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Article

Selection of Recombinant Bacteria with High L-Asparaginase Production through Normal Pressure and Room Temperature Plasma Mutagenesis

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Abstract: Using Bacillus subtilis WB600 as the host, a recombinant bacterium capable of secreting and expressing L-ASNase was constructed, and the enzyme production of the recombinant bacterium was further increased through normal pressure and room temperature plasma mutagenesis. After fermentation of recombinant Bacillus subtilis WB600 (pMA5-wapA-ansZ) for 30 h, the extracellular enzyme activity reached 37.2 U/mL, indicating that L-ASNase can be secreted out of the cell mediated by the signal peptide wapA. Plasma mutagenesis was performed on the recombinant bacteria under the mutagenesis operating conditions of 120 W power, 10 L/min air flow, and 40 s mutagenesis time.

The enzyme activity of the mutant strain reached a maximum of 48.4 U/mL, which was increased by 30% before mutation. The above results show that normal pressure and room temperature plasma mutagenesis can effectively improve the enzyme activity of L-ASNase produced by recombinant bacteria. The research results provide an efficient production strain for the industrial production of L-ASNase.

Keywords: L-asparaginase; Bacillus subtilis; heterologous expression; normal pressure and room temperature plasma mutagenesis

Introduction

L-ASNase (Asparaginase, EC3.5.1.1) is an amide hydrolase that can catalyze the hydrolysis of asparagine to produce aspartic acid and helium. Clinically, L-ASNase is often used in combination with other drugs to treat cancers such as acute lymphoblastic leukemia and certain lymphomas and mast cell tumors [1-4]. In addition, L-ASNase can effectively decompose asparagine, the precursor of the carcinogen acryl, making it one of the hot enzymes that European and American countries have recently paid attention to in the field of food safety. In the study by Arindam Jana et al [5], the acrylamide content of potato slices treated with L-ASNase was reduced by 80% after high-temperature frying. Shpresa Musa et al [6]. added L-ASNase to wheat flour, and the acrylamide content was reduced by 85%. Therefore, achieving efficient production of L-ASNase is a hot research topic at home and abroad.

L-ASNase is widely present in microorganisms, including Erwinia carotovora, Enterobacter serogenes, Thermes thermophilus,Streptomyces griseus, Escherichia coli, Pseudomonas aeruginosa Pseudomonas aeruginosa [1]**.** Sweeta et al [2]**,** used response surface methodology to optimize the L-ASNase fermentation of wild bacteria Enterobacter aerogenes, and the enzyme activity was 40.36 U/mL. Studies on recombinant expression of L-ASNase have also been reported. After L-ASNase derived from Streptomyces and Flammulina velutipes were expressed in E. coli, the enzyme activities reached 16 U/mL [3]. Pourhossein et al [4]. expressed L-ASNase derived from Erwinia chrysanthemi in Escherichia coli and Erwinia Carotovor, and the enzyme activity was increased to 49 U/mL. In general, the yield of L-ASNase produced by microbial fermentation is low, and the host for recombinant expression is limited to non-food-safe E. coli.

Atmospheric pressure radio frequency glow discharge plasma using helium as the gas source can cause the breakage of DNA molecules. Based on this, researchers developed the Atmospheric and Room Temperature Plasma (ARTP)mutagenesis breeding instrument [5]. Compared with ultraviolet mutagenesis, Other traditional mutagenesis techniques, ARTP mutagenesis has many advantages: low temperature of ions, high concentration and variety of active ions, simple operation, low cost, no pollution to the environment, fast mutagenesis (within few minutes), mutation High rate, large mutation library capacity, etc. [6]. ARTP mutagenesis has been successfully used for the mutagenesis of high-yield strains of glutamine transaminase [7], butanol [8], lipase [9], oils [10] and other products has gradually become an important platform technology for rapid evolution and breeding of microorganisms.

The author expressed B. subtilis 168 L-ASNase derived from B. subtilis in the food-safe B. subtilis WB600, and mutated the recombinant bacteria through the ARTP mutagenesis system to obtain a high-yield mutant flavor of L-ASNase.

Materials and Methods

1. Materials

Strains and plasmids Escherichia coli JM109.B.subtilis168, B.subtilis WB600 and plasmid pMA5-wapA (the pMA5 signal is inserted between the Ndel-BamHI sequences of the wapA plasmid) are all stored in the author's laboratory.

1.2 Enzymes, reagents, primers and DNA sequence determination restriction endonuclease DNA ligase, Prime STAR HS DNA polymerase, small fragment DNA recovery kit and agarose gel DNA recovery kit: Ta ka Ra company; ampicillin, sulfuric acid Kanamycin, plasmid extraction kit: Sangon Bioengineering Co., Ltd.; primer synthesis and DNA sequencing were also completed by this company. Protein Marker and SDS-PAGE gel preparation kit: Fermentas Company and Beyotime Company; L-asparagine: Sigma Company; Peptone and yeast powder: OXID Company; other reagents are domestic analytical grade.

1.3 Medium: LB medium for the cultivation of E. coli and B. subtilis WB600 seed culture (g/L): yeast extract powder 5, protein: 10,NaCl10; add 2 g/ L agar to the solid medium. Bacillus subtilis WB600 fermentation medium (g/L): sucrose 35, peptone 15, urea 0.8, corn steep liquor 12, K_2HPO_4 2.612, KH_2PO_4 2.041, $MgSO_4 \cdot 7H_2O$ 1.845, NaCl 3, L-asparagine 1; pH 7. Ampicillin 100 μg/mL, kanamycin sulfate 50μg/mL.

2. Method:

2.1 Construction of recombinant bacteria based on GenBank which provided Bacillus subtilis 168 , G19F and G19R primers were designed according to the gene sequence of L-ASNase and ansZ . The primers are detailed in Table 1. PCR 14: 98°C 3 min; 98°C 30 s; 50°C 30 s; 72°C70 s; 30 cycles; 72°C extension for 5 min. The amplified ansZ gene fragment was connected to pMD18-T-simple and transformed into E. coli competent cells JM109. Coat the plate, incubate at 37°C overnight, MD18-T-simple/ansZ and pMA5-wapA were double-digested with BamHI and EcoRI respectively, and then ligated with the ligase in the Takata DNA Ligation Kit to construct the recombinant plasmid pMA5 wapA-ansZ. After ligation overnight, the E. coli competent cells JM109 were transformed, and

positive clones were screened through colony PCR verification. Positive clones were selected and cultured in LB medium containing 100μg/mL ampicillin overnight at 37°C. Plasmids were extracted for restriction enzyme digestion verification and sequencing identification. Use chemical transformation method to transform The recombinant plasmid pMAS-wapA-ansZ into B. subtilis WB600, and select positive clones on a plate containing 50μg/mL kanamycin (K). The colonies were verified by PCR and enzyme digestion, and the successfully constructed recombinant bacteria were stored in -80°C glycerol tubes.

Table (1) Primer sequences

Primer name	Primer sequence (5'-3')	Primer length/bp
G19F	CGGAATTCTGTTCA CATTCCCTGAAACA	29
G19R	CGGGATCCTCAATACT CATTGAAATAAGCTTG	32

2.2 The recombinant bacteria were expressed at 37°C, 200 r/min , incubate the B.subtilis WB600 (pMA5-wapA-ansZ) in culture media for 10 hours, and then transferred the B. subtilis WB600 to fermentation medium. After 30 hours of fermentation, the cells were detected external enzyme activity.

2.3 ARTP mutagenesis method was used to mutagenize the recombinant bacterial liquid. Under certain conditions of mutagenesis power, working gas flow, and distance between the plasma emission source and the sample slide, the operable parameter of mutagenesis is the processing time. In order to obtain the optimal mutagenesis conditions, it is first necessary to obtain the lethality curve of the recombinant bacteria. The operating conditions are shown in Table 2. The mutagenized samples were diluted and spread on plates, and the lethality rate was calculated using the CFU method.

Table (2) ARTP mutagenesis conditions of B. subtilis WB600 (pMA5-wapA-ansZ)

Parameter	Operating conditions
Input power/W	120
Processing distance / mm	2
Air flow (L/ min)	10
Processing time / s	0,10,20,30,40,50,60
Sample	10 μL seed liquid, OD=0.6~0.8

1) Preliminary screening: Dilute the seed suspension after ARTP mutagenesis and spread it on the screening plate, and incubate at 37°C for 12 hours. Place on the tablet, A single colony was inoculated into a 96-well shallow-well culture plate containing seed culture medium, cultured at 37°C for 10 hours, and then transferred to a 96 deep-well culture plate containing fermentation culture medium. Place the 96 deep-well culture plate on a 96-well shaker for 30 hours and then measure the enzyme activity. Those strains whose enzyme activity is more than 10% higher than that of the original strain are recorded as positive mutants.

2) Reinstatement: Inoculate the initially obtained strain into the liquid seed culture medium and culture it at 37°C and 200r/min for 10h. Then the seeds were transferred to a 250 mL Erlenmeyer flask containing fermentation medium, and the inoculum volume fraction was 3%, cultured at 37°C, 200 r/min for 2 days. The fermentation supernatant was taken for measurement.

2.4 Genetic stability analysis: order to analyze the genetic stability of the mutant strain, 5 subcultures were carried out on solid plates, the strains of each generation were fermented, and then their L-ASNase activity was measured.

2.5 Determination of L-ASNase enzyme activity : Commercial use of Nessler's reagent method to determine L-ASNase. The reaction proceeds in two steps. 1) Enzymatic hydrolysis reaction; 1mL phosphate buffer (10mmol/L, pH7.5), 300μL enzyme solution, 100μL substrate, react at 37°C for 30 minutes, then add 100μL trichloroacetic acid to terminate the reaction. After mixing, centrifuge at 12000 r/min for 2 minutes. 2) Color reaction: In the first step, the enzymatic hydrolysis reaction solution is 200μL, 3.3 mL of deionized water, and 500μL Nessler's reagent. After mixing, measure the absorbance value at 436nm.

Definition of enzyme activity unit: The amount of enzyme required to hydrolyze L-asparagine to generate 1 μmol per minute is defined as L-ASNase activity unit (U/mL).

The standard curve uses $(NH_4)_2SO_4$. Determination: Prepare a standard solution of 18 mmol/L $(NH_4)_2SO_4$, and add 0.50, 100, 150, 200, 250, 300,350, 400 μL respectively, and make up with deionized water. to 400 μL, then add μ 1 mL of phosphate buffer (10 mmol/L, pH 7.5), react at 37°C for 30 min, and then add 100 μL of trichloroacetic acid to terminate the reaction. After mixing, centrifuge at 12000r/min for 2 minutes, and then perform a color reaction.

2.6 Determination of bacterial biomass: Use a spectrophotometer to detect the absorbance value of the appropriately diluted bacterial solution at a wavelength of 600 nm, ED OD_{600}

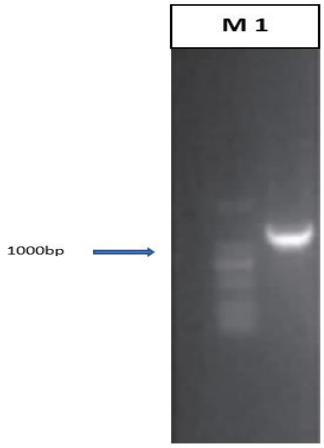
2.7 The mass concentration of sucrose was measured using the citrione-concentrated sulfuric acid method to determine the mass concentration of sucrose in the fermentation broth, see literature [21].

Results

1. Construction and expression of recombinant bacteria

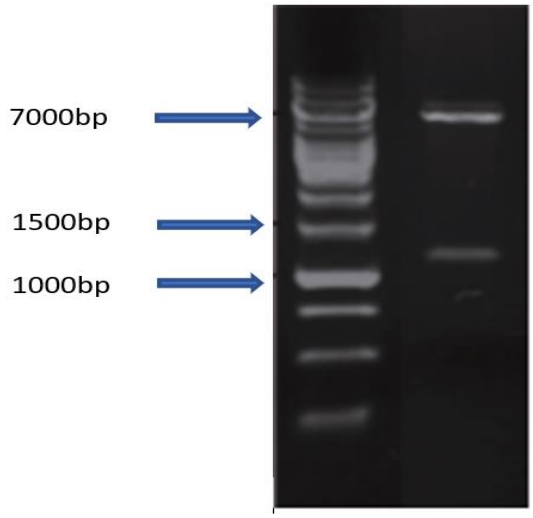
Using the synthesized B. subtilis 168 genome as a template, primers G19F and G19R were used to amplify the L-ASNase gene. The resulting PCR product size was 1071 bp, as shown in Figure 1-3. Connect the gene fragments Go downstream of the wapA signal peptide of the expression vector pMA5-wapA to obtain the recombinant vector pMA5-wapA-ansZ, as shown in Figure 2-3.

After sequencing verification, pMA5-wapA-ansZ was transformed into B. subtilis WB600 to obtain the recombinant bacterium B. subtilis WB600 (pMA5-wapA-ansZ).



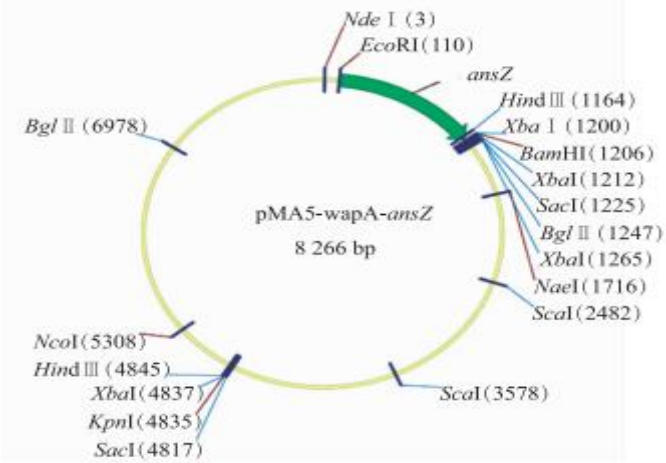
M: DNA standard; 1: L-ASNase PCR product

Figure (1) Electrophoresis of PCR products



M: DNA standard; 1: Recombinant plasmid EcoRI-BamHI double enzyme digestion product

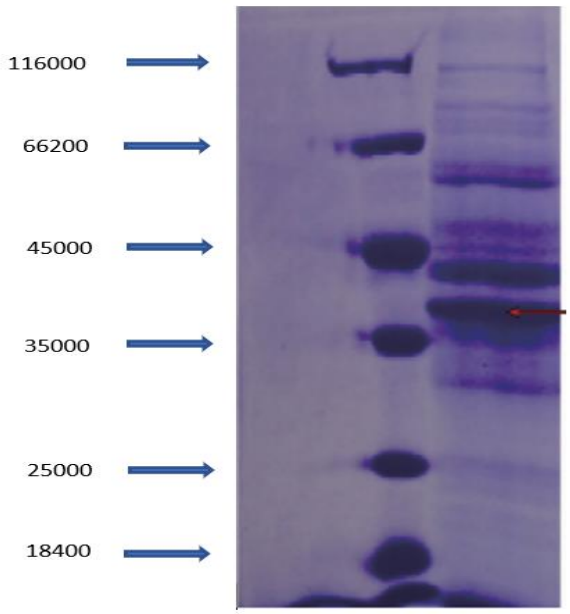
Figure (2) Plasmid double enzyme digestion electrophoresis



Construction of recombinant expression plasmid pMA5-wapA-ansZ

Figure (3) : construction of pMA5-wapA-ansZ

The recombinant strain B. subtilis was fermented at 37°C for 30 h, and the fermentation supernatant was taken for enzyme activity and SDS-PAGE analysis. In the fermentation supernatant of the recombinant bacteria, a protein band with a relative molecular mass of approximately 38,000 can be seen, which is consistent with the theoretical relative molecular mass of L-ASNase, as shown in Figure 4. This band was not seen in the control fermentation supernatant, indicating that L-ASNase was successfully secreted and expressed in B. subtilis WB600. After enzyme activity analysis, the recombinantly expressed L-ASNase can be secreted extracellularly, and the extracellular enzyme activity reached 37.2 U/mL.



M: protein standard relative molecular mass; 1: B. subtilis WB600 (MAS-wapA) fermentation supernatant; 2, B. subtilis WB600 (pMA5-wapA-ansZ) fermentation supernatant

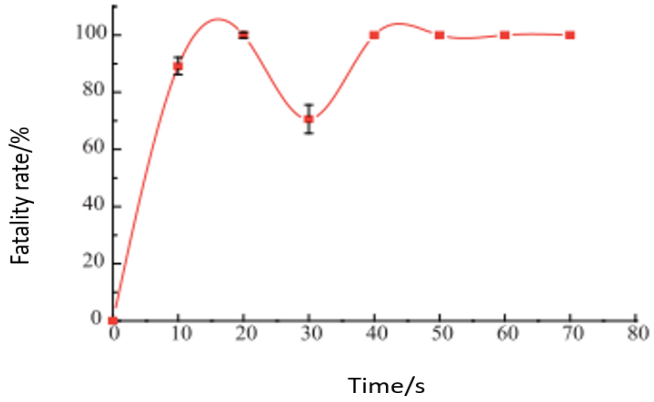
Figure (4): SDS-PAGE analysis of secreted expression of LASNase

2. Determination of ARTP mutagenesis lethality curve

Previous research by Yadav MK [21] found that plasma has a negative impact on microorganisms. Cell walls and membranes, as well as proteins, all have a significant impact on plasma. The daughter body contains a large number of high-energy free radicals and active ions, which can Destroys cell structure and continuously breaks down microorganisms causing their death. at the same time in the process of forming plasma, some of the ultraviolet rays produced It can also cause the death of bacteria. And only a few have been processed by ARTP. The remaining microorganisms survive through their own repair systems and remain alive [11].

A genetic mutation occurs in the process. Therefore, choosing the appropriate mutagenesis procedure The conditions are conducive to rapid and efficient selection of mutant strains.

As shown in Figure 5, as the processing time increases, B. subtilis. The lethality rate of WB600(pMA5-wapA-ansZ) bacteria continues to increase. The fatality rate reaches 89.2% in 10 seconds, and the fatality rate in 20 seconds Close to 100%. However, after 30 seconds of treatment, the fatality rate dropped to 70.6%. The reason for this phenomenon may be that the bacteria undergo a Quantitative plasma treatment stimulates the repair effect of the bacteria themselves. When the repair effect is stronger than the destructive effect of plasma, the lethality rate of the bacteria will decrease. However, as the plasma injection time increases and the plasma's damaging effect on the bacterial cells is greater than its repairing effect, the lethality of the bacterial cells increases. It can be seen that when plasma treatment is 40s, the fatality rate reaches more than 99%. After the treatment time is greater than 40 seconds, all bacteria will die. Taking the enzyme activity 10% higher than the control as a positive mutant strain, the positive mutation rate of B. subtilis WB600 (pMA5-wapA-ansZ) after mutagenesis for 40 s reached 26%, so the mutagenesis time in this study was set as 40 s [12, 13].

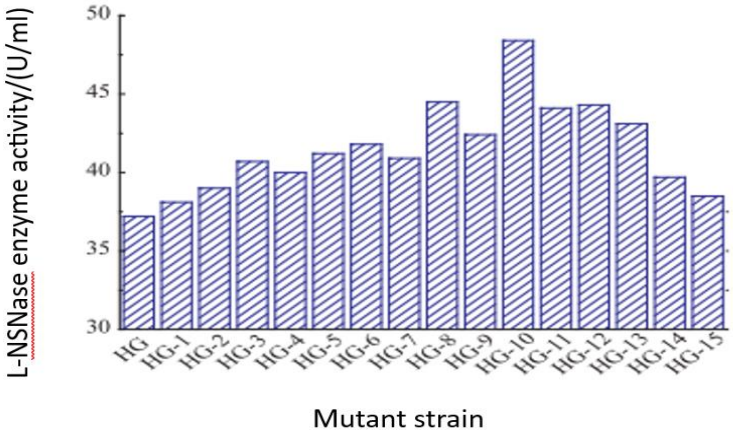


B. subtilis WB600 (pMA5-wapA-ansZ)'s verse is turned to death

Figure (5) Mutagenic lethality curve of B. subtilis WB600(pMA5-wapA-ansZ)

3. Screening of mutant strains

If the enzyme activity of the initial screening is increased by 10%, it will be regarded as a positive mutant strain. The positive mutation rate up to 26%. Then carry out re-screening on shake flasks, and test the mutant strains serial number. The results of re-screening are shown in Figure 6. A forward mutant strain was screened. HG-10, the enzyme activity is 48.4 U/mL, which is 1.3 times that of starting strain [14].



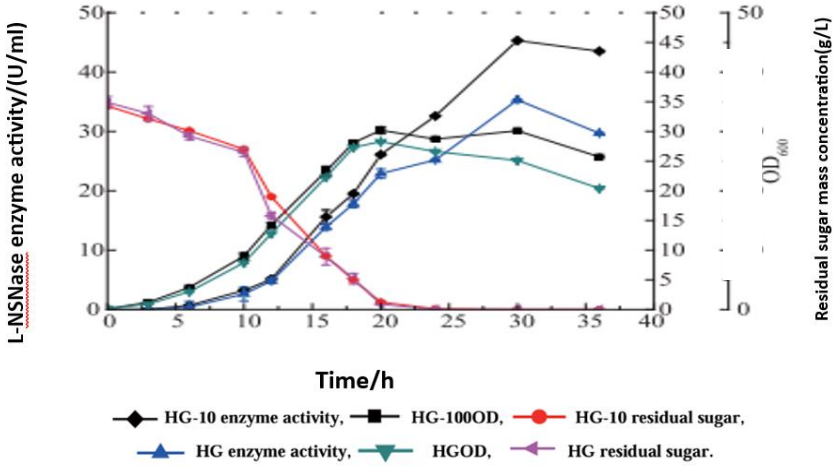
B. subtilis WB600 (pMA5-wapA-ansZ) (HG) and its mutations

Figure (6) L-ASNase activity analysis of 15 mutants and B. subtilis WB600(pMA5-wapA-ansZ) (HG)

4. Analysis of fermentation characteristics of mutant strains

In order to further understand the growth and fermentation of recombinant bacteria and mutant strains, their growth, sucrose consumption and enzyme production were studied as shown in Figure 7, the utilization of sugar by the recombinant strain B [15]. subtilis WB600 (pMA5-wapA-ansZ) and the mutant strain HG-10 is relatively consistent. In the early stage of 0-6 hours, the bacterial cells grow slowly, consume less sugar, and produce almost no enzymes [16]. From 6 to 18 hours, the bacterial growth enters the logarithmic phase. Due to the massive proliferation of the bacterial cells, the sucrose mass fraction in the fermentation culture medium drops sharply, and the enzyme production rate increases at this time. The mutant strain HG-10 grew faster than the original recombinant strain [17]. After 18 hours, the bacterial growth entered a stable phase, and the enzyme production continued to increase [18]. For most extracellular enzymes, the synthesis of the enzyme is roughly parallel to the growth star of the circle. When the growth stops, the enzyme production reaches the maximum, and the enzyme production may decrease if the culture continues **[19]**. The

stability period of the original recombinant bacteria is shorter and the bacteria decline faster. The mutant strain HG-10 has a longer stable period and slower bacterial cell death, which may be the reason for the increased enzyme activity of HG-10 [20].

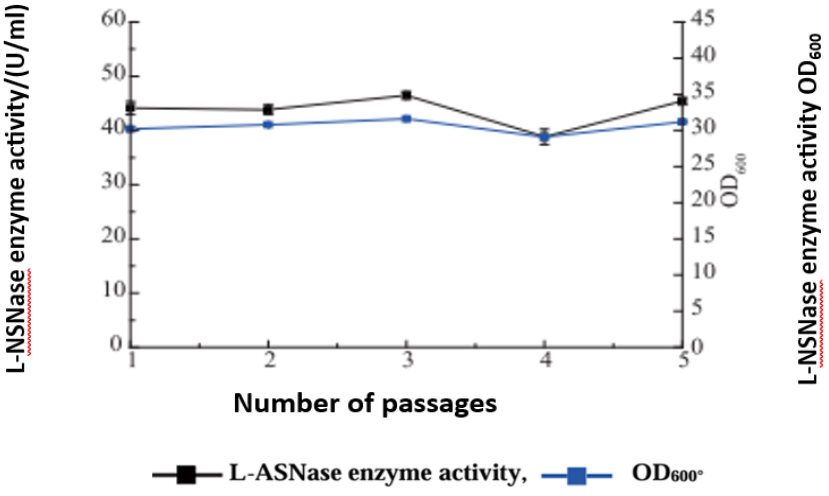


B. subtilis WB600 (pMA5-wapA-ansZ) (HG) Fermentation curve of strain HG-10 and mutations

Figure (7) B. subtilis WB600(pMA5-wapA-ansZ)(HG) and mutations Strain HG-10 fermentation curve.

5. Analysis of genetic stability of mutant strains

According to the method in 1.2.4, the mutant strain HG-10 was used to produce L-ASNase. The genetic stability of L-ASNase was investigated, see Figure 8. enzyme activity. The analysis results showed that after five consecutive passages, the enzyme activity range of the mutant strain was within 43.8~46.5 U/mL, the enzyme activity changes slightly in each generation [21]. The above results this shows that the mutant strain HG-10 has good genetic stability and is consistent with this study. The results are similar. The mutant strains obtained after hydrocarbon plasma mutagenesis of wild bacteria. The genetic stability is high. The enzyme activity levels of the mutant strains decreased by less than 10% [22].



Genetic stability analysis of mutant strain HG-10

Figure (8) Genetic stability analysis of mutant strain HG-10

Conclusion

The author used B. subtilis WB600 as the host and constructed a recombinant strain of B. subtilis WB600 (pMA5-wapA-ansZ) that efficiently secretes L-ASNase, with an extracellular enzyme activity of 37.2 U/mL. A mutant strain of B. subtilis WB600 (pMA5- wapA-ansZ) was obtained through ARTP mutagenesis technology. The extracellular enzyme activity increased to 48.4U/mL, 30% higher than before mutagenesis, and the mutant strain has high genetic stability. The above results show that constructing L-ASNase-producing recombinant bacteria from food-safe B. subtilis and performing ARTP mutagenesis is an effective method to obtain high-yield L-ASNase bacteria, and provides a high-yield strain for L-ASNase fermentation production. In addition, the strategy of physical mutagenesis of recombinant bacteria also provides a useful reference for the optimization of other recombinant producing bacteria.

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