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# Cloning of a xylanase gene *xyn2A* from rumen fungus *Neocallimastix* sp. GMLF2 in *Escherichia coli* and its partial characterization

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**Abstract:** Anaerobic fungi belonging to the family Neocallimastigaceae are native inhabitants in the rumen of the most herbivores, such as cattle, sheep and goats. A member of this unique group, *Neocallimastix* sp. GMLF2 was isolated from cattle feces and screened for its xylanase encoding gene using polymerase chain reaction. The gene coding for a xylanase (*xyn2A*) was cloned in *Escherichia coli* and expression was monitored. To determine the enzyme activity, assays were conducted for both fungal xylanase and cloned xylanase (Xyl2A) for supernatant and cell-associated activities. Optimum pH and temperature of the enzyme were found to be 6.5 and 50 °C, respectively. The enzyme was stable at 40 °C and 50 °C for 20 min but lost most of its activity when temperature reached 60 °C for 5-min incubation time. Rumen fungal xylanase was mainly released to the supernatant of culture, while cloned xylanase activity was found as cell-associated. Multiple alignment of the amino acid sequences of Xyl2A with published xylanases from various organisms suggested that Xyl2A belongs to glycoside hydrolase family 11.

**Key words:** Neocallimastigaceae; *Neocallimastix*; xylanase; gene expression; rumen; *E. coli*.

**Abbreviations:** CTAB, N-cetyl-N,N,N-trimethyl ammonium bromide; GH, glycoside hydrolase; LB, Lysogeny broth; ORF, open reading frame; PCR, polymerase chain reaction.

## Introduction

Plant cell wall is the major carbon source in nature and it is composed mainly from cellulose, hemicellulose, pectin and lignin (Thomson 1993). Hemicelluloses are the second abundant polysaccharide after cellulose in the world (Wong et al. 1988) and xylan is the basic polymeric compound of hemicellulose. Xylans possess a backbone consisting of $\beta$-1,4-linked xylose units with varying degrees of polymerization (Hespell & Whitehead 1990). Their structures are relatively more heterogeneous compared with cellulose. Because of this complex structure, complete degradation of xylan requires the synergistic actions of more than one enzyme to generate xylose (Biely 1985). Degradation of xylan by ruminant herbivores is mainly occurred in the rumen by the synergistic activity of the fibrolytic gut microorganisms, such as protozoa, bacteria and fungi which have symbiotic association with the host animals (for a review, see Orpin & Joblin 1997).

In these habitats rumen microbes, in particular anaerobic fungi, play an important role in the physical and chemical breakdown of ingested plant cell walls by means of their rhizoidal systems and powerful arrays of glycoside hydrolases (GH). This unique group of fungi secretes various plant biomass degrading enzymes, such as cellulase (Barichievich & Calza 1990), hemicellulase (Lowe et al. 1987), amylase (Mountfort & Asher 1989) and protease (Wallace & Joblin 1985). These enzymes are either localized on thalli (Lowe et al. 1987), or are released into the culture medium (Williams & Orpin 1987). Xylanase is one of the most active enzymes among the endo-acting polysaccharide hydrolases studied from the *Neocallimastix frontalis* and activity of this enzyme mainly resulted with the releasing of xylobiose as end-product (Mountfort & Asher 1989). Conversion of xylose from the xylooligosaccharides had also been reported for the isolates of the genus *Neocallimastix* (Pearce & Bauchop 1985).

Remarkably high enzymatic activity of rumen fungi was received great impetus by the researchers to express and characterize the genes encoding hemicellulolytic enzymes in *Escherichia coli*. Xylanase genes of *xynA* (Gilbert et al. 1992), *xynB* (Black et al. 1994), *xyn3* (Durand et al. 1996), *xynC* (Liu et al. 1999), *xyn11A* and *xyn11B* (Huang et al. 2005) from *Neocallimastix* sp. were studied extensively.

In current study, a fungal isolate *Neocallimastix* GMLF2, isolated from freshly collected fecal sample of cattle (purified culture has been deposited into the cul-

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ture collection of Biotechnology and Gene Engineering Laboratory of Kahramanmaras Sutcu Imam University, Bahcelievler, Kahramanmaras, Turkey), was used to obtain a xylanase gene. The gene encoding xylanase (proposed as *xyn2A*) was extracted from the isolate GMLF2 and cloned into *E. coli*. This enzyme was characterized for its pH and temperature optima, thermal stability, and substrate specificity, too. Xylanase activity of the fungal isolate GMLF2 was also studied and compared with activity of the cloned gene product *xyn2A*.

## Material and methods

*Strains and culture media*

*Neocallimastix* sp. GMLF2 was isolated from freshly collected cattle feces. The anaerobic medium contains 15% clarified rumen fluid and wheat straw as sole energy source and stock culture of the isolate has been kept in our fungal culture collection. For isolation and maintenance of fungi, anaerobic medium was prepared according to Orpin (1976) and dispensed into Hungate tubes under strictly anaerobic conditions (Theodorou et al. 1996). Purification of the fungal isolate was carried out in agar containing roll tubes as described by Joblin (1981) and partial characterization at genus level was conducted according to morphological data as seen under light microscope (Ho & Barr 1995). Growth media for enzyme production contained oat spelt xylan with a concentration of 0.5% (w/v). To obtain relatively higher amount of cell for DNA extraction, glucose (0.5% w/v) was used as sole energy source in anaerobic medium (Orpin 1976). *E. coli* EC1000 strain was used for plasmid constructions, and the cultures of *E. coli* cells were maintained in Lysogeny broth (LB) – Lennox medium and incubated at 37 °C in a gently shaking (150 rpm) incubator.

*Genomic DNA extraction*

Fungal isolate was grown on glucose-containing anaerobic medium for 2 days and the fungal biomass was harvested by centrifugation at $1250\times g$ for 10 min. Approximately 10 mg of biomass was frozen using liquid nitrogen and immediately broken down by Mixer Mill (Retsch MM301). DNA extraction was carried out using N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB) method of Doyle & Doyle (1987) with slight modifications as described earlier (Ozkose 2001). The DNA samples were dissolved in 50 µL TE (Tris EDTA, pH 8.0) buffer and stored in −20 °C for further analysis.

*Amplification and cloning of xylanase gene*

The xylanase encoding gene was amplified from genomic DNA of *Neocallimastix* sp. GMLF2 with the aid of polymerase chain reaction (PCR) using the forward 5'-TTGAAGAACCAACTACCG-3' and reverse 5'-CAGCAA CAATACCAC-3' primers. The primers were designed using published data of anaerobic fungal xylanase sequences derived from National Center for Biotechnology Information web-server (http://www.ncbi.nlm.nih.gov/). MoFavor Cloning Kit (Favorgen Biotech Corp., Taiwan) was used for cloning the PCR product into pCT vector (Favorgen Biotech Corp., Taiwan) with T sticky ends according to manufacturer's protocol and constructed vector was named as pCTX2A. The constructed plasmids were transformed into *E. coli* by $CaCl_2$ method (Mandel & Higa 1970) to express the xylanase protein. Transformed *E. coli* cells were plated on ampicillin (50 µg/mL) containing LB-Lennox agar plates. Recombinant strains were screened for xylanase activity by overlaying of 0.4% (w/v) xylan and 0.4% (w/v) agarose in 25 mM potassium phosphate buffer (pH 6.5). After incubation at 37 °C for 4 hours, xylanase activity was examined with the aid of Congo red staining. The colonies surrounded by yellow halo indicated the xylanase activity of the clones. Xylanase activity-positive strains were selected and stored in 15% (v/v) glycerol at −20 °C until required.

*Sequencing and alignment procedures*

The resultant plasmid, pCTX2A, was extracted using Plasmid DNA Extraction Kit (Favorgen Biotech Corp., Taiwan) according to manufacturer's protocol and xylanase gene (*xyn2A*) insert was sequenced on both strands by a commercial company using automatic sequencer (Iontek, Istanbul, Turkey). Sequence alignment and data analysis were performed using the computer programs ChromasPro V1.34 and Clone Manager 5, respectively, whilst similarity analysis was conducted using CLUSTAL W (http://www.ebi.ac.uk/clustalw/; Thompson et al. 1994). Published data of various xylanases: *Aspergillus fumigatus* (GenBank Accession No.: ABA40419), *Penicillium* sp. 40 (BAA88421), *Hypocrea jecorina* (AAB29346), *Trichoderma viride* (CAB60757), *Fusarium oxysporum* (AAK27974), *Streptomyces* sp. S38 (CAA67143), *Bacillus subtilis* (AAZ17392), *Paenibacillus* sp. DG-22 (ABI96991), *Clostridium saccharobutylicum* (AAA23287), *Ruminococcus albus* (AAA85198), *Neocallimastix frontalis* (AAN07082), *Piromyces communis* (ABY52795), *Orpinomyces* sp. PC-2 (AAD04194) derived from the CAZy web-server (http://www.cazy.org/; Cantarel et al. 2009) were used for similarity comparison.

*Enzyme assays*

The methodology used for determination of xylanase activity was based on the technique described by Miller (1959) using either 0.5% (w/v) oat-spelts or 0.5% (w/v) birchwood xylan as sole substrate in 25 mM potassium phosphate buffer. Reducing sugar, resulted from the enzymatic reaction, was screened by measuring the absorbance at the wavelength of 540 nm by a micro-plate reader (Spectromax 384 Plus, UK) using xylose as standard. One unit of xylanase activity was defined as the quantity of the enzyme required to release 1 µmol of reducing sugar per min under assay conditions. All standard assay procedures were conducted in triplicate and repeated twice.

*Determination of pH, temperature and thermostability characteristics*

The effect of pH on xylanase activity was assayed for different pH values ranging from 3.5 to 9.0 with 0.5 unit increments by preparing the substrate in 50 mM acetate buffer (for pH 3.5–5.5), phosphate buffer (for pH 6.0–7.5) and Tris-HCl buffer (for pH 8.0–9.0) solutions. Optimum temperature was determined as the enzymes were incubated with substrate at different temperatures ranging between 30 and 80 °C with 10 °C increments. For determining thermal stability, the crude enzyme extract was pre-incubated at different temperatures: 40, 50, 60 and 70 °C for 5, 10, 15 and 20 min (for each temperature figure) without addition of substrate. Test tubes containing crude enzyme samples were taken after incubation at various temperatures (40, 50, 60 and 70 °C) and immediately cooled on ice for 2 min. The activities were then measured with the addition of relevant substrates using standard assay procedure. All enzymatic measurements were performed by carrying out the standard assay procedures as described above.

*Sequence data*
The sequence of the *xyn2A* gene reported in this paper has been deposited with the GenBank (Benson et al. 2008) under the accession number FJ586243.

## Results

*Cloning of Xyn2A gene*
Xylanase encoding gene of *Neocallimastix* sp. GMLF2 was amplified by PCR and a ~1 kb long DNA fragment inserted into the pCT vector. The new construction vector was numbered as pCTX2A and transformed into *E. coli* EC1000 as described in Figure 1.

Plasmid survival and gene stability was proved by growing of the recombinant *E. coli* EC1000 in a sequential sub-culturing system for both various temperature and pH values (data not shown).

*Xylanase activities*
Cell-associated and extracellular total enzyme activities of both the isolate GMLF2 and *xyn2A* containing transformant *E. coli* are presented in Table 1 for different substrates. *Neocallimastix* sp. GMLF2 released xylanase mainly into the culture medium and approximately 10% of the enzyme activity was found as cell-associated when oat-spelt xylan was used as sole substrate. In contrast, main xylanase activity was observed as cell-associated for the *xyn2A* containing transformant *E. coli* cells regardless of substrates. Both oat-spelts xylan and birchwood xylan were hydrolyzed by Xyl2A. Some other carbon sources, such as carboxymethyl cellulose, medium fibrous cellulose and lichenan, were also used as substrate, however, no detectable activity of the cloned gene could be observed against those carbon sources (data not shown). The total activity of *xyn2A* was found 269 U on the oat spelt xylan, while total activity of *xyn2A* was 357 U on the xylan derived from birchwood. Specific activities of Xyl2A against oat-spelt and birchwood xylans were calculated as 5096 U and 6756 U, respectively.

The pH, temperature and thermostability characteristics of the enzymes were also studied for both original isolate *Neocallimastix* sp. GMLF2 and cloned *xyn2A* gene in *E. coli* (Fig. 2).

The pH profiles of transformant *E. coli* and isolate GMLF2 were similar, and maximum activities were observed when the pH figures were 6 and 6.5 (Fig. 2a). Remarkable relative enzyme activities were still observed at the pH values of 5.0 (78%) and 7.0 (79%) while alkali pH values (pH 8.5–9.0) caused a dramatic decline in enzyme activity. Low pH values exhibited however, relatively less adverse effects on the enzyme activities in particular for transformant *E. coli* xylanase.

The effect of the temperature on the enzyme activity was also evaluated between 30℃ and 80℃ with 10℃ increments. Results indicated that xylanase activity increased in parallel to increment of temperature and the maximum enzyme activity was recorded when the enzyme was incubated at 50℃ (Fig. 2b). Enzymes of both the isolate GMLF2 and transformant strain preserved their ~50% activity when incubated at 60℃ and lost most of their activity when incubation temperature reached 70℃ (Fig. 2b).

Xylanase of transformant *E. coli* strain was studied to determine thermal stability at different temperatures ranging from 40℃ to 70℃ at various time courses (5, 10, 15 and 20 min for each temperature). These results are displayed in Figure 2c. Xyl2A showed maximum activity when pre-incubated at 40℃ and 50℃ for 5 min and lost its ~20% activity at 20-min pre-incubation. Further thermostability studies concluded that pre-incubation temperature for 60℃ and 70℃ af-

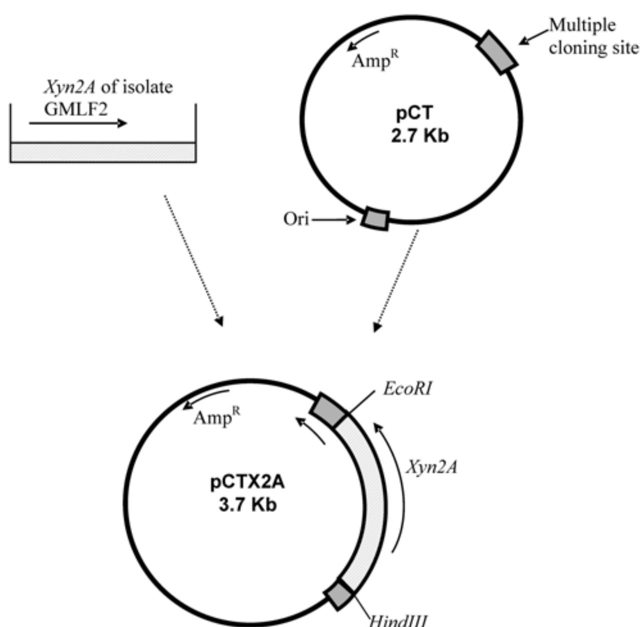

Fig. 1. Illustration of the strategy followed to construct the novel pCTX2A vector system.

Table 1. Total enzyme activities (μmol/min/mL culture) belonging to cell-associated and extracellular fractions of *Neocallimastix* sp. GMLF2 and transformant *E. coli* cells containing a xylanase gene carrying plasmid (pCTX2A) on various substrates.

| Substrate | *Neocallimastix* sp. GMLF2 | | *E. coli* / pCTX2A | |
|---|---|---|---|---|
| | Cell-associated | Extracellular | Cell-associated | Extracellular |
| Oat spelt xylan | 15.33 ± 0.27 | 140.39 ± 1.58 | 269.06 ± 3.52 | 7.23 ± 0.31 |
| Birchwood xylan | 28.29 ± 0.72 | 125.80 ± 3.64 | 356.73 ± 2.58 | 8.14 ± 0.39 |

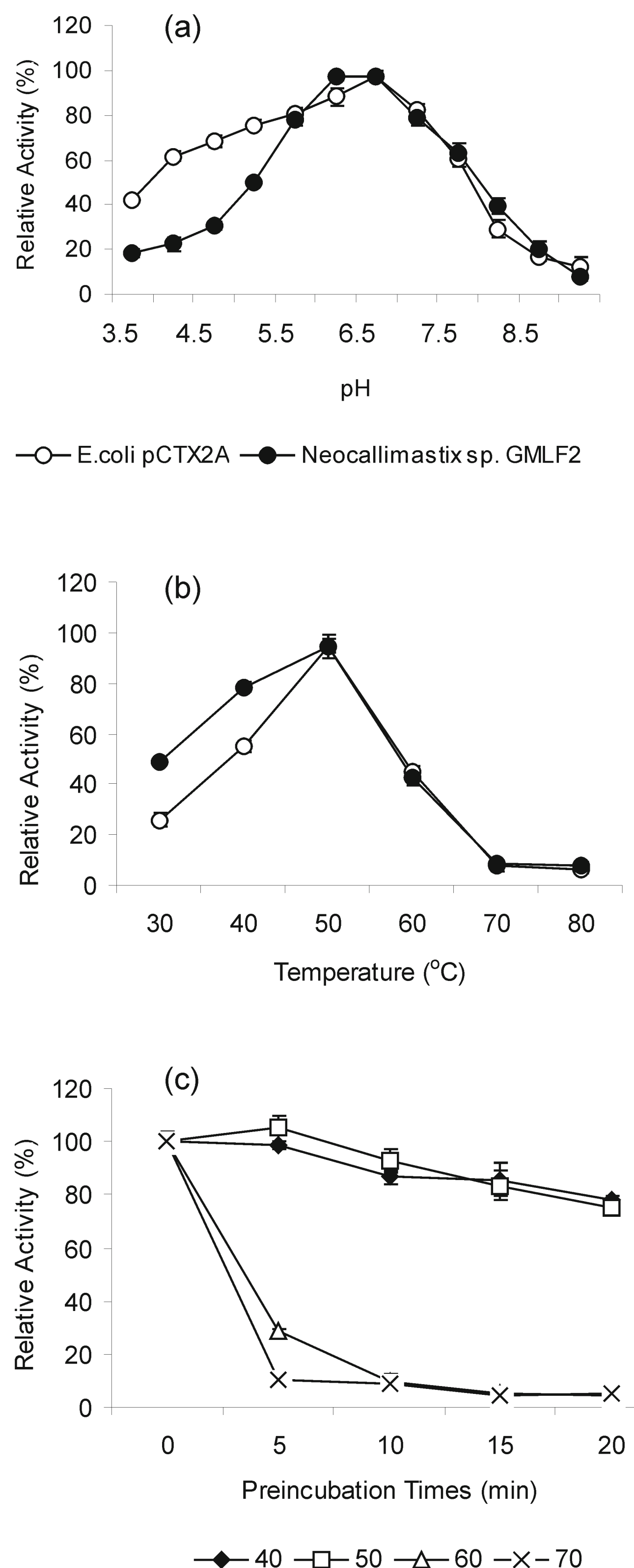


Fig. 2. (a) Temperature and (b) pH profiles of xylanase enzyme of *Neocallimastix* sp. GMLF2 and transformant *E. coli*. (c) Thermostability of Xyl2A for different temperatures and preincubation times.

fected the enzyme activity saliently. The enzyme lost its 71% and 91% activity in 5$^{th}$ min and 10$^{th}$ min at 60 ℃, respectively, whereas 90% activity lost was determined for 5-min pre-incubation at 70 ℃.

*Nucleotide and deduced amino acid sequence of xyn2A*
*Xyn2A* inserted pCT plasmid, designated as pCTX2A, was isolated from *E. coli* and *xyn2A* insert was sequenced on both ends. The complete sequence of *xyn2A* has been submitted to GenBank (Acc. No. FJ586243) and deduced primary structure of the encoded protein is presented in Figure 3. It was 805 base length fragment containing an open reading frame (ORF) of 219 amino acids with a molecular mass of ∼33 kDa. GC content of the entire product and ORF of *xyn2A* were 44.10% and 44.03% respectively.

The bias in codon usage was also investigated in the *xyn2A,* and 43.9% and 10.7% of codons involved T and G, respectively, in the third position. The codons have A and C in the third position with a proportion of 20.7% and 23.2%, respectively. The nucleotide sequence similarity comparison with web-based CLUSTAL W showed that nucleotide sequence of *xyn2A* exhibits a 73% similarity with *xyn11B* of *N. frontalis* and 75% one with both*xyn11A* of *N. frontalis* and *xynA* of *N. patriciarum.* Multiple alignment of the amino acid sequences of Xyl2A with other published xylanases suggested that Xyl2A belongs to the family GH11. Three conservative regions, thought to be specific for the family GH11 members, were detected as YGW, EYY and SVR (Fig. 3). A specific 12 bp insert, including four amino acid residues (NLTT) of Xyl2A, has been determined.

## Discussion

Rumen fungal enzymes have much interest for scientific investigation (Qiu et al. 2000), because of the wide substrate specificity of these enzymes (Trinci et al. 1994) and remarkably powerful fibrolytic activity (Liu et al. 2005). Since the production of fibrolytic enzymes of anaerobic fungi is under the control of catabolite repression (Mountfort & Asher 1983), in order to stimulate the xylanase expression in our isolate *Neocallimastix* sp. GMLF2, oat spelt xylan was used as sole carbon source in growth and maintenance medium. *Neocallimastix* sp. GMLF2 released the xylanolytic enzyme mainly into the culture supernatant and relatively lower xylanase activity was found with thallus associated. Xylanase activity of *N. frontalis*, similarly, was found mainly in culture fluid (Pearce & Bauchop 1985; Mountfort & Asher 1989), however, findings of Lowe et al. (1987) were disputed to the results of that reports. Different substrates in enzyme assay were used to evaluate the enzyme production of *Neocallimastix* sp. GMLF2 in the xylan-containing medium. Xylan (regardless of origin whether oat spelts or birchwood) was degraded relatively in large extent in comparison with carboxymethyl cellulose, medium fibrous cellulose and lichenan. This result suggests that xylanase of *Neocallimastix* sp. GMLF2 was considerably inducible with its appropriate substrates.

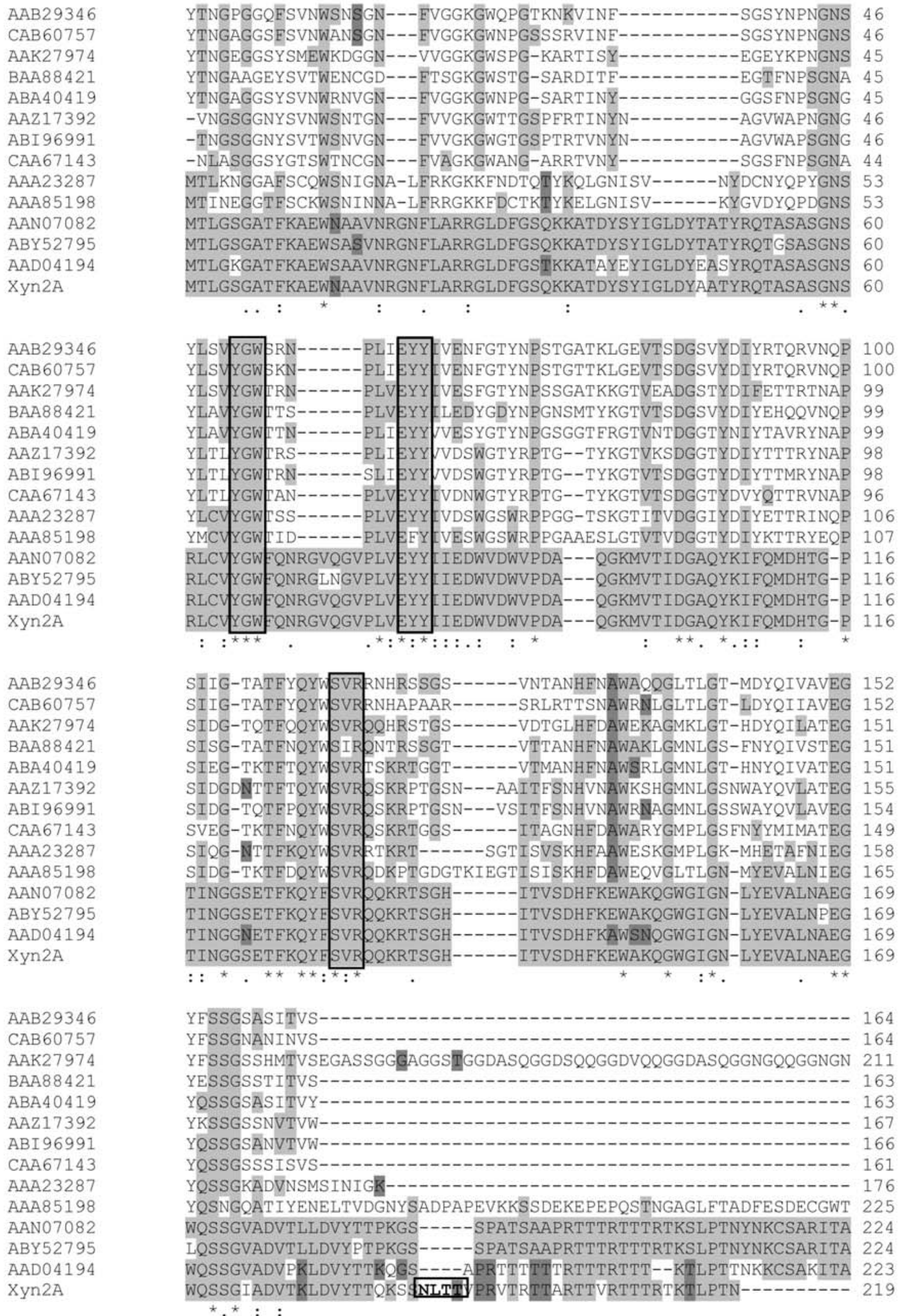

Fig. 3. Multiple alignment of some GH11 amino acid sequences. Conservative regions are signified by rectangles, the different stretch of *Neocallimastix* xyn2A is bolded and matching residues are shown on shaded background. The sources of the enzymes: *Aspergillus fumigatus* (GenBank Accession No. ABA40419), *Penicillium* sp. 40 (BAA88421), *Hypocrea jecorina* (AAB29346), *Trichoderma viride* (CAB60757), *Fusarium oxysporum* (AAK27974), *Streptomyces* sp. S38 (CAA67143), *Bacillus subtilis* (AAZ17392), *Paenibacillus* sp. DG-22 (ABI96991), *Clostridium saccharobutylicum* (AAA23287), *Ruminococcus albus* (AAA85198), *Neocallimastix frontalis* (AAN07082), *Piromyces communis* (ABY52795), *Orpinomyces* sp. PC-2 (AAD04194), Xyn2A (current study).

The findings about pH and temperature optima (Fig. 2) were in parallel to previous reports. Optimum pH and temperature of rumen fungal xylanases have been found at 5.5–6.5 and 50–55 ℃, respectively, by several researchers (Lowe et al. 1987; Mountfort & Asher 1989; Gomez De Segura & Fevre 1993). Maximum activity was obtained at 50 ℃ and significant decline was observed from this point to 80 ℃ (Fig. 2b), suggesting that enzyme lost its activity at temperatures above 50 ℃.

Although anaerobic fungi are true eukaryotic microorganisms, previous reports suggested that some rumen fungal enzyme genes were originated from bacteria (Gilbert et al.1992; Zhou et al. 1994; Chen et al. 1997). Therefore, *xyn2A* was successfully expressed in *E. coli* under the control of bacterial promoter lacZ. Although *xyn2A* had a signal sequence, *E. coli* did not recognize the signal peptide of the XylA so the protein did not excreted from the cell to culture supernatant (reviewed by Orpin & Joblin 1997). As seen from Table 1, vast majority of the Xyl2A activity was found as cell-associated.

Xyl2A was tending to be more active in acidic pHs in contrast to fact that it lost its activity more rapidly in alkali pH values. Showing relatively higher activity in acidic pH ranges could be due to the adaptation of Xyl2A to rumen conditions where pH figure ranges from 5.0 to 7.0 under normal conditions and in some cases could even decline below pH 5.0 due to intensive bacterial activity.

Thermostability of *xyn2A* gene product was investigated with different temperatures and different preincubation times. It seems that enzyme lost its activity at temperatures more than 50 ℃ (Fig. 2c). According to this result, dramatic decrease in Xyl2A activity above the optimum incubation temperature could be explained by the instability of enzyme for higher temperatures. Rumen temperature is more stable (38–40 ℃) than rumen pH, therefore rumen enzyme could show limited thermal flexibility. Because of the high competition between microorganisms of rumen, gut fungi seem to be adapted considerably to the rumen conditions.

The substrate specificity of the Xyl2A was evaluated using different substrates. Both oat spelt xylan and birchwood xylan were hydrolyzed by Xyl2A, but other substrates were not hydrolyzed. In contrast to specific activity, the total activity of Xyl2A was found much higher on the oat-spelt xylan compared to the birchwood xylan, and that different degradation ratios for the two types of carbon sources could be explained by the different carbohydrate composition of these substrates (Li et al. 2000). Microorganisms have been known for their different strategies for hydrolyzing the fibrolytic plant biomass. Some microorganisms, such as *Trichoderma reesei*, tend to release much enzyme to solubilize the cellulose and hemicellulose, while anaerobic rumen fungi produce less enzyme that have remarkably high specific activities (Weimer 1996), as suggested by the results of current study, too.

The sequence similarity analysis of the nucleotide sequence of *xyn2A* showed ∼25% difference compared with *xyn11B* of *N. frontalis*, *xyn11A* of *N. frontalis* and *xynA* of *N. patriciarum*. The *xynA* and *xyn11A* possess two homologous catalytic domains and also two dockerin domains, whereas *xyn11B* has only one catalytic domain and two dockerin domains (Gilbert et al. 1992; Huang et al. 2005). In order to investigate the effects of dockerin domains on the enzyme activity, Huang et al. (2005) deleted the dockerin domains from the *xyn11A* and *xyn11B*. The optimum pH was slightly affected but optimum temperature was remarkably influenced by the deletion of dockerin domains. Furthermore in the present study, it was observed that the optimal pH and temperature values of *xyn2A* and xylanase of the fungal isolate GMLF2 were considerably similar.

The predicted amino acid sequence contains a conserved domain of the family GH11. The coding region of *xyn2A* in this study has similar GC content with the other fungal xylanases reported so far (Black et al. 1994; Durand et al. 1996), whereas untranslated region has notably higher GC content. Anaerobic fungi have been known for their AT rich genome and particularly some part of *Neocallimastix* genome have even less than 20% GC content (Billon-Grand et al. 1991). Non protein coding regions of *xynA* of *N. patriciarum* and *xyn11A* of *N. frontalis* have significantly low GC content (Gilbert et al. 1992; Huang et al. 2005). This difference was due to the primers which were designed for amplifying the ORF of *xyn2A* from *Neocallimastix* sp. GMLF2 genome. The bias in codon usage for *xyn2A* was also studied and our results show that a relatively low amount of T was involved in the third position. Important preference for T in the third position was found in *xynB* from *N. patriciarum* and G was excluded from the wobble position (Black et al. 1994). The reiterated sequences of RTTT determined in fungal *xynR8* (Liu et al. 2005) were also partially observed in the xylanase investigated here at the 197, 204 and 209 position of the deduced amino acid sequence (Fig. 3) with valin and arginine substitution in first two reiterated regions.

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