

Cloning and Sequence Analysis of Xyla Gene from Xylose Isomerase

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Keywords: Xylose Isomerase, Cloning, Vector Construction, Evolutionary Tree

Abstract: Xylose isomerase is an important industrial enzyme for preparing fuel ethanol and high fructose syrup. In this paper, acidophilic and thermostable microorganisms are used as xylose isomerase gene source bacteria. By means of molecular cloning and pet-28a(+) as vector plasmid, recombinant plasmid is constructed and expression strains are obtained. The result of sequencing showed that the gene was identical with the known gene in ncbi. It provides the basis for the expression and purification of recombinant protein.

1. Introduction

Xylose isomerase catalyzes the conversion of d-xylose from pentacarbon sugar to d-xylose in vivo, and also catalyzes the isomerization of d-glucose to d-fructose in vitro, so it is also called glucose isomerase [1]. The extremely important industrial application value of xylose isomerase makes it an important industrial enzyme together with protease and amylase. Early chan err cheng[2] and others introduced e. Coli xyla gene into schizosaccharomyces pombe, which measured the final concentration of ethanol to reach 3.7%, and proposed that the rate-limiting step of ethanol production is the conversion of xylose to xylulose. Zhang et al. [3] cloned xyla, xylb, tkta, and talb genes in e. Coli into zymomonas mobilis and expressed them. The recombinant bacteria can ferment xylose and produce ethanol. Since the 1970s, it has been mainly used in the preparation of fuel ethanol and high fructose syrup. In recent years, some foreign scientists have found that under certain conditions, xylose isomerase can transform and produce rare sugars, which are important raw materials for the pharmaceutical industry. Such as ribose, mannose, arabinose, lyxose, etc., can still be used in the research and development of nutritional feed and veterinary products in animal husbandry. The above findings open up new ideas for the study of xylose isomerase. In basic research, because the structure of the enzyme is very stable, it is also used as one of the models for studying the structure and functional relationship of proteins [4].

Due to slow growth, harsh culture conditions, low enzyme production and difficult separation and purification of strains obtained in natural environment. The development of genetic engineering and protein engineering technology has provided a new approach for the acquisition and transformation of gene cloning enzymes [5-8]. Based on the above conditions, this article uses *A. Cellulolyticus* 11b genome as a template to perform amplification, cloning, vector construction, and expression and bioinformatics research, laying a foundation for the application of enzyme preparations in biomass transformation.

2. Materials, Equipment and Methods

2.1 Test Materials and Equipment

A. cellulolyticus 11B(ATCC 43068) is the target gene source strain (deposited in laboratory), *E.coil* DH5 α , *E.coil* BL21, DNA marker(2000 -15000), 6 \times loading Buffer, Nde I, Hind III, T₄ DNA ligase, Q5 ultra-fidelity DNA polymerase. Other chemical reagents are analytically pure.

SW-079 PCR Analyzer (Bao Bio-Engineering Dalian Co., Ltd.), JY600t Electrophoresis

Instrument (Beijing Junyi Dongfang Electrophoresis Equipment Co., Ltd.), LAS 500 Biomolecular Imager (Beijing Green Cotton Technology Co., Ltd.), FA1004A Electronic Balance (Shanghai Jingtian Electronic Instrument Co., Ltd.), SW-073 Double One-sided Cleaning Workbench (Suzhou Cleaning Equipment Co., Ltd.), HH-8 Digital Thermostatic Water Bath Pot (Jintan Scientific Analysis Instrument Co., Ltd.), 5424R (Freezing) Ebonde Desktop High Speed Centrifuge (Shanghai Queensland Biotechnology Development Co., Ltd.), PHB-10 Selydos Acidimeter (Shanghai Danding International Trade Co., Ltd.), etc.

2.1.1 Medium Formula

1473 LPBM medium: NH_4Cl 1.0g, KH_2PO_4 1.0g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.02 g, Yeast extract 1.0 g, Cellbiose 0.5 g, Sigmacell alpha Type 50 5.0 g, Distilled water 1.0 L. Before adding the carbon source, adjust the pH to 5.2 with H_3PO_4 . Sterilize at 121 °C for 15min.

2.2 Experimental Method

2.2.1 Culture of *a.Cellulolyticus* 11b

The 1473 LPBM medium was prepared according to the medium formula provided by the American Strain Preservation Center ATCC, and 500 μL of bacterial liquid was inoculated into 20ml of medium at a culture temperature of 55 °C and 180 rpm. After 72 hours of culture, it was transferred to 100mL 1473 LPBM acid-heat medium for expanded culture.

2.2.2 Extraction of *a. Cellulolyticus* 11b Genome

The target gene was extracted by CTAB method, and the cultured *a. Cellulolyticus* 11b was centrifuged for 8 minutes at 6000 rpm. Add 800 μl CTAB buffer solution preheated at 65 °C, mix it well, shake it gently three times every 5 minutes, after 20 minutes of water bath at 65 °C, 12000 rpm, centrifugation for 15 minutes. Gently aspirate the supernatant, add an equal volume of phenol: chloroform (1: 1) solution for extraction, mix gently, and centrifuge at 4 °C, 13000 rpm for 10 min. Gently aspirate the supernatant, add an equal volume of chloroform, mix, mix at 4 °C, 13000 rpm, and centrifuge for 10 min. Extracting again with chloroform, collecting supernatant, adding 1/10 volume of 3mol/L sodium acetate buffer, mixing up and down, adding diploid precooled anhydrous ethanol, mixing up and down, precipitating at -20 °C for 1 h, 4 °C, 12000 rpm, centrifuging for 10 min. Discard the supernatant and wash the pellet twice with 70% ethanol; vacuum-dried and dissolved in 30-50 μL deionized water, and detected the genomic DNA by 1% agarose gel electrophoresis, and stored at -80 °C .

2.2.3 Pcr Amplification of Target Genes

Primers were designed based on the predicted *Acxyl1* gene sequence in the *A. cellulolyticus* 11B genome, and restriction sites were introduced at the 5' ends of the upstream and downstream primers, respectively. PCR was performed using the extracted target gene as a template. Amplification conditions were: pre-denaturation at 98 °C for 2 min; denaturation at 98 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and performing 30 cycles. Finally, it was extended for 8 min at 72 °C. After the reaction was completed, 1% agarose gel electrophoresis was performed, and the remaining samples were stored at 4 °C.

2.2.4 Construction and Identification of Expression Vectors

The basic gene operation technologies such as DNA enzymolysis, linkage, transformation and bacterial receptivity preparation refer to literature [9] and the instructions provided by relevant companies. The purified PCR products and plasmids were digested with *NDE I* and *hind III* respectively. After purification, the plasmids were connected with *T₄* DNA ligase. After incubation at 25 °C for 10 min, 5 μl of the connection products were added to 100 μl of *E. coli*. DH5 α sensitive cells. Mix it gently, put it on the ice for 20 min, heat it in the 42 °C water bath for 90 s, then put it on the ice for 2 min, add 800 μL LB liquid medium, put it on the 37 °C shaker for 120

rpm and shake it for 45 min. The bacterial solution was spread on an LB plate containing Kan, and cultured at 37 ° C for 16 hours. Pick 6 single colonies from the cultured plate into 5ml medium containing Kan, and culture at 37 ° C overnight. After the strains are stored, the plasmids are extracted, and then detected by PCR and electrophoresis. It was preliminarily determined to be a positive recombinant plasmid, and a strain was selected and sent for sequencing by Biotech Biotechnology (Shanghai) Co., Ltd. After sequencing, the obtained positive recombinant plasmid was named pAcXyl1.

Finally, the positive cloned plasmid was transferred to the expression host strain Ecoli. BL21 (DE3) for later protein expression and purification.

2.2.5 Induced Expression of Recombinant Bacteria

Take recombinant bacteria, plate them into LB solid medium containing kanamycin, and after overnight culture at 37°C, select single colony into 5mLLB liquid medium, and culture overnight at 37°C. 200μL of bacterial liquid was taken to expand into 20mLLB medium, cultured at 180rpm at 37°C under shaking until the OD value was 0.6-0.8, IPTG with a final concentration of 1mM/L was added, and induced to culture overnight at 16°C. Collecting thallus, adding 10 times volume of Binding Buffer (0.01mol/L PBS, pH 7.4) buffer, and resuspending. Ultrasonically disrupt bacterial cells (250W, 3S ultrasound, 3S interval). The samples after sonication were whole bacteria samples, and the remaining samples were centrifuged at 4 ° C and 3000 rpm for 2 min, and the supernatant was aspirated, centrifuged at 12,000 rpm, and centrifuged for 5 min. The supernatant was taken as a supernatant sample, and the remaining precipitate was a precipitated sample. Mix the sample with the loading buffer 1: 4 and use as a sample. Recognized by SDS-PAGE electrophoresis, the recombinant protein was expressed in the supernatant.

3. Results and Analysis

3.1 Pcr Amplification, Cloning and Identification of Target Genes

Genomic DNA was used as a template to amplify a 900bp band (fig. 1), which was consistent with the expected product size.

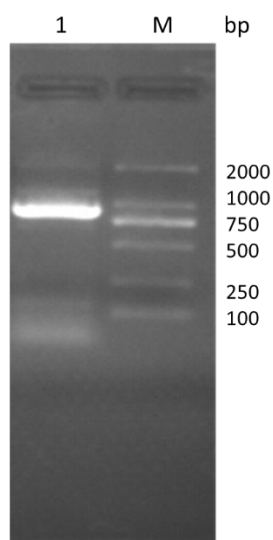


Fig.1 Electrophoresis of the Pcr Amplified Fragment of the Target Gene

M: DL 2000 DNA Marker; 1: PCR amplification products

3.2 Construction of Recombinant Plasmid

The purified PCR product was linked to the cloning vector pET-28a(+) with T₄ DNA ligase. In order to avoid the occurrence of false positive clones, 6 single colonies were selected and tested for extraction plasmid size (fig. 2) to obtain recombinant plasmid pAcXyl1. Sent to Bioengineering

(Shanghai) Co., Ltd. for sequencing. The sequencing results show that the obtained genes are completely consistent with the known genes in NCBI.

1 2 3 4 5 6

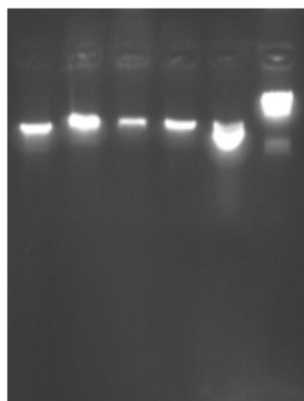


Fig.2 Electrophoresis Results of the Recombinant Plasmid Pacxy11

1-5: Preliminary positive recombinant plasmid; 6: False Positive Recombinant Plasmid

3.3 Bioinformatics Analysis of Xylose Isomerase

The whole gene sequencing project of thermophilic bacterium *A. cellulolyticus* 11B has been completed [10]. NCBI is used to analyze the predicted xylose isomerase (WP_011719960.1) in the genome. The results show that it encodes 304 amino acids, and the amino acid sequence is as follows:

- 1 mvsirvaaap vsfgvfelta snglpaads vldavvqcy qgidlgpagy lgdlslrrr
 - 61 ladrelalag gwleahfadd vllqreleti ratldlmaaa atngawrpkp tlavtgdarr
 - 121 rsaigqaahr tdlaldegw rrlvknlrsv velcrernle ptfhhhlgtf ieapeeierl
 - 181 leatdvglcl dtghllggg dpvkawhdwt drinqihvkd arlavmrsvm tdgcdmteaw
 - 241 rrgifcrlgd gdvdldafia avsasdygw vvveqdvipd sstpfealva dqrrnreflr
- 301 argw

Submit the amino acid sequence of xylose isomerase on SWISS-MODEL online server (<http://swissmodel.expasy.org/>), and select the protein structure that has been analyzed and has similar Identity (identity is 44%) as the template for homology modeling.



Fig.3 Schematic Diagram of the Molecular Modeling Structure of the Recombinant Protein Acxyl

4. Conclusion

As one of the three major enzymes used in industry, xylose isomerase has great industrial value. Through genetic engineering technology, the gene of xylose isomerase can be cloned into the vector. The gene source strain selected in this paper has heat-resistant acidity. Using *A. cellulolyticus* 11B gene as a template, after PCR amplification and gene cloning, the target gene was successfully constructed into the vector pET-28a (+), and the recombinant plasmid was obtained. It was transformed into the expression strain *E.coli* BL21 (DE3) to obtain a genetically engineered recombinant strain. After induced by IPTG, the recombinant bacteria can express the target

recombinant protein.

Due to the complexity of *A. cellulolyticus* 11B in the culture process, the concentration of the bacterial solution was low, and the concentration of the extracted genome was not ideal, which brought certain difficulties to subsequent experiments. According to discussion and analysis, the main reason is that the strain has eosinophilic and heat resistance [11], and it is not artificially domesticated, so it grows slowly. Therefore, it is improved in the method of genome extraction. In addition, the GC content of the gene is high, reaching 63.6%, so ordinary DNA polymerases cannot be smoothly amplified during the gene amplification process. NEB's Q5 super-fidelity DNA polymerase was used to successfully amplify the gene. Recombinant strains have been successfully constructed to provide support for subsequent studies on the expression and activity of recombinant proteins. The designed protocols and test methods still have reference value.

Acknowledgment

Fund projects: National University Student Science and Technology Innovation and Entrepreneurship Training Program (201811439040), Key Construction Disciplines of Biology, Jilin Agricultural Science and Technology University ([2019] No. SW10), Jilin Provincial Department of Education “Thirteenth Five-Year” Science and Technology Project (JJKH20180737KJ)

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