Journal of Microbiology (2013) Vol. 51, No. 1, pp. 82–87
DOI 10.1007/s12275-013-2370-z

Identification of *Petriella setifera* LH and Characterization of Its Crude Carboxymethyl Cellulase for Application in Denim Biostoning

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(Received July 19, 2012 / Accepted October 3, 2012)

The phylogenetic tree of the partial elongation factor-1 alpha gene fits better than the partial 18S rDNA for generic classification. From the results of the molecular tree and analysis of morphological characters, *Petriella setifera* LH was identified. It can be induced to produce carboxymethyl cellulase (CMCase). The crude CMCase only shows a 44.1-kDa band by activity staining after SDS-PAGE. It is optimally active at 55°C and pH 6.0, and is stable from pH 5.0–8.0 and at 45°C or below. The crude CMCase, which is not affected by Co^{2+}, is strongly activated in the presence of 10 mM Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, EDTA, and Mn^{2+}. It is strongly inhibited by 10 mM Fe^{2+}, Pb^{2+}, Al^{3+}, Zn^{2+}, Ag^{+}, Fe^{3+}, and Cu^{2+}. When compared with denim treatment by Novoprime A800 (a commercial neutral cellulase), crude CMCase exhibits a similar fabric weight loss and indigo dye removal. These results indicate that crude CMCase has potential application in denim biostoning.

***Keywords*:** neutral carboxymethyl cellulase, *Petriella setifera* LH, biostoning

Introduction

Cellulase, consisting of endo-1,4-β-D-glucanase or carboxymethyl cellulase (CMCase, EC 3.2.1.4), exo-1,4-β-D-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21), is a multicomponent enzyme system capable of cleaving the β-1,4 linkages of cellulose. Three types of cellulase act synergistically to completely decompose crystalline cellulose (Murashima *et al*., 2002a). Cellulases are usually divided according to their optimal pH into acidic cellulases (optimum pH 3.0–5.0), neutral cellulases (optimum pH 6.0–8.0), and alkaline cellulases (optimum pH 8.0–11.0) (Miettinen-Oinonen *et al*., 2004).

Cellulases have broad industrial application in: medicine, pulp and paper industries, detergent industry, and wastewater treatment (Mou *et al*., 1991; Schülein, 2000; Xie *et al*., 2010). In recent years, special attention has been given to the application of biotechnology in the textile industries (Chen *et al*., 2007). Neutral crude carboxymethyl cellulases (CMCases) work especially well in biostoning; they exhibit lower backstaining and less reduction in fabric strength compared with acidic CMCases (Klahorst *et al*., 1994; Campos *et al*., 2002; Hirvonen and Papageorgiou, 2003; Miettinen-Oinonen *et al*., 2004). Although some acidic cellulases (including some genetically modified products used for biostoning denim) have been produced, neutral CMCases have not yet been commercialized in China (Chen *et al*., 2007). Therefore, studies to find high-quality neutral CMCases suitable for denim biostoning are highly desirable.

CMCases have been found in fungi, bacteria, plants, animals, and actinomycetes. CMCases from some fungi such as *Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Humicola* sp., and so on, have been characterized (Murashima *et al*., 2002b). But, to the best of our knowledge, CMCases from many other fungi have not been characterized, especially from the Microascaceae.

The elongation factor-1 alpha (*EF-1α*) is a key protein in the translational elongation process, and it does not hold internal repeated sequences and other obvious features that would complicate phylogenetic analyses. *EF-1α* gene sequences have been successfully applied in generic classification by phylogenetic tree construction (Cho *et al*., 1995; Rehner and Buckley, 2005). In the present study, *Petriella setifera* LH, which can produce neutral CMCases, was identified by phylogenetic analysis of partial *EF-1α* sequences along with observation of morphological characteristics. The crude CMCases from *P. setifera* LH were characterized, and denim biostoning using the crude CMCase was investigated.

Materials and Methods

Organism

A 1-g soil sample from Mount Sanqingshan in Jiangxi Province, China, was suspended in a 250-ml Erlenmeyer flask with 20 ml 0.90% (w/v) of NaCl solution rotating at 200 rpm for 10 min, and diluted in distilled water up to 10^{-4}. Then, 100 μl of suspension were spread onto a screening medium (pH 6.0) containing 1% filter paper powder, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% NaCl, 0.05% KCl, 0.05% $MgSO_4$, 0.03% $CaCl_2$, 0.1% Mandel salt solution (v/v), and 1.8% agar at 28°C for 7 days. The screened culture was isolated and preserved in PDA medium at 4°C.

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Production of the crude CMCase

The isolate was grown at pH 8.0 for 3–4 days, rotating at 200 rpm in a 500-ml Erlenmeyer flask containing 200 ml of the following medium: 2.0% corn stalk powder, 2.0% bran powder, 1.5% microcrystalline cellulose, 0.5% peptone, 0.5% yeast extract, 0.5% corncob powder, 0.2% $(NH_4)_2SO_4$ and 0.05% KH_2PO_4.

Concentration of the crude CMCase

The broth was centrifuged at 10,000×*g* for 15 min at 4°C. Proteins from a 65-ml volume of supernatant were precipitated by adding $(NH_4)_2SO_4$ to a final concentration of 60% (w/v) and were left overnight at 4°C. Precipitated proteins, which were dried at 25°C after centrifugation at 10,000×*g* for 30 min at 4°C, were dissolved in 10 ml of double-distilled H_2O and then were desalinated using a 5-kDa exclusion dialysis bag immersed in 500 ml double-distilled H_2O, renewed every 12 h (total for 48 h) at 4°C. The desalinated protein solution was reduced to approximately 1 ml by Millipore 3,000 NMWL at 4,000×*g* for 10 h at 4°C. The desalinated crude enzymes were used for characterization and molecular weight determination.

Activity staining on SDS-PAGE

After the desalinated crude enzymes were boiled for 5 min, the denatured enzymes were separated using an SDS-PAGE gel containing 0.15% CMC. The SDS-PAGE gel was soaked in solution A (50 mM sodium phosphate buffer, pH 6.0) containing 25% isopropanol for 2 h to renature the denatured enzymes, and subsequently in solution B (50 mM sodium phosphate buffer, pH 6.0) for 30 min at 50°C (Coral *et al.*, 2002) to allow the enzymes to react with CMC. Then the gel was stained with 1% Congo Red solution for 20 min and destained with 1 M NaCl until yellow bands appeared. The molecular weights of the cellulases were determined using SDS-PAGE gels stained with Coomassie Brilliant blue and Congo Red.

Assay of the crude CMCase activity

The crude CMCase activity was determined using the dinitrosalicylic acid (DNS) method in the following way: 50 µl the diluted crude CMCase and 450 µl 1.5% CMC were incubated with 50 mM sodium phosphate buffer (pH 6.0) at 50°C (Miller *et al.*, 1960). One endoglucanase activity unit (ECU) was defined as the release of 1 µg glucose/min.

Amplification of partial 18S rDNA and the partial *EF-1α* sequences

The genomic DNA from the screened culture was extracted and purified using a Column Fungal DNAout Kit (Tiandz, Inc., China). The partial 18S rDNA was amplified using two primers: 5′-TTAGCATGGAATAATRRAATAGGA-3′ and 5′-ATTGCAATGCYCTATCCCCA-3′ (Borneman and Hartin, 2000). According to the conserved region of *EF-1α* sequences from fungi, we designed two primers: 5′-GGTG AGTTCGAGGCTGGTATC-3′ and 5′-GAGGRGGGTAG TCAGTGAAGGC-3′. PCR was conducted in a final 50-µl volume containing 5 µl 10× pfu reaction buffer, 0.3 µl of 100 ng template genome DNA, 2 µl each of 10 µM primers, 1 µl of 2.5 U pfu DNA Polymerase (Fermentas, Lithuania), 4 µl of 2.5 mM dNTP each, and 25.7 µl filter-sterilized water. The PCR thermal cycling conditions for the two sequences were as follows: initial denaturation of template at 95°C for 4 min followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1.3–2.0 min, and a final extension step at 72°C for 10 min. The two sequences reported in this study have been submitted to GenBank database (Accession nos. JN408257 and JN408258).

Phylogenetic tree construction

The partial *EF-1α* sequences of the screened culture and 14 cultures selected from GenBank were aligned using Clustal X (1.83) (Thompson *et al.*, 1997). The phylogenetic tree was constructed using the neighbor-joining method, and distances were calculated using the Kimura two-parameter (K2P) model for nucleotide substitutions in MEGA 4.0 software (Kimura, 1980; Saitou and Nei, 1987; Tamura and Dudley, 2007), with a standard error calculated from a bootstrap analysis using 1,000 replicates (Felsenstein, 1985).

Morphological observation

The isolate was cultured on solid PDA medium at room temperature for 3 weeks in the dark. During this period, morphological characters (e.g., ascoma, ascus, and ascospores) were identified and observed.

Optimal temperature and enzymatic thermostability

To determine optimal temperature, the crude CMCase was incubated with a mixture of 1.5% CMC and 50 mM sodium phosphate buffer (pH 6.0) for 30 min at different temperatures (30–80°C). To test thermostability, it was preincubated with 50 mM sodium phosphate buffer (pH 6.0) at different temperatures (30–80°C) for 1 h. Then the samples were tested for the residual crude CMCase activity as described above.

Optimal pH and pH stability

To determine optimal pH, the crude CMCase was incubated with the appropriate buffers: 50 mM citric acid buffer (pH 3.0–5.0), 50 mM phosphate buffer (pH 6.0–8.0), and 50 mM NaOH-glycine buffer (pH 9.0–10.0) at 50°C for 30 min. To determine pH stability, the crude CMCase was preincubated at different pH values (pH 3.0–10.0) for 1 h at 40°C. The residual CMCase activity was determined as described above.

Inhibition or activation by cations and chelators

To determine the effects of EDTA and metal ions (i.e., Na^+, K^+, Ag^+, Pb^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Al^{3+}, and Fe^{3+}) on the activity of the crude CMCase, each reagent was added separately to the CMCase–CMC reaction buffer (50 mM sodium phosphate buffer, pH 6.0) at a final concentration of 10 mM. The relative activity in the presence of metal ions was calculated by setting the control value (i.e., no metal ions nor EDTA in the mixture) as 100%. The residual CMCase activity was determined as described above.

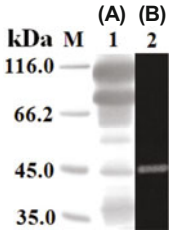

Fig. 1. Molecular weight was determined by SDS-PAGE, with gels stained for protein pattern (A) and activity pattern (B). Lanes: M, Marker; 1, The SDS-PAGE gel stained with Coomassie Brilliant blue; 2, The SDS-PAGE gel activity stained with Congo Red.

Biostoning

Denim cloth was treated with the crude CMCase in a 250-ml Erlenmeyer flask with 10 glass beads (diameter 0.7 cm). At a solid:liquid ratio of 1:10, a 6×8-cm denim swatch, was immersed into 20 ml of 50 mM sodium phosphate buffer (pH 6.0) rotating at 200 rpm at 40°C for 1 h. The dosage of crude CMCase and Novoprime A800 was 540 ECU per g of fabric. The amount of indigo dye released into solution was determined by assaying the absorbance at 370 nm (Wu *et al.*, 2007). Denim dry weights were determined before and after enzymatic treatment.

Statistical analysis

The data are presented as mean±SD. Statistical analysis was conducted using SPSS 11.5 software.

Results and Discussion

Fungal isolation

After 7 days at 28°C, a fungal culture covered an entire plate and completely degraded the filter paper powder. Efficient degradation of cellulase is mainly attributed to synergy of cellulases (Murashima *et al.*, 2002b). In fact, we isolated the fungal culture and found it could readily be induced to produce β-1, 4-exoglucanase, and β-1, 4-glucosidase (data not shown).

Molecular weight of the crude CMCase

The gel stained with 1% Congo Red showed a single clear band (Fig. 1). The molecular weight of the crude CMCase was estimated to be 44.1 kDa by SDS-PAGE, which was obviously different from molecular weights reported in some other studies (Schülein, 1997; Onsori *et al.*, 2005).

Analysis of partial 18S rDNA

The 760 bp partial 18S rDNA from the screened fungus, has a 100% maximum identity with those from *P. setifera* (GenBank accession no. U43908), *Lomentospora prolificans* (GenBank accession nos. U43910 and U43909), and *Graphium tectonae* (GenBank accession no. U43907). *P. setifera* is closely related to *G. tectonae* and *L. prolificans* (synonym: *Scedosporium prolificans*) (Issakinen *et al.*, 1999), and they all belong to the family Microascaceae. Some studies have demonstrated that highly conserved sequences are not phylogenetically informative at lower taxonomic levels (Cho *et al.*, 1995; Rehner and Buckley, 2005), which is in agreement with our finding that the screened fungus could not be distinguished from other cultures for generic classification in the present study.

Phylogenetic tree analysis

The 894 bp partial *EF-1α* sequence from the screened fungus has 95% maximum identity with *P. setifera* (DQ836911). The cultures grouped together into *P. setifera* (Fig. 2). Phylogenetic analysis demonstrated five distinct clades: *Petriella* sp., *Microascus* sp., *Colletotriclum* sp., *Aspergillus* sp., and *Ceratocystis* sp. Bootstrap values of the five clades reached 100%, indicating that the molecular tree of the partial *EF-1α* sequences strongly supports the morphological classification. On the basis of our results, the partial *EF-1α* sequences were more suitable than partial 18S rDNA for the generic classification as described previously by Cho *et al.* (1995). Based on this result, the screened fungus was identified as *Petriella* sp.

Morphological identification

The morphology of the screened culture was almost identical to that of *P. setifera* (Barron *et al.*, 1961) (Fig. 3). Colonies of *Petriella* sp. grew rapidly at room temperature on PDA. The colonies were white when they were young. As they aged they turned a pale grayish and finally a blackish color. Sporotricha were the first structures evident for the conidial stage, and then conidia appeared, usually united to form

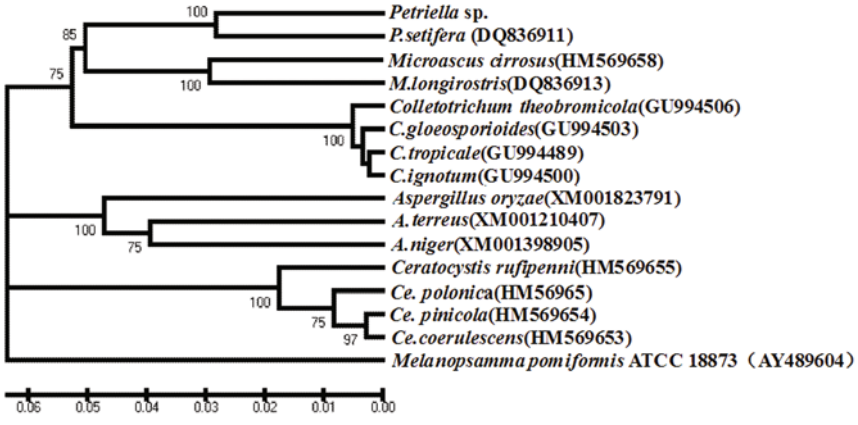

Fig. 2. Phylogenetic tree based on *EF-1α* gene sequences from *Petriella* sp. and other cultures. The tree was constructed by the Neighbor-Joining method of MEGA 4.0. Distances were calculated using the Kimura two-parameter (K2P) model for nucleotide substitutions in MEGA 4.0. The alignment was resampled 1,000 times using the Bootstrap method. Bootstrap percentages >50% were shown above respective branches. The tree was rooted using *Melanopsamma pomiformis* ATCC 18873 (AY489604) as an outgroup.

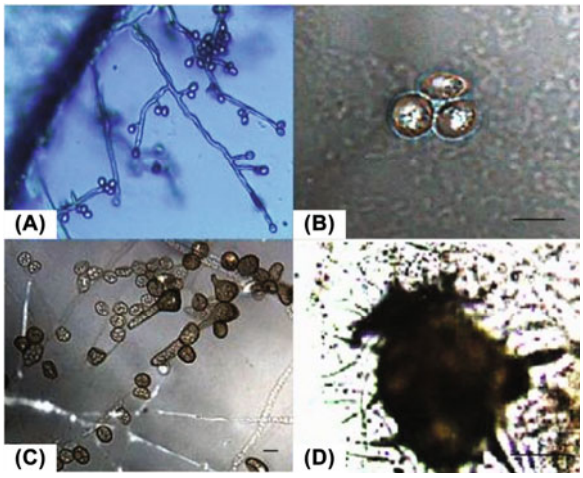

Fig. 3. Morphological characters of *Petriella setifera* LH. (A) The conidial stage Sporotrichum, (B) Ascospores, (C) Asci, (D) Ascoma. Scale bars: B, C=10 μm; D=50 μm.

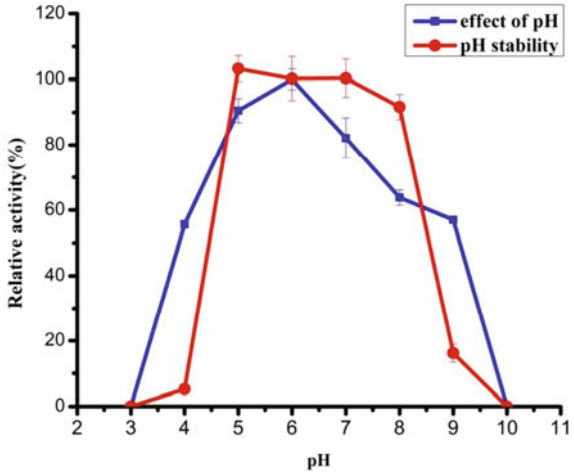

Fig. 5. Effect of pH on the stability and activity of crude CMCase from *P. setifera* LH.

loose balls. Ascospores (7.5–11.5×5–6 μm) were asymmetrically convex and were pointed at the ends. Their walls were red brown in color and thinner at the ends, forming apical germinal pores. The ascospores contained numerous oil droplets, which was a feature distinct from other cultures. The shapes of asci (21–25×12–15 μm) were ovoid to clavate, sometimes like a well-marked foot. Perithecia (75–125 μm) with short necks covered with scattered hairs were membranous, spherical, and pale brown to dark brown. Based on the molecular tree position and the morphological characters, the screened fungus was identified as *P. setifera* and named as *P. setifera* LH.

Thermal stability and effect of temperature on the activity of crude CMCase

Presently, CMCases with a wide range of temperature and pH requirements are in increasing demand for the denim biostoning process, due to their low energy use. In this connection, the crude CMCase from *P. setifera* LH showed promise, as it retained more than 80% activity from 40 to 70°C (Fig. 4). The optimal temperature was 55°C, the same as that of *Rhizopus oryzae* (Murashima *et al.*, 2002b). The optimal temperatures of CMCases from *H. grisea*, *P. occitanis* and some prokaryotic microorganisms are even higher (Mawadza *et al.*, 2000; Huang and Monk, 2004; Chaabouni *et al.*, 2005). However, for the denim biostoning process, a higher optimal temperature is not required.

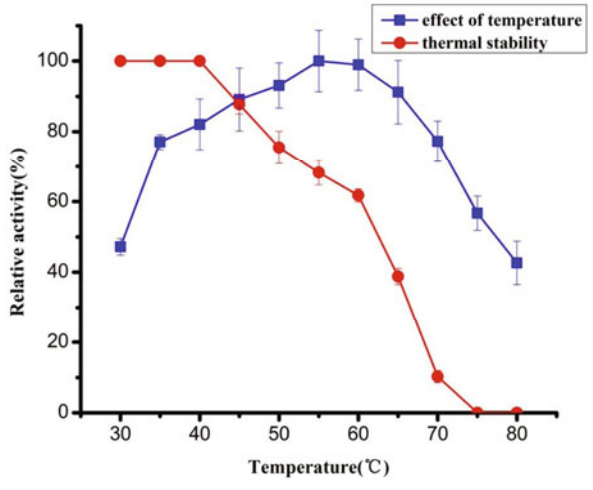

Fig. 4. Effect of temperature on the stability and activity of the crude CMCase from *P. setifera* LH.

For testing thermostability, the crude CMCase was preincubated in 50 mM buffer of different temperatures for 1 h at pH 6.0. The crude CMCase showed 100% relative residual activity between 30°C and 40°C, and it possessed high stability at 45°C, whereas a rapid decrease in stability was observed above 45°C. These results are consistent with the previous observation that enzymes can quickly lose their activity in the absence of substrate (Mawadza *et al.*, 2000).

pH stability and effect of pH on the activity of crude CMCase

The crude CMCase exhibits more than 80% relative activity between pH 5.0 and pH 7.0 (Fig. 5). The activity decreased more slowly above pH 6 than below pH 6.0. More than 50% of maximal activity was retained at pH 4.0 and pH 9.0, but the crude CMCase completely lost activity at pH 3.0 and pH 10.0.

pH stability was assayed by preincubating the crude CMCase in 50 mM buffer of different pH values for 1 h at 40°C. The crude CMCase retained more than 90% relative residual activity between pH 5.0 and pH 8.0, which is appropriate for biostoning. The crude CMCases also displayed rapid decreases in stability above pH 8.0 or below pH 5.0, with less than 20% relative residual activity observed at pH 4.0 and pH 9.0. There was no activity after preincubation at pH 3.0 and pH 10.0.

Effect of various metal ions and chelator on the crude CMCase activity

Ag^{+}, Cu^{2+}, and Fe^{3+} almost completely inhibited the activity

Table 1. Effect of 10 mM of various metal ions and EDTA on crude CMCase activity

Metal ions	Relative activity (%)
None (control)	100
K^+	148.96±3.48[a]
Na^+	119.35±4.83[a]
Ag^+	7±0.21[b]
Ca^{2+}	145.52±3.03[a]
Zn^{2+}	10.10±1.54[a]
Mn^{2+}	200.81±4.56[b]
Co^{2+}	102.30±5.53[a]
Pb^{2+}	24.79±2.52[b]
Fe^{2+}	55.24±3.16[b]
Mg^{2+}	162.10±4.21[a]
Cu^{2+}	4±0.13[a]
Fe^{3+}	5±0.28[a]
Al^{3+}	12.34±2.21[a]
EDTA	161.20±8.29[b]

[a] $p<0.05$, [b] $p<0.01$

of crude CMCase (Table1). Partial inhibition resulted from the presence of some metal ions: Zn^{2+}, Pb^{2+}, Al^{3+}, and Fe^{2+}, but Co^{2+} had no effect on the activity. Some studies have concluded that the heavy metals attack certain groups such as the thiol groups and inhibit CMCase activity (Murashima *et al*., 2002a; Huang and Monk, 2004).

The crude CMCase was strongly activated by Na^+, K^+, Ca^{2+}, Mg^{2+}, and Mn^{2+} with the latter causing the greatest increase in activity. Mn^{2+} enhances the substrate binding affinity of the enzyme, and stabilizes the conformation of the catalytic site (Lee *et al*., 2008). Surprisingly, EDTA strongly stimulated activity, indicating the possibility that EDTA bound an inhibitor present in the crude preparation.

Biostoning

Compared with only buffer treatment (Table 2), crude CMCase increased denim weight loss 133.3% and indigo dye removal 127%. In contrast, corresponding values for weight loss and indigo dye removal by Novoprime A800 treatment were 151.5% and 131.8%, respectively. Denim treated with crude CMCase had a similar weight loss and indigo dye removal compared with the commercial neutral cellulase, indicating that it is an excellent candidate for denim biostoning applications.

The biostoning process aims at giving denim a more uniformly aged appearance. In fact, along with an external mechanical agitation, CMCase is mainly responsible for an effective indigo dye removal and weight loss from denim (Murashima *et al*., 2002a). *P. setifera* LH can be induced to produce mostly CMCase and secrete only a very small amount of exoglucanase and glycosidase (data not shown), which resembles the general proportion of cellulases in the biostoning process. For further research, we suggest that the CMCase gene from *P. setifera* LH be cloned and expressed in systems such as *Thrichoderma reesei* or *P. pastoris* to obtain this CMCase for testing in the denim stoning process.

Table 2. The result of biostoning denim fabric with crude CMCase and Novoprime A800 treatment, respectively

Preparation	Dosage (ECU/g)	Weight loss (%)	A_{370nm}
Contral (Buffer only)	0	0.33±0.15[a]	0.211±0.123[a]
Crude CMCase	540	0.77±0.45[a]	0.479±0.246[b]
Novoprime A800	540	0.83±0.37[b]	0.489±0.227[a]

[a] $p<0.05$, [b] $p<0.01$

Conclusion

Using traditional and molecular classification approaches, we identified *P. setifera* LH. The crude CMCase from *P. setifera* LH exhibited optimum activity at 55°C and pH 6.0, showed broad temperature and pH ranges and meets the conditions needed for a biostoning agent. Denim treatment with crude CMCase gives a weight loss and indigo dye removal similar to that of current commercial neutral cellulases. Therefore, the crude CMCase appears to be appropriate for the textile biostoning industry.

Acknowledgements

This work was supported by National Basic Research Program of China (973, Program NO.2012CB721103), and the National High Technology Research and Development Program of China (863, Program NO.2012AA101806).

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