PROGRESS IN BIOLOGICAL SCIENCES

Comparison of biochemical properties of recombinant endoglucanase II of *Trichoderma reesei* in methylotrophic yeasts, *Pichia pastoris* and *Hansenula polymorpha*

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Abstract

Bioconversion of cellulosic material into bioethanol needs cellulase complex enzymes that contain endoglucanase, exoglucanase and beta glucosidase. One of the most important organisms that produce cellulases is the filamentous fungi, *Trichoderma reesei* which able to secrete large amounts of different cellulases. These enzymes are probably the most widely used cellulases industrially, however, the cellulases excreted from fungi are not stable at high pH or high temperatures. In this study methylotrophic yeasts, *Pichia pastoris* and *Hansenula polymorpha* were used for the comparative heterologous production of endoglucanase II. Two synthetic *egII* genes with *P. pastoris* and *H. polymorpha* codon preferences were transferred into the yeasts. In addition, both expression vectors contained the pre-pro-sequence of *Saccharomyces cerevisiae* mating factor alpha to allow secretion of protein. Enzymes characterization demonstrated increasing thermal stability in both recombinants EGII compare with native enzyme from *T. reesei* and the *Hansenula* enzyme was more stable than *Pichia* in higher temperature. Biochemical properties determination on different substrates showed higher binding site affinity in *Pichia* than *Hansenula* and native one. We can conclude that *P. pastoris* and *H. polymorpha* are appropriate hosts for expression and production of endoglucanase with improved thermal stability.

Keywords: *Hansenula polymorpha*, *Pichia pastoris*, Recombinant protein, endoglucanase

Introduction

For production of recombinant proteins, scientists use various hosts such as bacteria (1), yeast (2-4), plant (5) or insect cells (6). Recombinant protein expression in *E. coli* usually makes inactive inclusion bodies (7). In different expression systems, yeast expression system besides providing high expression level, simplicity of techniques needed, eukaryotic posttranslational modifications such as glycosylation, can be utilized for increasing the stability of proteins (8, 9). However *Saccharomyces cerevisiae* for recombinant expression of heterologous proteins, has some problems such as hyperglycosylation, failure in secretion of the recombinant proteins and low levels of expression limit its use, especially for biotechnological production (10).

Manipulation of methylotrophic yeasts is easy and they have a high growth rate on simple and inexpensive medium. Meanwhile, it has many of the advantages of eukaryotic expression systems (e.g., protein processing, folding, and post-

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translational modifications) (11) and they express recombinant proteins in high-density fermentor without loss of product yield and efficiently secrete heterologous proteins (12). The methylotrophic yeasts like *Pichia pastoris* and *Hansenula polymorpha* (*Pichia angusta*) are powerful systems for the heterologous expression of active and soluble mammalian proteins (11). Both of them be able to perform posttranslational modifications such as glycosylation and proteolytic processing (13). *P. pastoris* has become a popular academic tool whereas *H. polymorpha* has been used for industrial production of recombinant proteins.

One of the most important organisms that produce cellulases is the filamentous fungi, *Trichoderma reesei* which able to produce several different cellulases (1, 14). These enzymes can be utilized in several industries such as food, animal feed, textile, pulp and paper, printing and dyeing and chemical industries (15, 16). Among enzyme secreted from fungi *T. reesei*, endoglucanase II (EG II) is one of the most common endoglucanases which accounts for 5-10% of the total cellulase that randomly degrades internal β-1,4-glycosidic bonds within amorphous region of cellulose and has got most catalytic proficiency among others endoglucanases and has the optimal activity at pH 4-6 (17). Only in a few studies comparison of recombinant expression (and secretion) of proteins in several yeasts was carried out (11, 18). In this study we have sought to express synthetic EG II gene (GenBank Accession No. JF340120 and JN193556) from *T. reesei* in the yeasts *P. pastoris* and *H. polymorpha* and the extracellular expression of recombinant enzyme was investigated. Fig. 1 shows the amino acid sequence of the native endoglucanase II with their native signal peptide and the amino acid sequence of recombinant proteins contained the pre-pro-sequence of *S. cerevisiae* mating factor alpha as they were produced in yeast. The *egII* gene is 1197 bp long comprising one open-reading frame encoding a polypeptide of 397 amino acid residues. This gene with two different codon preferences was synthesized and it was cloned under the control of AOX1 inducible promoter in *P. pastoris* and FMD promoter in *H. polymorpha*. Our result showed that active form of EGII was successfully expressed and secreted in both yeasts.

A. Endoglucanase II native sequence (GenBank DQ178347)

```
01    MNKSVAPLLL AASILYGGAV AQQTVWGQCG GIGWSGPTNC APGSACSTLN PYYAQCIPGA
61    TTITTSTRPP SGPTTTTRAT STSSSTPPTS SGVRFAGVNI AGFDFGCTTD GTCVTSKVYP
121   PLKNFTGSNN YPDGIGQMQH FVNEDGMTIF RLPVGWQYLV NNNLGGNLDS TSISKYDQLV
181   QGCLSLGAYC IVDIHNYARW NGGIIGQGGP TNAQFTSLWS QLASKYASQS RVWFGIMNEP
241   HDVNINTWAA TVQEVVTAIR NAGATSQFIS LPGNDWQSAG AFISDGSAAA LSQVTNPDGS
301   TTNLIFDVHK YLDSDNSGTH AECTTNNIDG AFSPLATWLR QNNRQAILTE TGGGNVQSCI
361   QDMCQQIQYL NQNSDVYLGY VGWGAGSFDS TYVLTETPTG SGNSWTDTSL VSSCLARK
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B. Endoglucanase II synthesized in P. pastoris and H. polymorpha

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01    MRFPSIFTAV LFAASSALAA PVNTTTEDET AQIPAEAVIG YSDLEGDFDV AVLPFSNSTN
61    NGLLFINTTI ASIAAKEEGV SLEKRQQTVW GQCGGIGWSG PTNCAPGSAC STLNPYYAQC
121   IPGATTITTS TRPPSGPTTT TRATSTSSST PPTSSGVRFA GVNIAGFDFG CTTDGTCVTS
181   KVYPPLKNFT GSNNYPDGIG QMQHFVNEDG MTIFRLPVGW QYLVNNNLGG NLDSTSISKY
241   DQLVQGCLSL GAYCIVDIHN YARWNGGIIG QGGPTNAQFT SLWSQLASKY ASQSRVWFGI
301   MNEPHDVNIN TWAATVQEVV TAIRNAGATS QFISLPGNDW QSAGAFISDG SAAALSQVTN
361   PDGSTTNLIF DVHKYLDSDN SGTHAECTTN NIDGAFSPLA TWLRQNNRQA ILTETGGGNV
421   QSCIQDMCQQ IQYLNQNSDV YLGYVGWGAG SFDSTYVLTE TPTGSGNSWT DTSLVSSCLA
481   RK
```

Figure 1. Amino acid sequences of secretory endoglucanase II. A. The primary structure of the native protein and its N-terminal native signal peptide (underlined) is shown. B. The amino acid sequence of recombinant endoglucanase II synthesized in *P. pastoris* and *H. polymorpha* (the alpha mating factor signal peptide of *S. cerevisiae* is underlined).

Materials and methods

strains, plasmids, culture conditions and DNA techniques

Escherichia coli strain DH5α was used for cloning, transformation, and propagation. *E. coli* bacteria were grown in Luria and Bertani (LB) medium at appropriate conditions (37ºC with 200 rpm shaking). Restriction enzymes, T_4 DNA ligase and *Taq* DNA polymerase were purchased from Fermentas (USA). The hosts, *P. pastoris* strain GS115 and *H. polymorpha* (*Pichia angusta*) strain RB11 (*ura3*$^-$) were obtained from Invitrogen (USA) and ARTES (Germany), respectively. Yeasts were cultured on Yeast extract Peptone Dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar). For expression of recombinant proteins, *P. pastoris* cells were cultured on Buffered Glycerol-complex (BMGY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.00004% biotin, 1% glycerol) to generate biomass and induced in Buffered Methanol-complex (BMMY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.00004% biotin, 0.5% methanol). YNB-1% Glycerol (0.17% Yeast Nitrogen Base, 0.5% ammonium sulphate, 1% glycerol) was used for protein expression in *H. polymorpha*. The growth conditions for *P. pastoris* was 28-30ºC with 300 rpm shaking and 37ºC with the same shaking conditions for *H. polymorpha*. pPinkα-HC (7.9 kb) and pFPMT-MFa (7.2 kb) expression vectors were used for *P. pastoris* and *H. polymorpha* transformation, respectively. Unless otherwise stated, standard DNA methodologies were used (19). Native endoglucanase II from *T. reesei* was purchased from Sigma Aldrich (C8546) and used as positive control enzyme in kinetic experiments.

Construction of the endoglucanase expression vectors and yeast transformation

A gene encoding endo-1,4-beta-glucanase *(egII*) from *T. reesei* was designed according to its protein sequence published at GenBank (Accession No. DQ178347). The codon usage was adapted to the codon bias of *P. pastoris* and *H. polymorpha* genes and the negative *cis*-acting sequence motifs were avoided. The designed sequences were synthesized by GeneArt Company (Germany) (GenBank Accession No. JF340120 and JN193556). The synthetic *egII* gene for *P. pastoris* was flanked by *Xho*I and *Kpn*I restriction sites at the 5′ and 3′ ends, respectively. It was subcloned into the *Xho*I/*Kpn*I cleavage sites of an expression vector pPinkα-HC, placing under the control of a methanol-inducible promoter (AOX1) (Fig. 2a). The synthetic *egII* gene for *H. polymorpha* was cloned into the *Hind*III/*Bam*HI restriction sites of pFPMT-MFa expression vector downstream of FMD promoter (Fig. 2b). Both proteins contained the pre-pro-sequence of *S. cerevisiae* mating factor alpha to allow secretion.

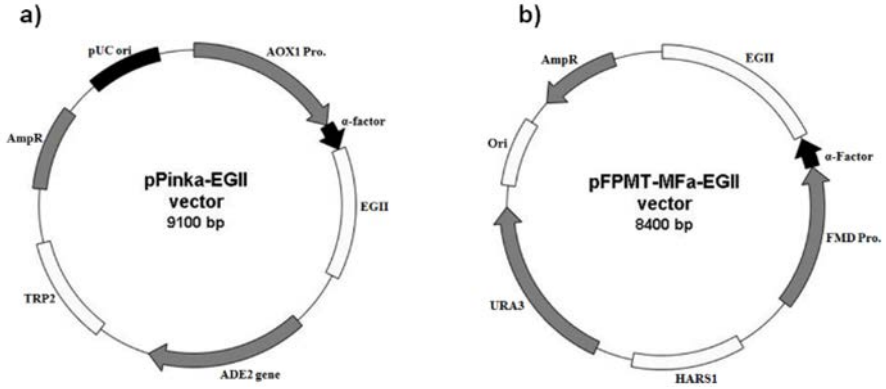

Figure 2. **(a)** Construction of expression vectors pPinkα-EGII and **(b)** pFPMT-MFa-EGII.

For *P. pastoris* transformation, recombinant expression plasmid pPinkα-EGII was linearized by digestion with *Bsp*T1 restriction enzyme to target the integration of the expression cassette into the TRP2 locus. The linearized pPinkα-EGII was transformed into *P. pastoris* GS115 by electroporation (BTX, ECM630 at 1800 V, 200 Ω and 25 µF with 0.2 cm cuvette) following

Invitrogen protocol. After electroporation, 1 mL of ice-cold 1 M sorbitol was immediately added to the cuvette. After incubation for 60 min at 28ºC, the cuvette contents were spread on PAD plates (*Pichia* Adenine Dropout) and incubated at 28°C for 3-10 days. As a negative control, the vector of pPinkα-HC was also linearized by *Bsp*T1 and transformed into *P. pastoris*.

H. polymorpha RB11 is a uracil auxotrophic (*Ura*$^-$) strain and can be converted into a prototroph (*Ura*$^+$) by uptake of plasmids that carry the URA3 expression cassette. Transformation of circular recombinant pFPMT-MFa-EGII plasmid into the *H. polymorpha* was carried out by electroporation (BTX, ECM630 at 2000 V, 200 Ω and 25 µF with 0.2 cm cuvette). Selectable marker of pFPMT-MFa plasmid is URA3 so transformants was selected on the YNB-Glucose medium without uracil. Transformants were grown for approximately 30-80 generations by successive cultivation steps under selective conditions. During this period, plasmid copy number was increased and integration was occurred. After passaging, stabilization and final selection was done in non-selective and selective media, respectively.

Confirmation of yeast integrants

To determine if the *egII* gene has integrated into the genome of *P. pastoris* and *H. polymorpha*, genomic DNA was extracted from selected grown yeast colonies by glass beads method as described by Hoffman and Winston (20). The genomic DNA is then used directly as a template for *Taq* DNA polymerase PCR using specific primers for *P. pastoris* (EGIIF-Pp: 5′-TCTCTCGAGTCAAA GGCAACAAACTGT-3′ and EGIIR-Pp: 5′-GGT ACCGCATGCCTACTACTTTCTT-3′) and *H.polymorpha* (EGIIF-Hp: 5′-AGACTGTTTGGG GTCAGTGTGGTG-3′ and EGIIR-Hp: 5′-AGAC AAGACGAAACCAGAGAGGTGTC-3′). The standard cycling conditions used for PCRs were as follows: 5 min at 95ºC, 30 cycles of 1 min at 95ºC, 1 min at 60ºC, and 2 min at 72ºC, and 1 cycle for 5 min at 72ºC.

Recombinant endoglucanase expression in yeast and deglycosylation analysis

For EGII expression in *P. pastoris*, the positive colonies were inoculated in 5 mL BMGY medium and the cultures were incubated at 30°C for 48 h with constant shaking (300 rpm) until an OD600 value of 2-5 had been reached. For induction, the cells were collected by centrifugation and resuspended in 1 mL BMMY induction medium and allowed to grow at 28°C. For continuous expression, methanol was added to the flasks every 24 h in order to keep the final methanol concentration at 1%. In *H. polymorpha*, selected positive colonies were cultured in YNB-1% glycerol to growth and increase biomass at 37°C for 48 h with 300 rpm shaking. After 2 days derepression of FMD promoter in absence of glycerol was occurred. The supernatants were analyzed on 12% SDS-PAGE.

Recombinant proteins were deglycosylated by Endo H enzyme (*BioLabs*). 1 mg of protein in citrate buffer (pH 4.8) was denatured in boiling water after 10 minutes. Then it was treated with 0.1 U EndoH for 12 hours at 37ºC and analyzed by SDS-PAGE.

Endoglucanase assay

Endoglucanase activity measurement was done as method of Ghose (21) that based on the release of reducing sugar from carboxymethyl cellulose (CMC) as substrate and the dinitrosalicyclic acid (DNS). In this study, recombinant enzymes were added to 1% CMC-Na in sodium acetate buffer 50 mM (pH 4.8). After 30 min incubation of the mixture (250 µl) at 50ºC, the reaction was stopped by the addition of 750 µl of DNS reagent. The resulting mixture was placed in boiling water for 5 min for color development and reducing sugar content measured by absorbance at 540 nm. As a negative control, enzyme was replaced by citrate buffer. One IU (International Unit) of CMCase activity was specified as the amount of enzyme that released 1 mg of reducing sugar per minute per ml in the above reaction condition. The amount of total protein was determined by the Bradford method (22) and bovine serum albumin (BSA) was used as a standard.

Activities measurement on different carbohydrate polymer substrates such as CMC (high viscosity), CMC-4M (low viscosity), Avicel and Filter paper was done.

Characterization of recombinant EGII

The optimal temperature for recombinant enzyme activity was determined using the standard assay at temperatures ranging from 30 to 85ºC in 50 mM citrate buffer, pH 4.8. The optimal pH was tested at 50ºC for pH range 3 to 7. For determination of thermal stability, the recombinant enzymes and native endoglucanase from *T. reesei* (Sigma) were incubated at various temperatures from 60 to 80ºC and remaining activity was measured by the standard assay method.

For hydrolysis kinetics determination, Michaelis-Menten constants were checked by measuring the rate of CMC and Avicel hydrolysis under the standard assay conditions using eight different substrate concentrations, rang from 0.5 to 30 mg ml^{-1} in triplicate. The apparent kinetic constants K_m, V_{max} and k_{cat} were calculated using the equation for enzyme kinetics. Digestion of Whatman no. 1 filter paper (3 mg) was assayed at 50ºC for exactly 60 min. One FPU (Filter Paper Unit) is the quantity of enzyme activity that will produce reducing sugar equivalent to 2.0 mg of glucose.

Results and Discussion

Cloning and transformation of *egII* in *P. pastoris* and *H. polymorpha*

Recently, the codon optimization technique has been widely used to increase the expression yield of recombinant proteins in yeast (4, 23). In addition researches show that rare codons decrease mRNA stability and its translation rate and high G+C content could cause translational yields decline or even result in failed expression (24). During the optimization of *egII* gene for expression in *P. pastoris* and *H. polymorpha* GC-rich sequence, RNA instability motifs and other negative *cis*-acting sequences were avoided. Gene optimization was done by GeneOptimizer software.

The coding sequence of *egII* was synthesized with codon optimization of *P. pastoris* and *H. polymorpha*. For *P. pastoris* transformation, recombinant plasmid (pPinkα-EGII) was linearized with *Bsp*T1 restriction enzyme in TRP2 region to facilitate integration of plasmid into the *Pichia* genome by homologous recombination. In *H. polymorpha*, optimized *egII* gene was ligated into expression vector pFPMT-MFa in *Hind*III/*Bam*HI sites. The recombinant plasmid was designated as pFPMT-MFa-EGII, and was then transformed into *H. polymorpha* strain RB11. *H. polymorpha* can be able to keep plasmids as episomal DNA and replicate it independently. During several passaging, in selective medium, the plasmid copy number increases and some integration can occur as a tandem head-to-tail into the genomic DNA. After passaging, 2 steps stabilization was done in non-selective medium. In this phase, yeast cells lose their episomal plasmids so in selection step on selective medium, just cells with integrated plasmids can growth. PCR amplification with *egII* gene specific primers (EGIIF-Pp/EGIIR-Pp for *P. pastoris* and EGIIF-Hp/EGIIR-Hp for *H. polymorpha*) for both *P. pastoris* and *H. polymorpha* showed that only one band appeared at 1200 bp using the genomic DNA from positive recombinant colonies as the template that confirmed *egII* gene integration into the yeast genome (Fig. 3).

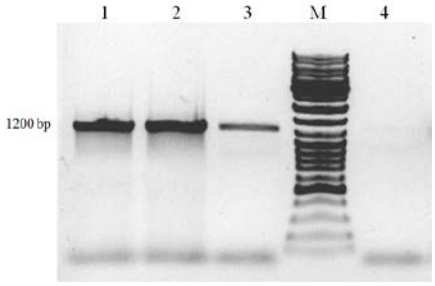

Figure 3. PCR analysis of the egII gene integrated in the chromosome of methylotrophic yeasts P. pastoris and H. polymorpha by egII specific primers. Lane 1: PCR on transformed colonies of P. pastoris. Lane 2: PCR on transformed colonies of H. polymorpha. Lane 3: pPinkα-egII plasmid as a positive control. Lane M: DNA marker. Lane 4: P. pastoris transformed with pPinkα-HC as a negative control.

SDS-PAGE analysis of deglycosylated recombinant EGII

Endoglucanase II of *T. reesei* is a 48 kDa glycoprotein with native glycosylation and its molecular weight is about 44 kDa without glycosylation (2). SDS-PAGE analysis of recombinant EGII protein expressed in *P. pastoris* showed a fat band with larger molecular mass

(about 53 kDa) because of its glycosylation (Fig. 4a). In *H. polymorpha* an unclear spread band with higher molecular weight (in region of 63 kDa) was observed on SDS-PAGE (Fig. 4b) like previous heterologous expression experiments (25, 26). Glycosylation studies on *H. polymorpha* demonstrated that different high mannose glycosylation patterns in *H. polymorpha* were occurred on recombinant proteins (25) in contrast of *P. pastoris*. There is just one N-glycosylation site (N103-F-T) in amino acid sequence of EGII. Recombinant EGII proteins were treated with Endoglycosidase H to remove the asparagine-linked N-glycosylation. Deglycosylated proteins in *P. pastoris* and *H. polymorpha* were stood lower than glycosylated form in SDS-PAGE but not at 44 kDa (Fig. 4). It seems, higher molecular weight of deglycosylated forms comes from O-glycosylations, because EndoH just remove carbohydrates in N-glycosylation forms. Deglycosyleted EGII in *H. polymorpha* had a spread band on SDS-PAGE, too, indicate that it is possibly do different O-glycosylation patterns. On the other hand, our results confirmed that recombinant proteins in *H. polymorpha* become more glycosylated than *P. pastoris* (27) and it can influent other biochemical properties of protein.

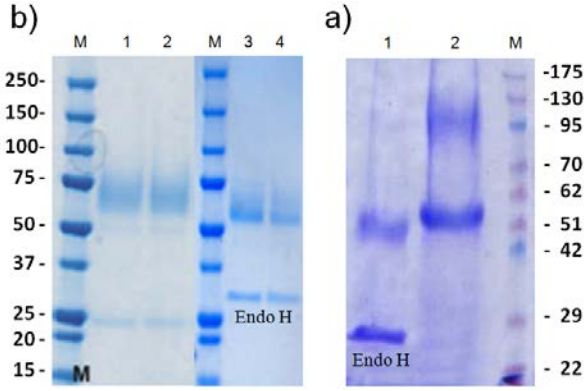

Figure 4. SDS-PAGE analysis of recombinant endoglucanase II from *Pichia* pastoris **(a)** and *Hansenula polymorpha* **(b)** and protein treatment with Endo H.

Effect of pH on enzyme activity

The effect of pH on enzymatic hydrolysis of CMC was studied (Fig. 5a). Recombinant endoglucanases from *P. pastoris* has a pH optimum at 4.2 and it was shift to 4.8 in *H. polymorpha*. Acosta and coworkers (27) in their comparison of invertase expression in yeast reported 0.5 unit shift in pH optimum from *P. patoris* to *H. polymorpha*. It seems that different high mannose glycosylation patterns in *H. polymorpha* increase protein stability in higher pH. Yeast recombinant EGII enzymes kept more than 90% of the maximal activity within pH range 4 to 5 as same as native enzyme and both of them have the same pH pattern. It is according to Masarova researches (2001) that showed recombinant proteins expressed in yeast maintained their activity in wide pH range. It demonstrated that heterologous expression of EGII in yeast has no considerable effect on its pH profile activity that previously reported by Qin et al. (2) in *S. cerevisiae*.

Effect of different temperature on enzyme activity

Enzyme activity of recombinant endoglucanase from *P. pastoris* and *H. polymorpha* in different temperature on 1% CMC was measured (Fig. 5b). Both of recombinant enzymes had maximum activity in 75°C (optimum temperature) and they showed similar temperature profile pattern (Fig. 5b). Yeast recombinant enzymes kept more than 75% of the maximal activity within the temperature range of 60 to 75ºC, but then suddenly lose activity at 85ºC maybe because of conformational disruption.

Comparison of recombinant endoglucanases thermal stability

Thermal stability of recombinant endoglucanases from *Pichia* and *Hansenula* and native endoglucanase II from *T. reesei* (Sigma) were checked at 60, 70 and 80ºC (Table 1). Qin (2) demonstrated that higher glycosylation of recombinant protein in yeast increases thermal stability. SDS-PAGE analysis of recombinant endoglucanases showed higher glycosylation in *Hansenula* than *Pichia* (Fig. 4). Our results showed recombinant endoglucanases expressed in *P. pastoris* and *H. polymorpha* were more stable than native form and the *H. polymorpha* endoglucanase with higher glycosylation showed higher thermal stability than *P. pastoris*. Comparison of recombinant endoglucanases demonstrated that *Pichia* endoglucanase half life at 60°C is 88% of *Hansenula* endoglucanase whereas in 80ºC it

decreases to 69%. Results indicated that thermal stability differences increase in higher temperatures and recombinant *Hansenula* endoglucanase is more stable in higher temperat ures than recombinant *Pichia* endoglucanase and native enzyme. Some studies suggested that glycosylation has been identified as one important way to increase thermal stability of proteins (2, 9). It seems that higher glycosylation in *Hansenula* compare with *Pichia*, caused increasing in thermal stability. Depending on the results, our enzyme can be classified as a thermophilic acidic endoglucanase, which can be widely used in industrial applications, especially in feed, textile, wastewater treatment and paper making industries.

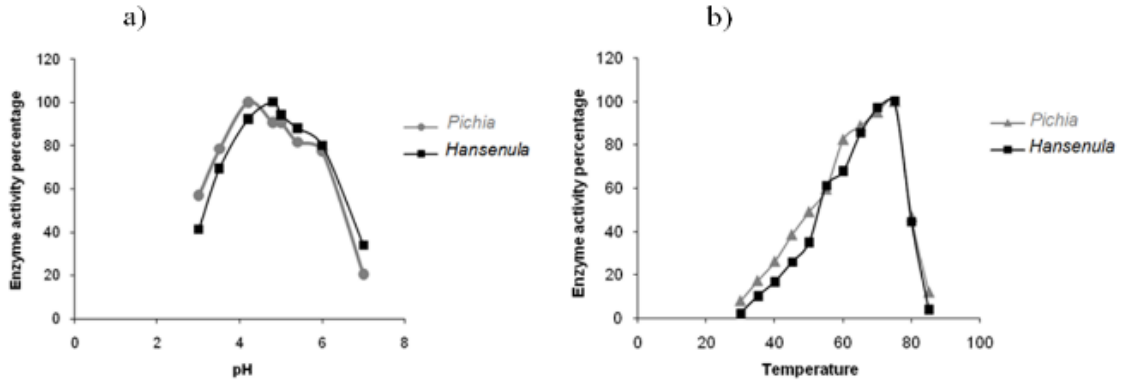

Figure 5. characterization of recombinant endoglucanases expressed in *P. pastoris* and *H. polymorpha*. **(a)** Effects of different pH on recombinant endoglucanases activity. **(b)** Effects of temperature on enzyme activity of recombinant enzymes

Table 1. Thermal stability comparison of recombinant and native endoglucanases in different temperatures.

Thermal stability comparison	Recombinant *P. pastoris* endoglucanase	Recombinant *H.polymorpha* endoglucanase	*T. reesei* Native endoglucanase
Half life in 60ºC (Min)	122.41	139.2	98.91
Half life in 70ºC (Min)	36.86	51.33	24.63
Half life in 80ºC (Min)	1.94	2.82	1.13

Enzymatic properties of recombinant endoglucanases on different substrates

Endoglucanase activity of recombinant enzymes was determined on three different substrates. The kinetic parameter values of the EGII on high and low viscosity CMC and Avicel were calculated from the Michaelis-Menten equation (Table 2). Comparison of recombinant EGII specific activity on CMC and Avicel indicate that EGII has negligible activity on crystalline cellulose (Avicel). Considerably, comparing the apparent K_m values of native and recombinant enzymes on different substrates showed the active site of native EGII and *P. pastoris* expressed enzyme have similar affinity to carbohydrate substrates, and it is significantly decreased in *H. polymorpha*. This demonstrated that the recombinant EGII had better affinity for low viscosity CMC substrate. In previous research, de Castro et al. (28) demonstrated that the higher viscosity of cellulose sources cause the lower endoglucanase activity, which is might because of diffusivity limitations. The k_{cat}/K_m value of three enzymes on low viscosity CMC were more than 2 folded than high viscosity CMC, suggesting that in the enzymatic reaction, low viscosity CMC is more specific substrate for recombinant endoglucanase than high viscosity CMC. In addition, low value of k_{cat}/K_m in *H. polymorpha* indicates that different high glycosylation pattern in *H. polymorpha* affect binding site conformation of enzyme and decreases its affinity to cellulosic substrates.

On the other hand, hydrolysis activity of native and recombinant endoglucanases on filter paper was investigated. Native and recombinant *H. polymorpha* EGIIs showed 0.341 and 0.356 FPU activity, respectively, that was relatively considerable (29, 30), but it was increased to 0.7 FPU in *P. pastoris*. It demonstrated that *P. pastoris* EGII could better act on natural cellulose

Table 2. The apparent kinetic parameter values comparison of the native and recombinant endoglucanases on high and low viscosity CMC and Avicel.

Substrate	Species	V_{max}	K_m (g l^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m	Percentage
CMC (Low viscosity)	*P. pastoris*	2.564	2.829	287.0	101.41	100
	H. polymorpha	2.108	1.653	22.46	13.59	13.4
	T. reesei	7.844	2.248	98.93	44.01	43.4
CMC (High viscosity)	*P. pastoris*	2.152	4.827	299.4	47.5	100
	H. polymorpha	2.144	3.143	28.51	9.07	19.1
	T. reesei	6.741	4.183	94.58	22.61	47.6
Avicel	*P. pastoris*	3.484	12.38	ND	ND	ND
	H. polymorpha	2.404	8.43	ND	ND	ND
	T. reesei	12.084	12.20	ND	ND	ND

In the present work, we have successfully expressed and characterized the endoglucanase II produced in two yeasts *Pichia pastoris* and *Hansenula polymorpha*. These two methylotrophic yeasts can be used as an economically competitive expression/secretion system for low-cost production of endoglucanase. To the best of our knowledge, this is the first report of expression and biochemical comparison of optimized synthetic *egII* gene from *T. reesei* in *P. pastoris* and *H. polymorpha*.

According to characterization of recombinant enzymes, the optimal pH and temperature of both enzyme forms did not differ significantly. EGII expressed in *Hansenula* demonstrated considerable decrease in k_{cat}/K_m value. But On the other hand, thermal stability comparison of recombinant endoglucanases and native EGII from *T. reesei* demonstrated higher stability of recombinant enzymes compare with native one. Although, recombinant enzyme expressed in thermo-tolerant *Hansenula* was more stable than *Pichia* enzyme in higher temperature which is important from the biotechnological point of view. As the two forms of the enzyme differ only in their carbohydrate contents (35% for *H. polymorpha* and 25% for *P. pastoris*), it is reasonable to assume that the carbohydrate moiety is responsible for the observed differences.

In conclusion, our results demonstrate that endoglucanase expressed in *P. pastoris* is more attractive enzyme with good activity and stability properties than EGII expressed in *H. polymorpha*, and it can be useful for several industrial applications.

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