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Cloning and expression of an endoglucanase gene from the thermotolerant fungus *Aspergillus fumigatus* DBiNU-1 in *Kluyveromyces lactis*

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ABSTRACT

An intronless endoglucanase from thermotolerant *Aspergillus fumigatus* DBiNU-1 was cloned, characterized and expressed in the yeast *Kluyveromyces lactis*. The full-length open reading frame of the endoglucanase gene from *A. fumigatus* DBiNU-1, designated Cel7, was 1383 nucleotides in length and encoded a protein of 460 amino acid residues. The predicted molecular weight and the isoelectric point of the *A. fumigatus* Cel7 gene product were 48.19 kDa and 5.03, respectively. A catalytic domain in the N-terminal region and a fungal type cellulose-binding domain/module in the C-terminal region were detected in the predicted polypeptide sequences. Furthermore, a signal peptide with 20 amino acid residues at the N-terminus was also detected in the deduced amino acid sequences of the endoglucanase from *A. fumigatus* DBiNU-1. The endoglucanase from *A. fumigatus* DBiNU-1 was successfully expressed in *K. lactis*, and the purified recombinant enzyme exhibited its maximum activity at pH 5.0 and 60 °C. The enzyme was very stable in a pH range from 4.0 to 8.0 and a temperature range from 30 to 60 °C. These features make it suitable for application in the paper, biofuel, and other chemical production industries that use cellulosic materials.

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Introduction

Cellulose, the most abundant organic and renewable biomass, has been considered a good candidate for the production of alternative biofuels and chemicals.¹ Cellulose consists of

a linear chain of several glucose units that are linked by β (1→4) glycosidic bonds.^{2–4} It is one of the major structural constituents of plant cell walls and is commonly found in the stalks, stems, trunks and all the woody portions of the plant body.⁵ Conversion of cellulose into fermentable sugar,

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i.e., glucose, requires the synergistic action of many cellulolytic enzymes known as cellulases. Cellulases refer to a multienzyme complex composed of endoglucanase (endo-1,4- β -glucanase, EC 3.2.1.4), exoglucanase (exo-1,4- β -glucanase, EC 3.2.1.91), and cellobiase (β -glucosidase, EC 3.2.1.21).^{6,7} The enzymes are widely found in many organisms, such as actinomycetes, bacteria, fungi and plants. Among the three major groups of cellulases, endoglucanase is considered to be the most important enzyme because its action results in a non-reducing end that is necessary for the action of exoglucanase and cellobiase.⁸

There are several sources of endoglucanase such as bacteria, fungi and plant,^{7–10} but fungal endoglucanase is the major source of enzyme for industrial applications. Although several endoglucanases have been cloned, identified and characterized, most efforts have focused on the enzymes from the filamentous fungi, such as *Aspergillus usami*,⁷ *A. kawachii*,¹² *Trichoderma reesei*,¹³ *A. niger*,¹⁴ *Volvariella volvacea*,^{15,16} *Penicillium canescens*,¹¹ *P. funiculosum*¹⁷ and *Verticillium dahlia*.¹⁸ Nierman et al.¹⁹ reported that there are approximately eighteen different genes that encode endoglucanases in the entire sequence of the *A. fumigatus* Af293 genome. Most recently, the endoglucanase gene belonging to the family AA9 (formerly known as GH61) from the alkali-tolerant *A. fumigatus* MKU1, which is isolated from paper and pulp industry wastes, has also been cloned and sequenced.³ Das et al.²⁰ reported the kinetic properties of the purified halostable endoglucanase from a culture extract of *A. fumigatus* ABK9, and the authors demonstrated that the calculated molecular weight of the enzyme was approximately 56.3 kDa. The enzyme displayed its maximum activity at a temperature of 50 °C and a pH value of 5.0. This enzyme is highly stable in a pH range from 4.0 to 7.0 and at NaCl concentrations up to 3.0 M. Xu et al.²¹ reported the expression and secretion of fungal endoglucanase II and chimeric cellobiohydrolase I in the oleaginous yeast *Lipomyces starkeyi* and the authors demonstrated that the engineered *L. starkeyi* was capable of expressing and secreting both enzymes with high activity toward cellulose.

Currently, *Kluyveromyces lactis*, an ascomycetous budding yeast belonging to the endoascomycetales, is considered as one of the most favored systems for the expression of several heterologous proteins because it has several advantages including protein folding, protein processing and posttranslational modification. It has been reported that *K. lactis* produces and secretes heterologous proteins at a higher level than *Saccharomyces cerevisiae*. It also secretes few native proteins and a secreted recombinant product is therefore easily recovered and purified from the culture medium.^{22,23} Most importantly, *K. lactis* can be grown in inexpensive media. Furthermore, it has been asserted as a “Generally Recognized As Safe” microorganism by the US FDA and can thus be applied in several industries such as food, beverage, flavor, vitamin and feed production.²⁴

In the current study, the cloning and molecular characterization of the gene encoding endoglucanase from the thermotolerant fungus *A. fumigatus* DBiNU-1 were investigated. In addition, the heterologous expression of the *A. fumigatus* endoglucanase gene in yeast *K. lactis* GG799 and the enzymatic properties of the gene product were also described.

We demonstrated in the current study that the endoglucanase from *A. fumigatus* DBiNU-1 is a thermostable enzyme.

Materials and methods

Strains, plasmid vectors and culture conditions

The thermotolerant fungus *A. fumigatus* DBiNU-1 used in this work was obtained from Department of Biology, Faculty of Science, Naresuan University. It was isolated from a soil sample collected in the northern part of Thailand, and was identified by morphological analysis and DNA sequencing of the internal transcribed spacer (ITS) and large subunit (LSU) of rDNA region. It was used as a source for genomic DNA isolation. The fungus was maintained on potato dextrose agar (PDA) and stored at 4 °C. *K. lactis* GG799 (New England Biolabs, US) was used as a host system for protein expression, while *Escherichia coli* DH5 α was used as a host system for gene cloning. The pGEM-T Easy (Promega, Madison, WI, USA) and pKLAC2 vectors were used for gene cloning in *E. coli* and for gene expression in *K. lactis*, respectively.

Genomic DNA extraction

A. fumigatus DBiNU-1 was cultured in YPD medium on a rotary incubator shaker (220 rpm) at 30 °C for 24 h. The fungal mycelia were harvested by filtration and washed twice with sterile distilled water. Genomic DNA was isolated from the fungal mycelia using the GF-1 nucleic acid extraction kit (Vivantis, USA), and the purified genomic DNA was used as a DNA template for gene amplification using the polymerase chain reaction (PCR) technique.

PCR amplification, cloning and nucleotide sequencing of the endoglucanase gene from *A. fumigatus* DBiNU-1

Based on the alignment of the multiple amino acid sequences of the endoglucanases from *A. fumigatus* MKU1 (AFJ54162), *A. niger* ATCC10574 (ADC84000) and *A. oryzae* KBN616 (BAA22588), the conserved regions at the N-terminus (GALYLSEM) and C-terminus (IRWGDIG) were found. The design of the degenerate primers Cel7asper_1F (5'-GGNGCNYTNWSNGARATG-3') and Cel7asper_1R (5'-NCCDATRTCNCNCCCANCKDAT-3') were based on these two regions, and the primers were used to amplify a partial sequence of the endoglucanase gene from *A. fumigatus* DBiNU-1. Since the partial sequence of the endoglucanase gene from *A. fumigatus* DBiNU-1 was highly homologous to that from *A. fumigatus* MKU1 (JQ796069), the amplification of the full length open reading frame (ORF) of the endoglucanase gene from *A. fumigatus* DBiNU-1 was performed using specific primers Cel7full-F (5'-ATGGACTCCAAAAGAGCGTCGT-3') and Cel7full-R (5'-CTACAGACACTGAGATTACCACGGA-3'), which were synthesized based on the nucleotide sequences of the endoglucanase gene from *A. fumigatus* MKU1. The PCR reaction mixture (25 μ l) consisted of 2 $\times$ KOD XtremeTM PCR buffer, 2 mM dNTPs, 10 pmol/ μ l each forward and reverse primer, 1.0 U/ μ l KOD XtremeTM Hot Start DNA polymerase, and 100 ng/ μ l template DNA, as per the manufacturer's instructions (Merck, Germany). The template DNA was first denatured

at 95 °C for 3 min. Thirty cycles of the amplification reaction were then performed using of the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 50 °C and extension for 2 min at 72 °C. A final extension was performed at 72 °C for 7 min. The PCR product was visualized on 0.7% agarose gel, extracted and purified from the gel using the NucleoSpin Extract II Kit (Macherey-Nagel, Germany). The Rapid DNA Ligation and Transformation Kit (Fermentas, USA) was used to clone the purified PCR product into the pGEM T-easy vector and to transform the resulting plasmid into *E. coli* DH5 α . Transformants were screened at 37 °C on LB medium containing 100 μ g/ml ampicillin. The recombinant plasmid DNA was isolated from the selected transformants and subjected to DNA sequencing by the Sanger method using the MegaBACE 1000 automated DNA sequencer (Pharmacia Biotech, Sweden). The nucleotide and the deduced amino acid sequences of the endoglucanase gene product from *A. fumigatus* DBiNU-1 were analyzed using GENETYX (Software Development, Tokyo, Japan), while a homology analysis was carried out using the FASTA and BLAST program in the GenBank database.

Construction of the expression vector and transformation of *K. lactis*

The ORF of the endoglucanase gene from *A. fumigatus* DBiNU-1 was amplified by PCR using the following primer pairs: CEL7PKLAC.F: 5' CGCTCGAGAAAAGAATGG ACTCCAAAAGAGGC-3' (the underline bases represent the *Xho*I restriction site) and CEL7PKLAC.R: 5'-CCGGAATTCCTACAGACACTGAGAGTACCA-3' (the underline bases represent the *Eco*RI restriction site). All PCR primers were synthesized by Integrated DNA technology (IDT, Singapore). The PCR reaction mixture (25 μ l) consisted of 2 $\times$ KOD Xtreme™ PCR buffer, 2 mM dNTPs, 10 pmol/ μ l each forward and reverse primer, 1.0 U/ μ l KOD Xtreme™ Hot Start DNA polymerase and 20 ng/ μ l template DNA, as per the manufacturer's instruction (Merck, Germany). The thermocycling conditions consisted of a 94 °C step for 2 min, followed by 35 cycles of successive incubation at 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min. After the thermocycling, a final extension was performed at 72 °C for 7 min. To construct the expression cassette pKLAC2-Cel7, the purified amplified fragment was digested with *Xho*I and *Eco*RI and then ligated into the *Xho*I/*Eco*RI site of the *K. lactis* expression vector pKLAC2. The accuracy of the nucleotide sequence of the cloned endoglucanase gene was confirmed by DNA sequencing as described above.

The transformation of the expression cassette into *K. lactis* GG799 was performed using lithium acetate with the heat shock method.²⁵ To integrate the cloned endoglucanase gene into the LAC4 locus of the *K. lactis* chromosome, the expression cassette pKLAC2-Cel7 was linearized with *Bst*XI before transformation. The transformants harboring the pKLAC2-Cel7 plasmid were screened and selected at 30 °C using yeast carbon base (YCB) agar medium supplemented with 5 mM acetamide. The selected transformants were further analyzed on YPD agar medium containing 1% carboxymethyl cellulose (CMC) (Fluka, Germany).

Detection of the recombinant endoglucanase using 1% Congo red

The selected transformants were grown in YPGal medium at 30 °C in an incubator shaker (200 rpm) for 24 h. Thereafter, 50 μ l of the yeast culture was transferred onto the YPD agar medium that contained 1% CMC and incubated at 30 °C. Sterile distilled water was used as a control in this study. After 7 days of incubation, the agar medium was flooded with 1% Congo red and subsequently incubated at room temperature (RT) for 15 min. The agar medium was rinsed twice with sterile distilled water and then washed with a 1 M NaCl solution for 15 min. The clear zone that appeared on the agar medium was monitored and photographed.

SDS-polyacrylamide gel electrophoresis and zymogram analysis of the recombinant endoglucanase

The selected transformant was grown in YPD liquid medium supplemented with 1% CMC at 30 °C in an incubator shaker (200 rpm) for 7 days. The culture supernatant was collected by centrifugation at 13,000 rpm and 4 °C for 15 min, and the endoglucanase activity in the clear supernatant (crude enzyme) was assayed. The protein concentration in the culture supernatant was measured by the Bradford method²⁶ using bovine serum albumin (BSA) as the standard. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the crude enzyme was prepared based on the method described by Eriksson and Pettersson²⁷ and Béguin,²⁸ using a sample buffer containing 2% SDS, 0.5 M Tris-HCl (pH 6.8), 10% glycerol and 0.01% bromophenol blue. The SDS-PAGE was carried out based on the standard method described by Li,²⁹ using 30 μ g of protein sample and a 12% polyacrylamide gel, and the separation of the protein bands in the gel was visualized by Coomassie brilliant blue R-250 (Sigma) staining.

The zymogram analysis was based on the method described by Grigorevski-Lima et al.,³⁰ and was carried out to determine the enzyme activity. The crude enzyme was subjected to SDS-PAGE using a 12% polyacrylamide gel that contained 1% CMC as substrate. After electrophoresis, the gel was soaked in 2.5% (v/v) Triton X-100 for 2 h and then incubated in 50 mM sodium acetate buffer (pH 5.0) for 1 h at RT in order to remove the SDS and renature the enzyme. The resulting gel was washed with 50 mM sodium acetate buffer (pH 5.0) at 45 °C for 30 min, stained with 1% (w/v) Congo red dye solution at RT for 30 min, and subsequently destained with 1 N NaCl until the excess dye was removed. The clear zone, which corresponded to enzyme activity, was monitored and documented with a gel documentation system (Bio-Rad, Singapore).

Purification of protein using an aqueous two-phase system

The recombinant endoglucanase was purified using an aqueous two-phase system.³¹ Various concentrations of polyethylene glycol 6000 (PEG 6000) and dextran T70 were used in this system. To prepare the two-phase system, a stock solution composed of 20% (w/w) dextran and 40% (w/w) PEG and the enzyme solution were mixed and left to settle for at least

1.5 h. Protein samples in the top and bottom phases were taken, and the enzyme activity in both phases was further determined.

The activity of the purified recombinant endoglucanase was assayed using the method described by Li et al.,¹⁶ using CMC as substrate. One unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing 1 μ mol of reducing sugar from CMC per minute under the assay conditions. D-Glucose was used as the standard.

Effect of pH on enzyme activity and stability

The effect of pH on the activity of the purified recombinant endoglucanase was evaluated using the standard method described by Li et al.,¹⁶ with different buffers including sodium acetate buffer (pH 3.0–6.0) and phosphate buffer (pH 7.0–11.0). The stability of the purified endoglucanase at different pH values was evaluated by incubating the purified enzyme at 50 °C for 1 h in the same buffers in the pH range from 3.0 to 11.0 and measuring the remaining enzyme activity under standard conditions.

Effect of temperature on enzyme activity and stability

The effect of temperature on the activity of the purified recombinant endoglucanase was evaluated at the optimum pH at temperatures ranging from 30 to 80 °C. The thermal stability of the purified enzyme was evaluated by preincubating the enzyme solution in 50 mM sodium acetate buffer (pH 5.0) at various temperatures (30–80 °C) for 1 h. The remaining enzyme activity was assayed under standard conditions.

Results

Cloning and nucleotide sequencing analysis of the endoglucanase gene from *A. fumigatus* DBiNU-1

The ORF of the gene encoding the endoglucanase in *A. fumigatus* DBiNU-1 was amplified by PCR using a genomic DNA template that was isolated from *A. fumigatus* DBiNU-1 and specific primers whose design was based on the nucleotide sequences of the endoglucanase gene from *A. fumigatus* MKU1, as described in the Materials and methods section. A PCR product of approximately 1.3 kb was detected after agarose gel electrophoresis analysis, and it was purified and cloned, and the resulting vector was transformed into *E. coli* DH5 α . The recombinant plasmid DNA that was isolated from the selected transformants was then subjected to DNA sequencing. As found in the present study, the complete nucleotide sequence of the endoglucanase gene from *A. fumigatus* DBiNU-1, designated *Cel7*, was 1,383 nucleotides in length and encoded a polypeptide of 460 amino acid residues (Fig. 1). The predicted molecular weight and the isoelectric point of the *A. fumigatus* *Cel7* gene product were found to be 48.19 kDa and 5.03, respectively. Based on the sequence similarities in the carbohydrate-active enzyme (CAZy) database (<http://www.cazy.org/>), the deduced amino acid sequence of the endoglucanase gene product from *A. fumigatus* DBiNU-1 was classified into

glycoside hydrolase family 7 (GH7).^{32–34} These comprise two domains, i.e., a catalytic domain at the N-terminus, which contains the glycosyl hydrolase family 7 active-site residues (amino acids at positions 22–398), and a fungal type cellulose-binding domain/module (CBD/CBM) at the C-terminus (amino acids at positions 428–456), which is involved in substrate binding, as observed in most fungal cellulases.³⁵ The prediction of the amino acid sequences by the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) revealed the signal peptide (MDSKRGVVAVLALLPLVSA) at the N-terminal region and a signal peptide cleavage site between Ala²⁰ and Gln²¹.

The homology analysis of the deduced amino acid sequence showed that the endoglucanase from *A. fumigatus* DBiNU-1 shared 98%, 74%, 59%, 51%, 46% and 34% identity with the GH7 endoglucanases from *A. fumigatus* KMU1 (AFJ54162), *A. terreus* MS-31 (ADR78837), *T. reesei* M5 (ADM08177), *A. oryzae* KBN616 (BAA22589), *A. nidulans* FGSCA4 (EAA63386), and *Fusarium oxysporum* (AAA65586), respectively (Fig. 2). The putative catalytic amino acid residues Asp¹⁹¹ and Glu³⁶⁹ were found to be highly conserved among the GH7 endoglucanases and are similar to those reported in the GH12 and AA9 family.^{3,7} A three-dimensional structure of the predicted protein sequence of the endoglucanase from *A. fumigatus* DBiNU-1 was predicted using the Swiss-PdbViewer (<http://www.expasy.org/spdbv/>), and the result is shown in Additional information section, Fig. 1. The protein structure was observed at the tertiary level, and two completely distinct structures corresponding to the catalytic domain and CBM1 domain were detected.

Expression of the endoglucanase gene from *A. fumigatus* DBiNU-1 in *K. lactis* GG799

The expression vector pKLAC2-Cel7 was constructed and transformed into *K. lactis* GG799, and the transformants were selected using YCB agar medium, as described in the Materials and methods section. The selected transformants were further analyzed on YPD agar medium containing 1% CMC. Transformants harboring the pKLAC2-Cel7 exhibited a clear zone on agar plates after Congo red staining, suggesting that the cloned endoglucanase gene from *A. fumigatus* DBiNU-1 was expressed in the *K. lactis* (Fig. 3).

The crude enzyme in the cell-free supernatant was analyzed by SDS-PAGE using a 12% polyacrylamide gel, and the resolved proteins were visualized using Coomassie brilliant blue R-250 staining. As shown in Fig. 4A, a protein band of approximately 48 kDa that corresponded to the predicted molecular weight of the endoglucanase from *A. fumigatus* DBiNU-1 was detected. The endoglucanase activity was also determined based on the degradation of CMC in the polyacrylamide gel using a zymogram analysis. A protein band of approximately 48 kDa that coincided with the band that was observed in the SDS-PAGE analysis was detected on a polyacrylamide gel after Congo red staining (Fig. 4A), suggesting that this protein band was the recombinant endoglucanase from *A. fumigatus* DBiNU-1. The endoglucanase activity in the cell-free supernatant from *K. lactis* harboring the pKLAC2-Cel7 plasmid was measured, and the highest enzyme activity was found to be 0.80 U/ml with a specific activity of 3.08 U/mg protein.


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ATGGACTCCAAAGAGGCGTCGTGGCCGCGGTGCTGGCCTTCTCTCTCGTTTCTGCGCAACAACCCGCGGAGTTCTGCTGGTAAC 90
M D S K R G V V A A V L A L L P L V S A Q Q P A A S S A G N
CCCAAGCTGACGACATACAAATGTACCACTGCTGGCGGCTGTGTTGCGCAGGATACATCTGTAGTTCTAGATTGGGGCTACCACTGGATC 180
P K L T T Y K C T T A G G C V A Q D T S V V L D W G Y H W I
CACACGGTCGATGGGTATACATCATGCACCACATCGTCCGGAGTCGACAGCACCCTGTGCTGATGCGGGCCACCTGCGGAAGAAGTGT 270
H T V D G Y T S C T T S S G V D S T L C P D A A T C A K N C
GTGATCGAGCCGCCCACTACACCAGCGCCGGTGTGACCACCTCGGGTGACTCTCTGACGATGTACCAATATGTTTCAGAGCAACGGCGTC 360
V I E P A N Y T S A G V T T S G D S L T M Y Q Y V Q S N G V
TATACCAACGCCTCGCCTCGCCTCTACCTCTCGGCCCCGACAAGAACTATGTCATGCTGAAGCTGCTAGGCCAGGAGCTCACCTTCGAC 450
Y T N A S P R L Y L L G P D K N Y V M L K L L G Q E L T F D
GTGGACCTGTCCACACTCCCCTGCGGCGAAACGGCGCCCTGTATCTCTCCGAAATGAGCGCCACCGGTGGTCGCAACGAATACAACACC 540
V D L S T L P C G E N G A L Y L S E M S A T G G R N E Y N T
GGCGGCGCGAGTACGGCTCTGGCTACTGTGACGCCAATGCCAGTGATCGCCTGGAAGAAGCGCACCCCTCAACACGAGCGCGCAAGC 630
G G A E Y G S G Y C D A Q C P V I A W K N G T L N T S G A S
TACTGCTGCAACGAGATGGACATCCCCGAGGCCAACTCCCGCGCCAACTCGTACACCCCGCACCCCTGCAGTGCAACGGACTGTGACAAG 720
Y C C N E M D I P E A N S R A N S Y T P H P C S A T D C K
GGCGGATGCGGCTTCAACCCATACGCTCTCGGCGAAAGAGCTACTGGGGGCCCGCGGCCACCGTAGACACGTCTAAGCCCTTCACCATT 810
G G C G F N P Y A L G Q K S Y W G P G G T V D T S K P F T I
ACCACGAGTTTCATCACCAACGAGCGCCACCACCGCGCCCTGTCCGAAATCCGCGGACAGTACATGCAGAACGGCAAGGTGATCGCC 900
T T Q F I T N D G T T T G T L S E I R R Q Y M Q N G K V I A
AATGCCGTTTCTCCACTGGTGTCAACTCCATTACCGAGGACTGGTGACGTCGCTCGACGGCTCGGCGGCCACCTTTGGCGGACTGACC 990
N A V S S T G V N S I T E D W C T S V D G S A A T F G G L T
ACCATGGGCAAGCGCTGGGCGCGGGATGGTCTCATCTTACGATCTGGAACGATGCCAGCGCCCTATGAAGTGGCTCGACAGCGGC 1080
T M G K A L G R G M V L I F S I W N D A S G L M N W L D S G
AACGCAGGCCCTTGACGAGCAGCAGGAGGAAACCGGACTTGATCAGGGCGCAGAATCCACGACCCACGTGGTCTTCTCGAATATCCGC 1170
N A G P C S S T E G N P D L I R A Q N P T T H V V F S N I R
TGGGGAGATATCGGATCGACTTTCAAGGGTCTGATGGCTCGGTGACGACGACTACGTCACCACGTCGACCAAGACCACCACTAGCACC 1260
W G D I G S T F K G S D G S V T T T T S T T S T K T T T S T
GCGCCTGGGCCAACGAGACTCACTATGGACGGTGGGACAGGCTGGACTGGACCCACGGCTTGTGCATCGCCCTACACCTGCCAG 1350
A P G P T Q T H Y G R C G G Q G W T G P T A C A S P Y T C Q
GTTCTGAATCCGTGCTACTCTCAGTGTCTGTAG 1383
V L N P W Y S Q C L *

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Fig. 1 – Nucleotide and deduced amino acid sequences of an intronless endoglucanase from thermotolerant *A. fumigatus* DBiNU-1. The signal peptide from Met¹ to Ala²⁰ is underlined. The conserved amino acid regions, GALYLSEM and IRWGDIG, are shown in bold letters. The putative catalytic amino acid residues Asp¹⁹¹ and Glu³⁶⁹ are shown in the boxes.

There was no endoglucanase activity in the cell-free supernatant from the negative control (*K. lactis* transformed with pKLAC2) under the same cultural conditions.

Purification and characterization of the purified recombinant endoglucanase

The recombinant endoglucanase from the *K. lactis* strain that harbored pKLAC2-Cel7 was purified using the aqueous two-phase system, as described in the Materials and methods section. A maximum enzyme activity and specific activity of 1.57 U/ml and 15.39 U/mg protein, respectively, were achieved using the two-phase system that contained 7% PEG and 5% dextran. Fig. 4B shows the SDS-PAGE analysis of the purified recombinant endoglucanase that was obtained from aqueous two-phase system. Most of the protein band at approximately 48 kDa, which corresponded to the monomer of the Cel7 gene product from *A. fumigatus* DBiNU-1, was observed, while those low-molecular weight proteins that were contained in the cell-free supernatant were mostly removed.

The effect of pH on the activity and stability of the purified recombinant endoglucanase was evaluated, and the results

are summarized in Fig. 5A. The purified recombinant endoglucanase displayed its highest activity at pH 5.0, and more than 80% of its original activity remained in the pH range from 4.0 to 8.0.

The effect of temperature on the recombinant endoglucanase activity and stability is illustrated in Fig. 5B. The maximal activity of the purified recombinant endoglucanase at pH 5.0 was attained at a temperature of 60 °C. The activity of the purified enzyme remarkably decreased at temperatures above 60 °C. More than 80% of the original activity of the purified enzyme remained in a temperature range from 30 to 60 °C, whereas only half of the original activity was detected at 70 °C.

Discussion

Based on the whole genome sequence of *A. fumigatus* Af293, 18 different genes encoding endoglucanases have been reported.¹⁹ They belong to various families, such as GH5, GH7, GH12, GH45, AA9 (formerly known as GH61), and GH81, based on their amino acid sequence similarities. According to a nucleotide sequence analysis, three endoglucanase genes from *A. fumigatus* are intronless (one from GH7 and

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A.fumigatus-DBiNU-1 --MDSKRGVVAAVLALLPLVSAQQPAASS-AGNPKLTTYKCTTAGGCVADQTSVVDLWGY
A.fumigatus-MKU1 --MDSKRGVVAAVLALLPLVSAQQPAASS-AGNPKLTTYKCTTAGGCVADQTSVVDLWGY
A.terreus-MS-31 -MMSLFKSI IAAAGLVFHLVQAQQPSSSS-PGDPSLTWTKCTGGGCLQDDTSVVDLWGV
T.reesei-M5 MAPSVTLPLTTAILAARLVAAQQPGTSTPEVHPKLTITYKCTKSGGCVADQTSVVDLWNY
A.nidulans -----MALLLSLSLLATTISAQQIG-TPEIRPLTTYHCTSSANGCTEQNTSVVLDAAAT
A.oryzae-KBN616 -----MIWTLAPFVALLPLVTAQQVGTADAHPRLTITYKCTSQNGCTFRQNTSLVLDAAAT
F.oxysporum -----MKSLSLSLSALAVQVAVAQTPDKAKEQHPKLETYRCKTASGCKKQNTYIVADAGI
* : * * * : * * * : * * * : * * *

A.fumigatus-DBiNU-1 HWIHTVDGYTSCCTSS-GVDSTLCPDAATCAKNCVIEPAN---YTSAGVTTSGDSLTMQ
A.fumigatus-MKU1 HWIHTVDGYTSCCTSS-GVDSTLCPDAATCAKNCVIEPAN---YTSAGVTTSGDSLTMQ
A.terreus-MS-31 HWIHTSN-SQSCCTSS-GVNSALCPDEATCAKNCVIDPAN---YATAGVATSGDSTLTMHQ
T.reesei-M5 RWMHDAN-YNSCTVNG-GVNTTLCPEATCGKNCFIEGVD---YAASGVTTSGSSLTMNQ
A.nidulans HPIHDASNPSVSCCTSSNGLNFPALCPDKQTCADNCVIDGITD---YAAHGVETHGSRLLTLTQ
A.oryzae-KBN616 HFIHKKG-TQTCTNSNGLDTAICPDKQTCADNCVIDGITD---YASYGVQTKNDTTLTQQ
F.oxysporum HGIRQKN-GAGCGDWGQKPNATACPEASCACNCLSGMDSNAYKNAGITTSNKLRLQQ
: : . . . : : * * : : * * : : * * : : * * : : * * : : * *

A.fumigatus-DBiNU-1 YVQSN--GYTNASPRLYLLGP----DKNYVMLKLLGQELTFDVLSTLPCGENGALYLSE
A.fumigatus-MKU1 YVQSN--GYTNASPRLYLLGP----DKNYVMLKLLGQELTFDVLSTLPCGENGALYLSE
A.terreus-MS-31 YYQNG--VITNASPRLYLLGS----DKNYVMLKLLGQELTFDVLSTLPCGENGALYLSE
T.reesei-M5 YMPSSSGYSSVSPRYLLDS----DGEYVMLKLLGQELTFDVLSTLPCGENGALYLSE
A.nidulans YRNVNG-ALSSVSPRYLVDESDEPDEQYRALSLLAQEFTFTVNVNVALPCGMNGALYLSE
A.oryzae-KBN616 YLQTN--ATKSLSPRYLLAE---DGENYSMLKLLNQEFTFDVASTVLCGMNGALYLSE
F.oxysporum LINNQL----VSPRYVLEE---NKKYEMHLTGTEFSFVEMEKLPFGMNGALYLSE
* * * : * * * : * * * : * * * : * * * : * * * : * * * : * * *

A.fumigatus-DBiNU-1 MSATGGRNEYN--TGGAEGSGYCDACQCPVIAWKNGTLNTSGASYCCNEMDILEANSRAN
A.fumigatus-MKU1 MSATGGRNEYN--TGGAEGSGYCDACQCPVIAWKNGTLNTSGASYCCNEMDILEANSRAN
A.terreus-MS-31 MSATGGRNEYN--TGGAQYSGYCDACQSPVMAWRNGTLNTSGQFGCNCNEMDILEANSRAN
T.reesei-M5 MDENGGAQYN--TAGANYSGYCDACQCPVQVTRNGTLNTSHQFGCCNEMDILEANSRAN
A.nidulans MSPSGGRSALN--PAGASYGTGYCDACQCVNPNWINGEGNNGYAGCCNEMDILEANSRAN
A.oryzae-KBN616 MEASGGKSSLN--QAGAKYGTGYCDACQCYTPWINGEGNTESVGCCQEMDILEANSRAN
F.oxysporum MPQDGKSTSRNSKAGAYYGAGYCDACQCYVTPFINGVNIKGQVCCNELDILEANSRAN
* * * . . . * * * : * * * : * * * : * * * : * * * : * * * : * * *

A.fumigatus-DBiNU-1 SYTPHPCSAT-----DCDKGGCGFNYPALGQKSYWPGG
A.fumigatus-MKU1 SYTPHPCSAT-----DCDKGGCGFNYPALGQKSYWPGG
A.terreus-MS-31 SFTPHSCNGN-----DCDKGGCGFNYPALGQKSYWPGG
T.reesei-M5 ALTPHSCNAT-----ACDSAGCGFSPYSGYKSYWPGG
A.nidulans GFTPHACLYEPEETEGRGVYECASEDECDGAGENDGICDKWCGFNYPALGNTYGYGRGQ
A.oryzae-KBN616 GLTPHPCNTT-----GLYECGSG-----SGCGDSGVCDKAGCGFNYPGLGAKDYGYG-
F.oxysporum HIAPHPCSKPGLYG-----CTGDECGSSGICDKAGCGWNNHNRINVDYFGRGK
: * * * * * * * * * * * * * * * * * * * * * * * * * * * *

A.fumigatus-DBiNU-1 --TVDTSKPFTTITQFITNDGTTTGLTSEIRRYQMONGKVIANAVSSTG---VNSITED
A.fumigatus-MKU1 --TVDTSKPFTTITQFITDDGTTTGLTSEIRRYQMONGKVIANAVSSTG---VNSITED
A.terreus-MS-31 --TVDTSKPFTTITQFITDDGSKTGTLEIRRYQIQNGKIIANAKSSAG---VDSITEP
T.reesei-M5 --TVDTSKFTTITQFNTDNGSPSGNLVSIIRKYQQNGVDVPSAQPGG-----
A.nidulans GFEVDTKPFTTITQFLTDGTTSTGALTEIRRYQIQNGQVIENAVVSSG---ADSLTDS
A.oryzae-KBN616 -LKVTNETFTTITQFLTDNDNTSGQLSEIRRYQIQNGQVIAAVTSGGKT-VDSITKD
F.oxysporum QYKVDSTRKFTTITQFVANK---QGDLELHRHYIQDNKVIESAVVNISGPPKINFINDK
* : : . . * * : * * : : . * * : * * * : : . * * : * *

A.fumigatus-DBiNU-1 WCTSV-DGSAATFGGLTTMGKALGRGMVLIFSIWNDSAGLMNWLDSGNAGPCSTEGNPD
A.fumigatus-MKU1 WCTSV-DGSAATFGGLTTMGKALGRGMVLIFSIWNDSAGFMNWLDSGNAGPCSTEGNPD
A.terreus-MS-31 RCESV-DGAAATYGGGLKRMGEALGRGMVLIFRIWNDAGGFMNWLDSGAGPCSTEGNPS
T.reesei-M5 DTISS-CPSASAYGGGLATMGKALSSGMVLVFSIWNDSQYMNWLDSGNAGPCSTEGNPS
A.nidulans LCAST-ASWFDSYGGMEGMGRALGRGMVLAMSIWNDSAGGYMQWLDGGDAGPCNATEGAPE
A.oryzae-KBN616 FCSGE-GSAFNLRLGLEEMGHALGRGMVLALSIWNDSAGFMQWLDGGSAGPCNATEGNPA
F.oxysporum YCAATGANEYMRLLGGTKQMGDAMSRGMVLAMSVWVWSEGDFAWLDQGVAGPCDATEGDPK
. * * * * * : * * * : * * * : * * * : * * * : * * * : * * *

A.fumigatus-DBiNU-1 LIRAQNPPTHVFSNIRWGDIGSTFKGSDGS-----VTTTTSTSTKTTTSTAP
A.fumigatus-MKU1 LIKAQNPPTHVFSNIRWGDIGSTFKGSDGS-----VTTTTSTSTKTTTSTAP
A.terreus-MS-31 LIQSAHPDTHVFSNIRWGDIGSTYTNPGSGSSSSSTTTTSTSTATTKITTTTSTAG
T.reesei-M5 NILANNPPTHVFSNIRWGDIGSTTNSTAPP-----PPPASSTTFSTTRSSSTSSSP
A.nidulans FIEHTPWTTRVVFEDLKWGDIGSTFQAS-----
A.oryzae-KBN616 LIEKLYPDTHVKFSKIRWGDIGSTRH-----
F.oxysporum NIVKVQPNPEVTFNIRIGEIGSTSSVKAPAYPGPHRL-----
* * * * * : * * * : * * * : * * * : * * * : * * * : * * *

A.fumigatus-DBiNU-1 GPTQTHYGRCGGQGTGTACASPYTCQVLNFWYSQCL
A.fumigatus-MKU1 GPTQTHYGRCGGQGTGTACASPYTCQVLNFWYSQCL
A.terreus-MS-31 GATQTHWQCGGQGTGTACASPYTCQKQNEWYSECL
T.reesei-M5 SCTQTHWQCGGIGYSGCKTCTSGTTCQYSNDYYSQCL
A.nidulans -----
A.oryzae-KBN616 -----
F.oxysporum -----

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Fig. 2 – Alignments between the *A. fumigatus* DBiNU-1 endoglucanase sequence and the sequences of other fungal endoglucanases from GH7. The putative catalytic amino acid residues Asp¹⁹¹ and Glu³⁶⁹ are shown in the boxes.

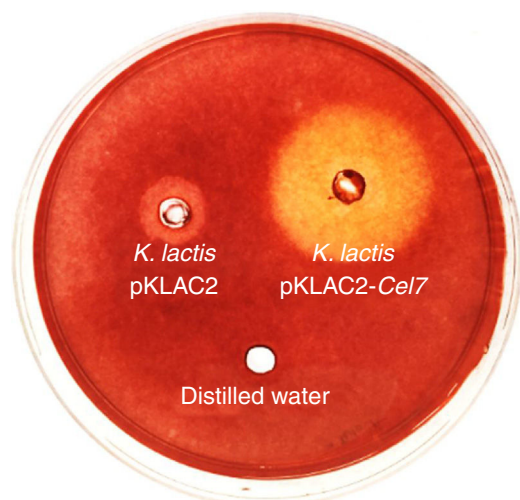


Fig. 3 – The activity of the endoglucanase from *A. fumigatus* DBiNU-1 that was expressed in the yeast *K. lactis*, as determined using YPD agar medium containing 1% CMC.

two from AA9), and two out of these three known intronless endoglucanase genes are predicted to have a cellulose-binding domain/module in the C-terminal region. In the current study, a gene that encodes an intronless endoglucanase in *A. fumigatus* DBiNU-1 was cloned, and its nucleotide and putative amino acid sequences were analyzed using GENETYX (Software Development, Tokyo, Japan). The results showed that the complete sequence of the gene that encodes the endoglucanase in *A. fumigatus* DBiNU-1, designated *Cel7*, was 1383bp in length and encoded a polypeptide of 460 amino acid residues (Fig. 1). Based on the amino acid sequence similarity in the CAZy database (<http://www.cazy.org>), the

endoglucanase from *A. fumigatus* DBiNU-1 was classified into the GH7 family,^{32–34} similar to endoglucanases from *Aspergillus* spp. such as *A. terreus* MS-31, *A. oryzae* KBN616, and *A. nidulans* FGSC44, and endoglucanases from *T. reesei* M5 and *Penicillium* sp. C7. According to the Pfam tool (<http://pfam.sanger.ac.uk/>), two domains, i.e., a catalytic domain in the N-terminal region (amino acids from position 22 to 398) and a fungal type CBD/CBM in the C-terminal region (amino acids from position 428 to 456), were found in the deduced amino acid sequences of the *A. fumigatus* *Cel7* gene product. As demonstrated by Sugimoto,³⁶ the CBD/CBM is important for the binding of cellulose. Cellulases that lack the CBD/CBM exhibited a reduction in cellulose degradation. It should be noted from the current study that a signal peptide containing 20 amino acid residues was detected in the N-terminal region, suggesting that the endoglucanase from *A. fumigatus* DBiNU-1 is an extracellular enzyme because the same feature has been found in an intronless endoglucanase from *A. fumigatus* MKU1.³ In addition to a signal peptide, two regions, GALYLSEM and IRWGDIG, that are highly conserved in all fungal endoglucanases in the GH7 family were detected in the endoglucanase from *A. fumigatus* DBiNU-1. This finding also verified that the *A. fumigatus* *Cel7* gene product was a member of the fungal GH7 family. No putative N-glycosylation site (N-W-T) was found in the predicted protein sequences, suggesting that the endoglucanase from *A. fumigatus* DBiNU-1 is not an N-glycosylated protein, which is distinct from the reported case of the endoglucanase from *A. ussuriensis* E001.⁷

The predicted molecular weight of the endoglucanase from *A. fumigatus* DBiNU-1, as confirmed by SDS-PAGE analysis, was approximately 48 kDa, which was distinct from other endoglucanases from other *Aspergillus* strains, e.g., *A. terreus* M11 (25 kDa),³⁷ *A. awamori* VTCC-F099 (32 kDa),³⁸ *A. fumigatus* KMU1 (35 kDa),³ *A. oryzae* AG1 (45 kDa),³⁹ *A. ussuriensis* E001 (24 kDa),⁷ and *A. fumigatus* ABK9 (56 kDa).²⁰ Many

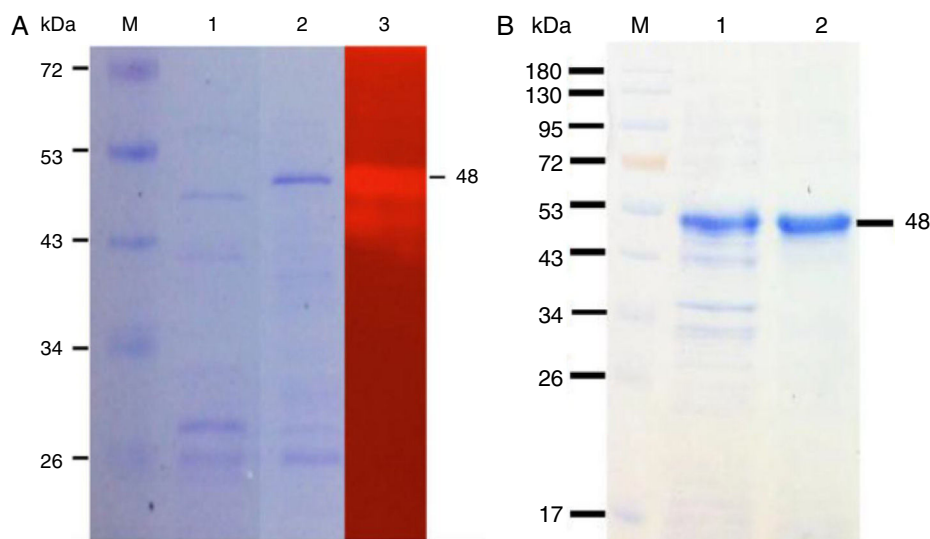


Fig. 4 – SDS-PAGE and zymogram analysis of the endoglucanase (A) and the purified recombinant endoglucanase (B) from *A. fumigatus* DBiNU-1. The protein samples (30 µg) were loaded on the gel and subjected to electrophoresis. (A) M, protein marker; 1, the cell-free supernatant from *K. lactis* GG799; 2, the cell-free supernatant from *K. lactis* harboring pKLAC2-*Cel7*; 3, zymogram analysis of the endoglucanase after Congo red staining. (B) M, protein marker; 1, crude recombinant endoglucanase; 2, purified recombinant endoglucanase.

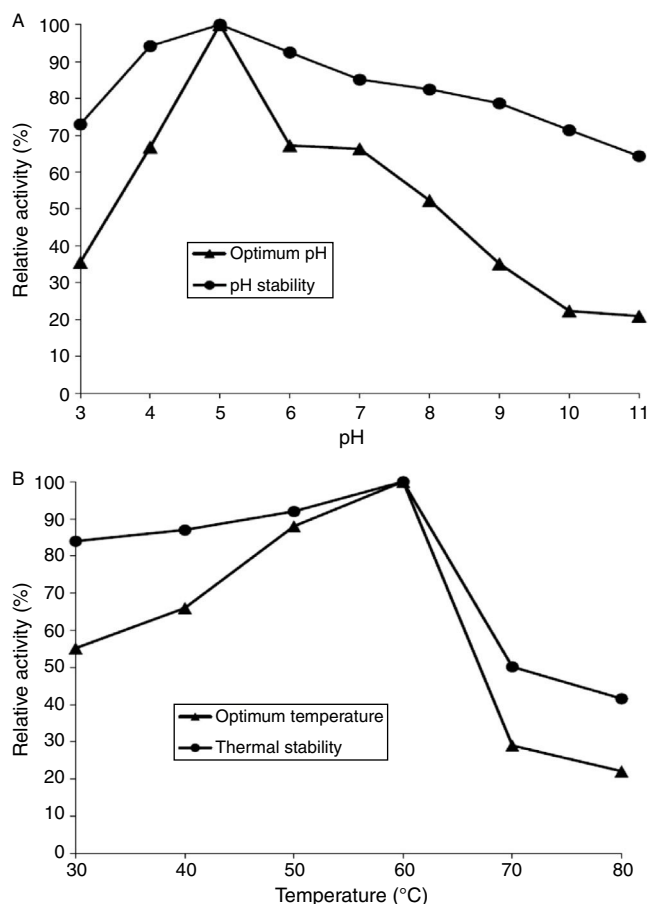


Fig. 5 – The effect of pH (A) and temperature (B) on the activity and stability of the purified recombinant endoglucanase from *A. fumigatus* DBiNU-1. The maximum enzyme activity (1.57 U/ml) assayed under the standard condition (60°C, pH 5.0) was taken as a control and recorded as 100%.

glycoside hydrolases such as GH7, GH12, GH16 and AA9 have a modular structure that consists of a catalytic domain and a substrate-binding domain.⁴⁰ The Swiss-PdbViewer (<http://www.expasy.org/spdbv>) was used to predict the three-dimensional structure of the putative amino acid sequence of the endoglucanase from *A. fumigatus* DBiNU-1 (Additional information section, Fig. 1). The GH7 endoglucanase from this thermotolerant fungus exhibited an arrangement of catalytic residues and a fold that are similar to those of the β -1,3-glucanases and β -1,3-1,4-glucanases of family 16.⁴¹

The aqueous two-phase system has been widely used to purify several enzymes. Compared with conventional purification methods, this technique provides a high yield of recovery with high level of enzyme activity and stability.³¹ In the current study, the recombinant endoglucanase from *A. fumigatus* DBiNU-1 that was expressed in *K. lactis* with the pKLAC2-Cel7 vector was purified using the aqueous two-phase system. A maximum enzyme activity and specific activity of 1.57 U/ml and 15.39 U/mg protein, respectively, were achieved; these values were approximately 2- and 5-fold greater than the corresponding original activities. The optimum pH of the activity

of the purified recombinant endoglucanase from *A. fumigatus* DBiNU-1 was 5.0, and the enzyme was highly stable in a pH range from 4.0 to 8.0 (Fig. 5A). The purified recombinant endoglucanase also exhibited its highest activity at 60°C, and it over 80% of its original activity in a temperature range from 30 to 60°C (Fig. 5B). The results of the current study were in good agreement with those reported for most of the endoglucanase from *Aspergillus* strains, such as *A. terreus* DSM 826,⁴² *A. awamori* VTCC-F099,³⁸ *A. usamii* E001⁷ and *A. fumigatus* ABK9.²⁰ However, a high thermal stability (70°C) was also reported for endoglucanases from *T. aurantiacus*⁴³ and *A. fumigatus* MKU1.³ The differences in thermal stability indicated the different molecular properties of the enzymes, including the bonding that stabilizes in the structures and the conformations among the various species. It has been reported that endoglucanases with optimal activities between 55 and 80°C are considered thermostable enzymes.⁴⁴ The high thermal stability of the endoglucanase from *A. fumigatus* DBiNU-1 (60°C) make this enzyme a good candidate for application in the paper industry and in saccharification processes for the production of biofuel and other chemicals using cellulosic materials.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bjbm.2017.10.001](https://doi.org/10.1016/j.bjbm.2017.10.001).

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