolle 3020; M_w 1.4×10^5), PBS (Bionolle 1020; M_w, 1.4×10^5), poly(ε-caprolactone) (PCL; M_w, $0.7–1.0\times10^5$; Wako Pure Chemical Industries, Ltd., Osaka, Japan), poly[(*R*)-3-hydroxybutyrate] (PHB; M_w, not described; Sigma-Aldrich, St. Louis, MO), poly(L-lactic acid) (PLLA; M_w, 1.0×10^5; Polyscience Inc. Warrington, PA), and poly(DL-lactic acid) (PDLLA; M_w, 2×10^4; Wako Pure Chemical Industries, Ltd.). The degradation activities of BP films by the purified enzyme were evaluated by measuring the water-soluble total organic carbon (TOC) released from the BP cast film as described previously (Shinozaki et al. 2012a, b) with the following modifications. To prepare cast films onto each well (7-mm diameter) of a water-repellent printing glass slide (Matsunami Glass Ind. Ltd., Osaka, Japan), BP pellets (except those of PHB) were dissolved in dichloromethane individually as described previously. Chloroform was used to dissolve PHB because it does not dissolve in dichloromethane. Each of the BP cast films (0.265 mg/7-mm diameter) was treated with 50 μl of purified enzyme solution (9.2 μg ml^{-1} in 25 mM HEPES-NaOH buffer, pH 7.4, containing 2.5 mM $CaCl_2$) at 30 °C for 24 h. The reaction solution was collected, and the TOC was measured using a TOC-V CSH analyzer (Shimadzu Co., Kyoto, Japan). After subtracting the values of controls containing no enzyme for each of the BPs, the corresponding film-degrading activities were evaluated.

Nucleotide sequence accession numbers

The nucleotide sequences of ITS1-5.8S-ITS4 region of rDNA determined in this study were deposited in the GenBank/EMBL/DDBJ databases under accession numbers AB727344 for *C. magnus* JCM 9038, AB727347 for *F. floriforme* JCM 10631, AB727345 for BPD1A, and AB727346 for BPD2A.

Results

Isolation of BP-degrading microorganisms from the larval midgut of stag beetle, *A. laevicollis*

Among the emulsified PBSA-degrading microorganisms present in the midgut derived from 72 stag beetle larvae, 18 colonies were selected on NA-PBSA-oil medium with cycroheximide and 11 on FMM-PBSA-oil-agar medium with chloramphenicol. From integuments, two colonies were selected on FMM-PBSA-oil-agar medium, and none on NA-PBSA-oil medium. Likewise, no strains were isolated from the hindguts on both selective media.

Among these strains, two strains named BPD1A and BPD2A, which were both isolated from midguts on NA-PBSA-oil medium, degraded over 75 % of both PBSA and PBS films at 25 °C in 10 days. Other tested strains showed no or weak degradation activity for the BP films tested.

Identification of strains

The morphology of BPD1A and BPD2A was observed under the microscope. Although both strains were isolated on NA-PBSA-oil medium containing 50 μg ml^{-1} cycloheximide, they both showed yeast-like shape. The nucleotide sequences of D1/D2 and ITS1-5.8S-ITS2 regions of both strains were analyzed and were confirmed to be completely identical to each other and with those of the type strains of *C. magnus* CBS 140 (JCM 9038) as well as those of the type strain of *F. floriforme* CBS 6241. It is known that the nucleotide sequences of D1/D2 and ITS1-5.8S-ITS2 regions of the type strains of *C. magnus* and *F. floriforme* are identical, but the length of ITS1-5.8S-ITS4 region of *C. magnus* was found to be more than 20 nucleotides shorter than that of *F. floriforme* strains (Fonseca et al. 2011, Kwon-Chung 2011)). Comparison of the ITS1-5.8S-ITS4 region revealed that the DNA sequences of both strains were identical to that of *C. magnus* JCM 9038, and 26 nucleotides shorter than that of *F. floriforme* JCM 10631. As both strains exhibited highly similar microbial characteristics and BP degradation activities, the morphology and emulsified PBSA degradation activity on NA-PBSA-oil medium with and without cycloheximde of strain BPD1A are presented (Fig. 1). BPD1A was selected and used in subsequent analyses.

BP film-degrading ability of isolated strains

We already reported that *P. antarctica* JCM10317 showed strong BP film-degrading ability at 30 °C on

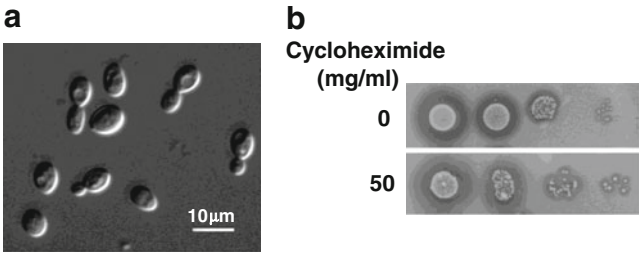

Fig. 1 The phenotype of the isolated biodegradable plastic-degrading strain BPD1A. **a** Morphology of cells under the microscope and **b** emulsified PBSA-degradation activity on NA-PBSA-oil medium without cycloheximide (at 2 days) and with 50 μg ml^{-1} of cycloheximide (at 7 days)

FMM agar plate (Kitamoto et al. 2011). However, since the growth characteristic of *C. magnus* strains was observed to vary at 30 °C (Fonseca et al. 2011), the PBS film-degrading abilities of BPD1A and the type strains were evaluated on FMM medium at 28 °C. BPD1A and *P. antarctica* JCM10317 degraded PBS films (2×2 cm) at similar rate; however, no film degradation was observed with the type strain *C. magnus* JCM 9038 (Fig. 2).

Production and affinity purification of BP-degrading enzyme

PBSA-degrading activity was successfully detected in the liquid culture broth of strain BPD1A using medium of the same composition as that used by Kamini et al. (2000) (Table 1). The crude enzyme solution was considerably cloudy, but with the addition of $CaCl_2$ to a final concentration of 10 mM, the suspended materials precipitated and were then removed by filtration. Because the enzymatic activity was stably observed in the clarified solution, it was used for enzyme purification. The BP-degrading enzyme purified from the culture medium of BPD1A using a newly developed affinity purification method gave a single 21-kDa band in SDS-PAGE (Fig. 3). It was estimated that approximately 65 % of the total original BP-degrading activity was recovered and that the purified enzyme solution had a specific activity that was 13.9-fold that of the crude enzyme solution (Table 1).

Partial internal amino acid sequences

Internal peptide sequences of three digested fragments (fragment 1 to 3) of the purified enzyme were analyzed. The tFAST*x* search for GenBank database exhibited that no known sequences completely matched the partial sequences of our BP-degrading enzyme, demonstrating that this is a novel enzyme. Figure 4 shows the partial sequence alignments of three fragments of this enzyme and two cutinase-like enzymes, CLE and PaE, from *Cryptococcus* sp. S-2 (accession No. AB102945) (Masaki et al. 2005) and *P. antarctica* JCM 10317 (DM067526) (Shinozaki et al. 2012a), respectively, both are classified into yeast. Although only three residues out of twelve matched for fragment 3, the two other fragments possess high similarity: nine out of eleven for fragment 1; and ten out of twelve for

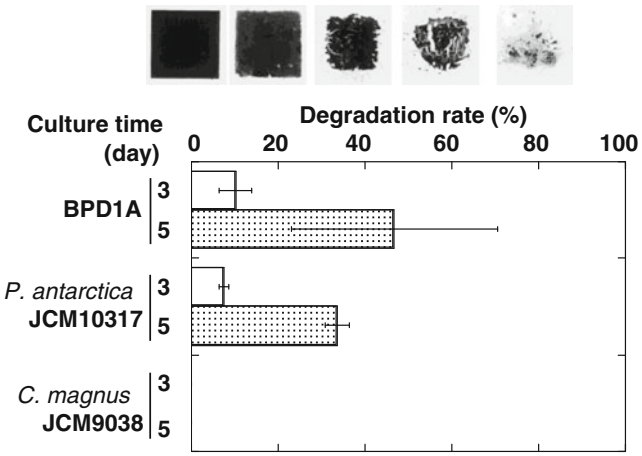

Fig. 2 Evaluation of PBS film degradation. Degradation of 2×2-cm squares of PBS mulch film at 28 °C by strain BPD1A and the type strains of *P. antarctica* (JCM10317) and *C. magnus* (JCM9038). PBS degradation was observed at 3 days (*open bar*), and 5 days incubation (*hashed bar*). Typical image of films reflecting the respective degradation rates are displayed. Values are expressed as the mean and SD obtained from triplicate experiments

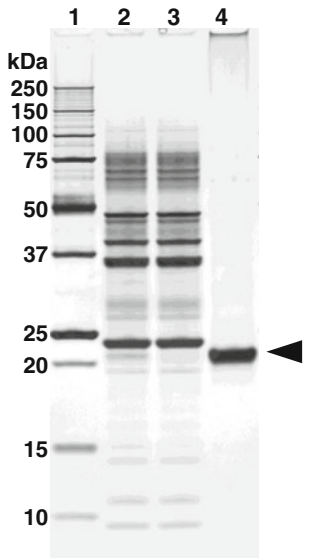

Fig. 3 SDS-PAGE of the purified BP-degrading enzyme. *Lanes 1*, molecular weight standard; *2*, crude enzyme solution; *3*, PBSA-unadsorbed fraction; and *4*, purified enzyme solution. The *arrow* indicates the position of the purified enzyme. The *lanes from 2 to 4* originated from one purification experiment

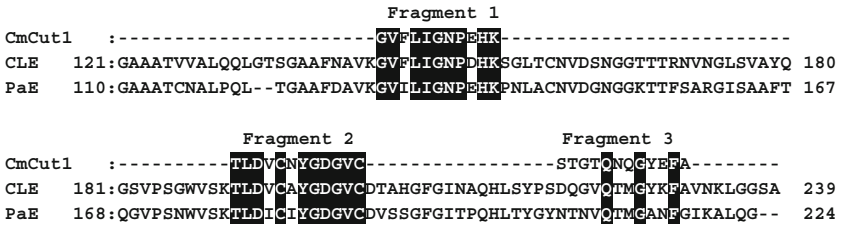

Fig. 4 Amino acid sequence alignment of the partial sequences of CmCut1 and two cutinase-like enzymes, CLE and PaE. Amino acid residues that matched in the three sequences are indicated in *reversed letters*. The origins of the enzymes and their GenBank accession numbers are as follows: CLE (*Cryptococcus* sp. S-2, AB102945) and PaE (*P. antarctica* JCM 10317, DM067526)

fragment 2. These results suggest that this BP-degrading enzyme from BPD1A belongs to the same family of cutinase-like enzymes. Thus, we named this enzyme as CmCut1.

Enzyme characterization

The effect of cations on CmCut1 activity was analyzed (Fig. 5a). The PBSA-degrading activity of CmCut1 was strongly affected by Ca^{2+} and Mg^{2+} concentrations. Both ions have optimal concentrations at around 2.5 mM and enhanced the activity even at low concentrations, but were rather inhibitive at higher concentrations. The effect of Na^{+} was remarkably weaker than that of Ca^{2+} and Mg^{2+}. CmCut1 exhibited maximum PBSA-degrading activity at pH 7.5, which sharply decreased to approximately 20 % as the pH rose to 9; a drop in pH to 6 inactivated the enzyme (Fig. 5b). Its activity was maximum at an optimum temperature of 40 °C, but sharply decreased to approximately 10 % at 20 °C, and almost got inactivated as the temperature rose to 50 °C (Fig. 5c).

pNP-dodecanoate (C12) was the most preferred substrate chain length of CmCut1 (Fig. 6); however, the enzyme had broad substrate specificity ranging from pNP-C4 to pNP-C18, retaining approximately 56.0 % (pNP-C4) and 76 % (pNP-C18) of the activity against pNP-C12, whereas its degradation activity against pNP-acetate (C2) was only 4 % of that against pNP-C12. Degradation activities in buffers without $CaCl_2$ and $MgCl_2$ were also assayed for C4 and C12. In contrast to emulsified PBSA degradation, these activities were significantly lower for both substrates in buffers containing $CaCl_2$ or $MgCl_2$ than in buffers without $CaCl_2$ and $MgCl_2$. The relative degradation rates of C4 in the presence of $CaCl_2$ and $MgCl_2$ were 86 and 79 %, and

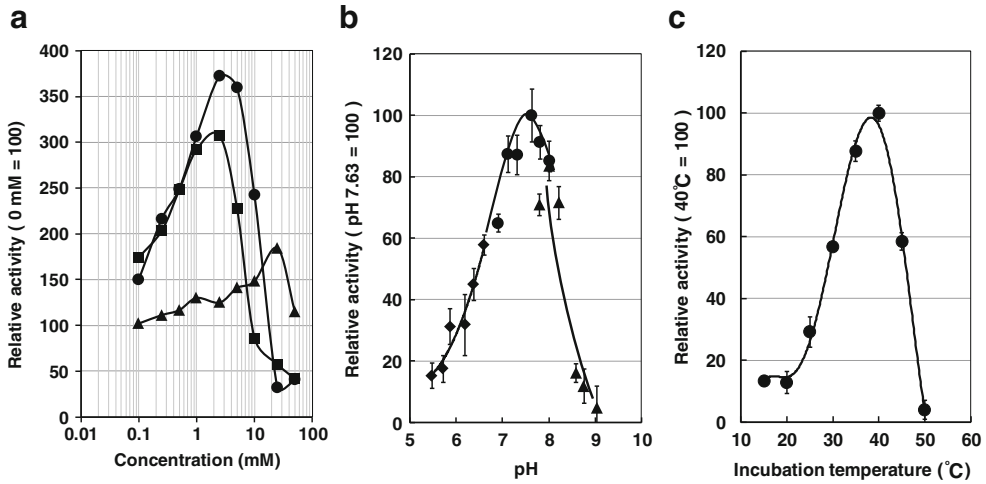

Fig. 5 Effect of cations, pH, and temperature on the PBSA-degrading activity of CmCut1. **a** Effect of cations: $CaCl_2$ (*closed circles*), $MgCl_2$ (*closed squares*), and NaCl (*closed triangles*). The cross axle of the graph is expressed as logarithm of concentrations. The relative activities are represented, with the activity in 0 mM cations (8.8 U/mg) set at 100. The activity was measured in 25 mM HEPES-NaOH buffer (pH 7.4) containing cations at various concentrations. The data shown were obtained from a single assay. **b** Effect of pH. The buffers used were MES-NaOH (*closed diamonds*), HEPES-NaOH (*closed circles*), and TAPS-NaOH (*closed triangles*). The relative activities are represented, with the activity at pH 7.63 (39.3 U/mg) set at 100. The data shown are means and SDs of triplicate assays. **c** Effect of temperature. The relative activities are represented, with the activity at 40 °C (64.3 U/mg) set at 100. The data shown are means and SDs of triplicate assays

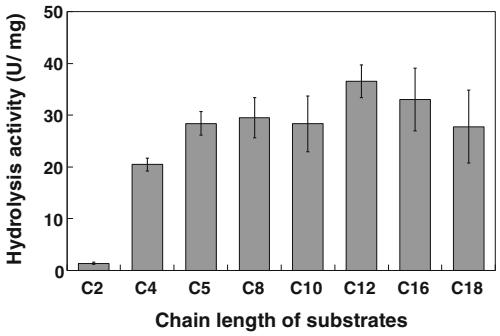

Fig. 6 Hydrolysis of pNP-acetate (*C2*), pNP-butyrate (*C4*), pNP-valerate (*C5*), pNP-octanoate (*C8*), pNP-decanoate (*C10*), pNP-dodecanoate (*C12*), pNP-palmitate (*C16*), and pNP-stearate (*C18*) by CmCut1. The average and SD of the hydrolysis activities were obtained from triplicate experiments

those of C12 were 83 and 81 %, respectively, compared with the rates in the absence of cations.

In 25 mM HEPES-NaOH buffer (pH 7.4) without $CaCl_2$ and $MgCl_2$, the activities of CmCut1 and porcine pancreatic lipase were 18.6±0.59 and 12.4±0.70 μmol mg^{-1} min^{-1}, respectively; thus, significant and almost comparable lipase activities were detected. However, the activities of both CmCut1 and porcine pancreatic lipase considerably declined to 5.0±0.33 and 4.5±0.06 μmol mg^{-1} min^{-1}, respectively, in the presence of 2.5 mM $CaCl_2$. Furthermore, the lipase activity of only CmCut1 declined to 5.0±0.30 μmol mg^{-1} min^{-1} in the presence of 2.5 mM $MgCl_2$, whereas the activity of porcine pancreatic lipase was stable at 12.9±0.39.

The protease activities of CmCut1 in 25 mM HEPES-NaOH (pH 7.4) buffers without $CaCl_2$ and $MgCl_2$, with 2.5 mM $CaCl_2$, and with 2.5 mM $MgCl_2$, were 105.3±13.9, 66.7±5.0, and 46.1±9.9 ΔRFU μg^{-1} min^{-1}, respectively. These activities seemed almost negligible compared with the activities of TPCK-trypsin in the same buffers (33,021±1,905, 71,192±1,398, and 64,893±439 ΔRFU μg^{-1} min^{-1}, respectively).

Evaluation of the enzymatic degradation of BP films

The degradation spectrum of CmCut1 for BP cast films based on released water-soluble TOC from the films is shown in Fig. 7. Though strict quantitative comparison cannot be achieved because of the uneven carbon contents of the films, CmCut1 revealed evident degradation activity for all the BP films tested here, except PHB. In particular, the PBSA film almost completely vanished visually from the surface of glass slide, and nearly all the carbon in the cast film (ca. 150 μg) was detected as water-soluble TOC (133 μg). In contrast, CmCut1 did not degrade PHB film at all.

Discussion

Two yeast strains (BPD1A and BPD2A) with strong BP-degrading ability were isolated from the midgut of a stag beetle, found to be identical, and identified as most closely related to the basidiomycetous yeast, *C. magnus*.

In recent years, insects have become the focus of interest as habitats of diverse yeasts (Vega and Dowd 2005). For example, Suh et al. (2005) isolated over 650 yeast strains from the guts of various coleopteran insects. Various strains of *C. magnus* have been isolated from trees, such as the wattle tree, Portuguese oak, Montpellier maple, lemon tree, and from shrubs (Fonseca et al. 2011). Thus, it is supposed that the isolated strains may have become concentrated in the midguts of stag beetle larvae collected from brown-rotted woods.

The genus *Cryptococcus* is polyphyletic and occurs in four clades (Fell et al. 2000, Fonseca et al. 2011). Among them, in the order Tremellales, the BP-degrading activities of *Cryptococcus* sp. S-2 (Masaki et al. 2005), which is closely related to *C. flavus* (Iefuji et al. 1994; Masaki et al. 2012), and of rice husk-derived *Cryptococcus flavus*, *Cryptococcus laurentii*, and *Cryptococcus rajasthanensis* (Kitamoto et al. 2011) have already been reported. However, *C. magnus* belongs to the order Filobasidiales (Fell et al. 2000, Fonseca et al. 2011). This fact suggests that BP-degrading ability may be widespread in the genus *Cryptococcus*, and that the structure and characteristics of the enzyme present may vary. However, because the type strain *C. magnus* JCM 9038 did not degrade PBSA and PBS films, further analysis has to be carried out in order to accurately identify the isolated strains.

A BP-degrading enzyme purified from the culture medium of the strain BPD1A was characterized. In our preliminary study, the addition of $CaCl_2$ in the reaction mixture was found to significantly enhance the enzymatic activity of the BPD1A culture medium, with maximal activity observed in the pH range 7–8 (data not shown). For this reason, 25 mM

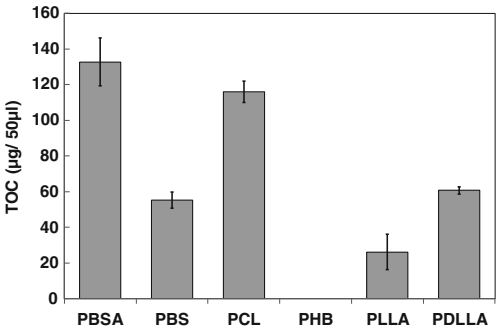

Fig. 7 The eluted TOC from BP cast films after treatment with 25 mM HEPES-NaOH buffer (pH 7.4) containing 2.5 mM $CaCl_2$ with or without CmCut1 (9.2 μg ml^{-1}). The averages and SDs were obtained from triplicate experiments

HEPES-NaOH buffer (pH 7.4) containing 2.5 mM $CaCl_2$ was used to study emulsified PBSA-degradation activity.

Since BP-degrading enzymes are expected to have specific and strong affinities for their BP substrates, we developed a method to purify BP-degrading enzymes using emulsified PBSA as the ligand for affinity chromatography. Our method involves a simple single-step operation and does not require any special instruments. Whereas enzymes are usually eluted from enzyme-ligand complexes with buffers containing detergents or salts at high concentrations, in our method, the ligand was digested completely by the substrate-degrading activity of the bound enzyme. After the removal of the digested products by dialysis, the concentrated purified enzyme was obtained in the clarified appropriate buffer with a high recovery yield of 65 % of the initial content (Table 1). The efficiency of our method is comparable to those obtained in other similar reported studies that include the final elution step. To date, PHB has also been used for the purification of its depolymerase, with activity recovery of 8.2–86.11 % (Iyer et al. 2000; Elbanna et al. 2004; Papaneophytou et al. 2009; Papaneophytou and Pantazaki 2011). Furthermore, excluding the final dialysis step, the major operation can be completed within 1 h. The method may also be applied for the purification of other enzymes with PBSA-binding and -degrading activities (e.g., some lipases and cutinases). Further investigation to determine suitable purification conditions for increased enzyme recovery yield will be conducted.

The partial amino acid sequences of purified CmCut1 revealed some similarity to those of cutinase-like BP-degrading enzymes of basidiomycetous yeast, CLE from *Cryptococcus* sp. S-2 (Masaki et al. 2005), and PaE from *P. antarctica* (Shinozaki et al. 2012a). In fragment 2 of CmCut1, there were two cysteine residues corresponding to Cys195 and 202 of CLE, which are reported to form a disulfide bond, and an aspartic acid residue corresponding to Asp199 in CLE that is reported to be a catalytic residue (Kodama et al. 2009) (Fig. 4). These residues are also conserved in PaE (Shinozaki et al. 2012a) (Fig. 4).

It is widely known that enzyme activity is greatly affected by a variety of factors. In this study, the emulsified PBSA degrading activity of CmCut1 was found to be significantly affected by Ca^{2+} and Mg^{2+} concentrations. At 2.5 mM, both ions enhanced CmCut1 activity by approximately 3- to 4-fold. However, the lipase activity of CmCut1 was considerably decreased in the presence of 2.5 mM Ca^{2+} or Mg^{2+}, whereas the lipase activity of CLE is reportedly not affected by most metal ions including Ca^{2+} and Mg^{2+}. The mechanism(s) by which these cations affect function are unknown; this knowledge will be useful for the development of degradation control techniques for biodegradable plastic materials.

Based on the similarity of the partial amino acid sequences of CmCut1 with cutinase-like enzymes, the ability to degrade a wide spectrum of pNP-fatty acids and BP cast films (PBSA, PBS, PCL, PLLA and PDLLA) could be considered a unique characteristic of the cutinase-like enzymes of basidiomycetous yeasts, though their structures and characteristics are not completely identical. Kodama et al. (2009) resolved the crystal structure of CLE to a 1.05-Å resolution and predicted that the hydrophobic aromatic residues exposed to solvent are important for its specificity towards long-chain substrates. To confirm this hypothesis, the elucidation and comparison of the CmCut1 and PaE crystal structures would be of great interest.

A thorough understanding of the differences and commonalities in the characteristics of these BP-degrading enzymes will enable us to develop technologies to control the degradation speed of BP materials in various field environments. Further investigation of CmCut1, including determination of its full-length cDNA sequence and structural analysis, will provide more information about its nature and characteristics.

Acknowledgments We thank Showa Denko K. K. for generously supplying the Bionolle materials (PBS and PBSA) and Ms. C. Xiao-Hong for the technical assistance. We also acknowledge Dr. H. Iefuji and Dr. T. Nakajima-Kambe as well as Dr. E. Suto for the valuable comments in this research. This research was financially supported by the Ministry of the Environment, KAKENHI (235658083), and the National Institute for Agro-Environmental Sciences, Japan.

References

Acero EH, Ribitsch D, Steinkellner G, Gruber K, Greimel K, Eiteljoerg I, Trotscha E, Wei R, Zimmermann W, Zinn M, Cavaco-Paulo A, Freddi G, Schwab H, Guebitz G (2011) Enzymatic surface hydrolysis of PET: Effect of structural diversity on kinetic properties of cutinases from *Thermobifida*. Macromolecules 44(12):4632–4640. doi:10.1021/Ma200949p

Araya K (1993) Relationship between the decay types of dead wood and occurrence of lucanid beetles (Coleoptera: Lucanidae). Appl Entomol Zool 28(1):27–33

Breznak JA, Brune A (1994) Role of microorganisms in the digestion of lignocellulose by termites. Annu Rev Entomol 39:453–487. doi:10.1146/annurev.en.39.010194.002321

Chatterjee S, Roy B, Roy D, Banerjee R (2010) Enzyme-mediated biodegradation of heat treated commercial polyethylene by *Staphylococcal* species. Polym Degrad Stab 95(2):195–200. doi:10.1016/j.polymdegradstab.2009.11.025

Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: Aphid and their symbiotic bacteria *Buchnera*. Annu Rev Entomol 43:17–37. doi:10.1146/annurev.ento.43.1.17

Elbanna K, Lütke-Eversloh T, Jendrossek D, Luftmann H, Steinbüchel A (2004) Studies on the biodegradability of polythioester copolymers and homopolymers by polyhydroxyalkanoate (PHP)-degrading bacteria and PHA depolymerases. Arch Microbiol 182:212–225. doi:10.1007/s00203-004-0715-z

Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int J Syst Evol Microbiol 50:1351–1371. doi:10.1099/00207713-50-3-1351

Fonseca Á, Boekhout T, Fell JW (2011) *Cryptococcus* Vuillemin (1901). In: Kurtzman CP, Fell JW, Boekhout T (eds) The yeasts: a taxonomic study, 5th edn. Elsevier, New York, pp 1661–1737

Gross RA, Kalra B (2002) Biodegradable polymers for the environment. Science 297(5582):803–807. doi:10.1126/science.297.5582.803

Iefuji H, Iimura Y, Obata T (1994) Isolation and characterization of a yeast *Cryptococcus* sp. S-2 that produces raw starchdigesting alpha-amylase, xylanase, and polygalacturonase. Biosci Biotechnol Biochem 58(12):2261–2262. doi:10.1271/bbb.58.2261

Iyer S, Shah R, Sharma A, Jendrossek D, Desai A (2000) Purification of *Aspergillus fumigatus* (Pdf1) poly(β-hydroxybutyrate) (PHB) depolymerase using a new, single-step substrate affinity chromatography method: characterization of the PHB depolymerase exhibiting novel self-aggregation behavior. J Polym Environ 8 (4):197–203. doi:10.1023/A:1015249811314

Kamini NR, Fujii T, Kurosu T, Iefuji H (2000) Production, purification and characterization of an extracellular lipase from the yeast, *Cryptococcus* sp S-2. Process Biochem 36(4):317–324. doi:10.1016/S0032-9592%2800%2900228-4

Kasuya K, Ishii N, Inoue Y, Yazawa K, Tagaya T, Yotsumoto T, Kazahaya J, Nagai D (2009) Characterization of a mesophilic aliphatic-aromatic copolyester-degrading fungus. Polym Degrad Stab 94(8):1190–1196. doi:10.1016/j.polymdegradstab.2009.04.013

Kitamoto HK, Shinozaki Y, Cao XH, Morita T, Konishi M, Tago K, Kajiwara H, Koitabashi M, Yoshida S, Watanabe T, Sameshima-Yamashita Y, Nakajima-Kambe T, Tsushima S (2011) Phyllosphere yeasts rapidly break down biodegradable plastics. AMB Express 1 (1):44. doi:10.1186/2191-0855-1-44

Kodama Y, Masaki K, Kondo H, Suzuki M, Tsuda S, Nagura T, Shimba N, Suzuki E, Iefuji H (2009) Crystal structure and enhanced activity of a cutinase-like enzyme from *Cryptococcus* sp. strain S-2. Proteins 77(3):710–717. doi:10.1002/prot.22484

Koitabashi M, Noguchi MT, Sameshima-Yamashita Y, Hiradate S, Suzuki K, Yoshida S, Watanabe T, Shinozaki Y, Tsushima S, Kitamoto HK (2012) Degradation of biodegradable plastic mulch films in soil environment by phylloplane fungi isolated from gramineous plants. AMB Express 2:40. doi:10.1186/2191-0855-2-40

Kwon-Chung KJ (2011) *Filobasidium* Olive (1968). In: Kurtzman CP, Fell JW, Boekhout T (eds) The yeasts: a taxonomic study, 5th edn. Elsevier, New York, pp 1457–1465

Kyrikou I, Briassoulis D (2007) Biodegradation of agricultural plastic films: a critical review. J Polym Environ 15(3):227–227. doi:10.1007/s10924-007-0063-6

Lange K-B (2011) Bioplastics to pass the one million tonne mark in 2011. Press Release, European Bioplastics, Berlin

Maeda H, Yamagata Y, Abe K, Hasegawa F, Machida M, Ishioka R, Gomi K, Nakajima T (2005) Purification and characterization of a biodegradable plastic-degrading enzyme from *Aspergillus oryzae*. Appl Microbiol Biotechnol 67(6):778–788. doi:10.1007/s00253-004-1853-6

Masaki K, Kamini NR, Ikeda H, Iefuji H (2005) Cutinase-like enzyme from the yeast *Cryptococcus* sp. strain S-2 hydrolyzes polylactic acid and other biodegradable plastics. Appl Environ Microbiol 71 (11):7548–7550. doi:10.1128/AEM.71.11.7548-7550.2005

Masaki K, Tsuchioka H, Hirano T, Kato M, Ikeda H, Iefuji H (2012) Construction of a new recombinant protein expression system in the basidiomycetous yeast *Cryptococcus* sp. strain S-2 and enhancement of the production of a cutinase-like enzyme. Appl Microbiol Biotechnol 93(4):1627–1636. doi:10.1007/s00253-011-3680-x

Papaneophytou CP, Pantazaki AA, Kyriakidis DA (2009) An extracellular polyhydroxybutyrate depolymerase in *Thermus thermophiles* HB8. Appl Microbiol Biotechnol 83:659–668. doi:10.1007/s00253-008-1842-2

Papaneophytou CP, Pantazaki AA (2011) A novel affinity chromatographic material for the purification of extracellular polyhydroxybutyrate depolymerases. J Polym Environ 19:876–886. doi:10.1007/s10924-011-0345-x

Park DS, Oh HW, Jeong WJ, Kim H, Park HY, Bae KS (2007) A culture-based study of the bacterial communities within the guts of nine longicorn beetle species and their exo-enzyme producing properties for degrading xylan and pectin. J Microbiol 45(5):394–401

Ronkvist AM, Lu WH, Feder D, Gross RA (2009) Cutinase-catalyzed deacetylation of poly(vinyl acetate). Macromolecules 42 (16):6086–6097. doi:10.1021/Ma900530j

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68(5):850–858. doi:10.1021/ac950914h

Shinozaki Y, Morita T, Cao XH, Yoshida S, Koitabashi M, Watanabe T, Suzuki K, Sameshima-Yamashita Y, Nakajima-Kambe T, Fujii T, Kitamoto HK (2012a) Biodegradable plastic-degrading enzyme from *Pseudozyma antarctica*: cloning, sequencing, and characterization. Appl Microbiol Biotechnol. doi:10.1007/s00253-012-4188-8

Shinozaki Y, Watanabe T, Nakajima-Kambe T, Kitamoto HK (2012b) Rapid and simple colorimetric assay for detecting the enzymatic degradation of biodegradable plastic films. J Biosci Bioeng. doi:10.1016/j.jbiosc.2012.08.010

Suh SO, McHugh JV, Pollock DD, Blackwell M (2005) The beetle gut: a hyperdiverse source of novel yeasts. Mycol Res 109(Pt 3):261–265. doi:10.1017/S0953756205002388

Tanahashi M, Kubota K, Matsushita N, Togashi K (2010) Discovery of mycangia and the associated xylose-fermenting yeasts in stag beetles (Coleoptera: Lucanidae). Naturwissenschaften 97:311–317. doi:10.1007/s00114-009-0643-5

Uchida H, Nakajima-Kambe T, Shigeno-Akutsu Y, Nomura N, Tokiwa Y, Nakahara T (2000) Properties of a bacterium which degrades solid poly(tetramethylene succinate)-*co*-adipate, a biodegradable plastic. FEMS Microbiol Lett 189(1):25–29. doi:10.1111/j.1574-6968.2000.tb09201.x

Vega FE, Dowd PF (2005) The role of yeasts as insect endosymbionts. In: Vega FE, Blackwell M (eds) Insect–fungal associations: ecology and evolution. Oxford University Press, New York, pp 211–243