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Optimization of Enzymatic Clarification of Sapodilla Juice: A Statistical Perspective

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Abstract Response surface methodology (RSM) was employed to establish optimum conditions for enzymatic clarification of sapodilla juice. Polygalacturonase obtained from *Streptomyces lydicus* had been purified to homogeneity and was used for the treatment. The independent variables were temperature (30–45 °C), enzyme concentration (0.5–1.5 U), and treatment time (30–90 min), whose effects on viscosity and clarity of the juice were evaluated using a Box–Behnken design. Significant regression models describing the changes of viscosity and clarity with respect to the independent variables were obtained, with the coefficient of determination, R^2, greater than 0.8. Based on response surfaces and contour plots, the optimum conditions for clarifying sapodilla juice were enzyme concentration 1.15 U, incubation temperature 34 °C, and incubation time 70 min.

Keywords *Streptomyces lydicus* · Polygalacturonase · Sapodilla juice · Enzymatic clarification · Response Surface Methodology

Introduction

Sapodilla is a nutritious, fleshy berry, with a scurfy brown peel and light brown, brownish yellow to reddish brown pulp, with a texture varying from gritty to smooth. The pulp has a very sweet pleasant flavor. Sherbets, milk shakes, ice cream, and juices can be made from fresh pulp. Crude sapodilla juice is turbid, yellowish brown in color, very viscous, and tends to settle during storage, necessitating the use of enzymes to clarify the juice [1]. Fruit juices contain colloids that may lead to fouling problem during filtration process, and these colloids are basically polysaccharides such as pectin and starch [2].

Pectinases play a key role in fruit juice industry as they can degrade pectin and can cause pectin protein complexes to flocculate. Pretreatment of juices with pectinases is performed

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to lower the amount of pectin present and to decrease the viscosity of the juice, which in turn accelerates the subsequent filtration process. Enzymatic degradation of pectic substances depends on several physicochemical factors such as treatment time, enzyme concentration, and incubation temperature and so it is necessary to optimize the levels of these parameters. Adjustment of pH of fruit juices is not practiced during pretreatment with enzymes [2], as it may affect the quality of juices and so it is necessary to select enzymes with suitable pH for a particular juice.

In common practice, optimization of a process is carried out by varying one variable at a time while fixing the other parameters at a constant level. The major disadvantage of the approach is that it does not consider interactive effects among the variables. An alternative method is Response surface methodology (RSM), which is defined as the statistical tool that uses quantitative data from appropriate experimental design to determine and simultaneously solve multivariate equations [3]. RSM reduces the number of experiments required and so it is less time-consuming than the other approaches. RSM has been used widely for optimizing process variables for fruit juice clarification [1, 2, 4].

The purpose of the present investigation was to optimize the conditions for sapodilla fruit juice clarification using purified polygalacturonase produced by a novel strain of *Streptomyces lydicus*. According to the literature and to the best of our knowledge, this is the first attempt on enzymatic clarification of sapodilla juice using polygalacturonase produced by an actinomycete strain.

Materials and Methods

Enzyme Source

The novel strain of *Streptomyces lydicus* MTCC 7505 [5] was maintained on starch casein agar slants at 4 °C. Inoculum preparation was carried out as described in the earlier report [6]. Solid-state fermentation was carried out in 250-mL Erlenmeyer flasks with 10 g of wheat bran with a particle size of 500–1000 μm. The solid substrate was moistened with a solution containing (g/L) K_2HPO_4, 4 and KH_2PO_4, 4, autoclaved for 45 min and inoculated with 1.25×10^5 colony forming units (CFU). The final moisture content of the flask was 70%. Flasks were incubated for 3 days at 30 °C. After fermentation, the fermented material was mixed with distilled water and was centrifuged for 20 min at 10,000 rpm and 4 °C. The crude enzyme was purified [7] and stored at 4 °C until use.

Enzyme Assay

Polygalacturonase activity was determined by measuring the reducing ends liberated as a result of enzyme action [8]. Reaction mixture containing 0.5 mL of suitably diluted enzyme was incubated with 0.5 mL of 0.5% polygalacturonic acid (Sigma, St. Louis, MO, USA) in 100 mM McIlvaine buffer (pH 6.0) for 30 min at 50 °C. The reaction was stopped by adding 2 mL of 100 mM borate buffer (pH 9.0) followed by 1 mL of 1% cyanoacetamide. The mixture was immersed in a boiling water bath for 10 min, cooled and read against a suitable blank at 276 nm using a spectrophotometer (Model UV PC 2401, Shimadzu Corporation, Japan). One unit (U) of polygalacturonase was defined as 1 μmol of galacturonic acid released per minute under the assay conditions.

Juice Preparation

Fresh ripe sapodilla (*Manilkara zapota*) was purchased from local market and was used for juice preparation. Five grams of peeled and deseeded sapodilla was ground in a mixer grinder to obtain a homogenous fruit pulp. Juice extraction was carried out at 60 °C for 60 min after adding distilled water. The juice was filtered and centrifuged at 10,000 rpm for 15 min and was made up to 100 mL. The pH of the juice obtained was 5.64 with a viscosity of 1.07 MPa s and a clarity of 0.048 (absorbance at 660 nm).

Enzymatic Treatment

The extracted juice was subjected to different enzyme treatment conditions. For each experimental trial, 12 mL of juice was taken and treated as given in Table 1. The incubation temperature was maintained using a water bath. After incubation, the mixture was heated for 5 min at 90 °C to inactivate the enzyme, centrifuged at 10,000 rpm for 15 min and was taken for analysis.

Viscosity

The viscosity of the clarified sapodilla juice was measured using an Ostwald viscometer. Viscosity was calculated using the equation $V_s = R_s/R_w \cdot V_w$, where, V_s is the viscosity of

Table 1 The Box–Behnken experimental design together with the responses (dependent variables) employed for enzymatic clarification of sapodilla juice.

Independent variables							Dependent variables	
	Coded variables			Uncoded variables				
Run	X_1	X_2	X_3	*A*: Time (min)	*B*: Temp. (°C)	*C*: Enzyme conc. (U)	Clarity (abs)	Viscosity (MPa s)
1	0	1	1	60	45	1.5	0.063	1.0519
2	1	0	1	90	37.5	1.5	0.014	1.0469
3	1	−1	0	90	30	1	0.023	1.0511
4	0	0	0	60	37.5	1	0.022	1.046
5	0	0	0	60	37.5	1	0.022	1.046
6	0	−1	−1	60	30	0.5	0.033	1.052
7	−1	−1	0	30	30	1	0.031	1.0498
8	−1	1	0	30	45	1	0.063	1.0538
9	0	−1	1	60	30	1.5	0.027	1.0539
10	1	0	−1	90	37.5	0.5	0.052	1.0597
11	−1	0	−1	30	37.5	0.5	0.056	1.0581
12	1	1	0	90	45	1	0.062	1.0541
13	0	0	0	60	37.5	1	0.022	1.046
14	0	1	−1	60	45	0.5	0.056	1.0538
15	0	0	0	60	37.5	1	0.022	1.046
16	0	0	0	60	37.5	1	0.022	1.046
17	−1	0	1	30	37.5	1.5	0.058	1.0581

The independent variables were analyzed at three different levels, viz, low, medium, and high coded as −1, 0, and +1 in a total of 17 runs.

juice in MPa s, R_s is the run time of juice in s, R_w is the run time of water in s, and V_w is the viscosity of water in MPa s.

Clarity

The clarity of the juice obtained was determined by measuring the absorbance at 660 nm using a spectrophotometer (Model UV PC 2401, Shimadzu Corporation, Japan). Distilled water was used as the reference.

Experimental Design and Statistical Analysis

The pH of the juice was kept constant at its natural pH and it was excluded from the experimental design. The independent variables optimized were treatment time, temperature, and enzyme concentration. A Box–Behnken design [9] was employed for optimization where each selected parameter was analyzed at three different levels, viz, low, medium, and high coded as −1, 0, and +1 in a total of 17 runs. The behavior of the system can be explained by a second-order polynomial equation (Eq. 1)

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

where, Y is the predicted response, β_0 is offset term, β_i is linear effect, β_{ii} is squared effect, β_{ij} is interaction effect, and X_i is dimensionless coded value of independent variables under study. The main effects, interaction effects, and quadratic effects were evaluated using this design and the levels of the parameters were optimized for juice clarification. The statistical software, Design Expert (version 6.0.6, Stat-Ease, Minneapolis, MN, USA), was used for data analysis.

Results and Discussion

Statistical Analysis

Statistical optimization of enzymatic clarification of sapodilla juice was conducted to identify the optimal levels of factors, which showed a predominant influence on clarification process. The levels of these factors were selected based on previous experience (Table 1). Polygalacturonase produced by *Streptomyces lydicus* had been purified to homogeneity and was used for the clarification of sapodilla juice. The optimum activity pH of the purified enzyme was 6.0 [7] and was close to the juice pH (5.64). This indicated the suitability of the polygalacturonase produced by *S. lydicus* for use in the clarification of sapodilla juice, as it is undesirable to adjust the pH of the juice.

The combinations of the three independent variables together with the responses (dependent variables) are shown in Table 1. Coefficient of determination, R^2, is defined as the ratio of the explained variation to the total variation and is a measure of the degree of fit [10]. It is also the proportion of the variability in the response variables, which is accounted for by regression analysis [11]. The analysis of variance (ANOVA) for the selected quadratic model showed that the model was adequate (Tables 2 and 3), with no significant lack of fit and with very satisfactory values of the R^2 for both the responses. The R^2 values for clarity and viscosity were 0.9291 and 0.8792, respectively, indicating that the statistical model can explain 92.91% and 87.92% of variability in the responses. The closer the value of R^2 to the unity, the better the empirical model fits the actual data.

Table 2 Analysis of variance for the response surface quadratic model (response–clarity).

Source	Sum of squares	*df*	Mean square	*F* value	Prob>*F*
Model	4.967×10^{-3}	9	5.518×10^{-4}	10.19	0.0029
Time (*A*)	4.061×10^{-4}	1	4.061×10^{-4}	7.50	0.0290
Temperature (*B*)	2.113×10^{-3}	1	2.113×10^{-3}	38.99	0.0004
Enzyme conc. (*C*)	1.531×10^{-4}	1	1.531E-004	2.83	0.1366
Time × Time (A^2)	5.568×10^{-4}	1	5.568×10^{-4}	10.28	0.0149
Temperature × Temperature (B^2)	5.329×10^{-4}	1	5.329×10^{-4}	9.84	0.0165
Enzyme conc. × Enzyme conc. (C^2)	5.568×10^{-4}	1	5.568×10^{-4}	10.28	0.0149
Time × Temperature (*AB*)	1.225×10^{-5}	1	1.225×10^{-5}	0.23	0.6489
Time × Enzyme conc. (*AC*)	4×10^{-4}	1	4×10^{-4}	7.38	0.0299
Temperature × Enzyme concentration (*BC*)	4.225×10^{-5}	1	4.225×10^{-5}	0.78	0.4065
Residual	3.793×10^{-4}	7	5.418×10^{-5}		
Corrected total	5.346×10^{-3}	16			

The linear effects of treatment time (*A*) and temperature (*B*), the quadratic effects of treatment time (A^2), temperature (B^2) and enzyme concentration (C^2) and the interactive effect of treatment time and enzyme concentration (*AC*) were identified as the significant model terms.

Adequate precision measures the signal-to-noise ratio and a value greater than 4 is desirable. The model had adequate precision of 8.281 for clarity and 7.132 for viscosity, which indicated an adequate signal to navigate the design space.

Clarity of the Juice

Clarity of the juice is an important factor regarding the quality of the juice as it fetches consumer attention for the product in the market. Pectinolytic enzyme treatment results in the breaking down of pectin present, which facilitates the formation of pectin–protein flocs, leaving a clear supernatant and removes the colloidal aspect of the juice significantly [12].

Table 3 Analysis of variance for the response surface quadratic model (response–viscosity).

Source	Sum of squares	*df*	Mean square	*F* value	Prob>*F*
Model	3.104×10^{-4}	9	3.449×10^{-5}	5.66	0.0162
Time (*A*)	8×10^{-6}	1	8×10^{-6}	1.31	0.2895
Temperature (*B*)	5.78×10^{-6}	1	5.78×10^{-6}	0.95	0.3625
Enzyme conc. (*C*)	2.048×10^{-5}	1	2.048×10^{-5}	3.36	0.1094
Time × Time (A^2)	8.526×10^{-5}	1	8.526×10^{-5}	14.00	0.0073
Temperature × Temperature (B^2)	1.217×10^{-5}	1	1.217×10^{-5}	2.00	0.2004
Enzyme conc. × Enzyme conc. (C^2)	1.139×10^{-4}	1	1.139×10^{-4}	18.69	0.0035
Time × Temperature (*AB*)	2.500×10^{-7}	1	2.5×10^{-7}	0.041	0.8452
Time × Enzyme conc. (*AC*)	4.096×10^{-5}	1	4.096×10^{-5}	6.72	0.0358
Temperature × Enzyme concentration (*BC*)	3.61×10^{-6}	1	3.61×10^{-6}	0.59	0.4666
Residual	4.264×10^{-5}	7	6.091×10^{-6}		
Corrected total	3.53×10^{-4}	16			

The quadratic effects of treatment time (A^2) and enzyme concentration (C^2) and the interactive effect of treatment time and enzyme concentration (*AC*) were identified as the significant model terms.

Regression analysis of the data on clarity of the juice was performed and the following second-order polynomial equation was derived (Eq. 2)

$$Y = 0.022 - 7.125\text{E} - 003X_1 + 0.016X_2 - 4.375\text{E} - 003X_3 + 0.012X_1^2 + 0.011X_2^2 + 0.012X_3^2 + 1.750\text{E} - 003X_1X_2 - 0.010X_1X_3 + 3.250\text{E} - 003X_2X_3, \quad (2)$$

where, Y is the predicted response and X_1, X_2, and X_3 are the coded values for the variables. The interaction of treatment time and temperature is shown in Fig. 1.

Treatment time and temperature significantly affected the clarity of the juice in both linear and quadratic manners (Table 2). Clarity of the juice increased with increase in treatment time and temperature. However, high temperature and extended incubation were not preferred. This may be caused by the fact that prolonged incubation time can cause the formation of haze particles that consisted of protein–carbohydrates or protein–tannin complex [1]. Also when the treatment temperature is very close to the optimum activity temperature of the enzyme, degradation of pectic substances may be faster than flocculation and settling down. The optimum activity temperature of polygalacturonase produced by *S. lydicus* was 50 °C [7]. Figure 2 shows the interactive effect between treatment time and enzyme concentration.

The interactive effect between time and enzyme concentration was significant with Prob>F value of 0.0299. The linear effect of enzyme concentration was not significant, while the quadratic effect was significant and the Prob>F value was 0.0149. Increase in enzyme concentration resulted in an increase in the clarity of the juice. Increasing the enzyme level in the treatment mixture may increase the rate of clarification, by exposing part of the charged protein beneath, thus reducing the electrostatic repulsion between cloud

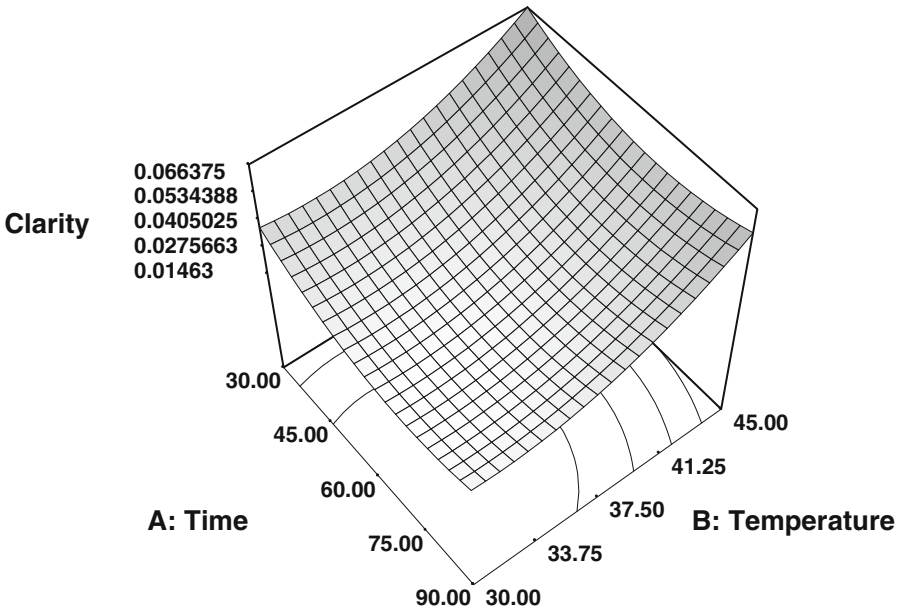

Fig. 1 Response surface for clarity of the juice according to the experimental design. The three-dimensional plot shows the clarity of the juice as a function of treatment time and temperature

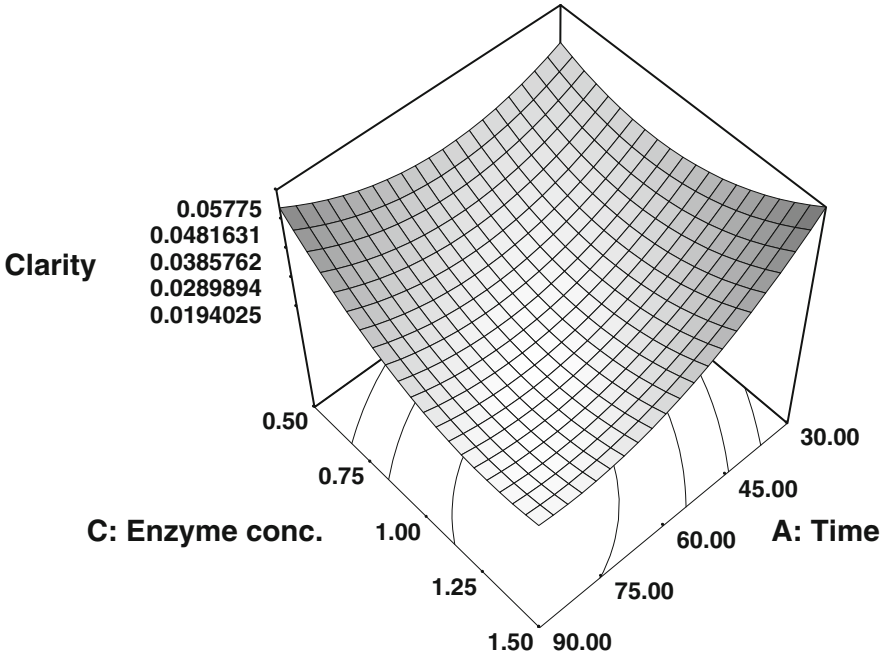

Fig. 2 Response surface for clarity of the juice according to the experimental design. The three-dimensional plot shows the clarity of the juice as a function of treatment time and enzyme concentration

particles, which cause them to aggregate and to settle down eventually [1]. While increasing the enzyme concentration, at a particular point, there will not be enough pectin to form pectin–protein flocs and thus the protein particles will remain in solution decreasing the clarity of the juice. Thus, high levels of enzyme concentration were not suitable for the process. The interactive influence of temperature and enzyme concentration exhibited a similar pattern as the interactions between time and temperature and time and enzyme concentration (Fig. 3).

Viscosity of the Juice

The viscosity of fruit juices becomes important during the preparation of juices as it can cause decrease in the rate of filtration and the cloud particles may result in the clogging of the filtration apparatus. Regression analysis of the data on viscosity of the juice was performed and the following second-order polynomial equation was derived (Eq. 3)

$$\begin{aligned} Y = {} & 1.05 - 1.000\text{E} - 003X_1 + 8.500\text{E} - 004X_2 - 1.600\text{E} - 003X_3 + 4.500\text{E} \\ & - 003X_1^2 + 1.700\text{E} - 003X_2^2 + 5.200\text{E} - 003X_3^2 - 2.500\text{E} - 004X_1X_2 \\ & - 3.200\text{E} - 003X_1X_3 - 9.500\text{E} - 004X_2X_3, \end{aligned} \tag{3}$$

where, Y is the predicted response and X_1, X_2, and X_3 are the coded values for the variables. The quadratic effects of treatment time and enzyme concentration were significant statistically. Also, the interactive effect of treatment time and enzyme concentration was

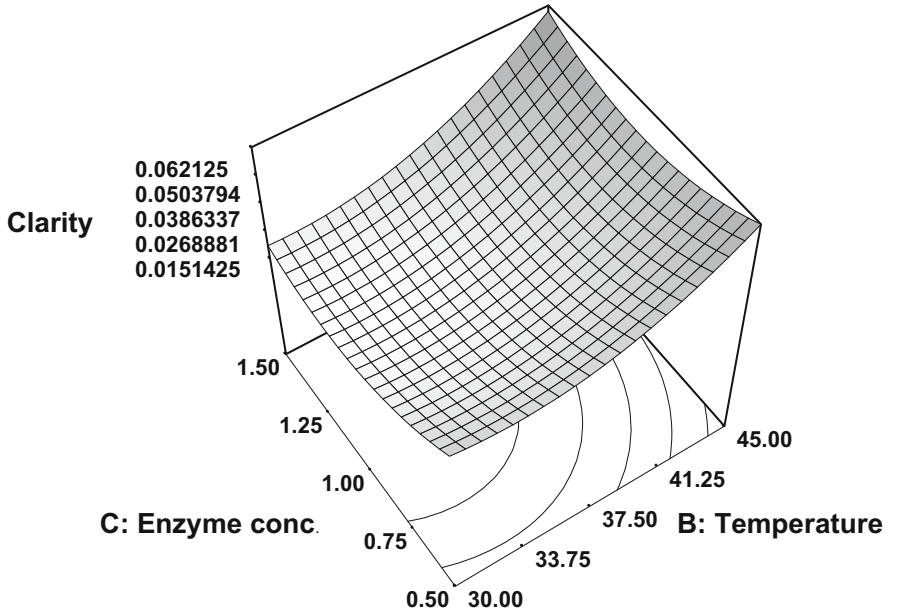

Fig. 3 Response surface for clarity of the juice according to the experimental design. The three dimensional plot shows the clarity of the juice as a function of temperature and enzyme concentration

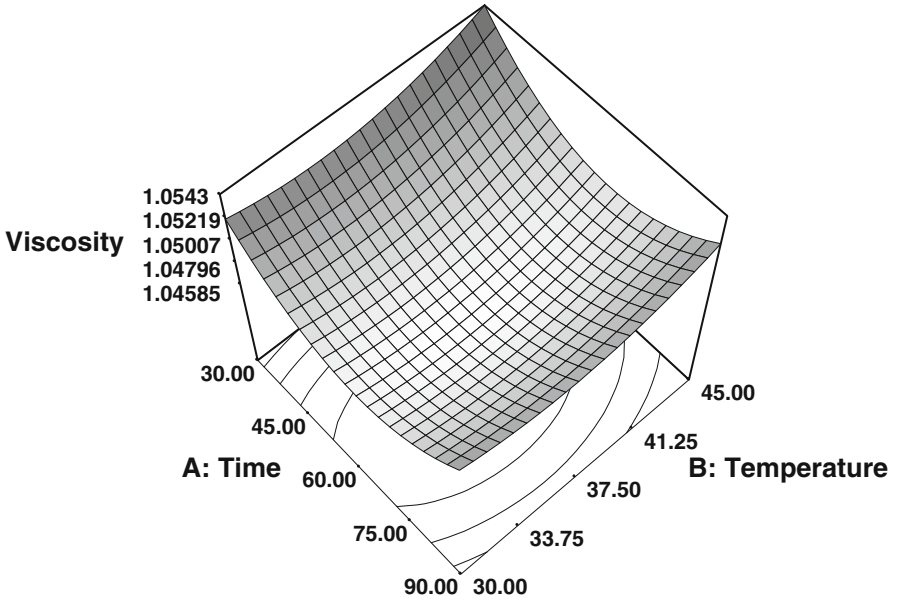

Fig. 4 Response surface for viscosity of the juice according to the experimental design. The three dimensional plot shows the viscosity of the juice as a function of treatment time and temperature

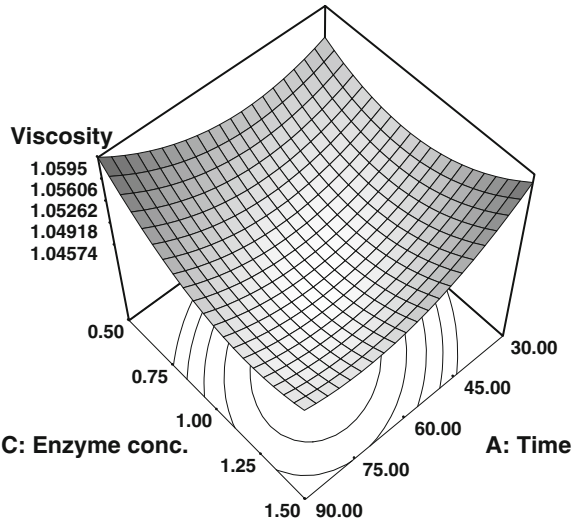

Fig. 5 Response surface for viscosity of the juice according to the experimental design. The three dimensional plot shows the viscosity of the juice as a function of treatment time and enzyme concentration

significant with Prob>*F* value of 0.0358. The viscosity of the juice as a function of treatment time and temperature is depicted in Fig. 4.

The viscosity of the juice decreased with increase in treatment time and temperature; however, prolonged incubation time and high temperature were not suitable. The interactive effect of treatment time and enzyme concentration (Fig. 5) and temperature and enzyme concentration (Fig. 6) exhibited a similar tendency as observed for the clarity of the juice. A

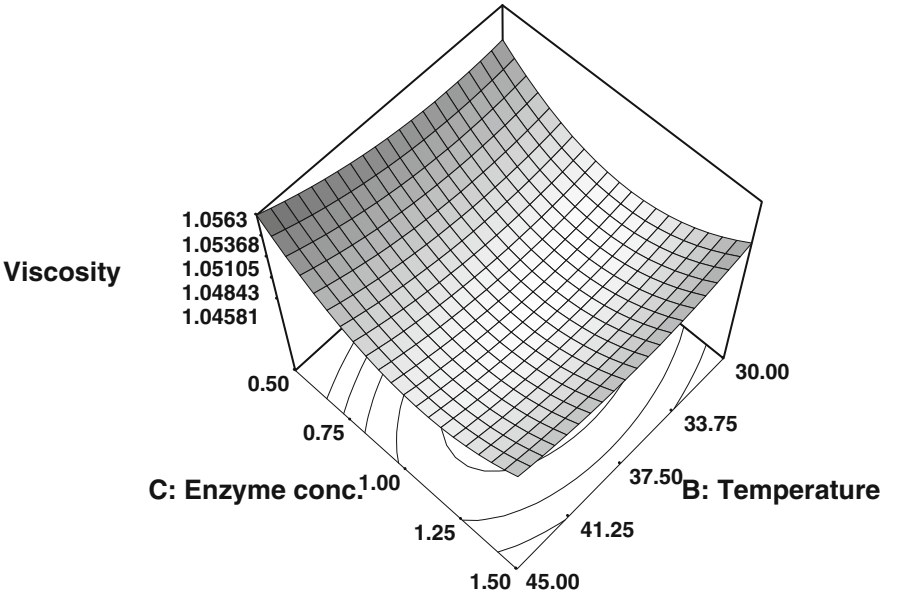

Fig. 6 Response surface for viscosity of the juice according to the experimental design. The three dimensional plot shows the viscosity of the juice as a function of temperature and enzyme concentration

high or low level of enzyme concentration was not appropriate for reducing the viscosity of the juice as it is evident from the present study. At high levels of enzyme concentration, protein particles may remain in solution because of insufficient concentration of pectin to flocculate with the enzyme thus increasing the viscosity of the juice.

Optimization

After analyzing the response surface quadratic model for enzymatic clarification of sapodilla juice, it was necessary to determine the optimum conditions for the process. The process variables were considered to be optimum, when the absorbance value at 660 nm and the viscosity of the juice were the lowest. Based on response surfaces and contour plots, the optimum conditions for clarifying sapodilla juice were enzyme concentration 1.15 U, incubation temperature 34 °C, and incubation time 70 min. The optimum process conditions depend on the concentration of the juice to some extent. Clarification of sapodilla juice has been performed using Pectinex 3X L from *Aspergillus niger* and the optimum process conditions have been reported as temperature 40 °C, incubation time 120 min, and enzyme concentration 0.1% [1]. Banana juice clarification, employing Pectinex Ultra SP-L, has been reported to be optimum at 43.2 °C for 80 min, with an enzyme concentration of 0.084% [4].

Conclusions

Response Surface Methodology was found to be an efficient tool for the optimization of enzymatic clarification of sapodilla juice. Polygalacturonase produced by *S. lydicus* was suitable for the process as its optimum activity pH was in close proximity to the juice pH. Generally, enzymatic clarification of fruit juices is carried out by employing commercial pectinase preparations, especially from *Aspergillus niger*. Fungal pectinases are acidic in nature and are suitable for juices with very low pH. In the present study, polygalacturonase produced by the filamentous bacteria, *S. lydicus*, has been proved to be suitable for less acidic juices. Given the potential of polygalacturonase produced by *Streptomyces lydicus* for use in enzymatic clarification of sapodilla juice, it can be applied for the depectinization of other juices also.

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