The Journal of Microbiology (2012) Vol. 50, No. 3, pp. 518–525
DOI 10.1007/s12275-012-1207-5

Characterization of *Trichoderma reesei* Endoglucanase II Expressed Heterologously in *Pichia pastoris* for Better Biofinishing and Biostoning

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(Received April 21, 2011 / Accepted January 26, 2012)

The endoglucanase II of *Trichoderma reesei* is considered the most effective enzyme for biofinishing cotton fabrics and biostoning denim garments. However, the commercially available preparation of endoglucanase II is usually mixed with other cellulase components, especially endoglucanase I, resulting in hydrolysis and weight loss of garments during biofinishing and biostoning. We thus isolated the endoglucanase II gene from *T. reesei* to express this in *Pichia pastoris,* under the control of a methanol-inducible AOX1 promoter, to avoid the presence of other cellulase components. A highly expressible Mut$^+$ transformant was selected and its expression in BMMH medium was found most suitable for the production of large amounts of the recombinant protein. Recombinant endoglucanase II was purified to electrophoretic homogeneity, and functionally characterized by activity staining. The specific activity of recombinant endoglucanase II was found to be 220.57 EU/mg of protein. Purified recombinant endoglucanase II was estimated to have a molecular mass of 52.8 kDa. The increase in molecular mass was likely due to hyperglycosylation. Hyperglycosylation of recombinant endoglucanase II secreted by *P. pastoris* did not change the temperature or pH optima as compared to the native protein, but did result in increased thermostability. Kinetic analysis showed that recombinant endoglucanase was most active against amorphous cellulose, such as carboxymethyl cellulose, for which it also had a high affinity.

Keywords: endoglucanase, hyperglycosylation, methanol inducible promoter, *Trichoderma reesei*, thermostability

Introduction

Trichoderma reesei is the most widely studied and industrially important cellulolytic fungus. This fungus produces two genetically different cellobiohydrolases (CBH I and CBH II, E.C.3.2.1.91), eight genetically different endoglucanases (EG I to EG VIII, E.C.3.2.1.4), and one β-glycosidase (Foreman *et al*., 2003). These enzymes have been used in laundry detergents and have potential uses for bioenergy production, and in the textile and pulp and paper industries. Among eight different endoglucanases, endoglucanase I and endoglucanase II are predominant. Lack of endoglucanase II production reduces the endoglucanase activity in the culture supernatant of *T. reesei* by as much as 55%, whereas lack of EG I reduces it by only 25% (Suominen *et al*., 1993). Thus, endoglucanase II appears to account for most of the endoglucanase activity of *T. reesei*. Endoglucanase I represents 5% to 10% of the secreted proteins (Penttila *et al*., 1987). *T. reesei* endoglucanase II has the highest catalytic efficiency among the endoglucanases (Macarron *et al*., 1993; Medve *et al*., 1998).

The use of cellulase in biofinishing or biopolishing cellulose-based fabrics was initiated in the 1980s and early 1990s, and resulted in superior hand-feel and novel finishes on rayon, linen, and cotton knits by removing the surface fuzz (cellulose fibrils and micro fibrils). *T. reesei* cellulase is also used in biostoning, in place of, or in addition to, the use of pumice stones to produce stonewash effects on denim fabrics. The efficiency of cellulase in stonewashing or biopolishing is judged by the CMCase activities (efficiency in hydrolysis of carboxy-methyl cellulose) of endoglucanases and cellobiohydrolases. If the CMCase activities of purified CBH I, CBH II, EGI, EGII of *T. reesei* are compared separately, it is found that EGII is the most active, followed by EG I, CBH II, CBH I in decreasing order (Gama *et al*., 1998). The presence of cellobiohydrolases and endoglucanase I in cellulase preparations from *T. reesei* make it unsuitable for biofinishing and biostoning, as endoglucanase I and cellobiohydrolases lead to loss of the strength and weight of cellulosic materials (Gusakov *et al*., 2000). It has been shown that the loss of strength and weight is mainly caused by endoglucanase I (EG I), whereas improved biofinishing and biostoning with minimum loss of weight and strength can be achieved by using endoglucanase II (Miettinen-Oinonen *et al*., 2001). It has also been found that purified *T. reesei* endoglucanase II was the most effective at removing colour from denim garments producing a good stone-wash effect with the lowest hydrolysis level (Heikinheimo *et al*., 2000). Therefore, the endoglucanase II of *T. reesei* is the best enzyme for biofinishing and biostoning denim fabrics.

It has been reported that a *T. reesei* strain producing high levels of EG I and II activity, without CBH I and II, has been constructed by replacing the *cbh2* locus with the coding region of the *egl2* gene in the EGI-overproducing CBHI-negative strain. Production of endoglucanase activity (both

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endoglucanases I and II) by the EG-transformant strain was increased fourfold above that of the host strain (Miettinen-Oinonen and Souminen, 2002). No wild type nor any existing mutant strain of *T. reesei* can produce endoglucanase II as the only endoglucanase component in *T. reesei* cellulase. Therefore, a system with sole production of *T. reesei* endoglucanase II would be in great demand by the textile industry for biostoning and biofinishing and for other applications.

Pichia pastoris provides an efficient heterologous gene expression system that is extensively used for production of a large number of eukaryotic proteins of commercial interest (Romanos *et al*., 1992; Cregg *et al*., 1993). Our aim was thus to obtain heterologous expression of the *endoglucanase II* gene of *T. reesei* in *P. pastoris*, to produce endoglucanase II in large quantities without exoglucanases or endoglucanase I, for use in the textile industry and for other applications.

Materials and Methods

Materials

Restriction endonucleases, ligases, and Deep Vent Polymerase were purchased from New England Biolabs (USA). Q-sepharose was purchased from Amersham Pharmacia Biotech (UK). Media for bacterial, fungal, and *P. pastoris* culture were purchased from HiMedia (India). Dinitrosalisylic acid (DNS) and carboxy-methyl cellulose (CMC) were purchased from Merck (Mumbai, India). The antibiotic zeocin, was purchased from Invitrogen (USA). Avicel and Cellobiose were purchased from Sigma (USA).

Strains and plasmids

Strains: *T. reesei* (local source), *E. coli* DH10B (Stratagene, CA, USA), *Pichia pastoris*, GS115 (*his 4*) (Invitrogen).
Plasmids: pUC18 (Stratagene), pPICZαA (Invitrogen).

Recombinant PCR, cloning, and sequencing of the endoglucanase gene

The published nucleotide sequence (M19373) of the *endoglucanase II* gene of *T. reesei* shows that the *endoglucanase II* gene has one intron between exon I and exon II. Hence, the coding region of the gene was isolated in two fragments. The full-length gene without intron was obtained by recombinant PCR using two pairs of primers designed on the basis of the reported nucleotide sequence.

The primer pairs used for isolation of exon I were:
5′-CCGG*GAATTC*CAGCAGACTGTCTGGGGCC-3′ (5′ start primer)
5′-GAGGTAACGCAAGTGCCA**TCTGTGGTACAGCCAAAG**-3′ (3′ middle primer)

The primer pairs used for isolation of exon II were:
5′-**CTTTGGCTGTACCACAGA**TGGCACTTGCGTTACCTC-3′ (5′ middle primer)
5′-CGCCGC*GGATCC*CTACTTTCTTGCGAGACACGAGC-3′ (3′ end primer)

In the 5′ middle and 3′ middle primers bold lettering represents the area from exon I and normal lettering that from exon II. Italics represent the *Eco*RI and *Bam*HI restriction sites added into the primers to allow directional cloning of the *endoglucanase II* ORF into pUC18. The PCR was set up as follows: 100 ng of genomic DNA was used as template for a 25 µl reaction with 2.5 µl of 10× thermopile buffer, 0.5 units of Deep Vent DNA Polymerase (New England Biolabs, USA), 2.5 µl of 2 mM dNTPs, 1 µl of each primer at 10 µM concentrations, and 0.3 µl of 100 mM magnesium sulphate. The thermal profile used was: initial heat denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 40 sec, 58°C for 40 sec and 75°C for 1 min with a final extension at 75°C for 7 min. In the first cycle of normal PCR, exon I and exon II were generated separately. The full-length gene was generated by recombinant PCR from the first round of PCR products using the same thermal profile as above. The recombinant PCR product was cloned following standard techniques (Maniatis *et al*., 1982) and sequenced following Sanger's dideoxy chain termination method (Sanger *et al*., 1977) using a DNA sequencing kit (T7v 2.0, Amersham Pharmacia Biotech).

Construction of the *endoglucanase II* gene expression vector, and its electro-transformation into *P. pastoris*, GS115

The *endoglucanase II* gene was excised from recombinant pUC18 by *Eco*RI/*Xba*I restriction digestions, ligated into the pPICZαA vector along with its *S. cerevisiae* α-factor signal sequence, without fusion to the his-tag, and then transformed into the *E. coli* strain Top10F. The expression construct, pPICZαA/*endoglucanaseII* was linearized by restriction digestion with *Bst*XI. Six microgram of linearized recombinant DNA were used for electroporation using an electroporator 2510 (Eppendorf). Transformants were selected on YEPDS plates with zeocin, 100 µg/ml.

Selection of multi-copy transformants

The recombinant clones obtained were streaked on YEPD plates with increasing concentrations of zeocin i.e. 500, 1000, and 2000 µg/ml, and incubated at 28°C for 2 days. Transformants were examined for their expression of endoglucanase, measured by a plate diffusion assay (Farkas *et al*., 1985). Colonies were streaked onto plates containing minimal medium with 1.0% (w/v) carboxyl methylcellulose and 0.5% (v/v) methanol, and incubated at 28°C for 12 h. Thereafter, the plates were stained with 0.1% Congo red solution for 30 min, and washed with 1 M sodium chloride solutions several times over 1 h.

Expression of endoglucanase II in BMMY and BMMH media

For expression in BMMY medium, an initial inoculum was prepared by inoculating a single colony in 5 ml BMGY medium, and growing at 28°C overnight until the cell density reached OD_{600}=4.0. The inoculum was added to 25 ml BMMY medum in such a way that the final OD_{600} became 1.0. Methanol (0.5%, v/v) was added at 24 h intervals for induction, and the colony was allowed to grow at 150 rpm and 28°C for 60 h. Every 12 h, 1.5 ml of culture supernatant was withdrawn. Endoglucanase activity was measured by the dinitrosalicylic acid (DNS) method (Wood and Bhatt, 1988) using CMC as substrate (CMCase assay). Expression in BMMH medium was carried out in the same way.

Endoglucanase assay

The endoglucanase assay was done following the DNS method. 100 µl of 1.0% CMC solution, 200 µl of 100 mM Tris-HCl buffer (pH 6.8) and 300 µl of culture supernatant were added to the assay mixture. As a blank, 300 µl of Tris-HCl buffer and 300 µl of culture supernatant from the vector control for the same induction time were used. The assay mixture was incubated at 40°C for 20 min and then 450 µl of DNS solution was added. The total reaction mixture was incubated in a boiling water bath for 5 min. The optical density was measured at 540 nm to estimate the quantity of reducing sugars produced in the assay mixture. The endoglucanase activity was measured as the number of micromoles of reducing sugar produced per min, per ml of total protein, or per mg of total protein.

Purification of recombinant endoglucanase II protein

Secreted proteins were enriched by ammonium sulfate fractionation. The active fraction (40–80%) was purified using a Q-sepharose anion exchange column. One milliliter of column material was used and the column was equilibrated with 20 mM Tris-HCl (pH 6.8) buffer overnight. 5.2 mg of the ammonium sulfate fraction was loaded onto the equilibrated column. A five-bed volume of the same buffer was passed through the column before elution with a step-wise increase of a sodium chloride gradient (25, 50, 75, and 100 mM) in the same buffer, using a flow rate of 4 ml/h. One mililiter of the eluant was collected in each 1.5 ml centrifuge tube. The protein content in each tube was estimated by the Bradford's dye binding method, and the activity of endoglucanase in each tube was measured by the endoglucanase assay.

SDS-PAGE and activity staining

The SDS PAGE of the purified protein was performed following Laemmli (1970). Activity staining of purified protein was performed following Chen and Buller (1994) to detect the functional activity of the recombinant endoglucanase. A 10% SDS-PAGE, with 0.1% CMC in an acrylamide-bisacrylamide mixture was carried out using the purified protein. After electrophoresis, the gel was kept in 25% isopropanol at 16°C overnight, and then washed with 20 mM of Tris-HCl buffer several times for 3 h. Subsequently the gel was kept in 20 mM Tris-HCl at 37°C for another 3 h. Finally, the gel was stained with 0.1% Congo red solution for 30 min and destained with 1 M sodium chloride.

Determination of temperature optimum and temperature stability

To determine the temperature optimum of purified recombinant endoglucanase, the endoglucanase activity was estimated at different temperatures (40°C, 50°C, 55°C, 60°C, 70°C, and 80°C) under the standard assay conditions, using 1.0% CMC solution as substrate.

To determine the thermal stability of purified recombinant endoglucanase, equal quantities of purified endoglucanase were pre-incubated in 20 mM Tris-HCl buffer (pH 6.5) for 10, 20, 30, 40, 50, and 60 min, at 40°C, 50°C, 55°C, 60°C, 70°C, and 80°C. After pre-incubation, relative endoglucanase activities were measured under standard assay conditions. Considering the highest endoglucanase activity as 100%, residual endoglucanase activities were measured and graphically represented.

Determination of optimum pH

To determine the optimum pH, an endoglucanase assay was carried out under standard assay conditions while keeping equal amounts of purified endoglucanase II at different pH (4.0, 5.0, 6.0, 6.5, 7.0, 8.0, and 9.0).

Determination of substrate specificity and kinetics for the purified recombinant endoglucanase II

The specificity of purified recombinant endoglucanase towards different substrates was determined using different substrates e.g. CMC (1%), Avicel (1%), cellobiose (1%), and filter paper (50 mg). 100 µl of specific substrate, 400 µl of 100 mM Tris-HCl buffer (pH 6.8), and 100 µl of purified enzyme were added to the assay mixture and incubated at 40°C for 20 min. The Michaelis-Menten constant (K_m) and maximum velocity of substrate hydrolysis (V_{max}) were determined from a Lineweaver-Burk plot.

Results and Discussion

Isolation of the *endoglucanase II* gene

The *endoglucanase II* gene, without the secretion signal, was successfully isolated from the genomic DNA of *T. reesei* by recombinant PCR. DNA fragments of 0.264 kb for exon I and 0.927 kb for exon II were initially generated separately. These two exon fragments were then ligated by recombinant PCR to generate the full length (1,191 bp) coding region of the *endoglucanase II* gene (Fig. 1). The gene was successfully cloned into the *Eco*RI and *Bam*HI restriction sites in pUC18. The authenticity of the *endoglucanase II* gene was verified by sequencing and restriction analysis (data not shown). Expression of any gene in a heterologous system such as *P. pastoris* requires some modifications, as (1) the

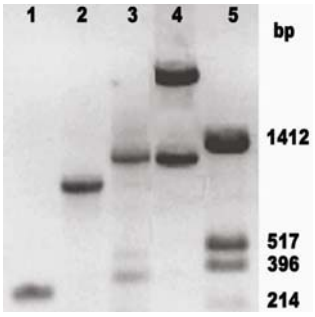

Fig. 1. 1% agarose gel showing PCR products and one of the clones containing *endoglucanase II* gene. Lanes: 1, Exon I; 2, Exon II; 3, Full length PCR product representing *endoglucanase II* gene; 4, *Eco*RI and *Bam*HI digested clone, Upper fragment is *Eco*RI and *Bam*HI digested pUC18 and lower fragment is *endoglucanase II* gene; 5, Molecular weight marker (pUC 18 digested with *Hin*fI).

yeast splicing apparatus may not recognize higher eukaryotic intron sequences (2) the yeast secretory apparatus may not recognize the native signal sequences of higher eukaryotes efficiently. Therefore, the 1,191 bp coding region of the *eg II* gene, lacking the intron, was isolated without the native secretory signal.

Construction of chimeric *endoglucanase II* gene expression vector

The *endoglucanase II* gene was cloned in the *P. pastoris* expression vector, pPICZαA, at the *Eco*RI and *Xba*I restriction sites, in-frame with the *S. cerevisiae* α-factor secretion signal, under the control of a methanol-inducible AOX1 promoter. The *endoglucanase II* gene was prevented from fusing with the his-tag or the Myc epitope of the pPICZαA expression vector by incorporating the termination codon at the end of the coding sequence of the gene. It was important to terminate transcription at this point because modification of the C-terminus by the addition of the Myc epitope or polyhistidine tagged amino acids might have rendered the recombinant protein biologically inactive. Proper orientation of the *endoglucanase II* gene with respect to the α-factor secretion signal was verified through restriction analysis (data not shown) and sequencing.

Transformation of chimeric *endoglucanase II* gene expression vector in *P. pastoris* and selection of multicopy transformants

The linearized chimeric DNA was transformed into *P. pastoris*, GS115 cells, through electroporation. Integration of the transgene into the *Pichia* genome took place as planned at the AOX1 locus using pPICZαA as the expression vector. To facilitate the integration, the chimeric gene expression vector had to be linearized within the AOX1 locus. To achieve this, the vector was linearized by *Bst*XI restriction. This digestion can cleave only at the AOX1 region of the pPICZαA vector, which increases the efficiency of transformation and may allow the integration of more than one copy of the gene into the *Pichia* genome.

The recombinant clones were selected on YEPD plates containing zeocin (100 μg/ml) as a selection drug. A total of 1000 recombinant clones were picked and used for screening, after growth at 28°C for 48 h. Identification of multi-copy recombinant clones was done through stepwise screening against increasing concentrations of zeocin in YEPD plates, assuming they would show increased resistance against zeocin. For this, all the clones were first exposed to YEPD medium containing 500 μg/ml of zeocin, and 902 clones were found to grow normally. The concentration of zeocin in the YEPD medium was then increased to 1,000 μg/ml and transformants were still found to grow properly. Finally, the surviving clones were exposed to 2,000 μg/ml of zeocin and only 5 transformants were able to survive (Fig. 2).

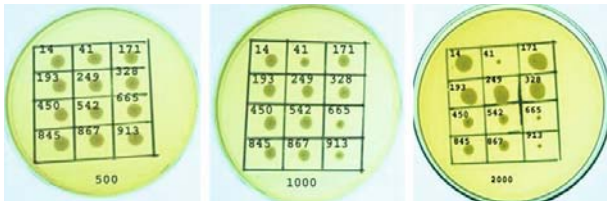

Fig. 2. Representative figure for detection of transformants containing multicopy integration of *endoglucanase II* gene. 500, 1000, and 2000 represent amount of zeocin in μg/ml on YEPD plates and other numbers written in the box represent clone number.

Fig. 3. Plate diffusion assay showing the expression profile of different recombinant transformants: No. indicates the respective no. of transformants, VC stands for vector control.

Identification of high-expression recombinant clones was further carried out through the plate diffusion assay method. Clones that could grow properly in each of the different concentrations of zeocin were selected randomly. Varying numbers of clones from each drug concentration were chosen for the plate diffusion assay: clones 1, 41, 542, 724, 892, 901, and 913 from 500 μg/ml; 22, 450, 665, 845, 867, and 1000 from 1000 μg/ml and 14, 171, 193, 249, and 328 from 2000 μg/ml of zeocin were selected. This experiment was carried out to determine their level of expression according to the copy number of the *endoglucanase II* gene present in the transformants.

As CMC was present as the sole carbon source in minimal medium, on induction with 0.5% methanol, transformants secreted endoglucanase, which hydrolyzed CMC for their survival in minimal medium. After 12 h of growth at 28°C, the plates were stained with 0.1% Congo red to detect hydrolysis of the CMC. High-expression clones were selected on the basis of the size of the orange-coloured halo zone of clearance produced by endoglucanase II. Five transformants (no.s 14, 171, 193, 249, 328) were observed to express at high levels in this experiment (Fig. 3). These five transformants were chosen to test their expression in liquid medium (BMMY). The results from this experiment confirmed that the transformants that survived at highest zeocin concentration, also produced the highest levels of endoglucanase II protein. The size of the halo produced through the degradation of CMC correlated well with increasing zeocin resistance of the transformants.

Expression of transformants in BMMY medium

All the selected clones were Mut+ (data not shown). Production levels of recombinant endoglucanase in the culture supernatants, by each selected transformant on induction with 0.5% methanol (v/v) in BMMY medium, were estimated at 12 h intervals by the endoglucanase assay. Maximum endoglucanase productivity was obtained at 60 h following methanol induction. Productivity of each transformant with regard to 0–80% ammonium sulfate-fractionated, secretory proteins after 60 h of growth, was estimated by an endoglu-

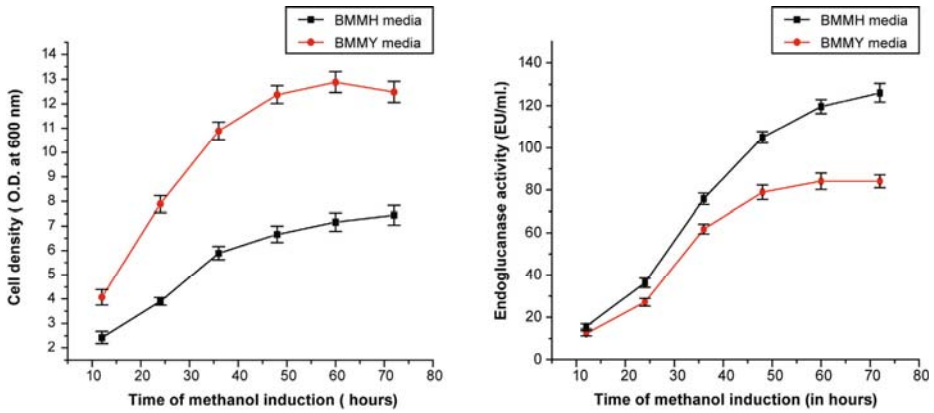

Fig. 4. Growth and expression profile of highest expressible recombinant transformant no. # 249 in BMMY (—●—) media and in BMMH media (—■—). (A) Growth curve of recombinant transformant (B) Expression of endoglucanase by recombinant transformant.

canase assay in terms of EU/mg of total protein (data not shown). Productivities differed among all five transformants. The best endoglucanase-producing transformant (249) was selected for further study.

Selection of the best medium for optimum production of endoglucanase

The transformant yielding the highest production of endoglucanase was allowed to grow in two different media, BMMH (a minimal medium) and BMMY. Maximum expression of endoglucanase II in the recombinant clone was reached after 72 h in BMMH and after 60 h in BMMY medium (Fig. 4). The growth of this transformant was better in BMMY medium, but the production of the recombinant endoglucanase was found to be 1.5 fold higher in BMMH medium than in BMMY (Fig. 4).

The change of growth medium from BMMY to BMMH resulted in an increase in endoglucanase production of ~1.5 fold for the best producing clone. Time course analysis of growth and expression optimization of the best recombinant clone showed a higher expression level of endoglucanase II despite its reduced growth in minimal medium (BMMH). This indicates that the production of recombinant endoglucanase II does not depend on cell density, which is supported by earlier evidence that culturing *P. pastoris* in minimal media increases the production of recombinant protein, and that production of the recombinant protein is not proportional to cell growth (Cregg *et al.*, 1993). We found that the maximum amount of recombinant endoglucanase was produced in a shake flask of BMMH medium with 1% methanol induction, at 25°C; the amount was estimated to be 14.4 g/L.

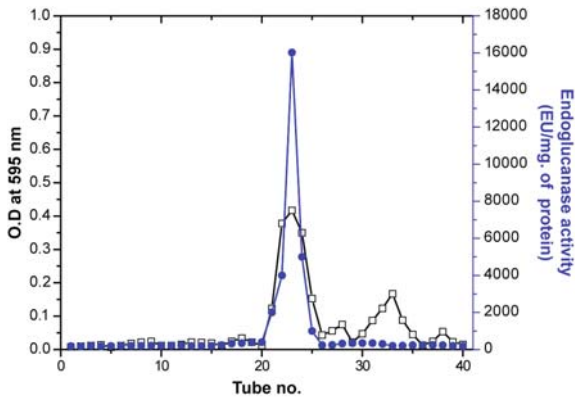

Fig. 5. Elution profile of proteins charged onto the anion-exchanger, Q-Sepharose Fast Flow column at different sodium chloride concentration. Protein amount in each tube (—□—) and activity profile of recombinant Endoglucanase (—●—) in each tube. Tube number 1-10=25 mM, 11-20=50 mM, 21-30=75 mM and 31-40=100 mM Sodium Chloride concentrations.

Purification of endoglucanase protein

Recombinant *P. pastoris* culture filtrate was fractionated with two concentrations of ammonium sulfate (0–40% and 40–80% saturation). Measurement of the endoglucanase activity in both of the fractions indicated that the 40–80% fraction retained 90% of the total endoglucanase activity in the culture filtrate. The endoglucanase-rich fractions were further purified through an anion-exchange column, Q-Sepharose Fast Flow at pH 6.8 (in 20 mM Tris-HCl buffer). Elution of protein from the column was performed using a step gradient of sodium chloride (25, 50, 75, and 100 mM). A typical elution pattern of the Q-Sepharose chromatography is shown in Fig. 5. This exhibited a single activity peak. The recombinant endoglucanase was highly purified, as shown by the single protein band in the SDS-PAGE analysis, corresponding to a molecular mass of about 53 kDa (Fig. 6, lane 2). A clear band signifying cellulolytic activity (Fig. 6, lane 1) produced by activity staining, confirmed the

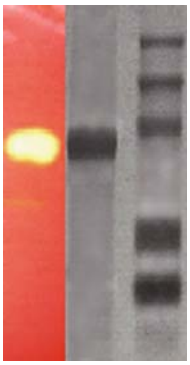

Fig. 6. SDS-PAGE analysis and activity staining of purified recombinant Endoglucanase II. Lanes: 1, Activity staining of purified protein; 2, SDS-PAGE of purified protein; 3, Molecular weight markers.

Table 1. Purification of recombinant Endoglucanase II from transformant of *P. pastoris*, GS115

Purification step	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Recovery	Fold of purification
Culture broth	18	466.92	25.94	100	
40%-80% Ammonium sulphate fractionation	5.2	380.38	73.15	81.46	2.82
Q-Sepharose	0.96	211.75	220.57	45.35	8.50

EU: One unit of CMCase activity, defined as the amount of enzyme releasing one micromole of reducing sugar per min.

production of functionally active recombinant endoglucanase II.

The specific activities to CMC and the recovery yields of recombinant enzyme after purification are summarized in Table 1. The specific activity of recombinant endoglucanase II was 220.57; higher than the CMCase activity of the endoglucanase II catalytic domain expressed in *E. coli* (Nakazawa *et al*., 2008), and the CMCase activity of endoglucanase II expressed in *S. cerevisiae* (Qin *et al*., 2008).

Characterization of recombinant endoglucanase II

Temperature optimum and thermal stability: Enzyme activity of the recombinant endoglucanase was assayed at different temperatures ranging from 40°C to 80°C, and the optimum temperature was found to be 55°C (Fig. 7), which is the same as the temperature optimum of native endoglucanases (Saloheimo *et al*., 1988).

We also studied the effect of temperature on enzyme stability (Fig. 8). Pre-incubation of equal quantities of recombinant endoglucanase at different temperatures for different time intervals, followed by estimation of residual activity, indicated that the activity at 55°C could be retained for 60 min. Most of the endoglucanase activity was retained for up to 40 min at 60°C, but after 40 min endoglucanase activity was appreciably reduced. During pre-incubation of purified endoglucanase at 70°C and 80°C, the activity was reduced drastically even after 10 min of pre-incubation. The native protein is known to be stable - up to 50°C for 60 min, but beyond 50°C the activity of the native protein decreases sharply (Saloheimo *et al*., 1988). More than 80% of the activity of recombinant endoglucanase is retained even at 60°C, a temperature at which native endoglucanase II is rapidly destroyed. The molecular mass of the purified recombinant endoglucanase, determined by the R_f value method, was found to be 52.8 kDa (data not shown). There was an almost 4.0 kDa increase in the molecular weight of recombinant endoglucanase over the native endoglucanase (Saloheimo *et al*., 1988), possibly due to hyperglycosylation (Cereghino and Cregg, 1999; Han and Lei, 1999; Ogawa *et al*., 1999).

Determination of optimum pH: The measurement of endoglucanase activity at different pHs in the range of 4–9 indicated that the recombinant endoglucanase was most active at pH 5.0. About 50% of the maximal activity of the recombinant enzyme was retained at pH 7.0 and more than 60% of its maximal activity was retained at pH 4.0. Thus the recombinant endoglucanase is appreciably stable in an

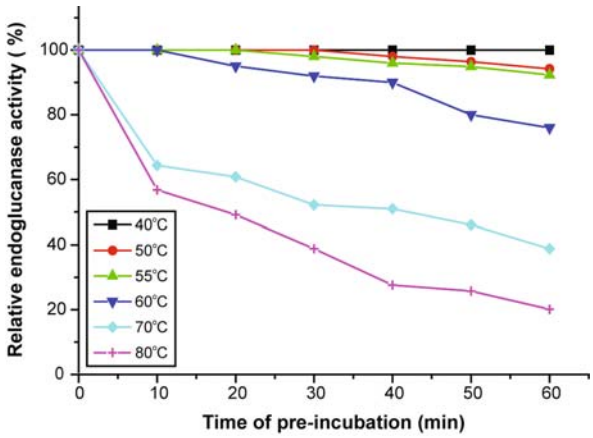

Fig. 8. Determination of thermal stability of recombinant Endoglucanase II at different temperatures.

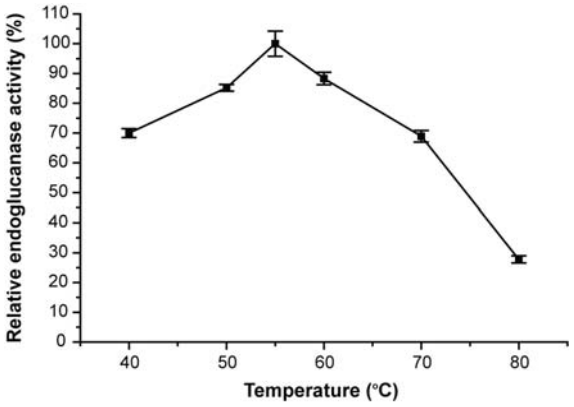

Fig. 7. Determination of temperature optimum of recombinant Endoglucanase II. Values are averages of triplicate assays, bars indicate SD.

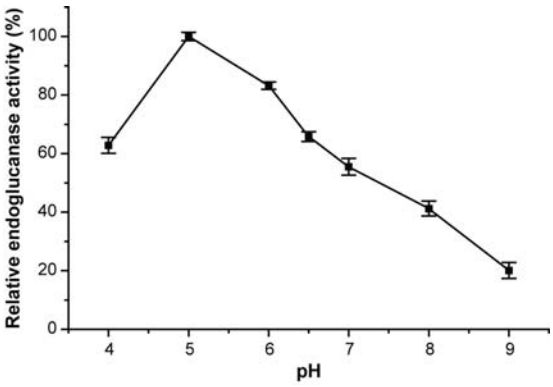

Fig. 9. Determination of pH optimum of recombinant Endoglucanase II. Values are averages of triplicate assays, bars indicate SD.

Table 2. Enzymatic activity of recombinant endoglucanase on various cellulosic substrates

Substrate	Enzymatic activity (EU/mg of protein)
Carboxymethyl cellulose (CMC)	220.18
Cellobiose	14.12
Avicel	No detectable activity
Filter paper (Whatman filter paper no. 1)	No detectable activity

acidic pH range; however it loses its activity quickly above pH 7.0 (Fig. 9). The native endoglucanase also showed the same pH optimum (Saloheimo *et al.*, 1988).

Substrate specificity and kinetic analysis: The substrate specificity of the purified recombinant endoglucanase II was ascertained by measuring its specific activity on different cellulose-derived substrates, such as CMC, cellobiose, filter paper, and avicel (Table 2). The enzyme was found to be most active towards CMC with a specific activity of 220.57 EU/mg. The enzyme showed very low activity towards cellobiose, and no detectable activity towards avicel or filter paper. These results clearly indicate that the recombinant enzyme had less activity on shorter molecules of cellulose (dimers) and no activity against crystalline cellulose (filter paper and avicel) and, like the native enzyme, it preferentially hydrolyzed longer chain amorphous cellulose - the hallmark of a Cel 5A endoglucanase, the family to which endoglucanase II belongs (Karlsson *et al.*, 2002a, 2002b).

Enzyme kinetic analysis was performed by the measurement of the initial rate of hydrolysis of CMC by recombinant endoglucanase. The initial reaction rates were analyzed using a Lineweaver-Burk plot. The enzyme had a Michaelis Menten constant (K_m) of 2.1 mg/ml and maximum reaction velocity (V_{max}) of 220.57 micromole/min/mg of total protein for CMC hydrolysis. The lower value of the K_m indicates that the recombinant enzyme has a high affinity towards CMC (Aboul-Enein *et al.*, 2010).

Heterologous expression of the *endoglucanase II* gene of *T. reesei* in *P. pastoris* yields an enzyme that does not cause weight loss of cellulosic fibers when it is used in denim washes and biopolishing, a great improvement over the use of commercially available *T. reesei* cellulase. When previously the *T. reesei endoglucanase II* gene was expressed in *S. cerevisiae*, the yield of enzyme was low (Qin *et al.*, 2008). Using shake flasks, 14.4 mg of recombinant endoglucanase was produced in 1 L cultures of *P. pastoris*, an expression level that would be considered high.

The biochemical properties of the native and recombinant endoglucanases (pH and temperature optima, and substrate specificity) are almost identical. Thus, the recombinant endoglucanase II is suitable for commercial use in denim washing and biopolishing. The increased thermostability of the recombinant endoglucanase is an added advantage, as increased thermostability is a trait much sought after, especially by the textile and detergent industries.

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