CLONING, EXPRESSION AND PURIFICATION OF AN XYLANASE GENE FROM *ASPERGILLUS NIGER* DSM1957 IN *PICHIA PASTORIS* GS115

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SUMMARY

A gene coding an endo-β-1,4-xylanase (XlnA) from *Aspergillus niger* DSM1957 was cloned and sequenced. The cDNA sequence of 978 bps encodes for a putative endoxylanase of 326 aa with a predicted molecular mass of 36 kDa. The cDNA was overexpressed in *Pichia pastoris* GS115 under the control of an AOX1 promoter. Conditions were first optimized to offer the highest production yield of the recombinant protein. A series of methanol induction concentrations (0.5 to 2.5% v/v) was tested. The induction by 0.5% (v/v) of methanol showed the highest production level of the recombinant xylanase. The xylanase activity reached to 21.5 U/ml culture supernatant, after 144 h of cell growth in YP medium induced with 0.5% (v/v) of methanol. The culture supernatants were collected and used for enzyme activity assay. The recombinant xylanase was purified from the culture supernatant by the affinity chromatography with ProBond resin. The molecular mass of the purified XlnA, determined by SDS-PAGE, was 36 kDa, with the specific activity of 246.9 U/mg toward 0.5% (w/v) xylan. The recombinant XlnA (rXlnA) was stable over a temperature range of 25-40°C. The activity of rXlnA was declined to 66% activity when it was heated at 50°C for 240 min. Residual activities of about 45% was observed after prolonged heating at 60°C for 60 min. However, the rXlnA enzyme heated at 80°C for only 2 min showed no activity. The rXlnA was stable and highly specific toward xylan and is expected to show high potential for downstream biotechnological applications.

Keywords: *Aspergillus niger DSM1957, xylanase gene, cloning, expression*

INTRODUCTION

Xylanase (1,3-1,4-β-D- xylanase) is a special catalytic endoenzyme for hydrolysis reaction of xylan (to break the β-1,4 bond among xylose molecules). Xylan is the long chain carbohydrate of pentoses (the polymer of xylose) which is mainly found in wheat and black oat. Xylanases are produced by many organisms, such as bacteria, fungi, algae, and arthropods (Dekker, Richards 1976). Xylanase has been extensively used as additive of animal feed in order to improve feed digestion and the performance of poultry and pigs. The acidic environment of the proximal digestive system, the stomach may be attractive location for xylanase function since they have an acidic pH optimum. Xylanases reduce feed viscosity by breaking polysaccharide in feed.

In previous studies from our laboratory, we optimized culture conditions for *A. niger* DSM1957 producing xylanase (Do Thi Tuyen *et al.* 2008). Furthermore, we purified and biochemically characterized xylanase (Do Thi Tuyen *et al.* 2009a). The xylanase gene *xlnA* of *A. niger* was cloned (GenBank accession number: EU848304) by reverse transcription-polymerase chain reaction (PCR) (Nguyen, Quyen, 2008). Sequence analysis showed that *xlnA* is composed of 978 nucleotides that encode a 36 kDa protein. The protein has high amino acid identity to xylanase of *A. niger*. So far, *xlnA* genes from several *Aspergilli* strains have been cloned and expressed at various levels in different expression systems including *E. coli* (Zhou *et al.* 2008), *Plectosphaerella cucumerina* (Zhang *et al.* 2007), *Bacillus* (Gat *et al.* 1994, Tabernero *et al.* 1995), *Aspergillus* (Takada *et al.* 2002) and *P. pastoris* (Ferreira *et al.* 2006, Ferreira V. 2006, Liu *et al.* 2006).

In this study, we cloned and heterologously expressed a xylanase gene from *A. niger* DSM1957 in *P. pastoris* GS115 under the control of AOX1 promoter for expression and secretion of XlnA. Purified protein was subsequently assayed for its sensitivity towards different temperature ranges.

MATERIALS AND METHODS

Chemicals and agents

Restriction enzymes, *Taq* DNA polymerase and

T4 ligase were obtained from Fermentas, part of Thermo Fisher Scientific Inc. (Waltham, USA); kit ProBond™ Nickel-Chaleting Resin was from Invitrogen Corp (Carlsbad, USA); 3,5-initrosalisilic acid was from Fluka (China); Birch wood xylan was from Biochemical (France); Peptone and yeast extract were purchased from Bio Basic Inc. (New York, USA)

Vectors, strains and culture conditions

The *A. niger* DSM1957 strain purchased from the German Microorganism Collection Center (DSMZ) was used as the source for the xylanase gene (*xlnA*). *E. coli* DH5α and pJET1.2/blunt vector (Fermentas, Thermo Fisher Scientific Inc., W altham, USA) were used for DNA manipulations and amplification. *P. pastoris* host strain GS115 and pPICZαA (Invitrogen Corp., Carlsbad, USA) were used for expression of the xylanase. Luria-Bertani medium (LB) containing 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, at a pH of 7-7.5 was used for the cultivation of *E. coli*. LB agar contained additionally 2% (w/v) agar and 100 µg ampicillin/ml; low salt LB (1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl) agar contained 25 µg zeocin/ml.

DNA manipulations

Genomic and plasmid DNA isolations were carried out by methods as previously described (Quyen *et al.*, 2007). DNA fragments and PCR products were excised from a 0.8% agarose gel and purified by a gel extraction kit (Qiagen, Venlo, Netherlands) according to manufacturer's instruction. DNA sequencing was performed on ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, USA) *E. coli* DH5α cells were transformed using heat shock method that has been previously described (Quyen et al., 2007).

DNA amplification and plasmid construction

Based on the nucleotide sequence of *xlnA* encoding xylanase from *A. niger* DSM1957(EU848304), two oligonucleotides XlnAF (5'- GC GAA TTC GTT CAG ATC AAG GTA GCT -3') and XlnAR (5'-GC TCT AGA GAG AGC ATT TGC GAT A -3') were designed as primers to amplify the gene *xlnA* from *A. niger* DSM1957 underlined *Eco*RI and *Xba*I restriction sites introduced to the primers. Polymerase chain reaction (PCR) for the amplification of the 978 bps fragment of *A. niger* DSM 1957 *xlnA* gene from genomic DNA was carried out using *Taq* DNA polymerase. The PCR mixture contained 2.5 µl 10xPCR buffer, 2 µl of 2.5 mM dNTP, 2 µl of 25 mM $MgCl_2$, 1 µl genomic DNA (50 ng), 0.5 µl 5 unit *Taq* polymerase, and 1 µl each primer (10 pmol), supplemented with 15 µl distilled water to a final volume of 25 µl. The thermocycler conditions were as following: 94°C/5'; 35 cycles of 95°C/45",52°C/1', 72°C/1'; 72°C/10'. The PCR products were inserted into the pJET1.2/blunt vector. Then the cloned *xlnA* gene in pJET1.2/blunt vector was restricted by *Eco*RI and *Xba*I and inserted into pPICZαA to generate pPXlnA.

Yeast transformation and screening

The plasmid pPXlnA linearized using restriction enzyme *Sac*I and subsequently then transformed into *P. pastoris* GS115 according to the manufacturer's instructions for the Easy select *Pichia* expression Kit (Invitrogen Corp., Carlsbad, USA). Transformants were screened on YPDS [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, 2% (w/v) agar] plates containing zeocin at a final concentration of 1 mg/ml. The presence of the *xlnA* gene in the transformants was confirmed by PCR using yeast genomic DNA as template and XlnA- specific primers. The clones that showed the right size of the PCR product and zeocin resistance were chosen for the expression assay.

Gene expression

P. pastoris transformants were grown in 20 ml of YP medium [1% (w/v) yeast extract, 2% (w/v) peptone] supplemented with 1% (w/v) glycerol at 30°C with agitation at 220 rpm until an OD600 nm reached 5 to 6. The cell pellet was harvested by centrifugation at 4500 rpm for 5 min. For AOX1 promoter-controlled expression of XlnA, the cell pellet was resuspended in 25 ml of YP medium. Cultivation was performed at 30°C and 220 rpm. The culture supernatant was collected periodically to detect XlnA activity.

Purification of recombinant rXlnA

The purification of the recombinant His-tagged rXlnA was carried out according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, USA). The culture supernatant containing rXlnA was harvested from 25 ml culture by centrifugation at 8000 rpm and 4°C for 5 min and 8 ml of culture supernatant was applied to a

ProBondTM Ni^{2+}-affinity column and rinsed with wash buffer. The rXlnA was eluted with 5x1 ml of native elution buffer.

Xylanase activity estimation

Xylanase activity was determined by measuring the increase in concentration of reducing sugars formed by enzymatic hydrolysis of birchwood xylan. A reaction mixture of 100 µl of the crude or purified xylanase containing 0.045 µg total protein was incubated with 400 µl of 0.5% (w/v) birch wood xylan in 20 mM potassium phosphate buffer pH 6.5 at 55°C for 5 min. To arrest the reducing sugar released in the reaction mixture, 1.25 ml of 3,5,-dinitrosalicylic acid (DNS) was added. The reduced sugars were determined by measuring the absorbance at 540 nm (Miller 1959). D-xylose was used as standard. One unit (IU) of xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose per min under the standard assay conditions. All measurements were carried out three times and from these values the average value was taken.

SDS-PAGE and protein concentration

The homogeneity and molecular mass of XlnA were determined by 12.5% (w/v) SDS polyacrylamide gel electrophoresis (Laemmli, 1970) with Biometra (Göttingen, Germany). Proteins were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R-250. Protein concentrations were estimated by Braford assay with the bovine serum albumin as standard (Bradford 1976).

Temperature stability

For the determination of temperature stability, purified XlnA was preincubated at different temperatures 25, 37, 40, 50, and 60°C for 1-8 h in 20 mM potassium phosphate buffer pH 6.5. The residual activity was then determined at 50°C and pH 6.5.

RESULTS AND DISCUSSION

Construction of constitutive expression vector and expression in *P. pastoris*

In this experiment, a constitutive expression vector pPICzαA of *P. pastoris* was used. This plasmid facilitates heterologous expression, secretion and subsequent purification of recombinant proteins. The synthetic *xlnA* was ligated into pPICzαA using *EcoR*I and *Xba*I sites introduced in the primers for amplification. The transformants obtained through zeocin selection were cultured in 200 mL of YPD liquid medium and incubated with shaking at 30°C. The supernatant of the culture was analyzed by SDS-PAGE (Fig. 3a). Our results indicated the presence of a single protein band with a molecular weight of proximately 36 kDa. Protein concentration of the dialyzed supernatant was 0.2 mg/mL. The activity of the recombinant xylanase in the supernatant was 21.5 U/mL (50°C and pH 6.5 for 5 min). As a control, supernatant of wild-type *P. pastoris* GS115 showed no detectable activity. The linearized plasmid (pPICzαA/XlnA) was transformed into the *P. pastoris* GS115 by electrophoresis. To check for integration of pPICzαA/XlnA into the *P. pastoris* genome, the putative transformants were cultured in YPD medium and total DNAs were isolated. These DNA were used as templates to amplify *xlnA* gene with primers XlnAF and XlnAR. A fragment of 978 bps was observed (Fig. 1A).

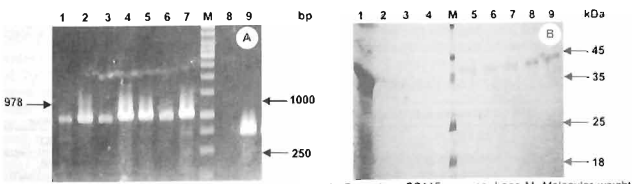

Figure 1. (A) PCR detection of the recombinant xylanase gene in *P. pastoris* GS115 genome. Lane M, Molecular weight marker. Lane 1- 7, recombinant *xlnA*, Lane 8, *P. pastoris* GS115 without foreign gene transformed. (B) SDS-PAGE analysis of rXlnA samples. Lane 1, Culture supernatant of *P. pastoris* GS115/pPXlnA. Lane 2, through-column sample. Lane 3-4, column-washing. Lane 5 -9, fraction 1, 2, 3, 4 and 5. Lane M, standard proteins (Fermentas, Thermo Fisher Scientific Inc.,Waltham, USA).

Pichia pastoris, a methylotropic single-cell yeast strain, has been routinely used as a heterologous expression system because of its efficient secretion, high expression level, proper protein folding, and very high cell density (Kim *et al.* 1997; Reverter *et al.* 1998). In the *P. pastoris* inducible expression system, expression of a foreign gene is under the control of alcohol oxidase I (AOX I, E.1.1.3.13) promoter and the expressed protein can be secreted out of the yeast cell. Many proteins, including phytase and xylanase, have been efficiently expressed with methanol induction in *P. pastoris* (Berrin *et al.* 2000; Luo *et al.* 2004) However, in this system, protein production must be induced with methanol, and the fermentation course must be carried out over a long period.

Recently, another *P. pastoris* expression system was highlighted for the foreign gene under the control of the PPICzαA gene promoter that the protein was constitutively expressed (Waterham *et al.* 1997). In our laboratory, a modified xylanase gene *xln*B from *A. niger* was secreted using this system (Do Thi Tuyen *et al.* 2009b)

P. pastoris GS115 was transformed with pPXInA, the resulting transformants were grown in YP medium for the xylanase production. After 144 h of methanol induction, the culture supernatants were collected and used for enzyme activity assay. The *P pastoris* GS115/pPXInA transformant showing the highest production level of xylanase (15.3 U/ml, Tab. 1) was used for enzyme production, purification and characterization.

Table 1 XlnA production curve

Induced time	Recombinant XlnA in *P. pastoris* GS115		
	Xylanase activity (U/ml) X ± SD	Protein content (mg/ml) X ± SD	OD_{600nm}
24h	5.1 ± 0.23	0.031 ± 0 0037	1 290
48h	7 4 ± 0 13	0.033± 0.0015	1.472
72h	10 6 ± 0 42	0 028 ± 0 0004	1 469
96h	11 8 ± 0.2	0.023 ± 0 0003	1 537
120h	11 9 ± 1.0	0.026 ± 0 0001	1.447
144h	**15.3 ± 0.04**	**0.027 ± 0.0018**	**1.547**
168h	14.0 ±0 09	0 027 ± 0 0004	1.411

Conditions were first optimized to offer the highest production yield of the recombinant protein. A series of methanol induction concentrations (0.5 to 2,5% v/v) was tested. The induction by 0.5% (v/v) of methanol showed the highest production level (13,3U/ml) of the recombinant xylanase (Tab. 2).

The recombinant xylanase was purified from the culture supernatant by the affinity chromatography with ProBond resin. On a SDS-PAGE, only one protein band of about 35.5 kDa was observed (Fig. 1B, lane 5-9). The purified xylanase had a specific activity of 246.9 U/mg protein (Tab. 3)

Table 2. Methanol induction on XlnA production

Methanol (%)	Xylamase activity (U/ml) X ± SD	Protein content (mg/ml) X ± SD	OD_{600nm}
0.5	**13.3 ± 1.1**	**0.037 ± 0.00036**	**1.653**
1	11 4 ± 0.4	0 032 ±0.00009	1.466
1.5	10.2 ± 0.1	0.031 ±0 00063	1 814
2	9.7 ±0.5	0.033 ± 0 00145	1 806
2.5	9 1 ±0.1	0.033 ± 0 00054	1 798

Table 3. Purification produce of rXlnA from the culture supernatants of *P. pastoris* GS115/pPXlnA

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Culture supernatant	21.55±0.4	0.201±0.00	107.2±2.9	100	
Ni^{2+}-ProBond™ resin column	11.85±0.8	0.048±0.00	246.9±16.5	55	2.3

The mature peptide of *Bacillus licheniformis* xylanase A (BlxA) was successfully expressed in *P. pastoris* under the control of AOX1 promoter. After 96 h 0.25% methanol induction, the activity of recombinant *B. licheniformis* xylanase A (reBlxA) in culture supernatant was 122.9 U/mg (Liu, Liu, 2008). The endo- β- 1, 4-xylanase gene *xynA* from *Aspergillus sulphureus*, encoded a lack-of-signal peptide protein of 184 amino acids, was de novo synthesized by splicing overlap extension polymerase chain reaction according to *P. pastoris* protein's codon bias. The synthetic DNA, composed of 572 nucleotides, was ligated into the downstream sequence of an alpha-mating factor in a constitutive expression vector pGAPzalphaA and electrotransformed into the *P. pastoris* X-33 strain. The recombinant enzyme was expressed with a yield of 120 units/mL under the flask culture at 28°C for 3 days (Cao *et al.* 2007). The mature peptide of *A niger* xylanase A (AnxA) was successfully expressed in *P pastoris* at high levels under the control of AOX1 promoter. The recombinant AnxA (reAnxA) was secreted into culture medium. After 96h of 0.25% methanol induction, the activity of reAnxA in the culture supernatant reached the peak, 175 U/mg, which was 1.9 times as high as that of the native AnxA (92 U/mg) (Liu et al. 2006). These data suggested that *P. pastoris* is a suitable host for overexpression of functional xlnA.

Although the xylanase has been expressed as an active enzyme in several systems such as *Streptomyces olivaceoviridis, E. coli, A. niger* (Yang *et al.* 2005, Yang *et al.* 2007; Sharma *et al.* 2012), however the recombinant protein produced in these systems still have some limitations such as production yield and protein activity and stability

Temperature stability

Temperature stability of xylanase plays important role for their commercial effectiveness. To determine temperature stability of the recombinant protein, the residual activities were measured at 50°C and pH 6.5 after it was treated for 480 min at temperatures ranging from 25°C to 60°C for 480 min. The enzyme was shown to be very stable (Fig. 2). The enzyme displayed no less than 71% to 111% activity after being heated at 25 to 40°C for 480 min.

However, the activity of rXlnA was declined to 66% activity when it was heated at 50°C for 240 min. Residual activities of about 45% was observed after prolonged heating at 60°C for 60 min. However, the rXlnA enzyme heated at 80°C for only 2 min showed no activity (data not shown).

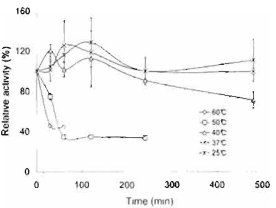

Figure 2. Temperature stability of the recombinant enzyme

CONCLUSIONS

In the present study, we successfully expressed a gene coding for XlnA isolated from *A niger* DSM1957 in *P. pastoris* and have demonstrated that *P pastoris* is a good system to express the XlnA gene at industrial level. The rXlnA was stable and highly specific toward xylan and is expected to show high potential for downstream biotechnological applications.

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NHÂN DÒNG GEN, BIỂU HIỆN VÀ TINH SẠCH XYLANASE TÁI TỔ HỢP TỪ CHỦNG *ASPERGILLUS NIGER* DSM 1957 TRONG *PICHIA PASTORIS* GS115

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TÓM TẮT

Gen mã hóa xylanase A từ chủng *Aspergillus niger* DSM1957 đã được nhân dòng và phân tích trình tự DNA và trình tự amino acid trong một nghiên cứu trước trong vector pJET (pJXlnA). Trong nghiên cứu này, gene *xlnA* được đưa vào vector biểu hiện pPICZαA (pPXlnA) sau khi được nhân dòng phụ trong vector pJET/1.2 blunt (pJbXlnA). Vector pPXlnA mở vòng bằng enzyme hạn chế *SacI* và được biến nạp vào nấm men *Pichia pastoris* GS115 bằng phương pháp xung điện tạo ra hoạt tính biểu hiện *P pastoris* GS115/pPXlnA. Xylanase tái tổ hợp XlnA (rXlnA) được biểu hiện ở dạng ngoại bào với hoạt tính đạt 21,5 U/ml (107,2 U/mg protein), hàm lượng protein đạt 0,201 mg/ml. Dịch sau lên men 144h ở 30°C và lắc 200rpm trong môi trường YP (cảm ứng bằng methanol ở nồng độ 0,5%) được ly tâm 5000 vòng/phút trong 10 phút ở 4°C. Dịch enzyme thô được tinh sạch qua cột sắc ký ái lực Nickel Xylanase tinh sạch thu được có khối lượng phân tử là 36 kDa, hàm lượng protein tinh sạch đạt 0,048 mg/ml, với hiệu suất tinh sạch đạt 55%, độ sạch là 2,3 lần. Hoạt tính xylanase tái tổ hợp cao nhất là 246,9 U/mg protein, bền ở nhiệt độ 25- 40°C, sau 8 giờ ủ hoạt tính còn lại gần 80%. Hoạt tính xylanase vẫn còn duy trì được hơn 45% sau khi ủ 1 h ở 60°C. Ở nhiệt độ 80°C, hoạt tính bị mất hoàn toàn sau ủ 2 phút.

Từ khóa: *Aspergillus niger DSM1957, biểu hiện, Pichia pastoris, xylanase*

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