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ENZYMATIC PROCESS PARAMETERS OPTIMIZATION FOR ENHANCED CARROT (*Daucus carota* L.) JUICE QUALITY USING RESPONSE SURFACE METHODOLOGY

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Abstract: The effects of enzyme concentration (50-650 mg/kg grated carrot), pectolytic and cellulolytic enzyme ratio (3:7-7:3), incubation time (30-150 min) and temperature (25-65^{o}C) on TSS and colour index of juice from grated carrot were studied. A central composite rotatable design (CCRD) was used in designing the treatment combinations of four variables at five levels. The process involved treating the blanched grated carrot with mixture of crude pectolytic enzyme from *Aspergillus foetidus* and crude cellulolytic enzyme from *Trichoderma ressi*, keeping the samples at the desired time, followed by extraction of juice. Enzyme treated grated carrot sample resulted juice with higher TSS and colour index as compared to control. A second order response surface model adequately fitted the data. All the variables affected responses significantly. The optimum condition was at enzyme concentration, 385.1 mg/kg of grated carrot; pectolytic and cellulolytic enzyme ratio, 4:6; incubation time, 146 min and incubation temperature 32^{o}C. Under the optimal conditions, TSS of the juice extracted from enzyme treated grated carrot was 8.3^{o}Brix and having colour index value 0.2922, correspond to the increase in TSS by 1.3 ^{o}Brix and in colour index by 0.1190.

Keywords: Grated carrot, crude pectolytic enzyme, crude cellulolytic enzyme, TSS, colour index.

Introduction

Carrot (*Daucus carota* L.) is a rich source of vitamin, specially pro-vitamin A, and minerals and generally consumed directly as in salad, juice and other preparations at home level. Due to its seasonal and perishable nature, efforts have been made to extend the shelf life to use it during off season by drying, freezing, canning, pickling and manufacturing various products such as *murabba,* soup, fabricated baby foods and juice (Kalra et al. 1987). Juice from carrot is one of the most acceptable products and is reported to have various medicinal values (Peto et al. 1981). Total soluble solids and colour value are important quality attributes (Sharma 2002).

The juice expression process essentially involves a size reduction unit operation i.e. crushing,

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grinding, pulping which facilitate the recovery and depends on mechanical and physical properties of press and material. The expression process produces juice and pomace i.e. slurry of solids having high dietary fiber, lesser amount of juice and almost similar nutritive values as in whole. The pomace is difficult to utilize as food purposes and generally used for feed or discarded as waste.

The juice yield from fruits and vegetables can be increased using various pre-treatments i.e. crushing, pulping, hydrothermal treatment and enzymatic treatment etc. Enzymatic treatment alone or in combination with other, is one of the potential pretreatment which results in increased yield with better juice quality and acceptability (Sharma et al. 2005, Chadha et al. 2003, Bezusov et al. 1989, Lazano et al. 1988, Traversi et al. 1988, Birch et al. 1981, Rombouts and Pilnik 1978). Enzymes, either crude or pure, are widely used in disintegration of fruit and vegetable pulp for more juice yield and/or for the clarification of juices (Pilnik and Voragen 1993).

The crude enzyme system, a complex of many different enzymes with less purity and activity, derived from fungal sources and with minimum downstream processing may be a step towards cutting costs in comparison of pure enzyme preparation. Baumann (1981) and Dominguez et al (1994) reported that synergetic effect of different enzymes such as cellulase and pectinase also reduces the enzyme cost and increases the yield.

In developing, analyzing and optimizing of such complex processes, Response surface methodology (RSM) consisting of a group of mathematical and statistical procedure has been reported as a convenient method. It also leads to cost minimization of enzymes with an efficient screening and optimization of process parameters (Sharma et al. 2005, Chadha et al. 2003, Sharma 2002, Pandey 2000, Sarkar et al. 1998).

It is evident from the literature that work relating to the enzyme assisted juice expression from grated carrot is scant. This research was, therefore, undertaken with an overall objective to investigate the use of enzymatic hydrolysis on grated carrot as a pre-extraction treatment before the juice expression and to optimize the process parameters to produce juice with maximum TSS and colour index, using RSM by adopting a five-level, four- variables central composite rotatable design (CCRD).

Materials and Methods

Experimental material: Fresh carrots (Variety: '*Pusa Kesar*') of uniform size, color, maturity and firm texture were selected for the experiments. Material was washed and trimmed with the help of a vegetable knife. The carrots were then grated (thickness ~2 mm and width 3-4

mm) using a hand grater. The grated carrots were immediately blanched by dipping it in boiling water for 5 min according to the general commercial practice.

Enzyme source: Crude pectolytic and cellulotic enzymes were then prepared in the laboratory on pectin rich and cellulose rich media (Table 1), using *Aspergillus foetidus* (MTCC-151) and *Trichoderma reesi* (MTCC-*164) respectively (Sharma et al. 2005, Chadha et al. 2003, Bhatnagar and Johri 1987). The fungal strains were obtained from Institute of Microbial Technology (IMTech), Chandigarh and sub-cultured on standard media following standard procedures (IMTech 2000) before using it in crude enzymes preparation. The crude enzymes were assayed for protein content (Lowry et al. 1951), pectolytic (Bergmeyer 1983) and cellulolytic activities (Mandels et al. 1976). The enzymes were stored in a refrigerator and used within two days.

Methods:

The enzyme added juice expression process was carried out according to the flow diagram shown in Fig. 1. The hydrolysis parameters: enzyme concentration, pectolytic and cellulolytic enzymes ratio, incubation time and temperature were varied according to the experimental design (Table 2). The range of the parameters were carefully chosen, based on the limited literature available on enzymatic hydrolysis of oilseed, fruit and vegetable (Sharma et al. 2005). The details of variable selection were as follows.

Enzyme concentration: Enzyme concentration was selected by considering protein content and activity of crude enzymes. Trial runs were conducted to establish the level of this parameter. The overall experimental range decided was from 50 to 650 mg enzyme protein per kg of carrot with a centre point at 350 (Sharma et al. 2005).

Pectolytic and cellulolytic enzyme ratio: The combined effect of pectolytic and cellulolytic enzymes gave good results in enzymatic fruit juice processing (Bezusov et al. 1989, Traversi et al. 1988). Massiot et al (1992) reported that the hydrolysis of the polysaccharides by pectinase was facilitated by the presence of cellulase which was necessary for complete liquefaction of tissues. Chadha et al. (2003) and Bezusov et al. (1989) found optimum ratio of pectolytic and cellulolytic enzymes 1.16:1 and 1:1, respectively, for maximum juice yield. In the present study, crude pectolytic and cellulolytic enzymes were prepared using microorganism of *Aspergillus* and *Trichoderma* species and were mixed in the proportions of 3:7 to 7:3 considering 5:5 (i.e. 1:1) as the centre point value.

Incubation period: The incubation time varied from 30 to 150 min for various enzyme preparations used in fruit and vegetable processing at various temperatures (Lazano et al.

1988, Traversi et al. 1988, Bezusov et al. 1989, Chadha et al. 2003, Sharma et al. 2005). Therefore, the range of incubation period of 30-150 min was considered with 90 min as the centre point.

Application temperature: The optimum temperature for the activity of enzyme was 25-45°C for pectolytic enzymes from *Aspergillus spp* and 40-65°C for cellulolytic enzymes from *Trichoderma spp,* a thermophilic mold strain (Sarkar et al. 1998, Biocatalysts 2003, Sharma et al. 2005). Therefore, 45°C was chosen as the center point of the design and the range was 25-65°C.

The increments of variation for each variable spaced around the center point along with the equation relating the actual and coded ratios are presented in Table 3. By substituting these equations, process variables were coded for solution of the multiple regression equation.

Response measurement techniques:

TSS: The soluble solids in the juice extracted were determined with the help of a hand refractometer (Erma, 0-32^{o}Brix) at room temperature (20$\pm$2°C) (Ranganna 1991).

Colour index: An Agilent Diode-Array (Model-8453) spectrophotometer was used to measure the absorbance value of juice in the visible range (350-800 nm) and thereby to study the effect of enzymatic liquefaction on apparent colour change of the juice. The absorbance readings were recorded at characteristic wavelengths of 430 nm, 420 nm and 400 nm at which absorbency peaks were observed. Colour of the juice was calculated on the basis of alcohol soluble pigments (Chadha et al. 2003). The following equation of the standard reference colour method was used:

Colour Index = $A_{Sample} - A_{60\%}$

Data analysis: Responses are function of independent variables and assumed to be approximated by a second-order polynomial equation:

$$Y = f(X_1, X_2, X_3, X_4) = \beta_o + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \quad \ldots.(1)$$

Where, Y is response; β_o, β_{ii}, β_{ij} are regression coefficient and X_i, X_j are variables

Data were analyzed (Khuri and Cornell 1987, Mayers 1971) using statistical software (Minitab V-6.1.1) to develop response function and analyzing regression coefficients for desired optimization. Optimizations of the process parameters for maximum TSS and colour index were calculated by partially differentiating the response surface model with respect to each parameter, equating to zero and simultaneously solving the resulting functions. Multiple response package (MR) optimization software, which takes into account all these steps

(Conlon and Khuri, 1988) was used for this purpose. The contour plots for these models were plotted, using Surfer V-6 software, as a function of two variables, while keeping other variables at the optimum level.

Results and Discussion

TSS in carrot juice and colour index varied from 6.3 to 8.1°Brix and 0.2045 to 0.2996, respectively in enzyme treated grated carrots. In control, i.e. without enzymatic treatment, TSS and colour index were in the range of 6.9-7.1°Brix and 0.1700-0.1764 respectively (Table 4). Thus the TSS increased upto 1°Brix and colour index by 0.0345-0.1232 due to enzymatic action. Variation in TSS and increase in colour index are mainly due to the action of pectolytic enzyme action on carrot cell (containing juice) wall constituents consisting of protopectin and pectin thereby increasing uronic acid, methanol content and total acidity of the product; and cellulolytic enzyme on cellulose, hemicellulose and starch while protein are partially hydrolyze (Sharma et al 2005, Siliha et al 1990, Chadha et al 2003).

Multiple regression analysis (analysis of variance) was carried out considering full quadratic model (eq 1) on the responses to evaluate the adequacy of fit and results are reported in Table 5. Calculated F-values, for TSS 7.89 and colour index 9.92 were higher than that of tabulated F-value at 1% level of significance. The coefficient of determination (R^2-values) showed that developed models (eq 2, 3) explained 87.0% and 90.0% variation in the responses (Table 5) further R^2(adj)-values 76.0% and 81.0% were also found in agreement with the R^2-values. Therefore, the models were found to be adequate in representing the response data of the juice yield and colour index and can be further used for analysis and prediction purposes.

Multiple regression equations relating juice yield and colour index to coded levels of the variables so developed are as follows:

$$Y_1 = 7.39 + 0.38\ X_1 + 0.087\ X_2 + 0.096\ X_3 - 0.10\ X_4 - 0.04\ X_1^2 - 0.0188\ X_1X_2 + 0.0313\ X_1X_3 - 0.0683\ X_2^2 + 0.0188\ X_2X_3 + 0.0438\ X_2X_4 + 0.144\ X_3^2 - 0.0308\ X_4^2 \quad \ldots.(2)$$

$$Y_2 = 0.25 + 0.011\ X_1 + 0.01\ X_2 + 0.017\ X_3 + 0.016\ X_4 + 0.004\ X_1^2 - 0.004\ X_1X_2 - 0.002075\ X_1X_3 - 0.00269\ X_1X_4 + 0.00323\ X_2^2 - 0.00373\ X_2X_4 + 0.00254\ X_3^2 + 0.00365\ X_3X_4 + 0.0028\ X_4^2 \quad \ldots.(3)$$

Where, Y_1 = Total soluble solids, °Brix; Y_2 = Colour index; X_1 = enzyme concentration, mg protein/1000 g grated carrot sample; X_2 = Pectolytic: cellulotic enzyme ratio; X_3 = Incubation time; X_4 = Incubation temperature.

All the effects, at linear, interactive and quadric level at various predictors' level were also calculated for both models. The effects were used to plot a standardized Pareto chart. The

chart consists of bars of which lengths are proportional to the absolute values of the estimated effects divided by their standard errors. The charts include a vertical line at the critical t value at 95% confidence level. A bar crossing this vertical line corresponds to a factor or combination of factors that has a significant contribution in variation of response values. The charts (Fig. 2 and Fig. 3) and eqs 2 and 3 clearly indicate that the all variables, expect incubation temperature, has a positive linear effect on TSS. Except interactive effect of incubation time and temperature, all variables have significant effect on colour index ($p \leq 0.01$). There was higher positive linear effect of the enzyme concentration and quadratic effect of incubation time on TSS ($p \leq 0.01$).

The total effect of the variables at linear, quadratic and interactive level on TSS and colour index was also calculated and summarised in Table 6. All variables affected TSS significantly at linear and square levels. Effect of the enzyme concentration on TSS was about five times that of the enzyme ratio and incubation time. Similarly, the effects of enzyme ratio and incubation time on colour index are almost equal and about twice that of enzyme concentration and incubation temperature. Effect of incubation temperature is least on both the responses and insignificant on TSS. The total effect at linear level was more pronounced than quadratic on TSS. Colour index significantly explained by all linear, interactive and square terms.

Condition for optimum responses

For a juice expression process highest possible soluble solid content and coloring agents with better suspension in the juice is desired. Therefore, eq 2 and 3 were solved for maximum TSS and colour index. The optimum conditions to the above responses are presented in Table 7. Optimum values of the responses lie in the experimental range indicate the validity of the selection of the variable range. At the optimum condition TSS and colour index were 8.3 and 0.3223 respectively. This condition was experimentally verified in the laboratory and the response were found to be 8.24 and 0.3217 respectively, which are in good agreement with the predicted values.

The contour plots were obtained by selecting two variables and keeping others at their optimum condition for the desired level of response which leads to the optimum range of operation. The higher yield of soluble solids and colour index were occurred at higher level of enzyme concentration and pectolytic cellulotic enzyme ratio (Fig. 4a). The superimposed contour plot (Fig. 4b) of the responses as a function of incubation time and enzyme concentration also show higher values of responses at higher level of variables. Fig. 4c

clearly shows higher recovery of solids at higher level of enzyme concentration and lower level of incubation temperature and increased colour index at the higher level of both variables. Overall, enzyme concentration shows more pronounced effect on responses. Similarly, enzyme ratio effected (Fig. 4d,e). This may be attributed to more pectolytic activity of enzyme. The contour plot of incubation temperature and incubation time (Fig. 4f) does show increasing effect at above mid level of incubation temperature. This may be due to higher enzyme interaction with cell material and, in turn, disintegrate into lower molecules of smaller size and weight, thereby ease in the removal of juice droplets with higher soluble and coloring substances. The present observation agrees with the result reported by Sharma *et al.*, (2005) wherein the effect of enzymes was higher on the macerated carrots as compared to grinded carrots as reported by Chadha et al. (2003). The difference in finding may be attributed to the effect of size and shape of the substrate, used for the enzymatic actions.

It is some time difficult to maintain the recommended condition during processing and some deviation may occur. Therefore, optimum conditions were varied as enzyme concentration $\pm$20.0 mg protein/kg of grated carrot (610-640), enzyme ratio $\pm$5% (5.7:4.3-6.3:3.7), incubation time $\pm$5 min (125-135) and incubation temperature $\pm$2^{o}C (58-62) and then TSS and colour index were predicted using equations 2 and 3. It was observed that juice yield ranged from 8.08-8.30^{o}Brix and colour index 0.2981-0.3211 respectively. The minimum and maximum values were corresponding to the low and high levels of above processing conditions, respectively.

The study indicated that about 1^{o}Brix higher soluble solids was obtained due to enzymatic hydrolysis of grated carrot sample, corresponding to increase in colour index level to 0.3073. The study indicated more pronounced effect of crude cellulolytic enzymes than crude pectolytic enzymes and having synergetic effect.

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FIGURES

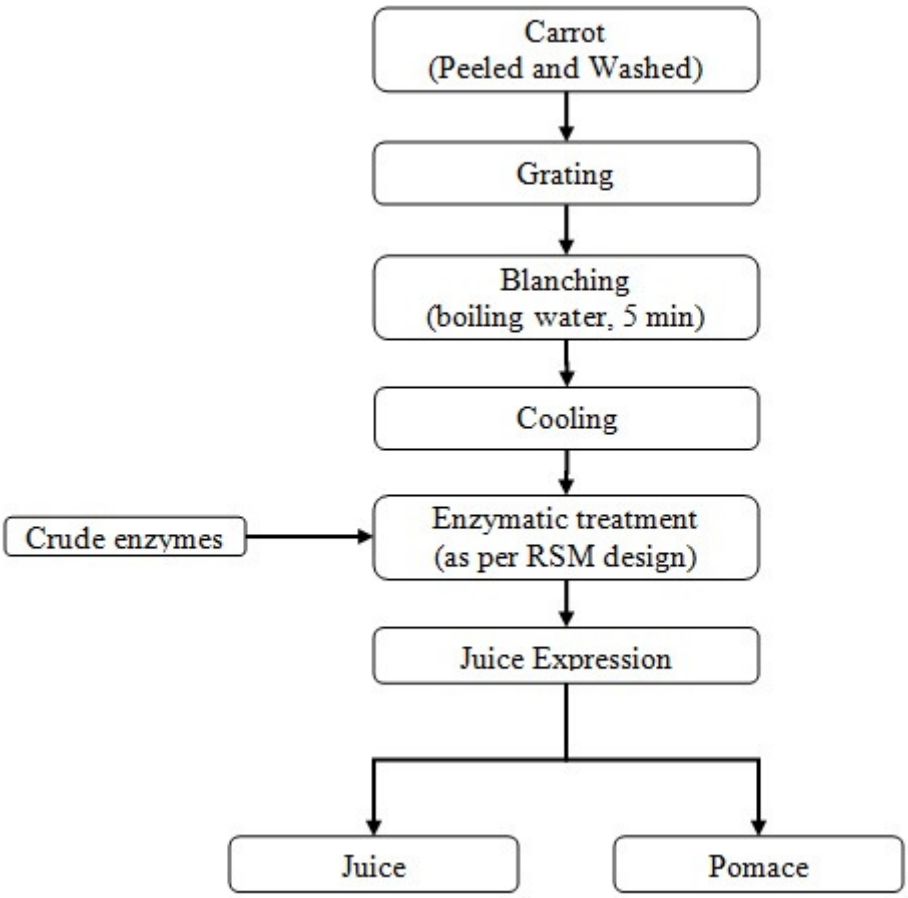


Fig. 1: Flow-sheet for the enzyme assisted carrot juice expression

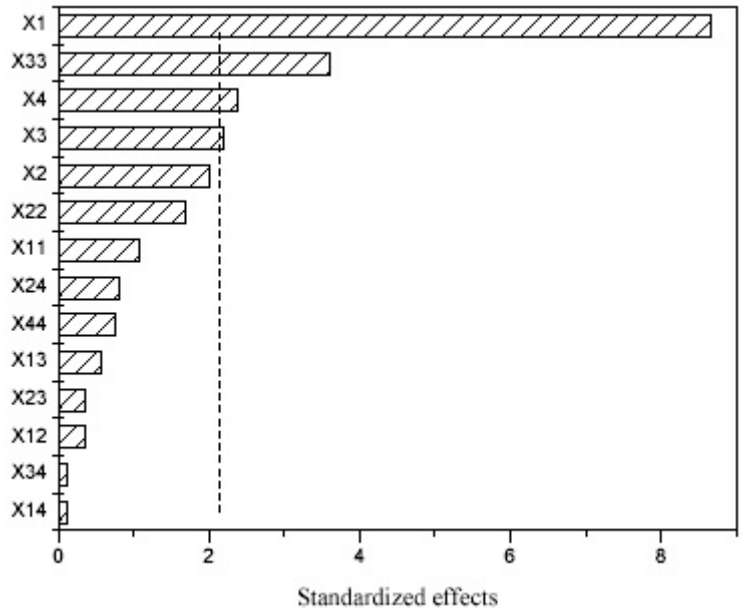


Fig. 2 Pareto chart for the estimated effects (TSS)

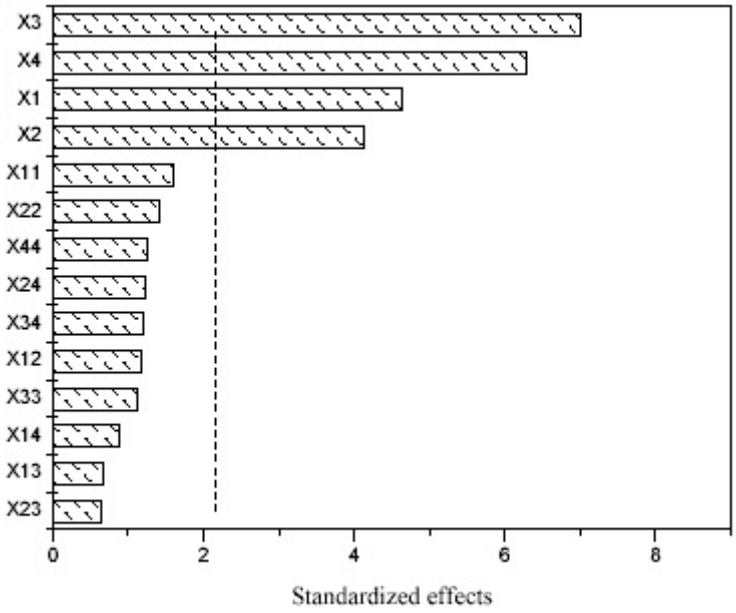
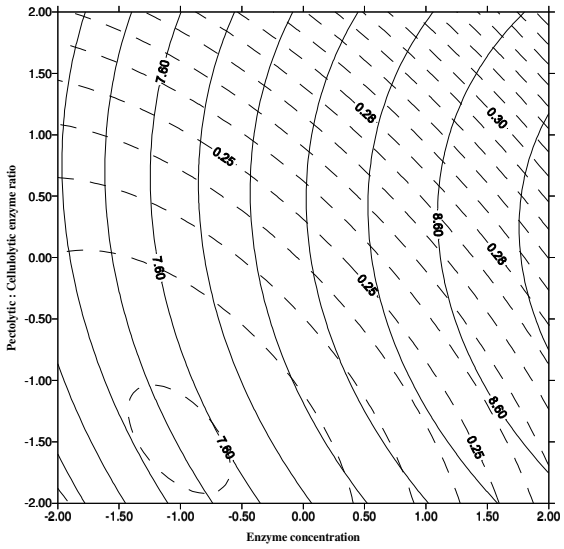
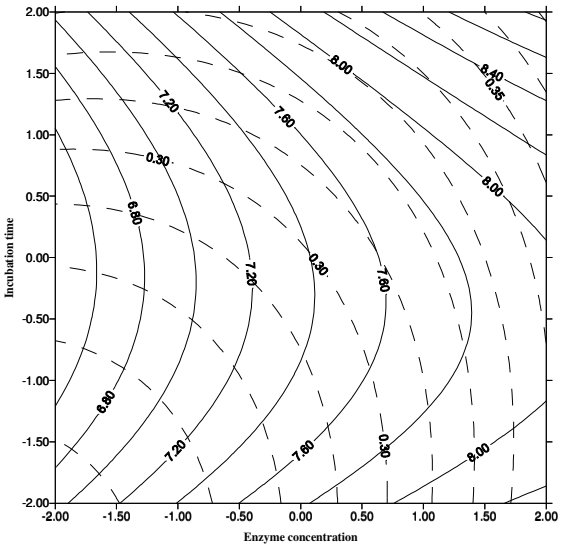


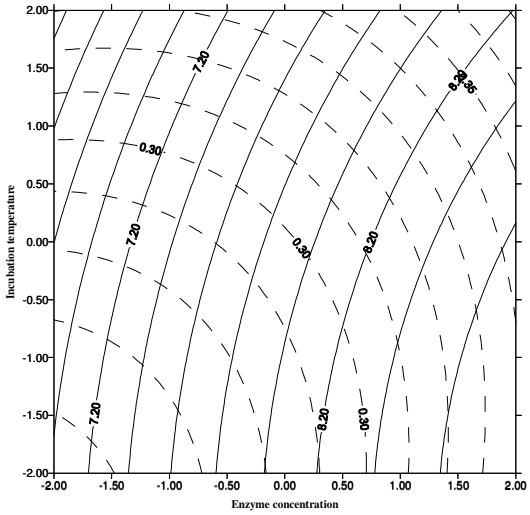
Fig. 3 Pareto chart for the estimated effects (colour index)



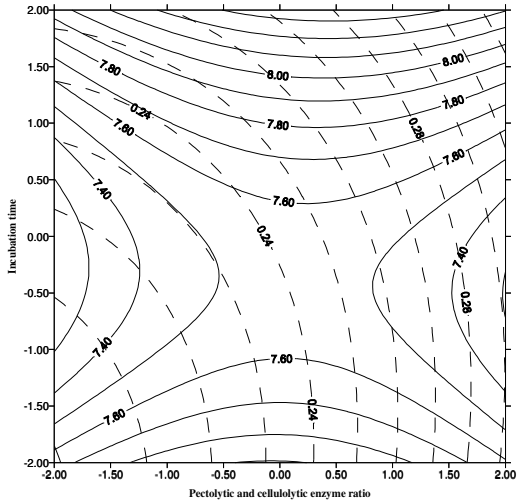
(a) Enzyme concentration and Enzyme ratio



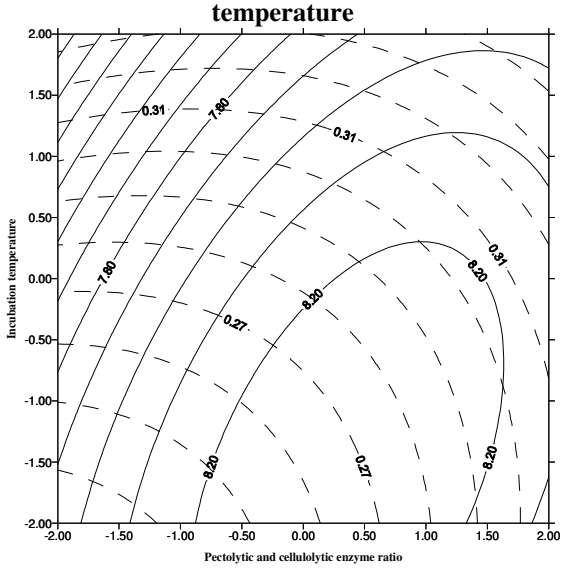
(b) Enzyme concentration and Incubation time



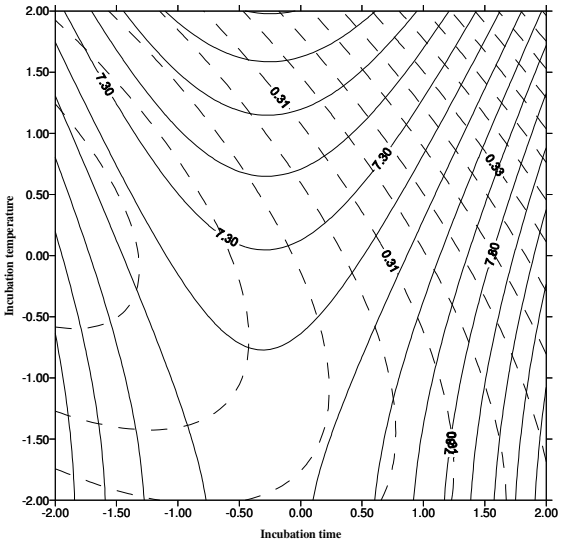
(c) Enzyme concentration and Incubation temperature



(d) Enzyme ratio and Incubation time



(e) Enzyme ratio and Incubation temperature



(f) Incubation time and Incubation temperature

Fig. 4 TSS and colour index as affected by variables

Table 1 Pectin and cellulose rich media

Pectin rich media, 1000 ml	
Pectin	12.5 g
Peptone	10.0 g
K_2HPO_4	0.5 g
NH_4NO_3	0.5 g
$MgSO_4.7H_2O$	0.2 g
FeCl, 2%w/v	0.3 ml
pH	5.4 using 1N HCl
Cellulose rich media, 1000 ml	
Wheat bran	40 g
pH	7.0 using 1N NaOH

Table 2 Experimental design in coded* form for response surface analysis

Coded variables				Combinations	Replications	Number of experiments
X_1	X_2	X_3	X_4			
0	0	0	0	1	7	7
±1	±1	±1	±1	16	1	16
±2	0	0	0	2	1	2
0	±2	0	0	2	1	2
0	0	±2	0	2	1	2
0	0	0	±2	2	1	2
				Total number of experiments		31

*Code "0" is for centre point of the parameter range investigated, "±1" for factorial points, and "±2" for augmented points; X_1, Enzyme concentration; X_2, pectolytic and cellulolytic enzyme ratio; X_3, Incubation time; X_4 Incubation temperature.

Table 3 Coded, uncoded parameter levels and equations relating actual and coded values

	Code				
	-2	-1	0	1	2
Experimental parameters					
Enzyme concentration, mg/Kg of matter (X_1)[a]	50	200	350	500	650
Pectolytic and cellulolytic enzyme ratio, (X_2) [b]	3:7	4:6	5:5	6:4	7:3
Incubation time, min, (X_3) [c]	30	60	90	120	150
Incubation temperature, ^oC, (X_4) [d]	25	35	45	55	65

[a] $X_1 = (x_1-350)/150$
[b] $X_2 = (x_2-5)/1$
[c] $X_3 = (x_3-90)/30$
[d] $X_4 = (x_4-45)/10$, where 'X' and 'x' are coded and actual values respectively.

Table 4 Responses at different experimental combinations for enzyme-assisted juice expression process

Experiment No.	Coded Variable				Responses	
	X_1	X_2	X_3	X_4	TSS, ^{O}Brix	Colour Index
1.	-1	-1	-1	-1	7.1	0.2045
2.	1	-1	-1	-1	7.7	0.2131
3.	-1	1	-1	-1	7.1	0.2137
4.	1	1	-1	-1	7.8	0.2397
5.	-1	-1	1	-1	7.2	0.2167
6.	1	-1	1	-1	8.0	0.2461
7.	-1	1	1	-1	7.3	0.2447
8.	1	1	1	-1	8.0	0.2919
9.	-1	-1	-1	1	6.8	0.2113
10.	1	-1	-1	1	7.5	0.2473
11.	-1	1	-1	1	7.0	0.2412
12.	1	1	-1	1	7.6	0.2769
13.	-1	-1	1	1	6.8	0.2996
14.	1	-1	1	1	7.7	0.2869
15.	-1	1	1	1	7.2	0.2839
16.	1	1	1	1	7.9	0.2931
17.	-2	0	0	0	6.3	0.2361
18.	2	0	0	0	8.0	0.2843
19.	0	-2	0	0	6.8	0.2371
20.	0	2	0	0	7.3	0.2803
21.	0	0	-2	0	7.7	0.2309
22.	0	0	2	0	8.1	0.2810
23.	0	0	0	-2	7.4	0.2313
24.	0	0	0	2	7.0	0.2832
25.	0	0	0	0	7.5	0.2391
26.	0	0	0	0	7.3	0.2428
27.	0	0	0	0	7.5	0.2468
28.	0	0	0	0	7.6	0.2390
29.	0	0	0	0	7.2	0.2391
30.	0	0	0	0	6.8	0.2401
31.	0	0	0	0	7.8	0.2393
Control (untreated sample)					7.0+0.1	0.1955+0.002

Table 5 Analysis of variance of full second order models 3 and 4

Responses	Sources of variation	Degree of freedom	Sum of squares	Mean sum of squares	F-value (calculated)
TSS	Regression	14	5.07877	0.3628	7.89**
	Error	16	0.7361	0.046	
	Total	30	5.81484		
	$R^2 = 0.87$; R^2(adj) = 0.76; R^2(pre) = 0.75; s = 0.2145				
Colour index	Regression	14	0.02041	0.001458	9.92**
	Error	16	0.00235	0.000147	
	Total	30	0.02276		
	$R^2 = 0.90$; R^2(adj) = 0.81; R^2(pre) = 0.42; s = 0.0121				

*P<0.05 (2.37), **P<0.01 (3.46), for degree of freedom (14, 16)

Table 6 Analysis of variance for total effect of individual parameter and combined effect at linear, interactive and square level

Responses	Parameter	Degree of freedom	Sum of squares	Mean sum of squares	F-Value (calculated)
TSS	X_1	5	3.538	0.708	15.38**
	X_2	5	0.852	0.170	3.70*
	X_3	5	0.872	0.174	3.79*
	X_4	5	0.319	0.064	1.39
	All linear terms	4	4.110	1.028	22.34**
	All interactive terms	6	0.060	0.010	0.22
	All square terms	4	1.353	0.338	7.35*
Colour index	X_1	5	0.090	0.018	68.78**
	X_2	5	0.141	0.028	107.67**
	X_3	5	0.137	0.027	104.92**
	X_4	5	0.052	0.010	39.66**
	All linear terms	4	0.126	0.031	119.77**
	All interactive terms	6	0.102	0.017	64.62**
	All square terms	4	0.092	0.023	87.66**

*P<0.05 (2.74), **P<0.01 (4.20), for degree of freedom (6, 16)
*P<0.05 (2.85), **P<0.01 (4.44), for degree of freedom (5, 16)
*P<0.05 (3.01), **P<0.01 (4.77), for degree of freedom (4, 16)

Table 7 Predicted and observed responses value at optimum level of variables

Response	Predicted (Observed) value of response	Uncoded (Coded) values of variables at maximum TSS			
		x_1 (X_1)	x_2 (X_2)	x_3 (X_3)	x_4 (X_4)
TSS	8.3°Brix (8.24°Brix)	629.56	6:4	131.57	60.89
Colour index	0.3223 (0.3217)	(1.86)	(1)	(1.39)	(1.59)