

Optimization of pectinase and protease clarification treatment of pomegranate juice



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ABSTRACT

Clarification is a fundamental step in the processing of pomegranate juice to improve its appearance and marketability. The objective of this work was to develop a tailored pectinolytic and proteolytic enzymatic clarification process for pomegranate juice. Response surface methodology was used to analyse the effects of incubation time (30–120 min), temperature (25–50 °C) and complex enzyme amount (0.1–0.4 g/100 g of juice) on physical characteristics of pomegranate juice. R^2 and adjusted R^2 for chill haze, turbidity, potential turbidity and clarity regression models were greater than 0.9 and 0.8, respectively. The complex enzyme amount was the most important factor influencing the clarification of pomegranate juice. Moreover, it was found that an increase of the complex enzyme amount caused a substantial loss of protein and phenol haze forming activity. The optimum enzymatic treatment conditions were: temperature 25–30 °C, time 100–110 min, protease-pectinase complex enzyme amount (the ratio of protease:pectinase was 1:2) 0.22–0.25 g/100 g of pomegranate juice.

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1. Introduction

Pomegranate fruit juice has come to be regarded as an important functional and healthy drink all over the world (Viuda-Martos, Fernandez-Lopez, & Perez-Alvarez, 2010; Fadavi, Barzegar, & Azizi, 2006). The current interest and consequently the increasing market demand by consumers are supported by its antioxidant properties (Kalaycıoğlu & Erim, 2016). Moreover, it has also been revealed that pomegranate fruit contains anti-carcinogenic (Bell & Hawthorne, 2008), antimicrobial (Reddy, Gupta, Jacob, Khan, & Ferreira, 2007) and anti-atherosclerotic compounds (Aviram et al., 2004). Tannins and anthocyanins are responsible for the higher antioxidant activity of the juice (Gil, Tomas-Barberan, Hess-Pierce, Holcroft, & Kader, 2000). However, the same molecules contribute to the formation of the undesirable haze and sediment in the bottle during storage, which are often wrongly perceived as deterioration of quality (Mirzaaghaei, Goli, & Fathi, 2016; Onsekizoglu, 2013; Vardin & Fenercioglu, 2003). Thus, clarification could be considered as a necessary step in the processing of fruit juice to improve its appearance and marketability. This is even more so due to the fact

that pomegranate juice shows excessive turbidity (Erkan-Koç, Türkyılmaz, Yemiş, & Özkan, 2015; Mirzaaghaei et al., 2016). Conventional clarification methods involve simple filtration and centrifugation (to achieve a clarified but not stabilized juice), fining agents such as bentonite, gelatine and silica sol (Yousefnezhad, Mirsaedghazi, & Arabhosseini, 2016; Mirzaaghaei et al., 2016; Erkan-Koç et al., 2015). These latter compounds could produce an acceptable clarified and stabilized juice, however these also lead to a substantial loss of phenolic compounds and antioxidant activity (Erkan-Koç et al., 2015; Bağcı, 2014). An alternative approach to the fining treatment would be a more targeted enzymatic treatment that can be considered as an advanced step in the industrial production of pomegranate juice. Several studies reported on the depectinization of fruit juices with the aim to increase its clarity (Ghosh, Pradhan, & Mishra, 2016; Pinelo, Zeuner, & Meyer, 2010). As demonstrated by Cerreti, Liburdi, Benucci, and Esti (2016), turbidity in pomegranate juice can be removed by pectinase-catalyzed electrostatic destabilization of suspended cloud-causing pectin particles, and by modification of haze active protein-polyphenol complexes via enzyme catalysis using proteases without altering the anthocyanin composition and juice colour. A significant and synergistic effect of the combined use of pectinase and protease enzymes in terms of turbidity and potential haze formation of the pomegranate juice was revealed (Cerreti et al.,

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2016). This result motivated us to investigate and find a new optimized enzymatic procedure to clarify pomegranate juice. In fruit juice processing, the rate of enzymatic hydrolysis depends on several physical-chemical factors such as incubation time, temperature and enzyme concentration (Chen, Xu, Qin, Ma, & Zheng, 2012; NufAliaa, Siti Mazlina, Taip, & Liew Abdullah, 2010). Any optimization procedure implies improving the performance of a process in order to obtain the maximum benefit from it. Traditionally, in order to optimize the operating conditions in a process, the influence of one factor at a time on an experimental response was investigated, while all other factors were kept constant. This optimization technique (one-variable-at-a-time) showed various disadvantages: it did not include interactive effects among the variables, it did not depict the complete effects of the parameter on the response, and it required a large number of experiments and consequently led to an increase of time and expense as well as an increase in the consumption of reagents and materials.

Response surface methodology (RSM) is a collection of statistical and mathematical techniques which are advantageous towards overcoming the above-mentioned problems (Khuri & Mukhopadhyay, 2010; Myers & Montgomery, 2002). RSM has been widely applied and used for developing, improving and optimizing food industry processing, in which a response is influenced by several variables and the objective is to optimize this response (Ahdno & Jafarizadeh-Malmiri, 2016; Ghosh et al., 2016; Tastan & Baysal, 2015).

To our knowledge the effects of incubation time, temperature and enzyme concentration on chill and permanent haze, heat turbidity potential and clarity of pomegranate juice and their further optimization have not been previously reported. In this regard, the objective of this work was to establish the optimal enzymatic treatment conditions to clarify pomegranate juice using RSM. Different clarification parameters such as time, temperature and protease-pectinase complex enzyme amounts were studied, as well as the effect of protease-pectinase treatment on haze active proteins and phenols present in the juice.

2. Materials and methods

2.1. Materials

2.1.1. Fruits

Fresh pomegranate (*Punica granatum* L., var Wonderful) were provided by Fruittaweb.com (Bologna, Italy). Fruits were stored at 4 °C until use.

2.1.2. Enzymes and other chemicals

Native plant cysteine protease, papain from papaya latex (A) was purchased from Sigma Aldrich (Milan, Italy), while Klerzyme 150

pectinase preparation (B) from *Aspergillus niger* was purchased from DSM (Barcelona, Spain). The powder of papain was solubilized to give the same final concentration of protein as the Klerzyme 150 liquid preparation (8 g/L). All other reagents used in this study were purchased from Sigma Aldrich (Milan, Italy) and were of analytical grade.

2.2. Methods

2.2.1. Pomegranate juice process

Fresh fruits were selected, washed manually to eliminate any particles and drained. Then, pomegranate fruits were cut into two pieces and processed into juice with a laboratory-type press. The pH, titratable acidity and soluble solids content of juice obtained were 3.3, 16.3 g/L (as anhydrous citric acid) and 15.2 °Brix, respectively.

The pomegranate juice obtained was divided into equal parts in test tubes and subjected to different clarification treatments, firstly to investigate the optimal ratio of protease and pectinase in the complex enzyme solution and then to further optimize the clarification conditions of pomegranate juice: time, temperature and concentration of complex enzyme preparation. The temperature of each clarification treatment was adjusted to the desired level using a thermostat bath (Kottermann, Hänigsen, Germany). At the end of each enzymatic treatment, the juice was heated at 85 °C for 2 min to inactivate the enzymes and then centrifuged at 15,000 rpm for 15 min. The supernatant was filtered through PES membrane filter with a pore size of 0.45 µm and placed in dark cold storage at 4 °C until further analysis.

2.2.2. Orthogonal test design to optimize protease:pectinase ratio mixture

An orthogonal $L_9(3)^4$ test design was applied to investigate the optimal papain:pectinase ratio in the complex enzyme preparation. As seen from Table 1, nine experiments were conducted with 2 factors and 3 levels. The samples were shaken and placed in a water bath at 50 °C for exactly 2 h. Following the enzymatic treatment each sample was treated as reported above. The turbidity level results were determined after 1 d of cold storage (4 °C) and for up to 21 d.

2.2.3. Optimization of pomegranate clarification conditions

Optimization of the process conditions for the enzymatic clarification of pomegranate juice was performed with RSM. Experimental design and statistical analysis were performed using Minitab 17 software (Minitab Inc., State College, PA, USA). Central composite design (CCD) was employed to study the combined effect of three independent variables namely incubation time, temperature and complex enzyme amount, coded as x_1 , x_2 , and x_3 .

Table 1
Orthogonal experiment design using protease (A), papain from papaya latex, and pectinase (B) Klerzyme 150.

No.	(A) Papain concentration (g/100 g)	(B) Pectinase concentration (g/100 g)	Turbidity (NTU)
1	0 (0)	0 (0)	18.3
2	0	1 (0.125)	14
3	0	2 (0.25)	11.5
4	1 (0.125)	0	11.3
5	1	1	11
6	1	2	10.3
7	2 (0.25)	0	25
8	2	1	11
9	2	2	10
k_1	-23.13	-24.76	
k_2	-20.72	-21.53	
k_3	-22.93	-20.49	
R	2.41	4.27	

respectively. Each independent variable had 3 levels which were -1, 0 and +1.

The minimum and maximum actual values for incubation time (X_1) were set to 30 and 120 min, incubation temperature (X_2) at 25 °C and 50 °C, while for complex enzyme amount (the ratio of papain:pectinase was 1:2) ranging from 0.1 to 0.4 g/100 g of juice (X_3).

A total of 18 combinations were chosen randomly according to a CCD configuration for three factors. The dependent variables (y), reversible turbidity or chill haze (y_1), turbidity (y_2), heat turbidity or potential turbidity (y_3), and clarity (y_4), were chosen as the response function. These values were related to the coded variables by a second order polynomial function as follows:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

where b_0 is the constant term, b_1 , b_2 and b_3 the linear coefficient, b_{11} , b_{22} and b_{33} the quadratic coefficient, b_{12} , b_{13} , and b_{23} the cross product coefficient.

The analysis of variance (ANOVA) table was reported and regression coefficients of individual linear, quadratic, and interaction terms were determined. The regression coefficients were used to generate contour maps from the regression models. The three dimensional response surface plots were plotted by keeping one variable constant at the central point and varying the other two variables.

2.2.4. Turbidity measurement and heat stability test

The turbidity of the juice was measured with a HD 25.2 turbidimeter (Delta Hom, Padua, Italy) and expressed in nephelometric turbidity units (NTU). The formation of chill haze (reversible turbidity) in pomegranate juice stored at 4 °C was revealed after the clarification treatment (1 d of cold storage) and for up to 21 d of cold storage. After each measurement pomegranate juice samples were warmed to room temperature and the irreversible turbidity was revealed. Instead, the potential turbidity of pomegranate juice was determined by heat test as reported by Cerreti et al., 2016.

2.2.5. Clarity

Clarity was determined by measuring the absorbance at 660 nm using a UV-Vis spectrophotometer (UV-2450 Spectrophotometer, Shimadzu) (Sin, Yusof, Sheikh Abdul Hamid, & Abd. Rahman, 2006). Distilled water was used as a reference.

2.2.6. Assessment of haze-active proteins and phenols

Haze forming activity was assessed by adding a known amount of gelatin (3 g/L) or tannic acid (2.5 g/L) to the treated juice after 1, 7 and 21 d of cold storage. Then all the samples were incubated for 30 min in a water bath at 25 °C and haze determinations were carried out as reported above. The corresponding turbidity indicates the relative amounts of haze-active (HA) phenols and HA proteins that are present in the pomegranate juice after adding the enzyme (Siebert, Carrasco, & Lynn, 1996).

3. Results and discussion

3.1. Effect of different ratios of papain:pectinase on pomegranate juice turbidity

Orthogonal analysis results shown in Table 1 revealed that the influence (R) of enzyme factor on the turbidity level was $R_B > R_A$, therefore pectinolytic enzymes play the most important role for the turbidity level ($R_B = 4.27$). Indeed, an increased concentration of

pectinase enzyme used in this study reduces the turbidity level, improving the clarification of pomegranate juice (Table 1). However, a further reduction in turbidity is observed when proteolytic activity is coupled with pectinolytic activity, confirming the results reported of Cerreti et al. (2016). The advantage of using two enzymes in combination is revealed by means of synergistic enzymatic activities (pectinolytic and proteolytic). This is due to the pectinolytic activity that degrades the pectin chains, thus exposing positively-charged proteins. Secondly, proteolytic activity breaks down haze-active proteins, thus preventing new protein-phenol interaction.

Data indicated that the optimal enzyme combination, within the tested range, to obtain the lowest value of turbidity was A_2B_3 , using papain from papaya latex (0.125 g/100 g of juice) and pectinase Klerzyme 150 (0.25 g/100 g of juice) corresponding to enzyme ratio of 1:2. In this condition the minimum turbidity level was achieved soon after the enzymatic process.

Although there was no remarkable difference after enzymatic treatment of juice samples by using either protease or pectinase and juice treated with the optimal enzyme combination (A_2B_3 , protease and pectinase), the juice samples treated with a single enzyme showed a strong increase in turbidity during cold storage. This is in sharp contrast with the sample treated with a combined enzyme. During cold storage, turbidity of juice treated with both enzymes, protease and pectinase (A_2B_3) in combination, remained approximately unchanged (at 14 d about 9 NTU), whereas the turbidity of juice samples treated with only protease or pectinase, increased up to 16.5 and 17 NTU, respectively (data not shown). Therefore, the sample treated with protease and pectinase (enzyme ratio of 1:2) exhibited the minimum turbidity level after the enzymatic treatment and particularly during the entire storage time (data not shown). No further positive effect was gained at a 2:2 enzyme ratio of papain:pectinase, thus the ratio of 1:2 appeared to be the best combination towards minimizing cost, and so it was adopted for further study.

3.2. Analysis of experimental pomegranate clarification process

RSM is a suitable method to study and optimize a complex process. In this study RSM was employed for optimizing the enzymatic clarification process in pomegranate juice. The experimental design in the coded (x) and actual (X) levels and the corresponding results are shown in Table 2. The results were fitted with a second order polynomial equation and the estimated regression coefficient of the variables along with the corresponding R^2 values and lack of fit tests were calculated (Table 3).

Analysis of variance for the four response variables indicated that the response surface models developed for all responses were adequate. The R^2 value for all variables were higher than 0.9 indicating that the empirical models fit the actual data. The value of the adjusted determination coefficients ($Adj R^2$) were higher than 0.8, confirming that the models were highly significant. The significance of each coefficient of equations was determined using p-value (Table 3). The smaller the p-value was, the more significant the corresponding coefficient (Hou & Chen, 2008). The p-value of lack of fit was also given in Table 3, the models did not show any lack of fit.

3.3. Influence of time, temperature and complex enzyme amount on chill haze

Most industrially fruit based beverages are clarified in order to avoid haze turbidity and sediment in the bottle (Bagci, 2014; Anuradha, Naga Padma, Venkateshwar, & Reddy, 2016). Fresh juices have common colloidal suspension that can affect the clarity of

Table 2

Effect of incubation time, incubation temperature, and complex enzyme amount (independent variables) on chill haze, turbidity, heat turbidity and clarity (dependent response variables).

Trial	Independent variables			Dependent variables			
	Time (min)	Temperature (°C)	Complex enzyme amount (g/100 g)	Chill haze (NTU)	Turbidity (NTU)	Heat Turbidity (NTU)	Clarity (Abs)
	$X_1(x_1)$	$X_2(x_2)$	$X_3(x_3)$				
1	75 (0)	37.5 (0)	0.25 (0)	27.0	0.30	3.30	0.094
2	30 (-1)	50.0 (+1)	0.40 (+1)	43.0	0.20	4.10	0.089
3	30 (-1)	25.0 (-1)	0.10 (-1)	36.0	7.30	9.40	0.109
4	75 (0)	37.5 (0)	0.40 (+1)	35.0	0.05	3.20	0.093
5	120 (+1)	37.5 (0)	0.25 (0)	20.0	0.10	2.50	0.090
6	75 (0)	37.5 (0)	0.25 (0)	23.0	0.16	2.90	0.091
7	120 (+1)	25.0 (-1)	0.10 (-1)	36.0	6.70	9.20	0.103
8	75 (0)	25.0 (-1)	0.25 (0)	32.5	0.24	3.50	0.098
9	75 (0)	37.5 (0)	0.25 (0)	27.3	0.16	3.00	0.094
10	75 (0)	37.5 (0)	0.10 (-1)	43.5	7.00	10.00	0.104
11	75 (0)	37.5 (0)	0.25 (0)	27.4	0.27	2.77	0.094
12	75 (0)	50.0 (+1)	0.25 (0)	29.0	0.90	3.30	0.096
13	120 (+1)	25.0 (-1)	0.40 (+1)	19.5	0.03	2.99	0.092
14	120 (+1)	50.0 (+1)	0.10 (-1)	28.5	7.90	10.90	0.105
15	30 (-1)	50.0 (+1)	0.10 (-1)	34.5	7.50	10.00	0.111
16	120 (+1)	50.0 (+1)	0.40 (+1)	26.0	0.50	3.15	0.098
17	30 (-1)	25.0 (-1)	0.40 (+1)	30.0	0.15	3.27	0.092
18	30 (-1)	37.5 (0)	0.25 (0)	23.0	0.35	3.60	0.093

Table 3

Regression coefficients, model p -value, lack of fit, R^2 and adjusted R^2 for the different polynomial models.

Regression coefficient	Chill haze (NTU)	Turbidity (NTU)	Heat Turbidity (NTU)	Clarity (Abs)
b_0	61.4	17.701	18.08	0.1565
b_1	0.679**	-0.01184	0.0099	-0.000038
b_2	-1.042	-0.1387***	-0.076	-0.001779
b_3	-285.4*	-94.60***	-92.36***	-0.1834***
b_{12}	-0.00278	0.000316**	0.000096	0.000002
b_{13}	-0.398*	0.00704	-0.0357	0.000389*
b_{23}	1.900**	-0.0587*	-0.0873	-0.000067
b_{11}	0.003713**	-0.000016	-0.000054	-0.000001
b_{22}	0.0111	0.002005**	0.00154	0.000022*
b_{33}	454.8***	145.25***	152.9***	0.2228**
p -value of model	0.000***	0.000***	0.000***	0.000***
p -value for lack of fit	0.245	0.163	0.106	0.306
R^2	0.916	0.999	0.991	0.961
Adj R^2	0.822	0.998	0.981	0.918

Subscripts: 1 = incubation time; 2 = incubation temperature; 3 = enzyme complex amount.

*Significant at 0.05 level.

**Significant at 0.01 level.

***Significant at 0.001 level.

the beverage. Haze can be reversible (chill haze) or irreversible (turbidity). Chill haze is a reversible turbidity and it is soluble when the liquid returns to room temperature. After enzymatic treatment, the formation of chill haze in pomegranate juice stored at 4°C was revealed. Chill haze was found to depend on the enzyme concentration and incubation time in both linear and quadratic manners. The concentration of the enzyme mixture showed a negative effect on the linear term but showed a positive effect on its quadratic terms. As shown in Table 3 the interaction effect between incubation time and enzyme concentration was negative ($P < 0.05$), whereas it was significant and positive between incubation temperature and enzyme concentration ($P < 0.01$).

The 3D response surfaces (Fig. 1a–b) and contour plots (Supplementary Figs. 1a–b) were plotted to better visualize the significant ($P < 0.05$) interaction effects of independent variables on chill haze of pomegranate juice. The plots are drawn as a function of two factors at a time, holding the third factor at fixed levels (at the mid-level).

As shown in Fig. 1a–b the presence of curvatures in the chill haze curves confirmed that the variation of chill haze (Y_1) was

explained as a nonlinear function of pomegranate juice. At fixed temperature, increase in enzyme concentration and incubation time (as reported in Fig. 1a), may decrease the chill haze of the pomegranate juice. It is well-known that this involves polyphenols and proline-rich proteins. The lowest chill haze of pomegranate juice was observed when enzyme concentration was up to 0.29 g/100 g followed by a strong increase in turbidity as enzyme concentration was increased. This phenomenon may be due to an excessive amount of enzyme, which could be involved in the formation of reversible haze.

It was demonstrated that protein concentration could influence protein–polyphenol interactions in model systems (Siebert et al., 1996). Therefore, if the amount of polyphenol is held constant as the protein increases, haze increases up to a certain point. The same in this regard was stated by Landbo, Pinelo, Vikbjerg, Let, and Meyer (2006) and Ghosh et al. (2016) who found that, after adding enzymatic preparations to elderberry and jamun juice at the highest enzyme dose level, juice samples significantly increased in turbidity immediately after treatment.

Moreover, it is worth noting that this turbidity trend as a

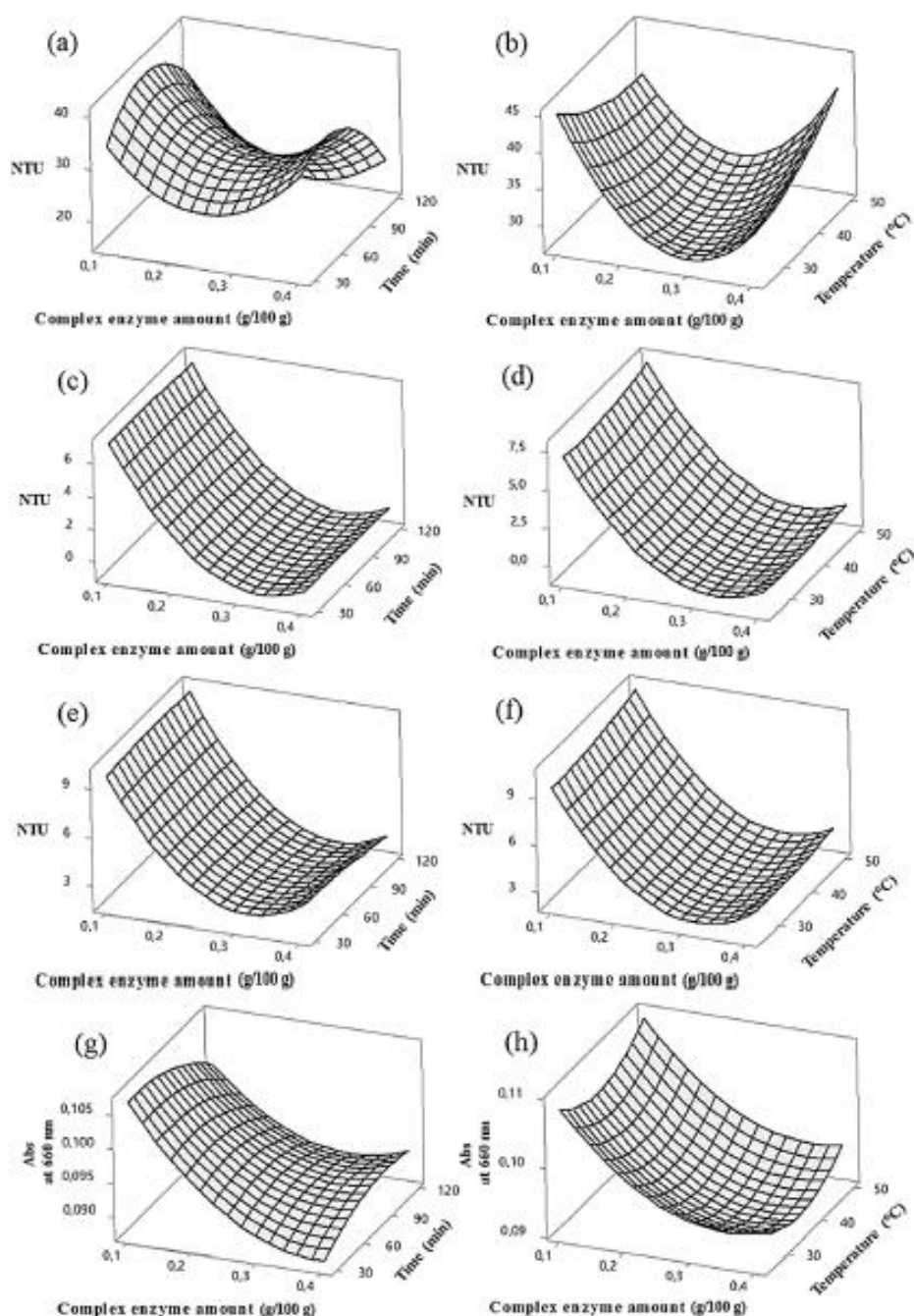


Fig. 1. 3D response surfaces for chill haze (a–b), turbidity (c–d), heat turbidity (e–f), clarity (g–h) of pomegranate juice as a function of complex enzyme amount and incubation time at 37.5 °C (left-side) and of complex enzyme amount and temperature at 75 min (right-side).

function of complex enzyme amount is evident (Fig. 1a–b) in chill haze. However the same trend is only hinted at in the irreversible turbidity values (Fig. 2c–d). Therefore this turbidity trend could be correlated to low temperature storage. Usually, temperature exerts considerable influence on the turbidity results, a striking example is provided by the chill-haze of beer. When a system is cooled, the withdrawal of energy can result in the settling of colloidal particles and it may also cause the formation of additional particles through a reduction in solubility (Siebert, 2006).

3.4. Influence of time, temperature and complex enzyme amount on turbidity and on heat turbidity

Most haze caused by chilling partially dissolved when pomegranate juice was warmed at room temperature. It was observed that among three factors, temperature and enzyme mixture concentration significantly affected the irreversible haze or permanent turbidity still present when pomegranate juice was returned to room temperature, in both linear and quadratic terms (Table 3).

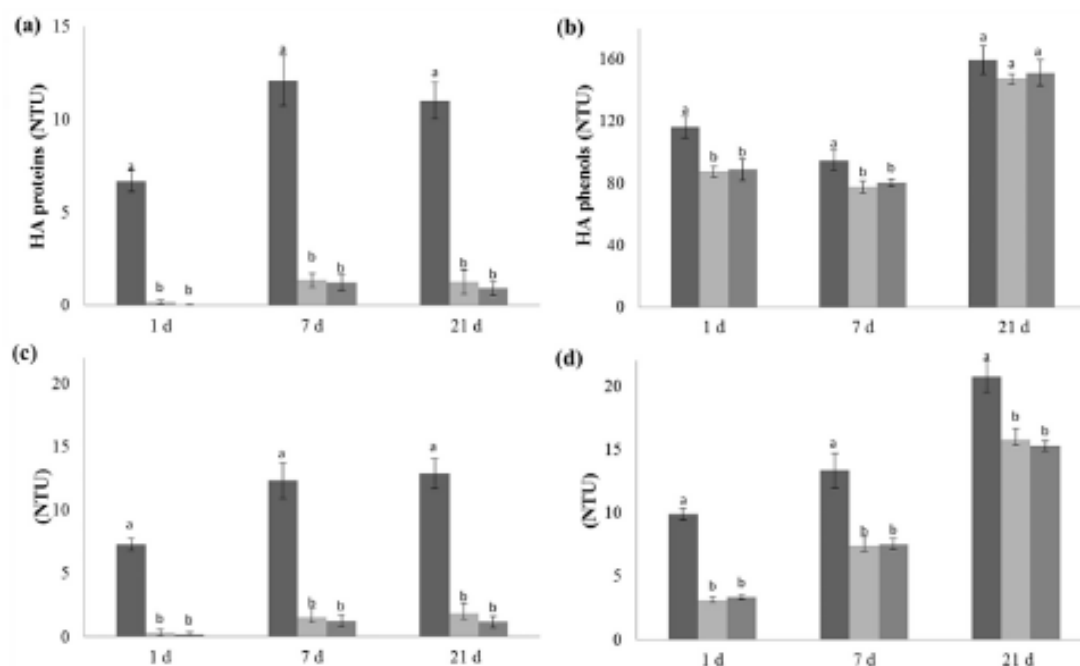


Fig. 2. Haze active proteins (a) and phenols (b) developed by adding tannic acid or gelatin respectively, turbidity (c) and potential turbidity (d) of treated juice with 0.1 0.25 0.4 g of enzyme complex per 100 g of juice after 1, 7 and 21 d of storage at 4 °C. The values are mean $\pm$ SD of triplicate analysis of juice samples. For each time of storage (1, 7 and 21 d) data with different superscript letters indicate significantly different ($P < 0.05$).

The interaction effect between incubation time and temperature was positive, whereas it showed a negative interaction effect between incubation temperature and enzyme concentration. Again from 3D response surfaces (Fig. 1c–d) and contour plots (Supplementary Figs. 1c–d) it is observed that, as the enzyme complex concentration increases up to 0.25 g/100 g of juice, the turbidity decreases to its lowest level. A new index of haze stability was obtained using heat test (6 h, 80 °C and 12 h, 4 °C), a forcing method to develop haze, widely used for testing protein instability in wine but also in apple juice (Wu & Siebert, 2002). After heat treatment on pomegranate juice, the potential haze was measured. From Table 3, it is observed that complex enzyme concentration had a negative effect on potential turbidity in terms of NTU level after heat treatment, showing a significant level of $P < 0.001$. Quadratic terms of enzyme concentration were also significant $P < 0.001$. Heat haze formation is mainly associated with low molecular weight protein (less than 30 kDa) as revealed in beer and apple juice (Wu & Siebert, 2002), thus an increase in enzyme concentration may lead to a modification of this kind of haze active protein via enzyme catalysis using proteases. As observed in Fig. 1e–f and Supplementary Figs. 1e–f, potential turbidity was significantly reduced with higher enzyme mixture concentration. A similar trend was observed in irreversible turbidity level where temperature and enzyme concentration were the most influential independent variables.

3.5. Influence of time, temperature and complex enzyme amount on clarity

The performance of the clarification process with protease-pectinase treatment was also evaluated using clarity index. Lower absorbance values indicate a clearer juice. Clarity was found to depend mainly on the concentration of complex enzyme as its

linear effect was negative and its quadratic effect was positive, showing a high significance level of $P < 0.001$ and $P < 0.01$, respectively.

The other factors that also contributed to clarity included the quadratic effects ($P < 0.01$) of temperature, and the interaction effect between time and enzyme concentration (Table 3). This interaction was significant at $P < 0.05$ and its effect was positive on clarity, indicating that the action of complex enzyme was dependent on the incubation time during the enzymatic treatment of pomegranate.

Fig. 1g–h shows a three dimensional plot of effect of the independent variables, time and complex enzyme amount, on the juice clarity. It was observed that the absorbance value decreases (clarity increase) with the increase in enzyme concentration at a fixed temperature (37.5 °C). It was clear from 3-D response surface plot (Fig. 1g) and contour plot (Supplementary Fig. 1g) that the lowest absorbance value is obtained at the highest enzyme concentration and lower absorbance values indicated a clearer juice. Similar results for clarity were reported for the changes of enzyme concentration and time in sapodilla juice (Sin, Yusof, Sheikh Abdul Hamid, & Abd, 2006). Instead, Fig. 1h and Supplementary Fig. 1h describe the dependence of clarity on complex enzymes and temperature at fixed time. The lowest absorbance value of pomegranate juice was observed when enzyme concentration was up to 0.3 g/100 g of juice. It can be seen from Fig. 1h that the absorbance values decreased as temperature increased and reached a minimum between 32 and 43 °C. Any further increase in temperature increased the absorbance values. Similar results were reported for the changes of enzyme concentration and temperature in banana juice (Lee, Yusof, Hamid, & Baharin, 2006), mosambi juice (Rai, Majumdar, DasGupta, & De, 2004) and also in the clarification of green asparagus juice (Chen et al., 2012).

3.6. Influence of enzymatic clarification on haze active proteins and phenols

As reported by Siebert (2006), the addition of gelatine and tannic acid provokes an increase of turbidity (NTU level) resulting from the presence of haze active phenols and haze active proteins, respectively, in the fruit juice. Due to the high influence of enzyme mixture concentration on turbidity, potential turbidity and clarity of pomegranate juice in respect to processing time and temperature, it was also important to assess the effect of enzymatic treatment on haze causing proteins and haze causing phenols (Fig. 2a–b) present in pomegranate juice.

Adding large amounts of tannic acid (an HA phenol) to bottled pomegranate juice produced an increase in turbidity. The haze developed by adding tannic acid was strictly correlated with the complex enzyme amount (Fig. 2a), much more than that revealed after the addition of gelatine (Fig. 2b). It was possible to see that an increase of enzyme mixture percentage from 0.1 to 0.25 g/100 g of juice reduced the haze causing proteins, developed by adding tannic acid almost totally at 1 d (Fig. 2a). The same increase of enzyme complex slightly decreased the reactivity of phenols (Fig. 2b). However, for both turbidity causing molecules, any further effect was observed if the enzyme concentration increased up to 0.4 (g/100 g of juice). This finding revealed that this kind of treatment worked mainly on the haze active protein component, confirming the high selectivity of enzyme catalysis. However, both pectinolytic and proteolytic activities could lead to a subsequent destabilization of the pectin coat and protein-polyphenol core thus decreasing the reactivity of phenol aggregates.

In addition, Fig. 2a–b shows an increase in the haze forming activity of proteins and phenols that remain in the treated pomegranate juice, probably induced during cold storage by a consecutive molecular rearrangement. The increase in HA proteins showed a different trend and intensity as those observed for HA phenols. After enzyme treatment, the reactivity of proteins increased considerably within the first 7 d of cold storage, and then stabilized and grew no further. Instead the reactivity of phenols tended to increase successively between the 7 and 21 d of cold storage.

The corresponding irreversible turbidity after warming at room temperature and potential turbidity evaluated by heat stability test are reported in Fig. 2c–d. It is evident that juices treated with 0.25 g/100 g of juice of enzymes and more are well clarified and stabilized, showing turbidity level close to zero (Fig. 2c) and a potential turbidity level of 3 NTU (Fig. 2d) at 1 d. The enzyme

concentration of 0.25 g/100 g of juice allowed reaching the lowest turbidity and heat turbidity (potential turbidity) also throughout the residual storage time. Therefore it appears that the largest decrease of haze active proteins compared to polyphenols decrease were sufficient to cause a reduction of turbidity and potential turbidity levels of pomegranate juice.

3.7. Optimization of enzymatic pomegranate juice clarification

With multiple responses, the optimum condition where all parameters simultaneously meet the desirable values could be visualized graphically by superimposing the contours of the response surfaces in an overlay plot. Graphical optimization displays the area of feasible response values in the factor space. Regions that do not fit the optimization criteria are shaded.

The minimum constraints were chosen relatively close to the acquired minimum value of chill haze (<24 NTU), turbidity (<1 NTU), heat turbidity (<4 NTU) and absorbance (clarity) value, in order to obtain a moderately precise optimum zone. Based on overlay plots the optimum clarifying conditions of pomegranate juice are shown in a nonshaded area (Fig. 3).

Fig. 3b shows the superimposed contour plot for optimization of chill haze, turbidity and heat turbidity, and clarity keeping the temperature constant at the central point. The zone of optimization, as shown in the superimposed contour plot, depicts complex enzyme concentration to be in the range of 0.22–0.25 g/100 g of juice and incubation time between 100 and 115 min.

Fig. 3c shows the superimposed contour plot of all four dependent variables, keeping the time constant at 110 min. The zone of optimization, as shown in the nonshaded area, depicts enzyme concentration to be in the range of 0.22–0.25 g/100 g of juice and process temperature between 25 °C and 47 °C. Thus, keeping the complex enzyme concentration constant as determined from Fig. 3c and b, the best combination of response function can be finally determined in Fig. 3a.

Therefore, taking into account the effect of high temperature on the overall quality of fruit based beverages, these results demonstrated that a clarified pomegranate juice could be obtained under the optimum clarification conditions: i.e. at temperature 25–30 °C for 100–110 min using 0.22–0.25 g of enzyme mixture (the ratio of protease:pectinase was 1:2) per 100 g of juice.

The same four dependent variables were also determined during cold storage at 7 and 21 d. Chill turbidity decreased over time, instead there was an increase in irreversible turbidity, heat

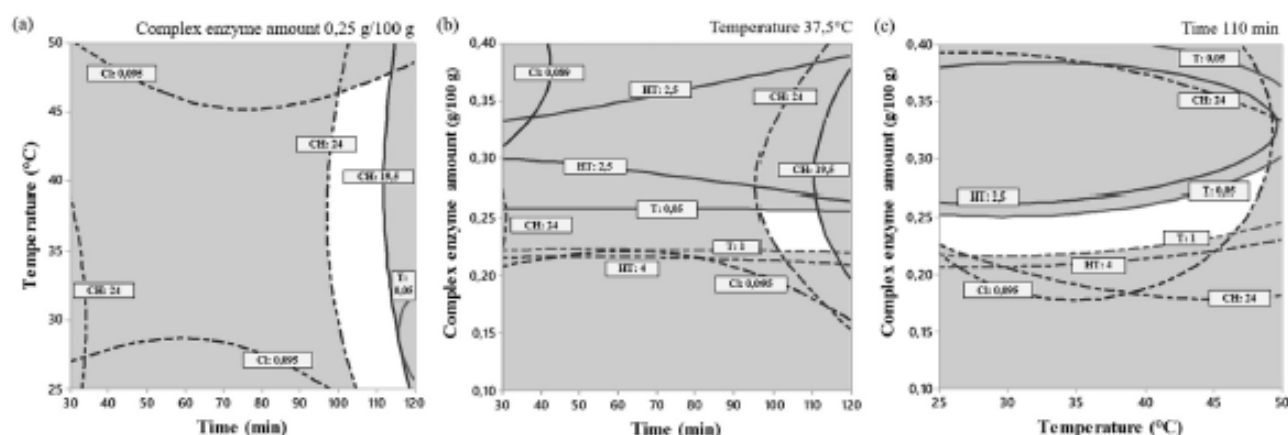


Fig. 3. The optimum region by overlaying contour plots of the four responses (chill haze - CH, turbidity - T, heat turbidity - HT, clarity - Cl) evaluated as a function of incubation time and temperature (a), incubation time and complex enzyme amount (b), incubation temperature and complex enzyme amount (c).

turbidity as well as absorbance values at 660 nm. The optimal clarification conditions were defined also during pomegranate storage by superimposing the contours of the response surfaces in an overlay plot. In Supplementary Figs. 2a–b both overlaying plots are shown attained at 7 and 21 d of cold storage, respectively. Results demonstrated that the optimum clarification conditions revealed immediately after pomegranate clarification treatment remain generally unchanged.

4. Conclusion

The optimum enzyme reaction conditions for the clarification of pomegranate juice were evaluated using response surface methodology. These were: incubation time (30–120 min), temperature (25–50 °C) and complex enzyme amount (0.1–0.4 g/100 g of juice). All these variables markedly affect the chill haze, turbidity, potential turbidity and clarity of the pomegranate juice. Generally, complex enzyme concentration was the most important factor influencing the clarification of pomegranate juice. Using the contour plots, the optimum set of the operating conditions were graphically obtained: temperature 25–30 °C for 100–110 min using 0.22–0.25 g of protease-pectinase complex enzyme amount (the ratio of protease:pectinase was 1:2) per 100 g of juice. Moreover this kind of clarification treatment caused a substantial decrease of protein and phenol haze forming activity, thus reducing the potential juice turbidity.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.lwt.2017.04.022>.

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