Screening and Characterization of Polygalacturonase as Potential Enzyme for Keprok Garut Orange (*Citrus nobilis var. chrysocarpa*) Juice Clarification

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Abstract. Use of thermostable enzyme from bacilli for industrial application is significant. This research aimed to isolate thermophilic pectinolytic bacteria from orange peel and vegetable waste which produced thermostable polygalacturonase, to investigate the polygalacturonase ability in clarifying keprok Garut orange juice, and to characterize polygalacturonase based on pH optimum, temperature optimum, enzyme stability, enzyme kinetics $K_M$ and $V_{max}$. Obtained, 14 isolates that further selected to 4 best isolates based on highest polygalacturonase activity and keprok Garut orange juice clarification ability. Four selected enzyme isolates were AR 2, AR 4, KK 4, and KK 5 had ability to increase juice transmittance, decrease juice viscosity and also reduce total soluble solid. Furthermore 4 selected isolates were partially purified by ammonium sulphate precipitation and dialysis method. Four partially purified enzymes were known that enzyme character of AR 2 optimum at pH 6; AR 4 optimum at pH 5.5; KK 4 optimum at pH 6; and KK 5 optimum at pH 4.5. Four enzymes were optimum at temperature 60$^\circ$C thus stable at temperature 50-60$^\circ$C, this characteristic indicate that enzymes were thermostable. AR 2 showed active activity stable at pH 4-7; AR 4 showed active activity stable at pH 6-7; KK 4 showed active activity stable at pH 4-6; however KK 5 stable at pH 4-5. Enzyme AR 2 and KK 4 was getting inactive at pH 11, thus AR 4 and KK 5 inactive at pH 12. $K_M$ value of AR 2, AR 4, KK 4, and KK 5 was 0.0959; 0.0974; 0.0966; and 0.178 mg/ml respectively. $V_{max}$ of AR 2, AR 4, KK 4, and KK 5 was 0.0203; 0.0202; 0.0185; and 0.0229 U/ml respectively. Enzyme AR 2 was the most compatible enzyme to be applied in keprok Garut orange juice clarification for it had the lowest $K_M$ value.

1. Introduction

Keprok Garut orange (*Citrus nobilis var. chrysocarpa*) belongs to keprok orange (*Citrus nobilis*) species which has best quality among other orange species in Indonesia [18;21] particularly cultivated in Garut and it has been recognized as superior variety. Highest pectin percentage in keprok Garut orange is about 3-3.5% [1]. Orange has been commonly processed into orange juice.

Orange juice with a high content of pectin cause turbidity and viscosity effect in juice. This condition allows difficulty of extraction and filtration, as pectin particle blocks filtration machine where the juice must pass. Pectinase (especially polygalacturonase) addition in the extraction process improves fruit juice yield through an easier process, decreases juice viscosity and degrades the gel structure, thus improving juice concentration capacity. Enzymatic clarification can increase yields by
more than 90% compared to conventional mechanical juicing, besides improving the organoleptic (color, flavor) and nutritional (vitamins) properties and technological efficiency (ease of filtering) [14]. Industry prefers to higher temperature for juice clarification condition. For high temperature decrease viscosity of the solution, increase substrate solubility and reduce the risk of microbial contamination. Thermostable enzymes isolated from thermophilic organisms are needed to adapt that juice clarification condition in the industry.

Polygalacturonase is one of many well known pectinolytic enzyme that catalyzes hidrolysis of polygalacturonic acid producing D-galacturonic acid, has thermostable characteristic, optimum pH 3-6 [17]. Pectin depolimerisation with polygalacturonase can decrease juice viscosity and contribute in improving flavor of juice [14;25]. The most commonly bacteria produced polygalacturonase are Bacillus sp. [14] dan Pseudomonas sp. [7].

This research was done to screen and characterize polygalacturonase from pectinolytic bacteria found in keprok Garut peel and vegetable waste which had potential in keprok Garut orange juice clarification.

2. Material and Methods

2.1. Material

Pectin citrus and D-galacturonic acid used in this research were from Sigma Aldrich. Various isolate sources like keprok Garut peel and vegetable waste (darker colored mustard green and cabbage) were collected from local market (Surakarta, Indonesia). Those samples were rotted for five days then incubated at 55°C for three days. Keprok Garut orange for polygalacturonase clarification ability test was also obtained from the same local market.

2.2. Isolation and screening of pectinolytic bacteria

Isolates of pectinolytic bacteria were isolated from decaying like keprok Garut peel and vegetable waste and repeatedly sub cultured on pectin plates, then purified colonies obtained from quadrant streak method. The medium composition consist of yeast extract 1g/L; Na2HPO4 0.9 g/L; KH2PO4 1g/L; MgSO4.7H2O 0.5 g/L; KCl 0.2 g/L; Pectin citrus 1g/L; and Bacteriological Agar 15g/L. The isolates were maintained in pectin agar slant at 4°C for further studies. Morphological characteristics of isolates were identified using Gram staining, endospore staining, catalase test and colony morphology. Isolates which were isolated from decayed keprok Garut orange peel designated as KK, then from decayed vegetable waste designated as AR.

2.3. Enzyme production

The bacterial isolated were separately cultured at 55°C for 24 hours in pectin citrus broth (yeast extract 1g/L; Na2HPO4 0.9 g/L; KH2PO4 1g/L; MgSO4.7H2O 0.5 g/L; KCl 0.2 g/L; and Pectin citrus 1g/L). The pH of the medium was adjusted at range 4-6 (must be acid condition to ensure the only pectinolytic enzyme produced was polygalacturonase). The broth from each culture was centrifuged (Hettich) at 6000 rpm for 15 min at 4°C and the supernatant was collected as crude enzyme extract.

2.4. Enzyme assay

Polygalacturonase activity was assayed using D-galacturonic acid as standard. To measure the activity of polygalacturonase, the assay mixture (1 cc) containing 0.1 cc volume of enzyme and 0.9 cc (0.7% w/v pectin dissolved in 0.025 M asetat buffer (pH 4.8) was incubated at 55°C for 30 min. The reducing sugar released was measured by the dinitrosalicylic acid method (Miller, 1959). The reaction in the test tube was stopped by adding 1 cc of DNS, boiled during 10 min at 90°C, cooled and added 0.5 ml potassium sodium tartrate 40% [15;12]. Optical density (OD) of the solution was measured by spectrophotometer (UV-Vis Shimadzu), at 540 nm. A standard curve was prepared using D-galacturonic acid (0 to 90 µg/ml) and a linear regression analysis was used to determine the total
reducing sugar. Blanks were prepared with inactivated enzymes. One unit of enzymatic activity (U) was defined as one µmol of galacturonic acid released per minute.

2.5. Juice clarification
Keprok Garut orange that was already peeled then extracted without additional water. Orange juice was filtered by the manual filter to separate from big orange dreg. 30 cc juice was treated with different enzymes (3 cc) and incubated for 60 min at 55°C. Enzymes were also given to 1% pectin solution. Physical characteristic was measured by viscosity by relative viscosity method [27], transmittance (%T) using spectrophotometer (UV-Vis Shimadzu) at 600nm while 1% pectin solution at 1100nm, total soluble solid (°Brix) determined by Hand Refractometer (ATAGO).

2.6. Growth rate and enzyme production curve
Growth rate and enzyme activity was determined by taking samples aseptically from submerged fermentation at the interval 15 minutes for 2 initial hours of fermentation, start from 3rd hour till 13th hour samples were taken every 1 hour.

2.7. Polygalacturonase purification
Polygalacturonase was purified using ammonium sulphate followed by dialysis. Different levels of ammonium sulphate saturation were used (50, 60, 70, and 80%). Known volume and units of crude enzyme filtrate were treated with these levels of ammonium sulfate saturation overnight at 4°C and then centrifuged (Hettich) at 12,000 rpm for 10 minutes. The obtained precipitate was dialyzed in a selophan membrane against sodium acetate buffer (pH 5.2; 0.05 M), overnight at 4°C in cool chamber under agitation, with change of the buffer every 8 hours.

2.8. Enzyme specific activity determination
Protein concentrations in crude enzyme, and the partially purified one were determined as described by Lowry (1951) using bovine serum albumin as standard. Enzyme specific activity was defined as enzyme units per mg protein.

2.9. Characteristics of partially purified Polygalacturonase:

2.9.1. Optimum pH
0.1 cc volume of enzyme and 0.9 cc (0.7% w/v pectin dissolved in 0.025 M asetat buffer) pH 4.0; 4.5; 5.0; 5.5 and 6.0 was incubated at 55°C for 30 min. Enzyme activity was measured by DNS method.

2.9.2. Optimum temperature
0.1 cc volume of enzyme and 0.9 cc (0.7% w/v pectin dissolved in 0.025 M asetat buffer pH 4.8) was incubated at 35, 40, 45, 50, 55 and 60°C for 30 min. Enzyme activity was measured by DNS method.

2.9.3. Thermal stability
0.1 cc volume of enzyme dissolved in 0.1 cc 0.025 M asetat buffer in optimum pH of each enzyme then incubated at 30, 40, 50, 60, 70, 80, and 90°C for 30 min. Enzyme activity was measured by DNS method.

2.9.4. pH stability
0.1 ml volume of enzyme dissolved in 0.1 ml 0.025 M asetat buffer pH 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 then incubated at optimum temperature of each enzyme for 30 min. Enzyme activity was measured by DNS method.
2.9.5. **Enzyme kinetics**
The Michaelis constant ($K_M$) of the purified polygalacturonase (PG) was determined by measuring the reaction velocities ($\mu$ mol galacturonic acid /min) at various concentrations of pectin 0.05; 0.1; 0.2; 0.4; 0.8; and 1 (mg.ml$^{-1}$) dissolved in 0.025 M acetate buffer pH 4.8 at 55ºC for 30 min. The data were plotted according to Lineweaver-Burk plot [16].

3. **Result and Discussion**

3.1. **Isolation and characterisation of pectinolitic bacteria**
Pectinolytic bacteria was isolated from decaying like keprok Garut peel and vegetable waste and incubated at 55ºC to obtain thermophilic pectinolytic bacteria. High temperature of incubation was needed to screen potential thermostable polygalacturonase produced by the pectinolitic bacteria so it will be appropriate for industry clarification process which uses high temperature. According to [2] the temperature of clarification of orange juice is 37-55ºC. Nine isolates from vegetable waste (designated as AR) and five isolates from keprok Garut orange peel (designated as KK) were already obtained.

Gram staining showed that all isolate was Gram negative except AR 5. Endospore staining with malachite green and safranin solution showed no isolate had endospore. Catalase test showed all isolate positive (found forming CO$_2$), it means all isolates were aerob as reported by [26] all isolates collected from sweet orange peel also showed had no endospore, positive in catalase test, and had characteristics as Gram negative bacteria.

3.2. **Screening potential isolates for juice clarification.**
First priority choosing four best-potential isolates was from four highest polygalacturonase activity by DNS method to ensure that dominant enzyme act in juice clarification (decreasing viscosity and increasing transmittance) was polygalacturonase. The activity of PG in crude enzymes was studied (Table 1) the range of PG activity in crude enzyme produced was 0.001 Unit/ml to 0.123 Unit/ml. Enzyme isolate AR 2 had highest PG activity 0.123 Unit/ml. AR 4 was second highest PG activity 0.075 Unit/ml, KK 4 as the third with 0.074 Unit/ml and KK 5 as the fourth with 0.064 Unit/ml. Screened four isolates from 14 isolates, those are AR 2, AR 4, KK 4 and KK 5. Orange juice that already clarified by pectinolitic enzyme will show clearer and less viscous. [9] pectic enzyme are used to reduce haze or gelling of juice. The addition of pectinases is preferred since it increases the volume of free-run juice and reduces pressing time. Range of juice viscosity with crude enzyme addition was 0.896 cp – 1.211 cp, whereas in control (orange juice without addition of crude enzyme) had viscosity value 0.946 cp, it showed decreasing viscosity. Similar result had been reported by [8] pectinase addition could reduce mango juice viscosity caused by depolimerisation of soluble pectin. Pectinase can hydrolyze protopectins and pectins to smaller chains like galacturonic acid [22], leading to a reduction of water holding capacity. As a consequence, free water was released to the system caused a reduction of viscosity [10]. It promoted the reduction of waste loss because the less viscous pure was easier for the filtration, showing a significant increase in juice yield [19]. Range of orange juice %transmittance with enzyme addition was 2.10 – 2.90 whereas in control %transmittance was 1.90. All crude enzyme could increase transmittance value of orange juice. It had been reported by [26] that enzyme addition could increase % transmittance in both orange juice and 1% pectin solution. All orange juice added with crude enzyme show 0°Brix 8, where it was less than control with 0°Brix 9 for pectinase degraded pectin substance into simple reduction sugar as stated by [8].
Table 1. Enzyme activity and clarification ability in juice

| Isolate | Crude Enzyme Activity (Unit/ml) | Total Cell (cell/ml) | Orange Juice Viscosity (cp) | % T | TPT (°Brix) | 1% Pectin Solution Viscosity (cp) | % T |
|---------|--------------------------------|----------------------|-----------------------------|-----|-------------|----------------------------------|-----|
| AR 1    | 0.059                          | 1.190. 10^6          | 0.938                       | 2.60| 8           | 1.234                           | 83.10|
| AR 2    | 0.123                          | 0.540. 10^6          | 0.912                       | 2.90| 8           | 1.124                           | 83.80|
| AR 3    | 0.036                          | 0.548. 10^6          | 0.896                       | 2.60| 8           | 1.173                           | 83.80|
| AR 4    | 0.075                          | 0.654. 10^6          | 0.930                       | 2.70| 8           | 1.197                           | 83.20|
| AR 5    | 0.031                          | 0.688. 10^6          | 1.211                       | 2.10| 8           | 1.211                           | 83.00|
| AR 6    | 0.039                          | 1.288. 10^6          | 1.078                       | 2.10| 8           | 1.178                           | 83.00|
| AR 7    | 0.019                          | 1.142. 10^6          | 0.923                       | 2.50| 8           | 1.222                           | 83.40|
| AR 8    | 0.046                          | 0.756. 10^6          | 0.905                       | 2.60| 8           | 1.221                           | 83.40|
| AR 9    | 0.027                          | 0.786. 10^6          | 0.925                       | 2.40| 8           | 1.174                           | 83.80|
| KK 1    | 0.053                          | 0.618. 10^6          | 0.904                       | 2.50| 8           | 1.198                           | 83.40|
| KK 2    | 0.001                          | 0.552. 10^6          | 0.951                       | 2.50| 8           | 1.203                           | 83.20|
| KK 3    | 0.058                          | 0.744. 10^6          | 0.917                       | 2.40| 8           | 1.464                           | 83.30|
| KK 4    | 0.074                          | 0.442. 10^6          | 0.945                       | 2.70| 8           | 1.193                           | 83.30|
| KK 5    | 0.064                          | 0.218. 10^6          | 0.900                       | 2.50| 8           | 1.173                           | 83.00|
| Control |                                | 0.946                | 1.90                        | 9   | 1.199       | 82.90                           |  

3.3. Determination of bacterial growth period and pectinase productivity

Determination of bacterial growth period and pectinase productivity was used to determine time of product recovery. In brief enzyme synthesis period with highest activity was used as time production of partially purified polygalacturonase. The highest activity was seen at the end of exponential phase, before the initial stationary phase. AR 2 and AR 4 isolate showed log total cell/ml at 10th hour after inoculation, the highest activity of enzyme AR 2 at 9th hour; AR 4 at 10th hour. KK 4 showed log total cell/ml at 11th hour after inoculation, the highest enzyme activity at 12th hour; whereas KK 5 showed log total cell/ml and highest enzyme activity at 12th hour. This result has similar time production with [26] which reported pectic enzyme isolated from sweet orange peel (designated as KJ-9) had time production at 10th hour.

3.4. Enzyme Characterization

3.4.1. Optimum pH

Palmer stated that the changes of enzyme tertiary structure caused hydrophobic group contact with water thus solubility of enzyme decreased. Decreasing enzyme solubility caused decreasing enzyme activity gradually[13]. Polygalacturonase activity found to be in optimum condition at several specific pH. Changes in pH have an effect on the affinity of the enzyme for the substrate [4]. According to [5] polygalacturonases have a relatively higher optimum temperature 50-60°C and a high tolerance to low pH 4.5-6.0. These properties could lead to their becoming acceptable in food industry for fruit juice sprocessing. Enzyme isolate AR 2 optimum at pH 6; AR 4 optimum at pH 5.5; KK 4 optimum at pH 6; and KK 5 optimum at pH 4.5 (figure 1).
3.4.2. **Optimum Temperature**

Effect of temperature is related with reaction rate that catalysed by enzyme. This could be due to the breakage of secondary, tertiary and quaternary bonds that maintain the three dimensional structure of enzymes at high temperature and there by would lead to conformational changes of the enzyme active site. It is known that enzyme activity increases with increasing temperature up to the optimum temperature as the result of increasing kinetic energy, which can favor rate of collisions between substrate and enzyme during hydrolysis process [20]. Optimum temperature condition of all enzymes was found to be at 60°C (figure 2). This optimum activity was similarly determined in the previous study by [3] which showed highest polygalacturonase activity of *Bacillus* sp. AD 1 was found to be at 60°C. Regarding bacterial pectinases, the optimal temperature for a PGase from *Streptomyces* sp. QG-11-3 is 60°C [23]. Higher optimum temperature offers some advantages for industrial process such lower viscosity of the substrate, reducing the risk of microbial contamination and provides better solubility at high temperature [24].

![Figure 1](image-url)
3.4.3. **pH and thermal stability**

Enzyme AR 2 isolate showed active activity and constantly stable at pH 4-7; AR 4 showed active activity and constantly stable at pH 6-7; while KK 4 at pH 4-6; and KK 5 showed active activity and constantly stable at pH 4-5. Enzyme AR 2 and KK 4 isolates start being completely inactive at pH 11, while AR 4 and KK 5 start being completely inactive at pH 12 (figure 3). Generally pH showed enzymes still active and stable at pH 4-10. Based on thermal stability result, enzyme AR 2, AR 4, KK 4 and KK 5 isolates were stable at 50-60°C (figure 4). Thermal stability results of PG indicated that all enzymes were thermostable enzyme. The result tended similarly with previous research. The optimal pH pectic enzymes may act range between 3.5-11, while the optimal temperatures vary between 40-75°C [6; 9].

**Figure 2.** Optimum temperature of enzyme AR 2 (a), AR 4 (b), KK 4 (c), KK 5 (d).
Figure 3. pH stability of enzyme AR 2 (a), AR 4 (b), KK 4 (c), KK 5 (d) at incubation 60°C.

Figure 4. Thermal stability of enzyme AR 2 at pH 6 (a); AR 4 at pH 5.5 (b); KK 4 at pH 4 (c); KK 5 at pH 4.5 (d).
3.4.4. Enzyme kinetics

Table 2. K_M and V_max Enzyme AR 2, AR 4, KK 4, and KK 5 Isolate

| Isolate | K_M (mg/ml) | V_max (U/ml) |
|---------|-------------|--------------|
| AR 2   | 0.0959      | 0.0203       |
| AR 4   | 0.0974      | 0.0202       |
| KK 4   | 0.0966      | 0.0185       |
| KK 5   | 0.178       | 0.0229       |

The K_M and V_max of AR 2, AR 4, KK 4, and KK 5 enzymes is shown on Table 2. Enzyme isolate AR 2 had lowest K_M value compared with others, was found to be 0.0959 mg/ml. Therefore, this low K_M value signified strong affinity of this enzyme for the substrate. For the lower K_M value, determines the higher affinity between substrate and enzyme. Higher affinity between polygalacturonase with pectin (pectic acid) as substrate makes higher potential to degrade pectin. [16] stated that the difference of V_max and K_M value was related with enzyme purification level. With result above, obtained AR 2 as the most ideal enzyme to be applied in keprok Garut orange juice clarification. The highest K_M value was found in KK 5 to be 0.178 mg/ml; the highest V_max was found in KK 5 to be 0.0229U/ml; whereas the lowest V_max was found in KK 4 to be 0.0185U/ml. This range of K_M and V_max was in line with range of K_M and V_max respectively reported by [26] to be 0.0170-0.025mg/ml and 0.0386-0.040U/ml.

4. Conclusion

14 were screened up to four best isolates based on highest polygalacturonase activity and keprok Garut orange juice clarification ability. Four selected enzyme isolates are AR 2, AR 4, KK 4, and KK 5 had ability to increase juice transmittance, decrease juice viscosity and also reduce total soluble solid. Four partially purified enzymes had each properties AR 2 optimum at pH 6; AR 4 optimum at pH 5.5; KK 4 optimum at pH 6; and KK 5 optimum at pH 4.5. Four enzymes were optimum at temperature 60°C thus stable at temperature 50-60°C, this characteristic indicates that enzymes were thermostable. All screened enzymes were stable in acidic condition, while enzyme AR 2 and KK 4 was getting inactive at pH 11, thus AR 4 and KK 5 inactive at pH 12. K_M value of AR 2, AR 4, KK 4, and KK 5 was 0.0959; 0.0974; 0.0966; and 0.178 mg/ml respectively. V_max of AR 2, AR 4, KK 4, and KK 5 was 0.0203; 0.0202; 0.0185; and 0.0229 U/ml respectively. Enzyme AR 2 was the most compatible enzyme to be applied in keprok Garut orange juice clarification for it had the lowest K_M value.

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