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Response Surface Optimization and Impact of Immobilized Enzymes Naringianse and Tannase on the Quality Parameters of *Citrus maxima* Juice

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Abstract: Pomelo has been reported as a rich source of flavanone glycoside with antioxidants and exhibits favorable health effects such as antimicrobial, anti-inflammatory, antiatherogenic, antitumor, and anti-clotting activity. Despite all the beneficial health impacts of Citrus maxima, it still has lower commercial value because of its juice's bitterness due to the presence of naringin and tannic acid. Therefore, an attempt has been made for the cost-effective and economic debittering process using naringinase and tannase enzymes. The 17 experiments were planned according to RSM, BBD to analyze the effect of independent variables with three levels of each, i.e., Enzyme ratio ((Naringinase: tannase) (100:0, 50:50, 0:100)), incubation temperature (30°C, 40°C, 50°C) and incubation time (2, 3, 4 hrs) on physicochemical quality of *Citrus maxima* Juice. The study's result indicated that independent variables affected the responses (pH, TSS, TA, Naringin content, Tannin content, TPC, and Vitamin C content). Optimization was done using Design Expert 10.0.1 software to debitter and clarify *citrus maxima* juice by immobilized enzymes. The optimum values were found to be 54.55, 50°C, and 4 hrs. The values for pH, TSS, TA, Naringin content, Tannin content, TPC and Vitamin C content were found to be 3.17, 6.256 °Brix, 0.885 %citric acid , 220.549 µg/ml ,0.311mg/ml, 1256.721 mg GAE/L,30.309 mg/100ml respectively. From the study, it could be concluded that the maximum debittering and clarification of *citrus maxima* juice could be done under processing conditions, i.e., enzyme ratio 50:50, incubation temperature 50°C and incubation time 4 hrs.

Keywords: *Citrus maxima*; naringinase; tannase; total phenolic content; tannin content; naringin content.

1. Introduction

Citrus maxima (or *Citrus grandis*), commonly known as Chakotra, pomelo, and jabong [1] has been reported as a rich source of naringin, a bitter flavored, flavanone glycoside with antioxidant [2], and exhibit favorable health effects such as antimicrobial, anti-inflammatory, antiatherogenic, antitumor, and anti-clotting activity [3-5]. *Citrus maxima* juice contains anti-nutrients like phytic acid, tannin, and oxalate [6]. Bitter compounds are present in a different part of a single fruit [7]. Naringin is the source of undesirable bitterness [8], especially in the citrus fruit juice industry; therefore, it must be removed or reduced from processed products [9-11]. Also, upon storage of the juices, due to tannin content, certain factors such as sedimentation, haziness, color, astringency [12-13], and bitterness get increased [14-15]. These problems can be solved using the enzymes naringinase produced by different microbial strains

[16] and tannase produced by several microbial strains [17]. Naringinase hydrolyzes naringin to naringenin, a non-bitter derivative, which cannot be reconverted to naringin [18-19] and glucose [20-21], resulting in an improvement in the taste of citrus juice [22]. Besides, naringinase plays an important role in modifying flavonoids to yield highly bioactive compounds [23-24]. Similarly, tannin acyl hydrolase, i.e., tannase enzyme (EC 3.1.1.20), has been used to produce gallic acid and glucose from tannins [25]. This enzyme has found its role in various applications world-wide, especially in pharmaceuticals, tannery, beverage, and alcohol industries for clarification purposes [26-27].

Despite all the beneficial health impacts of Citrus maxima, it still has lower commercial value because of its juice's bitterness. Therefore, a cost-effective and economic debittering process could be achieved if naringinase and tannase produced industrially using microorganisms [28]. Hence, this study's objective was debittering and clarifying *Citrus maxima* juice by immobilized enzyme naringinase and tannase produced from *Aspergillus sp.* isolate SK01 isolated from rotten *Citrus maxima*.

2. Materials and Methods

2.1. Materials.

Citrus maxima of sound quality were brought from Dehradun's local market and washed properly 2-3 times with tap water and then with distilled water to remove dirt. The flavedo and albedo were peeled and separated from the pulp. The pulp was blended and filtered through a muslin cloth to obtain a clear juice and stored in a refrigerator at 4°C for further analysis. All the chemicals of the analytical grade used in this study were purchased from Hi-Media, Mumbai, India.

2.2. Production of enzymes.

Enzymes naringinase and tannase were produced through submerged fermentation by the method of [29-30] and [31] respectively from *Aspergillus sp*. isolate SK01 and partially purified by ammonium sulfate (80%) ppt method. Naringinase activity was assayed with respect to naringin using Davis's method [32] with little modification. Tannase activity was determined calorimetrically using the method of Mondal [33]**.** The activity of partially purified crude enzymes naringinase and tannase was 1.63 IU/ml and 1.18 IU/ml, respectively.

2.3. Immobilization of naringinase.

Immobilization of Naringinase was done by [34] with some modifications. Briefly, a 5 ml suspension containing crude naringinase at a concentration of 1 g/ l and 3% sodium alginate was extruded into a 0.2 M $CaCl_2$ solution at a temperature of 4°C to form the gel beads. After 4 h the beads were washed 2-3 times with 0.1 M sodium acetate buffer, pH 4.0, and used for naringinase assay.

2.4. Immobilization of tannase.

Tannase was done by [35] with some modifications. Briefly, a 5 ml suspension containing tannase at a concentration of 1 g/ l and 3% sodium alginate was extruded into a 0.2 M $CaCl_2$ solution at a temperature of 4°C to form the gel beads. After 4 h the beads were washed 2-3 times with water and kept at 4°C.

2.5. Identification and selection of the most important variables.

Several experiments were carried out to standardize the parameters for debittering and clarification of *Citrus maxima* juice by immobilized enzyme naringinase and tannase produced from Aspergillus sp. isolate SK01 isolated from rotten *Citrus maxima*.

Enzyme ratios were selected to show the effect of naringinase and tannase individually or in a combination of both enzymes on debittering and clarification characteristics of *Citrus maxima* juice as an individual or in a combination of both. The enzyme ratios range (100:0, 50:50, 0:100) were selected. Based on preliminary experiments, incubation temperature (30°C, 40°C, 50°C) and incubation time (2, 3, 4 hrs) were selected as variables.

2.6. Physiochemical analysis.

pH was measured by handy pH meter (Eutech), TSS was measured by using a hand refractometer (ERMA). Titrable acidity was measured by [36], Vitamin C (mg/100ml) was measured by the method of [37-38]. Total Phenolic Content (mg GAE/L) was measured as described[39]. Tannin content (mg/ml) and naringin content (µg/ml) in *Citrus maxima* juice were determined by the method of [40- 41], respectively.

2.7. Statistical analysis.

Design-Expert 10.0.1 was used for data analysis and process optimization. To evaluate the effect of process parameters, i.e., enzyme ratios (naringinase: tannase), incubation temperature (°C) and incubation time (hrs.) on the responses, i.e., pH, TSS, titrable acidity, naringin content, tannin content, TPC and vitamin C content a second-order response function was implemented for three independent variables having following general form Equation (1) [42-43].

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j + \sum_{i=1}^{3} \beta_{ii} X_i^2 \quad (1)$$

Where,
Y= Response
β0, βii, βij = Coefficients
Xi and Xj = Independent Variables

ANOVA was used for determining the statistical significance of the independent parameters and their relative interactions. The model's adequacy was explained in terms of R^2 (coefficient of determination), F-value (Fisher's value), and LOF (lack-of-fit)

3. Results and Discussion

A total of 17 experiments were designed by using the Box-Benkhen design of RSM. These runs were performed to see the effect of the selected process parameters- enzyme ratio, temperature & time on the said responses, i.e., naringin content, tannin content, TPC, and vitamin C. The results were statistically analyzed for being either significant or non-significant. The results of the experiment are given in Table 1. Table 2 and Table 3 show the ANOVA and regression analysis performed to check the model's adequacy. Constraints for optimization for independent variables/ dependent variables are given in Table 5.

3.1 Effect on pH

Full second order equation to show the effect of X_1, X_2 and X_3 on pH could be explained by the equation given below.

$$pH = 3.28 + 0.0375\ X_1 + 0.0375\ X_2 - 0.050\ X_3 - 0.075\ X_1 X_2 - 0.15\ X_1 X_3 + 1.08\times 10^{-16} X_2 X_3 - 0.10250\ X_1^2 - 0.0525\ X_2^2 - 0.0275 X_3^2$$

Where,

X_1, X_2 and X_3 are Enzyme ratios (naringinase: tannase), temperature, and time respectively.

The total effect of individual parameters on pH at linear, quadratic, and interactive levels is reported in table 3. The F value (16.22) for the model was significant at 1% ($p<0.01$) level of. Correlation coefficient R^2 measures the generosity of fit of the model. The R^2 value for pH was 95.42%, which means that the model could account for 95.42% of the data. The model does not elucidate the variation of 4.58%. The R^2 value higher than 90% showed that the regression model explained the reaction well. The Pred R-Squared of 77.12% was in equitable agreement with the Adj R-Squared of 89.54 %. Lack of fit, i.e., LOF was insignificant; hence, the second-order model was acceptable in describing pH. The pH value for debittered juice was varied from 3.0 to 3.4 (Table 3). The highest pH score was found maximum (3.4) at the level of X_1(0:100), X_2 (40°C), and X_3(2 hrs).

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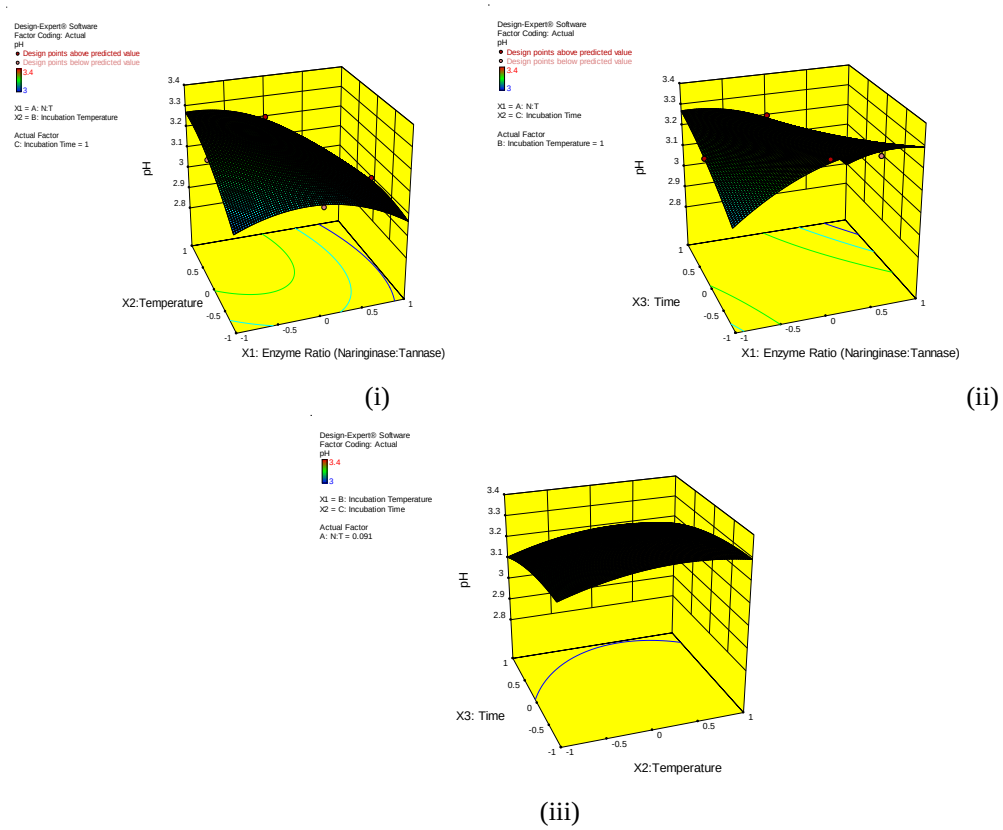

Figure 1. 3D Response surface showing interaction effect of variables on pH of debittered *Citrus maxima* juice i) temperature and enzyme ratio ii) time and enzyme ratio and iii) time and temperature.

The 3D response surface curve for pH was presented in Figure 1. (i, ii & iii). The values presented in Table 3 show that there were no significant changes in the pH of the debittered juice. Figure1 (i) shows that as temperature increase (30°C to 50°C), pH was increased in the presence of enzyme ratio.

Table 1. Experimental design.

Expt. No.	Coded Levels			Real Levels		
	X_1	X_2	X_3	***Enzymes Blend ratios*** (Naringinase: Tannase)	Temperature (°C)	***Time (***hrs***)***
1	-1	-1	0	100:0	30	3
2	1	-1	0	0:100	30	3
3	-1	1	0	100:0	50	3
4	1	1	0	0:100	50	3
5	-1	0	-1	100:0	40	2
6	1	0	-1	0:100	40	2
7	-1	0	1	100:0	40	4
8	1	0	1	0:100	40	4
9	0	-1	-1	50:50	30	2
10	0	1	-1	50:50	50	2
11	0	-1	1	50:50	30	4
12	0	1	1	50:50	50	4
13	0	0	0	50:50	40	3
14	0	0	0	50:50	40	3
15	0	0	0	50:50	40	3
16	0	0	0	50:50	40	3
17	0	0	0	50:50	40	3

Figure 1 (ii) showed that the pH increase as time increases (2-4 hrs) with different enzyme ratio levels. Figure 1. (iii) depicted that the pH increased along with an increasing level of time and temperature. A similar finding was observed by[44] in the case of the enzymatic treatment of pomegranate juice.

From table 6 of coefficient, it was observed that X_1 (enzyme ratio) and X_2 (temperature) had a positive effect on pH at 5% level, i.e., $p<0.05$ of significance at linear level, while X_3 (Time) had a negative effect at 1% level, i.e., $p<0.01$ of significance. Interactive effect of X_2 (temperature), X_3 (time) had significant positive effect at 10% level i.e. $p<0.1$ of significance while X_1 (enzyme ratio), X_2 (temperature) and X_1 (enzyme ratio), X_3 (time) had negative effect at 1% level i.e. $p<0.01$ of significance. Quadratic effect of X_1 (enzyme ratio), X_2 (temperature) and X_3 (time) was negative at 1% i.e. $p<0.01$, 5% level i.e. $p<0.05$ and more than 10% level i.e. $p<0.1$ of significance respectively.

Table 2. Independent variables (coded and actual value).

Independent variables		Coded Levels		
Name	**Code**	-1	0	1
		Actual Levels		
Enzymes Blend ratios (Naringinase: Tannase)	X_1	100:0	50:50	0:100
Temperature **(**°C**)**	X_2	30	40	50
Time (hrs**)**	X_3	2	3	4

3.2. Effect on TSS.

The effect of X_1, X_2, and X_3 on TSS could be explained by the equation given below.

$$TSS = 7.26 – 1.1625\ X_1 - 0.0375\ X_2 – 0.0250\ X_3 + 0.075\ X_1\ X_2 + 0.150\ X_1\ X_3 - 0.200\ X_2\ X_3 + 0.482\ X_1^2 - 0.217\ X_2^2 - 0.44250 X_3^2$$

Table 3. Experimental data for debittering and clarification of pomelo juice by using immobilized enzyme naringinase and tannase and combination of both.

Variables				Responses						
Exp. No.	***Enzymes Blend ratios* (Naringinase: Tannase)**	**Temp. (°C)**	**Time (hrs)**	**pH**	**TSS (°Brix)**	**TA (% citric acid)**	**Naringin content (µg/ml)**	**Tannin content (mg/ml)**	**TPC mgGAE/L**	**Vitamin C content (mg/100ml)**
1	100:0	30	3	3.0*	8.8**	0.98	505.8	0.68**	1819.8	58.2**
2	0:100	30	3	3.2	6.3	0.97	686.5**	0.63	1632.8	55.2
3	100:0	50	3	3.2	8.6	0.99**	213.8	0.60	1822.8**	52.2
4	0:100	50	3	3.1	6.4	0.92	544.3	0.41	1518.8	47.6
5	100:0	40	2	3.0*	8.7	0.85	419.5	0.65	1660.7	53.4
6	0:100	40	2	3.4**	6.1*	0.84*	659.8	0.62	1177.4	53.1
7	100:0	40	4	3.2	8.2	0.86	414.2	0.68**	1695.7	54.4
8	0:100	40	4	3.0*	6.2	0.85	612.4	0.55	1362.7	45.4
9	50:50	30	2	3.2	6.4	0.89	415.5	0.52	1218.6	44.5
10	50:50	50	2	3.3	6.7	0.88	226.5	0.39	1230.3	37.5
11	50:50	30	4	3.1	6.9	0.91	354.3	0.55	1389.6	38.8
12	50:50	50	4	3.2	6.4	0.89	216.9*	0.31*	1265.7	29.3
13	50:50	40	3	3.3	7.3	0.97	365.5	0.50	1125.3	26.8
14	50:50	40	3	3.3	7.3	0.95	304.2	0.49	1048.0*	24.8
15	50:50	40	3	3.3	7.3	0.96	305.3	0.50	1125.3	24.8
16	50:50	40	3	3.3	7.2	0.95	305.5	0.50	1124.3	24.5*
17	50:50	40	3	3.2	7.2	0.97	363.5	0.48	1125.3	26.8

*Minimum value **Maximum value

Table 4. ANOVA for different responses.

Source	Degree of freedom	pH		TSS (°Brix)		TA (% citric acid)		Naringin content (µg/ml)		Tannin content (mg/ml)		TPC (mgGAE/L)		Vitamin C content (mg/100ml)	
		F value	**P Value Prob>F**	**F value**	**P Value Prob>F**	**F value**	**P Value Prob>F**	**F value**	**P Value Prob>F**	**F value**	**P Value Prob>F**	**F value**	**P Value Prob>F**	**F value**	**P Value Prob>F**
Model	9	16.22	0.0007	170.08	< 0.0001	37.17	< 0.0001	42.54	< 0.0001	76.04	< 0.0001	47.76	< 0.0001	147.92	< 0.0001
X_1	1	7.50	0.0290	1271.91	< 0.0001	10.00	0.0159	123.20	< 0.0001	80.23	< 0.0001	83.07	< 0.0001	18.87	0.0034
X_2	1	7.50	0.0290	1.32	0.2877	4.90	0.0625	79.03	< 0.0001	225.09	< 0.0001	2.42	0.1636	59.86	0.0001
X_3	1	13.33	0.0082	0.59	0.4682	2.50	0.1579	2.08	0.1921	4.06	0.0837	8.85	0.0207	28.04	0.0011
X_1X_2	1	15.00	0.0061	2.65	0.1478	7.20	0.0314	6.13	0.0425	19.66	0.0030	1.33	0.2865	0.34	0.5791
X_1X_3	1	60.00	0.0001	10.59	0.0140	0.000	1.0000	0.48	0.5090	10.03	0.0158	2.20	0.1819	10.00	0.0159
X_2X_3	1	0.000	1.0000	18.82	0.0034	0.20	0.6682	0.73	0.4219	12.13	0.0102	1.79	0.2230	0.83	0.3937
X_1^2	1	29.49	0.0010	115.32	< 0.0001	11.84	0.0108	167.84	< 0.0001	304.42	< 0.0001	253.51	< 0.0001	972.52	< 0.0001
X_2^2	1	7.74	0.0272	23.43	0.0019	19.00	0.0033	4.77	0.0653	39.32	0.0004	62.52	< 0.0001	104.57	< 0.0001
X_3^2	1	2.12	0.1885	96.99	< 0.0001	280.47	< 0.0001	0.21	0.6638	0.18	0.6854	1.38	0.2789	58.56	0.0001
Lack of fit	3	0.42	0.7510	5.28	0.0709	1.58	0.3258	0.68	0.6108	5.94	0.0591	3.72	0.1184	1.97	0.2612
Std. Dev.		0.039		0.092		0.011		30.25		0.016		50.71		1.38	
Mean		3.19		7.18		0.92		406.68		0.53		1373.12		41.02	
R^2		0.9542		0.9954		0.9795		0.9820		0.9899		0.9840		0.9948	

R^2_{adj}		0.8954		0.9896		0.9532		0.9590		0.9769		0.9634		0.9880	
Pred R- Squared		0.7712		0.9404		0.8074		0.8848		0.8648		0.8047		0.9468	
Adeq Precision		13.887		39.421		17.784		20.062		30.451		19.377		31.503	
CV (%)		1.21		1.28		1.22		7.44		2.96		3.69		3.55	
Model		Sig		Sig		Sig		Sig		Sig		Sig		Sig	
Lack of Fit		NOT Sig		NOT Sig		NOT Sig		NOT Sig		NOT Sig		NOT Sig		NOT Sig	

Table 5. Constraints for optimization for independent variables/ dependent variables by design Expert 10.0.1.

Constraints Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
X_1:Enzyme Ratio (Naringinase:Tannase)	is in range	-1	1	1	1	3
X_2: Temperature (°C)	is in range	-1	1	1	1	3
X_3: Time (hrs)	is in range	-1	1	1	1	3
pH	is in range	3	3.4	1	1	3
TSS (°Brix)	minimize	6.1	8.8	1	1	3
TA (% citric acid)	is in range	0.84	0.99	1	1	3
Naringine content (µg/ml)	minimize	213.8	686.5	1	1	5
Tannin content (mg/ml)	minimize	0.31	0.68	1	1	4
TPC (mgGAE/L)	is in range	1048	1822.8	1	1	3
Vitamin C Content (mg/100ml)	is in range	24.5	58.2	1	1	3

Table 6. Coefficients for different responses by design Expert 10.0.1.

Factor	Coefficients						
	pH	**TSS °Brix**	**TA %**	**Naringine Content (µg/ml)**	**Tannin Content (mg/ml)**	**TPC (mgGAE/L)**	**Vit C Content (mg/100ml)**
Intercept	3.28	7.26	0.96	328.8	0.494	1109.64	25.54
X_1 **Enzyme Ratio (Nringinase: Tannase)**	0.0375**	-1.162***	-0.012**	118.713***	-0.05***	-163.412***	-2.1125***
X_2 **(Incubation Temperature)**	0.0375**	-0.037	-0.0087*	-95.075***	-0.08375***	-27.9	-3.7625***
X_3 **(Incubation Time)**	-0.05***	-0.025	0.0062	-15.4375	-0.01125*	53.3375**	-2.575***
X_1X_2	-0.075***	0.075	-0.015**	37.45**	-0.035***	-29.25	-0.4
X_1X_3	-0.15***	0.15**	-1.124×10^{-18}	-10.525	-0.025**	37.575	-2.175**
X_2X_3	1.088×10^{-16}	-0.2***	-0.0025	12.9	-0.0275**	-33.9	-0.625
X_1^2	-0.1025***	0.4825***	-0.0187**	190.988***	0.13425***	393.492***	20.905***
X_2^2	-0.0525**	-0.217***	0.02375***	-32.1875*	-0.04825***	195.417***	6.855***
X_3^2	-0.0275	-0.442***	-0.09125***	6.6875	-0.00325	-29.0075	5.13***

***, **,* Significant at 1,5and 10% level of significance respectively

The F value (170.08) for the model was significant at 1%, i.e., $p<0.01$ level of significance. Correlation coefficient R measures the generosity of fit of the model. R^2 value for TSS was 99.54%, which means that the model could account for 99.54% of data. The model does not elucidate 0.46% variation. The Pred R-Squared of 94.04 % was in equitable agreement with the Adj R-Squared of 98.96 %. LOF was insignificant; hence, the second-order model was acceptable in describing TSS. The variation in TSS value for debittered juice was observed from 6.1 to 8.8 (table 3). The TSS score was found minimum (6.1) at the level of X_1(0:100), X_2 (40°C), and X_3(2 hrs).

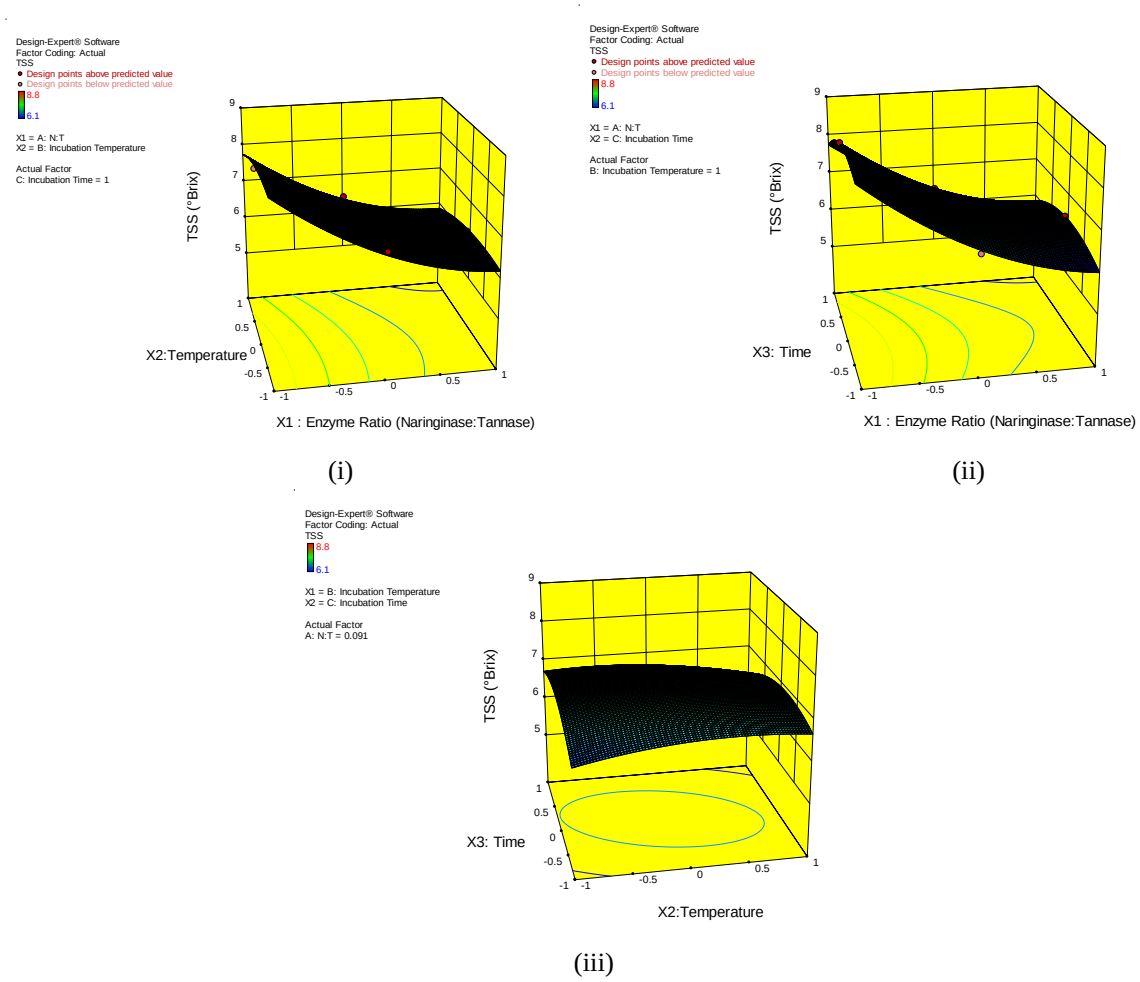

Figure 2. 3D Response surface showing interaction effect of variables on TSS of debittered *Citrus maxima* juice i) temperature and enzyme ratio ii) time and enzyme ratio and iii) time and temperature.

The 3D response surface curve for TSS was presented in figure. 2 (i, ii & iii). The values presented in Table 3 show that there were significant changes in TSS of the debittered juice (6.1-8.8). Figure 2 (i) shows that as temperature increase (30°C to 50°C), TSS was decreased in the presence of enzyme ratio. Figure 2 (ii) showed that the TSS decreased as time increased (2-4 hrs) with different enzyme ratio levels. Figure 2 (iii) depicted that the TSS increased along with an increasing level of time and temperature. Our finding favors the finding of [45].

From table 6 of the coefficient, it was observed that X_1 (enzyme ratio) at 1% level, i.e., $p<0.01$ of significance, X_2 (temperature), and X_3 (time) had a negative effect on TSS at a linear level. Interactive effect of X_2 (temperature), X_3 (time) had significant negative effect at 1% level i.e. $p<0.01$ of significance while X_1 (enzyme ratio), X_2 (temperature) had positive effect at more than 10% level ($p<0.1$) of significance whereas X_1 (enzyme ratio), X_3 (time) had positive effect at 5% level ($p<0.05$) of significance. Quadratic effect of X_1 (enzyme ratio) was

positive at 1% level ($p<0.01$) of significance, while X_2 (temperature) and X_3 (time) was negative at 1 level % ($p<0.01$).

3.3. Effect on TA.

Full second order equation to show the effect of X_1, X_2, and X_3 on TA could be explained by the equation given below.

$$TA = 0.96 - 0.012\ X_1 - 8.75 \times 10^{-3}\ X_2 + 6.250 \times 10^{-3}\ X_3 - 0.015\ X_1 X_2 - 1.124 \times 10^{-18}\ X_1 X_3 - 2.50 \times 10^{-3}\ X_2 X_3 - 0.018750\ X_1^2 + 0.023750 X_2^2 - 0.091250 X_3^2$$

The total effect of individual parameters on TA at linear, quadratic, and interactive levels is reported in Table 4 The F value (37.17) for the model was significant at 1%, i.e., $p<0.01$ level of significance. Correlation coefficient R^2 measures the generosity of fit of the regression model. R^2 value for TA was 97.95%, which means that the model could account for 97.95% of data, and 2.05% variation is not elucidated by the model. The Pred R-Squared of 80.74 % was an in equitable agreement with the Adj R-Squared of 95.32 %. LOF was insignificant; hence, a second-order model was adequate in describing TA. The variation in TA value for debittered juice was observed from 0.84 to 0.99 (table 3). The TA score was found maximum (0.99) at the level of X_1(100: 0), X_2 (50°C), and X_3(3 hrs).

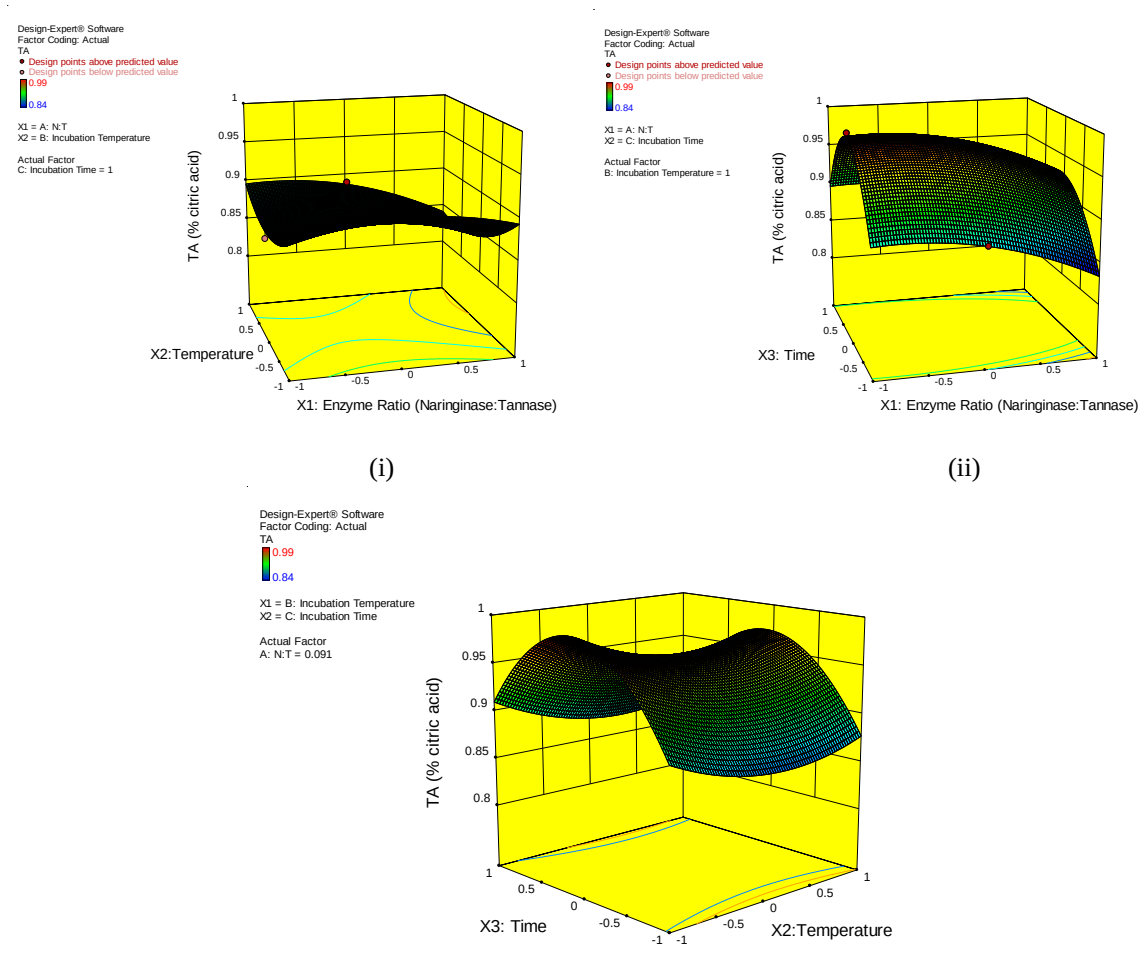

Figure 3. 3D Response surface showing interaction effect of variables on TA of debittered *Citrus maxima* juice i) temperature and enzyme ratio ii) time and enzyme ratio and iii) time and temperature.

The 3D response surface curve for TA was presented in the figure. 3 (i, ii & iii). The values presented in Table 3 show that there were significant changes in TA of the debittered juice (0.84- 0.99). Figure 3 (i) shows that as temperature increase (30°C to 50°C) TA was first decreased slightly and then increased slightly in the presence of enzyme ratio. Figure 3 (ii) showed that the TA increased as time increase and maximum at a central point (X_3=3 hr) and then decreased very rapidly as the time increased (3-4 hrs.) with different levels of enzyme ratio. From figure 3 (iii), it is clearly depicted that the TA increased as time increase and maximum at the central point (X_3=3 hr) and then decreased very rapidly as the time increased (3-4 hrs.) with an increasing level of temperature. Our finding favors the finding of [44], who observed no significant TA changes during enzymatic treatment of pomegranate juice.

From table 6 of coefficient, it was observed that X_1 (enzyme ratio) at 5% level, i.e., $p<0.05$ of significance, X_2 (Temperature) at 1% level, i.e., $p<0.01$ of significance had a negative effect on TA at a linear level while X_3 (Time) had a positive effect on TA at a linear level. Interactive effect of X_1 (enzyme ratio), X_2 (Temperature) had a significant negative effect at 5% level i.e., $p<0.05$ of significance and X_1 (enzyme ratio), X_3 (Time) and X_2 (Temperature), X_3 (Time) also had a significant negative effect at more than 10% level ($p<0.1$) of significance. Quadratic effect of X_1 (enzyme ratio) and X_3 (Time) was negative at 5% level ($p<0.05$) and 1% level ($p<0.01$) of significance respectively, while X_2 (Temperature) and was positive at 1 level % ($p<0.01$) of significance.

3.4 Effect on naringin content.

Full second order equation to show the effect of X_1, X_2, and X_3 on naringin content could be explained by the equation given below.

Naringin Content = $328.80 + 118.7\ X_1 - 95.08\ X_2 - 15.44\ X_3 + 37.45\ X_1\ X_2 - 10.52 X_1\ X_3 + 12.90\ X_2\ X_3 + 190.99 X_{12} - 32.19 X_{22} + 6.69 X_{32}$

The F value (42.54) for the model was significant at 1%, i.e., $p<0.01$ level of significance. Correlation coefficient R measures the generosity of fit of the regression model. The R_2 value for naringin content was 98.20%, which means that the model could account for 98.20% of data, and 1.8% variation is not explained by the model. The Pred R-Squared of 88.48 % was in equitable agreement with the Adj R-Squared of 95.90 %. Lack of fit was insignificant; therefore, a second-order model was acceptable in describing naringin content. The variation in naringin content value for debittered juice was observed from 686.5 to 213.8 (table 3). The naringin content was found minimum (213.8) at the level of X_1(100: 0), X_2 (50°C), and X_3(3 hrs).

The 3D response surface curve for naringin content was presented in figure 4 (i, ii & iii). The values presented in Table 3 show that there were significant changes in the naringin content of the debittered juice (686.5-213.8). Figure 4 (i) shows that as temperature increase (30°C to 50°C), naringin content was decreased significantly. With respect to enzyme ratio, naringin content was lower with naringinase enzyme. There was no decrement in naringin content with tannase enzyme in enzyme ratio. Figure 4 (ii) showed that the naringin content value was lower with the naringinase enzyme with time with respect to enzyme ration. Figure 4 (iii) clearly depicted that the naringin content decreased as time and temperature increased. A similar finding was observed by [46-49], who observed that the naringin content in grapefruit juice and pomelo juice respectively was decreased when the juice sample was treated with naringinase.

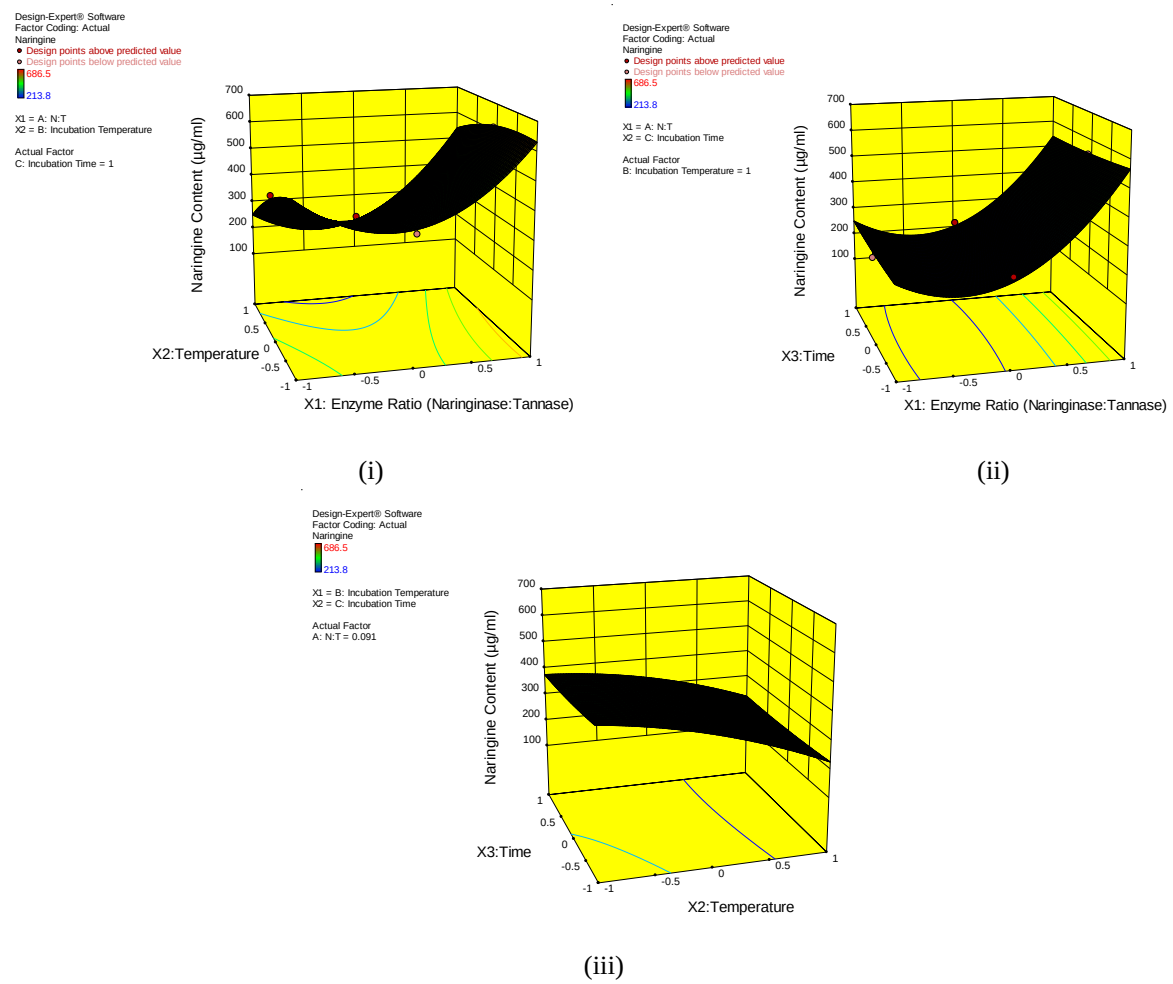

Figure 4. 3D Response surface showing interaction effect of variables on naringin content of debittered *Citrus maxima* juice i) temperature and enzyme ratio ii) time and enzyme ratio and iii) time and temperature.

From table 6 of coefficient, it was observed that X_1 (enzyme ratio) had a positive effect on naringin content at 1% level, i.e., $p<0.01$ of significance at a linear level, while X_2 (Temperature) and X_3 (Time) had a negative effect at 1% level, i.e., $p<0.01$ and more than 10 % level, i.e., $p<0.1$ of significance respectively. Interactive effect of X_1 (enzyme ratio), X_2 (Temperature), and X_2 (Temperature), X_3 (Time) had a significant positive effect at 5% level ($p<0.05$) and more than 10 % level, i.e., $p<0.1$ of significance respectively. Quadratic effect of X_1 (enzyme ratio) and X_3 (Time) was positive at 1% level ($p<0.01$) and more than 10 % level ($p<0.1$) of significance respectively, while X_2 (Temperature) and was negative at 10 % level ($p<0.1$) of significance.

3.5 Effect on tannin content.

Complete second-order equation produced in terms of X_1, X_2, and X_3 for the effect of enzyme ratio, temperature, and time on tannin content could be explained by the equation given below.

$$\text{Tannin Content} = 0.49 - 0.050\,X_1 - 0.084\,X_2 - 0.011\,X_3 - 0.035\,X_1X_2 - 0.025\,X_1X_3 - 0.028\,X_2X_3 + 0.13X_1^2 - 0.048X_2^2 - 3.250\times10^{-3}\,X_3^2$$

The F value (76.04) for the model was significant at a 1% ($p<0.01$) level of significance. Correlation coefficient R^2 measures the generosity of fit of the regression model. The R^2 value for tannin content was 98.90 %, which means that the model could account for 98.90% of data, and the model does not elucidate 1.1% variation. The R^2 value higher than 90% meant that the

regression model explained the reaction well. The Pred R-Squared of 86.48 % was in equitable agreement with the Adj R-Squared of 97.69 %. Lack of fit was insignificant; hence, a second-order model was acceptable in describing naringin content. The tannin content value variation for debittered juice was observed from 0.68 to 0.31 (table 3). The tannin content was found minimum (0.31) at the level of X_1(50: 50), X_2 (50°C), and X_3(4 hrs).

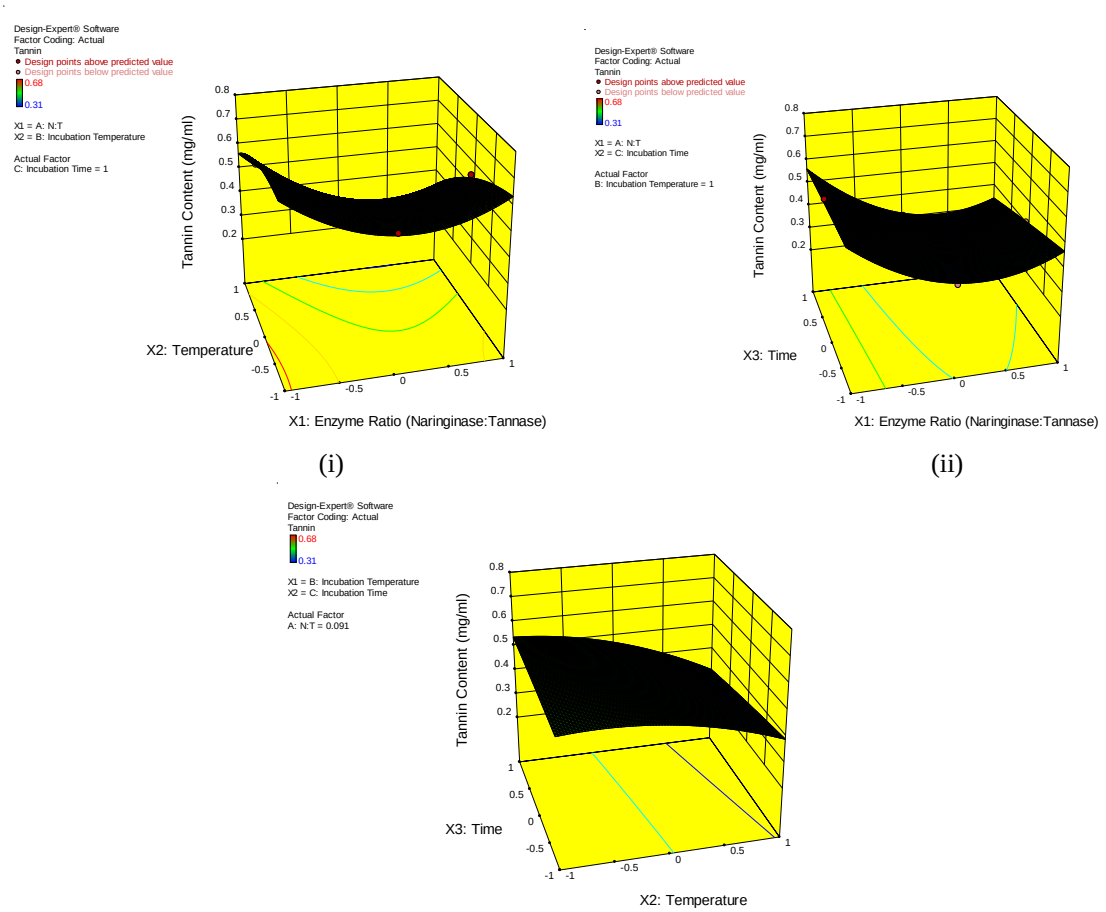

Figure 5. 3D Response surface showing interaction effect of variables on the tannin content of debittered *Citrus maxima* juice i) temperature and enzyme ratio ii) time and enzyme ratio and iii) time and temperature.

The 3D response surface curve for tannin content was presented in figure 5 (i, ii & iii). The values presented in Table 3 show that there were significant changes in the tannin content of the debittered juice (0.68- 0.31). Figure 5 (i) shows that as temperature increase (30°C to 50°C), tannin content was decreased significantly. With respect to enzyme ratio, there was no change in tannin content with the naringinase enzyme. There was a significant change in tannin content with tannase enzyme in enzyme ratio. Figure 5 (ii) showed that the tannin content decreased significantly with an increase in time, and tannin content was also decreased with tannase enzyme in enzyme ratio. Figure 5 (iii), it is depicted that the tannin content decreased as temperature increased. Our findings favor the findings of [50-52].

From table 6 of coefficient, it was observed that X_1 (enzyme ratio), X_2 (Temperature), and X_3 (Time) had a negative effect on tannin content at 1% level ($p<0.01$), 1% level ($p<0.01$) and 10 % level ($p<0.1$) of significance respectively. Interactive effect of X_1 (enzyme ratio), X_2(Temperature) and X_1 (enzyme ratio), X_3 (Time) and X_2 (Temperature), X_3 (Time) had significant negative effect at 1% level ($p<0.01$), 5% level ($p<0.05$) and 5 % level ($p<0.05$) of significance respectively. Quadratic effect of X_1 (enzyme ratio) was positive at 1% level

($p<0.01$) of significance. In comparison, X_2 (Temperature) was negative at 1% level ($p<0.01$) of significance, but the quadratic effect of X_3 (Time) was positive at more than 10 % level ($p<0.1$) of significance.

3.6. Effect on TPC.

Full second order equation to show the effect of X_1, X_2, and X_3 on TPC could be explained by the equation given below.

$$TPC = 1109.64 - 163.41\ X_1 - 27.90\ X_2 + 53.34\ X_3 - 29.25\ X_1 X_2 + 37.57\ X_1 X_3 - 33.90\ X_2 X_3 + 393.49\ X_1^2 + 195.42\ X_2^2 - 29.01\ X_3^2$$

The F value (47.76) for the model was significant at 1%, i.e., $p<0.01$ level of significance. Correlation coefficient R measures the goodness of fit of the regression model. R^2 value for TPC was 98.40%, which implies that the model could account for 98.40% of data, and the model does not explain 1.6% variation. The Pred R-Squared of 80.47 % was in equitable agreement with the Adj R-Squared of 96.34 %. Lack of fit was insignificant; therefore, a second-order model was acceptable in describing naringin content. The variation in TPC value for debittered juice was observed from 1048 to 1822.8 (table 3). The TPC was found minimum (1048.0) at the level of X_1(50: 50), X_2 (40°C), and X_3(3 hrs).

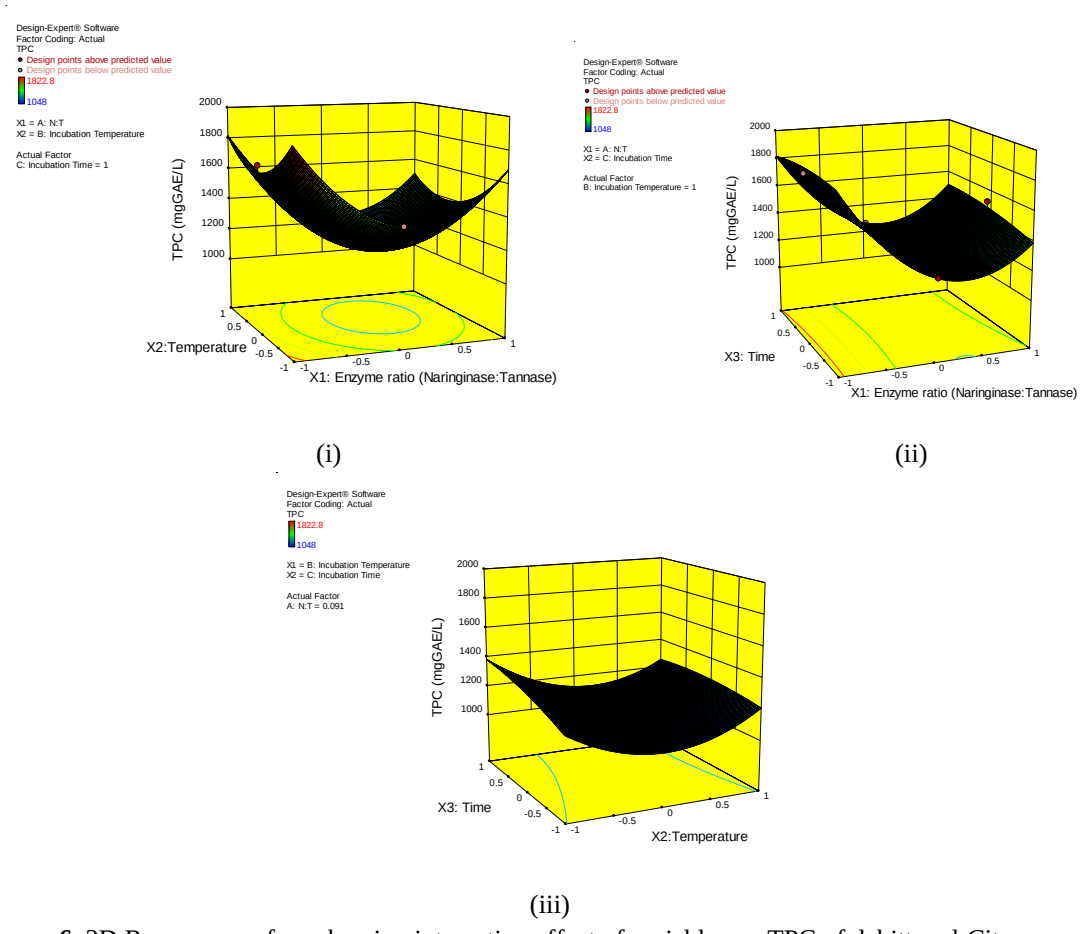

Figure 6. 3D Response surface showing interaction effect of variables on TPC of debittered *Citrus maxima* juice, i) temperature and enzyme ratio ii) time and enzyme ratio and iii) time and temperature.

The 3D response surface curve for TPC was presented in figure 6 (i, ii & iii). The values presented in Table 3 show that there were significant changes in TPC of the debittered juice (1048.0 – 1822.8). Figure 6 (i) shows that TPC increased with the enzyme ratio as temperature increase (30°C to 50°C). Figure 6 (ii) showed that the TPC increased significantly with an

increase in time while decreased with tannase enzyme with respect to enzyme ratio. Figure 6 (iii) depicted that the TPC increased as incubation time and incubation temperature increased. This increment in TPC could be co-related with the presence of naringenin (i.e., a metabolite produced from naringin with hydroxyl groups) or, due to the possibility of overestimation of polyphenolic content with respect to lack of selectivity of Folin-Ciocalteu's reagent [53], when analyzed through the spectrophotometric method. It also shows that Folin-Ciocalteu's reagent reacts with reducing compounds like sugars (e.g., glucose) and not only reacts with phenols[54]. Our findings favor the findings of Cavia-Saiz [55, 49], who observed that the total phenolic content was increased as the naringinase concentration increases.

From table 6 of coefficient, it was observed that X_1 (enzyme ratio) and X_2 (Temperature) had a negative effect on TPC at 1% level ($p<0.01$) and more than 10 % level ($p<0.1$) of significance, respectively while X_3 (Time) had positively effect on TPC at 5% level ($p<0.05$) of significance. Interactive effect of X_1 (enzyme ratio), X_2 (Temperature) and X_2 (Temperature), X_3 (Incubation Time) had a significant negative effect at more than 10% level ($p<0.1$) of significance while X_1 (enzyme ratio), X_3 (Incubation Time) was positive at more than 10% level ($p<0.1$) of significance. Quadratic effect of X_1 (enzyme ratio) and X_2 (Temperature) was positive at 1% level ($p<0.01$) of significance, while X_3 (Time) was negative at more than 10 % level ($p<0.1$) of significance.

3.7. Effect on vitamin C content.

Complete second-order equation produced in terms of X_1, X_2, and X_3 for the effect of enzyme ratio, temperature, and time on Vitamin C content could be explained by the equation given below.

$$\text{Vitamin C Content} = 25.54 - 2.11\ X_1 - 3.76\ X_2 - 2.58\ X_3 - 0.4\ X_1 X_2 - 2.17\ X_1 X_3 - 0.63\ X_2 X_3 + 20.91\ X_1^2 + 6.85\ X_2^2 + 5.13\ X_3^2$$

The F value (147.9) for the model was significant at 1% ($p<0.01$) level of significance. Correlation coefficient R^2 measures the generosity of fit of the model. The R^2 value for TPC was 99.48%, which means that the model could account for 99.48% of data, and the model does not elucidate 0.52% variation. The Pred R-Squared of 94.68 % was in equitable agreement with the Adj R-Squared of 98.80 %. Lack of fit was insignificant; hence, a second-order model was acceptable in describing Vitamin C content. The variation in vitamin C content value for debittered juice was observed from 58.2 to 24.5 (table 3). The vitamin C content was found maximum (58.2) at the level of X_1(100: 0), X_2 (30°C), and X_3(3 hrs).

The 3D response surface curve for vitamin C content was presented in figure 7 (i, ii & iii). The values presented in table 4 show that there were significant changes in vitamin C content of the debittered juice (24.5 - 58.2). Figure 7 (i) shows that as temperature increase (30°C to 50°C), vitamin C content was decreased with enzyme ratio. Figure 7 (ii) showed that vitamin C content decreased significantly with an increase in enzyme ratio time. Figure 7 (iii) depicted that the vitamin C content decreased as time and temperature increased. The vitamin C content was found to increase near the central valve of enzyme concentration. The decrease in response (ascorbic acid content) may be due to the denaturation of the enzyme at high temperatures. A similar finding was reported by [56-57], who observed that the ascorbic acid content decreases as the temperature and time increases, respectively.

From table 6 of the coefficient, it was observed that X_1 (enzyme ratio), X_2 (Temperature), and X_3 (Time) had a negative effect on vitamin C content at a 1% level ($p<0.01$) of significance. The interactive effect of X_1 (enzyme ratio), X_3 (Time) had a significant

negative effect at a 5% level ($p<0.05$) of significance. Quadratic effect of X_1 (enzyme ratio), X_2 (Temperature), and X_3 (Time) were positive at 1% level ($p<0.01$) of significance.

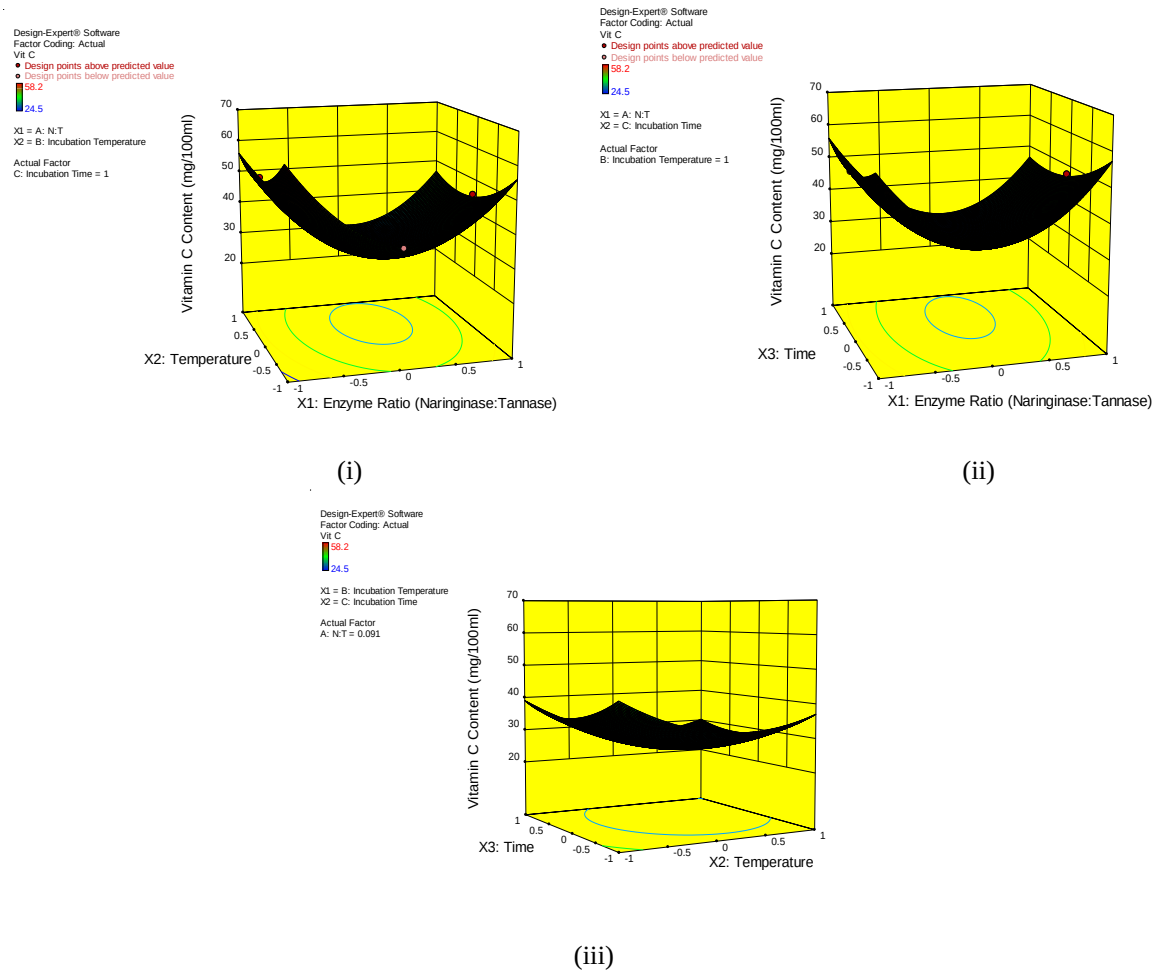

Figure 7. 3D Response surface showing interaction effect of variables on Vitamin C content of debittered *Citrus maxima* juice i) temperature and enzyme ratio ii) time and enzyme ratio and iii) time and temperature.

4. Conclusions

The model F value was found to be highly significant at a 1% level of significance for all the responses. The second-order model could be fitted to predict the entire dependent parameters. The optimum values for debittering and clarification of citrus maxima juice were found to be 54.55:45.45, 50°C, and 4 hrs. The values for pH, TSS, TA, Naringin content, Tannin content, TPC and Vitamin C content were found to be 3.17, 6.256 °Brix, 0.885 % citric acid, 220.549 µg/ml ,0.311mg/ml, 1256.721 mg GAE/L,30.309 mg/100ml respectively. It could be concluded that the enzymatic debittering and clarification of citrus maxima juice could be done by immobilized enzymes naringinase and tannase, which is produced from *Aspergillus* sp. SK01 isolated from rotten Citrus maxima under optimum processing conditions.

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Conflicts of Interest

The authors declare no conflict of interest.

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