Clarification of Guava (Psidium Guajava) Fruit Juice using Multi-Enzyme (Pectinase, Cellulase and Hemicellulase) Treatment: Optimization of Processing Parameters

Ravi Kumar, Alak Kumar Singh

Abstract: Clarified juices are making remarkable place in the market as these are preferred by a significant portion of the population apart from making ready to serve drinks, clear juice cocktails, cordials, clear nectars, translucent jelly products, candies, clear juice blends, etc. from it. There exist several market opportunities, not only for the traditional clear juice from apple, but also for clarified juices produced from fruits with high pulp content. This study was carried out with an objective of producing high quality clarified guava juice with high yield by multi enzyme system. Guava juice was treated with various concentration levels of commercial enzymes pectinase, cellulase and hemicellulase for different incubation periods (30-150 min) at 55 ^{0}C. The effect of treatment conditions was studied on turbidity and yield of clarified juice obtained for each enzyme separately and optimum process conditions were determined. Based on these findings guava juice was given simultaneous treatment of all three enzymes with compromised process conditions (Pectinase 1.00%, Cellulase 0.50%, and Hemicellulase 0.80% with 90 min incubation time at 55 ^{0}C) which produced clarified juice with turbidity 18 NTU and yield 62%. Response Surface Methodology (RSM) employing a second order central composite design was used to obtain optimum process conditions for simultaneous treatment with the range of variables for enzymatic treatment conditions (Enzyme concentration: 0.20-1.40% w/w, 0.20-0.80% w/w and 0.20-1.00% w/w for pectinase, cellulase and hemicellulase respectively, Incubation time: 30-150 min and Incubation temperature 55 ^{0}C) based on previous individual experiments, which showed results very close to that obtained in previous experiments giving the optimum values as 0.96%, 0.57% and 0.77% enzyme concentration for Pectinase, Cellulase and Hemicellulase respectively, and incubation period of 99 min at incubation temperature of 55 ^{0}C. Under this condition, the juice was obtained with turbidity value as 17 NTU and 64.7% of yield.

Keywords: Guava Juice, Enzymatic Clarification, Response Surface Methodology, Pectinase, Cellulase, Hemicellulase.

I. INTRODUCTION

Guava is one of the important commercial tropical fruits in India. It is known as the poor man's apple of the tropics [1]. Except during the summer season, it is available throughout the year. It gives an assured crop even with very little care. Its requirements for fertilizer, irrigation and plant protection are not much and so its cost of production is also low. Further it is of very high nutritive value and so it is often considered as super fruit. It is rich in vitamins A and C (in the pericarp), omega-3 and -6 polyunsaturated fatty acids (in the seeds) and has high levels of dietary fibre. A single guava fruit weighing 160-170 g contains over four times more of vitamin 'C' compared to a single orange (220-230 mg per 100g) and also has adequate levels of dietary minerals, potassium, and magnesium [2]. Along with its nutritional properties, this fruit is very appetizing due to its sensory (flavour and colour) properties [3]. Guava is also grown as a backyard fruit to great extent. India ranks first in production of guavas [4]. In India, the best quality guavas are produced in Uttar Pradesh, particularly in Allahabad region. Guava fruits are consumed either fresh or processed. However, only 0.05% of the produce is being exported to foreign countries [5]. There is ample scope for production of high-value clarified juices from guava to minimize the wastage and to earn higher foreign revenue by increasing the export of such valuable products.

The ripened guava is highly perishable when kept at ambient temperature. Therefore, it is processed in various commercial guava products that include paste, puree, juice, canned slices. The guava juice has become economically important in the market among these products. The consumption of tropical fruit juice like guava juice has been increasing currently as it is natural, high in nutritional value and may be used as an alternative to other beverages such as soft drinks, coffee and tea [6]. A lot of people prefer a grit-free, haze-free and clear guava juice. Clarified guava juice may be more acceptable by the general population, and may be used in the manufacturing of products that are based on clear juice e.g. clear guava nectar, clear jelly, clear guava powder or a mixed juice blend [7]. The fruit-based juice market is one of the fastest-growing categories in the beverage sector. It is growing at a CAGR (compound annual growth rate) of 25-30 per cent in the past decade. The juice market in India is estimated to be around Rs. 10,781.62 crores (16 per cent of the total soft drink market in India). The fruit juice market in India is projected to grow at a compound annual growth rate of 22 per cent over the next five years, and is expected to grow more than double in the next few years [8]. The flavour and aroma of guava are highly appreciated and is able to compete in the market, either as guava juice or as mixtures with other fruit juices.

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However, the fresh guava juice is gray in colour, turbid, very viscous and tends to settle during storage [9]. Fruit juices are generally extracted by crushing and grinding. Juices obtained by these operations are viscous, turbid and cloudy. This happens due to the presence of pulp particles and colloidal suspensions. Yield of this kind of juices is low and it is very difficult to concentrate and pasteurize them. The conventional guava juice processing can be made by mechanical pressing of guava mash. Raw guava juice is gray in colour, very viscous and turbid. Pectin substances, that are composed of partially methyl-esterified galacturonic acid residues linked by α-1,4-glycosidic bonds, are responsible for the turbidity and viscosity of guava juice [10]. Juices with unacceptable cloud and muddy turbidity are undesirable for marketing [11]. A variety of products based on clarified juice such as ready to serve drinks, sparkling clear beverages (soft drinks, clear juice cocktails, cordials, clear nectars, alcoholic beverages, nutritional carbonated beverages, cold teas with clear juice), translucent jelly products, candies, clear juice blends, fruit honey or fruit sugar, 100% canned fruit (with clear juice as syrup) etc. are making place in the market.

Clarification is the process of breaking the semistable emulsion of colloidal plant carbohydrates that support the insoluble cloud material in a freshly extracted juice. Enzymatic treatment of juices results in degradation of pectin and viscosity reduction which facilitates separation through filtration or centrifugation giving the juice higher clarity. Currently pectinases, cellulases and hemicellulases, collectively called macerating enzymes are used for extraction and clarification of fruit juices. Pectinases hydrolyses α-1,4-glycosidic linkages of pectin molecules and produce polygalacturonic acid monomers [12]. Cellulases cleaves β-1,4-D-glucan linkages of cellulose to yield oligosaccharides, cellobiose and glucose [13,14] while hemicellulases are a diverse group of enzymes that hydrolyse hemicelluloses, one of the most abundant group of polysaccharide found in nature [15]. The use of pectinolytic and cellulolytic enzymes in combination for juice clarification enhances the yield and clarity because of simultaneous degradation of polysaccharides [16]. The effect of hydrolytic enzymes on juice extraction and clarification have been reported in guava [6,10,17,18,19,20], banana [21, 22,23,24,25,26,27,28,29], litchi [30,11], mango [31,32], kiwifruit [33,34], papaya [35,36], apple [37,38], pineapple [39,40], asparagus [41], peach [42], pear [43], carrot [44,45] and plums [46].

II. MATERIALS AND METHODS

A. Fruits: Fully ripened fresh guavas *(Psidium guajava)* were purchased from the local market and used immediately or stored in a refrigerator (4^0 C) for not more than 5 days before being used.

B. Enzyme Source: Commercial enzymes, BL-Pectinase, BL-Cellulase and BL-Hemicellulase obtained from Biolaxi Corporation, Bhiwandi, India, were used for enzymatic treatment of fruit juice. BL-Pectinase was a food grade enzyme preparation specially designed for cell wall degradation and pectic substance extraction. The activity of BL-Pectinase was 1200 PGU/g. The recommended optimum enzyme reaction conditions were pH 3.5 to 6.0 (Optimum 3.8) and temperature 40 ^{0}C to 60 ^{0}C (Optimum 55 ^{0}C). BL-Cellulase and BL-Hemicellulase were also food grade enzyme preparations designed for cell wall degradation and extraction, with the enzyme activities of 1,00,000 CMCU/g and 1,00,000 HCU/g respectively. The recommended optimum enzyme reaction conditions for BL-Cellulase were pH 4.5 to 6.0 (Optimum 4.8) and temperature 40 ^{0}C to 60 ^{0}C (Optimum 55 ^{0}C) and for BL-Hemicellulase were pH 4.5 to 6.5 (Optimum 4.5) and temperature 40 ^{0}C to 60 ^{0}C (Optimum 50 ^{0}C).

C. Juice Preparation: Ripened guavas were washed, peeled and cut in small pieces. Based on previous works, a ratio of 1:1 (guava: water; w/w) [18] was used for juice preparation in electric blending and juicing machine. A homogeneous mixture was obtained free of seeds from juicing machine. The pH of the juice obtained was 5.3. Figure 1 shows the steps involved in extraction and clarification of guava juice by enzyme treatment.

D. Enzymatic Treatment and Optimization: The juice was treated with each enzyme separately and optimum conditions for enzyme dose and incubation time were determined. For optimization of enzyme dose the fruit juice was treated with different doses (from 0.10% w/w to 1.40% w/w for pectinase, from 0.10% w/w to 0.80% w/w for cellulase and from 0.10% w/w to 1.00% w/w for hemicellulase) keeping the incubation time and temperature fixed (60 min and 55 ^{0}C respectively) in each experiment, and for optimization of incubation time, the fruit juice was treated for different time periods (from 30 min to 150 min) keeping the enzyme dose (optimum as obtained from previous experiment) and incubation temperature (55 ^{0}C) fixed in each experiment. The reaction is carried out at incubation temperature of 55 ^{0}C, based on the optimum temperature recommendations for commercial enzymes. The fruit juice without addition of enzyme was taken as control for enzyme dose experiments and fruit juice without addition of enzyme and no retention time was taken as control for incubation time experiment. At the end of the enzymatic treatment, the enzyme in the sample was inactivated by heating the suspension at 90 ^{0}C for 5 min in a water bath and immediately cooled to room temperature. The treated juices were centrifuged at 1100 RPM for 20 min and the supernatant was collected. The effect of simultaneous addition of all the three enzymes on juice was also studied at the adjusted optimum conditions obtained from the above experiments.

To obtain optimum process conditions for simultaneous treatment of all the three enzymes by Response Surface Methodology (RSM), a four variable (five level of each) second order central composite rotatable design (CCRD) was employed. Minitab 17.1.0 (Minitab Inc.) statistical software (trial version) was used for data analysis. The independent variables were concentration of pectinase (x_1), concentration of cellulase (x_2), concentration of hemicellulase (x_3) and incubation time (x_4). Based on the previously mentioned experiments with the individual enzymes, the ranges of variables selected are: Enzyme Concentration (Pectinase), X_1: 0.20-1.40% w/w, Enzyme Concentration (Cellulase), X_2: 0.20-0.80% w/w, Enzyme Concentration (hemicellulase), X_3: 0.20-1.00% w/w and Incubation Time, X_4: 30-150 min. The experimental design is shown in Table-1 in coded (x) and actual (X) levels of variables.

The response functions (y) were the turbidity and juice yield. The values of response functions were related to the coded variables (x_i, i=1, 2, 3) by a second-degree polynomial using the equation below (Eq. 1):

$$Y_k = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1x_1 + b_{22}x_2x_2 + b_{33}x_3x_3 + b_{44}x_4x_4 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 + \varepsilon \quad \ldots[1]$$

The coefficients of polynomial were represented by constant term b_0; linear effects b_1, b_2, $b_3$and b_4; quadratic effects b_{11}, b_{22}, $b_{33}$and b_{44}; interaction effects b_{12}, b_{13}, b_{14}, b_{23}, $b_{24}$and b_{34} and random error ε. The Analysis of Variance was performed and the effects of individual linear, quadratic and interaction terms were determined. The significance of all the terms in the polynomial equation were judged statistically by computing the probability (p) at level of 0.001, 0.01 and 0.05 [6, 18].

E. Turbidity Determination: Turbidity was determined using Insif India Digital Turbidity Meter (Labpro International, Ambala, Haryana). The results were reported as Nephelometric Turbidity Units (NTU).

F. Percentage Yield Determination: Percent yield was estimated as percentage of the clarified juice obtained based on the initial fruit pulp.

III. RESULTS AND DISCUSSION

The guava juice was first treated with each enzyme separately to study the effect and optimum process conditions for individual enzymes and then simultaneous treatment was given to juice based on the optimum conditions obtained for individual enzymes. Response surface methodology was used to obtain optimum for simultaneous treatment based on the range of concentration of enzymes and range of incubation time obtained during the experiments performed with individual enzymes.

A. Optimization of enzyme reaction conditions for individual enzyme treatments

Effect of Enzyme Dose

For determination of optimum enzyme dose of pectinase, cellulase and hemicellulase, concentrations of the enzymes were varied starting from 0.10% (w/w), as shown in Table 2, 4 and 6 respectively, at temperature of 55 ^{0}C for 60 min of incubation. Table 2 shows the effect of pectinase concentration on juice turbidity and yield. On increasing the pectinase concentration, the turbidity of juice decreased and minimum value of turbidity 10 NTU was obtained at the pectinase concentration value of 1.0%. No juice was obtained at pectinase concentration of 0.10%. The decrease in turbidity value on increasing the enzyme concentration was due to decreased amount of pectin in the juice, reducing the turbidity [47]. Guez et al. [48] also reported negative effect of pectinase concentration on turbidity for caja-manga (*Spondiascytherea* Sonn.) pulp making the juice clearer. Drastic decrease in turbidity was also reported by Alam et al. [45] on increase in pectinase concentration for clarification of carrot juice. Abdullah et al. [49] and Landbo et al. [50] also used pectinase for reduction in juice turbidity in carambola and elderberry juice. The variation in turbidity values is due to breakdown of protein molecules and formation of pectin-protein flocs that results in removal of colloidal suspension leaving a clear supernatant [47]. Juice yield increased with the increasing concentration of pectinase up to the corresponding pectinase concentration of 1.0% and remained almost constant thereafter. Based on these results the optimum pectinase concentration was taken as 1.0%. The turbidity of control sample which was found to be more than 1000 NTU was reduced to a minimum value of 10 NTU after the treatment with pectinase. Per cent yield was also significantly increased after enzyme treatment. These effects are graphically represented in Fig.2. Akesowan et al. [13], Sevda et al. [10], Kaur et al. [18] also reported the increase in guava juice clarity and yield on increasing pectinase enzyme concentration. Table 4 shows the effect of cellulase concentration on juice turbidity and yield. The turbidity of juice decreased on increasing the cellulase concentration up to a minimum value of 94 NTU at the concentration value of 0.50 % and thereafter it remained almost constant. Juice yield increased markedly with the increasing concentration of cellulase up to the enzyme concentration of 0.50% and only slight increase was observed thereafter. As compared to control the turbidity was greatly reduced from the value greater than 1000 NTU and to a minimum value of 94 NTU at the cellulase concentration of 0.5%. Yield was found slightly decreased as compared to control at this cellulase concentration. Based on these results the optimum cellulase concentration was taken as 0.50%. These effects are graphically represented in Fig.4. Jori et al. [51] studied the effect of combined treatment of cellulase and pectinase on blended pineapple and mango pulp and reported increase in clarity and yield on increasing the enzyme concentration and juice clarity and yield were found more sensitive with respect to cellulase. By using the enzymatic pool Cellulase FNC-1. Sreenath et al. [40] recovered up to 86% pineapple juice by treatment with cellulase and pectinase. Table 6 shows the effect of hemicellulase concentration on juice turbidity and yield. No significant change was observed in turbidity up to the hemicellulase concentration of 0.50%. After that a significant decrease in turbidity was observed up to the enzyme concentration of 0.8% and slight increase thereafter. Yield was also not markedly changed up to the hemicellulase concentration of 0.5%. After that it markedly increased up to the enzyme concentration of 0.8% and remained almost constant thereafter. Significant decrease in turbidity was observed in juice as compared to control but there was also a decrease in yield of juice than that of control. Based on these results the optimum hemicellulase concentration was taken as 0.80%. These effects are graphically represented in Fig.6. Shah and Nath [11] reported significant increase in litchi juice clarity and yield by the treatment of pectinase, cellulase and hemicellulase. The optimum concentration values for the enzymes were used in next experiment for optimization of incubation time.

Effect of Incubation Time

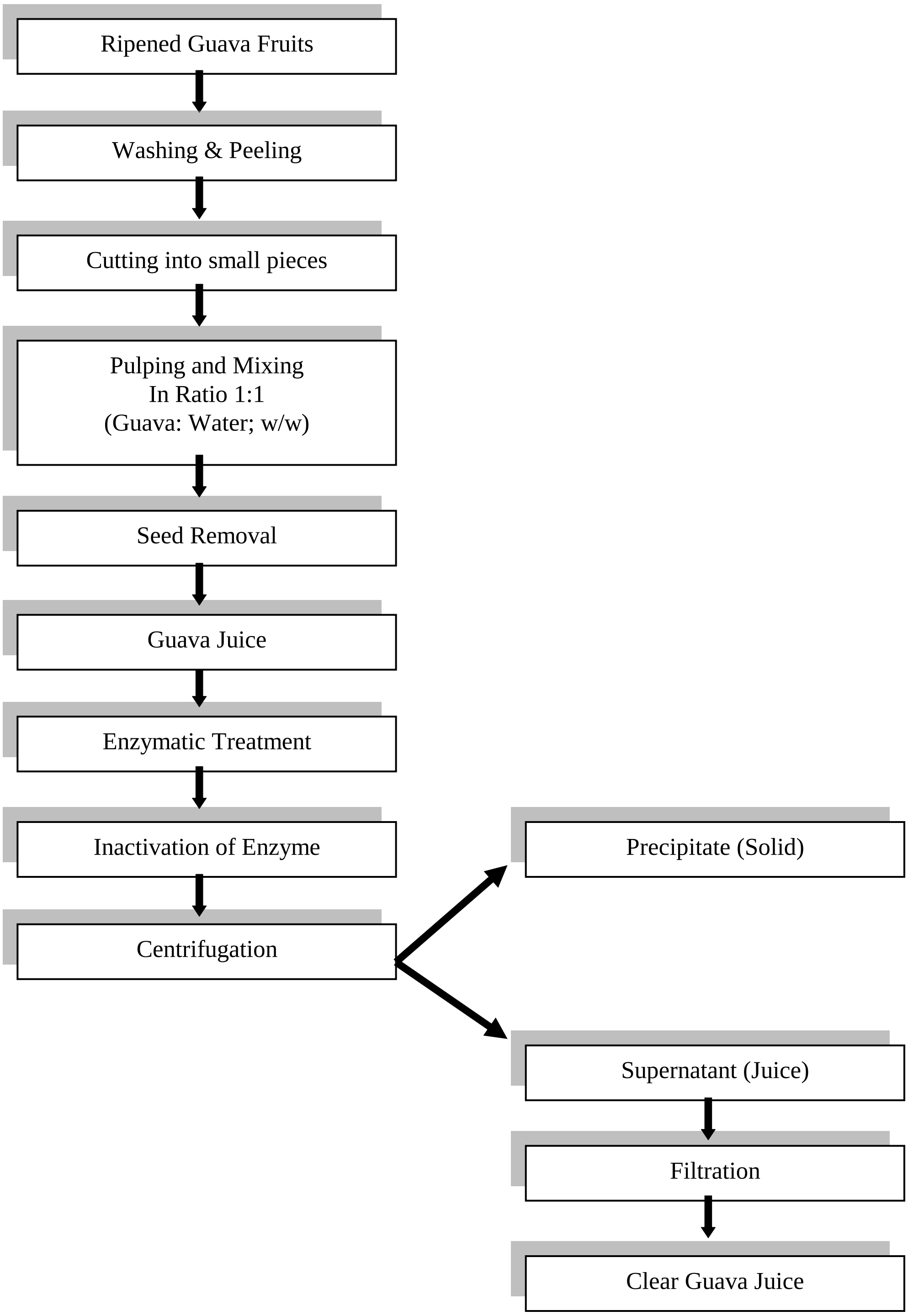

Fig 1: Steps for Guava Juice Extraction and Subsequent Clarification by Treatment with Enzymes

The effect of varying incubation time for pectinase, cellulase and hemicellulase (from 30 min to 150 min as shown in Table 3,5 and 7 respectively), on the efficiency of enzymes was studied at a fixed enzyme dose of 1.0% (w/w) for pectinase, 0.05% for cellulase and 0.8% for hemicellulase based on above experiments at temperature of 55 ^{0}C. Table 3 shows the effect of incubation time on juice turbidity and yield for pectinase. Turbidity was found minimum at 60 min incubation time. There was a slight decrease in yield at this incubation time and again increase was observed at 90 min incubation time that remained almost same at 150 min incubation time. The turbidity and yield were significantly affected with the variation of incubation time as compared to control. Based on minimum turbidity value the compromised optimum incubation time was taken as 60 min for pectinase. In Fig. 3, these effects are graphically represented. Table 5 shows the effect of incubation time on juice turbidity and yield for cellulase. Turbidity was significantly decreased from the value of 146 NTU at 30 min incubation time to 95 NTU at 60 min incubation time and a very slight decrease was observed thereafter.

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Yield was found maximum at 90 min incubation time. Turbidity and yield both are significantly affected by changing the incubation time

Table-1: The Central Composite Rotatable Experimental Design (in coded and actual level of four variables) employed for treatment of Guava juice with enzymes

Central Composite Design

Factors: 4 Replicates: 1
Base runs: 31 Total runs: 31
Base blocks: 1 Total blocks: 1
Two-level factorial: Full factorial
Cube points: 16
Center points in cube: 7
Axial points: 8
Center points in axial: 0
α: 2

Design Table

Experiment No.	Enzyme Concentration (% w/w)						Incubation Time (min)	
	Pectinase		Cellulase		Hemicellulase			
	X_1	x_1	X_2	x_2	X_3	x_3	X_4	x_4
1	0.50	-1	0.35	-1	0.40	-1	60	-1
2	1.10	1	0.35	-1	0.80	1	120	1
3	1.10	1	0.35	-1	0.40	-1	120	1
4	0.50	-1	0.35	-1	0.80	1	60	-1
5	0.80	0	0.50	0	0.60	0	90	0
6	0.80	0	0.50	0	0.60	0	30	-2
7	0.20	-2	0.50	0	0.60	0	90	0
8	1.40	2	0.50	0	0.60	0	90	0
9	0.80	0	0.50	0	0.60	0	90	0
10	1.10	1	0.65	1	0.80	1	120	1
11	0.80	0	0.50	0	0.20	-2	90	0
12	1.10	1	0.65	1	0.40	-1	120	1
13	0.80	0	0.20	-2	0.60	0	90	0
14	0.80	0	0.50	0	0.60	0	90	0
15	0.80	0	0.50	0	0.60	0	90	0
16	1.10	1	0.65	1	0.40	-1	60	-1
17	0.80	0	0.50	0	0.60	0	90	0
18	0.50	-1	0.65	1	0.80	1	120	1
19	1.10	1	0.35	-1	0.40	-1	60	-1
20	0.50	-1	0.35	-1	0.80	1	120	1
21	0.50	-1	0.65	1	0.80	1	60	-1
22	0.50	-1	0.65	1	0.40	-1	60	-1
23	0.50	-1	0.35	-1	0.40	-1	120	1
24	0.50	-1	0.65	1	0.40	-1	120	1
25	0.80	0	0.50	0	0.60	0	90	0
26	1.10	1	0.35	-1	0.80	1	60	-1
27	0.80	0	0.80	2	0.60	0	90	0
28	0.80	0	0.50	0	0.60	0	90	0
29	1.10	1	0.65	1	0.80	1	60	-1
30	0.80	0	0.50	0	0.60	0	150	2
31	0.80	0	0.50	0	1.00	2	90	0

as compared to control. Based on these results the optimum incubation time was taken as 90 min for cellulase. In Fig. 5, these effects are graphically represented. The effect of incubation time on juice turbidity and yield for hemicellulase are shown in Table 7. Turbidity decreased markedly with the increasing incubation time up to 90 min and very slight change was observed thereafter. There was a significant increase in yield up to incubation time of 90 min, and a very slight increase thereafter. Turbidity was significantly decreased with the increase in incubation time as compared to control. Yield also increased with the increase in incubation time but as compared to control it decreased.

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Based on these results the compromised optimum value for incubation time was taken as 90 min for hemicellulase. In Fig. 7, these effects are graphically represented. Thus decrease in turbidity and increase in juice yield was observed with increase in treatment time but high incubation time was not preferred because of the fact that increased incubation time can cause the formation of protein-carbohydrate or protein-tannin complex [52].

Table 2. Effect of pectinase concentration on guava juice turbidity and yield

S. N.	Enzyme Conc. (%w/w)	Turbidity (NTU)	Yield (%)
1	0.00	>1000	47.2
2	0.10	-	-
3	0.20	19	10.6
4	0.30	18	21.3
5	0.40	18	31.7
6	0.50	16	40.0
7	0.60	16	46.3
8	0.70	15	47.9
9	0.80	13	50.4
10	0.90	12	52.9
11	1.00	10	55.0
12	1.10	14	55.0
13	1.20	18	55.2
14	1.30	23	55.3
15	1.40	26	55.6

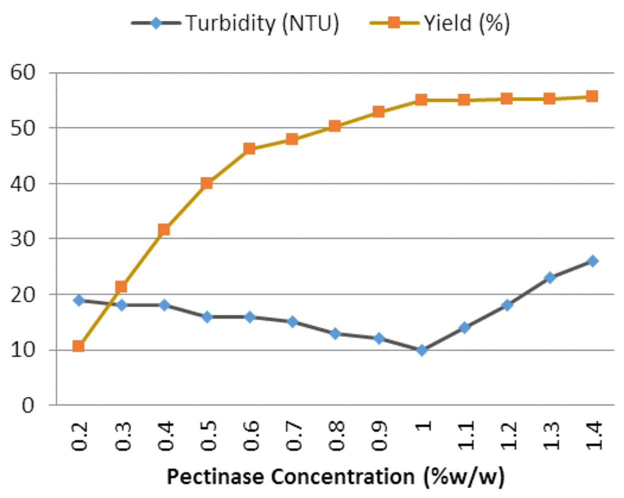

Fig.2. Effect of pectinase concentration on guava juice turbidity and yield

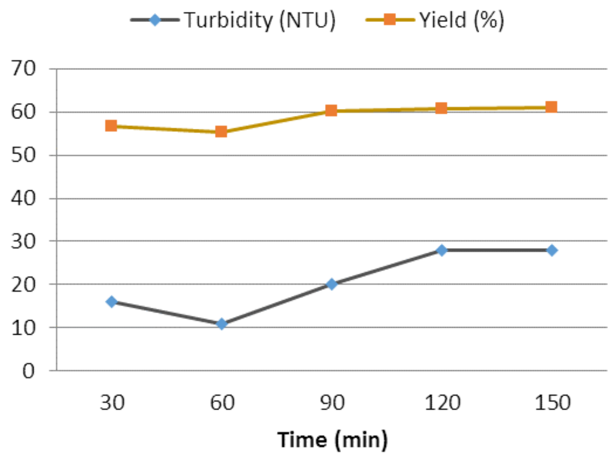

Fig. 3. Effect of incubation time on pectinase treatment on guava juice

Table 3. Effect of incubation time on pectinase treatment on guava juice

S. N.	Enzyme Conc. (%w/w)	Time (min.)	Turbidity (NTU)	Yield (%)
1	0.00	00	>1000	46.3
2	1.00	30	16	56.6
3	1.00	60	11	55.4
4	1.00	90	20	60.3
5	1.00	120	28	60.8
6	1.00	150	28	60.9

Table 4. Effect of cellulase concentration on guava juice turbidity and yield

S. N.	Enzyme Conc. (%w/w)	Turbidity (NTU)	Yield (%)
1	0.00	>1000	47.2
2	0.10	294	29.8
3	0.20	168	41.6
4	0.30	130	44.0
5	0.40	110	45.6
6	0.50	94	46.6
7	0.60	94	47.1
8	0.70	95	47.4
9	0.80	94	47.6

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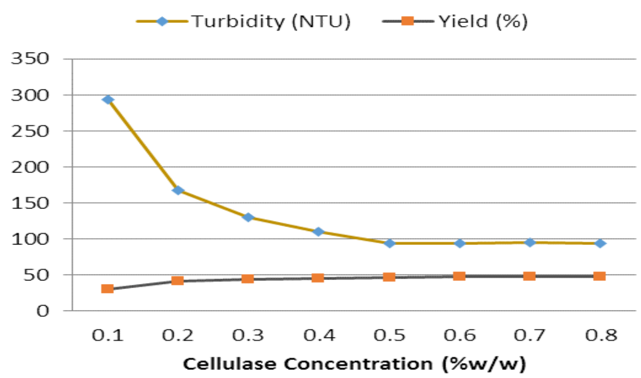


Fig.4 Effect of cellulase concentration on guava juice turbidity and yield

Table 5. Effect of treatment time on cellulase treatment on guava juice

S. N.	Enzyme Conc. (%w/w)	Time (min.)	Turbidity (NTU)	Yield (%)
1	0.00	00	>1000	46.3
2	0.50	30	146	44.5
3	0.50	60	95	46.4
4	0.50	90	94	52.6
5	0.50	120	91	52.0
6	0.50	150	91	51.9

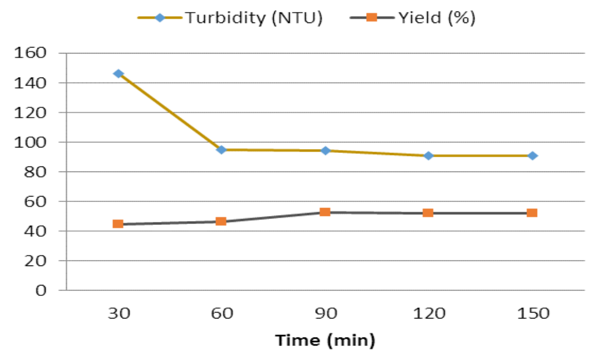


Fig.5 Effect of treatment time on cellulase treatment on guava juice

Table 6. Effect of hemicellulase concentration on guava juice turbidity and yield

S. N.	Enzyme Conc. (%w/w)	Turbidity (NTU)	Yield (%)
1	0.00	>1000	47.2
2	0.10	526	19.3
3	0.20	524	19.6
4	0.30	525	19.2
5	0.40	525	19.4
6	0.50	524	19.1
7	0.60	418	28.5
8	0.70	280	33.6
9	0.80	140	38.2
10	0.90	142	38.4
11	1.00	146	38.6

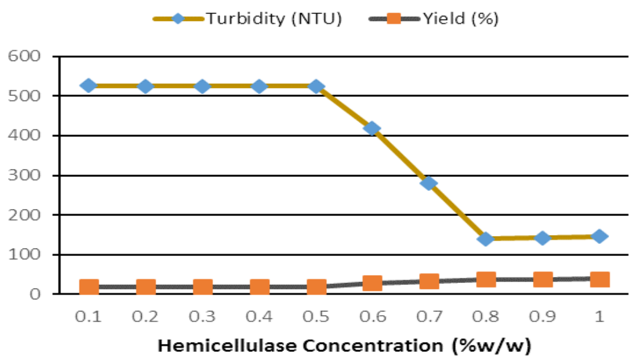
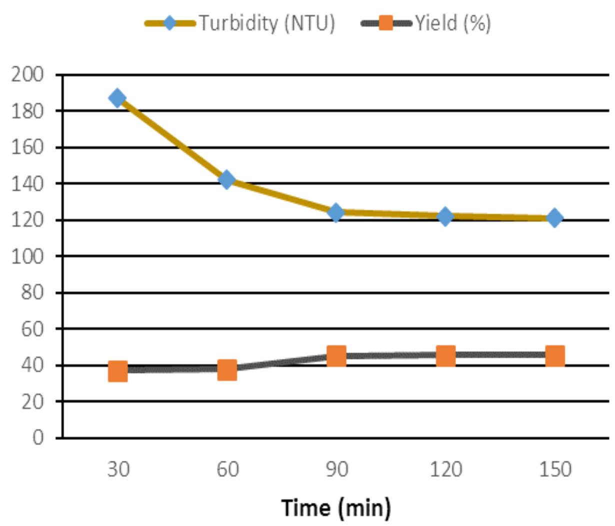


Fig.6. Effect of hemicellulase concentration on guava juice turbidity and yield

Table 7. Effect of treatment time on hemicellulase treatment on guava juice

S. N.	Enzyme Conc. (%w/w)	Time (min.)	Turbidity (NTU)	Yield (%)
1	0.00	00	>1000	46.3
2	0.80	30	187	37.2
3	0.80	60	142	37.8
4	0.80	90	124	45.4
5	0.80	120	122	45.6
6	0.80	150	121	45.7

Fig.7. Effect of treatment time on hemicellulase treatment on guava juice



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B. Effect of simultaneous treatment of fruit juice with Pectinase, Cellulase and Hemicellulase

The optimum enzyme reaction conditions were obtained from the above experiments for the three enzymes separately. The optimum reaction conditions obtained for pectinase, were 1.00% enzyme concentration and 60 min incubation time. The optimum reaction conditions obtained for cellulase and hemicellulase, were 0.50% enzyme concentration with 90 min incubation time and 0.80% enzyme concentration with 90 min incubation time respectively. The compromised optimum treatment conditions for simultaneous treatment were taken as Pectinase 1.00%, Cellulase 0.50%, Hemicellulase 0.80% with 90 min incubation time at a temperature of 55 ^{0}C. The effect of treating the fruit juice simultaneously with all the three enzymes is shown in Table 8. The fruit juice without addition of enzyme was taken as control.

Table 8. Effect of treatment of fruit juice with compromised optimum treatment conditions of pectinase, cellulase and hemicellulase simultaneously.

	Control	Test
Enzyme Concentration (% w/w)		
Pectinase	0	1.00
Cellulase	0	0.50
Hemicellulase	0	0.80
Incubation Temperature (^{0}C)	55	55
Incubation Time (min)	90	90
Results		
Turbidity (NTU)	>1000	18
Yield (%)	47.4	62

Simultaneous treatment of guava juice with pectinase, cellulase and hemicellulase produced clarified juice with turbidity 18 NTU and yield 62 %.

C. Response Surface Optimization

The experimental results of effect of the independent variables viz. concentrations of pectinase, cellulase and hemicellulase and incubation time on the responses (turbidity and juice yield) are shown in Table 9. The regression coefficients and R^2 values for second order polynomial equation are presented in Table 10 for the responses. The adequacy and fitness of these equations were tested by analysis of variance (ANOVA) [53]. Table 11 shows Analysis of Variance of regression models for responses. It suggests that linear and quadratic terms contribute significantly to the models for almost all responses. The values of R^2 for turbidity and yield were 96.02 and 94.63 respectively. The R^2 value close to 100% suggests that the model fitted to the actual data. Thus the analysis of variance shows that the predicted 2nd order models are statistically valid.

Table 9: Responses for clarified guava juice during RSM optimization studies

Experiment No.	Factors				Responses	
	Enzyme Concentration			Incubation Time	Turbidity	Yield
	Pectinase	Cellulase	Hemicellulase			
	(% w/w)	(% w/w)	(% w/w)	(min)	(NTU)	(%)
1	0.50	0.35	0.40	60	43	42.5
2	1.10	0.35	0.80	120	31	57.5
3	1.10	0.35	0.40	120	39	50.3
4	0.50	0.35	0.80	60	24	48.9
5	0.80	0.50	0.60	90	25	68.2
6	0.80	0.50	0.60	30	24	39.2
7	0.20	0.50	0.60	90	32	46.7
8	1.40	0.50	0.60	90	28	60.9
9	0.80	0.50	0.60	90	24	64.4
10	1.10	0.65	0.80	120	16	61.8
11	0.80	0.50	0.20	90	48	45.3
12	1.10	0.65	0.40	120	33	52.9
13	0.80	0.20	0.60	90	39	49.0
14	0.80	0.50	0.60	90	25	63.8
15	0.80	0.50	0.60	90	24	63.9
16	1.10	0.65	0.40	60	40	50.2
17	0.80	0.50	0.60	90	22	63.0
18	0.50	0.65	0.80	120	20	52.6
19	1.10	0.35	0.40	60	42	48.6
20	0.50	0.35	0.80	120	23	52.3
21	0.50	0.65	0.80	60	31	54.2
22	0.50	0.65	0.40	60	47	43.8
23	0.50	0.35	0.40	120	40	48.3
24	0.50	0.65	0.40	120	38	48.1
25	0.80	0.50	0.60	90	23	63.5
26	1.10	0.35	0.80	60	27	54.7
27	0.80	0.80	0.60	90	30	53.0
28	0.80	0.50	0.60	90	21	62.9
29	1.10	0.65	0.80	60	20	59.2
30	0.80	0.50	0.60	150	25	57.0
31	0.80	0.50	1.00	90	21	56.4

Table 10: Regression Coefficients and R^2 values for dependent variables for Guava

Term	Regression Coefficient	Turbidity	Yield
Constant	b_0	23.429*	64.243*
x1	b_1	-1.083***	3.038*
x2	b_2	-1.750**	1.154***
x3	b_3	-7.667*	3.279*
x4	b_4	-1.333***	2.387*
x1.x1	b_{11}	1.851*	-2.497*
x2.x2	b_{22}	2.976*	-3.197*
x3.x3	b_{33}	2.976*	-3.235*
x4.x4	b_{44}	0.476	-3.922*
x1.x2	b_{12}	-2.250***	0.394
x1.x3	b_{13}	0.625	0.369
x1.x4	b_{14}	0.875	-0.131
x2.x3	b_{23}	-0.750	0.569
x2.x4	b_{24}	-1.750**	-0.356
x3.x4	b_{34}	0.625	-0.456
R^2		96.02	94.63

* Significant at 0.001 level
** Significant at 0.01 level
*** Significant at 0.05 level

Table 11: Analysis of Variance (ANOVA) for 2nd Order Model

Source	DF	Turbidity		Juice Yield	
		Adj Sum of Squares	P Value	Adj Sum of Squares	P Value
Model	14	2225.65	<0.001	1591.28	<0.001
Linear	4	1555.00	<0.001	648.28	<0.001
Square	4	506.90	<0.001	927.54	<0.001
2-Way Interaction	6	163.75	<0.01	15.47	0.830
Error	16	92.55	-	90.35	-
Lack-of-Fit	10	78.83	0.072	70.46	0.184
Pure Error	6	13.71	-	19.90	-
Total	30	2318.19	-	1681.64	-
R^2	-	96.02%	-	94.63%	-
R^2 (adj)	-	92.51%	-	89.93%	-

Turbidity

Juice turbidity was significantly ($p \leq 0.05$) affected by the first order and second order of variables (Table 10). From the table 10 it was observed that turbidity has a negative linear effect with all the variables (significant at $p \leq 0.05$) and a positive effect at quadratic level for almost all variables (significant at $p \leq 0.001$). There were some interactions also found among the variables for juice turbidity. Turbidity value indicates impurities or unsettled matter in water suspension, e.g. colloidal polysaccharide particles in fruit juices [54]. A juice that is to be marketed as clear should have no unstable cloud and no turbidity that is considered ''muddy'', otherwise it will be unacceptable to be marketed [55]. The decrease in turbidity on increasing pectinase enzyme concentration was also reported by Alam et al. [45] for carrot juice, Karangwa et al. [56] for blended carrot-orange juice, Vinjamuri and Bhavikatti [57] for mixed fruit juices, Umsza-Guez et al. [58] for caja-manga pulp, Abdullah et al. [49] for carambola fruit juice and Sin et al. [52] for sapodilla juice. The effect of experimental variables on juice turbidity is also shown in Fig 8a-8c as response surface and contour plots generated from fitted model. The application of Response Surface Methodology yielded following regression model (after removing non-significant terms), which is empirical relation between response (turbidity) and the test variables in coded units:

$$\text{Turbidity (NTU)} = 23.429^{\dagger} - 1.083\,x1^{\dagger\dagger\dagger} - 1.750\,x2^{\dagger\dagger} - 7.667\,x3^{\dagger} - 1.333\,x4^{\dagger\dagger\dagger} + 1.851\,x1{*}x1^{\dagger} + 2.976\,x2{*}x2^{\dagger} + 2.976\,x3{*}x3^{\dagger} - 2.250\,x1{*}x2^{\dagger\dagger\dagger} - 1.750\,x2{*}x4^{\dagger\dagger\dagger}$$

† Significant at 0.001 level
†† Significant at 0.01 level
††† Significant at 0.05 level

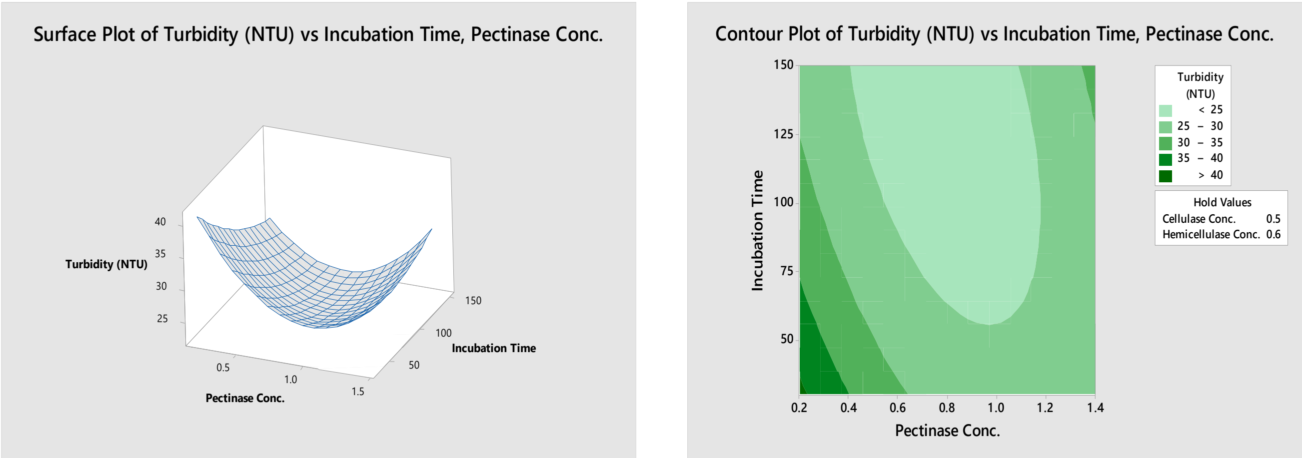
Juice Yield

From Table 10 it is clear that juice yield was significantly affected by concentration of enzymes at first order and a positive effect was observed ($p \leq 0.05$). Yield was also found to be effected by all the variables at second order but a negative effect was observed (significant at $p \leq 0.001$). Interaction effects were not found significant for juice yield. Similar results were reported by Diwan and Shukla [59] and Kaur et al. [60]. Akesowan and Choonhahirun [6] and Sevda et al. [10] also reported the significant effect of pectinase enzyme on guava juice yield**.** Influence of enzyme(s) concentration and incubation time on juice yield is shown in Fig 9a-9c as response surface and contour plots generated from fitted model. The following regression model (after removing non-significant terms) was obtained by the application of RSM, which shows empirical relation between response (yield) and the test variables in coded units:

$$\text{Yield (\%)} = 64.243^{\dagger} + 3.038\,x1^{\dagger} + 1.154\,x2^{\dagger\dagger\dagger} + 3.279\,x3 + 2.387\,x4^{\dagger} - 2.497\,x1*x1^{\dagger} - 3.197\,x2*x2^{\dagger} - 3.235\,x3*x3^{\dagger} - 3.922\,x4*x4^{\dagger}$$

† Significant at 0.001 level
††Significant at 0.01 level
†††Significant at 0.05 level

The effect of multi-enzyme treatment on juice clarity and yield was also studied by Shah and Nath [11] who reported juice clarity and yield as a function of linear and quadratic effects of concentrations of pectinase, cellulase, hemicellulase and incubation time for litchis. Jori et al. [51] showed the effects of multi-enzyme (Pectinase and cellulase) treatment for clarification of blended pineapple and mango pulp. Koffi et al. [29] also reported the effect of different combinations of pectinase, cellulase and hemicellulase in reducing viscosity and improving filterability of green and ripe banana purees.



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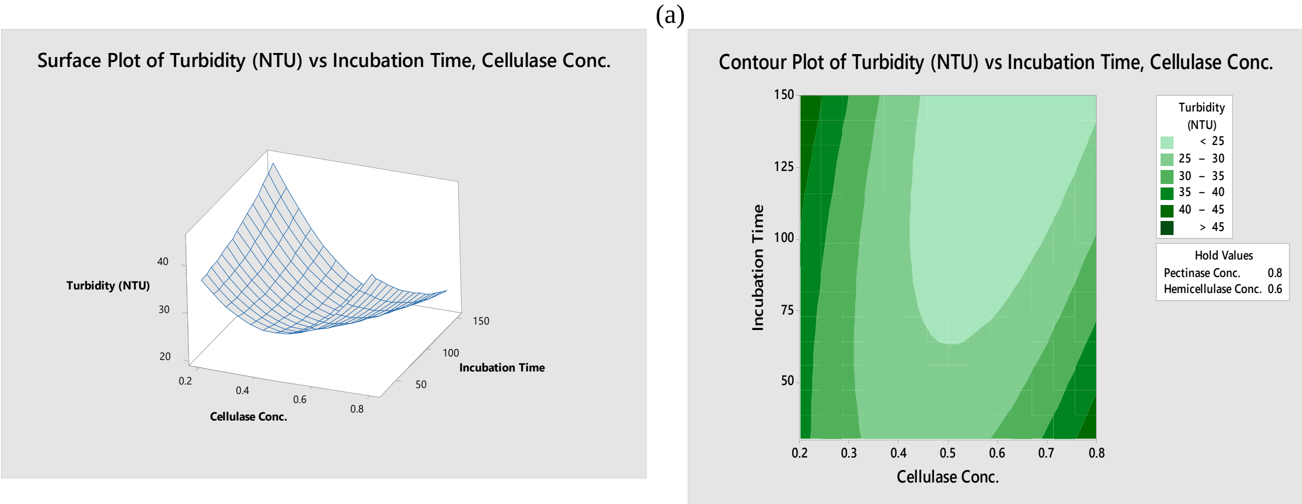

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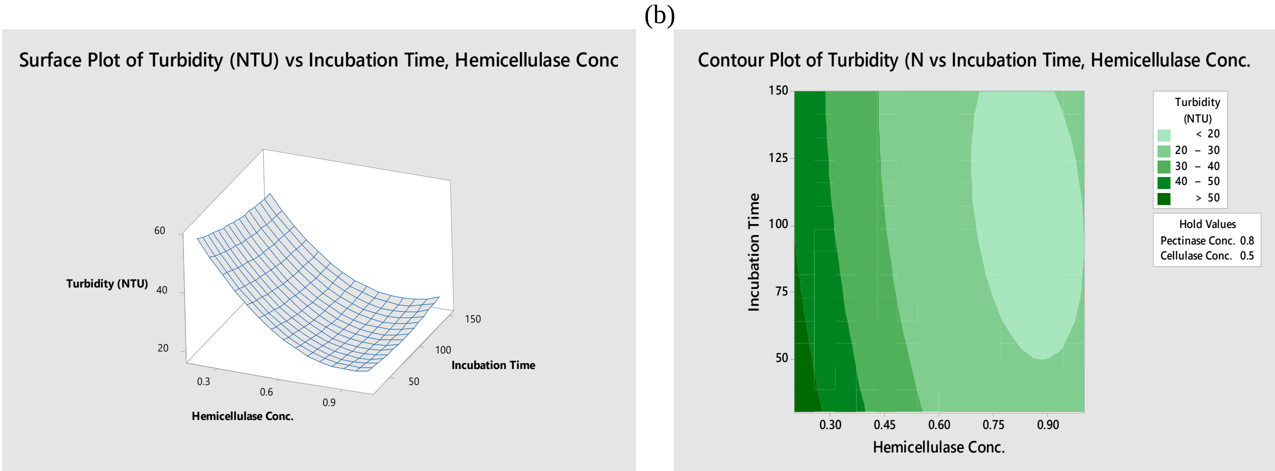

(c)

Fig 8 (a,b,c): Surface and Contour plots showing interaction of Juice Turbidity with enzyme (s) concentration and incubation time

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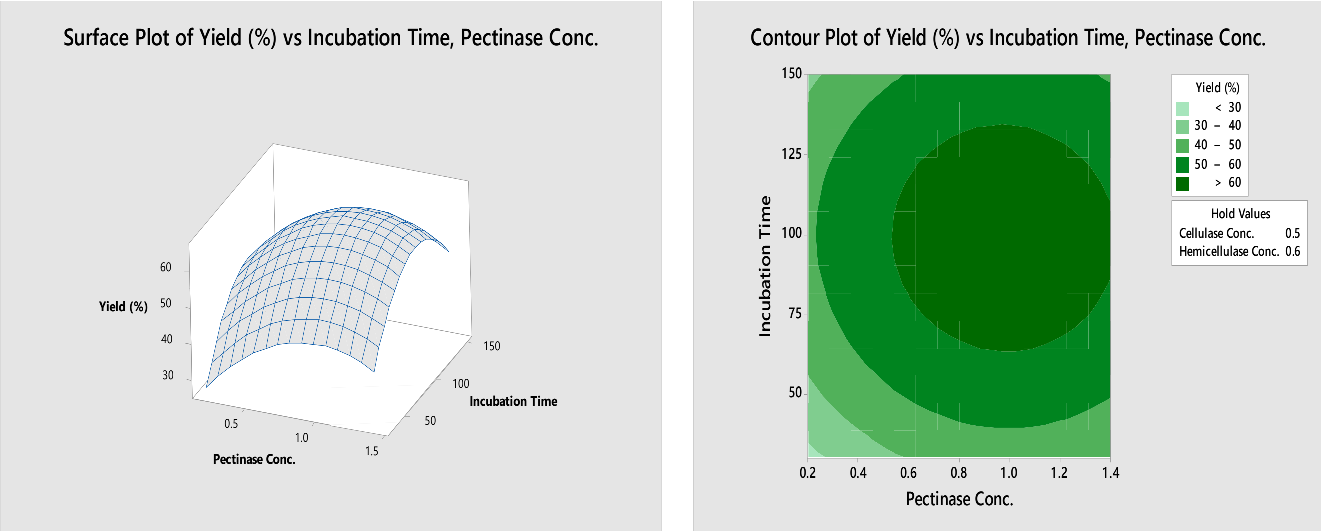

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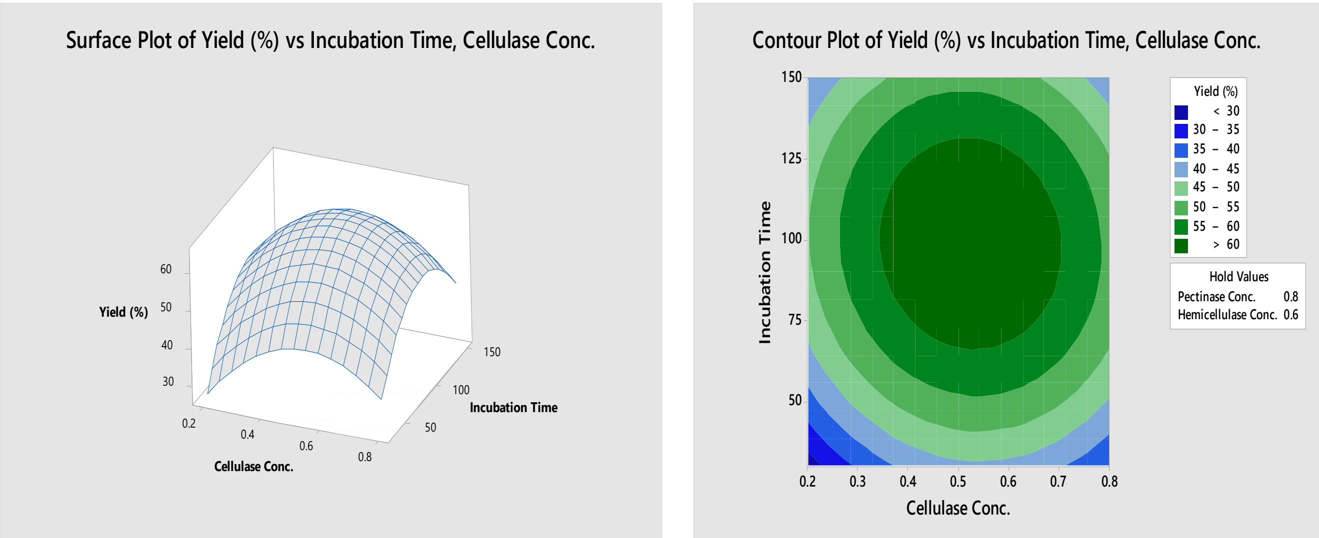

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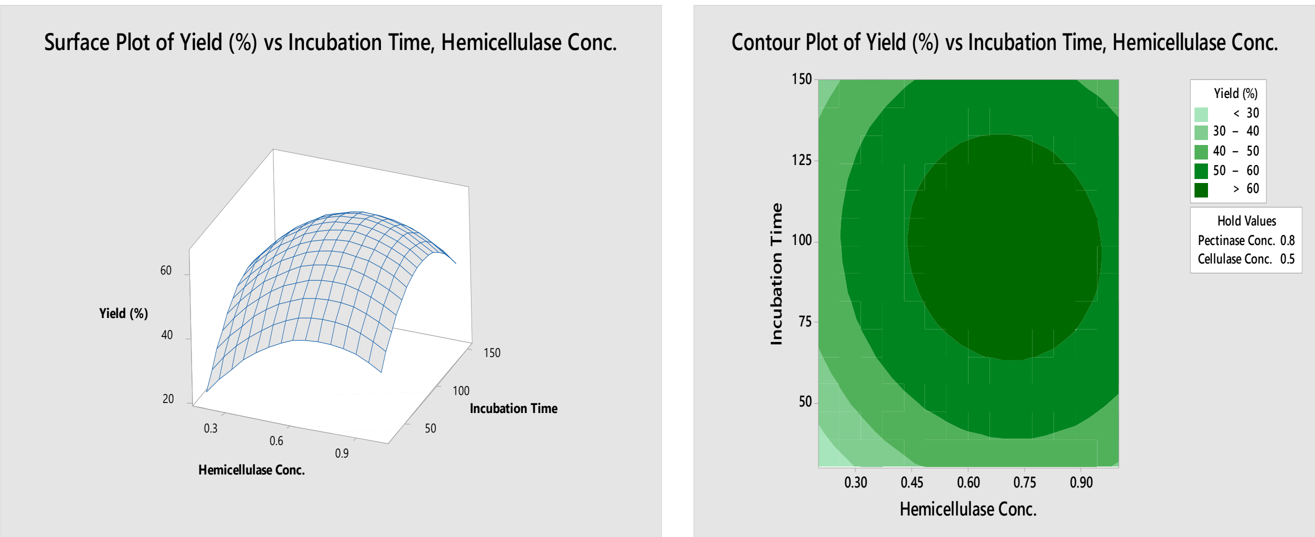

(c)

Fig 9 (a,b,c): Surface and Contour plots showing interaction of Juice Yield with enzyme concentration and incubation time

Multiple Response Optimizations

For determining the overall optimum conditions in multi-response situation of this study, a two- sided desirability function was used with the responses juice turbidity to be minimized and juice yield to be maximized (Table 12). Similar importance was given to both the responses. Table 13 gives the optimum parameters for multiple response juice turbidity and yield along with the predicted responses which are also shown in optimization plot (Fig 10). The optimization solution for multiple responses juice turbidity and yield showed treatment of guava juice with 0.96 % (w/w) pectinase, 0.57% (w/w) cellulase and 0.77% (w/w) hemicellulase for 99 min incubation time at 55° C with predicted values of turbidity 17.9 NTU and yield 66.09% and composite desirability as 0.93. These optimum conditions obtained with the response optimizer were very close to the compromised optimum conditions obtained for simultaneous treatment based on the optimum treatment conditions obtained for individual enzymes as shown in table 8. When the juice was treated with the optimized set of conditions obtained with response optimizer, the juice turbidity and yield were obtained as 17 and 64.7% which were in good agreement with the predicted values.

Table 12: Desirability Functions:

Response	Goal	Lower	Target	Upper	Weight	Importance
Turbidity (NTU)	Minimum		16	48	1	1
Yield (%)	Maximum	39.2	68.2		1	1

Table 13: Solution (Uncoded Value)

Variables				Multiple Response Prediction		Composite Desirability
Pectinase Concentration	Cellulase Concentration	Hemicellulase Concentration	Incubation Time	Turbidity (NTU)	Yield (%)	
0.963636	0.569697	0.773737	99.0909	17.929	66.086	0.933380

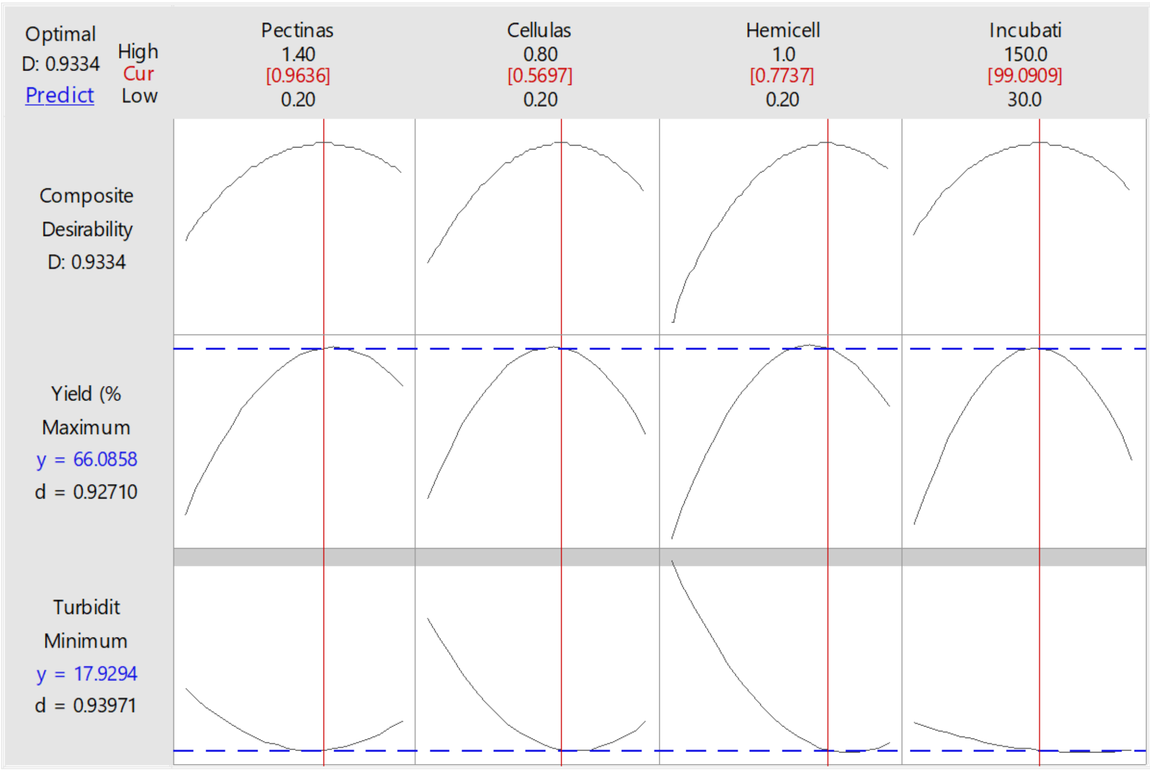

Fig. 10: Optimization Plot

IV. CONCLUSION

The present study showed the effects of separate treatments of guava juice with commercial enzymes pectinase, cellulase and hemicellulase as well as the effect of simultaneous treatment with all three enzymes. The turbidity and % yield improved significantly by simultaneous enzymatic treatment of guava juice. The treatment of guava juice with enzymes was effectively optimized using response surface methodology with a four factor and five level central composite rotatable design which involved thirty-one experiments. Based on Response Surface Optimization, the recommended enzymatic clarification conditions for simultaneous treatment of guava juice were 0.96%, 0.57% and 0.77% enzyme concentration for Pectinase, Cellulase and Hemicellulase respectively and incubation period of 99 min, at incubation temperature of 55 ^{0}C. Under these conditions, the juice obtained experimentally was having turbidity of 17 NTU and yield obtained was 64.7%.

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