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Full Length Research Paper

Mutation breeding of *Bacillus subtilis* YTB4 with high yield of multienzyme complex

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Helium-neon (He-Ne) laser irradiation is a highly efficient mutation breeding technology and is widely applied to various fields of biological science. Using *Bacillus subtilis* YTB4 with high yield of multienzyme complex as original strain, mutation breeding was carried out by He-Ne laser irradiation in this study. Based on the study of the strains' survival rate and positive mutation frequency, the appropriate irradiation dose was determined. The mutant YTB4A was selected through see-through circle way and enzyme activity determination. Compared with the original strain the activities of cellulase, protease and amylase increased by 265.14, 35.42 and 172.78%, respectively. In addition, the hereditary stability test showed that the activities of multienzyme complex were stable and that there was no reverse mutation after 25 generations.

Key words: *Bacillus subtilis*, multienzyme complex, He-Ne laser, mutation, hereditary stability.

INTRODUCTION

Mutation breeding is a technology that employs physical or chemical mutagen treatment to increase mutation rate of strains, and then applies simple, fast and efficient screening methods to select a small number of mutant lines; the major purposes of this technology are to increase the production of metabolites, to improve the product quality, to diversify breeds and to simplify the processing technique (Ma et al., 2011). As one of the physical methods, He-Ne laser irradiation is a highly efficient mutation breeding technology and has been widely applied to various fields of biological sciences (El Batanouny et al., 2002; Gulsoy et al., 2006; Perveen et al., 2011). It is also characterized by high energy density, small target, good directivity, and the occurrence of genetic mutations right after irradiation of the same generation (Jiang et al., 2006). Recently, the use of He-Ne laser irradiation technology to mutate the biological strains has been attracting considerable attention. Karu et al. (1994) determined the mechanisms of the effects of irradiation on *E. coli* with a He-Ne laser and the quantity of viable cells changed in the irradiated culture. Kohli et al. (2001) reported that irradiation with a He-Ne laser (632.8 nm) could stimulate *E. coli* strain KY706/pPL-1 and lead to the induction of *phr* gene expression; the optimal irradiation parameters were also suggested.

However, no effort has been made to apply He-Ne laser technology to screen microorganisms such as those involved in multienzyme complex activities improvement (Van et al., 2009). Despite being of excellent protease activity, bacteria of *Bacillus* are generally of poor cellulase and amylase activities. Furthermore, there are few reports about the strain of *Bacillus* of multienzyme complex. In this study, using *Bacillus subtilis* YTB4 with high yield of multienzyme complex as original strain, we reported our recent attempt to screen mutant strains of *B. subtilis* YTB4 with higher activities of the three enzymes and stable passage by using He-Ne laser irradiation technology.

MATERIALS AND METHODS

Microorganisms

B. subtilis YTB4, a newly isolated strain with high yield of multienzyme complex, was used in this study. It was identified and deposited in College of Agriculture, Henan University of Science and Technology, Luoyang, China.

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Media and growth conditions

Bacteria were preserved on nutrient agar medium (peptone 10 g / l; beef extract 3 g / l; NaCl 5 g / l; agar 18 g / l; pH 7.2 to 7.4) at 4°C. Cellulase screen medium (carboxymethyl cellulose sodium 10 g / l; peptone 10 g / l; yeast extract 5 g / l; glucose 2 g / l; agar 18 g / l; pH 7.0) was used to screen the target strains. Three different liquid media were used to determine enzyme activities. The media were cellulase fermentation medium (bran 20 g / l; corn starch 10 g / l; beef extract 3 g / l; peptone 2 g / l; $MgSO_4 \cdot 7H_2O$ 0.5 g / l; pH 7.0), protease fermentation medium (soybean meal 20 g / l; corn starch 10 g / l; peptone 2 g / l; glucose 5 g / l; $MgSO_4 \cdot 7H_2O$ 0.7 g / l; $CaCl_2 \cdot 2H_2O$ 0.3 g / l; pH 7.0), and amylase fermentation medium (bran 10 g / l; soybean meal 20 g / l; peptone 2 g / l; $MgSO_4 \cdot 7H_2O$ 0.5 g / l; pH 7.0) (Acharya et al., 2008; Sun et al., 2010).

Preparation of bacteria suspension

The strain was inoculated to nutrient agar liquid medium by the volume fraction of 1% and incubated with a shaker at 100 rpm at 30°C for 20 h. Then, 10 ml culture was centrifuged at 3000 rpm for 20 min, and the supernatant was discarded. The cell pellet was washed twice with sterile saline, and suspended in sterile saline with the concentration of about 10^6 CFU / ml for laser mutagenesis experiments.

He-Ne laser irradiation procedure

The irradiation condition was varied by adjusting output power (15 mW) and exposure time (5, 10, 15, 20, 25 and 30 min) (Kohli and Gupta, 2003). The laser output power was set to 15 mW and 0.2 ml of bacterial suspension was placed in sterile test tubes and located at 30 cm under the laser for different laser exposure time (Jiang et al., 2007). After mutagenesis, bacterial suspension with serial dilutions was prepared, and three 0.1 ml parallel samples were taken out from each dilution. 0.1 ml suspension was coated on both beef extract peptone agar and cellulose filter media, and the same operation without the laser irradiation was conducted as a control. All these were placed upside-down in the dark and cultured at 30°C for 20 h. After colonies emerged from the beef extract peptone medium, the numbers of colonies with irradiation and the control were counted, respectively and their survival rates were calculated. On the cellulase screening plate, selected strains with the transparent circle diameter ratio (that is, HC value) higher than the original value of starting strain were considered as positive mutations and inoculated onto the slant medium, and the mutation rate was calculated. Survival rate and positive mutation rate were calculated as:

$$\text{Survival rate} = \frac{\text{Number of colonies after mutagenesis}}{\text{Number of colonies of control group}} \times 100\% \quad (1)$$

$$\text{Positive mutation rate} = \frac{\text{Number of positive mutant strains}}{\text{Number of colonies after mutagenesis}} \times 100\% \quad (2)$$

Determination of mutant enzyme activity and stability analysis of genetic mutants

The positive mutant strains with higher HC values were selected from cellulase screening plate. Based on comprehensive analysis of various enzyme activities, the best positive mutant strain was obtained. Positive mutant strains had continuously passages on the slant medium for 25 times, and the strains were inoculated onto the fermentation medium for enzyme activity determination every 5 generations with three repetitions. The Statistical Package for Social Sciences (SPSS) v13.0 (SPSS. Inc., USA) was employed to analyze data and study the genetic stability of strains.

Determination of mutant enzyme activity

Strains were inoculated onto the beef extract peptone medium and cultured at 30°C for 22 h; then, they were inoculated with 5% inoculum onto three kinds of fermentation media and cultured with a shaker at 160 rpm at 30°C for 36 h. The fermentation broth was centrifuged, and the supernatant was measured to determine the corresponding enzyme activity. Cellulase and amylase activities were determined by means of 3, 5-dinitrosalicylic acid colorimetric method and protease activity by Folin-phenol method (Ponpium et al., 2000; Van et al., 2009).

RESULTS AND DISCUSSION

Effects of laser irradiation on strain survival and positive mutation rates

As indicated in Figure 1, *B. subtilis* YTB4 was irradiated by He-Ne Laser for 30 min, and the survival and positive mutation rates were measured, respectively.

The survival rates of strains increased with the decrease of irradiation time. The bacterial survival rate was 20% after 30 min irradiation. In contrast with the change in survival rates, strains showed irregular changes on the positive mutation rates (Jiang et al., 2007). The highest positive mutation rate appeared in the 15~20 min exposure time range, and strains' positive mutation rate is higher than 40%. Considering the effects of laser irradiation on bacterial survival and positive mutation rates, the output power was set to 15 mW and exposure time to 20 min. The mutagenesis was repeated three times, and HC positive mutant strains were screened and stored on the slant medium based on their sizes. According to HC values of transparent circle and the growth rate, ten strains were selected from positive mutant strains and their enzyme activities were determined. The numbers of specific strains are shown in Table 1 and the transparent circles of some mutants and original strain are demonstrated in Figure 2.

Determination of mutants' enzyme activities

As shown in Table 2, ten strains were inoculated onto the fermentation medium and cultured at 30°C for 36 h; then the activities of three enzymes were determined.

The enzymes of positive mutant strains exhibited iregular change of activity. The cellulase activity increased significantly, while the activities of protease and amylase just increased slightly and some decreased. All three enzymes' activities increased and reached a significant level only for YTB4A. Compared with the original strain the activities of cellulase, protease and amylase

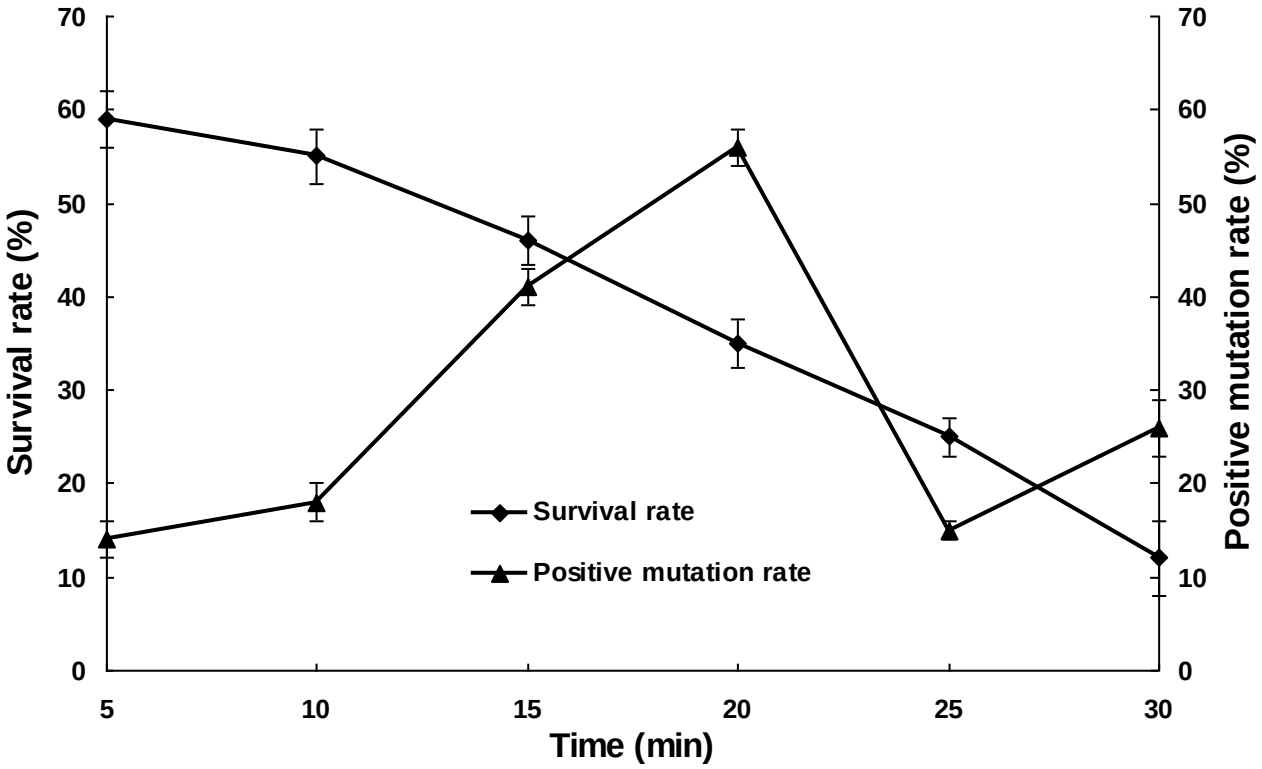

Figure 1. The influence of exposure time on the strains' survival rate and positive mutation frequency. Each experiment was repeated three times.

Table 1. The numbers and the HC value of each positive mutant strains.

Mutant strain	HC value	Mutant strain	HC value
YTB4	1.6 ± 0.08	YTB4E	2.0 ± 0.09**
YTB4A	2.3 ± 0.02**	YTB4F	1.9 ± 0.12*
YTB4B	1.7 ± 0.10*	YTB4G	1.8 ± 0.14*
YTB4C	1.9 ± 0.16*	YTB4H	2.0 ± 0.15**
YTB4D	2.1 ± 0.13**		

*, $P < 0.05$, significant difference level; **, $P < 0.01$, highly significant difference level. All experiments were done three replicates

increased by 265.14, 35.42 and 172.78%, respectively. Thus, YTB4A was selected for further analyses.

In this study, HC values of the other two enzymes were not examined, which may miss strains that only increased in protease or amylase activity. However, it may explain the phenomenon that cellulase activity increased significantly, while the increase of the other two enzymes' activities was not obvious. Further studies would be conducted to solve this issue.

Multienzyme complex was produced mainly by *Aspergillus* (Mamma et al., 2008). The enzyme activities of cellulase, protease and amylase from fungus have been significantly improved by mutagenesis, while few studies on multienzyme complex production with *B. subtilis* were conducted. Although enzyme activities are lower in YTB4A than in *Aspergillus*, the research is still prominent in the study of enzyme production with bacteria.

Stability analysis of genetic mutants

Positive mutant strain YTB4A had continuous passages on the slant medium, and the activities of three enzymes were determined at every five generations for genetic stability evaluation. The results are shown in Table 3. The ANOVA analysis indicated no significant difference between generations ($P = 0.850$), suggesting that the positive mutants has good genetic stability, and steadily transmit high enzyme activity to the next generation.

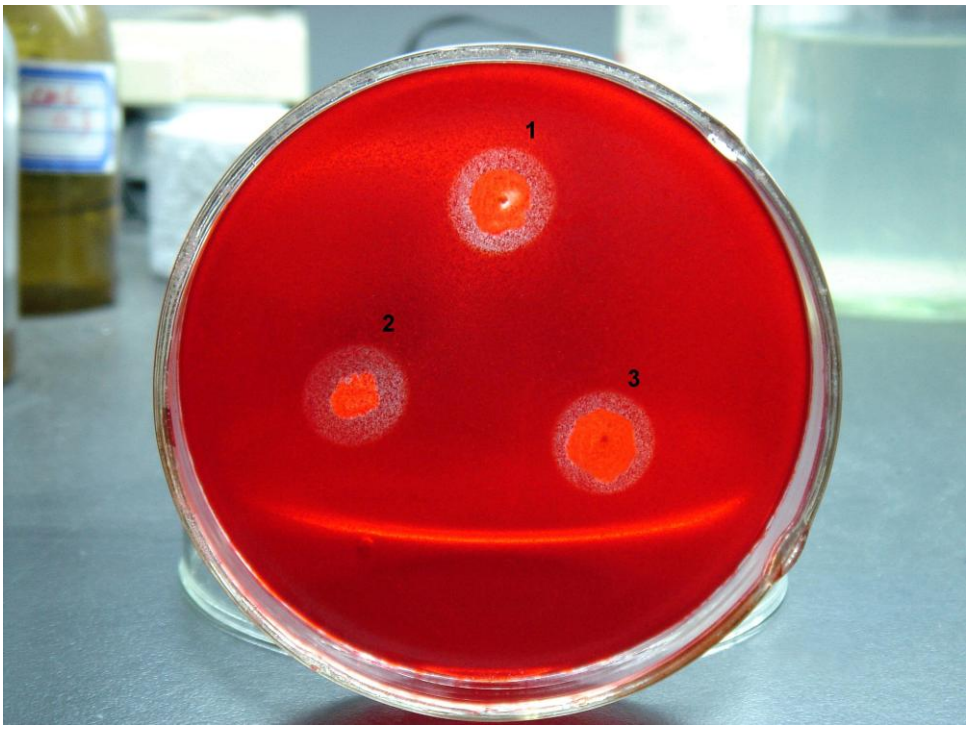

Figure 2. The transparent circles of some mutants and original strain in cellulase screen medium. 1. YTB4F; 2. YTB4A; 3. YTB4.

Table 2. The enzymes activities of each mutant strains.

Mutant strain	Enzyme activity (U / ml)		
	Cellulase activity	Protease activity	Amylase activity
YTB4	198.23 ± 6.33	432.85 ± 8.46	9.92 ± 1.91
YTB4A	723.82 ± 26.73**	586.18 ± 14.05**	27.06 ± 2.65**
YTB4B	250.12 ± 23.43	550.23 ± 10.50**	21.35 ± 2.08*
YTB4C	311.75 ± 13.05**	356.37 ± 5.77	32.43 ± 2.89**
YTB4D	403.53 ± 8.50**	626.59 ± 10.41**	3.90 ± 1.65
YTB4E	585.07 ± 16.07**	311.67 ± 2.89	18.27 ± 2.52
YTB4F	631.96 ± 11.02**	611.39 ± 19.70**	11.04 ± 1.22
YTB4G	452.38 ± 18.61**	476.09 ± 8.96*	21.55 ± 1.46**
YTB4H	600.87 ± 25.17**	500.74 ± 14.43*	7.87 ± 1.89

*, $P < 0.05$, significant difference level; **, $P < 0.01$, highly significant difference level. Data is analyzed statistically only on the strains that enzymes activities was improved compared to *B. subtilis* YTB4 and each experiment was repeated three times.

Laser irradiation on strain can result in mutation, which has been applied to breeding practices of micro-organisms and achieved great success. But only a few industrial practices have employed this technology. One main reason is the reverse mutation which finds expression in the degeneration of some important characteristics after several generations. In this study, the activities of multienzyme complex were stable and no reverse mutation occurred after 25 generations, sug-esting its potential practical utility in industrial production.

Conclusion

In this study, the best irradiation exposure time was set at 20 min by calculating the survival rate of strains and positive mutation rate after He-Ne laser irradiation. Ten positive mutant strains with greater HC values of cellulose transparent circle were selected for significance tests which indicated that YTB4A's enzyme activities all increased and reached a significant level. Compared with the original strain the activities of cellulase, protease and

Table 3. Analysis of the hereditary stability of strain YTB4A.

Generation	Enzyme activity (U / ml)		
	Cellulase activity	Protease activity	Amylase activity
1	723.82 ± 26.73	586.18 ± 14.05	27.06 ± 2.65
5	713.67 ± 9.63	590.23 ± 10.60	26.25 ± 3.02
10	742.05 ± 11.46	578.55 ± 12.69	27.77 ± 2.19
15	731.97 ± 12.35	581.64 ± 8.13	26.64 ± 1.85
20	698.78 ± 27.61	595.67 ± 11.06	27.27 ± 2.32
25	719.62 ± 10.98	590.82 ± 15.44	27.36 ± 2.73

All experiments were done three replicates.

amylase increased by 265.14, 35.42 and 172.78%, respectively. YTB4A has a good genetic stability without back mutation by 25 generation of genetic stability test, suggesting its practical utility.

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