DIBAF

Department for Innovation in Biological, Agro-food and Forest systems

Ph.D. Course in Science, Technology and Biotechnology for Sustainability

XXIX Cycle

TITOLO TESI DI DOTTORATI DI RICERCA

MULTIPLE ENZYME-ASSISTED CLARIFICATION PROCESS OF FRUIT JUICE

(AGR/15)

Tutor

Prof. Marco Esti

PhD Student

Martina Cerreti

PhD Program Coordinator

Prof. Mauro Moresi

Anno Accedemico 2016-2017

Short Abstract

The aim of this PhD thesis research project was to create a new approach to conventional clarification processing of fruit juice, using tailored enzymatic treatment in free and/or immobilized form. The first part of the work provided new insight into the individual and interactive effects of pectinase, protease, or multiple (pectinase-protease) treatments on juice turbidity and on haze formation. A significant and synergic effect of the combined use of pectinase, Klerzyme 150 DSM and protease, papain from Papaia latex, was demonstrated. Excellent results in terms of turbidity levels (during cold storage at 4 °C for 14 days) and potential haze formation in juice were obtained. Moreover, in the second part of the research, the optimum conditions of the clarification process were achieved using response surface methodology. The recommended enzymatic treatment conditions were: temperature 25-30 °C for 100-110 min using 0.22-0.25% (v/v) of protease-pectinase complex enzyme amount (the ratio of protease : pectinase was 1:2).

In the last part of this PhD project the use of PVA gel in the form of LentiKats®, a biocompatible and non-toxic enzymatic carrier, to immobilize pectinases was investigated. A biocatalyst, Panzym YieldMash, a polygalacturonase, showed the possibility of practical application in juice. Indeed, the efficient depolymerisation of apple pectin was performed. The immobilized polygalacturonic enzyme was capable of producing an 80% reduction in turbidity in a pectin solution compared to 95% as found with the soluble enzyme. The value could be considered acceptable for the application of this immobilized system in fruit juice processing, as well as considering that the same percentage of turbidity reduction was observed in the following two cycles.

Keywords: Fruit juice clarification, Pomegranate juice, Pectinase, Protease, Turbidity, Response surface methodology, Immobilization.

***

Riassunto

Questo progetto di dottorato ha riguardato lo sviluppo di una nuova strategia di chiarifica di succhi di frutta, attraverso trattamenti a base di enzimi pectinolitici e proteolitici in forma libera e/o immobilizzata. La prima parte del progetto di ricerca ha permesso di

acquisire nuove conoscenze circa l'efficienza di trattamenti a base di pectinasi, proteasi e loro combinazioni, sulla torbidità e sulla formazione di *haze* durante la conservazione del prodotto. E' stato dimostrato in succo di melograno un effetto sinergico nell'utilizzo di pectinasi (Klerzyme 150 DSM) in combinazione con proteasi (papaina da lattice di papaia). Tale trattamento risulta essere il più efficace sia nell'abbattimento dei livelli di torbidità, riscontrati conservando il prodotto a 4 °C per 14 giorni, sia rispetto alla torbidità potenzialmente esprimibile dal prodotto durante tutta la sua *shelf-life*.

Inoltre, mediante la metodologia delle superfici di risposta, è stato possibile determinare le ottimali condizioni di chiarifica del succo: temperatura di processo 25-30 °C per un tempo di trattamento di 100-110 minuti utilizzando 0.22-0.25% (v/v) di una complesso enzimatico a base di proteasi (papaina) e pectinasi (Klerzyme 150 DSM) nel rapporto 1:2.

In ultimo, è stato valutata l'applicazione del PVA gel sotto forma di particelle lenticolari, LentiKats®, biocompatibili e *food-grade*, come *carrier* enzimatico, per l'immobilizzazione di differenti enzimi pectinolitici. La migliore performance è stata ottenuta con l'utilizzo dell'enzima poligalatturonasi, Panzym YieldMash, il quale ha ridotto di circa l'80% la torbidità presente nel succo di mela contro il 95% ottenuto utilizzando l'enzima in forma libera. Tale risultato può essere considerato accettabile per una potenziale applicazione in un sistema reale, considerando, inoltre, che la percentuale di riduzione del livello di torbidità (80%) si è mantenuta costante per due cicli operativi.

Parole chiave: Chiarifica di succhi di frutta, Succo di melograno, Pectinasi, Proteasi, Torbidità, Metodologia delle superfici di risposta, Immobilizzazione.

M**ultiple enzyme-assisted clarification process of fruit juice**

Martina Cerreti (cerreti@unitus.it)
Dept. for Innovation in the Biological, Agrofood and Forestry systems,
University of Tuscia, Viterbo, Italy
Tutor: Prof. Marco Esti

This PhD thesis was undertaken to examine the efficiency of various alternative enzymatic clarification strategies on fruit juice, in particular on pomegranate juice in order to obtain a new insight into individual and interactive effects of pectinase, protease, or multiple (pectinase-protease) treatments on juice turbidity and on haze formation.

Trattamenti enzimatici multipli per la chiarifica di succhi di frutta

Questo progetto di dottorato ha riguardato lo sviluppo di strategie alternative di chiarifica di succhi di frutta, con particolar riguardo al succo di melograno, e l'ottenimento di nuove conoscenze circa l'efficienza di trattamento di pectinasi, proteasi e loro combinazioni, sulla torbidità e sulla formazione di *haze* durante la conservazione.

Keywords: Fruit juice clarification, Pomegranate juice, Pectinase, Protease, Turbidity, Response surface methodology, Immobilization.

1. Introduction

One of the major problems encountered and actually unsolved in the industrial preparation of fruit-based beverages, including both fruit juices and wines but excluding citrus juices, is undesirable turbidity in the final product. A cloudy visual perception is the result of light scattering caused by particles suspended in fruit juice. Many different sources of haze in beverages have been recently described. Immediate turbidity in freshly produced juices is provided by suspension of polysaccharide particulates (i.e., pectin, cellulose, hemicellulose, starch and lignin) arising from primary and inner cell walls (Sorrivas *et al*., 2006). Instead, the turbidity development during cold storage or after reconstitution with water from fruit juice concentrates, generally known as haze formation, is caused by formation of protein-polyphenol insoluble complexes (Siebert, 2006).

The main purpose of the clarification procedures employed in industrial juice processing is to eliminate any substances responsible for the turbidity and to avoid cloudiness and sediments in the final produced juice.

Conventional clarification methods involve simple filtration and centrifugation (to achieve a clarified but not stabilized juice), fining agents such as bentonite, gelatin, silica sol (Yousefnezhad *et al*. 2016; Mirsaeedghazi *et al*. 2016, Erkan- Koç 2015). As it has been reported, these latter compounds could produce an acceptable stabilized juice, however these also cause a substantial losses of phenolic compounds and antioxidant activity (Erkan- Koç 2015; Alper *et al*. 2011).

The fruit juice industry investigated several methods to optimize fruit juice clarification process, because consumer's opinion is mainly driven by juice visual appearance. There is an increased interest towards pectolytic enzymes as clarifying agents (Sandri *et al*. 2013), also in the immobilized form (Esawy *et al*. 2013), yet few studies have so far relied on proteases to clarify fruit juices (Pinelo *et al*. 2010; Landbo *et al*. 2006).

The aim of this PhD thesis research project was to create a new approach to conventional clarification processing of fruit juice, using tailored enzymatic treatment in free and/or immobilized form.

In particular this work was structured in three specific sections, according to the following objectives:

- to enhance the colloidal stability of pomegranate juice with tailored enzymatic (protease and pectinase) treatments, by examining pectins, proteins and phenol amount and their haze forming activity;
- to establish the optimal enzymatic treatment conditions, time, temperature and complex enzyme amounts (protease and pectinase) to clarify pomegranate juice using response surface methodology;
- to develop a novel alternative enzymatic clarification strategy consisting of pectinases immobilized into PVA gel capsules, LentiKats®, for fruit juice clarification (this phase was performed at the Institute of Biotechnology, Slovak University of Technology, SK).

2. Materials and Methods

2.1 Pectinase and protease clarification treatment in pomegranate juice.

Pomegranate juice, obtained from fresh pomegranates using a laboratory-type press, was divided in test tubes and was subjected to various experimental enzymatic clarification treatments using Klerzyme 150 (DSM) as pectinase and two proteases, purchased by Sigma Aldrich, bromelain and papain, as follows: (Control) no added enzyme preparation, (A) pectinase, (B_1) bromelain, (B_2) papain, (AB_1) pectinase and bromelain, (AB_2) pectinase and papain (AB_1) and pectinase with both proteases (AB_1B_2). Each tube with sample and enzyme preparation was shaken and placed in a water bath at 50 °C for exactly 2 h. Following the enzymatic clarification treatment, the samples were immediately heated to 85 °C for 1 min in order to inactivate the enzyme and centrifuged at 15000 rpm for 10 min before being placed in darkness in cold storage at 4 °C for up to 14 days. The control was subjected to the same incubation time at 50 °C, then pasteurized and centrifuged as conventional industrial clarification treatment. 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 immediate turbidity was measured immediately after the clarification treatment (day 0) and the same measurement was performed after 1, 7, and 14 days of cold storage (chill haze). The potential turbidity of pomegranate juice was determined by heat test: juice samples were incubated at 80 °C for 6 h and then kept at 4 °C for 16 h. Haze formation was measured after equilibration at room temperature (approximately 25°C).

2.2 Impact of the enzymatic treatment on the overall quality.

During processing, pH, titratable acidity and soluble solid content (°Brix) of pomegranate juice were monitored. pH was measured potentiometrically with a Mettler Toledo pH meter (Steroglass, Perugia, Italy). Titratable acidity was determined as g anhydrous citric acid/L of juice by titrating 10 mL of pomegranate juice with NaOH 0.1M reaching pH 8.1 and °Brix measurements were carried out at 20°C with a digital refractometer HI 96801 (Hanna Instruments, Milan, Italy). The pH, titratable acidity and soluble solid content of juice samples ranged from 2.97 to 3.05, 15.60 to 15.68 g/L (as anhydrous citric acid) and 14.73 to 14.85 °Brix, respectively, thus indicating that enzymatic treatment did not affect the amounts of organic acid and sugar contained in the pomegranate juice. Moreover, several samples were withdrawn from any tube at various storage times (0, 1, 7, 14 days) and assayed to quantify the amount of pectins, proteins, phenols and anthocyanin level.

2.3 Response surface optimization of conditions for clarification of pomegranate juice using pectinase and protease.

Response surface methodology was employed for simultaneous analysis of the effect of multiple enzymatic treatment conditions (incubation time, temperature and complex enzyme amount) on physical characteristics on pomegranate juice, such as: chill haze, turbidity, potential turbidity and clarity. Fresh pomegranate juice was treated with protease-pectinase complex enzyme amounts (ratio 1:2) at different incubation time (30-120 min), incubation temperature (25-50 °C) and enzyme mixture (0.1-0.4%).

The complete design consisted of 18 combinations and were carried out in random order.

2.4 Immobilization of pectinases into PVA gel.

Six commercial pectinolytic enzymes (Pectinex Ultra Color (UC) and Pectinex BE xxl (PB) from Novozymes; Klerzyme 150 (K150) and Rapidase (RD) from DSM; Panzym Smash XXL (PS) and Panzym YieldMASH (PM) from Begerow) were immobilized into polyvinyl alcohol hydrogels using the LentiKats® technology in the form of lens-shaped particles (Rebroš *et al*. 2006). The enzyme preparation (2 ml) was mixed with PVA gel, extruded through a syringe onto a hard surface and dried at 35 °C for 1 h to produce solid gel particles (LentiKats®). The activity of pectin-lyase (PL) in 0.1 M acetate buffer pH 4.2 was spectrophotometrically determined by monitoring the increase of absorbance at 235 nm (Busto *et al*. 2006); while that of polygalacturonase (PG) in 0.1 M acetate buffer pH 6 was measured by assaying the galacturonic acid liberated from polygalacturonic acid via the 3,5'-dinitrosalicylic acid method. Free (40 µl as diluted 1⁄100) or immobilized enzyme (0.4 g) was mixed with 2 ml of 0.1 M acetate buffer enriched with 1% (w/v) of substrate, then the mixture volume was adjusted to 4 ml with the aforementioned buffer. Pectin and polygalacturonic acid from Sigma Chemical Co. (Milan, Italy) were used as substrates to assay enzyme activities.

3. Results and Discussion

3.1 The effect of pectinase and protease treatment on turbidity and on haze active molecules in pomegranate juice.

Pomegranate juice was subjected to various experimental enzymatic clarification treatments using Klerzyme 150 as pectinase and two proteases, bromelain and papain. The turbidity increase, during the cold storage (4°C) for 14 days, of centrifuged pomegranate juice (control) and of the experimentally clarified and centrifuged juices is shown in Figure 1a. The value was measured immediately after the clarification treatment on day 0, and on the following 1 to 14 days of cold storage. The initial turbidity of the control juice sample was approximately 10 NTU. The enzymatic clarification treatment on pomegranate juice significantly reduced the initial turbidity levels (0 day) from 10 NTU to 3-4 NTU in all treated samples ($P<0.05$), except those both treated with a single protease (B_1 or B_2 sample). A significant and positive effect of pectinase and protease synergic activity in samples AB_1 and AB_2, was already evident after 2 hours in presence of enzymes (day 0) and this significant clarifying effect was recorded throughout the residual storage time (14 days) ($P<0.05$).

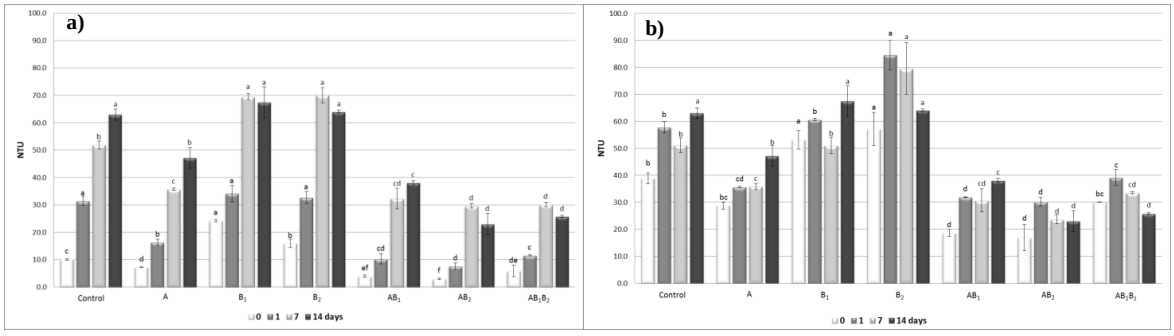


Figure 1: *Chill haze (a) and potential turbidity (b) determined up to the 14th day of storage at 4°C of enzymatically-treated pomegranate juice. For each enzymes, means ± SD (n=3) with different letters are significantly different (P≤0.05). (Control) no added enzyme preparation, (A) pectinase, (B_1) bromelain, (B_2) papain, (AB_1) pectinase and bromelain, (AB_2) pectinase and papain and pectinase with both proteases (AB_1B_2).*

During the 14 days of cold storage, the turbidity levels gradually increased in all enzyme–treated samples as well as for the control sample whose turbidity increased up to 63 NTU. However the synergic clarifying effect was observed in samples AB_1, AB_2 and AB_1B_2 also on 14th day, showing 38, 23 and 26 NTU levels, respectively. Pomegranate juice sample treated with pectinase and papain (AB_2) exhibited the lowest turbidity level with a 63% of haze decrease during cold storage.

Heat-induced haze (named potential juice turbidity) measured in all juice samples following heat treatments (6 hours at 80°C) and subsequent cold periods (16 hours at 4°C) is shown in Figure 1b. The cloudiness stability in the enzyme-treated samples, present from 0 day on, differed significantly according to the enzymatic treatment ($P<0.05$) applied, and thus showing a comparable trend and intensity to those observed for immediate turbidity (Figure 1a). Although potential turbidity in all samples increased over time during cold storage, the lowest NTU levels were observed in the samples treated with a combination of pectinase and proteases (AB_1, AB_2 and AB_1B_2), thus confirming that the latter were less susceptible to heat-induced haze formation than the control juice and other treated samples.

Therefore, these experimental data show that even a single pectinase treatment has a significant clarification effect on pomegranate juice, which notably increases if pectinase is combined with protease.

Data relative to pectin, protein and total phenol amounts immediately after enzymatic treatment on pomegranate juice and during the following 14^{th} day of cold storage show that pectinases and proteases most likely results in differences in size, shape and consequently in the reactivity of turbidity causing molecules, such as pectins, proteins and phenolics but not in their total amount.

Significant reductions or increases in the levels were not observed during the 14 days of storage, in which, pectin, protein and phenol total contents ranged from 867 to 1238 M, 0.63 to 0.81 g/L (as BSA equivalent) and 2.4 to 2.7 g/L (as gallic acid equivalent), respectively. However the enzymatic treatment affected the haze forming activity of turbidity-causing molecules like protein and phenol, estimated by measuring the cloudiness caused by adding gelatin and tannic acid (data not shown). A comparable effect was observed for cherry juice in the study carried out by Pinelo *et al*. 2010, in which the measured levels of total phenols, total pectin and total proteins were similar in the control and in the experimental enzyme- treated juices.

3.2 Optimization of pectinase and protease clarification treatment of pomegranate juice.

Firstly, in order to obtain the optimal ratio of protease and pectinase in the complex enzyme solution an orthogonal test design $L9(3)^4$ was applied. Analysis revealed that pectinolytic enzyme plays the most important role in the turbidity level and the optimal enzyme ratio, protease and pectinase, to obtain the lowest value of turbidity was 1:2.

Moreover, RSM study was employed for optimizing the enzymatic process.Significant regression models describing the change of chill haze, turbidity, potential turbidity and clarity with respect to independent variables were established, with R^2 coefficient greater than 0.9 and *adj* R^2 greater than 0.8. The results indicated that the complex enzyme amount was the most important factor influencing the characteristic of pomegranate juice as it exerted a significant influence on all the dependent variables. Moreover, it was found that an increase of protease-pectinase complex enzyme amount has a direct influence on haze forming activity of protein and phenols. 0.25% of enzyme mixture was the best concentration, reducing the haze forming activity of proteins almost totally at 1 day. Using the contour plots (Figure 2), the optimum set of the operating conditions are graphically obtained: temperature 25–30 °C for 100-110 min using 0.22-0.25% (v/v) of protease-pectinase complex enzyme amount (the ratio of protease:pectinase was 1:2).

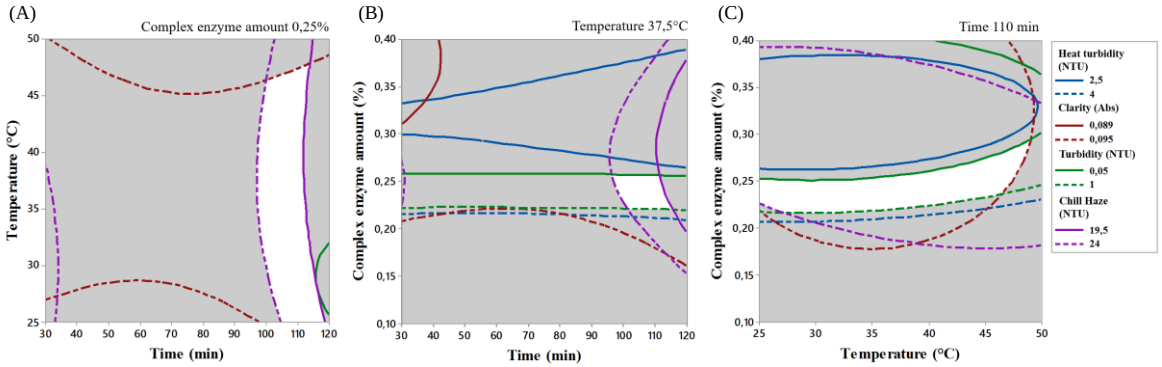


Figure 2: *The optimum region by overlaying contour plots of the four responses evaluated (chill haze, turbidity, heat turbidity, clarity) as a function of incubation time and temperature (A), incubation time and complex enzyme amount (B), incubation temperature and complex enzyme amount (C).*

3.3 Immobilization of pectinases into PVA gel for fruit juice application.

In this study, the use of PVA gel in the form of LentiKats® to immobilize pectinase was investigated. The six commercial enzymes, both in free and immobilized form, were clearly different with respect to the pectinase activity profiles ($P<0.05$). Panzym Smash XXL and Panzym YieldMASH showed the highest PL and PG activity, respectively, in free and also in immobilization form. In SDS-PAGE profile, a dominant band was observed in Panzym YieldMASH and Panzym Smash XXL, which indicated a mono-component enzyme in both commercial preparations. The immobilization procedure affected both enzymatic activities, showing lower values with respect to the free enzymes.

Enzyme	Type of enzyme	PG activity (U/ml)	PL activity (U/ml)
Pectinex Ultra Color	Free	$21.44 \times 10^3 \pm 144^{b}$	388 ± 20^{d}
	Immobilized	75.12 ± 3.21	0.47 ± 0.01^{BC}
Pectinex Be XXL	Free	$9.51 \times 10^3 \pm 402^{d}$	827 ± 32^{b}
	Immobilized	35.60 ± 0.24^{D}	0.58 ± 0.01^{B}
Klerzyme 150	Free	$20.33 \times 10^3 \pm 575^{b}$	359 ± 13^{d}
	Immobilized	65.04 ± 0.60^{B}	0.25 ± 0.02^{D}
Rapidase C80	Free	$13.08 \times 10^3 \pm 473^{c}$	553 ± 8^{c}
	Immobilized	52.14 ± 3.52^{C}	0.35 ± 0.04^{CD}
Panzym Smash XXL	Free	0 ± 0^{e}	1256 ± 2^{a}
	Immobilized	0 ± 0^{E}	2.75 ± 0.1^{A}
Panzym YieldMASH	Free	$62.10 \times 10^3 \pm 1293^{a}$	0 ± 0^{e}
	Immobilized	214.89 ± 4.41	0 ± 0^{E}

Table 1: *Polygalacturonase (PG) and pectin lyase (PL) activities of free and immobilization pectinases at 40°C in 0.1 M acetic-acetate (pH 4.2 or 6.0, respectively), containing 1% (w/v) of substrate (polygalacturonic acid or pectin). Different capital letters (Immobilized enzymes) or small letters (free enzymes) indicate significantly differences ($P<0.05$) according Tukey's posthoc test.*

According to these data, all the following experiments were performed with Panzym YieldMASH and Panzym Smash XXL, the two mono-component enzymes that exhibited maximum PG and PL activity in immobilization forms, respectively. Moreover, the modulation of enzyme properties by immobilization needs to be studied using pure enzymes, to precisely understand the occurring phenomena.

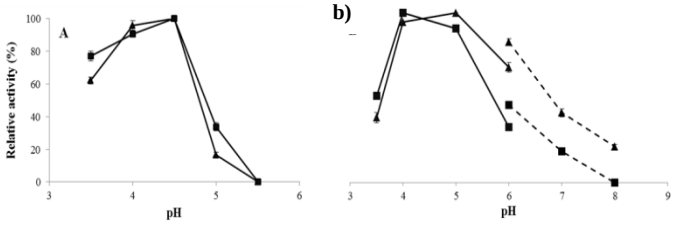


Figure 3: *pH profile of free (▲) and immobilization (□) pectinases. Effect of pH on Panzym YieldMASH (a) and Panzym Smash XXL (b) activity. Buffer system: acetic-acetate in a continuous line; phosphate in a dotted line. Values are given as the mean of three experiments ± standard deviation.*

A good adaptability of immobilization PG to an acidic solution as fruit juice was showed: at pH 3.5, the immobilization form preserved 77% of its initial activity, whereas the free form retained 62% of its original activity (Figure 3a). The pH profiles of the free and immobilization PL are plotted in Figure 3b. The optimum pH of immobilization enzyme was shifted to 4, one unit lower than that of the free enzyme and, in particular at pH 3.5, that corresponds to the pH of acidic fruit juice like citrus and apple juice, the immobilized biocatalyst retained 51% of its initial activity, whereas the free form retained 40% of its original activity.

Despite several recent studies about pectolytic enzymes immobilization, the performance of immobilized biocatalyst on real matrix, like fruit juice, has been tested in only few cases and has been measured using different evaluation methods (Diano *et al*. 2008; Esawy *et al*. 2013). This makes a direct comparison of the results more difficult.

Figure 4 shows the effect of enzymatic hydrolysis on the decrement of turbidity due to the flocculation of the pectin-protein complex in apple and pomegranate.

The best result was obtained using polygalacturonase enzyme, Panzym YieldMASH, in apple juice, with about 80% of turbidity reduction in three following cycles.

Instead, the enzymatic treatment with immobilization Panzym Smash XXL showed a lower level of clarification in both juice samples, however, also in the free form. The decrease in turbidity level indicates that only the polygalacturonase in immobilized form may destabilize the colloidal system present in the raw apple juice via pectin depolymerisation.

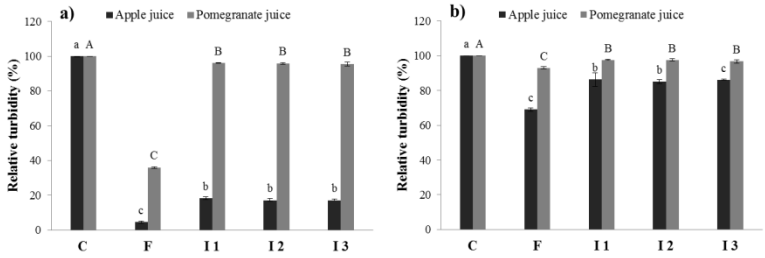


Figure 4: *Effect of immobilization and free Panzym YieldMASH (a) and Panzym Smash XXL (b) treatment on apple and pomegranate juice turbidity added with pectin at 40°C for 120 min. C: control without enzymes; F: free enzymes; I_n: immobilization enzymes (n, cycle number).*

4. Conclusions

Juice clarification strategies have been developed in order to optimize the visual appearance of fruit juices which strongly affects the consumer behaviour. The experience done with this PhD project is an effort to enhance the colloidal stability and to improve the overall quality of fruit juices using tailored enzymatic treatment in free and immobilized form. In particular, the effect of pectinolytic and/or proteolytic clarification treatment on turbidity and on haze-active molecules in pomegranate juice was evaluated. A significant and synergic effect of the combined use of pectinase and protease enzymes were thus demonstrated and the best results in terms of turbidity levels and potential haze formation of the juices were consequently obtained. Moreover, the optimum conditions of the clarification process were achieved using response surface methodology, a mathematical and statistical tool widely used for developing, improving and optimizing food industry processes. The recommended enzymatic treatment conditions, graphically obtained using the contour plots, was: temperature 25–30 °C for 100-110 min using 0.22-0.25% (v/v) of protease-pectinase complex enzyme amount (the ratio of protease : pectinase was 1:2). Pectinases enhance the clarification of various fruit juices, immobilization of these enzymes provides several advantages for the food processing industry. For this reason, the use of PVA gel in the form of LentiKats® to immobilize pectinase for its application in the industrial fruit juice clarification process has been investigated. Despite the scarce immobilization yields, the immobilized enzymes revealed a good adaptability to an acidic solution like fruit juice, and exhibited good reusability for pectin hydrolysis in apple juice. The best result in term of turbidity reduction was obtained in apple juice by immobilized Panzym YieldMASH (about 80%), and the same % of turbidity reduction was successfully retained in the two following uses. The results of the present work can be used

towards a rational selection and an optimization of novel clarification treatments in large-scale juice processing development.

INDEX

INDEX OF FIGURES

INDEX OF TABLES

1. GENERAL CONTEXT OF RESEARCH AND STATE OF THE ART

1.1 Sources of haze in beverages

Turbidity could be considered desirable or undesirable, depending on whether the resulting visual appearance of the final product fits well within consumer expectations. For example, a hazy apple juice characterized by stable cloud particles was produced and marketed in Canada by Sun-Rype Products Ltd. of Kelowna, to an enthusiastic marketplace. This product is no longer produced by this company, but recently turbid juices have been produced for the Japanese market, where they are well-liked and popular (Beveridge 1997).

It is well known that clarified apple juice is the predominant style on the market and the appearance of haze in this type of juices is perceived by consumers as a sign of inferior quality or spoilage. Thus, one of the major problems encountered in the preparation of industrially processed fruit based beverages, including fruit juices and wines, except for citrus juices since they are mostly consumed and appreciated as a cloudy drink, is the undesirable turbidity of the final product and after-bottling haze formation. Haze occurring in packaged and bottled juice during distribution or retailing is an expensive problem for the industry, because the haze appearance is often unpredictable and unexpected.

Visual perception of turbidity and haze in juice, but also in wine and beer is the result of light scattering caused by suspended particles (Siebert, 2006). Opalescence and sediments in beverages usually involve macromolecule components such as starch, proteins, polyphenols, pectins and a number of inorganic constituents such as copper and iron. If these complexes reach a level that they become insoluble, they can cause scatter light (Figure 1.1), thus as a result an increase in turbidity can be seen (Siebert *et al*. 1996).

Figure 1.1: Cloudy (left side) and clarified (right side) fruit juice.

Many different sources of hazes in beverages have been described, that include both inorganic and organic matter. Among organic haze, oxalate and tartrate were found to be the main causes of haze in wine and beer (Siebert 2006). Moreover, a number of inorganic constituents such as Cu2+ and Fe2+, as well as other ions Ca2+, Mg2+, K+ have been reported in juice sediments. In particular, metal ions such as Fe2+ and Cu2+ catalyzed juice phenolics lead to precipitation of oxidized phenolic polymers (Beveridge 1997). Moreover, both of them have been implicated in the formation of protein haze in white wines, but their role in protein haze formation is very poorly understood (Pocock *et al*. 2007). However, the common sources of these ions are contaminations from piping, tank, and iron or copper fittings but their presence does not necessarily indict them as the haze causative agents. Some organic hazes have been associated with the growth of microorganisms, whose cells scatter light directly or through formation of particles caused by metabolic activity. In any case, the most frequent causes of haze in beverages are starch, cell wall material and protein–polyphenol complexes (Beveridge 1997; Wu and Siebert 2002; Siebert *et al*. 1996). Generally, suspension of polysaccharide particulates (pectin, cellulose, hemicellulose, starch and lignin) arising from the primary and inner cell wall may cause immediate turbidity in freshly produced juice (Sorrivas *et al*. 2006); otherwise, the turbidity development during cold storage or after reconstitution of the juice concentrate, commonly referred to as haze formation, is caused by protein-polyphenol insoluble complexes (Siebert *et al*. 1996; Siebert, 2006; Pinelo *et al*. 2010). Most haze caused by

chilling partially dissolved when the beverage is warmed at room temperature. This turbidity (chill haze) is a soluble and reversible, instead the haze that characterizes the beverages when the liquid is at room temperature is considered an irreversible turbidity.

1.1.1 Microbial hazes

Microorganisms, dead or alive, may produce haze in bottled juice. Rapid increase in haze indicated the presence of live organisms. Actively growing microorganisms can alter the character of the juice, they may lower the pH and also produce anomalous aroma. This kind of haze does not decrease when juice is warmed. Dead microorganisms indicate defective filters or contamination between the filters and the pasteurization step, instead live organisms indicate faulty pasteurization or introduction of the live organisms during

bottling or during other steps after pasteurization. Thus, the absence of microbial haze depends on good hygiene and sanitation practices.

1.1.2 Starch

Starch is a polymeric carbohydrate consisting of a large number of glucose units joined by glycosidic bonds. This biopolymer consists of two major components: amylose and amylopectin (Pérez *et al*. 2010). Amylose that builds up to 15–35% of the granules in most plants, is a primarily linear polysaccharide with α-(1–4)-linked D-glucose units. Some amylose molecules, particularly those of large molecular weight may have up to ten or more branches. Amylopectin, is a highly branched molecule, with α-(1–4)-linked D-glucose backbones and exhibits about 5% of α-(1–6)-linked branches, which have a profound effect on the physical and biological properties. Indeed, amylose and amylopectin have different properties. Amylose has a high tendency to retrograde and produce tough gels and strong films. Amylopectin, dispersed in water, is more stable and produces soft gels and weak films (Pérez *et al*. 2010). Starch is a common constituent of fruit such as apple as small spherical insoluble granules with a diameter ranging from 1 to 13 μm (Figure 1.2).

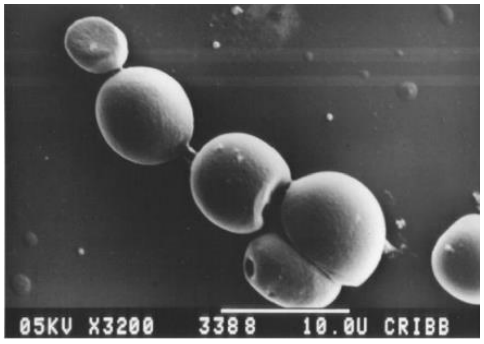


Figure 1.2: Scanning electron micrograph of isolated apple starch granules (5 kV × 3200) (Sorrivas *et al*. 2006).

These granules, when heated above 60 °C may solubilize and gelatinize, thus after heat treatment, mainly gel-like starch fragments were detected. Haze can be produced by retrogration of solubilized starch molecules (Tajchakavit *et al*. 2001). This problem normally occurs in cases where under-ripe or early harvested fruits are used; in fact apple juice prepared from early-season apples may contain up to 15% starch. Moreover, dextrins produced by natural process or by amylase enzymes to avoid starch-haze formation may

also contribute as haze precursors, to post-bottling haze formation by aggregating among themselves or through the formation of protein-starch complexes.
Indeed, it was observed that, during retrogradation, starch and dextrins can complex protein and polyphenol (Beveridge 1997).

1.1.3 Pectin substances

Pectin substances (pectins) located primarily in the middle lamella between cells are complex polysaccharides in which the backbone consists of 1,4-linked α-D-galacturonic acid residues. These substances involve protopectin, pectin polysaccharides, and the concomitant arabinans, galactans, and arabinogalactans. Protopectin is an insoluble high-molecular-weight pectin complex; together with cellulose and hemicelluloses it forms the backbone of a cell wall and, when treated with diluted acids, gives soluble pectin. Pectin polysaccharides include both components of insoluble protopectin and soluble biopolymeric constituents of plant juices. Arabinans, galactans, and arabinogalactans have, as a rule, a complex branched structure. The fine structures of distinct pectic elements and complex pectins formed from these are not fully known (Figure 1.3). It is widely believed that three to four pectic element kinds, namely, homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II) and/or xylogalacturonan (XGA) are covalently inter-linked to form a pectin-complex, but the way different blocks of these pectic polysaccharides are positioned relative to one another in such a macromolecular pectin-complex is still a matter of controversy.

Homogalacturonan (HG)

Homogalacturonan (HG), a linear homopolymer of α-1,4-linked galacturonic acid that comprises 65% of pectin, is the most abundant pectic polysaccharide (Mohnen 2008). The other pectic polysaccharides are considerably more complex in structure than HG and include the substituted HGs rhamnogalacturonan II (RG-II), xylogalacturonan (XGA), and apiogalacturonan (AP), along with the structurally more variable pectic polysaccharide rhamnogalacturonan I (RG-I).

Substituted HG: rhamnogalacturonan II (RG-II)

The most structurally complex pectin, RG-II, makes up 10% of pectin. Its structure consists of an HG backbone of at least 8 (and most probably more) 1,4-linked α-D-GalA

residues decorated with side branches consisting of 12 different types of sugars in over 20 different linkages (Mohnen 2008).

Other substituted HGs

Two other substituted galacturonans are xylogalacturonan (XGA) and apiogalacturonan (AP). XGA is an HG substituted at *O*-3 with a blinked xylose. The 3-linked xylose has occasionally been found to be further substituted at *O*-4 with an additional blinked xylose (Mohnen 2008).

Rhamnogalacturonan I (RG-I)

RG-I represents 20–35% of pectin. It contains a backbone of the disaccharide repeat [-α-D-GalA-1,2-α-L-Rha-1-4-]$_n$ and exhibits a high level of variation in the type and number of sugars, oligosaccharides, and branched oligosaccharides attached to its backbone (Mohnen 2008).

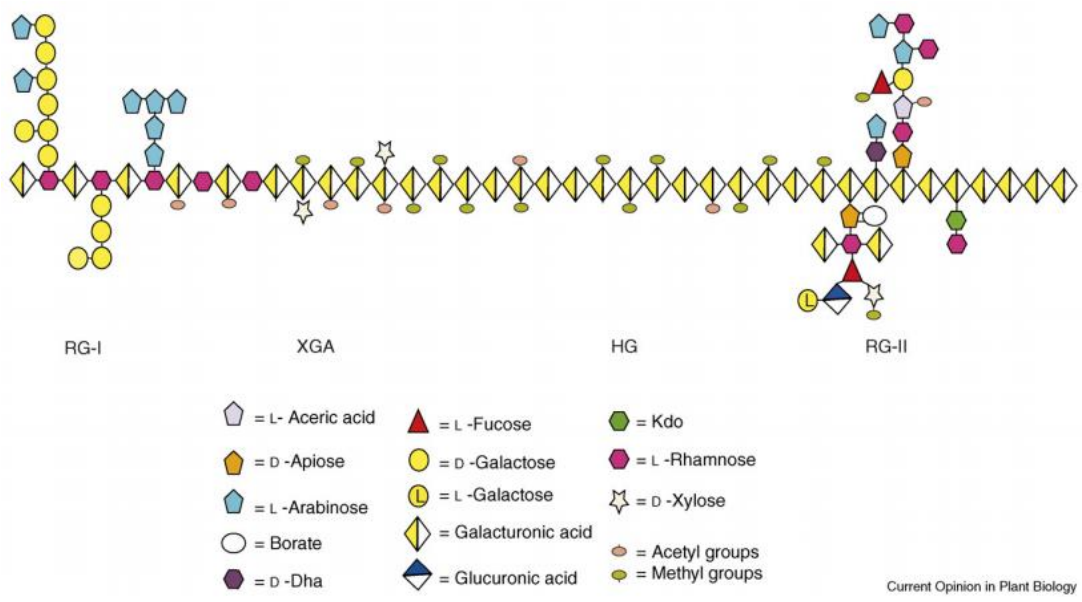


Figure 1.3: Schematic structure of pectin showing the four pectic polysaccharides homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) linked to each other (Mohnen 2008).

1.1.4 Proteins

Proteins are macromolecules consisting of one or more long chains of amino acid residues. Proteins differ from one another primarily in their sequence of amino acids, which is dictated by the nucleotide sequence of their genes, and which usually results in protein

folding into a specific three-dimensional structure that determines its activity. A linear chain of amino acid residues is called a polypeptide. A protein contains at least one long polypeptide. Short polypeptides, containing less than 20–30 residues, are rarely considered to be proteins and are commonly called peptides. Proteins are expressed ubiquitously in all living organisms. Biological structures consist mainly of structural proteins. Not surprisingly, plant cell walls, the major structural part of the plant cell, also contain structural proteins. Five plant cell wall proteins classes have been identified to date: extensins, the glycine-rich proteins (GRPs), the proline-rich proteins (PRPs), the solanaceous lectins, and the arabinogalactan proteins (AGPs). Each of them, with the exception of the GRPs, contains hydroxyproline (Showalter 1993). These proteins are not the only cell wall proteins that are known, others exist such as: cysteine-rich thionins, 28- and 70-kD water-regulated proteins, a histidine-tryptophan-rich protein, and many cell wall enzymes (i.e.peroxidases, phosphatases, invertases, glucanases, polygalacturonase, pectin methylesterases, arabinosidases, proteases).

Extensins

Extensins are a family of hydroxyproline-rich glycoproteins (HRGPs) found in the cell walls of higher plants. In dicots, extensins are particularly abundant and are generally characterized by hydroxyproline and serine and some combination of the amino acids: valine, tyrosine, lysine, and histidine.

GRPs

Glycine-rich proteins are a relatively newly discovered class of plant proteins that are characterized by their repetitive primary structure, which contains up to 70% glycine arranged in short amino acid repeat units.

PRPs

Proline-rich proteins represent another relatively newly identified class of plant cell wall proteins of which at least some, and perhaps all members contain hydroxyproline. There are at least two broad subclasses of PRPs: those that are components of normal plant cell walls and those that are plant nodulins (i.e., proteins produced in response to infection by nitrogen-fixing bacteria) and constitute part of the nodule cell wall. All of the PRPs are characterized by the repeating occurrence of Pro-Pro repeats that are contained within a

variety of other larger repeat units. For example, many of the normal cell wall PRPs and some nodulin PRPs, are characterized by the presence of the repeating sequence Pro-Pro-X-Y-Lys, where X and Y can be valine, tyrosine, histidine, and glutamic acid.

Solenaceous lectins

Lectins are carbohydrate binding proteins or glycoproteins of diverse origin. The solanaceous lectins represent a unique class of plant lectins that can be distinguished from other lectins by their restricted occurrence in solanaceous plants, their predominantly extracellular location, and their unusual amino acid and carbohydrate composition in which hydroxyproline and arabinose are major constituents.

Arabinogalactan proteins (AGPs)

AGPs are HRGPs are generally very soluble and highly glycosylated. AGPs are widely distributed in plants and typically comprise only 2 to 10% protein by weight. Their molecular weights are extremely heterogeneous, presumably reflecting different extents of glycosylation. AGPs are readily solubilized during tissue extraction with low ionic strength aqueous solutions. These solubility properties have greatly facilitated their isolation and characterization. The protein moiety of AGPs is typically rich in hydroxyproline, serine, alanine, threonine, and glycine and is resistant to proteolysis in its native state, a property that is presumably conferred by extensive glycosylation.

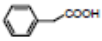
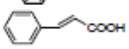
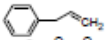
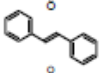
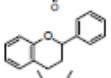
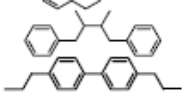
Protein - tannin interaction are the most frequent cause of haze in juice after the bottling. The principal cause of this haze is the introduction of proteins during some steps of the juice processing. Proteins that have a high affinity for binding polyphenols have been shown to contain proline and the relative haze-forming activity of a protein has been shown to be largely a function of the mol% proline content (Siebert, 1999). Presumably the prolines are involved in the sites where the polyphenols attach to the proteins. Proline prevents the formation of an alpha helix and favors a more open protein structure; this likely facilitates access by polyphenols, but proline is also involved in the binding sites. For instance, the proteins in beer that are haze active (HA) have been shown to originate in the barley hordein (Asano *et al*. 1982). Hordein is a prolamin, proline-rich proteins of grains, and contains approximately 20 mol% proline. Gliadin, the wheat prolamin, is also HA. Fruit proteins associated with haze formation have also been shown to contain proline, although not as much as the grain HA proteins. An HA protein isolated from apple juice

was shown to contain 5% proline (Wu and Siebert, 2002). A grape seed protein that formed haze had 9.5% proline (Wu and Lu, 2004).

1.1.5 Polyphenol

Phenolic compounds are secondary products which possess an aromatic ring bearing a hydroxyl substituent and most are of plant origin. Plant phenolics are a chemically heterogeneous group: some are soluble only in organic solvents, some are water-soluble carboxylic acids and glycosides. Another group of phenolics are insoluble polymers. Phenolics play a variety of important roles in the plant. A majority of phenolic substances have important effects on defence against herbivores and pathogens. Others play some role in mechanical support, in attracting pollinators and in reducing the growth of nearby competing plants (Özeker 1999). The term "polyphenol" includes more than 8,000 compounds with great structural diversity (Garcia-Salas *et al*. 2010). They can be divided into 10 different classes depending on their basic chemical structure. Table 1.1 shows the main families of phenolic compounds, most of which are found in nature associated with mono- or polysaccharides (glycosides) or functional derivatives such as esters or methyl esters (Garcia-Salas *et al*. 2010).

Table 1.1: Classification of families of phenolic compounds (Garcia-Salas *et al*. 2010).

Carbon numbers	*Class*	*Basic structure*	*Sources*
C_6	Simple phenols		
	Benzoquinones		
C_6-C_1	Benzoic acid		Cranberry, cereals
C_6-C_2	Acetophenones		Apple, apricot, banana, cauliflower
	Phenylacetic acid		
C_6-C_3	Cinnamic acid		Carrot, citrus, tomato, spinach, peaches, cereal, pears, eggplant
	Phenylpropene		
	Coumarins		Carrot, celery, citrus, parsley
	Chromones		
C_6-C_4	Naphthoquinones		Nuts
C_6-C_1-C_6	Xanthones		Mango, Mangosteen
C_6-C_2-C_6	Stilbenes		Grapes
	Anthraquinones		
C_6-C_3-C_6	Flavonoids		Widely distributed
$(C_6$-$C_3)_2$	Lignans, neolignans		Sesame, rye, wheat, flax
$(C_6$-$C_1)_n$	Hydrolysable tannins	Heterogeneous polymer composed of phenolic acids and simple sugars	Pomegranate, raspberry
$(C_6$-$C_3)_n$	Lignins	Highly crosslinked aromatic polymer	

Phenolic compounds are present in all plants so all fruit and vegetable based foods, as well as beverages (tea, wine, beer and fruit juice) contain various phenolic compound amounts (Aguilera *et al*. 2016). The most abundant polyphenols are phenolic acids (benzoic and cinnamic acids) and flavonoids. The common structure of flavonoids consists of two aromatic rings linked by three carbons that usually form an oxygenated heterocycle. In plants, flavonoids can be found as aglycones, although they are usually found as glycosides contributing to the color (blue, scarlet, orange) of leaves, flowers, and fruits. Flavonoids can be subdivided in 13 classes: chalcones, dihydrochalcone, auron, flavones, flavonols, dihydroflavonol, flavanones, flavanols (catechins), flavandioles or leucoanthocyanidins, anthocyanidins (its glycoside is called anthocyanin), isoflavononas, flavonoids, and condensed tannins or proanthocyanidins (Escarpa and Gonzalez 2008; Wu and Prior 2005).

Wine, beer and fruit juices each contain a spectrum of polyphenols ranging from fairly small molecules to much larger structures (Guyot *et al*. 2003; Siebert, 2006). Of these, the flavanols (catechins and procyanidins) have the strongest association with haze-forming activity, binding to protein. These molecules are large enough to encompass two attachment sites (each an aromatic ring with at least two phenol groups). This has been demonstrated in both model systems and actual beverages. It has been shown that phenol binding to protein depends very much on the number and location of the hydroxy groups on an aromatic ring. Monophenols hardly attach to proteins, metadiphenols bind proteins weakly, ortho-diphenols bind with moderate strength and vicinal triphenols bind quite strongly (Siebert, 2006).

Removal of soluble flavanols in beverages, using fining agents such as gelatin, PVPP, although it could produce an acceptable clarified and stabilized juice, causes flavour and color changes and leads to a substantial loss of phenolic compounds and antioxidant activity (Erkan- Koç 2015; Alper *et al*. 2011).

1.2 Haze and precipitate formation

Raw juice obtained after pressing is generally turbid, viscous and tends to settle during storage. This primary turbidity in fresh fruit juice is caused mainly by polysaccharides such as pectin, cellulose, and starch stemming from the plant cell walls. The production of clear fruit juice requires firstly, the removal of this suspended material and secondly, the prevention of turbidity developed after bottling the juice. This latter, usually referred to as haze formation, is assumed to be caused by interaction between haze active proteins and polyphenols that form insoluble multi-molecular structures. Moreover, pectin could form a protective coat around suspended proteins, thus forming and stabilizing cloud particles.

In beverages initially free of turbidity, the tendency of pectin, starch, protein and polyphenols to aggregate, results in particle formation. In well-filtered or centrifuged juice the particles are so small with a diameter less than 0.1 μm, they do not cause significant haze. Over time, the molecules aggregate and get larger and larger. They may reach 0.3-1 μm diameter, thus scatter light and develop maximum haze (Figure 1.4). If the particles grow still larger, they may settle down more rapidly and leave a clear supernatant liquid. After particles settling, haze can reform in the supernatant juice, if some haze precursors remain in suspension.

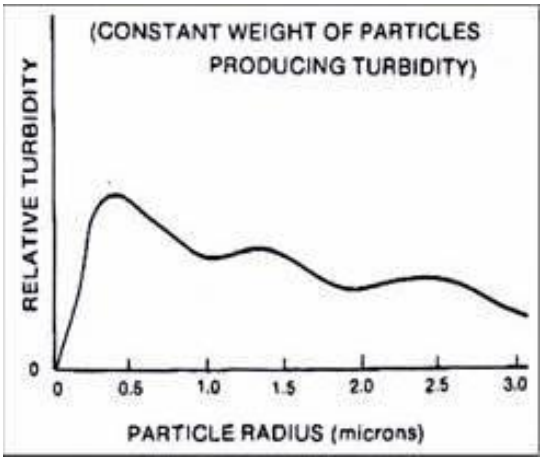


Figure 1.4: Relationship of particle size to relative turbidity (Van Buren 1989).

Precipitation generally takes place with particles above 0.5 mm in diameter. The smaller the particle, the slower the settling. The collisions of molecules are important in slowing or preventing the settling of finer particles, in particular at a higher storage temperature. Haze

can be persistent if particles are too small to settle at an appreciable rate. The aggregation of cloud particles and consequently the increase in the size occur through both covalent bonding and electrostatics interactions. The polymerization of tannins involves covalent bonding, instead electrostatic interactions are characteristics of the increase in particle size. For example, hydrogen bonds, involving positively charged hydrogen atoms (hydroxyl group of a phenol) and negatively charged oxygen atoms (of peptide group of protein) are fundamental in the growth of tannin-protein particles. Moreover, dipole interactions are present in all molecules and play an important role in haze particle increase. The increase in size and the settling of particles are delayed when the net charge on the particles is the same and when protective colloids are present. In juices, after a little time to permit equilibration, all particles tend to have the same net electric, positive or negative charge. Since like charges repel each other, these net charges normally delay aggregation of particles. Protective colloids coating the particles slow aggregation. Usually, they prevent the close approach of one to another needed for the formation of hydrogen bonds or dipole interactions. In addition, they may give particles a large enough charge that significant electrostatic repulsion takes place between particles. Pectin is a protective colloid and it is widely present in fresh juice (Figure 1.5), thus preventing the aggregation and precipitation of small insoluble particles. Instead, in clear juice, the presence of pectin can retard haze development.

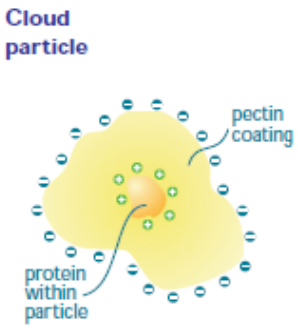


Figure 1.5: Cloud particle, pectin coat the protein core.

In clarified juice, where proteins and polyphenols can gradually form insoluble complexes, haze formation commonly occurs and the sediment is usually dark and brown in color (Figure 1.6). Analysis of sediments formed indicated the involvement of catechins and procyanidins that were suggested to be the precursors of phenolic haze formation. Oxidation of these phenolic precursors results in high molecular weight polymeric form.

Continued oxidation results in further polymerization of these molecules, resulting in particles large enough to be perceived as haze. Protein may be incorporated into these oxidizing complexes by covalent attachment or by hydrophobic interaction. It was demonstrated that four factors influence protein–polyphenol interaction in model systems: protein concentration, polyphenol concentration, pH and alcohol content (Siebert *et al*. 1996). If protein content is kept constant and polyphenol concentration is increased, the amount of haze increases, but only to a point, then declines. Similarly, if the amount of polyphenol is held constant as the protein increases, haze increases up to a point and then declines. Moreover, the protein/polyphenol ratio affects particle size, and the largest particles were observed with intermediate protein/polyphenol ratios (Siebert and Lynn, 2000).

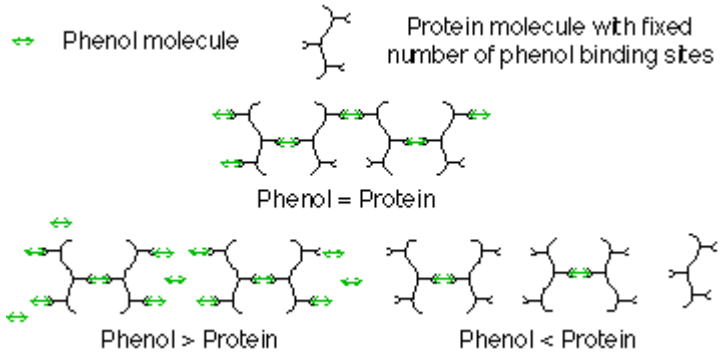


Figure 1.6: Proteins – polyphenols interaction.

pH and ethanol also influence the amount of haze. The ethanol effect was more modest, near the pH of apple juice or beer, increasing ethanol concentration which led to a slight decline in haze followed by an increase at higher ethanol levels. pH had a deep effect on haze, with the same amounts of protein and polyphenol, increasing the pH from almost 3 to slightly above 4 increasing the amount of light scattering by a factor of 7.

1.3 Turbidity test procedures

Tests on fresh juice is fundamental for assessing the suitability of the juice making process and for designating any corrective action that may be needed to improve the visual appearance of the final product. Sometimes the results could give useful information about the cause of any haze and sediments that develop during the storage period.

A numerical measure of haze could be made using a dedicated instrument, called turbidimeter and nephelometer. When particles are suspended in a solution, they make the solution unclear.

Incidental light entering the sample in a cuvette have the following three reactions;

- some of the light will be absorbed by the particles;
- some will be transmitted through the cuvette;
- some will be scattered in various directions.

Turbidimetry is involved with measuring the amount of transmitted light (and calculating the absorbed light) by particles in suspension to determine the turbidity of the sample. Measurements are made using light spectrophotometers, at wavelengths above 600 nanometers (nm), spectrophotometer is highly sensitive and minimizes interference from the normal coloration juice.

Nephelometry is concerned with measurement of scattered light from a cuvette containing suspended particles in a solution. The components of a nephelometer are the same as a light spectrophotometer except that the detector is placed at a specific angle from the incident light.

Haze can be revealed by eye, but the eye has difficulty in detecting low haze levels and in making comparisons of haze amount in various juices, at different times of storage. Instead, haze instrumental values are usually expressed as nephelos turbidity units (NTU) which are based on standard formazin suspension (Van Buren 1989).

The quantitative determination of molecules involved in haze formation (pectin, polyphenols, protein) requires sophisticated equipment and time, and the interpretation of the results is difficult due to the transformation of these components during storage and due to the various haze forming activity of these molecule classes.

As reported by Siebert and Linn (1997), most protein analysis methods do not provide information about haze formation in the product. In beer and wine, results from the

commonly used Coomassie blue dye binding method (Bradford, 1976), for example, provide no useful information about haze formation in beer (McMurrough *et al*. 1992). Dye response is strongly biased in favor of peptides rich in basic and aromatic amino acids. In apple juice that contains much less protein than beer and is high in free amino acids, the Kjeldahl method is not specific for proteins, and methods based on protein hydrolysis, followed by amino acid analysis have limited usefulness. Only polypeptides that contain proline, have been shown to cause haze (Siebert and Linn 1997).

A method for specifically measuring the amount of haze active (HA) proteins or "sensitive proteins" is based on the addition of a known amount of tannic acid (TA), which is a very HA polyphenol. After adding a fixed volume of the TA solution to the sample, the mixture is incubated. This leads to the development of haze between the TA and any protein in the sample capable of haze formation with polyphenols. The turbidity is then measured and presumed to be proportional to the amount of HA protein in the sample. For example, adding tannic acid (a gallotannin preparation) to bottled, commercial apple juices resulted in a slight increase in haze, indicating that the bottled juices had relatively little haze-active protein (Siebert *et al*. 1996).

A test to measuring the amount of HA polyphenol in a sample is based on the addition of a known amount of a haze active protein (for example gelatine) to cause haze (Siebert *et al*. 1996). This is conceptually the same as the HA protein method except that the additive is an HA protein rather than an HA polyphenol. After adding fixed volumes of gelatin solution to the samples, the mixtures were incubated. This led to the development of haze between the gelatin and any HA polyphenol. The measured turbidity indicates the relative amounts of HA polyphenols in the juice.

1.4 Prevention of haze and sediment formation in fruit juice

Except for citrus fruit juices, where the maintenance of a stable cloud is desirable, most industrially processed fruit based beverages are clarified and stabilized during processing.

The production of clear juice requires the removal of suspended material responsible for the turbidity and sediments in freshly produced juice. This step is called clarification. Other measures are often taken to remove soluble materials that have the potential to form hazes during storage, at reconstitution of the concentrate or after bottling of the juice (Tajchakavit *et al*. 2001). These measures are often called fining and they are necessary to stabilize the juice. Moreover, in some kind of juices, all these procedures led to a significant and positive reduction of bitterness and astringency (Alper and Acar 2004).

The clarification-stabilization of fruit juices can be performed using physical, chemical and/or enzymatic methods. Physical methods for the clarification act by separating the suspended molecules according to their size and molecular weight (filtration and ultrafiltration), or in the case of the pasteurization technique, it acts by destabilizing the colloidal particles in juices in order to facilitate settling (Van Buren 1989). Instead, chemical methods (gelatin, bentonite, silica sol etc.)are based primarily on the ionic charge of the turbidity causing molecules, while the enzyme clarification approach is based upon proteins and polysaccharides hydrolysis reactions or pholyphenols oxidation (Benitez and Lozano 2007; Neifar *et al*. 2011; Alper and Acar 2004).

1.4.1 Physical methods

1.4.1.1 Heat treatment

Pasteurization is a process by which foods are heated to a specific temperature for a specific amount of time to kill or deactivate a target number of potentially harmful bacteria. Many different beverage products such as milk, fruit juices, cider, and beer may be pasteurized at least one time.

In order to obtain a prolonged shelf life, the freshly squeezed juices must be pasteurized. Generally this step is performed prior to centrifugation, filtration and any clarification treatments and it is carried out at temperatures between 62 °C and 85 °C for a time which can vary from a few seconds to several minutes.

Two common types of pasteurization techniques used for pasteurizing beverages are:

- Low Temperature Long Time (LTLT) LTLT involves heating to 62.5ºC and holding this for 30 minutes,

- High Temperature Short Time (HTST). HTST involves heating to 72ºC for 15 seconds.

HTST also called flash pasteurization is the commonly method of heat pasteurization of perishable beverages like fruit and vegetable juices, beer, kosher wine.

Pasteurization in fruit juices, as in other drinks, generally does not alter the physical-chemical properties or the sensory quality of the product, if properly conducted. However, in some cases, it is possible to note a partial darkening of the beverage (post-pasteurization), due to the development of interactions between sugars and amino acids present in solution (Maillard reaction) (Jaeger *et al*. 2010).

Regarding clarification treatment, each heat treatment has the purpose to facilitate the coagulation of proteins, so that they can be removed more easily during the following filtration process (Yi *et al*. 2013). Various combinations of time and temperature need to be explored in order to arrive at a set of conditions that will give the desired coagulation. Incorrect heat treatments could led to a persistent fine haze. Heat treatment should be used after removal of starch granules; otherwise an amylase treatment will be needed to digest solubilized starch.

1.4.1.2 Centrifugation

The purpose of centrifugation is to separate insoluble particulates based on their density by a centrifugal force, generated by high speed rotations. This method of clarification can easily be applied to fruit juices, allowing the separation of insoluble molecules, such as large protein complexes-polyphenols-polysaccharides, from liquid. In the last 15 years, the growing uses for centrifuges industrially has resulted in a plethora of special centrifuges designed and adapted to particular uses. However, centrifuges fall into two general

classifications, termed sedimentation centrifuges and filter centrifuges. In sedimentation centrifuges, solids are transported to the periphery wall of the rotating machine bowl and collected against this surface; liquid is removed from the solids by the close packing of their individual particulates. In filter centrifuges the solids are transported to the surface of a filter element and the solids trapped on this filter, while the liquid drains through the particulates and exits through the filter surface (Beveridge 2000). The stream should be settled or coarse strained prior to centrifugation in order to reduce the sludge load in the feed going to the centrifuge (Bates 2001). The clarification by centrifugation is a highly efficient method to reduce the immediate turbidity levels. As reported by Meyer *et al*. (2001), this step induced significantly decreased turbidity with increasing centrifugation speed (g). However the centrifugation does not stabilize the colloidal fraction of juices, which can cause new turbidity and opalescence during storage time.

1.4.1.3 Filtration of juice

The purpose of filtration is to remove solid particles from a liquid sample.

In fruit juice processing, like apple juice processing, the largest particles are removed by passing the juice from the press through a mesh screen or a continuous self-cleaning centrifuge. This step is sufficient for an unclarified juice product. In order to obtain a completely clear product, juice must go through a second mechanical step, also known as filtration. However, filtration can produce a clear juice, but the final product may show again a little tendency to become turbid.

There are many filtration systems well suited to various juices. These range from plate and frame filters, fitted with porous cellulose pads, to plastic, ceramic, or metal membranes. Diatomaceous earth mixed with the liquid serves to greatly increase the surface area and porosity of the filter bed and hence the particulate absorbing capacity of the filter. In

extreme cases, a filter can have small enough pores to physically remove microorganisms from the juice (sterile filtration) or even remove macromolecules such as proteins and carbohydrate polymers (ultrafiltration- UF).

The system can be made continuous by a rotary vacuum filter. The juice passes into the rotating drum through the filter bed that is constantly renewed by scraping the spent material off the surface after a rotation. Various other filters use durable ceramic or metal porous membranes with backflush capabilities and flow patterns that minimize clogging.

These systems operate in parallel to ensure continuous operation and can reduce or eliminate the need for filter aids; a purchase and disposal expense.

Recent advances have been shown regarding membrane technology. In the fruit juice industry, this technology is used mainly to clarify the juice by means of ultrafiltration and microfiltration. Both processes occur under pressure: in the case of ultrafiltration (UF) membrane pores can be between 1-100 μm, while in the microfiltration pore sizes ranging from 0.1-10 μm. Nowadays, UF is commonly used to stabilize fruit juice, but the disadvantage is that ultrafiltered juices are not always stable, but rather tend to produce pronounced subsequent haze caused by reactive phenolic compounds that cannot be retained by the UF membrane (Stutz 1993). Recent research reported that hyperoxidation of raw juice with laccase prior to UF, as an alternative to treatment with physical-chemical adsorbents, could effectively clarify and stabilize the fruit juice. However this technique decreases the total phenolics of about 50% and the antioxidant capacity of about 20% (Neifar *et al*. 2011)

1.4.2 Chemical methods

Chemical methods are based mainly on the use of fining agents, they work on the principle that all of the particles responsible for the clouding or haze in beverages have an electrical charge. As an example, gelatin has a positive charge meaning that it can attract negatively charged materials. In binding to the negatively charged materials the combined weight increases resulting in settling to occur. In practice it is necessary to have fining agents of different charges added sequentially to the beverage in order to remove the materials of various charges contained in the liquid sample.

As shown below there are a lot of materials that can be used to clarify a fruit juice.

1.4.2.1 Bentonite

Bentonite is a clay mineral of the montmorillonite type. It consists of tiny platelets that carry a negative charge. This colloidal clay has a high affinity to bind with proteins.

Proteins form a coagulum with bentonite that settles more rapidly than bentonite alone. Such coagulums can also entrap particles in suspension thus reducing the haze level.

Bentonite is widely used in the wine industry, to remove undesirable proteins from white wine. The use of bentonite in beverages shows a disadvantage, the formation of rather

bulky lees. Bentonite is usually prepared for use as a thick 4-5% suspension, in which the platelets are well hydrated and separated (Van Buren, 1989).

1.4.2.2 Gelatin

Gelatin is a soluble protein that is derived from the collagen of animal skins. Treatment with gelatin is commonly used for clarification of juice. There are two basic types, depending on the method of manufacturing; one is produced by acid digestion of collagen (type A), the other type, type B, results from an alkaline digestion of collagen. Both types are available as products with a wide range of molecular weights depending on the extent of chemical digestion of the collagen. The gelatin is allowed to swell in cold water or juice for several hours, then vigorously stirred while the temperature is brought to near the boiling temperature. The solution should be used soon thereafter. Holding it for several days at 5 °C may be possible but then it should rewarmed to 95 °C before using in juice. This is necessary because the molecules of gelatin tend to aggregate with each other during storage, and when added to the juice, the gelatin should be in the nonaggregated form (Van Buren, 1989). Gelatin has a partially negative charge at acid pH, like fruit juice, and it is able to bind positive haze active molecules like polyphenols and also some proteins. In juice, these molecules form a colloidal suspension in which the dispersed phase is so small (~1-1000nm) that gravitational forces are negligible and interactions are dominated by short-range forces, such as van der Waals attraction and surface charges. Gelatin as well as bentonite work either by sticking to the particles, or by using charged ions to cause particles to stick to each other, in any case making them heavy enough to sink to the bottom by the action of gravity (Figure 1.7) (Benitez and Lonzano 2007).

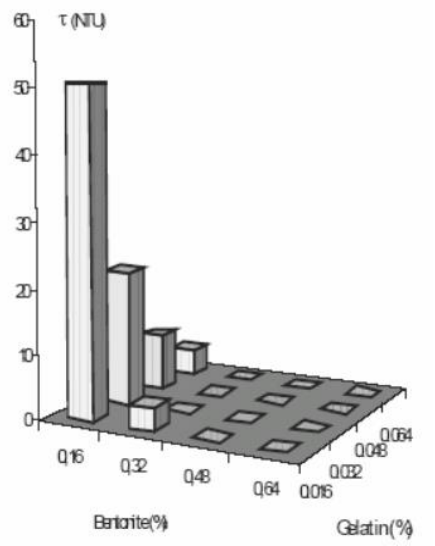


Figure 1.7: Effect of bentonite and gelatin on the turbidity of a modelled cloudy juice (juice clarified by ultrafiltration, with a known fraction of particles) (Benitez and Lozano 2007).

1.4.2.3 Polyvinylpyrrolidon

Polyvinylpyrrolidon (PVP) is a polymer which consists of vinylpyrrolidon units with a molecular weight ranging above 10,000 daltons. It is widely used as a fining agent. PVPP is a highly cross-linked version of PVP, making it insoluble in water, though it still absorbs water and swells very rapidly generating a swelling force. The insoluble form can be used to remove polyphenols from beverages. The fining agent after treatment could be completely removed by filtration. In beer brewing, where proteins are considered fundamental to give foam stability, for example, the removal of polyphenols is the best choice for avoiding the development of haze in beer (Mitchell *et al*. 2005). One such commercial product is called Polyclar and it can be used to remove polyphenol from beverages. Polyclar preferentially absorbs smaller polyphenols, whereas gelatin binds larger polyphenol better. It acts by preferentially complexing haze-active polyphenol through the mechanism of hydrogen bonding. In its chemical structure, it is similar to amino acid proline, which explains its high affinity and selectivity for haze polyphenols (Rehmanji *et al*. 2002).

1.4.2.4 Silica Sols

Silica sols, composed of specially prepared colloidal silicon dioxide (SiO_2) particles, is another fining agent that absorb proteins. Silica sol and protein if present in suitable

proportion, form a light curdy coagulum. Usually, companies supply the sols as aqueous suspensions, that contain about 30% solids. Various types are available, differing in the sizes of the silicon dioxide particles. In beverages, particle sizes around 20 nm showed the high efficiency for removing proteins. The small size of silicon dioxide particles makes them difficult to remove by filtration. Frequently, a small amount of gelatin should be used in order to completely remove the silica gel from the juice, improving the flocculation (Oszmiański and Wojdyło 2007).

1.4.2.5 Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is made by treating the chitin shells of shrimp and other crustaceans with an alkaline substance, such as sodium hydroxide (Figure 1.8).

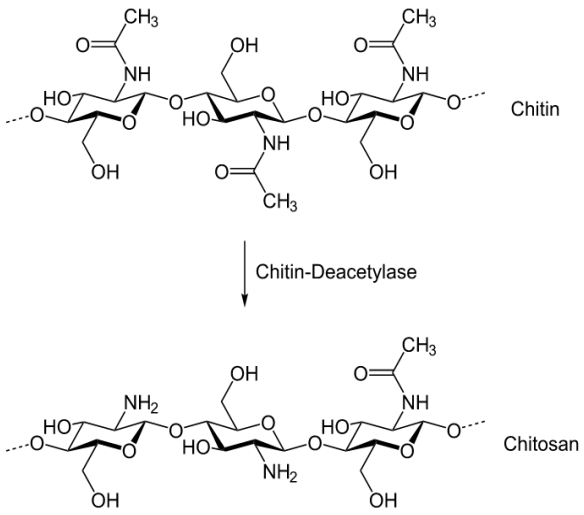


Figure 1.8: Forming chitosan by partial deacetylation of chitin.

The amine-groups of D-units has a rather low pKa-value of 6.5, meaning that the amine-groups are predominantly positively charged at pH-values below 6.5. The cationic nature of chitosan led to interactions with the negatively charged molecules in solutions. Deacetylated chitin, being polycationic in nature, nontoxic and biodegradable, has been found to be an effective coagulating agent in aiding the separation of suspended particles from beverages (Domingues *et al*. 2012). Several works have reported the successful

application of chitosan as a clarifying aid for apple (Chatterjee *et al*. 2004; Rungsardthong 2006), grape, lemon, orange (Chatterjee *et al*. 2004), pomegranate juice (Tastan and Baysal 2015), besides wine (Spagna *et al*. 2006) and green tea (Rao *et al*. 2011). Moreover, Domingues *et al*. (2012) recently studied the application of chitosan in tropical fruit juice clarification such as passion fruit juice, verifying that coagulation with chitosan is a suitable process to reduce passion fruit turbidity. Moreover chitosan is able to reduce passion fruit juice viscosity at the same level as enzymatic treatment.

1.4.3 Enzyme technology

Any constituent of fruit cell walls has been widely present in the fresh juice, soluble pectin, cellulose, non-cellulosic polysaccharides and other polysaccharides and proteins. All these cell wall components are degraded during fruit ripening by endogenous enzymes in the fruit itself. Actually, through the progress in biochemistry, many microorganisms produce enzymes that can degrade fruit cell walls. Only these enzymes, from food grade microorganisms, may be used to produce fruit juice. These enzymes are exocellular and generally produced by fermentation in surface and submerged cultures (Grassin and Fauquembergue 2002). Commercial preparations contain several different enzymes of which pectinases are usually the main activities; however numerous interesting side activities are naturally present or specially selected. Commercial enzymes are new tools used to clarify and stabilize fruit juices bringing a range of potential benefits to both producers and consumers. They are used to help extract, clarify, modify juices from many crops including berries, stone and citrus fruit, grape, apple, pear and even vegetable juices (Park and Kim 2009; Landbo and Meyer 2004; Pinelo *et al*. 2010). The enzyme clarification treatment is based on hydrolysis (Sorrivas *et al*. 2006) or oxidation (Neifar *et al*. 2011; Alper and Acar 2004) reactions of all turbidity causing particles that naturally occur in fruit juice such as polysaccharides (pectin, cellulose, hemicelluloses, lignin and starch), proteins and tannins (Vaillant *et al*. 2001).The addition of exogenous enzymes allows more specific action on colloids and at the same time preserves aroma, vitamins, pigment and color (Sandri *et al*. 2011).

Clarification technology using pectinases, in conjunction with amylase, proteinases and several other complementary enzymes is explained from viscosity reduction prior to

concentration of stable turbid juices, right through to the stabilization of clarified juices to prevent haze formation and sedimentation in the finished product (Baumann 1981).

The most widely and commercially used enzymes available to clarifying juices are pectinases. The high concentration of pectin leads to colloid formation, which constitutes one of the main problems during the processing of clear fruit juices. However, although the suspended pulp particles can be removed through filtration, the presence of pectin may make this method difficult (Sulaiman *et al*. 1998). Indeed industrial juice clarification procedures typically involve enzyme catalyzed depectinization by addition of pectinases to accomplish pectin degradation and subsequent physical-chemical precipitation of sediments and haze-active components. The depectinisation of fruit juices through the use of pectinases has been presented as an efficient alternative to reduce turbidity, in many studies (Kashyap *et al*. 2001; Landbo and Meyer 2004; Vaillant *et al*. 1999). Pectinases degrade pectin hence resulting in viscosity reduction and cluster formation, which facilitates separation through centrifugation or filtration. As a result, the juice presents higher clarity, as well as more concentrated flavour and color (Abdullah *et al.* 2007; Kaur *et al*. 2004; Mutlu *et al*. 1999). However, the successful use of pectinolytic enzymes in fruit juice clarification depends on the involved substrate, which may not only present different pectin concentrations, but also may include other substances such as cellulose, hemicelluloses, lignin and other components (Vaillant *et al*.1999). Thus the combined use of other enzyme activities could enhance the clarifying effect.

The efficiency of protease, another enzyme type, was extensively demonstrate to stabilize and eliminate post-bottling haze. Acid proteases extensively hydrolyze proline-rich proteins, yielding a peptide fraction that is unable to form a haze. Despite pectolytic enzyme are commonly used by brewers to chill-proof beer, only recent researches has demonstrated their efficiency in the fruit juice industry (Pinelo *et al*. 2010; Landbo *et al*, 2006).

The application of enzymes in fruit juice technology will be discussed in detail in the next chapter.

1.5 Application of enzymes in fruit juice technology

Commercial enzyme preparations are currently used as processing aids in fruit (and vegetable) juice processing to improve the process efficiency (such as juicing and clarification) and product quality, because enzymes show activity on specific substrates under mild processing conditions. Therefore, there has been a striking growth in the enzyme market for the fruit and vegetable industry.

In fruit juice processing, the enzymes used are mainly pectinase, cellulase, hemicellase etc. These enzymes facilitate maceration, liquefaction, and clarification during fruit juice processing with the benefit of reducing processing costs and improving yields. Thus, the quality of juices manufactured and their stability have been enhanced through the use of enzymes. For example, enzyme addition increases the release of various phenolics and other nutritionally important components in the juice. Juice appearance is also improved by enzymatic clarification. Juice quality in terms of reduced viscosity, decreased turbidity, and improved filterability is also improved. Enzymes are also used for debittering of citrus fruit juices and in preventing darkening of juices.

Enzymes as safe processing aids can be obtained mainly from pure cultures of selected strains of food-grade microorganisms. Enzymes may be discovered by screening microorganisms sampled from different environments or developed by modification of known enzymes using modern methods of protein engineering or molecular evolution. Many commercial enzymes used in fruit juice processing are exocellular enzymes that come from selected strains of *Aspergillus niger* or *Trichoderma* spp produced by fermentation.

An enzyme preparation typically contains the enzyme of interest and several added substances such as diluents, preservatives, and stabilizers. Enzyme preparations may also contain other enzyme activities and metabolites from the production organism and the residues of raw materials used in fermentation media. All these materials are expected to be of appropriate purity consistent with current good manufacturing practice.

1.5.1 Nature of enzymes

Proteins are the most versatile molecules in the biological world meant for several functions including structural organization and catalytic capabilities (Srilakshmi *et al*. 2015). Enzymes are effective protein catalysts for biochemical reactions. The structural components of proteins are L-α-amino acids with the exception of glycine, which is not chiral. The four levels of protein structure are primary, secondary, tertiary, and quaternary structures. Primary structure is related to the amino acid sequence. The amino acids are linked by covalent bonding, known as a peptide bond, where the amino group of one amino acid is joined to the carboxyl group of the next amino acid. Secondary structure refers to regular, local structure of the protein backbone, stabilized by intramolecular and sometimes intermolecular hydrogen bonding of amide groups. There are two common types of secondary structures, alpha helix and β strand. Tertiary structure refers to the three dimensional structure of folded protein. Presence of disulfide bridges, hydrogen bonding, ionic bonding, hydrophobic and van der Waals interactions maintain the protein conformation. Folding the protein brings sequence of the polypeptide chain to form the enzyme active site that consists of a few amino acid residues and occupies a relatively small portion of the total enzyme volume. The rest of the enzyme is important for the three-dimensional integrity. The quaternary structure of a protein results from the association of two or more polypeptide chains (subunits).

Specificity and catalytic power are two characteristics of an enzyme, which are the biocatalyst with higher precision and accuracy while performing biochemical reactions. These amazing molecules offer a high degree of substrate specificity which leads to an efficient biochemical process with negligible error in product formation (Xu *et al*. 2011).

The enzyme-substrate binding is generally explained by lock-and-key model (conformational perfect fit) or induced fit model (enzyme conformation change such as closing around the substrate). The lock-and-key model has been modified due to the flexibility of enzymes in solution. The binding of the substrate to the enzyme results in a distortion of the substrate into the conformation of the transition state, and the enzyme itself also undergoes a change in conformation to fit the substrate (Figure 1.9). This dynamic binding arrangement between the enzyme and the substrate maximizes the enzyme's ability to catalyze its reaction (Cornish-Bowden 2012).

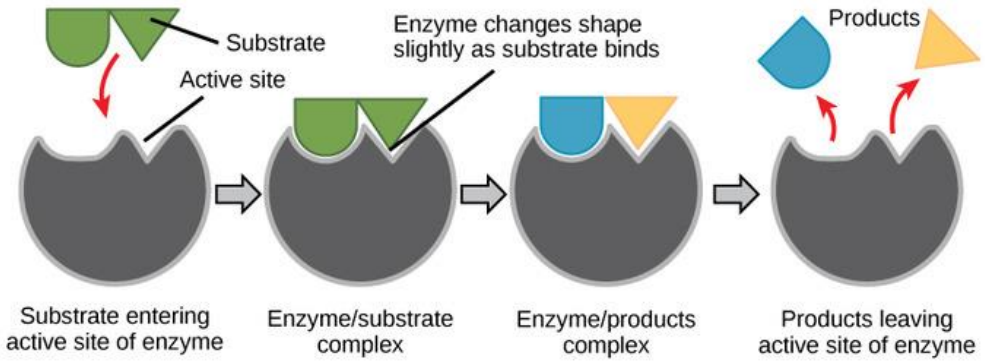


Figure 1.9: Induces fit model (https://www.wikipremed.com/image.php?img=040101_68zzzz248600_648px-Induced_fit_diagram.svg_68.jpg&image_id=248600).

Many enzymes exhibit stereochemical specificity in that they catalyze the reactions of one conformation but not the other. Catalytic power is increased by use of binding energy, induced-fit, proximity effect, and stabilization of charges in a hydrophobic environment. The catalytic activity of many enzymes depends on the presence of cofactor for catalytic activity. If the organic compound as cofactor is loosely attached to the enzyme, it is called a coenzyme. It is called a prosthetic group when the organic compound attaches firmly to the enzyme by covalent bond.

1.5.2 Enzyme kinetics

Enzymes are protein catalysts that, like all catalysts, speed up the rate of a chemical reaction without being used up in the process. They achieve their effect by temporarily binding to the substrate and, in doing so, lowering the activation energy needed to convert it to a product. The rate of enzyme catalyzed reactions is generally modelled by the Michaelis-Menten approach (Cornish-Bowden 2012).

The Michaelis-Menten model is the one of the simplest and best-known approaches to studying the enzyme kinetics. It takes the form of an equation relating reaction velocity to substrate concentration for a system where a substrate S binds reversibly to an enzyme E to form an enzyme-substrate complex ES, which then reacts irreversibly to generate a product P and to regenerate the free enzyme E. This system can be represented schematically as follows:

$$E + S \underset{k_3}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightleftharpoons} E + P$$

The Michaelis-Menten equation for this system is:

$$V_0 = \frac{V_{\max}[S]}{(K_M + [S])}$$

V_{max} represents the maximum velocity achieved by the system, at maximum (saturating) substrate concentrations. K_M (the Michaelis constant) is the substrate concentration at which the reaction velocity is 50% of the V_{max} and it is a measure of the affinity of the enzyme for the substrate. [S] is the concentration of the substrate.

If the enzyme has a high affinity for the substrate, then the reaction will occur faster and K_M has a lower value instead high K_M value means less affinity. K_M varies considerably from one enzyme to another and also with different substrates for the same enzyme.

The Michaelis-Menten plot (Figure 1.10) is used for kinetic analyses of data.

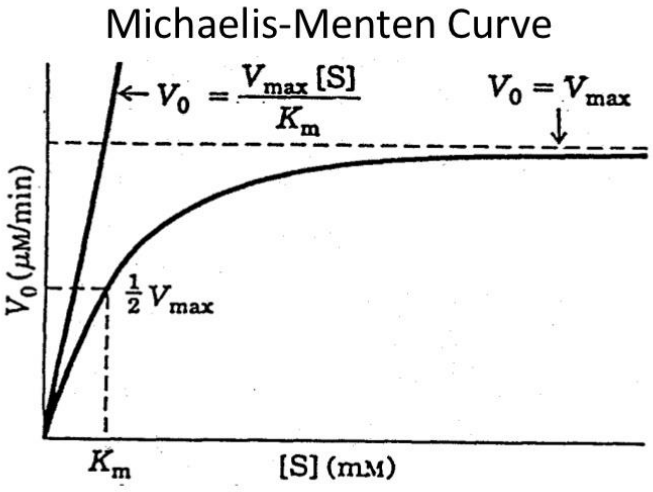


Figure 1.10: Michaelis-Menten plot.
(https://www.slideshare.net/nithinaneesh/enzyme-kinetics-10903597)

The activity of an enzyme is deeply affected by its environmental conditions. Changing these alter the rate of reaction caused by the enzyme (Purich 2010). In nature, organisms

adjust the conditions of their enzymes to produce an optimum rate of reaction, where necessary, or they may have enzymes which are adapted to function well under extreme conditions.

Besides the presence of enzyme and substrate, several other factors affect the rate at which enzymatic reactions proceed such as temperature, pH and the presence of any inhibitors or activators (Purich 2010). Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases with increase in temperature up to a maximum and then declines. A bell-shaped curve is usually observed (Figure 1.11). Increasing temperature increases the kinetics. Since enzymes catalyze reactions by randomly colliding with substrate molecules, increasing temperature increases the rate of reaction, forming more product. However, increasing temperature also increases the vibrational energy that molecules have, specifically in this case enzyme molecules, which puts strain on the bonds that hold them together. As temperature increases, more bonds, especially hydrogen and ionic bonds, will break as a result of this strain. Breaking bonds within the enzyme will cause the active site to change shape. This change in shape means that the active site is less complementary to the shape of the substrate, so that it is less likely to catalyze the reaction. Eventually, the enzyme will become denatured and will no longer function (Sizer 2006).

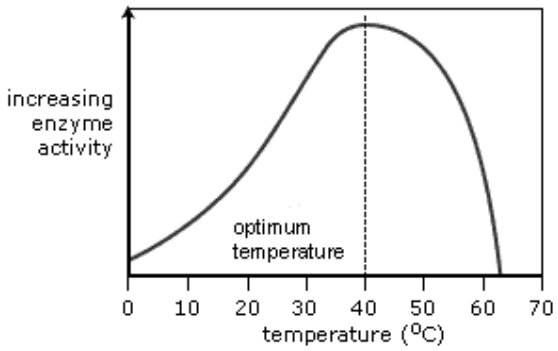


Figure 1.11: Effect of temperature on enzyme activity.
(http://www.bbc.co.uk/schools/gcsebitesize/science/add_aqa/proteins/proteinsrev3.shtml)

Moreover, increase in the hydrogen ion concentration may considerably influence the enzyme activity. H^+ and OH^- ions are charged and therefore interfere with hydrogen and ionic bonds that hold together an enzyme, since they may be attracted or repelled by the charges created by the bonds. This interference causes a change in the shape of the enzyme, and consequently of its active site.

Each enzyme shows an optimum pH at which the velocity is maximum, any change in pH above or below the optimum may quickly cause a decrease in the rate of reaction (a bell-shaped curve is normally obtained Figure 1.12) (Purich 2010).

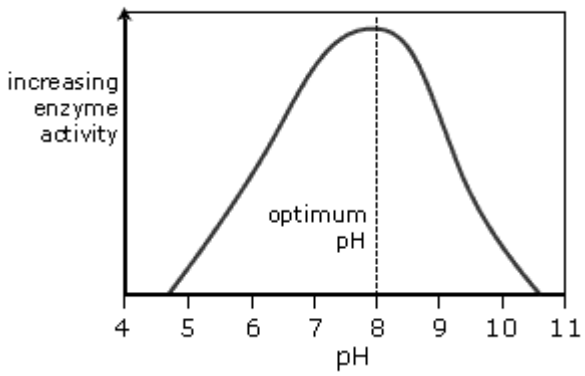


Figure 1.12: Effect of pH on enzyme activity.
(http://www.bbc.co.uk/schools/gcsebitesize/science/add_aqa/proteins/proteinsrev3.shtml)

It is well known that the enzymatic process is influenced by several factors therefore various tools are developed to estimate the effects of different variables and their interaction on enzyme activity. A central composite design (CCD) is one of the most useful approaches in determining optimum conditions of many processes. It explores the response surfaces covered in the experimental design, thus making the optimization process more efficient and effective (Montgomery 2008). In fruit juice production, a statistical tool extensively used to optimize the enzymatic process is response surface methodology (RSM) (Abdullah *et al*. Nur'Aliaa *et al*. 2010)

1.5.3 Immobilization of enzymes

In addition to the unquestionable advantages, there exists a number of practical problems in the use of enzymes. To these belong: the high cost of their isolation and purification, the instability of their structures once they are isolated from their natural environments, and enzymes sensitivity. Moreover, unlike conventional heterogeneous chemical catalysts, most enzymes operate dissolved in water in homogeneous catalysis systems, contaminating the product. Consequently, they cannot be recovered in the active form from reaction mixtures for further use. Several methods have been proposed to overcome these limitations, one of the most successful is enzyme immobilization (Cantone *et al*. 2013; Tischer and Wedekind, 1999; Krajewska, 2004). An immobilized enzyme is an enzyme that

is attached to or within an inert, insoluble material. Since in food industry it is preferable to avoid the presence of extraneous compounds in the final products, the possibility to remove the enzymes is a significant advantage.

Basically, three traditional methods of enzyme immobilization can be distinguished, binding to a support (carrier), entrapment (encapsulation) and cross-linking. Support binding can be physical (such as hydrophobic and van der Waals interactions), ionic, or covalent in nature (Figure 1.13). However, physical bonding is generally too weak to keep the enzyme fixed to the carrier under industrial conditions of high reactant and product concentrations and high ionic strength. Ionic binding is generally stronger and covalent binding of the enzyme to the support even more so, which has the advantage that the enzyme cannot be leached from the support. However, this also has a disadvantage: if the enzyme is irreversibly deactivated, both the enzyme and the (often costly) support are rendered unusable. The support can be a synthetic resin, a biopolymer or an inorganic polymer. Entrapment involves inclusion of an enzyme in a polymer network such as an organic polymer or a silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule. Entrapment requires the synthesis of the polymeric network in the presence of the enzyme. The last category involves cross-linking of enzyme aggregates or crystals, using a bifunctional reagent, to prepare carrier-free macroparticles (Mateo *et al*. 2007; Sheldon and van Pelt 2013).

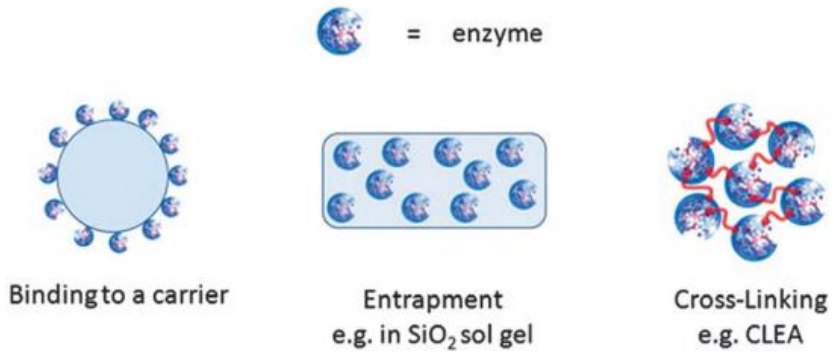


Figure 1.13: Different methods for immobilizing enzymes (Sheldon and van Pelt 2013).

Traditionally, the industrial utilization of enzymes in fruit juice processing has been conducted in conventional batch reactors using soluble enzymes. Unfortunately, after each cycle of operation the enzymes cannot be recovered and reused, and are inevitably present in the final product altering organoleptic properties. In this context, it is shown that the immobilization of enzymes may be very advantageous for continuous industrial use (Ceci

and Lonzano *et al*. 2010). In the literature there is data about immobilization of pectinolytic enzymes on different supports by various methods. Most published research focused on immobilized pectic enzymes can be classified into two groups: those concerning the immobilization of the whole complex of pectic enzymes (Rajdeo *et al*. 2016; Dianto *et al*. 2008; Demir *et al*. 2001), and those showing the immobilization of purified pectic enzymes (Busto *et al*. 2006; Amin *et al*. 2017; which were further studied for the effect of immobilization on their properties and mode of degradation of polymeric substrates. Until now, pectinases have been immobilized on various supports including nylon (Lozano *et al*. 1987; Vaillant *et al*. 2000), ion exchange resin (Kminkova and Kucera 1983), chitosan (Lei and Bi 2007) and sodium alginate (Li *et al*. 2007) among others. Diano *et al*. (2008) studied the catalytic behaviour of a mixture of pectic enzymes, covalently immobilized on different supports [glass microspheres, nylon 6/6 pellets, and polyacrylnitrile (PAN) beads], in a pectin aqueous solution that simulates apple juice. From all these papers, and others not mentioned here, it can be noted that the most frequent immobilized enzyme is polygalacturonase although pectin lyase and pectinesterase have also been immobilized.

In spite of this amount of research on immobilized pectic enzymes, the procedures proposed still show that low satisfactory results without any limitations have rarely been achieved. The coupling of the enzyme usually results in a decrease in enzyme activity and, in some cases, some other characteristics of the enzyme action mode are also altered. Nonetheless, there are in the literature some positive results suggesting the vast potential of immobilized pectic enzymes for the clarification of fruit juices and other similar applications. Once certain problems, such as diffusional constraints and the decrease in the enzyme activity usually found after immobilization are overcome, the field will benefit greatly from the advantages intrinsic to immobilized systems. In spite of the development of an increasing number of works on immobilized enzymes, before considering a future commercial application, much research is still necessary. Thus, the preparation of a library of biocatalysts as broad as possible may be a key turning point towards finding a suitable immobilized biocatalyst with improved properties when compared to the free enzyme.

1.5.4 Pectinases

Pectinases were some of the first enzymes to be used at home. Primarily, these enzymes are responsible for the degradation of the long and complex pectin molecules that occur as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells (Kashyap *et al*. 2001).

The pectinolytic enzymes may be divided in three broader groups as follows (Jayani *et al*. 2005):

- Protopectinases: these enzymes solubilize the insoluble protopectin and give rise to highly polymerized soluble pectin;

- Esterases: catalyze the hydrolysis of pectin methyl ester to pectic acid. The enzyme acts preferentially on a methyl ester group of galacturonate unit next to a non-esterified galacturonate unit;

- Depolymerases: catalyze the hydrolytic cleavage of the α-(1 → 4)-glycosidic bonds in the D-galacturonic acid moieties of the pectic substances.

This latter group, depolymerases, act on pectic substances by two different mechanisms, hydrolysis, in which they catalyze the hydrolytic cleavage with the introduction of water across the oxygen bridge and trans-elimination lysis, in which they break the glycosidic bond by a trans-elimination reaction without any participation of water molecule.

Depending on the preference of enzyme for the substrate, the mechanism of cleavage and the splitting of the glycosidic bonds, depolymerases can be subdivided into four different categories: polygalacturonase, polymethylgalacturonase, polygalacturonate lyase and polymethylgalacturonate lyase (Kashyap *et al*. 2001; Jayani *et al*. 2005).

Polygalacturonase and polymethylgalacturonase breakdown pectate and pectin, respectively by the mechanism of hydrolysis. However, polygalacturonate lyase and polymethylgalacturonate lyase breakdown pectate and pectin by β elimination, respectively (Pedrolli *et al*. 2009).

Over the years, pectinases have been used in several conventional industrial processes, such as textile (Hoondal *et al*. 2002), plant fiber processing (Kapoor *et al*. 2001), tea, coffee, oil extraction (Pasha *et al*. 2013), treatment of industrial wastewater, containing pectinacious material (Hoondal *et al*. 2002), etc. The largest industrial application of

pectinases is in fruit juice processing. Considering that pectic enzymes alone account for about one-quarter of the worlds food enzyme production, it is possible to assert that this huge market could benefit dramatically from the application of technological innovations designed to reduce economical costs and increase the productivity of the system (Kashyap *et al*. 2001).

Almost all the commercial preparations of pectinases are produced from fungal sources (Jayani *et al*. 2005). *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Patil and Dayanand, 2006; Patil *et al*. 2006; Diaz-Godinez *et al*. 2001).

In fruit juice processing, pectinolytic enzymes, may be added in two phases: in fruit juice extraction and/or in fruit juice clarification (Chauhan *et al*. 2001, Joshi *et al*. 2011). Pectinases are often added during the extraction phase, to facilitate pressing or juice extraction and to aid in the separation of a flocculant precipitate by sedimentation, filtration or centrifugation. During incubation the pectinase degrades soluble pectin in the pulp, which would otherwise hamper juice extraction in two ways. First, these slimy pectin particles become saturated with juice, which is then difficult to extract from the pulp and second, they block drainage channels in the pulp through which the juice must pass (Kashyap *et al*. 2001).

A second step which involves pectinases is the clarification-stabilization phase to remove suspended particles. This step is commonly affected by several variables such as pH, temperature, contact time and enzyme concentration. A juice with low pH will clarify more readily than one with a higher pH. Moreover, as the temperature increases the rate of clarification also increases as long as the temperature is below the denaturation temperature for the enzyme (40–60 °C) (Kilara, 1982). In general, the time required to obtain a clarified juice is inversely proportional to the concentration of enzyme used at constant temperature over the range of 5–50 °C and treatment time of 2–16 h. Stability of clarified juice towards haze formation is a consequence of an efficient depectinization, given that pectate flocculations may produce post-bottling haze during storage at low temperature (Baumann 1981). Usually a blend of pectinases is used in the depectinization process to degrade pectic substances.

Sorrivas *et al*. (2006) proposed that enzyme depectinization causes two effects:

1) It destroys the weak soluble pectin net, and reduces juice viscosity Beveridge (2002);

2) It causes the aggregation of cloud particles (Figure 1.14). Insoluble pectin forms a protective coat around proteins in suspension (Lozano 2003). In an acidic environment (for example apple juice has a pH of 3.5), pectin molecules carry a negative charge. This causes them to repel one another. Pectinase degrades the pectin molecule and exposes part of the positively charged protein beneath. The electrostatic repulsion between cloud particles is thereby reduced so that they clump together.

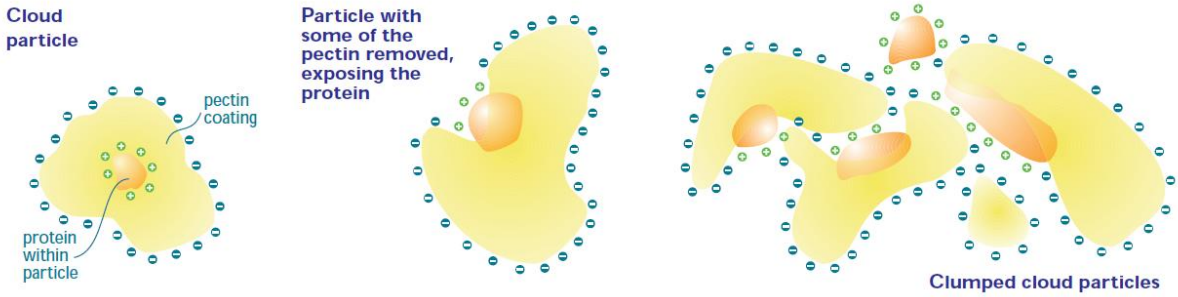


Figure 1.14: Mechanism of floc formation due to electrostatic interactions during juice clarification with pectic enzymes. (http://www.ncbe.reading.ac.uk/MATERIALS/Enzymes/PDF/JAM01.pdf)

Once the enzyme begins its action, there is a reduction in viscosity depending on the temperature, amount of enzymes, and type of juice. The fine haze in the juice slowly begins to agglomerate and form a floc, which eventually settles out, leaving the clear supernatant juice. As a finishing step the juice could be centrifuged or filtered with diatomaceous earth (Baumann 1981).

1.5.5 Other enzymes for fruit juice clarification

Clarification technology could be enhanced by using pectinases in combination with other complementary enzymes: amylase, proteinase, cellulase. The enzyme treatment not only facilitates easy pressing and increases in juice recovery but also ensures the highest possible quality of the end products. Enzymatic hydrolysis of the cell walls increases the extraction yield, reducing sugars, soluble dry matter content, galacturonic acid content and titrable acidity of the products (Sharma *et al*. 2015). Currently, cellulases and hemicellulases, with pectinases, collectively called macerating enzymes, are used for

improvement in pressing, extraction and clarification of fruit and vegetable juices (Bath 2000). Cellulases are defined as a family of enzymes which perform the process of degradation of cellulose and of some related polysaccharides into glucose. They are widespread in nature and are particularly common in the world of bacteria and fungi. Cellulases break down the cellulose molecules into monosaccharides such as β-glucose, or shorter polysaccharides and oligosaccharides. The specific reaction involved is the hydrolysis of the 1,4-beta-D-glycosidic linkages in cellulose, hemicellulose, lichenin, and cereal beta-D-glucans (Zhang *et al*. 2006). Because cellulose molecules bind strongly to each other, cellulolysis is relatively difficult compared to the breakdown of other polysaccharides such as starch.

The widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic actions by endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Henrissat, 1994; Lynd *et al*. 2002; Teeri, 1997; Wood and GaricaCampayo, 1990; Zhang and Lynd, 2004b).

Hemicellulases are a diverse group of enzymes that hydrolyze hemicelluloses. Hemicellulases including endo- and exo-xylanases, galactanases, xyloglucanases and mannanases. Xylanases (EC 3.2.1.8) hydrolyze the ß-1,4 bond in the xylan backbone, yielding short xylooligomers. ß-Mannanases (EC 3.2.1.78) hydrolyze mannan-based hemicelluloses and liberate short ß-1, 4-mannooligomers, which can be further hydrolyzed to mannose by ß-mannosidases (EC 3.2.1.25) (Shallom and Shoham 2003).

In addition, α-amylase and amyloglucosidase, active at acidic pH, were used to process starch containing fruits, especially early harvested apples in order to prevent haze formation (Grassin and Fauquembergue 1996). It catalyzes the hydrolysis of starch into smaller carbohydrate molecules such as maltose (a molecule composed of two glucose molecules). Two categories of amylases, denoted alpha and beta, differ in the way they attack the bonds of the starch molecules (Sharma *et al*. 2015).

Amylases may be required after pressing to degrade starch for clear apple juice production. Before amylase treatment, the juice must be pasteurised to gelatinize the starch and then cooled down 50 °C to avoid enzyme inactivation by heat in an excess of water. SEM studies revealed that amylase normally used in fruit juices clarification quickly reduced starch contents in pasteurized juices to an undetectable level. Starch was not detected by iodometry either (Sorrivas *et al*. 2006).

A new approach to prevent haze formation in fruit juice processing could be the use of proteases. Various food processing, are driven by microbial, animal and vegetable proteases. Papain, bromelain are some of the well-known proteases of plant origin (Divakar and Ellaiah 2006; Ferid and Ferid 2008). Nowadays proteases like papain are widely used and commercialized to prevent chill haze in beer (Stewart 2016).

Protease, also called a peptidase or proteinase, is any enzyme that performs proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain. Proteases may detach the terminal amino acids from the protein chain (exopeptidases, such as aminopeptidases, carboxypeptidase A); others attack internal peptide bonds of a protein (endopeptidases, such as trypsin, chymotrypsin, pepsin, papain, elastase). Proteases have evolved multiple times, and different classes of protease can perform the same reaction by completely different catalytic mechanisms (López-Otín and Bond 2008).

Proteases can be classified into seven broad groups:

Serine proteases - using a serine hydroxyl group;

Cysteine proteases - using a cysteine thiol group;

Threonine proteases - using a threonine secondary alcohol;

Aspartic proteases - using an aspartate carboxylic acid;

Glutamic proteases - using a glutamate carboxylic acid;

Metalloproteases - using a metal, usually zinc;

Asparagine peptide lyases - using an asparagine to perform an elimination reaction (not requiring water).

Alternatively, they may be classified by the optimal pH in which they are active:

- Acid proteases;
- Neutral proteases;
- Basic proteases (or alkaline proteases).

Current juice clarification strategies using fining agents such as gelatin–silica sol and/or bentonite work by allowing unspecific binding of both proteins and polyphenols during the formation of colloid flocs (Pinelo *et al*. 2010). Instead, thee application of proteolytic enzymes able to catalyze the degradation of protein would be an alternative and more targeted approach to remove proteins thus decreasing turbidity level and haze forming during storage (Figure 1.15).

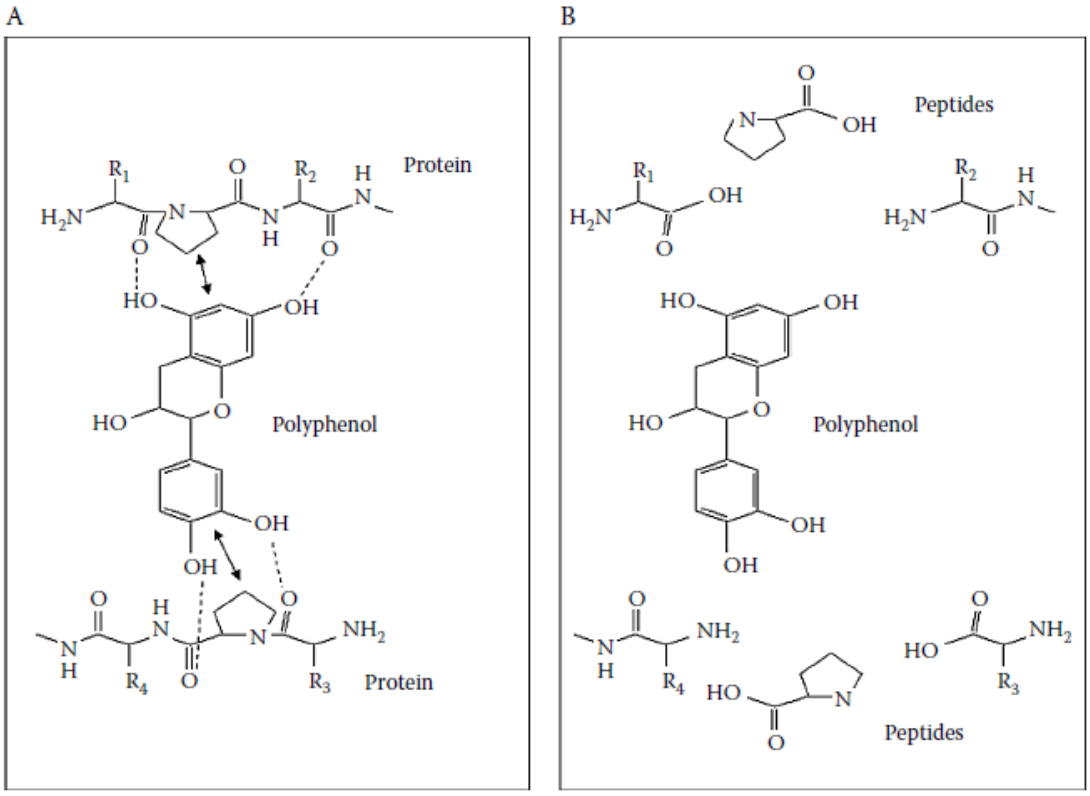


Figure 1.15: (A) Hypothesized chemistry between proteins and polyphenols of haze-causing hydrogen (dashed lines) and hydrophobic bonding (double headed arrows), respectively, in beer, wines, and fruit juices. (B) Proteases destroying the proteins prevent haze formation (Van de Berg *et al*. 2010).

Recently, Landbo *et al*. (2006) showed that addition of gallic acid in combination with various proteases in black currant juice reduced the haze formation drastically, but retained much more of the beneficial phenolics. The best-performing enzyme was Enzeco Fungal Acid Protease (Enzyme Development Corp) produced from *A. niger*, which reduces only 12% of the phenolics, while the traditional methods reduce up to 30%. However, as early as the 1990s Dawes *et al*. (1994) demonstrated that addition of a fungal acid protease to kiwifruit juice decreased the immediate turbidity and retarded the haze formation during cold storage.

Despite the detailed knowledge about pectinolytic enzymes to clarify juice, only few studies have evaluated the use of proteolytic enzymes in fruit juice.

2. AIMS OF STUDY

Juice clarification strategies have been developed in order to optimize the visual appearance of juice which strongly affects consumer behaviour.

In some fruit based beverages, clarity during post-bottling storage, in addition to nuance and intensity of juice color, are attractive quality descriptors that may improve their marketability.

After-bottling hazes are one of the most bothersome and expensive problems encountered in the industrial fruit juice processing. The corrective techniques used depend strictly on the composition of haze particles present. Industrial juice clarification is accomplished by a combination of physical methods, to remove after pressing any sediments and particles, and subsequent fining treatment (gelatin–silica sol, and/or bentonite) to avoid the formation of haze during storage. The fining treatment step is particularly slow, mischievous, and requires comprehensive downstream processing to obtain clarified juice. Moreover it leads to a substantial loss of phenolic antioxidant amount.

This PhD thesis was undertaken to examine the efficiency of various alternative enzymatic clarification strategies on fruit juice, in particular on pomegranate juice, in order to obtain a new insight into individual and interactive effects of pectinase, protease, or multiple (pectinase-protease) treatments on juice turbidity and on haze formation.

In particular this work was structured in three specific sections, according to the following different objectives:

1. **The effect of pectinase and protease treatment on turbidity and on haze active molecules in pomegranate juice.**

   The aim of this section was to enhance the colloidal stability of pomegranate juice with tailored enzymatic treatments, focused on pectinolytic and/or proteolytic enzymes, by examining pectins, proteins and phenol amount and their haze forming activity. Only very little is known about the identity of the substances responsible for cloud, turbidity, and about mechanism of haze development in pomegranate juice.

   The present study was conducted to obtain further information on the events and the possible causes of pomegranate juice turbidity and haze. Moreover, in this work, the effect of pectinase and protease enzyme treatments on the anthocyanin level and chromatic parameters of the pomegranate juice turbidity was investigated.

2. Optimization of pectinase and protease clarification treatment of pomegranate juice

The objective of this part was to develop a clarification process under optimum conditions for pomegranate juice, using pectinolytic and proteolytic enzymes. Response surface methodology was used to optimize the process parameters for the clarification of pomegranate juice. Different conditions such as incubation time, temperature and enzyme concentration mixture were studied, as well as the effect of multiple enzymatic treatments on haze forming activity of proteins and phenols. To our knowledge the effect of these parameters on various physical properties and its further optimization has not been previously reported.

3. Immobilization of pectinases into PVA gel for fruit juice application.

Objective of this last section was to develop a novel alternative enzymatic clarification strategy consisting of pectinases immobilized into PVA gel capsules, LentiKats®, for fruit juice clarification in collaboration with Slovak University Technology. Although pectinolytic enzymes in free form play an important role in fruit juice technology, enzyme immobilization may help in the development of continuous bioprocesses reusing the biocatalyst. Nevertheless, the immobilized enzymes are still today the object of many research projects. In this study, firstly, six different commercial pectinases were tested for their polygalacturonase and pectin lyase activities, secondly, the use of polyvinyl alcohol gel in the form of LentiKats® to immobilize pectinase was investigated as well as its application in apple and pomegranate juice clarification.

3. THE EFFECT OF PECTINASE AND PROTEASE TREATMENT ON TURBIDITY AND ON HAZE ACTIVE MOLECULES IN POMEGRANATE JUICE

In the last decade much attention has been paid to pomegranate fruit and juice due to the biological properties and health benefits of the large amount of polyphenols it contains, such as punicalin, punicalagin, hydrolyzable tannins and anthocyanins (Gil *et al*. 2000; Mousavinejad *et al*. 2009; Seeram *et al*. 2005). Nowadays, there is an increasing demand for pomegranate juice worldwide which has consequently led to an increase in the production of pomegranate fruits (Tehranifara *et al*. 2010; Turfan *et al*. 2011). Conventional processing of pomegranate fruit into juice is laborious and involves several steps such as fruit washing, cleaning (removal of peel and separation of arils), pressing, clarification, pasteurization and filtration. As for other types of clear juice production, clarification is a fundamental step of the pomegranate juice process in order to eliminate substances responsible for turbidity in the fresh juice and to avoid the development of turbidity during storage, usually known as haze formation (Cassano *et al*. 2011; Mirsaeedghazi *et al*. 2010; Vardin and Fenercioglu 2003).

Removing these particles is a critical industrial problem that improves clarity as well as quality and stability of color. The fruit juice industry has investigated various methods to solve this problem since consumer choice is mainly driven by the visual appearance of juice (Costell *et al*. 2010).

Nowadays, conventional clarification procedures rely on the hydrolysis of pectin and starch with pectinases and amylases, respectively; clarifying agents, such as bentonite, gelatin or silica-sol to induce the physico-chemical precipitation of sediments and/or haze-active components and filtration or centrifugation processes (Mirsaeedghazi *et al*. 2010; Pinelo *et al*. 2010; Rinaldi *et al*. 2013).

In particular, pectinases remove immediate turbidity. Any depectinizing action has two effects: to degrade the viscous soluble pectins and to induce the aggregation of cloud particles. Pectin forms a protective coat around suspended proteins and carries a negative charge in acidic environments which causes them to repel each other. Pectinases degrade the chain of pectins, thus exposing positively-charged proteins. The electrostatic repulsion between the cloud particles is thereby reduced so that they aggregate together (Kashyap *et al*. 2001; Sorrivas *et al*. 2006).

Moreover, in beverages that are initially free of turbidity, it is assumed that the haze formation is caused by interactions between proteins and polyphenols (Siebert, 2006).

Proteases break down haze-active proteins, thus preventing protein-phenol interaction and reducing turbidity level without removing phenolics (Pinelo *et al*. 2010).

There is an increase in interest towards pectolytic enzymes as clarifying agents (Sandri *et al*. 2013), also in the immobilized form (Esawy *et al*. 2013), yet few studies have so far relied on proteases to clarify fruit juices (Meyer *et al.* 2001; Landbo *et al*. 2006). Recently, Pinelo *et al*. (2010) and Landbo *et al*. (2006) found that treatment with acid protease improved clarity and had a haze-retarding effect on cherry and black currant juice, respectively.

The aim of this study was to enhance the colloidal stability of pomegranate juice with tailored enzymatic (protease and pectinase) treatments, by examining pectins, proteins and phenol amount and their haze forming activity.

To date, few studies have reported changes in antochyanins and color of pomegranate juice during the clarification process (Turfan *et al*. 2011, 2012; Vardin and Fenercioglu 2003), and no data have been published regarding the influence of the enzymatic clarification treatment on the anthocyanin level and chromatic parameters of the juice.

To our knowledge, this is the first study on pomegranate juice, which highlights the individual and interactive effects of pectinase, protease, pectinase-protease treatments on turbidity and haze formation during cold storage, as well as on haze-active molecule content and color stability.

3.1 Materials and methods

3.1.1 Chemicals

Folin-Ciocalteau reagent, tannic acid, gelatin, bovine serum albumin (BSA) and the other chemicals used for analytical purposes were purchased from Sigma Aldrich (Milan, Italy). Klerzyme 150 pectinase preparation (A) from *Aspergillus niger* was purchased from DSM company (Barcelona, Spain), while the two native plant cysteine proteases, bromelain (B_1) and papain (B_2) were purchased from Sigma Aldrich (Milan, Italy).

3.1.2 Pomegranate juice preparation

Pomegranate juice was obtained from fresh pomegranates using a laboratory-type press, avoiding the seeds crushing. Before juice extraction, the pomegranates were washed and drained then cut into two pieces and processed into juice. The pomegranate juice was then divided into equal parts (325 mL in triplicate) in test tubes and was subjected to various experimental enzymatic clarification treatments as follows: (Control) no added enzyme preparation, (A) pectinase, (B_1) bromelain, (B_2) papain, (AB_1) pectinase and bromelain, (AB_2) pectinase and papain, (AB_1B_2) pectinase with both proteases, according to Table 3.1.

Table 3.1: Enzyme preparation dosage (g protein/L of juice).

Enzyme	**Clarification treatment**						
	Control	A	B_1	B_2	AB_1	AB_2	AB_1B_2
Klerzyme 150	-	0.02	-	-	0.02	0.02	0.02
Bromelain	-	-	0.02	-	0.02	-	0.02
Papain	-	-	-	0.02	-	0.02	0.02

'-'no added enzyme preparation.

The tubes with samples were then shaken and placed in a water bath at 50 °C for exactly 2 h. Following the enzymatic clarification treatment, the samples were immediately heated to 85 °C for 1 min in order to inactivate the enzyme. All juice samples were centrifuged at 15000 rpm for 10 min before being placed in darkness in cold storage at 4 °C for up to 14 days. During processing, pH, tritable acidity and soluble solid content (°Brix) of pomegranate juice were monitored. pH was measured potentiometrically with a Mettler

Toledo pH meter (Steroglass, Perugia, Italy). Tritable acidity was determined as g anhydrous citric acid/L of juice by tritating 10 mL of pomegranate juice with NaOH 0.1M reaching pH 8.1 and °Brix measurements were carried out at 20 °C with a digital refractometer HI 96801 (Hanna Instruments, Milan, Italy). The pH, tritable acidity and soluble solid content of juice samples ranged from 2.97 to 3.05, 15.60 to 15.68 g/L (as anhydrous citric acid) and 14.73 to 14.85 °Brix, respectively, thus indicating that enzymatic treatment did not affect the amounts of organic acid and sugar contained in the pomegranate juice. Moreover, several samples were withdrawn from any tube at various storage times (0, 1, 7, 14 days) and assayed to quantify the amount of pectins, proteins and phenols.

3.1.3 Turbidity measurement and heat stability test

The turbidity of the juice was measured with a HD 25.2 turbidimeter (Delta Hom, Padua, Italy) (Figure 3.1) and expressed in nephelometric turbidity units (NTU). The turbidity was measured immediately after the clarification treatment (day 0). The same measurement was performed to evaluate the chill haze level developed at 1, 7, and 14 days of cold storage at 4 °C.

The potential turbidity of pomegranate juice was determined by heat test: juice samples were incubated at 80 °C for 6 h and then kept at 4 °C for 16 h (Vincenzi *et al*. 2011). Haze formation was measured after equilibration at room temperature (approximately 25 °C).

Figure 3.1: HD 25.2 turbidimeter (Delta Hom, Padua, Italy).

3.1.4 Induction of haze in pomegranate juice

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 following 14 days of storage. 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 in the pomegranate juice, after adding the enzyme (Siebert, Carrasco and Lynn, 1996).

3.1.5 Determination of pectin content

The total pectin content was determined with the K-PECID 11/11 kit (Megazyme International Ireland Ltd., Wicklow, Ireland). The pectate is incubated with pectate lyase, which cleaves the polygalacturonic acid, releasing unsaturated oligosaccharides, which absorb strongly at 235 nm. From the increase in absorbance (ΔA) the amount of unsaturated product produced can be calculated as:

Unsaturated product = $\Delta A/L \times \varepsilon$

where:

ΔA = Reaction Absorbance (after 30 min) – Blank Absorbance.

L = path length of the reaction cuvette (= 1 cm).

ε = the molar extinction coefficient of the reaction product (4600 M^{-1} cm^{-1}).

3.1.6 Determination of total protein

Following enzymatic treatment, the total protein content of the above-mentioned samples was determined using the potassium dodecyl-sulphate (KDS) protein precipitation followed by bicinchoninic acid assay (Vincenzi *et al*. 2011). Proteins were precipitated from 1 mL of juice by adding 10 μL of 10% (w/v) sodium dodecyl-sulphate and 250 μL of 1 mol KCl and then quantified with a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, Saint Louis, USA) according to the manufacturer's instructions. Total proteins were calculated against a calibration curve obtained with bovine serum albumin and results were reported as g_{BSA}/L (Figure 3.2).

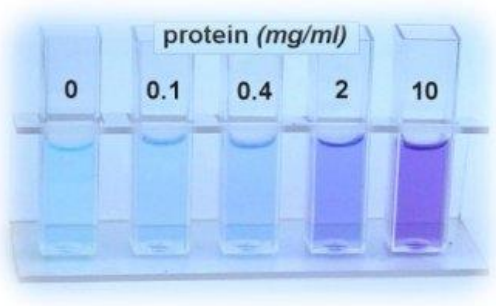


Figure 3.2: BCA protein assay using bovine serum albumin (BSA) solutions as protein concentration reference standard.

3.1.7 Determination of total phenol and anthocyanins

The overall content of phenol compounds was quantified using the Folin Ciocalteau method (Singleton and Rossi, 1965). Briefly, 1 mL of pomegranate juice was exactly diluted to 10 mL with distilled water, then 1000 µL of the diluted juice was mixed with 2.5 mL of Folin–Ciocalteu reagent, alkalinized with 5 mL of 20 g/100 g sodium carbonate solution and taken to 50 mL with distilled water. The mixture was allowed to stand for 60 min at room temperature, then the absorbance was measured by a UV/vis spectrophotometer (UV-2450 Spectrophotometer, Shimadzu) at 760 nm. Total phenolics were calculated against a calibration curve (Figure 3.3) obtained with gallic acid and results were reported as gallic acid equivalents (g/L).

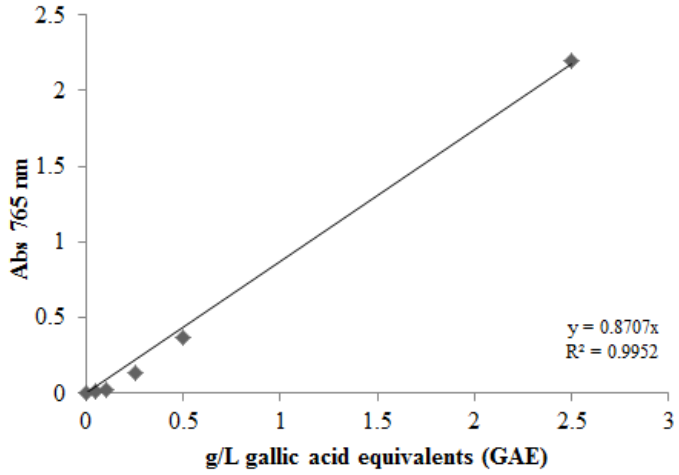


Figure 3.3: Folin Ciocalteau gallic acid standard curve.

Total and monomeric contents of anthocyanins were determined with the spectrophotometric method described by Di Stefano *et al*. (1989) and Genova *et al*. (2012). The results were calculated as mg per litre of cyanidin-3-glucoside equivalents with a molar extinction coefficient of 29,600 and a molecular weight of 449.2 g/mol.

The HPLC-DAD analysis and the individual identification and quantification of the anthocyanins was carried out as reported by Dae-Ok and Chang (2005) using an apparatus provided by Dionex Corp. (Sunnyvale, CA), composed of a P680A pump coupled to a PDA-100 diode array detector. Prior to injection, each juice sample was filtered with a 0.45 μm PTEF filter and 50 μl of sample was used to inject. The column was a Nova-Pak C18 cartridge, 4 μm - 250 x 4.6 mm - protected by a guard column packed with the same material (Waters Corp., Milford, MA). The different anthocyanins were identified by their UV-vis spectra recorded with a diode array detector (520 nm) and expressed as a percentage of their peak areas in the chromatograms with respect to total anthocyanin peak area.

3.1.8 Spectrophotometric color analysis

A UV–vis spectrophotometer (UV-2450 Spectrophotometer, Shimadzu) was used to measure the chromatic characteristics of the juice samples. A direct measurement of absorbance of the juice in 420 and 520 nm was carried out with a 1 mm quartz cell and the following variables were then calculated: color intensity [CI = A420 + A520 + A620], tonality [A420/A520], and percentage of yellow [A420/CI × 100], red [A520/CI × 100] and blue [A620/CI × 100] pigments (Sepúlveda *et al*. 2010). The measurements were taken in triplicate.

3.1.9 Statistical analysis

Three replicates of juice were performed and the results are given as mean ± standard deviation (SD). The turbidity values were analyzed with one-way analysis of variance (ANOVA) and the *post hoc* Tukey's test, with significance set at $P < 0.05$, using EXCEL® Add-in macro DSAASTAT program.

3.2 Results and Discussion

3.2.1 Effect of pectinolytic and proteolytic treatments on pomegranate juice turbidity during cold storage

Figure 3.4 shows the increase in turbidity of pomegranate juice stored at 4 °C for 14 days. The turbidity was measured immediately after the clarification treatment (immediate turbidity) on day 0, and following 1, 7, and 14 days of cold storage.

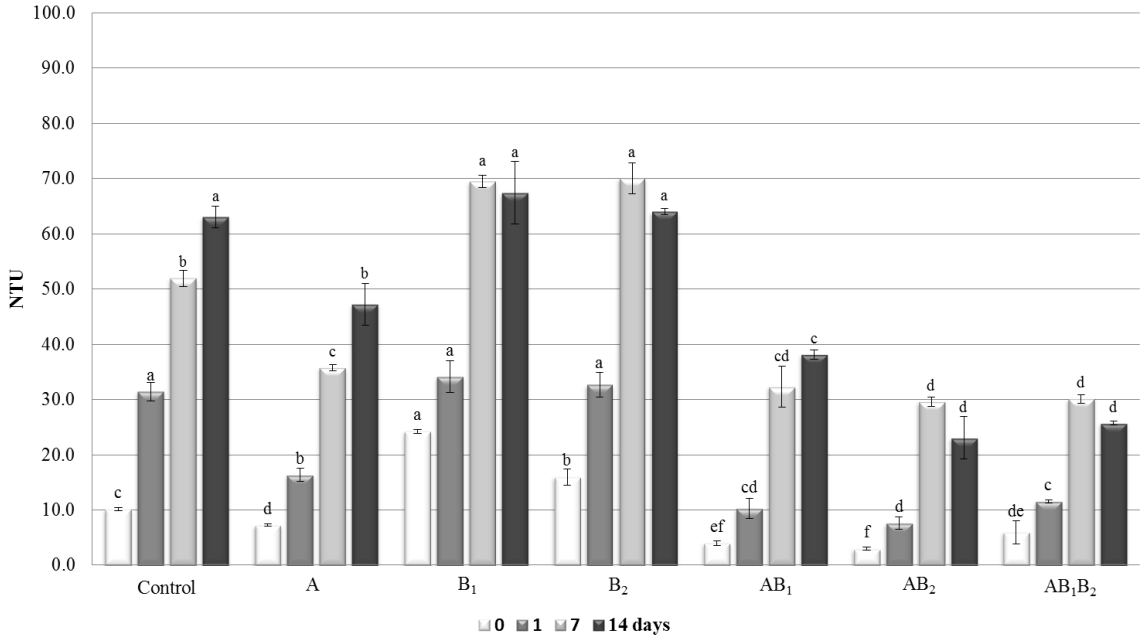


Figure 3.4: Chill haze (NTU) up to the 14th day of storage at 4 °C of enzymatically-treated pomegranate juice. (Control) no added enzyme preparation, (A) pectinase, (B_1) bromelain, (B_2) papain, (AB_1) pectinase and bromelain, (AB_2) pectinase and papain, (AB_1B_2) pectinase with both proteases.The values are mean ± SD of triplicate analysis of juice samples. For each time of storage (0, 1, 7 and 14 days) data with different superscript letters indicate significantly different ($P < 0.05$).

The initial turbidity of the control juice sample was approximately 10 NTU. The enzymatic clarification treatment on pomegranate juice significantly reduced the initial turbidity levels (0 day) from 10 NTU to 3-4 NTU in all treated samples ($P<0.05$), except those treated with both proteases alone. A significant and positive effect of pectinase and protease synergic activity in samples AB_1 and AB_2, was already evident after 2 hours in presence of enzymes (day 0) and this significant clarifying effect was recorded throughout the residual storage time (14 days) ($P<0.05$). Moreover, any further effects were observed if pectinase was added at the same time as both proteases. Surprisingly, immediately after the enzymatic treatment, the pomegranate juice samples treated with bromelain and papain

(B_1 and B_2), showed a higher initial turbidity (24 and 16 NTU, respectively) than the control sample and the other clarified juices.

Several laboratory research studies have explored the various clarification treatments carried out on pomegranate juice for reducing juice turbidity, improving the extraction of anthocyanins and stabilizing the color of the juice (Turfan *et al*. 2011; Vardin and Fenercioglu, 2003; Alper and Acar, 2004), yet up to now, little insight has been gained into the mechanisms involved in the evolution of pomegranate juice turbidity during cold storage. During the 14 days of cold storage, the chill haze levels gradually increased in all enzyme-treated samples as well as for the untreated sample whose turbidity increased up to 63 NTU. On the other hand, lower levels (47, 38, 23 and 26 NTU, respectively) were observed in the samples treated with pectinase (A) and pectinase and protease (AB_1, AB_2, and AB_1B_2) compared to the initial level. During cold storage a turbidity-promoting effect was surprisingly observed in the juices added with both proteases (B_1 and B_2) where the highest turbidity levels, approximately 70 NTU, were reached after 7 days of storage. Landbo *et al*. (2006) found that adding several types of proteases to black current juice samples significantly increased turbidity levels immediately after the treatments. Considering that generally for fruit juice the first potential contributor to haze formation consists in a proteinaceous core surrounded by pectin molecules (Pinelo *et al*. 2010; Grassin and Fauquembergue, 1996), the addition of bromelain and papain (B_1 and B_2) without pectinase, could lead to the formation of new positively charged protein-tannin colloid complexes which would increase turbidity in cloud pomegranate juice. Only after the break down of pectic envelope exerted by pectinases, thus exposing part of underlying positively-charged proteins, the proteases would be able to carry out their proteolytic action on the haze active protein preventing the following binding with haze active polyphenols. Therefore, these experimental data show that even pectinases alone have a significant clarification effect on pomegranate juice, which notably increases if pectinase is combined with protease. This positive synergism was not observed in the study carried out by Landbo *et al*. (2006) on experimental cherry juice in which a combined use of pectinase and protease lead to a turbidity-enhancing effect.

3.2.2 Effect of pectinolytic and proteolytic treatments on pomegranate juice potential turbidity

The effect of pectinase and protease enzyme treatments on the stability of pomegranate juice was also evaluated by means of a heat stability test. Heat-induced haze (named potential juice turbidity) measured in all juice samples following heat treatments (6 hours at 80 °C) and subsequent cold periods (16 hours at 4 °C) is shown in Figure 3.5.

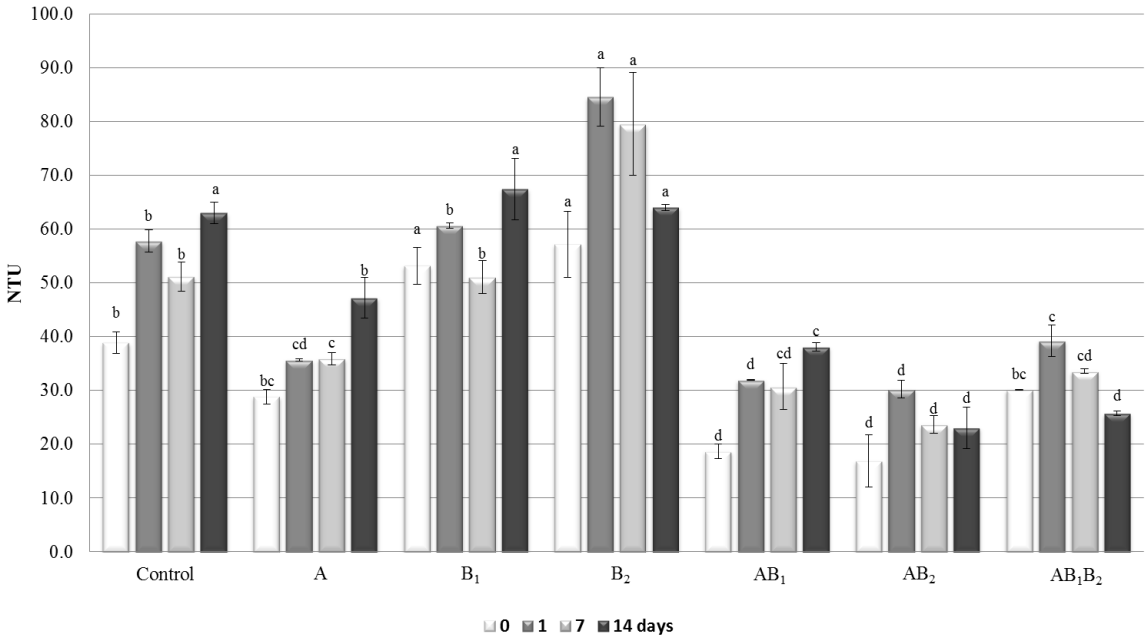


Figure 3.5: Potential turbidity (NTU), measured with the heat stability test, determined up to the 14th day of storage at 4 °C of enzymatically-treated pomegranate juice. (Control) no added enzyme preparation, (A) pectinase, (B_1) bromelain, (B_2) papain, (AB_1) pectinase and bromelain, (AB_2) pectinase and papain, (AB_1B_2) pectinase with both proteases. The values are mean ± SD of triplicate analysis of juice samples. For each time of storage (0, 1, 7 and 14 days) data with different superscript letters indicate significantly different ($P < 0.05$).

The cloudiness stability in the enzyme-treated samples, from 0 day differed significantly according to the enzymatic treatment ($P<0.05$) applied, showing a similar trend and intensity as those observed for immediate turbidity (Figure 3.4). In fact the induced turbidity level of the control juice, immediately after treatment (0 day), was 39 NTU while the samples treated with pectinase and both proteases (AB_1, AB_2) showed the highest haze stability, with turbidity levels of 19 and 17 NTU, respectively. Although potential turbidity in all samples increased over time during cold storage, the lowest NTU levels were observed in the samples treated with a combination of pectinase and proteases (AB_1, AB_2 and AB_1B_2), thus confirming that the latter were less susceptible to heat-induced haze

formation than the control juice and other treated samples. Therefore, the combined use of pectinase and protease reduces the immediate turbidity level in pomegranate juice (as represented in Figure 3.4) as well as its cloudiness stability over time. As already discussed concerning the cold turbidity enhancing effect observed after adding both proteases (B_1 and B_2), the same phenomenon was observed if the potential (heat) turbidity was determined.

3.2.3 Pectinase and protease effects on pectins, proteins, and phenols

The results show that pectin, protein and total phenol amounts were not sensitive to pectinase and protease enzyme treatments (Table 3.2). The analyses carried out on pectins, proteins and phenols during the storage period did not show significant differences among the various enzymatic treatments.

Table 3.2: Range of the total amounts of pectin (M), protein (g/L) and phenols (g/L) in treated pomegranate juice after adding various enzymes from day 0 to the 14th day of cold storage at 4 °C. (Control) no added enzyme preparation, (A) pectinase, (B_1) bromelain, (B_2) papain, (AB_1) pectinase and bromelain, (AB_2) pectinase and papain, (AB_1B_2) pectinase with both proteases. *Pectin is expressed as unsaturated product (M), protein as g bovine serum albumin/L and phenols as g gallic acid equivalent/L.

Clarification treatment	Pectin range* (M) Days 0-14	Protein range (g/L) Days 0-14	Phenol range (g/L) Days 0-14
Control	892-1110	0.69-0.72	2.4-2.7
A	883-1316	0.61-0.67	2.5-2.6
B_1	872-1074	0.62-0.76	2.5-2.6
B_2	872-1139	0.77-1.20	2.5-2.6
AB_1	867-1212	0.60-0.81	2.5-2.6
AB_2	872-1238	0.63-0.73	2.5-2.6
AB_1B_2	867-1063	0.66-0.77	2.4-2.6

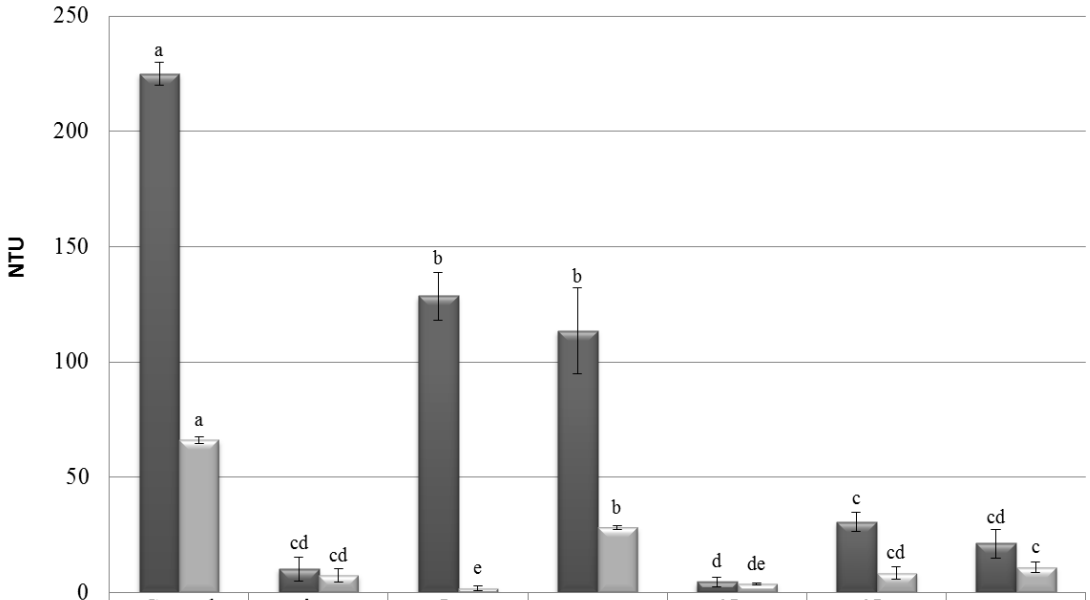
Therefore the ranges were similar in all cases including the control sample. Significant reductions or increases in the levels were not observed during the 14 days of storage, in which, pectin, protein and phenols total contents ranged from 867 to 1238 M, 0.63 to 0.81 g/L (as BSA equivalent) of juice and 2.4 to 2.7 g/L (as gallic acid equivalent), respectively,

thus indicating that enzymatic treatment did not affect their total amount. Moreover, it is important to note that the total phenol level was not affected by enzymatic treatments of this kind, which is in contrast to what was observed in fruit juices clarified with fining agents, where phenol depletion occurred (Turfan *et al*. 2012; Lee *et al*. 2007; Miguel *et al*. 2004).

A comparable effect was observed for cherry juice in the study carried out by Pinelo *et al*. 2010; in which the measured levels of total phenols, total pectin and total proteins were similar in the control and in the experimental enzyme-treated treated juices but in the meantime an individual turbidity reducing effect of pectinase and protease treatment was observed.

The above results suggest that the influence of pectinases and proteases most likely lead to differences in size, shape and consequently in the reactivity of turbidity causing molecules (for example via depolymerisation and via demethylation in the case of pectin molecules) but not in their total amount. In this regard Jiang *et al*. (2001) and Croak and Correding (2006) observed a change in pectin particle size and in pectin particle charge upon different pectinesterase treatments. The authors thus demonstrated that the pectinases acting on the pectins, which are in the cloud particles, make decrease their stability.

As previously described by Siebert *et al*. (1996), the corresponding turbidity, resulting from the addition of a known amount of gelatin (an haze-active (HA) protein) and tannic acid (an haze-active (HA) phenol), indicates the relative amounts of HA phenols and HA proteins in beverages. After each enzymatic treatment, the retained reactivity of phenol and protein molecules, commonly referred to as haze forming activity, was assessed in each pomegranate juice sample (Figure 3.6). The gelatin and tannic acid presence produced significant amounts of haze in the control juice, which had not been treated with any type of enzyme, thus revealing a high level of both haze active phenol and protein in the juice. On the other hand, all the enzyme-treated juices contained lower levels of haze forming phenols and proteins compared to the control sample.



	Control	A	B_1	B_2	AB_1	AB_2	AB_1B_2
Gelatin	225	10	129	114	5	31	21
Tannic acid	66	8	2	28	4	8	11

Figure 3.6: Effect of pectinolytic and proteolytic treatment on pomegranate juice haze (NTU) resulting from the addition of gelatin and tannic acid on the 14th day of storage at 4 °C. (Control) no added enzyme preparation, (A) pectinase, (B_1) bromelain, (B_2) papain, (AB_1) pectinase and bromelain, (AB_2) pectinase and papain, (AB_1B_2) pectinase with both proteases.

The juice samples treated with bromelain, either alone or combined with pectinase, showed the smallest increases (2-4 NTU) in turbidity after adding tannic acid. A slightly larger increase was observed in the other samples (A, AB_2), except for the juice treated with papain alone for which the haze value increased significantly with tannic acid addition (approximately 28 NTU level). This last result seems to indicate that sample treated with papain as protease, is characterized by high haze active protein level probably due to protein complexes that could be caused by this specific enzyme preparation.

The relative amount of haze active phenols in enzyme-treated juices, estimated by measuring the cloudiness caused by adding gelatin, is reported in Figure 3.6. The data show that both samples treated with proteases gave a considerable phenol haze response (approximately 120 NTU), even if less than the control juice due to a new protein-phenol complex arrangement. A less pronounced haze phenol response was observed in all the other samples in which pectinolytic activity was exerted. This phenomenon may be attributed to pectinolytic action on the pectin layer resulting in the subsequent destabilization of the pectin cloud and protein-polyphenol core that could lead to a new stable aggregation.

It is evident that AB_1 juice had the lowest turbidity response determined after adding both tannic acid (5 NTU) and gelatin (4 NTU) which was significantly lower than that measured in juice only treated with pectinase or bromelain. This result shows a synergic effect between pectinase and protease which modified the haze-forming behaviour, in accordance with the above-mentioned results relative to cold storage and potential juice turbidity. Instead, pomegranate juice treated with pectinase with papain (AB_2) showed different NTU levels (31 and 8 NTU, after adding tannic acid and gelatin, respectively). The synergic turbidity reducing effect, using pectinase with papain could be disguised since the high haze-active protein turbidity level was already showed in B1 sample after tannic acid addition. However the clarifying efficiency of pectinase and papain is demonstrated combining the result about turbidity level during storage and potential turbidity.

3.2.4 Effect of enzymatic treatments on anthocyanins and color of pomegranate juice

The nuance and the intensity of red color, in addition to clarity during post-bottling storage of pomegranate juice, are attractive quality descriptors. The color key factors, mainly due to the amount and composition of anthocyanin, strongly affect consumer choice. Therefore, the stability of anthocyanin throughout juice processing and storage is of great importance.
An HPLC-DAD analysis of treated pomegranate juices showed the same anthocyanic composition at 0 and 14^{th} days of storage, in the following order: delphinidin 3,5-diglucoside, the major anthocyanin, as reported by Mousavinejad *et al*. (2009) for several Iranian varieties, with approximately 30% of total peak area, pelargonidin 3,5-diglucoside (25%), cyanidin 3-glucoside (22%), cyanidin 3,5-diglucoside (21 %), pelargonidin 3-glucoside (1%), while delphinidin 3-glucoside was not found in the juice (Figure 3.7).

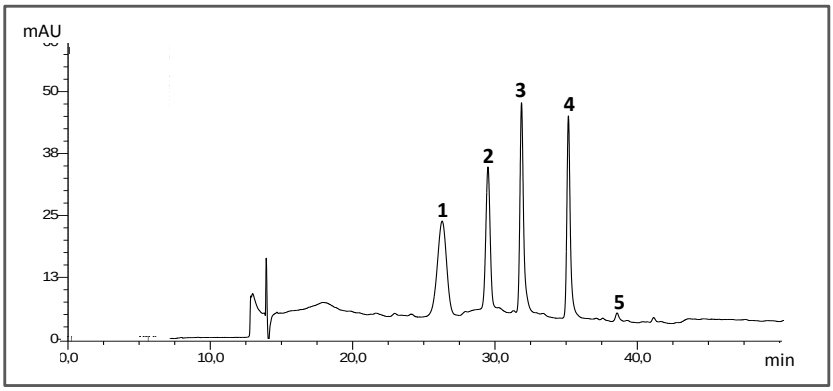


Figure 3.7: HPLC- DAD chromatogram for the anthocyanins of pomegranate juice (520 nm). 1: Dp-3,5-diglu, 2: Cy-3,5-diglu, 3: Pg-3,5-diglu, 4: Cy-3-glu, 5: Pg-3-glu.

As reported in literature, the anthocyanin composition in pomegranate juice greatly depends on both variety and ripening stage (Miguel *et al*. 2004; Martí *et al*. 2002). The results show that the enzymatic treatments did not significantly affect the percentage of anthocyanin composition (Table 3.3).

Table 3.3: Anthocyanin composition (%) of control and treated pomegranate juice at 0 and 14th day of storage at 4 °C. (Control) no added enzyme preparation, (A) pectinase, (B_1) bromelain, (B_2) papain, (AB_1) pectinase and bromelain, (AB_2) pectinase and papain, (AB_1B_2) pectinase with both proteases.

	Stored time (days)	**Dp-3,5-diglu (%)**	**Cy-3,5-diglu (%)**	**Pg-3,5-diglu (%)**	**Cy-3-glc (%)**	**Pg-3-glc (%)**
Control	0	29.2	20.4	26.0	23.4	0.9
	14	33.4	22.4	25.9	21.3	0.9
A	0	29.6	21.6	26.4	23.0	0.8
	14	31.8	20.4	25.9	22.6	*nd
B_1	0	29.4	20.3	25.6	22.1	0.6
	14	30.9	20.1	25.8	22.2	0.7
B_2	0	29.1	20.0	25.6	20.5	0.7
	14	30.8	21.6	24.1	21.8	0.7
AB_1	0	30.1	20.0	25.6	20.5	0.6
	14	33.5	21.3	25.4	21.6	0.7
AB_2	0	31.1	21.4	24.7	22.7	0.4
	14	35.1	19.7	25.2	21.2	0.4
AB_1B_2	0	31.2	20.7	24.8	19.5	nd
	14	35.0	20.2	24.4	19.8	0.5

Total and monomeric anthocyanin amounts and color indexes, at 0 and 14th day of juice cold storage are reported in Table 3.4.

Table 3.4: Total, monomeric anthocyanins and chromatic parameters of control and treated pomegranate juice at 0 and 14th day of storage at 4 °C. (Control) no added enzyme preparation, (A) pectinase, (B_1) bromelain, (B_2) papain, (AB_1) pectinase and bromelain, (AB_2) pectinase and papain, (AB_1B_2) pectinase with both proteases.

	Stored time (days)	**Total anthocyanins** (mg cyanidin-3 -glucoside/L)	**Monomeric anthocyanins** (mg cyanidin-3 -glucoside/L)	**CI**[1]	**CT**[2]	**%Ye**[3]	**%Rd**[4]	**%Bl**[5]
Control	0	77.8±1.9	61.0±2.2	0.30	0.59	35.9	60.5	3.6
	14	25.8±2.0	27.0±1.5	0.25	0.85	43.8	51.8	4.4
A	0	76.7±4.3	55.4±3.6	0.31	0.58	35.5	60.8	3.7
	14	27.5±1.6	29.7±0.9	0.26	0.84	43.7	51.8	4.5
B_1	0	75.5±3.6	54.0±3.0	0.32	0.59	35.9	60.3	3.8
	14	28.0±0.7	30.0±3.9	0.27	0.84	44.6	50.8	4.6
B_2	0	76.4±3.2	57.5±3.6	0.31	0.58	35.5	61.0	3.5
	14	26.3±0.6	28.5±1.7	0.27	0.86	44.4	51.3	4.3
AB_1	0	74.7±0.5	60.4±2.1	0.31	0.58	35.6	60.9	3.6
	14	27.5±1.6	30.8±1.1	0.26	0.83	43.4	52.3	4.3
AB_2	0	73.3±1.4	58.9±3.0	0.31	0.59	35.6	60.8	3.6
	14	26.3±0.5	28.1±4.9	0.25	0.82	43.2	52.4	4.4
AB_1B_2	0	75.1±0.5	59.8±0.9	0.31	0.58	35.5	60.8	3.8
	14	31.9±3.4	24.9±1.7	0.25	0.82	43.0	52.6	4.3

The values represent mean of three replicates ± standard deviation.
[a] Color Intensity.
[b] Tonality.
[c] Percentages of yellow pigment.
[d] Percentages of red pigment.
[e] Percentages of blue pigment.

Immediately after carrying out the enzymatic and pasteurization treatments (0 day), the total and monomeric anthocyanin content of all juice samples remained almost the same, approximately 76 and 26 mg cyanidin-3-glucoside/L, respectively, in accordance with the data reported by Tzulker *et al*. (2007) (from 100 up to 300 mg cyanidin-3-glucoside/L in Israeli pomegranate juices) and Sepúlveda *et al*. (2010) from 61 up to 483 mg cyanidin-3-glucoside/L in Chilean pomegranate juice). A decrease in the content of both anthocyanin classes was observed during storage at 4 °C, however the concentration was similar in all treated samples as well as the evolution profile over time. After 14 days of storage, a loss

of approximately 63% and 50% of total and monomeric anthocyanins respectively, caused the final amount of both classes to even out, while the various enzymatic treatments had no significant effects. Therefore, the substantial loss of anthocyanins may be attributed to storage time and not to the enzymatic clarification process.

As reported by Landbo *et al*. (2006) for cherry juice samples, a direct influence of enzymatic clarification on anthocyanin decrease by comparing the anthocyanin data from the protease-clarified samples with the data of the corresponding control was not demonstrated. On the other hand, as previously reported in literature, other clarification treatments of pomegranate juice, based on fining agents such as gelatin, polyvinylpolyprrolidone or bentonite, caused a significant loss of anthocyanin (Vardin and Fenercioglu 2003; Turfan *et al*. 2012).

It is well known that changes in the phenolic fraction can strongly influence the chromaticity of juice. Color intensity (CI), tonality (CT), and the percentages of yellow (% Ye), red (% Rd) and blue (% Bl) pigments, were determined in all samples, at 0 and 14th day (Table 3.4). Therefore, color intensity decreased from 0.30 to 0.25 during 14 days of storage and simultaneously the red tones decreased and tonality increased from 0.60 to 0.85 in all juice samples. In all untreated and treated juices, the loss of red tones (9% on average), is mainly caused by the degradation and loss of anthocyanins, and the increase of the yellow tone (8% on average) determined a color shift from red to orange. Moreover, since there were no significant differences between chromatic parameters when comparing control and enzymated juices on the same day (0 or 14th day), the chromatic modification may be not attributable to the enzymatic clarification treatment, but could indicate a natural anthocyanin evolution during storage mainly due to an oxidation process.

In light of the results of this section, it can be assumed that both pectin and protein, as well as phenol molecules may strongly increase immediate turbidity and haze formation in pomegranate juice, during cold storage in darkness at 4 °C for up to 14 days. The data showed that although the pectinolytic and proteolytic treatments did not modify the total amount of pectins, proteins and phenols, they affected the haze-forming activity of turbidity causing molecules. It was observed that a synergic clarifying effect of the pectinase and protease activity took place in the juice immediately after the enzymatic treatment and was recorded throughout the residual storage time, showing the best results in terms of the turbidity levels of the juice and potential haze formation. Considering that

the intensity of the shade of red in addition to clarity are important sensorial attributes which provide quality information for human perception, it is important to note that this kind of enzymatic clarification treatment did not affect anthocyanin composition and color of pomegranate juice.

4. OPTIMIZATION OF PECTINASE AND PROTEASE CLARIFICATION TREATMENT OF POMEGRANATE JUICE

In recent years, pomegranate fruit juice has become an important functional and healthy drink, all over the world (Viuda- Martos *et al*. 2010; Fadavi *et al*. 2006). The current interest and consequently the increasing market demand by consumers are supported due to the antioxidant properties demonstrated. Recently, it has also been revealed that pomegranate fruit contains anti-carcinogenic (Bell and Hawthorne, 2008), antimicrobial (Reddy *et al*. 2007), antiviral (Kotwal, 2007), anti-inflammatory (Giménez-Bastida *et al*., 2012), and anti-artherosclerotic compounds (Aviram *et al*. 2004). Tannin and anthocyanins are responsible for the higher antioxidant activity of the juice (Gil *et al*. 2000; Tzulker *et al*. 2007), however the same molecules contribute to undesirable haze and sediment formation in the bottle during storage, which are often wrongly perceived as deterioration of quality (Vardin and Fenercioglu, 2003; Mirsaeedghazi *et al*. 2016; Onsekizoglu, 2013). Thus clarification treatment could be considered as a fundamental step in the processing of fruit juice to improve its appearance and marketability, in particular in pomegranate juice that presents an excessive turbidity (Erkan- Koç 2015;Mirsaeedghazi *et al*. 2016). Conventional clarification methods involve simple filtration and centrifugation (to achieved a clarified but not stabilized juice), fining agents such as bentonite, gelatin, silica sol (Yousefnezhad *et al*. 2016; Mirsaeedghazi *et al*. 2016, Erkan- Koç 2015).

As it has been reported, these latter compounds could produce an acceptable clarified and stabilized juice, however these also led to substantial losses of phenolic compounds and antioxidant activity (Erkan- Koç 2015; Alper *et al*. 2011). An alternative approach to 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 works have reported on the depectinization of various fruit juices with the aim to increase its clarity and homogeneity (Rinaldi *et al*. 2013; Pinelo 2010; Ghosh *et al*. 2016). As demonstrated in the previous chapter, 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 color. A significant and synergic effect of the combined use of pectinase and protease enzymes in terms of turbidity and potential haze formation of the pomegranate juice was revealed. 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, enzyme concentration and temperature (Uzuner *et al*. 2015; Chen *et al*. 2012; Nur ‘Aliaa *et al*. 2010). Optimizing 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 others factors were kept at a constant level. 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 expenses 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 problems above mentioned (Myers *et al*. 201; Khuri *et al*. 2010). 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 (Frank 2001; Tastan *et al*. 2015).

To our knowledge the effect of these parameters (incubation time, enzyme concentration and temperature) on various physical properties 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 multiple enzymatic treatments on haze active proteins and phenols present in the juice.

4.1 Materials and Methods

4.1.1 Materials

Fresh pomegranate (*Punica granatum* L) were provided by Fruttaweb.com (Bologna, Italy). Samples of fruit were stored at 4 °C until use.

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 company (Barcelona, Spain). The powder of papain was solubilized to give the same final concentration of protein revealed in Klerzyme preparation (8 $g_{protein}$/L). All other reagents used in this study were purchased from Sigma Aldrich (Milan, Italy) and were of analytical grade.

4.1.2. Pomegranate juice process

The processing steps of pomegranate juice are outlined below. 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, tritable acidity and soluble solid 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 treatment, firstly to investigate the optimal ratio of protease and pectinase in the complex enzyme solution (Section 4.1.3) and then to further optimize the clarification condition of pomegranate juice: time, temperature and concentration of complex enzymes using RSM (Section 4.1.4). The temperature of each clarification treatment was adjusted to the desired level using a thermostat bath (Kottermann). 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 15000 rpm for 15 min. The supernatant was filtered through PES membrane filter with a pore size of 0.45 mm and placed in dark cold storage at 4 °C until further analysis.

4.1.3 Orthogonal test design to optimize protease:pectinase ratio mixtures

An orthogonal L9 $(3)^4$ test design was applied to investigate the optimal papain:pectinase ratio in the complex enzyme preparation. As seen from Table 4.1, 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 2h. Following the enzymatic treatment each sample was treated as reported above. The turbidity levels resulted were determined after 1 day of cold storage and for up to 21 days.

Table 4.1: Orthogonal experiment design using protease (A) from Papaia latex (papain) and pectinase (B) Klerzyme 150 DSM, solutions.

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

4.1.4 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 process time, process temperature and complex enzyme amount, coded as x_1, x_2, and x_3, respectively. Each independent variable had 3 level which were -1, 0 and +1.

The minimum and maximum actual values for incubation time (X_1) were set at 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% v/v (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:

$$Y= b_0 + b_1x_1+ b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x^2_1 + b_{22}x^2_2 + b_{33}x^2_3$$

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.

4.1.5 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 (day 1 of cold storage) and for up to 21 days of cold storage. After each measurement pomegranate juice samples were warmed at room temperature and the irreversible turbidity was revealed.

Instead, the potential turbidity of pomegranate juice was determined by heat test: juice samples were incubated at 80 °C for 6 h and then kept at 4 °C for 16 h (Vincenzi, *et al*. 2011). Haze formation was measured after equilibration at room temperature (approximately 25 °C).

4.1.6 Assessment of haze-active proteins and phenolics

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 days 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 *et al*. 1996).

4.1.7 Clarity

Clarity was determined by measuring the absorbance at 660 nm using a UV–Vis spectrophotometer (UV-2450 Spectrophotometer, Shimadzu) (Sin *et al*. 2006). Distilled water was used as a reference.

4.2 Results and Discussion

4.2.1 Effect of different ratio of papain: pectinase on pomegranate juice turbidity

In the enzymatic clarification process of fruit juice various parameters play an important role in the reduction of haze level. In order to obtain the optimal ratio of protease and pectinase in the complex enzyme solution an orthogonal test design $L9(3)^4$ was applied.

Orthogonal analysis results shown in Table 4.1 revealed that the influence (R) of enzyme factors on the turbidity level was $R_B > R_A$, therefore pectinolytic enzyme play the most important role in 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 4.1). However, a further reduction in turbidity level is observed when proteolytic activity is coupled with pectinolytic activity, confirming the results reported in the previous chapter. Thus, 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% v/v) and pectinase Klerzyme® (0.25% v/v) corresponding to enzyme ratio of 1:2. In this condition the minimum turbidity level was achieved after the enzymatic process and during the whole storage time.

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.

4.2.2 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 4.2. 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 and given in Table 4.3.

Analysis of variance for the four response variables indicated that the response surface model 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 were determined using *p*-value (Table 4.3). The smaller was the *p*-value, the more significant was the corresponding coefficient (Hou and Chen, 2008). *p*-value of lack of fit was also given in Table 4.3 in order to check the quality of the fitted models and for all responses (Y); the models did not show any lack of fit.

Table 4.2: Effect of incubation time, incubation temperature, and complex enzyme amount (independent variables) on four dependent variables.

Trial	Independent variables			Dependent variables			
	Time (min) $X_1(x_1)$	Temperature (°C) $X_2(x_2)$	Complex enzyme amount(%) $X_3(x_3)$	Chill haze (NTU)	Turbidity (NTU)	Heat Turbidity (NTU)	Clarity (Abs)
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 4.3: 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= complex enzyme amount.

*** Significant at ≤ 0.05 level.**

**** Significant at 0.01 level.**

***** Significant at 0.001 level.**

4.2.3 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. Fresh juices have common colloidal suspension that can affect the clarity of the beverage. It is well known that haze can be reversible (chill haze) or irreversible (turbidity). Chill haze is a reversible turbidity. It is soluble when the liquid is returned 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 be dependent on the enzyme concentration and incubation time in both linear and quadratic manners. The concentration of enzyme mixture showed a negative effect on the linear term but showed a positive effect on its quadratic terms. As shown in Table 4.3 the interaction effect between incubation time and enzyme concentration was negative ($P < 0.05$); whereas it showed a

significant and positive effect between incubation temperature and enzyme concentration ($P<0.01$).

The 3D response surfaces 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) (Figure 4.1).

As shown in Figure 4.1 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 Figure 4.1(A), may decrease the chill haze of the pomegranate juice, which, as is it well-known, involves polyphenols and proline-rich proteins. The lowest chill haze value in pomegranate juice was observed when the enzyme complex concentration was 0.29 %; subsequently, when the enzyme complex concentration increased, the value of the chill haze increased. This phenomenon may be due to an excessive amount of enzyme, which could be involved in the formation of reversible haze.

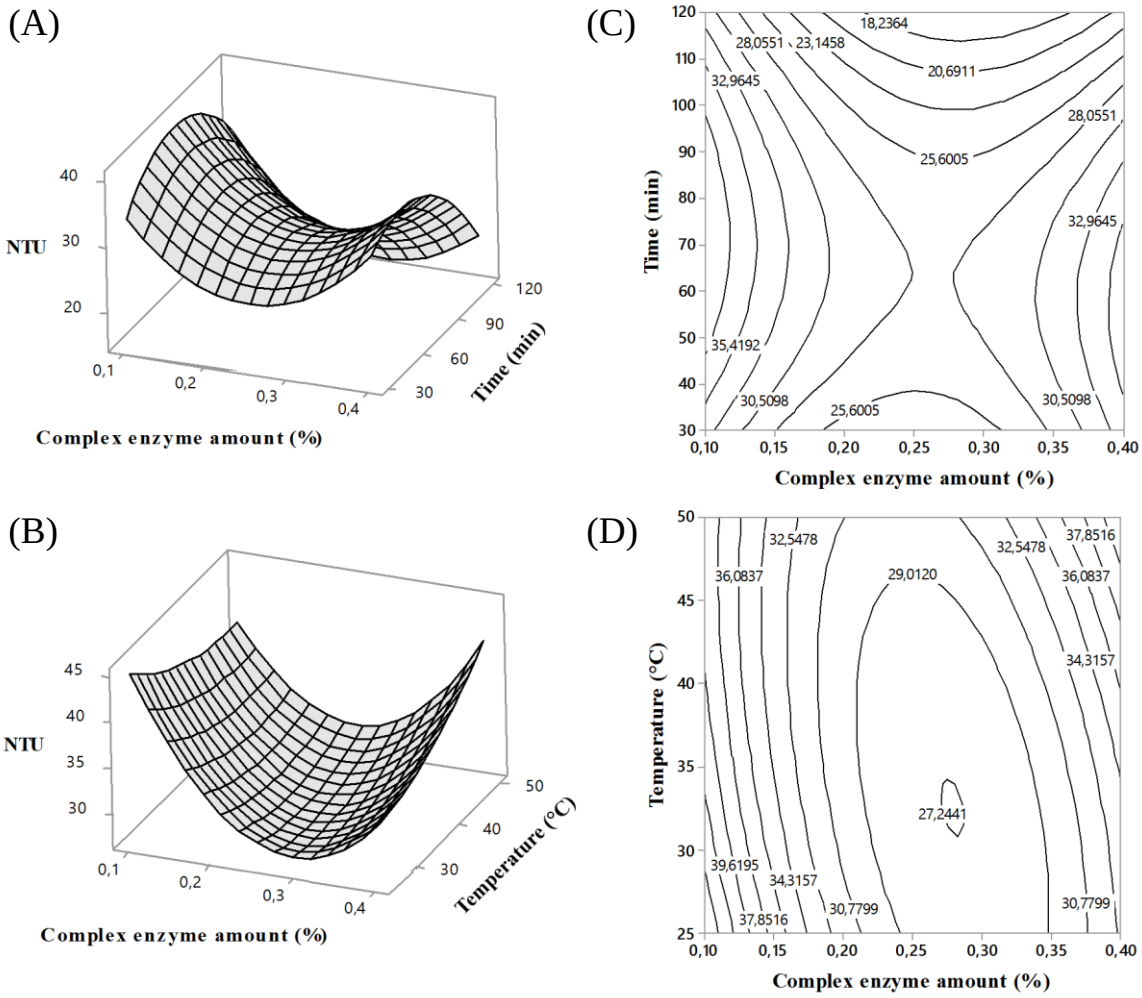


Figure 4.1: 3D response surfaces (A, B) and 2D contour plots (C, D): for chill haze of pomegranate juice as a function of complex enzyme amount and incubation time at 37.5 °C and of complex enzyme amount and temperature at 75 min.

4.2.4 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 can be inferred that any parameters had a negative linear effect on irreversible turbidity level (Table 4.3). 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.

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 in Figure 4.2, it is observed that as the enzyme complex concentration increases up to 0.25% v/v the turbidity decreases at its lowest level. A new index of haze stability was obtained using heat test (6 h 80 °C; 12 h, 4 °C), a forcing method to develop a haze, widely used for testing protein instability in wine but also in apple juice (Wu and Siebert 2002). After heat treatment on pomegranate juice the potential haze was measured. From Table 4.3, 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 term of enzyme concentration was 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, 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 Figure 4.3, 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.

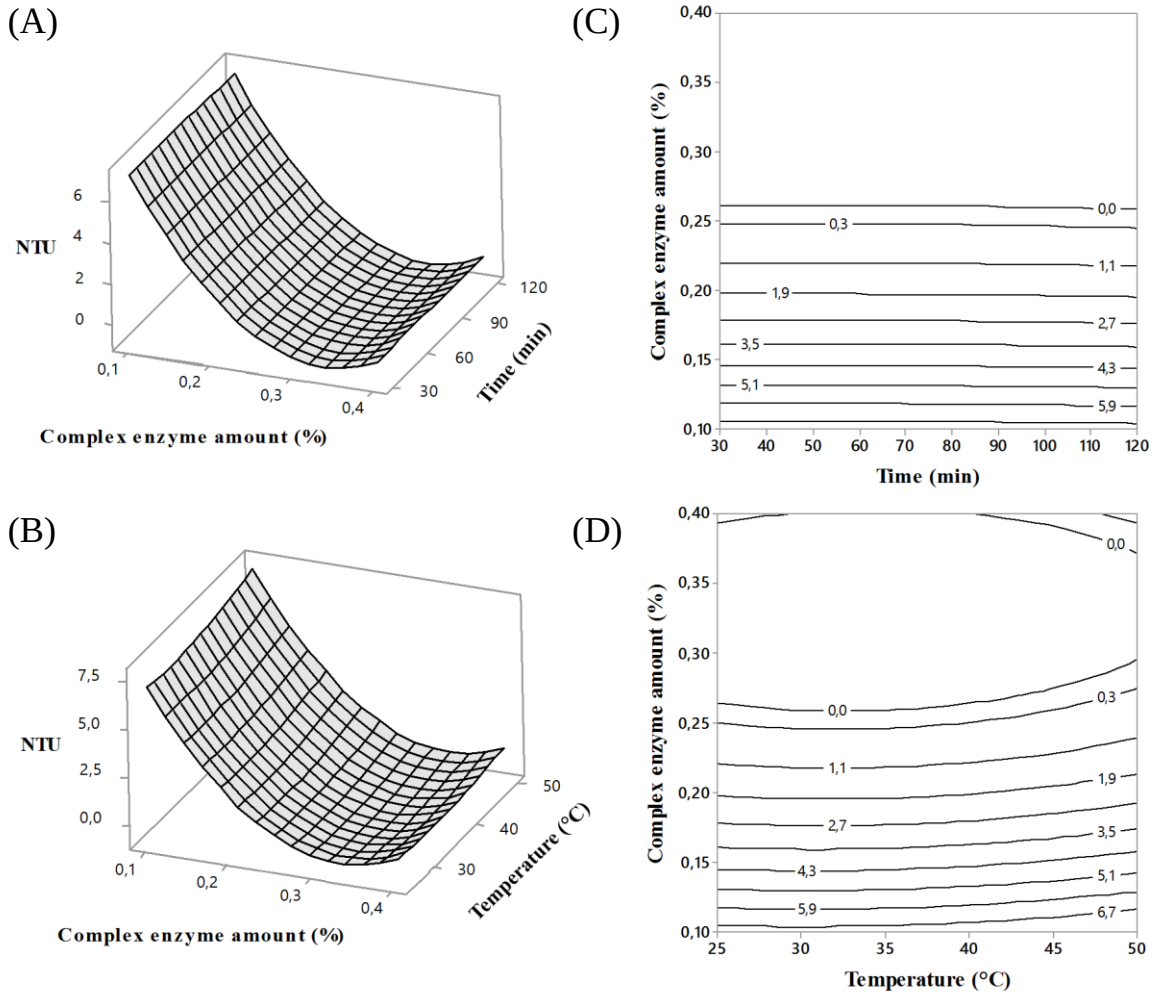


Figure 4.2: 3D response surfaces (A, B) and 2D contour plots (C, D)for turbidity of pomegranate juice as a function of enzyme complex amount and incubation time at 37.5 °C and of enzyme complex amount and temperature at 75 min.

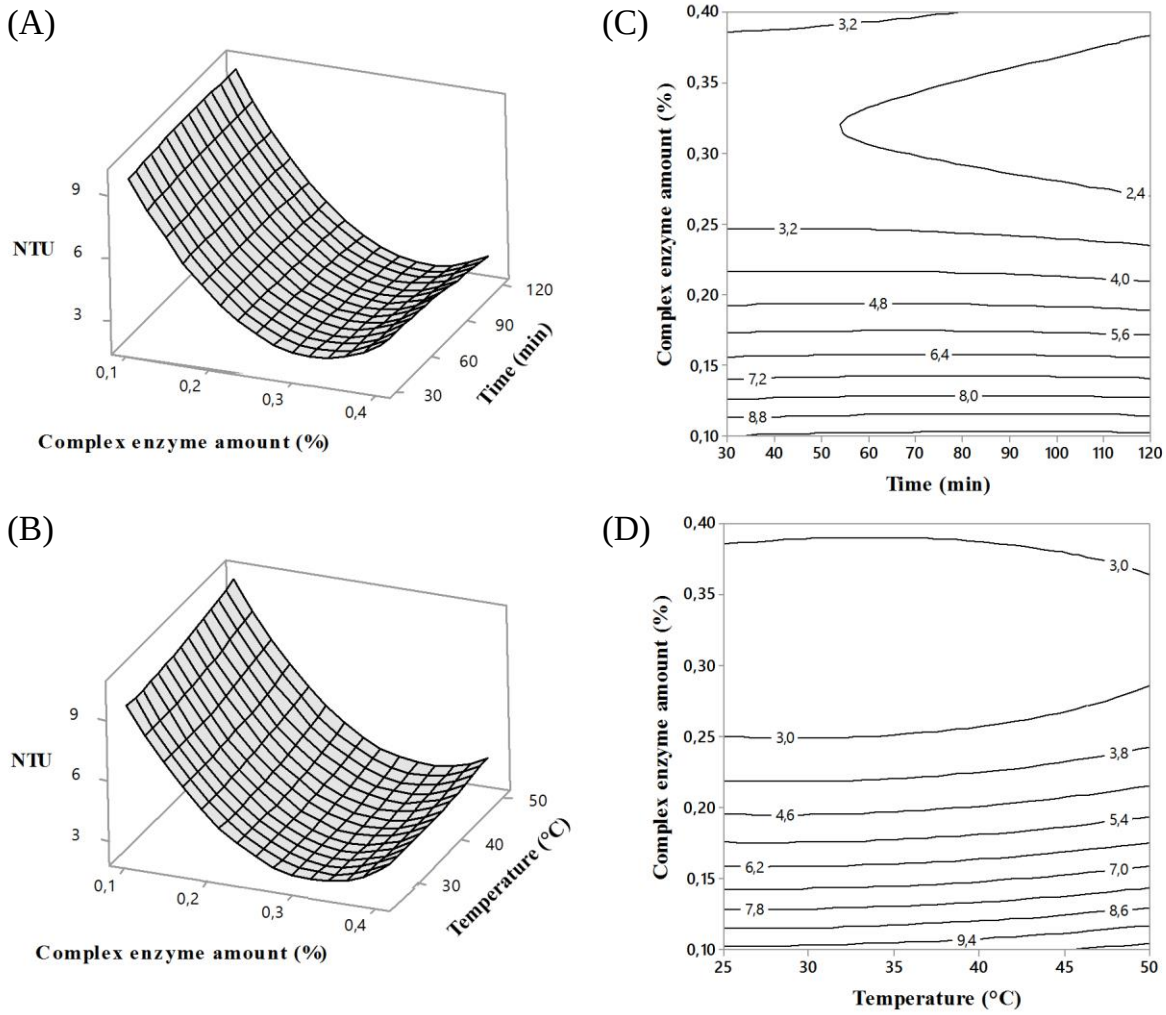


Figure 4.3: 3D response surfaces (A, B) and 2D contour plots (C, D) for heat-turbidity of pomegranate juice as a function of enzyme complex amount and incubation time at 37.5 °C and of enzyme complex amount and temperature at 75 min.

4.2.5 Influence of time, temperature and complex enzyme amount on clarity

Clarification is an important industrial step to improve clarity as well as overall quality of fruit juice. Actually, clarity is an important index to evaluate the performance of the clarification process. Clarified juices do not have cloudy visual appearance and sediment.

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 4.3). 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

Figure 4.4(A) 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 the Figure 4.4(A) 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 *et al*. 2002). Instead, Figure 4.4(B) describes the dependence of clarity on complex enzyme and temperature at fixed time. The lowest absorbance value of pomegranate juice was observed when enzyme concentration was up to 0.3% v/v. It can be seen from the Figure 4.4(B) that the absorbance values decreased as temperature increased and reached a minimum between 32-43 °C. Any further increase in temperature increased the absorbance values.

Analogous results were reported for the changes of enzyme concentration and temperature in banana juice (Lee *et al*. 2006), mosambi juice (Rai *et al*. 2004) and also in the clarification of green asparagus juice (Chen *et al*. 2012).

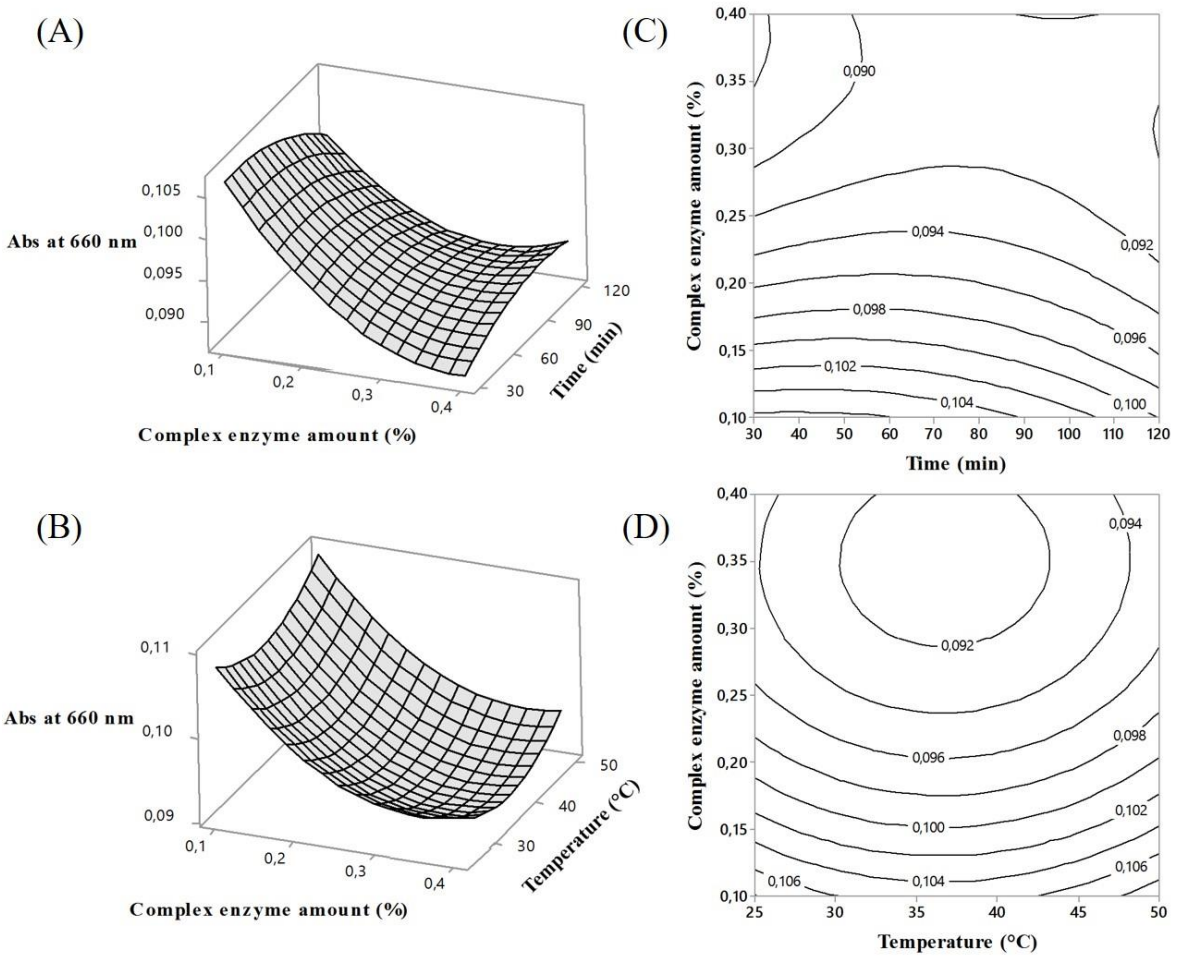
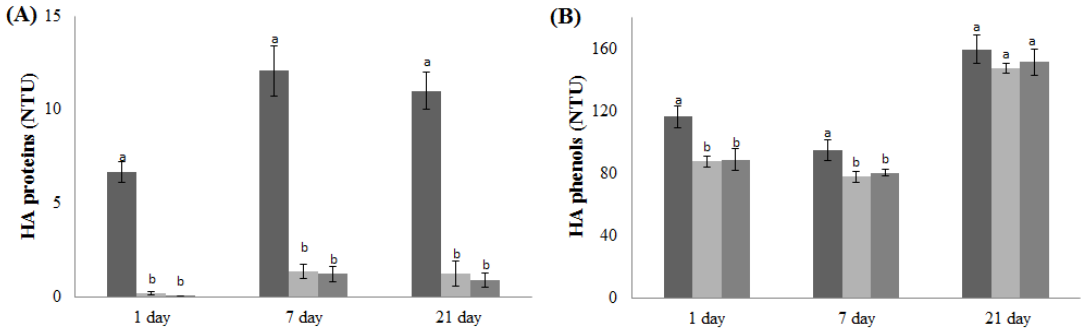


Figure 4.4: 3D response surfaces (A, B) and 2D contour plots (C, D) for clarity of pomegranate juice as a function of enzyme complex amount and incubation time at 37.5 °C and of enzyme complex amount and temperature at 75 min.

4.2.6 Influence of enzymatic clarification on haze active proteins and phenolics

As reported by Siebert *et al*. 2006, the addition of gelatin 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 protein (Figure 4.5A) and haze causing phenols (Figure 4.5B) present in pomegranate juice.

Figure 4.5: Haze (NTU) developed by adding tannic acid (A) or gelatin (B) to pomegranate juice treated with ■ 0.1 %■ 0.25 % ■ 0.4 % v/v of enzyme complex after 1, 7 and 21 day of storage at 4 °C. The values are mean ± SD of triplicate analysis of juice samples. For each time of storage (1, 7 and 21 days) data with different superscript letters indicate significantly different (P < 0.05).



Adding large amount of tannic acid (an HA phenol) to bottled pomegranate juices has been shown to produce an increase in turbidity. The haze developed by adding tannic acid is strictly correlated with the complex enzyme amount (A), much more than that revealed after the addition of gelatin due to haze active proteins (B). Instead, it is possible to see that an increase of enzyme mixture percentage from 0.1% to 0.25 % reduces the haze turbidity developed by adding tannic acid almost totally at 1 day (A). Instead, the same increase % of enzyme complex slightly decreases the reactivity of phenols (B). However, for both turbidity causing molecules, any further effect was observed if the enzyme concentration increased up to 0.4%. This finding revealed that this kind of treatment works mainly on the haze active protein component, confirming the high selectivity of enzyme catalysis. However, both pectinolytic and proteolytic activities on colloidal molecules could lead to a subsequent destabilization of the pectin coat and protein-polyphenol core thus decreasing the reactivity of phenol aggregates.

In addition, Figure 4.5 (A) and (B) shows, during the cold storage (at 4 °C for 21 days) an increase in the haze forming activity of proteins and phenols that remain in the treated pomegranate juice, probably induced by a consecutive molecular rearrangement. The increase in HA proteins showed a different trend and intensity as those observed for HA phenols. After the enzyme treatment, the reactivity of proteins increases considerably within the first 7 days of cold storage, and then stabilizes and grows no further. Instead the reactivity of phenols tends to increase successively between 7th and 21st days of storage.

The corresponding irreversible turbidity after warming at room temperature and potential turbidity evaluated by heat stability test are reported in Figure 4.6. It appears that the juices

treated with 0.25% v/v of enzymes and more result well clarified and stabilized, showing for example at 1st day turbidity level close to zero (Figure 4.6A) and a potential turbidity level of 3NTU (Figure 4.6B). The enzyme concentration of 0.25% v/v allows to reach the lowest turbidity and heat turbidity (potential turbidity) also throughout the residual storage time. Therefore it appears that the largest decrease of haze active protein compared to polyphenols decrease is sufficient to cause a reduction of turbidity and potential turbidity levels of pomegranate juice. Thus, the juices after enzymatic treatment contained little protein that could react with added tannic acid.

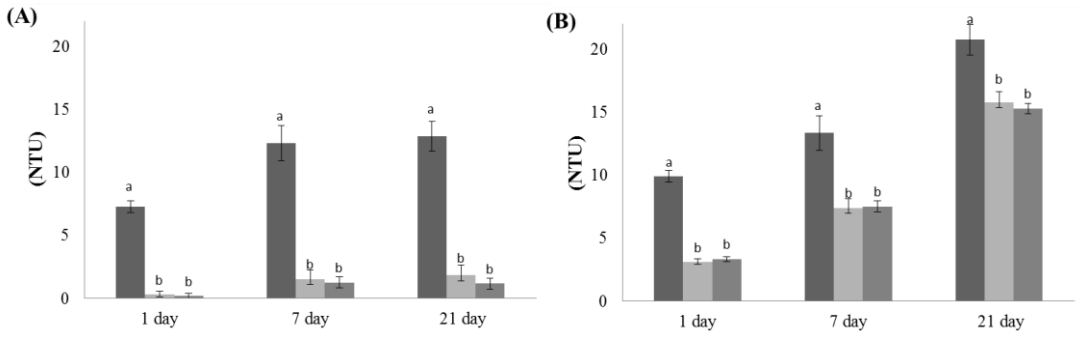


Figure 4.6: Turbidity (A) and potential turbidity (B) in NTU, up to the 21st day of storage at 4 °C of pomegranate juice treated with ■ 0.1 %■ 0.25 %■ 0.4 % v/v of enzyme complex. The values are mean ± SD of triplicate analysis of juice samples. For each time of storage (1, 7 and 21 days) data with different superscript letters indicate significantly different ($P < 0.05$).

4.2.7 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.

These 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 (Figure 4.7).

Figure 4.7 (B) 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% v/v and 0.25% v/v and incubation time between 100 and 115 min.

Figure 4.7(C) 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% v/v and 0.25% v/v and process temperature between 25 °C and 47 °C. Thus, keeping the complex enzyme concentration constant as determined from Figures 4.7(C) and 4.7(B), the best combination of response function can be finally determined in Figure 4.7(A).

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 % v/v of enzyme mixture (the ratio of protease:pectinase was 1:2).

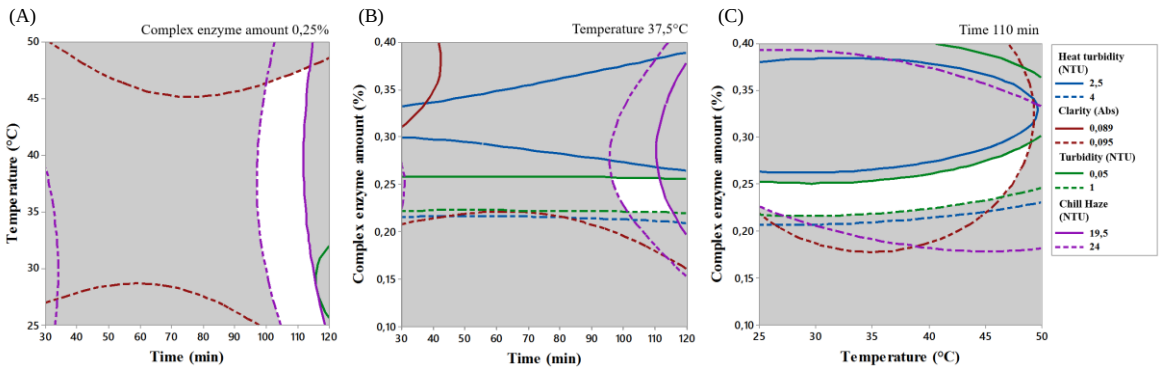


Figure 4.7: The optimum region by overlaying contour plots of the four responses evaluated (chill haze, turbidity, heat turbidity, clarity) as a function of incubation time and temperature (A), incubation time and complex enzyme amount (B), incubation temperature and complex enzyme amount (C).

The same four dependent variables were also determined during cold storage at 7 days and 21 days. Chill turbidity decreased over time, instead there was an increase in irreversible turbidity, heat 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 Figure 4.8 (A) and (B) are shown both overlaying plots attained at 7^{th} and 21^{st} days of cold storage, respectively. Results demonstrated that the optimum clarification conditions revealed immediately after pomegranate clarification treatment remain generally unchanged.

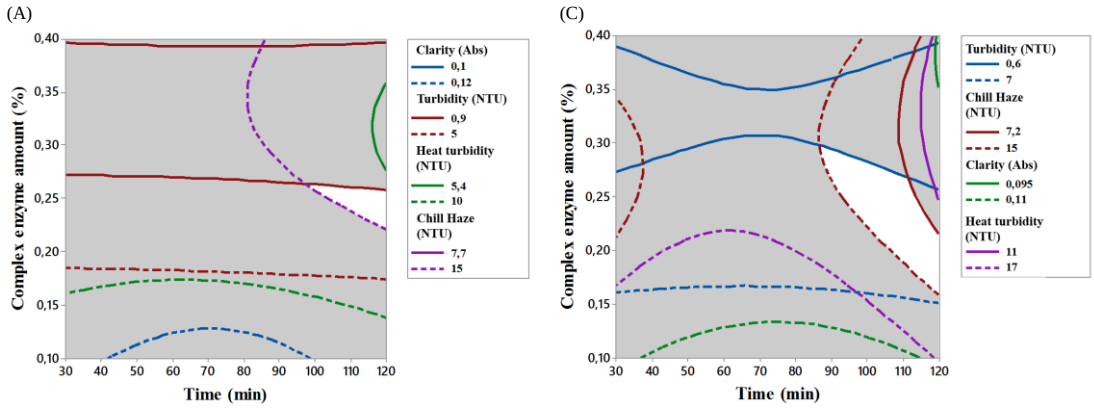


Figure 4.8: The optimum region by overlaying contour plots of the four responses evaluated (chill haze, turbidity, heat turbidity, clarity) as a function of incubation time complex enzyme amount evaluated at 7th (A) and 21st (B) days of storage at 4 °C.

In conclusion, the different conditions (complex enzyme amount, temperature and incubation time) for enzyme treatment revealed that all these variables markedly affect the chill haze, turbidity, heat or potential turbidity and clarity of the pomegranate juice. Generally, enzyme concentration was the most important factor influencing the clarification of pomegranate juice. Its effect on all the dependent variables was significant at linear and quadratic regression.

Using the contour plots, the optimum set of the operating conditions are graphically obtained: temperature 25–30 °C for 100-110 min using 0.22-0.25% (v/v) of protease-pectinase complex enzyme amount (the ratio of protease : pectinase was 1:2). These parameters allow to obtain the suitable clarification properties of the pomegranate juice by a multiple enzyme based clarification process. Moreover this kind of clarification treatment causes a substantial decrease of protein and phenol haze forming activity, thus reducing the potential juice turbidity.

5. IMMOBILIZATION OF PECTINASES INTO PVA GEL FOR FRUIT JUICE APPLICATION

One of the major problems encountered in the preparation of industrially processed fruit based beverages, including fruit juices and wines, except for citrus juices, is the undesirable turbidity of the final product. Cloudy visual perception is the result of light scattering caused by particles suspended in fruit juice (Siebert, 2006). Many different sources of haze in beverages have been described in recent research. Immediate turbidity in freshly produced juice is caused by suspension of polysaccharide particulates (pectin, cellulose, hemicellulose, starch and lignin) arising from the primary and inner cell wall (Sorrivas *et al*. 2006). Otherwise, the turbidity development during cold storage or after reconstitution of the juice concentrate, commonly referred to as haze formation, is caused by protein-polyphenol insoluble complexes (Siebert *et al*. 1996; Siebert, 2006; Pinelo *et al*. 2010).

The main purpose of the clarification procedures employed in industrial juice processing is to eliminate substances responsible for the turbidity, avoiding cloudiness and sediments in the final produced juice. Commercial pectinolytic enzymes play an important role in fruit juice technology, and exert a favourable effect in improving the yield, clarity and filterability of juices (Uzuner and Cekmecelioglu 2015; Khandare *et al*. 2011; Sandri *et al*. 2011; Sandri *et al*. 2013). Pectinases degrade the viscous soluble pectins; in particular, pectin lyase (PL, EC 4.2.2.10) and polygalacturonase (PG, EC 3.2.1.15) act on pectin and polygalacturonic acid, respectively, causing also the aggregation of cloud particles and the clarification of fruit juices.

It is well know that free pectinases are characterized by excellent catalytic properties, however free enzymes always present some drawbacks such as poor stability under operational conditions, impossibility of multiple reuses in an industrial process, presence in the final food product of compounds arising from enzyme preparation (Sheldon *et al*. 2005). There is an increasing interest in the preparation of immobilization pectinases for the clarification and depectinization of fresh juice to overcome these problems. Enzyme immobilization helps in the development of continuous bioprocess reusing the biocatalyst and offers several other advantages such as, better control over enzymatic reactions and high volumetric productivity (Diano *et al*. 2008; Kaur *et al*. 2014; Li *et al*. 2008).

Nevertheless, while free pectinases are being extensively used not only in the fruit juice industry, the immobilized forms are still today the object of many research projects (Rajdeo *et al*. 2016; Rehman *et al*. 2013; Fang *et al*. 2016).

Several studies on the preparation of immobilized pectinases have been carried out on various support synthetic polymers (Diano *et al*. 2008; Esawy *et al*. 2013; Rajdeo *et al*. 2016) or natural macromolecules (Busto *et al*. 2006; Lei and Bi 2007; Rehman *et al*. 2014). Natural polymers, such as alginate, chitosan and agarose show low mechanical strength and durability, although they are biocompatible and non-toxic; otherwise, synthetic polymers have good mechanical stability but have some disadvantages such as the imperfect biocompatibility and hydrophobicity.

Polyvinyl alcohol (PVA) is a synthetic polymer of great interest, offering several significant advantages such as its low cost, non-toxicity, high stability and durability, and mechanical strength. For this reason, PVA hydrogel is widely used as a microbial and enzymatic immobilizing material (Takei *et al*. 2011; Rebroš *et al*. 2007; Wang and Hsieh, 2008). LentiKats® technology is an immobilization method based on a porous PVA carrier with a unique lentil-like shape (Ding and Vorlop, 1995). This immobilization procedure on LentiKats® has already been tested to immobilize invertase (Rebroš *et al*. 2007), glucoamylase (Czichocki *et al*. 2001; Rebroš *et al*. 2006), *β*-galactosidase (Grosová *et al*. 2009) and dextransucrase (Gómez de Segura *et al*. 2003), and if compared with other entrapment methods using PVA hydrogel, its results were not only inexpensive but also easy to carry out. Moreover, the macroscopic dimensions (diameter: 3–4 mm, thickness: 200–400 μm) of the biocatalyst allows for an easy separation, multiple reuse, and continuous automatic operation in industrial processing (Grosová *et al*. 2009; Rebroš *et al*. 2006).

Thus, the objective of this last part was to investigate, for the first time, the use of PVA gel in the form of LentiKats® to immobilize pectinase for its application in the industrial, fruit juice clarification process.

In this section, firstly, six different commercial pectinases used for fruit juice clarification were tested for their PG and PL activities toward model substrates. Secondly, the immobilization of pectinases into LentiKats® and the properties of immobilized enzymes, such as kinetic parameters, pH and temperature profiles and operational stability, were investigated and compared with those of free pectinases. Finally, the application of free and immobilized enzymes were evaluated in apple and pomegranate juice.

5.1 Materials and methods

5.1.1 Materials

The enzyme preparations used in this work were the following (Table 5.1): Pectinex Ultra Color and Pectinex BE XXL from Novozymes, Monza (Italy); Klerzyme 150 and Rapidase from DSM, Barcelona (Spain); Panzym Smash XXL and Panzym YieldMASH from Begerow, Altlussheim (Germany). Citrus pectin (galacturonic acid content 76% and methoxy content 8.6%) and polygalacturonic acid from Sigma Chemical Co. (Milan, Italy) were used as substrates to assay enzyme activities. Other reagents were purchased from commercial sources and used without further purification.

Table 5.1: Pectin enzymes and suppliers.

Enzyme		**Company**
Pectinex Ultra Color	UC	Novozymes
Pectinex BE xxl	PB	
Klerzyme 150	K_{150}	DSM
Rapidase C80	RD	
Panzym Smash XXL	PS	Begerow
Panzym YieldMASH	PM	

5.1.2 Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

The commercial pectinase samples were electrophoresed through a 10% SDS polyacrylamide linear resolving gel overlaid with a 5% stacking gel. The gel runs on a constant voltage of 200 V inside an electrophoresis tank connected to a Consort EV243 electrophoresis power supply. Protein bands in the gels were stained with Coomassie Blue G-250.

5.1.3 Pectin lyase assay

The determination of the PL activity was carried out spectrophotometrically (Agilent 8453 model) by monitoring the increase of absorbance at 235 nm due to the formation of a double conjugate bond of the Δ4:5 unsaturated uronide formed during the reaction (Busto

et al. 2006). One unit of enzyme activity was defined as the amount of enzyme that produced an increase of one unit of A_{235} per minute at 40 °C.

Free (40 μL diluted 100-fold) or immobilized enzyme (0.4 g) was mixed with 2 mL of 1% (w/v) of pectin (dissolved in 0.1 M acetate buffer, pH 6) and filled up to 4 mL with 0.1 M acetate buffer at pH 6. Solutions were preincubated at 40 °C for 10 min.

5.1.4 Polygalacturonase assay

PG activity of enzyme preparations was measured by assaying the galacturonic acid liberated from the polygalacturonic acid (Miller, 1959). Free (40 μL diluted 100-fold) or immobilized enzyme (0.4 g of LentiKats®) was mixed with 2 mL of 1% (w⁄v) polygalacturonic acid (dissolved in 0.1 M acetate buffer, pH 4.2) and filled up to 4 mL with the aforementioned buffer. The reaction mixture was incubated at 40 °C. The resulting galacturonic acid was determined by the 3',5'-dinitrosalicylic (DNS) acid method, 400 μL of the DNS reagent was added to 200 μL of the reaction mixture, as reported by Kashyap *et al*. (2000). After heating at 100 °C for 15 min, the mixture was finally diluted to 5 mL with deionised water (4.4 mL). The absorbance of the reaction mixture was measured at 530 nm against the substrate blank and the values were corrected with the absorbance of the enzyme blank. Calibration standards of galacturonic acid (0–1 mg mL^{-1}) were prepared in distilled water. One unit (U) of pectinase activity was defined as the amount of enzyme required to liberate 1 μmol of galacturonic acid from polygalacturonic acid per minute under the assay conditions.

5.1.5 Enzyme immobilization

The above-mentioned enzymes were immobilized into PVA hydrogels using the LentiKats® technology in the form of lens-shaped particles (Figure 5.1), according to Rebroš *et al.* (2007). The enzyme (5 mL) was mixed with 100 mL of PVA gel in liquid form (10% w/v), extruded through thin nozzles onto a hard surface and dried at 40 °C for 1 h. According to the manufacturer, solid lens-shaped particles were swollen in the hardening solution (www.lentikats.eu).

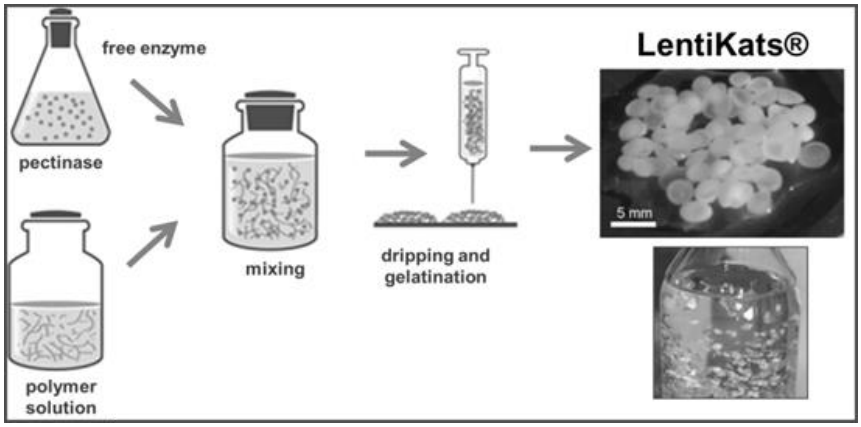


Figure 5.1: Scheme of LentiKats® laboratory preparation. (www.lentikats.eu).

5.1.6 Characterization of free and immobilized enzymes

To determine the maximum reaction rate (V_{max}) and the Michaelis-Menten constant (K_M) for both free and immobilized enzymes, the PG and PL activity assays were determined at 40 °C in 0.1 M acetate buffer at pH 4.2 and 6, respectively, using different substrate (polygalacturonic acid or pectin) concentrations between 0.25% and 1.5% (w/v). Kinetic parameters (K_M, V_{max}) were estimated using the non-linear regression analysis software, GraphPad Prism version 5.00 for Windows, (GraphPad Software). The best-fit value, for each data set, was attested by the squares of the correlation coefficients (r^2).

The effect of pH on PG activity was studied using an acetate buffer in the pH range 3.5–5.5 at 40 °C. Instead, the effect of pH on PL activity was assayed using two different buffers: acetate buffer (pH 3.5–6) and phosphate buffer (pH 6–8). The enzyme assays were prepared as described previously. The effect of temperature on immobilized pectinase was determined by performing both enzyme assays at different temperatures varying between 30 and 70 °C under the same experimental conditions described before.

5.1.7 Operating stability assay

The operational stability of the immobilized pectinase was determined by quantifying its activity in consecutive cycles of repeated use of the enzyme. After each cycle, immobilized particles were recovered, washed with deionised water and used for the next batch run: this process was repeated eight times.

5.1.8 Application of immobilized enzyme in apple and pomegranate juice clarification

Golden Delicious apples and pomegranates were purchased from a local market and washed with water to remove any adhering substances. Apples were cut into small pieces, instead pomegranate arils were separated from the husks and pericarp by hand. Fruits were put into a household electric extractor machine juicer (Clatronic AE 3532 at low speed setting) in order to obtain the fresh juices. The hazy juices thus obtained were subsequently pasteurised at 85 °C for 30 sec to inactivate the fruit enzymes and microorganisms, followed by filtration through a cheese cloth. The extracted juices were stored at 4 °C until used. The clarification assay was performed with 0.6% v/v of free enzyme preparation or the same concentration of immobilized enzyme (0.1 g mL^{-1}) at 40 °C for a reaction time of 120 min in the above-mentioned juice with 0.5% added pectin. At the end of the reaction period, the immobilized enzyme was removed and washed with distilled water before being used in freshly extracted juice to start a new run. The process was repeated three times. Control samples, in which the enzyme preparations were replaced by distilled water, were made for all assays.

Clarity was determined by measuring the absorbance at 660 nm using an Agilent 8453 model UV–VIS spectrophotometer.

5.1.9 Statistical analysis

All analytical measurements were performed in triplicates, and the results were expressed as mean ± SD. Differences in enzyme activities, among commercial enzyme preparations, were determined using a one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) posthoc test (P=0.05) with the EXCEL® Add-in macro DSAASTAT (Onofri, 2006).

5.2 Results and discussion

5.2.1 Catalytic properties of free and immobilized pectinase preparations

The six commercial enzymes were clearly different with respect to the pectinase activity profiles.PG or PL activities towards the aforementioned substrates were revealed in all enzymatic preparations, except Panzym Smash XXL and Panzym YieldMASH, respectively (Table 1).

On the contrary, Panzym Smash XXL and Panzym YieldMASH showed the highest PL and PG activity, respectively. All enzyme activities were found to be significantly different ($P<0.05$) except for Pectinex Ultra Color and Klerzyme 150, which had almost the same PG and PL activities ($P>0.05$). The SDS-PAGE profile (Figure 5.2) provided the fingerprint of different enzymes present in the pectinolytic preparations and corroborated the activity results shown in Table 1. A dominant band was observed in Panzym YieldMASH and Panzym Smash XXL, which indicated a mono-component enzyme in both commercial preparations. On the contrary, the other commercial samples showed many protein bands, probably attributable to different enzymatic activities.

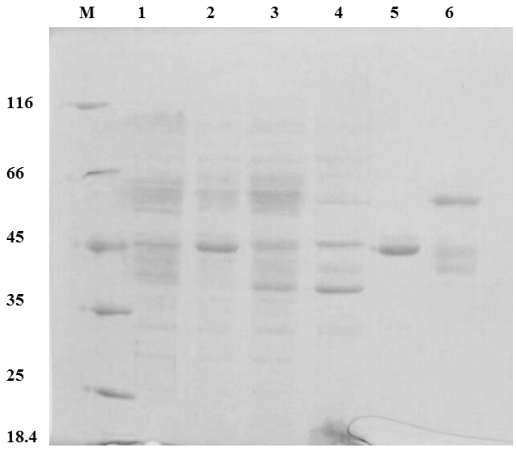


Figure 5.2: SDS-PAGE of commercial pectinases: Pectinex Ultra Color (1), Pectinex Be XXL (2), Klerzyme 150 (3), Rapidase C80 (4), Panzym Smash XXL (5) and Panzym YieldMASH (6). Molecular masses of protein marker (M), in kD, are shown on the left side of the gel.

All enzymes were immobilized into lens-shaped PVA hydrogel particles LentiKats®, and the PG and PL activities were tested (Table 5.2). It is worth to note that, also in immobilized form, pectinases showed significant differences for the PG and PL activity ($P<0.05$), even if the immobilized Panzym YieldMASH and Panzym Smash XXL preparations retained the highest PG and PL activity, respectively.

Table 5.2: Polygalacturonase (PG) and pectin lyase (PL) activities of free and immobilized pectinases.

Enzyme	Type of enzyme	PG activity (U mL^{-1})	PL activity (U mL^{-1})
Pectinex Ultra Color	Free	$21.44 \times 10^3 \pm 144^{b}$	388 ± 20^{d}
	Immobilized	75.12 ± 3.21^{B}	0.47 ± 0.01^{BC}
Pectinex Be XXL	Free	$9.51 \times 10^3 \pm 402^{d}$	827 ± 32^{b}
	Immobilized	35.60 ± 0.24^{E}	0.58 ± 0.01^{B}
Klerzyme 150	Free	$20.33 \times 10^3 \pm 575^{b}$	359 ± 13^{d}
	Immobilized	65.04 ± 3.06^{C}	0.25 ± 0.02^{D}
Rapidase C80	Free	$13.08 \times 10^3 \pm 473^{c}$	553 ± 8^{c}
	Immobilized	52.14 ± 3.52^{D}	0.35 ± 0.04^{CD}
Panzym Smash XXL	Free	0 ± 0^{e}	1256 ± 2^{a}
	Immobilized	0 ± 0^{F}	2.75 ± 0.1^{A}
Panzym YieldMASH	Free	$62.10 \times 10^3 \pm 1293^{a}$	0 ± 0^{e}
	Immobilized	141.68 ± 4.75^{A}	0 ± 0^{E}

Data are expressed as mean ± standard deviation SD (n=3). Means in a column followed by different small letters (for free enzymes) or capital letters (for immobilized enzymes) are significantly different ($P<0.05$) according Tukey's posthoc test.

However, the immobilization procedure affected both enzymatic activities, showing lower values with respect to the free enzymes. The immobilized yields obtained in this study are in the value range described by other authors, for example Dinnella *et al*. (1997) with Pectolyase Y23 (0.31%) and Vaillant *et al*. (2000) immobilizing Citolase PCL5 (about 5 and 2 % yields on chitosan and nylon, respectively). Table 1 shows that the amount of enzyme immobilized in a catalytic active form represents, in all cases, a minimal percentage of that theoretically bound about 0.35% for PG activity in Panzym YieldMASH and 0.22% for PL activity in Panzym Smash XXL preparations. Since the biocatalyst is porous, the very low yield could be due to the existence of diffusional constraints that impaired both substrate access to the enzyme and reaction product release from the gel. The entrapped enzymes are probably affected by the availability of substrate (lower substrates concentration within PVA matrix) and consequently, the catalytic site of the immobilized enzyme is empty for most of the time. Thus, the rate at which the product can be formed is limited by the concentration of substrate which is available. According to these data, all the following experiments were performed with Panzym YieldMASH and Panzym Smash XXL, the two mono-component enzymes that exhibited maximum PG and PL activity in immobilized forms. Moreover, the modulation of enzyme properties by immobilization needs to be studied using pure enzymes, to precisely understand the occurring phenomena.

Kinetic parameters of polygalacturonase and pectin lyase enzymes

Michaelis constant and maximum velocity, characteristic properties of the enzyme and substrate, were calculated by GraphPad Prism and are shown in Table 5.3.

Table 5.3: The K_M and V_{max} values of free and immobilized Panzym YieldMASH and Panzym Smash XXL.

Enzyme	***K_m*** ***($g\ L^{-1}$)***		***V_{max}*** ***($U\ mL^{-1}$)***		***r^2***	
	Free	Immobilized	Free	Immobilized	Free	Immobilized
Panzym YieldMASH	0.83	1.45	77789	273.8	0.99	0.99
Panzym Smash XXL	6.1	11.6	2551	9.1	0.98	0.96

The kinetic analysis revealed that both the soluble and immobilized enzyme followed pure Michaelis-Menten kinetics (Figure 5.3) in the two tested commercial enzymes, as reported in PL preparation by Busto *et al*. (2006) and Ortega *et al*. (2004a) and also in PG enzymes (Ortega *et al*. 2004b; Buga *et al*. 2010).

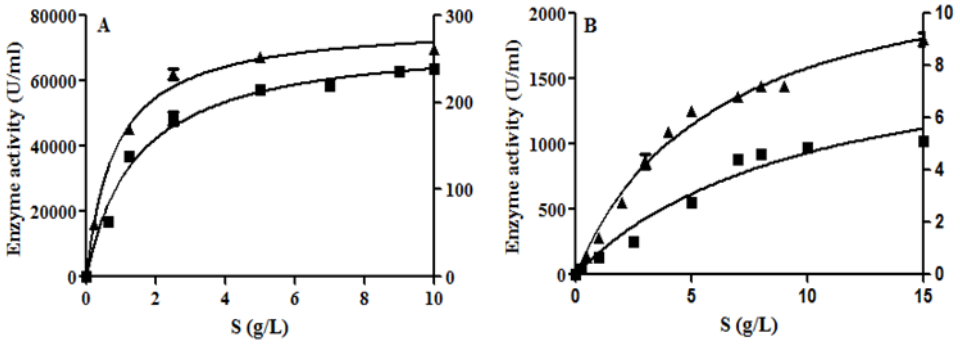


Figure 5.3: Enzyme activity of free and immobilized Panzym YieldMASH (A-left) and Panzym Smash XXL (B-right). On left Y axis: (▲) free enzymes; on right Y axis: (■) immobilized enzymes.

The K_M value of both the immobilized enzymes in LentiKats® increased with respect to the free enzyme, indicating a substantial decrease in substrate affinity to the active site; for example Buga *et al*. (2010), Esawy *et al*. (2013) and Rehman *et al*. (2014) reported a higher K_M value for entrapped pectinases into different supports such as, sodium alginate beads, PVA sponges and agar matrix. The K_M value was shifted from 0.83 to 2.45 g/L free and immobilized Panzym YieldMASH and from 6.1 to 11.1 g/L free and immobilized Panzym Smash XXL. The increase in K_M of both immobilized enzymes may be due to a

limitation in the diffusion of substrates into the lens-shaped particles, especially in the case of high molecular weight substrates such as pectin complexes. A decrease of V_{max}, the highest possible velocity when all of the enzyme is saturated with substrate, was observed in both immobilized enzymes, compared with V_{max} of the free enzymes, maybe due to the chemical modification as reported by Busto *et al*. (2006) and Rehman *et al*. (2014). In this concern, Esawy *et al*. (2013) showed a similar trend in K_M and Vmax value: 2 mg mL^{-1} and 5 mg $mL^{-1}min^{-1}$, for free enzyme, respectively, and 3.2 mg mL^{-1} and 3.8 mg $mL^{-1}min^{-1}$, respectively, for immobilized pectinase.

5.2.2 Effect of pH and temperature on polygalacturonase and pectin lyase

The effect of pH on the activity of the free and the immobilized pectinase was assayed in the pH range of 3.5–5.5 and 3.5–8, for Panzym YieldMASH and Panzym Smash XXL enzymes, respectively.

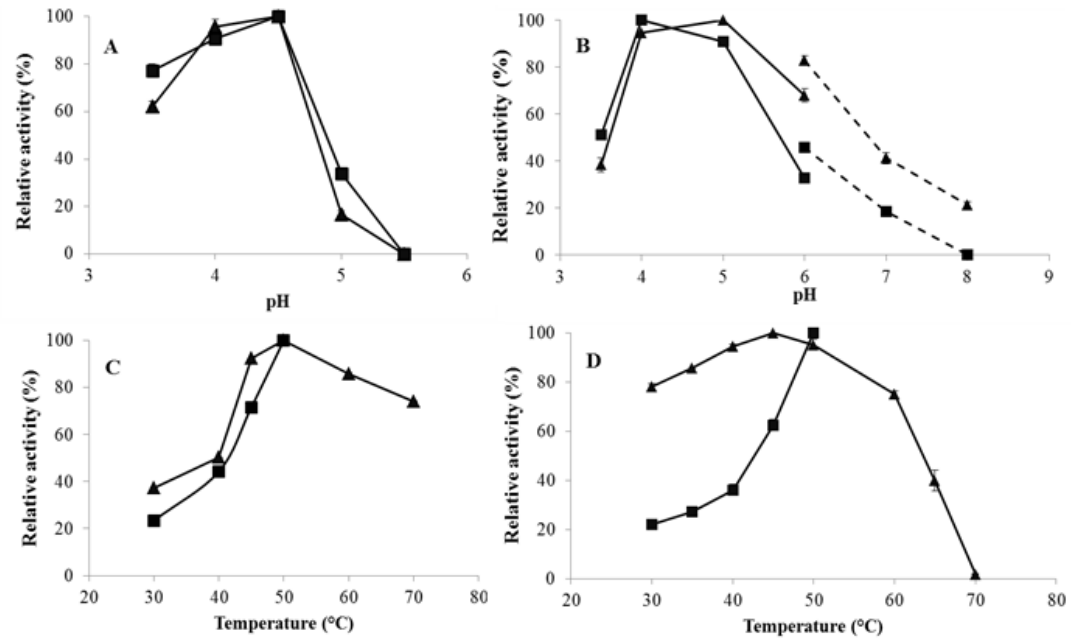


Figure 5.4: pH and temperature profile of free (▲) and immobilized (■) Panzym YieldMASH (A, C- left) and Panzym Smash XXL (B, D-right). The values are means ± SD (n=3).

Figure 5.4A shows that both immobilized and free PG exhibited maximum activity at pH 4.5. According to Ortega *et al*. (2004b), in different free commercial PG enzymes, the optimum pH values were assayed between 4.0 and 4.7. In addition, no change in pH optima of PG was observed when the enzyme was immobilized on polyethylene matrix (Saxena *et al*. 2008). However, the nature of the enzyme, the immobilization procedure and the structure and composition of the matrix used for immobilization could modify the

optimum pH and pH profile, as previously reported (Rebroš *et al*. 2006). As it can be seen in Figure 5.4A, above and below the optimum pH, the relative activity of the immobilized enzyme is slightly higher than that of the free enzyme. A good adaptability of immobilized polyglacturonase to an acidic solution as fruit juice was showed: at pH 3.5, the immobilized form preserved 77% of its initial activity, whereas the free form retained 62% of its original activity. The pH profiles of the free and immobilized PL are plotted in Figure 5.4B. The optimum pH of immobilized enzyme was shifted to 4, one unit lower than that of the free enzyme and the relative activity below the optimum pH was slightly higher than that of the free enzyme. In particular at pH 3.5, that corresponds to the pH of acidic fruit juice like citrus and apple juice, the immobilized biocatalyst retained 51% of its initial activity, whereas the free form retained 40% of its original activity. These findings can be attributed to the new microenvironment, due to the entrapment of the enzyme in the PVA polymer matrix, which may have buffered and, consequently, protected the protein structure of the enzymes. In this way, the enzymes were less affected by the acidity of the solution and, as reported by Vaillant *et al*. (2000), Lei and Bi (2007), Rehman *et al*. (2014) a higher response at low pH values can be an advantage in fruit juice processing industries, because the pH of juices is often less than 4.5.

The effect of temperature on the activity of free and immobilized Panzym YieldMASH and Panzym Smash XXL is shown in Figure 5.4C and 5.4D, respectively. Maximum activity of free PG and PL was observed at 50 and 45 °C, respectively. The rate of enzymatic reaction, in both immobilized enzymes, increased with the reaction temperature and showed maximum activity at 50 °C.

However, at temperatures higher than 55 °C, the LentiKats® were found to be mechanically unstable and started to melt (Rebroš *et al*. 2006); for this reason, the activity of both immobilized forms was investigated in the range between 30 and 50 °C.

The effect of temperature on the activity of the two enzymes in immobilized forms was varied; even if the immobilization procedure by entrapment method in PVA gel is the same for both types of pectinolytic enzymes, Panzym YieldMASH (polygalacturonase) and Panzym Smash XXL (pectin lyase) show different catalyzed chemical reactions, different active sites and substrates specificity. Consequently, for example, at 30 °C, immobilized Panzym YieldMASH and Panzym Smash XXL exhibited about 22% of their optimal activity, whereas the free enzyme showed 37% and 80%, respectively. At this temperature,

PG activity showed only a 15% difference between the free and immobilized form, which seems less temperature dependent than immobilized PL activity. The relative activity of immobilized Panzym Smash XXL dropped significantly at the same temperature (30 °C). Probably the immobilization procedure, and consequently the new microenvironment generated, modified the pectin lyase enzyme behaviour, thus the dependence of pectin lyase activity on temperature. A drop in enzymatic activity of pectolytic enzyme was observed by Diano *et al*. 2008; their results showed a higher dependence of immobilized enzyme mixture on the temperature below its optimum (50 °C).

5.2.3 Operational stability of the pure polygalacturonase and pectin lyase preparation

The reusability of immobilized biocatalyst is an important aspect in industrial applications. Eight consecutive cycles were conducted in the presence of polygalacturonic acid and pectin solution at 40 °C to assess the reusability of PG and PL enzyme entrapped into LentiKats® particles, respectively. The residual activity following each cycle is shown in Figure 5.5.

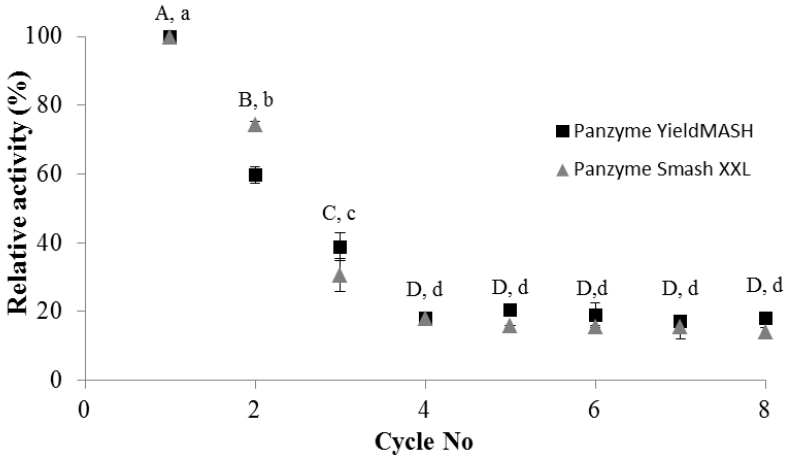


Figure 5.5: Operational stability of immobilized Panzym YieldMASH (■) and Panzym Smash XXL (▲). Values are given as the mean ± SD (n=3). Different capital letters (Panzym YieldMASH) or small letters (Panzym Smash XXL) indicate significantly differences ($P < 0.05$) according Tukey's posthoc test.

It was observed that, the immobilized Panzym YieldMASH and Panzym Smash XXL retained about 60% and 74% residual activity in the second cycle and more than 30% during the third cycle. After that, Panzym YieldMASH and Panzym Smash XXL retained about 20% of their initial activity even after repeating eight times without a significant decrease in the observed activity ($P<0.05$). Even if this value was not as high as that

reported by Esawy *et al*. (2013), entrapped enzymes usually suffer a gradual loss of activity after consecutive catalytic cycles. However, the results obtained are better than those earlier reported by Rehman *et al*. (2013) about pectinase entrapment in calcium alginate instead, a similar result, about 20% of retained activity, was observed by Buga *et al*. (2010) when pectinases were immobilized using the entrapment technique. The initial decrease in activity may be due to the denaturation of the protein, conformational changes of the enzymes, or the leaching of the enzyme from the PVA gel by excessive washing after each cycle (Ipsita *et al*. 2003; Demir *et al*. 2001). However, as an outcome, these immobilized enzymes allow repeated uses with a significant reduction of bioprocesses cost whereas the free enzymes can only be used one time.

5.2.4 Juice clarification with polygalacturonase and pectin lyase

The free and immobilized PG (Figure 5.6A) and PL (Figure 5.6B) enzymes were applied for apple and pomegranate fruit juice clarification with an additional pectin as a substrate in a batch reactor. Despite several recent studies about pectolytic enzymes immobilization (Rehman *et al*. 2013, 2014; Lei and Bi 2007), the performance of immobilized biocatalyst on real matrix, like fruit juice, has been tested in only few cases and has been measured using different evaluation methods (Chauhan *et al*. 2015; Esawy *et al*. 2013; Diano *et al*. 2008). This makes a direct comparison of the results more difficult.

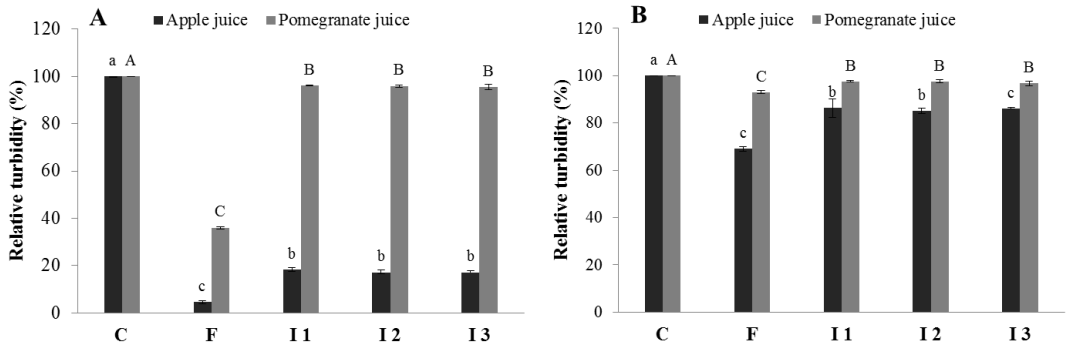


Figure 5.6: Effect of immobilized and free Panzym YieldMASH (A) and Panzym Smash XXL (B) treatment on juice turbidity. C: control without enzymes; F: free enzymes; In: immobilized enzymes (n, cycle number).The values are means ± SD (n=3). Different small letters (apple juice) or capital letters (pomegranate juice) indicate significantly differences ($P < 0.05$) according Tukey's posthoc test.

Figure 5.6 shows the effect of enzymatic hydrolysis on the decrement of turbidity due to the flocculation of the pectin-protein complex (Lee *et al*. 2006). In spite of the low activity

showed, the reduction of turbidity with immobilized Panzym YieldMASH in apple juice was about 80%, compared to 95% found with the soluble enzyme. The value could be considered acceptable for the application of this immobilized system in fruit juice processing, considering also that the same percentage of turbidity reduction was successfully retained in the two following uses. Indeed, immobilization of enzymes on support, with macroscopic dimensions such as LentiKats®, may facilitate separation of biocatalyst, enzyme multiple reuse, and continuous automatic operation in industrial processing, so avoiding the presence in the final food product of compound/protein arising from enzyme mixture. However, the same immobilized enzyme, Panzym YieldMASH, showed a lower level of clarification in pomegranate juice (about 4%) compared to apple juice.

The enzymatic treatment with immobilized Panzym Smash XXL showed a lower level of clarification in both juice samples: about a 15% reduction of turbidity in apple and only a 2.5% reduction in pomegranate juice. Nevertheless, also the performance of Panzym Smash XXL in the free form was significantly lower than the free Panzym YieldMASH.

The most significant results for clarification were observed in both juices treated with the PG enzyme, indicating that the immobilized polygalacturonase may destabilize the colloidal system present in the raw apple juice via pectin depolymerisation.

The best result of the treatment regarding the Panzym YieldMASH compared with the Panzym Smash XXL might be related to the degree of esterification of added pectin used as a substrate, since PL is specific for methyl-esterified galacturonide linkages in pectin molecules.

By comparing the effect of pectinases on fruit juice clarity, the best results were obtained in apple juice, which confirms that a successful clarification process depends on the matrix (i.e., reaction medium, presence of inhibitors) as reported by Sandri *et al*. (2011), Sandri *et al*. (2013) and Pilnik and Voragen (1993). Moreover, the low activity of both immobilized biocatalysts in pomegranate juice can be attributed to the presence of a high quantity of high molecular weight polyphenolic compounds, that probably affected the pore size and structure of PVA gel.

However, further research could examine various strategies to improve the performance of immobilized enzymes in pomegranate juice. It would be possible to modify the immobilization procedure by the entrapment of cross linked enzymes in Lentikats® (pre-

activated). It would also be possible to modify PVA gel composition thus creating a matrix with different structural characteristics (porosity, pore size).

To conclude, in the present chapter, commercial pectinases were immobilized, for the first time, into PVA gel in the form of LentiKats®.

From these results it may be concluded that, despite the scarce immobilization yields, the immobilized enzymes revealed a good adaptability to an acidic solution like fruit juice, and exhibited good reusability for pectin hydrolysis. The best result was obtained in apple juice using a pure polygalaturonase enzyme (Panzym YieldMASH), which resulted in an 80% reduction in turbidity in three following cycles. LentiKats® technology, a simple and cheap immobilization method, revealed promising perspectives for practical applications in the clarification of fruit juice. Taking into account there is no single enzyme that would completely breakdown complex pectins, the results obtained from this research would be useful to develop co-immobilized pectolytic enzyme studies.

6. CONCLUSIONS

Juice clarification strategies have been developed in order to optimize the visual appearance of fruit juices which strongly affects consumer behaviour. The endeavour of this PhD project is to enhance the clarity and to improve the overall quality of marketable fruit juices using tailored enzymatic treatments in free and immobilized forms.

Specifically, the effect of pectinolytic and/or proteolytic clarification treatment on turbidity and on haze-active molecules in pomegranate juice was evaluated immediately after the clarification treatment and during cold storage. A significant and synergic effect of the combined use of pectinase and protease enzymes was thus demonstrated and the best results in terms of turbidity levels and potential haze formation of the juices were consequently obtained. In particular, pomegranate juice samples treated with pectinase and papain showed the lowest turbidity level with a 63% of haze decrease during cold storage.

Data indicated that although pectinolytic and proteolytic treatments did not modify the total amount of pectins, proteins and phenols, they affected the haze forming activity of turbidity-causing molecules. Moreover, it is important to note that enzymatic treatments of this kind did not modify anthocyanin composition and juice color.

These observations motivate us to investigate the effect of complex enzyme concentration (papain and pectinase), incubation temperature and time on clarity and stability of pomegranate juice. Thus, RSM was successfully used to establish optimum process conditions for clarifying pomegranate juice. Significant regression models describing the change of chill haze, turbidity, potential turbidity and clarity with respect to independent variables were established, with R^2 coefficient greater than 0.9 and *adj* R^2 greater than 0.8. The results indicated that the complex enzyme amount was the most important factor influencing the characteristic of pomegranate juice as it exerted a significant influence on all the dependent variables. Moreover, it was found that an increase of the protease-pectinase complex enzyme amount has a direct influence on haze forming activity of protein and phenols. 0.25% of enzyme mixture was the best concentration, reducing the haze forming activity of proteins almost totally at 1 day. The recommended enzymatic treatment condition, graphically obtained using the contour plots, was: temperature 25–30 °C for 100-110 min using 0.22-0.25% (v/v) of protease-pectinase complex enzyme amount (the ratio of protease : pectinase was 1:2).

These results could be used towards a rational selection and an optimization of novel clarification treatments in large-scale pomegranate juice processing development.

Although the enzymes could enhance the clarification of various fruit juices as above demonstrated, immobilization of these enzymes provides several advantages for the food processing industry. Thus, the last part of this PhD project was carried out to examine the possibility of application of PVA-gel, as an immobilized support, with a unique lentil-like shape, biocompatible and non-toxic, in the industrial apple and pomegranate fruit juice process. Despite the scarce immobilization yields, the immobilized enzymes revealed a good adaptability to an acidic solution like fruit juice (pH 3.5), and exhibited good reusability for pectin hydrolysis. However, the best result was obtained only in apple juice using a pure polygalaturonase enzyme (Panzym YieldMASH), which resulted in an 80% reduction in turbidity in an apple pectin solution compared to 95% as found with the soluble enzyme. The value could be considered acceptable for the application of this immobilized system in fruit juice processing, as well as considering that the same % of turbidity reduction was observed in the following two cycles.

Acknowledgment

I would like to thank particularly my supervisor Prof. Marco Esti who gave me the opportunity to carry out this research. I sincerely thank him for his patient guidance, encouragement and advice throughout this study. I particularly thank my fellow researchers and colleagues for their prompt, sympathetic and unconditional help they gave me any time it was needed.

I notably want to acknowledge Prof. Martin Rebros for giving me the opportunity of spend six months in his laboratory, at Institute of Biotechnology, Slovak University of Technology (SK). Thanks to his research team for their dedication to share with me their experience in biotechnology field.

I would like, in conclusion, to thank all persons, whose enthusiasm and academic passion in the field of food biotechnology, made this research possible.

References

Abdullah AGL, Sulaiman NM, Aroua MK., Noor MMM (2007) Response surface optimization of conditions for clarification of carambola fruit juice using a commercial enzyme. *J Food Process Eng* **81**: 65-71.

Aguilera Y, Martin-Cabrejas MA, de Mejia EG (2016) Phenolic compounds in fruits and beverages consumed as part of the mediterranean diet: their role in prevention of chronic diseases. *Phytochem Rev* **15**: 405-423

Alper N, Acar J (2004) Removal of phenolic compounds in pomegranate juices using ultrafiltration and laccase-ultrafiltration combinations. *Food/Nahrung* **48**: 184-187.

Alper N, Onsekizoglu P, Acar J (2011) Effects of various clarification treatments on phenolic compounds and organic acid compositions of pomegranate (*Punica granatum* L.) juice. *J Food Process preserv* **35**: 313-319.

Amin F, Bhatti HN, Bilal M, Asgher M (2017) Improvement of activity, thermo-stability and fruit juice clarification characteristics of fungal exo-polygalacturonase. *Int J Biol Macromol* **95**: 974-984.

Asano K, Shinagawa K, Hashimoto N (1982) Characterization of haze-forming proteins of beer and their roles in chill haze formation. *J Amer Soc Brew Chem* **40**: 147-154.

Aviram M, Rosenblat M, Gaitini D, Nitecki S, Hoffman A, Dornfeld L, Volkova N, Presser D, Attias J, Liker H, Hayek T (2004) Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. *Clinical Nutrition* **23**: 423-433.

Bates RP, Morris JR, Crandall PG (2001) Principles and practices of small-and medium-scale fruit juice processing. FAO Agricultural Services Bulletin 146.

Baumann JW (1981) Application of enzymes in fruit juice technology. In *Enzymes and food processing* Springer Netherlands, pp. 129-147.

Bell C, Hawthorne S (2008) Ellagic acid, pomegranate and prostate cancer: a minireview. *J Pharm Pharmacol* **60**: 139–144.

Benitez EI, Lozano JE (2007) Effect of gelatin on apple juice torbidity. *Latin Am App Research* **37**: 261-266

Beveridge T (2000) Large-scale centrifugation. In: Wilson ID, Adlard ER, Cooke M, Poole CF, *Encyclopedia of Separation Science*, Academic Press, London, pp. 320-342.

Beveridge T, Tait V (1993) Structure and composition of apple juice haze. *Food Struct* **12**: 195-198.

Bhat MK (2000) Cellulases and related enzymes in biotechnology. *Biotechnol adv* **18**: 355-383.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

Buga ML, Ibrahim S, Nok AJ (2010) Partially purified polygalacturonase from *Aspergillus niger* (SA6). *Afr J Biotechnol* **9**: 8944-8954.

Busto MD, García-Tramontín KE, Ortega N, Perez-Mateos M (2006) Preparation and properties of an immobilized pectinlyase for the treatment of fruit juices. *Bioresource Technol* **97**: 1477-1483.

Cantone S, Ferrario V, Corici L, Ebert C, Fattor D, Spizzo P, Gardossi L (2013) Efficient immobilisation of industrial biocatalysts: criteria and constraints for the selection of

organic polymeric carriers and immobilisation methods. *Chem Soc Rev*, **42**: 6262-6276.Cassano A, Conidi C, Drioli E (2011) Clarification and concentration of pomegranate juice (*Punica granatum* L.) using membrane processes. *J Food Eng* **107**: 366-373.

Ceci LN, Lozano JE (2010) Use of enzymes for non-citrus fruit juice production. In Bayindirli A (Ed) *Enzymes in Fruit and Vegetable Processing*, Taylor and Francis Group, pp175.

Chatterjee S, Chatterjee S, Chatterjee BP, Guha AK (2004) Clarification of fruit juice with chitosan. *Process Biochem* **39**: 2229-2232.

Chauhan S, Vohra A, Lakhanpal A, Gupta R (2015) Immobilization of commercial pectinase (Polygalacturonase) on celite and its application in juice clarification. *J Food Process Pres* **39**: 2135-2141.

Chauhan SK, Tyagi SM, Singh D (2001) Pectinolytic liquefaction of apricot, plum, and mango pulps for juice extraction. *International Journal of Food Properties*, **4**: 103-109.

Chen X, Xu F, Qin W, Ma L, Zheng Y(2012) Optimization of enzymatic clarification of green asparagus juice using response surface methodology. *J Food Sci* 77:C665-C670.

Cornish-Bowden A, (2012) Fundamentals of enzyme kinetics, 4th ed, Weinheim:Wiley–Blackwell

Costell E, Tárrega, A, Bayarri S (2010) Food acceptance: The role of consumer perception and attitudes. *Chemosens Percept* **3**: 42-50.

Croak S, Corredig M (2006) The role of pectin in orange juice stabilization: Effect of pectin methylesterase and pectinase activity on the size of cloud particles. *Food Hydrocoll*, **20**: 961-965.

Czichocki G, Dautzenberg H, Capan E, Vorlop KD (2001) New effective entrapment of polyelectrolyte-enzyme-complexes in LentiKats. *Biotechnol Lett* **23**: 1303-1307.

Dae-Ok K, Chang YL (2005) Unit 11.3. HPLC separation of polyphenolics. In Wrolstad RE, Acree TE, Decker EA Penner MH Reid DS, Schwartz, SJ *et al*. (Eds.), *Handbook of food analytical chemistry*, New Jersey:Hoboken, pp. 483-498

Demir N, Acar J, Sarioglu K, Mutlu M (2001) The use of commercial pectinase in fruit juice industry: immobilized pectinase for mash treatment. *J Food Eng* **47**: 275-280.

Di Stefano R, Cravero MC, Gentilini N (1989) Metodi per lo studio dei polifenoli dei vini. *L'enotecnico* 83-89.

Diano N, Grimaldi T, Bianco M, Rossi S, Gabrovska K, Yordanova G, Godjevargova T, Grano V, Nicolucci C, Mita L, Bencivenga U, Canciglia P, Mita DG (2008) Apple juice clarification by immobilized pectolytic enzymes in packed or fluidized bed reactors. *J Agric Food Chem* **56**: 11471-11477.

Diaz-Godinez G, Soriano-Santos J, Augur C, Viniegra-González G (2001) Exopectinases produced by *Aspergillus niger* in solid-state and submerged fermentation: a comparative study. *J Ind Microbiol Biot* **26**: 271-275.

Ding WA, Vorlop KD (1995) Gel aus polyvinylalcohol und verfahren zu seiner herstellung. *DE Pat*. 4327923.

Dinnella C, Stagni A, Lanzarini G (1997) Pectolytic enzymes co-immobilization on γ-alumina spheres via organophosphate compounds. *Process Biochem* **32**: 715–722.

Divakar G, Ellaiah P (2006) Optimization of process parameters for alkaline protease production under solid-state fermentation by *Thermoactinomyces thalpophilis*. *Indian Journal of Biotechnology*, **5**:80-83.

Domingues RCC, Junior SBF, Silva RB, Cardoso VL, Reis MHM (2012) Clarification of passion fruit juice with chitosan: Effects of coagulation process variables and comparison with centrifugation and enzymatic treatments. *Process Biochem* **47**: 467-471.

Erkan-Koç B, Türkyılmaz M, Yemiş O, Özkan M (2015) Effects of various protein-and polysaccharide-based clarification agents on antioxidative compounds and colour of pomegranate juice. *Food Chem* **184**: 37-45.

Esawy MA, Gamala AA, Kamel Z, Ismail AM, Abdel-Fattah AF (2013) Evaluation of free and immobilized *Aspergillus niger* NRC1ami pectinase applicable in industrial processes. *Carbohyd Polym* **92**: 1463-1469.

Escarpa A, Gonzalez MC (2008) An overview of analytical chemistry of phenolic compounds in foods. *Crit Rev Anal Chem* **75**: 57-139.

Fadavi A, Barzegar M, Azizi HM (2006) Determination of fatty acids and total lipid content in oilseed of 25 pomegranates varieties grown in Iran. *J Food Comp Anal* **19**:676–80.

Fang G, Chen H, Zhang Y, Chen A (2016) Immobilization of pectinase onto Fe3O4@SiO2-NH2 and its activity and stability. *Int J Biol Macromolec* **88**: 189-195.

Ferid A, Ferid L (2008) Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: Assay as biodetergent. *Process Biochem* **43**:1202-1208.

Garcia-Salas P, Morales-Soto A, Segura-Carretero A, Fernández-Gutiérrez A (2010) Phenolic-compound-extraction systems for fruit and vegetable samples. *Molecules* **15**: 8813-8826.

Genova G, Iacopini P, Baldi M, Ranieri A, Storchi P, Sebastiani L (2012) Temperature and storage effects on antioxidant activity of juice from red and white grapes. *Int J Food Sci Tech* **47**: 13-23.

Ghosh P, Pradhan RC, Mishra S (2016) Low-temperature extraction of Jamun Juice (Indian Black Berry) and optimization of enzymatic clarification using box-behnken design. *J Food Process Eng* doi: 10.1111/jfpe.12414

Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA (2000) Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J Agric Food Chem* **48**: 4581-4589.

Giménez-Bastida JA, Larrosa M, Gonzaález-Sarrıas A, Tomás-Barberán F, Espín JC, García-Conesa MT (2012) Intestinal ellagitannin metabolites ameliorate cytokine-induced inflammation and associated molecular markers in human colon fibroblasts. *J Agric Food Chem* **60**:8866-8876

Godfrey T, Reichelt J (1983) Industrial enzymology. Surrey, U.K.: Macmillan Publishers Ltd.

Gómez de Segura A, Alcalde M, Plou JF, Remaud-Siméon M, Monsan P, Ballesteros A (2003) Encapsulation in LentiKats of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299, and its effect on product selectivity. *Biocatal Biotransform*, **21**: 325-331.

Grassin C, Fauquembergue F, (1996) Fruit juices. In Godfrey T, West S, (Eds) *Industrial Enzymology*, 2nd ed, Stockholm Press: New York, USA pp. 225–264.

Grassin C, Fauquembergue P (1996) Application of pectinases in beverages. *Progress in Biotechnology* **14**: 453-462.

Grosová Z, Rosenberg M, Gdovin M, Sláviková L, Rebroš M (2009) Production of D-galactose using β-galactosidase and *Saccharomyces cerevisiae* entrapped in poly(vinylalcohol) hydrogel. *Food Chem* **116**: 96-100.

Guyot S, Marnet N, Sanoner P, Drilleau JF (2003) Variability of the polyphenolic composition of cider apple (*Malus domestica*) fruits and juices. *J Agric Food Chem* **51**: 6240-6247.

Henrissat B, Driguez H, Viet C, Shulein M (1985) Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose. *Nat Biotechnol* **3**:722-726.

Hoondal G, Tiwari R, Tewari R, Dahiya N, Beg Q (2002) Microbial alkaline pectinases and their industrial applications: a review. *Appl Microbiol Biotechnol* **59**:409-418.

Hou XJ Chen W (2008) Optimization of extraction process of crude polysaccharides from wild edible BaChu mushroom by response surface methodology. *Carbohyd Polym* **72**: 67-74.

Ipsita R, Meryam S, Munishwar NG (2003) Evaluation of a smart bioconjugate of pectinase for chitin hydrolysis. *Biochem Eng J* **16**: 329–335.

Jaeger H, Janositz A, Knorr D (2010) The Maillard reaction and its control during food processing: the potential of emerging technologies. *Pathol Biol* **58**: 207-213.

Jayani RS, Saxena S, Gupta R (2005) Microbial pectinolytic enzymes: a review. *Process Biochem*, **40:** 2931-2944.

Jiang CM, Wu MC, Chang WH, Chang HM (2001) Change in particle size of pectin reacted with pectinesterase isozymes from pea (*Pisum sativum* L.) sprout. *J Agric Food Chem* **49**: 4383-4387.

Joshi VK, Parmar M, Rana N (2011) Purification and characterization of pectinase produced from Apple pomace and evaluation of its efficacy in fruit juice extraction and clarification. *Indian J Nat Prod Resour* **2**:189-197.

Kapoor M, Beg QK, Bhushan B, Singh K, Dadhich KS, Hoondal GS (2001) Application of an alkaline and thermostable polygalacturonase from *Bacillus* sp. MG-cp-2 in degumming of ramie (*Boehmeria nivea*) and sunn hemp (*Crotalaria juncea*) bast fibres. *Process Biochem* **36**: 803-807.

Kashyap DR, Chandra S, Kaul A, Tewari R (2000) Production, purification and characterization of pectinase from a *Bacillus* sp. DT7. *World J Microbiol Biotechnol* **16**: 277-282.

Kashyap DR, Vohra PK, Chopra S, Tewari R (2001) Applications of pectinases in the commercial sector: a review. *Bioresource Technol* **77**: 215- 227.

Kaur G, Kumar S, Satyanarayana T (2004) Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould *Sporotrichum Termophile*. *Bioresource Technol* **94**: 239-243.

Kaur I, Gupta R, Lakhanpal A, Kumar A (2014) Investigation of immobilization and hydrolytic properties of pectinase onto chitosan-PVA copolymer. *International Journal of Advanced Chemistry* **2**: 117-123.

Khandare V, Walia S, Singh M, Kaur C (2011) Black carrot (*Daucus carota* ssp. *sativus*) juice: Processing effects on antioxidant composition and color. *Food Bioprod Process* **89**: 482-486.

Khuri AI, Mukhopadhyay S (2010) Response surface methodology. *WIREs Comp Stat* **2**: 128-149.

Kilara A (1982) Enzymes and their uses in the processed apple industry: A Review. *Process Biochem* **23**: 35-41.

Kilara A, Van Buren JP (1989) Clarification of apple juice. In Downing DL (Ed) *Processed apple products*. Springer US, pp. 83-96.

Kminkova M, Kucera J (1983) Comparison of pectolytic enzymes using different methods of binding. *Enzyme Microb Technol* **5**:204-208.

Kotwal GJ (2007). Genetic diversity-independent neutralization of pandemic virus-es (e g. HIV), potentially pandemic (e.g. H5N1 strain of influenza) and carcinogenic (e.g. HBV and HCV) viruses and possible agents of bioterrorism (variola)by enveloped virus neutralizing compounds (EVNCs). Vaccine **26**: 3055-3058.

Krajewska B (2004) Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. *Enzyme Microb Technol* **35**: 126-139.

Landbo AK, Meyer AS, (2004) Effects of different maceration treatments on enhancement of anthocyanins and other phenolic in black currant juice. *Innov Food Sci Eme* **5**: 503-513.

Landbo AKR, Pinelo M, Vikbjerg AF, Let MB, Meyer AS (2006) Protease-assisted clarification of black currant juice: synergy with other clarifying agents and effects on the phenol content. *J Agric Food Chem* **54**: 6554-6563.

Lauwers A, Scharp ES (1997). Pharmaceutical enzymes, drugs and pharmaceutical sciences. New York-Basel-Hong Kong: Marcel Dekker, Inc.

Lee WC, Yusof S, Hamid NSA, Baharin BS (2006) Optimizing conditions for enzymatic clarification of banana juice using response surface methodology (RSM). *J Food Eng* **73**: 55-63.

Lee WC, Yusof S, Hamid NSA, Baharin BS (2007) Effects of fining treatment and storage temperature on the quality of clarified banana juice. LWT-*Food Sci Technol* **40**: 1755-1764.

Lei Z, Bi S (2007) The silica-coated chitosan particle from a layer-by-layer approach for pectinase immobilization. *Enzyme Microb Technol* **40**: 1442-1447.

Li T, Li S, Wang N, Lirui T (2008) Immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support. *Food Chem* **109**: 703-708.

Li T, Wang N, Li S, Zhao Q, Guo M, Zhang C (2007) Optimization of covalent immobilization of pectinase on sodium alginate support. *Biotechnol Lett* **29**: 1413-1416.

López-Otín C, Bond JS (2008) Proteases: multifunctional enzymes in life and disease. *J Biol Chem* **283**: 30433-30437.

Lozano JE (2003) Separation and clarification. In Encyclopedia of Food Science and Nutrition. Caballero B, Trugo L, Finglas P, eds.) London, England: AP Editorial Elsevier pp. 5187-5196.

Lozano P, Manjón A, Romojaro F, Canovas M, Iborra JL (1987) A cross flow reactor with immobilized pectolytic enzymes for juice clarification. *Biotechnol Lett* **9**: 875-880.

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol Mol Biol Rev* **66**:506-77.

Martí N, Perez-Vicente A, García-Viguera C (2002) Influence of storage temperature and ascorbic acid addition on pomegranate juice. *J Sci Food Agric* **82**: 217-221.

Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R (2007) Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme Microb Technol* **40**:1451-1463.

McMurrough I, Kelly R, Byrne J (1992) Effect of the removal of sensitive proteins and proanthocyanidins on the colloidal stability of lager beer. *J Amer Soc Brew Chem* **50**: 67-76.

Meyer AS, Köser C, Adler-Nissen J (2001) Efficiency of enzymatic and other alternative clarification and fining treatments on turbidity and haze in cherry juice. *J Agric Food Chem* **49**: 3644-3650.

Miguel G, Fontes C, Antunes D, Neves A, Martins D (2004) Anthocyanin concentration of "Assaria" pomegranate fruits during different cold storage conditions. *J Biomed Biotechnol* **5**:338-342.

Miller GL (1959) Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Anal Chem* **31**: 426-428.

Mirsaeedghazi H, Emam-Djomeh Z, Mousavi SM, Aroujalian A, Navidbakhsh M (2010) Clarification of pomegranate juice by microfiltration with PVDF membranes. *Desalination* **264**:243-248.

Mitchell AE, Hong YJ, Calemay J, Wright CA, Bowforth CW (2005) A comparison of polyvinypolypyrrolidone (PVPP), silica xerogel and polyvinylpyrrolidone (PVP)-silica co product for their ability to remove polyphenols from beer. *J Inst Brew* **111**: 20-25.

Mohnen D (2008) Pectin structure and biosynthesis. *Curr Opin Plant Biol* **11**: 266-277.

Montgomery DC (2008) Design and analysis of experiments. John Wiley & Sons.

Mousavinejad G, Emam-Djomeh Z, Rezaei K, Hossein M, Khodaparast H (2009) Identification and quantification of phenolic compounds and their effects on antioxidant activity in pomegranate juices of eight Iranian cultivars. *Food Chem* **115**: 1274-1278.

Mutlu M, Sarioglu K, Demir N, Ercan MT, Acar J (1999) The use of commercial pectinase in fruit juice industry. Part I: viscosimetric determination of enzyme activity. *J Food Eng* **41**: 147-150.

Myers RH, Montgomery DC, Anderson-Cook CM (2016) Response surface methodology: : process and product optimization using designed experiments. 2nd ed, John Wiley & Sons.

Neifar M, Ellouze-Ghorbel R, Kamoun A, Baklouti, S, Mokni, A, Jaouani A, Ellouze-Chaabouni S (2011). Effective clarification of pomegranate juice using laccase treatment optimized by response surface methodology followed by ultrafiltration. *J Food Process Eng* **34**: 1199-1219.

Nur'Aliaa AR, Siti Mazlina MK, Taip FS, Liew Abdullah AG (2010) Response surface optimization for clarification of white pitaya juice using a commercial enzyme. *J Food Process Eng* **33**: 333-347.

Onofri A (2006) Enhancing Excel capability to perform statistical analyses in agriculture applied research. In International Association for statistical Computing (eds) *Computational statistics and data analysis - Statistical Software Newsletters*.

Onsekizoglu P (2013). Production of high quality clarified pomegranate juice concentrate by membrane processes. *J Membr Sci* **442**: 264-271.

Ortega N, de Diego S, Perez-Mateos M, Busto MD (2004a) Kinetic properties and thermal behaviour of polygalacturonase used in fruit juice clarification *Food Chem* **88**: 209-217.

Ortega N, de Diego S, Rodríguez-Nogales JM, Perez-Mateos M, Busto MD (2004b) Kinetic behaviour and thermal inactivation of pectinlyase used in food processing. *Int J Food Sci Tech* **39**: 631-639.

Oszmiański J, Wojdyło A (2007) Effects of various clarification treatments on phenolic compounds and color of apple juice. *Eur Food Res Technol* **224**: 755-762.

Park MK, Kim CH (2010) Extraction of polyphenols from apple peel using cellulose and pectinase and estimation of antioxidant activity. *Korea Agricultural Science Digital Library*, **38**: 535-540.

Pasha KM, Anuradha P, Subbarao D (2013) Applications of pectinases in industrial sector. *Int J Pure Appl Sci Technol* **16**:89-95

Patil SR, Dayanand A (2006) Production of pectinase from deseeded sunflower head by *Aspergillus niger* in submerged and solid-state conditions. *Bioresource Technol* **97**:2054-2058.

Pedrolli DB, Monteiro AC, Gomes E, Carmona EC (2009) Pectin and pectinases: production, characterization and industrial application of microbial pectinolytic enzymes. *Open Biotechnology J* **3**:9-18.

Pilnik W, Voragen AGJ (1993) Pectic enzymes in fruit and vegetable juice manufacture. In: Nagodawithana T, Reed G (eds) *Enzymes in Food Processing* New York, Academic Press, pp. 363-399.

Pinelo M, Zeuner B, Meyer AS (2010) Juice clarification by protease and pectinase treatments indicates new roles of pectin and protein in cherry juice turbidity. *Food Bioprod Process* **88**: 259-265.

Pocock KF, Alexander GM, Hayasaka Y, Jones PR, Waters EJ (2007) Sulfate a candidate for the missing essential factor that is required for the formation of protein haze in white wine. *J Agric Food Chem* **55**: 1799-1807.

Purich DL, (2010) *Enzyme kinetics: catalysis & control: a reference of theory and best-practice methods.*1st ed, Elsevier.

Rai P, Majumdar GC, Gupta DS, De S. (2004)Optimizing pectinase usage in pretreatment of mosambi juice for clarification by response surface methodology. *J Food Eng* **64**:397-403.

Rajdeo K, Harini T, Lavanya K, Fadnavis NW (2016) Immobilization of pectinase on reusable polymer support for clarification of apple juice. *Food Bioprod Process* **99**: 12-19.

Rao L, Hayat K, Lv Y, Karangwa E, Xia SQ, Jia C, Zhonga F, Zhang X (2011) Effect of ultrafiltration and fining adsorbents on the clarification of green tea. *J Food Eng* **102**: 321-326.

Rebroš M, Rosenberg M, Mlichová Z, Krištofíková Ľ (2007) Hydrolysis of sucrose by invertase entrapped in polyvinyl alcohol hydrogel capsules. *Food Chem* **102**: 784-787.

Rebroš M, Rosenberg M, Mlichová Z, Krištofíková Ľ, Palúch M (2006) Entrapment of glucoamylase into LentiKats®: properties and effective application to maltodextrin hydrolysis. *Enzyme Microb Technol* **39**: 800-804.

Reddy MK, Gupta SK, Jacob MR, Khan SI, Ferreira D (2007) Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. *Planta Med* **73**:461-467.

Rehman HU, Aman A, Silipo A, Qader SAU, Molinaro A, Ansari A (2013) Degradation of complex carbohydrate: immobilization of pectinase from *Bacillus licheniformis* KIBGE-IB21 using calcium alginate as a support. *Food Chem* **139**: 1081-1086.

Rehman HU, Aman A, Zohra RR, Qader SAU (2014) Immobilization of pectin degrading enzyme from *Bacillus licheniformis* KIBGE IB-21 using agar-agar as a support. *Carbohyd Polym* **102**: 622- 626.

Rehmanji M, Gopal C, Mola A (2002) A novel stabilization of beer with Polyclar BrewbriteTM. *Master Brewers Association of the Americas*, **39**: 24-28.

Rinaldi M, Caligiani A, Borgese R, Palla G, Barbanti D, Massini R (2013) The effect of fruit processing and enzymatic treatments on pomegranate juice composition, antioxidant activity and polyphenols content. LWT-*Food Sci Technol* **53**: 355-359.

Rungsardthong V, Wonputtanakul N, Kongpien N, Chotiwaranon P (2006) Application of fungal chitosan for clarification of apple juice. *Process Biochem* **41**:589-93.

Sandri IG, Fontana RC, Barfknecht DM, da Silveira MM (2011) Clarification of fruit juices by fungal pectinases. LWT-*Food Sci Technol* **44**: 2217-2222.

Sandri IG, Fontana RC, Barfknecht DM, Silveira MM (2011) Clarification of fruit juices by fungal pectinases. LWT-*Food Sci Technol* **44**: 2217-2222.

Sandri IG, Lorenzoni CMT, Fontana RC, Silveira MM (2013) Use of pectinases produced by a new strain of *Aspergillus niger* for the enzymatic treatment of apple and blueberry juice. LWT-*Food Sci Technol* **51**: 469-475.

Sarvamangala PR Dayanand A (2006) Exploration of Regional Agrowastes for the Production of Pectinase by *Aspergillus niger. Food Technol Biotechnol* **44**: 289-292.

Saxena S, Shukla S, Thakur A, Gupta R (2008) Immobilization of polygalacturonase from *Aspergillus niger* onto activated polyethylene and its application in apple juice clarification. *Acta Microbiol Immunol Hung* **55**: 33-51.

Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, Heber D (2005) In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *J Nutr Biochem* **16**: 360-367.

Sepúlveda E, Saenz, C, Peña A, Robert P, Bartolomé B, Gómez-Cordovés C (2010) Influence of the genotype on the anthocyanin composition, antioxidant capacity and color of Chilean pomegranate (*Punica granatum* L.) juices. Chil J Agric Res **70**: 50-57.

Shallom D and Shoham Y (2003) Microbial hemicellulase. *Curr Opin* Microbiol **6**: 219-228.

Sharma HP, Patel H, Sugandha SP (2016) Enzymatic Extraction and Clarification of Juice from Various Fruits–A Review. Crit Rev Food Sci Nutr **5**: 0.

Sheldon RA (2007) Enzyme Immobilization: The Quest for Optimum Performance. *Adv Synth Catal* **349**: 1289-1307.

Sheldon RA, van Pelt S (2013) Enzyme immobilization in biocatalysis: why, what and how. Chem Soc Rev **42**: 6223-6235.

Showalter AM (1993) Structure and function of plant cell wall proteins. *Plant Cell* **5**: 1-9

Shulein CM (1985) Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose. *Nat Biotechnol* **3**:722-726.

Siebert KJ (1999) Effects of protein–polyphenol interactions on beverage haze, stabilization, and analysis. *J Agric Food Chem* **47**: 353–362.

Siebert KJ (2006) Haze formation in beverages. LWT-*Food Sci Technol* **29**: 987-994.

Siebert KJ, Carrasco A, Lynn PY (1996) Formation of protein-polyphenol haze in beverages. *J Agric Food Chem* **44**: 1997–2005.

Siebert KJ, Lynn PY (1997) Haze-active protein and polyphenols in apple juice assessed by turbidimetry. *J Food Sci* **62**: 79-84.

Siebert KJ, Lynn PY (2000) Effect of protein/polyphenol ratio on the size of haze particles. *J Amer Soc Brew Chem* **58**: 117-123.

Sin HN, Yusof S, Sheikh Abdul Hamid N, Rahman R Abd (2006) Optimization of enzymatic clarification of sapodilla juice using response surface methodology *J Food Eng* **73**: 313–319.

Sizer IW (2006) Effects of temperature on enzyme kinetics. *Enzymol Relat Areas Mol Biol* **3**: 35-62.

Sorrivas V, Genovese DB, Lozano JE (2006) Effect of pectinolytic and amylolytic enzymes on apple juice turbidity. *J Food Process Pres* **30**: 118-133.

Spagna G, Pifferi PG, Rangoni C, Mattivi F, Nicolini G, Palmonari R (1996) The stabilization of white wines by adsorption of phenolic compounds on chitin and chitosan. *Food Res Int* **29**:241–8.

Srilakshmi J, Madhavi J, Lavanya S, Ammani K (2015) *J Pharm Chem Biol Sci* **2**:218-234.

Stewart GG (2016) Beer Shelf Life and Stability. In Subramaniam P and Wareing P (eds) *The Stability and Shelf Life of Food*, pp. 293-309.Stutz C (1993) The use of enzymes in ultrafiltration. Fruit processing (Germany).

Sulaiman MZ, Sulaiman, NM, Liew SY (1998) Limiting permeate flux in the clarification of untreated starfruit juice by membrane ultrafiltration. *Chem Eng J* **69**: 145-148.

Tajchakavit S, Boye JI, Couture R (2001) Effect of processing on post-bottling haze formation in apple juice. *Food Res Int* **34**: 415-424.

Takei T, Ikeda K, Ijima H, Kawakami K (2011) Fabrication of poly (vinyl alcohol) hydrogel beads crosslinked using sodium sulfate for microorganism immobilization. *Process Biochem* **46**: 566-571.

Tastan O, Baysal T (2015) Clarification of pomegranate juice with chitosan: Changes on quality characteristics during storage. *Food Chem* **180**: 211-218.

Tehranifara A, Zarei M, Nemati Z, Esfandiyaria B, Vazifeshenas MR (2010) Investigation of physico-chemical properties and antioxidant activity of twenty Iranian pomegranate (*Punica granatum* L.) cultivars. *Sci Hort* **126**: 180-185.

Tischer W, Wedekind F (1999) Immobilized enzymes: methods and applications. In Fessner WD, Archelas A, Demirjian DC, Furstoss R, Griengl H, Jaeger KE, Morís-Varas E, Öhrlein R, Reetz MT, Reymond JL, Schmidt M, Servi S, Shah PC, Tischer W, Wedekind F (eds) *Biocatalysis-from discovery to application* Berlin: Springer, pp. 95-126.

Turfan Ö, Türkyılmaz M, Yemis O, Özkan M (2011) Anthocyanin and colour changes during processing of pomegranate (*Punica granatum* L., cv. Hicaznar) juice from sacs and whole fruit. *Food Chem* **129**: 1644-1651.

Turfan Ö, Türkyılmaz M, Yemis O, Özkan M (2012). Effects of clarification and storage on anthocyanins and color of pomegranate juice concentrates. *J Food Quality* **35**: 272-282.

Tzulker R, Glazer I, Bar-Ilan I, Holland D, Aviram M, Amir R (2007) Antioxidant activity, polyphenol content, and related compounds in different fruit juices and homogenates prepared from 29 different pomegranate accessions. *J Agric Food Chem*, **55**: 9559-9570.

Uzuner S, Cekmecelioglu D (2015) Optimising clarification of carrot juice by bacterial crude pectinase. *Int J Food Sci Tech* **50**: 2707-2712.

Vaillant F, Millan A, Dornier M, Decloux M, Reynes M (2001) Strategy for economical optimisation of the clarification of pulpy fruit juices using crossflow microfiltration. *J Food Eng* **48**: 83-90.

Vaillant F, Millan A, Millan P, Dornier M, Decloux M, Reynes M (2000) Co-immobilized pectinlyase and endocellulase on chitin and nylon supports. *Process Biochem* **35**:989-996.

Vaillant F, Millan P, Brien GO, Dornier M, Decloux M, Reynes M (1999) Crossow microfiltration of passion fruit juice after partial enzymatic liquefaction. *J Food Eng* **42**: 215-224.

Van Buren JP (1989) Causes and prevention of turbidity in apple juice. In Downing DL (Ed) *Processed apple products* Van Nostrand Reinhold, pp. 97-120.

Van de Berg MA, Roubos JA, Pařenicová L (2010) Enzymes in fruit and vegetable processing. Future Trends in Enzyme Discovery, Design, Production, and Application. In Bayindirli A (Ed) *Enzymes in Fruit and Vegetable Processing*, Taylor and Francis Group pp 341-358.

Vardin H, Fenercioglu H (2003) Study on the development of pomegranate juice processing technology: Clarification of pomegranate juice. *Nahrung*, **47**: 300-303.

Vincenzi S, Marangon M, Tolin S, Curioni A (2011) Protein evolution during the early stages of white winemaking and its relations with wine stability. *Aust J Grape Wine Res* **17**: 20-27.

Viuda-Martos M, Fernandez-Lopez J,. Perez-Alvarez JA (2010) Pomegranate and its many functional components as related to human health: A Review. *Comp Rev Food Sci Food* **9**: 635–654.

Wang Y, Hsieh YL (2008) Immobilization of lipase enzymes in polyvinyl alcohol (PVA) nanofibrous membranes. *J Membr Sci* **309**: 73-81.

Wood TM, Garica-Campayo V (1990) Enzymology of cellulose degradation. *Biodegradation* **1**:147–61.

Wu LC, Lu YW (2004) Electrophoretic method for the identification of a haze-active protein in grape seeds. *J Agric Food Chem* **52**: 3130-3135.

Wu LC, Siebert KJ (2002) Characterization of haze-active proteins in apple juice. *J Agric Food Chem* **50**: 3828-3834.

Wu X, Prior RL (2005) Identification and characterization of anthocyanins by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry in common foods in the United States: vegetables, nuts, and grains. *J Agric Food Chem* **53**: 3101-3113.

Xu, X., Yan, H., Chen, J., Zhang, X. (2011). Bioactive proteins from mushrooms. Biotechnology advances, **29**: 667-674.

Yi XX, Wang F, Pang MX, Tan F, Qi JH (2013) Effect of Processing on the Secondary Haze Formation in Clarified Apple Juice. In Advanced Materials Research, Trans Tech Publications, pp. 1464-1468.

Yousefnezhad B, Mirsaeedghazi H, Arabhosseini A (2016) Pretreatment of pomegranate and red beet juices by centrifugation before membrane clarification: a comparative study. *J Food Process Preserv* doi:10.1111/jfpp.12765.

Zhang YHP, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies. *Biotechnol Adv* **24**:452-481.

Zhang Y-HP, Lynd LR (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulose systems. *Biotechnol Bioeng* **88**:797-824.

Appendix

List of Scientific Publications

1. **Cerreti M,** Liburdi K, Benucci I, Emiliani Spinelli S, Lombardelli C, Esti M (2017) Optimization of pectinase and protease clarification treatment of pomegranate juice. *LWT- Food Sci Tec* **82**: 58-65.
2. **Cerreti M**, Markošová K, Esti M, Rosenberg M, Rebroš M (2017) Immobilization of pectinases into PVA gel for fruit juice application. *Int J Food Sci Tech* 52: 531-539.
3. **Cerreti M**, Ferranti P, Benucci I, Liburdi K, de Simone C, Esti M. (2017). Thiol precursors in Grechetto grape juice and aromatic expression in wine. *Eur Food Res Technol* 243: 753-760.
4. **Cerreti M**, Liburdi K, Benucci I, Esti M (2016) The effect of pectinase and protease treatment on turbidity and on haze active molecules in pomegranate juice. *LWT- Food Sci Tec* **73**: 326-333.
5. **Cerreti M**, Fidaleo M, Benucci I, Liburdi K, Tamborra P, Moresi M (2016) Assessing the potential content of ethyl carbamate in white, red, and rosé wines as a key factor for pursuing urea degradation by purified acid urease. *J Food Sci* **81**: C1603-C1612.
6. Benucci I, Liburdi K, **Cerreti M**, Esti M (2016) Characterization of active dry wine yeast during starter culture (pied de cuve) preparation for sparkling wine production. *J Food Sci* **81**: M2015-M2020.
7. **Cerreti M**, Esti M, Benucci I, Liburdi K, de Simone C, Ferranti P (2015) Evolution of S-cysteinylated and S-glutathionylated thiol precursors during grape ripening of *Vitis vinifera* L. cvs Grechetto, Malvasia del Lazio and Sauvignon Blanc. *Aust J Grape Wine Res* **21**: 411-416.

Poster and Oral Communications to Congresses

1. **Oral Presentation** on "Multiple enzyme-assisted clarification process of fruit juice " in 21st Workshop on the Developments in the Italian PhD Research in Food Science and Technology" organized by Italian Network of the PhD Courses in Food

Science Technology & Biotecnology (University of Naples Federico II) Portici (NA), 14th-16 th September, 2016.

2. **Presented poster** on "Application of immobilized acid urease in red wine using fluidized-bed reactor" in 42nd International Conference of Slovak Society of Chemical Engineering (SSCHE) - organized by SSCHE in Vysoké Tatry, Slovak Republic on 23th to 27th May, 2015.
3. **Presented poster** on "Fruit juice clarification by immobilized pectinases on PVA-gel support" in 20st Workshop on the Developments in the Italian PhD Research in Food Science and Technology" organized by Italian Network of the PhD Courses in Food Science Technology & Biotecnology (University of Perugia) Perugia (PG) 23rd-25th September, 2015.
4. **Presented poster** on "Fruit juice clarification using immobilized enzymes" in 19th Workshop on the Developments in the Italian PhD Research in Food Science and Technology" organized by Italian Network of the PhD Courses in Food Science Technology & Biotecnology (University of Bari Aldo Moro) Bari (BA) 24th-26th September, 2014.
5. **Presented poster** on **"**Tioli volatili in vino da uve Grechetto (Vitis vinifera L.)" in Enoforum 2013 organized by Società Italiana di Viticoltura e Enologia, Arezzo (AR) 7th-9 th May, 2013.