academicJournals

Vol. 7(27), pp. 3517-3525, 5 July, 2013
DOI: 10.5897/AJMR2013.5607
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation of *Lecythophora* sp. YP363, a secretor of various thermostable plant cell wall-degrading enzymes with high activity

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Accepted 3 July, 2013

A fungal strain that produces high activities of various plant cell wall degrading enzymes such as CMCase, filter paperase, β-glucosidase, avicelase, xylanase and pectinase with high activities was isolated from soil surrounding rotten wood. The strain was identified as *Lecythophora* sp. YP363 based on its ITS, 18S and 26S sequences. The specific activities of the various plant cell wall-degrading enzymes of *Lecythophora* sp. YP363 were, depending on the enzyme, identical to or much higher than those of *Trichoderma reesei* Rut C-30. Notably, the specific activities of the β-glucosidase, xylanase and pectinase of *Lecythophora* sp. YP363 were 4.6, 1.9 and 3.2 times higher, respectively, than those of *T. reesei* Rut C-30. The residual activities of CMCase, FPase and avicelase of *Lecythophora* sp. YP363 after the heat treatment at 60°C for 1 h were 100% or close to 100%, whereas the enzymes of *T. reesei* Rut C-30 are not resistant to heat treatment, that is, the residual activities of all the *T. reesei* Rut C-30 enzymes were only 36.9 ~ 41.9%. This result indicated that the enzymes of *Lecythophora* sp. YP363 were quite stable to heat treatment. Therefore, *Lecythophora* sp. YP363 has a potential as an alternative fungal strain to *T. reesei* in the lignocellulosic biomass-based industry.

Key words: *Lecythophora* sp., thermostable, plant cell wall degrading enzymes, *Trichoderma reesei* Rut-C30.

INTRODUCTION

Lignocellulosic plant biomass is the earth's most abundant renewable bioresource with great potential for the production of bioenergy and commodity chemicals. Cellulose and hemicellulose are the major constituents of plant biomass. By enzymatic hydrolysis, these polysaccharides are transformed into glucose and other fermentable sugars, which might further be converted to liquid fuels such as bioethanol and many other useful chemicals.

Due to the recalcitrant nature of lignocelluloses, the high cost of hydrolyzing biomass polysaccharide to fermentable sugars remains a major obstacle that must be overcome before lignocellulose-based products can be effectively commercialized. One of the most important obstacles to achieve the cost-effective production of fuels and chemicals, is the availability of highly active cellulolytic enzymes.

Cellulases are a family of enzymes that hydrolyze the ß-1,4 linkages of cellulose. Cellulases are also used commercially in many applications, for example, in detergents, in the pulping and textile industries, as animal feed additives, and for the clarification of fruit and vegetable products (Rolle, 1998). Filamentous fungi are the major sources of commercial cellulases. Among them, *Trichoderma reesei* is the most well-known cellulase-producing strain, and commercial cellulase preparations based on mutant strains of *T. reesei* (also known as *Hypocrea jecorina*) are produced on an industrial scale by many companies worldwide (Gusakov, 2011; Merino and Cherry, 2007; Nieves et al., 1998). When the fungus

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was first discovered during World War II, it was identified as *T. viride* strain QM 6a, and later, it was renamed *T. reesei*. Different cellulase hyperproducing mutant strains of *T. reesei* have been developed; the RUT C30 strain is one of the most powerful and best-characterized strains, and it has become a reference strain among the *T. reesei* high-cellulase producers (Callow and Ju, 2012; Le Crom et al., 2009; Peterson and Nevalainen, 2012, Wang et al., 2013).

In contrast to pure cellulose, the plant cell wall is a heterogeneous matrix of polysaccharides with a diverse composition and linkages, such that the hydrolysis of plant biomass is much more complex than that of pure cellulose (Berlin et al., 2005, 2007; Schmer et al., 2008). In most plant cell walls, cellulose microfibrils are embedded in a matrix of pectin, hemicelluloses, lignin and structural proteins. Efficient degradation requires endocellulase and exocellulase activities, as well as the activities associated with ß-glucosidase, xylanase, pectinase and other plant cell wall degrading enzymes (PCWDEs) (Berlin et al., 2005, 2007; Selig et al., 2008). Virtually, all fungi produce a great abundance and variety of PCWDEs that may be needed to soften plant cell walls for penetration by fungal hyphae, as well for the provision of nutrients for growth (Tonukari, 2003).

The major cost in the lignocellulose-based industry is the production of cellulase and the enzymatic hydrolysis of cellulose; therefore, screening and isolation of powerful cellulase-secreting microorganisms that are superior to *T. reesei* have been attempted. Using these microorganisms in the production process to decrease costs has always been the focus of bio-liquid fuel and biorefinery research. In fact, the cellulase of *T. reesei* shows relatively low specific activity, low thermal stability and high sensitivity to product-inhibition (Adsul et al., 2007; Chand et al., 2005; Lynd et al., 2002; Viikari et al., 2007). It is well known that the ß-glucosidase of *T. reesei* is not sufficient for cellulose degradation (Lynd et al., 2002; Merino and Cherry, 2007; Nieves et al., 1998). The analysis of genomic sequence of *T. reesei* revealed that it contains the smallest number of PCWDEs as compared to those found in 13 other fungal genomes (Martinez et al., 2008).

The present study reports the isolation of a fungal strain that produces various PCWDEs with specific activities identical to or much higher than, depending on the enzyme, those of *T. reesei* Rut-C30. The enzymes of the isolate also have a higher thermal stability when compared with those of *T. reesei* Rut-C30.

MATERIALS AND METHODS

Culture medium

Potato dextrose agar (PDA, Difco, USA) was used to isolate fungi from soil. To detect their cellulase production, carboxymethyl cellulose agar (Mandels et al., 1981) containing (g/L): $NaNO_3$ 2.0, KH_2PO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, carboxymethyl cellulose (CMC, Sigma C5678, USA) 2.0, peptone 0.2 and agar 15.0 was used. The medium used for liquid culture to prepare the various crude PCWDEs was ATCC cellulose medium 907 (g/L): $(NH_4)_2SO_4$ 0.5, L-asparagine 0.5, KH_2PO_4 1.0, KCl 0.5, $MgSO_4.7H_2O$ 0.41, $CaCl_2$ 0.1, yeast extract 0.5 and CMC 5.0. The medium used for fungal morphological observations was malt extract agar (MEA) containing (g/l): malt extract 20, glucose 20, peptone 1 and agar 20.

Isolation of fungal strains producing cellulase

One gram of the soil surrounding rotten wood was transferred to 9 ml of sterile saline in a test tube. It was subjected to serial dilutions, spread onto PDA plates and incubated for 4 to 6 days at 30°C. Fungal colonies were picked up and cultured on PDA plates. Cellulase-producing fungi were screened on carboxymethyl cellulose agar. The plates were spot-inoculated with pure cultures and incubated at 30°C. After several days of incubation, the plates were flooded with 1% Congo red solution for 30 min, then destained with 1 M NaCl solution for 15 min. The diameter of the zone of decolorization around each colony was measured. The fungal colonies that produced a relatively large zone of decolorization were collected.

Enzyme assays

For the liquid cultures to prepare the crude enzyme solutions, one colony (diameter, 7 mm) of each isolate was inoculated into a 50 ml of ATCC cellulose medium 907 in a 250 ml Erlenmeyer flask and cultured at 30°C in a rotary shaking incubator operated at 200 rpm for 7 days. The culture broth was filtered through a Millipore filter (pore size, 0.2 µm) and the filtrate was used as the crude enzyme solution.

The activities of the PCWDEs were determined by measuring the release of reducing sugar (King et al., 2009) from an appropriate substrate using an appropriately diluted culture filtrate as the enzyme source, and the amount of reducing sugars was determined using the 3,5-dinitrosalicylic acid method (Miller, 1959).

The endoglucanase activity was assayed using 0.5 ml of 1% substrate (CMC) suspended in 50 mM citrate buffer (pH 5.2); 0.5 ml of the culture filtrate was added as the enzyme source, the solution was incubated for 30 min at 50°C. Avicel (FMC type PH-101, *50* µm) was used in place of CMC to determine the avicelase activity; xylan (from Birchwood, Sigma X0502, USA) was used for xylanase activity, pectin (Junsei Chemical, Japan) was used for pectinase activity, and cellobiose (Sigma C7252, USA) was used for β-glucosidase activity. One unit of enzyme activity was defined as the amount of enzyme releasing 1 *µ*mole of reducing sugar in a 1 min reaction.

To assay the filter paperase (FPase) activity that represents the total cellulase activity, aliquots of culture filtrate, the enzyme source, were added to a strip of Whatman No. 1 filter paper (1 × 6 cm; 50 mg) immersed in 1 ml of 0.05 M sodium acetate buffer, pH 5.0 and incubated at 50°C for 1 h. One unit of FPase activity was defined as above.

The enzyme level produced in the culture broth was expressed as U/ml and the specific enzyme activity was expressed as U/mg protein. All the enzyme assays were performed in triplicate.

DNA isolation and amplification

The 18S rDNA sequence of the strain was determined using genomic DNA isolated from pure cultures. DNA extraction was performed using an Accuprep Genomic DNA extraction kit (Bioneer Co. Korea) according to the methods of Kurtzman et al. (1998). The resulting isolated DNA was used as a template in PCR reactions to amplify several regions, using the following primers: NL1 (5'-GCA

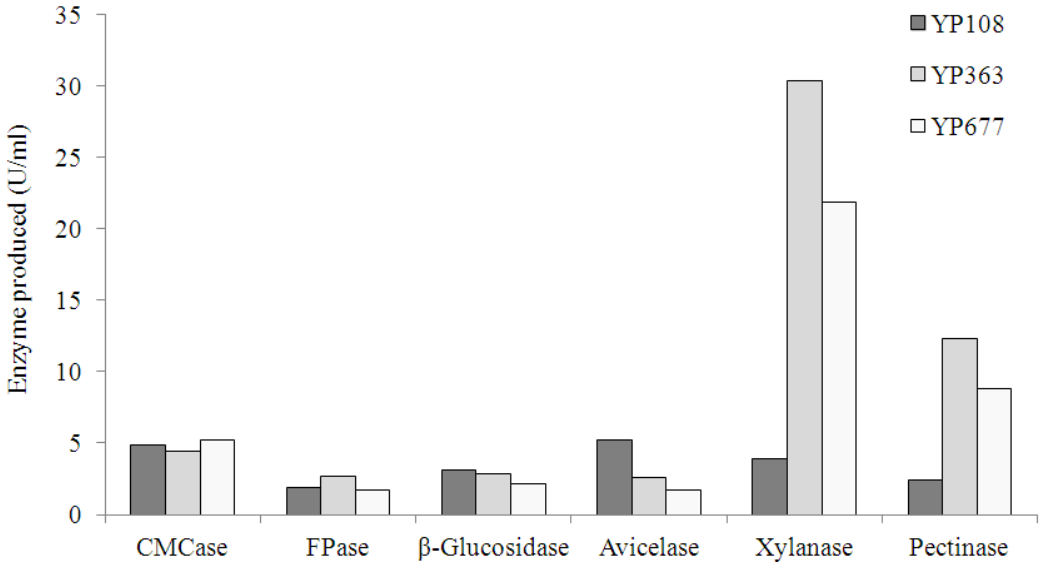

Figure 1. The activities of the various PCWDEs produced by three isolates.

TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TTTC AAG ACG G-3') (O'Donnell, 1993) for the 26S D1/D2 region, NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') and NS8 ('5-TCC GCA GGT TCA CCT ACG GA-3') (Thompson et al., 1994) for the 18S region; and ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Innis et al., 1990) for the ITS region.

PCR amplification was performed using a Maxime PCR pre-mix kit (i-StarTaq, Intron, Co, Korea). The following thermal profile was used for PCR: denaturation at 94°C for 2 min, primer annealing at 60°C for 20 s and extension at 72°C for 1.5 min. The final cycle included extension for 5 min at 72°C to ensure full extension of the products. Then, the PCR products were purified using a QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and sequenced using an ABI 310 DNA sequencer (Applied Biosystems, Foster City, California, USA).

Identification and relatedness of the isolate

The DNA sequences were compared with reference sequences available in the NCBI GenBank and aligned for phylogenetic analysis. To determine the phylogenetic relatedness of the different species, they were aligned using the UPGMA algorithm, which considers the rate of evolution to be constant between species.

Heat stability of various enzymes

A 10 ml test tube containing 5 ml of an enzyme solution was incubated for 1 h at 60°C in a water bath, and then rapidly cooled by immersing the tube in a water bath at room temperature. Each enzyme activity was analyzed, and the percent of residual activity of each enzyme was calculated.

Protein assay

The concentration of protein was determined using the dye binding method of Bradford (1976) using the Bio-Rad dye reagent concentrate (Bio-Rad, USA) in microtiter plates. A standard curve was generated using a solution of 1 µg/ml of bovine serum albumin. The absorbance at 595 nm was measured following 5 min of incubation at room temperature; the measurement was performed in triplicate.

RESULTS AND DISCUSSION

Isolation and identification of a fungal strain producing PCWDEs with high activity

A total of 494 fungal strains with cellulase activity, as judged by the formation of a clear zone with Congo red around the colony, were isolated from the soil surrounding rotten wood. One hundred and nineteen strains that produced a relatively larger clear zone were selected and subjected to analysis of CMCase and FPase. Among the ten strains with relatively higher CMCase and FPase activities, three strains with the highest CMCase and FPase activities were again selected and their various PCWDEs were analyzed (Figure 1). Although, YP108 exhibited the highest avicelase activity, the YP363 strain produced significantly higher levels of FPase, xylanase, pectinase and a fairly high level of β-glucosidase (Figure 1), and therefore this strain was selected for further study. By microscopic observation (x400, x1000), YP363 (Figure 2) and YP677 had very similar fungal morphology, whereas YP108 was a bacillus-type bacterium. Due to their high level of protein expression, fungal strains are preferred to bacterial strains as commercial cellulase producers (Gusakov, 2011).

According to its ITS sequence (accession number JX910080), the YP363 isolate showed 93% similarity to *Lecythophora savoryi*. In the next step, by comparing its 18S rRNA sequence (accession number JX910082), the strain was located in the genus *Lecythophora* and the clade including *Lecythophora*. However, the 18S rRNA

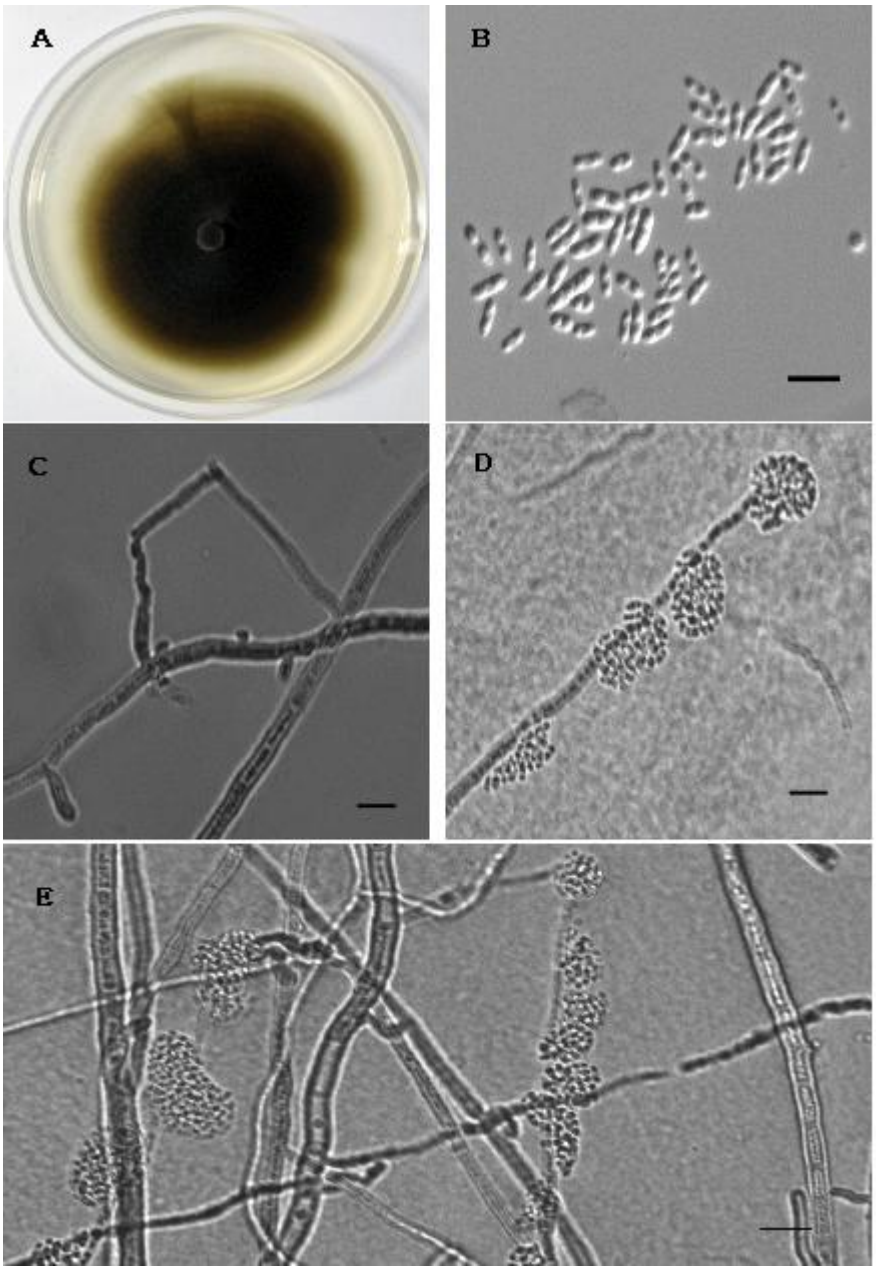

Figure 2. Fungal strain YP363 that was isolated from soil surrounding rotten wood. A, colony grown on MEA medium; B, conidia; C, conidia formed on hyphal cell; D, conidiogenous cells on hyphal cells; E, conidia formed on hyphal cells. Scale bar: 10 μm.

sequence of the strain was not identical to that of any species in GenBank. Based on an analysis of its 26S rRNA sequence (accession number JX910081), the strain showed 98% similarity to *Lecythophora subcorticalis* CBS 551.75 in GenBank (Figure 3). Because there is no species with 100% similarity to it, the isolate was named *Lecythophora* sp. YP363. *Coniochaeta* and *Lecythophora* are names for the sexual and asexual states, respectively, of the same genus included in the family Coniochaetacea of Ascomycetes. Since the strain was isolated from soil as the form of the conidia of asexual state, not from the ascocarps of the sexual state, *Lecythophora* was chosen instead of *Coniochaeta*. The characteristics of *Lecythophora* are hyaline hyphae and mostly intercalary phialides with very short lateral necks, periclinal wall thickening and flaring collarettes (Gams, 2000). The shape of the conidia of *Lecythophora* sp. YP 363 (Figure 2) is highly similar to that of previously reported species belonging to *Lecythophora* (Perdomo et al., 2011).

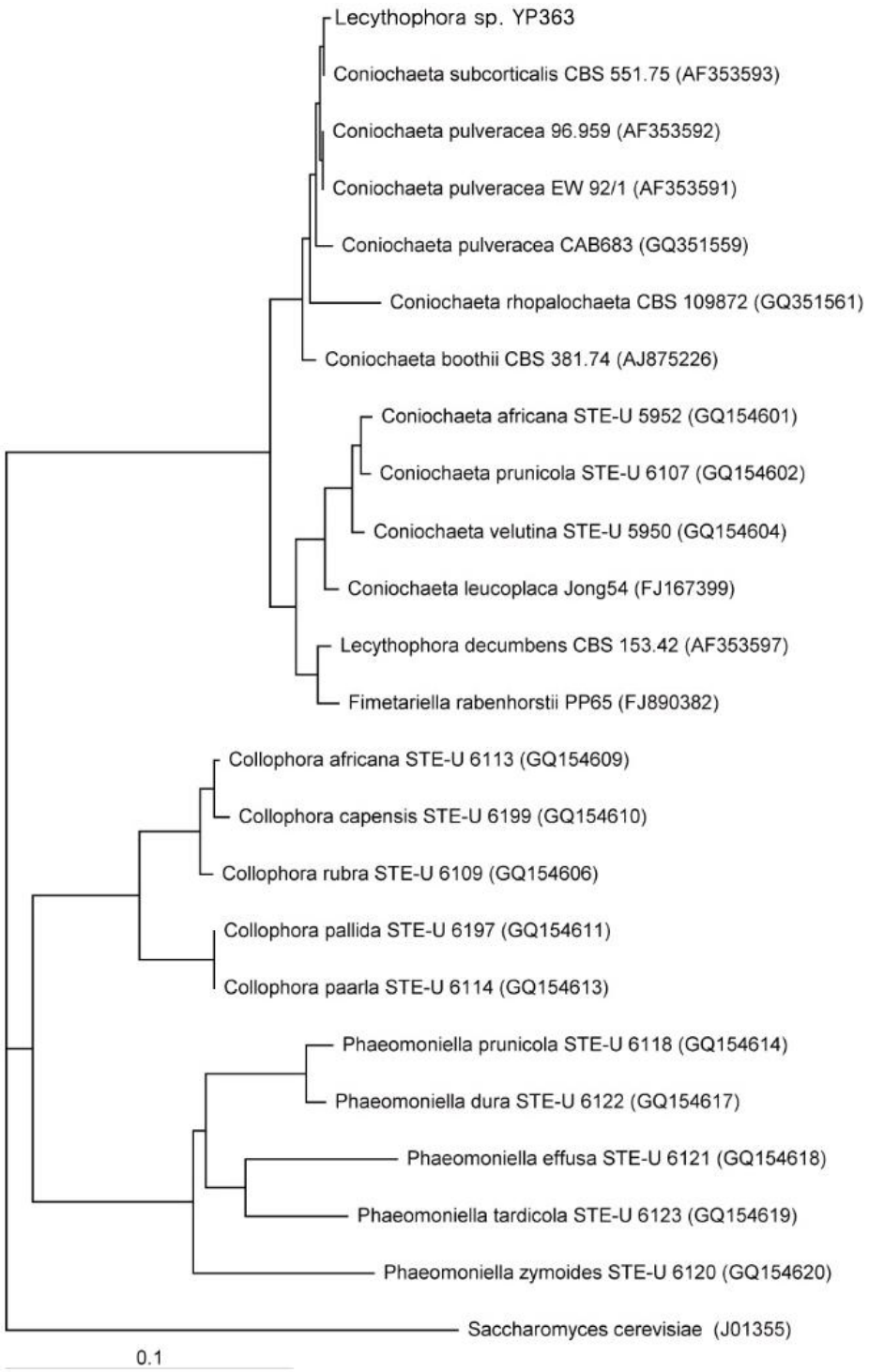

Figure 3. Phylogenetic tree based on the 26S sequence of the YP363 strain.

This strain was isolated from soil surrounding rotten wood, and it has been reported that the *Lecythophora* strains inhabit decomposing wood and soil (Weber, 2002). Some species of *Coniochaeta* are associated with wood necroses of *Prunus* trees (Damm et al., 2010).

Activities of the various PCWDEs produced by *Lecythophora* sp. YP363

The enzyme production of the two fungal strains in the culture broth was analyzed and compared. The results

(Figure 4A) showed that the amounts of CMCase, FPase, avicelase, xylanase and pectinase produced by *Lecythophora* sp. YP363 were 30.7, 28.9, 33.1, 33.2 and 97.4% of the amount of each corresponding enzyme by *T. reesei* Rut-C30, whereas the amount of β-glucosidase produced by *Lecythophora* sp. YP363 was 138.4% of that produced by *T. reesei* Rut-C30. These results suggest that *Lecythophora* sp. YP363 produced smaller amounts of some enzymes in the culture broth than did Rut-C30. However, when QM6a, the mother strain of Rut-C30, was originally isolated, its FPase activity was only 1/15 to 1/20 that of the Rut-C30 strain (Montenecourt and Eveleigh, 1979); its endoglucanase activity was 1/30 and its β-glucosidase activity was 1/3 those of the Rut-C30 strain (Ghosh et al., 1984). Therefore, *Lecythophora* sp. YP363 produced much higher levels of FPase, endoglucanase and β-glucosidase activity than does *T. reesei* QM6a. Considering the improvement of Rut-C30 resulting from several rounds of mutagenesis of QM6a (Montenecourt and Eveleigh, 1979), *Lecythophora* sp. YP363, which was directly isolated from soil, could be developed into a much higher cellulase-producing strain by a similar mutagenesis process. Furthermore, the process of optimizing the PCWDEs production by *Lecythophora* sp. YP363 will increase its enzyme production to much higher levels.

Lopez et al. (2007) previously reported that lingocelluloses-degrading enzymes are produced by species related to *Lecythophora*, but they assayed only the endoglucanase (from 0.02 to 0.34 U/ml) and xylanase activities (from 0.02 to 0.34 U/ml) and the activity were very low as compared to those of *Lecythophora* sp. YP363 (CMCase 4.0 U/ml and xylanase 24.1 U/ml). Ravindran et al. (2012) isolated *Coniochaeta* sp. LF2 from soil beneath rotten wood and measured the enzyme activities in the supernatant of the cell culture. While the maximum β-glucosidase activity of *Coniochaeta* sp. LF2 was comparable to that of our strain, the exoglucanase (0.03 to 1.34 U/mL), endoglucanase (0.45 to 3.34 U/mL) and xylanase activities (0.68 to 5.45 U/mL) were lower than those of our strain. The authors did not measure the FPase and pectinase activities.

The specific activities of the various PCWDEs of *Lecythophora* sp. YP363 and *T. reesei* Rut-C30 were determined and compared (Figure 4B). The specific activities of the various enzymes of *Lecythophora* sp. YP363 were, depending on the enzyme, identical to or much higher than those of *T. reesei* Rut C-30 (Figure 4B). Notably, the specific activities of the β-glucosidase, xylanase and pectinase of *Lecythophora* sp. YP363 were 4.6, 1.9 and 3.2 times higher, respectively, than those of *T. reesei* Rut C-30 (Figure 4B). The FPase activity of *T. reesei* Rut C-30 determined in the present investigation, that is, 9.4 U/ml (Figure 3A) or 3.2 U/mg protein (Figure 4B), was comparable to the FPase activity of *T. reesei* Rut C-30 described in the literature, which varied from 1.63 U/ml or 2.7 U/mg protein in a shaken flask (Schimenti et al., 1983) to 15 U/ml under controlled fermenter conditions (Montenecourt and Eveleigh, 1979).

T. reesei RUT C-30, a hypersecretor of cellulases that are resistant to catabolite repression, does not appear to possess any new or altered enzymes as compared to those of the parental strain QM6a (Sheir-Neiss and Montenecourt, 1984). In the case of Rut C-30, improving the specific activities by conventional mutagenic procedures appears to be very difficult. In a review paper on cellulase producers, fungi belonging to the genera *Penicillium, Acremonium* and *Chrysosporium* were suggested as the alternatives to *T. reesei* because they are competitive with *T. reesei* in some important para-meters, such as levels of protein production and the cellulose hydrolytic performance per unit of activity or milligram of protein (Gusakov, 2011). Because the speci-fic activities of the various enzymes of *Lecythophora* sp. YP363 were, depending on the enzyme, identical to or much higher than those of *T. reesei* Rut C-30 (Figure 4B), *Lecythophora* sp. YP363 could also be an alternative to *T. reesei* Rut C-30.

In both *Lecythophora* sp. YP363 and *T. reesei* Rut C-30 strains, the amount of xylanase produced and specific xylanase activity were distinctly higher than those of the other PCWDEs. Among the various PCWDEs of the two strains, both the lowest level of enzyme produced and the lowest specific enzyme activity were those of the β-glucosidase of *T. reesei* Rut-C30. The ß-glucosidase of *T. reesei* was also reported in other literature to be insufficient for cellulose degradation (Lynd et al., 2002; Merino and Cherry, 2007; Nieves et al., 1998).

In contrast to pure cellulose, the plant cell wall is a heterogeneous matrix of polysaccharides with diverse composition and linkages, such that the hydrolysis of plant biomass is much more complex than that of pure cellulose (Berlin et al., 2005, 2007; Schmer et al., 2008). The ability of a commercial *T. reesei* cellulase preparation (Celluclast 1.5 L) to hydrolyze the cellulose and xylan components of pretreated corn stover was significantly improved by supplementation with xylanase, pectinase and ß-glucosidase (Berlin et al., 2007). It was suggested that xylanase and pectinase facilitate cellulose hydrolysis by removing the non-cellulosic polysaccharides that coat the cellulosic fibers (Berlin et al., 2007). Therefore, with higher activities of xylanase, pectinase and ß-glucosidase in addition to comparable activities of CMCase, filter paperase and avicelase, *Lecythophora* sp. YP363 has the potential to degrade a lignocellulosic biomass more efficiently than *T. reesei* Rut-C30, when it is developed into a much higher cellulase-producing strain by a mutagenesis process similar to that applied to the parental strain of *T. reesei* Rut-C30. Moreover, it was recently reported that the lignin degradation potential of *Coniochaeta* sp. LF2 was higher than that of *T. reesei* (Ravindran et al., 2012). Lignin-degrading enzymes initially deconstruct a lignocellulosic biomass, and make cellulose and hemicellulose accessible to cellulolytic and hemicellulolytic enzymes.

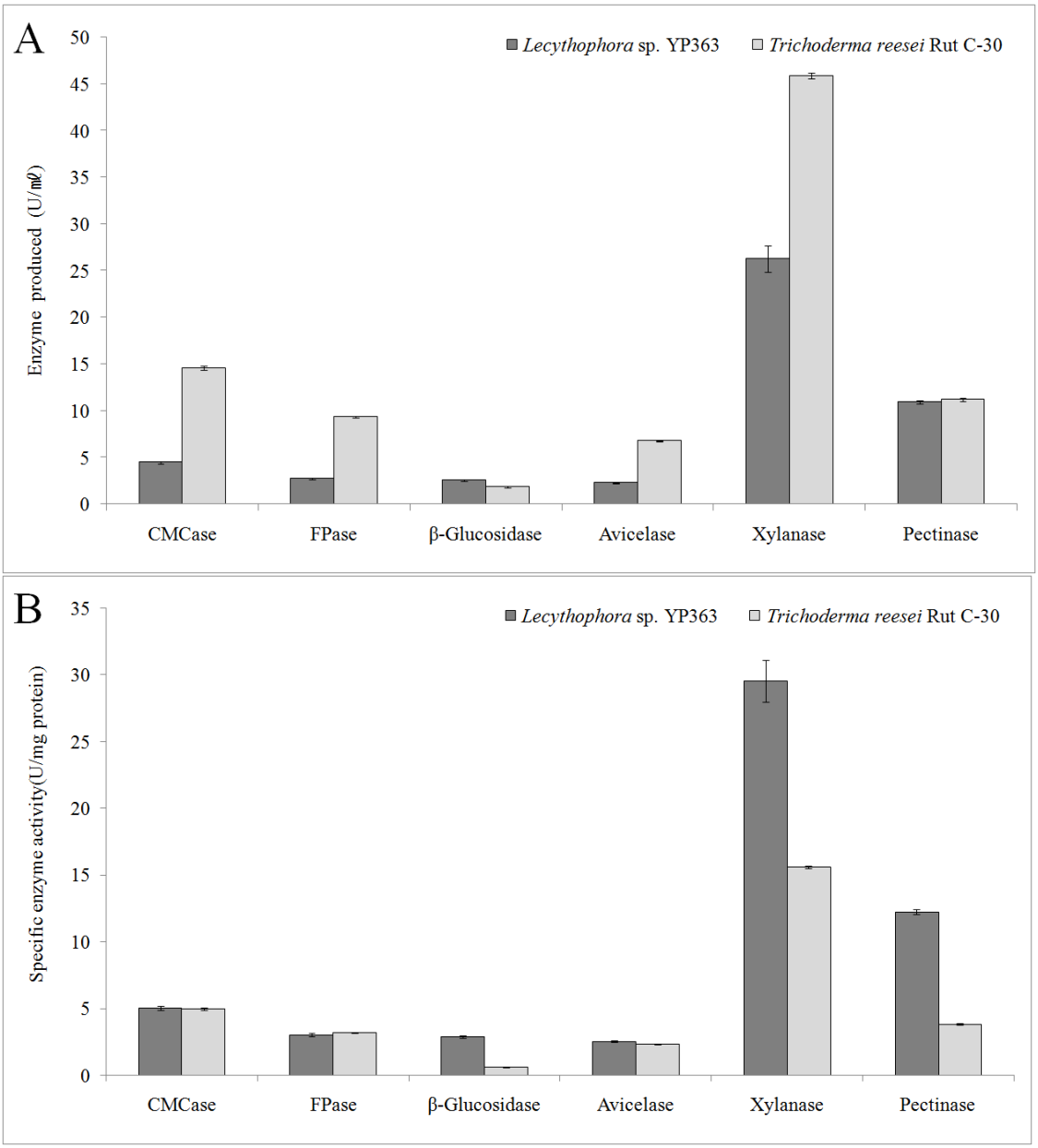

Figure 4. The activities of various PCWDEs produced by *Lecythophora* sp. YP363 and *T. reesei* Rut C-30. A, activities (U/ml) of the enzymes produced in culture broth; B, enzyme activities expressed as the specific enzyme activity (U/ mg protein).

Thermostability of the various PCWDEs produced by *Lecythophora* sp. YP363

The residual activities of the various PCWDEs of *Lecythophora* sp. YP363 and *T. reesei* Rut-C30 after heat treatment at 60°C were examined; the results are shown in Figure 5. The residual activities of the CMCase, FPase and avicelase of *Lecythophora* sp. YP363 were 100% or close to 100%, whereas those of β-glucosidase, xylanase and pectinase were 97.5, 94.6 and 96.0%, respectively. Although there are some variations in the heat stability of the various PCWDEs, the enzymes of *Lecythophora* sp. YP363 were quite stable after the heat treatment. However, the various enzymes of *T. reesei* Rut C-30 were very sensitive to heat treatment, that is, the residual activities of all of the *T. reesei* Rut C-30 enzymes were only 36.9 ~ 41.9%. The thermal-instability of *T. reesei* cellulase has been described in the literature.

Viikari et al. (2007) reported that *T. reesei* cellulases were rapidly inactivated during the first 2 h of hydrolysis of pretreated spruce substrates at temperatures above 60°C and that the hydrolytic yield of sugars from spruce

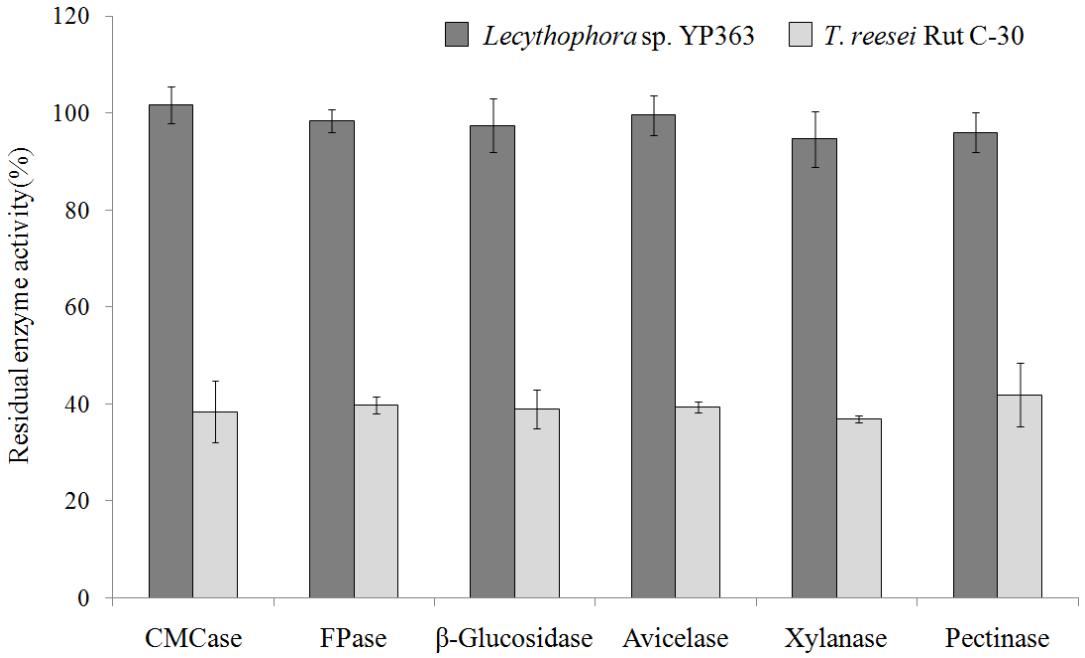

Figure 5. The residual activities of the various PCWDEs of *Lecythophora* sp. YP363 and *T. reesei* Rut-C30 after heat treatment at 60°C for 1 h. The protein concentrations in the crude enzyme solutions of *Lecythophora* sp. YP363 and *T. reesei* Rut C-30 were 0.89 and 2.94 mg/ml, respectively.

at 60°C during the first 2 h was only 33% of the theoretical maximum. It was also reported that the residual activity of the CMCase of *T. viride* was 60% after 1 h of treatment at 60°C (Li et al., 2010).

Thermostable enzymes offer potential benefits in the hydrolysis of lignocellulosic substrates, including higher specific activity that decreases the amount of enzymes needed, enhanced stability that improved hydrolytic performance and increased flexibility with respect to process configurations, which all led to improvements of the overall economy of the process (Viikari et al., 2007). Other potential advantages are higher mass-transfer rates that lead to better substrate solubility and a lowered risk of potential contamination (Rastogi et al., 2010).

In conclusion, we have isolated *Lecythophora* sp. YP363, which produces various PCWDEs important in lignocelluose degradation that have an identical or much higher specific activity, depending on enzymes, when compared with those of the well-known reference strain *T. reesei* Rut-C30. The *Lecythophora* sp. YP363 enzymes also have a higher thermal stability when compared with those of *T. reesei* Rut-C30. Therefore, *Lecythophora* sp. YP363 is a very promising alternative fungal strain to *T. reesei* in the lignocellulosic biomass-based industry.

ACKNOWLEDGEMENTS

We thank Professor Jae-Jin Kim, Department of Environmental Science and Ecological Engineering, Korea University, Korea for identification of *Lecythophora* sp. YP363 and for providing us with the fungal photomicrographs. This study was supported by the Technology Development Program (309016-5) of the Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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