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Full Length Research Paper

Enhanced production of intracellular dextran dextrinase from *Gluconobacter oxydans* using statistical experimental methods

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Optimization of the fermentation medium for DDase production by *Gluconaobacter oxydans* M5 was carried out in the shake flasks using two kinds of statistical methods. Four variables, namely glucose, tryptone, yeast extract and sodium chloride, were found to influence DDase production significantly by the Plackett-Burman screening. A four-factor five-level central composite design (CCD) was chosen to explain the combined effects of the four medium constituents. The optimum medium consisted of glucose (17.670 g/L), maltobiose (30 g/L), tryptone (12.198 g/L), yeast extract (13.528 g/L), ammonium nitrate (15 g/L), copper sulfate (0.01 g/L), zinc sulfate (0.01 g/L), and sodium chloride (0.009 g/L); the initial pH 6.0 was set prior to sterilization. The DDase yield obtained from optimized medium increased by 17-fold (0.238 U/mL) or so. Under these optimal conditions, the experimental values agreed with the predicted values, indicating that the chosen method of optimization of medium composition was efficient, relatively simple, time reducing and material saving.

Key words: Dextran dextrinase, *Gluconobacter oxydans,* medium optimization, Plackett-Burman design, central composite design.

INTRODUCTION

Dextran, which is synthesized from sucrose by the dextran sucrase (EC 2.4.1.5, DSase) of *Leuconostoc mesenteroides*, is widely used in pharmaceutical and biochemical fields (Mountzouris et al., 1999; Robyt and Walseth, 1979; Sidebotham, 1974). Dextran dextrinase (EC2.4.1.2), produced by *Gluconobacter oxydans*, could also convert maltooligosaccharides to dextran (Hehre, 1951), which have consecutive α(1,6)-linked glucose residues in the main chains and a wealth of α(1,4), α(1,3) and α(1,2)-branch linkages (De Muynck et al., 2007; Mountzouris et al., 1999). Yamamoto et al. purified the intracellular DDase from *G. oxydans* ATCC11894 and reported three different transglucosylation modes of the DDase enzyme (Yamamoto et al., 1992, 1993a).

The difference in structure between *Gluconobacter* and *Leuconostoc* dextran had been further studied and *G.* dextran could be used as a dietary fibre (Yamamoto et al., 1993b). Furthermore, *G.* dextran was used not only as a fat replacer in acceptable low-fat foods but also for taste improvement of the bitter-sweet compound stevioside (Sims et al., 2001; Yamamoto et al., 1994). What's more, *G.* dextran displayed shear-thinning flow behavior (Naessens, 2003). According to the former research, the novel polysaccharide displayed lower viscosity than *L. mesenteroides* dextran of similar molecular weight as a consequence of its higher degree of branching, and it might be suitable for certain food applications not asso-

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Abbreviations: DDase, Dextran dextrinase; **CCD,** central composite design; **DSase,** dextran sucrase; **NPG,** *p*-nitrophenyl-α-D-glucopyranoside; **R^2,** determination coefficient; **RSM,** response surface methodology.

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ciated with thickening functionality, such as a source of dietary fibre or a low-calorie bulking agent for sweeteners (Naessens et al., 2005). Above all, *G. oxydans* DDase could promise alternatives to *L. mesenteroides* DSase as biocatalysts for the synthesis of dextran and oligodextrans. But the enzyme system had been studied far less extensively than the glucosyltransferase of *L. mesenteroides*.

In our group, *G. oxydans* have been studied for about 20 years in the applications of incomplete oxidation, such as synthesizing L-ribulose, D-tagatose, miglitol and chiral aldehydes (Gao et al., 2006; Gao and Wei, 2006; Yang et al., 2008). In the process of these studies, we have found the strain also has the potential ability of interconverting between maltodextrins and dextran. It is known to all that changing environmental factors, such as the medium composition and the concentration of nutrient, can lead to an increase of DDase production. The development of an economically productive medium requires selecting carbon, nitrogen, ion and trace element sources. And there were two ways by which the problem of medium component limitations may be addressed: classical and statistical (Mao et al., 2007). As for the classical experimental design, there are several limitations towards complete optimization. Moreover, medium optimization by single dimensional search needs a great deal of experiments to determine optimum levels, which are unreliable. Furthermore, it was hard to evaluate the relative significance and the presence of complex interactions of several affecting factors (Kammoun et al., 2008; Mao et al., 2007; Tan et al., 2010). Compared with the classical method, the statistical experimental design in the fermentation medium optimization exhibits its advantages, including more advanced results with less process variability, closer confirmation, less development time and less overall costs.

In this study, medium optimization for production of intracellular DDase by *G. oxydans* was reported to make it clear that fermentation factors influenced the DDase yield under statistical experimental design.

MATERIALS AND METHODS

Microorganism

The strain *G. oxydans* M5 was stored as 1 mL aliquots in 20% glycerol at -72°C. The frozen cultures were plated periodically to control their viability.

Medium and growth conditions

The *G. oxydans* grew in a medium containing 80 g of D-sorbitol, 20 g of yeast extract, 2 g of KH_2PO_4 and 0.5 g of $MgSO_4 \cdot 7H_2O$ in 1 L of deionized water. The initial pH was set at 6.0 prior to sterilization.

The organism was incubated at 30°C at 200 rpm in a rotary shaker for 24 h. After 24 h cultivation, 5 mL of the seed culture was used to inoculate 50 mL of production medium in a 250 mL glass flask and this culture was grown under the same conditions as the seed culture. In the optimization process, the amounts of every component were changing in different experimental processes. All the experiments were carried out in three parallel tests.

DDase assay

The extraction method of *G. oxydans* DDase was as described in the former research by Yamamoto et al. (1992). The *G. oxydans* DDase activity was determined in accordance with the method described by Naessens et al. with some modifications (Naessens et al., 2005). It was based on the release of nitrophenol from *p*-nitrophenyl-α-D-glucopyranoside (NPG), mimicking G2 utilization by the transglucosidase, which was developed. The DDase specificity of the test was proven via an NPG zymogram. All results represented an average of three separate determinations. One unit of DDase activity was defined as the amount of enzyme which liberates 1 µmol of nitrophenol from NPG per min under the standard assay conditions.

Experimental design and response surface methodology

Screening for the important factors

For screening, the Plackett-Burman design was applied with which 12 runs would enable us to estimate all main effects for up to 11 factors. Table 1 showed the Plackett-Burman design used in this study (Plackett-Burman, 1946). Since there were only 9 factors in this case, less than the maximum allowable factors, the remained 2 factors became “dummy factors” which would be used to estimate errors. The signs -1 and +1 represented the lower and higher levels of the corresponding components under investigation. In this experiment, the higher levels of the components were chosen to equal 1.25 times of their lower levels (Table 2).

Central composite design (CCD) method

Sodium chloride, tryptone, glucose and yeast extract were four effective nutrients in the Plackett-Burman design. These variables were selected to find the optimum condition for higher DDase production using central composite design (CCD). In this case, a 2^4 full factorial central composite design for four independent variables each at five levels with eight star points and six replicates at the centre points was employed to fit a second order polynomial model, in which 30 experiments were required in this procedure (Box et al., 1978, Box, 1951). Each variable was designed at five levels (–2, –1, 0, 1, 2) and the lowest and the highest concentration were: sodium chloride, 0 and 0.02 g/L; tryptone, 2 and 18 g/L; glucose, 5 and 55 g/L; yeast extract, 2 and 18 g/L , respectively. Table 3 showed the CCD design for the values and the observed response for the DDase activity. The central values (zero level) chosen for experimental design were same as the lower level (designated -1) in the Plackett-Burman design.

For statistical calculation, the relationship between the coded and actual values is described as the following equation:

$$X_i = \frac{U_i - U_i^0}{\Delta U_i} \quad (1)$$

where X_i is the coded value of the ith variable, U_i is the actual value of the ith variable, U^0_i is the actual value of the ith variable at the center point and ΔU_i is the step change of variable.

The response variable (DDase activity) suitable to a quadratic

Table 1. Plackett-Burman design for 11 variables with coded values.

S/N	A	B	C	D	E	F	G	H	J	K	L	DDase (U/mL)
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.171
2	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.174
3	1	1	-1	1	1	-1	1	-1	-1	-1	1	0.193
4	1	-1	-1	-1	1	1	1	-1	1	1	-1	0.178
5	1	-1	1	1	-1	1	-1	-1	-1	1	1	0.192
6	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.171
7	1	1	1	-1	1	1	-1	1	-1	-1	-1	0.144
8	-1	1	1	1	-1	1	1	-1	1	-1	-1	0.166
9	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.153
10	-1	1	-1	-1	-1	1	1	1	-1	1	1	0.165
11	-1	-1	-1	1	1	1	-1	1	1	-1	1	0.138
12	1	-1	1	-1	-1	-1	1	1	1	-1	1	0.169

Table 2. Assigned concentrations of variables at different levels in Plackett-Burman design.

Factor	Variables with designate	Lower level (-1) (per litre)	Higher level (+1) (per litre)
1	A Maltobiose	30 g	37.5 g
2	B Glucose	30 g	37.5 g
3	C Tryptone	10 g	12.5 g
4	D Yeast extract	10 g	12.5 g
5	E Ammonium nitrate	15 g	18.75 g
6	F Copper sulfate	0.01 g	0.0125 g
7	G Zinc sulfate	0.01 g	0.0125 g
8	H Sodium chloride	0.01 g	0.0125 g
9	J Prior pH	6	7.5
10	K Dummy		
11	L Dummy		

equation for the variables was as following:

$$Y = b_0 + \sum_i b_i x_i + \sum_i \sum_j b_{ij} x_i x_j + \sum b_{ii} x_i^2 + e \quad (2)$$

where Y is the measured response, X_i, X_j are input variables which influence the response variable Y; b_0 is a constant; b_i is the ith linear coefficient; b_{ii} is the squared coefficient and b_{ij} is the ijth cross-product coefficient.

The 'Design Expert' software (version 7.1.6, Stat-Ease, Inc., Minneapolis, USA) was utilized to analyze the significance of experimental results. The central composite design (CCD) employed to fit a second order polynomial model, had four variables, of which everyone was at five levels with eight star points and six replicates at the centre points and the result indicated that 30 experiments were required for this procedure (Cochran, 1957).

RESULTS AND DISCUSSION

Screening for the important factors

The effects of different carbon sources on the growth of *G. oxydans* and DDase activity were studied in the media containing yeast extract (2%) as the nitrogen source. The results showed that glucose and maltobiose were better than other carbon sources for the DDase production; the effects of nitrogen sources on the DDase production by *G. oxydans* were investigated using glucose (2%) as carbon source and it exhibited that tryptone was the best and yeast extract and ammonium nitrate was inferior (data not shown). Then the DDase yield was determined for fermentation containing glucose (2%) and tryptone (1%) as carbon and nitrogen sources and one of the following ions (0.001, 0.01 and 0.1%) were used as additives: KCl, NaCl, $MgCl_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $BaCl_2 \cdot 2H_2O$, $CaCl_2$, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$. High yield of DDase was observed when $CuSO_4$ or $ZnSO_4$ or NaCl was used as trace mineral "cocktails" for fermentation (data not shown). According to above study, which was under the premise of keeping the other factors constant, we optimized the composition of the production medium as follows: glucose 30 g/L, maltobiose 30 g/L, tryptone 10 g/

Table 3. The CCD design for the values in coded and the observed values in response.

S/N		Sodium chloride (g/L)	Tryptone (g/L)	Glucose (g/L)	Yeast extract (g/L)	DDase (U/mL)
1	CENTER	0.01	10	30	10	0.177
2	CENTER	0.01	10	30	10	0.173
3		0	10	30	10	0.114
4		0.005	14	17.5	14	0.207
5		0.005	6	17.5	14	0.202
6	CENTER	0.01	10	30	10	0.171
7		0.01	10	30	18	0.141
8		0.015	6	42.5	14	0.077
9		0.005	6	42.5	6	0.092
10		0.01	10	30	2	0.068
11		0.005	14	17.5	6	0.178
12		0.01	18	30	10	0.148
13		0.005	14	42.5	14	0.103
14	CENTER	0.01	10	30	10	0.175
15		0.005	6	17.5	6	0.115
16	CENTER	0.01	10	30	10	0.170
17		0.02	10	30	10	0.105
18		0.01	2	30	10	0.113
19		0.015	14	42.5	14	0.102
20		0.005	6	42.5	14	0.099
21		0.015	14	17.5	6	0.171
22		0.015	6	42.5	6	0.069
23	CENTER	0.01	10	30	10	0.177
24		0.015	14	17.5	14	0.184
25		0.015	14	42.5	6	0.078
26		0.01	10	5	10	0.157
27		0.015	6	17.5	14	0.166
28		0.005	14	42.5	6	0.097
29		0.01	10	55	10	0.067
30		0.015	6	17.5	6	0.111

L, yeast extract 10 g/L, ammonium nitrate 15 g/L, copper sulfate 0.01 g/L, zinc sulfate 0.01 g/L, sodium chloride 0.01 g/L and the initial pH 6.0 was set prior to sterilization. The culture was incubated at 30°C at 200 rpm in a rotary shaker for 62 h.

After above optimization, the Plackett-Burman technique was used to select the most important factors which would influence the DDase yield. In this section, twelve experiments were minimum runs to screen the importance of nine complications, namely glucose, maltobiose, tryptone, yeast extract, ammonium nitrate, copper sulfate, zinc sulfate, sodium chloride and pH. The Model F-value of 20.84 implied the model was significant. There was only a 4.66% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicated model terms were significant. The experimental date analysis showed that four variables, namely glucose, tryptone, yeast extract and sodium chloride out of nine variables influenced the DDase yield significantly.

Central composite design (CCD) method

With the central composite design method, the experiments with different combination of glucose, tryptone, yeast extract and sodium chloride were performed. A four-factor five-level central composite design was used based on the "Design Expert" software (version 7.1.6) and experimental data is presented in Table 3.

By statistical analysis, a mathematical model was obtained to describe the relationship between the DDase production (Y) and the test variables in coded factors (concentration of glucose, tryptone, yeast extract and sodium chloride, respectively):

Table 4. ANOVA for response surface quadratic model.

Source	Sum of Squares	DF	Mean Square	F Value	R^2
Model	0.052	14	3.695×10^{-3}	13.82	0.9280
Residual	4.011×10^{-3}	15	2.674×10^{-4}		
Lack of Fit	3.970×10^{-3}	10	3.970×10^{-4}	48.97	
Pure Error	4.054×10^{-5}	5	8.109×10^{-6}		
Cor Total	0.056	29			

Table 5. The least-squares fit and coefficient estimate.

Factor	Coefficient estimate	Standard error	95% CI Lower limit	95% CI Upper limit	F Value	p-value Prob > F
Intercept	0.17	6.676×10^{-3}	0.16	0.19		
X_1	-6.386×10^{-3}	3.338×10^{-3}	-0.014	7.290×10^{-4}	3.66	0.0750
X_2	0.011	3.338×10^{-3}	3.700×10^{-3}	0.018	10.50	0.0055
X_3	-0.033	3.338×10^{-3}	-0.040	-0.026	99.46	< 0.0001
X_4	0.016	3.338×10^{-3}	8.584×10^{-3}	0.023	22.12	0.0003
$X_1 X_2$	2.243×10^{-3}	4.088×10^{-3}	-6.470×10^{-3}	0.011	0.30	0.5913
$X_1 X_3$	3.192×10^{-4}	4.088×10^{-3}	-8.394×10^{-3}	9.033×10^{-3}	6.095×10^{-3}	0.9388
$X_1 X_4$	-1.888×10^{-3}	4.088×10^{-3}	-0.011	$6.826\times10^{-}$	0.21	0.6509
$X_2 X_3$	-6.383×10^{-3}	4.088×10^{-3}	-0.015	2.331×10^{-3}	2.44	0.1393
$X_2 X_4$	-5.339×10^{-3}	4.088×10^{-3}	-0.014	3.375×10^{-3}	1.71	0.2113
$X_3 X_4$	-8.808×10^{-3}	4.088×10^{-3}	-0.018	-9.439×10^{-3}	4.64	0.0479
X_1^2	-0.014	3.122×10^{-3}	-0.020	-7.089×10^{-3}	19.38	0.0005
X_2^2	-8.551×10^{-3}	3.122×10^{-3}	-0.015	-1.896×10^{-3}	7.50	0.0152
X_3^2	-0.013	3.122×10^{-3}	-0.020	-6.466×10^{-3}	17.66	0.0008
X_4^2	-0.015	3.122×10^{-3}	-0.022	-8.330×10^{-3}	23.03	0.0002

CI = Confidence interval; Prob = probabaility; F = frequency.

$$Y = 0.17 - 6.386\times10^{-3} X_1 + 0.011 X_2 - 0.033 X_3 + 0.016 X_4 + 2.243\times10^{-3} X_1 X_2 + 3.192\times10^{-4} X_1 X_3 - 1.888\times10^{-3} X_1 X_4 - 6.383\times10^{-3} X_2 X_3 - 5.339\times10^{-3} X_2 X_4 - 8.808\times10^{-3} X_3 X_4 - 0.014 X_1^2 - 8.551\times10^{-3} X_2^2 - 0.013 X_3^2 - 0.015 X_4^2 \quad (3)$$

The coefficient values of the model and factors were calculated by Design Expert Software and their values are shown in Tables 4 and 5. The results of the second order response surface model fitting in the form of ANOVA were given in Table 4.

In Table 5, the Model F-value of 13.82 implied that the model was significant. There was only a 0.01% chance that a “Model F-Value” this large could occur due to noise. The “Lack of Fit F-value” of 48.97 implied the Lack of Fit was significant. The well fit of the model was checked by determination coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0.9280) indicated that it was reasonable to use the regression model to analyze the tendency (Akhnazarove, 1982; Khuri and Cornell, 1987). Table 5 shows the significance of each coefficient determined by F-value and P-value. Values of “Prob > F” less than 0.0500 indicate model terms were significant. In this case X_2, X_3, X_4, X_3 X_4, X_1^2, X_2^2, X_3^2, X_4^2 were significant model terms.

Figures 1 - 6 shows the response surface for the variation in the production of DDase, from which the values of DDase activity for different concentrations of the variables could be predicted. Each contour curve represented an infinite number of combinations of two test variables with another maintained at their zero level. Figures 1 - 3 show that increasing the sodium chloride concentration from 0.003 to 0.01 g/L increased DDase production. The maximum production was obtained with the concentration of sodium chloride at about 0.01 g/L. The two nitrogen sources, tryptone and yeast extract, were important to increase the DDase yield. From the response surface contour plot, the concentrations of tryptone and yeast extract were around 12 - 14 g/L, respectively, to result in the maximum DDase yield. In this study, glucose was also a significance parameter. Figures 2, 4 and 6 have shown that the maximum DDase activity appeared at the concentration of 17.5 - 18 g/L.

According to the optimized mathematical model, the optimal values of the test variables in coded unit were as follows: X_1 = -0.156, X_2 = 0.549, X_3 = -0.986, X_4 = 0.882

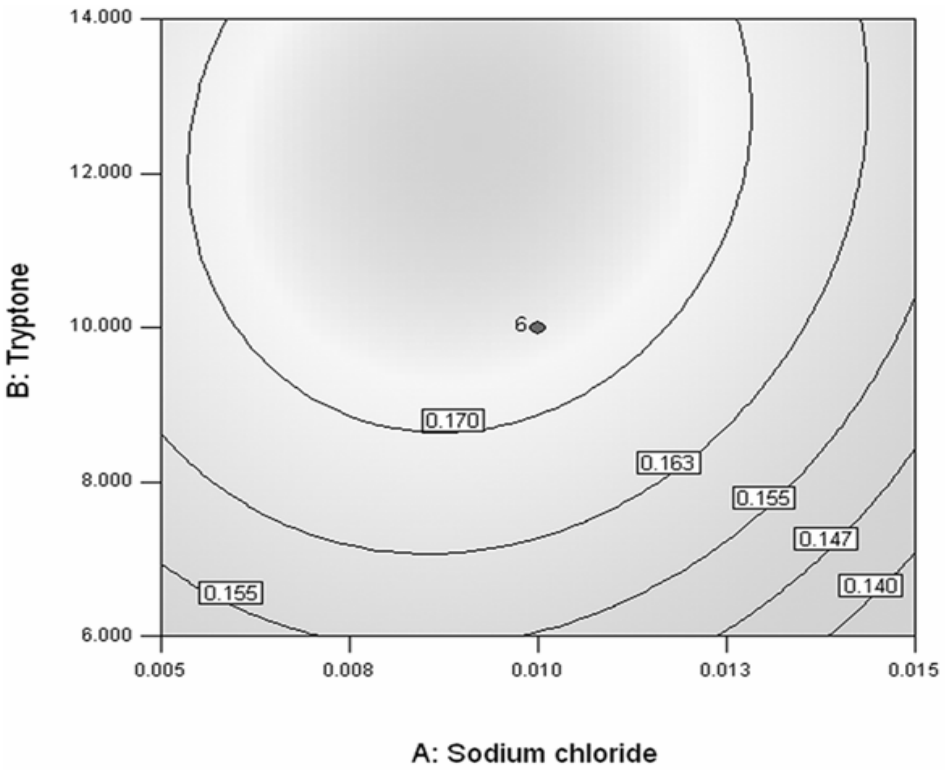

Figure 1. Effect of sodium chloride and trypotone concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

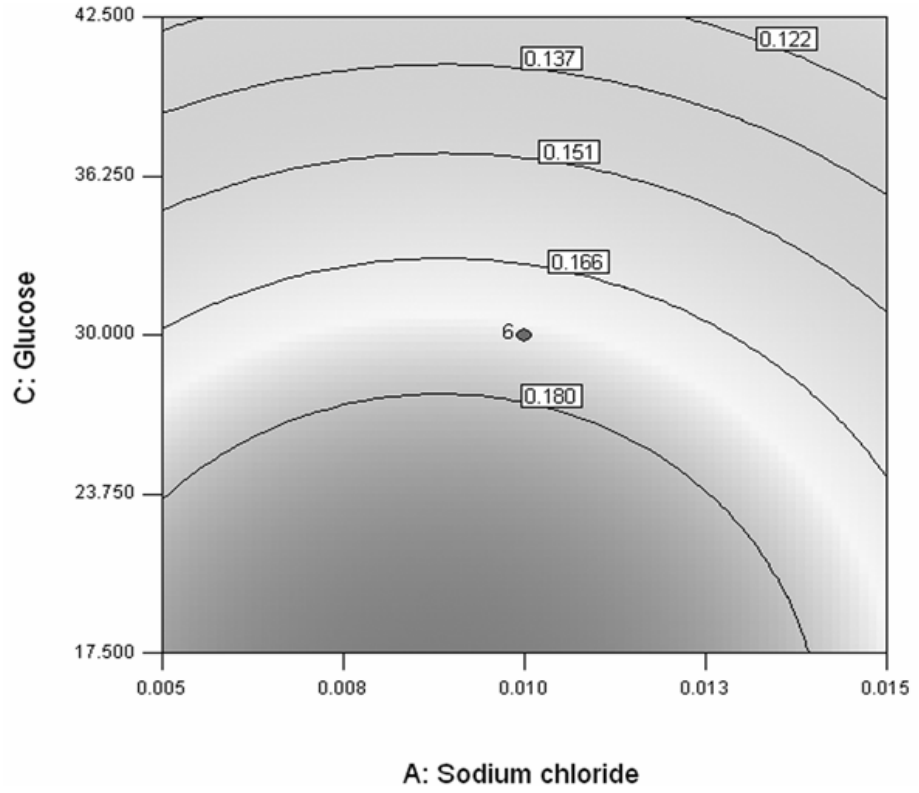

Figure 2. Effect of sodium chloride and glucose concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

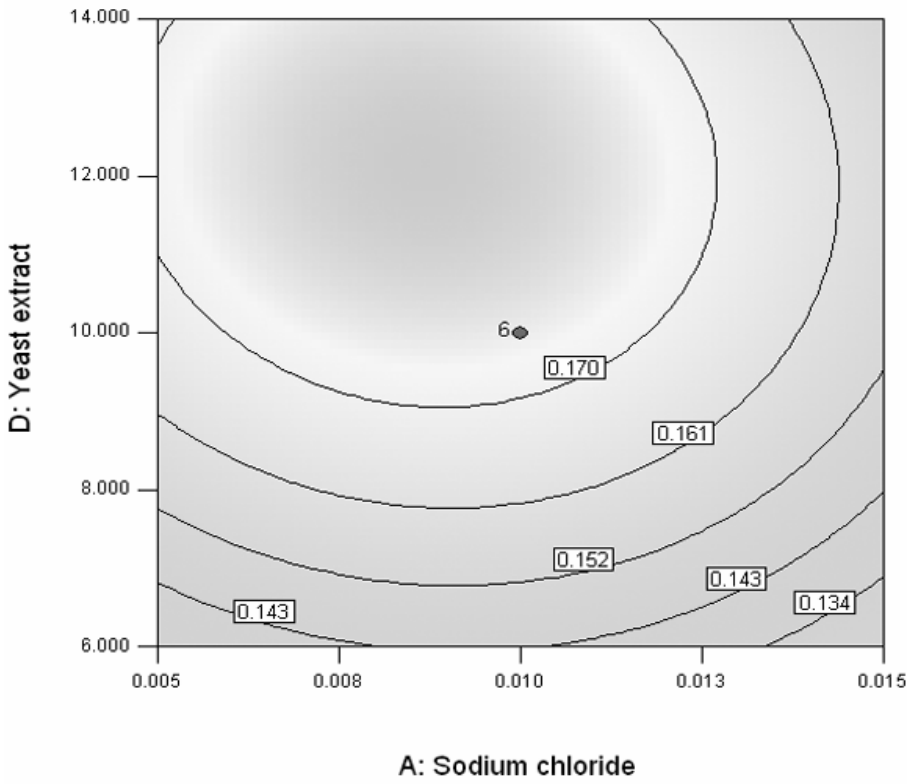

Figure 3. Effect of sodium chloride and yeast extract concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

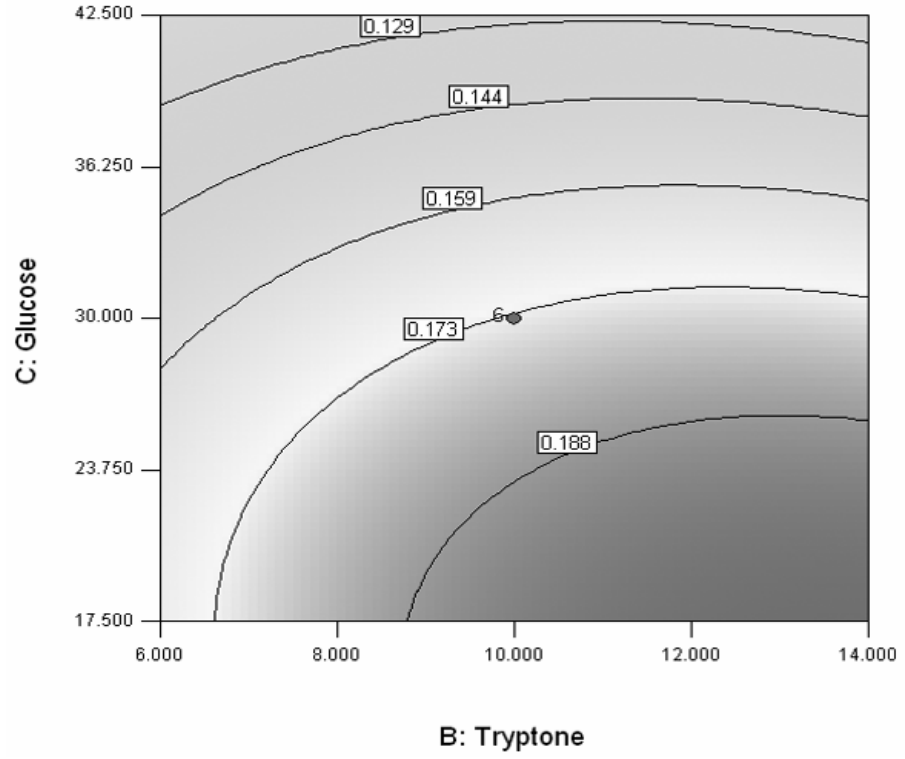

Figure 4. Effect of trypotone and glucose concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

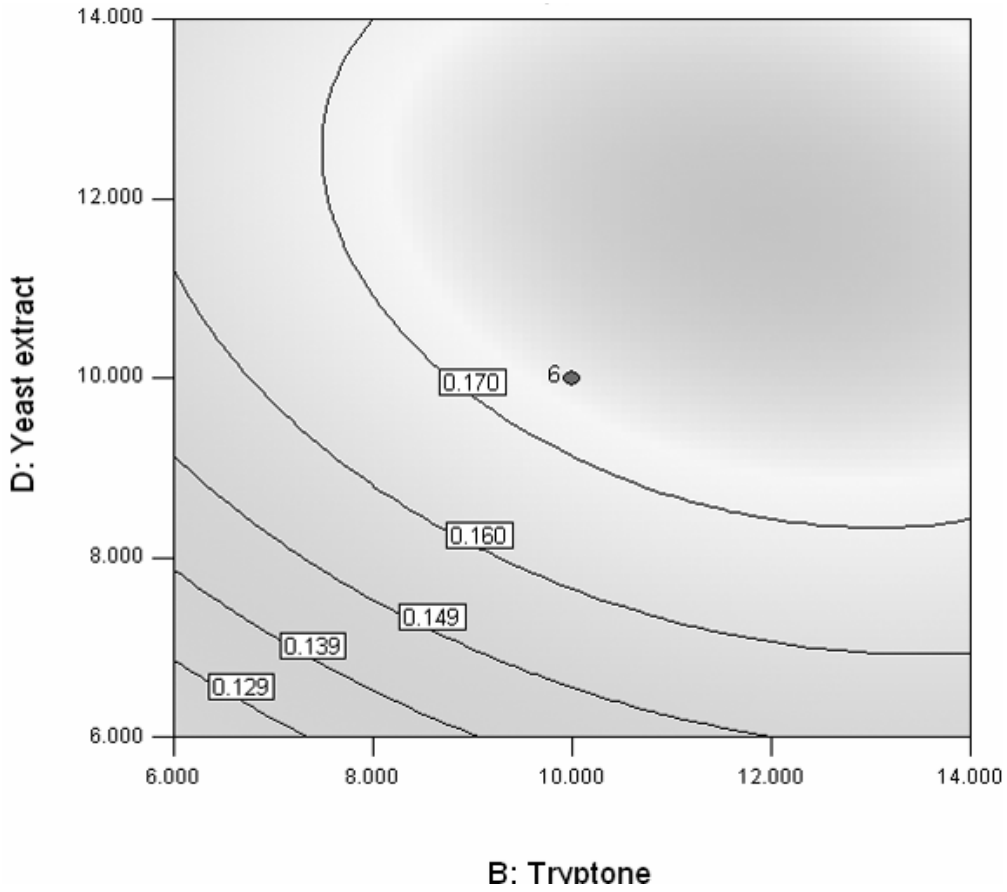

Figure 5. Effect of trypotone and yeast extract concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

with the corresponding maximum DDase production Y = 0.209 U/mL (a possible variation is between 0.17 and 0.25 mg/L) in the confidence range of 95%. Putting the respective values of X_i in Equation (1), the concentration of sodium chloride, tryptone, glucose and yeast extract were at 0.009, 12.198, 17.670 and 13.528 g/L, respectively. It was clear that the optimal values from response surface plots were almost consistent with those from optimized mathematical equation.

In order to verify the predicted results, the experiment was performed with the optimized medium and the maximum DDase production was found to be 0.238 U/mL, which was obvious in close agreement with the model prediction. After optimization, compared with the minimum medium, the production of DDase was enhanced about 17 fold, experimentally (Table 6). To our best knowledge, the DDase production in this research is higher than any other reported results (Naessens et al., 2005).

Conclusions

Plackett-Burman screening and response surface methodology (RSM) for medium optimization were employed for the DDase production from *G. oxydance*. The method used was effective in the screening for nutritional requirements in a limited number of experiments. It enabled us to screen a large number of experimental factors and was useful to determine the optimum levels of medium components concentration that significantly influenced the DDase production in a perfect way. The chosen method of optimization of medium composition was efficient, relatively simple, time reducing and material saving. The final composition of definite medium after the optimization was as follows: glucose 17.670 g/L, maltobiose 30 g/L, tryptone 12.198 g/L, yeast extract 13.528 g/L, ammonium nitrate 15 g/L, copper sulfate 0.01 g/L, zinc sulfate 0.01 g/L, sodium chloride 0.009 g/L, respectively, and the initial pH was set at 6.0. The optimization of the medium resulted in an about 17-fold (0.238 U/mL) increase in DDase yield. This work would provide a stable foundation for the industrialization of DDase.

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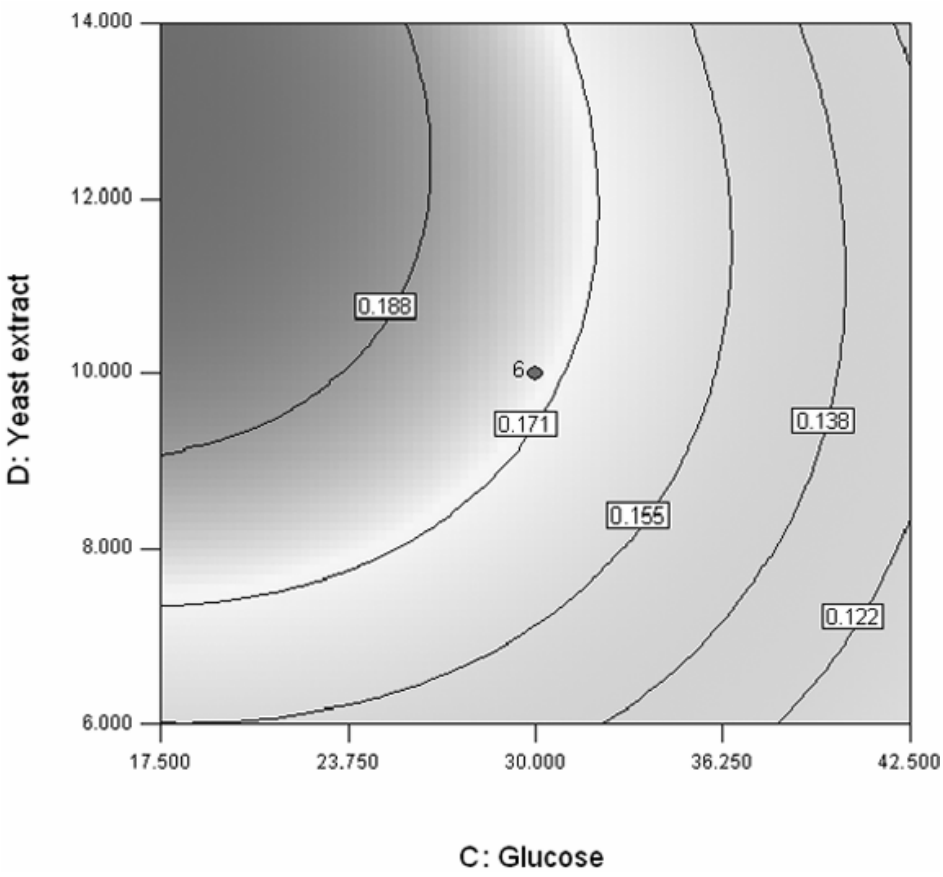

Figure 6. Effect of glucose and yeast extract concentration on the DDase production by *G. oxydans*. Other variables are held at zero level.

Table 6. Experimental verification of combined effect of optimized medium on the response of DDase production.

Variables	Levels after optimization		DDase production (U/mL)		
	Coded	Uncode (g/L)	Unoptimized medium	New medium	
				Predicted	Experimental
Sodium chloride	-0.156	0.009	0.013	0.209	0.238
Tryptone	0.549	12.198			
Glucose	-0.986	17.670			
Yeast extract	0.882	13.528			

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