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Yield Enhancement of Recombinant α-Amylases in *Bacillus amyloliquefaciens* by ARTP Mutagenesis-Screening and Medium Optimization

(Hasil Peningkatan Rekombinan α-Amilase dalam *Bacillus amyloliquefaciens* ARTP Mutagenesis-Penyaringan dan Pengoptimuman Sederhana)

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ABSTRACT

α-Amylase is the most extensively applied enzyme in industry. There is *an urgent need for improvement on the yield of α-amylases currently. Herein, a strategy which combined Atmospheric and Room Temperature Plasma (ARTP) mutagenesis tool for construction of mutant library of* Bacillus amyloliquefaciens *with a 24-well plates screening technique was adopted to improve the yield of recombinant* Bacillus amyloliquefaciens *α-amylases (BAA). A mutant strain named* B. amyloliquefaciens *ZN mut-7# was obtained, and the activity of BAA produced by this mutant strain was 86.92% higher than that of the original strain.* B. amyloliquefaciens *ZN mut-7# has an unchanged BAA gene and genetic stability. This successful application proved that ARTP can be applied to the genetically engineering strains that contain recombinant plasmid. Furthermore, response surface methodology offers an achievable and efficient strategy to optimize the composition of medium used to generate BAA in* B. amyloliquefaciens *ZN mut-7#. A 1.28-fold increase had been obtained compared to the production of non-optimized fermentation medium. This study demonstrates that ARTP mutagenesis and medium optimization are efficient and feasible methods for increasing recombinant enzyme production in the genetically engineering strains.*

Keywords: ARTP mutagenesis; Bacillus amyloliquefaciens *α-amylases; medium optimization; response surface methodology*

ABSTRAK

α-Amilase adalah enzim yang diaplikasi secara meluas dalam industri. Terdapat keperluan segera untuk menambah baik hasil daripada α-amilase pada masa ini. Di sini, satu strategi yang menggabungkan alat mutagenesis atmosfera dan plasma suhu bilik (ARTP) untuk pembinaan perpustakaan mutan Bacillus amyloliquefaciens *dengan teknik saringan plat 24-telaga yang telah diguna pakai untuk meningkatkan hasil recombinan* Bacillus amyloliquefaciens *α-amylases (BAA). Terikan mutan yang dipanggil* B. amyloliquefaciens *ZN # mut-7 telah diperoleh dan aktiviti BAA yang dihasilkan oleh terikan mutan ini adalah 86.92% lebih tinggi daripada terikan asal.* B. amyloliquefaciens *ZN mut-7 # mempunyai gen BAA tidak berubah dan kestabilan genetik. Aplikasi ini berjaya membuktikan bahawa ARTP boleh digunakan untuk kejuruteraan genetik terikan yang mengandungi plasmid recombinan. Selain itu, kaedah gerak balas permukaan menawarkan strategi yang boleh dicapai dan cekap untuk mengoptimumkan komposisi medium yang digunakan untuk menjana BAA dalam* B. amyloliquefaciens *ZN # mut-7. Peningkatan sebanyak 1.28-lipatan telah diperoleh berbanding pengeluaran sederhana penapaian tidak optimum. Kajian ini menunjukkan bahawa mutagenesis ARTP dan pengoptimuman sederhana adalah kaedah yang cekap dan boleh dilaksanakan untuk meningkatkan penghasilan enzim recombinan dalam kejuruteraan genetic terikan.*

Kata kunci: Bacillus amyloliquefaciens *α-amilase; mutagenesis ARTP; pengoptimuman sederhana; kaedah gerak balas permukaan*

INTRODUCTION

Amylase as the main catalyst plays a significant role in the fields of brewing, textile, food processing and medicine (Souza & Magalhaes 2010). The common industrial amylases are divided into four categories (α-amylase, β-amylase, γ-amylase, debranching enzyme), one of which can catalyze the hydrolysis of internal α-1,4-glycosidic linkages is α-amylase (Rivera et al. 2003). Amylase is widely considered to be the earliest industrialized and most broadly applied enzyme at present (Vijayaraghavan et al. 2011). *Bacillus amyloliquefaciens* α-amylase (BAA) is a liquefying-type mesophilic α-amylase, with an optimum pH around 6.0 and optimum temperature at 50-70°C and inactivated temperature at 90-100°C (Wu et al. 2017). Therefore, it has been widely applied into special processes where the liquefaction degree must be strictly controlled (Fan 2014).

The BAA producing strains are mainly derived from *B. amyloliquefaciens* BF7658 (Fan 2014). *B. amyloliquefaciens*, a gram-positive bacterial strain, has a

clear genetic background and is generally recognized as safe. Thus, it is regarded as a well identified and popular host for recombinant enzyme expression (Yang et al. 2010). To improve the expression of α-amylase in *Bacillus amyloliquefaciens*, large amounts of strategies have been employed, including mutagenesis and genetic engineering. Li (2008) confirmed that α-amylase of *B amyloliquefaciens* mutant strain YL1-5 was improved by 19.3% compared with the original strain after treatment with UV, microwave and LiCl.

Atmospheric and Room Temperature Plasma (ARTP) is an effective mutation method that can increase the yield of industrial commodities, as a result of it has the distinguished feature of destroying DNA chains at atmospheric pressure and room temperature momentarily (Zhang et al. 2015, 2014). The principle of ARTP mutagenesis tool is that helium radio-frequency Atmospheric-Pressure Glow Discharge (APGD) plasmas breaks the double chains of DNA; The genetic influences depend on the plasma operating parameters such as helium flow rate, power input, processing distance and time (Li et al. 2008). Cao et al. (2017) reported that the lipid and dry weight productivity of the ARTP-induced mutagenesis strain II-H6 was increased by 16.85% and 22.07% compared with the original oleaginous microalgae Chlorella. ARTP has also been reported to increase the yield of α-amylase in *Bacillus* spp. Wang et al. (2016) reported that the mesophilic α-amylase activity of the *B. subtilis* mutant strain BS-12 was 801 U/mL, which was 32.2% higher than that of the original strain. Up to now, ARTP mutagenesis has been successfully used for microbial breeding, mainly for non-genetic engineering strains, but rarely applied for engineering bacteria breeding. Ma et al. (2016) reported that the growth of *B. subtilis* 168 with recombinant plasmids was improved by ARTP mutagenesis, the highest production rate of alkaline amylase in recombinant plasmids was enhanced from 1.31 U/(mg•h) to 1.57 U/(mg•h).

In previous studies, *B. amyloliquefaciens* ZN was achieved by genetic engineering techniques to introduce recombinant plasmids containing the BAA gene into the wild type of *B. amyloliquefaciens* (Li 2016). After a series of modification by promoter element and signal peptide of vectors comprising a gene encoding BAA, the BAA activity of *B. amyloliquefaciens* ZN fermentation is about 200 U/mL in 250 mL shake-flask (unpublished data). However, the strain *B. amyloliquefaciens* ZN grows rapidly in moderate fermentation temperatures and has a good BAA production stability, and there is still more effort needed to improve the yield of BAA.

In this study, a strategy integrated ARTP mutagenesis and high throughput screening technique was conducted to improve the yield of BAA. A higher BAA expression levels strain named *B. amyloliquefaciens* ZN mut-7# was obtained. The strain has good genetic stability and is a useful object for further research. Moreover, response surface methodology was adopted to optimize the fermentation medium and improve the production of BAA in *B. amyloliquefaciens* ZN mut-7#.

Materials and Methods

Microorganisms, medium and cultures

B. amyloliquefaciens ZN was kept in the culture collection center of School of Minerals Processing and Bioengineering, Central South University, China. The Lucia-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) was used for the seed culture. The fermentation medium was described as follows: 12 g/L soluble starch, 10 g/L casein tryptone, 3 g/L beef extract, 2 g/L $CaCl_2$ and 2 g/L NaCl (Li 2016). All media were sterilized at 121°C for 15 min.

The working volumes of seed and fermentation cultures were 20 and 50 mL in 250 mL shake-flask, respectively. Flasks containing seeds culture were shaken at 180 rpm at 32°C for 12 h, then transferred to the fermentation medium by 2% inoculation, and cultured for extra 72 h to produce the BAA.

ARTP mutation operational procedure

ARTP mutation operational procedure was conducted following the method described by Ma et al. (2015). With 99.99% helium, the working radio-frequency power input, treatment distance and gas flow was set to 100 W, 2.0 mm and 8.0 SLM, respectively. *B. amyloliquefaciens* ZN samples were treated with ARTP mutagenesis for 0-60 s. After proper dilution, 100 μL of cell suspension was incubated on LB agar to construct a mutant library for 12 h at 37°C.

Screening and verification of the mutants

To increase the screening efficiency, clones with the larger sizes were picked into 24-well plates containing 2 mL LB medium and shaken over night at 600 rpm and 32°C. Overnight cultures were transferred by 2% inoculation rate into another 24-well plates with 2 mL fermentation medium and fermented for another 72 h to produce recombinant enzymes. After screening by 24-well plates fermentation, the highest BAA yield *B. amyloliquefaciens* ZN mutant was selected by comparing the enzyme activity. Afterwards, the highest BAA yield mutant strain and the original strain were activated and fermented in three 250 mL shake-flasks with fermentation medium simultaneously. The BAA production capacity was tested during the 120 h course of fermentation. Moreover, the sequence of BAA gene in the recombinant plasmid was verified by DNA sequence alignment and the genetic stability of *B. amyloliquefaciens* ZN mutant was detected by subculturing mutants for 10 generations.

Analysis of a-amylase activity

α-Amylase activity was determined by modified 3,5-dinitrosalicylic acid (DNS) method (Fuwa 1954). One unit (U) of α-amylase activity was defined as the enzyme releasing 1 mg reducing sugar per minute at pH6.0 and at 60°C.

SODIUM DODECYL SULFATE (SDS)-POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS (PAGE)

The cultures which fermented for 72 h were centrifuged at 12,000 g for 5 min at 4°C. The supernatants which contained α-amylase were determined by SDS-PAGE (Laemmli 1970). The gels were formed as 81×74×1.5 mm slabs which consisted of separating gels containing 4%-20% gradient acrylamide and stacking gels had a gel concentration of 5%. Samples were boiled before added to gels and gels were run at 180 V. The proteins were stained with 0.25% Coomassie Brilliant Blue for 60 min and then elution in distilled water overnight.

SINGLE FACTOR EXPERIMENT

In single factor experiments, five kinds of carbon sources and nitrogen sources were selected as the experimental factors. For carbon-related single factor experiment, basic fermentation medium (10 g/L casein tryptone, 2 g/L $CaCl_2$, 2 g/L NaCl) was supplemented with 10 g/L five different carbon sources (CH_3COONa, glucose, corn flour, bran and soluble starch). For nitrogen-related single factor experiment, basic fermentation medium (10 g/L soluble starch, 2 g/L $CaCl_2$, 2 g/L NaCl) was supplemented with 10 g/L five different nitrogen sources (casein typtone, beef extract, soybean meal, yeast extract and NH_4Cl). *B. amyloliquefaciens* ZN mutant was cultivated in 250 mL shake-flasks with the modified fermentation medium at 32°C at 180 rpm for 72 h, and BBA accumulation were monitored as mentioned before.

EXPERIMENTAL DESIGN OF OPTIMUM MEDIUM

The medium for *B. amyloliquefaciens* ZN mutant was optimized using Minitab17.0 software (Minitab Inc., Pennsylvania, USA) systematically, including the Plackett-Burman design and response surface analysis (Gao et al. 2009).

PLACKETT-BURMAN DESIGN

Each independent factor is tested at high and low levels, defined as (+) and (-) respectively, in the Plackett-Burman experiment. As shown in Table 1, the high level was 1.5 times of the low level. Six factors (bran, beef extract, NaCl, $CaCl_2$, yeast extract and soluble starch) with significant influence were preferred according to the results of previous experiments (data not shown), others (pH, temperature, speed) were constant. The Plackett-Burman design with N=12 (containing dummy variables were conducted to evaluate the standard error) was adopted. α-Amylase production average value (three parallel experiments) was taken as the response (Table 1 shows the experimental design data and attached list showed design details). Only variables with a confidence level

TABLE 1. Design matrix and experimental results of Plackett-Burman design

Run	A	B	C	D	E	F	G	H	I	activity (U/mL)
1	1	1	-1	1	1	1	-1	1	1	323.70
2	1	1	1	1	-1	1	-1	-1	-1	336.64
3	1	1	1	-1	1	-1	-1	-1	1	277.88
4	1	1	1	1	1	-1	1	1	-1	388.38
5	1	1	-1	-1	-1	1	1	1	-1	296.35
6	1	1	-1	-1	1	1	1	-1	1	287.48
7	1	1	-1	1	1	-1	1	-1	-1	319.64
8	1	1	-1	-1	-1	-1	-1	-1	-1	237.22
9	1	1	1	1	-1	1	1	-1	1	366.20
10	1	1	1	-1	-1	-1	1	1	1	321.85
11	1	1	-1	1	-1	-1	-1	1	1	292.66
12	1	1	1	-1	1	1	-1	1	-1	287.12

Attached list of design details:

Factors		level	
		-1	1
A	bran (g/L)	12	18
B	beef extract (g/L)	6	9
C	-		
D	soluble starch (g/L)	1	1.5
E	yeast extract (g/L)	4	6
F	-		
G	NaCl (g/L)	2	3
H	$CaCl_2$ (g/L)	1	1.5
I	-		

above 90% can be determined to have compelling effect on the production of BAA. These factors were worth taken optimization further.

METHODS OF PATH STEEPEST ASCENT

Based on Plackett-Burman experimental results, the steepest ascent experiment was adopted to move towards the optimum response vicinity rapidly. Design details are shown in Table 3.

BOX-BEHNKEN EXPERIMENTAL DESIGN

On the basis the results of Plackett-Burman and steepest ascent experiment, three factors (bran, beef extract and yeast extract) in the Box-Behnken experiment were selected and each factor required for three levels. The experiment had 15 experimental points, including 12 factorial points and 3 zero (zero experiment was conducted to evaluate the error).

STATISTICAL ANALYSIS

Minitab 17.0 software was applied for the experimental data regression analysis, including the confident of determination R-Sq, F-test and t-test.

RESULTS AND DISCUSSION

CONSTRUCTION OF THE MUTANT LIBRARY

In this experiment, the *B. amyloliquefaciens* ZN was treated with ARTP for 0, 20, 25, 30, 35, 40, 50 and 60 s, while other parameters and condition maintained unchanged. After cell suspension was properly diluted and cultured on LB agar, the number of colonies with different ARTP treatment time was calculated to discover the relationship between lethality rate of *B. amyloliquefaciens* ZN and ARTP treatment time. The lethality rate is calculated as follows:

$$\text{Lethality rate } (\%) = (x\text{-}y)/x^{*}100$$

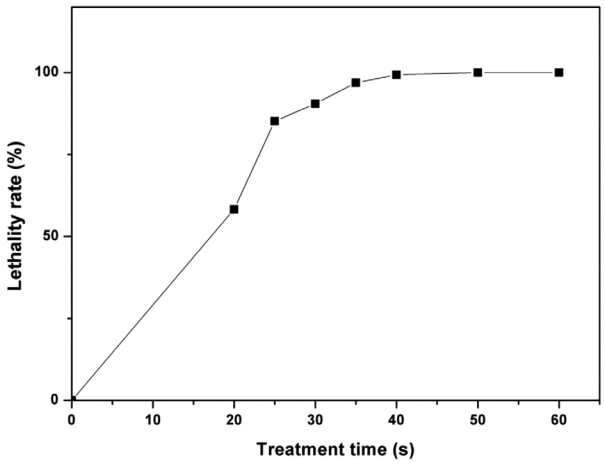

FIGURE 1. Relationship between ARTP treatment time and lethality rate of *B. amyloliquefaciens* ZN

In the formula, x is the average number of colonies on the plate after ARTP treatment, CFU; y is the number of colonies on the plate without ARTP, CFU.

The results showed that lethality rate increased with the increase of mutagenesis treat time (Figure 1). When the mutagenesis time was 25 s, the lethality rate was above 80%. When the mutagenesis time was 30 s, the kill rate was about 89.5%. The lethality was above 99%, when the treatment time reached 40 s. It has been accepted that the fatality rate between 70%-90% is beneficial for researchers to screen positive mutants (Zhang et al. 2012; Zhang & Pan 2009). In this experiment, the lethality rate can be set as 80%-90%, mutagenesis processing time was determined at 30 s.

SCREENING AND VERIFICATION OF THE MUTANTS

The mutant library was constructed by about 1000 bacterial mutants. The relative α-amylase activities (the α-amylase activities in *B. amyloliquefaciens* ZN was assumed as 100% relative α-amylase activity) of parts of mutants which were fermented in 24-well plates for 72 h were given in Figure 2. The No. 7 mutant strain which named *B. amyloliquefaciens* ZN mut-7# had the greatest increase in α-amylase activity, increased by 26.29% compared to the original strain in 24-well plates fermentation.

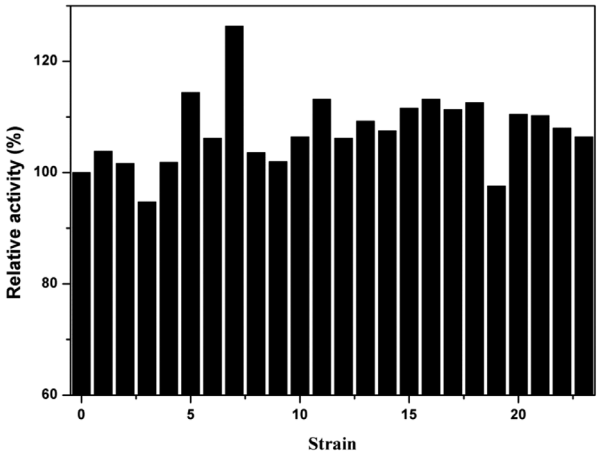

FIGURE 2. High throughput screening of mutants *B. amyloliquefaciens* ZN by using 24-well plates

In 250 mL shake-flask, the yield of BAA of *B. amyloliquefaciens* ZN mut-7# had the highest activity (385.05 U/mL), which was 86.92% higher than the original strain (Figure 3). It was found that the maximum cell growth rate and final biomass yield were nearly the same during amounts of *E. coli* DH5α cultures at similar $K_L a$ values under the same shaking conditions in shake flask and 24-well plates (Zhang et al. 2008). In this study, The BAA yield increase rate of *B. amyloliquefaciens* ZN mut-7# in 250 mL shake-flasks was greater than in 24-well plates. This is probably due to the difficulty of shaking the fermentation broth in a 24-well plates to achieve the

same dissolved oxygen as in shake flask experiments at 600 rpm. Although the α-amylase activity in 24-well plates fermentation was lower than that in flask fermentation, 24-well plates fermentation screening method provided reliable results of mutants relative enzyme activities. Thus, the 24-well plates high throughput screening method that can be used to screen the BBA producing strains was established. This method is suitable for high throughput screening for mutant libraries produced by ARTP mutagenesis. The integration of ARTP mutagenesis and high throughput screening strategy is an efficient way to obtain strains which have a high yield of the desired metabolic or enzyme, or have a high cell density in the culture system.

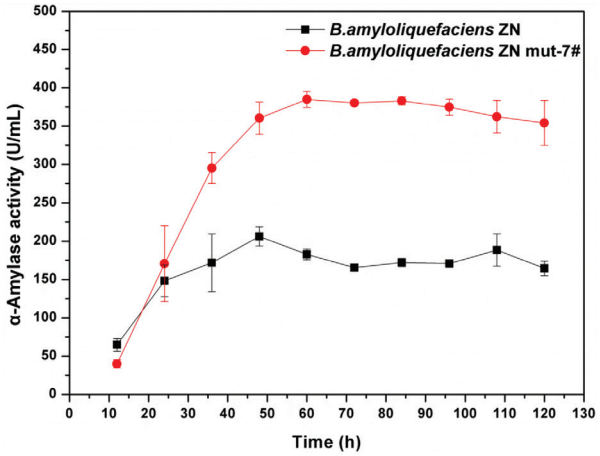

FIGURE 3. Comparison of *B. amyloliquefaciens* ZN mut-7# with the *B. amyloliquefaciens* ZN for α-amylase activity

Moreover, the sequence of BAA gene in the recombinant plasmid was examined and verified, which showed that the sequence of the amylase gene (GenBank accession nos. J01542.1) did not change. Figure 4 shows that both *B. amyloliquefaciens* ZN mut-7# and the original strain had one obvious electrophoresis band whose molecular weight were around 58 kDa. The band of *B. amyloliquefaciens* ZN mut-7# was strong, while the band of the original strain was weak at 58 kDa on the gel. This result demonstrated that the BAA was overexpressed in the mutant strain. Then, the genetic stability of *B. amyloliquefaciens* ZN mut-7# mutant was examined by subculturing for 10 generations in 250 mL shake-flask. The activity of BAA was kept in 380.16 ± 5.25 U/mL for 10 generations. It turned out that *B. amyloliquefaciens* ZN mut-7# has good genetic stability.

In 2010, Wang et al. first successfully implemented the helium ARTP mutagenesis system to obtain mutants of *Streptomyces avermitilis* with high productivity of avermectins. Subsequently, the ARTP has been successfully employed to improve or decrease the different properties of various bacteria, fungi and microalgae (Jiang et al. 2017; Liu et al. 2017; Tan et al. 2015), including the metabolite, biochemical and enzyme activities (Guo et al. 2011; Lu et al. 2011; Xu et al. 2012; Zong et al. 2012).

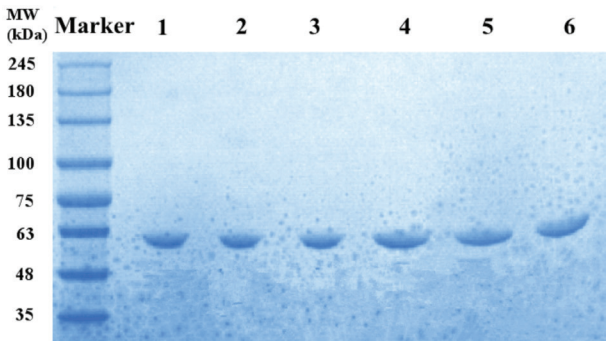

FIGURE 4. Analysis of molecular weight and yield of BAA in fermentation supernatant by SDS-PAGE. Marker: protein marker; MW: molecular weight; 1, 2, 3: supernatant of *B. amyloliquefaciens* ZN; 4, 5, 6: supernatant of *B. amyloliquefaciens* ZN mut-7#

In this study, ARTP was utilized to improve the yield of recombinant BAA in *B. amyloliquefaciens*. A large number of positive mutants were obtained from a mutant library containing approximately 1000 recombinant *B. amyloliquefaciens* ZN mutants by using 24-well plates high throughput screening. This experiment demonstrated that the integration of ARTP mutagenesis-high throughput screening strategy had the ability to rapidly and efficiently obtain positive mutants. Finally, the BAA yield in the best positive mutant strain (*B. amyloliquefaciens* ZN mut-7#) was 86.92% higher than that in the original strain. This result confirmed that through the ARTP mutagenesis-screening strategy, the mutant strain with the highest enzyme activity and genetic stability was successfully obtained. There is no change in BAA gene after ARTP mutagenesis, the mutant sites of *B. amyloliquefaciens* ZN mut-7# may be located on the genes involved in secretion of a heterologous protein. Therefore, ARTP can be utilized to increase the enzyme yield in strains that contain recombinant plasmids, and the desired enzyme property keep constant.

SINGLE FACTOR EXPERIMENT OF MEDIUM OPTIMIZATION

The influence of different carbon or nitrogen sources on α-amylase activity in *B. amyloliquefaciens* ZN mut-7# was compared in 250 mL shake-flask fermentation. Different carbon and nitrogen sources tested had different impact on the yield of BAA (Figure 5). When bran or soluble starch was used as carbon source, the yield of BAA in *B. amyloliquefaciens* ZN mut-7# reached a higher level (Figure 5a). When using beef extract as nitrogen source, *B. amyloliquefaciens* ZN mut-7# achieved the highest level of BBA. While using yeast extract, *B. amyloliquefaciens* ZN mut-7# achieved the second highest level of BBA (Figure 5b).

The results showed that the major factors influencing the BAA yield were bran, soluble starch, beef extract and yeast extract. These components were chosen for further optimization.

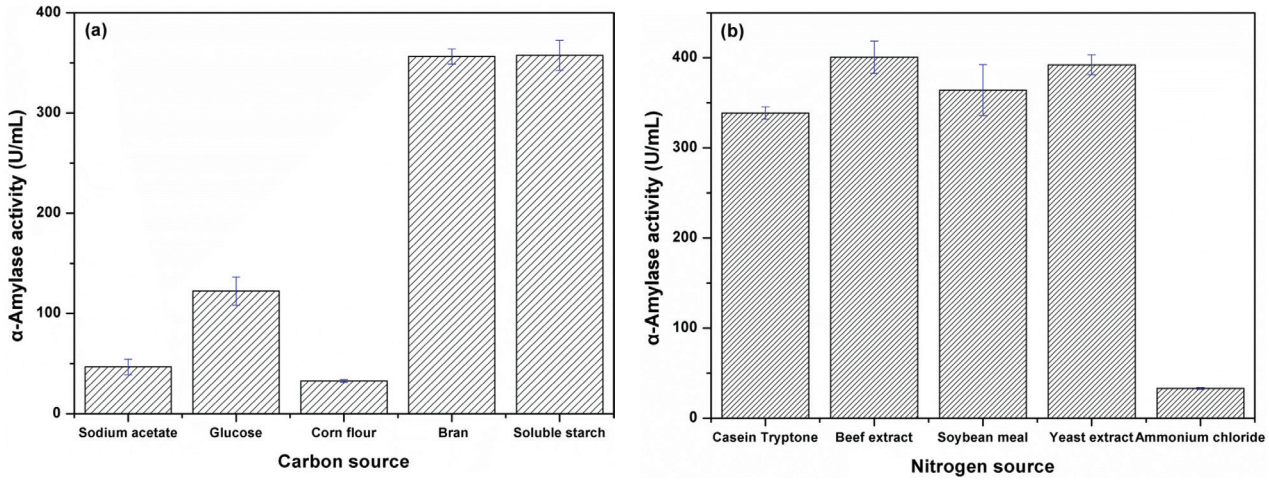

FIGURE 5. Comparison of different carbon (a) or nitrogen (b) sources in *B. amyloliquefaciens* ZN mut-7# shake flask fermentation for α-amylase activity

OPTIMIZATION BY PLACKETT-BURMAN DESIGN

The Plackett-Burman design designed six components significance of the fermentation medium for BAA production. Table 2 shows the effects of these components on the response and levels of significance. According to the statistical analysis, the influence of three components (bran, beef extract and yeast extract) had confidence levels above 95%, therefore, these were determined as important influential variables. Others (soluble starch, $CaCl_2$ and NaCl) had low confidence levels and were seen as insignificant factors. In the following experiment, the three factors were taken from the median value.

OPTIMIZATION BY THE PATH STEEPEST ASCENT EXPERIMENT

The effect of bran, beef extract and yeast extract was positive, for the value of effect in Plackett-Burman design was above 0. The origin of the path is considered as the center-point of Plackett-Burman design. The data of Table 3 indicated that the maximum yield of BAA was obtained when the media contained 21 g/L bran, 12 g/L beef extract and 7 g/L yeast extract. It showed that it may be close to the highest range of BAA yield.

TABLE 2. The analytic result of Plackett-Burman experiment design

Factors	Effect	Coef	SE Coef	*T*	*P*	Significance
Constant	36.84	311.26	4.18	74.42	0.00	
A	53.22	18.42	4.18	4.4	0.048	*
B	5.55	26.61	4.18	6.36	0.024	*
C	9.98	2.77	4.18	0.66	0.575	
D	37.45	4.99	4.18	1.19	0.355	
E	14.17	18.72	4.18	4.48	0.046	*
F	0.74	7.08	4.18	1.69	0.232	
G	3.94	0.37	4.18	0.09	0.938	
H	2.83	1.97	4.18	0.47	0.684	
I		1.42	4.18	0.34	0.767	
S = 14.4877			R-Sq = 97.70%		R-Sq (adj) = 87.36%	

TABLE 3. Design matrix and experimental result of steepest accent

Number	bran (g/L)	beef extract (g/L)	yeast extract (g/L)	activity (U/mL)
1	12	6	4	208.01
2	15	8	5	303.127
3	18	10	6	376.18
4	21	12	7	470.63
5	24	14	8	468.67

OPTIMIZATION BY BOX-BEHNKEN EXPERIMENTAL DESIGN

The data of Table 4 were analyzed by Minitab 17.0 software. The model terms are significant for having P-values less than 0.05. And the model is applicative to the production of BAA, for there was a perfect agreement between the predicted reactions and observations of the R-Sq value and R-Sq value more than 0.9 (Table 5). The adjusted coefficient of determination (R-Sq=95.86%) was sufficient to prove the significance of the model. The model can be shown as follows:

$$Y = 468.93 + 14.14\,X_1 - 21.04\,X_2 - 5.38\,X_3 + 10.15\,X_1^2 - 34.43\,X_2^2 - 10.78\,X_3^2 + 7.02\,X_1{*}X_2 + 6.19\,X_1{*}X_3 - 23.05\,X_2{*}X_3$$

In the equation, Y refers to the predicted production of BAA, X_1 is bran, X_2 is beef extract and X_3 is yeast extract. Furthermore, the analysis of model variance of response surface was presented in Table 6. The table showed that P value of Lack-of-Fit of this model is 0.23 (above 0.05). The result suggests that the model can predict within the factors used reliably, for the model has no significant Lack-of-Fit item. For the sake of indicating the effect of three factors on the yield of BAA, the 3D response surface analysis was conducted. Figure 6(a) shows the response for interactive effects of bran (X_1) and beef extract (X_2), when X_3 (yeast extract) code level was kept at 0. Maximum BAA yield was predicted to be 494.63 U/mL when code level of X_1 was +1 and X_2 was -0.21. Figure 6(b) shows the response for the interactive effects of bran and yeast extract when X_2 code level was 0. The yield of BAA was increased continuously as the increase of X_1, reached the highest level (493.22 U/mL), when X_3 code level was 0.03. Figure 6(c) shows maximum BAA yield was expected to be 472.24 U/mL, when the code level of X_3 was 0 and that of X_2 was -0.33 and X_3 was 0.11.

VALIDATION OF THE OPTIMIZED CONDITION

Base on medium optimization, the model predicted that the maximum code Y (yield of BAA) was 495.73 U/mL, when X_1 code level was 1 (24.00 g/L bran), X_2 code level was -0.33 (11.34 g/L beef extract) and X_3 code level was 0.39 (7.39 g/L yeast extract). And the average activity (three parallel experiments) of BAA was 494.94 ± 2.29 U/mL in

TABLE 4. Design matrix and experimental results of Box-Behnken design

Number	bran (g/L)	beef extract (g/L)	yeast extract (g/L)	activity (U/mL)
1	18	14	7	405.19
2	18	12	6	469.31
3	21	14	8	375.44
4	21	12	7	468.17
5	18	12	8	438.64
6	21	12	7	465.43
7	24	10	7	470.05
8	21	14	6	424.78
9	21	10	8	468.76
10	18	10	7	456.19
11	21	12	7	473.18
12	24	12	6	485.57
13	24	14	7	447.14
14	24	12	8	479.66
15	21	10	6	425.89

TABLE 5. Significance test of regression coefficient

Term	Coef	SE Coef	*T*	*P*	Significance
Constant	468.93	3.63	129.35	0	*
X_1	14.14	2.22	6.37	0.001	*
X_2	-21.04	2.22	-9.48	0	*
X_3	-5.38	2.22	-2.42	0.06	
$X_1{*}X_1$	10.15	3.27	3.11	0.027	*
$X_2{*}X_2$	-34.43	3.27	-10.54	0	*
$X_3{*}X_3$	-10.78	3.27	-3.3	0.022	*
$X_1{*}X_2$	7.02	3.14	2.24	0.076	
$X_1{*}X_3$	6.19	3.14	1.97	0.106	
$X_2{*}X_3$	-23.05	3.14	-7.34	0.001	*
S = 6.27922		R-Sq = 98.52%		R-Sq (adj) = 95.86%	

TABLE 6. ANOVA for the regression equation

Source	*df*	Adj SS	Adj MS	*F*	*P*
Regression	9	13139.5	1459.95	37.03	0
Linear	3	5372.5	1790.82	45.42	0
Quadratic	3	5291.1	1763.69	44.73	0
Interactions	3	2476	825.34	20.93	0.003
Residual error	5	197.1	39.43		
Lack of fit	3	166.3	55.42	3.59	0.226
Pure error	2	30.9	15.44		
Total	14	13336.7			

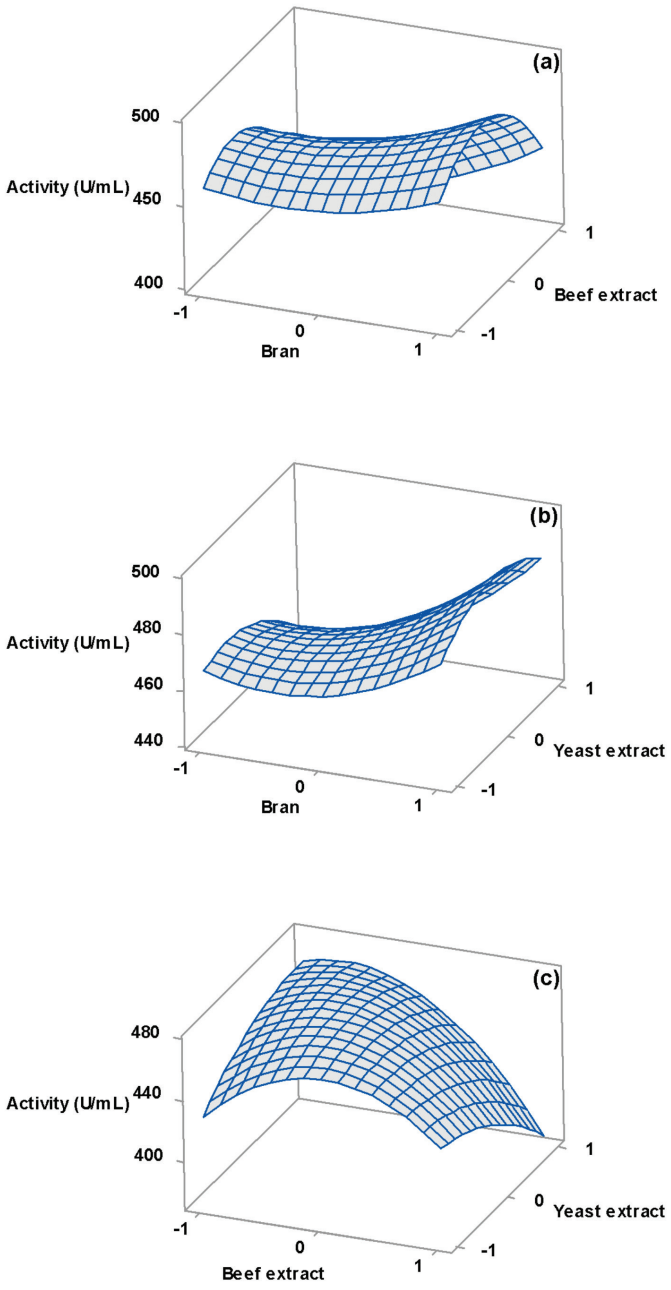

FIGURE 6. Response surface plot for the interaction effects of bran, beef extract and yeast extract on the activity of α-amylase production from *B. amyloliquefaciens* ZN mut-7#: (a) Response surface curve between bran and beef extract, (b) Response surface curve between bran and yeast extract, and (c) Response surface curve between beef extract and yeast extract

250 mL shake-flask under the optimized fermentation medium. The confirmatory experiment activity value of BAA yield was concordant with the predicted value (495.73 U/mL). The two similar values (494.94 and 495.73 U/mL) certified effectiveness of the model. Besides, the activity of BAA was 385.05 U/mL in non-optimized medium. Therefore, a 1.28-fold increase of BAA activity was reached after medium optimization by using response surface methodology.

A group of mathematical and statistical techniques were used to obtain the diversification of an adequate functional relationship between a response of factors in response surface methodology (Khuri 2006). To research interaction and select optimum conditions of variables for a desirable response, the response surface methodology contributed to build models and evaluate the effective factors compared to the ordinary methods (Puri et al. 2002). This study provided an efficient and feasible method for BAA fermentation medium optimization. The consequence resolved that the predicted value agreed with the experimental values well, and a 1.28-fold increase was obtained compared to the non-optimized medium. The results also give a basis for further study with larger scale fermentation for production of BAA.

Conclusion

B. amyloliquefaciens ZN mut-7#, whose BAA yield was 86.92% higher than that of *B. amyloliquefaciens* ZN, was obtained by using ARTP mutagenesis tool to construct a mutant library and 24-well plates screening method in this study. Furthermore, *B. amyloliquefaciens* ZN mut-7# exhibited good genetic stability. This work proved that ARTP can be utilized in the genetically engineering strain that contains recombinant plasmid to enhance its enzyme protein expression. The 24-well plates fermentation screening method provided a high throughput reliable approach to screen the mutant library. The strategy which combined ARTP and 24-well plates screening is rapid and effective for obtaining positive mutants.

For further improving the yield of BAA, the statistical experimental designs offer an efficient and feasible method for BAA medium optimization. The optimized fermentation medium was used including the following components: 1.25 g/L soluble starch, 24.00 g/L bran, 11.34 g/L beef extract and 7.39 g/L yeast extract, 1.25 g/L $CaCl_2$ and 1.50 g/L NaCl. In comparison to the production of non-optimized fermentation medium, it turned out that a 1.28-fold increase had been obtained in 250 mL shake-flask.

Using ARTP mutagenesis-screening and medium optimization strategy, the BAA production in *B. amyloliquefaciens* increased to 494.94 U/mL in 250 mL shake-flask. It provides a basis for studies to improve production of α-amylase in various strains.

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