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Xylose induces the phyllosphere yeast *Pseudozyma antarctica* to produce a cutinase-like enzyme which efficiently degrades biodegradable plastics

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There is a need to speed up the degradation of used agricultural mulch films that are made of biodegradable plastics (BPs) in the field. Treating them with BP-degrading enzymes could be a solution to this problem. A cutinase-like enzyme of yeast *Pseudozyma antarctica* (PaE) has wide specificity of BPs degradation, but needs to be produced efficiently. Here we report that the production of PaE by *P. antarctica* can be increased by using xylose as carbon source. BP-degradation activity was analyzed using a polybutylene succinate-*co*-adipate (PBSA) emulsion as the substrate. Strain *P. antarctica* GB-4(1)W was found to be the best PaE producer among the tested strains. Using a 5-L jar fermentor with xylose fed-batch cultivation, high PaE productivity could be maintained and about 21 U/ml of PaE was obtained in 120 h. This amount was 100 times higher than the amount that we obtained previously (0.21 U/ml by flask cultivation using glycerol as carbon source). Under repeated xylose fed-batch cultivation with 24 h intervals, the maximum PaE production rate (0.34 U/ml/h) was maintained and the highest PaE productivity (28,000 U/2 L/d) was repeatedly obtained for 7 intervals. The activity of filtered jar-culture (crude PaE) was stable over 12 weeks at 4°C. Commercially available BP mulch films (20 μm thickness, cut into 1-cm-squares) were completely degraded by submerging them in crude PaE (2 U/ml) at 30°C in 24 h. These results indicated that concentrated PaE can rapidly degrade the strength of BP mulch films in the field so that they do not interfere with plowing.

[**Key words:** Biodegradable plastics; Agricultural mulch film; Degradation; Phyllosphere yeast; *Pseudozyma antarctica*; Cutinase-like enzyme; Xylose; Jar-fermentor]

Many farmers cover the soil with polyethylene mulch films to keep the soil warm, retain water and suppress weed growth. However, after harvesting, recovering the used mulch films requires a lot of labor and results in a large volume of plastic waste. These problems can be avoided by using biodegradable plastics (BPs) for mulch films (1). To be effective, BP mulch films should have the strength of polyethylene mulch film, but should be degradable enough for them to be plowed into the field after use. However, the speed of degradation of BP mulch films can be very slow under certain environmental conditions (2). Farmers say that undecomposed BP mulch films slow down plowing by becoming entangled in rotary plows. There is thus a need for improved methods for degrading undecomposed BPs, and treatment with BP-degrading enzymes is expected to accelerate the reaction.

BPs are made from aliphatic polyesters, which are hydrolyzed by esterases in the environment. Several microorganisms have been shown to produce such esterases. One of them is the bacterium *Acidovorax delafieldii* strain BS-3 which produces BPs depolymerase (*pbsA*) that degrades polybutylene succinate (PBS) and polybutylene succinate-*co*-adipate (PBSA) (3). The filamentous fungus *Aspergillus oryzae* strain RIB40 produces a cutinase (CutL1p) that degrades PBSA and PBS (4). The basidiomycete yeast *Cryptococcus* sp. strain S-2 produces a cutinase-like enzyme (CLE) that degrades PBSA, PBS, poly ε-caprolactone (PCL), and polylactic acid (PLA) (5). For practical use, low cost and efficient technique for producing BP-degrading enzymes are needed. Although these enzymes well degrade BPs in laboratory-scale experiments, it is difficult to produce large amounts of the enzymes without recombinant techniques. In such cases, steps to prevent the release of recombinants into the environment are necessary and drive up production costs.

Both the cuticular layer of the plant surface (phyllosphere) and BPs are composed of esterified fatty acids (6,7). We therefore expected that the enzymes produced by phyllosphere microorganisms would also degrade BPs. We isolated several phyllosphere *Pseudozyma* spp. yeast strains from plant surfaces as candidate producers of BP-degrading enzymes (8). The ones with the strongest abilities to degrade PBS and PBSA films were strains of *Pseudozyma antarctica* that were isolated from rice leaves and husks. The responsible 22-kDa enzyme (accession no. DM067526), isolated from the type strain *P. antarctica* JCM10317 (isolated from lake

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sediment of Antarctica), designated PaE, efficiently degraded BPs such as PBS, PBSA, PCL, and PLA (9).

Carbon sources that induce bacteria and fungi to produce BP-degrading enzymes include triacylglycerols, PBS and PBSA polymers, tributyrin, casein, and gelatin (4,10,11). However, the cultivation conditions that induce *P. antarctica* to produce PaE are unknown. Only 0.07 and 0.21 U/ml of PaE were produced by *P. antarctica* JCM10317 when it was cultivated with 1% soybean oil and 6% glycerol, respectively (8).

The main goal of our study was to increase production of PaE from *P. antarctica* by selecting the appropriate carbon sources, strains, and cultivation conditions. Because we previously had success with producing heterologous proteins by *Pichia pastoris* with a fed-batch system (12), we developed jar-fermentor cultivation conditions (fed-batch cultivation and repeated fed-batch cultivation) to increase the production of concentrated native PaE. Finally, we examined the stability of crude PaE and its ability to degrade 3 types of BP mulch films.

MATERIALS AND METHODS

Strains Type strains of *P. antarctica* JCM10317, *Pseudozyma aphidis* JCM10318, *Pseudozyma rugulosa* JCM10323 and *Pseudozyma tsukubaensis* JCM10324 were purchased from the Japan Collection of Microorganisms (JCM) of the Riken Bioresource Center (Japan). *P. antarctica* NRL-A and NRL-B, isolated from rice leaves and GB-2, GB-3, GB-4(0), GB-4(1)W, GB-5W, GB-7, GB-8, and GB-11W, isolated from rice husks, were stocked in our laboratory (8). These strains were maintained on YM plates (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar) at 4°C.

Investigation of culture conditions in flask cultivation Yeast strains were pre-cultivated in test tubes containing 5 ml of YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose) at 30°C with reciprocal shaking at 160 rpm for 24 h. The pre-cultures (600 µl) were added to 300 ml flasks containing 60 ml of fungal minimum medium (FMM) with an appropriate carbon source. Basal FMM contains 0.1% yeast extract, 0.2% $NaNO_3$, 0.02% KH_2PO_4, and 0.02% $MgSO_4 \cdot 7H_2O$. Two types of enriched FMM (3× FMM and 5× FMM) contained 3 and 5 times, respectively, the above amounts of yeast extract, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ in FMM. Carbon source and other nutrients were separately autoclaved (121°C, 20 min). The cultures were cultivated at 30°C with rotary shaking at 200 rpm for 96 h.

At various times during the growth, 1 ml of aliquots was harvested and centrifuged at 15,000 rpm for 5 min. The pellets were dried at 105°C for 2 h and their dry cell weights were measured to investigate cell growth. At the same time, the enzymatic activities of the supernatant were measured as described below.

PBSA emulsion degradation activity BP-degradation activity was analyzed using a PBSA emulsion as the substrate at 30°C for 15 min (8). The reaction mixture contained 20 mM Tris–HCl buffer (pH 6.8), 0.045% (w/v) emulsified PBSA (Bionolle EM-301, Showa Denko K. K., Japan) and 100 µl of crude enzyme solution in total volume of 2 ml. The initial absorbance of 660 nm (OD_{660}) was about 0.65. One unit (U) of PBSA degradation activity was defined as a 1 OD_{660} decrease per min in the reaction mixture.

Sequence analysis of PaE gene Yeast strains were cultivated in 5 ml of YM medium overnight. The cultures were centrifuged, and genomic DNA was isolated from the cell pellets using a Dr. GenTLE for Yeast (Takara, Kyoto, Japan).

A primer pair for PCR amplification of the PaE gene of *P. antarctica* GB-4(1)W, PaE forward (5′-ATGCAGTTCAAGTCGACCTTTGCCGC-3′) and PaE reverse (5′-TTATCCCTGAAGAGCCTTGATACCGA-3′), was designed based on the DNA sequence of PaE cDNA of *P. antarctica* JCM10317 (accession no. DM067526) in GenBank. The PCR reaction was performed using KOD-plus (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Amplified DNA fragments were purified using a PCR purification kit (Qiagen, Hilden, Germany). The sequences of the PCR products were determined using a BigDye Terminator Cycle Sequence V3.1 Kit with the same primers and an Applied Biosystems 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Scale-up cultivation using a jar fermentor A 30 ml culture of *P. antarctica* GB-4(1)W was grown in a 300 ml flask at 30°C with rotary shaking at 200 rpm for 24 h in YM medium. The culture was then used to inoculate the 5-L jar fermentor containing 3 L of basal medium (0.2% yeast extract, 0.2% $NaNO_3$, 0.04% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 8% xylose). Xylose and other nutrients were separately autoclaved (121°C, 20 min). The batch cultivation conditions were as follows: aeration rate was 8 LPM (2.66 vvm); agitation value was 500 rpm; temperature was 30°C. The effect of 0.08% NH_4NO_3 addition in basal medium was investigated. The effect of 0.2% $(NH_4)_2SO_4$ addition in basal medium and pH control at 6.0 with 14% ammonia solution, which also provided a nitrogen source for the culture, was also investigated.

Fed-batch cultivation was performed to limit the concentration of xylose in the medium to induce PaE production. A 30-ml culture of *P. antarctica* GB-4(1)W was grown in a 300 ml flask at 30°C with rotary shaking at 200 rpm for 24 h in YM medium. The culture was then used to inoculate the 5-L jar fermentor containing 3 L of PaE production medium (0.2% yeast extract, 0.2% $NaNO_3$, 0.2% $(NH_4)_2SO_4$, 0.04% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 2% xylose). The batch cultivation was performed until entire xylose has been depleted (around 24 h). Then, xylose fed-batch cultivation was performed to induced PaE production by adding fed medium (0.2% yeast extract, 0.085% YNB w/o amino acid & ammonium sulfate (Difco) and 20% xylose) at 500 ml/d of the feeding rate. Xylose and other nutrients were separately autoclaved (121°C, 20 min). The cultivation conditions were as follows: aeration rate was 8 LPM; agitation value was 500 rpm; dissolved oxygen (DO) was maintained around 25% of saturated value; pH was controlled at 6.0 with 14% ammonia solution, which also provided a nitrogen source for the culture; temperature was 30°C.

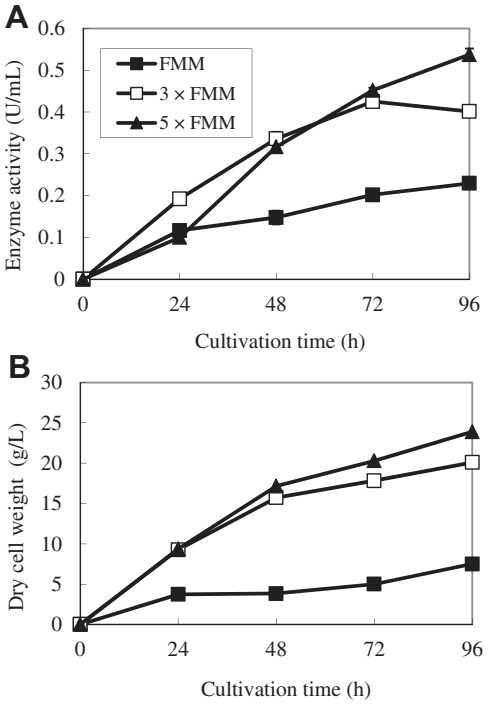

FIG. 1. Effects of FMM concentration with 6% glycerol on PaE production (expressed as activity against a PBSA emulsion) (A) and cell growth (B) of *P. antarctica* JCM10317. See Methods for the compositions of FMM (closed squares), 3× FMM (open squares), and 5× FMM (closed triangles).

Repeated fed-batch cultivation with 24 h-cycle was also performed. After 72 h cultivation as described above, 2 L of culture was replaced with 2 L new PaE production medium every 24 h. The cell growth and PaE production of *P. antarctica* GB-4(1)W was induced by adding fed medium (700 ml/d).

Stability of crude enzyme Crude PaE (30 ml) was added to 50 ml tubes and maintained at −30°C, 4°C, 15°C, 23°C, and 30°C. Periodically, 500 µl aliquots were sampled and residual enzyme activities were analyzed as described above. Crude PaE (500 µl) was also added into a 1.5 ml tube and freeze-dried using a Bench Top K Freeze Dryers (SP Industries, NY, USA). Each tube was maintained at 4°C and 30°C and residual enzyme activities were periodically analyzed as described above.

Degradation of BP mulch films Filtered jar-culture (crude PaE) was obtained using a cellulose acetate membrane filter (Advantec; pore size 0.45 µm, Toyo Roshi Kaisha Ltd., Japan). PBSA film (Bionolle 3001G) and PBS film (Bionolle 1001G), both of which has an average molecular weight of $20–25 \times 10^4$, were obtained from Showa Denko K. K. (Tokyo, Japan). Commercially available BP mulch film, made of PBSA, PBS and polybutylene adipate-*co*-terephthalate (PBAT) at weight-ratio of 47:37:17 (13), was also used (molecular weight could not be analyzed). Each mulch film (20 µm thickness) was cut into 1-cm-squares and one square film (23 mg) was put in each well of a 12-well micro-well plate (Multidish 12 wells, Nunc, Denmark) containing 1 ml of 20 mM Tris–HCl buffer (pH 8.0). Crude enzyme (1 U or 2 U) was added into the each well. The micro-well plate was shaken at 30 rpm with the Deep Well Maximizer (Bioshaker M-BR-022up, TAITEC, Japan) at 30°C. Degradations of mulch films were periodically observed by visually.

RESULTS AND DISCUSSION

Effect of FMM concentration for PaE production *p*-Nitrophenyl esters are the substrates most commonly used to determine esterase and lipase activity. However, because *P. antarctica* produces

two lipases, CALA and CALB, which have esterase activities (14,15), *p*-nitrophenyl esters are not suitable substrates for determination of crude PaE activity. On the other hand, the PBSA emulsion degradation activities of CALA and CALB are very low (5,8). Thus, PBSA emulsion degradation activity was assayed to evaluate crude PaE activity. One U of PBSA emulsion degradation activity of PaE was found to correspond to 3.84 U of *p*-nitrophenyl butyrate esterase activity in 50 mM Tris–HCl buffer (pH 8.0) (9).

In order to screen for phyllosphere microorganisms that produce BP-degrading enzymes, the composition of FMM was adjusted to poor nutrient conditions to simulate the conditions on the plant surface (8). *P. antarctica* strains were isolated in this way from rice leaves and husks. However, to produce concentrated PaE, nutrients would have to be added to the FMM. Therefore, we first investigated the effect of FMM concentration on PaE production.

With 6% glycerol, the PaE activity against PBSA emulsion (expressed as the decrease in OD_{660}) and cell growth of *P. antarctica* JCM10317 were greatly increased by switching FMM to 3× FMM, but were not greatly improved by switching from 3× FMM to 5× FMM (Fig. 1). Therefore, 3× FMM was used in the following experiments. These results indicated that cell growth is an important factor for enhancing PaE production.

Effect of carbon source for PaE production The PaE activity of *P. antarctica* JCM10317 against PBSA emulsion was increased by switching 3× FMM with 6% glycerol (0.40 U/ml) to 3× FMM with 10% glycerol (0.51 U/ml) (Table 1). Among the various carbon sources tried (six sugars, glycerol, soybean oil, and dextrin), the PaE activity of *P. antarctica* JCM10317 was highest with xylose (1.26 U/ml), being 2.5–25 times higher than that with the other carbon sources (Table 1). The dry cell weight was little affected by the carbon source. Therefore, the increased PaE activity was due to an increased activity per cell rather than an increased cell mass.

Selection of strain to produce PaE For all strains of *P. antarctica* used in the present study, PaE production (expressed as the activity against PBSA emulsion) was much higher with xylose than with glucose (Fig. 2). These results showed that xylose induced *P. antarctica* strains to produce PaE. On the other hand, xylose did not induce *P. aphidis* JCM10318, *P. rugulosa* JCM10323 and *P. tsukubaensis* JCM10324 to produce BPs degrading enzymes (expressed as the activity against PBSA emulsion) (Fig. 2).

To our knowledge, xylose has not previously been used to produce a BPs-degrading enzyme. At present, we have no idea about the reason why xylose induces *P. antarctica* to produce PaE. Xylose is the main component of hemicellulose (xylan) which is the structural carbohydrate of plant tissue. Although the role of PaE in the life cycle of *P. antarctica* on the plant surface is unclear, plant-derived xylose may induce PaE production of *P. antarctica* in the phyllosphere.

In the following experiments, we used the strain with the highest PaE activity, *P. antarctica* GB-4(1)W isolated from rice husk.

TABLE 1. Effects of carbon sources on PaE production by a type strain, *P. antarctica* JCM10317, cultivated with 3× FMM for 96 h.

Carbon source	Enzyme activity (U/mL)	Dry cell weight (g/L)
6% glycerol	0.40 ± 0.01	20.1 ± 0.4
10% glycerol	0.51 ± 0.01	20.5 ± 0.8
3% soybean oil	0.05 ± 0.01	17.9 ± 1.7
8% glucose	0.07 ± 0.01	16.0 ± 1.7
8% fructose	0.09 ± 0.01	18.2 ± 0.3
8% galactose	0.31 ± 0.05	14.1 ± 1.5
8% xylose	1.26 ± 0.04	14.8 ± 1.2
8% arabinose	0.43 ± 0.03	16.3 ± 0.8
8% sucrose	0.25 ± 0.04	20.2 ± 0.2
8% lactose	0.08 ± 0.01	16.3 ± 0.9
8% dextrin	0.12 ± 0.02	18.1 ± 1.6

The experiments were performed in triplicate.

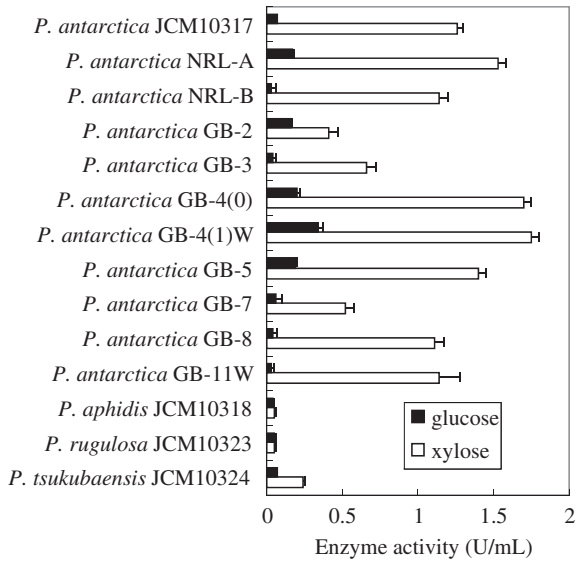

FIG. 2. Biodegradable plastics (BPs)-degrading enzyme activities of various *Pseudozyma* species (based on the activity against a PBSA emulsion) cultivated with glucose (closed bars) and xylose (open bars). The results show the average of three different experiments. Error bars show standard deviations.

The strain GB-4(1)W has been deposited in the National Institute of Technology and Evaluation- International Patent Organism Depositary (NITE-IPOD) (accession no. FERM P-22155). With flask cultivation using *P. antarctica* GB-4(1)W, we also confirmed that 8% xylose is the optimal concentration for PaE production among the various concentrations (6%, 8%, and 10%) (data not shown).

Comparison of the PaE properties of *P. antarctica* GB-4(1)W and JCM10317 The nucleotide sequences of the PaE genes of *P. antarctica* JCM10317 (accession no. DM067526) and GB-4(1)W (accession no. AB739034) differed by only one nucleotide (C164G), which did not change the deduced amino acid sequence. The specific activities of purified PaE of *P. antarctica* GB-4(1)W [52.1 ± 4.2 U/mg: purified by the method of Shinozaki et al. (9)], and that of JCM10317 (54.8 ± 6.3 U/mg) (9) were almost the same.

Based on these results, it can be speculated that the PaE properties of these two strains were almost the same. Thus, the higher PaE productivity of *P. antarctica* GB-4(1)W could result in the higher PaE activity of GB-4(1)W culture than that of JCM10317 culture (Fig. 2).

Scale-up of batch cultivation using a 5-L jar fermentor PaE productivity after 72 h was 1.3 U/ml with basal medium containing 8% xylose for scale-up of batch cultivation (Fig. 3A) and 2.4 U/ml with basal medium adding 0.08% NH_4NO_3 (Fig. 3B) which suggests that the basal medium was lacking in nitrogen. In the case of heterologous enzyme production by *P. pastoris*, inorganic acids, such as H_3PO_4 and H_2SO_4 were added to medium and pH value was maintained using ammonia solution (which was also used as nitrogen source) (12). Therefore, 0.2% $(NH_4)_2SO_4$ was added to the basal medium and the pH value was maintained at 6.0 using 14% ammonia solution. As a result, PaE productivity was significantly increased and 9.4 U/ml of PaE was obtained after 72 h (Fig. 3C).

Effect of the xylose feeding on PaE production When the initial xylose concentration was high (8%), the DO value dropped below 10% of saturation during 24–56 h (data not shown). In the case of *P. pastoris*, over 25% of saturation DO value was important to induce heterologous enzyme production, and the methanol fed-batch cultivation was used (16). Therefore, to maintain high PaE productivity, we tried to maintain the DO value over 25% of saturation by continuous xylose feeding.

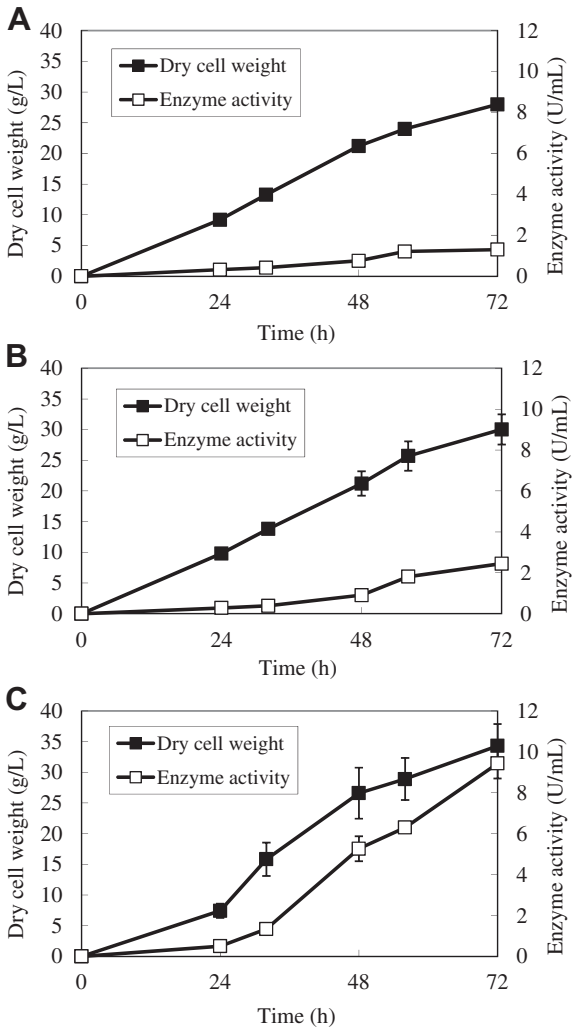

FIG. 3. Effects of nitrogen sources on cell growth (closed squares) and PaE production (open squares) by batch cultivation of *P. antarctica* GB-4(1)W using a jar fermentor. (A) Basal medium. (B) Basal medium plus 0.4% NH_4NO_3. (C) Basal medium plus 0.2% $(NH_4)_2SO_4$ plus 14% ammonia solution to keep pH at 6.0. The results show the average of two different experiments. Error bars show standard deviations.

After the initial low concentration of xylose (2%) was consumed (24 h), xylose was fed into the PaE production medium using a peristaltic pump. This resulted in a DO value around 50% of saturation (data not shown) and 13.8 U/ml of PaE was obtained after 72 h (Fig. 4A). This concentration was about 1.5 times higher than that of batch cultivation (Fig. 3C). Moreover, 21.4 U/ml of PaE was obtained after 120 h (Fig. 4A). This concentration and the total amount of PaE were 100 times and 6000 times higher, respectively, than those obtained previously (0.21 U/ml in 50-ml flasks) (8).

On the xylose fed-batch cultivation, PaE production started after 24 h (cells were grown to a density of over 9 g/l dry cell weight) and the maximum PaE production rate (0.34 U/ml/h) was obtained during 48 to 72 h (Fig. 4A). Therefore, we next tried to maintain the maximum PaE production rate by the repeated fed-batch cultivation by reusing the grown cells as described in Materials and methods. Two liters of PaE (about 14 U/ml) was stably obtained in every interval for 7 cycles (Fig. 4B). Accordingly, the PaE production efficiency of the repeated fed batch cultivation (28,000 U/d, Fig. 4B) was about 2.5 times higher than that of the fed batch cultivation (12,600 U/d, Fig. 4A).

Stability of crude PaE The optimal pH of crude PaE was 9.5 and almost the same as that of purified PaE (9). On the other hand,

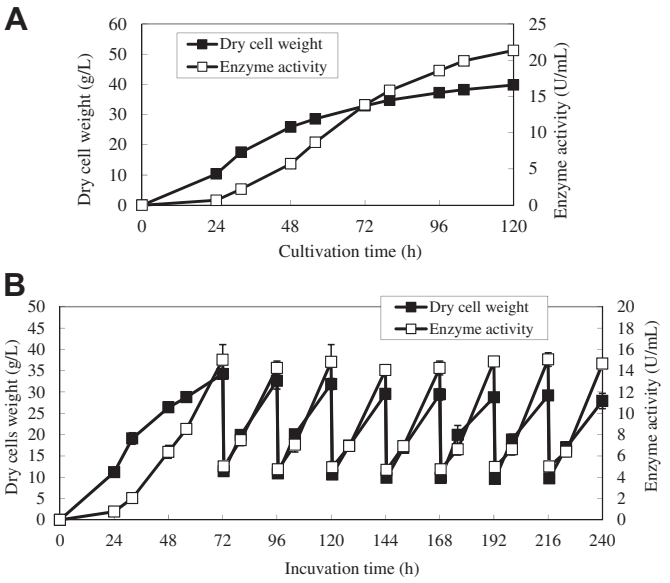

FIG. 4. Effect of xylose feeding on cell growth (closed square) and PaE production (open square) of *P. antarctica* GB-4(1)W. (A) Initial low xylose concentration (2%) and xylose feeding after 24 h. (B) Repeated fed-batch cultivation at 24-h intervals. The results show the average of two different experiments. Error bars show standard deviations.

the optimal temperature of crude PaE (50°C) was slightly higher than that of purified PaE (45°C) (9). Solutions of filtered jar-culture crude PaE were stored at various temperatures. After 12 weeks, the activity was 90% of the initial activity for samples stored at 4°C, decreasing to about 55% of the initial activity as the temperature was increased to 30°C (Fig. 5). On the other hand, freeze-dried samples remained stable, even when stored at 30°C (Fig. 5).

Degradation of BP mulch films Two units of crude PaE per 23 mg film (20 μm thickness, cut into 1-cm-squares) completely degraded PBSA film in 3 h at 30°C with shaking, and completely degraded PBS and a commercially available BP mulch film in 24 h (Fig. 6).

In conclusion, we found that xylose induces *P. antarctica* to produce PaE. PaE productivity was further increased by using a jar fermentor with xylose fed-batch cultivation. A possible inexpensive source of xylose is the waste products of bioethanol production. Ethanol can be produced from lignocellulosic biomass by the yeast *Saccharomyces cerevisiae* (17). Xylose constitutes 20–35% dry weight in lignocellulosic biomass in the form of hemicellulose (18)

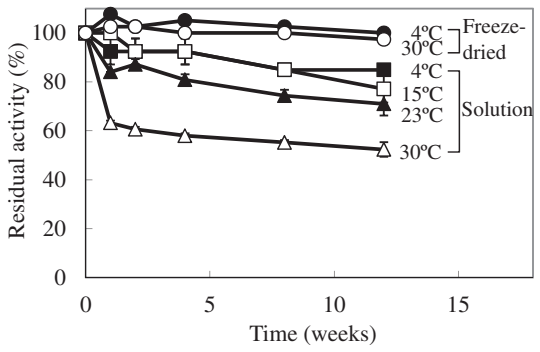

FIG. 5. Stability of crude PaE under various storage conditions. Crude PaE was maintained with filtrated solution at 4°C (closed squares), 15°C (open squares), 23°C (closed triangles), and 30°C (open triangles) and with freeze-dry powder at 4°C (closed circles) and 30°C (open circles). The results show the average of three different experiments. Error bars show standard deviations.

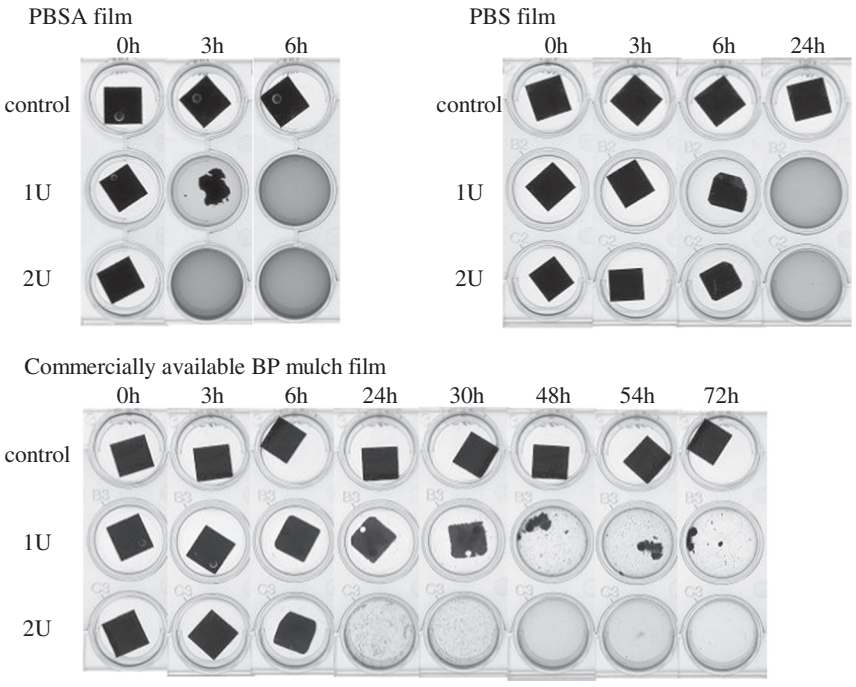

FIG. 6. Degradation of BP mulch films (PBSA, PBS, and commercially available BP mulch film) by submerged treatment with crude PaE. Each film was cut into 1 × 1 cm^2 (23 mg) squares and one square film was put in each well.

but is not consumed by *S. cerevisiae*. Further study is needed to test the feasibility of using this source of xylose for PaE production.

The goal of this study was to obtain highly concentrated PaE for rapidly degrading the strength of BP mulch films after harvesting crops. Further study is needed to test the efficiency of the concentrated PaE to degrade BPs mulch film in field experiments.

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