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Construction of the highly secreted endochitinase *Pichia pastoris* strain and the optimization of chitin-degrading conditions

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ABSTRACT

The endochitinase has a very important application in the preparation of bioactive chitooligosaccharides by chitin degradation. In this paper, the mature endochitinase cDNA from *Trichoderma* sp. was overexpressed in *Pichia pastoris* GS115 in order to enhance its activity. The screened recombinant strain could secrete the endochitinase into the culture broth highly on induction with methanol of 0.5%. The activity of the endochitinase was up to 89.3 U/mL, corresponding to its concentration of 365 mg/L, at 72 h after induction. The optimal chitin-degrading conditions with it were as follows: powder chitin concentration 4%, pH 7.0 and temperature 30 °C. Under above conditions, the content of the reducing sugar obtained was up to 398.4 μg/mL at 10 h. These results indicated that the expressed endochitinase exhibited an exceptionally excellent chitin-degrading ability, and could be used for the preparation of bioactive chitooligosaccharides in future.

1. Introduction

Chitin, after cellulose, is one of the most abundant and renewable biopolymers in nature, with a constantly recycled mass in the biosphere of 10^{11} tons per year (Muzzarelli et al., 2012). It is a structural constituent of some organisms, including crustaceans, fungi and invertebrates, etc. (Jayakumar, Prabaharan, Nair, & Tamura, 2010; Muzzarelli, 2011; Zhang, Zhang, Jiang, & Xia, 2012). Some developments and applications are those by Parameswaran, Chandran, and Pradeep (2007), Ramirez-Coutino, Marin-Cervantes, Huerta, Revah, & Shirai (2006) and Zhang et al. (2012). Among its depolymerization products, chitooligosaccharides which include 2–10 units of N-acetyl-glucosamine have received more and more attention owing to their many important physiological functions, such as enhancing the body immunity, promoting the intestinal health, and eliminating toxins from the body and inhibiting the growth of tumor cells. Therefore, they have many potential applications in the fields of foods, cosmetics and medicines, etc. (Aranaz et al., 2009; Harish Prashanth & Tharanathan, 2007; Jayakumar et al., 2010; Tao et al., 2006). At present, however, chitooligosaccharides were mainly prepared by the acid hydrolysis of chitin, it has some disadvantages, including the acid waste by the usage of the highly concentrated hydrochloric acid, the high production cost and the heavily environmental pollution, etc. Therefore, the enzymatic method has been studied as an alternative to the conventional acid hydrolysis (Yu & Li, 2008).

Chitin can be degraded by chitinases (EC 3.2.1.14). They are a group of complex hydrolytic enzymes that catalyze the depolymerization of chitin and divided into two categories: exochitinases, demonstrating activity only for the non-reducing end of the chitin chain and endochitinases, which hydrolyze internal β-1,4-glycoside bonds to produce reducing oligosaccharides (Aam et al., 2010; Horn et al., 2006). Therefore, chitooligosaccharides can be prepared by chitin degradation using the endochitinase. However, the present activities of endochitinases from naturally isolated microorganisms are often low and not satisfied with the requirement for the industrial production (Tao et al., 2006; Wu, 2008). Thus, the enhancement of the endochitinase activity becomes a key problem in the preparation of chitooligosaccharides by chitin degradation.

Trichoderma sp. is a common soil fungus used as the biological control agent due to its ability to parasitize phytopathogenic fungi. The endochitinase from it has deserved at least a decade of studies (Yu & Li, 2008), most of which focused on its production by the traditional fermentation, structure, function, biocatalytic mechanism and kinetics (Anand & Reddy, 2009; Perez-Martinez, Deleon-Rodriguez, Harris, Herrera-Estrella, & Barba de la Rosa, 2007; Wu, 2008; Yu & Li, 2006). Despite these, the activity of the endochitinase from *Trichoderma* sp. by the traditional fermentation is still low (Wu, 2008).

An efficient method for enhancing the enzyme activity is the usage of the recombinant DNA technology. In the past twenty years, a number of enzyme genes have been cloned from different

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organisms, overexpressed and industrialized by the recombinant DNA technology (Chen, Shen, & Wu, 2009; Yamabhai et al., 2008; Zhou, Zhang, & Anthony, 2008). *Pichia pastoris* has been developed as a host for a high level heterologous gene expression using a strong methanol-controlled alcohol oxidase promoter. It has a potential for the high protein expression level, the efficient secretion of products and the easy culture to a high cell density (Cereghino & Cregg, 2000). Therefore, it is very attractive for investigating whether the endochitinase from *Trichoderma* sp. can be expressed in *P. pastoris* host cells for enhancing its activity, and further exploring optimal powder chitin-degrading conditions with it for future use.

In the present study, the mature endochitinase cDNA from *Trichoderma* sp. was cloned and overexpressed in *P. pastoris* strain GS115, and the optimal conditions of the powder chitin degradation, such as the substrate concentration, pH, the temperature and the degradation time, were also reported.

2. Materials and methods

2.1. Strains, plasmids and reagents

Trichoderma sp. strain was purchased from the China Center for Type Culture Collection (CCTCC, No. AF93252). *Escherichia coli* strain DH5α was purchased from the Novagen Co. Ltd., USA and used for the routine plasmid amplification. *P. pastoris* strain GS115 and the vector pPIC9K were purchased from the Invitrogen Co. Ltd., USA. The plasmid pET-1 bearing the mature endochitinase cDNA from *Trichoderma* sp. was constructed and kept in our laboratory (Yu & Li, 2008). PCR reagents and restriction endonucleases were purchased from the TaKaRa Biotech Co. Ltd., Japan. G418, chitin, 4-methylumbelliferyl (4-MU) and 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside (4-MUChT) were purchased from the Sigma, Co. Ltd. All other reagents were analytical grade and they were used as supplied.

2.2. Construction of the expression vector

The degenerated primer set, F_1: 5′-CATGAATTCGCCAGCGGATATGCAAACGCC-3′ and R_1: 5′-AAGCGGCCGCCTAGTTGAGACCGCTTCGGATGTT-3′, was designed for the amplification of the mature endochitinase cDNA from the constructed plasmid pET-1. Restriction sites *Eco*RI and *Not*I were added to the upstream from the primer F_1 and the downstream from the primer R_1, respectively. PCR conditions consisted of an initial denaturation at 94 °C for 5 min, 35 cycles of the amplification consisted of the denaturation at 94 °C for 1 min, annealing at 58 °C for 1.5 min, and extension at 72 °C for 1 min. Then the further extension at 72 °C was performed for 10 min. The resultant PCR product was purified, digested with *Eco*RI and *Not*I, and ligated into the *Eco*RI–*Not*I digested vector pPIC9K to construct the recombinant plasmid pPIC9K-ECH. The plasmid pPIC9K-ECH was transformed into the *E. coli* strain DH5α by the $CaCl_2$-heat shock method (Sambrook, Fritsch, & Maniatis, 1989). The recombinant plasmid was isolated from the positive transformant using a High Pure Plasmid Isolation Kit (Roche, Germany). The presence and the correct orientation of the insert sequence were confirmed by DNA sequencing.

2.3. P. pastoris transformation and identification

Competent *P. pastoris* GS115 cells were prepared according to the specification provided by the manufacturer. Transformants were streaked on YPD plates containing G418 with different concentrations (0.5, 1.00, 2.00, 4.00 mg/mL), and incubated at 30 °C until the single colony appeared. The transformant with the fastest growth rate on the YPD plate containing G418 of the highest concentration was screened. The genomic DNA of this transformant was isolated according to the Multicopy *Pichia* Expression Kit provided by the manufacturer. The PCR amplification was done to confirm whether the mature endochitinase cDNA was integrated into the genomic DNA of *P. pastoris* strain according to the specification provided by the manufacturer. PCR amplification primers used, 5′ AOX_1 (5′-GACTGGTTCCAATTGACAGC-3′) and 3′ AOX_1 (5′-GCAAATGGCAT TCTGACATC C-3′), were provided by the manufacturer.

2.4. Time-course analysis of the recombinant endochitinase expression

The screened transformant was inoculated into a 15 mL BMGY medium (10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base with ammonium sulfate and without amino acids, 4×10^{-5}% (w/v) biotin, 1.0% (v/v) glycerol) followed by incubation at 28 °C with a vigorous agitation in a shaking incubator until the culture reached an OD_{600} = 3.0. Cells were harvested by centrifugation at 12,000 rpm for 20 min and resuspended in 15 mL BMMY medium (10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base with ammonium sulfate and without amino acids, 4×10^{-5}% (w/v) biotin, 0.5% (v/v) methanol). Cultures were incubated with a constant agitation at 250 rpm for 120 h in total at 28 °C. During the incubation, 75 μL of the methanol was added to BMMY cultures to maintain a continuous induction and 500 μL of cultures was taken out and centrifuged at 12,000 rpm for 10 min at 4 °C every 24 h. The supernatant was stored at −80 °C until use.

The qualitative analysis of the endochitinase activity was performed according to the method described by Tao et al. (2006) with minor modifications. Holes with a diameter of 5 mm were dug in a water-agar plate containing 1% (w/v) colloidal chitin. Two hundred microliters of the sample was dripped into the hole in the water-agar plate. The plate was incubated at 37 °C for 24 h. The endochitinase activity was assayed by observing clear zones. The quantitative analysis of the endochitinase activity was determined according to the method of Kudan and Pichyangkura (2009) with 4-MUChT as the substrate. One unit of the endochitinase activity was defined as 1 ml of the enzyme which produced 1 ng of 4-MU per minute at pH 5.6 and 40 °C. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on a 15% running gel. The resolved proteins were visualized by staining with Coomassie Brilliant Blue R250. The concentration of the recombinant endochitinase was analyzed according to the specification of the Quantity One Software from Bio-Rad Laboratories after SDS-PAGE. The zymogram for the endochitinase activity was assayed by a fluorometric method using 4-MUChT as the substrate (Perez-Martinez et al., 2007).

2.5. Determination of optimal power chitin-degrading conditions with the recombinant endochitinase

To determine the optimal concentration of the powder chitin, the content of the reducing sugar was studied using 4 mL reaction mixture containing 2 mL crude enzyme and 2 mL citric acid–disodium hydrogen phosphate buffer (pH 5.6) at 40 °C for 3 h. The powder chitin concentration ranged from 1% to 8% (w/v). The optimal temperature was investigated by incubating 4 mL reaction mixture for 3 h at different temperatures ranging from 20 to 80 °C. Reaction mixtures contained 2 mL crude enzyme, 2 mL citric acid–disodium hydrogen phosphate buffer (pH 5.6) and the optimal concentration of the powder chitin. The optimal pH was investigated by incubating 4 mL reaction mixture for 3 h at the optimal temperature. Reaction mixtures contained 2 mL crude enzyme,

2 mL buffer and the optimal concentration of the powder chitin. Buffers used were a 0.1 M citric acid–disodium hydrogen phosphate buffer ranging from pH 3 to 8. The time course of the powder chitin degradation was investigated by incubating 4 mL reaction mixture at the optimal temperature. Reaction mixtures contained 2 mL crude enzyme, 2 mL buffer and the optimal concentration of the powder chitin. Water was used as the control and samples were analyzed in duplicate. The content of the reducing sugar was detected by DNS method with *N*-acetyl-glucosamine (GlcNAc) as the standard (Zhang, Zhang, & Li, 1997).

3. Results and discussion

3.1. Construction of the expression vector pPIC9K-ECH

A PCR product was amplified with the pET-1 plasmid as the template which was constructed before in our laboratory (Yu & Li, 2008). The PCR product was purified, digested with *Eco*RI and *Not*I, and ligated into the *Eco*RI–*Not*I digested pPIC9K vector to construct the recombinant plasmid pPIC9K-ECH. The correct clone was confirmed by digestion with *Eco*RI and *Not*I. The recombinant plasmid was also confirmed by PCR amplification and DNA sequencing. All these results indicated that the expression vector pPIC9K-ECH was successfully constructed (Fig. 1). In order to make endochitinase secreted highly, its mature cDNA was designed to fuse to the downstream of the strong α-factor signal peptide from *Saccharomyces cerevisiae* when the plasmid pPIC9K-ECH was constructed.

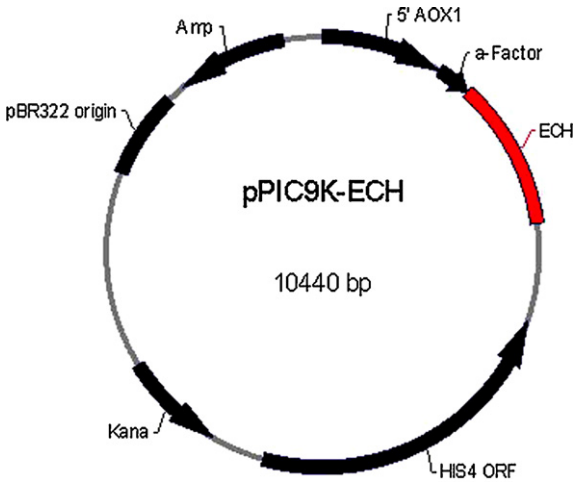

Fig. 1. The schematic map of the constructed expression vector pPIC9K-ECH bearing a mature endochitinase cDNA sequence. Amp: ampicillin resistance gene; Kana: kanamycin resistance gene; 5′AOX1: 5′ alcohol oxidase promoter 1; α-Factor: α-factor secretion signal; HIS4 ORF: histidine open reading frame; pBR322 origin: the origin of replication; ECH: the mature endochitinase cDNA sequence.

3.2. P. pastoris transformation and identification

After being linearized by *Bpu*1102I, the plasmid pPIC9K-ECH was integrated into the genome of the strain *P. pastoris* GS115 by the electroporation method. Transformants were screened on YPD plates containing different concentrations of G418 ranging from 0.5 to 4 mg/mL. Transformants which were resistant to the highest G418 concentration were screened. After induction with 0.5% (v/v) methanol, a transformant with the highest endochitinase activity was selected directly. The PCR analysis based on the specification provided by the manufacturer showed that the screened transformant had bands of expected sizes, 1293 bp (endochitinase, 1193 bp plus the terminal sequence of 3′ AOX_1, 100 bp) or 1573 bp (endochitinase, 1193 bp plus the terminal sequence of 5′AOX_1, 380 bp) in 1% (w/v) agarose gel (Fig. 2A), but no band was found with the genome from the control strain transformed with the primitive plasmid pPIC9K as the template, which indicate that the linear construct pPIC9K-ECH has been integrated into the genome of *P. pastoris* GS115. When the fermentation broth from each of nine selected recombinant strains was dripped separately into the hole in the chitin agar plate, clear zones (from number 1 to 9) could be observed (Fig. 2B). This indicates that the expressed endochitinase from each transformant is active and can degrade colloidal chitin to produce a pronounced clear zone. However, this did not appear when the fermentation broth from the control strain (number 10) was used. Compared to that of the control strain GS115/pPIC9K, the protein with the molecular weight of about 45 kDa appeared clearly in the SDS-PAGE analysis (Fig. 2C). The zymogram was carried out to further confirm the chitinolytic activity, which was visualized as a pronounced fluorescent band (Fig. 2C).

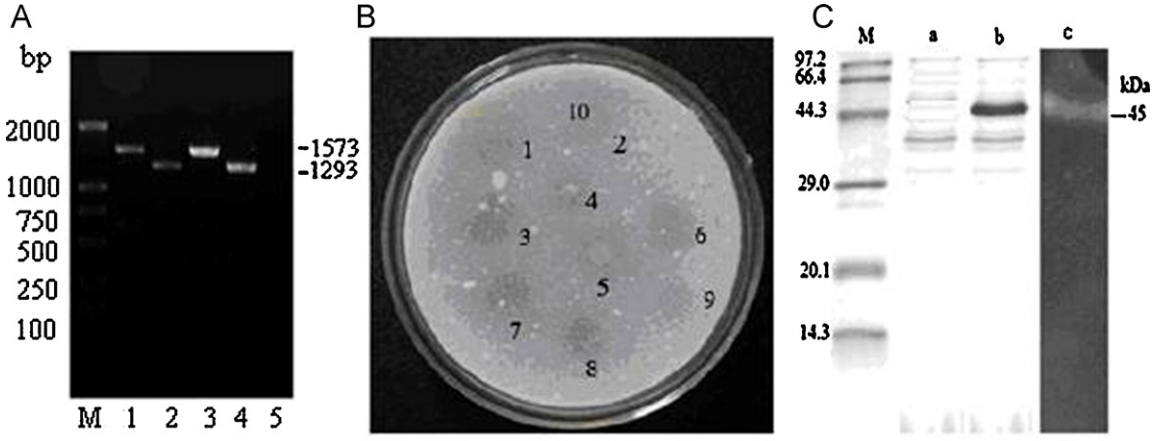

Fig. 2. The identification of the recombinant endochitinase by PCR, the colloidal chitin-hydrolyzing activity, the SDS–PAGE and the zymogram analysis. (A) Identification of the recombinant strain with PCR. *Lane M*: DL2000, *lanes 1* and 3: PCR products using the primer 5′AOX_1 and R_1, *lanes 2 and 4*: PCR products using the primer F_1 and the antisense 3′AOX_1, *lane 5*: PCR product using the primer F_1 and R_1 with the genome of the control strain transformed with the primitive vector pPIC9K. (B) Analysis of the colloidal chitin-hydrolyzing activity with the recombinant endochitinase. Number 1–9: screened transformants containing a mature endochitinase cDNA. Number 10: the control strain GS115 transformed with the primitive plasmid pPIC9K. (C) SDS–PAGE and zymogram analysis of the endochitinase expression. *lane M*: protein molecular weight standard, *lane a*: SDS–PAGE analysis of the supernatant of the control strain GS115 transformed with the primitive plasmid pPIC9K, *lane b*: SDS–PAGE analysis of the supernatant of the recombinant strain GS115 transformed with the expression plasmid pPIC9K-ECH, *lane c*: zymogram analysis of the recombinant endochitinase.

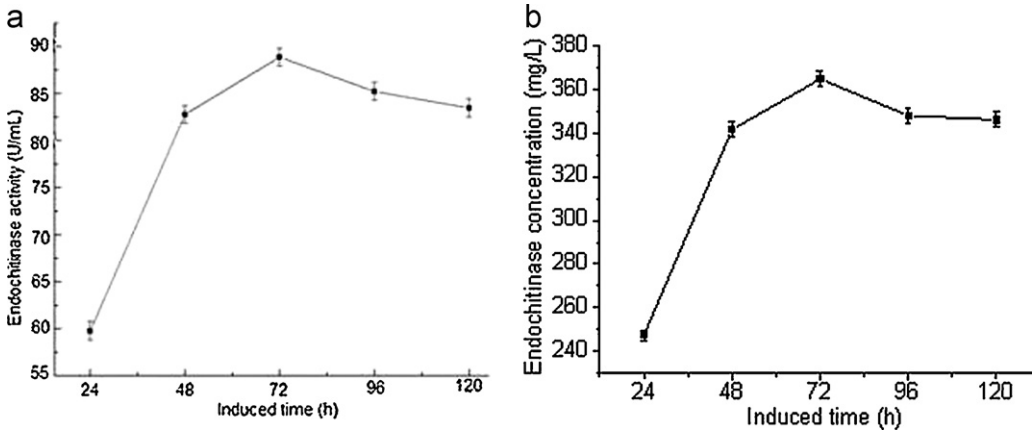

Fig. 3. The time-course analysis of the expression of the recombinant endochitinase by the recombinant *P. pastoris*. (A) Change in endochitinase activities with the induced time, and (B) change in endochitinase concentrations with the induced time. Experiments were repeated three times and data are expressed as the mean ± standard error. Control strains, *P. pastoris* GS115 and *P. pastoris* GS115/pPIC9K, do not contain a mature endochitinase cDNA from *Trichoderma* sp. and an endochitinase DNA in their genome, and hence cannot degrade the powder chitin.

3.3. Time-course analysis of the expression of the recombinant endochitinase

The time-course analysis of the expression of the recombinant endochitinase is shown in Fig. 3. Fig. 3A showed that the endochitinase activity increased quickly after the recombinant strain was induced with 0.5% (v/v) methanol. At 72 h after induction, its activity reached a maximum value of 89.3 U/mL. This activity is 4.57-fold higher than that in the primitive strain *Trichoderma* sp. (Yu & Li, 2006). Thereafter, the slight decrease in its activity appeared when the induction time increased. This is probably due to the degradation of the recombinant endochitinase by the protease secreted by the host strain itself, implying that the protease-deficient *P. pastoris* strain may be a better choice. Fig. 3B indicated that the concentration of the expressed endochitinase showed the same varying trend as its activity, and the maximum endochitinase

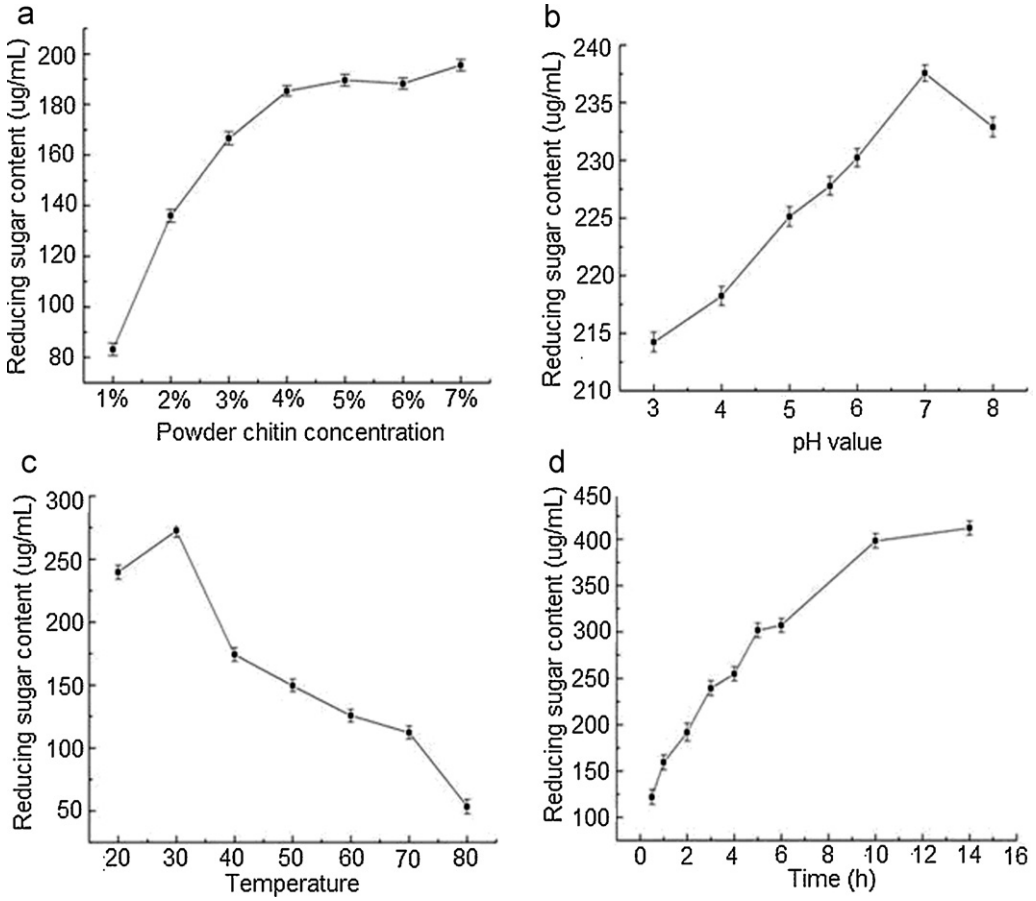

Fig. 4. Determination of optimal chitin-degrading conditions for the recombinant endochitinase. (A) Effect of the powder chitin concentration on the enzymatic reaction. (B) Effect of pH on the enzymatic reaction. (C) Effect of the temperature on the enzymatic reaction. (D) Change in the content of the reducing sugar during chitin degradation by the recombinant endochitinase. Experiments were repeated three times and the content of the reducing sugar is expressed as the mean ± standard error.

concentration obtained was 365 mg/L at 72 h after induction with the methanol.

The maximum value of 89.3 U/mL of the endochitinase activity (corresponding to its concentration of 365 mg/L) from this recombinant strain screened by G418 of 4 mg/mL and induced for 72 h was comparable with a previous study conducted by Arriaga, Acosta-Munguía, Pérez-Martínez, León-Rodríguez and Barba de la Rosa (2010) in which 317 mg/L of the endochitinase concentration was obtained from the recombinant strain integrated with the endochitinase cDNA from *T. atroviride* and screened by Geneticin of 250 μg/mL and induced for 96 h. The difference in the endochitinase concentration could be explained well by different antibiotic concentrations used when transformants were screened. Higher antibiotic concentration may increase the copy number of the endochitinase expression cassette, and hence enhance its activity. This was also confirmed by a previous study conducted by Long, Liu, Liu, and Wang (2006) in which the hirudin was overexpressed in *P. pastoris*.

3.4. Optimal powder chitin-degrading conditions with the recombinant endochitinase

The optimal powder chitin-degrading conditions with the recombinant endochitinase were determined and the results are shown in Fig. 4. Firstly, the optimal concentration of the powder chitin was found to be 4% (w/v) (Fig. 4A). The optimal temperature was found to be 30 °C (Fig. 4B). Secondly, a pH-activity optimum was found to be 7.0 under the optimal substrate and temperature conditions (Fig. 4C). Finally, the optimal reaction time for the powder chitin degradation was found to be 10 h and the content of the reducing sugar obtained was up to 398.4 μg/mL (Fig. 4D).

4. Conclusion

The results of the present research demonstrate that *P. pastoris* is a more convenient and excellent expression system for an efficient production of the endochitinase. The expression vector bearing the mature endochitinase cDNA was constructed and integrated into the genome of *P. pastoris* GS115 strain, and the recombinant strain which could secrete the endochitinase effectively was screened by PCR, SDS-PAGE, zymogram and the qualitative and quantitative analysis of the endochitinase activity. The endochitinase activity was up to 89.3 U/mL, corresponding to its concentration of 365 mg/L, after 72 h induction with 0.5% methanol. The content of the reducing sugar was up to 398.4 μg/mL under the optimal conditions: the powder chitin concentration 4%, pH 7.0, the temperature 30 °C and the induction time 10 h. All these results lay a good foundation for the preparation of chitooligosaccharides by chitin degradation using the recombinant endochitinase. Continued efforts should be given to further prepare chitooligosaccharides with a highly physiological activity with this recombinant endochitinase.

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