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Strain mutagenesis and initial conditions of solid-state fermentation soybean meal using *Bacillus subtilis* (ACCC 01746)

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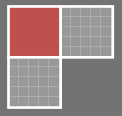
ABSTRACT

To enhance the protease activity effectively, during the solid-state fermentation (SSF) the *Bacillus subtilis* ACCC 01746 (*B. subtilis*) exhibiting protease secretion ability was irradiated by the 10-30 mW output power and 10-30 min exposure time under the He-Ne laser with a wavelength λ = 632.8 nm and laser beam 1.3 mm in diameter, respectively. The optimum irradiation dosages were obtained at the 20 mW output power and 20 min exposure time. Upon the optimum mutagenesis conditions, the maximum protease activity of a stable positive mutant strain *B. subtilis*-3 was obtained, and displayed maximum protease activity in the soybean protein-agar slant medium, 656.88 U/mL, compared with the parent strain, 467.43 U/mL. The SSF with *B. subtilis*-3 was studied to evaluate the effects of the optimal single-factor conditions on the activity of protease containing the temperature, time, moisture content, inoculum size and pH as initial fermentation conditions for the mutant. The optimum initial conditions of SSF soybean meal and the maximum protease activity respectively were shown at the range, where the temperature, time, moisture content, inoculum size and pH were 34°C, 43 h, 59%, 7% and 6.8, whereas the enzyme activities were 642.61, 658.94, 667.07, 683.73 and 693.45 U/mL for *B. subtilis*-3, respectively. The results suggest that the mutant *B. subtilis*-3 SSF soybean meal had beneficial effects on the production of protease.

KEYWORDS

Bacillus subtilis ACCC 01746; Laser irradiation; Protease; Solid-state fermentation; Soybean meal.



INTRODUCTION

The protease from *Bacillus subtilis* has been successfully applied for processing of soybean products containing soybean protein and soybean meal[7], and its enzyme production conditions have been better research[5,15,18]. However, there is a need to further improve the study methods to achieve a better result like mutation, along with culture conditions. Laser-induced breeding is a promising technology and has played an important role in increasing the production of microbial enzyme[4,16,20], and attracted more and more attention in recent years. In order to enhance the protease production efficiency, single-factor analysis and application of polynomial model are necessary. For example, Song et al. reported that the mutant *B. subtilis* QLB6 protease increased more than 38%, while the complex enzymes increased by 328% based on the results of single-factor analysis, using soybean meal as the fermentation medium. The method for mutating and breeding through laser possess obvious advantages, such as easy operation, safety in use, high energy density, good direction and genetic stability, etc compared to the used UV-light and chemical mutations[3,8].

The *B. subtilis* is most frequently used microorganism for solid-state fermentation (SSF) soybean meal due to its high proteolytic activity. On the other hand, utilizing the strain to hydrolysis soybean proteins through SSF is a more efficient method to increase nutritional quality of the products like high content of small-size bioactive peptide, low concentration of trypsin inhibitor and better palatability[7]. We also have previously reported the *B. subtilis* ACCC 01746 SSF soybean meal which secretes protease, but the yield of protease was relatively low[6]. Convert soybean proteins into superior peptide by *B. subtilis* and its enzymes is a good choice, and efficient protease-producing the microbe and perfected SSF method are needed to meet the amid rising marketable demand for its enzyme and fermented product. In the present study, the SSF of soybean meal was performed using laser mutagenesis *B. subtilis* (ACCC 01746) for the high-level production of protease and optimize the conditions of fermentation.

MATERIALS AND METHODS

Bacillus subtilis

B. sublitis (ACCC 01746) as a probiotics producing protease was used in our experiment, and was received as a present from Dr. Guan (College of Bioengineering, Henan University of Technology) and maintained in the cryogenic refrigerator at −80°C.

Mutagenesis and mutant selection

The strain was cultivated (at 30 °C and 150 rpm for 20 h) on seed medium having composition: 3.0 g beef extract, 10.0 g peptone, 5.0 g NaCl and 1.0 L water (pH7.0). The whole culture substrate was centrifuged at 3000 rpm and 4°C for 20 min, and the precipitates was collected, diluted and quantitated with sterile saline solution in sterilized test-tubes, and then the number of bacteria was determined (contain at least 1×10^8 cell/ml) and used for mutagenesis.

He-Ne laser (Guangzhou Institute of Laser Application Technology, Guangzhou, China) with a wavelength λ = 632.8 nm and a beam of diameter 1.3 mm was used as a mutagenic source. The test-tubes with a 0.2 ml suspension were exposed under the He-Ne laser 30 cm away at 30°C with a 10-30 mW varying the output power and the10-30 min exposure time. In order to further optimize the irradiation conditions for the positive mutation rate, the validation tests were performed by means of the varying rates of the output power to the exposure time (14/25, 16/23, 18/21, 20/20, 22/18 and 24/16 mW/h). The irradiation procedure was performed in triplicates as the more fortuitous mutagenic option. After each mutagenesis, the suspension was 10-fold serial dilution and 0.1mL of each dilution was plated on the soybean protein-agar medium and cultivated in dark at 30°C for 24 h. The medium having 10.0 g soybean protein, 5.0 g yeast powder, 10.0 g NaCl and 10.0 g agar in 1.0 L ultra-filtered water was prepared and then the pH was adjusted autoclaved with phosphate buffer solution before being used.

The best protease-producing *B. sublitis* was selected as positive mutant strain based on the clear hydrolysis zone surrounding the colonies, in which the ratio of the hydrolysis zone (Hz) to the diameter of the colonies (D) over 1.6. Next, the genetic stability of the selected mutants was tested for more than 30 generations by serial passage on the soybean protein-agar slant medium. The activity of protease was analyzed every five generation as the reliable indicator ($P>0.05$).

Inoculum preparation

The soybean meal used was obtained from the Zhengzhou Jinbaihe Biology Engineering CO., Ltd (Zhengzhou, China) and analysed for protein (44.5%, N×6.25) by Kjedhal method. It was ground using a FW100 grinder machine (Tianjin Taisite Instrument Co., Ltd., Tianjin, China) provided with a 0.2 mm screen. The 3000 g meal was mixed with mineral salts (g/kg: KH_2PO_4 1.0, Na_2HPO_4 2.0, $MnSO_4$ 0.005, $MgSO_4$ 0.1, $CuSO_4$ 0.002 and $FeSO_4$ 0.001) and autoclaved at 105°C, 1.02 atmospheres (105 kPa) for 30 min, and cooled to 30°C and stored for SSF use.

The selected mutant was inoculated in the medium in 1.5 L Erlenmeyer flask containing 10.0 g soybean protein, 5.0 g yeast powder and 10.0 g NaCl in 1.0 L ultra-filtered water at 30°C, 120 rpm for 20 h. The solution was centrifuged and then the cells were harvested, resuspended and adjusted to 10^8 CFU/mL, and used as a stock solution for SSF.

Single-factor experiments

The SSF was carried out in a 250 ml conical flask, which was sealed with sterile gauze that contained 50.0 g of soybean meal of mixed nutrient salt per bottle. The humidity and initial pH were adjusted to 52% and 7.0, respectively. The conditions of single-factor experiment, such as pH, temperature, inoculum size, moisture content and time were tested independently to select the optimization for protease production. The triplicates results for each single-factor were then averaged.

Survival and positive rate

Survival rate (%) = 100 × colony after the mutagenesis (CFU)/colony of control group (CFU); Positive rate (%) = 100 × colony of the mutagenesis (CFU)/colony of total (CFU).

Proteinase assays

The activity of the protease was determined using folin-phenol method according to GB/T23527-2009 of Chinese national standards. One unit of the enzyme activity (U) was defined as the amount of the enzyme which released 1μmol tyrosine per min under assay conditions.

Statistical analysis

The results of SSF are expressed as mean ± S.D. The data of single-factor experiment and SSF were determined at the $P<0.05$. A polynomial model on optimizing single-factor was fitted to explore the effects of the variables to the result, and selected as the optimum conditions of single-factor culture.

RESULTS AND DISCUSSION

The results of laser mutagenesis and selection

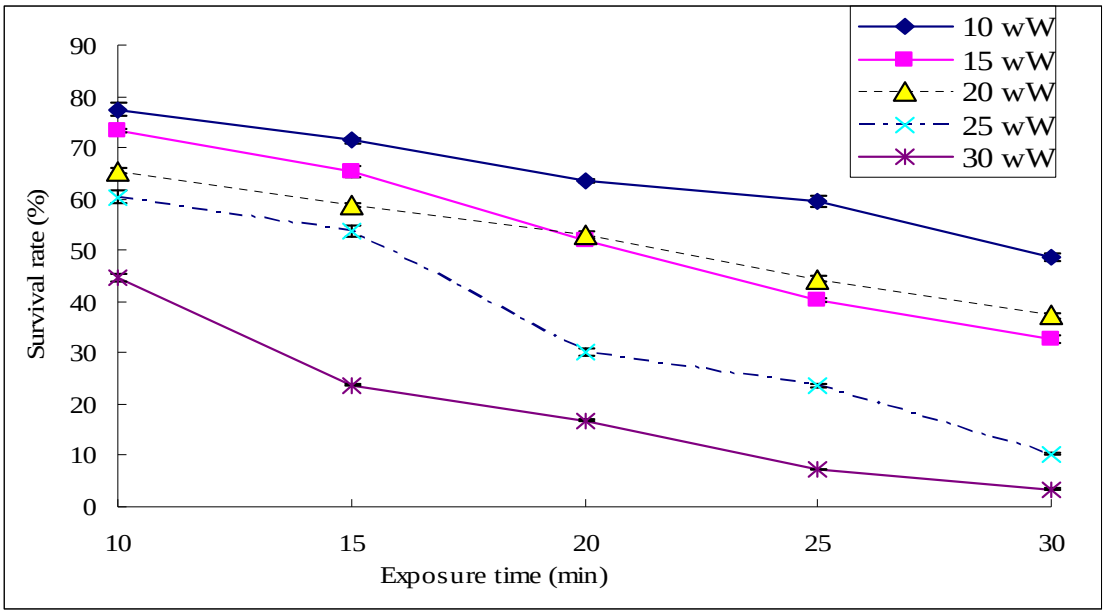


Figure 1 : The results of survival rate under different laser exposure time.

Figure 1 reveals the survival rate of the irradiated *B. subtilis* under different irradiation parameters. The percentage cell survival decreased with the increase in power output and/or exposure time. It was noted that the maximum cell survival rate presented at the output power of 10 mW, indicating that condition with shorter-term and lower-power had little impact on the *Bacillus*. At the same time, we have also taken note that low positive mutation rate could be found at the high survival rate (Figure 2). At 20 mW, the positive mutation rate increased gradually up to the optimum (50.32%) in 20 min, which was followed by a slight decrease, while the survival rate reached only 53.15%.

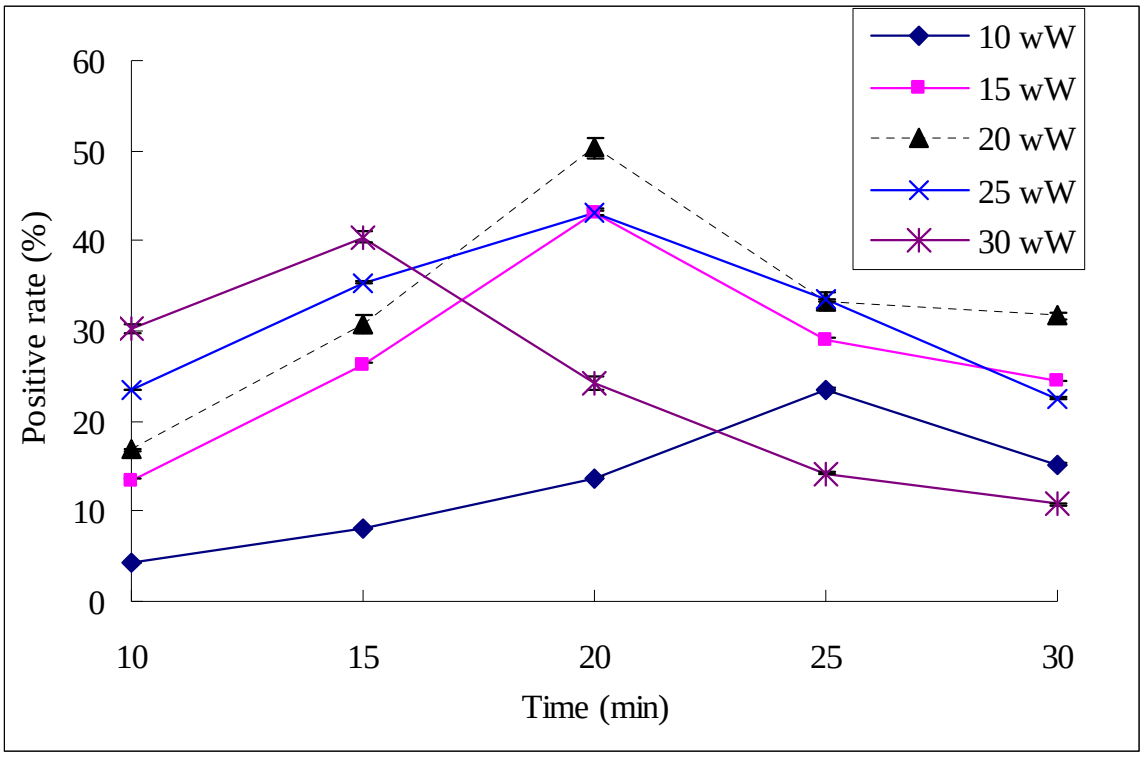


Figure 2 : The results of positive rate under different laser exposure time.

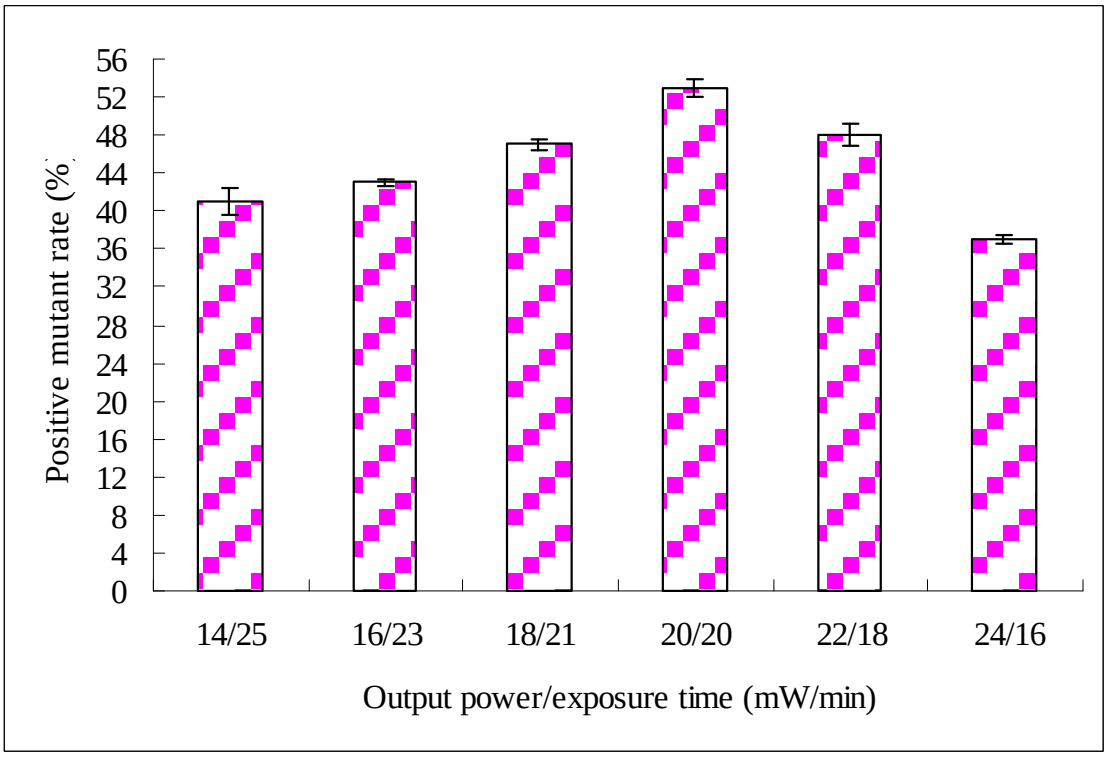


Figure 3 : Effects of the different the output powers (mW) to the exposure time (min) on the positive mutation rates.

Figure 3 showed the optimal irradiation conditions on positive mutation rate of the mutant strains, and the best result of 53.1% obtained at the rate of 20/20 mW/min. It was indicated that the optimum output power and the exposure time were 20 mW and 20 min under the laser beam with a 1.3 mm-diameter, respectively.

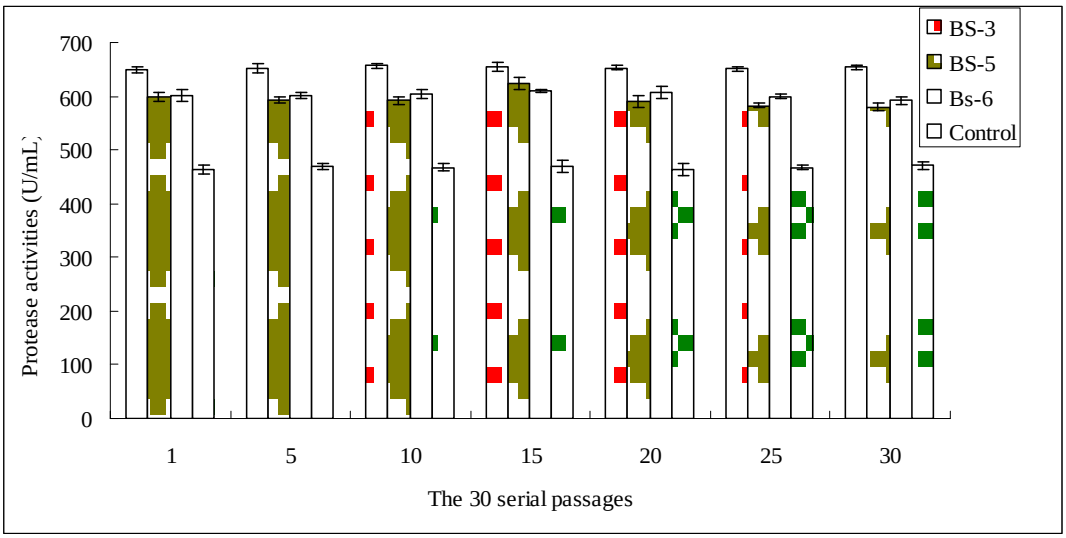


Figure 4 : The effects of the 30 serial passages on genetic stability of the mutant strain *B. subtilis* -3, *B. subtilis* -5, *B. subtilis* -6 and the parent strain.

The soybean protein-agar medium as the source of nutrition was used to screen for the Hz/D and protease activity of the mutants from the parent strain of *B. subtilis* (ACCC 01746). 10 mutants (TABLE 1) with the Hz/D over 1.6 of *B. subtilis* were selected, in which the highest protease activities were *B. subtilis*-3, 5, 6 (P<0.01). After being selected as the preferred mutants for protease yield, the results of genetic stability were showed in Figure 4. The positive mutant strain with the highest protease activity was *B. subtilis*-3, which the protease yield ability (649.23- 656.88 U/mL) was verified to be stable within 30 generations.

Table 1 : Initial and further screenings for mutants of the parent strain of *Bacillus subtilis* [a] (ACCC 01746).

Strain	Initial screening		Further screening		
	Hydrolysis zone (mm)	Rate	Protease activity (U/mL)	SEM	P=0.05
B. subtilis	4.4[c]		467.43	10.26	
B. subtilis-1	8.1	1.84	438.47	9.37	0.162
B. subtilis-2	7.4	1.68	501.44	11.22	0.052
B. subtilis-3	9.1	2.07	649.23	13.41	<0.01[b]
B. subtilis-4	7.3	1.66	526.46	7.98	0.068
B. subtilis-5	9.3	2.11	598.65	10.03	<0.01[b]
B. subtilis-6	8.8	2.0	601.42	13.21	<0.01[b]
B. subtilis-7	7.6	1.73	478.53	8.58	0.276
B. subtilis-8	8.9	2.02	554.71	9.06	0.002
B. subtilis-9	7.1	1.61	439.58	10.17	0.015
B. subtilis-10	8.4	1.91	579.44	12.63	0.001

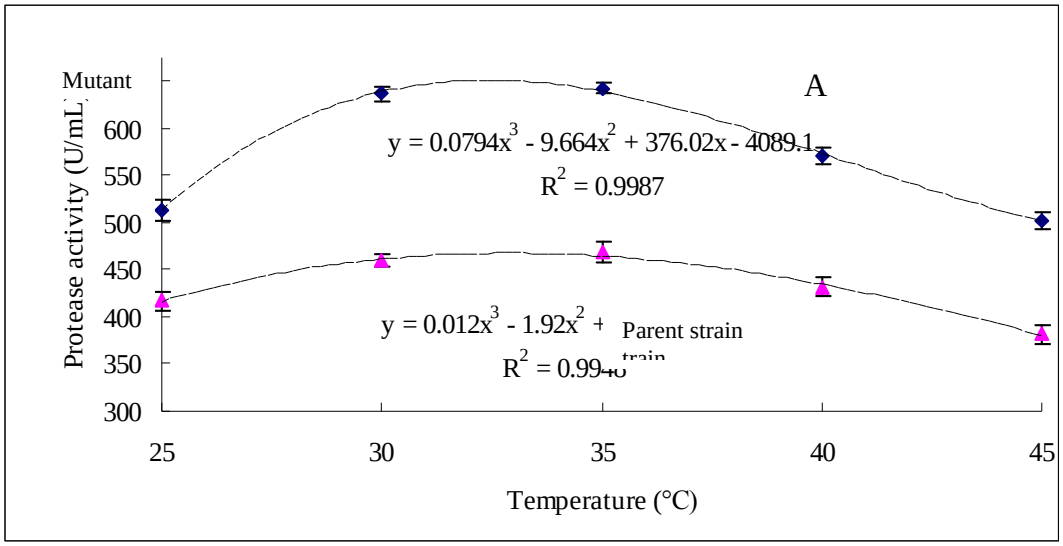
[a] Result are presented as the mean values of three deter; [b] Significant at 1% as a selected strain; [c] The diameter of the strain.

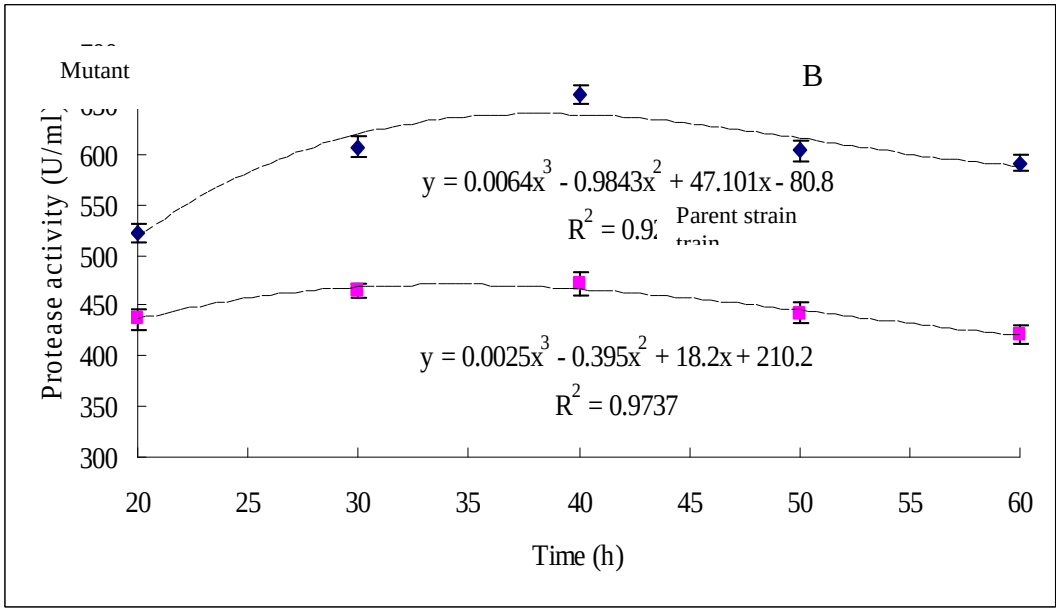
He-Ne laser irradiation was used as a useful tool to mutate[11], and an effective method to increase the enzyme activity and protein synthesis of microorganisms[23,24]. In the study, the highest protease activity of the mutant strain was 656.88 U/mL, which was 40.43% higher than that of the parent strain (467.43 U/mL). The results of laser mutation further confirm the formerly reported by Song et al. who found that the high activity of protease was showed on the radiation of *B. subtilis* with a He-Ne laser at 632.8 nm, output power 20 mW and exposure time 20 min. The radiation can be considered as inducible factor to mediate the cell reaction containing the rearrangements in the membrane lipid phase, modification of the enzyme system and formation of the second messengers[22], and the resulting generated more genes encoding enzymes to increase the protease activity for the mutant[10]. The extension/bandwidth of irradiation time/output power over the optimum

got low enzyme activity of the mutant in the tests. Higher mutational parameters alter the cellular structure and secretive function, and then obstruct protein synthesis and even death of mutant, as described by Zhang[25].

The results of optimizing single-factor culture

According to the polynomials model, the data provided by Figures 5 (A, B, C, D and E) indicate that the ideal single-factor SSF conditions were temperature 34°C (R^2=0.9987), time 43 h (R^2=0.9267), inoculums 7% (R^2=0.9848), moisture content 59% (R^2=0.9999) and pH 6.8 (R^2=0.9981), respectively. The best protease activity of the *B. subtilis*-3 was obviously higher than its parent strain. The conclusion is coupled with that reported more previously by Song et al. who found that the exposure of the mutant QLB6F to 15 mW of He-Ne laser radiation at λ = 632.8 nm for 20 min caused a 38% higher protease activity production, which is also similar to the results reported by Baysal et al[1]. Chutmanop et al[2]. reported that the medium composition can affect the production of enzymes, and higher amino acids produce higher enzyme production. The differences likely interpret the higher protease activity on soybean meal and slightly lower the activity on soy flour. Similarly, more researchers have observed a similar phenomenon for temperature, time, inoculums, moisture content and pH, different conditions of fermentation had different results[12,14]. The differences of the result are to be attributed to the different components of the culture medium and difference of inter-species[17]. However, *B. subtilis*, a member of probiotic species, is a most extensively used microorganism for SSF soybean meal. In order to enhance production efficiency of protease, the mutation screens of the *B. subtilis* of higher production enzyme ability is an ideal method using He-Ne Laser.





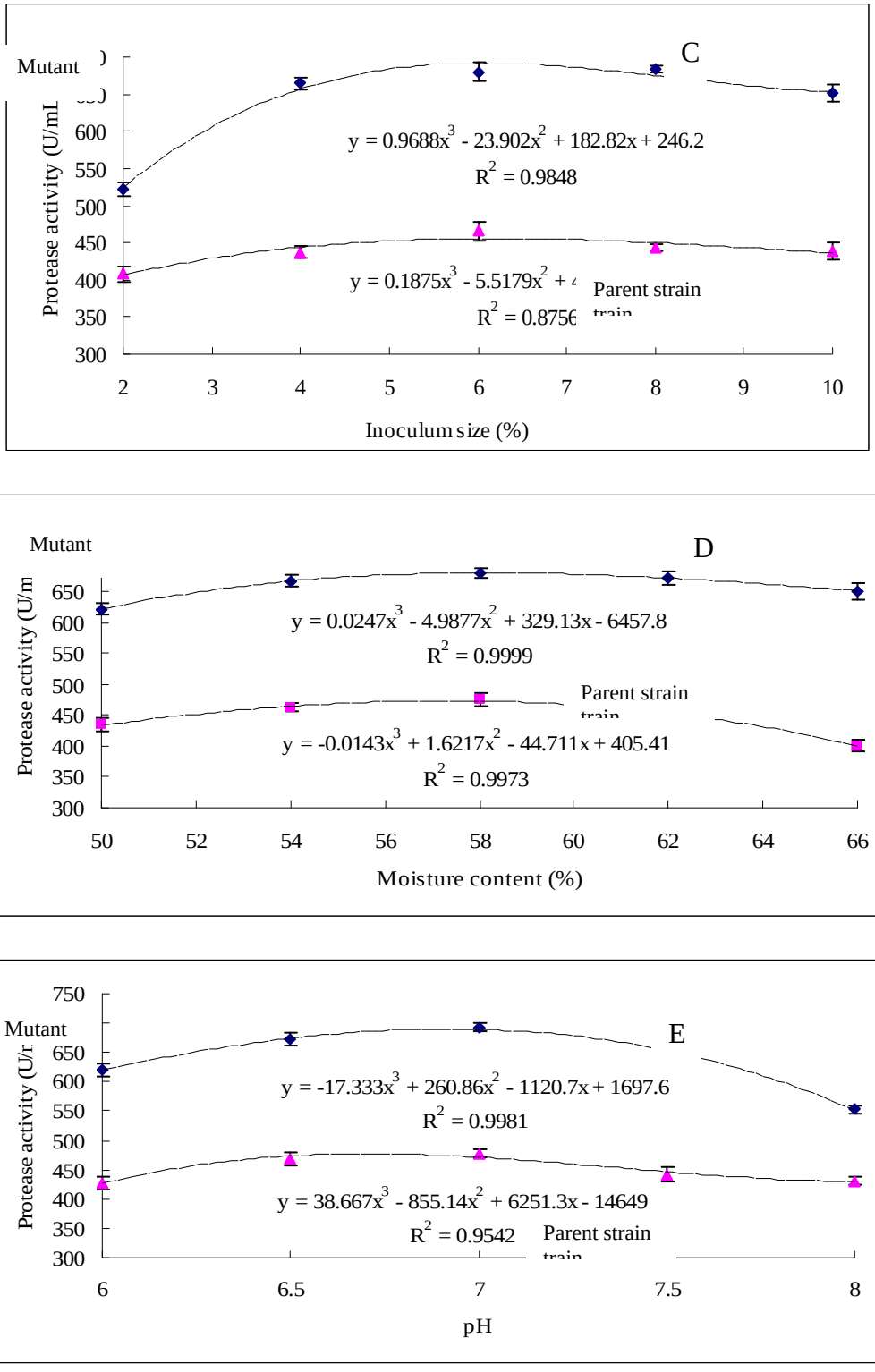


Figure 5 : Effects of temperature (A), time (B), inoculum size (C), moisture content (D) and pH (E) on the protease activity (U/mL).

CONCLUSION

The He-Ne laser irradiation can be successfully utilized to enhance the protease-producing ability of the strain *B. subtilis* ACCC 01746 and obtain the mutant *B. subtilis-3*. Single-factor experiments were applied to evaluate the activity of protease with the mutant and got the optimal culture conditions: pH 6.8, temperature 31.5°C, time 46 h, inoculums 7.5% and

relative humidity 52%, respectively. The *B. subtilis* -3 is more efficient for protease-producing as compared with its parent strain.

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