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Optimizing conditions for enzymatic clarification of tender palmyra (*Borassus flabellifer*) endosperm juice

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Abstract

Palmyra is seasonal fruit with rich nutritional and medicinal properties. The tender palmyra is seasonal, highly perishable and rich in pectin due to which it is underutilized. Thus, palmyra pulp was clarified to obtain a ready-to-serve palmyra juice. The tender palmyra pulp was treated with pectinase at different substrate concentrations (10-50 %), enzyme concentration (0.1-0.4%), temperature (32-50 ˚C) and time (60-240 min). The effect of this enzyme treatment on pH, TSS, Viscosity and colour of juice was studied using response surface methodology. It was found that the coefficient of determination, R^2 was greater than 0.900 for all the dependent variables. Based on the 3D surface graph, the desired combination of juice was obtained at 10.84 % substrate and 0.33 % enzyme concentration treated at 43.84 ˚C for 229.05 min. Thus the current study undoubtfully help in promoting the utilisation of tender palmyra for the production of the ready-to-serve drink.

Keywords: Tender palmyra endosperm, Response surface methodology, pectinase, juice clarification

Introduction :

Borassus flabellifer commonly called Palmyra is a tropical palm tree that is native to tropical Africa and grown mostly in India, Burma (Myanmar) and Cambodia. In India, it is found in dry semi-arid regions of Tamil Nadu, Andra Pradesh, Odisha, West Bengal, Bihar, Karnataka and Maharashtra. This tree is versatile and its different parts have been used for several purposes including food, beverage, fodder, medicine and timber. A delicious drink from the sap called Neera is becoming famous as it is a health drink with nutritional and medicinal properties. Natural fermentation of the sugar sap by wild yeasts and bacteria produces 'Toddy' with an alcohol maximum of 5%. The mature ripe palmyra fruit pulp is yellow in colour, rich in Vitamin A and C and also fibre rich.

The tender palmyra endosperm is very young, hollow and translucent like ice with sweetish liquid within the endosperm. It is consumed usually during summer in southern and eastern parts of India. Previous studies on the palmyra endosperm emphasized the presence of calcium, magnesium, and iron with less amount of fat and protein (Thivya et al., 2020). In addition to the nutritional properties, it has high phenolic and Vitamin C content contributing to antioxidant properties (Tharmaratnam et al., 2020). Despite the high nutritional properties, this is not much explored as it is seasonal and cannot be transported or preserved under natural conditions due to the natural sugars present which renders them susceptible to oxidation and fermentation (Mathanghi et al., 2020). Though there are studies on the production of ready to serve (RTS) drink from mature palmyra fruit pulp (Mohanty et al., 2018a; Nithiyananthan et al., 2018), there are no studies form the tender palmyra endosperm as they are rich in pectin content which hinders the production of juice. Hence, the current research was conducted to study the effect of the pectinase enzyme on the production of palmyra juice.

Materials and methods :

Sample collection :

The tender palmyra samples are available only during the summer season in tropical regions. Fresh samples were collected from the local cultivars of Thanjavur, India which was used in the current study.

Chemicals:

The chemicals used for the analysis viz., ethanol, hexane, acetic acid, hydrochloric acid, sulphuric acid, trichloroacetic acid, copper sulfate, sodium sulfate and sodium hydroxide were of analytical grade and were procured from Himedia.

Enzyme :

Pectinase (EC-232-885-6) from *Aspergillus niger* with activity 8000-12,000 U/mg used for the experiment was procured from Hi-media Laboratories Pvt. Ltd, Mumbai.

Pulp extraction :

Tender palmyra endosperm (TPE) was removed carefully using a sterilized knife from the whole fruit to avoid water loss. The translucent part of the jellies was separated from the white outer covering and bitter compounds and enzymes responsible for browning. The TPE was then made to a pulp using a blender. The pulp obtained was used for further studies.

Enzymatic extraction :

Enzyme assisted extraction of juice was carried out and the optimisation was conducted using a central composite design. The selection of variables was based on the previous study by Mohanty et al. (2018b). The pulp was diluted at different concentrations with water with substrate concentrations X_1 from 10-50 % and subjected to different concentrations of enzymes X_2 (0.1-0.4 %) at varying temperature X_3 (32-50 ˚C) and time X_4 (60-240 min). The experimental design is given in table 1 and the results are repeated thrice and the mean values are considered as responses.

Pectin :

The pectin content in the palmyra pulp was estimated pre and post pectinase treatment at various conditions. 50g of sample was boiled with 300 mL of 0.01 N HCl for 30 min followed by collecting the filtrate after washing the residue with hot water. To the residue, 100 mL of 0.05 N and 0.3 N HCl was added and boiled for 20 min and 10 min respectively and the filtrate was collected as above. Then the filtrates were made up to 500 mL volume and from this aliquots of 100 to 200 mL were pipetted out in a 1L beaker. To this 250 mL of water was added and to neutralise 1 N NaOH with phenolphthalein indicator. Also, 10 mL of 1N NaOH was added with constant stirring and allowed to stand overnight. 50 mL of 1N acetic acid was added to this mixture and 25 mL of 1 N of calcium chloride solution and allowed to stand for 1 h. After boiling for 2 min the solution was filtered through pre-weighed Whatman filter no. 1 paper and washed with boiling water until the filtrate was free from chloride. The filter paper containing calcium pectate was dried at 100 ˚C overnight and weighed after cooling down in a desiccator. The total pectin content was measured using the following equation (2) Jadhav et al. (2018).

$$\%\ calcium\ pectate = \frac{Wt.of\ calcium\ pectate \times 500 \times 100}{mL\ of\ filtrate\ taken \times Wt.of\ the\ sample\ for\ estimation} \ldots (2)$$

RSM optimisation :

Central composite design (CCD) was used to determine the concentration of the substrate, enzyme, and time-temperature combinations (RSM) for the treatment. The range of enzyme concentration was decided based on the studies conducted by Mohanty et al., (2018). RSM was conducted using Design expert software file version 11.1.0.1. To optimise a process, it is necessary to have lower variance and a narrower experimental range. They were statistically evaluated, and regression models were constructed as a result. Enzyme concentration (X1); substrate concentration (X2); incubation temperature (X3) and time (X4); were selected as factors. Each value was performed in triplicates for independent variables and the mean values were taken as a response. The experimental data analysis was performed using analysis of variance (ANOVA) and the results were fitted in the following second-order polynomial equation (1).

$$Y = \beta_0 + \sum \beta_i X_i + \sum b_i^2 X_i^2 + \sum b_{ij} X_{ij} \ldots (1)$$

where, Y is the predicted response (pH, TSS, viscosity and colour), Xi and Xj are the levels of independent variables and β_0, is the constant, β_i is a linear coefficient, β_{ii} is the quadratic term and β_{ij} is the coefficient of the interaction terms. Experimental design, regression and graphical analysis were performed using the design expert software version 11. The fitness of the polynomial equation was analysed statistically by computing the F-value at $p<0.05$ and the 3D response surface graphs were generated using Design Expert-11.

Tests for model validity :

The independent variables such as substrate concentration, enzyme concentration, temperature and treatment time were optimised using response surface methodology (RSM) based on pH, TSS, Viscosity and colour for the enzyme-treated samples. The model developed was confirmed by comparing the predicted and the experimental values.

pH and Total soluble solids (TSS) :

TSS was measured using a hand-held pocket refractometer (Atago3810 pal-1, Japan) and pH was measured using microprocessor pH meter model KLPHM-114 (KINGLAB INSTRUMENTS PVT LTD, Tamil Nadu, India).

Viscosity :

The apparent viscosity was measured using Rapid Visco Analyser (RVA) Model RVA 3D+, Newport Scientific Australia. The enzyme-treated palmyra juice (0.5 g) was heated and the viscosity was measured at the shear rate of 501/s with a linear increase in temperature from 20-50 ˚C at the rate of 2 ˚C/min. The apparent viscosity was recorded in mPa at 50 ˚C and the experiments were performed in triplicates (Larsen et al., 2018).

Colour :

The colour of the treated palmyra juice was measured in terms of L*, a* and b* values with the help of Hunter colour lab model D-25 optical sensor (Hunter Associates Laboratory, Reston, VA). The calibration of the equipment was initially performed with black and white tiles. 20 mL of juice

sample was then added to the clean glass sample cup and the analysis was carried out (Kochadai et al., 2021).

Table 1. Experimental values of Pectinase assisted extraction of palmyra juice by central composite design (CCD)

Sl.No	Substrate concentration (%)	Enzyme concentration (%)	Temp (˚C)	Time (min)
1	(-1) 10	(-1) 0.1	(-1)32	(+1)240
2	(0) 30	0.46	(0)41	(0)150
3	(-1) 10	(+1) 0.4	(+1) 50	(+1) 240
4	(-1) 10	(+1) 0.4	(+1) 50	(-1) 60
5	(0) 30	(0) 0.25	(0) 41	(0) 150
6	(-1) 10	(-1) 0.1	(-1) 32	(-1) 60
7	(-1) 10	(-1) 0.1	(+1) 50	(+1) 240
8	(0) 30	(0) 0.25	53.73	(0) 150
9	(0) 30	(0) 0.25	(0) 41	(0) 150
10	(0) 30	(0) 0.25	(0) 41	(0) 150
11	(+1) 50	(-1) 0.1	(-1) 32	(-1) 60
12	(0) 30	(0) 0.25	(0) 41	(0) 150
13	(-1) 10	(-1) 0.1	(+1) 50	(-1) 60
14	(+1) 50	(+1) 0.4	(+1) 50	(-1) 60
15	(+1) 50	(-1) 0.1	(-1) 32	(+1) 240
16	(0) 30	0.038	(0) 41	(0) 150
17	(0) 30	(0) 0.25	(0) 41	277.28
18	(+1) 50	(+1) 0.4	(-1) 32	(-1) 60
19	(+1) 50	(-1) 0.1	(+1) 50	(-1) 60
20	(+1) 50	(-1) 0.1	(+1) 50	(+1) 240
21	58.28	(0) 0.25	(0) 41	(0) 150
22	(0) 30	(0) 0.25	28.27	(0) 150
23	1.71	(0) 0.25	(0) 41	(0) 150
24	(+1) 50	(+1) 0.4	(+1) 50	(+1) 240
25	(0) 30	(0) 0.25	(0) 41	22.72
26	(0) 30	(0) 0.25	(0) 41	(0) 150
27	(0) 30	(0) 0.25	(0) 41	(0) 150
28	(-1) 10	(+1) 0.4	(-1) 32	(+1) 240
29	(+1) 50	(+1) 0.4	(-1) 32	(+1) 240
30	(-1) 10	(+1) 0.4	(-1) 32	(-1) 60

Statistical analysis :

The treatment and statistical analysis were performed in Design expert software (Ver. 11.1.0.1).

Results and discussion :

Optimisation of pulp clarification using a central composite design :

The experimental values for pH, viscosity and colour are represented in table 2. The regression coefficient for the second-order polynomial equations and the results for linear quadratic and interaction terms are presented in table 3. Moreover, the statistical analysis results indicate that the proposed model was adequately possessing a lack of it insignificant for the responses. The R^2 having satisfactory results ie. closer the values to the unity, 0.9172, 0.9632, 0.9579, 0.9773, 0.9634, 0.9372 for pH, TSS, viscosity and L*, a* and b* values respectively. The *p* values for all regression models were less than 0.000 with no lack of fit.

Table 2. Effect of substrate concentration, enzyme concentration, time and temperature on 4 dependent variables

Sl. No.	Substrate conc. (%)	Enzyme conc. (%)	Temp. (˚C)	Time (min)	pH	TSS (˚Brix)	Viscosity (m.Pa)	Colour		
								L*	a*	b*
1	(-1) 10	(-1) 0.1	(-1) 32	(+1) 240	5.64	1.16	119	18.97	-0.88	2.04
2	(0) 30	0.46	(0) 41	(0) 150	5.91	3.36	106.6	31.74	0.6	9.76
3	(-1) 10	(+1) 0.4	(+1) 50	(+1) 240	5.66	1.36	66.8	16.89	-0.54	4.84
4	(-1) 10	(+1) 0.4	(+1) 50	(-1) 60	5.8	1.33	199.2	17.61	0.29	7.43
5	(0) 30	(0) 0.25	(0) 41	(0) 150	5.89	2.96	251	34.76	-0.81	5.84
6	(-1) 10	(-1) 0.1	(-1) 32	(-1) 60	6.9	1.86	124	22.68	-0.1	2.14
7	(-1) 10	(-1) 0.1	(+1) 50	(+1) 240	5.	1.1	116	16.57	-0.79	0.67

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8	(0) 30	(0) 0.25	53.73	(0) 150	5.85	3.33	255.6	29.73	-0.71	5.64
9	(0) 30	(0) 0.25	(0) 41	(0) 150	6.62	2.96	191.3	34.76	-0.88	5.84
10	(0) 30	(0) 0.25	(0) 41	(0) 150	6.62	2.96	191.3	34.76	-0.88	5.84
11	(+1) 50	(-1) 0.1	(-1) 32	(-1) 60	6.8	4.43	530	39.72	0.04	4.9
12	(0) 30	(0) 0.25	(0) 41	(0) 150	6.05	2.96	191.3	34.76	-0.81	5.84
13	(-1) 10	(-1) 0.1	(+1) 50	(-1) 60	7.08	1.86	95.68	18.5	-0.923	1.69
14	(+1) 50	(+1) 0.4	(+1) 50	(-1) 60	5.89	4.5	269.5	33.25	1.2	9.12
15	(+1) 50	(-1) 0.1	(-1) 32	(+1) 240	5.69	4.26	689	43.52	-1.93	3.46
16	(0) 30	0.038	(0) 41	(0) 150	6.12	2.76	298.8	37.1	-1.62	5.9
17	(0) 30	(0) 0.25	(0) 41	277.28	4.8	3.13	212.13	34.75	-1.15	4.57
18	(+1) 50	(+1) 0.4	(-1) 32	(-1) 60	6.08	5.13	328	33.63	2.12	9.42

19	(+1) 50	(-1) 0.1	(+1) 50	(-1) 60	5.91	4.63	677	39.68	-1.71	2.11
20	(+1) 50	(-1) 0.1	(+1) 50	(+1) 240	4.03	4.63	712	37.55	-0.51	4.3
21	58.28	(0) 0.25	(0) 41	(0) 150	7.1	5.4	742.2	44.07	-0.3	8.17
22	(0) 30	(0) 0.25	28.27	(0) 150	5.3	3.33	335.4	28.41	0.04	5.7
23	1.71	(0) 0.25	(0) 41	(0) 150	7.3	1.54	67.1	10.6	-0.87	3.56
24	(+1) 50	(+1) 0.4	(+1) 50	(+1) 240	6.32	4.33	220	30.64	0.31	8.43
25	(0) 30	(0) 0.25	(0) 41	22.72	5.86	3	176	33.66	0.01	5.51
26	(0) 30	(0) 0.25	(0) 41	(0) 150	6.82	2.96	191	34.76	-0.81	5.84
27	(0) 30	(0) 0.25	(0) 41	(0) 150	6.52	2.96	191	34.76	-0.81	5.84
28	(-1) 10	(+1) 0.4	(-1) 32	(+1) 240	5.03	1.86	101	18.63	-0.74	4.72
29	(+1) 50	(+1) 0.4	(-1) 32	(+1) 240	5.65	4.76	248.66	38.8	-0.62	8.47
30	(-1) 10	(+1) 0.4	(-1) 32	(-1) 60	4.	1.46	70.16	19.34	2.03	8.62

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Table 3. Coefficient of regression and R^2 values for different responses at different treatment conditions

Regression coefficient	**pH**	**TSS (˚Brix)**	**Viscosity (m.Pa)**	**Colour**		
				L*	**a***	**b***
b_0	6.39	3.076	215.306	34.73286	-0.8321	6.117714
b_1	0.0098	1.5066***	186.8528***	9.746686***	0.067955	1.228976***
b_2	-0.1158	0.0821	-91.5586****	-1.7990***	0.6996***	2.2599***
b_3	0.0039	-0.0593	1.675288	-1.13666*	-0.18268**	-0.26324
b_4	-0.3769***	-0.0774	1.500777	-0.06493	-0.5143***	-0.49147*
b_{12}	0.315****		-95.27***	-1.24375*	0.211688**	0.1
b_{13}	-0.215*		1.2075	-0.28125	0.122063*	0.0375
b_{14}	0.0037		9.4625	0.70625	-0.00956	0.42
b_{23}	0.3213***		-8.1875	0.03625	-0.02919	0.1475
b_{24}	0.4025***		-27.4825	0.31875	-0.36331****	-0.485*
b_{34}	-0.07		-14.505	-0.74625	0.492063***	0.2675
b_1^2			84.055***	-3.67857****	0.122625	-0.33464
b_2^2			-16.92	-0.13607	0.160125*	0.647857*
b_3^2			29.48	-2.81107****	0.247625**	-0.43214
b_4^2			-21.2375	-0.24357	0.130125	-0.74714*
R^2	0.9172	0.9632	0.9579	0.9773	0.9634	0.9372
p or probability	0.0000	0.0000	0.0000*	0.0000	0.0000***	0.0000

Note: *b represents the regression coefficient with b_0 being the constant; b_1, b_2, b_3, b_4 are the linear effects with superscripts 1- substrate concentration, 2- enzyme concentration, 3- temperature and 4- time; b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{34} are the different interactions and b_1^2, b_2^2, b_3^2, b_4^2 are the quadratic effects. ***p≤0.001,**p≤0.01, *p≤0.05*

Effect of substrate and enzyme concentration, temperature and time :

The effect of different concentrations of substrate and enzyme treatments conditions on pH, TSS, viscosity and colour are represented in table 3 by the coefficient of second-order polynomials. The results and their interactions are visualized in 3D graphs (Fig. 1).

Figure 1, a& b represents the effect of enzyme-substrate concentration and temperature- time on pH of the palmyra juice after each treatment. It was observed that there was no significant change in pH with a change in enzyme-substrate concentration and also with a change in time and temperature. Although the change was insignificant, there was a reduction in pH with the enzymatic treatment which may be due to the formation of galacturonic acid as a product of enzyme treatment when pectic substances start to break down (Bhatkar et al., 2021). Similar results were obtained on the enzymatic treatment of banana and carrot juice where the pH reduction was observed with the enzymatic treatment (Bora et al., 2017; Sharma & others, 2013). Also, the pH was maintained in the range of 4-6 where the activity of the enzyme was found to be intact. Also according to Suresh & Viruthagiri, (2010), it was observed that the activity of the pectinase enzyme was found to be optimum at pH 5. It was found that the palmyra pulp had a pH of 5.6 ± 0.0045 which falls under the favourable range of the pectinase enzyme (Bhatkar et al., 2021).

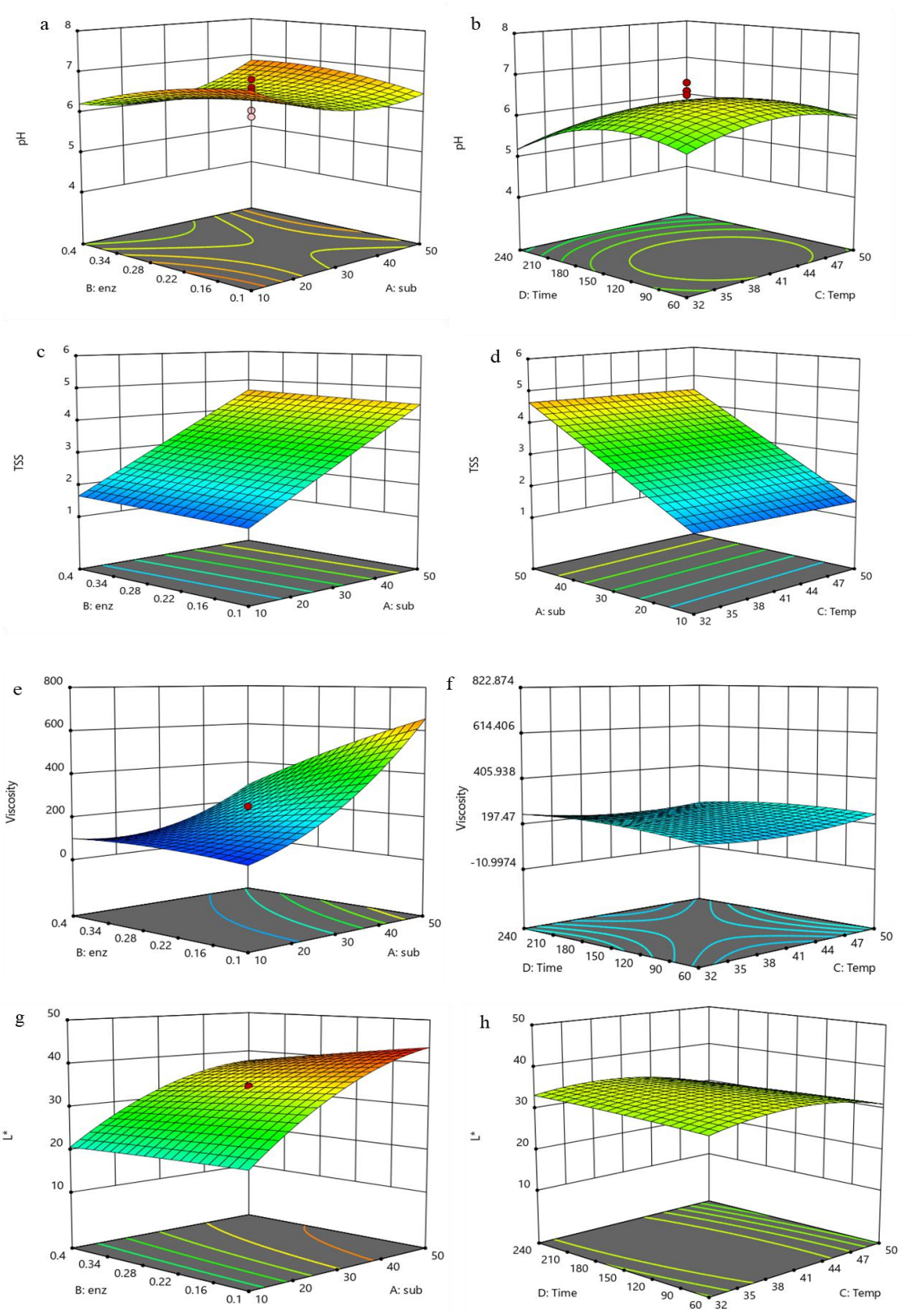
The regression models representing the effect of enzyme and substrate concentration; time and temperature on pH and TSS are given as final equations (1) & (2) in terms of coded factors where A, B, C and D is substrate, enzyme, temperature and time

pH =

+6.04 +0.1530 A -0.3565 B +0.1578 C -0.3065 D +0.1027 AB -0.0111 AC +0.0148 AD -0.1486 BC +0.1173 BD +0.2486 CD -0.2478 A² +0.0922 B² +0.4572 C² -0.0828 D² …(1)

***TSS (˚Brix)** =*

+3.08 +1.51 A +0.0821 B -0.0593 C -0.0775 D … (2)


a
pH
B: enz
A: sub
b
pH
D: Time
C: Temp
c
TSS
B: enz
A: sub
d
TSS
A: sub
C: Temp
e
Viscosity
B: enz
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B: enz
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C: Temp

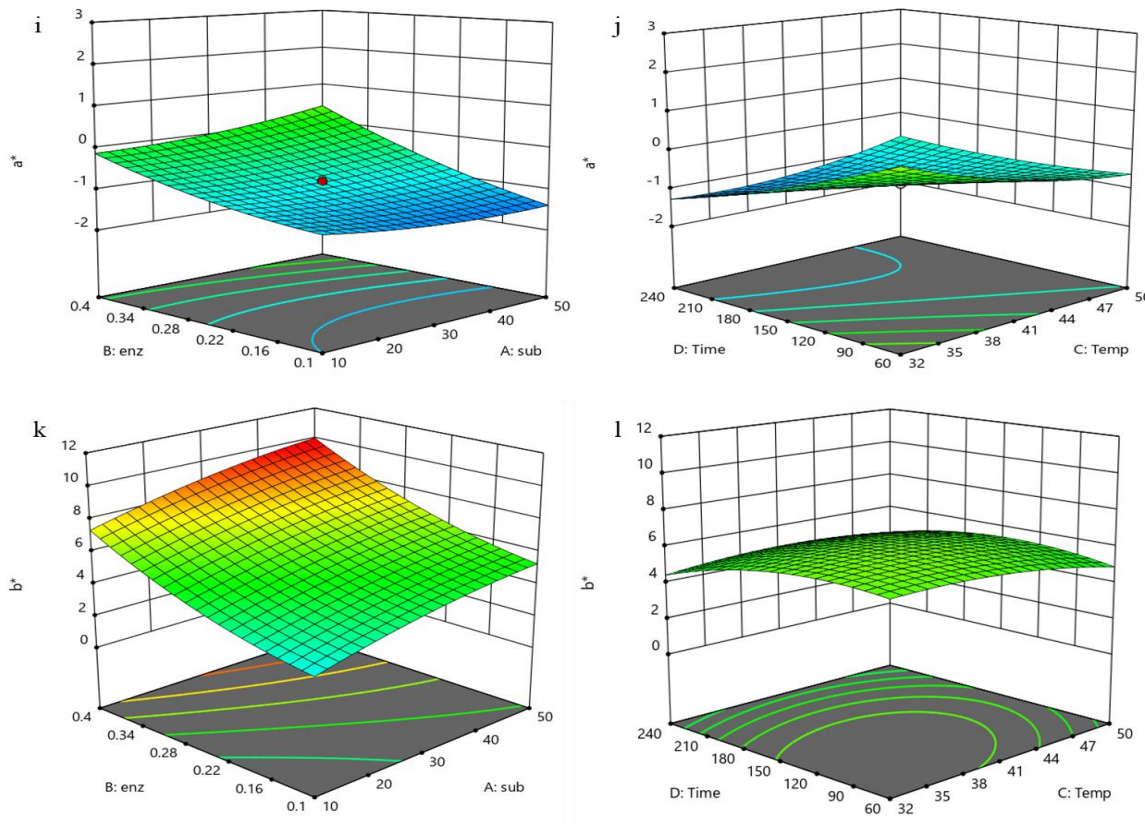

Figure 1. Response surfaces for pH (a), TSS (b), Viscosity (c) and color [L* (d), a* (e), b* (f)] as a function of enzyme-substrate concentration and time-temperature combinations keeping the constant variable at the centre point.

The TSS was found to increase with an increase in substrate concentration. It is obvious from figure 1 c & d that TSS was positively related to the linear effect of enzyme and substrate concentration ($p<0.0001$). Also, there was no significant change in the effect of treatment time and temperature on TSS. A similar effect was observed in Jamun fruit processing where the TSS increases with an increase in enzyme concentration (Ghosh et al., 2017; Mohanty et al., 2018a). This is because the enzyme acts onto the substrate leading to the breakdown of complex molecules to simpler ones thus reducing the viscosity and increasing the TSS. It was clear from the study that more the substrate concentration more will be the TSS. This is due to the amount of total suspended solids present in the medium.

The viscosity of the juice decreased with the addition of enzymes. The pectin which is responsible for the viscosity of the juice is degraded by the activity of the enzyme thus decreasing the viscosity of juice. Fig 1 e & f shows that the increase in enzyme concentration decreases the viscosity and the treatment time and temperature also contributed to the viscosity. At the highest level of treatment time, the viscosity of the juice decreased non-linearly and as the temperature increased, the viscosity decreased to a certain extent. Although no significant change in viscosity was observed with a temperature change, the maximum activity was observed in the range 37- 43 ± 0.13 ˚C after which a reduction in activity was observed. Similar results were observed in the extraction of juice from banana using pectinase enzyme where the viscosity decrease was observed maximum up to 35-40 ˚C and the increase in temperature caused a decrease in enzyme activity (Sagu et al., 2014).

The regression models representing the effect of enzyme and substrate concentration; time and temperature on viscosity and color equation (3 to 6) are given as final equations in terms of coded factors where A, B, C and D is substrate, enzyme, temperature and time

Viscosity (m.Pa) *=*

+147.42 +107.65 A +42.56 B +17.68 C -19.50 D -30.27 AB +68.71 AC -26.79 AD +43.06 BC -4.98 BD +105.50 CD +38.91 A² +68.44 B² +106.84 C² +56.12 D² …(3)

L* *= +34.73 +9.75 A -1.80 B -1.14 C -0.0649 D -1.24 AB -0.2812 AC +0.7063 AD +0.0363 BC +0.3187 BD -0.7462 CD -3.68 A² -0.1361 B² -2.81 C² -0.2436 D² …(4)*

a* *= -0.4004 +0.1545 A +0.6514 B -0.0625 C -0.4284 D +0.2236 AB +0.1552 AC -0.0039 AD +0.1527 BC -0.2089 BD +0.3927 CD …(5)*

b* *= +5.14 +1.33 A +2.91 B +0.1160 C -1.14 D …(6)*

The enzyme and substrate had a significant effect ($p<0.001$) on the colour of the juice. With a higher concentration of pulp the L* value was also higher as the viscosity was high Fig 1 (g & h). It was observed that when the temperature has increased the lightness (L*) reduced and the redness (a*) increased (i & j). At higher temperatures, the caramelization of sugars present in the pulp occurs which contributes to the change in colour. Also as the enzyme concentration and temperature was higher, the lightness value was less (Mohanty et al., 2018a). Similarly, Thi Le et al., (2021) observed the reduction in L* and b* values and increase in a* when subjected to thermal and ultrafiltration processes. The analysis of variance (ANOVA) for the responses was performed to evaluate the quadratic polynomial model. The low value of p (<0.0001) and high F values showed that all the models were significant. Also, it was observed that the adjusted and predicted values of R^2 are less than 0.2 demonstrating the well-fitting of the model.

Optimisation of juice clarification process :

The optimum conditions for the clarification process using the pectinase enzyme were determined to obtain the juice with increased TSS, less viscosity and maintaining higher L* values. In the current investigation, one solution having the highest desirability of 0.99 was chosen. The optimum conditions were 10.84 % and 0.33 % for pulp and enzyme concentration respectively at 43.84 ˚C for 229.05 min with pH (5.65 ± 0.051), TSS (3.6 ± 0.057), Viscosity (203.47 ± 17.11) and L* (18.77 ± 1.732), a* (-0.62 ± 0.777) and b* (4.57 ± 2.945). The pulp after treatment with pectinase enzyme at optimum showed a decrease in pectin content from 45.51 ± 0.145 % to 12.27 ± 0.003 %.

Conclusion :

Tender palmyra endosperm is known for its micro minerals and nutrients that are essential for human health. This fruit is seasonal and is consumed only in tropical countries. Hence the development of ready-to-drink beverages contributes to their year-round availability. But the presence of high pectin in the pulp makes it difficult to use. To address the issue enzymatic clarification of juice was carried out and optimisation was done using response surface methodology using substrate concentration (10.84%), enzyme concentration (0.33%), temperature (43.84 ˚C) and time (229.05 min). These conditions were related by second-order polynomials and the major responses obtained were pH (5.65 ± 0.051), TSS (3.6 ± 0.057), viscosity (203.47 ± 17.11)

and colour (L*:18.77 ± 1.732). With these operating variables obtained a palmyra juice with desired properties that were suitable to serve as a ready-to-serve drink was obtained.

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