



Research article

Pectinase hydrolysis of guava pulp: effect on the physicochemical characteristics of its juice

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ABSTRACT

The objective of this research is to assess the effect of enzymatic treatment of guava puree on the physicochemical parameters of the juice. Pectinases from *Aspergillus niger* were applied to the puree at 43 ± 3 °C under constant stirring. Enzyme concentrations used were: 0.033 % (w/w), 0.055% (w/w), 0.078 % (w/w) and 0.1 % (w/w). For each enzyme concentration, the treatment times were varied from 3 – 90 min. Physicochemical parameters of raw puree and enzymatically treated juice were determined. These were: viscosity, pH, electric conductivity, protein and polyphenol content, galacturonic acid content, color, TSS, and antioxidant capacity. Particle distribution, homogeneity of raw puree and juice samples dried extracts were assessed using a Field Emission Scanning Electron Microscopy (FESEM). A 91% viscosity decrease was recorded for each enzyme concentration after 3 min of enzyme reaction. That decrease was accompanied by an increase in galacturonic acid content with increasing depectinization factors. Enzyme treatment of guava puree led to a decrease in pH, protein and polyphenol contents and an increase in conductivity and color. Analysis of FESEM images of guava samples bestowed a decrease in particle size, a scattering of particles in the medium, an increase in continuous phase proportion and an improvement of sample homogeneity with increasing values of processing parameters, due to the breaking-down of bigger particles and the solubilization during depectinization.

1. Introduction

Native from Mexico, Peru, USA and Columbia, Guava known as *Psidium guajava*, belonging to family of *Myrtaceae*, is a tropical and subtropical fruit widely found in different countries throughout South America, Asia, Europe, and Africa (Ninga et al., 2018). Guava is well appreciated for antioxidant capacity of its phenolic compound and its vitamin A and C content. It contains high concentration of vitamin C (100–200 mg/100 g), more than fresh orange juice (60–80 mg/100 g) (Akesowan and Choonhahirun, 2013; Surajbhan et al., 2012). It is a highly perishable climacteric fruit with a shelf-life of 3–4 days when stored at room temperature. It can be consumed freshly or processed into different products: juice, puree, jam, jelly, concentrate, nectar (Wu et al., 2005).

Guava is rich in pectin, a complex polysaccharide made of galacturonic acid units linked by α – (1–4) galactosidic bonds. Two different chains can be found pectin structure, homogalacturonan, also known as

“smooth” region, and rhamnogalacturonan I, known as “hairy” region, branched to the former one (Combo). Due to its molecular weight, its degree of methoxylation (DM) and its hydroxyl groups, pectin exhibit a high water retention capacity yielding to a high viscosity of the puree. Moreover during guava juice clarification, pectin molecules cause membrane fouling, resulting in low juice recovery yield (Lee et al., 2006). To increase that yield, clarification process may require several preliminary extraction steps that are: hot, cold and enzymatic extraction. Compared to the first two preliminary steps, the enzymatic one is known to be that with an appreciable juice recovery yield (Sharma Harsh et al., 2016).

Pectinases, used during enzymatic treatment of guava puree, will break-down pectin molecules into smaller oligagalacturonans causing pectin-protein complexes to flocculate, so that the resultant juice has much lower pectin and viscosity. The resulting enzymatically treated can therefore be easily clarified through centrifugation or filtration. Attributes of the juice such as clarity, aroma and flavor increase after

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clarification (Sharma Harsh et al., 2014; Ninga et al., 2018). Research works published on enzymatic treatment of guava puree highlighted a reduction in viscosity, turbidity, and pH, an increase in the juice extraction efficiency, increased color attributes, titratable acidity, total soluble solids, and clarity. There was also an increase in the concentration of neutral sugar, uronic acids, and methanol (Akesowan and Choonhahirun, 2013; Kaur et al., 2011; Marcelin et al., 2017; Nguyen et al., 2013; Nso et al., 1998; Surajbhan et al., 2012; Thi Thuy Le et al., 2012). Guava juice contains proteins and polyphenols which are susceptible of haze formation due to interactions between them, leading to cloud and tannin appearance. The effect of enzymatic treatment of guava puree on these two parameters as well as the forementioned ones will help in assessing the storage stability of the juice. It is also essential to correlate changes in physicochemical parameters with morphological modifications of the particle due to macromolecular network break-down during the enzymatic depectinisation of guava juice. Hence, this paper aims to assess the impact of enzyme hydrolysis of guava puree on the physicochemical characteristics of its juice and visualize the morphological modifications that occur all along the enzymatic depectinisation.

2. Materials and methods

2.1. Biological material

Guava fruits (*Psidium guajava*), used for the experiments, were purchased from local market in Kharagpur, West Bengal, India. They were selected based on their degree of maturity and ripeness.

Pectinase used in the present study (isolated from *Aspergillus niger*, activity: 8000–12000 U/g, dry extract) was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Other chemicals are: bovine serum albumin; pectin; sodium potassium tartrate; gallic acid; D-galacturonic acid monohydrate; 2,2-Diphenyl-1-picrylhydrazyl (DPPH); copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$); Folin Ciocalteu's phenol reagent. All chemicals were of analytical grade.

2.2. Methods

2.2.1. Guava juice extraction

Guava juice was prepared following the procedure described by Ninga et al. (2018). Mature and ripened guava fruits were processed into guava puree following these steps: washing followed by rinsing using tap water, removal of blemishes by trimming, manual deseeding by a 16 – mesh sieve, blending using a food processor and mixing of the puree with demineralized water at a ratio of 35% w/w. The guava fruits of proper maturity and ripeness were washed using tap water, rinsed, trimmed to remove blemishes, cut in small pieces and deseeded manually by using a 16-mesh sieve. The puree was blended using a food processor without addition of water. The bulk solution was packaged in plastic bottles and stored in the freezer for further experiments.

2.2.2. Enzyme treatment of the pulp

The guava pulp was thawed before use, and 100 g of pulp was weighed in a 250 mL beaker, as described by Ninga et al. (2018). The beaker was put in a water bath at $45 \pm 2^\circ\text{C}$ temperature for 5 min under continuous stirring. Dried *A. niger* pectinase extract was added in each sample under continuous stirring using a Remi Motor agitator, type RQ 122 (supplied by Elektrotechnik Ltd, Kolkata, India) at 1500 rpm. The depectinization parameters were as followed: enzyme concentrations: 0.033%, 0.055%, 0.078% and 0.1% weight of dried extract/weight of initial guava pulp; incubation time: 3, 5, 8, 12, 15, 18, 20, 40, 60, 80 and 90 min. The depectinisation was stopped by a heat treatment at 95°C for 5 min. That required the transferring of the solution into a glass sampling bottle followed by heat treatment in boiling water bath. After cooling at the room temperature, the mixture was filtered by a cheese cloth (200 μm mesh size). The filtrate was collected and kept in polyethylene

terephthalate (PET) bottles, and stored in the freezer for further analysis. The experiments were performed in triplicate.

2.3. Analysis of physicochemical parameters of guava juice samples

For each sample of juice, some analyzes were executed to estimate the parameters which are: the viscosity, the reducing sugar content, the polyphenol content, the protein content, the antioxidant capacity. To these parameters are added the pH, the conductivity, total soluble solids, and the color.

2.3.1. Determination of viscosity

The juice viscosity was estimated at ambient temperature ($30 \pm 2^\circ\text{C}$) using capillary viscometer said Ostwald (Pisco, Calcutta, India) (Jain and De, 2016).

Knowing the dynamic viscosity of water at room temperature, the dynamic viscosity of the juice was determined from the equality of the flow time ratio and dynamic viscosity as shown in Eq. (1).

$$\mu_{\text{jus}}(\text{mPa.s}) = \frac{t_1}{t_0} \times \mu_{\text{eau}} \quad \text{Eq. 1}$$

with μ_{jus} μ_{eau} the dynamic viscosity of the juice and water in mPa.s, respectively.

To evaluate the effect of processing parameters on the viscosity of the juice, the viscosity value was plotted against time and the enzyme concentration.

2.3.2. Determination of pH, electric conductivity and total soluble sugars

The pH and conductivity were measured using a pocket tester (Eutech Instruments Ltd, Singapore) at room temperature ($30 \pm 2^\circ\text{C}$). Demineralized water (obtained from Millipore reverse osmosis device, Surepro pre-filtration System Merck Life Science Private Limited, Bangalore, India) was utilized as blank. The calibration of the apparatus required a preliminary step electrode washing with deionized water. Then, in a beaker containing deionized water, the electrodes were immersed. The electrical conductivity and pH were measured of the juice samples were measured by introducing the electrode in the sample. After this determination, electrodes were rinsed with deionized water.

The total soluble solids (TSS) were calculated by a laboratory refractometer with digital display (Digital Lab Refractometer Salinity - 300034, Sper Scientific, Scottsdale, Arizona, United States of America). Demineralized water was therefore used as blank. For this, a few droplets of water were put on the playback interface. The determination of the value of TSS was made by pressing the Read and the value of TSS was posted on the screen. The playback interface has been cleaned with kimwipe to remove any trace of water that may influence the reading. Some guava juice droplets were also filed on the playback interface and the value of TSS measured as in the case of deionized water.

2.3.3. Determination of color

The color of guava juice was established using the spectrophotometric method by reading the absorbance at 420nm using a UV-visible spectrophotometer (M/s Perkin Elmer, Connecticut, USA) (Jain and De, 2016). For this, the sample was diluted to one-tenth with deionized water in 25mL test tubes. The sample color value was obtained multiplying the absorbance value by 10. The deionized water was utilized as blank.

2.3.4. Determination of protein content

Sodium bicarbonate solution (Lowry Solution A) was realized by dissolving 1 g of Na_2CO_3 (Merck Specialties Private Limited, Mumbai, India) in 50 mL of a 0.1N NaOH solution. The Lowry B solution was obtained by dissolving in water (5 mL), a mixture of 25 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Merck Specialties Private Limited, Mumbai, India), and 50 mg of $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ (Loba Chemie Pvt. Ltd., Mumbai, India). The two solutions were subsequently mixed gradually to prepare the solution of

Lowry. The commercial Folin-Ciocalteu reagent (Loba Chemie Pvt. Ltd., Mumbai, India) was diluted to half (1/2) using distilled water. The calibration range was achieved as follows. From a BSA solution (bovine serum albumin) (HiMedia Laboratories Pvt. Ltd., Mumbai, India) standard of 2.5 g/L, a suitable concentration of the standard assay solution was prepared to achieve a range from 0 to 250 mcg of protein. Each tube was filled to 1 mL with distilled water. 5 mL of Lowry reagent was thereafter adjoined to each tube. After stirring, the mixture was permitted to rest for 10 min. The Folin-Ciocalteu solution (0.5 mL) was then introduced and the tube was homogenized, then permitted to stay in the dark for 30 min. Absorbance was obtained at 660 nm against the blank prepared by replacing 1 mL of the sample by deionized water (1 mL). The concentration of protein guava juice was obtained using the previous protocol and, replacing the BSA solution by 1 mL of the sample. The amount of protein in each test sample was calculated as in Eq. (2):

$$[\text{protein}] (\text{mg.l}^{-1}) = \frac{\text{Abs}}{\alpha} \quad \text{Eq. 2}$$

with: [Protein]: protein concentration; Abs: sample absorbance; α : calibration curve slope.

2.3.5. Determination of galacturonic acid

The determination of the concentration of galacturonic acid in the samples was obtained using the method cyanoacetamide (OIV, 2009) with some modifications. The supernatant (2 mL) from the centrifugation of the sample mixture of ethanol (99 % v/v) was diluted in a volumetric flask. This dilution (2 mL) was inserted in a test tube, and then augmented with 4 mL of borate buffer (100 mM; pH 9.0). (Loba Chemie Pvt Ltd., Mumbai, India). and 2 mL of cyanoacetamide solution (1% w/v) (Spectrochem Pvt. Ltd. Mumbai, India). The test tube was deposited in a water bath (Remi model RSB - 12, Remi Elektronik Ltd, Maharashtra, India) at 95 °C for 10 min. After heating, the test tubes were cooled to room temperature by putting them in a water bath (30 ± 2 °C). The optical density of samples was read at 273 nm. The concentration of galacturonic acid was calculated by considering the calibration curve using the galacturonic acid at concentrations of 0–250 µg/mL, with a dilution factor of 50 and using Eq. (3).

$$[\text{AcG}] (\text{mg.l}^{-1}) = \frac{\text{Abs}}{\alpha} \times 50 \quad \text{Eq. 3}$$

with: [AcG]: the galacturonic acid content; Abs: absorbance of the sample; α : the slope of the calibration curve.

2.3.6. Determination of the total polyphenol content

The protocol used for the estimation of the total polyphenol content is related to the literature (Sagu et al., 2014). The juice/blank/standard (0.5 mL) was introduced in 25 mL tubes and supplemented with 0.5 mL of Folin Ciocalteu reagent. The mixture was incubated for 5 min to facilitate the reaction with stirring. The anhydrous sodium carbonate (75 g/L, 10 mL) was introduced and mixed. The tube was then filled to 25 mL with distilled water. After mixing, the tubes were permitted to rest at room temperature (30 ± 2 °C) for 1 h. Absorbance was measured at 750 nm using UV spectrophotometer - Visible. Gallic acid (Sigma Aldrich, Slovakia) was utilized for the calibration curve. The results were formulated as mg gallic acid equivalents per 100 mL (See Equation 4).

$$[\text{polyphenols}] (\text{mgGAE} / 100\text{mL}) = \frac{\text{Abs}}{\alpha} \times 50 \quad \text{Eq. 4}$$

with: [polyphenols]: the phenolic compounds content; Abs: absorbance of the sample; α : the slope of the calibration curve.

2.3.7. Determination of the antioxidant capacity

The antioxidant capacity determination protocol (Brand-Williams et al., 1995) of every sample was utilized with some modifications. The

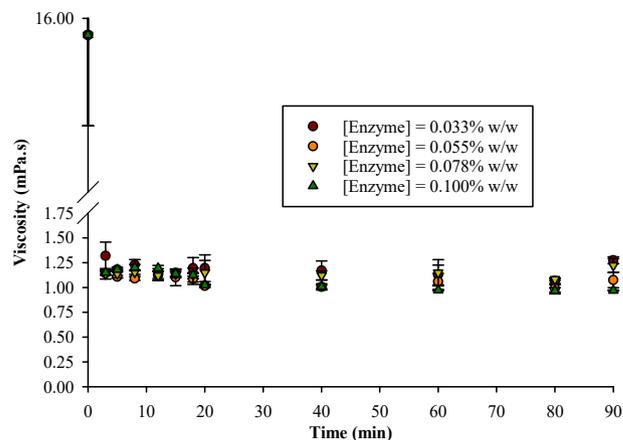


Figure 1. Evolution in the viscosity of guava juice with time and enzyme concentration.

reaction mixture was composed of 2.5 mL of supernatant from the centrifugation of the sample-ethanol (99 % v/v), 4.5 mL absolute alcohol, and 0.6 mL of DPPH solution (0, 5 mM in pure ethanol) (Sigma Aldrich, Slovakia). When the DPPH reacts with antioxidants which may form hydrogen, it is reduced. This modification of color (from dark purple to light yellow) was characterized by absorbance at 517nm after a reaction of 100 min. The blank was composed of 2.5 mL of supernatant and 5.1 mL of pure ethanol. The control solution was achieved using a mixture of 7 mL ethanol and 0.6 mL of DPPH solution. The antioxidant capacity was determined as in Eq. (5).

$$\text{AC}(\%) = 100 - \left[\frac{(\text{Abs}_{\text{éch}} - \text{Abs}_{\text{blanc}})}{\text{Abs}_{\text{contrôle}}} \times 100 \right] \quad \text{Eq. 5}$$

AC: Antioxidant capacity of the sample; $\text{Abs}_{\text{éch}}$: Absorbance of the sample; $\text{Abs}_{\text{blanc}}$: Absorbance white; $\text{Abs}_{\text{contrôle}}$: Absorbance of the control solution.

The ANOVA was done using the software Statgraphics Centurion Version XV.II and 5 % significance level was utilized to check the difference between samples.

2.3.8. Morphological study of the juice samples by Field Emission Scanning Electron Microscopy (FESEM)

The imaging technique made it possible to visualize the structure of the suspension by studying the morphology of the particles, the profile of the mixture, and the distribution of the granules and particles. The image analysis required the untreated or treated juice to be dehydrated. For this, juice samples were previously spread on microscope slides and dried under vacuum in a desiccator for 10 days. The images were produced by FESEM (JEOL, model JSM-7610F, Tokyo, Japan).

3. Results and discussion

3.1. Effect of enzymatic treatment parameters on viscosity

The reduction of viscosity with treatment time for each enzyme concentration used is observed in Figure 1. The viscosity of untreated juice is 15.90 ± 0.522 mPa.s. At the end of the third minute of treatment (after deactivating the enzyme in a boiling bath for 5 min), it decreased significantly to a viscosity of 1.32 ± 0.2 mPa.s for all enzyme concentrations; a decrease of more than 90% of the initial viscosity. This observation is similar to that made by the literature (Akesowan and Choonhahirun, 2013; Kaur et al., 2011; Nso et al., 1998; Surajbhan et al., 2012). This could be elucidated by the hydrolysis of pectin macromolecules by pectinases. Industrial pectinase preparation of *Aspergillus niger* is a conglomerate of several isoforms with different action mechanisms and

kinetic parameters (Benen et al., 2003). Hydrolysis of pectin macromolecules and subsequent accumulation of the reaction products would occur in the first min of the reaction (Combo et al., 2012). These scientists have demonstrated that at the fifth minute of the enzyme reaction, products of degree of polymerization (DP) 1 to 10 are already formed. These low degree of polymerization products compared to pectin macromolecule would exhibit lower hydraulic radius and water retention capacity than the substrate (pectin) from which they were derived. Therefore, the resulting mixture would get a better flowability, thus justifying the low viscosity values obtained from the treated samples. PGII isoform is a highly active enzyme that hydrolyzes pectin molecules in the first few min of reaction. Without substrate inhibition, this isoform would easily attack pectin or polygalacturonic acid macromolecules (its natural substrate would be pectin) and release oligomers (Benen et al., 1999). The rapid decrease in viscosity from the third minute of reaction may be due to the low affinity of PGII isoform for pectin or polygalacturonic acid molecules and its high activity.

It is also observed that after the sudden decrease in viscosity, another more moderate and non-significant decrease occurs between the 3rd and 80th min (1.32–1.27; 1.15 to 0.97 0.033% and 0.1% w/w of enzyme respectively) with the asymptotically tending viscosity value (0.9 mPa.s for the enzyme concentration of 0.1% m/m; 1 mPa.s for other concentrations). The phenomenon observed during this phase may be due to both the complementarity between isoforms and the phenomenon of processivity that would occur both during the treatment period. Some isoforms (PGI and PGD) would be sensitive to steric hindrance due to substrate size and therefore the molecular weight. As a result, these isoforms would prefer low molecular weight substrates from the hydrolysis of oligomers by other isoforms (Benen et al., 1999; Pařenicova et al., 2000). Besides, the phenomenon of processivity would be accompanied by a gradual decrease in the number of oligomers in the decreasing order of their molecular weight and an accumulation of lower molecular weight products. This phenomenon is described in the evolution profile of reaction products for all isoforms (Benen et al., 1999, Benen et al., 2003; Pařenicova et al., 1998, 2000). It was shown with the increase in enzyme processing time (from the 15th-minute reaction), there was a gradual decrease in the concentration of oligomers of polymerization degree between 4 and 10 with the formation of mono, di, and trimers (Combo et al., 2012; Nikolić and Mojovic, 2007). These secondary substrates and their derivatives would form the compounds encountered during the slow decrease in viscosity. The non-significant change in viscosity observed is because these compounds would describe a range of hydraulic radii that would not significantly affect the viscosity of the samples. Also, the hydrolysis of pectin molecules (a highly hydroxylated compound) leads to the release of oligomers of galacturonic acid weakly hydroxylated compared to pectin. Hydrolysis would be accompanied by a decrease in water retention capacity; therefore, the release of water molecules into the system would result in a decrease in viscosity (Akesowan and Choonhahirun, 2013).

After the non-significant decrease, the same figure shows a non-significant increase in viscosity after 90 min of enzyme treatment (about 10% of the viscosity value at 80 min). This could be due to the formation of particles after cooling the juice. These particles are reported to be the result of the coagulation phenomenon observed by the interaction between protein or polyphenol molecules with mono, di, and certain galacturonic acid oligomers (Shomer et al., 1999). The heat treatment of the samples to stop the enzyme reaction has the effect of denaturing protein molecules. The latter being positively charged to the natural pH of the juice (3.5 ± 0.2) could form ionic interactions with the negatively charged galacturonic monomers and oligomers. The result would be the creation of particles known as "cloud" (Rai et al., 2004; Shomer et al., 1999). The formation of these particles could be promoted by the high monomer content after 90 min of enzymatic treatment. The particles obtained would affect the viscosity of the medium due to their higher hydraulic radius than those of galacturonic acid monomers and protein molecules taken individually.

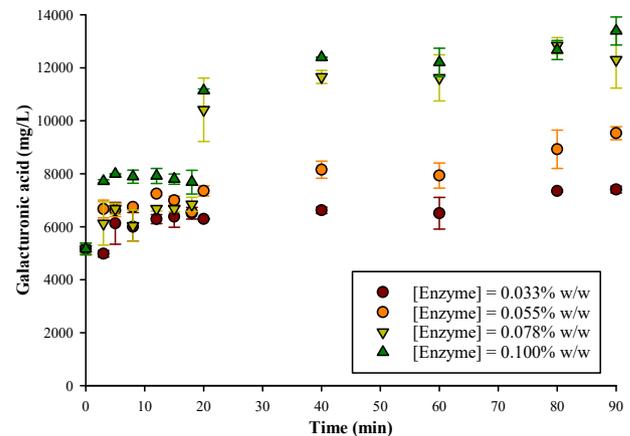


Figure 2. Evolution of the galacturonic acid content as a function of time for the different enzyme concentrations.

Finally, this figure shows that an increase in enzyme concentration goes hand in hand with a decrease in viscosity value (Akesowan and Choonhahirun, 2013; Kaur et al., 2011; Surajbhan et al., 2012). This could be because increased enzyme concentration would mean an increase in the number of catalysts. The rate of reaction is a function of enzyme concentration. The increase in enzyme concentration would be followed by an increase in the number of products formed (Nikolić and Mojovic, 2007).

3.2. Effect of enzymatic treatment parameters on the galacturonic acid content

The raw juice has a galacturonic acid content of 5161.510 ± 216.461 mg/L. For each enzyme concentration, increased treatment time was followed by a significant increase in galacturonic acid content (Figure 2). The rise in galacturonic acid content as a function of contact time may be due to the liberation of galacturonic acid following the reaction of pectin hydrolysis by pectinases. Galacturonic acid is the smallest galacturonate which can be released after the enzymatic treatment of guava puree by pectinases. In most of the mechanisms involved (simple attack on single chain, multiple attack on single chain or multiple chain of pectin or oligogalacturonates) during that treatment, that product is known to be the final product. In the single attack mechanism, an α (1–4) linkage of the pectin substrate is hydrolyzed by the enzyme with the liberation of two low molecular weight products. Processivity or multiple attack on a single chain involves the release of the lower molecular weight product after hydrolysis and the binding of the bigger product with the enzyme. That product will later shift to a new position on the enzyme for another hydrolysis reaction to take place. The release of low molecular weight oligogalacturonates would be followed by their hydrolysis by the various PG isoforms; the last product released regardless of the polymerization degree of the substrate is the galacturonic acid monomer (Benen et al., 2003).

For an enzyme concentration of 0.033% w/w, the galacturonic acid content progressively increases in a non-significant way over the contact time. This trend is obtained between 3 and 90 min for 0.055% w/w enzyme concentration. For 0.078% and 0.1% w/w enzyme concentrations, three distinct zones can be obtained: an area of non-significant variation in galacturonic acid content between 3 and 18 min and between 20 and 90 min and also, a significant increase between 0 and 3 min.

Findings on each PG isoform showed that the progression curve of products formed during the hydrolysis of pectin exhibited a rise in oligomeric levels followed by a decline in these contents justifying the

phenomena of processivity and/or simple attack. This decrease was accompanied by a rise in monomer content (Jacques A. E. Benen et al., 1999; Pařenicova et al., 1998, 2000). PG conglomerate of *A. niger* for polygalacturonic acid hydrolysis showed the appearance of galacturonic acid monomers and products of DP = 2 to DP = 10 at the end of the fifth minute of treatment (Combo et al., 2012). These researchers also showed that with an increase in treatment time, there was a progressive decrease (in descending order of polymerization) in the content of oligomers of molecular weight between 2 and 10. The release of galacturonic acid at the third minute of reaction may be due to the enzyme activity of the commercial preparation used in this study. That was between 8000 and 12000 U/g; it is, therefore, higher than that used (3160 U/ml) in the literature (Combo et al., 2012). This high value of enzyme activity would result in the acceleration of the above phenomena. The progressive increase in galacturonic acid content for enzyme concentrations of 0.033% and 0.055% m/m could be due to the progressive hydrolysis of the DP oligomers above 1 with the progressive release of galacturonic acid monomers. The same phenomenon could explain the variation in concentrations of 0.078 % and 0.1 % w/w above 20 min. Indeed, Combo et al. (2012) observed that after the 60th-minute reaction, the medium was saturated mainly with galacturonic acid monomers with very small amounts of dimer and trimer of galacturonic acid. They also showed an increase in the monomer/dimer ratio over the treatment time reflecting progressive hydrolysis of dimers to monomers. With the predominance of these two oligomers, PGD isoform is the one that would be most active since it is the only one to hydrolyze the galacturonic acid dimer. Hydrolysis would occur at a relatively low rate ($0.67 \cdot 10^{-3}$ μ kat/mg and $0.67 \cdot 10^{-4}$ μ kat/mg for 50 and 500 μ M dimer, respectively) (Pařenicova et al., 2000). This dimer hydrolysis may explain the nonsignificant increase in galacturonic acid content observed with enzyme concentrations of 0.078 % and 0.1% w/w.

For enzyme concentrations of 0.078% and 0.1% w/w, a significant and abrupt increase was observed between 18 and 40 min of reaction. This increase could be the result of the processivity phenomenon involving the processes of hydrolysis of substrates, the release of low DP products, migration of high DP products along the enzyme molecule to the active site, and secondary hydrolysis. The simple attack mechanism for the subsequent release of products following hydrolysis could also occur during this period. Since the amount of enzyme was high compared with the other two concentrations, the accumulation of low-polymerization oligomers for the first 20 min of these two enzyme concentrations would be faster. This accumulation would be followed by hydrolysis of these oligomers into others of lower degrees of polymerization. These new products would undergo secondary hydrolysis with the progressive release of monomers. These sequenced hydrolysis reactions would occur between 18 and 40 min. It was highlighted the release of oligomers of DP between 3 and 8 from 15 min of reaction (Nikolić and Mojovic, 2007). The amount of these oligomers increased over time and after 120 min of treatment, the amount of DP oligomer between 6 and 8 showed a decrease. These phenomena would be observed in this study and more accelerated concerning the activity of the enzyme preparation used (8000–12000 U/g), i.e. at least 8 times that of the enzyme preparation used by these two researchers (1000 U/g).

For a given duration of treatment (Figure 2), increased enzyme concentration results in a significant increase in galacturonic acid content (Wang et al., 2007). These researchers have shown that increased pectinase concentration leads to more production of galacturonic acid for a given period of cotton fiber treatment. For a period of treatment of guava juice, increased enzyme concentration would lead to an increase in the amount of reducing ends as shown by (Nikolić and Mojovic, 2007) with apple pectin. These reductive ends are those of the galacturonic acid molecules and their oligomers. All released after the enzyme reaction. Increasing enzyme concentration and processing time would result in an acceleration of the reaction of enzyme hydrolysis of pectin, and thus of simple attack and processivity. Progressive spacing between the evolutionary curves of the number of galacturonic acids would reflect the fact

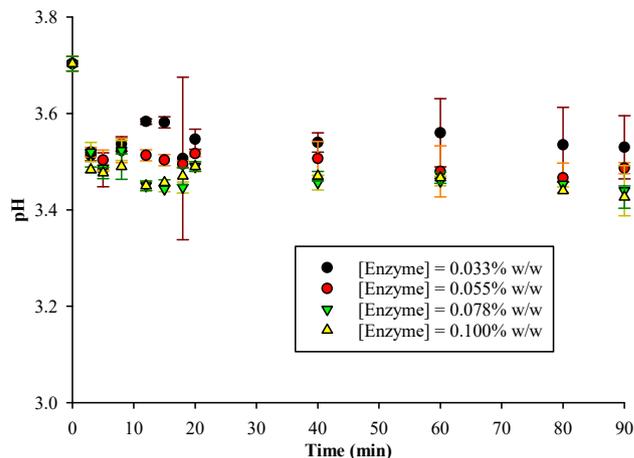


Figure 3. Guava juice pH evolution based on the enzymatic processing time.

that increased treatment time would result in the progressive release of the reaction products with an increase in enzyme concentration. A gradual disappearance of oligomers of a degree of polymerization between 2 and 10 coupled with an increase in the quantity of galacturonic acid monomers was observed. It is this acceleration of the reaction that may explain the fact that, as the treatment duration increases, the gap between the galacturonic acid content curves increases with increasing enzyme concentration. As a consequence of these different observations, the effect of inhibition by substrate molecules could be reduced with increasing enzyme concentration.

3.3. Effect of operating conditions on the parameters of juice

3.3.1. Effect of enzymatic treatment on the pH of guava juice

The initial pH was 3.70 ± 0.02 , and it was found to decrease significantly with treatment at the third minute of treatment (Figure 3). This decrease is accompanied by near-constant variation (plateau presence) between 3 and 90 min. It was shown that the enzyme treatment of guava juice resulted in a decrease in the pH value for all factor combinations (Ahmed et al., 2014; Combo et al., 2012).

Figure 3 also shows that, with increasing enzyme concentration, the pH value decreases over a given treatment period. This could be explained that with increased enzyme concentration there would be a more significant production of galacturonic acid monomers (Nikolić and Mojovic, 2007; Wang et al., 2007). The decrease in pH with increased enzyme concentration may also be due to the release of ascorbic acid molecules during pectin hydrolysis (Akesowan and Choonhahirun, 2013). The non-significant difference between enzyme concentrations of 0.078% and 0.1% m/m corroborates the observation for time-dependent variation of galacturonic acid content for both concentrations.

A decrease in pH during enzyme treatment could also be attributed to the release of carboxylic groups (acid groups) during pectin hydrolysis. Pectin is a complex macromolecule consisting of so-called "hairy" zones built around the "smooth" zones. The smooth areas that make up the skeleton of the pectin are a polymer of galacturonic acids bound by an α -(1–4) galactosidic bond. Pectin hydrolysis by pectinases is accompanied by the release of galacturonic acid oligomers. Mono (Figure 4a), di, tri, and oligomer (Figure 4b) all have a reductive end that may cause a decrease in pH. Besides, galacturonic acid has two carboxylic groups, including C1 and C6 carbon, as shown in Figure 4a. Hydrolysis of methoxyl groups by Pectin Esterase (PE) results in the formation of a carboxylic group in the C6 position as shown in Figure 4c (Garg et al., 2016). This demethoxylation reaction is essential for the activity of polygalacturonases which will hydrolyze the oligomers of galacturonic acid at the level of galactosidic

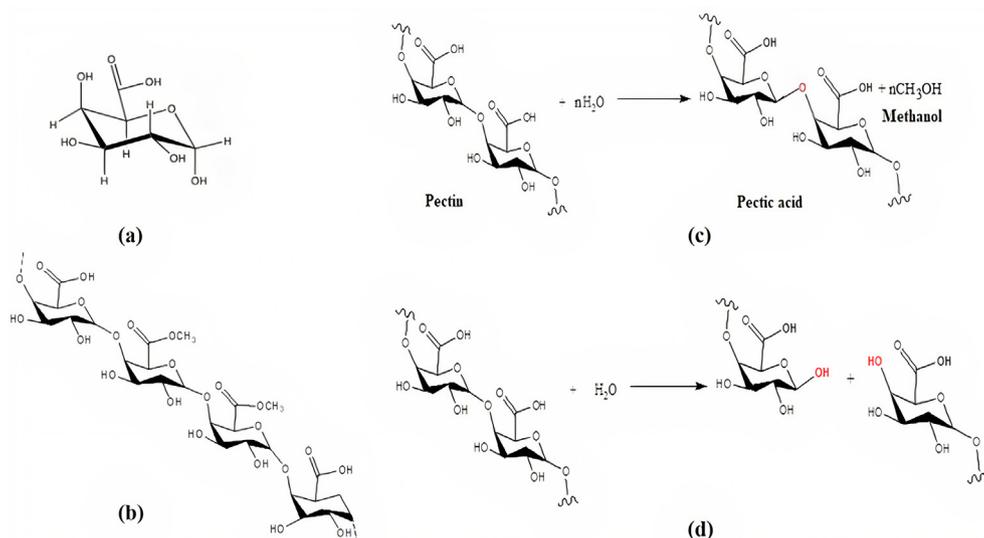


Figure 4. Identification of carboxylic groups in the reaction medium. (a) and (b) schematic representations of galacturonic acid and a segment of the pectin chain with certain methoxylated residues of galacturonic acid, respectively; (c) hydrolysis reaction of the methoxyl bond by Pectin esterase with the formation of a carboxylic group; (d) hydrolysis reaction of the galactoside bond by PG with the release of a reducing end at C1 carbon.

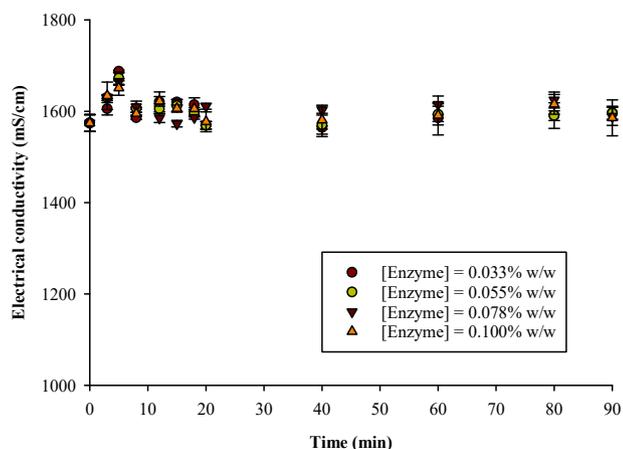


Figure 5. Evolution of electrical conductivity during the enzymatic treatment.

bonds with the release of a reducing end (Figure 4d). Some PG isoforms would prefer partially demethoxylated pectin substrates; methoxyl group describing an inhibitory effect on their activity due to its conformation (Benen et al., 2003).

The near-constant pH variation observed between 3 and 90 min for all enzyme concentrations could be explained by the buffer effect exerted by galacturonic acid molecules. Hydrolysis of pectin molecules by the synergistic action of pectinases would be accompanied by the release and accumulation of galacturonic acid molecules (Combo et al., 2012). This monomer is a molecule with two carboxylic groups and could, therefore, be considered a low organic acid (Figure 4a). In the reaction medium, this acid would coexist with its conjugate base.

Depending on the pH value, the acidic species could release an H⁺ ion in the medium or the basic species could capture an H⁺ ion from the reactive medium to maintain stable pH values. This buffer effect is optimally expressed when the pH value of the medium is in the order of pK_a ± 0.5; however, the maximum pH limit for expression of the buffer effect is pK_a ± 1 (Dennison, 2002; Stoll and Blanchard, 2009). Galacturonic acid pK_a is 3.47 (Holvik and Høiland, 1977). The buffer effect of galacturonic acid and the base species may be explained by the fact

that the pH values of the juice obtained after enzymatic treatment are all within the range of optimal expression of activity ($2.97 \leq \text{pH} \leq 3.97$).

3.3.2. Effect of enzyme treatment on the electrical conductivity of guava juice

The electrical conductivity of the initial juice is 1574.33 ± 18.48 mS/cm. It appears that for all enzyme concentrations, three trends are generally observed according to time intervals (Figure 5). The first-time range is between 0 and 5 min where the enzyme treatment causes a significant increase in the conductivity values of the juice compared to the untreated juice. The second is the one between 5 and 20 min where the treatment causes a non-significant decrease in the conductivity. The third range is from 20 to 90 min; in this, there is a non-significant increase in conductivity for all enzyme concentrations, except for 0.078% and 0.1% w/w where there is a decrease between 80 and 90 min of treatment.

The increase in electrical conductivity for the first five min could be due to the release of calcium ions (Ca²⁺) during hydrolysis. Pectin is present in biological systems as a complex network with an “egg-box” structure. Pectin chains due to their negative charge can be chelated by calcium ions (Prasanna et al., 2007). During the hydrolysis of pectin, the appearance of the products of the reaction results from the destruction of these networks with the possible release of calcium ions in the reaction medium. This release would increase the conductivity value observed with the samples having undergone the enzymatic treatment.

The decrease of the conductivity between 5 and 20 min of treatment could be due to the chelation phenomena that may exist between certain cations and the anionic groups of the reaction products. These chelation phenomena could be the result of ionic interactions between these groups of opposite charge. Guava is a fruit that has a high mineral content; it contains inter alia several types of cations (K⁺, Ca²⁺, Mg²⁺, Cu²⁺, Na⁺, Fe²⁺). The magnesium content is the highest compared to other cations (Flores et al., 2015). These cations would contribute to the ionic strength. These different cations could be involved in interactions with a galacturonic acid monomer or oligomer molecules or protein molecules present in the medium. These interaction phenomena could be driven by the buffering effect of monomers, galacturonic acid oligomers, and proteins. At the pH of the juice samples, conjugated species of galacturonic acid could be dominant. This is negatively charged and could be associated with ionic interactions with cations. These interactions would have the effect of regulating the buffering effect by promoting the release

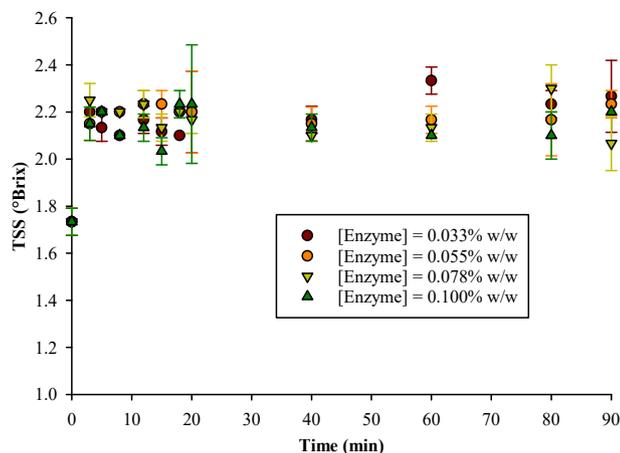


Figure 6. Evolution of TSS content of guava juice during the enzymatic treatment.

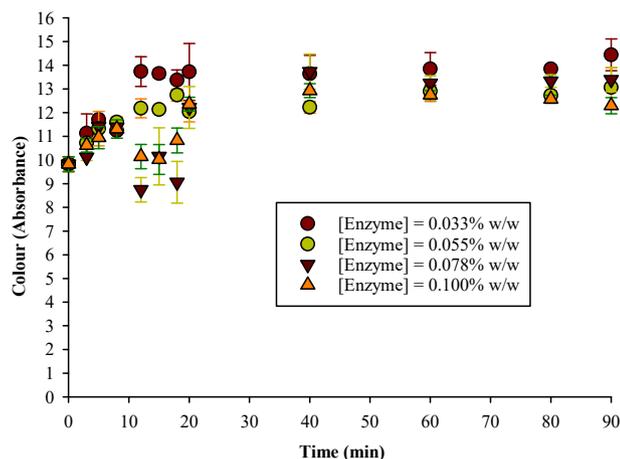


Figure 7. Changes in the color of the juice during the enzymatic treatment.

of H^+ ions in the reaction medium, as they would help to reduce the quantity of the negative species (Dennison, 2002).

The increase in conductivity between 20 and 90 min could be explained by the presence of other types of interaction involving galacturonic acid oligomers and proteins as mentioned above (Shomer et al., 1999). These could render some monomer or oligomer molecules unavailable for cation chelation reactions. Moreover, the decrease in pH that occurs in this time range and the predominance of the conjugated species of galacturonic acid due to the pH of the samples greater than the pKa of the galacturonic acid buffer, there would be an increase of conductivity as shown by (Dennison, 2002).

3.3.3. Effect of enzyme treatment on the value of TSS (total soluble sugar) of guava juice

The TSS content of the initial juice is 1.7 ± 0.1 . It appears from Figure 6 that the enzymatic treatment leads to a significant increase in total soluble sugar content from the third minute of reaction for all enzyme concentrations. Beyond 3 min and up to 90 min, the TSS content does not describe significant variation during treatment. This increase in TSS content compared to initial juice was also observed (Ahmed et al., 2014; Akesowan and Choonhahirun, 2013). It could be explained by the hydrolysis of pectin which releases galacturonic acid monomers that can be assimilated to glucose molecules and thereby lead to an increase in the

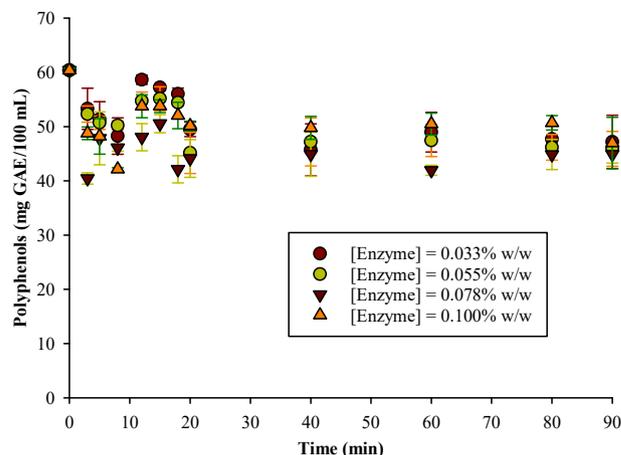


Figure 8. Evolution of juice polyphenols in the enzymatic treatment.

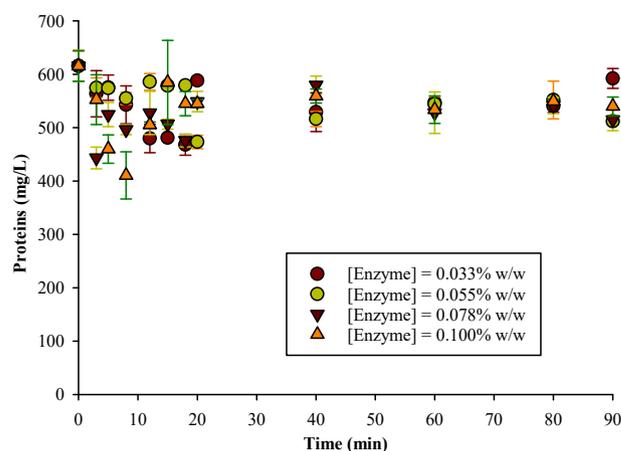


Figure 9. Evolution of the juice protein content during the enzymatic treatment.

TSS value. It could also be due to the release of sugar due to the hydrolysis of guava starch by endogenous amylases. It was demonstrated the presence of α and β - amylases and starch during the ripening of guava (Jain et al., 2001). During enzymatic treatment of guava juice, these endogenous amylases hydrolyze the starch resulting in the release of glucose molecules that can lead to an increase in the value of TSS.

3.3.4. Effect of enzymatic treatment on color samples of juice

The initial juice color value is 9.8 ± 0.3 . Figure 7 shows that for each enzyme concentration, two-time ranges are distinct according to the observed trends in color: a range where there is an increase in color and a second where it does not record significant variation. Overall the increase in color occurs during the first 12 min. Beyond this time, the color is almost constant for all enzyme concentrations.

The increase in the color attribute during the treatment could be due to the enzymatic browning of the initial juice, resulting from the oxidation of the phenolic compounds by the polyphenol oxidases (PPO) present in the initial juice. PPOs are enzymes that catalyze the conversion of compounds with a phenolic ring to o-quinone. They possess in their structure a copper atom as a prosthetic group which is very indispensable for their activity.

The conversion of phenolic compounds to quinone would require the presence of oxygen and could occur in two specific steps (Taranto et al., 2017; Yoruk and Marshall, 2003). The first is the hydroxylation of the

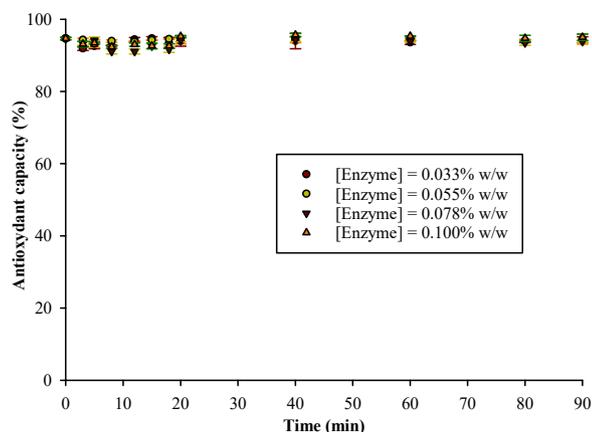


Figure 10. Evolution of the antioxidant capacity of guava juice during the enzymatic treatment.

phenolic nucleus in the ortho position of a hydroxyl group of a monophenolic compound. During this reaction, which has phenol and oxygen as substrates, one oxygen atom is introduced into the monophenolic compound while the other oxygen atom is reduced to water. This reaction is known as monophenolase activity or hydroxylase activity or cresolase activity. It is facilitated by the presence of ascorbic acid or *o*-diphenol compounds that act as electron donors. The product of this first reaction is an *o*-diphenolic compound. The second step is known as diphenolase activity or oxidase activity or catecholase activity. It is an oxidation reaction of the preceding *o*-diphenolic compound with the formation of an *o*-quinone compound which would be highly active. It would undergo non-enzymatic condensation reactions with amino acid molecules, proteins, and other phenolic compounds to give colored complex polymers known as melanin or to form polymers with protein residues such as -SH or -NH₂ groups. During this reaction where two *o*-diphenol compounds are oxidized to two *o*-quinone, the two oxygen atoms are reduced to a water molecule. The first reaction would necessarily involve the second, but the opposite would not always be true. PPOs, include three enzyme groups: tyrosinases, catechol oxidase, and laccases. The latter would be the only ones to catalyze the oxidation of a *p*-diphenolic compound. Tyrosinases possess both cresolase and catecholase activities. Catechol oxidases catalyze the conversion of *o*-diphenols to *o*-quinone (Taranto et al., 2017; Yoruk and Marshall, 2003). Before enzymatic treatment, the guava juice did not undergo

preliminary heat treatment to inactivate endogenous enzymes, therefore stopping enzymatic reactions occurring. As a result, the polyphenol oxidases present in the juice would still be active during enzymatic treatment that took place under aerobic conditions (the reactor was not closed). Stirring during the treatment would have allowed the mixing of the ambient oxygen in the reaction medium. This mixing would have increased the dissolved oxygen content in the juice, which would have increased the substrate content for the oxidation of polyphenols. In addition to this dissolution of oxygen, ascorbic acid present in the medium would also have played a role in browning by promoting monophenolase activity. To these aerobic conditions coupled with the presence of ascorbic acid could be added the temperature and the reaction time. The enzymatic treatment was done at a temperature of 43 ± 2 °C. This temperature is substantially equal to the optimum activity temperature of the guava polyphenol oxidase which is 48 °C (Razzaque et al., 2000).

Also, during processing, the color value increases for a given enzyme concentration. This could reflect the progressive oxidation of phenolic compounds with the appearance of melanins. The increase in enzyme concentration has no significant effect on the color of the samples of given treatment time. This could be because the industrial preparation used is mainly composed of pectinase. Increasing the pectinase concentration would not lead to a change in the polyphenol oxidase concentration already present in the juice.

3.3.5. Effect of enzymatic treatment on the levels of polyphenols and protein of guava juice

The content of phenolic compounds of the initial juice is 60.3 ± 0.4 mg GAE/100mL. Figure 8 shows a decrease in polyphenol content during enzymatic treatment for all enzyme concentrations compared to that of the initial juice. Between 20 and 90 min, the polyphenol content does not change significantly with time for all enzyme concentrations. The decrease in the polyphenol content in the treated samples was reported in the case of enzymatic treatment of banana juice (Sagu et al., 2014). The decrease in the polyphenol content could, among other things, be explained by their oxidation to *o*-quinone by the PPO. By this oxidation, the hydroxyl groups of the preliminary phenolic compounds would be oxidized to a quinone. This new grouping would have low reactivity with a mixture of phosphotungstic and phosphomolybdic acids; it would have little influence on the reduction of this mixture. This would lead to the formation of a less colored blue complex than that of the unoxidized phenolic compound (Blainski et al., 2013). The formation of this *o*-quinone would lead to condensation reactions and complexation with amino acids and proteins resulting in the formation of melanins. The decrease in the polyphenol content could also be due to the

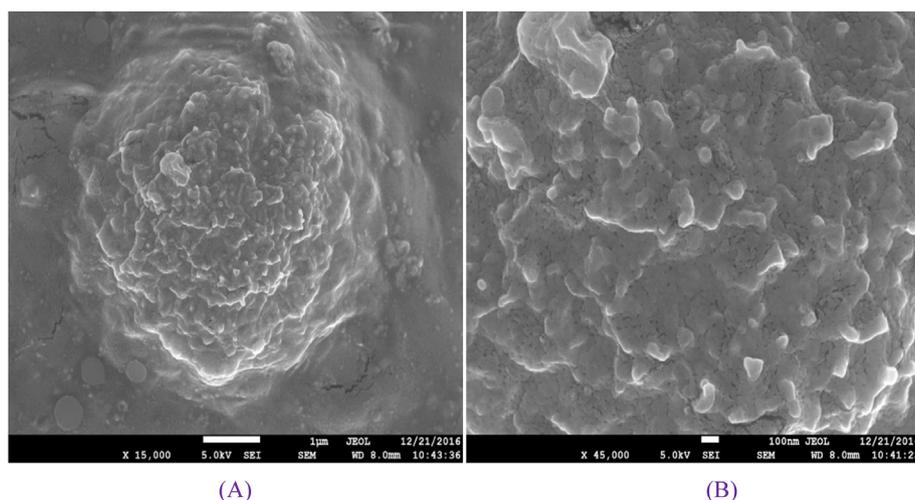


Figure 11. Images FESEM guava puree without any enzymatic treatment (A) and (B) Enlargement 15000 and 45000 respectively.

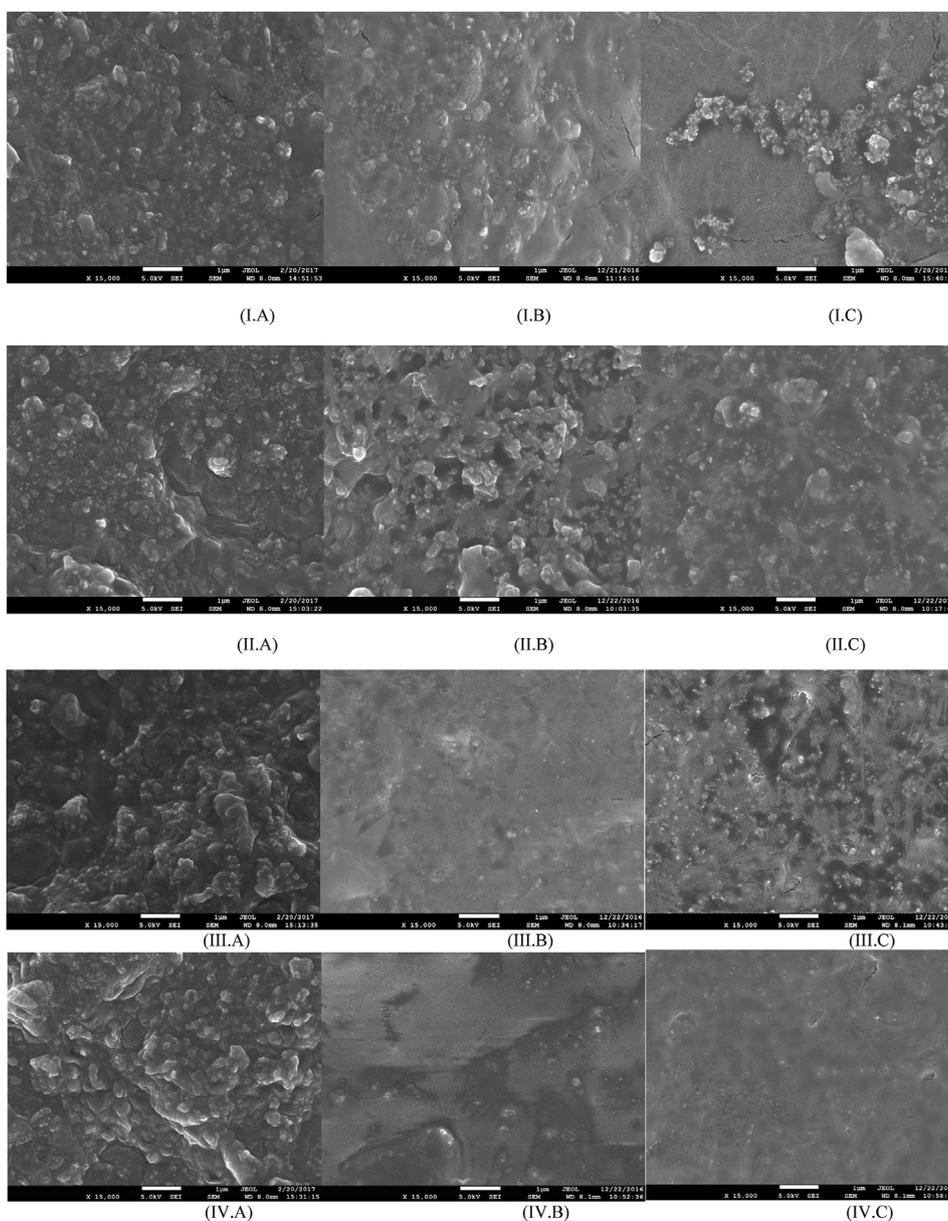


Figure 12. Images FESEM of guava juice samples. (I), (II), (III), and (IV) Images guava samples having undergone an enzymatic treatment with 0.033% w/w, 0.055% w/w, 0.078% w/w, and 0.1% w/w enzyme concentration, respectively. The indices A, B, and C represent respectively the time of enzyme treatment of 8, 20, and 90 min for a given concentration of enzyme.

non-reactivity of the polyphenols involved in the formation of complexes with other molecules such as proteins and other polyphenols. The non-reactivity of these polyphenols would reflect the non or slight reduction of the Folin Ciocalteu reagent because of the involvement of their hydroxyl groups in the formation of the complexes. Indeed, polyphenol molecules act as bridge and the "glue" to promote the precipitation of protein chains, resulting in the formation of tannin or tannin (McLellan and Padilla-Zakour, 2005). This phenomenon could be accelerated by the addition of enzymes during treatment, increasing protein content. Also, polyphenols could be involved in the formation of clouds due to their polymerization to form larger complexes (McLellan and Padilla-Zakour, 2005).

The protein content of the initial juice is 615.6 ± 28.5 mg/L. Figure 9 shows that the enzymatic treatment results in a decrease in the protein content of the samples compared to that of the raw juice for all enzyme concentrations. During processing, the protein content does not describe a monotonous trend with increasing time for all enzyme concentrations.

The decrease in the protein content of the treated samples compared to the initial one could be explained by the non-reactivity with the reagents (the Folin Ciocalteu Reagent and the Lowry solution) used for their detection and the complexes that these proteins form.

These complexation phenomena would lead to unavailability in the reaction medium of the protein molecules. This decrease could, among other things, be attributed to the oxidation of certain tyrosine residues of proteins by PPOs (polyphenol oxidases) (Taranto et al., 2017; Yoruk and Marshall, 2003). Indeed, this amino acid has in its structure an aromatic ring having a hydroxyl group in para position concerning the carbon 1 of the ring. This position of the hydroxyl group would make tyrosine an ideal substrate for the expression of monophenolase activity. Since the reduction of the Folin Ciocalteu reagent is, among other things, dependent on the presence of tyrosine, a group of tyrosine oxidized to o-quinone by the PPOs would, therefore, be unavailable for the blue color formation characteristic of the reduced Folin Ciocalteu reagent. The decrease in protein content could also be due to the formation of

melanins between o-quinone compounds derived from the oxidation of polyphenols and protein molecules. The formation of these melanins would involve -SH or -NH₂ residues. These SH residues are essential for the reduction of Folin Ciocalteu reagent; their involvement in melanin formation would reduce the intensity of the blue color (Noble and Bailey, 2009). The decrease in protein content could further be explained by the complexation phenomena involving protein molecules and galacturonic acid monomers (Shomer et al., 1999). These phenomena could be dominant with the reaction products (monomers and oligomers) than with pectin macromolecules. Guava contains several types of phenolic compounds: anthocyanins, flavonoids, and proanthocyanidins. These compounds contain several hydroxyl groups (at least 3) likely to be involved in several types of reactions (Flores et al., 2015).

3.3.6. Effect of enzymatic treatment on the antioxidant capacity of guava juice

The antioxidant capacity of the initial juice is $94.671 \pm 0.325\%$. Figure 10 shows that the enzymatic treatment does not possess a significant effect on the antioxidant capacity of the samples compared to that of the untreated juice. The antioxidant capacity is that exerted by the soluble compounds in the ethanol used for the precipitation of the particles. The antioxidant capacity would be a function of the phenolic compounds soluble in alcohol and ascorbic acid which would be one of the major contributors.

The phenolic compounds that contribute to the antioxidant capacity would be those that are free since those complexed in particulate form would be removed by centrifugation. Twenty-one (21) phenolic compounds have previously been identified in guava cultivars. Among these compounds are two anthocyanins, ten flavonoids, two proanthocyanidins, two sesquiterpenoids, and five triterpenes. All of these compounds would have abilities to donate hydrogen atoms or to transfer electrons; thus, contributing to the antioxidant capacity (Flores et al., 2015).

A strong positive correlation was observed between antioxidant capacity, phenolic compounds, and ascorbic acid showing that these two families of compounds are essential contributors to the antioxidant capacity of guava. This correlation is characteristic of fruit with high levels of ascorbic acid such as guava and orange (Thaipong et al., 2006).

3.4. Analysis of samples by Field Emission Scanning Electron Microscopy (FESEM)

To visualize the different suspension, these were analyzed by Field Emission Scanning Electron Microscopy (FESEM). This technique enabled to obtain images of the dehydrated samples. The images allowed the samples to be analyzed for the particles they contain, the distribution of these particles, and the homogeneity of the samples. It also makes it possible to compare these samples with untreated puree (Figure 11A).

Figure 11A shows particles of different sizes that can vary between 60 nm and 5200 nm. The particle distribution is heterogeneous. Some particles are in the form of clusters with irregularities (asperities). Other particles, on the other hand, have a round shape; however, their magnification (Figure 11B) reveals that they are also provided with several asperities. These particles represent a cluster of tissues consisting of several polysaccharides and proteins forming a complex network. Pectin is the major constituent of this network in the primary cell wall (35% w/w). It tends, because of its solubility in water, as well as proteins, to form a gel around the other constituents of the wall and thus acting as a binder (Prasanna et al., 2007).

Figure 12 shows that the enzymatic treatment causes the disintegration of the particles observed on the images of the raw sample (Figure 12A) with the consequence of reducing the sizes of these particles. It also shows that juice samples after 8 min of treatment for all enzyme concentrations show an almost identical particle distribution. This treatment time is not fit to highlight the differences due to the variation of the enzyme concentration. For a given enzyme concentration, the increase in the treatment duration is accompanied by a gradual

appearance of homogeneous layers surrounding the particles. These layers represent the liquid phase, showing that the enzymatic treatment is accompanied by liquefaction of the mixture due to the release of water molecules. This release of the water molecules would occur during the hydrolysis of pectin (a compound of high-water retention capacity and hydraulic radius compared to the monomers that constitute them) with the consequent appearance of a liquid phase leading to lower viscosity (Akesowan and Choonhahirun, 2013). The hydrolysis of pectin would also be accompanied by a destruction of the macromolecule network with the result of a release of these in the medium. Images from 90 min samples show a higher proportion of the liquid phase. Analysis of the 90 min samples reveals that the one corresponding to the 0.1% w/w concentration has a higher proportion of the liquid phase. This sample has fewer particles and has better homogeneity. This can be explained by the fact that this sample corresponds to the combination of maximum values of treatment time and enzyme concentration. Samples corresponding to all enzyme concentrations still show particles after 90 min of treatment. However, the size of the particles, their distribution or their grouping vary with the increase of the enzyme concentration. With the enzyme concentration, the particle size decreases, the latter is more dispersed.

4. Conclusion

In sum, this paper examined the effect of the enzyme treatment of guava puree on the physicochemical parameters of the juice. The enzymatic treatment of the puree resulted in a significant decrease in the viscosity of the juice from the third minute of treatment. Viscosity decreased with increasing enzyme concentration. This enzyme treatment generated a significant increase in galacturonic acid content over time. Parameters such as pH, protein, and polyphenol levels decreased significantly during treatment; while others such as conductivity and color increased. However, enzyme treatment did not have a significant effect on the antioxidant capacity of the samples. Images of the dried extracts from the samples showed the decay of the particles and liquefaction of the mixture during enzymatic treatment. Images also showed that increased enzyme concentration led to an increase in the proportion of the liquid phase and improved homogeneity of the samples. It was also observed that particle size decreased during treatment and, with increased processing time, particles were more dispersed.

Declarations

Author contribution statement

Kombele Aime Ninga: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Zangue Steve Carly Desobgo: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sirshendu De, Emmanuel Jong Nso: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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