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Statistical Optimization of the Medium Components for the Production of Protopectinases by *Bacillus subtilis*

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Abstract In this study *Bacillus subtilis* PTCC 1023 was used for the production of protopectinase using soybean based media. The use of isolated soybean protein (ISP) and soybean flour resulted in similar protopectinase production and growth rates. The effect of medium composition on protopectinase production was studied using central composite design (CCD) methodology. The change in the concentration of ISP (1-7%), glucose (0-10%), and phosphate (0.1-0.3 M) was found to affect the protopectinase activity (response variable) after 24 hr of cultivation. In the range studied, ISP and glucose had a negative effect on the response variable, whereas phosphate had a positive effect. A statistically significant interaction was identified between phosphate and ISP, suggesting that correct optimization of medium formulation in this case can only be obtained using factorial design of experiments. Protopectinase activity exceeding 215 U/mL was obtained in a medium containing 4% ISP, 0.3M phosphate, and no added sugar.

Keywords: pectinolytic enzyme, protopectinase, *Bacillus subtilis*, isolated soybean protein, central composite design

Introduction

Protopectinases (PPases) are a heterogeneous group of enzymes with different catalytic activities that catalyzes the solubilization of protopectin, the insoluble form of pectin present in plant tissues. They are classified into 2 types depending on their reaction mechanism (1-5). The enzymes that cleave the polygalacturonic acid region in protopectin are called A-type PPases, whereas those that cleave the polysaccharide chains that may connect the polygalacturonic acid chain and cell wall constituents are called B-type PPases, an example of which is PPase-C. Furthermore A-type PPases are divided into 2 types (A1 and A2): A1-type PPase has polygalacturonic acid hydrolyzing activity, and A2-type PPase has polygalacturonic acid transeliminase activity. A2-type PPases are further divided into those which are active on polygalacturonic acid (PPase-N) and those which are active on methoxylated polygalacturonic acid (PPase-R).

Protopectinases are used in various industries such as fruit juice extraction and clarification (1,2,6,7) and the refinement of cotton fiber (5,8). Amongst other uses, they have also been employed in the production of single-cell foods (3,5,7), in waste water treatment (2,6), vegetable oil extraction (2,6), tea and coffee fermentation (2,6,7), and production of alcoholic beverages (2).

PPases have been found in the culture filtrate of some microorganisms including *Tramete* (3,4), *Bacillus* (5,8-12), *Geotrichum* (13), *Trichosporon* (3,14), and *Aspergillus* (8,15,16).

The medium based on soybean flour is reported to be effective in producing larger amounts of PPases compared with conventional media using *Bacillus subtilis* (3,5,8,16, 17). It has been reported that the presence of phosphate in the media is essential to ensure high PPase productivity by *B. subtilis* (5,17). Phosphate is actually needed for the induction of PPase (8), although high concentration of this nutrient has an inhibitory effect (5). It has also been reported that the production of different PPases is inhibited by the addition of a rapidly metabolized carbon sources such as glucose and fructose to the soybean based media (5,8). Initial soybean flour concentration has also been found to affect the production of PPases enzyme (8).

The previous studies that have used soya based media to produce protopectinases have employed defatted soybean. Furthermore the effect of medium formulation on proptopectinase production by *B. subtilis* has been studied using one factor at a time experimental methodology. However, such methodology does not enable the identification of interactions between factors, which may well be important. The only experimental design methodology that enables all interaction between factors to be identified is full factorial methodology. However, higher order interactions are rarely significant and therefore a more efficient approach would be the use of fractional factorial experimental designs which allow the estimation of the effect of factors on the response variable together with first order interactions (18).

One such fractional factorial experimental methodology is response surface method (RSM). This methodology has been employed to optimize the mineral formulation and pH for the production of protopectinases using *Geotrichum klebahnii* (19). It has also been extensively employed in medium optimization for the production of other fermentation metabolites (20-22).

The objective of the present study was 2-fold: 1) comparison of the type of soybean (soybean flour or isolated soybean protein) employed in a soybean based media on the production of protopectinases by *B. subtilis*

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PTCC 1023; 2) study of the effect of change in concentration of glucose, isolated soybean protein (ISP), and phosphate in the media on the production of protopectinase by the aforementioned *Bacillus* strain using a response surface methodology called central composite design (CCD).

Materials and Methods

Chemicals D-Galacturonic acid monohydrate (GALA) and carbazole were from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals employed were commercially available and of analytical grade.

Microorganism and culture media *Bacillus subtilis* PTCC 1023 (Persian Type Culture Collection, IRSOT, Iran) was used throughout this investigation. Strain maintenance was performed at 4°C on agar slants containing 1% glucose, 0.5% peptone, 0.5% meat extract, 0.5% NaCl. The media used for the production of protopectinases in the preliminary studies had the following formulation: 4% low fat soybean flour (SF) (Soya Sun Co., Tehran, Iran) or isolated soybean protein (ISP) (Soypro Co., Shandong, China), 2.8% K_2HPO_4, and 1.2% KH_2PO_4. The composition of low-fat soybean flour and ISP used in the present study is presented in Table 1. In the preliminary studies the media, excluding glucose, was autoclaved at 121°C (15 psi) for 15 or 45 min. Glucose solution was sterilized at 121°C (15 psi) for 15 min separately, to prevent browning reactions, and then mixed aseptically with the rest of the heat treated media. In further studies the length of heat treatment the media, excluding glucose, was fixed at 45 min; and the concentration of the 3 components was varied according to CCD experiments explained below.

Shake flask cultivations All the experiments were carried out in 250-mL Erlenmeyer flasks containing 50 mL of soybean based medium, for 24 hr at 37°C, on a rotary shaker at 120 rpm. Each run was performed at least 3 times, and the enzyme titers reported are the mean of all the replicates.

Analytical techniques To observe the effect of cultivation conditions on cell growth, insoluble materials have to be removed from the fermentation media. Therefore in runs aimed at obtaining data on the growth of biomass, after autoclave treatment for 45 min, the medium was centrifuged at 6,000×g for 20 min and then inoculated prior to the fermentation runs. In these runs, the cell dry weight was determined by drying the samples, after centrifugations, in an oven at 85-90°C for 48 hr and weighing the resulting dried biomass.

Reducing sugar concentration in culture medium was measured by phenol-H_2SO_4 method (23).

Total pectin-releasing activity in the samples of fermentation broth was assayed by measuring the pectic substances liberated from protopectin by carbazole-H_2SO_4 method (24). The reaction was performed at 37°C for 30 min in a mixture containing 10 mg protopectin, 2 mL of acetate buffer containing 50 μg/mL of bovine serum albumin, and 0.5 mL of enzyme containing sample, in a total volume of 2.5 mL. One unit of enzyme activity was defined as the activity that liberates soluble pectic substances corresponding to 1 μmol of D-galacturonic acid/mL of reaction mixture at 37°C in 30 min (24). The protopectin used as a substrate was prepared by the following procedure: the albedo layer of lemon peel was scooped out, pooled, washed with 2% sodium hexametaphosphate solution (pH=4) until the soluble substances that reacted with carbazole-H_2SO_4 were washed off, and then the remaining material was freeze-dried (4,16,25).

Table 1. Low fat soybean flour (SF) and isolated soybean protein (ISP) analysis (wt%)

	Chemical analysis[1)]	
Material	Low fat SF	ISP
Moisture	8.63	4
Protein	48-50	90
Fat	6	0.2
Fiber	16.69	0.2
Carbohydrate	27.72	5.6

[1)]Analysis provided by the manufacturers.

Experimental design and optimization Statistical methodology was used to study the effect different components in a soybean based media on the enzyme production by *B. subtilis*. The effect of type of soybean on the production of protopectinase was assessed using 2 factors analysis of variance (ANOVA), with the second factor being taken as the time of cultivation. The effect of concentration of carbon source (ISP), nitrogen source, and phosphorous source was investigated using CCD.

The CCD is one of the most important experimental designs used in process optimization studies (21). In this study a Box-Wilson CCD with 3 levels for each of the 3 factors (ISP, phosphate, and glucose) was used to evaluate the quadratic effects and two-way interactions among these variables. In CCD, the variable levels X_i were coded as x_i according to Eq. 1:

$$x_i=(X_i-X_0)/\Delta X_i,\ i=1,2,3,\ \ldots,\ k \qquad (1)$$

Where x_i and X_i are the dimensionless (codified) value and the actual value of an independent variable, respectively, X_0 is the real value of an independent variable at center point, and ΔX_i is the step change.

These 3 factors levels (denoted −1 for low level, 0 for middle level, and +1 for high level) are presented in Table 2. It should mentioned the high level for the factor phosphate concentration was set at 0.3 M, since concentrations of phosphate equal to and higher than 0.4 M resulted in the formation of precipitate in the media which interfered with the enzyme activity measurements.

Table 2. Level of the 3 independent variables used in the CCD experiments

Variable	0	+1	-1
(X_1) Glucose (g/L)	5	10	0
(X_2) ISP[1)] (g/L)	40	70	10
(X_3) Phosphate (mol/L)	0.2	0.3	0.1

[1)]Isolated soybean protein.

RSM was used to analyze the data from this experimental design. The second-degree model used to fit the response to the independent variables is shown in Eq. 2:

$$Y=\beta_0+\sum_{i=1}^{k}\beta_i x_i+\sum_{j=2}^{k}\sum_{i=1}^{j-1}\beta_{ij}x_i x_j+\sum_{i=1}^{k}\beta_{ii}x_i^2 \quad (2)$$

where Y is the predicted response, x_i and x_j are the input variables that influence the response variable Y, β_0 is the intercept, β_i is the *i*th lineal coefficient, β_{ii} is the *i*th quadratic coefficient, and β_{ij} is the *ij*th interaction coefficient (22).

All the experimental runs were carried out at least 3 times with the order of the runs being randomized. Statistical and numerical analyses were carried out by means of the ANOVA and regression analysis, using the statistical software package Minitab, ver 14 (Minitab Inc., State College, PA, USA).

Results and Discussion

Preliminary studies Previous reports on the microbial production of protopectinases have used only defatted soybean as the C&N-source in the cultivation media. In this study it was decided to first compare the use of isolated soybean protein (ISP), and low fat soy bean flour (SF) as C&N source, on the production of protopectinase and growth of *B. subtilis*. Figure 1 compares production of pectin-releasing enzymes using media containing 4% low-fat SF or 4% ISP. The results show that in the first stages of fermentation low-fat SF results in higher enzyme titers, but this trend is reversed in the final stages of the fermentation. To see whether the difference indicated in the data in Fig. 1 for the 2 soy types is statistically significant or not, the data were subjected to a two-way ANOVA analysis (Table 3). The results of the analysis showed no significant difference between the enzyme titers for the 2 different soy during the course of the 24 hr fermentation at $p<0.05$, although as expected time did have a significant effect on enzyme titers.

The growth curve of *B. subtilis* when grown on media containing 4% SF or ISP is presented in Fig. 2. Consistently higher dry cell weights throughout the fermentation have been obtained when ISP has been used as the main substrate, although the difference is not very considerable. Comparison of the enzyme production (Fig. 1) and growth curve (Fig. 2) shows that protopectinase production by *B. subtilis* is growth dependent. This has also been previously reported by Matsumoto *et al*. (8).

Figure 3 shows the time course of the variation of reducing sugar content in these 2 culture media during the fermentation. The reducing sugar content decreased during initial 10 hr of fermentation and reached constant values of around 3 and 1 g/L in the case of runs with low-fat SF and ISP, respectively. This result indicates reducing sugars extracted from soybean flour were metabolized as nutrients by microorganisms, and termination of cell growth was not caused by deficiency of reducing sugars but of other nutrients or accumulation of growth inhibitors, which is in agreement with results reported by Matsumoto *et al*. (8), who varied the initial reducing sugar concentration by changing the concentration of soybean flour in their experiments. However, unlike that study in which higher initial reducing sugar concentration yielded higher final dry cell weight, in the present study although in the runs with

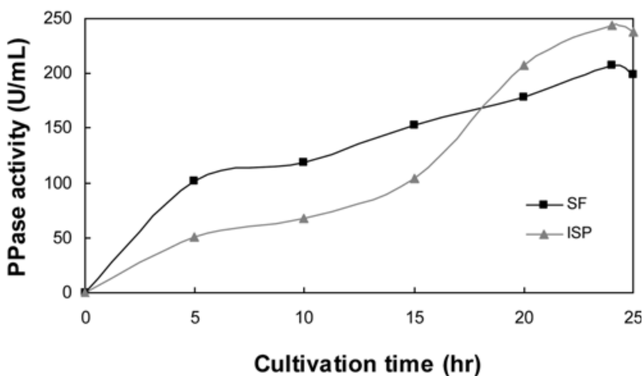

Fig. 1. PPase activity during fermentation of soybean based media with *B. subtilis*. Media composition: 4% soybean flour or ISP, 2.8% K_2HPO_4, and 1.2% KH_2PO_4.

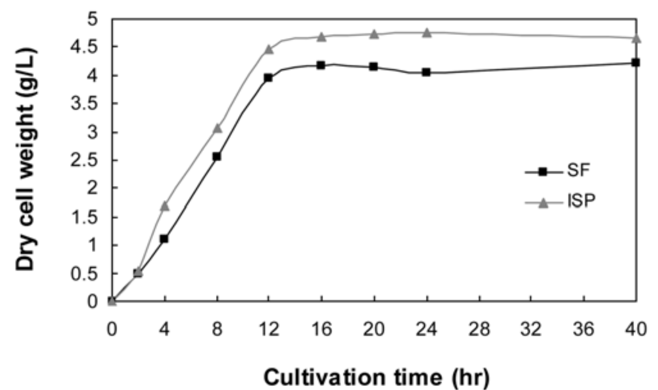

Fig. 2. Dry cell weight during fermentation of soybean based media with *B. subtilis*. Media composition: 4% soybean flour or ISP, 2.8% K_2HPO_4, and 1.2% KH_2PO_4.

Table 3. Results of two-way ANOVA for enzyme activity as a function of time (C1) and type of carbon source (C2)[1)]

Source	DF	SS	MS	F-value	*p*-value
C1	5	0.431009	0.0862018	30.89	0.000
C2	1	0.000052	0.0000523	0.02	0.892
Interaction	5	0.050597	0.0101194	3.63	0.014
Error	24	0.066981	0.0027909		
Total	35	0.548639			

[1)]DF, degrees of freedom; SS, sum of square; MS, mean square; S=0.05283, R^2=0.8779, R^2(adj)=0.8000.

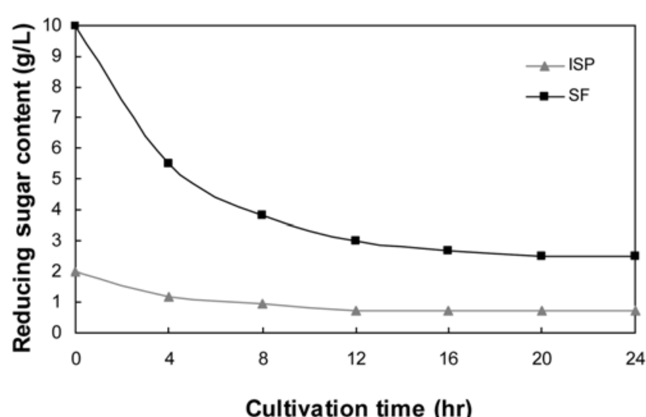

Fig. 3. Reducing sugar content during fermentation of soybean based media with *B. subtilis*. Media composition: 4% soybean flour or ISP, 2.8% K_2HPO_4, and 1.2% KH_2PO_4.

soybean flour the initial reducing sugar concentration was around 5 times more than the runs with ISP, but nevertheless the final dry cell weight was actually slightly higher in the latter case. This seems to suggest that in the case of runs with ISP, protein instead of reducing sugar has been employed by the bacterial culture as C source.

In the next stage of the study, 2 different periods of heat treatment (15 and 45 min) were applied to medium containing ISP. Larger amount of PPase titers were obtained after 24 hr of fermentation with the medium autoclaved for 45 min (about 236 U/mL) compared with that autoclaved for 15 min (about 78 U/mL). This result indicates that the nutrients contained in soybean are degraded and extracted into the medium by autoclaving for a sufficiently long time length. Similar results were obtained by Matsumoto *et al.* (8) for defatted SF. For this reason, in the rest of the study, the higher heat treatment time was employed to prepare the medium.

Effect of medium composition on PPase activity The effect of concentration of ISP, glucose, and phosphate on the activity of protopectinase at the end of 24 hr fermentation was studied using central composite design methodology. The design matrix containing the value of the response variable (protopectinase activity in units of U/mL) is presented in Table 4. One repeat was included at the center of the design, and the total number of this design was 15. The results of the experiment with CCD were fitted with a second-order polynomial function.

The statistical significance of the second-order model

Table 4. CCD Design matrix: Independent variables (coded units) and values of the enzyme activity (Enz. Act.) (mean±SD)

Std. order	ISP	Phosphate	Glucose	Enz.Act. (U/mL)
1	-1	-1	-1	163.5(±3.3)
2	+1	-1	-1	116.0(±2.0)
3	-1	+1	-1	174.3(±4.4)
4	+1	+1	-1	205.0(±1.8)
5	-1	-1	+1	126.4(±2.6)
6	+1	-1	+1	77.8(±2.7)
7	-1	+1	+1	170(±3.0)
8	+1	+1	+1	154.8(±2.4)
9	-1	0	0	161.9(±2.4)
10	+1	0	0	115.6(±2.9)
11	0	-1	0	99.3(±2.5)
12	0	+1	0	203.6(±2.7)
13	0	0	-1	221.1(±3.7)
14	0	0	+1	119.9(±2.5)
15	0	0	0	143.2(±2.7)

equation was checked by an F-test. It was found that it adequately approximated to the experimental data at the 5% probability level. The coefficient of determination R^2, was calculated to be 0.9064, which showed that the regression model was suitable for describing protopectinase production. The equation obtained for PPase production was:

$$Y=151.58-23.39x_1-12.36x_2+32.16x_3+14.67x_1^2-17.09x_2^2+14.34x_2x_3 \quad (3)$$

Where x_1, x_2, and x_3 represent the codified levels of glucose, ISP, and phosphates, respectively.

Table 5 shows the results of ANOVA, in which the model was tested for adequacy. The regression model for protopectinase production was highly significant (p<0.001). At the other hand, the lack of fit of the regression model was not significant, and Fischer's F-test demonstrated a high significance (p<0.05) for the regression. The significance of each coefficient was determined by t-values and p-values which are presented in Table 6. According to the magnitude of the t-test and the p-values presented in this Table, it can be concluded that the first order and second order main effects of glucose and ISP and the first order main effects of phosphate are significant. This equation also indicates that the interaction terms of ISP×phosphate is significant whereas the 2 other first order interaction terms ISP×glucose and phosphate×glucose are not significant.

Table 5. Analysis of variance for the second degree polynomial model

Source	DF	Sq SS	Adj SS	Adj MS	F-value	p-value
Regression	9	125.439	125.439	20.907	24.43	0.000
Linear	3	111.644	111.644	37.215	43.48	0.000
Square	3	5.915	5.915	5.915	6.91	0.015
Interaction	3	7.880	7.880	3.940	4.6	0.021
Residual error	20	19.686	19.686	0.856		
Lack-of-fit	5	4.929	4.929	0.616	0.63	0.744
Pure error	15	14.757	14.757	0.984		
Total	29	145.125				

Table 6. Final estimated regression coefficients

Model term	Parameter estimate	Standard error	Computed t-value	p-value
Intercept	151.58	5.114	29.639	0.000
x_1	-23.39	3.189	10.084	0.000
x_2	-12.36	3.189	-3.876	0.000
x_3	32.16	3.189	10.084	0.000
x_1^2	14.67	6.027	2.434	0.020
x_2^2	-17.09	6.027	-2.835	0.007
x_2x_3	14.34	3.566	4.021	0.000

The negative sign of the coefficients for ISP and glucose in Table 6 indicate negative influence of these variables, in the range studied, on protopectinase production by *B. subtilis*. This relationship is graphically illustrated in Fig. 4A, and shows that the negative influence of ISP concentration on enzyme activity is only evident when the concentration of ISP is increased from 4 to 7%, whereas similar enzyme activities have been obtained with 1 and 4% ISP. Matsumoto *et al*. (8), using concentrations of 1, 4, and 7% defatted SF, found that the maximum protopectinase activity by *B. subtilis* after 24 hr is obtained at 4% concentration; they attributed the lower enzyme activity at 7% soybean flour concentration to the negative influence of extracted excess nutrients on the enzyme productivity of the bacterial cells. The results of the present study shows the same negative influence of ISP concentration when concentration is increased to 7%, but unlike the case with defatted SF there is no appreciable difference in the enzyme productivity of the cells for 1 and 4% ISP concentration.

The negative effect of glucose on protopectinase production (Fig. 4B) has also been observed previously and attributed to catabolite repression (21). According to Sakamoto *et al.* (5) PPase-C and -N production is repressed by addition of rapidly metabolizable carbon source such as glucose whereas it has the opposite effect on PPase-R production. These authors report that the maximum PPase-R activity is obtained at glucose concentration of 0.5%. The method used in the present study to quantify protopectinase level does not distinguish between the different types of protopectinases. It is also worth noting that in the run at the middle level of glucose the initial reducing sugar concentration was around 3 g/L lower than the corresponding value for runs with low fat SF, whereas in the runs at the high level it was around 2 g/L lower, but in both cases the enzyme productivity was significantly higher in the runs with low fat SF. The possible explanation for this is that the reducing sugar in the runs with low fat SF was mainly in the form of oligosaccharides and not the catabolite repressor glucose.

The positive sign for the coefficient of phosphate in Table 6, and the corresponding graphical relationship presented in Fig. 4C, shows that increasing phosphate concentration, in the range 0.1-0.3 M, increases protopectinase production by *B. subtilis*. Sakamoto *et al.* (16) found that protopectinase is only produced by *B. subtilis* under high phosphate concentrations. Sakamoto *et al.* (17) identified the genetic mechanism for phosphate induction in *B. subtilis*. These authors furthermore found that increasing the concentration of KH_2PO_4 in the range 1.0-5% (corresponding to 73-370 mM KH_2PO_4) had an enhancing effect on PPase-R production although it suppressed growth. Sakamoto *et al.* (5) found that increase in phosphate concentration up to around 0.3 and 0.5 M increased PPase-C and PPase-R activity, respectively by *B. subtilis* whereas further increases lead to decrease in the activity of both these enzymes. On the other hand, increase in phosphate concentration up to 0.1 M increased the corresponding PPase-N activity but

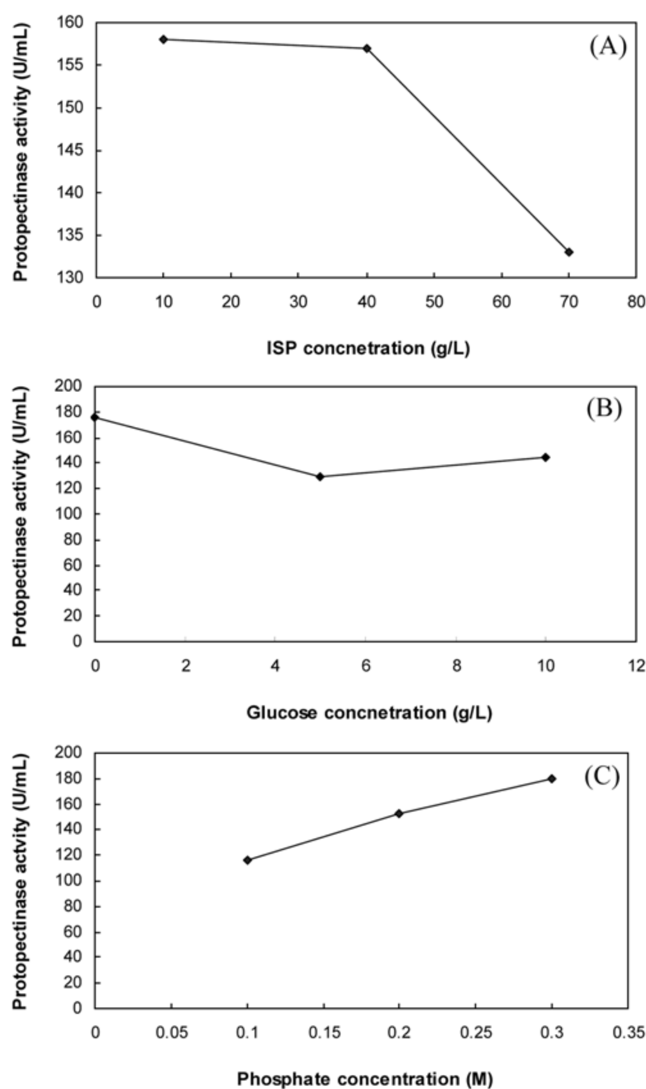

Fig. 4. The relationship between protopectinase activity (response variable) and independent variables based on the central composite experimental results. ISP concentration (A), glucose concentration (B), and phosphate concentration (C).

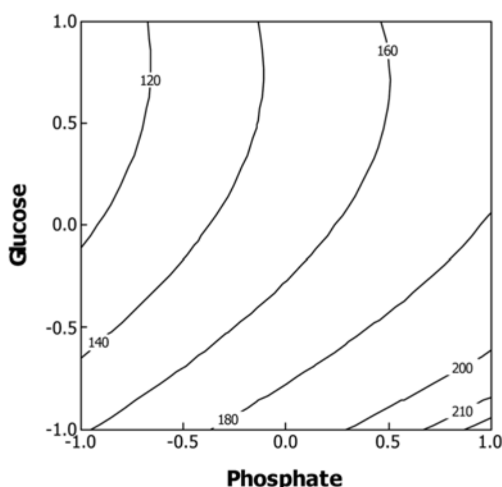

Fig. 5. Contour plot of the combined effect of glucose and phosphate (coded units) on protopectinase production (U/mL) by *B. subtilis* PTCC 1023. Fixed level: ISP (middle level).

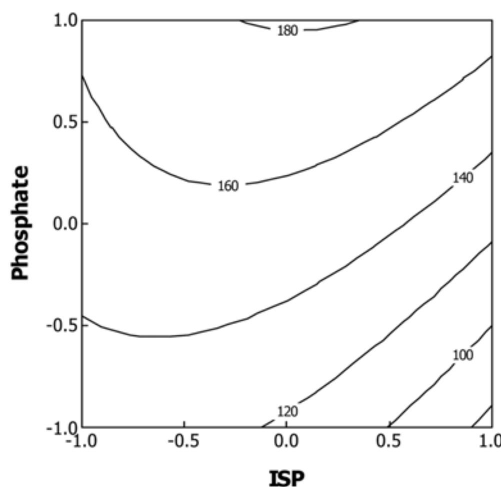

Fig. 7. Contour plot of the combined effect of ISP and phosphate (coded units) on protopectinase production (U/mL) by *B. subtilis* PTCC 1023. Fixed level: glucose (middle level).

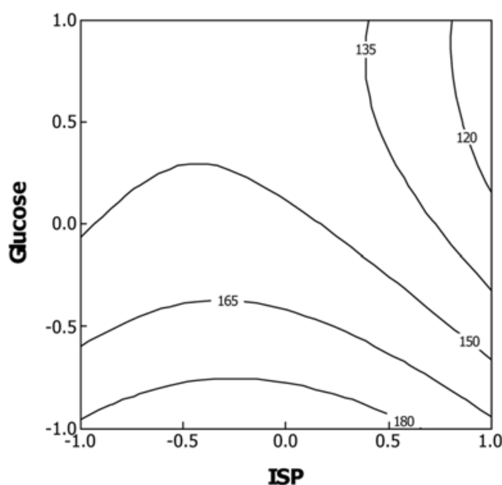

Fig. 6. Contour plot of the combined effect of glucose and ISP (coded units) on protopectinase production (U/mL) by *B. subtilis* PTCC 1023. Fixed level:phsphate (middle level).

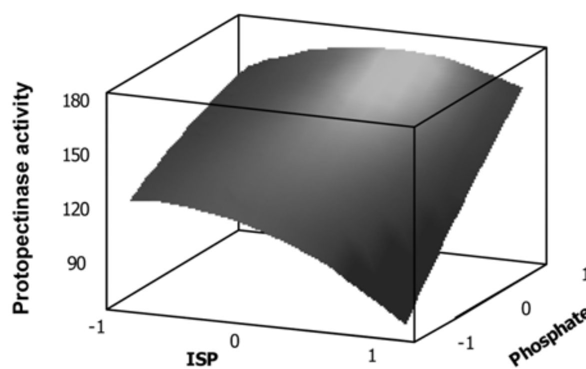

Fig. 8. Response surface plot of protopectinase concentration (U/mL) produced by *B. subtilis* after 24 hr cultivations as a function of ISP and phosphate concentrations (in coded units). Fixed level: glucose (middle level).

further increases suppressed it. In the present study increase in phosphate concentration up to 0.3 M lead to increase in PPase activity; runs with higher phosphate concentrations could not be carried out. Also, as mentioned before the method employed in the present study did not distinguish between the different types of PPases.

The 2D contour plots for the optimization of medium components for the production of protopectinase by *B. subtilis* are presented in Fig. 5-7. Each Fig. presents the effect of 2 variables on the production of protopectinase, with the other variable being held constant at the zero level. The Figs. show that the maximum productivity of >215 U/mL protopectinase activity is obtained at low level of glucose concentration (0 g/L), middle level of ISP (4%), and high level of phosphate (0.3 M).

The 3D surface response plot for the effect of ISP and phosphate on protopectinase activity is presented in Fig. 8. The sharp twist in the response plane illustrates the interaction between these 2 parameters (18). Observation of Fig. 8 shows that the positive effect of phosphate on protopectinase activity becomes more pronounced with increase in the concentration of ISP, in the range 1-7%. Also the negative influence of ISP on protopectinase activity becomes much less pronounced with increase in the concentration of phosphate. This suggests that probably relatively high phosphate concentrations mask the inhibitory effect of extracted nutrients on protopectinase activity, which was observed at high carbon source concentration (7%) in this work for ISP and previously by Matsumoto *et al*. (8) with defatted SF.

Based on the results obtained in the present study the following conclusions can be made:

The use of soybean flour or ISP as carbon source did not lead to statistically significant difference in protopectinase production although the growth yield on reducing sugar was significantly higher with ISP, suggesting that protein can be as effectively used as sugars by *B. subtilis* PTCC 1023 for growth and protopectinase production.

CCD methodology was used to study the effect of change in ISP, phosphate, and added glucose concentration on protopectinase production, and a second order polynomial

equation was developed for predicting protopectinase activity at different concentration of these 3 components. ISP and glucose were found to have a negative influence on protopectinase production whereas phosphate was found to have a positive effect. A significant interaction was detected between 2 of the independent variables, namely phosphate and ISP. Maximum protopectinase activity was obtained with media containing 4% ISP, 0.3 M phosphate, and no glucose.

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