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ORIGINAL ARTICLE

Identification of a fungus able to secrete enzymes that degrade regenerated cellulose films and analyses of its extracellular hydrolases

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Abstract The LW03 strain was isolated from Chinese farmland soil and found to be able to secrete certain enzymes degrading regenerated cellulose films at low temperature. The LW03 strain was systematically identified as *Rhizopus arrhizus* var. *arrhizus* by morphological, physiological, and molecular methods. Incubation of regenerated cellulose films with the extracted crude enzyme of LW03 was done to measure morphological changes by using scanning electron microscopy. Microscopic observations showed that the morphology of the regenerated cellulose films changed drastically due to enzymatic hydrolysis. The extracellular hydrolases of LW03 strain incubated on bran medium were also assessed. The predominant activity in the crude enzyme was glucoamylase activity, followed by acid proteinase, phytase and pectinase activity. Interestingly, activities of β-glucosidase, endoglucanase, exoglucanase, and cellulase were also observed, but at a much lower extent. Based on initial evidence, the crude enzyme is most likely to contain some new constituents capable of degrading regenerated cellulose films.

Keywords Regenerated cellulose films · Degradation · Enzymes · *Rhizopus arrhizus*

Introduction

It is well-known that cellulose, the oldest and the richest natural polymer on earth, can be regenerated or derived from various useful products (Yang et al. 2002). The viscose route for producing regenerated cellulose fibers and films has dominated the current processing of cellulose for more than 100 years (Li et al. 2012). Regenerated cellulose films, such as cellophane and dialysis tubing, are widely known for being applied in packaging and many technologies, such as dialysis, ultra-filtration and fractionation of polymer mixtures (Yang et al. 2002; Chen et al. 2004). Nevertheless, the fate of these films in the environment is unknown. Despite their biodegradable nature, the observation of the degradation process is difficult and time-consuming. Their residues can persist in the environment for a long time. Hence, an increasing interest is focused on the degradation mechanisms conducted by microbes to process these macromolecular materials (Kleeberg et al. 2005).

Chemically, cellulose consists of a homopolysaccharide of β-D-glucopyranose residues linked by β-(1→4)-glycosidic bonds, and cellobiose is its smallest repetitive unit (Nascimento et al. 2010). Cellulolytic fungi and bacteria produce a family of different cellulolytic enzymes that act synergistically to degrade cellulose to glucose (Limam et al. 1995). In fungi, these cellulose degrading systems usually contain various endo-glucanases and exo-glucanases and at least one β-glucosidase. The number of produced enzymes depends on the fungus and culture conditions (Zyani et al. 2009). The majority of cellulose hydrolysis research has focused on the genetics, structure, function, and interaction of components of cellulose enzyme systems (Lynd et al. 2002; Medie et al. 2012; Sukharnikov et al. 2012; Zhang and Lynd 2006). However, there are few reports to date on the nature of regenerated cellulose biodegradation. Furthermore, there have been few reports concerning the species that degrade regenerated cellulose films in mild conditions.

In this study, we identified a fungal strain, LW03, capable of secreting regenerated cellulose films that degrade enzymes by molecular analysis, microscopic examination and physiological

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tests and investigated its extracellular hydrolases production. To our knowledge, this is the first report on the finding of a microbial metabolite efficiently degrading regenerated cellulose films at low temperature.

Materials and methods

Materials

Regenerated cellulose films (dialysis tubing and cellophane) are commercial materials. Dialysis tubing (MW 8000–14000, produced by the Viskase Company, a hydrophilic material made from regenerated cellulose prepared using the viscose process) was purchased from Biosharp Company. Cellophane was obtained from Shanghai Yuanye Biotechnology Co., Ltd. The cellophane composition was regenerated cellulose 80 %, glycerin 12 %, water 7.5 %, and abherent 0.5 %. The commercial cellulase ("Onozuka" R-10) was an extract of *Trichoderma* and purchased from Biosharp Company.

Microorganism

Soil samples were collected in August 1999 in Laiwu, Shandong province, China, for the screening phytase producing fungi. The fungal strain, LW03, was isolated from a farmland soil using a culture enrichment method as described by Shieh and Ware (1968), which can produce phytase and other extracellual hydrolases. The strain was conserved in the laboratory at 4 °C, on slants of PDA medium.

Identification of LW03 strain

Morphological and physiological characteristics The LW03 strain was cultivated routinely on potato-dextrose-agar (PDA) at 30 °C for the morphological investigations. Macroscopic colony characters, such as shape, color, surface texture and other physiological properties were described and investigated according to the method of Wei (1979). Microscopic features were observed following the method of Zheng et al. (2007). A Nikon Eclipse E600 microscope with digital camera was employed to capture images.

Sequences analysis Total genomic DNA of LW03 strain was extracted by the CTAB method (Sambrook and Russell 2001). The ITS regions of rDNA of strain LW03 were amplified and sequenced using primers ITS1, 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4, 5′-TCCTCCGCTTATTGATATGC-3′ (Gonthier and Garbelotto 2011). The 18S rDNA sequences were amplified using primers 18SF, 5′-ACCTGGTTGATCCTGCCAGT-3′ and 18SR, 5′-TCACCTACGGAAACCT

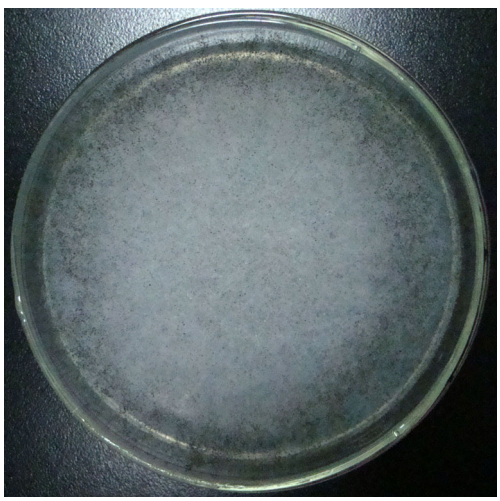

Fig. 1 Growth of LW03 strain on a PDA plate at 30 °C for 3 days

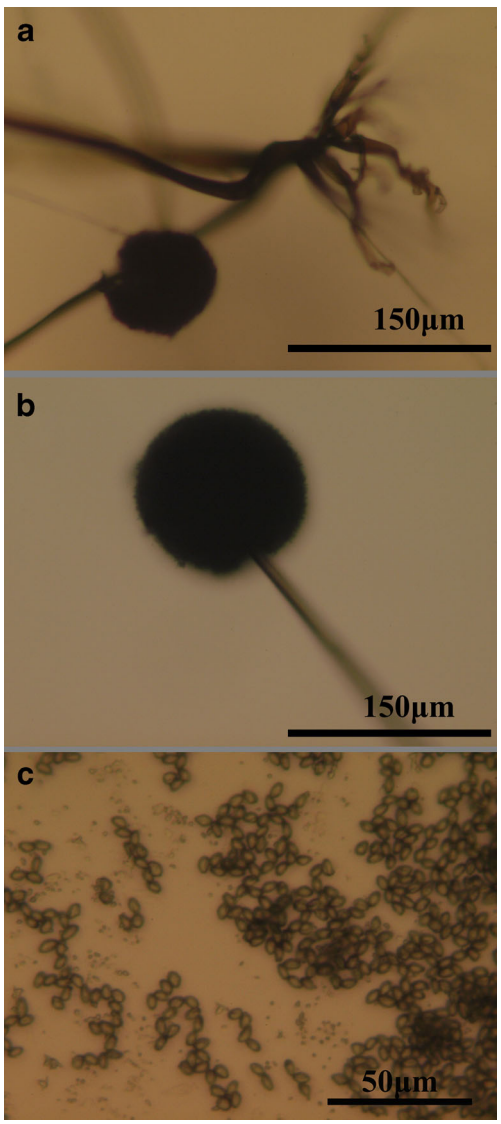

Fig. 2 Light micrographs of LW03 strain. **a** rhizoids. **b** sporangia. **c** sporangiospores

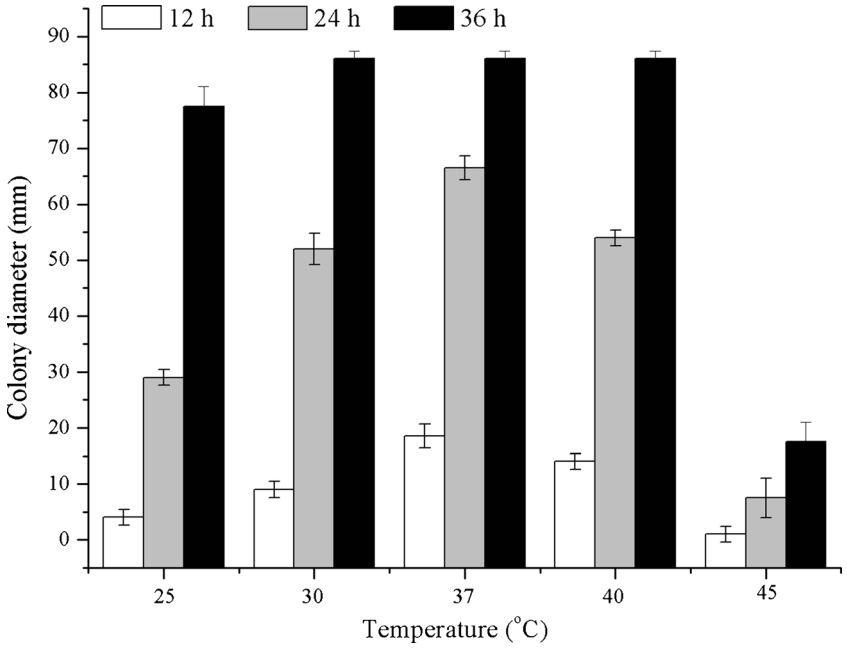

Fig. 3 Effect of incubation temperature on the growth of LW03 strain

TGT-3′ (Zhen et al. 2008). The PCR program was 5 min of pre-denaturation at 94 °C, followed by 30 cycles of PCR reaction (denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 40 s, per cycle) and a final extension for 7 min at 72 °C. PCR products were purified using the EasyPure Quick Gel Extraction Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instruction. The size of the PCR products using two different pair of primers, 18SF/18SR and ITS1/ITS4, were 1713 and 582 bp, respectively. Sequencing work was performed on both strands by Nanjing Jinsirui Biotechnology Co. The sequences were aligned using the CLUSTAL X program (Thompson et al. 1997). A phylogenetic tree was reconstructed by using the neighbor-joining method, with bootstrap values based on 1000 pseudoreplicates (Saitou and Nei 1987; Felsenstein 1985). Genetic distances were calculated using the Kimura two-parameter model (Kimura 1980).

Preparation of crude enzyme

For enzyme production, the LW03 stain was cultivated in the bran medium (60 % moisture content of bran). One milliliter of the LW03 spore suspension (approximately 10^6 spores per ml) was inoculated for 20 g of sterilized bran medium in 500-mL Erlenmeyer flasks and incubated at 30 °C for 5 days. After incubation, 1 kg of the incubated bran medium was mixed with water (1:5 volume) at 10 °C in a rotary shaker at 100 rpm for 2 h. The mixture was filtered through six sheets of gauze, centrifuged at 4,000×*g* for 20 min at 4 °C, and the supernatant was used as the crude enzyme preparation.

Enzymatic hydrolysis of regenerated cellulose films

The enzymatic hydrolysis of regenerated cellulose films performed by the crude enzyme was carried out at 4 °C for hours in an aqueous solution. The regenerated cellulose films (initial weights: 60 mg, initial film dimension: 20×70 mm) were placed in plates containing 0.02 % sodium azide. The reaction was started by adding an aqueous solution of LW03 crude enzyme. The control experiments were conducted without adding the LW03 crude enzyme. Additional control experiments were also performed, in which the films were subjected to the same experimental protocol by adding the commercial cellulase enzyme.

Enzyme assays

The activities of β-glucanase (Molina et al. 1987), xylanase (Kosugi et al. 2001), α-amylase (Ramachandran et al. 2004), endoglucanase, exoglucanase, β-glucosidase and cellulase (Gunjikar et al. 2001) were determined by measuring the rate of release of reducing sugars from pure substrates. The enzyme units were expressed as the quantity of reducing sugars released per unit of time per unit of enzyme (Beauchemin et al. 2003). The production of reducing sugars was estimated using the dinitrosalicylic acid (DNS) method, as described by Miller (1959). The activity of acid proteinase was assayed following the method previously described by Fernandez-Lahore et al. (1999). Glucoamylase activity was evaluated according to the procedure of Cereia et al. (2000). Pectinase activity was measured by the method of Ricard and Reid (2004). Phytase

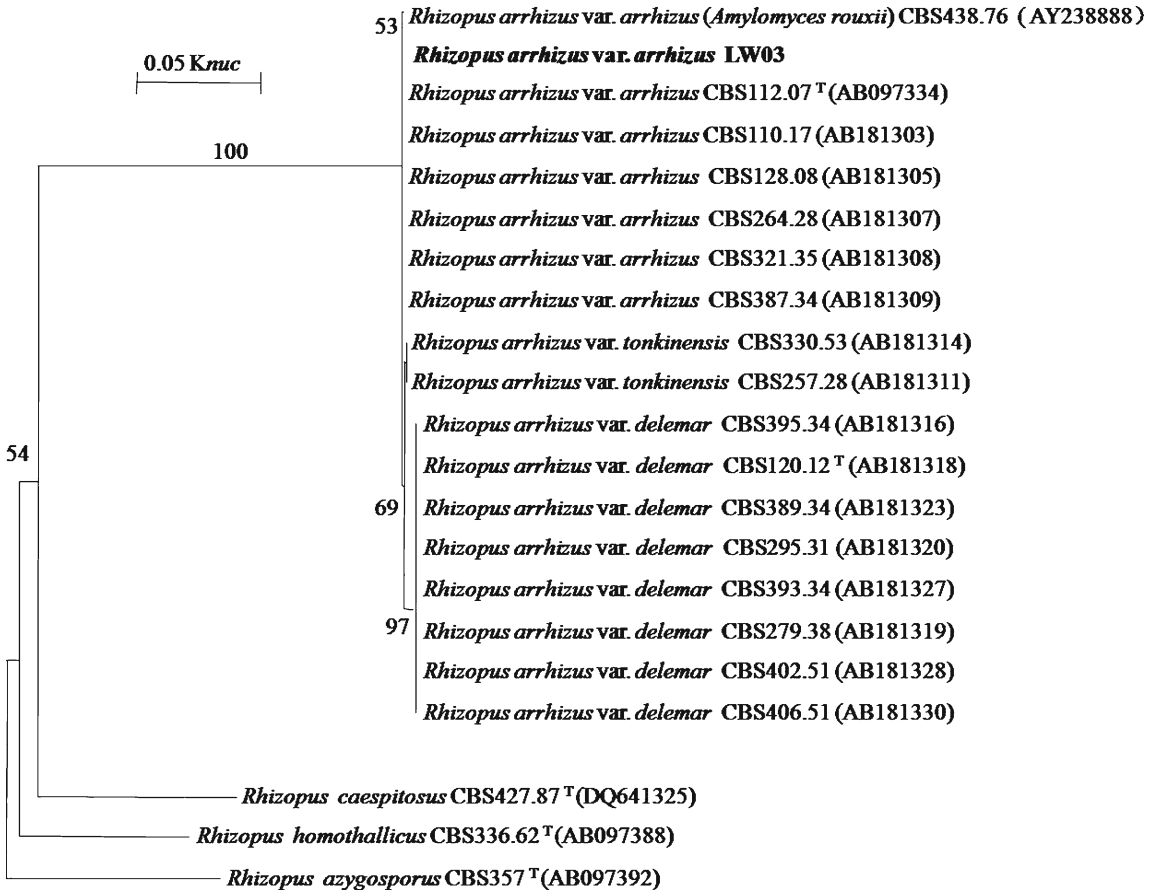

Fig. 4 Phylogenetic tree reconstructed by the neighbor-joining method based on ITS region. Bootstrap percentages values over 50 % are shown. Reference sequences were retrieved from GenBank, and accession numbers displayed. *Rhizopus azygosporus* was used as an out-group species. The scale bar indicates 5 % sequence divergence

activity was determined using the colorimetric method, as described by Ou et al. (2011).

Scanning electron microscopic observation

Scanning electron micrographs (SEM) were taken on a JSM-6390 scanning electron microscope with 20 kV accelerating voltage and at a magnification of 7000. The wet regenerated cellulose films were frozen and then vacuum-dried. The surface of the films were sputtered with gold, and then observed and photographed.

Results

Identification of LW03 strain

Colonies of LW03 strain could reach 50–55 mm diameter (Fig. 1) on a PDA plate at 30 °C for 24 h. Three days later, aerial hyphae filled the plate. The texture was from loose to dense. The colony surface of LW03 strain was lanose. The color varied with state of sporulation from white to dark brown. As shown in Fig. 2, the distinctive features of the genus *Rhizopus* such as the formation of rhizoids (Fig. 2a) and characteristics of the *Rhizopus* species such as the formation of sporangia (Fig. 2b), and sporangiospores (Fig. 2c) were observed. Zygospores were absent. The LW03 strain was able to grow well between 25 and 40 °C on PDA plates, and the optimum temperature for growth was 37 °C (Fig. 3).

Phylogenetic analyses based on sequences from 18S rDNA and ITS regions were performed to identify the LW03 strain. The 18S rDNA sequences of LW03 strain were analyzed by the GenBank nucleotide database using BLAST search. The blast search indicated that LW03 strain was a member of the genus *Rhizopus* or *Amylomyces*, as it showed the highest sequence similarities to *Rhizopus oryzae* AB250164 (100 %) and *Amylomyces rouxii* AB250171 (100 %). The phylogenetic tree reconstructed by the neighbor-joining method, based on ITS sequences, showed that the LW03 strain formed a cluster with species of *R. oryzae* and *A. rouxii* with 100 % bootstrap of branch support (Fig. 4). The LW03 strain has an identical sequence as the *R. oryzae* type strain, CBS112.07, and has 1 nt difference with the *A. rouxii* type strain, CBS438.76.

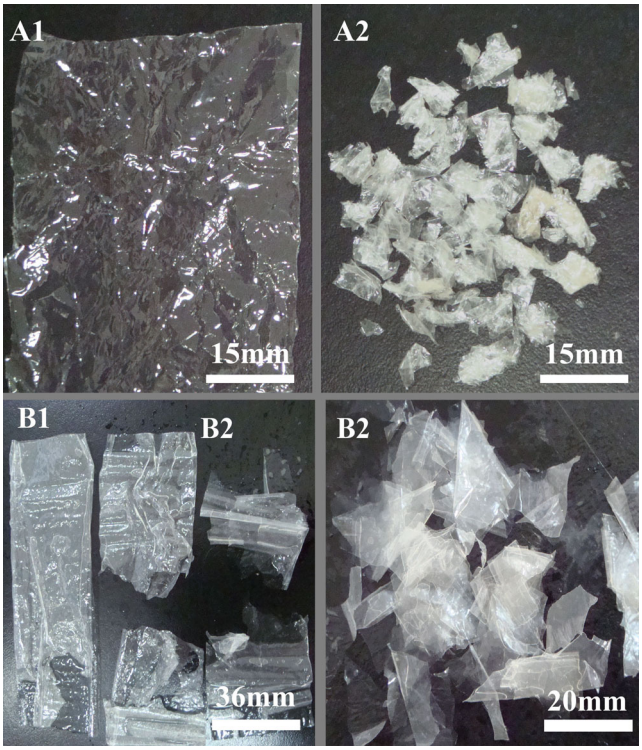

Fig. 5 Photographs of regenerated cellulose films including cellophane (**A**) and dialysis tubing (**B**). (1): Control films. (2): Pieces of films after 12 h suspending in the crude enzyme solution extracted from the LW03 strain

The analyses of the 18S rDNA and ITS region sequences revealed that LW03 strain belongs to the species *R. oryzae* or *A. rouxii*. However, *R. oryzae* and *A. rouxii* had been synonymized with *Rhizopus arrhizus* var. *arrhizus* (Zheng et al. 2007; www.indexfungorum.org). Hence, LW03 strain was identified as *R. arrhizus* var. *arrhizus* based on the rDNA sequences analyses (Liu et al. 2007).

Regenerated cellulose films degradation

This section describes the degradation of regenerated cellulose films (cellophane and dialysis tubing) exposed to the crude enzyme solution, which was extracted from the LW03 strain. After 12 h of in vitro assay along with control films, the test films were broken into small pieces (Fig. 5). The scanning electron microscopy photographs of the regenerated cellulose films showed that the surface before degradation was very smooth (Fig. 6a, b). However, the occurrence of corrosive structure was observed and many fine visible holes began to occur on the surface after 12 h of enzymatic hydrolysis with the crude enzyme from the LW03 strain (Fig. 6c, d). Meanwhile, the smooth region gradually disappeared. However, the surface treatment with the commercial cellulase was still smooth as well as that before degradation (Fig. 6e, f).

Enzyme activities

The LW03 strain produced an extracellular hydrolase system with predominantly glucoamylase activity (129.096 U/ml) (Table 1). The crude enzyme extracted from the LW03 strain also displayed relatively high acid proteinase (40.392 U/ml), phytase (7.384 U/ml) and pectinase (3.696 U/ml) activity. β-glucosidase (0.286 U/ml), endoglucanase (0.158 U/ml), cellulase (0.078 U/ml), xylanase (0.045 U/ml), α-amylase (0.044 U/ml), β-glucanase (0.032 U/ml), as well as exoglucanase (0.004 U/ml) activity were also observed. About its ability to degrade regenerated cellulose films, the extremely low activities of cellulase and exoglucanase make this crude enzyme, extracted from the LW03 strain, unusual. Based on initial evidence, this crude enzyme is most likely to contain some new constituents capable of degrading regenerated cellulose films.

Discussion

Filamentous fungi are by far the main sources of a great diversity of industrial enzymes. In this study, the LW03 strain grew optimally at 37 °C and could produce multiple extracellular hydrolases (Table 1). Although further study is needed, these results indicate that it may be possible to exploit its enzymes such as glucoamylase and acid proteinase for a variety of industrial applications.

Over the years, the majority of investigations had demonstrated that efficient degradation of native cellulose would generally require a complete set of enzymes, including three major types of activity: endoglucanase, exoglucanase and β-glucosidase (Limam et al. 1995; Zyani et al. 2009; Lynd et al. 2002; Medie et al. 2012; Sukharnikov et al. 2012; Zhang and Lynd 2006). Many studies have shown that the mechanism of enzymatic cellulose degradation is extremely complex (Ahola et al. 2008; Fierobe et al. 2002; Gunnars et al. 2002; Mohan et al. 2013; Wilson 2009). The properties of the cellulose substrate (crystallinity, morphology, degree of polymerization, etc.) markedly influenced the dynamics of enzymatic degradation (Jeoh et al. 2006; Himmel et al. 2007; Turon et al. 2008). The component of cellulase is also a very important factor for cellulose degradation. Each component in cellulase systems plays a different role (Ahola et al. 2008; Cheng et al. 2011; Mohan et al. 2013; Josefsson et al. 2008; Wilson 2009). For example, Josefsson et al. (2008) demonstrated that EGs produced swelling and new end groups in the cellulose substrate. By way of contrast, CBHs degraded the cellulose films rather fast.

It is worth noting that the enzymatic hydrolysis of various cellulose films in the above investigations was generally caused by the action of cellulase enzymes at temperatures of 20–40 °C. In this study, the LW03 strain is an interesting strain

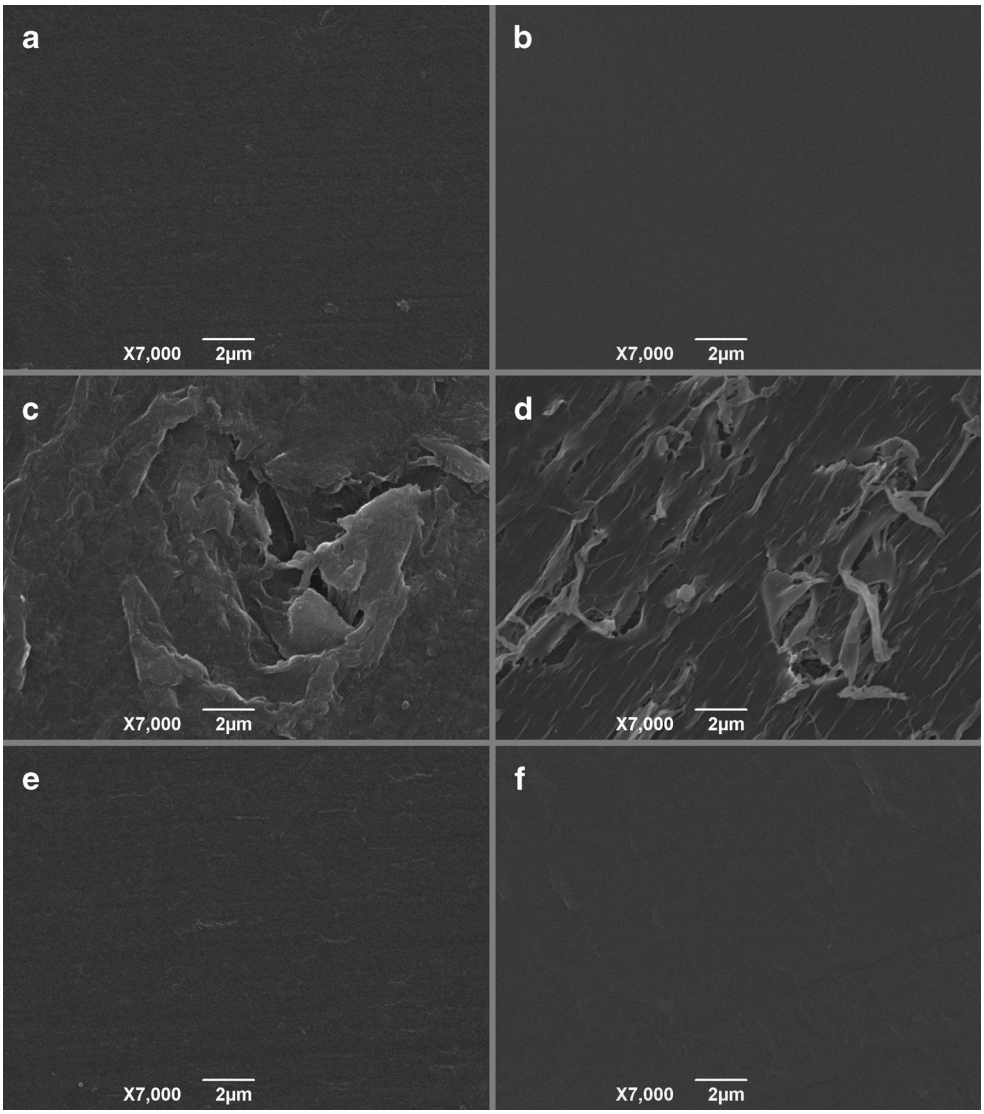

Fig. 6 Scanning electron microscopic images of regenerated cellulose films including cellophane and dialysis tubing. **a** cellophane surfaces before degradation; **b** dialysis tubing surfaces before degradation; **c** cellophane surfaces after degradation at 4 °C by the crude enzyme from the LW03 strain; **d** dialysis tubing surfaces after degradation at 4 °C by the crude enzyme from the LW03 strain; **e** cellophane surfaces after treatment with the commercial cellulase enzymes at 4 °C; **f** dialysis tubing surfaces after treatment with the commercial cellulase enzyme at 4 °C

that produces extremely low activities of cellulase, endoglucanase, exoglucanase and β-glucosidase. However, certain enzymes secreted by it could degrade commercial regenerated cellulose films (cellophane and dialysis tubing) drastically with a short time and a wide temperature range of 4–30 °C. More interestingly, in our case, the commercial cellulase ("Onozuka" R-10) extract from *Trichoderma* cannot degrade the cellulose films at 4 °C in comparison to the crude enzyme extracted from the LW03 strain. The cellulase enzymes can completely or mostly degrade the different types of cellulose films at room or high temperature (Ahola et al. 2008; Turon et al. 2008; Eriksson et al. 2005; Mohan et al. 2013; Cheng et al 2011; Kargl et al. 2013), but the knowledge about the degradation of cellulose films at low temperature is still unknown. The property of degrading cellulose films at low temperature for the LW03 strain enzyme, to our knowledge, has not been reported for any other *Rhizopus* strain or any other mesophilic fungal. Further studies are recommended with the aim of purifying the cellulose films that degrade enzymes and investigating their properties and special applications.

Table 1 Enzymatic activities of several extracellular hydrolases extracted from the LW03 strain

Enzyme	Activity (U/ml)
α-Amylase	0.044±0.004
Glucoamylase	129.096±18.487
Exoglucanase	0.004±0.000
Endoglucanase	0.158±0.005
β-Glucosidase	0.286±0.040
Cellulase	0.078±0.003
Acid proteinase	40.392±0.448
Pectinase	3.696±1.601
Xylanase	0.045±0.005
β-Glucanase	0.032±0.005
Phytase	7.384±0.085

Values are shown as mean values±SD ($n=3$)

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