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Full Length Research Paper

Expression of chitosanase gene from *Aspergillus fumigatus* JXSD-97 in *Pichia pastoris*

Yang Li, Yang Ping and Wang Manying*

School of Life Science, Jiangxi Normal University, 437 Beijing Road, Nanchang 330027, PR China.

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In this study, the plasmid pPIC9K-CSN was transformed into *Pichia pastoris* strain GS115 by electroporation and the high expression transformants with G418 resistance were obtained. The expression conditions for CSN in *P. pastoris*, such as the expression time, pH value and methanol concentration in the BMMY were optimized. The maximum activity of CSN is about 100 mg/L under optimized condition (96 h of 0.5% methanol induction). The Chitosanase exhibited a molecular mass of approximately 25 kDa on 12% SDS–PAGE. The results showed that the coding sequence of CSN was successfully obtained and inserted into *P. pastoris* GS115 vector. This study would provide a new opportunity for large-scale expression and purification of CSN, which might facilitate studies on the biological activity of CSN.

Key words: Chitosanase, *Pichia pastoris,* expression.

INTRODUCTION

Chitosanase (CSN, EC 3.2.1.132) can hydrolyze β-1,4-glycosidic linkages of the chitosan backbone to produce short chain chitooligosaccharides of various length. Chitosanases are produced by a wide range of organisms including bacteria (Boucher et al., 1992; Izume et al., 1992; Mitsutomi et al., 1998; Okajima et al., 1994, 1995; Omumasaba et al., 2000; Park et al., 1999), fungi (Ak et al., 1998; Chih Yu and Li, 2000; Kim et al., 1998; Shimono et al., 2002), insects (Yamada et al., 1999), viruses (Sun et al., 1999) and plants (El Ouakfaoui et al., 1992). The characteristic of CSN and its mechanism of positive effect on broiler performance were comparatively studied by using a series of investigations such as the structure and characteristic of CSN, antimicrobial effect and anti-adhesion *in vitro*, cell culture and feed trial (Seo et al., 2000).

In recent years, the demand for CSN is tending to increase. However, the high price of CSN limits its applications. Therefore, it is of great importance to develop an efficient way to produce CSN. Compared with screening and genetic engineering methods, fermentative production of CSN is an efficient approach in practical process. To date, CSN from *Bacillus sp* strain K17 has been cloned and expressed in *Escherichia coli* (Yatsunami et al., 2002). However the activity of CSN is every low. Many attempts have focused on screening CSN over-producers by mutagenesis and process optimization to achieve high content of CSN.

Pichia pastoris is being widely used as a host-cell system for the production of heterologous proteins (Eckart et al., 1996). The most advantageous characterristic of this microorganism is that it is able to grow to high biomass in simple minimal defined media. Since *P. pastoris* has the ability to reach higher cell density than that of *E. coli* during fermentation, the recombinant *P. pastoris* has the potential to produce higher volumetric yield of CSN.

In the previous study, cDNA was amplified by RT-PCR from total RNA of *Aspergillus fumigatus* and cloned into *E. coli* expression vector DH5a. The recombinant expression plasmid was designated as pPIC9K-CSN. After identification by PCR, restriction enzyme digestion and DNA sequencing, the positive recombinants were transformed into *E. coli*. In this work, a recombinant *Pichia pastoris* expressing the genes of CSN was

*Corresponding author. E-mail: wangmy88@yahoo.com.cn. Fax: +86-791-8507365.

Abbreviations: CSN, Chitosanse; ***P. pastoris***, *Pichia pastoris*; **BMMY,** buffered methanol-complex medium; **MD,** minimal dextrose medium; **MM,** minimal methanol medium; **PYD,** yeast extract peptone dextrose; **PCR,** polymerase chain reaction; **BMGY,** buffered glycerol-complex medium; **SDS-PAGE,** SDS-polyacrylamide gels electrophoresis.

successfully constructed into GS115. This is a good example of CSN production by recombinant *P. pastoris.*

MATERIALS AND METHODS

Strains, plasmids and media

The plasmid pPIC9K, host strain *P. pastoris* GS115 were purchased from America invitrogen. The expression plasmid pPIC9K-CSN was constructed by our laboratory. JXSD-97 strain was screened from the soil. The host strain used for all plasmid construction was cultivated in Luria Bertani (LB; 1% tryptone, 1% NaCl, 0.5% yeast extract; pH 7.5). For *P. pastoris* cultivations, the following media were used at appropriate steps: YPD (1% yeast extract, 2% peptone, 2% dextrose), MD (1.34% YNB, 4 × 10^{-5}% biotin, 2% dextrose), MM (1.34% YNB, 4 × 10^{-5}% biotin, 0.5% methanol), BMGY (1% yeast extract, 2% peptone, 100 mM phosphate buffer pH 6.0, 1.34% YNB, 4 × 10^{-5}% biotin, 1% glycerol), and BMMY (1% yeast extract, 2% peptone, 100 mM phosphate buffer pH 6.0, 1.34% YNB, 4 × 10^{-5}% biotin, 0.5% methanol).

Transformation of *P. pastoris*

The secreted expression plasmid pPIC9K-CSN of *P. pastoris* was constructed and digested with SaII and transformed into *P. pastoris* GS115 by electroporation using a micropublisher (BioRad) according to the *P. pastoris* vector manual. Approximately 20 µL of linearized plasmid DNA was used for electroporation in 0.2 cm cuvettes, using a Gene Purser (BioRad, Hercules, CA) at 1.5 kV, 25 µF and 200 Ω. Immediately after pulsing, 1 M ice-cold sorbitol (1 ml) was added to the cuvette cells and the cuvette contents were incubated at 30°C without shaking for 1 h. The mixture was spread on MD for these elections of His^+ transformants. The parent pPIC9K without the insert, lineralized with SaII, was also transformed into *P. pastoris* and used as a negative control. To screen for methanol utilization, each colony on the MD plate was first spotted on to MM agar and then on to a new MD plate. After 48 h, Mut^s and Mut^+ colonies were identified. Transformants bearing the chromosomally integrated copies of the pPIC9K-CSN were detected by a genomic PCR assay using the 5'AOX1 and 3'AOX1 primer (Table 1).

Screening of recombinants His^+ transformants were selected on His^- minimal dextrose (MD). To improve the expression levels, multiple inserts were screened *in vivo* according to the instructions provided in the pPIC9K manual and G418 was used at final concentrations of 0.25, 1.0, 2.0, and 4.0 mg/ml.

Expression of CSN in recombinant *P. pastoris*

According to the instructions provided in the pPIC9K manual, a single colony of His^+ transformants exhibiting G418 resistance in different degrees was inoculated into 20 ml of buffered glycerol-complex medium BMGY in 100 ml flasks. The suspension was incubated at 30ºC with shaking at 230 rpm until an OD_{600} value of 2 - 6 was attained. The cells were harvested by centrifugation at 3,000 × g and 4ºC for 5 min, resuspended in 100 ml buffered methanol-complex medium BMMY, transferred to a 250 ml flask and grown at 30ºC for 4 days in a shaking incubator at 230 rpm. To maintain induction of the culture, methanol at a final concentration of 0.5% was fed to the culture every 24 h for induction and samples were withdrawn at intervals. The cells were removed by centrifugation at 10,000 × g and 4ºC for 10 min and the supernatant was analyzed for CSN expression by forming sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Optimization expression of CSN in *P. pastoris*

By the fermentation in shake flask, it was studied that the influence of temperature, pH values and concentration of methanol on the production of enzyme activity by fermentation of the recombinants. Six time points were monitored at 24, 48, 72, 96 and 120 h (pH = 7.0, methanol concentration 1.5%) by electrophoresis analyzes to determine the optimal harvest time. To determine the optimal cells density, the clone was cultured to OD_{600} ≈ 6.0 in BMGY medium, centrifuged and resuspended the cell pellet to BMMY medium to induce expression. We compared the yields of CSN in 6 pH value mediums which have been adjusted to pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 with phosphoric acid or KOH. Moreover, the expression levels were analyzed at different methanol induction concentration of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%. In all of the methods, 1 mL medium supernatant (1.5 mL) for SDS-PAGE analysis was aspirated from each culture every day till 5 days.

Enzyme assays

CSN activity was assayed according to Poulton et al. (2006). A suitably diluted medium supernatant was incubated with 1% chitosan in 5% sodium acetate buffer pH 7.0 at 60°C for 10 min. The released reducing sugars were assayed after adding 100 µl of DNS (3, 5-dinitrosalicylic acid) reagent and then boiled for 5 min. The absorbance was measured at 520 nm (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of reducing sugar per min under the experimental conditions. Protein purification scale-up expression was carried out in four 250 mL baffled flasks. The CSN clone was grown in 50 mL BMGY medium at 30°C with constant shaking at 250 rpm to OD600 ≈ 6.0. Cell was harvested and resuspended in 100 mL BMMY medium (OD600 ≈ 2.0), then cultured for 96 h. Methanol was added to the medium to a final concentration of 1.5% every 24 h for induction. The culture was centrifuged at 12,000 rpm for 5 min and the supernatant was collected. The supernatant was precipitated with ammonium sulfate (70% saturation) and centrifuged at 12,000 rpm for 20 min. Then the protein has been purified to approximate homogeneity by ammonium sulfate fractionation. The precipitate was dissolved in 3 mL 0.02 mmol/L potassium phosphate buffer then dialyzed against 1 L of the same buffer for 48 h.

RESULTS

Polymerase chain reaction of pPIC9K-CSN/GS115

The Chitosanse gene was inserted into contain methanol promoter and yeast signal peptide to construct expression plasmid pPIC9K-CSN and the plasmid was linearized by restriction enzyme SAII digestion, then transformed into *P. pastoris* GS115 strain by electroporation. Six positive recombinant colonies were obtained. Four recombinant colonies and one colony (pPIC9K/GS115) were checked by PCR (Figure 1).

Expression of CSN in *P. pastoris*

The Chitosanase gene was inserted into contain methanol promoter and yeast signal peptide to construct expression plasmid pPIC9K-CSN. The recombinant pPIC9K-CSN plasmid was linearized by restriction enzyme SaII digestion, then transformed into *P. pastoris* GS115 strain by electroporation. The His^+ Mut^+ recombintant

Table 1. Oligonucleotide primers used in this study.

Primer	Sequence (5'→3')	Length (bp)
5'AOX1	TTT ACG TAG CAG GTG GAG TTG ATG GTC CTA	30
3'AOX1	GCA AAT GGC ATT CTG ACA TCC	21

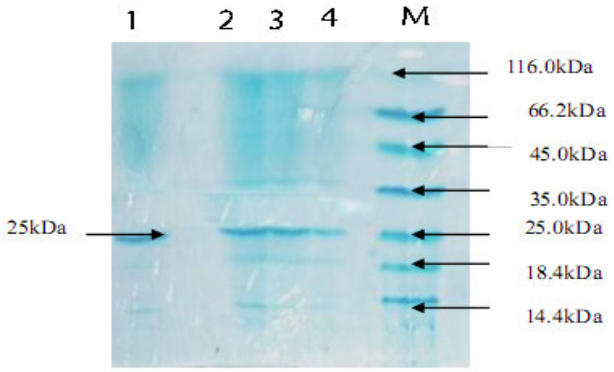

Figure 1. SDS-PAGE analysis. SDS-PAGE was performed on a 12% gel and stained with Coomassie brilliant blue. Lanes 1 - 4, supernatant from the positive strain transformed with *P. pastoris* plasmids , Lane M, protein molecular weight marker.

was selected for expression optimization and scale-up expression of CSN. The condition of transformant was induced by methanol to identify colonies capable of secreting CSN. The expressed protein from *P. passoris* strain GS115 was concentrated by decentralization and the result of SDS-PAGE indicated that the expression protein was about 25kDa (Figure 2).

Optimization of CSN expression

The productions of the secreted CSN by *P. pastoris* were performed under several different conditions. The supernatant from each experiment was collected and analyzed by SDS–PAGE. The gel was analyzed by software of Quantity One 4.62 (Bio-Rad). BMMY at pH 6.0-7.0 was the optimized culture condition for expressing CSN (Figure 3c), and 72 - 120 h was the most suitable methanol induction time (Figure 3a). The optimized methanol concentration and cell density were 1.5% (Figure 3b).

One of the recombinants was studied for CSN expression under shake flask conditions. The maximum level of CSN activity (100 U/ml) was obtained in the culture filtrate of *P. pastoris* GS115 (pPIC9K-CSN) after 96 h of growth (Figure 4).

Protein purification with ammonium sulfate

In this study, CSN protein expressed in *P. pastoris* was secreted and soluble in the culture supernatant. The supernatant was only precipitated by ammonium sulfate fractionation. The results showed that 60% ammonium sulfate was the best to remove impurity protein and 70 - 80% saturation was suitable to collect the CSN protein (Figure 5).

DISCUSSION

A chitosanase producing *A. fumigatus* JXSD-97 was isolated from soil in China. By using the RT-PCR method, the cDNA gene fragment about 666 bp in size was amplified corresponding to the nucleotide JXSD-97 genome. The amplified full-length CSN gene fragment was then inserted into the expression vector pPIC9K to generate a recombinant plasmid pPIC9K-CSN. After comparing the nucleotide of CJ 22-326 CSN gene derived from the recombinant plasmid pPIC9K-CSN and its deduced amino acid sequence with those of A. *fumigatus* strain, the nucleotide sequence of the cloned CSN gene was found to be similar (98%) to that of *A. fumigatus* strain retrieved from GenBank AY190324, with 98% homology of the deduced amino acids.

The open reading frame of CSN encodes a mature protein consisting of 220 amino acids with a predicted molecular weight of 25 kDa on SDS-PAGE, which is apparently smaller than the weight of known other chitosanase (of molecular masses ranging from 6 to 140 kDa), for example, 41 kDa for *Bacillus cereus* D-11 (Gao et al., 2009), 27 kDa for *Amycolatopsis* sp. CsO-2 (Saito et al., 2009).

The Chitosanase gene from *Aspergillus* sp. CJ22-326 was successfully expressed in *E. coli* with its own signal peptide, but Chitosanase activity of the recombinant protein was much lower than other heterologous expression fungal proteins expressed in *P. pastoris* (Li et al., 2008). Hence, in order to improve the Chitosanase activity, we chose the *P. pastoris* as microbial expression systems which has become a vital host organism for the production of foreign proteins (Macauley-Patrick et al., 2005; Daly and Hearn, 2005). The results showed that the heterologous expression of CSN in *P. pastoris* strain GS115 conferred a high level of Chitosanase activity which succeeds in improving the enzyme yield by high cell-density fermentation. After optimization of the media, the Chitosanase activity from *A. fumigatus JX*SD-97 reached to 100 U/mL in 250 mL flasks, which was nearly 10-fold increase of Chitosanase production at the parent lowest level (You, unpublished).

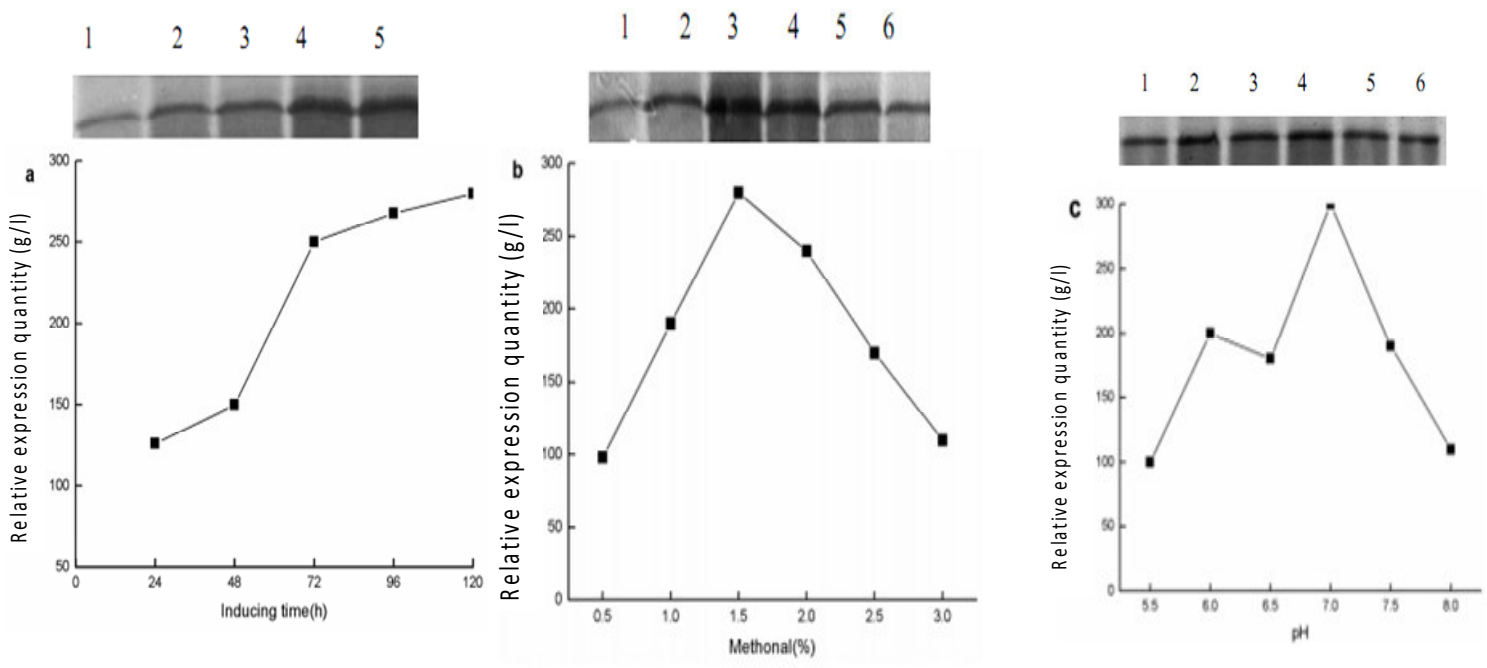

Figure 2. Optimization expression of CSN in *P. pastoris*. (a) Effect of culture times on CSN expression. Lanes 1 – 5: 24, 48, 72, 96, 120 h; (b) Effect of methanol concentration on CSN expression. Lanes 1 – 6: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0%; (c) Effect of culture pH value on CSN expression. Lanes 1 – 6: 5.5, 6.0, 6.5, 7.0, 7.5, 8.0.

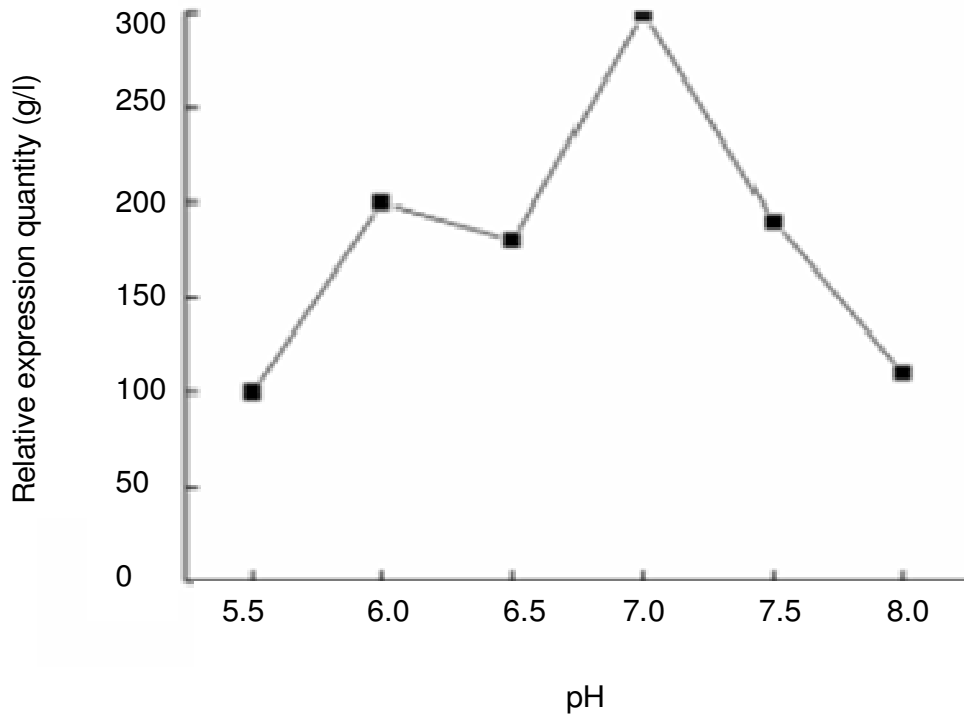

Figure 3. Optimization expression of CSN in *P. pastoris*. (a) Effect of culture times on CSN expression. Lanes 1–5: 24 h, 48 h, 72 h, 96 h, 120 h; (b) Effect of methanol concentration on CSN expression. Lanes 1–6: 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%; (c) Effect of culture pH value on CSN expression. Lanes 1–6: 5.5, 6.0, 6.5, 7.0, 7.5, 8.0.

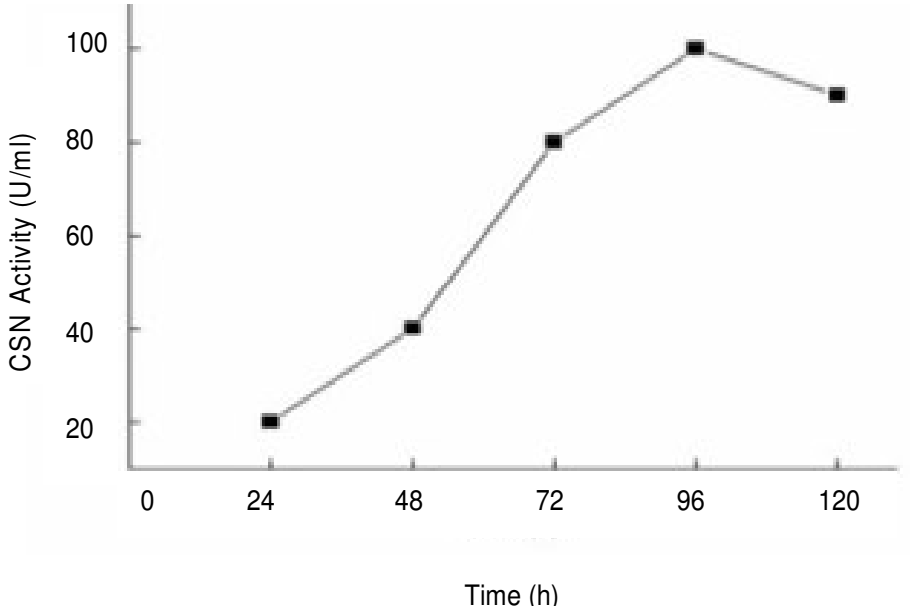

Figure 4. Kinetics of CSN production by recombinant *P. pastoris* GS115 was grown in minimal medium with 1.5% methanol. Samples were taken at 24 h intervals and assayed for CSN activity in culture supernatant.

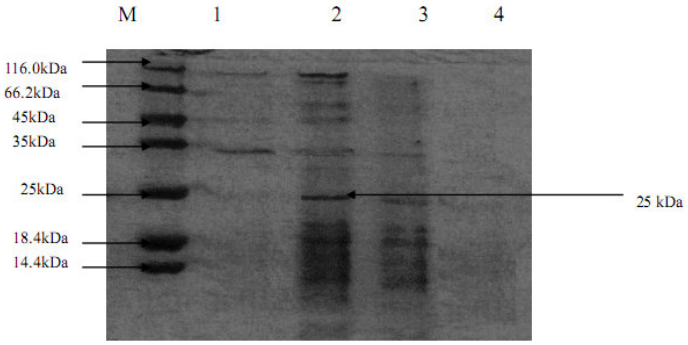

Figure 5. SDS–PAGE of CSN purified by ammonium sulfate fractionation. Lane M, protein molecular standard; Lanes 1 – 4, the ammonium sulfate saturation was 60, 70, 80, 90%, respectively.

Conclusion

The efficient procedure of expression and purification may be useful for the mass production of Chitosanase. Of course, further optimization of production condition is needed to use *P. pastoris* as an expressing system of CSN and higher activity of enzyme may be acquired.

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