Effect of Partially Purified Polygalacturonase and Cellulase on Red Guava Juice Clarification at Various Incubation Times and Temperatures

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ABSTRACT

Red guava juice has some undesirable characteristics including high viscosity and cloudiness as well as sediment formation due to the presence of polysaccharide in the juice. Hence, enzymatic treatment for juice clarification is needed to overcome this problem. The current study was aimed to evaluate the effect of incubation times and temperature on juice clarification using partially purified polygalacturonase from *Bacillus licheniformis* and cellulase from *Bacillus subtilis*. The incubation times of 60,90, and 120 minutes and temperatures of 35 °C, 47.5 °C, and 60 °C were used in this study. pH value, total dissolved solids (TDS), transmittance, viscosity, and yield were analyzed. The results showed that incubation temperature had a significant effect on all the parameters except for pH value. The interaction between incubation temperature and time on decreasing pH value and viscosity was observed. The best clarification condition was observed at incubation time of 47.5 °C for 90 minutes, which resulted in pH, TDS, transmittance, viscosity, and yield of 4.98±0.13, 7.67±0.21 °Brix, 23.27±0.24%T, 36.37±3.46 cP, and 77.51±1.95% respectively.

Keywords: Cellulase; clarification; incubation; polygalacturonase; red guava juice

INTRODUCTION

Red guava *(Psidium guajava* L.) tree is one of the horticultural plants that grow in tropical areas. It contains several nutrients beneficial to the body and the vitamin C content almost doubles those in sweet oranges which only contain 49 mg of vitamin C in each 100 g. Guava can be consumed as fresh or in processed form. Processed red guava generates various products such as syrup, juice, nectar, jelly, jam, confectionery, etc. Among those products, guava juice is the most common product. Processing red guava into juice increases the economic value while still maintaining its vitamin, mineral and taste (Sharma *et al*., 2014; Unal and Sener, 2015; Widowati *et al*., 2019). In general, the red guava juice is viscous and also forms sediment in two separate phases when left untreated. This appearance is less preferred by consumers (Ahmed *et al*., 2014; Sharma *et al*., 2015) and is mainly caused by several polysaccharide components (Barbalho *et al*., 2012).

Red guava contains 1.228% cellulose and 0.11% pectin. Enzymatic degradation of polysaccharides such as pectin could reduce water holding capacity thereby increasing the yield and transmittance (clarity) of fruit juice (Kumar, 2015; Robin *et al*., 2013). Polygalacturonase (EC 3.2.1.15) is an enzyme that hydrolyzes alpha -1,4 glycosidic bonds between galacturonic acid residues and degrades pectin. The hydrolysis rate is dependent on polysaccaharide chain length (Widowati *et al*., 2017). Cellulase (EC 3.2.1.4) is an enzyme produced by cellulolytic bacteria or fungi. It degrades cellulose molecule into monosaccharides such as beta-glucose

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or shorter polysaccaharides and oligosaccharides. The specific reaction involved is 1,4-beta-D-glycosidic linkages hydrolysis in cellulose, hemicellulose, lichenin, and beta D-glucan (Kaur *et al*., 2011; Widowati *et al*.,2016). Therefore, this study was aimed to evaluate the effect of partially purified polygalacturonase and cellulase on red guava juice clarification at different incubation time and temperatures.

RESEARCH METHODS

Tools

The equipments for enzyme production included pH meters (Ohaus) (USA), incubators (Selecta, Spain), micro-centrifuge (Hettich, Germany), autoclaves (Selecta, Spain), Laminar Air Flow (Labconco, USA), hot plate and stirrer (IKA Labortechnick, Japan), binocular microscope (Yazumi, Japan), coolbox, and 12 kDa cut off cellophane membrane (Carolina, USA). The equipments used for making fruit juice included juicers, knives, fruit juice containers, filter cloths, and beaker glass. The equipments for analysis were pH meter (Hanna, USA), spectrophotometer (UV-mini-1240 Shimadzu, Japan), vortex (Heidolph, Germany), analytical scale (Mettler Toledo, USA), hand refractometer (ATAGO, Japan), and a viscometer (Falling Ball, HAAKE, Germany).

Materials and Methods

The red guava, a bacterial isolate of *Bacillus subtilis* Krakarya_1 S6 and *Bacillus licheniformis* GD2a AR2 were used in this study. The chemicals included $(NH_4)_2SO_4$ (Merck, Germany), buffer acetate 0.05M pH 5.2, CH_3COOH (Merck, Germany), and sodium acetate CH_3COONa (Merck, Germany). The other components were $BaCl_2$, yeast extract (Merck, Germany), Na_2HPO_4 (Merck, Germany), KH_2PO_4 (Merck, Germany), $MgSO_4.7H_2O$ (Merck, Germany), KCl (Merck, Germany), citrus pectin (Sigma-Aldrich, Singapore), bacteriological agar (Difco, USA), CMC, KNO_3 (Merck, Germany), K_2SO_4 (Merck, Germany), $FeSO_4.7H_2O$ (Merck, Germany), $CaCl_2$ (Merck, Germany), and $C_6H_{12}O_6$ (Merck, Germany).

Methods

Polygalacturonase and cellulase enzymes production

Enzyme production commenced by inoculating 100 mL inoculum of *Bacillus licheniformis* GD2a AR2 in 900 mL liquid pectin media and 100 mL inoculum of *Bacillus subtilis* Krakarya_1 S6 in 900 mL liquid carboxymethyl cellulose media. Then, *B. licheniformis* GD2a AR2 pectinolytic isolates were incubated in shaker incubators at 144 rpm and 55 °C, while *B. subtilis* Krakarya_1 S6 cellulolytic isolates were incubated at 37 °C for 10 hours. The media was centrifuged at 6000 rpm and 4 °C to obtain supernatant containing crude enzymes (Widowati *et al*., 2017).

Partial purification of polygalacturonase and cellulase enzymes

Partial purification was performed through the ammonium sulfate precipitation process according to each enzyme saturation level. The ammonium sulfate saturation fraction for cellulase enzyme was 40% and 50% for polygalacturonase (Widowati *et al*., 2017). Furthermore, the precipitate produced was centrifuged at 12, 000 rpm and 4 °C for 10 minutes. Then, dialysis was carried out in a 12 kDa cut off cellophane membrane bag.

Enzyme activity test

Polygalacturonase and cellulase activity was determined by analyzing reducing sugar levels using the DNS method. About 0.1 mL of the enzyme was added to 0.9 mL of reagent media consisting of 0.7% citrus pectin and 0.025 M sodium acetate buffer at pH 4.8 for polygalacturonase, while 0.1 mL enzyme was added to 0.9 mL carboxymethyl cellulose reagent media for cellulase (Widowati *et al*., 2019). Afterwards, incubation was carried out at 55 °C for polygalacturonase and that of cellulase was at 37 °C for 30 minutes. Enzyme activity was measured by adding 1 mL of the DNS reagent and then heated at 90 °C for 10 minutes. The solution was cooled and given 0.5 mL of 40% K-Na-Tartarat, well being put on the vortex. The control and blank treatments were carried out simultaneously with the same methods and stages as the sample, where the control was the reagent medium that has been incubated and added following DNS method before enzymes addition and heating at 90 °C, while the blanks did not use enzymes but buffers reacted with the reactant media. Then, the absorbance of the sample obtained was measured using a spectrophotometer with a wavelength of 540 nm and the value was substituted into the standard curve equation. Enzyme activity (Unit/mL) was obtained from Equation 1 below.

$$\text{Enzyme activity (Unit/mL)} = \frac{X}{t \times BM} \quad (1)$$

Notes:
X = the amount of reducing sugar in sample
t = incubation time (minute)

MW = glucose molecular weight ($C_6H_{12}O_6$)
(Widowati *et al*., 2019)

Enzyme specific activity was calculated by first measuring the protein content following the Lowry method using BSA as a standard.

Table 1**.** Polygalacturonase and cellulase enzyme activity

Enzyme	Enzyme activity (U/mL)		Specific activity (U/mg)	
	Crude	Partially pure	Crude	Partially pure
PG	0.02635	0.12746	0.38344	1.48854
Cellulase	0.03996	0.12065	0.34679	0.60389

The crude and partially pure enzyme activity as well as specific activity analysis used only one sample for each enzyme. Consequently, there was no standard deviation for these data.

Red guava juice clarification

Polygallacturonase and cellulase with 0.15% (v/v) and 0.12% (v/v) concentrations were added to red guava juice, respectively. The juice samples were incubated at 60, 90, and 120 min as well as 35 °C, 47.5 °C, and 60 °C at 150 rpm. Then, the enzymes were inactivated at 90-95 °C for 2 minutes and filtered using a filter cloth (Ahmed *et al*., 2014; Sassi *et al*., 2016) followed by performing the pH, TDS, viscosity, transmittance and yield tests (Nakkeeran *et al*., 2011; Yadav *et al*., 2017).

Characterization of Clarified fruit juice

The clarified fruit juice test investigated 5 parameters including pH using pH meter (Ahmed *et al*., 2014), total dissolved solids using hand refractometer (Widowati *et al*., 2019), viscosity using falling ball viscometer (Widowati *et al*., 2017), transmittance using a spectrophotometer at λ 660 nm (Widowati *et al*., 2019), and yield by comparing samples before and after filtering (Diniz *et al*., 2015).

Statistical Analysis

Data were presented as mean ± deviation. There were triplicate of experiments and analysis. Therefore to compare treatments, analysis of variance (ANOVA) was used. The $p < 0.05$ was considered as statistically significant, along with the Duncan Post-Hoc Test which was significant and at a 95% confidence interval. SPSS version 20.0 program was used for statistical analysis.

RESULTS AND DISCUSSION

Clarification Results of Red Guava (*Psidium guajava* L.) Juice

pH Value

Based on Table 2, the pH of clarified fruit juice ranged from 4.82 ± 0.04 to 5.23 ± 0.16, while the one without clarification was 5.1. The polygalacturonase is stable in the pH range of 4-7, while cellulase is stable in 3-9, therefore both enzymes still functioned on the tested red guava juice (Widowati *et al*., 2016; Widowati *et al*., 2017). Moreover, Incubation at 47.5 °C and 60 °C produced a lower pH of clarified guava juice than at 35 °C. Akesowan and Choonhahirun (2013) reported a pH decrease which is also shown by Guava nectars with pectinase. pH determination is important since it is a limiting factor for the growth of pathogenic and spoilage bacteria, while the value affects red guava juice taste.

The polygalacturonase produced by *Bacillus licheniformis* GD2A AR2 was stable in the 50-60°C, while the cellulase from *Bacillus subtilis* Krakarya_1 S6 was stable in 30-60 °C (Widowati *et al*., 2016; Widowati *et al*., 2017). Besides, the incubation temperature that increased to 47.5 °C reduced the red guava juice pH (Table 2). This decrease was caused by an increased enzyme activity during incubation with temperatures close to the optimum of polygalacturonase enzyme, thereby increasing D-galacturonic acid formation and reducing the juice pH. While cellulose hydrolysis did not contribute much to the pH decrease compared to D-galacturonic acid. Glucose as the main product of cellulose breakdown, has a low dissociation constant (Ka) and a low acidity level of around 10-12 Pka (Carvalho and Silva, 2010; Widowati *et al*., 2019). Incubation at 60 °C produced a juice with lower pH compared to 47.5 °C.

The pH value is a negative logarithm of hydrogen ions concentration, hence a large hydrogen ions amount is needed to change the value. Also, the galacturonic acid from pectin degradation is a weak acid that had no significant effect on pH even though incubation time increased. Polygalacturonases only work on the natural polygalacturonate (pectic acid) substrate of red guava juice which is the cutting result of the fruit's endogenous pectinesterase enzyme, and this involves hydrolyzing the α-1,4 glycosidic bond to form D-galacturonic acid (Akesowan and Choonhahirun, 2013). Therefore, the substrates number available is limited and has possibly contributed to the decreased red guava juice acidity despite the elevated incubation time.

Total dissolved solids (TDS)

Increasing the clarification temperature elevated the speed of substrate formation *i.e.,* galacturonic and glucose. Based on Table 3, incubation at 47.5 °C led to higher ° Brix than that of obtained at 35 °C, while at 60 °C, the TDS tended to decrease. This might be due to differences in enzymes activity at different temperatures. Generally, enzyme activity increases following the temperature until it reaches the optimum. Afterwards, the activity decreases as the temperature increases until it finally becomes inactive. This is due to enzyme denaturation at higher temperatures (Akesowan and Choonhahirun., 2013; Widowati *et al*., 2016).

The partially purified cellulase produced from *B. subtilis* Krakarya_1 S6 isolate was optimum at 35 °C and stable at 30-60 °C. Meanwhile, partially purified polygalacturonase from *B. licheniformis* GD2a AR2 was optimum at 60 °C and stable at 50-60 °C (Widowati *et al*., 2016; Widowati *et al*., 2017). At 35 °C, the cellulase worked due to being its optimum temperature, while polygalacturonase tended not to function well. TDS increased with an increase in temperature to 47.5 °C or near 60 °C because of an increase in polygalacturonase and cellulase activity, which is still in the stable range. This occurred might be due to a decreased cellulase activity at 60 °C despite being considered stable at that temperature.

The difference in incubation time significantly affected red guava juice TDS. Furthermore, the time is related to the hydrolyzable substrate amount at that moment. Widowati *et al*., (2019) reported an increasing red guava juice TDS with a clarification time of about 2.5 hours. This is in line with the results for 90 and 120 minutes incubation which increased the red guava juice TDS compared to 60 minutes.

Table 2**.** pH of clarified red guava juice

Incubation temperature (°C)	pH		
	60 minutes	90 minutes	120 minutes
35	5.23 ± 0.16^{b}	5.03 ± 0.10^{ab}	5.03 ± 0.10^{ab}
47.5	4.90 ± 0.89^{a}	4.98 ± 0.13^{a}	4.87 ± 0.82^{a}
60	4.82 ± 0.04^{a}	4.85 ± 0.14^{a}	4.85 ± 0.10^{a}

The pH value followed by the same uppercase and lowercase notation shows no significant difference at the significance level (α) = 0.05.

Transmittance Value

Fruit juice transmittance shows the clarity level. The clarified fruit juice usually produces higher transmittance due to the breakdown of the fruit juice components. The red guava juice transmittance value ranged from 19.44 to 82.8% (Le *et al*, 2012; Widowati *et al*., 2019). Based on Table 4, the incubation temperature had a significant effect on red guava juice transmittance. The increase in incubation temperature increased the enzymatic reaction rate on the juice clarification. This was marked by increased transmittance after incubation at 47.5 °C compared to 35 °C, while the biggest value was yielded at 47.5 °C. However, incubation at 47.5 °C and 60 °C did not show a significantly different transmittance value. These results are similar to TDS parameters, which are related to the optimum temperature and stability of the enzyme used.

Table 3**.** Total dissolved solids of clarified red guava juice

Incubation temperature (°C)	Total dissolved solids (°Brix)		
	60 minutes	90 minutes	120 minutes
35	7.22 ± 0.26^{a}	7.40 ± 0.31^{ab}	7.40 ± 0.18^{ab}
47.5	7.40 ± 0.19^{ab}	7.67 ± 0.21^{b}	7.62 ± 0.18^{ab}
60	7.27 ± 0.16^{ab}	7.50 ± 0.17^{ab}	7.52 ± 0.75^{ab}

The TDS value followed by the same uppercase and lowercase notation shows no significant difference at the significance level (α) = 0.05.

Partially purified cellulase from *Bacillus subtilis* Krakarya_1 S6 isolates was optimum at 35 °C and stable at 30-60 °C, while the partially purified polygalacturonase from *Bacillus licheniformis* GD2a AR2 was optimum at 60 °C and stable at 50-60 °C (Jori *et al*, 2015; Widowati *et al*., 2016; Widowati *et al*., 2017). Polygalacturonases function better at 47.5 °C which is closer to their optimum temperature (60 °C) than at 35 °, while cellulase activity tended to decrease at 60 °C even though it is considered stable.

Incubation for 90 minutes yielded greater red guava juice transmittance than 60 minutes. The longer incubation delayed substrate degradation by the enzyme, hence the juice obtained becomes clearer. However, incubation for 120 minutes showed no significant difference in clarified red guava juice transmittance. This result is possibly related to the polygalacturonate substrate limitation and cellulase inhibition by the product, hence the transmittance had little changes as the incubation time increased.

Viscosity

The temperature used had a significant effect on red guava juice viscosity. Based on Table 5, incubation at 47.5°C produced a lower viscosity than at 35 °C. Red guava juice viscosity decreased from 46.34 ± 9.99 cP

Table 4. The transmittance of clarified red guava juice

Incubation temperature (°C)	Transmittance (%)		
	60 minutes	90 minutes	120 minutes
35	18.13 ± 1.96^{a}	19.67 ± 1.83^{a}	20.18 ± 1.82^{ab}
47.5	22.13 ± 0.60^{bc}	23.27 ± 0.24^{c}	23.45 ± 0.64^{c}
60	23.25 ± 0.41^{c}	23.77 ± 0.56^{c}	23.75 ± 0.32^{c}

The transmittance value followed by the same uppercase and lowercase notation shows no significant difference at the significance level (α) = 0.05.

at 35 °C and to 40.68 ± 5.47 cP at 47.5 °C. The results were not significantly different at 47.5°C and 60 °C. In addition, increasing temperature to 47.5 °C led to increased TDS.

The incubation time had a significant effect on red guava juice viscosity. Furthermore, incubation for 90 and 120 minutes led to lower clarified red guava juice viscosity compared to 60 minutes. Similar results were also obtained by Widowati *et al.*, (2019) which found the incubation time length had a linear effect on reducing the sapodilla juice viscosity. Enzymes used in fruit juice clarification reduce viscosity generally due to pectins fiber particles breakdown (Ninga *et al.*, 2018). The longer the incubation time in the clarification process, the more pectin and fiber components are broken down, hence the viscosity becomes lower (Sinatari *et al.*, 2013; Bonnin and Lahaye, 2013; Ongratto and Viotto, 2016). The lowest viscosity values were obtained by 90 and 120 minutes incubation. Based on statistical calculations of interaction between incubation temperature and viscosity time, high temperature, led to longer incubation and decreased viscosity.

Yield

Incubation temperature had a significant effect on clarified red guava juice yield (Table 6). The largest value was obtained by incubating at 47.5 °C and 60 °C, while 76.38 ± 1.50% yield was produced at 47.5 °C which was higher than the 73.09 ± 1.69% at 35 °C. However, incubation at 60 °C produced similar yield with that of incubated at 47.5 °C. The result of the yield is in accordance with the result of viscosity. In addition, fruit juice the decrease of viscosity affected the ease of filtration process due to insoluble juice component degradation (Golan, 2011; Arsad *et al.*, 2015; Izadi *et al.*, 2015).

The result of analysis on several parameters (pH, TDS, transmittance, viscosity, and yield of red guava juice) were ranked to determine the be best treatment condition which is presented in Table 7.

Table 5. The viscosity of clarified red guava juice

Incubation temperature (°C)	Viscosity (cP)		
	60 minutes	90 minutes	120 minutes
35	55.62±5.58^{d}	48.32±6.51^{cd}	35.07±2.49^{a}
47.5	46.61±4.33^{bcd}	36.37±3.46^{a}	39.07±1.79^{abc}
60	47.15±7.92^{bcd}	35.67±7.88^{a}	37.73±1.81^{ab}

The viscosity value followed by the same uppercase and lowercase notation shows no significant difference at the significance level (α) = 0.05.

Table 6. The yield of clarified red guava juice

Incubation Temperature (°C)	Yield (%)		
	60 minutes	90 minutes	120 minutes
35	71.64±2.14^{a}	73.72±1.10^{ab}	73.92±0.34^{abc}
47.5	75.53±0.30^{cd}	77.51±1.95^{d}	76.08±1.12^{bcd}
60	75.57±0.37^{bcd}	76.83±1.07^{d}	76.29±0.72^{cd}

The yield value followed by the same uppercase and lowercase notation shows no significant difference at the significance level (α) = 0.05.

Table 7 showed the selected red guava juice pH values were 5.23 ± 0.16 and 5.03 ± 0.10, which were chosen because it is the closest pH value of clarified red guava to the pH value of unclarified fresh red guava juice. The fruit juice pH is related to the amount of acid present, while the acid amount and TDS (sugar) in fruit juice affect red guava juice sensory. Therefore, red guava juice with a high brix/acid ratio had the best acceptance level according to Widowati *et al.*, (2019) that stated high brix value and low acidity in red guava juice increased consumer acceptance level.

Furthermore, the best incubation treatment was obtained at 47.5 °C and 60 °C for 90 and 120 minutes. These were selected based on the highest TDS, transmittance, and yield value of fruit juice and the lowest clarified red guava juice viscosity. The selected treatment was incubated at 47.5 °C for 90 minutes with energy efficiency and time consideration. This incubation treatment produced fruit juice with pH, TDS, transmittance, viscosity, and yield of 4.98±0.13, 7.67±0.21 °Brix, 23.27±0.24%T, 36.37±3.46 cP, and 77.51±1.95% respectively.

CONCLUSIONS

Increasing temperature in red guava juice clarification decreased the pH and viscosity while

Table 7. Selection of best treatment

No.	Temperature	Time	pH	TDS	Transmittance	Viscosity	Yield	Score
1.	35	60	**5.23±0.16^{b}**	7.22±0.26^{a}	18.13±1.96^{a}	55.62±5.58^{d}	71.64±2.14^{a}	1
2.	47.5	60	4.90±0.89^{a}	**7.40±0.19^{ab}**	**22.13±0.60^{bc}**	46.61±4.33^{bcd}	**75.53±0.30b^{cd}**	3
3.	60	60	4.82 ±0.04^{a}	**7.27±0.16^{ab}**	**23.25±0.41^{c}**	47.15±7.92^{bcd}	**75.57±0.37^{bcd}**	3
4.	35	90	**5.03±0.10^{ab}**	**7.40±0.31^{ab}**	19.67±1.83^{a}	48.32±6.51^{cd}	73.72±1.10^{ab}	2
5.	47.5	90	4.98±0.13^{a}	**7.67±0.21^{b}**	**23.27±0.24^{c}**	**36.37±3.46^{a}**	**77.51±1.95^{d}**	**4**
6.	60	90	4.85±0.14^{a}	**7.50±0.17^{ab}**	**23.77±0.56^{c}**	**35.67±7.88^{a}**	**76.83±1.07^{d}**	**4**
7.	35	120	**5.03±0.10^{ab}**	**7.40±0.18^{ab}**	20.18±1.82^{ab}	**35.07±2.49^{a}**	73.92±0.34^{abc}	3
8.	47.5	120	4.87±0.82^{a}	**7.62±0.18^{ab}**	**23.45±0.64^{c}**	**39.07±1.79^{abc}**	**76.08±1.12^{bcd}**	**4**
9.	60	120	4.85±0.10^{a}	**7.52±0.75^{ab}**	**23.75±0.32^{c}**	**37.73±1.81^{ab}**	**76.29±0.72^{cd}**	**4**

The value followed by the same letter notation shows no significant difference at the significance level (α) = 0.05

increased the TDS, transmission and yield. Longer incubation in red guava juice clarification decreased the viscosity while increased TDS, transmittance, and yield. The interaction between incubation temperature and time decreased pH and viscosity. Conclusively, the best incubation treatment based on all parameters of clarified red guava juice was at 47.5 °C for 90 minutes.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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