Statistical Optimization of Medium Compositions for Alkaline Protease Production by Newly Isolated *Bacillus amyloliquefaciens*

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The nutritional medium requirement for alkaline protease production was optimized by newly isolated *Bacillus amyloliquefaciens* B7. The variables significantly influencing the protease activity were screened through one-factor-at-a-time experiments and Plackett–Burman design, by which fructose and yeast extract were identified as the most significant variables. The steepest ascent method was employed to approach the experimental design space, followed by an application of central composite design and response surface methodology for further optimization of the protease production. The protease production was found to increase from 242.45 U mL^{-1} to 951.49 U mL^{-1}, approx. a 3.92-fold increase compared with that using the original medium. Validation experiments were also carried out to verify the adequacy and accuracy of the model, and the results showed that the predicted value agreed well with the experimental values. 3120.55 U mL^{-1} of the maximum enzyme activity was obtained by batch cultivations in a 10 L bioreactor.

Key words:
Alkaline protease, *Bacillus amyloliquefaciens*, fermentation optimization, medium composition, response surface methodology

Introduction

As one of the most important commercial enzymes, proteases account for nearly 60 % of the total sales of industrial enzymes,[1] and enzymes were expected to increase at the rate of 7.6 % every year to about $ 6 billion by 2011 in the world market.[2] Proteases are used in food processing, detergents, dairy industry and leather making.[3] Furthermore, in recent years they have attracted a great deal of attention in pharmaceuticals and medical diagnosis, as well as in organic synthesis.[4,5] Protease production is an inherent property of all organisms, but only those that produce high-yield extracellular protease have been exploited commercially.[6] A large proportion of commercially available proteases are currently derived from *Bacillus* strains due to their high pH and temperature stability.[7]

Process optimization is important in industrial production processes, in which even small improvements can be decisive for commercial success.[8] Extracellular protease production in microorganisms is greatly influenced by media compositions, for example, variation in carbon[9] and nitrogen sources,[10] as well as presence of metal ions.[11] Besides this, physical factors such as dissolved oxygen, temperature, pH and incubation time also affect the yield of protease production.[12] Media compositions have great influence on the extracellular protease production and are differ for each microorganism,[13] about 30–40 % of the production costs is estimated to account for the cost of the growth medium.[14] Therefore, the required constituents and their concentrations have to be optimized accordingly.

This study attempted to optimize medium compositions for protease production by *Bacillus amyloliquefaciens* B7, which is a newly isolated strain in our laboratory. Effective and statistical optimization steps were carried out as follows: (1) screening medium compositions that affect protease production significantly using one-factor-at-a-time experiments and Plackett–Burman design; (2) accessing the optimal region of the significant variables with the steepest ascent method; (3) optimizing these significant variables by response surface methodology; (4) validating the model developed under optimized conditions.

Materials and methods

Microorganism

Bacillus amyloliquefaciens B7 producing alkaline protease was isolated from soil. It was identified on the basis of the biochemical properties and 1420 bp with 16S rDNA analysis

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(BankIt1478671 JN657265). This microorganism was cultivated at 37 °C in a bacteriological incubator for 24 h and subsequently maintained at 4 °C, while the maintained agar slants contained: 5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 3 g L^{-1} NaCl and 18 g L^{-1} agar.

Inoculum preparation

The strain was transferred from a slant culture into an Erlenmeyer flask (250 mL) containing 50 mL seed medium, which was the same as the above mentioned slant medium without agar, and initial pH = 7.5. The seed cultures were grown at 37 °C on a rotary shaker incubator at 200 rpm for 20 h.

Flask culture and batch cultivations in bioreactor

Inoculum (4 %, v/v) was transferred into an Erlenmeyer flask (250 mL) containing 50 mL of fermentation medium in which the composition was varied based on the experimental designs, and initial pH = 7.5. The fermentation cultures in the shake flask were carried out at 30 °C under shaking speed of 200 rpm for 24 h.

The bioreactor cultivation was carried out in a 10 L bioreactor (Baoxing Bio-Engineering Equipment, China) containing 6 L working volume of optimized medium. 300 mL of seed culture was used as inoculum. The medium was supplemented with 1 mL L^{-1} of antifoam to avoid excessive foaming. The temperature was controlled automatically at 30 °C. Filter-sterilized air at 3 L min^{-1} was purged into the culture for aeration, and the concentration of dissolved oxygen was kept above 10 % by increasing stirrer speed.

Determination of protease activity and cell density

Protease activity was determined by applying the modified Anson's method,[15] and using casein as substrate. One milliliter of the suitably diluted enzyme was mixed with 0.5 mL of 100 mmol L^{-1} phosphate buffer (pH = 7.8) containing 10 g L^{-1} casein, and incubated at 40 °C for 10 min. The reaction was stopped by adding 2 mL trichloroacetic acid (0.4 mol L^{-1}). The mixture was allowed to stand at 40 °C for 20 minutes and then centrifuged at 5,000×g for 10 minutes to remove the precipitate. The soluble fraction was added 5 mL Na_2CO_3 (0.4 mol L^{-1}) and l mL Folin reagent, and then estimated at 660 nm after standing at 40 °C for 20 minutes. A standard curve was generated using the solutions of 0–80 μg mL^{-1} tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used.

Growth of the microorganism was determined by optical density measurement at 600 nm (OD_{600}). The optical density was also converted to dry cell mass (DCM) using a standard curve. One OD_{600} unit corresponded to 0.29 g L^{-1}.

Experimental design and data analysis

Plackett–Burman design

The variables that significantly influence the protease production were screened using a fractional factorial Plackett–Burman (PB) design.[16] The carbon and nitrogen sources as well as metal salts, which had been screened earlier, were added to the culture medium for optimization. Eight independent medium compositions were evaluated at two levels (high and low) and designated as level + 1 and level –1 respectively (Table 1). The significant variables were screened in 12 combinations in accordance with the design matrix (Table 2), and the responses were measured according to the protease activity. For the selection of these factors, Design Expert 7.1.6 (Stat-Ease, Inc., Minneapolis, USA) was used to generate and analyze the experimental design of Plackett–Burman.

Table 1 – *Minimum and maximum ranges for the parameters selected in Plackett–Burman design*

Variables	Factors	Concentration (g L^{-1})	
		–1 level	1 level
A	Fructose	10	20
B	Yeast extraction	4	8
C	Peptone	2	4
D	$FeSO_4$	0.25	0.5
E	$MgSO_4$	0.4	0.8
F	Urea	1	2
G	$Na_2HPO_4 \cdot 12H_2O$	7.2	14.4
H	K_2HPO_4	1.81	3.62

Path of steepest ascent method

The significant variables screened with the Plackett–Burman design experiments were roughly optimized using steepest ascent method to determine the center points of variables for the next optimization step. The direction of steepest ascent was parallel to the normal contour line of the model response curve and passed through the center point of PB design experiment. Experiments were performed along the steepest ascent path until the re-

sponse no longer increased. This point would be near optimal point and could be used as center point to optimize.[17]

Central composite design and response surface methodology

Central composite design and response surface methodology was employed to optimize the two most significant factors, i.e. fructose and yeast extract for enhancing the protease production, which was studied at five different levels and thirteen experiments were carried out. The statistical software package Design Expert® 7.1.6 was used to analyze the results of the experimental design. The behavior of each variable, their interactions, and statistical analysis to obtain predicted responses was explained by the following second-order polynomial equation:[18]

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} b_{ij} x_i x_j \,,\ i<j \quad (1)$$

Where Y represents the response variable, b_0 is the interception coefficient, b_i is the coefficient of the linear effect, b_{ii} is the coefficient of quadratic effect, b_{ij} is the coefficient of interaction effect when $i < j$, and k are the numbers of involved variables.

Verification of the Quadratic Model

In order to validate the optimization of medium compositions, it was carried out using the optimized condition, to confirm the results from the response surface. The fermentations for the validation experiments were conducted at 30 °C and 200 rpm for 24 h in 250 mL Erlenmeyer flasks containing 50 mL aliquots of the optimized medium.

Results

Effect of carbon, nitrogen sources, and metal ions on protease activity

The optimization of culture medium was carried out by a combination of non-statistical methodology and statistical methodology-based experimental designs. A series of experiments were first carried out to study the effects of complex carbon and nitrogen sources as well as metal salts on the protease production by *Bacillus amyloliquefaciens* B7 with one-factor-at-a-time experiments. Protease activity was produced at a higher level in the presence of D-fructose (10 g L^{-1}), and 198.5 U mL^{-1} of protease activity was obtained, while relatively lower activity (136.43, 111.93, 104.36, 97.87, 73.73, 44.90 and 27.60 U mL^{-1} respectively) was obtained with other carbon sources (lactose, mannose, xylose, maltose, glucose, starch and dextrin). Different organic and inorganic nitrogen sources, at a concentration of 5 g L^{-1}, were tested. Higher protease activity was obtained with yeast extract (222.45 U mL^{-1}) compared to peptone, beef extract, ammonium nitrate, diammonium phosphate, ammonium chloride and urea (188.58, 106.59, 44.79, 45.33, 52.54, 63.53 U mL^{-1} respectively). The effect of metal ions on the protease activity was also tested with the concentration at 0.5 g L^{-1}, and higher level of protease production was obtained with $MgSO_4$ and $FeSO_4$ (242.45 and 241.91 U mL^{-1} repectively) compared to $MnSO_4$, $ZnSO_4$, $CuSO_4$ and $CaCl_2$ (only 143.25, 127.64, 77.76 and 75.52 U mL^{-1}).

One-factor-at-a-time experiments revealed that fructose, yeast extract, peptone, $MgSO_4$ and $FeSO_4$ were supposed to have more effect on protease activity, so these substrates were selected for further optimization. Furthermore, urea was added due to it being propitious to the cell grown, and $Na_2HPO_4 \cdot 12H_2O$ and K_2HPO_4 were also needed for the microorganism cultivation.

Selection of significant variables affecting protease activity

The matrix developed by the Plackett–Burman design and the results are presented in Table 2, and the regression analysis and variables evidencing statistically significant effects are shown in Table 3. The F-value of 10.15 implies the model is significant and there is only a 0.49 % chance that a

Table 2 – *Plackett–Burman design with coded values along with the observed results*

Trial	Coded variable level								Protease activity
	A	B	C	D	E	F	G	H	(U mL^{-1})
1	1	1	–1	1	1	1	–1	–1	540.76
2	–1	1	1	–1	1	1	1	–1	268.68
3	1	–1	1	1	–1	1	1	1	314.81
4	–1	1	–1	1	1	–1	1	1	253.19
5	–1	–1	1	–1	1	1	–1	1	248.86
6	–1	–1	–1	1	–1	1	1	–1	203.10
7	1	–1	–1	–1	1	–1	1	1	269.77
8	1	1	–1	–1	–1	1	–1	1	394.09
9	1	1	1	–1	–1	–1	1	–1	307.60
10	–1	1	1	1	–1	–1	–1	1	280.58
11	1	–1	1	1	1	–1	–1	–1	302.20
12	–1	–1	–1	–1	–1	–1	–1	–1	208.86

Table 3 – *Regression analysis of the Plackett–Burman design for the protease activity*

Sources	Effects	Contribution (%)	Prob > *F*
Model			0.0049
Fructose	110.99	40.23	0.0033
Yeast extraction	82.88	22.43	0.0137
Peptone	–24.50	1.96	0.2492
$FeSO_4$	32.79	3.51	0.2192
$MgSO_4$	29.07	2.76	0.2357
Urea	58.02	10.99	0.0560
$Na_2HPO_4 \cdot 12H_2O$	–59.70	11.64	0.0508
K_2HPO_4	–11.65	0.44	0.1000

"Model F-Value" this large could occur due to noise. Values of "Prob > *F*" less than 0.05 were considered to have significant effect on the response, and were therefore selected for further optimization. Regression analysis determined that the compositions of fructose and yeast extract had a significant effect ($P < 0.05$) on the enzyme activity, so these compositions were evaluated in the further optimization experiments. After the neglect of insignificant terms (on the basis of *P*-values higher than 0.05), a modified first-order equation in terms of actual factors was developed to describe enzyme activity:

$$Y = 8.56 + 11.10\ A + 20.72\ B \qquad (2)$$

According to the Plackett–Burman design, the optimum medium compositions were as follows: 20 g L^{-1} fructose, 8 g L^{-1} yeast extract, 2 g L^{-1} peptone, 0.5 g L^{-1} $FeSO_4$, 0.8 g L^{-1} $MgSO_4$, 2 g L^{-1} urea, 7.2 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$ and 1.81 g L^{-1} K_2HPO_4.

Steepest ascent method for determination of center point

The Plackett–Burman design was a valuable tool for screening variables that significantly affected the protease production, but it was unable to predict the optimum levels of the variables. Based on Table 4, the path of the steepest ascent was determined to find the proper direction of changing variables by increasing the concentration of fructose and yeast extract to improve protease activity. Regarding the results from the steepest ascent path, it was apparent that the yield profile showed a maximum 884.04 U mL^{-1} at run 2. Consequently, this point was close to the region of maximum protease activity response. So these variables, i.e. 40 g L^{-1} fructose and 16 g L^{-1} yeast extract, were chosen for further optimization.

Table 4 – *Experimental design and results of the steepest ascent path*

Trial	Fructose (g L^{-1})	Yeast extract (g L^{-1})	Protease activity (U mL^{-1})
Origin	20	8	574.49
1	30	12	749.98
2	40	16	884.04
3	50	20	749.98
4	60	24	574.49
5	70	28	469.62
6	80	32	364.40

Central composite design and response surface methodology

Once the ranges of relevant variables were selected through the Plackett–Burman and steepest ascent experiment, two compositions i.e. fructose and yeast extract that significantly affected protease activity were optimized by response surface methodology (RSM) using a 2-factor-5-level central composite design (CCD). Therefore, a group of 13 treatment combinations with different combinations of fructose and yeast extract were performed (Tables 5 and 6).

Table 5 – *Coded and real values of variables in central composite design*

Factors	Levels of factors				
	–1.414	–1	0	1	1.414
Fructose (g L^{-1})	25.86	30	40	50	54.14
Yeast extract (g L^{-1})	10.34	12	16	20	21.66

The highest activity of the protease observed was 956.59 U mL^{-1} at run 6 (Table 6). The model *F*-value of 175.03 implies that the model is significant, because there is only a 0.01 % chance that the "Model *F*-Value" could occur due to noise (Table 7). The regression equation obtained after ANOVA indicated that the *R*-Squared value of 0.9921 (a value of *R*-Squared > 0.75 indicated the aptness of the model) ensured a satisfactory adjustment of the quadratic model to the experimental data. The "Lack of Fit F-value" of 3.30 implies that the Lack of Fit is non-significant relative to the pure error. Non-significant lack of fit is good, and there is a 13.95 % chance that a "Lack of Fit F-value" this large could occur due to noise. The "Pred

Table 6 – *Observed and predicted values of protease activity for the CCD matrix*

Trial	Coded variable level		Protease activity (U mL^{-1})	
	X_1	X_2	observed	predicted
1	–1	–1	593.56	578.62
2	1	–1	806.89	805.94
3	–1	1	846.53	826.77
4	1	1	781.32	775.55
5	–1.41421	0	812.29	832.54
6	1.41421	0	956.59	957.06
7	0	–1.41421	514.71	521.65
8	0	1.41421	661.86	675.63
9	0	0	905.20	909.14
10	0	0	899.26	909.14
11	0	0	919.62	909.14
12	0	0	898.65	909.14
13	0	0	922.95	909.14

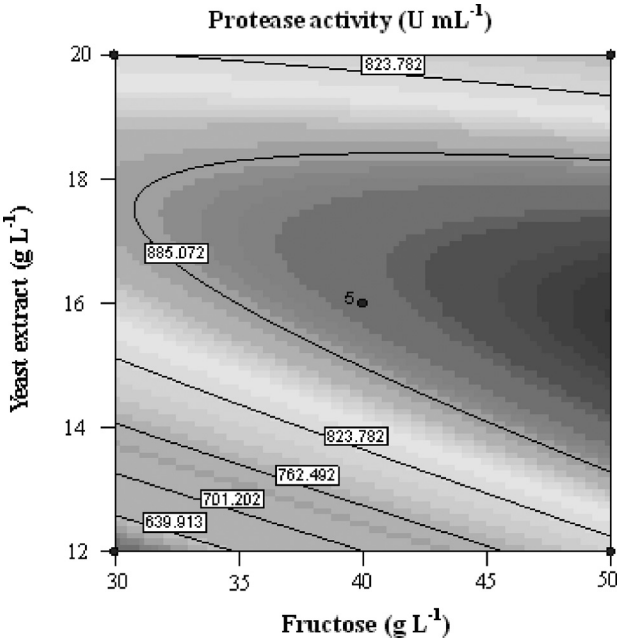

Fig. 1 – *Contour plot of protease production from Bacillus amyloliquefaciens B7 showing interaction between fructose and yeast extract*

R-Squared" of 0.9562 is in reasonable agreement with the "Adj R-Squared" of 0.9864. "Adeq Precision" measures the signal to noise ratio, a ratio greater than 4 is desirable, so the ratio of 39.751 indicates an adequate signal. A second-order polynomial function was fitted to the experimental protease activity, resulting in the following regression equation in terms of actual factors:

$$\text{Protease activity} = -3197.55 + 38\,X_1 + \\ + 393.74\,X_2 - 1.74\,X_1X_2 - 0.07\,X_1^2 - 9.7\,X_2^2 \quad (3)$$

Fig. 1 shows the relative effects of two factors, viz. fructose and yeast extract on the contour plot of protease activity. Evidently, the protease activity varied significantly with the concentration of fructose and yeast extract. The optimum value of each variable was identified based on the hump in the three-dimensional plot, or from the central point of the corresponding contour plot. The results predicted by the model equation from RSM indicated that a combination of adjusting the concentration of fructose to 50 g L^{-1} and yeast extract to 15.8 g L^{-1} would favor maximum protease activity, giving 946.36 U mL^{-1}.

Table 7 – *Analysis of variance for the second-order polynomial model for optimization of protease activity*

Source	SS	DF	MS	*F*-value	Prob>*F*
Model	227483.7	5	45495.95	175.0076	< 0.0001
Residual	1819.61	7	259.9656		
Lack of Fit	1295.88	3	431.9563	3.2981	0.1396
Pure Error	523.72	4	130.9726		
Total	229303.3	12			

R^2 = 0.9921; Pred-R^2 = 0.9562; CV% = 1.99; Adeq Precision = 39.751; Adj-R^2 = 0.9864; SS, sum of squares; DF, degrees of freedom and MS, mean square.

Validation of the model equation in shake-flask culture and batch cultivations in a 10 L bioreactor

In order to determine the accuracy of the optimized results, the culture of *Bacillus amyloliquefaciens* B7 was studied using predicted medium compositions. Maximum rate of protease activity was observed as 951.49 U mL^{-1} at 24 h, which was almost equal to the predicted value. By means of optimizing medium compositions, the activity of protease was enhanced from 242.45 U mL^{-1} to 951.49 U mL^{-1}, thus a 3.92-fold increase had been obtained. This result therefore corroborated the predicted values and the effectiveness of the model, which indicated that the optimized medium was propitious to the production of protease from *Bacillus amyloliquefaciens* B7.

Protease production was carried out in a 10 L bioreactor with the optimized medium compositions, and the results are summarized in Fig. 2. The cell density and protease activity both increased with the culture time, and the maximum enzyme activity obtained was about 3120.55 U mL^{-1} at

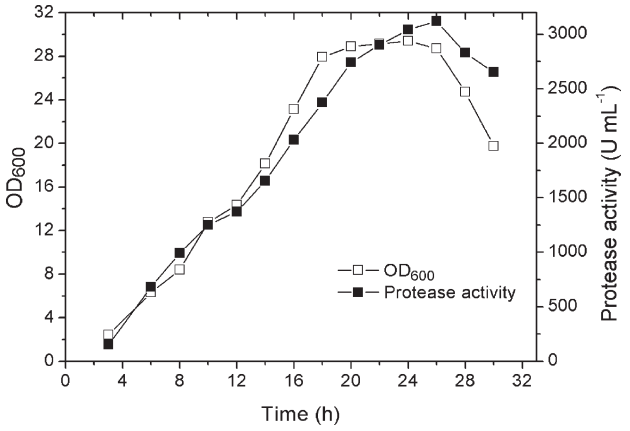

Fig. 2 – *Time course of alkaline protease production from Bacillus amyloliquefaciens B7 in a 10 L bioreactor with 6 L working volume*

26 h, while the highest cell density was 29.4 (OD_{600}) at 24 h. After 26 h, OD_{600} decreased gradually because of autolysis of the bacterial cells.

Discussion

The effect of environmental conditions on protease production plays an important role in the expression or repression of the enzyme,[19,20] and each organism has its own specific conditions for maximum production of enzyme.[21] The greatest difficulty in optimization of culture conditions is the presence of interactive effects of medium compositions and culture condition factors.[22] The "one-factor-at-a-time" method, in which one independent variable is studied while maintaining all the other factors at a fixed level, is laborious and time-consuming, and cannot provide the information about mutual interactions of the parameters on the desired outcome.[23] Statistical experimental design methods, such as response surface methodology, can provide a systematic and efficient plan for experimentation to achieve certain goals, so that several control factors are simultaneously studied.[24] The statistical experimental design, which is a collection of statistical techniques applicable to experimental design, model building, evaluating the effect of factors, and screening optimum conditions of factors for desirable responses, can overcome these shortages.[25]

The bacterium used in this study was shown to constitutively secrete extracellular proteolytic enzyme. The optimization of culture medium was carried out by a combination of non-statistical and statistical based experimental designs. The selection of medium compositions was carried out through the one-factor-at-a-time experiments, and 242.45 U mL^{-1} of the protease activity was observed. The Plackett–Burman experimental design indicated that fructose and yeast extract were the most significant factors, and then the steepest ascent method was employed to approach the experimental design space.

The three-dimensional response surface and contour presentations were then plotted to study the interaction among various physicochemical factors used and to find out the optimum concentration of each factor for maximum alkaline protease production from *Bacillus amyloliquefaciens* B7. The optimized values of variables for maximum protease activity were determined by solving the quadratic mode equation. The corresponding optimum values of each variable were determined to be 50 g L^{-1} fructose and 15.8 g L^{-1} yeast extract, at which conditions a maximum protease activity response of 946.36 U mL^{-1} was predicted. The results were validated by conducting a shake-flask culture under optimized conditions, and the maximum actual protease activity was 951.49 U mL^{-1}. This result shows that the regression model developed in this study resulted in good agreement between the actual and predicted responses.

Some researchers have previously reported about protease production with microorganisms, for example *Bacillus sp*. RKY3 (939 U mL^{-1}),[6] *Bacillus mojavensis* (2400 U mL^{-1}),[21] *Aspergillus clavatus* ES1 (770.66 U mL^{-1}),[26] *Bacillus clausii* (1520 U mL^{-1}),[27] *Bacillus aquimaris* (796 U mL^{-1}),[28] *Microbacterium sp.* (202.7 U mL^{-1}),[29] but few have reported the production of protease from *Bacillus amyloliquefaciens*. Furthermore, the maximum protease activity (3120.55 U mL^{-1}) of *Bacillus amyloliquefaciens* B7 was relatively high, and proved to be a suitable potential microorganism for use in protease production.

Conclusions

Statistical experimental approaches proved useful for the optimization of protease production by *Bacillus amyloliquefaciens* B7, which resulted in about 3.92 times higher enzyme activity than that using the original medium in a shake-flask culture. The optimal concentration for higher production of protease was as follows: 50 g L^{-1} fructose, 15.8 g L^{-1} yeast extract, 2 g L^{-1} peptone, 0.5 g L^{-1} $FeSO_4$, 0.8 g L^{-1} $MgSO_4$, 2 g L^{-1} urea, 7.2 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$ and 1.81 g L^{-1} K_2HPO_4. This study offers knowledge that may prove useful for large-scale production of protease in a batch or fed-batch bioreactor. However, less expensive carbon and nutrient sources, as well as scale-up culture parameters such as agitation speed or dissolved oxygen should be further investigated for the commercial applications of protease. In addition, purification

and characterization of the protease derived from *Bacillus amyloliquefaciens* B7 will be conducted in further research.

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List of symbols

b_0 – interception coefficient
b_i – linear coefficient
k – number of involved variables
b_{ii} – coefficient of quadratic effect
b_{ij} – coefficient of interaction effect ($i < j$)
R^2 – determination coefficient
Y – response variable, U mL^{-1}

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