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Full Length Research Paper

Medium optimization for protopectinase production by batch culture of *Aspergillus terreus*

Chuanhui Fan[1,2], Zhanmin Liu[1,2]* and Lifeng Yao[1,2]

[1]School of Life and Sciences, Shanghai University, 99 Shangda Road, Shanghai 200444, P. R. China.
[2]Experimental Center of Shanghai University, 99 Shangda Road, Shanghai 200444, P. R. China.

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Optimization of medium compositions for protopectinase production by *Aspergillus terreus* in submerged culture was carried out. The medium components having significant effect on protopectinase production were reported to be sucrose, yeast extracts and ammonium acetate by the Plackett-Burman design. Subsequently, the concentrations of the three factors were optimized using central composite design in response surface methodology. As a result, a quadratic model was found to fit for protopectinase production. The optimal medium compositions were determined as follows (g/L): sucrose 129.7, yeast extracts 9.90, ammonium acetate 6.0, and protopectinase activity reached the maximum value of 41.30 U/ml in the optimal medium.

Key words: *Aspergillus terreus*, protopectinase, medium optimization, Plackett-Burman design, central composite design.

INTRODUCTION

Pectin is a water-soluble, heteroploysaccharide that contains galacturonic and methoxylgalacturonic acids as its main components (Acikgoz, 2011; Vriesmann et al., 2011). Pectin is widely used as a functional ingredient in food industry, pharmacy and cosmetic manufacture due to its ability to form aqueous gels, dispersion stabilizer. There are two main types of pectin extraction processes: one is a traditional chemical extraction process, which has several disadvantages, including complex process, energy intensive and industrial wastes (Iglesias and Lozano, 2004). The other is microbial and enzymatic extraction processes, which overcome disadvantages of chemical processes and are attractive in research and different applications in the world.

Protopectinases (PPases) are used as heterogeneous group of enzymes, which produce the enzymatic solubilization of pectin from protopectin, the water-insoluble parental pectic substance present in plant tissues. Several PPases from different microorganisms (bacteria, yeast or fungi) have been purified, characterized and their genes have been expressed in *Escherichia coli* and *Pichia pastoris* (Iguchi et al., 1997; Liu et al., 2006; Nagai et al., 2000; Takao et al., 2000). It is well known that PPases have an important role in liberating protopectin to pectin, and it is important and necessary to improve protopectinase yields for microbial and enzymatic pectin extraction.

In order to utilize microbial and enzymatic pectin extraction from agricultural and agro-industrial wastes, a novel protopectinase-producing *Aspergillus terreus* was screened and identified in our laboratory, and this isolate is able to tolerate tannin (Liu and Fan, 2011). However, batch fermentation medium has not yet been optimized. Many investigations describe that cell growth and the accumulation of metabolic products are strongly influenced by medium compositions such as carbon sources, nitrogen sources, growth factors and inorganic salts. When compared with conventional method, statistical experimental design techniques are very useful tools for the screening of nutrient, as they can provide statistical models, which help in understanding the interactions among the factors at varying levels and calculation of the optimal level of each factor for a given

*Corresponding author. E-mail: zhmliu119@yahoo.com.cn. Tel: +86-21-66135166. Fax: +86-21-66135166.

target. The application of statistical experimental design in fermentation process investigation can improve product yield, reduce process variability, as well as reduce development time and overall costs (Pan et al., 2008; Ren et al., 2008). The aim of this study was to screen key medium components and optimize suitable medium components for protopectinase production by *A. terreus* using statistical experimental.

MATERIALS AND METHODS

A. terreus used in this study was provided by the Laboratory of Food Safety and Biotechnology, Shanghai University. The strain was cultured on the potato dextrose agar (PDA) slant at 28℃ for one day, and then maintained at 4℃. The seed culture medium contained (g/L): sucrose 30, yeast extracts 5, meat peptone 5, K_2HPO_4 3, KH_2PO_4 1, and MgSO4 0.5, initial pH at 7.0. The fermentation media were prepared as shown in Tables 1 and 3, respectively.

Culture conditions

Inoculum was prepared by transferring one loop full of culture from PDA slant to an Erlenmeyer flask (250 ml) containing 50 ml seed medium. The seed cultures were grown at 28 ± 2℃ on a rotary shaker incubator at 180 rpm for 25 h. After incubation, 1 ml of the seed culture was transferred into an Erlenmeyer flask (250 ml) containing 50 ml of fermentation. The fermentation cultures were then incubated at 28 ± 2℃ with shaking at 150 rpm for 48 h.

Measurement of protopectinase activity

In order to determine protopectinase activity, aqueous two-phase systems (ATPSs) were constructed by weighing 3.5 g of poly ethylene glycol (PEG) 6000, 1.5 g of tripotassium phosphate and 10 ml distilled water into a suitable vessel, equilibrated at room temperature. The previous fermentation cultures were centrifuged at 2000 ×g for 5 min at 4℃, and then 4 ml of the supernatant was added to ATPSs and mixed adequately to make the two phase system. After equilibration and phase separation, the upper phase was carefully removed with a pipette, leaving a small amount at the interface. The lower phase was then sampled and analyzed for protopectinase activity according to sulfate-carbazole method (McComb and McCready, 1954).

Screening of factors significantly by the Plackett-Burman design

For the selection of the key ingredients significantly affecting protopectinase production from *A. terreus*, Eleven nutrient factors considered for the design were glucose, fructose, soybean meal, yeast extracts, tryptone, NH_4Cl, ammonium acetate, ammonium oxalate, KH_2PO_4, K_2HPO_4 and $CaCl_2$, which were designated as X_1, X_2, X_3, X_4, X_5, X_6, X_7, X_8, X_9, X_{10} and X_{11}, respectively. The entire variable had two levels of the lower and the higher. The principal effects of each on protopectinase activity were estimated as the difference between both averages of measurements made at the higher level and at the lower level. The significance of each variable was determined via Student's t-test.

Optimization of significant nutrients using the Box-Behnken design

After screening the significant medium components by a Plakett-Burumn design, the formulation of the medium was used to investigate the optimum levels of significant variables for protopectinase production. A Box-Behnken design was employed for the response surface methodology (RSM) studies and the three factors chosen were sucrose, yeast extracts and ammonium acetate, which were newly designated as X_1, X_2 and X_3, respecttively. The role of each variable, their interaction and statistical analysis to obtain the predicted yield were explained by applying the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the response; X_i and X_j are the independent variables; β_0 is a constant; and β_i, β_{ii} and β_{ij} are the linear, squared and interaction coefficients, respectively. The statistical software package, JMP 7.02 (SAS Institute Inc., Cary, NC, USA) was used for the regression analysis of the experimental data. The statistical significance of the model equation and the model terms was evaluated via the Fisher's test. The coefficient of determination (R^2) and adjusted R^2 (Adj R^2) were used for the verification of the significance of the second order polynomial model (Aziz et al., 2010). Based on the analysis of variance, parameters with a significance level (P) greater than 5% were eliminated to obtain the final reduced model. This final model can be displayed as three-dimensional (3D) response surface plots by varying two-factor levels while holding the other factor at a constant level.

RESULTS AND DISCUSSION

Factors significantly affecting protopectinase production

In order to find out the key ingredients significantly affecting protopectinase production, a Plackett-Berman design was carried out, and the experimental results are shown in the Table 1. Table 1 depicted that protopectinase activity varied from 0 to 22.90 U/ml throughout the twenty runs, which indicated that optimization of the medium was important for protopectinase activity. A Plackett-Burman design is one of the screening designs, which is traditionally used for identifying important factors from among many potential factors. In the analysis of these designs, usually only main effects are estimated.

Based on the statistical analysis (Table 2), sucrose (X_1) and fructose (X_2) are the most positive significant variables for protopectinase production (effect of X_1 = 11.96 and effect of X_2 = 10.20), followed by yeast extracts (X_5, effect of X_5 = 4.67), ammonium acetate

Table 1. A Plackett-Burman design matrix for eleven factors with actual values together with the actual and predicted protopectinase activity.

Run order	Experimental value											Response	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Actual	predicted
1	5	5	20	2	2	5	5	5	6	2	3	5.37	3.596
2	5	2	20	5	5	2	5	2	6	2	1	7.3	7.072
3	5	20	5	2	2	2	5	5	2	2	3	6.46	7.61
4	5	20	20	2	2	5	2	2	6	6	3	7.2	8.494
5	5	5	5	2	5	5	2	2	6	2	1	4.33	6.148
6	5	5	20	5	2	2	5	2	2	6	3	*	-9.88
7	5	5	5	5	5	2	2	5	2	2	3	2.1	2.24
8	5	5	20	5	5	5	2	5	2	6	1	5.12	4.842
9	5	20	5	5	2	2	2	2	6	6	1	2.25	0.642
10	5	20	5	2	5	5	5	5	2	6	1	11.41	10.896
11	20	20	5	2	5	2	2	5	6	6	3	22.46	21.142
12	20	20	20	5	5	5	5	5	6	6	3	15.67	16.832
13	20	5	5	2	2	5	5	2	2	6	1	5.47	4.772
14	20	5	20	2	2	2	2	5	6	2	1	17.6	17.428
15	20	20	5	5	2	5	2	2	2	2	3	13.99	14.194
16	20	5	5	5	5	5	5	2	6	2	3	6.31	5.314
17	20	5	20	2	5	2	2	2	2	6	3	12.35	12.488
18	20	20	20	2	5	2	5	2	2	2	1	21.91	21.986
19	20	50	5	5	2	2	5	5	6	6	1	0	1.822
20	20	20	20	5	2	5	2	5	2	2	1	22.90	22.682

Table 2. Sorted parameters estimates.

Term	Estimate	Std error	t Ratio	t Ratio	Prob>|t|
Sucrose g/L (5, 20)	4.85	0.406	11.96		<0.0001
Fructose g/L (5, 20)	4.139	0.406	10.20		<0.0001
NH_4Cl g/L (2, 5)	-2.44	0.406	-6.02		0.0005
Peptone g/L (2, 5)	-2.014	0.406	-4.96		0.0016
Yeast extracts g/L (2, 5)	1.893	0.406	4.67		0.0023
Ammonium acetate g/L 2(2, 5)	1.88	0.406	4.63		0.0024
KH_2PO_4 g/L (2, 6)	-1.811	0.406	-4.46		0.0029
Soybean meal g/L (5, 20)	1.538	0.406	3.79		0.0068
$CaCl_2$ g/L (1, 3)	-0.813	0.406	-2.00		0.0851
Ammonium oxalate g/L (2, 5)	0.761	0.406	1.88		0.1028
K_2HPO_4g/L (2, 6)	-0.167	0.406	-0.41		0.6929

(X_6, effect of X_6 = 4.63) and soybean meal (X_8, effect of X_8 = 3.79). However, three variables, NH_4Cl (X_3, effect of X_3 = -6.02), peptone (X_4, effect of X_4 = 4.63) and KH_2PO_4 (X_7, effect of X_7 = -4.46) had a negative significant effect in protopectinase production. Among the significant variables identified, fructose and soybean meal were excluded for the economic and stable production of protopectinase, as yeast extracts is one of the most

Table 3. The Box-Behnken design arrangement and responses.

Trial number	Pattern	Sucrose (g/L)	Yeast extract (g/L)	Ammonium acetate (g/L)	PPase (U/ml)	
					Actual	Predicted
1	−−0	30	6	13	9.70	10.31
2	−+0	30	20	13	11.50	9.23
3	+−0	150	6	13	30.32	32.59
4	++0	150	20	13	17.64	17.03
5	0−−	90	6	6	41.02	39.345
6	0−+	90	6	20	25.16	23.955
7	0+−	90	20	6	29.32	30.525
8	0++	90	20	20	14.46	16.135
9	−0−	30	13	6	29.12	30.185
10	+0−	150	13	6	43.60	43.005
11	−0+	30	13	20	12.48	13.075
12	+0+	150	13	20	31.40	30.335
13	000	90	13	13	36.46	31.047
14	000	90	13	13	30.32	31.047
15	000	90	13	13	26.36	31.047

Table 4. Analysis of variance (ANOVA) for regression model.

Source	Sum of square	Degree of freedom	Mean square	F-value	Prob>F
Model	1520.215	9	168.913	11.3610	0.0007
Lack-of-fit	22.542	3	7.514	0.2901	0.8330
Pure error	51.798	2	25.899		
Corrected total	1594.554	14			

R^2 is 0.9530, Adj R^2 is 0.870 and R is 0.976.

expensive nutrients and soybean meal is not a consistent component. All other insignificant variables were neglected, and the optimum levels of the three variables, (sucrose, yeast extracts and ammonium acetate) were further investigated by an RSM design.

Optimization of significant medium components using RSM

Using the Box-Behnken design, fifteen experiments, including three center points were carried out with three variables and at three levels (-1, 0, 1). The design matrix of the variables together with the experimental responses is shown in Table 3. The ANOVA analysis of the optimization study indicated that the model terms, X_1, X_2 and X_3 were significant (P<0.05). The linear effects of sucrose and ammonium acetate (P<0.01) were determined to be similar effect and more significant than the effects of the yeast extracts. These results indicate that the concentration of the sucrose bears a direct relationship with protopectinase production. The interactions between variables were not significant, as were shown by the low P-value (P>0.05) for the interactive terms.

The regression equation coefficient was calculated, and the data was fitted to a second-order polynomial equation. The response, protopectinase production (Y) by *A. terreus*, can be expressed in terms of the following regression equation:

$$Y = 31.047 + 7.52X_1 - 4.16X_2 - 7.445X_3 - 3.62X_1X_2 + 1.11X_1X_3 + 0.25X_2X_3 - 6.048X_1^2 - 7.708X_2^2 + 4.152X_3^2$$

Where, Y is the predicted protopectinase production; X_1 is the coded of sucrose; X_2 is the coded yeast extracts and X_3 is the coded ammonium acetate. The relationship between the actual experimental data and the fitted values is shown by the regression model. A good fit of the data is indicated by R^2 that is close to 1.0 and the closer the value is to 1.0, the better the fit. The value of R^2 (0.97) in Table 4 suggests that 97% of the response variability can be explained by the model. The lack-of-fit measures the failure of the model to represent data in the experimental domain at points, which are not included in the regression (Rastogi and Rashmi, 1999), so it is clear that the value of lack-of-fit for regression model (Prob> F= 0.8330 > 0.05) is not significant.

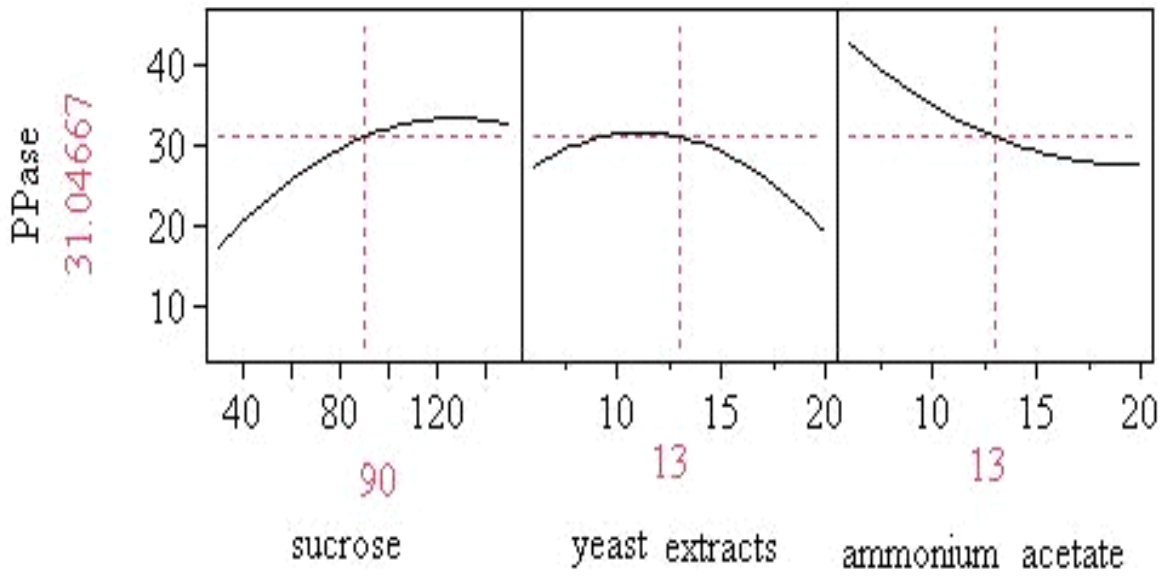

Figure 1. Prediction profiler of variable factors on protopectinase production.

Furthermore, the high F-value (11.3610), non-significant lack of fit, the P-values for the model (<0.0007) and lack of fit (0.8330) all suggested that the obtained experimental data was a good fit with the model, the model was adequate for predicting protopectinase production under any combination of values of the variables.

When compared with the traditional trial-and-error method, the prediction profiler provides an efficient and visual way of changing one variable while holding others constant to investigate the individual effects on the responses. It indicates how each variable influences the responses in the specified range, and the trends can be seen in prediction profiler. Figure 1 shows that sucrose and yeast extracts had significant positive effects on protopectinase production, but ammonium was in opposition to them. Furthermore, sucrose had more influence than yeast extracts. With the sucrose concentration increasing, protopectinase activity will increase gradually and reach the maximum value of 45.74 U/ml at 129.72 g/L of sucrose. It showed that sucrose was at high level, and high protopectinase production could be gotten. It is evident from Figure 1 that an increase in the concentration of yeast extracts causes protopectinase production to increase the maximum value of protopectinase production (31.61 U/m), first and decrease later, and the optimum concentration of yeast extracts is 11.13 g/L. However, with the ammonium acetate concentration increase, protopectinase activity will decrease gradually and the low level of ammonium acetate is suitable for protopectinase production.

Except for the individual effect contributed by each main variable, the responses are also influenced by the interaction variables, and the effects contributed by these interactions can be observed from the response surface plots generated by the contour profiler. Through these three-dimensional plots and their respective contour plots, it is very easy and convenient to understand simultaneously the interactions between two variables and also to locate their optimum levels. Figure 2 depicts the three-dimensional plot and its respective contour plot showing the effects of sucrose and yeast extracts on protopectinase production, while other variable, ammonium acetate, was fixed at the 13 g/L.

Yeast extracts contain a lot of vitamins, minerals and amino acids, which are usually used as growth stimulants or growth factors for microbial. Sucrose provides carbon source and energy for microbial growth, but high concentration of sucrose inhibits microbial metabolic production. As can be seen from Figure 2, an increase in yeast extracts resulted in an increase at first and a decrease later in protopectinase production. Protopectinase production was found to increase with an increase in sucrose until the toppoint of response surface was reached. The ranges of sucrose and yeast extracts for the highest protopectinase production were 110 to 140 and 10 to 12 g/L. The result shows that appropriate concentration of yeast extracts, and sucrose not only fulfilled protopectinase production, but also decreased production cost.

Figure 3 shows the interaction between sucrose and ammonium acetate while keeping yeast extracts at a zero level. The result demonstrates that with an increase in

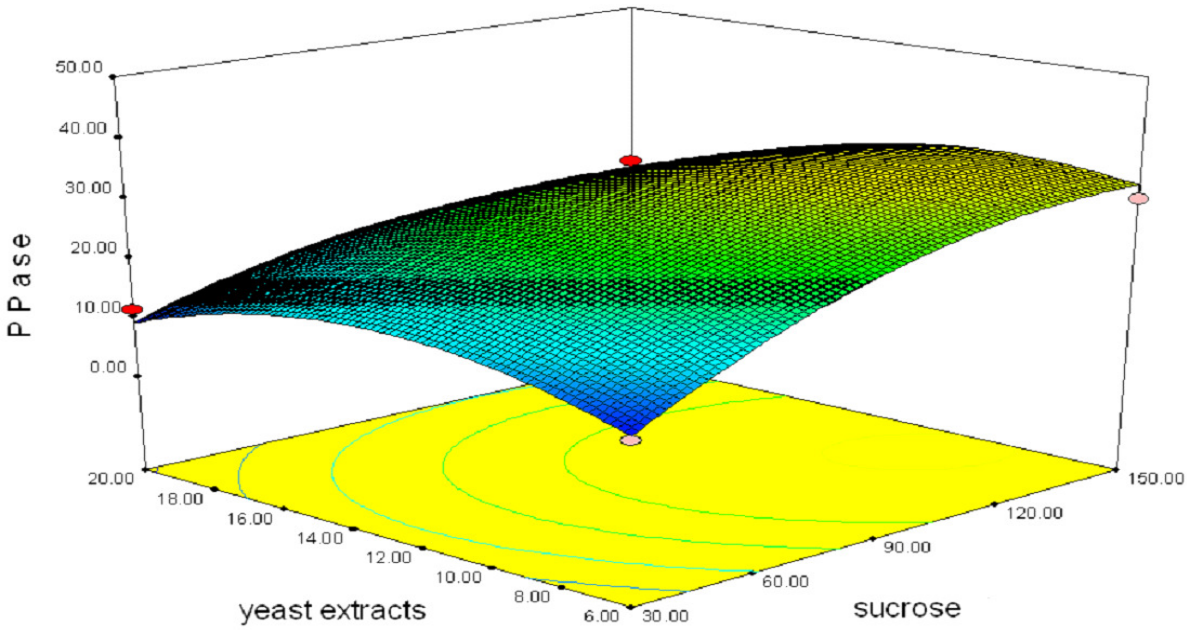

Figure 2. A 3-dimensional response surface and 2-dimensional contour plot of protopectinase production as a function of sucrose and yeast extracts at fixed ammonium acetate.

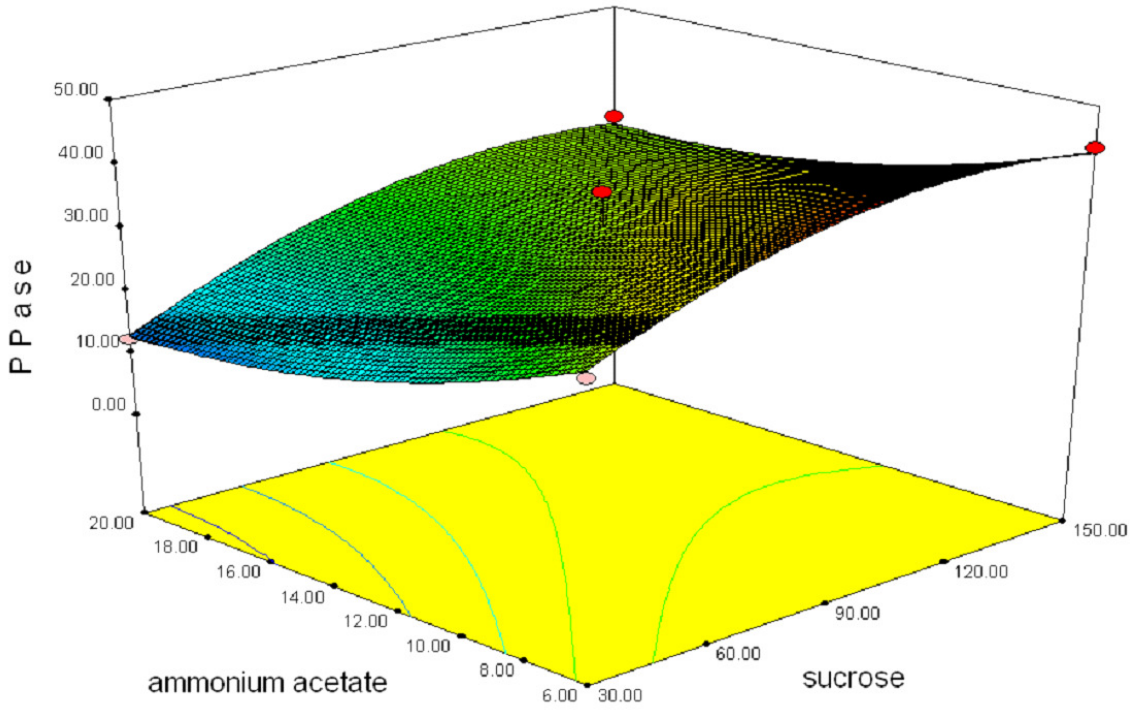

Figure 3. A 3-dimensional response surface and 2-dimensional contour plot of protopectinase production as a function of sucrose and ammonium acetate at fixed yeast extracts.

sucrose and a decrease in ammonium acetate to 120 and 13 g/L, respectively protopectinase production increased to 33.77 U/ml.

The interactive effect of yeast extracts and ammonium acetate was demonstrated in Figure 4, where sucrose was kept at a zero level. It is evident that with increasing ammonium acetate, protopectinase production steadily decreased, but the yeast extracts was not so. Figure 4

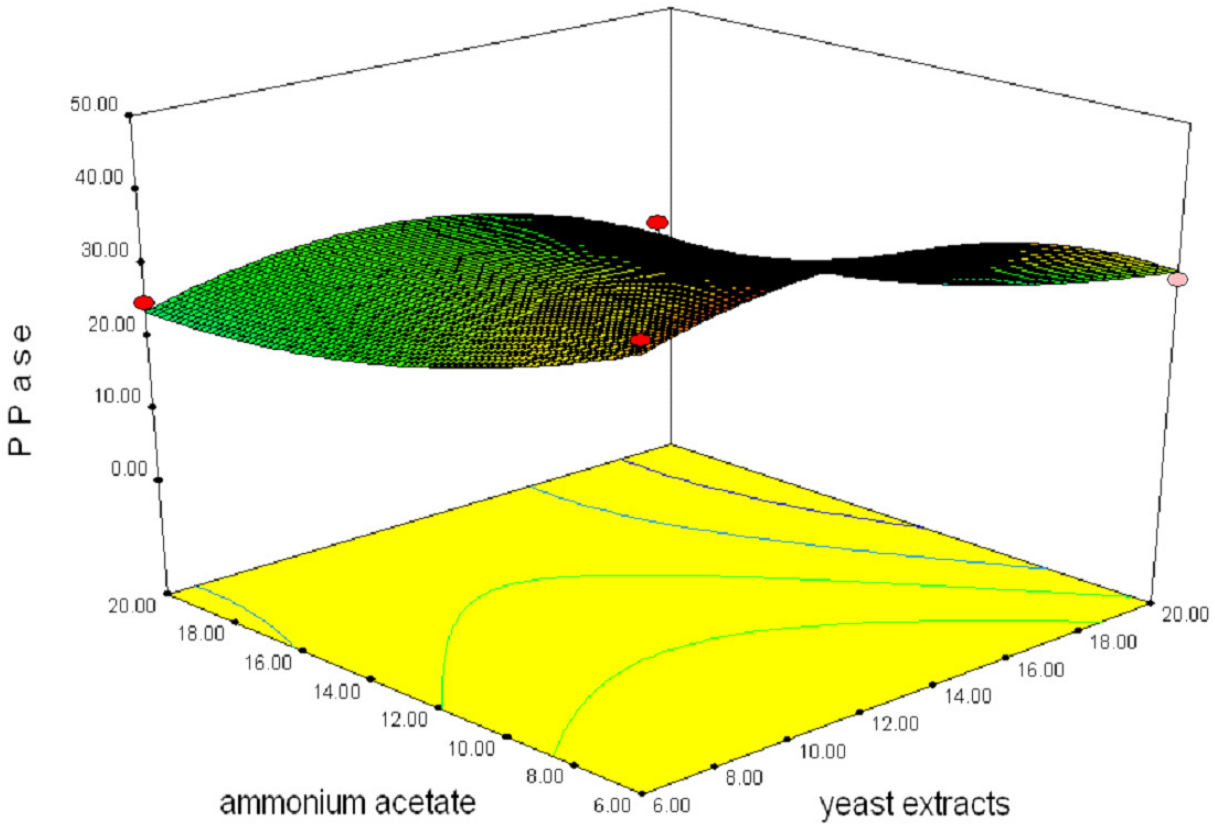

Figure 4. A 3-dimensional response surface and 2-dimensional contour plot of protopectinase production as a function of yeast extracts and ammonium acetate at fixed sucrose.

indicates that the maximum amount of protopectinase production (39.87 U/ml) could be obtained in a range of 9 to 12 g/L yeast extracts and 6 to 8 g/L ammonium acetate.

By solving the inverse matrix (from the regression equation) using JMP software, the maximum predicted protopectinase production of 45.739 U/ml was obtained. In this situation, the optimum sucrose, yeast extracts and ammonium acetate are 129.72, 9.91 and 6.0 g/L, respectively. According to the optimal medium, a verified experiment was carried out, and the actual experimental data was 41.30 U/ml. A comparison between the actual experimental data and predicted data (45.74 U/ml) was analyzed by using the SPSS software (version 13.0, SPPS Inc.). The result shows that experimental value is in good agreement with the predicted one, and suggested that this model is very satisfactory and accurate.

In the last decades, statistical experimental methods have been applied to media optimization for industrial purposes. As a favorite statistical experimental method, response surface methodology is suitable for describing a near optimum region and thus for exactly investigating conditions for a multifactorial system, which allows for the reduction of the number of experiments without neglecting the interaction among the parameters. In this study, an attempt was made to optimize medium compositions for maximal protopectinase production due to the increasing economic relevance of protopecatinase. The eleven variables were tested using the Plackett–Burman design, and three variables (sucrose, yeast extracts and ammonium acetate) exerted significant effects on protease production.

The optimal concentration ranges of the three factors were optimized subsequently using central composite design in response surface methodology. As a result, a quadratic model was found to fit for protopectinase production, and the optimal medium composition was determined as follows (g/L): sucrose 129.7, yeast extracts 9.90, ammonium acetate 6 and protopectinase activity reached 41.30 U/ml when compared with the predicted value of 45.74 U/ml. A successful and significant improvement (3.58-fold) in the production of protopectinase by *A. terreus* was accomplished using cheap carbon and nitrogen sources. The optimized medium established in this work might result in a significant reduction in the cost of medium constituents.

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