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Cloning and Characterization of Cellulase and Xylanase Coding Genes from Anaerobic Fungus *Neocallimastix* sp. GMLF1

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ABSTRACT

An anaerobic fungus was isolated from the cattle feces and identified as *Neocallimastix* sp. by using morphological approaches. Cellulase, xylanase, β-xylosidase and β-glucosidase production were assayed from culture supernatants and maximal activities were found 6.99, 10.68, 2.72 and 3.24 U/mL, respectively. Cellulase and xylanase coding genes were isolated by using polymerase chain reaction and expressed in *E. coli*. Nucleotide sequencing showed that the *cel1A* (1367 bp) and *xyn1B* (992 bp) had open reading frames encoding polypeptides of 393 and 259 amino acids, respectively. Cel1A showed highest activity on lichenan and followed by carboxymethyl cellulose but Xyn1B was found to be only active on xylan. The optimal conditions were pH 6.0 for Cel1A and pH 6.5 for Xyn1B and 50°C for both enzymes. The enzymes were stabile at 40-50°C but readily inactivation occurred at 60°C. Cel1A activity was enhanced more than 50% in the presence of 1 mM $MnCl_2$, $CoCl_2$ and dithiothreitol, however the same effect was only recorded for Xyn1B with $MnCl_2$. Application studies showed that Cel1A was found to be active on cereal grains such as barley and oat. Bio-bleaching trials by using Xyn1B reduced the kappa numbers of wheat straw and eucalyptus kraft pulps. © 2010 Friends Science Publishers

Key Words: *Neocallimastix*; Cellulase; Xylanase; Cereal; Kraft pulp

INTRODUCTION

Plant cell walls are the major stored carbon source in the nature and composed of cellulose, hemicellulose and lignin (Thomson, 1993). Cellulose is the most abundant polysaccharide that constitutes 40% of the plant material and followed by hemicellulose (Beguin, 1990). Synergistic action of β-1,4-endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) are required for the degradation of cellulose. Xylan constitutes the major component of hemicellulose and xylan breakdown is catalyzed by the enzymes of β-1,4-endoxylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37).

Plant structural polysaccharides provide an extensive renewable resource, therefore various forms of cellulases and xylanases have attracted the commercial interest in different agricultural and industrial applications such as enzymatic hydrolysis of industrial wastes, animal feed preparation and paper pulp pretreatment (Gilbert & Hazlewood, 1993). Numerous bacteria and fungi such as *Bacillus* (Aygan & Arikan, 2009), *Trichoderma* and *Aspergillus* produce xylanases and cellulases (Ahmed *et al*., 2003; Ahmad *et al*., 2009). Beside of these microorganisms, anaerobic rumen fungus *Neocallimastix* spp. has been reported to produce highly active diverse plant cell wall degrading polysaccharidases (Williams & Orpin, 1987).

In this study, an anaerobic fungus was isolated from the cattle feces and identified as *Neocallimastix* sp. by using morphological criters. Cellulolytic and xylanolytic activities of the *Neocallimastix* sp. GMLF1 were investigated in supernatant fraction of culture. Cellulase and xylanase coding genes of the isolate GMLF1 were cloned into *E. coli*, molecular characterization of the genes was accomplished and enzymatic characterization of cellulase and xylanase enzymes were also determined. Application studies were carried out on cereal grains and kraft pulps by using Cel1A and Xyn1B, respectively.

MATERIALS AND METHODS

Isolation, maintenance and identification of anaerobic fungus: Anaerobic fungus was isolated from the frozen (-20°C) cattle feces and the fungus was maintained in the anaerobic medium, which contained 15% clarified rumen fluid. For isolation and maintenance of fungi, anaerobic medium was prepared according to Orpin (1976) and maintenance anaerobic media contained wheat straw as the sole energy source. The fungal isolate was purified in agar containing roll tubes (Joblin, 1981) and the morphological characterization at genus level was examined under the light

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microscope according to Orpin (1994). Growth media for cellulase and xylanase production contained carboxymethyl cellulose (CMC) and oat spelts xylan, respectively with a concentration of 0.5% (w/v).

Bacterial strain, culture conditions and plasmids: *E. coli* strain EC1000 was used for plasmid construction and the cultures of *E. coli* cells were grown in LB broth (Sambrook *et al*., 2001) at 37°C in a shaking (150 rpm) incubator. pCT (Favorgen Biotech Corp., Taiwan) was used for cloning vector and the transformant *E. coli* strains were selected on LB plates containing 1.5% agar (w/v) and ampicillin with a final concentration of 50 μg/mL.

Molecular biology procedures: Genomic DNA isolation from anaerobic fungal biomass was performed by using Genomic DNA Extraction Kit (Favorgen Biotech. Corp., Taiwan) according to manufacturer's protocol. Cellulase and xylanase genes were amplified from *Neocallimastix* sp. genomic DNA by PCR using the primers, which were designed from *N. patriciarum* celD gene (AF053363; Xue *et al*., 1992; CelF: 5'-AATCCGTGATATTTCATC-3' & CelR: 5'-TTACTTTTTTTATTTGAAAG-3') and *N. frontalis* Xyn11B gene (AY131336; Huang *et al*., 2005; XynF: 5'-ACTGTTGCTAAGGCCCAATG-3' & XynR: 5'-ACCCCATTTACCATCGTCATC-3'). MoFavor Cloning Kit (Favorgen Biotech Corp., Taiwan) was used for cloning the PCR products into pCT vector with T sticky ends according to manufacturer's protocol. Recombinant plasmids were introduced into *E. coli* with the calcium chloride transformation method of Mandel and Higa (1970). Cellulase and xylanase positive *E. coli* strains were selected according to the method of Teather and Wood (1982).

DNA sequencing and computer analysis: Cellulase and xylanase inserts were sequenced on both strands by a commercial company (Iontek, Istanbul, Turkey) using automatic sequencer. The analyzes of the nucleotide and amino acid sequences were performed by Clone Manager 5 and homology search was carried out by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and similarity analysis was conducted by using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Non catalytic peptide domains belonging to carbohydrate binding module (CBM) family 10 derived from the CAZy web-server (http://www.cazy.org/fam/acc_CBM.html) were used for similarity comparison.

Enzyme assays: Cellulase and xylanase activities were determined as described by Miller (1959) using 0.5% (w/v) CMC (Sigma) and oat spelts xylan as the substrates, respectively in 50 mM sodium phosphate buffer. One unit of enzyme activity was defined as 1 μmol of reducing sugar released from the substrate per minute. The effects of pH on the activity were determined at 50°C with the following buffers: 50 mM acetate buffer (pH 3.5 to 5.5), phosphate buffer (pH 6.0 to 7.5) and Tris-HCl buffer (pH 8.0 to 9.0) solutions. The effect of temperature on enzyme activities was determined by assaying the enzymes at temperatures from 30 to 80°C. Thermal stability determination was performed as described before by Akyol *et al*. (2009). The ability of the enzyme to hydrolyze the various substrates was investigated by using oat spelts xylan (0.5%, w/v), *Cetraria islandica* lichenan (0.5%, w/v), avicel (0.5%, w/v), *p*-nitrophenyl-β-D-glucopyranoside (3 mM) and *p*-nitrophenyl-β-D-xylopyronoside (3 mM). Avicel was purchased from Merck and other substrates were obtained from Sigma.

Protein estimation: Protein concentrations in the enzyme preparations were determined by using Favorgen Protein Assay Kit (Favorgen Biotech. Corp., Taiwan) with bovine serum albumin as a standard.

The effects of chemicals: The effects of various ions and reagents on the activities of cloned enzymes were determined by adding the chloride salts of Co^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Hg^{2+}, Sn^{2+}, Ca^{2+}, Ni^{2+}, Cu^{2+} and ethylene diaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), dithiothreitol (DTT) at appropriate amounts to obtain 1 mM and 10 mM final concentrations into the enzyme assay systems. Enzymatic activities were measured at optimum pH and temperatures. The effects of chemicals on Cel1A and Xyn1B activities were compared by analysis of variance using the SPSS V12 statistical package program and a significance level of 0.01 was used.

Enzymatic treatments of cereal grains and kraft pulps: For the application studies of Cel1A and Xyn1B, these enzymes were used in enzymatic treatments of cereal grains and pulp materials, respectively. Barley, oat and wheat grains were ground and each grain was incubated with 0.5, 1.0, 1.5 and 2.5 U/g cereal of Cel1A for 1 h at 40°C. After incubation, cereals were removed by centrifugation at 14500 *g* and the release of reducing sugar was analyzed according to Miller (1959).

The enzymatic pretreatments of kraft pulps by using Xyn1B were performed at 5% pulp consistency in 50 mM sodium phosphate buffer (pH 6.0-6.5). Enzyme dose of 2 U/g pulp was used for all the studies and incubated for 3 h at 40°C. After incubation, pulps were then squeezed to collect the water extracts and then washed extensively with distilled water. Pulp filtrates analyzed for reducing sugar according to Miller (1959). The release of hydrophobic and phenolic compounds was determined by measuring the absorbances of filtrates at 465 and 237 nm, respectively (Gupta *et al*., 2000). The kappa number (T-236 cm-8) of kraft pulp was determined according to the standard methods of Technical Association of the Pulp and Paper Industry (Anonymous, 1992).

Accession numbers: DNA sequences for *cel1A* and *xyn1B* was deposited in the GenBank database under accession numbers HM625672 and EU909695, respectively.

RESULTS AND DISCUSSION

Characterization and enzyme production of *Neocallimastix* sp. GMLF1: Anaerobic fungal isolate GMLF1 was isolated from frozen cattle feces which was

stored for 10 months at –20°C. The isolate had a monocentric reproduction, relatively large sporangia with extensive rhizomycellium and released multiflagellate zoospores abundantly. According to Orpin (1994) the fungal isolate was identified as *Neocallimastix* sp.

Extracellular xylanase, CMCase, β-xylosidase and β-glucosidase production by *Neocallimastix* sp. GMLF1 were investigated. The β-xylosidase and β-glucosidase activities were detected in lower levels than the xylanase and CMCase activities. The highest xylanase and CMCase activity was 10.68 U mL^{-1} and 6.99 U mL^{-1}, respectively after 5 days of growth. However, the maximum β-xylosidase (2.72 U mL^{-1}) and β-glucosidase (3.24 U mL^{-1}) activities were obtained after 7 days of incubation. Gordon and Phillips (1989) reported the maximum levels of the extracellular activities of β-glucosidase, β-xylosidase, CMCase and xylanase in 5-7 day old cultures of *Neocallimastix* sp. LM1.

Cloning and analysis of cellulase and xylanase genes: Cellulase and xylanase encoding genes were amplified from *Neocallimastix* sp. GMLF1 genomic DNA by using CelF-CelR and XynF-XynR primers, respectively. Cellulase and xylanase primers produced ~1.5 and 1 kb long DNA fragments, respectively. The cellulase and xylanase fragments were inserted separately into the pCT vector. The new construction vectors were numbered as pCTC1A and pCTX1B for cellulase and xylanase plasmids, respectively and these plasmids were transformed into *E. coli* EC1000. pCTC1A and pCTX1B were isolated from the recombinant *E. coli* strains and cellulase and xylanase genes were re-amplified from the constructed plasmids. The isolated cellulase and xylanase genes were named as *cel1A* and *xyn1B*, respectively. The complete nucleotide sequences of cel1A and *xyn1B*, which were obtained from pCTC1A and pCTX1B, were determined in both strands. The nucleotide sequences of the *cel1A* and *xyn1B* were 1367 bp and 992 bp in length, respectively they contained complete open reading frames (ORF) encoding polypeptides of 393 and 289 amino acids with predicted molecular masses of 44,827 Da and 31,247 Da. Poly (A) tail was not found at 5' end for both genes. The GC content for the ORFs of *cel1A* and xyn1B were found 37.1% and 45.5%, respectively. The bias in the codon usage was also investigated and there is marked preference for T in the wobble position; 47.21% of cel1A and 43.9% of *xyn1B* codons end in T, whereas only 11.68% (*cel1A*) and 10.7% (*xyn1B*) end in G. The codon utilization of *xyn1B* and *cel1A* is similar to that of other xylanase and cellulase genes in *Neocallimastix* (Black *et al*., 1994; Denman *et al*., 1996).

The deduced amino acid sequences of Xyn1B and Cel1A from *Neocallimastix* sp. GMLF1 were used to search for homologous sequences by using BLAST (http://blast.ncbi.nlm.nih.gov/). Xyn1B contained one catalytic domain belongs to glycosyl hydrolase family 11 and no dockerin domain was observed although a linker sequence was found in the C terminal of the polypeptide.

Fig. 1: Multiple sequence alignment of the NCRPDs of *Neocallimastix* sp. GMLF1 Cel1A with the known NCRPDs belonging to CBM family 10 using the ClustalW. The enzymes included are *Orpinomyces joyonii* cellulase (AAB69348), *Pseudomonas aeroginosa* cellulase (ACX31080), *Teredinibacter turnerae* CelA (ACR12145), *Cellvibrio japonicus* cel5E (ACE83841), *C. japonicus* Xyn11A (CAA88763), *Saccharophagus degredans* Man5n (ABD79328), *N. frontalis* Xyn11A (AAT99015), *Anaeromyces* sp W-98 cellulase (AAQ09258), *Piromyces* sp. E2 ManA (AAP30747). Identical amino acids are indicated by solid black and the gaps (-) are inserted to improve the alignment. Dots and asterisks indicate similar and identical residues, respectively

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ACX31080     -NW-YGT-L  LV--------TQ  -  WSQS IS
ACE83841     QNW-YGT-R  LN--------TT  -  WSQS I-
CAA88763     QNW-WGT-F  LQ--------QT  -  WSRS IS
ACR12145     -NW-YGQGT  LN--------N-T  -  WNQS I-
ABD79328     QNW-YGS-V  LN--------NQA  -  WQQS I-
AAB69348     -WA--TELGFEC SEGNTRVVA DEN K  V GNW GI
AAT99015     KSARITAQG KC SDPNCVVYY DED T  V NDW GC
AAQ09258     SKF--EALG QC KNCNN-VVL DND A  I HEW GI
AAP30747     SWS--EALG QC VSTSD-VYY DND N  V GNW GL
Cel1A        -WS--EKYG EC SPNNTKVVV SDE N  V GNW GI
              *        :  *          .  .* ** **   *
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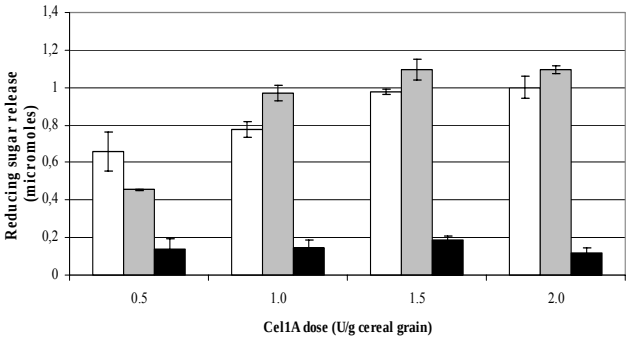
Fig. 2: The effect of different enzyme concentrations on the release of reducing sugar from barley (white bar), oat (gray bar) and wheat (black bar) grains. Grain samples were incubated for 1 h with Cel1A at pH 6.0 and 40°C. Error bars indicate ± SEM (n = 3)



Xyn1B indicated 85% similarity with Xyn11A from *N. frontalis* (Huang *et al*., 2005) and xylanase A from *N. patriciarum* (Gilbert *et al*., 1992). The amino acid sequence of Cel1A was found highly homologous (98%) with the third domain of CelD from *N. patriciarum* (Xue *et al*., 1992) and a cellulase gene from *N. frontalis* (Fujino *et al*., 1998). Analysis of the amino acid sequence revealed that Cel1A consisted of two distinct domains; a catalytic domain belongs to glycosyl hydrolase family 5 and two non catalytic repeated peptide domains (NCRPD). There was a linker sequence separating the catalytic domain from the NCRPD. NCRPDs of Cel1A have 51% homology to each other. Anaerobic fungal NCRPDs have been demonstrated

before in one to three copies at either N terminal (Li *et al*., 1997) or C terminal (Fujino *et al*., 1998) of polypeptide. NCRPDs function as docking domains, which involved in the interaction of polypeptides in cellulosomal complexes (Fanutti *et al*., 1995). The amino acid sequence alignment revealed that the dockerin domain of Cel1A had common amino acid residues with CBM family 10 non catalytic peptide domains (Fig. 1).

Enzyme characteristics: The pH profile of Cel1A and Xyn1B were studied at pH values between 3.5 and 9.0 in three buffer system and optimal pH values were found at 6.0 and 6.5, respectively. There was substantial activity between pH values 5.5 (90%) and 6.5 (78%) for Cel1A and between pH 6.0 (87%) and pH 7.0 (76%) for Xyn1B. The temperature profile revealed that optimum temperature was 50°C for both enzymes but activities decreased rapidly above 50°C. The findings about pH and temperature optimums were correlated with the previous studies. Optimum pH and temperature of rumen fungal xylanases and cellulases have generally found in the range of 5.5-6.5 and 40-50°C, respectively (Li *et al*., 1997; Akyol *et al*., 2009). The enzymes retained more than 95% of their activities after preincubation at 40°C for 2 h in the absence of substrate. Inactivation readily occurred after 5 min of preincubation at 60°C for both enzymes. Xylanase obtained from *N. frontalis* lost its 30% of activity after incubation at 50°C in 1 h (Mountfort & Asher, 1989). Instability is reported for CMCases of rumen fungi in the absence of substrate at the temperature of 50°C (Mountfort & Asher, 1985; Lowe *et al*., 1987).

The substrate specificity of the cellulase encoded by *cel1A* and xylanase encoded by *xyn1B* were investigated by incubating the enzymes with various substrates (Table I). The predominant enzymatic activities of Cel1A and Xyn1B were found to be as cell associated (>95% for Cel1A & 87% for Xyn1B) in *E. coli*. Cel1A exhibited the highest activity on lichenan, a polysaccharide with β-1, 3-1, 4 linkages and followed by CMC. Furthermore detectable reducing sugars were measured after hydrolysis of avicel and xylan by Cel1A. Xyn1B had high activity on xylan but no CMCase or lichenase activities were observed. No detectable hydrolysis was observed with pNP-*β-D*-glucopyranoside and pNP-*β-D*-xylopyranoside. Cel1A and Xyn1B showed high sequence similarity to the CelD from *N. patriciarum* and xylanase A from *N. frontalis*, respectively and similar substrate specificity was also observed with CelD and XylA (Xue *et al*., 1992; Gilbert *et al*., 1992).

Table II shows the effect of various ions and reagents on the activities of enzymes Cel1A and Xyn1B. Mn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+} were enhanced the enzyme activities whereas 10 mM concentration of these ions had negative effects on the Xyn1B ($P<0.01$). However, Xyn1B was found to be more resistant to 10 mM concentrations of Sn^{2+}, Zn^{2+} and Cu^{2+} than Cel1A. Cel1A was strongly inhibited by 10 mM concentrations of Sn^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+}. Complete inhibition was also observed for Xyn1B in the presence of 10 mM Hg^{2+}. Among the tested reagents EDTA stimulated Cel1A and Xyn1B at 1 mM concentration, while 10 mM concentration of EDTA reduced enzyme activities to 87% and 76%, respectively ($P<0.01$). DTT was also found as a stimulator for the enzymes, on the other hand enzyme activity reduced in the presence of SDS ($P<0.01$). Slight stimulation of EDTA was reported before for *Aspergillus* enzymes (Anthony *et al*., 2003). Activation by DTT was reflecting the presence of reduced form of cysteine residues in Cel1A and Xyn1B (Carmona *et al*., 2005).

Table I: Enzymatic activities of Cel1A and Xyn1B on various substrates[a, b]

Substrate	Specific activity (U/mg protein ± SEM)	
	Cel1A	Xyn1B
CMC	2.06 ± 0.07	ND[c]
Xylan	0.37 ± 0.09	8.12 ± 0.16
Lichenan	6.24 ± 0.17	ND
Avicel	0.22 ± 0.01	ND
β-glucosidase	ND	ND
β-xylosidase	ND	ND

[a]Assays were performed at optimum pH and temperature values for each enzyme for 30 min (CMC, xylan, lichenan), 4 h (avicel) and 1 h (p-NP-substrates)
[b]The cell extract of non-recombinant *E. coli* strain was used as control for checking the devoid of enzymatic activity on the substrates and no activity was detected
[c]ND: Not determined

Table II: Effect of metal ions and chemical reagents and their concentrations on the enzymes Cel1A and Xyn1B (% ± SEM)

Chemicals	Relative activity (% ± SEM)			
	Cel1A		Xyn1B	
	1mM	10mM	1 mM	10 mM
Control*	100 ± 1.0	100 ± 1.4	100 ± 2.4	100 ± 0.3
$MnCl_2$	156 ± 2.1	177 ± 2.6	157 ± 1.8	69 ± 3.7
$CoCl_2$	171 ± 1.7	130 ± 2.7	116 ± 1.7	92 ± 2.1
$CaCl_2$	127 ± 2.9	108 ± 2.3	107 ± 1.0	84 ± 2.9
$MgCl_2$	103 ± 3.3	116 ± 3.2	109 ± 0.8	87 ± 1.5
$SnCl_2$	99 ± 2.7	ND	96 ± 1.3	30 ± 1.1
$HgCl_2$	95 ± 1.6	3 ± 0.4	87 ± 2.1	ND
$ZnCl_2$	88 ± 1.7	5 ± 0.8	96 ± 1.5	67 ± 1.0
$NiCl_2$	86 ± 1.3	67 ± 1.7	101 ± 1.0	77 ± 0.2
$CuCl_2$	74 ± 2.6	2 ± 0.6	96 ± 0.5	61 ± 0.7
$BaCl_2$	65 ± 2.2	49 ± 2.6	97 ± 1.1	87 ± 2.2
EDTA	112 ± 3.0	87 ± 2.2	106 ± 2.9	76 ± 1.3
DTT	191 ± 1.4	156 ± 1.5	116 ± 2.6	113 ± 2.0
SDS	35 ± 0.7	35 ± 2.4	36 ± 2.0	19 ± 1.1

*No chemicals were added into the control assay

Enzymatic treatment of cereal grains by using Cel1A: The substrate specificity results indicated the activity of Cel1A was significantly higher towards lichenan, a polysaccharide structurally similar with β-glucan, which occur at different levels in variants of cereals (Genc *et al*., 2001). β-glucans are responsible for the poor nutritive value of these cereals (Annison & Choct, 1991) and the addition of β-glucan degrading enzymes improves the nutritive value of cereals (Wang *et al*., 2005). Accordingly, in order to examine the Cel1A activity on several cereal grains,

Table III: Effects of xylanase treatment on reducing sugar, hydrophobic and phenolic compound release from pulps and kappa numbers of pulps*

Pulp	Reducing sugar released (mg/g pulp)		Absorbance (237 nm)		Absorbance (465 nm)		Kappa Number	
	Control	Enzyme	Control	Enzyme	Control	Enzyme	Control	Enzyme
Wheat	1.57	7.99	0.93	1.68	0.014	0.022	23.05	22.00
Eucalyptus	1.38	3.13	0.38	0.51	0.24	0.25	18.12	17.41

*Standard errors were negligible that's why they were not shown

different concentrations of Cel1A was treated with different cereal grains and the reducing sugar released from cereals was analyzed after treatment. Of the three grains tested, the release of reducing sugar was markedly higher in the barley and oat grains than wheat grain (Fig. 2). Oat and barley have the highest concentrations of β-glucan, whereas low β-glucan content was known in wheat grains (Genc *et al*., 2001). Particularly reducing sugar increased with the increase of enzyme amount however a slight increase in reducing sugar release was observed after incubation with more than 1.5 U.

Bleaching potential of Xyn1B: For estimation of kraft pulp bleaching potential of Xyn1B, wheat straw and eucalyptus kraft pulps treated with cloned enzyme Xyn1B at pH 6 and 40°C for 3 h. Reducing sugar, kappa number, absorbance at 237 and 465 nm values showed variations as shown in Table III. Reducing sugars, hydrophobic (A_{237}) and phenolic (A_{465}) compounds have been released from wheat straw pulp more than eucalyptus pulp after Xyn1B treatment. Xyn1B found to be more effective in biobleaching of wheat straw and 1.05 points reduction was observed in kappa number. Wheat straw pulps pretreated with xylanases of *Thermomyces lanuginosus* and *Bacillus coagulans* reduces the kappa numbers 1.98 and 1.4 points, respectively (Li *et al*., 2005; Chauhan *et al*., 2006). XylA from anaerobic fungus *N. patriciarum* was used in enzymatic pretreatment of spruce kraft pulp at pH 8.0 and 40°C for 16 h and resulted in 1.5 unit decreases in kappa number (Clarke *et al*., 2000). Pretreatment of eucalyptus kraft pulp by Xyn1B resulted less kappa number reduction (0.71 units) than wheat straw pulp. Eucalyptus kraft pulp kappa number reduced 0.2 and 1.5 units after pretreatment by *Aspergillus* xyl I and xyl II, respectively at pH 6.0-6.5 (Sandrim *et al*., 2005). Eucalyptus kraft pulp was efficiently prebleached at higher pH values (8.5-9.5) by *Staphylococcus* sp. SG-13 (Gupta *et al*., 2000) and *Streptomyces* sp. QG-11-3 (Beg *et al*., 2000) xylanases. Incubation times, enzyme concentrations and pulp amounts were important variables in affecting the biobleaching of kraft pulps (Saleem & Akhtar, 2002).

CONCLUSION

Anaerobic ruminal fungus, as the producers of highly active wide range polysaccharidases, *Neocallimastix* is a promising microorganism for obtaining plant cell wall degrading enzyme coding genes. Cloning strategies enables to find applications for rumen fungal enzymes in pulp and paper industry and animal feed industry. Further research will explore full potential of the anaerobic fungal enzymes in several applications such as bio-bleaching of kraft pulp and pre-degradation of animal feeds.

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REFERENCES

Ahmed, S., Q. Ain, N. Aslam, S. Naeem, S. Rahman and A. Jamil, 2003. Induction of xylanase and cellulase genes from *Trichoderma harzianum* with different carbon sources. *Pakistan J. Biol. Sci.*, 22: 1912–1916

Ahmad, Z., M.S. Butt, F.M. Anjum and M. Asgher, 2009. Effect of wheat bran concentration on xylanase biosynthesis by *Aspergillus niger*. *Int. J. Agric. Biol*., 11: 571–576

Akyol, I., U. Comlekcioglu, B. Kar, M.S. Ekinci and E. Ozkose, 2009. Cloning of a xylanase gene *xyn2A* from rumen fungus *Neocallimastix* sp. GMLF2 in *E. coli* and its partial characterization. *Biologia*, 64: 664–670

Annison, G. and M. Choct, 1991. Anti-nutritive activities of cereal non-starch polysaccharides in broiler diets and strategies minimizing their effects. *World's Poult. Sci. J.*, 47: 232–242

Anonymous, 1992. *TAPPI Test Methods*. Atlanta, USA: TAPPI Press

Anthony, T., K.C. Raj, A. Rajendra and P. Gunasekaran, 2003. High molecular weight cellulase-free xylanase from alkali-tolerant *Aspergillus fumigatus* AR1. *Enz. Microbial Technol.*, 32: 647–654

Aygan, A. and B. Arikan, 2009. Production and characterization of multifunctional endoxylanase by *Bacillus* sp. X13. *Turkish J. Biol.*, 33: 231–237

Beg, Q.K., B. Bhushan, M. Kapoor and G.S. Hoondal, 2000. Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG-11-3 and its application in biobleaching of eucalyptus kraft pulp. *Enz. Microbial Technol.*, 27: 459–466

Beguin, P., 1990. Molecular biology of cellulose degradation. *Annul. Rev. Microbiol.*, 44: 219–248

Black, G.W., G.P. Hazlewood, G.P. Xue, C.G. Orpin and H.J. Gilbert, 1994. Xylanase B from *Neocallimastix patriciarum* contains a non-catalytic 455-residue linker sequence comprised of 57 repeats of an octapeptide. *Biochem. J.*, 299: 381–387

Carmona, E.C., M.B. Fialho, E.B. Buchgnani, G.D. Coelho, M.R. Brocheto-Braga and J.A. Jorge, 2005. Production, purification and characterization of a minor form of xylanase from *Aspergillus versicolor*. *Proc. Biochem.*, 40: 359–364

Chauhan, S., B. Choudhury, S.N. Singh and P. Ghosh, 2006. Application of xylanase enzyme of *Bacillus coagulans* as a prebleaching agent on non-woody pulps. *Proc. Biochem.*, 41: 226–231

Clarke, J.H., K. Davidson, J.E. Rixon, J.R. Halstead, M.P. Fransen, H.J. Gilbert and G.P. Hazlewood, 2000. A comparison of enzyme-aided bleaching of softwood paper pulp using combinations of xylanase, mannanase and α-galactosidase. *Appl. Microbiol. Biotechnol.*, 53: 661–667

Sandrim, V.C., A.C.S. Rizzatti, H.F. Terenzi, J.A.Jorge, A.M.F. Milagres and M.L.T.M. Polizeli, 2005. Purification and biochemical characterization of two xylanases produced by *Aspergillus caespitosus* and their potential for kraft pulp bleaching. *Process Biochem.*, 40: 1823–1828

Denman S., G.P. Xue and B. Patel, 1996. Characterization of a *Neocallimastix patriciarum* cellulase cDNA (*celA*) homologous to *Trichoderma reesei* cellobiohydrolase II. *Appl. Environ. Microbiol.*, 62: 1889–1896

Fanutti, C., T. Pony, G. Black, G.P. Hazlewood and H.J. Gilbert, 1995. The conserved non-catalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *J. Biol. Chem.*, 270: 29314–29322

Fujino, Y., K. Ogata, T. Nagamine and K. Ushida, 1998. Cloning, sequencing and expression of an endoglucanase gene from the rumen anaerobic fungus *Neocallimastix frontalis* MCH3. *Biosci. Biotechnol. Biochem.*, 62: 1795–1798

Genc, H., M. Ozdemir and A. Demirbas, 2001. Analysis of mixed-linked (1-3),(1-4)-β-D-glucans in cereal grains from Turkey. *Food Chem.*, 73: 221–224

Gilbert, H.J., G.P. Hazlewood, J.I. Laurie, C.G. Orpin and G.P. Xue, 1992. Homologous catalytic domains in a rumen fungal xylanase: evidence for gene duplication and prokaryotic origin. *Mol. Microbiol.*, 6: 2065–2072

Gilbert, H.J. and G.P. Hazlewood, 1993. Bacterial cellulases and xylanases. *J. Gen. Microbiol.*, 139: 187–194

Gordon, G.L.R. and M.W. Phillips, 1989. Degradation and utilization of cellulose and straw by three different anaerobic fungi from the ovine nunen. *Appl. Environ. Microbiol.*, 55: 1703–1710

Gupta, S., B. Bhushan and G.S. Hoondal, 2000. Isolation, purification and characterization of xylanase from *Staphylococcus* sp. SG-13 and its application in biobleaching of kraft pulp. *J. Appl. Microbiol.*, 88: 325–334

Huang, Y.H., C.T. Huang and R.S. Hseu, 2005. Effects of dockerin domains on *Neocallimastix frontalis* xylanases. *FEMS Microbiol. Lett.*, 243: 455–460

Joblın, K.N., 1981. Isolation, enumeration and maintenance of rumen anaerobic fungi in roll tubes. *Appl. Environ. Microbiol.*, 42: 1119–1122

Li, X.L., H. Chen and L.G. Ljungdahl, 1997. Two cellulases, CelA and CelC, from the polycentric anaerobic fungus *Orpinomyces* strain PC-2 contain N-terminal docking domains for a cellulose - hemicellulase complex. *Appl. Environ. Microbiol.*, 63: 4721–4728

Li, X.T., Z.Q. Jiang, L.T. Li, S.Q. Yang, W.Y. Feng, J.Y. Fan and I. Kusakabe, 2005. Characterization of a cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp. *Biores. Technol.*, 96: 1370–1379

Lowe, S.E., M.K. Theodorou and A.P.J. Trinci, 1987. Cellulases and xylanases of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose and xylan. *Appl. Environ. Microbiol.*, 53: 1216–1223

Mandel, M. and A. Higa, 1970. Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.*, 53: 159–162

Miller, G.L., 1959. Use of dinitrosalicylic acid regent for determination of reducing sugars. *Anal. Chem.*, 31: 426–428

Mountfort, D.O. and R.A. Asher, 1985. Production and regulation of cellulose by two strains of the rumen anaerobic fungus *Neocallimastix frontalis*. *Appl. Environ. Microbiol.*, 49: 1314–1322

Mountfort, D.O. and R.A. Asher, 1989. Production of xylanase by the ruminal anaerobic fungus *Neocallimastix frontalis*. *Appl. Environ. Microbiol.*, 55: 1016–1022

Orpin, C.G., 1976. Studies on the rumen flagellate *Sphaeromonas communis*. *J. Gen. Microbiol.*, 94: 270–280

Orpin, C.G., 1994. Anaerobic fungi: taxonomy, biology and distribution in nature. *In*: Orpin, C.G. and D.O. Mountfort (eds.), *The Anaerobic Fungi*, pp: 1–47. Marcel Dekker, New York

Saleem, M. and M.S. Akhtar, 2002. Biobleaching of kraft pulp by xylanase produced by Bacillus subtilis. *Int. J. Agric. Biol.*, 4: 242–244

Sambrook, J., E.F. Fritsch and T. Maniatis, 2001. *Molecular Cloning, a Laboratory Manual*, 2nd edition. C. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Teather, R.M. and P.J. Wood, 1982. Use of Congo red–polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.*, 43: 777–780

Thomson, J.A., 1993. Molecular biology of xylan degradation. *FEMS Microbiol. Rev.*, 104: 65–82

Wang, Z.R., S.Y. Qiao, W.Q. Lu and D.F. Li, 2005. Effects of enzyme supplementation on performance, nutrient digestibility, gastrointestinal morphology and volatile fatty acid profiles in the hindgut of broilers fed wheat-based diets. *Poult. Sci.*, 84: 875–881

Williams, A.G. and C.G. Orpin, 1987. Polysaccharide-degrading enzymes formed by three species of anaerobic rumen fungi on a range of carbonhydrate substrates. *Canadian J. Microbiol.*, 33: 418–426

Xue, G.P., K.S. Gobius and C.G. Orpin, 1992. A novel polysaccharide hydrolase cDNA (*celD*) from *Neocallimastix patriciarum* encoding three multi-functional catalytic domains with high endoglucanase, cellobiohydrolase and xylanase activities. *J. Gen. Microbiol.*, 138: 2397–2403

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