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# Kinetics of enzymatic hydrolysis of pectinaceous matter in guava juice

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ABSTRACT

The objective of the current study is to investigate the hydrolysis of pectinaceous matter in guava juice and to model the depectinization kinetics using commercial pectinase from *Aspergillus niger*. The procedure involves enzymatic treatment of guava juice at 45 °C with enzyme concentration (% w/w) 0.033, 0.055, 0.078 and 0.1. The degree of hydrolysis of pectinaceous matter and the rate of enzymatic reaction are determined. The results show an increase in the degree of hydrolysis with time for each enzyme concentration. Depectinization kinetics reveal allosteric behavior and is modeled using the Hill Equation. The image analysis of the sample using field emission scanning electron microscopy shows a change in the smoothness and homogeneity of the sample, indicating degradation of pectin network.



## 1. Introduction

Guava (*Psidium guajava*), belonging to family *Myrtaceae* and genus *Psidium*, is a tropical plant native to Mexico and available throughout South America, Asia, Europe, and Africa. It is one of the most cultivated fruit crops in many tropical and subtropical countries. The fruits contain large amount of vitamins and minerals, having high levels of polyphenolic antioxidants (referred to as "superfruits" in the popular literature because of their high antioxidant capacity). Guava contains flavonoids, triterpenoids, and other biologically active secondary compounds (Flores et al., 2015). 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).

Guava is extremely perishable, having shelf life of only two days at room temperature (Teixeira et al., 2016). Therefore, it is usually consumed as fresh, or processed into various commercial products including pulp, squash, nectar, puree, paste, canned slices in syrup and juice. Out of these, the juice has become economically important because of its high nutritional values, making it an alternative to other beverages such as carbonated and caffeinated drinks (tea and coffee) (Akesowan and Choonhahirun, 2013; Lee et al., 2006). In addition, guava juice is also used for the production of fruit wine by fermentation (Sevda and Rodrigues, 2011).

Guava juice contains colloids that are mainly polysaccharides (pectin, cellulose, hemicellulose, and lignin), protein, and tannins. These particles can settle during storage, leading to heterogeneity of the juice. Pectin makes the juice clarification difficult, as it is generally associated with plant polymers and cell debris having fiber-like molecular structure, thereby creating fouling during filtration. Presence of pectin makes the guava juice viscous and turbid (Liew Abdullah et al., 2007; McCook-Russell et al., 2012; Rai et al., 2004). Pectic polysaccharides are polygalacturonate molecules, in which 1–4-*d*-galacturonic acid chains are linked with branches of l-rhamnopyranosyl residues, and neutral side chains of L-arabinose, D-galactose and D-xylose. These are the major components of cell wall and middle lamella of plant tissues. Pectin undergoes structural changes during development and ripening of the fruits, thereby contributing significantly to textural softening of ripening fleshy fruits (Das and Majumdar, 2010). Pectin content of ripened guava ranges from 0.58%–0.66% (Surajbhan et al., 2012).

Viscosity and turbidity of guava juice can be decreased by enzymatic depectinization, degrading pectin and polysaccharides. Pectinase hydrolyzes pectin and causes pectin–protein complexes to flocculate, so that the resultant juice has much lower pectin and viscosity, facilitating the subsequent filtration process. Enzymatic hydrolysis of pectic substances depends on several operating

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factors such as enzyme concentration, incubation time and temperature (Lee et al., 2006). In most cases, pH is not taken as a parameter, as the hydrolysis is done at the natural pH of the fruit juice.

There has been study exploring the kinetics of enzymatic hydrolysis of pure pectin in general (Bélafi-Bakó et al., 2007). In addition, some studies exist on kinetics of depectinization of juice extracted from various fruits, like, apple, red currant, black currant, sugar beet, passion (Kiss et al., 2008; Klinchongkon et al., 2017). But, in all these studies, pectin was isolated from their sources before investigating on kinetics of hydrolysis. Hydrolysis kinetics of these extracted pectins as well as pure pectin showed Michaelis-Menten type behavior. In a practical industrial setting, extraction of pectin from the fruit juice and their hydrolysis become unnecessarily complex and cost intensive. Thus, in a real life scenario, depectinization needs to be undertaken on actual fruit juice. However, in case of depectinization of actual guava juice, pectin hydrolysis becomes complicated in presence of other polysaccharides, like, cellulose, hemicellulose, lignin, etc. These compounds may accelerate or inhibit the hydrolysis reaction. These complex interactions were not accounted in the previous studies (Bélafi-Bakó et al., 2007; Kiss et al., 2008; Klinchongkon et al., 2017). Based on these complex interactions, the hydrolysis of pectin molecules may or may not exhibit hyperbolic behavior, as described by the Michaelis-Menten kinetics. Researchers have studied depectinization in real life fruit juices (Akesowan and Choonhahirun, 2013; Kaur et al., 2009, 2011; Lee et al., 2006; Liew Abdullah et al., 2007; Rai et al., 2004; Surajbhan et al., 2012) as well, but they analyzed the system using response surface methodology to obtain the optimum operating conditions. Response surface methodology is a black box method fitting a polynomial equation to the experimental data, without providing any insight on fundamental understanding of depectinization kinetics. Baş and Boyaci (2007) reported that response surface methodology is not a suitable tool to predict the rate of enzyme reaction kinetics, as the generated model is inadequate and kinetic parameters of the enzyme lack high accuracy.

As mentioned, response surface based analyses did not elicit the underlying reaction mechanism, nor they established reaction kinetics, that is mandatory to design a reactor successfully. This has been attempted in present study and herein lies the novelty of the present work. To the best of knowledge of the authors, reports on the reaction mechanism of depectinization of actual guava juice are rare in literature. Therefore, the objective of the current work is to study the kinetics of the decay of pectinaceous substances during the enzymatic treatment of guava juice along with possible mechanism, and interpret the optimum operating conditions of depectinization.

## 2. Materials and methods

### 2.1. Materials

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

#### 2.1.1. Enzymes and other chemicals

The list of enzymes and other chemicals used in the present study are:

Pectinase (isolated from *Aspergillus niger,* activity: 8000–12000 U/g); bovine serum albumin; pectin; sodium potassium tartrate; gallic acid; D-galacturonic acid monohydrate; 2,2-Diphenyl-1-picrylhydrazyl (DPPH); copper sulfate pentahydrate ($CuSO_4.5H_2O$); Folin Ciocalteu's phenol reagent. All chemicals were of analytical grade.

### 2.2. Methods

#### 2.2.1. Guava juice extraction

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. Based on preliminary studies, a bulk solution of the guava pulp was prepared by mixing the pure pulp with water at a ratio of 35% w/w. The bulk solution was blended using a food processor, packaged in plastic cans and stored in the freezer for further analysis.

#### 2.2.2. Enzyme treatment of the pulp

The enzyme treatment of the pulp was performed following the protocol described by Akesowan and Choonhahirun (2013), with some modifications. The guava pulp was thawed before use, and 100 g of pulp was weighed in a 250 mL beaker. Literature survey reveals that most of the earlier studies for enzyme hydrolysis were performed for enzyme concentration up to 0.1% (Lee et al., 2006; Liew Abdullah et al., 2007). For enzymatic treatment of banana juice (Lee et al., 2006) and carambola fruit juice (Liew Abdullah et al., 2007), optimum enzyme concentrations were 0.084%, and 0.1% respectively. Hence, in the current study, four enzyme concentrations of 0.033%, 0.055%, 0.078% and 0.1% w/w were considered.

The preliminary study with 0.033%, 0.055%, 0.078% and 0.1% w/w of enzyme dose at 25 ± 2, 45 ± 2 and 55 ± 2 °C revealed 45 ± 2 °C as the temperature of optimal activity. Therefore, further experiments were conducted at 45 ± 2 °C. The beaker was put in a water bath at this temperature for 5 min under continuous stirring. Dried enzyme extract was added in each sample at different ratios (0.033%, 0.055%, 0.078% and 0.1% w/w of initial guava pulp). The mixture was then stirred using a Remi Motor agitator, type RQ 122 (supplied by Elektrotechnik Ltd, Kolkata, India) at 1500 rpm. For each enzyme concentration, different incubation times were applied (3, 5, 8, 12, 15, 18, 20, 40, 60, 80 and 90 min). After completion of respective incubation times, the mixture was transferred into a glass sampling bottle. The bottle was put in a boiling water bath (95 °C) and allowed to stand for 5 min to inactivate the enzymes. The mixture was cooled and filtered by a cheese cloth. 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. For each sample, the following parameters were analyzed: viscosity, pH, total soluble sugar (TSS), color, antioxidant activity and concentration of pectin, total polyphenol, protein and reducing sugar.

### 2.3. Analysis

#### 2.3.1. Viscosity

The viscosity of guava juice was determined at room temperature (30 ± 1 °C), as described by Jain and De (2016), using an Oswald capillary viscometer (Pisco, Kolkata, India).

#### 2.3.2. pH and total soluble sugar (TSS)

pH was determined using a multimeter pocket tester (EUTECH Instruments Ltd, Singapore). TSS was estimated using an ABBE type refractometer (Excel International, Kolkata, India).

#### 2.3.3. Color

The color of guava juice was determined by measuring the absorbance at 420 nm, using a UV-VIS spectrophotometer (M/s Perkin Elmer, Connecticut, USA).

#### 2.3.4. Pectin concentration

Pectin was extracted from guava juice following the protocol

described by Masmoudi et al. (2008) with some modifications. Pectin was precipitated by adding 15 mL of 96% w/w ethanol to 10 mL of guava juice under agitation. The obtained mixture was kept in the refrigerator overnight at 10 °C, and the pectin gels were centrifuged at 10,000 rpm for 15 min. The supernatant was collected in PET sampling bottles and stored in the freezer for further analysis. To remove the mono and disaccharides, the pectin precipitate was washed thrice with 50%, 75% and 96% v/v ethanol, and centrifuged at 10,000 rpm for 10 min at room temperature. Pectin concentration of guava juice samples was determined using carbazole method developed by McComb and McCready (1952) with some modifications. Firstly, the pectinaceous matters remaining in the centrifuge tubes were collected and dissolved in 0.05 N NaOH solution, and then the volume was made up to 100 mL. The samples were then allowed to stand for 30 min to de-esterify the pectin. 6 mL of $H_2SO_4$ was introduced in test tubes and the test tubes were cooled to 3 °C in an ice bath. Thereafter, 1 mL of the sample was added and the mouth of the test tube was covered. The tubes were heated for 10 min in a boiling-water bath, and then allowed to cool at room temperature. After that, 0.5 mL of 0.15% w/v carbazole reagent was added. The mixture was allowed to stand for 25 ± 5 min, and the transmittance was measured at 520 nm using a spectrophotometer (M/s Perkin Elmer, Connecticut, USA). Pectin concentration was calculated according to the calibration curve obtained using galacturonic acid monohydrate standard. The degree of hydrolysis of pectinaceous matters was determined using the following equation:

$$\textit{Degree of hydrolysis}\ (\%) = \frac{[S]_{t=0} - [S]_t}{[S]_{t=0}} \times 100 \tag{1}$$

where, $[S]$ is the pectin concentration and $t$ is the time. The rate of reaction was also determined using the following equation:

$$\textit{Rate of reaction}\ (\text{mg/L.min}),\ v = \frac{[S]_i - [S]_{i-1}}{t_i - t_{i-1}} \tag{2}$$

The rate of reaction ($v$) was plotted against pectin concentration, and it was modeled using Hill Equation (Marangoni, 2003). The use of Hill equation can be explained by the fact that the plot of reaction rate with pectin concentration describes a sigmoidal pattern, instead of the hyperbolic one typically exhibited by Michaelis-Menten behavior. It was observed that fitting the data with Michaelis-Menten equation gave a poor match, with $R^2$ values much less than 1. Thus, an attempt was made to have a detailed understanding of the mechanism that makes the current system deviate from usual Michaelis-Menten kinetics.

The Hill equation is given as

$$v = \frac{V_{\max}[S]^n}{K + [S]^n} \tag{3}$$

where $V_{\max}$ and $n$ refer to the maximum reaction rate and Hill coefficient, respectively. $K$ is expressed as $K = [S_{50}]^n$, where $[S_{50}]$ is the substrate concentration at which the reaction rate is half of its maximum value, i.e., $v = V_{\max}/2$. For each enzyme concentration, the values of the $V_{\max}$ and $n$ were obtained by minimizing the sum of squares at each experimental data, using MATLAB 2014b. The sum of squares is presented as,

$$s = \sum_{i=1}^{N} \left( \frac{v_{\exp,i} - v_{sim,i}}{v_{\exp,i}} \right)^2 \tag{4}$$

where, $v_{\exp,i}$ and $v_{sim,i}$ are the experimental and simulated values of the rate of reaction at $i$-th time point.

#### 2.3.5. Total polyphenol

Total polyphenol was measured using modified Folin and Ciocalteu method (Sagu et al., 2014). The results were expressed as mg of gallic acid equivalents per 100 mL.

#### 2.3.6. Protein concentration

The protein concentration of the guava juice was determined according to the method developed by Lowry et al. (1951) using bovine serum albumin as standard.

#### 2.3.7. Reducing sugar

The reducing sugar concentration of guava juice was estimated using cyanoacetamide method as described in the International Oenological Codex (2012), with some modifications. 2 mL of the supernatant (section 2.3.4) was introduced in a 100 mL volumetric flask and distilled water was added up to 100 mL. 2 mL of this diluted sample was mixed with 4 mL of cold borate buffer (100 mM, pH 9.0) and 2 mL of cyanoacetamide solution (1% w/v). Then, the test tube was put in boiling water bath for 10 min and cooled. After cooling, the absorbance was measured at 273 nm. The calibration curve was plotted using D-galacturonic acid (concentration ranging from 0 to 250 μg/mL) as standard.

#### 2.3.8. Antioxidant activity

The antioxidant activity was measured using the DPPH method. The percentage of antioxidant activity ($AA\%$) of each substance was assessed by DPPH free radical assay. The measurement of the DPPH radical scavenging activity was performed according to the methodology described by Mensor et al. (2001). The scavenging activity percentage ($AA\%$) was determined as:

$$AA\ (\%) = 100 - \left[ \frac{A_{sample} - A_{blank}}{A_{control}} \times 100 \right] \tag{5}$$

where, $A_{sample}$, $A_{blank}$ and $A_{control}$ are the values of absorbance of the sample, blank and control, respectively.

### 2.4. Morphology of the samples

The samples of guava juice were spread on a microscopic glass cover (18 mm) and vacuum dried in desiccator for 10 days. The image analysis was done using Field Emission Scanning Electron Microscopy (FESEM) (JSM-7610F, JEOL, Japan). FESEM analysis was performed to assess the morphology of the structures in guava juice samples.

## 3. Results and discussion

### 3.1. Effect of process parameters on the degree of hydrolysis

For each set of process parameters (enzyme concentration and time), the degree of hydrolysis was determined to quantify the extent of degradation of the pectinaceous matters in the guava juice, and the results are presented in Fig. 1a. From the figure, it appears that for each enzyme concentration, degree of hydrolysis increases with time. For enzyme concentration 0.033% w/w, there is a continuous increase in the degree of hydrolysis with decreasing slopes. This could be explained by the fact that the pectinaceous substances were still in juice and the extent of hydrolysis is incomplete. For 0.055% w/w and 0.078% w/w enzyme concentrations, there was a sharp increase in the degree of hydrolysis up to 20 min of reaction, followed by a sluggish change. Higher rate of hydrolysis within first 20 min indicates a significant decrease in pectin concentration, with progressive accumulation of reaction products. The slight increase in the degree of hydrolysis beyond

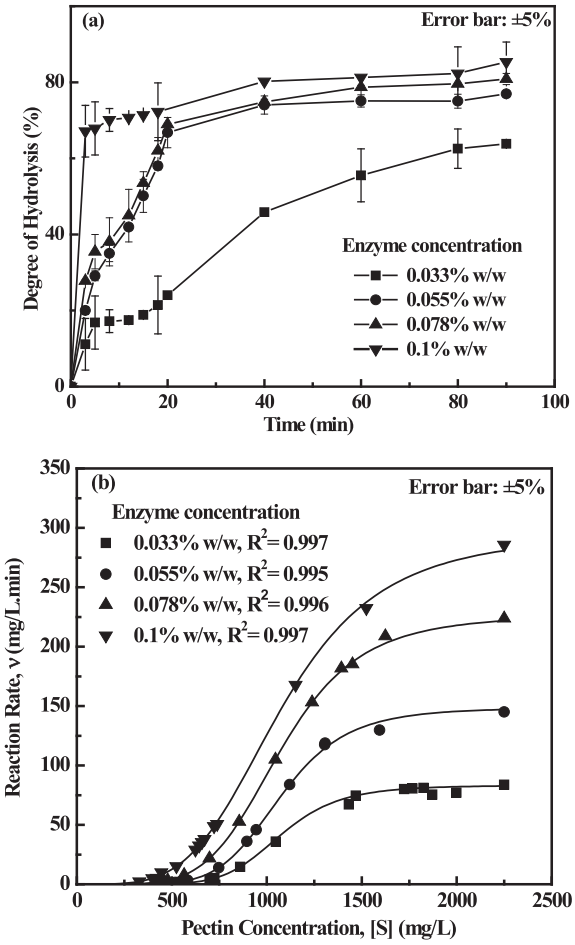


**Fig. 1.** (a) Degree of hydrolysis versus time for different enzyme concentrations; (b) reaction kinetics exhibited by pectinase with guava juice as the substrate for different enzyme concentrations (solid lines represent the model curves obtained from Hill equation).

20 min is due to decrease in the amount of substrates and the saturation of the reaction medium by the products formed. The same trend was observed for 0.1% w/w enzyme concentration, but the slope of the hydrolysis curve is higher in the initial stages of reaction. It is observed that the degree of hydrolysis after 5 min at 0.1% enzyme concentration is almost two folds, compared to 0.078% w/w, owing to the high enzyme concentration. Combo et al. (2012) reported an accumulation of mono, di and trimers of galacturonic acid after 5 min of hydrolysis of polygalacturonic acid using commercial pectinase from *A. niger*, thereby initiating inhibition of hydrolysis. As depicted in the figure, for 0.078% and 0.1% w/w enzyme concentration, variation of degree of hydrolysis is marginal beyond 40 min. The average difference in degree of hydrolysis between 0.078% and 0.1% enzyme concentration is 4.6%, i.e. well within the errors involved in the measurements (±5%). Hence, 0.078% w/w has been chosen as the optimal enzyme concentration. The optimal concentration of 0.078% w/w for guava juice is comparable with that of banana juice (0.084%) (Lee et al., 2006) and carambola juice (0.1%) (Liew Abdullah et al., 2007). Thus, 0.078% w/w of enzyme concentration and 40 min of treatment time can be considered as optimum one for guava juice depectinization.

### 3.2. Depectinization kinetics of guava pulp

Variation of the rate of reaction with pectin (i.e. the substrate) concentration for different enzyme concentrations is presented in Fig. 1b, exhibiting a sigmoidal type trend. This behavior deviates from the ideal Michaelis-Menten kinetics that was observed with pure pectin (Bélafi-Bakó et al., 2007). The deviation can be explained by the presence of some naturally occurring compounds which can act either as activators or inhibitors. The guava juice contains some sugar molecules (monomers, dimers, oligomers) originating from the maturation and ripening of guava. During maturation, insoluble protopectin molecules are degraded into monomers and oligosaccharides (Prasanna et al., 2007). These oligosaccharides have affinity with pectinase, as described below, thereby promoting an allosteric effect on the enzymes. The Hill Equation is adequate to model this phenomenon (Marangoni, 2003). Solid lines in Fig. 1b represent the model curves obtained by fitting the experimental data to Hill equation for different enzyme concentrations. The coefficient of determination ($R^2$) has been evaluated corresponding to each curve, in order to assess the goodness of fit, and provided in the same figure. Details regarding the values of $n$ and $V_{max}$ obtained from the model have been discussed later in sections 3.2.1 and 3.2.2.

The mechanism behind Hill kinetics has been illustrated in Fig. 2. The enzyme is assumed to have multiple substrates or ligand binding sites. The binding of one substrate molecule induces structural and/or electronic changes, resulting in altered substrate binding affinities in the remaining vacant sites (cooperativity) (Fig. 2a). The enzyme-substrate affinity can either increase in case of positive cooperativity, or decrease for negative cooperativity. On the other hand, binding of the substrate or non-substrate (activator or inhibitor) can affect the activity of the enzyme (allosterism) (Fig. 2b). The allosteric response can be homotropic in case of modulation of enzyme activity strictly by substrate molecules, or heterotropic for modulation of enzyme activity by non-substrate molecules or combinations of substrate and non-substrate molecules (Marangoni, 2003).

In case of polygalacturonases from *A. niger*, different possible reactions occurring on the substrate binding sites are represented in Fig. 3. Fig. 3a describes various possibilities of cleavage of a substrate molecule and the progress of depectinization. A substrate with a reduced end can be hydrolyzed at various locations of the glycosidic bond, thereby forming different products. Fig. 3b describes the case of a substrate bound to the enzyme at a precise binding site, where the catalysis occurs. One product (monomer) is released and the other one (oligomer) remains on the substrate, but the new complex formed is non-productive and exhibits an inhibition for the other substrates in the bulk. Fig. 3c describes the affinity of the substrate with a given binding site. On one hand, when the substrate is bound to the enzyme at a given binding site, the complex is non-productive and substrate inhibition takes place. On the other hand, the same substrate bound to another subsite may lead to a productive complex. Therefore, the mechanism of cleavage can be a single-attack with release of oligomers after the reaction, or multiple-attack (also known as processivity). It implies that the enzymatic hydrolysis of substrate may release one product, and the other product remains bound to the substrate, and the complex is also productive leading to release of other products (Benen et al., 1999, 2003; Pařenicová et al., 1998, 2000).

#### 3.2.1. Effect of enzyme concentration on Hill coefficient (n)

Fig. 4a presents the effect of enzyme concentration on the Hill coefficient. The figure exhibits a decrease in the value of $n$ from 7 to 4, as enzyme concentration is increased from 0.033% w/w to 0.1% w/w. This can explain the decrease in the extent of the sigmoidicity of the curve with enzyme concentration (Fig. 1b).

Decrease in the value of the Hill coefficient implies the reduction of cooperativity in the substrate binding process, thereby lowering

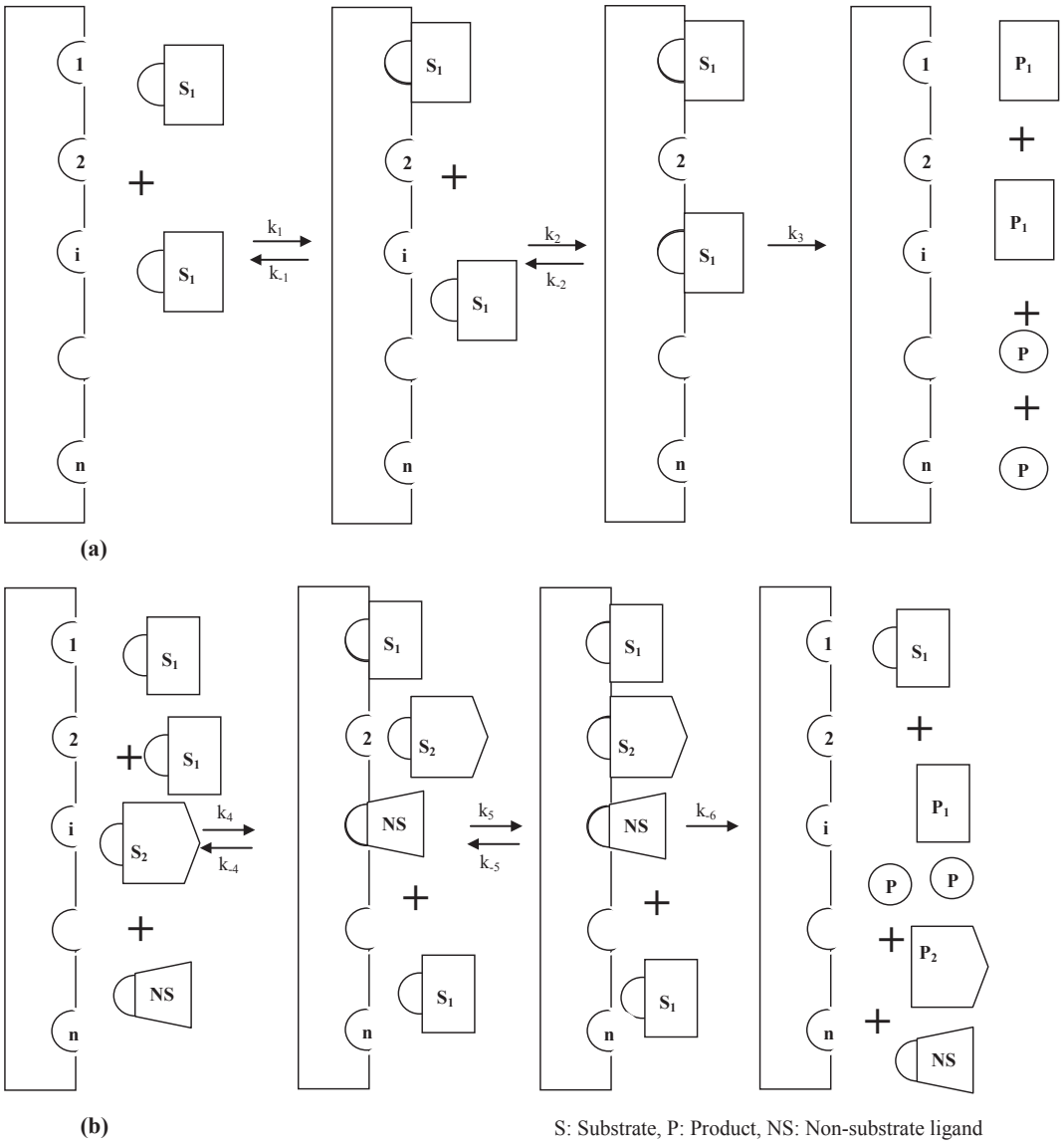


**Fig. 2.** Schematic for mechanism of Hill kinetics: (a) cooperativity mechanism; (b) allosteric mechanism.

the number of active substrate-binding sites with enzyme concentration (Marangoni, 2003). Decrease in the number of substrate-binding sites could be explained by the affinity between the substrate-binding sites and some oligomers as described below.

*A. niger* is a saprophytic filamentous fungus, having a broad arsenal of pectinolytic enzymes to break down the complex structure of pectins. The polygalacturonases from *A. niger* are known to be of endo type. The mode of action of the endopolygalacturonases consists of a random cleavage of the glycosidic bonds along the pectin chains, thereby releasing a broad range of products of different degrees of polymerization (Prasanna et al., 2007). *A. niger* possesses at least seven isoforms of polygalacturonases (PG) namely: PGI, PGII, PGA, PGB, PGC, PGD and PGE. Apart from the PGD isoform which possesses 4 binding sites (−3 to +1), the other isoforms possess at least 7 subsites suitable for the binding of the substrates. Therefore, multiple subsites are involved in the binding of the substrate due to length of the cleft found in the structure of polygalacturonases (Benen et al., 2003).

Release of large products due to random split of the pectin molecules by the isoforms of PG occurs in early stages of the reaction. These large products are degraded in course of reaction into low degree of polymerization (DP) oligoGalA (monomers, dimers, trimers etc.), until the smallest substrate is hydrolyzed. Extent of the reaction increases with enzyme concentration, increasing the rate of hydrolysis. The mechanism involved is mostly the processivity, in which a compound (usually the smallest) is released while the other one remains at the active site of the enzymes (Benen et al., 2003). Each isoform of PG has an affinity with a precise oligoGalA. PGI as well as PGA, PGC and PGD are processive enzymes with PGA and PGC being processive from DP 7 onward and PGD (the only isoform to hydrolyze dimer) from DP 4 onward. The oligoGalA can also have some preference with some binding sites. The hexamers will preferably bind to the subsites −5 to +1 with the release of pentamers that will remain bound to the subsites −5 to −1 because of their affinity to those subsites. That affinity can lead the pentamers to exhibit a competitive inhibition to the PGI when they are used as substrates (Fig. 3b). However, when they are bound to the subsites −4 to +1 or −3 to +2, the complex formed

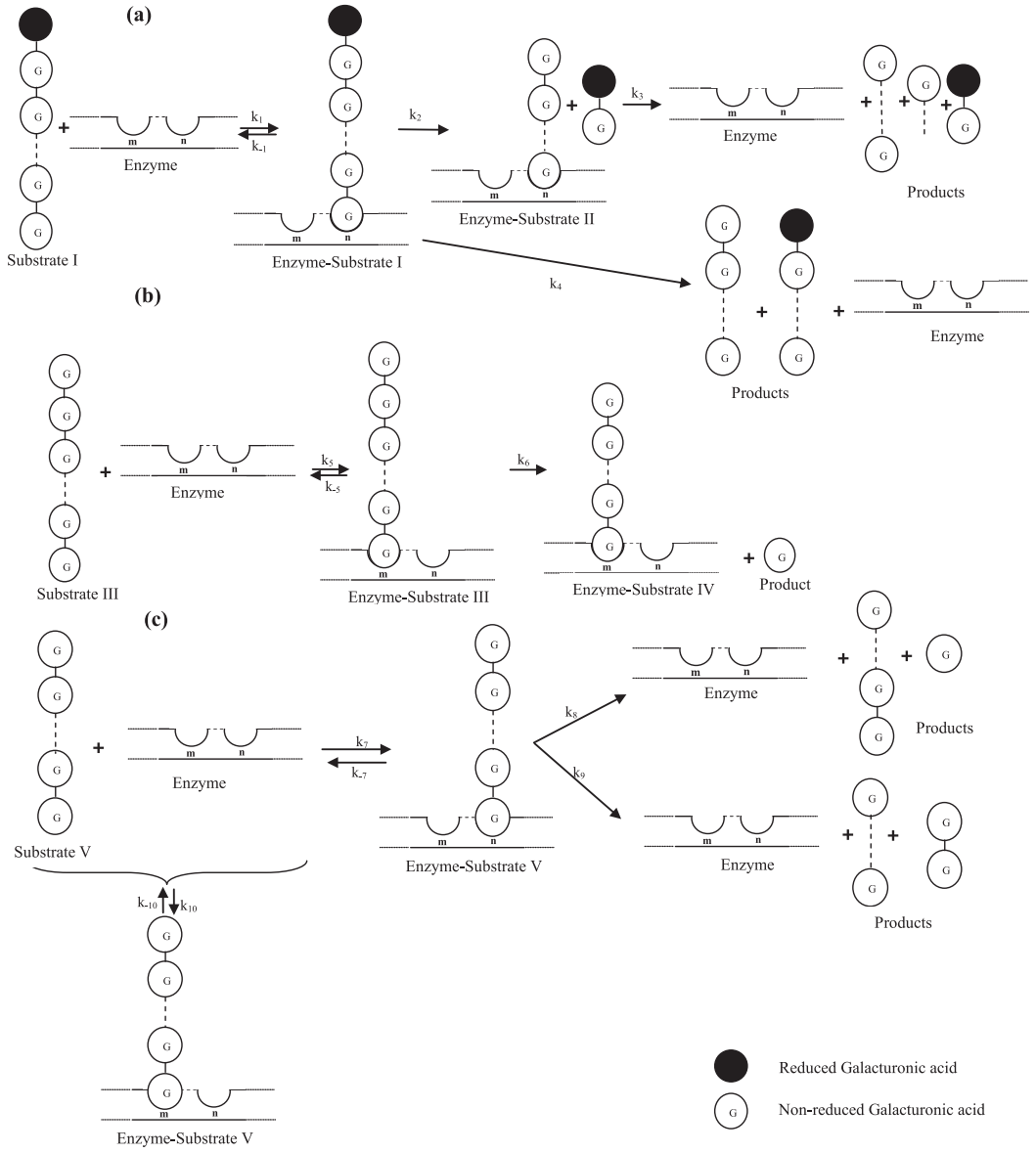


**Fig. 3.** Schematic for mechanism of the possible reactions taking place in the subsites of the pectinases for *A. niger* exhibiting an allosteric effect leading to the Hill Kinetics; (a) Processivity (b) Substrate Inhibition (c) Enzyme-substrate affinity with given binding sites; m and n = Substrate binding sites; k: reaction constants.

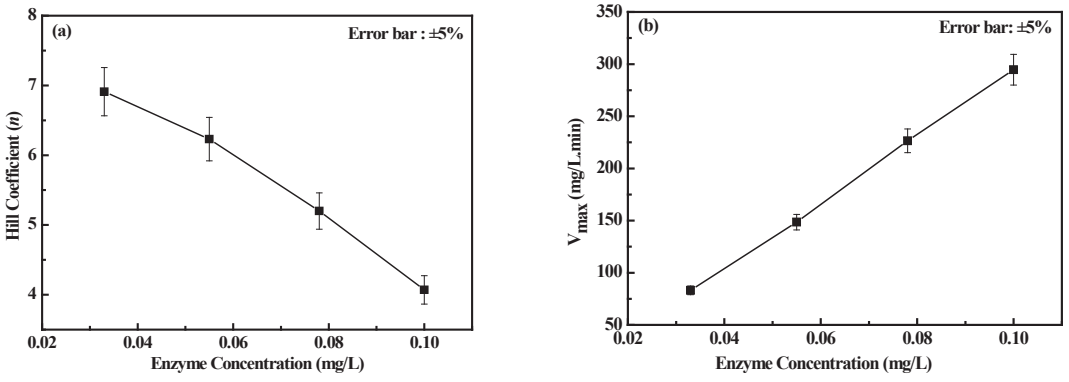


**Fig. 4.** Effect of enzyme concentration on (a) Hill coefficient ($n$); (b) maximum reaction rate ($V_{max}$).

will be productive with formation of products (Van Pouderoyen et al., 2003).

The trimer, which is usually the smallest substrate, will be degraded mostly at prolonged incubation time, making the hydrolysis slow (Benen et al., 2003). This could explain the decrease in the slope of degree of hydrolysis profile, at later time (40 min onwards) for each enzyme concentration (Fig. 1a).

In Hill equation, $n$ is either equal to or less than the number of active binding sites (Marangoni, 2003). Prinz (2010) reported high values of Hill coefficient (4 and above) in the analysis of dose response curves. The current study involves the use of pectinase and it is a mixture of seven polygalacturonase isoforms, each having several binding sites known as subsites. Apart from the isoform PGD which has four subsites, the other six isoforms has at least seven subsites each (Benen et al., 1996, 2003). It is already stated that $n$ is either equal to or less than the number of active binding sites. Thus, the value of $n$ obtained (4–7), although seemingly high, corroborates literature data.

#### 3.2.2. Effect of enzyme concentration on maximum rate of reaction ($V_{max}$)

Fig. 4b presents the effect of the enzyme concentration on the value of $V_{max}$. There is a monotonic increase in the value of $V_{max}$ from 83.2 mg/L.min to 294.7 mg/L.min with an increase in enzyme concentration from 0.033% w/w to 0.1% w/w. With increase in enzyme concentration, the rate of reaction increases leading to the formation of more products. Therefore, an increase in the enzyme concentration led to the increase of the maximum rate of reaction.

### 3.3. Effect of process conditions on other parameters of the juice

For each processing conditions (enzyme concentration, time), the following parameters were determined: viscosity, pH, TSS, color, total polyphenol, protein concentration, reducing sugar, and antioxidant activity. Table 1 shows the chemical, nutritional composition as well as the rheological parameters of raw guava juice and the sample treated after 90 min, at each enzyme concentration. It is observed that the samples undergoing enzymatic treatment exhibit higher values of reducing sugar and TSS, as compared to those of the raw sample. This is due to the fact that pectin molecules release reducing sugar during enzymatic hydrolysis. An increase in the enzyme concentration accelerates this process due to intensified degradation. Breakdown of pectin molecules causes a substantial decrease in viscosity (10% of the viscosity of raw sample). The enzymatic treatment did not show a significant effect on the antioxidant activity of the guava juice. The treated samples also underwent a decrease in protein and polyphenol concentration and pH values, similar to the findings of Sagu et al. (2014).

### 3.4. FESEM analysis of the samples

FESEM analysis of the samples was performed to assess the shape and structure of the particles in fruit juice as well as homogeneity of the samples on microscopic glass cover. For each enzyme concentration, the samples treated after 90 min were analyzed, and a comparison was made with the sample without enzymatic treatment. The results are shown in Fig. 5. It reveals that the guava juice without enzymatic treatment contained round-shaped particles of different sizes. Increase in enzyme concentration led to an increase in the smoothness of the surface. The image corresponding to 0.033% w/w enzyme concentration exhibits particles of different shapes at the surface. For higher enzyme concentration, homogeneity of the sample as well as smoothness of the surface was improved. This could be explained by the hydrolysis of pectin molecules resulting in disintegration of the pectin network with the release of low molecular weight products. As a result, more liquid phase was formed, spreading at the surface of the glass cover.

## 4. Conclusions

Kinetics of depectinization of guava juice was investigated in the present study. The optimum enzyme concentration and treatment time were 0.078% w/w and 40 min at 45 °C. The hydrolysis of pectinaceous matters was modeled using Hill equation, and the effect of enzyme concentration on Hill coefficient ($n$) and $V_{max}$ was assessed. The value of $n$ decreased with increase in enzyme concentration from 7 to 4, due to a decrease in binding sites of the enzyme. Reduction in the number of binding sites was due to allosteric effects, owing to naturally occurring compounds in the juice and the products of hydrolysis. The enzymatic treatment of the guava pulp led to an increase in the reducing sugar content of more than two folds for 0.078% and 0.1% w/w. For each enzyme concentration, viscosity decreased by 90% with enzymatic treatment. The values of pH, protein and polyphenol concentration also decreased with enzymatic treatment. FESEM images of the samples showed a change in surface morphology, indicating promotion of hydrolysis of pectin network.

Earlier studies on depectinization of pectin involved pectin isolated from fruit juice and not on the actual juice. The novelty of the present work lies in the fact that kinetic modeling of hydrolysis of the pectinaceous matters in actual guava juice was investigated in detail, providing an insight to inherent molecular mechanism. The results reveal that presence of polysaccharides in actual guava juice led to departure from the traditional Michaelis-Menten kinetics, and exhibited sigmoidal variation. On an industrial scale, depectinization needs to be undertaken on actual fruit juice itself and not the pectin extracted from it. Thus, the developed depectinization kinetic expression would help in identifying the optimum operating conditions and designing the scaled up

**Table 1**
Physicochemical, nutritional and rheological parameters of the raw guava juice as well as the samples treated at different enzyme concentrations at the end of 90 min.

| Sample Identity | pH | TSS (Bx) | Color (A) | Protein (mg/L) | Polyphenol (mg GAE/ 100 mL) | Antioxidant activity (%) | Viscosity (mPa.s) | Reducing sugars (mg/L) |
|---|---|---|---|---|---|---|---|---|
| Sample-1(Enzyme conc.-0.033% w/w) | 3.51 ± 0.01 | 2.27 ± 0.15 | 15.01 ± 1.09 | 554.3 ± 67.1 | 47.2 ± 4.9 | 94.3 ± 0.8 | 1.273 ± 0.04 | 5251 ± 91 |
| Sample-2(Enzyme conc.-0.055% w/w) | 3.34 ± 0.01 | 2.23 ± 0.06 | 13.06 ± 0.83 | 511.9 ± 6.7 | 45.9 ± 3.2 | 94.5 ± 1.4 | 1.074 ± 0.08 | 9534 ± 244 |
| Sample-3(Enzyme conc.-0.078% w/w) | 3.39 ± 0.01 | 2.07 ± 0.12 | 13.40 ± 0.51 | 514.9 ± 20.3 | 44.9 ± 1.6 | 93.8 ± 0.2 | 1.223 ± 0.07 | 12304 ± 1066 |
| Sample-4(Enzyme conc.-0.10% w/w) | 3.43 ± 0.02 | 2.20 ± 0.00 | 12.29 ± 0.34 | 502.4 ± 66.4 | 47.0 ± 4.7 | 95.0 ± 0.9 | 0.969 ± 0.01 | 13393 ± 5290 |
| Raw sample | 3.70 ± 0.02 | 1.7 ± 0.10 | 9.83 ± 0.32 | 615.7 ± 28.6 | 60.4 ± 0.4 | 94.7 ± 0.3 | 17.72 ± 3.18 | 5162 ± 216 |

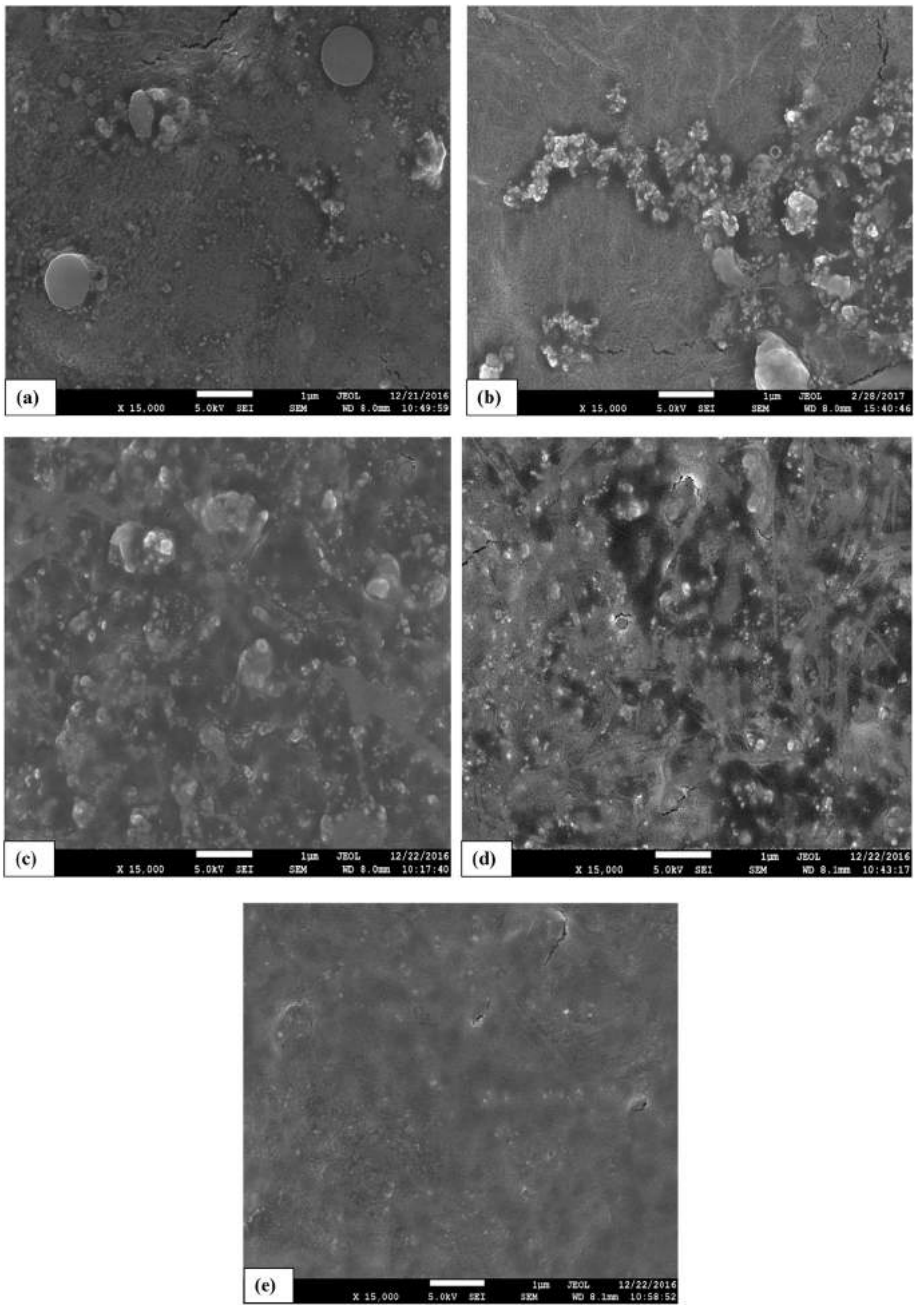


**Fig. 5.** FESEM images of the guava samples: (a) sample without enzymatic treatment; (b) sample treated with enzyme concentration of 0.033% w/w for 90 min; (c) sample treated with enzyme concentration of 0.055% w/w for 90 min; (d) sample treated with enzyme concentration of 0.078% w/w for 90 min; (e) sample treated with enzyme concentration of 0.1% w/w for 90 min.

reactor with fundamental understanding. Consequently, a precise knowledge of depectinization kinetics would aid in the designing and scaling up of subsequent juice clarification process equipments.

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## References

Liew Abdullah, A.G., Sulaiman, N.M., Aroua, M.K., Megat Mohd Noor, M.J., 2007. Response surface optimization of conditions for clarification of carambola fruit juice using a commercial enzyme. J. Food Eng. 81 (1), 65–71.

Akesowan, A., Choonhahirun, A., 2013. Effect of enzyme treatment on guava juice production using response surface methodology. J. Animal Plant Sci. 23 (1), 114–120.

Baş, D., Boyaci, İ.H., 2007. Modeling and optimization I: usability of response surface methodology. J. Food Eng. 78 (3), 836–845.

Bélafi-Bakó, K., Eszterle, M., Kiss, K., Nemestóthy, N., Gubicza, L., 2007. Hydrolysis of pectin by *Aspergillus niger* polygalacturonase in a membrane bioreactor. J. Food Eng. 78 (2), 438–442.

Benen, J., Parenicova, L., Kusters-van Someren, M., Kester, H., Visser, J., 1996. Molecular genetic and biochemical aspects of pectin degradation in *Aspergillus*. In: Visser, J., Voragen, A.G.J. (Eds.), Pectins and Pectinases. Elsevier, Amsterdam, The Netherlands, pp. 331–346.

Benen, J.A.E., Kester, H.C.M., Visser, J., 1999. Kinetic characterization of *Aspergillus niger* N400 endopolygalacturonases I, II and C. Eur. J. Biochem. 259 (3), 577–585.

Benen, J.A.E., van Alebeek, G.J.W.M., Voragen, A.G.J., Visser, J., 2003. Mode of action analysis and structure-function relationships of *Aspergillus niger* pectinolytic enzymes. In: Voragen, F., Schols, H., Visser, R. (Eds.), Advances in Pectin and Pectinase Research. Springer-Science+Business Media, B.V., The Netherlands, pp. 235–256.

Combo, A.M.M., Aguedo, M., Goffin, D., Wathelet, B., Paquot, M., 2012. Enzymatic production of pectic oligosaccharides from polygalacturonic acid with commercial pectinase preparations. Food Bioprod. Process. 90 (3), 588–596.

Das, A., Majumdar, K., 2010. Fractional changes of pectic polysaccharides in different tissue zones of developing guava (Psidium guajava L.) fruits. Sci. Hortic. 125 (3), 406–410.

Flores, G., Wu, S.B., Negrin, A., Kennelly, E.J., 2015. Chemical composition and antioxidant activity of seven cultivars of guava (Psidium guajava) fruits. Food Chem. 170 (1), 327–335.

International Oenological Codex, 2012. Determination of Polygalacturonase Activity in Enzymatic Preparations. http://www.oiv.int/public/medias/4352/e-coei-1-actpga-en.pdf. Accessed 03 August 16.

Jain, A., De, S., 2016. Aqueous extraction of bitter gourd (*Momordica charantia* L.) juice and optimization of operating conditions. Fruits 71 (6), 379–387.

Kaur, S., Sarkar, B.C., Sharma, H.K., Singh, C., 2009. Optimization of enzymatic hydrolysis pretreatment conditions for enhanced juice recovery from guava fruit using response surface methodology. Food Bioprocess Technol. 2 (1), 96–100.

Kaur, S., Sarkar, B.C., Sharma, H.K., Singh, C., 2011. Response surface optimization of conditions for the clarification of guava fruit juice using commercial enzyme. J. Food Process Eng. 34 (4), 1298–1318.

Kiss, K., Cserjesi, P., Nemestothy, N., Gubicza, L., Belafi-Bako, K., 2008. Kinetic study on hydrolysis of various pectins by *Aspergillus niger* polygalacturonase. Hung. J. Ind. Chem. 36 (1–2), 55–58.

Klinchongkon, K., Khuwijitjaru, P., Adachi, S., 2017. Degradation kinetics of passion fruit pectin in subcritical water. Biosci. Biotechnol. Biochem. 81 (4), 712–717.

Lee, W.C., Yusof, S., Hamid, N.S.A., Baharin, B.S., 2006. Optimizing conditions for enzymatic clarification of banana juice using response surface methodology (RSM). J. Food Eng. 73 (1), 55–63.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Marangoni, A.G., 2003. Enzyme Kinetics: a Modern Approach. John Wiley & Sons, Inc., New Jersey (Chapter 8).

Masmoudi, M., Besbes, S., Chaabouni, M., Robert, C., Paquot, M., Blecker, C., Attia, H., 2008. Optimization of pectin extraction from lemon by-product with acidified date juice using response surface methodology. Carbohydr. Polym. 74 (2), 185–192.

McComb, E.A., McCready, R.M., 1952. Colorimetric determination of pectic substances. Anal. Chem. 24 (10), 1630–1632.

McCook-Russell, K.P., Nair, M.G., Facey, P.C., Bowen-Forbes, C.S., 2012. Nutritional and nutraceutical comparison of Jamaican *Psidium cattleianum* (strawberry guava) and *Psidium guajava* (common guava) fruits. Food Chem. 134 (2), 1069–1073.

Mensor, L.L., Menezes, F.S., Leitão, G.G., Reis, A.S., Dos Santos, T.C., Coube, C.S., Leitão, S.G., 2001. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytotherapy Res. 15 (2), 127–130.

Pařenicová, L., Benen, J.A.E., Kester, H.C.M., Visser, J., 1998. pgaE encodes a fourth member of the endopolygalacturonase gene family from *Aspergillus niger*. Eur. J. Biochem. 251 (1–2), 72–80.

Pařenicová, L., Kester, H.C.M., Benen, J.A.E., Visser, J., 2000. Characterization of a novel endopolygalacturonase from *Aspergillus niger* with unique kinetic properties. FEBS Lett. 467 (2–3), 333–336.

Prasanna, V., Prabha, T.N., Tharanathan, R.N., 2007. Fruit ripening phenomena- an overview. Crit. Rev. Food Sci. Nutr. 47 (1), 1–19.

Prinz, H., 2010. Hill coefficients, dose–response curves and allosteric mechanisms. J. Chem. Biol. 3, 37–44.

Rai, P., Majumdar, G.C., DasGupta, S., De, S., 2004. Optimizing pectinase usage in pretreatment of mosambi juice for clarification by response surface methodology. J. Food Eng. 64 (3), 397–403.

Sagu, S.T., Nso, E.J., Karmakar, S., De, S., 2014. Optimisation of low temperature extraction of banana juice using commercial pectinase. Food Chem. 151, 182–190.

Sevda, S.B., Rodrigues, L., 2011. Fermentative behavior of Saccharomyces strains during guava (Psidium guajava L) must fermentation and optimization of guava wine production. J. Food Process. Technol. 2 (4), 1000118.

Surajbhan, S., Singh, A., Joshi, C., Rodrigues, L., 2012. Extraction and optimization of guava juice by using response surface methodology. Am. J. Food Technol. 7 (6), 326–339.

Teixeira, G.H.A., Júnior, L.C.C., Ferraudo, A.S., Durigan, J.F., 2016. Quality of guava (Psidium guajava L. cv. Pedro Sato) fruit stored in low-O2 controlled atmospheres is negatively affected by increasing levels of $CO_2$. Postharvest Biol. Technol. 111, 62–68.

Van Pouderoyen, G., Snijder, H.J., Benen, J.A.E., Dijkstra, B.W., 2003. Structural insights into the processivity of endopolygalacturonase I from *Aspergillus niger*. FEBS Lett. 554 (3), 462–466.