

Constitutive expression of barley α -amylase in *Pichia pastoris* by high-density cell culture

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Abstract α -amy gene amplified from barley genome was cloned into MCS of pGAP9K to generate pGAP9K- α -amy which was then transformed into *Pichia pastoris* GS115 by electroporation. Transformants with multi-copies and high expression for the foreign gene were selected on G418 containing plate and expression analysis. The fermentation was carried out in a 50 l bioreactor with 20 l working volume, using a high-density cell culture method by continuously feeding with 50% glycerol-0.8% PTM4 to the growing culture for 54 h at 30°C. Under the control of GAP promoter (pGAP), α -amy gene was constitutively expressed. At the end of the fermentation, the α -AMY expression reached 125 mg/l, while the biomass growth was 186 as measured by absorption of 600 nm. The secreted α -AMY was purified to 97.5% by SP-Sepharose FF ion-exchange chromatography and affinity purification. The recombinant α -AMY showed activity on hydrolysis of starch.

Keywords α -Amylase · Constitutive expression · *Pichia pastoris* · High-density cell culture · Bioactivity

Introduction

Many species of prokaryotes and eukaryotes can produce of α -amylase (α -1,4-D-glucan-glucanohydrolase, EC.3.2.1.1, α -amylase, α -AMY) which can specially digest α -1,4 indican bond to hydrolyze starch as maltose, maltotriose hydrate and other oligosaccharide [1]. α -AMY has larger sales volume each year in the world. Now the marketable α -AMY is general the crude product from natural α -AMY producing strains, such as the *Bacillus subtilis* [2], which has been playing important role on bio-ethanol production, paper making, light industry and rock oil exploitation. But the purified α -AMY requiring in some trades, such as bio-pharmaceuticals and analytical chemistry, is hard up because natural α -AMY is always with lower rate to stay in the fermentation broth along other proteins from the host to make it become difficult to be purified [3]. Since it's possible to construction engineering strain by using well productivity strain as host and setting stronger promoter to control the aim gene [4], the recombinant protein expression would be preponderantly and easily to be purified [5, 6].

The methylotrophic yeast *Pichia pastoris* (*P. pastoris*) is an efficient host for expression and secretion of heterologous proteins [7, 8]. It can grow to very high cell densities to high express and secrete recombinant proteins but only very few proteins coming from the host cell consist in the fermentation broth [5, 9]. Glyceraldehyde-3-phosphate dehydrogenase (GAP) is a key enzyme in glycolysis, its promoter constitutively expresses genes well while *P. pastoris* cells grown on glucose, glycerol or oleic acid [9, 10]. Here, we study of using *P. pastoris*'s pGAP expression

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system to constitutive expression of α -AMY in *P. pastoris* by high-density cell culture.

Materials and methods

Strains, plasmids, enzymes and reagents

The strain of *P. pastoris* GS115(his4) was purchased from Invitrogen. The pGAP9K [8] expression vector and α -amy gene were kept in our laboratory. Restriction and modified enzymes are the products of TaKaRa and Fermentas. PCR primers and DNA sequence were performed by Shanghai Invitrogen Company. Mediums using for yeast culture were prepared according to the operation manual of Invitrogen [5]. Peptone is the product of Beijing Aoboxing Universeen Bio-Tech CO., LTD.

Preparation of engineering strain

With cDNA of barley genome as template, α -amy gene was amplified and inoculated with the sequence of his6, a purification label for Ni, at the 3'-end by using the primers of 5'-CGCAGAATTCCAAGTCCTCTTTCAGGGGTTTC-3' and 5'-TAAGCGGCCGCTCAATGATGATGATGATGATGATGCTCCGTTGTAGTGTTCGCGGCACC-3', which was cloned into the MCS of pGAP9K [8] at *EcoR* I and *Not* I sites to generate pGAP9K- α -amy. The *Bgl*III linearized pGAP9K- α -amy in 10 μ g was transformed into GS115 of *P. pastoris* by electroporation. The treated GS115 was grown on the YPD plate containing 700 μ g/ml of G418, and the grown clones were moved to YPD plates with higher G418 [11] at 1,000–5,000 μ g/ml. Each of the eight recombinants from the different concentrations of G418 was moved to YPD medium in a shaking flask, fermented in 30°C for 2 days, and under went SDS-PAGE test and hydrolyzing starch to compare expression levels to select well-expressed clone as engineering strain.

Fermentation condition and metabolite determination for constitutive expression

Eight 1,000 ml flasks with 200 ml YPD medium each were inoculated with 1 ml GS115 (pGAP9K- α -amy) ($A_{600} = 1.5$) and incubated at 30°C and 250 rpm for 20 h. The culture was transferred to a 50 l fermentor with a 20 l working volume. A modified growth medium recommended by Invitrogen Corp was used. It consisted of (l) 26.7 ml of 85% H_3PO_4 , 9.3 g of $CaSO_4 \cdot 2H_2O$, 18.2 g of K_2SO_4 , 14.9 g of $MgSO_4 \cdot 7H_2O$, 4.13 g of KOH, 40 g of glycerol, 40 ml of PTM4, 20 g of peptone and 10 g of yeast extracts. The composition of PTM4 trace elements was described as mentioned above [5]. The fermentation

was carried out at 30°C and 100 to 750 rpm by continuously feeding with 50% glycerol-0.8% PTM4. The dissolved oxygen (DO) level was set between 20 and 30%. pH was controlled at five by adding 7 M NH_4OH . Samples were taken every 6 h to measure the biomass and α -AMY during the course of fermentation.

Purification of the expressed product

Biomass was removed from the fermentation broth by centrifugation. The supernatant was loaded on SP-Sepharose Fast Flow column (Pharmacia Biotech., NJ, USA). Then, the column was washed with an equilibrium buffer (50 mM Na_2HPO_4 –24 mM $C_6H_8O_7$). Bound proteins were eluted successively with 0.3, 0.6 and 1 M NaCl. Fractions containing α -AMY were collected, concentrated, and desalted using an ultrafiltration device (Millipore, Bedford, MA, USA), and purified again using a Ni-Agarose 6 \times Tagged Protein Purification Kit (Cwbio Co., LTD.). Reverse HPLC analysis was used in the purity assay for the protein with the high performance liquid chromatograph (SHIMADZU, LC-2010CHT).

Activity analysis for the recombinant α -AMY

For qualitative analysis of the α -AMY activity, 50 μ l sample and equal volume buffer (pH5.0, 0.1 mol/l $C_6H_8O_7$ - Na_2 - HPO_4) were added in each of the oxford cup stood on 0.5% solubility starch containing plate. After incubation at 37°C for 12 h, the plate was dyed by 1% KI for 5 min. The result was evaluated according the diameter of clarity circle under the oxford cup [12]. And the method of DNS [13] was using for quantitative analysis the activity of the recombinant α -AMY.

Result

Screening high gene copies and well-expressed strain

A total of 120 clones were grown on the 700 μ g/ml G418-containing YPD plate after 3 days incubation of the cell transformed with 10 μ g plasmid DNA of pGAP9K- α -amy. PCR proved that the α -amy gene was integrated in the genome of *P. pastoris* (Fig. 1). The results of the clones enduring G418 concentration after being moved to higher G418-containing plates are shown in Table 1. Each eight clones between the 1,000–3,000 μ g/ml of G418 concentrations were randomly selected to ferment in shaking flask, and a 50 μ l supernatant was taken from each fermentation broth to test the hydrolyzing starch. The dot values of the clarity circles analyzed by software of the Gel Imaging System (Tanon, Ver. 4.00) were shown in Table 2. The fermentation supernatants from the transformants with

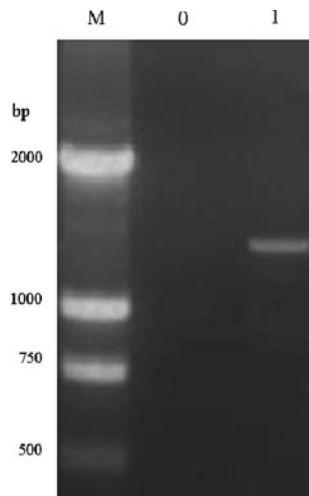


Fig. 1 Identifying the α -amy gene containing transformants by PCR. *M* DNA marker, *0* GS115(pGAP9K), *1* GS115(pGAP9K- α -amy)

Table 1 Numbers of transformants grown on different concentrations of G418

G418 concentration ($\mu\text{g/ml}$)	Clone numbers
1,000	56
2,000	33
3,000	15
4,000	3
5,000	0

Table 2 The dot values of fermentation supernatant hydrolyzing of starch

G418 conc. ($\mu\text{g/ml}$)	1,000	2,000	3,000
Clone 1	300241	49735	0
Clone 2	28075	305084	92805
Clone 3	0	35947	337322
Clone 4	219058	694	53547
Clone 5	130872	37392	405653
Clone 6	30681	372941	17043
Clone 7	192	16890	43077
Clone 8	893	8937	46540
Average	88751	103452	124498

same anti-G418 concentration were then mixed and run on the SDS-PAGE. The average expression levels of each of the eight clones from the 1,000–3,000 $\mu\text{g/ml}$ of G418 concentration are shown in Fig. 2. Table 2 and Fig. 2 indicated it is positive correlations of gene copy number and foreign protein average expression level. But the expression could be obviously difference between the recombinants in the same G418 resistance (Table 2; Fig. 3). No. 5, the best expressed clone as showing in Fig. 3 were selected as engineering strain.

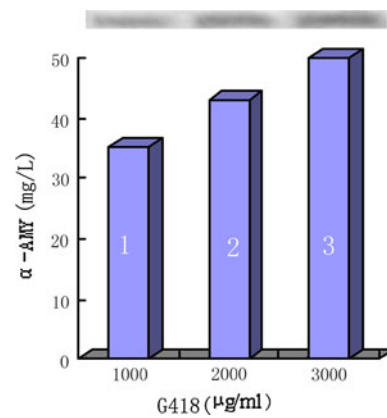


Fig. 2 Anti-G418 concentrations of GS115(pGAP9K- α -amy) and average α -AMY expression levels. The sample of each group is their mixing fermentation broth of the eight clones



Fig. 3 Test of hydrolysis starch of the fermentation broths from different transformants enduring 3,000 $\mu\text{g/ml}$ of G418 0.GS115 (pGAP9K); 1–8 different clones of GS115(pGAP9K- α -amy)

Peptone selection

GS115 (pGAP9K- α -amy) was inoculated into YPD medium with peptone sourced from three different companies which are the peptone of Beijing Aoboxing Universeen Bio-Tech CO., LTD., tryptone of Beijing Land Bridge Technology CO. LTD. and Tryptone of Oxoid LTD. to ferment for 2 days. The result of fermentation broth hydrolyzing starch is showing in Fig. 4. The dot values analyzed by software of the Gel Imaging System (Tanon, Ver. 4.00) indicated that the peptone from Beijing Aoboxing Universeen Bio-Tech Co., LTD. is better and it was chosen to use in the high-density fermentation.

α -AMY expression by high-density cell culture

The GS115 (pGAP9K- α -amy) cells were initially grown on glycerol and then fed-batch with 50% glycerol-0.8% PTM4 for 54 h. Fig. 5 shows the α -amy gene expression during high-density cell culture of GS115 (pGAP9K- α -amy). *P. pastoris* cell concentration/biomass (A_{600}), α -AMY

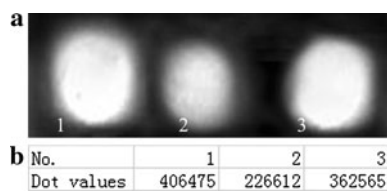


Fig. 4 Peptones affect on GS115(pGAP9K- α -amy) expression of α -AMY. **a** Fermentation broth hydrolyzed starch, **b** Dot values analyzed by software of the Tanon Gel Imaging System. 1 Peptone of Beijing Aoboxing Universeen Bio-Tech Co., LTD. 2 Tryptone of Beijing Land Bridge Technology Co. LTD. 3 Tryptone of Oxoid LTD

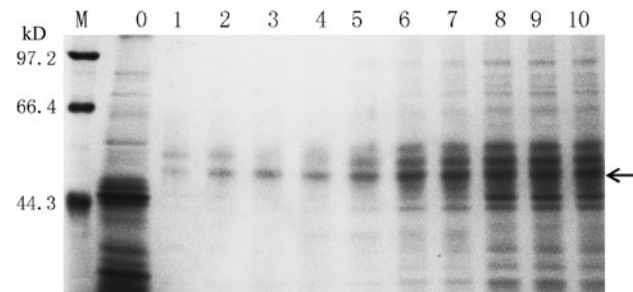


Fig. 5 SDS-PAGE analysis of the expression products by high-density cell culture. *M* protein marker, 0 GS115(pGAP9K), 1–10 GS115(pGAP9K- α -amy): 0, 6, 12, 18, 24, 30, 36, 42, 48, and 54 h

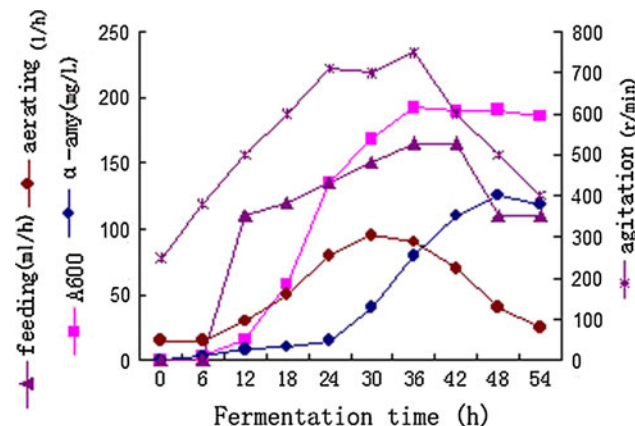


Fig. 6 Accumulation of biomass, α -AMY concentration, agitation, aeration and feeding velocity during the high-density cell culture of GS115 (pGAP9K- α -amy)

production, agitation, aeration and glycerol feeding during the fermentation were described in Fig. 6. At the end of fermentation, the culture volume increased from 21.6 to 33.3 l, accumulation of biomass is $A_{600} = 186$ while 125 mg/l of α -AMY was secreted into the fermentation broth.

Purification of recombinant α -AMY

After purification by the SP-sepharose fast flow, the mostly proteins from host were excluded in the process and the

foreign protein existed in the eluted buffer of 1 M NaCl with some hybrid proteins which were further excluded by the affinity purification and the purity quotient of the aim protein is 97.5% (Fig. 7).

Quantitative determination of the purified α -AMY

As shown in Fig. 8, the purified recombinant α -AMY and the α -AMY product (Shanghai Kayon Biological Technology Co. Ltd.) hydrolyzed starch as reducing sugar to react with 3,5-dinitrosalicylic acid, presenting a reddish brown color. 546 U/mg of the recombinant α -AMY had been harvested menstruated by ultraviolet Spectrophotometer (Shimadzu, UV-2550).

Discussion

Rabbani reported using *E. coli* to express α -AMY [14]. The expressed product stayed in the host cell in the way of inclusion body which is misfolding, poor solubility, and necessitates complicated purification methodologies [15, 16]. Wang reported expressing α -AMY in *Saccharomyces cerevisiae*, but the yield is very low [17]. The development of *S. cerevisiae* expression system was limited by the reasons of lacking strong promoter and being incapable high-density cell culture [18]. *P. pastoris* has been widely reported as an efficient expression system secreting very low levels of native proteins. The secreted heterologous protein comprises high ratio to hybrid protein in the medium serving as the first step in the purification of the protein [5]. And also it can grow to very high cell densities to express foreign protein [9]. The insufficiency of *P. pastoris* is its low expression level [7]. More attentions have been playing on the increase of foreign protein expression in *P. pastoris*. Generally speaking, mRNA level is parallel to the gene copy number in genome, and the amount of protein expression correlates with the mRNA level in *P. pastoris* [11]. Designing a resistance gene in expression vector is widely used for selecting more gene-copied recombinant of *P. pastoris* [11, 18]. Then, some authors reported that gene copy number does not correlate or is even contrary to foreign protein expression level [19, 20]. Our results indicated that gene copy number is positively correlated to foreign protein expression in the mass. However, the lower expressed, even unexpressed, strains may consist in the group of high gene copy numbers, which indicates that higher gene copies no mean certainly more expression. A second screening is necessary between the higher gene-copied recombinants.

High-density cell culture is beneficial for harvesting more products with unit volume of culture by cultivating the cell

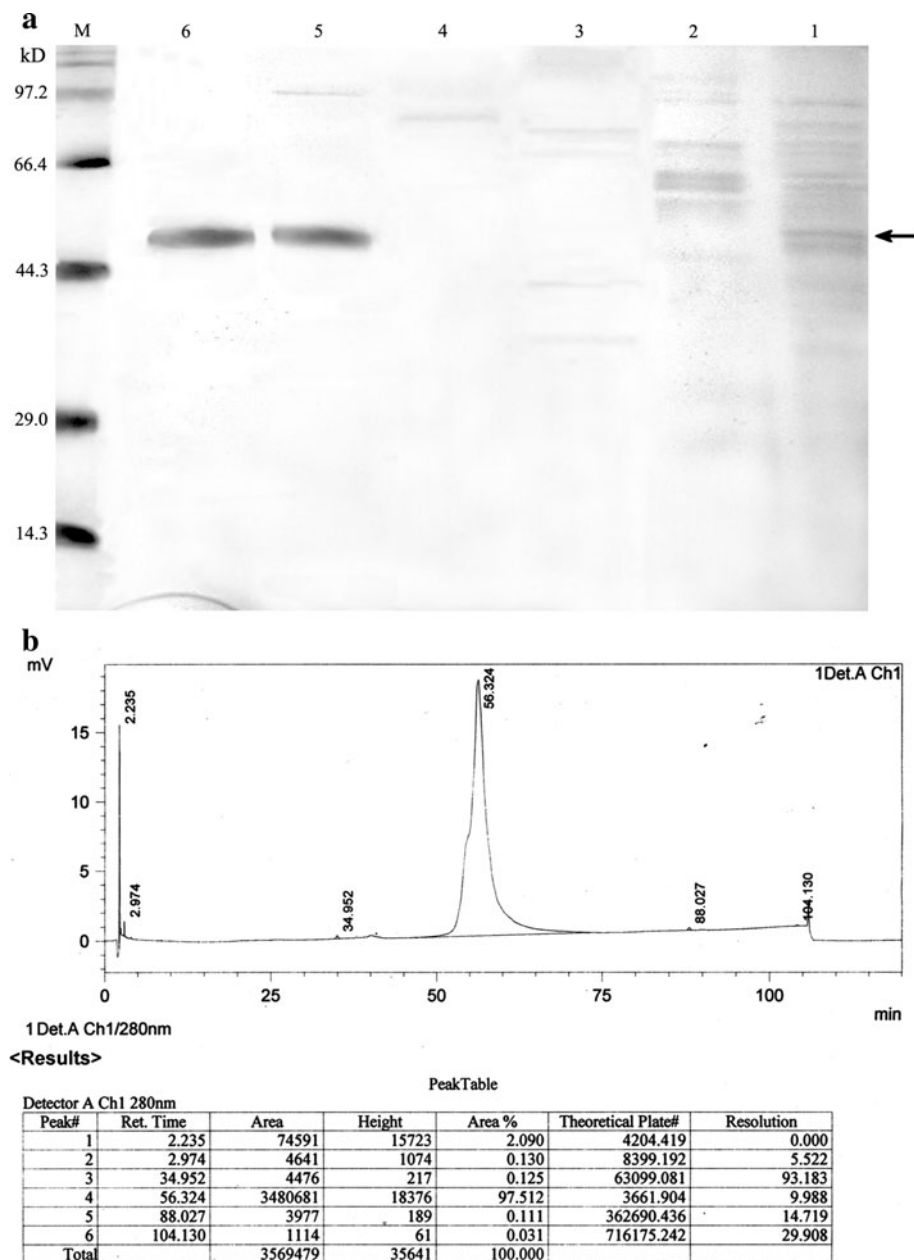


Fig. 7 Analysis of the eluted α -AMY **a** SDS-PAGE *l* fermentation broth of GS115(pGAP9K- α -amy), 2 flow of fluid through, 3 0.3 M NaCl, 4 0.6 M NaCl, 5 1 M NaCl, 6 eluted aim product by affinity

purification. **b** Reverse HPLC analysis the eluted aim product of the affinity purification, the peak 4 is the α -AMY



Fig. 8 The purified α -AMY hydrolysis of starch to generate reducing sugar. **a** Flow of fluid through from the ion-exchange, **b** Flow of fluid through from the affinity purification, **c** Purified recombinant α -AMY, and **d** α -AMY product

to high-density with high vigor in a bioreactor [9]. Using the screened strain to progress in 54 h fermentation in a

bioreactor, 125 mg/l of α -AMY was secreted by the cell of $A_{600} = 186$, which possesses about 20% of total proteins secreted by the *P. pastoris* and the enzyme activity is 546 U/mg. This expression yield is significantly higher than Yang used the same promoter to express α -AMY in *P. pastoris* [21].

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