

# Polygalacturonase production by AR2 pectinolytic bacteria through submerged fermentation of raja nangka banana peel (*Musa paradisiaca* var. *formatypica*) with variation of carbon source and pectin

R Utami<sup>1\*</sup>, E Widowati<sup>1</sup>, A Ivenaria<sup>1</sup>, E Mahajoeno<sup>2</sup>

<sup>1</sup>Department of Food Science and Technology, Universitas Sebelas Maret, Jl. Ir Sutami 36A, Surakarta, Central Java, Indonesia.

<sup>2</sup>Department of Biology, Universitas Sebelas Maret, Jl. Ir Sutami 36A, Surakarta, Central Java, Indonesia.

\*E-mail: rohulautami@staff.uns.ac.id

**Abstract.** Polygalacturonase (EC 3.1.2.15) catalyzes the hydrolysis of  $\alpha$ -1,4-glycosidic bonds on galacturonic acid. Polygalacturonase can be produced from AR2 pectinolytic bacteria isolated from orange peel and vegetable waste. Commonly cost production of enzymes were high. However, with the advancement of technology, enzymes can now be manufactured at a low cost. Production of enzymes in low cost media with agro-industrial waste is interesting. Raja nangka banana peel is agro-industrial waste that is uneconomic. Therefore, this material can be used as a pectin source in polygalacturonase production. Polygalacturonase was produced by AR2 pectinolytic bacteria with the addition of various carbon sources (1% glucose, 1% galactose, 1% lactose) and variation of pectin concentrations (5%; 7.5%; 10%). This study used submerged fermentation with a cultivation temperature of 55°C and an agitation speed of 144 rpm for a 48-h incubation time. The results showed that variation of carbon sources and pectin concentrations affected the production of polygalacturonase. After 48 h fermentation, the results showed that the number of cells of samples ranged from 8.3 to 9.445 log cells/mL; the used pectin of samples ranged from 87.170-93.745%; and the polygalacturonase activity of samples ranged from 0.030 to 0.151 U/mL. The highest polygalacturonase activity was obtained by production of polygalacturonase on 1% glucose and 10% pectin medium.

## 1. Introduction

Polygalacturonase (EC 3.2.1.15) catalyzes hydrolysis of  $\alpha$ -1,4-glycosidic bonds on polygalacturonic acid producing D-galacturonic [1]. Polygalacturonase has been used in foods, biofuels, and the textile industry due its thermostability; polygalacturonase remains stable at 50-60°C. Polygalacturonase is one of the most widely used enzymes in the food industry [2]. Therefore, the production of enzymes is necessary to fulfill the demand of the industry.

Polygalacturonase can be produced by microbes in the agricultural waste medium fermentation such as by *Fusarium moniliforme* on wheat bran and orange pulp substrate, *Penicillium viridicatum* on orange bagasse and wheat bran substrate, *Aspergillus awamori* on grape pomace substrate, *Candida utilis* on apple pomace substrate, and *Penicillium occitanis* on citrus pectin substrate [3]. Banana peels can also be used as a medium for fermentation in pectinase production by *Aspergillus niger* under laboratory



conditions [4]. Previous research [5] showed that on the sixth day of fermentation, *Aspergillus parasiticus* produced polygalacturonase that showed enzyme activity ranging from 0.1183 to 2.4683 U/mL using banana peel as the medium fermentation substrate. Previous study also reported that banana peel is best at producing enzyme substrate polygalacturonase compared with the substrate from orange peel and pineapple skin [4]. The pectic substances of agricultural waste are good inducer of polygalacturonase production.

Carbon and nitrogen sources in medium fermentation are needed in the production of pectinase at an optimum level. However, previous study reported that the addition of 5% pectin and 0.5% ammonium sulfate in polygalacturonase production using raja nangka banana peel waste by AR2 bacteria only produced polygalacturonase with 0.134 U/mL enzyme activity [6]. This activity was lower than the activity of polygalacturonase produced by the same bacteria (AR2) using pectin citrus medium [7] that achieved 0.209 U/mL. Therefore, to increase the activity of polygalacturonase the addition of a carbon source such as sugar or pectin is needed. Some of these sugars are glucose, galactose, and lactose. Previous research mentioned that the activity of polygalacturonase produced in medium with the addition of glucose, lactose and galactose were 2.4 U/mL, 2.8 U/mL, and 31.29 U/mL, respectively [8-9]. Pectin can also be used as medium fermentation substrate for polygalacturonase production and produced enzyme activity at 41.13 U/mL by *Rhizomucor pusillus* [9]. Medium fermentation for polygalacturonase production containing sugar and pectin can be expected to produce higher enzyme activity compared with medium fermentation only from sugar.

AR2 pectinolytic bacteria have been isolated from orange peel and vegetables waste. Polygalacturonase produced by AR2 bacteria showed a juice clarification ability [7]. Polygalacturonase produced by AR2 bacteria has also already been used in the clarification of super-red dragon fruit juice [10]. Additionally, previous study also conducted research by combining enzymes polygalacturonase from AR2 bacteria with cellulase and amylase in orange juice clarification [11]. Furthermore, to increase the juice clarification ability of polygalacturonase produced by AR2 bacteria, the enzyme activity of polygalacturonase must also increase. This research investigated the effect of variations in the carbon source and pectin concentration in the fermentation medium on polygalacturonase production by AR2 bacteria. After 48 h fermentation the number of bacteria, the pectin content, and enzyme activity of polygalacturonase were determined.

## 2. Material and methods

### 2.1. Material

The raja nangka banana peels were obtained from UPKKS Bakti Kencana Karanganyar (Indonesia). AR2 pectinolytic bacteria (further identified as *Bacillus licheniformis* strain AR2 by 16S rDNA gene sequencing analysis) were isolated from orange peel and vegetables wastes from Lokal Market (Surakarta, Indonesia) and collected by the Food Microbiology and Biotechnology Laboratory of Universitas Sebelas Maret. Other materials were also prepared such as ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) (Merck), glucose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) (Merck), galactose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) (Merck), and lactose (C<sub>12</sub>H<sub>22</sub>O<sub>11</sub>) (Merck).

### 2.2 Starter cultures preparation

Subcultures were made by scraping 1 loopfull from AR2 culture media collection into pectin agar slant. The subcultures were incubated at 55°C for 24 h. Preparation starter isolates AR2 done by scraping 1 loopfull from subculture into 10 mL of liquid pectin media. Incubation was carried out at 55°C for 24 h until the cell number reached 10<sup>8</sup> cells/mL [6].

### 2.3 Fermentation medium preparation

The cleaned banana peels (100 g) were steam blanched for 6 min at 100°C to soften the tissue. Furthermore, the steamed banana peels were crushed using mortar. The banana peels pure at various concentration (5% (13.186 g); 7.5% (19.778 g) and 10% (26.371 g) were diluted with distilled water to fulfill 100 mL. Furthermore, 0.5% ammonium sulfate as nitrogen source [6] and 1% carbon source such

as glucose, galactose, and lactose [12] were added to the medium. The medium was sterilized for 15 min at 121°C.

#### *2.4 Fermentation for polygalacturonase production*

Each medium (100 mL) was cultured with 1 mL AR2 bacteria ( $10^8$  cells/mL). Incubation was conducted for 48 h at 55°C and 144 rpm using an incubator shaker [7]. After 48 h fermentation, the number of bacterial cells were enumerated using Hemocytometer. Determination of residual pectin content in the medium after fermentation was conducted by taking 20 mL of fermented medium and followed the previous method [13].

#### *2.5 Enzyme partial purification*

After 48 h fermentation, each medium was centrifuged (*Eppendorf 5810 R*) at 6,000 rpm and 4°C for 15 min. The supernatants were precipitated with ammonium sulfate at 50% saturation fraction with agitation, and incubated for 24 h at 4°C to separate the enzyme. After 24 h, the precipitates were separated by centrifugation (*Eppendorf 5810 R*) at 12,000 rpm at 4°C for 10 min. The resulting pellets were dissolved in 0.05 M acetate buffer [14] and pH 5.2. The ratio of pellets and buffer was 1: 1. The mixtures were dialyzed in 12 kDa cut off cellophane membrane and soaked in 600 mL of 0.05 M acetate buffer solution and pH of 5.2 with magnetic homogenization stirrer for 24 h in a cool chamber, at a constant temperature of 4°C. The buffer solution was replaced and tested qualitatively using  $\text{BaCl}_2$  1% per h to ensure the ammonium sulfate particles and other impurities have been removed from the cellophane membrane [15].

#### *2.6 Determination of polygalacturonase activity and specific enzyme activity*

Polygalacturonase activity was determined by measuring the reducing sugar that released from pectin by using a 3,5-dinitrosalicylic acid reagent assay. The reaction mixture containing 0.1 mL enzyme and 0.9 mL 0.7% citric pectin in 0.0025 M sodium acetate buffer pH 4.8 [16] was incubated at 55°C for 30 min. The absorbance of the sample was determined using a spectrophotometer UV-Vis (Shimadzu) at wavelength of 540 nm [16]. One unit of enzyme activity (U/mL) was defined as the amount of enzyme which released one  $\mu\text{mole}$  of galacturonic acid per min per mL of enzyme solution. The specific enzyme activity is the number of enzyme units per mL divided by the concentration of protein in mg/mL. The concentration of protein enzyme was measured using the Lowry method with absorbance value at wavelength of 600 nm.

#### *2.7. Statistical analysis*

Completely Randomized Design was used to evaluate the effect of carbon sources (1% glucose, 1% galactose, 1% lactose) and pectin concentration (5%; 7.5%; 10%). All treatments were replicated twice. Data were analysed with ANOVA test (One Way ANOVA) followed with DMRT (Duncan Multiple Range Test) ( $\alpha=5\%$ ) if there were the variances between samples by SPSS Statistics 16 program.

### **3. Result and discussion**

#### *3.1 Bacterial cells count*

The result showed that the carbon source significantly affected the bacterial cells count, with the exception of the sample with 5% pectin. The bacterial cells counts of 1% glucose and 10% pectin concentration medium were significantly higher than those of 1% lactose and 1% galactose medium (Table 1). During fermentation, microbes utilize carbon sources for metabolism and cell proliferation [17]. Previous study reported that by the addition of glucose on medium, the biomass of *Aspergillus niger* was increased more than by the addition of galactose, fructose or lactose [12].

Pectin concentration (5; 7.5; 10%) on medium did not show any significant effect on bacterial cells count (Table 1). Previous research reported that pectin concentration (1-5%) on medium

significantly affected the bacterial cells [6]. That research indicated that bacterial cells utilized the carbon sources (glucose, galactose, lactose) more than pectin for cell growth.

The bacterial cells resulted at this research ranged from 8.310- 9.445 log cell/mL. This result was higher than the bacterial cell resulted by the fermentation using pectin as the single carbon source at 1-5% (6.000-6.738 log cell/mL) [6]. This indicated that the addition of other carbon source such as glucose, galactose or lactose on medium could increase the number of bacterial cells.

**Table 1.** Bacterial cells count (log cell/mL)

Carbon	Pectin		
	5%	7.5%	10%
Glucose 1%	9.267 <sup>a A</sup>	9.445 <sup>a B</sup>	9.444 <sup>a C</sup>
Galactose 1%	9.281 <sup>a A</sup>	9.366 <sup>a B</sup>	9.051 <sup>a B</sup>
Lactose 1%	8.972 <sup>a A</sup>	8.481 <sup>a A</sup>	8.310 <sup>a A</sup>

Lowercase different notation on the same line and the notation of different capital letters in the same column indicate significant difference at  $\alpha = 5\%$

**Table 2.** Pectin utilization during fermentation (%)

Carbon	Pectin		
	5%	7.5%	10%
Glucose 1%	91.185 <sup>a A</sup>	91.730 <sup>a A</sup>	93.745 <sup>a A</sup>
Galactose 1%	88.420 <sup>a A</sup>	90.293 <sup>a A</sup>	90.585 <sup>a A</sup>
Lactose 1%	87.170 <sup>a A</sup>	91.137 <sup>b A</sup>	92.333 <sup>b A</sup>

Lowercase different notation on the same line and the notation of different capital letters in the same column indicate significant difference at  $\alpha = 5\%$

### 3.2 Pectin utilization

The pectin content of raja nangka banana peel was 37.924%. This result was lower than the pectin content of raja nangka banana peel found in the previous research (68.47%) [6]. The pectin content of banana peels are related to the banana maturity levels [18].

The initial pectin content on medium were varied at 5; 7.5 and 10%. During fermentation, pectin was hydrolyzed into galacturonic acid by pectinase enzyme produced by microbes [19]. The result showed that the pectin utilization after fermentation ranged from 87.17-93.745 % (Table 2). Previous studies have shown that 86% pectin was hydrolyzed by AR2 during fermentation [6]. The decrease of pectin content was due to the AR2 bacteria activity to metabolize the pectin into galacturonic acid [6]. Previous study also reported that the pectin content of apple pomace was decrease during fermentation [20]. However, the carbon sources and pectin concentration variation have no significant effect on pectin utilization.

### 3.3 Enzyme activity

Different types of carbon source significantly affected ( $\alpha < 0.05$ ) the polygalacturonase activity. The polygalacturonase activity of enzyme produced on 1% glucose medium was significantly higher than that produced on 1% galactose or 1% lactose medium (Table 3). For most bacteria, glucose is the most common substrate and can be used directly for growth. Meanwhile, the lactose is polysaccharide which is required to breakdown into monosaccharides (glucose and galactose) in the fermentation process, which takes more time for metabolism [21]. Under the same conditions as glucose, galactose was not suitable as carbon source [22]. Previous research also reported that glucose performed as the best carbon source while lactose had no supporting role as a carbon source for pectinase production [23].

Pectin concentrations in medium also significantly affected the polygalacturonase activity (Table 3). However, higher pectin concentration did not always result in higher activity. A higher concentration of the substrate in media does not always increase the activity of produced enzymes [24]. Additionally, if the active site of enzyme has been completely filled out by the substrate, the enzyme can not degrade the other substrates [25]. So although the substrate concentration is increased, it will not increase the activity of enzymes.

**Table 3.** Polygalacturonase activity (U/mL)

Carbon	Pectin		
	5%	7,5%	10%
Glucose 1%	0.129 <sup>a C</sup>	0.147 <sup>a C</sup>	0.151 <sup>a C</sup>
Galactose 1%	0.108 <sup>b B</sup>	0.087 <sup>a B</sup>	0.089 <sup>a B</sup>
Lactose 1%	0.050 <sup>b A</sup>	0.030 <sup>a A</sup>	0.054 <sup>b A</sup>

Lowercase different notation on the same line and the notation of different capital letters in the same column indicate significant difference at  $\alpha = 5\%$

**Table 4.** Concentration of protein enzyme (mg/mL)

Carbon	Pectin		
	5%	7,5%	10%
Glucose 1%	0.021 <sup>a A</sup>	0.061 <sup>ab A</sup>	0.133 <sup>b A</sup>
Galactose 1%	0.032 <sup>a A</sup>	0.073 <sup>c A</sup>	0.059 <sup>b A</sup>
Lactose 1%	0.371 <sup>a B</sup>	0.668 <sup>b B</sup>	0.728 <sup>b B</sup>

Lowercase different notation on the same line and the notation of different capital letters in the same column indicate significant difference at  $\alpha = 5\%$

**Table 5.** Polygalacturonase Specific Activity (U/mg)

Carbon	Pectin		
	5%	7,5%	10%
Glucose 1%	6.317 <sup>b C</sup>	3.207 <sup>ab A</sup>	1.140 <sup>a B</sup>
Galactose 1%	3.423 <sup>b B</sup>	1.195 <sup>a A</sup>	1.524 <sup>a C</sup>
Lactose 1%	0.134 <sup>b A</sup>	0.045 <sup>a A</sup>	0.074 <sup>a A</sup>

Lowercase different notation on the same line and the notation of different capital letters in the same column indicate significant difference at  $\alpha = 5\%$

The results showed that the highest polygalacturonase activity was 0.151 U/mL which found with a 1% glucose-10% pectin concentration medium while the lowest enzyme activity was 0.030 U/mL that found with a 1% lactose-7.5% pectin concentration fermentation medium. This result showed higher activity than previous report [6] which produced polygalacturonase with 0.134 U/mL enzyme activity by the fermentation using pectin as single carbon source. This indicated that the addition of other carbon source such as glucose, galactose or lactose to the fermentation medium could increase the polygalacturonase activity.

### 3.4 Concentration of enzyme protein

Variations in the carbon type significantly affected the concentration of the enzyme protein (Table 4). Nutrients in the fermentation medium containing sugar will provide energy for metabolic processes of microorganisms [26]. If there are better nutrients in the substrate, the cell growth can be faster and will increase the protein content [27].

Variations of pectin concentration significantly affected the concentration of the enzyme protein (Table 4). The results showed that the higher concentration of pectin the higher concentration of the enzyme protein. However, the addition of 10% pectin on galactose medium decreased the protein concentration of enzymes.



### 3.5 Specific activity of enzyme

Table 5 showed that variations of carbon type significantly affected the specific activity of the polygalacturonase. The specific activity of the polygalacturonase produced on glucose medium was higher than the specific activity of the polygalacturonase produced on galactose and lactose medium. This result was different from previous study [28]. The specific activity of polygalacturonase enzymes produced by *Aspergillus niger* on lactose medium were higher than that produced on glucose or galactose medium. The lowest specific activity of polygalacturonase enzymes were produced on glucose medium [28].

Meanwhile, statistically (Table 5) the variation of pectin concentration in the medium affected the specific activity of the polygalacturonase. The results showed that if greater concentration of pectin was added to the fermentation medium, the specific activity of the polygalacturonase enzyme decreased.

The highest specific activity of the polygalacturonase (6.317 U/mg) was produced on glucose medium with the addition of 5% pectin. This specific activity was higher than the specific activity of polygalacturonase produced on pectin medium (2.679 U/mg) [6]. Other study [19] also stated that the specific activity of enzyme polygalacturonase produced by *Aspergillus utus* BL5 was 4.15 U/mg. This result indicated that the variety of carbon types and concentrations of pectin affected the specific activity of the polygalacturonase.

## 4. Conclusion

The results showed that different of carbon sources (glucose 1%, galactose 1%, lactose 1%) and pectin concentration (5%; 7.5%; 10%) affected the production of polygalacturonase. After 48 h fermentation, the result showed that the number of cells of samples ranged from 8.3 to 9.445 log cells/mL; the used pectin of samples ranged from 87.170-93.745%; and polygalacturonase activity of samples ranged from 0.030 to 0.151 U/mL. The highest polygalacturonase activity was obtained by using 1% glucose and 10% pectin.

## Acknowledgements

This work was financially supported by research project of Penelitian Unggulan Perguruan Tinggi (PUPT) No: 041/SP2H/LT/DRPM/II/2016 from Ministry of Research, Technology, and Higher Education of Republic of Indonesia.

## References

- [1] Pedrolli D B, Monteiro A C, Gomes E, and Carmona E C 2009 *TOBIOTJ* **3** 9-18
- [2] Wang S, Lian Z, Wang L, Yang X, and Liu Y 2015 *Beijing Bioresources and Bioprocessing* **2** (33) 1-13
- [3] Torres E F, Sepulveda T V, and Gonzales G F 2006 *FTB* **44** (2) 221-227
- [4] Durairajan B, and Sankari P S 2014 *J. Pharm. Biosci.* **2** 50-57
- [5] Kumar G P, and Suneetha V 2014 *Int. J. Drug Dev. & Res.* **6** (3) 109-118
- [6] Safitri N R D 2015 *Produksi Poligalakturonase Melalui Fermentasi Cair Limbah Kulit Pisang Raja Nangka (Musa paradisiaca) dengan Variasi Sumber Karbon dan Nitrogen Anorganik Oleh Bakteri Pektinolitik* (Surakarta : Ilmu dan Teknologi Pangan, Universitas Sebelas Maret)
- [7] Kalistiyatika K 2014 *Produksi dan Karakterisasi Enzim Poligalakturonase pada Klarifikasi Sari Buah Jeruk Keprok Garut (Citrus nobilis var. chrysocarpa)* (Surakarta : Ilmu dan Teknologi Pangan, Universitas Sebelas Maret)
- [8] Kumar A, and Sharma R 2012 *J. phytol.* **4** (1) 01-05
- [9] Siddiqui M A, Pande V, and Arif M *India Afr. J. Microbiol. Res.* **7** (3) 252-259
- [10] Muthoharoh 2015 *Penggunaan Enzim Poligalakturonase dan Gelatin dalam Klarifikasi Sari Buah Naga Super Merah (Hylocereus costaricensis)* (Surakarta : Ilmu dan Teknologi Pangan, Universitas Sebelas Maret)
- [11] Sulistyo A C B 2016 *Pengaruh Kombinasi Enzim Poligalakturonase, Selulase, dan Amilase pada Klarifikasi Jus Jeruk Keprok Garut (Citrus nobilis var. chrysocarpa)* (Surakarta : Ilmu dan

Teknologi Pangan, Universitas Sebelas Maret)

- [12] Mojsov K 2010 *ATI* **3** (3) 23-29
- [13] Sulihono A, Tarihoran B, and Agustina T E 2012 *Jurnal Teknik Kimia* **18** (4) 1-8
- [14] Barensse R I, Chellegatti M A S C, Fonseca M J V, and Said S 2001 *Braz. J. Microbiol.* **32** 327-330
- [15] Widowati E, Utami R, Nurhartadi E, Andriani M A M, dan Wigati A W 2014 *Jurnal Aplikasi Teknologi Pangan* **3** (1) 16-20
- [16] Ordonez R G, Morlon J, Gasparian S, and Guyot J P 1998 *Folia Microbiol.* **4.3** (6) 657-660
- [17] Oberman H, and Libudzisz Z 1998 *Fermented Milks*, In B.J.B. Wood (Ed.), *Microbiology of Fermented Foods second edition* (London: Blackie Academic & Professional)
- [18] Akili M S, Ahmad U, dan Suyatma N E 2012 *Jurnal Keteknik Pertanian* **26** (1)
- [19] Yopi, Rahmani N, Andriani A, dan Dewi F 2013 *Berita Biologi* **12** (3)
- [20] Villas-Boas S G, Esposito E, and de Mendonca M M 2002 *World J. Microbiol. Biotechnol.* **18** (6) 541-545
- [21] Todar, K 2008 *Textbook of Bacteriology: Regulation of Bacterial Metabolism*. (Madison: University of Wilconsin)
- [22] Chan V, Dreolini L F, Flintoff K A, Lloyd S J, and Mattenley A A 2002 *JEMI* **2** 130-137
- [23] Jagiasi R R 2013 *National Conference Biodiversity Status and Challenges Conservation 'Favea'*
- [24] Rahmani N, Andriani A, Anggraini Y S, dan Yopi 2013 *Teknologi Indonesia* **36** (3) 142-148
- [25] Oktavia Y, Andhikawati A, Nurhayati T, dan Tarman K 2014 *Jurnal Ilmu dan Teknologi Kelautan Tropis* **6** (1) 209-218
- [26] Buckle K A, Edwards R A, Fleet G H, and Wooton M 1985 *Ilmu Pangan* Translated by Purnomo, H. dan Adiono (Jakarta : UI Press)
- [27] Fardiaz S 1992 *Mikrobiologi Pangan* (Yogyakarta: Penerbit Kanisius)
- [28] Ogbonnaya N, and Chukwuemeka E I 2016 *Scientific Paper, Hemijska Industrija*
- [29] Sebayang F 2005 *Jurnal Komunikasi Penelitian* **17** (3)