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Enhanced Production of Vitamin K_2 from *Bacillus subtilis (natto)* by Mutation and Optimization of the Fermentation Medium

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ABSTRACT

The aim of this study was to enhance the production of vitamin K_2 by using N-methyl-N-nitro-N-nitroso-guanidine (NTG) and low energy ion beam implantation and optimizing the fermentation medium. Mutation resulted in 1.66-fold higher production of vitamin K_2 than that of the parentl strain. The production by the mutant BN-P15-11-1 was increased 55% and reached 3.593±0.107 mg/L by using the Plackett-Burman and Box-Behnken designs to optimize the fermentation medium. The optimal fermentation culture medium was composed of (g/L) glycerol 69.6, sucrose 34.5, K_2HPO_4 4.0, peptone 20, yeast extract 25 and fermented at 37 °C and 150 rpm for 72 h. The results showed that the NTG and low energy ion beam implantation mutations and optimizing fermentation medium were effective methods to enhance vitamin K_2 production.

Key words: *Bacillus subtilis (natto)*, Mutant, Optimization, Vitamin K_2

INTRODUCTION

Vitamin K was discovered almost 80 years ago to be an anti-hemorrhagic factor, capable of correcting dietary-induced bleeding disorders in chickens (Dam 1935) and blood clotting deficiencies caused by biliary diseases in humans (Almquist 1941). Studies in recent years have shown that vitamin K is an essential cofactor not only in blood coagulation but also in bone metabolism (Kruger and Horrobin 1997; Schurgers et al. 2007; Gast et al. 2009). Vitamin K is a series of compounds and there are two types of vitamin K occurring naturally: vitamin K_1 (phylloquinone/phytomenadione) and vitamin K_2 (menaquinones). Both the forms of vitamin K have a common 2-methyl-1,4-napthoquinone nucleus but differ in the structure of a side chain (Vermeer and Schurgers 2000). Vitamin K_1 is mainly formed in the plants (Meurer et al. 1996) and vitamin K_2 is mainly produced by the microorganisms (Collins and Jones 1981; Suttie 1995). The menaquinones have variable side chain lengths of 4-13 isoprene units, referred to as MK-*n*, where *n* denotes the number of isoprenoid residues.

It has shown that MK-7 may play an important role in reducing the risk of bone fractures (Tsukamoto et al. 2000; Schurgers et al. 2007) and specific cardiovascular disorders (Cranenburg et al. 2008; Gast et al. 2009). MK-7 is produced by fermentation with *Bacillus* species and can be obtained from food products such as cheese, meat and fermented soybean, but the concentration is

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very low. Natto is a traditional fermented soybean food in Japan; it contains comparatively high amounts of MK-7 (about 800-900 μg/100 g natto) compared with other foods (Sakano et al. 1988). Since the strains of *B. subtilis* used for manufacturing natto are edible, they are among the best sources of MK in the food industry. To improve the production of MK-7, studies have focused on genetic mutation and the nutrients in the fermentation media. Many previous studies have been carried out largely on microorganism isolation and process development in order to enhance vitamin K_2 production (Sato et al. 2001; Tsukamoto et al. 2001; Berenjian et al. 2011; Mahanama et al. 2011). At present, there are many mutagenic sources, such as ultraviolet rays (Said et al. 2010), γ-rays (Fatnassi et al. 2011), laser irradiation (Liu et al. 2012) and the N^+ ion beam (Nie et al. 2012). N^+ ion beam implantation has been increasingly used in various fields, especially in industrial microbial mutagenesis and mutation breeding (Yu et al. 1991). This is due to the controllable damage rate, higher mutation rate, and wider spectrum of mutations obtained by N^+ ion beam implantation compared to traditional mutation methods (Feng et al. 2006). In recent years, many achievements have been realized by using N^+ ion beam implantation (Ge et al. 2004; Gu et al. 2006; Gong et al. 2009; Wang et al. 2011). However, there are as yet few reports on improving the vitamin K_2 production of *B. subtilis (natto)* by N^+ ion beam implantation.

In this study, NTG was used as a chemical method and low energy ion beam implantation as a physical one to obtain the mutants and optimized the fermentation medium to enhance vitamin K_2 production.

MATERIALS AND METHODS

Microorganism and culture conditions

The strain *B. subtilis (natto)*-2-6 was isolated from the laboratory and named as BN-2-6. The medium was prepared as follows. Solid medium (%): 0.3 beef extract, 1.0 peptone, 0.5 sodium chloride, 2.0 agar, pH 7.0 to 7.2. Seed medium (%): 1.0 glucose, 1.0 peptone, 0.5 sodium chloride, pH 7.2 to 7.4. Fermentation medium (%): 7.0 glycerol, 2.5 yeast extract, 1.5 peptone, 0.05 K_2HPO_4, and the pH was adjusted to 7.3.

Ion beam implantation device

Ion beams was obtained by accelerating nuclei (ions) at a high, near-light speed using the ion implantation facility (Fig. 1) at ASIPP (Chinese Academy of Sciences, Institute of Plasma Physics). In this machine, ions were produced by a plasma generator, electrostatically extracted and accelerated, focused, and finally transported to the target chamber where a special bio-sample holder was installed.

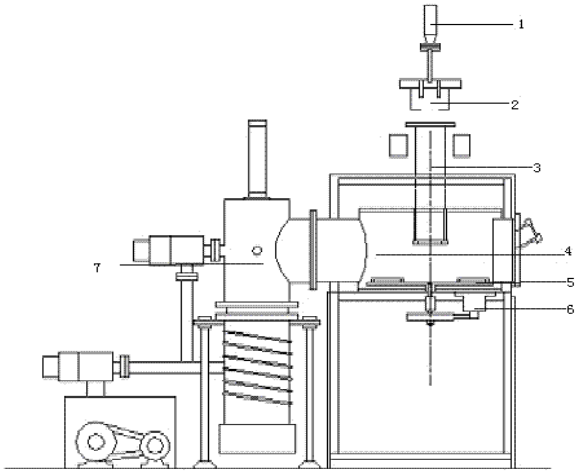

Figure 1 - Schematic of the ion beam implantation device. 1. Air supply 2. Plasma generator 3. Ion beam 4. Big target chamber 5. Rotatable scaffold 6. Small target chamber 7. several vacuum pump

Mutation and screening procedures

For the NTG mutation, strain BN-2-6 was first inoculated into fresh seed medium. The cells were harvested at the end phase of the exponential stage (15 h) by centrifuging at 6797 g for 10 min and the cells were suspended in physiological saline at a concentration of 10^8 cells/mL after washing. Cells were incubated in physiological saline containing NTG (200 μg/mL) at 37°C for 20 min. The cells were washed three times with fresh seed medium and shaken in seed medium for 3-4 h at 37°C. They were then spread on a plate containing the solid medium supplemented with 50 mg/L 1-hydroxy-2-naphthoic acid (HNA) (Sato et al. 2001; Tsukamoto et al. 2001).

In the low energy ion beam implantation procedure, the culture was prepared along similar lines as for the procedure described above. 100 μL cells suspension was spread over a sterilized plate and dried in an axenic workbench, then the plate was treated with low energy ion beam

implantation. The dose for implantation ranged from $0\times2.6\times10^{13}$ to $200\times2.6\times10^{13}$ ions/cm^2, and the energy was 0-20 Kev. At the same time, in order to evaluate the effects of vacuum on mutation, the mycelium of control group without N^+ beam implantation were also put into the target chamber. After irradiation, each treated plate was washed with 1.0 mL sterile physiological saline individually, diluted to an appropriate spore concentration, and spread on the plating medium according to the NTG procedure. Colonies that appeared during 2-4 days of incubation at 37°C were isolated. Colonies were chosen randomly for continued cultivation on a solid medium and they were inoculated into flasks to define the final vitamin K_2 production.

Analysis of vitamin K_2

The broth was centrifuged at 6797 g for 5 min after fermentation, and the resulting cells were mixed with 15 mL distilled water and stirred well. The mixed solution was ultrasonicated for 4 min in an ultrasonic cell disintegrator. Then 15 mL n-hexane were added to the solution, which was vigorously shaken for 2 min and then centrifuged at 6797 g for 5 min to separate the two phases. The organic phase was then separated and the absorbance was determined at a wavelength of 248 nm by UV spectrophotometer. The concentration of vitamin K_2 was derived by putting the optical density (OD) value into the equation of a standard curve of menaquinones. Each experiment was repeated three times.

Experimental design

To optimize the fermentation conditions of *B. subtilis (natto)*-P15-11-1, the conditions that had a significant effect on vitamin K_2 production were identified by Plackett-Burman (PB) design. In this design, eight components were selected for this study and the factors with a confidence level at or above 95% were selected to be optimized later. Then the Box-Behnken design was employed to optimize the selected effective factors chosen from the PB design, and the results were analyzed by response surface methodology (RSM).

RESULTS AND DISCUSSION

Mutation of *Bacillus subtilis (natto)*

In the mutation procedure, mutation efficiency less than 5.0% or higher than 5.0% was ruled as either negative mutation or positive mutation, respectively. Figure 2 shows the results of mutagenesis under different reaction times and NTG concentrations. The positive mutation rate for each set of NTG mutation conditions was calculated as the number of positive mutant strains divided by the number of colonies. It was observed that the cell survival rate decreased with increasing reaction time and NTG concentration. In addition, it was noted that a high survival rate could not be acquired simultaneously with a high positive mutation rate. Upon concentration with 200 mg/L NTG for 20 min, a 43.3% positive mutation rate was obtained, and the survival rate was 17.8%. Thus, the optimum NTG mutation parameters could be obtained when the NTG concentration was 200 mg/L and the reaction time was 20 min.

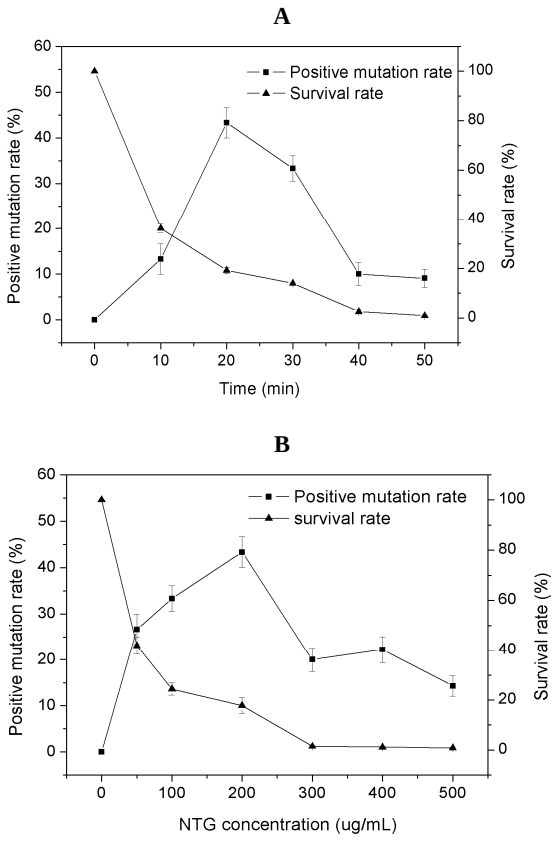

Figure 2 - The survival rate and positive mutation rate at different reaction times and NTG concentrations.

Figure 3 shows the results of mutagenesis under different doses and energies of N^+ implantation. According to statistical analysis, the cell survival rate decreased with increasing energies of N^+

implantation, and the maximum positive mutation rate was obtained when the energy of N^+ implantation was 15 keV (Fig. 3A). As for the doses of N^+ implantation, the survival curve exhibited a typical saddle shape as the dose of N^+ implantation increases, as shown in Figure 3B. The survival rate declined sharply to 11.1% when the dose of N^+ implantation ranged from 0 to 60 ($\times 2.6\times 10^{13}$ N^+/cm^2). After treatment with a dose of 80 ($\times 2.6\times 10^{13}$ N^+/cm^2), the survival rate increased to 23.3% and the maximum positive mutation rate of 40% was obtained; subsequently, the survival rate decreased with further increases in the dose of N^+ implantation. So, 15 keV and 80 ($\times 2.6\times 10^{13}$ N^+/cm^2) was selected as the optimum conditions for the energy and dose of N^+ implantation.

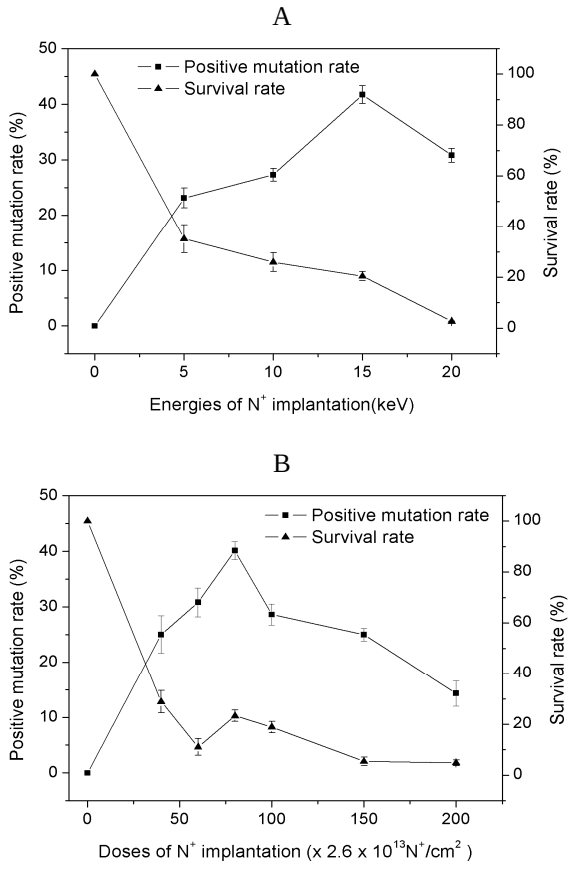

Figure 3 - The survival rate and positive mutation rate at different energies and doses of N+ implantation.

The saddle shape of the survival curve suggested that there were some obvious differences between this procedure and other traditional mutagen irradiations, such as UV and γ-rays. The following were regarded as the appropriate and important mutation effects of ion beam implantation, and this was confirmed by the results again. The biological effect of ion beam implantation may not only be induced by energy absorption, but also results from mass deposition and charge exchange. In the beginning, more ions reached the cellular cytoplasm; deposition of energy and mass in the cytoplasm might play an important role in breakage of the cytoskeleton and indirect induction of nucleolus damage, which could lead directly to the death of the cell. As the ion beam dose increases, further deposition of both energy and mass taking place in the nucleolus is likely to be the main factor that causes the cellular damage and activates the cell repair system. When the dose increases even further, the cells are subjected to serious damage that accumulates to an unrecoverable level that leads to a further decrease in the cell survival rate (Foti et al.1999; Wu et al. 1999; Yu et al. 2006).

Screening of high-production mutants

After the NTG mutation, a mutant strain named BN-N-30-1 was obtained and the production of vitamin K_2 increased by 53% over the original strain BN-2-6. Then the mutant strain was mutated further by using N^+ implantation. After a total of five N^+ implantation experiments and several generations of screening, one mutant strain named BN-P15-11-1 was obtained in which the production of vitamin K_2 increased by 74% over the strain BN-N-30-1 and by 166% over the original strain BN-2-6. These results suggested that NTG mutation and low energy ion beam implantation were effective methods for enhancing the production of vitamin K_2.

The genetic stability of mutant BN-P15-11-1 was detected. As shown in Figure 4, vitamin K_2 production remained relatively stable for five generation. Therefore, the mutant strain BN-P15-11-1 was selected for further studies.

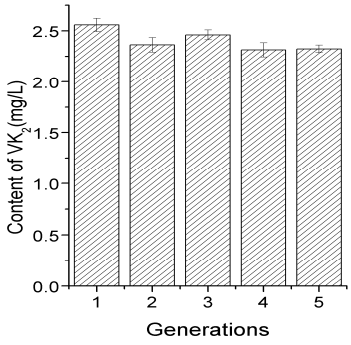

Figure 4 - Genetic stability of mutant BN-P15-11-1.

Optimization of the fermentation medium

Initial screening of the most important conditions affecting vitamin K_2 production by BN-P15-11-1 was performed by Plackett-Burman design. Table 1 represents the Plackett-Burman experimental design for 12 trials with two levels of values for each variable and the results of the design with respect to vitamin K_2 production. Table 2 represents the results of the Plackett-Burman experiment with respect to the effect, coefficient, *F*-value, *p*-value, and confidence level of each component. The components for which the confidence level was at or above 95% were screened.

Table 1 - Plackett-Burman design matrix with corresponding results.

Run	Variable											Production of vitamin
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	D_1	D_2	D_3	K_2(mg/L)
1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	2.556
2	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	2.62
3	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	1.582
4	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	2.25
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	2.664
6	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	1.696
7	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	2.232
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	2.434
9	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	2.034
10	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	2.844
11	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	3.23
12	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	3.118

X_1-X_8: variable; D_1-D_3: dummy variable; (+1): high level; (-1): low level.

Table 2 - Plackett-Burman regression analysis.

Term		Value		Effect	Coefficient	*F*-Value	P	Confidence level (%)
Variable	Component	Low (-)	High (+)					
X_1	Glucose (g/L)	30	50	0.12	0.06	1.30	0.337	66.3
X_2	Glycerol (g/L)	50	70	0.42	0.21	15.62	0.029	97.1
X_3	Sucrose (g/L)	50	70	-0.47	-0.23	19.64	0.021	97.9
X_4	Peptone (g/L)	15	20	0.30	0.15	8.26	0.064	93.6
X_5	Yeast Extract (g/L)	20	25	0.32	0.16	8.96	0.058	94.2
X_6	K_2HPO_4 (g/L)	0.5	1	-0.47	-0.23	19.58	0.021	97.9
X_7	NaCl (g/L)	0.5	1	-0.25	-0.12	5.50	0.101	89.9
X_8	$MgSO_4$ (g/L)	0.5	1	-0.21	-0.10	3.87	0.144	85.6

Glycerol, sucrose, and K_2HPO_4 were considered to be significant and optimized by using the Box-Behnken design. The experimental designs are given in Table 3. According to the Box-Behnken design matrix generated by Design-Expert software, a total of 17 experiments that included 12 factorial points and five replicates at the center point were performed as shown in Table 4, and the results were analyzed by ANOVA (Table 5). Results showed that the squared correlation coefficient R^2 and adjusted R^2 were 0.98 and 0.96, respectively, which indicated that the model could explain 98% variability in the response and showed the adequacy of the model to predict the response. Moreover, the value of the coefficient of variation (CV% =5.40) also indicated the precision and reliability of the model.

Predicted production of vitamin K_2 was calculated using the following second order polynomial equation:

$$Y = 3.48 - 0.2 \times X_1 - 0.19 \times X_2 - 0.36 \times X_3 - 0.15 \times X_1 \times X_2 + 0.53 \times X_1 \times X_3 - 0.16 \times X_2 \times X_3 - 0.85 \times X_1 \times X_1 - 0.54 \times X_2 \times X_2 - 0.47 \times X_3 \times X_3$$

Table 3 - Design of factors and levels.

	X_1 (K_2HPO_4)/(g/L)	X_2 (Glycerol)/(g/L)	X_3 (Sucrose)/(g/L)
-1	0.3	60	30
0	0.4	70	40
1	0.5	80	50

Table 4 - Arrangement and results of RSM.

	X_1	X_2	X_3	Y
1	-1	-1	0	2.280
2	1	-1	0	2.099
3	-1	1	0	2.369
4	1	1	0	1.589
5	-1	0	-1	3.113
6	1	0	-1	1.757
7	-1	0	1	1.507
8	1	0	1	2.255
9	0	-1	-1	3.018
10	0	1	-1	2.792
11	0	-1	1	2.478
12	0	1	1	1.599
13	0	0	0	3.396
14	0	0	0	3.488
15	0	0	0	3.549
16	0	0	0	3.524
17	0	0	0	3.437

Table 5 - ANOVA for Box-Behnken design.

Source	df	Sum of squares	Mean square	F-Value	P-Value(Prob>F)	Significance
X_1	1	0.31	0.31	15.55	0.0056	*
X_2	1	0.29	0.29	14.71	0.0064	*
X_3	1	1.01	1.01	50.98	0.0002	*
$X_1 \times X_1$	1	3.07	3.07	155.21	<0.0001	*
$X_1 \times X_2$	1	0.09	0.09	4.53	0.0708	
$X_1 \times X_3$	1	1.11	1.11	55.92	0.0001	*
$X_2 \times X_2$	1	1.23	1.23	62.13	0.0001	*
$X_2 \times X_3$	1	0.11	0.11	5.39	0.0533	
$X_3 \times X_3$	1	0.92	0.92	46.33	0.0003	*
Model	9	8.68	0.96	48.75	<0.0001	*
Pure Error	4	0.016	0.004			
Lack of Fit	3	0.12	0.041	10.46	0.0230	
Cor Total	16	8.82				
R^2	0.9843					
Adjusted R^2	0.9641					
CV. %	5.40					

*Significant at 99% confidence level.

According to the equation, the optimum levels for glycerol, sucrose, and K_2HPO_4 were determined to be 69.6, 34.5, and 0.4 g/L, respectively. Under these optimal conditions, the predicted value was 3.603 mg/L and the practical production of vitamin K_2 was 3.593±0.107 mg/L, which was in reasonable agreement with the predicted value. After optimization, the production of vitamin K_2 was 4.1 times that of the original strain BN-2-6 and 1.5 times that of the mutant strain BN-P-15-11-1. Figure 5 shows the results of a time-course study of cell growth and vitamin K_2 production of the strains BN-2-6 and BN-P-15-11-1.

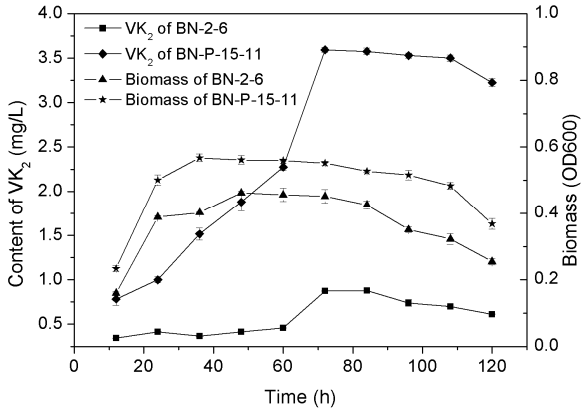

Figure 5 - The biomass concentration and vitamin K_2 production of the original and mutant strains at different cultivation times.

Fermentation was carried out in a 500 mL flask containing 50 mL fermentation medium on a shaker at about 150 rpm at 37°C. It was observed that the biomass and production of vitamin K_2 of the mutant were higher at the same fermentation time compared with the original strain. The production of vitamin K_2 increased sharply after the biomass reached a maximum, showing that the production of vitamin K_2 was partly growth associated.

CONCLUSIONS

The aim of this work was to enhance the production of vitamin K_2. The mutant BN-P15-11-1 was screened as a promising microbial producer and the production of vitamin K_2 was increased 166% over that of the original strain BN-2-6. In addition, the production of the mutant BN-P15-11-1 was increased by 55% using the Plackett-Burman and Box-Behnken designs to optimize the fermentation medium. The results showed that NTG and low energy ion beam implantation mutation technology and optimization of the fermentation medium were effective methods for enhancing vitamin K_2 production.

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