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Enhancement of NA of a Free Living Nitrogen-Fixing Bacteria by Combination of Dielectric Barrier Discharge Plasma Mutation and Optimization of Culture Conditions by Response Surface Methodology

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A free living nitrogen-fixing bacteria called SGD 07 was selected and identified as *Agrobacterium sp.* based on its 16S rRNA gene sequence analysis. Atmospheric Plasma — Dielectric Barrier Discharge Plasma (DBD) was used for mutation of SGD07 and a mutant called MU7-3 was obtained, whose NA increased by about 60% than its original strain SGD 07. Furthermore,the Response Surface Methodology (RSM) was adopted to optimize the culture medium of MU 7-3 and two significant effects on the NA were selected by Planckett-Burman Design. Through the analysis of the results of full factorial central composite design, a quadratic polynomial model for NA was established. The final optimal values of the four variables were as follows:glucose concentration (19.92 $g \cdot L^{-1}$), culture medium load (50ml), trace element ratio (0.014 $ml \cdot ml^{-1}$), buffer solution concentration (0.015 $mol \cdot L^{-1}$), the maximal NA in optimized culture medium could reach up to 0.63 $nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}$, increased by 57.5% than the NA of 0.40 $nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}$ in the original culture medium. In general, the NA of MU7-3 increased about 152% via DBD mutation and optimization of culture medium by RSM compared to SGD 07 .

Key words: Free living nitrogen bacteria, Nitrogenase activity, DBD mutation, RSM.

Nitrogen is an essential nutrient for plant growth (Allison *et al*. 1996), it's the main component of protein, nucleic acid and phospholipids, which are the most important element of protoplasm, nucleus and biofilm. When lack of nitrogen, plants are short and thin, old leaves turn to be yellow and fruits are easy to fall off , and thus resulting in low yield. Whereas in the past few decades, intensive agriculture entails the risk of excessive fertilization, the over fertilization of N leads to unhealthy food and pollution due to soil erosion and runoff water containing large amounts of N, the runoff from N-loaded soil is accepted as the main factor in eutrophication of natural water reservoirs, resulting in mass mortality of fish and shrimp. Microorganisms are important in agriculture for promoting the circulation of plant nutrients and reducing the need for chemical fertilizers as much as possible (Marschner *et al*. 2003). The inoculation of Nitrogen-fixing bacteria could enhance the yield of plant (Cakmakci *et al*. 2003) and Nitrogen-fixing bacteria had been considered as one of the possible alternatives for chemical nitrogen fertilizer (Ladha and Reddy, 2000). Recently, interest in the beneficial nitrogen fixing growth promoting rhizobacterial-plant association has increased due to their potential use as biofertilizers (Vessey, 2003). A significant reduction in the use of nitrogen-fertilizer could be achieved if biological nitrogen

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fixation is made available to crop plants (Dawe, 2000). Moreover, these organisms have been used to reduce plant stress associated with phytoremediation strategies for metal contaminated soils (Abdul G.K. 2005). In order to be inoculated to more plants roots, free living nitrogen-fixing bacteria were screening as PGPR (Lucy *et al.* 2004). *Agrobacterium* sp. was reported to have NA (Kanvinde and sastry. 1990) and could promote growth of pine and beech *et al.* (C. Leyval and J. Berthelin, 2003).

However, most of the wild free living nitrogen-fixing bacteria have low NA and ability for promoting growth of plants. So researchers turned their attentions to enhance the NA of strains. At present, there are two main methods for enhancement of NA of strains including gene level and optimization of culture conditions of strains(Holguin and Bashan, 1996). Gene level included construction of genetically engineered bacteria and various mutations such as physical mutation (typically UV mutagenesis) and chemical mutation . Atmospheric Plasma as a new method of mutation was increasingly applied to microbial mutation. Compared to other mutations, atmospheric plasma mutation had advantages as follows: low gas temperature , without any requirements of vacuum systems, high positive frequency of mutation, genetic stability of mutation, fast, safe, nontoxic, pollution-free. Atmospheric Plasma could easily form various mutants with high mutation frequency and high genetic stability, and could be a powerful tool for generating mutant library (Jiang *et al*., 2008). Construction of genetically engineered bacteria was also a good way except restrictions on its usage. *Dielectric Barrier Discharge Plasma* (DBD) is one kind of atmospheric plasma which had been used in Klebsiella pneumonia's mutation (Dong *et al*., 2008).

In this study, both the Atmospheric Plasma — Dielectric Barrier Discharge Plasma (DBD) and optimization of culture conditions were used for improvement of NA of the isolated free living nitrogen-fixing bacteria. The culture conditions of strains were optimized by *Response Surface Methodology* (RSM) which had been used in optimization culture medium (Sharma *et al*., 2009; Mu *et al*., 2009). The objectives of this paper were (i) to isolate and identify free living nitrogen-fixing wild type bacteria with high NA from the rhizosphere of plants;(ii) to use the DBD mutation to obtain mutants with higher NA ; (iii) to apply RSM to optimize the culture medium for the mutants to enhance the NA of strains and provide some theoretical guidance for the free living nitrogen-fixing bacteria used as micro biofertilizer to promote plant growth.

MATERIALS AND METHODS

Sampling and screening of strains

Soil samples of rhizosphere were collected from five distinct plants in Northern Shaanxi, China. The plants were as follows: *Mandala, Alfalfa, Kohlrabi Blue, Artemisia and Amorpha fruticosa*, respectively.

Plants were uprooted along with good amount of non-rhizosphere soil, brought immediately to the laboratory in polythene bags, then saved in refrigerator at 4°C.The non-rhizosphere soil was moved by gentle shaking, ten grams non-rhizosphere soil from each plant was aseptically weighed and transferred to an Erlenmeyer flask with 100ml sterile water, and was shaken for 10min with Vortex mixer. After shaken, a series of 10-fold dilutions of the suspension was made for each sample by pipetting 1 ml soil suspension into 9ml sterile water, and the final dilution was 10^5-fold. 0.2 ml of each dilution of series was placed onto a culture dish with N-free medium(NFM) comprising: 10 g glucose, buffer solution 0.006 mol·L^{-1}, 0.2 g $CaCl_2$, 0.1 g $MgSO_4 \cdot 7H_2O$, trace elements ratio 0.005 ml·ml^{-1} trace elements ratio were defined as trace elements volume divided by culture medium volume trace elements solution consisted of 0.1g $FeSO_4 \cdot 7H_2O$, 0.05 g $Na_2MoO_4 \cdot 2H_2O$ and 1000ml Distilled water. The pH of the medium was adjusted to 7±0.1 before autoclaving at 121°C for 15min. Three replicate plates were made for each dilution, and culture plates were placed in incubator at 28°C for 7 days. Isolates were selected according to differences in colony morphology including: colony form, elevation and pigment production, isolates had consecutive culture on NFM and were checked for purity (Park *et al*., 2005; Cakmakci *et al*., 2006).

NA determination

NA of isolates was determined in NFM by the acetylene reduction assay (ARA) (Hardy *et*

al., 1968). Pure cultures of all isolates were inoculated in 250ml Erlenmeyer flask with 100ml nitrogen free medium and were grown to the mid exponential phase at 28°C for 48h, The vials containing 40ml of the same medium were inoculated with 4ml aliquots of the above and incubated at 30°C till exponential phase. In following incubation, vials were sealed with rubber stoppers and 10% headspace was replaced with C_2H_2 by gas tight syringe. All the vials were again incubated at 28°C for 24h , the ethylene production was measured by Agilent gas chromatograph (Model 7890A, USA) fitted with a 2m by 1/8 mm packed column and hydrogen flame ionization detector (FID). The GC conditions were as follows: the temperature of inlet, detector and column were 200°C, 280°C and 180°C respectively; the flow rate of Carrier gas A r was 30ml/min. After completion of the ARA, the bacterial counts were measured by plate bacterial colony counting assay

Atmospheric plasma mutation

Through the determination of nitrogenase by ARA , two strains of high NA were selected. The two original strains were cultivated in Luria-Bertani (LB) liquid culture medium on a shaker at 28°C and a speed of 120 rpm for 12h, then culture solution was adjusted to about 10^5 cfu·ml^{-1}, 100μL aliquot of the above culture solution was spread on culture plate containing NFM , then the culture plate was placed into discharge chamber of DBD apparatus for a given time. The operational parameters were as follows: voltage is 30 V; electric current was 0.5A. A cell lethality percentage of 90% was set as the criterion for mutant generation. Based on those mentioned above, the treatment time was determined to be 3min.

Response Surface Optimization of Medium

One mutant of SGD 07 named as MU 7-3 was selected after determination of nitrogenase. The RSM was used to optimize the culture medium for MU 7-3. The response value was the NA, the independent variables were the factors that influenced the NA significantly, which were glucose concentration, culture medium load ,trace elements ratio and phosphate buffer concentration. NA were detected by ARA.

Planckett-Burman Design

The four factors glucose concentration (x_1), culture medium load (x_2), trace element volume(x_3), phosphate buffer concentration (x_4) were investigated. All experiments were performed in triplicate protocols. And the *Plackett-Burman* statistical design was employed using Design-expert 7.1.3 software to determine the significant factors.

Steepest Ascent Design

The significant factors (Glucose concentration, trace elements ratio) which influence the NA obviously were determined according to the results of Planckett-Burman experiments. The non-significant factors were kept at corresponding optimized levels based on the single factor experiments (culture medium load was 50ml, phosphate buffer concentration was 0.015 mol·L^{-1}). The orientation and step change of ascent were deduced from the sign and the size of f-value, respectively.

Full Factorial Central Composite Design

The optimal region that determined by steepest ascent experiments was applied to full factorial central composite design. A 2^2 factorial center composite design with four axis points (α=1.414) and six central points leading to a total 14 experiments were employed to optimize the NA. The variables are the significant factors that determined in the *Planckett-Burman* design. The code conversion Eq. is shown as below:

$$x_i = (X_i - m_i) / l \quad ...(1)$$

Where m_i is the optimal value of the significant factors that determined by steepest ascent; x_i is code value; X_i is conversion value; l is step change.

The second degree polynomial Eq. is

$$Y_i = \alpha_0 + \sum \alpha_i x_i + \sum \alpha_{ii} x_i^2 + \sum \alpha_{ij} x_i y_j \quad ...(2)$$

Where Y_i is the predicted response of human-like collagen concentration, x_i and y_j are the variables; α_0 is the offset term; α_i is the ith linear coefficient; α_{ii} is the ith quadratic coefficient; and α_{ij} is the ijth interaction coefficient. The Full Factorial Central Composite Design design and results were also conducted with Experiment Design Expert software.

Verification of NA

According to the results of RSM, optimal contents of the culture media were obtained. The experimental group was carried out in the optimized conditions. The control group was done under the

non-optimized conditions.

RESULTS AND DISCUSSION

Screening of nitrogen-fixing strains

Twenty five bacteria were isolated from the rhizosphere soil of the six kinds of plants on basis of colony morphology on nitrogen free medium. Their NA was determined by acetylene reduction assay. Eleven strains with NA were detected, the data were shown in Table 1.

According to the data shown in table 1, two nitrogen fixing bacteria SGD06 and SGD07 with higher NA from rhizosphere soil of Amorpha fruticosa were selected, their NA were (0.32 ± 0.05) $nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}$ and (0.25 ± 0.06) $nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}$, respectively.

Mutation Results—selection of Mutants

Isolates SGD06 and SGD07 with higher NA were chosen and treated with DBD mutation. Six mutants were selected . The NA of mutant MU6-8 was higher than its wild bacteria SGD 06 and the NA of mutants MU7-3 and MU7-4 was higher than their wild bacteria SGD 07, respectively. MU7-3 was chosen for the following RSM experiments because of its highest NA than any other strains. The results of their NA were shown in Table 2.

Results of RSM

Screening Experiments

According to the results of regression analysis of the *Planckett-Burman* design, glucose concentration and trace elements ratio showed significant effects on NA. The fitting Eq. of the first degree polynomial was shown as follows :

$$Y = 3.84 + 0.32X_1 + 0.33X_3 \quad ...(3)$$

R^2 value of the fitting analysis was 95.53% The "Lack of Fit F-value" of 2.20 implies the model was fitting. In order to obtain the maximal NA, these two significant factors were further optimized to approach the optimal region in RSM so as to establish an efficient Eq. The effect of X_1 and X_3 to the NA were both positive according to the *Planckett-Burman* design. The step change of glucose concentration and trace elements ratio was 2 and 0.002 , respectively.

In the light of the path of steepest ascent experiment, glucose concentration of 20 $g \cdot L^{-1}$ and trace elements ratio of 0.014 $ml \cdot ml^{-1}$ were selected for the extended optimization using a full factorial central composite design.

Full Factorial Central Composite Design

Based on the results of *Planckett-Burman* and Steepest Ascent design, the values of glucose concentration and trace elements ratio were further optimized using a full factorial central composite design. Experimental design and results were shown in Table 3. Design and results of full factorial central composite experiment

With Design-Expert software, a quadratic polynomial model for NA was obtained by multiple regression analysis of the results of full factorial central composite experiments (Table 4).

Table 1. NA of isolates from plants rhizosphere soil in Northern Shaanxi

Strain No.	Source(Rhizosphere soil of plants)	NA ($nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}$)
SGD 01	Artemisia	0.08 ± 0.02
SGD 02	Alfalfa	0.12 ± 0.03
SGD 03	Amorpha fruticosa	0.09 ± 0.02
SGD 04	Amorpha fruticosa	0.10 ± 0.03
SGD 05	Amorpha fruticosa	0.06 ± 0.01
SGD 06	Amorpha fruticosa	0.32 ± 0.10
SGD 07	Amorpha fruticosa	0.25 ± 0.05
SGD08	Amorpha fruticosa	0.15 ± 0.05
SGD 09	Amorpha fruticosa	0.13 ± 0.03
SGD 10	Mandala	0.09 ± 0.03
SGD 11	Mandala	0.08 ± 0.02

Table 2. NA of mutants of SGD 06 and SGD 07 and their mutants

Strain No.	Source	NA ($nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}$)
MU 6-2	Mutants of SGD 06	0.30 ± 0.03
MU 6-8	Mutants of SGD 06	0.35 ± 0.06
MU 6-10	Mutants of SGD 06	0.20 ± 0.02
MU 7-1	Mutants of SGD 07	0.27 ± 0.05
MU 7-3	Mutants of SGD 07	0.40 ± 0.10
MU 7-4	Mutants of SGD 07	0.33 ± 0.06
SGD 06	Amorpha fruticosa Rhizosphere soil	0.32 ± 0.05
SGD 07	Amorpha fruticosa Rhizosphere soil	0.25 ± 0.06

Table 3. Design and results of full factorial central composite experiment

Run	Glucose concentration ($g \cdot L^{-1}$)		Trace elements ratio ($ml \cdot ml^{-1}$)		nitrogenase activity ($nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}) \times 10^{-1}$
	Code x_1	X_1	Code x_3	X_3	
1	1	22.00	1	0.016	5.58 ± 1.36
2	0	20.00	0	0.014	6.18 ± 1.55
3	1.41	22.83	0	0.014	5.70 ± 1.39
4	0	20.00	0	0.014	5.95 ± 1.47
5	0	20.00	0	0.014	5.97 ± 1.47
6	-1	18.00	-1	0.012	5.62 ± 1.33
7	0	20.00	0	0.014	5.95 ± 1.45
8	0	20.00	1.41	0.017	5.40 ± 1.30
9	0	20.00	0	0.014	5.95 ± 1.50
10	0	20.00	-1.41	0.011	5.22 ± 1.22
11	1	22.00	-1	0.012	5.51 ± 1.37
12	-1.41	17.17	0	0.014	5.65 ± 1.36
13	-1	18.00	1	0.016	5.63 ± 1.39
14	0	20.00	0	0.014	5.97 ± 1.48

Table 4. Regression analysis of the full factorial central composite experiment

Term	Coefficient	Coefficient standard error	*F* value	*p*-value Prob>F
Intercept	6.001	0.039	19.587	0.0003
X_1	-0.012	0.033	0.122	0.7364
X_3	0.041	0.033	1.511	0.2539
$X_1 * X_3$	0.014	0.047	0.093	0.7683
$X_1 * X_1$	-0.139	0.035	15.895	0.004
$X_3 * X_3$	-0.322	0.035	85.413	< 0.0001
Lack of Fit			1.112	0.4266

$$Y = 6.001 - 0.012X_1 + 0.041X_2 + 0.014X_1X_2 - 0.139X_1^2 - 0.322X_2^2 \quad ...(4)$$

Y represents NA $\times 10^{-1}$ $nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}$. R^2 was 92.45%; it meant that the model could explain up to 92.45% of the total variation in response. According to the deducing partial derivative results, the maximal NA of 6.0×10^{-1} $nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}$ could be realized when x_1 was -0.04 and x_3 was 0 . According to Eq. (4), the conversion values of glucose concentration and trace element ratio were 19.92 $nmol \cdot 10^7 cfu^{-1} \cdot h^{-1}$ and 0.014 $ml \cdot ml^{-1}$, respectively.

According to Figure 1, glucose concentration and trace elements ratio had significant effect on NA. It was advantageous for the NA when the glucose concentration was 19.92 $g \cdot L^{-1}$, and trace element ratio was 0.014 $ml \cdot ml^{-1}$.

Verification

After optimization, the maximal NA could reach up to 6.3×10^{-1} $g \cdot L^{-1}$ which increased by 50% in comparison to the blank group 4.0×10^{-1} $g \cdot L^{-1}$.

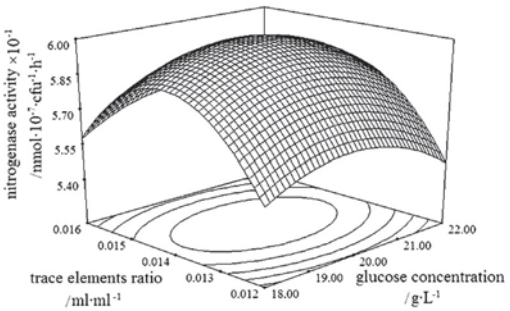

Fig. 1. Response surface plot and contour plot for NA

According to Eq.(4), the predict value of NA under the optimal condition was 6.0×10^{-1} g·L^{-1} with 5% tolerance to the actual NA value, which proved that the regression equation approached.

CONCLUSIONS

In this study, the wild-type strain Agrobacterium sp. SGD 07 was selected through screening of nitrogen-fixing strains by its highest NA whose mutant called MU 7-3 was obtained through DBD mutation, and the NA increased by 60% compared to SGD 07. Furthermore, the optimal medium for NA by RSM was examined by full factorial central composite and verification experiments. The final optimized culture medium components was as follows: Glucose concentration (19.92 g·L^{-1}), culture medium load (50ml), trace element volume(0.014 ml·ml^{-1}), buffer solution concentration (0.015 mol·L^{-1}). The maximal NA could be up to 0.63 nmol·10^{7}cfu^{-1}·h^{-1}, increased by 57.5% in comparison to the original 0.40 nmol·10^{7}cfu^{-1}·h^{-1}.

Under the optimal conditions, the NA could reach up to 0.63 nmol·10^{7}cfu^{-1}·h^{-1}, increased by 57.5% in comparison to the original 0.40 nmol·10^{7}cfu^{-1}·h^{-1}. The RSM could establish a model that could be used to optimize the culture medium efficiently According to the verification experiment. The culture medium optimization of MU 7-3 could provide some guidelines when MU 7-3 was used for producing micro biofertilizer.

The mechanism of DBD mutation for enhancing NA might be related to more access of trace element because of permeability increase of cell membranes after DBD mutation (Hua *et al*., 2009) or nitrogen-fixing gene change caused by DBD mutation (Korachi *et al*., 2010), further study is underway to investigate the underline mechanisms.

It was reported that the glucose concentration had effect on the population of nitrogen-fixing bacteria and their nitrogen-fixing ability (Keeling *et al*. 1998). In many culture medium formula, glucose concentration was 10 g·L^{-1}, but biology nitrogen-fixing was an energy-consuming process, because the amount of glucose was used to produce ATP which was critical to disintegration of the bond of N≡N. Whereas, glucose concentration higher than a particular value would lead to high osmotic pressure which would affect growth of bacteria and even caused bacteria death, so a suitable glucose concentration was important. Trace element volume was also a significant factor, bacteria which could fix nitrogen or not was decided by nitrogenase , most kinds of nitrogenase consisted of Mo-Fe protein or Fe-Fe protein , and the trace element Mo and Fe were active center of the two kinds of proteins. NA would be affected when lack of Mo and Fe, but excessive Mo and Fe would do harm to bacteria, for Mo and Fe were heavy metals.

The mutant MU 7-3 obtained in this study which had high NA might be used as micro biofertilizer for promoting growth of crops and vegetables, micro biofertilizer produced by nitrogen-fixing bacteria MU 7-3 could promote growth of oilseed rape significantly. It was reported by Yu Caiyun etc. (Yu *et al*, 2011) in natural science edition of Journal of northwest university.

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