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Mango juice clarification with polygalacturonase produced by *Aspergillus awamori* MTCC 9166 - Optimization of conditions

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Abstract

Pectin rich fruit juices need enzymatic treatment for clarification. As fruits are rich in pectin, pectinolytic treatment can efficiently reduce viscosity resulting in clarified fruit juice. Enzymatic treatment of mango pulp results in 80% recovery of total juice present in the fruit. Use of pectin degrading polygalacturonases increases both fruit juice extraction and clarification. Polygalacturonase (PG) produced by *Aspergillus awamori* MTCC 9166 is studied for mango juice clarification and conditions are optimized by Box-Behnken design. The conditions studied are incubation time (15-45 min), enzyme concentration (0.5-1.5 U/ml), and temperature (25-40^{0}C). Significant regression model describing the change on viscosity with respect to independent variables was established. Based on surface plots and contour plots, the optimum conditions for mango juice clarification were obtained. The recommended enzymatic treatment conditions are enzyme concentration 1.5 U/ml, incubation time 30 min at 40^{0}C. Significant (60%) reduction in viscosity was observed.

Introduction

Mango is one of the important fruits widely grown in tropical countries and is indigenous to India. The best quality mangoes originate in southern part of India. It is perishable, seasonal fruit which is rich source of pectin and also contains sucrose, glucose and maltose. Pectic substances and pectinolytic enzymes play important role in fruit juice processing (Fogarty and Kelly, 1983). Enzymatic treatment of mango mash results in 80% of total juice present in fruit. Pectinases along with cellulases and xylanases are used to degrade mango pulp (Kashyap *et al.*, 2001).

Pectinases play important role in processing plant materials to food products, such as de-pectinization of fruit juices, maceration of vegetables and fruits, and extraction of vegetable oils (Benen *et al*., 2003). Pectinolytic enzymes are used in fruit processing industry to increase yields, improve liquefaction, clarification and filterability of juices (Harsha *et al*., 2014). Commercial sources of fungal pectinases have been used in fruit juice processing since 1930's for clarifying fruit juices and disintegrating plant pulps to increase juice yields (Macmillan and Sheiman, 1974). Most enzymes are marketed on the basis that they are generally recognized as safe (GRAS) for their intended use in the juice process (Grassin and Fauquembergue, 1996; Heldt-Hansen *et al*., 1996). *Aspergillus niger* or *Aspergillus aculeatus* are widely used for industrial production of pectinolytic enzymes which are important in food and alcoholic beverage industry (Naidu and Panda, 1998). Both the naturally present (endogenous) enzymes and introduced enzymes (exogenous) catalyze the decomposition of pectin in fruit juice.

The exogenous pectinolytic enzymes improve and influence the efficiency of fruit juice process, which cannot be achieved by the endogenous enzymes alone that occur naturally in the fruit. Upon grinding of the raw fruit, pectinases are added which reduce viscosity of pectin-rich crude juice, also known as pulp enzyming, and therefore improve the processing capacity and yield of fruit juice. The production of fruit juices can also be achieved by liquefaction of fruit tissues. In this process the fruit tissues are solubilized by using a broad spectrum of polysaccharide degrading enzymes, such as pectinases, hemicellulases and cellulases (Benen and Voragen, 2003).

Pectinases assist in pectin hydrolysis, which cause reduction in pulp viscosity and a significant increase in juice yield (Solehah *et al*., 1964; Pilnik and Voragen, 1993;) reported action of commercial

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pectinolytic enzymes, Pectinex and Ultrazyme, in degradation and hydrolysis of anthocyanin pigments of raspberry juice. Ceci and Jorge Lozano (1998) reported simple method for testing of pectinolytic activities and determined enzymatic activity of commercial pectinases for clarification of apple juice. *Saccharomyces cerevisiae* was cultured in pine apple juice and its pectinolytic enzymes were used for extraction of pine apple juice (Dzogbefia *et al*., 2001). Conversion of fruits in to fruit juices was originally developed for utilizing the surplus fresh fruits, but now processing of fruit juices is firmly established as a major industry. As the demand for fruit juices is increasing, their production has increased considerably in recent years. Therefore there is a need to evolve better approaches for processing of fruits. Here we report the application of polygalacturonase (PG) produced by *Aspergillus awamori* MTCC 9166 for mango juice clarification and establish enzymatic conditions in preparation of clarified mango juice. The parameters like incubation time, enzyme concentration and temperature were studied and optimized by Box-Behnken statistical design to obtain maximum juice yield.

Material and Methods

Enzyme source and enzyme assay

Aspergillus awamori MTCC 9166 was isolated from vegetable dump yard soil and maintained on Potato Dextrose Agar (PDA) slants in refrigerator (Anuradha *et al*., 2010). Submerged fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml Czapek's broth with 1% commercial pectin (Citrus peel pectin, SD Fine Chemicals). The flasks were inoculated with 2.5% (v/v) spore suspension containing $1x10^6$ spores/ ml and incubated for production of polygalacturonase for 5 days at 27^0C in orbital shaker incubator at 200 rpm. Fermented broth was cold centrifuged at 4^0C, 5000 rpm for 10 minutes and supernatant was taken as crude enzyme source and maintained at 4^0C for further studies. The enzyme was assayed by measuring the D-galacturonic acid released from polygalacturonic acid as substrate. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µM of galacturonic acid per minute at 50^0C (Nelson, 1944; Collmer *et al*., 1988;).

Fruit juice preparation

Fresh ripe mangoes *(Mangifera indica)* were purchased from local market and used for juice preparation. Mangoes were peeled, deseeded, then ground in a blender to obtain homogenous mango pulp using distilled water in 1:1 (w/v) ratio. From this homogenate fruit juice about 100 ml was extracted by adding 40% distilled water and maintaining at 60^0C for 1 hour. After this treatment the juice was subjected to both centrifugation and filtration. The filtrate obtained was about 80 ml and it was made up to 100 ml. The pH was found to be 5, which is the pH of fruit juices in general (Jacob *et al.*, 2008).

Enzymatic treatment

10 ml of extracted mango juice (as above) was subjected to different enzymatic treatment conditions. The parameters tested were incubation time (15-45 min), enzyme concentration (crude enzyme 0.5-1.5 U/ml) and temperature ($25\text{-}40^0$C). After treatment the mango juice was heated to 90^0C for 5 minutes to inactivate the enzyme. The treated juice was centrifuged at 10,000 rpm for 15 min, filtered through Whatman no 1 filter paper and filtrate was collected for further analysis.

Viscosity studies

The viscosity of the mango juice was measured using DV-II+ Pro Viscometer Brook field LV-6.3V at 30^0C. The viscosity is expressed in milli Poise units (mPa.s).

Experimental design

Box-Behnken design was used for optimization of mango juice clarification. The parameters studied as independent variables were incubation time, enzyme concentration (crude enzyme) and temperature. The parameters selected were analyzed at three different (low, high and medium coded as -1, 0 and 1 levels (Box and Behnken, 1960). A total 17 combinations were used. A second order polynomial equation shown below was used to study the effect of variables in terms of main effects, quadratic effects and interactive effects. Statistical analysis of the data was done using INDOSTAT software.

$$Y=\beta 0+\sum \beta iXi+\sum \beta iiXi2++\sum \beta ijXiXi$$

Where Y is predicted response, Xi coded value of independent variable, β0 is constant term, βi coefficient of the linear terms, βi is the coefficient of the quadratic terms, and βii is the coefficient interactive terms.

Results and Discussion

Mango (*Mangifera indica* L.) is produced in over 90 countries worldwide with Asia accounting for approximately 77% of global production (Sudheer

Table 1. Box-Behnken experimental design for mango juice clarification using polygalacturonase produced by *Aspergillus awamori* MTCC 9166

Runs	Coded Variables			Uncoded Variables			Viscosity (mPa.s)
	X1	X2	X3	Time (min)	Temp (^{0}C)	Enzyme conc. (U/ml)	
1	0	1	1	45	40	1.5	1.998
2	1	0	1	60	37	1.5	1.055
3	1	-1	0	60	25	1	1.039
4	0	0	0	45	37	1	1.045
5	0	0	0	45	37	1	1.045
6	0	-1	-1	45	25	0.5	1.088
7	-1	-1	0	30	25	1	1.082
8	-1	1	0	30	40	1	1.999
9	0	-1	1	45	25	1.5	1.001
10	1	0	-1	60	37	0.5	1.812
11	-1	0	-1	30	37	0.5	1.912
12	1	1	0	60	40	1	1.996
13	0	0	0	45	37	1	1.045
14	0	1	-1	45	40	0.5	1.988
15	0	0	0	45	37	1	1.045
16	0	0	0	45	37	1	1.045
17	-1	0	1	30	37	1.5	1.921

kumar and Reddy, 2009). Among the Asian countries, India has good production of mango and it is mostly used for commercial production of juice. Mango pulp is rich in pectin, starch and xylan. The isolate under study has not only PG production but also amylase and xylanase activity (Anuradha et *al*., 2010) and so it could have ample application in mango juice clarification as done by Saisha and Shashidhar (2015). Clarification of mango juice was carried out using polygalacturonase produced by *Aspergillus awamori* MTCC 9166 using Box-Behnken statistical design to optimize conditions like temperature, enzyme concentration and incubation time, which showed significant influence on mango juice clarification. The enzymatic hydrolysis of pectic substances depends on several processing variables such as type of enzyme, hydrolysis time, enzyme concentration, incubation temperature and pH (Robert *et al*., 1972; Neubeck, 1975; Baumann, 1981). The crude polygalacturonase produced by *Aspergillus awamori* MTCC 9166 had been used for clarification of mango juice using Box-Behnken statistical design (Table 1).The combination 8 showed maximum viscosity and combination 9 showed minimum viscosity. Coefficient of determination, R2, is defined as the ratio explained variation to the total variation and is a measure of the degree of fit. It is also proportion of the variability in the response variables, which is accounted for regression analysis. When R2 reaches unity, the better the empirical model fits the actual data. The analysis of variance (ANOVA) was done using Indostat software which showed that the response surface models developed for all response variables were adequate. The R2 value for viscosity was 0.95163, indicating that regression model explained the reaction well (Table 2). Square

Table 2. Optimization of estimated effects and coefficients for mango juice clarification using polygalacturonase produced by *Aspergillus awamori* MTCC 9166 using Box-Behnken experimental design three variables (A - time, B - case temperature, C - enzyme concentration)

Source	Partial R2	Coef.	Std. Coef.	t value	t prob	Significance
Constant	0.0000	1.0450	0.0681	15.3531	0.0000	
A-Time	0.0382	-0.1265	0.0538	-2.3509	0.0510	*
B-Temperature	0.5303	0.4714	0.0538	8.7601	0.0001	Significant
C-Enzyme concentration	0.0254	-0.1031	0.0538	-1.9165	0.0968	*
A2	0.1401	0.3201	0.0742	4.3160	0.0035	Significant
B2	0.0412	0.1639	0.0742	2.2094	0.0629	*
C2	0.1319	0.3099	0.0742	4.1778	0.0041	Significant
AB	0.0001	0.0100	0.0761	0.1314	0.8991	*
AC	0.0438	-0.1915	0.0761	2.5165	0.0400	Significant
BC	0.0007	0.0243	0.0761	0.3187	0.7593	*

* Insignificant

interaction of time and enzyme concentration was found to be significant and adjacent interaction of three variables were found to be insignificant.

The interactive effect of time and incubation temperature on viscosity of mango juice using PG produced by *Aspergillus awamori* MTCC 9166 is indicated in response surface curve and its corresponding contour plot (Figure 1) and it shows that the temperature up to 40^{0}C has positive effect and beyond it has negative effect on clarification. Similarly treatment time has positive effect on clarification up to 30 min. The contour plot is semicircular and interaction between these parameters was insignificant. Enzyme concentration up to 1.5 U/ml has positive effect and less than that did not show much effect on clarification and treatment time has positive effect on clarification up to 30 min (Figure 2). The contour plot is circular and indicating significant interactive effect between these parameters. Figure 3 shows that enzyme concentration up to 1.5 U/ml and temperature up to 40^{0}C has positive effect on clarification and the semicircular contour plot indicated that interaction between enzyme concentration and temperature was insignificant.

Application of Box-Behnken statistical design in clarification of fruit juices has been reported in literature. Response surface methodology (RSM) was employed to establish optimum conditions for enzymatic clarification of sapodilla juice using polygalacturonase obtained from *Streptomyces lydicus* (Jacob *et al*., 2008). Diwan and Shukla (2005) developed a process for production of guava juice using purified enzyme at 2% concentration and 20 hours incubation time. Similar studies were done by

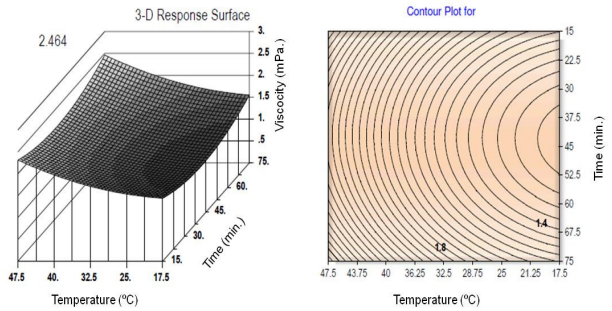

Figure 1. Surface and Contour plots showing interactive effect of time and temperature at various levels on mango juice clarification using polygalacturonase produced *Aspergillus awamori* MTCC 9166

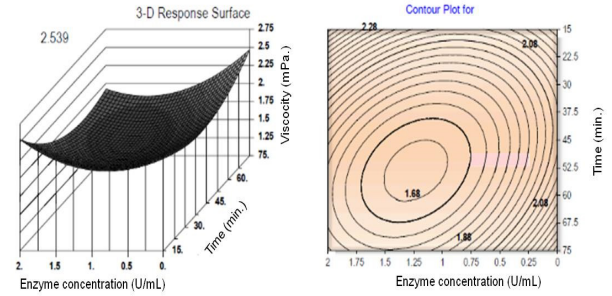

Figure 2. Surface and Contour plots showing interactive effect of time and enzyme concentration at various levels on mango juice clarification using polygalacturonase produced *Aspergillus awamori* MTCC 9166

(Rai *et al*., 2004) to optimize conditions for pectinase for clarification of sweet lime (Mosambi) juice to obtain maximum juice yield.

In view of several advantages offered by Box-Behnken design, it was applied for mango juice clarification studies using polygalacturonase produced from *Aspergillus awamori* MTCC 9166. The statistical analysis results were interpreted as response surface curves and its corresponding contour plots that indicated variation in viscosity of mango juice using PG produced by *Aspergillus awamori* MTCC 9166 (Table 2). In these interactions of treatment time and temperature; enzyme concentration and treatment time; temperature and enzyme concentration were interpreted (Figure 1, 2 and 3). Based on regression coefficient values, response plots and contour plots, the optimum conditions for clarifying mango juice were obtained as enzyme concentration 1.5 U/ml, temperature 40^0C and incubation time of 30 min using polygalacturonase produced by Aspergillus awamori MTCC 9166 and 60% viscosity reduction was achieved.

Conclusion

Box-Behnken design was found to be an efficient and valuable statistical tool for analyzing

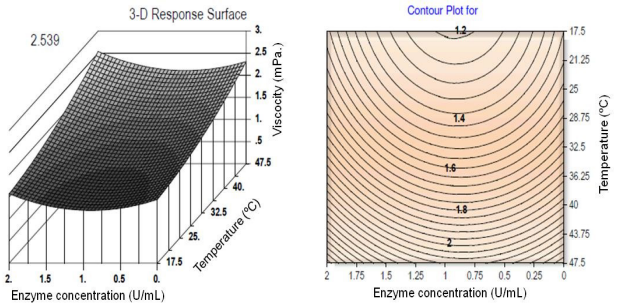

Figure 3. Surface and Contour plots showing interactive effect of temperature and enzyme concentration at various levels on mango juice clarification using polygalacturonase produced *Aspergillus awamori* MTCC 9166

and optimizing the effects of incubation time, enzyme concentration and temperature on enzymatic clarification of mango juice using polygalacturonase produced by *Aspergillus awamori* MTCC 9166. The recommended enzymatic treatment conditions were enzyme concentration 1.5 U/ml, incubation time 30 min and incubation temperature 40^0C. There was 60% viscosity reduction which is significant. The present study would be very useful for fruit juice industry especially mango juice industry as India is potential producer of mangoes and clarified mango juice has good demand for its known nutritive value and taste world wide.

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