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Screening, Identification, Cloning, Expression and Characterization of α-amylase from *Bacillus amyloliquefaciens*

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In order to obtain the strains of high α-amylase activity, 200 strains were obtained through isolation, the crude enzyme activity of 20 of the strains were higher than the others through the Yoo YJ's method. The strain L1-2, which had a highest enzyme activity, reaching 766 U/mL, was *Bacillus amyloliquefaciens* identified by 16S rRNA. The α-amylase gene was cloned and inserted into the pET32a vector, then transformed into *E. coli* BL21 (*ED3*). The enzyme activity of recombinant protein was 1747.20 U/mg. The optimal pH was 6, and it remained more than 80% activity between 5 and 7. The optimal temperature was 60° C, and there remained more than 80% activity between 40°C and 70°C. The recombinant protein was significantly activated by Ca^{2+}, significantly inhibited by Cu^{2+}; Co^{2+}; Fe^{3+}; K^{+}. The study was the genetically modification foundation of the α-amylase production strains and meeting the industrial production needs.

Key words: α-amylase, Screening, Identification, *Bacillus amyloliquefaciens*.

Alpha-Amylases [EC 3.2.1.1; α-(1,4)-d-glucan glucanohydrolase] are extracellular enzymes which randomly cleave the α-1,4 glycosidic linkages between adjacent glucose units in the linear amylose chain, generating dextrin and reducing sugar[1]. α-amylases are most widely distributed in various plants, animals, and microorganisms, they also play major roles in the utilization of polysaccharides; such as food processing, detergent manufacture, and other industries that use starch liquefaction[2]. Amylases make up approximately 25% of the enzyme market[3]. Although it can be derived from a lot of plants and animals, the enzymes from microorganisms are used extensively for the industrial demand[4].

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Large amounts of starch are produced every year; it is the most commonly used organic substrates in the fermentation industry. Most of the substrates are degraded chemically, which is high-cost and high-pollution. Degradation of starch using the amylases which derived from microbial fermentation is the superior than chemical method, and it is low-cost and environment protection. The microbial fermentation isn't limited by the raw material, only need low cost to achieve a large-scale industrial production with high efficiency. Therefore 100 kinds of enzymes which already large-scale industrialized production are mostly produced by microbial fermentation.

In order to obtain the strains of high α-amylase activity, the samples were collected from the flour factory (Chengdu, Sichuan, China) to screen the strains in this study, in order to lay foundation in the improvement of amylase production strains, and apply to the needs of industrial production[5,6].

MATERIALS AND METHODS

Soil samples and water samples were collected from the flour factory (Chengdu, Sichuan, China). Plasmid vector pET-32a, *E. coli* BL21-(*ED3*), *E. coli* JM109 are all stored in the Sichuan Centre of Typical Cultures Collection (SCTCC). *BamH* I, *Xho* I, T4 ligase, pMD 19-T vector kit (TaKaRa, Dalian, China), the primers were synthesized at Shanghai Invitrogen Biotechnology Co.Ltd.

Screening of the strains producing α-amylase

Ten grams sample (Chengdu, Sichuan, China) was suspended in 100 mL of sterilized water and incubated at 37°C for overnight. The 10 mL supernatant was spread in 100 mL liquid media [1% peptone, 0.5% NaCl, 0.3% beef extract, 0.2% starch pH 7.0 (W/V)] and cultured at 37°C at 200 rpm for 24 h. Then it was cultivated into a serial dilutions from $10^{-1}$to 10^{-8}. To obtain pure colonies, the highest dilution was spread on solid media plates [liquid media with agar 1% (W/V)] and incubated at 30°C for 3-5 days, then incubated at 4 °C for 3 days. The single colony would degradation circle after being dyed [0.5% I_2 and 5% KI (W/V)]. Subsequently, each colony with a circle was inoculated to liquid media at 37°C for 12 h, and then inoculated 0.5% of it to liquid fermentation medium at 37°C for 24 h. The supernate was centrifuged at 5000 rpm for 10 min, collecting the supernatant to test the crude enzyme activity.

Crude enzyme assay

The starch-iodine method has been widely used for the assay of amylase activity. We adopt the Yoo YJ's method for the assay of crude enzyme[7].

Identification of the bacterial strain

Partial 16s rRNA of strain L1-2 was amplified by PCR. The genomic DNA of strain L1-2 prepared by bacterial genomic DNA extraction kit (TaKaRa Code, DV810A, Japan) was used as PCR template. A set of primers (8), 27F(52 -GAGAGTTTGATCCTGGCTCAG-32) and 1541R(52 -AGAAAGGAGGTGATCCAGCC-32), and TaKaRa Ex Taq® (Takara Code: DRR001A, Japan) was used for PCR. The PCR was carried out at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min 30 s for a total of 30 cycles, and a final elongation step at 72 °C for 10 minutes. The PCR products were cloned into pMD 19-T vector. The plasmids were transformed into competent *E. coli* JM 109. The monoclonal was verified by DNA sequencing (Invitrogen, Shanghai). The sequence results were taken a homology comparison in the NCBI and BLAST analysis (http://blast.ncbi.nlm.nih.gov/).

Construction of expression strain

The α-amylase gene sequences of the strains L1-2 using is obtained by NCBI and BLAST comparison (http://blast.ncbi.nlm.nih.gov/). Then, the primers, PF(5'-CG*GGATCC*GTA AATGG CACGCTGATGCAGTA-3') and PR (5'-GGC*GAG CTC*TTATTTCTGAACATAAATGGAGACG-3') was designed for PCR to get the α-amylase gene. PrimeSTAR® Max DNA Polymerase (TaKaRa Code: DR045A) was used. The PCR was carried out at 98°C for 10s, 55 °C for 5s, and 72°C for 8s for a total of 30 cycles. The amplified fragments were digested by *BamH* I and *Xho* I after DNA extraction and then cloned into the multicloning site of the pET32a vector. The plasmids were transformed into competent *E. coli* BL21 (*ED3*) for protein expression. Recombinant strain was verified by DNA sequencing (Invitrogen, Shanghai).

Protein expression, purification and enzyme assay

The recombinant strain was cultured in 50 mL LB media (1% peptone, 0.5% yeast extract, 1% NaCl and 50 ug/mL ampicillin) at 37 °C until the OD_{600} reached 0.6-0.8, then added 0.1 mmol/L IPTG (Merk, Germany) to induce expressing at 16 °C for overnight. Suspending the cells in 8 mL NaH_2PO_4-$C_6H_8O_7$ buffer (pH 6.0) after centrifuging at 12000 rpm for 10 min; crushing cells by ultrasonic, then centrifuging at 12000 rpm for 10 min, collecting the supernatant for protein purification, which was carried out by BioLogic Duo Flow (BIO-RAD, America). Enzyme purity was verified by 10% SDS-PAGE and its concentration was determined by Bradford assay[9]. The enzyme assay was referred to Yoo YJ's method, and the enzyme unit was U/mg.

pH optimum

The pH optimum of amylase was determined at 40 °C using three different buffers: 0.2 M NaH_2PO_4-$C_6H_8O_7$ buffer for pH 3.0-6.0; 0.2 M Na_2HPO_4- NaH_2PO_4 buffer for pH 7.0 and 8.0; and 0.1 M Na_2CO_3-$NaHCO_3$ buffer for pH 9.0-11.0. The purified enzymes were diluted with each buffer with different pH, and the enzyme was assayed by Yoo YJ's method[7].

Temperature optimum

The optimal temperature was determined in 0.2 M NaH_2PO_4-$C_6H_8O_7$ buffer, pH 6.0 at 30° C, 40° C, 50° C, 60° C, 70° C, and 80° C.

Effects of metal ions

The NaH_2PO_4-$C_6H_8O_7$ buffer(pH 6.0) containing substrate activities were determined at 40 °C after reaction with Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and K^{+} (final concentration of 1 mM)

RESULTS AND DISCUSSION

Isolation and Identification

Flour factory is rich in Starch, so that the soil around contained a wealth of microorganisms which could degrade starch. It is good for screening strains, that's why the study chooses samples from the flour factory. At last, 200 strains were isolated, and it was in line with the intended purpose. Crude enzyme activity of 20 of the strains was higher than others through the Yoo YJ's method [Table 1], which were all stored at the Sichuan Centre of Typical Cultures Collection. Here in, enzyme activity of strain L1-2 was 766 U/mL, which named SCTCC100813. The 16S rRNA gene sequence from the L1-2 was similar to the 16S rRNA gene from *Bacillus amyloliquefaciens*. Consequently, it was possible to infer that L1-2 is the closest to *B. amyloliquefaciens* and it was named *B. amyloliquefaciens* L1-2[10]. Comparing of the α-amylase[11,12] from other *B. amyloliquefaciens* [Table 1], it found that the α-amylase activate of strain L1-2 is extremely higher than other *B. amyloliquefaciens*, and the other enzyme properties are similar with other strains that are employed for commercial applications. In addition, in china, Gao D and Ma G[13] reported that the α-amylase activity of mutant K211from *B. subtilis* BF-7658 was 527U/mL after optimization. Liu Z *et al*.[14] reported that the α-amylase activity from *B. amyloliquefaciens* was 603U/mL after optimization. In this study, the initial α-amylase activity of L1-2 was higher than them. Therefore it is the ideal strain for the further study. The *B. amyloliquefaciens* has been widely used in agriculture, industry, and environmental reclamation, and it also provides an interesting target for protein engineers, not only for improving its industrial performance but also for investigating the molecular basis. *B. amyloliquefaciens* structure is composed of the three distinct A, B, and C domains[15-19]. The N-terminus is the active site, which folds into an $(\alpha/\beta)_8$ barrel, named Domain A; Domain B is located between Aα3 and Aβ3, which created by α-strands; The C-terminal portion of the protein folds into a Greek-key motif, named Domain C.

Construction of expression strains

PCR amplify the α-amylase gene of *B. amyloliquefaciens* L1-2 under normal conditions and resulted in the production of a band in the expected region of 1452 bp as is shown in Fig. 1(a). Using agarose gel DNA extraction kit(TaKaRa Code, D823A Japan), PCR products were purified and then inserted into the *BamH* I and *Xho* I sites of the pET32a vector Fig. 1(b). Subsequently, it was transformed into competent *E. coli* BL21 (*ED3*). The expression strain was successfully constructed by DNA sequencing (Invitrogen, Shanghai). Before the design of primers, the signal peptide was predicted in the website (http://www.cbs.dtu.dk/services/SignalP/). The results displayed that 1-31 amino acids was the signal peptide of the *B. amyloliquefaciens* amylase. Since the removed of signal peptide is good for the recombinant protein expression[20], so the signal

Table 1. Comparison of α-amylases from *Bacillus amyloliquefaciens*

Strain	Optimum pH	pH stability[(a)]	Temperatur optimum	α-amylas activity	References
B.amyloliquefaciens ATCC 23842	5.0	5.0-6.0	50-60°C	470U/mL	Gangadharan, D.2009
B.amyloliquefaciens Kleistase M1	6.0	5.0-7.0	60°C	about 600U/mL	Lee, S.2006
*B.*amyloliquefaciens SCTCC100813	6.0	5.0-7.0	60°C	766 U/mL	this work

(a): the pH when remained more than 80% activity

peptide was removed, the amplified fragments of the PCR product, which was 1452bp, was less than ORF (Open Reading Frame) of *B. amyloliquefaciens* amylase.

Protein expression purification and enzyme assay

Growth and the expression of the fusion protein of the *E.coli* are impacted deeply by incubation and induction temperature. Schein found that 37 °C was the best temperature for *E. coli*, but some of the recombinant protein would fold errorly when the temperature was too high; instead, it was very easy for them to accumulate as inclusion bodies[21]. Reducing induced temperature is conducive to improve the soluble recombinant protein, and low temperature (15° C to 20° C) induction for overnight could make the production of soluble protein reaches the maximum. Thus the 16° C is used in this study for protein expression well. The recombinant α-amylase was purified by BioLogic Duo Flow (BIO-RAD, America) [Fig. 2]. From the figure, the recombinant α-amylase was 72.5 KDa and pure enough to detect enzyme assay. The theoretical molecular weight of α-amylase gene production is 54.8 KDa, but it was about 72.5 KDa in the SDS-PAGE. Because the expression vector pET32a in this experiment contains approximately 17.7 KDa fusion tag protein, the molecular weight of the recombinant protein is approximately 72.5 KDa, which is consistent with the expectation. According to the Formula (Yoo YJ 1986): Activity (U/mg) = D x $[(R_0-R)/R_0]$ x 100, the recombinant α-amylase activity of *E. coli* BL21 (*ED3*)-pET32a-amylase was detected, which was 1747.20 U/mg.

pH optimum

pH optimum was detected for the recombinant α-amylase and shown in Fig. 3. From the figure, the optimal pH of the recombinant protein was 6, and it remained more than 80% of the relative activity when the pH between 5 and 7. The activity decreased rapidly when the pH was raised to 10. In modern industry, two enzymes are frequently chosen to glucose production from starch. First of all, use amylase to dissolve starch

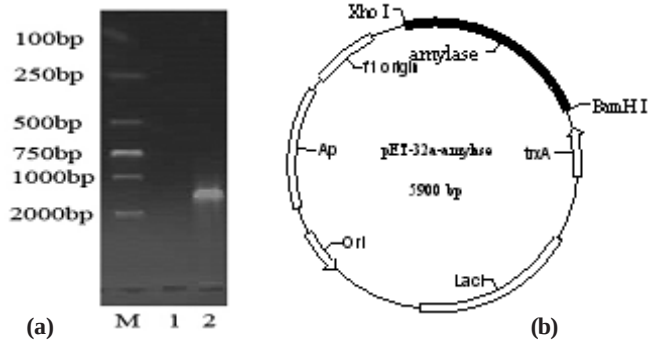

Fig. 1. The PCR production and map of expression vector, (a) M: DL2000 maker (2000; 1000; 750; 500; 250; 100bp) 1: the blank control; 2: the PCR production (b) Map of expression vector pET32a with the α-amylase gene

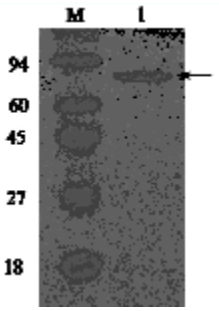

Fig. 2. SDS-PAGE of the purified recombinant α-amylase M: protein maker (94; 60; 45; 27; 18 KDa) 1: purified recombinant α-amylase

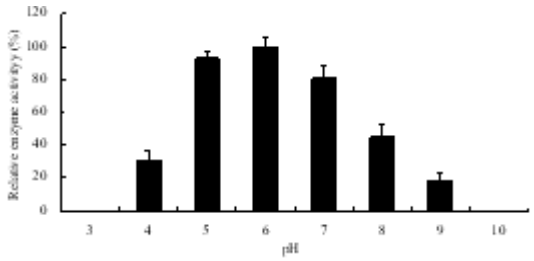

Fig. 3. The pH optimum of recombinant α-amylase. The pH optimum was determined by Yoo YJ's method at 40°C using three different buffers

to dextrin, and then saccharify dextrin to glucose via glucoamylase. During the process of amylase degradation, pH in the device was 6.0 which is the optimum pH of amylase. When begin to saccharify, the pH had to adjust to 4-5 which is the optimum pH of glucoamylase[22]. This process waste a lot of reagents, left bulk of salt in production, this went against later processing. In this study, the recombinant α-amylase remained extremely high relative activity (92.6%) when pH is 5.0. it can save a lot of reagents, and also reduce the salt generation. To a certain extent, the contradiction was alleviated, and it is conducive to the continuity of two enzymes processing.

Temperature optimum

Temperature optimum was detected for the recombinant α-amylase and shown in Fig. 4.

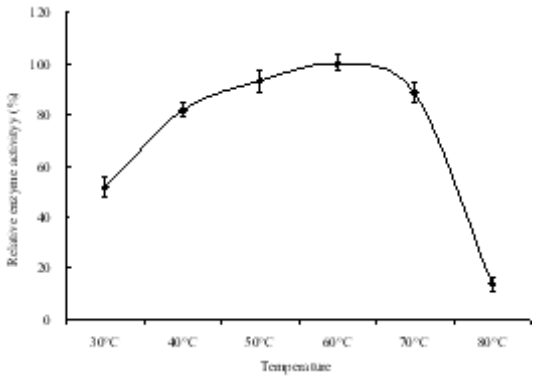

Fig. 4. The Temperature optimum of recombinant α-amylase, The temperature optimum was determined by Yoo YJ's method at pH optimum

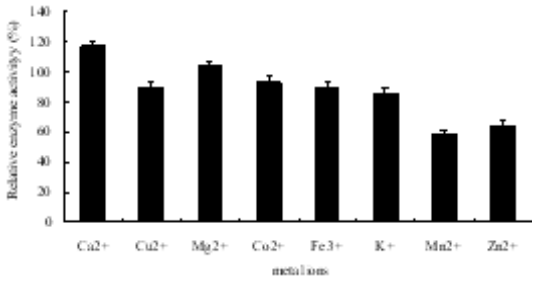

Fig. 5. Effect of Metal ions on recombinant α-amylase, The substrate included Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and K^{+} (ûnal concentration of 1 mM) and the activities were determined by Yoo YJ's method at 40 °C

From the figure, the optimal temperature was 60°C, and the recombinant protein showed the activity at a wide range of temperature. It showed the extremely high activity (>80%) at the temperature between 40°C and 70°C. It was conducive for the commercial applications.

Effects of metal ions

Effects of metal ions were detected for the recombinant α-amylase and shown in Fig. 5. The recombinant α-amylase was significantly activated by Ca^{2+}, significantly inhibited by Cu^{2+}; Co^{2+}; Fe^{3+} K^{+}, inhibited about 30% by Mn^{2+}; Zn^{2+}. The enzymatic activity was essentially unchanged when adding Mg^{2+}. The α-amylase is a Ca^{2+}-dependent enzyme. Adding 0.1 M Ca^{2+} can significantly improve the enzyme activity, consistent with many other literatures that indicated amylase is Ca^{2+}-dependent. The research shown that: Ca^{2+} can cause a conformational change in the α-amylase, thus accelerated the catalytic conversion reaction of the starch to glucose and maltose. But Zn^{2+} can competitively occupy the Ca^{2+}-binding sites to inhibit the combination of the enzyme and Ca^{2+}, thus enzyme activity decreased[5,23,24].

During starch saccharification and woven desizing, there is required adding a certain amount of á-amylase, which pH optimum is 6 and temperature optimum is about 60°C. Thus the α-amylase obtained from this study is basically meetting the requirements of starch industry and the textile industry with a prosperous application prospect.

CONCLUSION

We isolated the *B. amyloliquefaciens* L1-2 strain producing á-amylase, cloned its á-amylase gene using PCR, and then constructed the expression strain. Some enzymatic properties had been acquired. It was the foundation for the improvent of á-amylase production strains and meet the industrial production needs.

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