High level expression of *Saccharomyces cerevisiae* chitinase (*Sc*CTS1) in *Pichia pastoris* for degrading chitin

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Abstract: Chitin is the second most abundant renewable biopolymer in the world. Chitinases play important roles in the degradation of chitin. Chitinases are produced by different organisms for different purposes, which are widely expressed in the three domains of life, ranging from archaea, bacteria, to fungi, yeasts, plants, insects, and even vertebrates. But there are few reports about *Saccharomyces cerevisiae* chitinase (*Sc*CTS1). The aim of this study was to realize the high level expression of *Sc*CTS1. The *ScCTS*1 was cloned into the expression vector pPIC9K. The recombinant plasmid was linearized and transformed into competent *Pichia pastoris* GS115. After screening by G418 plate, the fermentation conditions were optimized. Ultimately, under the optimal fermentation conditions, *Sc*CTS1 enzymatic activity reached up to 94.6 U/mL. This paper presents the first report on the heterologous expression of a full-length *Sc*CTS1 with considerably high activity. The work will not only make a great stride towards its potential applications in biotechnology, but also facilitate elucidating the precise mechanism of yeast cell division.

Keywords: chitinases, heterologous expression, *Saccharomyces cerevisiae*, *pichia pastoris*, biotechnology

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1 Introduction

Chitin is a linear polymer of β-(1,4)- linked N-acetyl-D-glucosamine (GlcNAc) units[1]. It is the second most abundant renewable biopolymer after cellulose on earth and its estimated annual production is more than 10^{10}-10^{12} t in the aquatic ecosystems alone[2]. The derivatives of this biopolymer, including GlcNAc and chitin oligosaccharide (COSs), have a wide range of applications in food, medicine, agriculture and chemical industries[3]. Thus, the conversion of chitin into the above mentioned biologically active compounds has attracted increasing interests in the last several decades. This process can be realized by either chemical or enzymatic catalysis, and the latter is more environmentally sustainable and effective. Chitinases (EC 3.2.1.14) play important roles in the decomposition of chitin to renewable sources by enzymatically cleaving the β-1,4-glycosidic bonds of the chitin chain. Additionally, these enzymes can also be used in some other applications, such as rapid preparation of filamentous fungal protoplasts and serving as antifungal biocontrol agent to protect plants[4].

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Chitinases are widely expressed in the three domains of life, ranging from archaea, bacteria, to fungi, yeasts, plants, insects and even vertebrates[3-9]. Traditionally, chitinases can be divided into two categories of glycoside hydrolase (GH) family 18 and 19, with different structures and catalytic mechanisms. GH family 18 chitinases distribute in all kingdoms of life and thus can be subdivided into bacterial-type and plant-type; whereas GH family 19 chitinases are extensively found in plants[10]. Chitinases are produced by different organisms for different purposes. For bacteria, chitinases are solely involved in GlcNAc-containing carbohydrates degradation for supplying their nutrient source. Besides the nutritional purpose, fungal chitinases also participate in fungal cell wall degradation and remodeling. In yeast, chitinases are found to be closely related with cell separation[3,4,11]. So far, there have been a large amount of researches about heterologous expression of chitinases form bacteria, fungi, and even plant and animals in order to enhance chitinases production and their activity[12-15]. However, few chitinases from yeast have been cloned and heterologously expressed.

Saccharomyces scerevisiae has two chitinase-encoding genes, namely *ScCTS*1 and *ScCTS*2. *Sc*CTS1 contains four domains: a signal sequence, a catalytic domain, a Ser/Thr-rich domain, and a high-affinity chitin-binding domain[16]. It belongs to the class of plant-type family 18 chitinase with a molecular weight of 130 kDa. *Sc*CTS2 is similar to a bacterial-type family 18 chitinase and has been extensively studied. In contrast, the precise roles of *Sc*CTS1 for *S. scerevisiae* remain unclear. Thus, *Sc*CTS1 has been attempted to be expressed in *Aspergillus awamori*, but the activity of 0.141 U/mL was quite low and scaled-up from flask to 3-l pilot scale decreased *Sc*CTS1 production[17]. Later, Hurtado-Guerrero et al.[18] expressed the catalytic domain of *Sc*CTS1 in *Pichia pastoris* for its structure research. Recently, *Sc*CTS1 was heterologously expressed in *P. pastoris* but the enzyme activity remained very low[19]. Thus, production of highly active *Sc*CTS1 is very difficult. Meanwhile, it is necessary and urgent to over express the full-length *Sc*CTS1 with high activity, with an effort to understand its mechanism of action for yeast, improve its biotechnological applications, and speed up the design and synthesis of chemicals to significantly inhibit these plant-type chitinases. Besides *Escherichia coli*, *P. pastoris* has been developed as one of the favorite expression hosts due to its many advantages over other hosts[20,21]. This strain has been successfully employed to highly express chitinases[22-24].

Based on the currently available reports, *P. pastoris* with a closer relationship to *S. cerevisiae* was choosen as the expression host of full-length *Sc*CTS1 with full-length in this study.

The purpose of this study was to over express the full-length ScCTS1 of *S. scerevisiae* with high activity. In this work, we described the cloning and expression of *ScCTS1* gene in *P. pastoris*. The gene was expressed using the pPIC9K vector containing the AOX1-methanol inducible promoter. Meanwhile, the recombinant strain with the highest *Sc*CTS1 production was screened and employed as the stain for a series of optimization studies in both flask and fermenter.

2 Materials and methods

2.1 Materials

The strains, plasmids and primers used are listed in Table 1. *S. cerevisiae* W303-1A, a chitinases producer, and *P. pastoris* GS115 were deposited in the Laboratory of Applied Microbiology and Metabolic Engineering. *E. coli* JM109 was used as transformation host. *E. coli* strains were cultivated at 37°C in Lysogeny Broth (LB) medium, 100 μg/mL of ampicillin was added to the media when needed. The plasmids of pMD18-T (Takara) and pPIC9K were used as the cloning or expression vector. YPD, BMGY, BMMY and MD media were prepared as described by Multi-Copy Pichia Expression Kit (Invitrogen, USA). Chitin was purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

2.2 Methods

2.2.1 Plasmid construction

The genomic DNA of *S. cerevisiae* W303-1A was isolated according to the standard protocols. Primers of Sccts1F and Sccts1R for PCR amplification were

specifically designed based on the nucleotide sequence of *ScCTS1* gene (GenBank No. M74070).

Table 1 Strains, plasmids and primers used in this study

Strains, plasmids, or primers	Relevant characteristics	Source or reference
Strains		
Saccharomyces cerevisiae W303-1A	*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade 2-1 can1-100 gal suc2*	LAMME[a]
Pichia pastoris GS115	*his4 mut*$^+$	Invitrogen
Escherichia coli JM109	*recA*1 *supE*44 *endA*1 hsdR17 gyrA96 *relA*1 *thi*	LAMME[a]
Plasmids		
pMD18-T	2.7-kb vector for PCR product cloning, Amp^r	Takara
T- *ScCTS1*	4.4 kb, *ScCTS1*, Amp^r	This study
pPIC9K	9.3-kb expression vector, G418^r	Invitrogen
pPIC9K- *ScCTS1*	12.1 kb, *ScCTS1*, G418^r	This study
Primers[b]		
Scts1F	5'-ATCGAATTCATGTCACTCCTTTACA TCATTC-3'	This study
Scts1R	5'-TGACTGCGGCCGCTTAAAAGTAATT GCTTTCCAAAT-3'	This study

Note: [a] LAMME (Laboratory of Applied Microbiology and Metabolic Engineering).

[b] Sequences of the restriction sites are shown in bold.

The amplification product was subcloned into pMD18-T and sequenced. Subsequently the *ScCTS1* fragment was inserted between *Eco*RI and *Not*I sites of pPIC9K, resulting in pPIC9K-*ScCTS*1. The *ScCTS*1 gene was cloned in-frame to the secretory signal peptide on pPIC9K, which would enable the fusion chitinase to be secreted into the growth media. Both pMD18-T-*ScCTS*1 and pPIC9K-*ScCTS*1 were constructed in *E. coli* JM109.

2.2.2 Transformation of *P. pastoris* GS115

The pPIC9K-*ScCTS1* linearized with *Sal*I was introduced into *P. pastoris* GS115 (His$^-$/Mut$^+$) by the electro-transformation method[14]. The recombinant His$^+$ yeast strains were screened by plating on MD plate at 30°C. The positive recombinants were then subcultured on MD plate containing 1.0 mg/mL G418 and verified by restriction enzyme analysis and PCR using ScctsF1/ScctsR1. The MD plate containing 4.0 mg/mL G418 was used to screen multicopy transformants to improve *Sc*CTS1 expression level.

2.2.3 Cultivation conditions for *Sc*CTS1 expression in *P. pastoris*

A single colony of the recombinant *P. pastoris* GS115 was inoculated in 75 mL of BMGY medium in 250 mL flask, and grown at 28-30°C with shaking at 240 r/min until the OD_{600} of the culture reached 2. The cells were harvested by centrifugation at 3 000 *g* for 5 min. Subsequently the pellets were re-suspended in 25 mL BMMY medium (containing 1% chitin powder), and inoculated at 30°C in 100 mL flask. When OD_{600} of the culture reached 5, methanol was added at a final concentration of 1% (V/V). Every 24 h, methanol was fed into the medium to maintain 1% methanol concentration. After 72 h, the cells were pelleted by centrifugation and the supernatant of the expressed protein *Sc*CTS1 was enzymatically assayed and analyzed by SDS-PAGE.

2.2.4 Optimization of fermentation conditions for *Sc*CTS1 expression

One-variable optimization for maximum chitinase yield by *P. pastoris* was carried out in flask. One variable under this study was varied with keeping others constant. Each subsequent variable was tested after taking into account the previously optimized variable. The variables included chitin concentration (1%-6%), inoculation concentration (1%-5%), methanol concentration (0.5%-3%), pH (6-8) and induction time (24-120 h).

Based on the optimal parameters obtained in flask level, the subsequent scale-up experiments were carried out in a 5 L fermenter with a working volume of 2 L. The temperature, pH and agitation speed of fermenter system could be monitored. The vessel was equipped with pH electrode, dissolved oxygen (DO) probe, temperature probe, and four peristaltic pumps for feeding, antifoam, acid, and base mode. The standard bioprocess conditions were as follows: pH 6.4, temperature 30°C, initial DO 100% of saturation. The constant pH was maintained by periodic automatic addition of 30% phosphoric acid or 2 M KOH. When the DO began to rise, addition of glycerol feeding (100 mL 100% glycerol and 250 mL 10×YNB and biotin) was started. After 24 h of fermentation, the feeding was stopped. Then, 30-50 mL methanol was fed into medium. When the DO was stable, the coupling value of methanol adding and DO was set to 50% and the methanol adding was started. The effects of four factors (agitation, aeration,

methanol adding and induction temperature) on *Sc*CTS1 yields were investigated in bioreactor. Influence of agitation was evaluated at the speed of 400 r/min, 500 r/min, and 600 r/min; the effect of aeration was tested at 3 L/min, 4 L/min, and 5 L/min. Under the optimal agitation and aeration, the coupling values of methanol adding and DO were set to 30%, 40% and 50% to determine the effects. Through the optimal parameters obtained above, fermentation was carried out at 30°C or 28°C in induction phase to evaluate the influences of induction temperature.

Samples were taken out every 12 h and their biomass was monitored spectrophotometrically by measuring absorbance at 600 nm. After centrifugation (5000 *g* for 10 min), supernatants were stored at −4°C for the enzyme assay.

2.2.5 Measurement of enzyme activity

Chitinase activity was assayed colorimetrically using colloidal chitin as substrate which was described by Xiao et al[25]. One unit of chitinase activity was defined as the amount of enzyme that produced 1 μg of reducing N-acetyl-D-glucosamine per hour under the experiment condition. Colloidal chitin was prepared by the method described by Songsiriritthigul et al.[13].

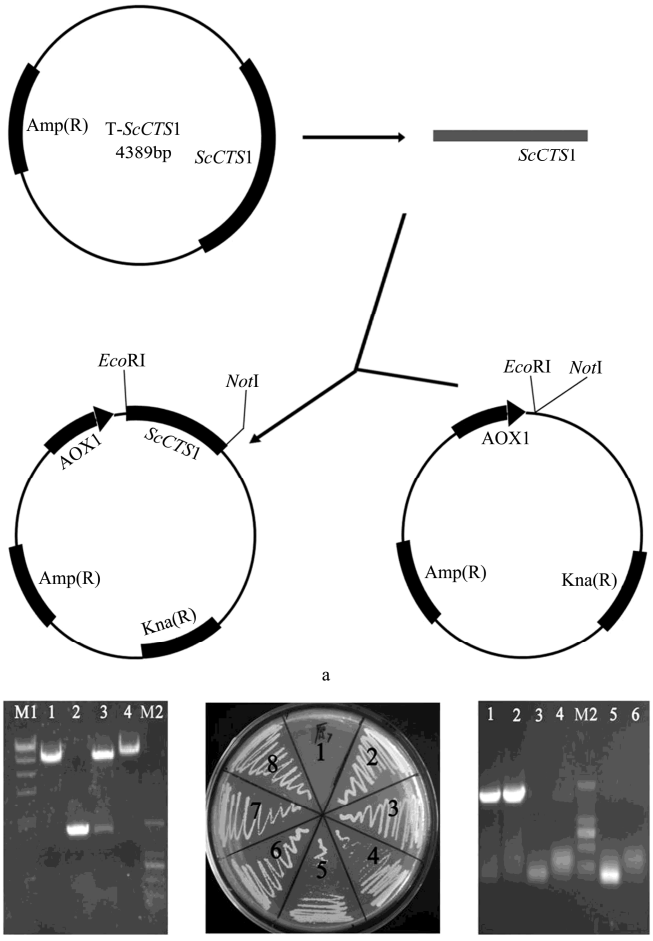
3 Results and discussion

3.1 Cloning of *ScCTS1* gene

ScCTS1 gene was amplified from the chromosomal DNA of *S. cerevisiae* W303-1A by PCR. As expected, a PCR product of approximately 1.7 kb in size was obtained. After recovery and purification, the fragment was ligated with pMD18-T (Figure 1a). The ligation product was transformed into *E. coli* JM109 to obtain the recombinant plasmid T-*ScCTS*1. After sequencing, the *ScCTS*1 gene digested by *Eco*RI and *Not*I was inserted into the pPIC9K linearized with the same restriction enzymes, resulting in pPIC9K-*ScCTS1* (Figure 1a). The plasmid of pPIC9K-*ScCTS*1 was confirmed by PCR and restriction enzyme analysis (Figure 1b), indicating pPIC9K-*ScCTS*1 has been successfully constructed.

3.2 Transformation of *P. pastoris* with *ScCTS*1

The *ScCTS*1 gene was placed under the control of the methanol-inducible AOX1 promoter in pPIC9K-*ScCTS*1. *P. pastoris* was transformed with the linearized pPIC9K-*ScCTS*1 which was digested by the unique site *Sal*I located in the promoter AOX1. The transformants were inoculated onto MD plate to screen positive transformants. *P. pastoris* and its transformants were subcultured onto MD+G418 (1 mg/mL) plates.



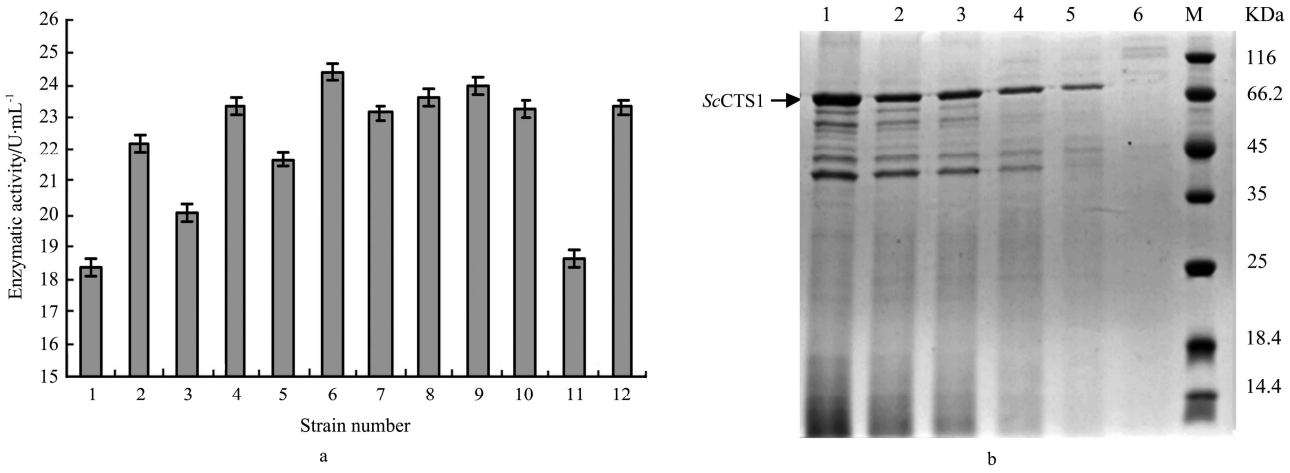
(a) Construction of recombinant plasmid pPIC9K-*ScCTS1*. *ScCTS1* products was inserted into pMD18-T vector and sequenced. The *ScCTS1* fragment with *Eco*RI and *Not*I sites was inserted into pPIC9K, resulting in pPIC9K-*ScCTS*1; (b) Verification of the plasmid pPIC9K-*ScCTS*1. M1: λDNA/*Hin*dIII markers, 1: pPIC9K/*Sal*I, 2: PCR products using pPIC9K-*ScCTS*1 and ScctsF1/ScctsR1 as templates and primers, respectively, 3: pPIC9K-*ScCTS*1 digestion products using *Eco*RI and *Not*I, 4: pPIC9K-*ScCTS*1 digestion products using *Sal*I, M2: DL2000 markers; (c) Phenotype validation of the *P. pastoris* GS115/pPIC9K-*ScCTS*1 on MD plates containing 1 mg/mL G418. 1: *P. pastoris* GS115, 2-8: *P. pastoris* GS115/ pPIC9K-*ScCTS*1; (d) Screening of the multicopy transformants of *P. pastoris* GS115/pPIC9K-*ScCTS*1 using PCR method. 1-2: transformants with multicopy *ScCTS*1, 3-4: transformants with low copy number of *ScCTS*1, 5. *P. pastoris* GS115, 6: *P. pastoris* GS115/pPIC9K, M2: DL2000 markers.

Figure 1 Construction and screening of recombinant *P. pastoris* GS115/ pPIC9K-*ScCTS*1

As shown in Figure 1c, *ScCTS*1 gene was integrated at AOX1 locus to form mono and multi-copied tandem integrants under the pressure of G418. Multicopied integrants of *P. pastoris*/pPIC9K-*ScCTS*1 were selected on plates with increased concentration of G418 (4 mg/mL) to increase *Sc*CTS1 expression level. Subsequently, the selected transformants were verified by PCR screening using ScctsF1/ScctsR1 as primers (Figure 1d). And the multicopied integrants of *P. pastoris*/pPIC9K-*ScCTS*1 with more PCR products as shown in Figure 1d were chosen for chitinase expression analysis.

3.3 Expression of *Sc*CTS1 in *P. pastoris*

One hundred and twenty-seven transformants were cultured in BMGY medium supplemented with 1% powdered chitin to prepare supernatants for *Sc*CTS1 enzyme activity analysis. Totally, there were 12 strains secreting considerably high levels of *Sc*CTS1, with activity ranging from 18.3 U/mL to nearly 25 U/mL using colloidal chitin as the substrate, into the liquid culture medium under the induction of 1% methanol for 72 h (Figure 2a). The strain, exhibiting maximum *Sc*CTS1 activity of 24.4 U/mL, was named *P. pastoris* GS115 *Sc*CTS1. SDS-PAGE results showed an apparently new band at 65 kDa compared with the sample with no methanol induction (Figure 2b), consistently with the theoretically predicted molecular weight. Notably, the *Sc*CTS1 yield produced by *P. pastoris* GS115 *Sc*CTS1 increased with the prolonging of methanol induction time. Herein, *P. pastoris* GS115 *Sc*CTS1 was used for further study about fermentation condition optimization.



(a) Enzymatic activity assay of *Sc*CTS1 expressed by 12 recombinants showing resistance against 4 mg/mL G418. The strain of No. 6 exhibited the highest activity and was named *P. pastoris* GS115 *Sc*CTS1; (b) SDS-PAGE analysis of total proteins in the supernatants of *P. pastoris* GS115 *Sc*CTS1. Samples were resolved on 15% polyacrylamide gel and then stained with Coomassie Blue R-250. Lane M, protein marker. Lanes 1-5, *P. pastoris* GS115 *Sc*CTS1 induced with 1% methanol for 120, 96, 72, 48 and 24 h, respectively. Lanes 6, negative control, *P. pastoris* GS115 *Sc*CTS1 with no methanol induction.

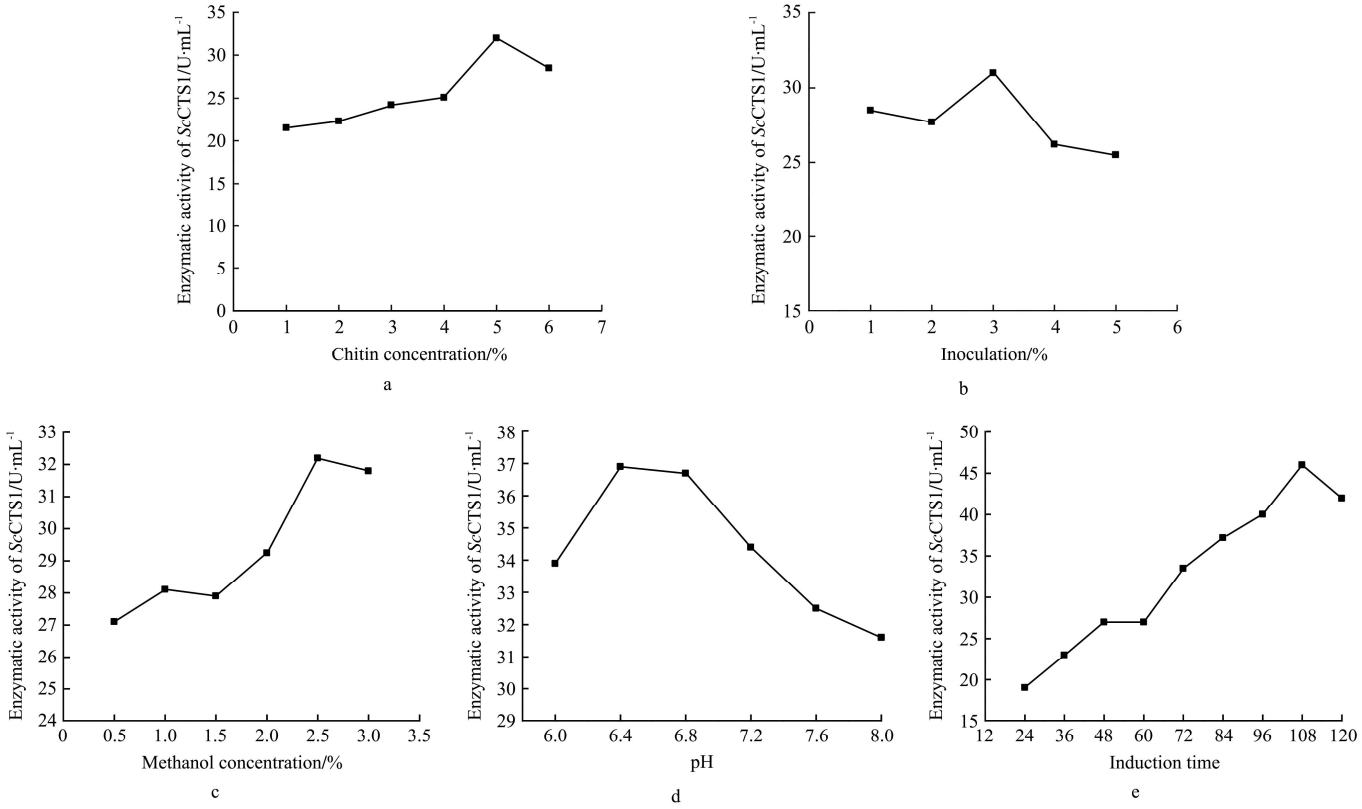
Figure 2 Analysis of *Sc*CTS1 expressed by *P. pastoris* GS115/pPIC9K-*ScCTS*1

3.4 Optimization of *Sc*CTS1 expression in shaking flask

The effects of five parameters on *Sc*CTS1 expression by *P. pastoris* GS115 *Sc*CTS1 were examined via one-variable optimization method. Colloidal chitin was supplied into the culture for the induction of *Sc*CTS1 expression. The chitinases activity significantly increased with the elevated chitin concentration from 1% to 5% and then decreased when chitin concentration reached 6% (Figure 3a). The *Sc*CTS1 activity exhibited upward trend in the range of inoculation concentration rising from 1% to 3% and then declined (Figure 3b). The maximum *Sc*CTS1 activity at 32 U/mL was observed when 3% methanol was used to induce protein expression (Figure 3c). As for the influence of inducing pH, the enzyme activity reached the peak (36.9 U/mL) at pH 6.4 (Figure 3d). The *Sc*CTS1 activity ultimately increased to 46.0 U/mL with the induction time of 108 h, and then decreased (Figure 3e). The optimal culture conditions for *Sc*CTS1 expression by *P. pastoris* GS115 *Sc*CTS1 in flask are as follows: 5% chitin concentration, 3% inoculum concentration, 2.5% methanol, pH 6.4 for induction, and 108 h induction duration.

The activity of heterologously expressed chitinases of *S. cerevisiae* reported by others was too low[17-18], which might result from low promoter transcription activity or

codon usage bias. In this study, the considerable activity of *Sc*CTS1 was obtained probably in that *ScCTS1* was strongly transcribed under the methanol-inducible AOX1 promoter. Interestingly, chitin effectively enhanced *Sc*CTS1 enzyme activity in the expression system. This phenomenon has never been reported and the relationship between the concentration of chitin and AOX1 promoter activity should be studied.

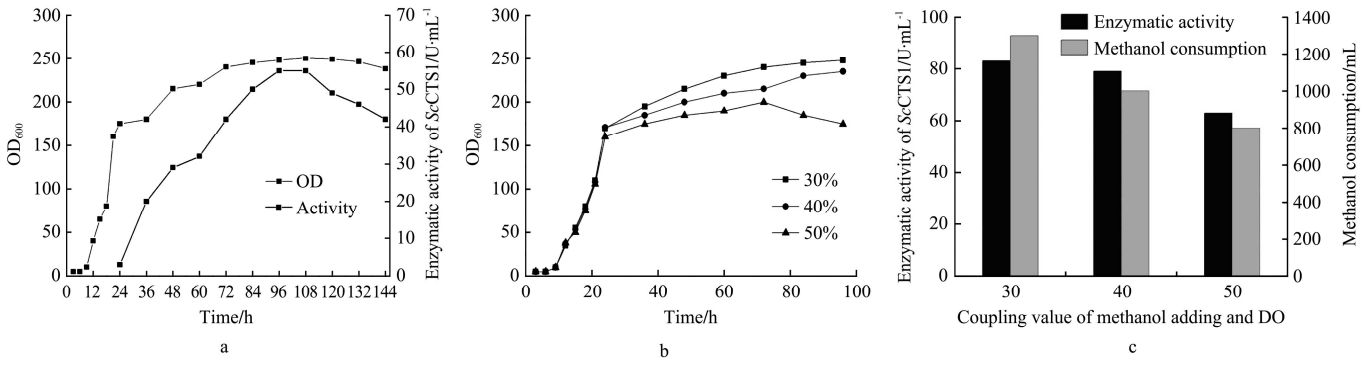


(a-e): Effects of chitin concentration, inoculation concentration, methanol concentration, pH and induction time of methanol on the enzymatic activity of *Sc*CTS1.

Figure 3 Optimization of culture conditions for *Sc*CTS1 expression by *P. pastoris* GS115 *Sc*CTS1 in flask

3.5 Optimization of *Sc*CTS1 expression in fermenter

Based on the optimal parameters obtained in flask, the process was further scaled up to a 5 L fermenter. The cell density was significantly enhanced compared with that of flask, while the chitinase activity was not (Figure 4a). There would be a great potential in increasing *Sc*CTS1 expression level by *P. pastoris* via high cell density fermentation. In order to improve the efficiency of the shake-flask expression system, the experiments were carried out in fermenter by studying the influences of agitation, aeration, methanol flow and induction temperature on *Sc*CTS1 expression.



(a) Time profiles of cell density and enzymatic activity of *Sc*CTS1; (b) Time profiles of cell density under different methanol and dissolved oxygen (DO) coupling values; (c) Effects of methanol flow on the activity of *Sc*CTS1 and methanol consumption.

Figure 4 Time profiles of *P. pastoris* GS115 *Sc*CTS1 fermentation and effects of methanol flow on the chitinase activity

Agitation and aeration would ultimately affect DO in the culture. The two fermentations were conducted in a similar manner. In the cell growth phase, the initial rotation (300, 400, 500 and 600 r/min) and air flow (2, 3, and 4 L/min) were examined, respectively. After 108 or 120 h fermentation, the cell density and *Sc*CTS1 activity in the supernatant were determined (Table 2). The time of fermentation was reduced against initial rotation, and the activity of chitinase increased to 75.6 U/mL with the initial rotation of 600 r/min. Similar results of air flow on the activity of chitinase were observed. When the air flow increased to 4 L/min, chitinases activity reached up to 76.8 U/mL.

Table 2 Effects of rotation speed and air flow on the activity of *Sc*CTS1 by *P. pastoris* GS115 *Sc*CTS1 in fermenter

Rotation and air flow	Fermentation time/h	Activity of *Sc*CTS1 /U·mL^{-1}	Cell density (OD_{600})
Rotation speed/r·min^{-1}			
300	120	48.3	151.7
400	108	61.4	173.4
500	108	72.8	199.8
600	108	75.6	212.0
Air flow/min^{-1}			
2	120	56.9	171.4
3	108	73.7	205.7
4	108	76.8	210.8

In the fermentation of *Sc*CTS1 in *P. pastoris*, glycerol and methanol were the carbon sources in different periods. Meanwhile, methanol was also the inducer for the AOX1 promoter. The method of methanol and DO coupling was used described in Section 2 for investigating the effects of methanol induction on chitinase expression. In the range from 30% to 50%, lower coupling value of methanol adding and DO resulted in higher cell density, chitinase activity (Figure 4b), and consumption of methanol (Figure 4c). The maximum chitinase activity reached 85.1 U/mL when the coupling value was 30%.

The suitable temperature for *P. pastoris* growth was 30°C in the first 24 h, but the temperature of induced phase for the optimal fermentation needed to be investigated. In the first 36 h, the cell density and the chitinase activity were almost not affected by temperature (Figures 5a and 5b), but with the continuous induction, the chitinase activity at 28°C got gradually higher than that at 30°C (Figure 5b). After 72 h induction, the chitinase activity was enhanced at 28°C (94.6 U/mL), compared with that at 30°C (83.3 U/mL). To our knowledge, this is the first report on the heterologous expression of the full-length *Sc*CTS1 with considerably high activity.

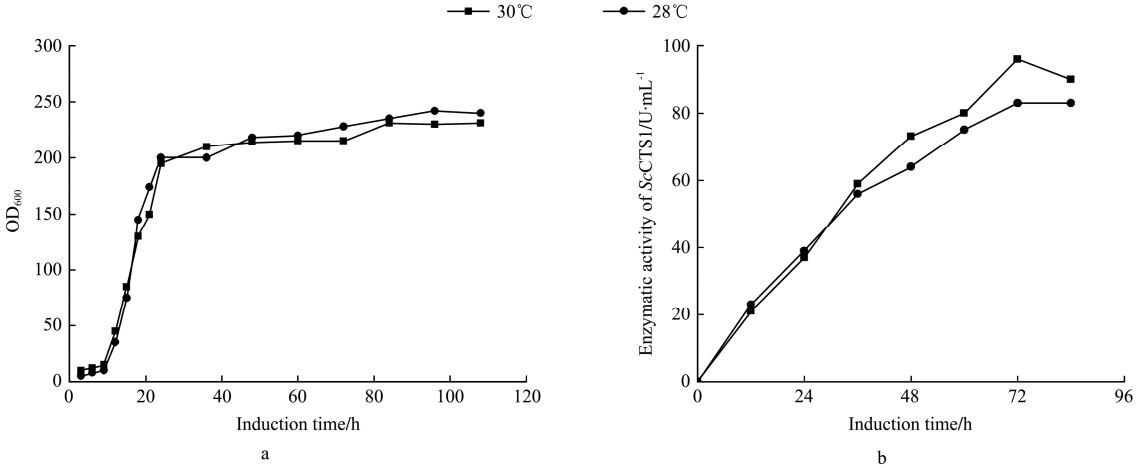


Figure 5 Time profiles of cell density of (a) *P. pastoris* GS115 *Sc*CTS1 and (b)enzymatic activity of *Sc*CTS1 under different induction temperatures using 1% methanol

P. pastoris has been developed as a notable system for high-level expression of heterologous proteins for more than 2 decades[26]. High cell density fermentation is recognized as an important and popular strategy for engineered strains to enhance heterologous protein expression levels. Up to date, high-level production of recombinant proteins have been successful using the high density fermentation of *P. pastoris*[27]. In the future research, we will investigate the effects of nitrogen source, initial glycerol concentration, and feeding mode on cell density and methanol feeding strategy on *Sc*CTS1 activity in order to further improve *Sc*CTS1 production

level by *P. pastoris*.

4 Conclusions

In this study, the chitinase of *Sc*CTS1 in *Pichia pastoris* was expressed. Under methanol induction conditions, *Sc*CTS1 was leaked into the medium. The *P. pastoris* transformant with the highest chitinase activity was selected for *Sc*CTS1 production in both shake flask and fermenter scale. At shake flask level, the maximal chitinase activity with 46.0 U/mL was obtained, in which the best fermentation conditions were as follows: 5% substrate concentration, 3% inoculum concentration, 2.5% methanol concentration, pH 6.4 for induction, and 108 h induction time. The optimal process parameters in a 5 L fermenter were as follows: initial stirring speed was 600 r/min, initial ventilation was 3 L/min, the coupling value of methanol adding and dissolved oxygen (DO) was 30%, and the induction temperature was 28°C.

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