African Journal of Food Science Vol. 5(12), pp. 668-675, 30 October, 2011
Available online at http://www.academicjournals.org/AJFS
ISSN 1996-0794 ©2011 Academic Journals

Full Length Research Paper

Cloning of the gene encoding acid-stable alpha-amylase from *Aspergillus niger* and its expression in *Pichia pastoris*

Qingmei Zeng, Chunyan Wei*, Jing Jin, Cong Wu and Boying Huang

Engineering Research Center of Bio-Process, Hefei University of Technology, Ministry of Education, 193 Tun Xi Road, Hefei, Anhui 230009, PR China.

Accepted 12 April, 2011

The gene of acid-stable alpha-amylase was amplified by PCR using *Aspergillus niger* genomic DNA as template, then the gene was cloned into the vector of pPIC9K, the recombinant vector pPIC9K-asAA was then transformed into *Pichia pastoris* SMD1168, high copy transformants were screened in G418 plates. Regulated by the α-Factor, promoter of *AOX*1 gene and termination signal of yeast genomic, the recombinant amylase was expressed and secreted out of the cells. The expression of the recombinant amylase was strictly induced by methanol, in shaking culture condition, after 168 h induction with 2% of methanol, the amylase reached maximal activity of 2838 U/ml. SDS-PAGE analysis showed that the molecular weight of the recombinant amylase was about 58 kDa. The recombinant amylase exhibited maximal activity at pH 4.0 and 70°C, the amylase was basically stable at the pH ranging from 3.0 to 6.0, and kept stable for a long time and with a high level of activity at common industrial temperature 50°C.

Key words: *Aspergillus niger*, acid-stable alpha-amylase, pPIC9K, *Pichia pastoris* SMD1168.

INTRODUCTION

Alpha-amylase (EC3.2.1.1) is an endo-type enzyme that hydrolyses α(1→4) glycosidic linkages in starch substrate backbone. It is widely used in food processing, food industry, brewing, fermentation, textiles industry, pharmaceutical industry and other industries, which accounts about 25% of the enzyme market.

China is a traditional agricultural country which is rich in starch resources (such as corn, potatoes) and has a strong starch processing industry. In traditional fermentation industries in China, most times amylase and glucoamylase work together to hydrolysis starch materials, the optimum pH of glucoamylase was about 4, but the two common alpha-amylase in market are moderate and high temperature enzyme, which optimum pH range is 6 to 7, and likely to lose activity below 4, so under acidic conditions they cannot work in some starch deep processing of raw materials. Therefore, the acid-stable alpha-amylase comes into being. The optimum pH range of acid-stable alpha-amylase is 4 to 5, the amylase shows higher activity in acidic conditions, that can be simplify liquefaction and saccharification process, production cost of starch processing is reduced, and can be used in textile desizing, high maltose syrup production, the development of new agents to aid digestion and industrial waste water treatment and other fields, so it has wide application prospect.

Current industrial acid-stable alpha-amylase mostly produced by microorganisms, *Bacillus* and *Aspergillus* are mainly microorganisms (Morimura et al., 1999), such as *Bacillus acidocaldarius* A-2 (Mutsuo, 1986), *B. acidocaldarius* ATCC2709 (Douglas and Colin, 1997), *Bacillus licheniformis*, *Bacillus docaldarius* 101 (Buonocore et al., 1976), *Aspergillus acidocaldarius* (Kelvin and Erwin, 2003), *Aspergillus niger* (Okolo et al., 1995), *Aspergillus cinnamoneus*, *Aspergillus kawachii* (Shigetoshi et al., 1993), *Thermomyces lanuginosus* (Petrova et al., 2000), *Bacillus steorothermopilius* (Hiroshi et al., 2002). *A. niger* is industrial strain of acid-stable alpha-amylase in China, Japan, Korea, Nigeria and some European Countries. Yasuji Minoda and other

*Corresponding author. E-mail: chunyanshandong@163.com.

researchers found that the acid-stable alpha-amylase from *A. niger* has a good acid stability (Mikami et al., 1987).

It is an effective way to achieve rapid over-expression of foreign genes by way of construction of engineering bacteria. *Pichia* expression system is widely used in recent years, people use the system for successful expression of acid-stable alpha-amylase from the extremely thermophilic anaerobic archaea *Pyrococcus furiosus* (Mikami et al., 2006), acid hot alicyclic acid bacteria *Alicyclobacillus acidocaldarius* (Tiezheng et al., 2005), thermophilic acidophilic solfataricus *Sulfolobus solfataticus* P2 (Yaying et al., 2009), but acid-stable alpha-amylase gene from *A. niger* expressed in *Pichia pastoris* has not been reported. SMD1168 is the protease-deficient yeast strain, which can effectively prevent the degradation of foreign proteins. We expressed acid-stable alpha-amylase from *A. niger* in *P. pastoris* SMD1168 with high activity.

MATERIALS AND METHODS

Strains and plasmids

A. niger (CICC40017, China), *Escherichia coli* DH5α (Sangon Biotech (Shanghai) Co., Ltd, China), *P. pastoris* SMD1168 (Guangzhou Institute of Respiratory Disease, China), pMD18-T simple vector plasmid (TaKaRa, China), plasmid pPIC9K (Invitrogen, USA).

Enzymes and reagents

T4 DNA ligase, restriction enzymes *Eco*RI, *Not*I, *Sac*I, UNIQ-10 column DNA gel recovery kit, ethidium bromide, PCR MasteMix (Sangon Biotech (Shanghai) Co., Ltd.).

Medium

(1) Luria bertani (LB): 10% pancreatic digest of casein, 5% yeast extract, 5% sodium chloride.
Yeast extract peptone dextrose medium (YPD): 1% yeast extract, 2% peptone, 2% dextrose.
(2) Regeneration dextrose medium (RD): 1 mol/L sorbitol, 2% Glucose, 1.34% YNB (w/o amino acids), 4×10^{-5}% biotin, 0.005% amino acid.
(3) Minimal methanol medium (MM): 1.34% YNB (w/o amino acids), 4×10^{-5}% biotin, 2% methanol.
(4) Minimal dextrose medium (MD): 1.34% YNB (w/o amino acids), 4×10^{-5}% biotin, 2% glucose.
(5) Buffered glycerol-complex medium (BMGY): 1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate (pH 6.0), 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol.
(6) Buffered methanol-complex medium (BMMY): 1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate (pH 6.0), 1.34% YNB, 4×10^{-5}%biotin, 1% methanol.

Construction of expression vector

Acid-stable alpha-amylase gene was amplified from *A. niger* genome by PCR. The forward and reverse primers were 5'-CCGGAATTCGACCTCAAGTCCCTCTCA-3' containing an *Eco*RI site (underlined) and 5'-ATTTGCGGCCGCTCGGCTACCCAGTCATAC-3' containing an *Not*I site (underlined), they were designed according to the structural part of acid-stable alpha-amylase gene (GenBank Accession No. XM001394298). The PCR product was digested with *Eco*RI and *Not*I, and then inserted into the expression vector pPIC9K linearized with the same enzymes. The recombinant plasmid pPIC9K-asAA was confirmed by restriction analysis and sequencing.

Transformation of *P. pastoris* and screening high-copy transformants

Expression vector pPIC9K-asAA linearized with *Sac*I was transformed into *P. pastoris* SMD1168 by protoplast transformation method (Yafeng et al., 2001). The obtained cells were spread onto MD plate and incubated at 30℃ for 2 to 3 days, the His^+ transformants were recovered on RD plate. Phenotypic identification for strains was performed by spreading colonies on MM and MD plates, the transformant which grew similarly on two plates was methanol utilization plus (Mut^+), which grew rapidly on MD plate, grew slowly on MM plate was methanol utilization slow (Mut^S).

High-copy transformants were screened by spreading colonies on YPD plates containing 0, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, 4.0 mg /mL of G418.

PCR identification of recombinant SMD1168/pPIC9K-asAA

To confirm whether or not the *P. pastoris* transformants contained the acid-stable alpha-amylase gene, PCR assay was performed. The genomic DNA was extracted from the recombinant *P. pastoris* SMD1168/pPIC9K, and identified by PCR.

Recombinant amylase expression and SDS-PAGE

SMD1168/pPIC9K-asAA was inoculated into 25 mL BMGY and cultured to OD_{600} 2 to 6 (about 16 to 18 h) at 30℃, after which cells were harvested by centrifuging at 1,500 r/min for 5 min at room temperature. The cells were then resuspended to OD_{600} 1.0 in BMMY medium to start induction. However, methanol was added every 24 h to continue induction.

After removing *P. pastoris* cells from fermentation culture, supernatant containing acid-stable alpha-amylase was analyzed by SDS-PAGE, and the gel was stained with Coomassie brilliant blue.

Recombinant acid-stable alpha-amylase activity determination and enzymatic characterization

P. pastoris cells were removed from the fermentation culture by centrifuging at 1,500 r/min for 10 min, and then amylase activity was measured.

Enzyme assays

The activity was determined according to the Chinese Industrial Standard (QB/T 1803 to 1993).

Optimum temperature

4 to 100℃ range, ph 6.0, enzyme activity was determined to

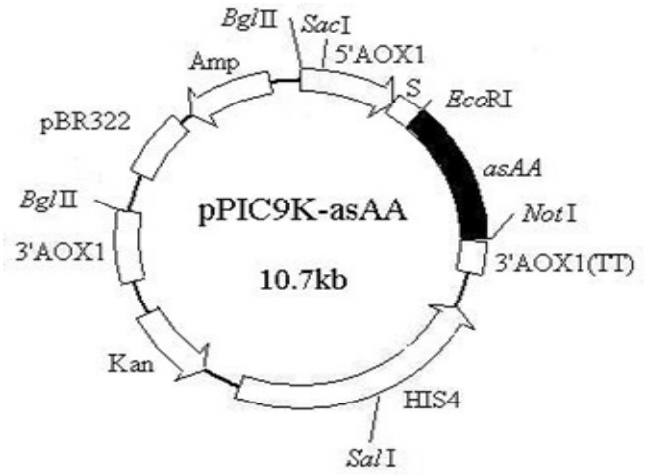

Figure 1. Construction of recombinant vector pPIC9K-asAA.

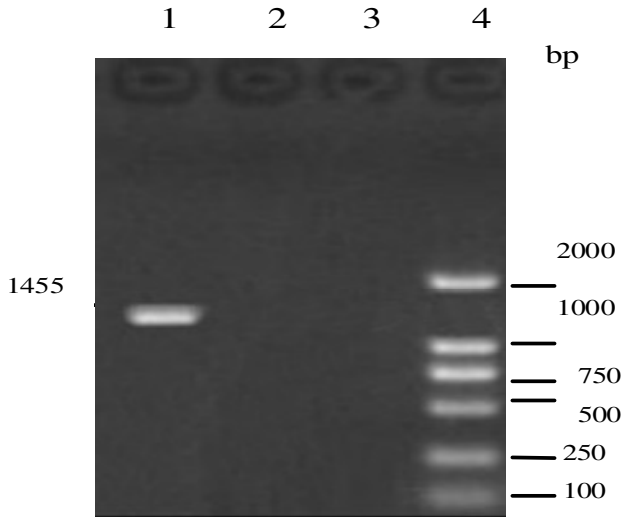

Figure 2. PCR assay of recombinant yeast genomic DNA. 1: Positive transformant (template: Yeast SMD1168/pPIC9K-asAA genomic DNA); 2: Negative transformant (template: Yeast SMD1168/pPIC9K genomic DNA); 3: Template: Yeast SMD1168 genomic DNA; 4: DNA marker DL2000.

calculate relative activity with maximum activity 100%.

Optimum pH

pH 2.2 to 7.0, enzyme activity was determined to calculate relative activity with maximum activity 100%.

Temperature stability

The enzyme incubated at different temperatures for different time, and determined to calculate relative activity after ice bath, we defined enzyme which incubated at 4℃ activity 100%.

pH stability

ph 2.2 to 7.0 were incubated at 37℃ for different times and were determined to calculate the relative activity. After ice bath, we defined enzyme which was incubated at 4℃ activity 100%.

RESULTS

Clone of acid-stable alpha-amylase

Acid-stable alpha-amylase gene from *A. niger* only has exons and no introns, P1, P2 as primers, *A. niger* genomic DNA as template, PCR amplified gene fragment was cloned into the vector pMD18-T, the sequencing results showed that the fragment has a complete open reading frame (ORF), gene size of 1455 bp, no original gene secretion signal peptide sequence and exactly the same as *A. niger* CBS 513.88 asAA (Accession No. XM001394298).

Construction of expression vector

To make acid-stable alpha-amylase gene inserted into expression vector pPIC9K in correct direction, the PCR product was digested with *Eco*RI and *Not*I, and then inserted into the expression vector pPIC9K linearized with the same enzymes, restriction analysis and sequencing results showed that acid-stable alpha-amylase gene integrated in the 3' end of pPIC9K the α-factor signal peptide sequence with correct reading frame. Figure 1 showed construction of expression vector pPIC9K-asAA.

PCR identification and high-copy transformants screened

Transformants grew similarly on MM and MD plates, so the phenotype was His^+ Mut^+. To increase the expression of foreign protein, we used the gradient plate G418 to screen high-copy transformants. Eight positive transformants SMD1168/pPIC9K-asAA were selected randomly with G418 resistant. To confirm whether or not the *P. pastoris* contained the acid-stable alpha-amylase gene, PCR assay was performed. As shown in Figure 2, The PCR results showed positive transformants gene fragment was amplified with the theoretical data while the control transformant and *P. pastoris* SMD1168 no amplification occurs, so we can prove the amylase gene integrated into the yeast genome.

Recombinant amylase expression and SDS-PAGE

Eight transformants randomly selected were induced in shake flask conditions (0.5% methanol, pH 6.0, 72 h), and a high-level expression of acid-stable alpha-amylase

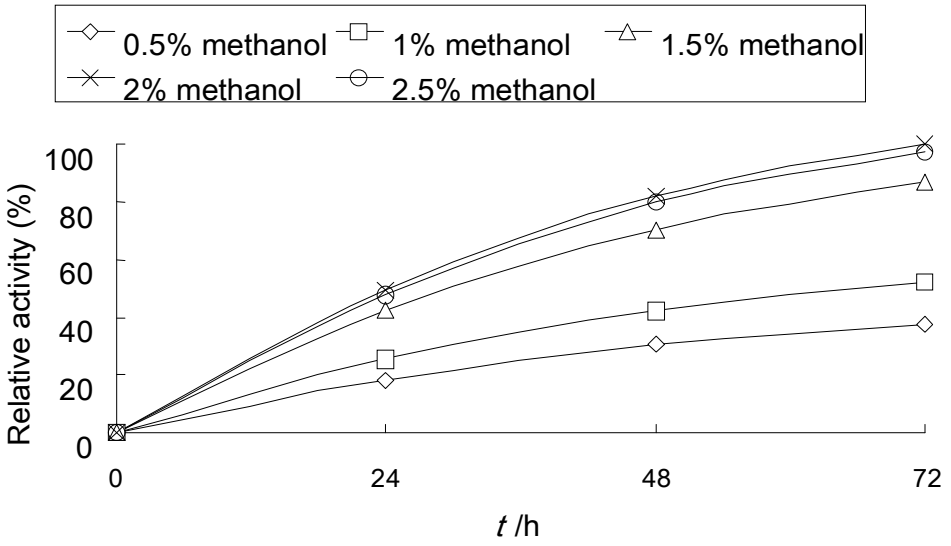

Figure 3. Effect of concentration of methanol on acid-stable alpha-amylase relative activity.

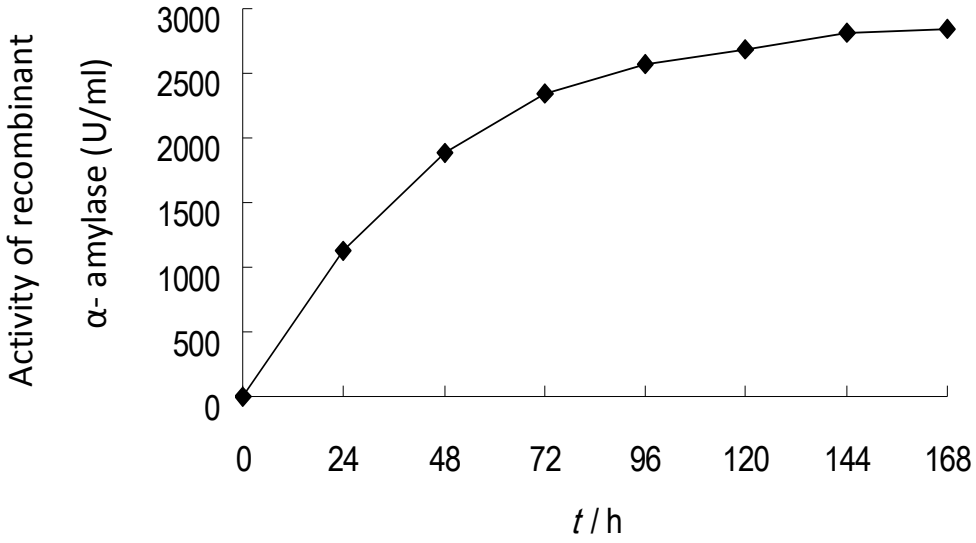

Figure 4. Effect of induction time on acid-stable alpha-amylase activity.

transformant was selected.

Transformant obtained in this study can consume methanol quickly. To get optimal induction condition, we added different concentrations of methanol to BMMY medium. As shown in Figure 3, the enzyme expression was regulated strictly by the regulation and induction of methanol, we can enhance the concentration of methanol to increase the expression of recombinant enzyme, but excessive concentrations of methanol was toxic to yeast cells and inhibit the expression of the recombinant enzyme. In this study, the best methanol concentration is 2%.

The transformant was induced by 2% methanol, effect of induction time on acid-stable alpha-amylase activity was studied. As shown in Figure 4, with the induction time increased, the unit volume activity corresponding increased and reached the maximum 2838 U/ml in 168 h, the result is much higher than the amylase produced by *A. niger* (Bo et al., 2007) and the recombinant enzyme expressed in *Saccharomyces cerevisiae* (Haiyan et al., 2004).

After 168 h of continuous methanol induction, supernatant was analyzed by SDS-PAGE (separating gel concentration of 12%, stacking gel concentration of 5%). The results showed in Figure 5, a protein band was visualized in lane 1, the size was 58 kDa, while there was

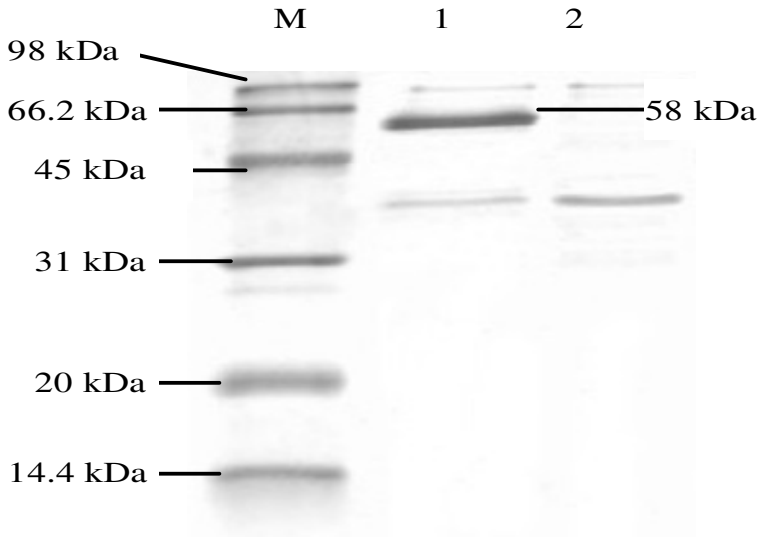

Figure 5. SDS-PAGE analysis of acid-stable alpha-amylase gene expressed in *P. pastoris*. M: Protein marker; 1: Positive transformant (template: Yeast SMD1168/pPIC9K-asAA genomic DNA); 2: Negative transformant(template: Yeast SMD1168/pPIC9K genomic DNA).

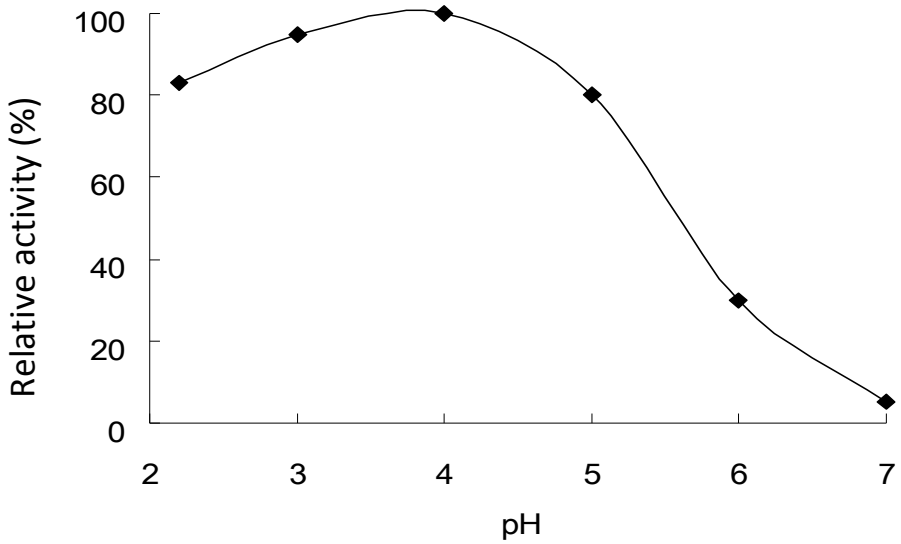

Figure 6. Optimum pH for acid-stable alpha-amylase activity.

no band in the corresponding position of Lane 2, indicating that the expressed protein was secreted. The protein size is greater than predicted date, suggesting protein glycosylation occurred.

Recombinant *P. pastoris* SMD1168/pPIC9K-asAA genetic stability study

The amylase activity of the tenth generation *P. pastoris* properties of the recombinant enzyme were basically can achieve 80% of the first generation, and the gene of acid-stable alpha-amylase still integration in the genome of the tenth generation, so recombinant *P. pastoris* has good genetic stability.

Characterization of recombinant enzymes

Enzymatic properties of recombinant enzymes were studied, the results showed in Figures 6 to 9. The same to nature of the amylase produced by *A. niger*

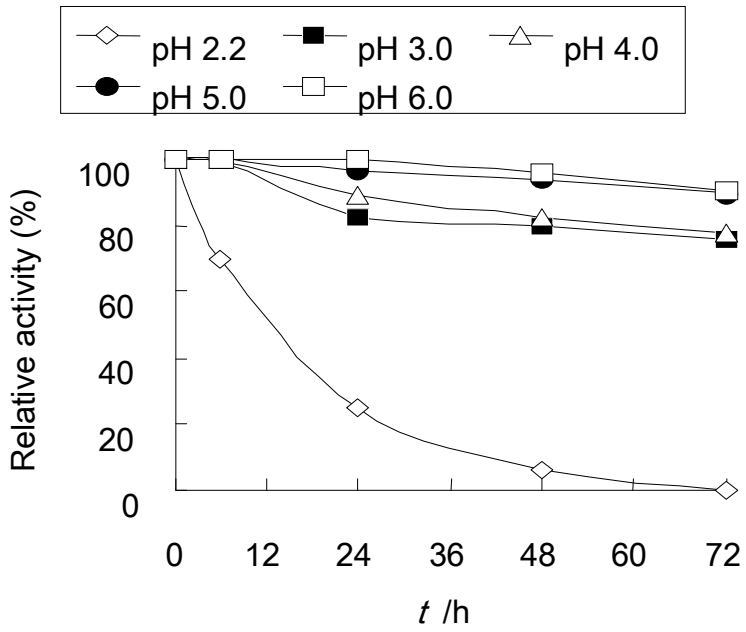

Figure 7. pH stability of acid-stable alpha-amylase.

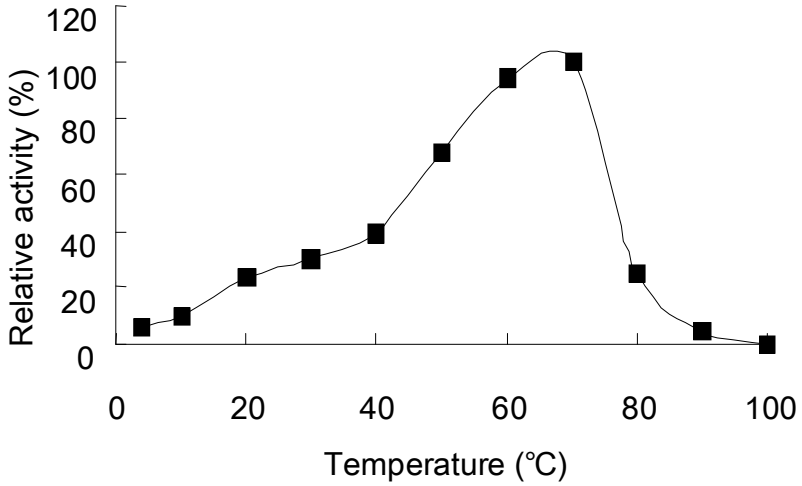

Figure 8. Optimum temperature for acid-stable alpha-amylase activity.

(Bo et al., 2008) and the recombinant enzyme expressed in *S. cerevisiae* (Haiyan et al., 2004). The optimum temperature and thermal stability results showed that the optimum temperature is 70℃, activity half-life of 60℃ is 2 h, the properties of the enzyme remained stable for a long time under industrial production temperature 50℃. The optimum pH and pH stability results showed that the optimum pH is 4.0, the enzyme activity is high between pH 2.2 to 5.0 and stable between pH 3.0 to 6.0. Therefore, the enzyme has good temperature and acid stability, can achieve liquefaction and saccharification of starch synchronously under acidic conditions, so it has a good prospect.

DISCUSSION

As previously mentioned, acid-stable alpha-amylase is a very important application enzyme, and there is an increasing concern about it. Domestic and foreign researchers did a lot of work and achieved some results in acid-stable alpha-amylase producing strain and

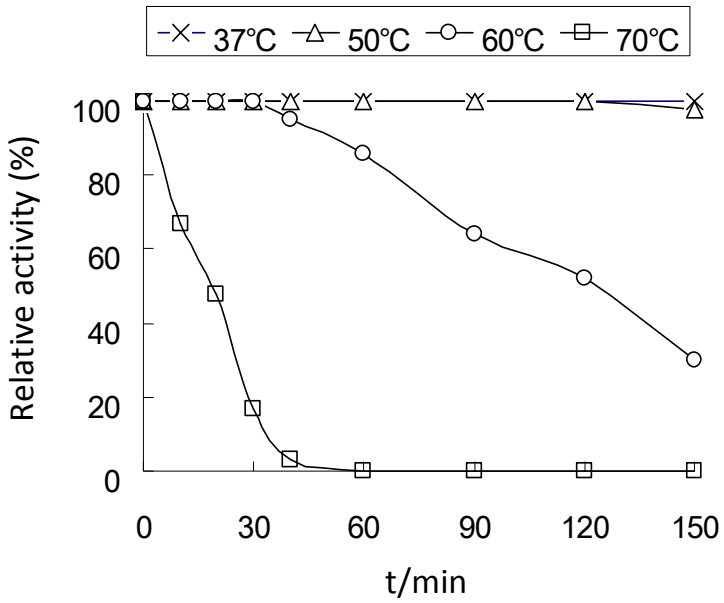

Figure 9. Thermal stability of acid-stable alpha-amylase.

optimization of fermentation conditions. However, *A. niger* as industrial production strain of acid-stable alpha-amylase in China, compared with other countries, enzyme activity remains low, so using gene recombination technology, we can express the amylase via a suitable vector in *P. pastoris* and lay the foundation for large-scale enzyme preparation and industrial application.

In this study, we cloned *A. niger* acid-stable alpha-amylase gene into the expression vector pPIC9K, and transformed into *P. pastoris* SMD1168, got high activity. In shaking culture condition with 2% of methanol, the activity of the acid-stable alpha-amylase reached the maximum of 2838 U/mL. The optimum activity of acid-stable alpha-amylase was determined at 70℃ at pH 4.0, the amylase was stable at the pH range of 3.0 to 6.0, with a half-life for almost 2 h at 60℃, the enzyme kept stable for a long time and possessed a high level of activity at 50℃. The enzyme can be used in starch processing industry, also used for textile desizing, citric acid fermentation of glucose production and other industries, so it has good application prospects.

GS115 is a widely used host strain of *P. pastoris*, but proteolytic enzyme KEX-2 existing in cell membrane degrades the expressed products, hindering the efficient expression of the product. Although, some measures can increase the stability of foreign proteins, such as changing the medium pH, adding the right amount of yeast extract and peptone or casein hydrolyzate, supplement with enzyme inhibitors, but the operation is cumbersome or ineffective. In this paper, we used protease deficient strain SMD1168 as the expression host to obtain highly expression of the product, provided a reference to study and application of the strain.

Acid and neutral alpha-amylase from *A. niger* are different in the acid resistance, heat resistance and the degree of starch hydrolysis. Researchers studied the mechanism of acid-stable alpha–amylase and found that the content of the acidic and basic amino acids in the acid-stable alpha–amylase was 30% less than in the neutral one, they suggested that the nature of acid-stable alpha-amylase was related with the isoelectric point in low pH condition. When pH is lower than isoelectric point, basic amino acids carry a large number of positive charge which mutually exclusive, leading to expansion of protein structure, thus affecting the catalytic center of activity (Liping et al., 2002). We found that the content of charged amino acids in the acid-stable alpha–amylase from *A. niger* was only 18%, so this may be the reason for the amylase with acid resistance, and our experiments provide data to support this inference.

In this study, the gene encoding acid-stable alpha-amylase from *A. niger* was expressed in *P. pastoris*, and enzymatic properties of recombinant enzyme were analyzed and determined, but the molecular mechanism of the enzyme was still in the exploratory stage. We believe that further study of the molecular mechanism will be beneficial to the development and utilization of the enzyme.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (Grant No. 31071556; 30871739) and Science and Technology Department of

Anhui province, PR of China (Grant No. 07010302117).

REFERENCES

Bo L, Yong D, Yu Ma, Lei Du (2008). Isolation of acid resistant α–amylase and its characteristic research. J. Northwest Univ., 38(3): 435-438.

Bo L, Zhengyou W, Yong D (2007). Breeding of Acid-resistant α–amylase Producing Strain. J. Microbiol., 27(4): 46-49.

Buonocore VC, Caporale de Rosa M, Gamwinbacorta A (1976). Stable, inducible thermoacidophilic α-amylase from *Bacillus acidocaldarius*. J. Bacteriol., (128): 515-521.

Douglas CW, Colin M (1997). Enzymes involved in the processing of starch to sugars. Trends in Biotechnol., 15 (9): 349-352.

Haiyan W, Junchuan Q, Aoquan W, Guomin T (2004). Integrations and Coexpression of Acid α–amylaseand Glucoamylase from *Aspergillus niger* in A Distiller's Yeast AS2.346. Acta Microbiologica Sinica, 44(4): 483-486.

Hiroshi K, Kazuhisa S, Hiroki T, Takahisa N, Takashi K (2002). *Bacillus stearothermophilus* neopullulanase selective hydrolysis of amylose to maltose in the presence of amylopectin. Appl. Environ. Microbiol., 68(4): 1658-1664.

Jianqiang G, Yunmin L, Lili Y, Yangsheng Q, Qinghua J (2006). Molecular Cloning and Expression of Extremely Thermostable and Acid-stable Amylase Gene in *Pichia pastoris*. Chin. J. Biotechnol., 22(2): 237-242.

Kelvin E, Erwin S (2003). A thermoacidophilic endoglucanase (CelB) from *Alicyclobacillus acidocaldarius* displays high sequence similarity to arabinofuranosidases belonging to family 51 of glycoside hydrolases. Eur. J. Biochem., 270 (17): 3593-3602.

Liping Z, Yan X, Jianzhong J (2002). The Application and Study of Acid ALFA-Amylase. Liquor Making, 29(3): 19-22.

Mikami S, Iwano K, Shiinoli S (1987). Purification and some properties of acid-stable α-amylase from shochu koji (*Aspergillus kawachii*). Agric. Biol. Chem., 51(9): 2495-2501.

Morimura S, Wen XZ, Ichimura T, Kida Y, Ibi Y, Kida K (1999). Genetic engineering of white *Shochu-Koji* to achieve higher levels of acid-stable α-amylase and glucoamylase and other properties when used for Shochu making on a laboratory scale. J. Inst. Brewing, 105(5): 309-314.

Mutsuo K (1986). A *Bacillus acidocaldarius* α-amylase that is highly stable to heat under acidic conditions. Agric. Biol. Chem., 50(1): 23-31.

Okolo BN, Ezeogu LI, Mba CN (1995). Production of raw starch digesting amylase by *Aspergillus niger* grown on native starch sources. J. Sci. Food Agric., 69(1): 109-115.

Petrova SD, Ilieva SZ, Bakalova NG, Atev AP, Bhat MK, Kolev DN (2000). Production and characterization of extracellular α-amylases from the thermophilic fungus *Thermomyces lanuginosus*(wild and mutant strains). Biotechnology Letters, 22(20): 1619-1624.

Shigetoshi S, Takeaki I, Yuko TS, Kazuo S, Toshiteru O (1993). Characteristics of acid-stable α-amylase production by submerged culture of *Aspergillus kawachii*. J. Ferment. Bioengr., 76(2): 105-110.

Tiezheng Y, Bin Y, Huiying L, Yaru W, Ningfeng W, Yunliu F (2005). Expression of Acidophilic α–amylase from *Alicyclobacillus acidocaldarius*. Chin. J. Biotechnol., 21(1): 78-83.

Yafeng Z, Xianen Z, Hong L, Chenggang Z, Anthony EGC (2001). Cloning and Expression of *Aspergillus niger* Glucose Oxidase Gene in *Methylotrophic Yeast*. Chin. J. Biotechnol., 17(4): 400-405.

Yaying G, Biao S (2009). Expression of thermophilic and acidophilic extracellular α–amylase gene from *Sulfolobus solfataticus* P2 in *E. coli* and *Pichia pastoris*. Ind. Microbiol., 39(3): 56-61.