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Production and Characterization of *Bacillus firmus* Pectinase

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ABSTRACT

Pectinase is an enzyme which functions to hydrolyze pectin become D-galacturonic acid unit. This enzyme is potential in various industries, especially in fruit juice industry. Pectinase can be isolated from various microorganisms. Thus, it produces various pectinase characters. This paper presents determination of optimum conditions of pectinase production and also characterization of the resulted pectinase including optimum conditions of pectinase activity and the influence of some metals ions. The optimum conditions of pectinase production was carried out by growing *Bacillus firmus* on basal media containing pectin as inducer with various conditions of pH (5, 6, 7, 8, 9, and 10), temperatures (30, 35, 40, 45, and 50 ^{o}C), and fermentation times (6, 12, 18, 24, 30, and 36 h). The optimum pectinase activities was performed at various pH (4, 6, 7, 8, and 10), temperatures (30, 35, 40, 45, and 50 ^{o}C), and reaction times (10, 20, 30, 40, and 50 min). The influence of metals ions was conducted using Zn^{2+}, Mg^{2+}, K^{+} at range concentrations from 2 to 10 mM. The isolated pectinase from *Bacillus firmus* provided optimum conditions of pectinase production at pH 7-8, temperatures of 40-50 ^{o}C and fermentation time of 18 h. While the optimum conditions of pectinase activity were obtained at pH 7, 50 ^{o}C and reaction times of 30 min. The present of Zn^{2+}, Mg^{2+}, and K^{+} ions significantly affected the enzyme activity. Furthermore, Mg^{2+} ion indicated as a non-competitive inhibitor; while K^{+} and Zn^{2+} as uncompetitive enzyme inhibitors.

Key word: *Bacillus firmus*, pectinase, optimum condition, production, activity, inhibitor

Introduction

Raw fruit juice contains colloids composed of pectin, starch and protein that can cause membrane fouling during filtration. Pectin can slow down the clarification because of its fibril structure. Therefore, an enzyme which can degrade pectin in raw fruit juice is needed. Generally, pectinase is used in fruit juice industry prior to fruit juice clarification in which pectinase enable to prevent flocculation of pectin-protein and also lower viscosity. The initial treatments of fruit juice with addition of pectinase have been investigated in many quantity in order to enhance permeation flux in microfiltration, ultra filtration, and also reversed osmosis [1].

The demand of industrial enzymes for food industry is getting increased, and about 10% from overall enzymes are pectinase. Naturally, pectinase can be obtained from various sources of microorganisms such as *Aspergilus niger*, *Penicillium spp* [2], *Bacillus spp.*, *clostridium spp., and Pseudomonas spp* [3]. However, increasing demand of pectinase requires further exploration sources of pectinase. Pectinase producing microorganisms has been reported and isolated, however each of them indicated producing different character from different sources. *Bacillus firmus* is bacteria found in fruits waste, which able to

produce a large amount of pectinase by using semisolid fermentation [4]. *Bacillus firmus* which was isolated from local cow milk indicated pectinase activity; however optimum condition of production and its character has not yet been investigated.

Optimum condition of pectinase production is affected by environmental factors such as pH, temperature, and fermentation time. Fermentation time correlates to the microorganism curve growth where its phase is able to produce primary and secondary metabolites. The pH and temperature affect the availability of nutrient used by cells and reaction rate. In addition, the optimum condition of pectinase activity is also important to be determined. In spite of enzymatic reaction occur at optimum pH; temperature and reaction time, the optimum condition of pectinase activity also depends on microorganism source. *Aspergilus niger* pectinase has maximum activity at pH 4 and temperature 50 ^{o}C, while *Penicillium dierckii* pectinase has maximum activity at pH 3.5 and temperature 60 ^{o}C [5]. Furthermore, the kinetic parameter including maximum rate (V_m) and Michaelis-Menten constant (K_M) needs to be determined as well. The Pectinex Ultra SP-L is commercial pectinase brand, produced from *Aspergillus niger* with certain strain has optimum condition activity at 35 ^{o}C, pH 4.5, V_m 0.0046% (w/v) pectin /s and K_M 1.137% w/v pectin[2].

In general, the pectinase activity can be inhibited or activated by addition of metal ions. The present of metal ions theoretically affects the bounding between side-active of pectinase and substrates. For example, activity of *Penicillium chrysogenum* pectinase can be enhanced 3.56% by addition of $CaCl_2$, in contrast , the addition of $MgCl_2$, $ZnCl_2$, $CoCl_2$, $HgCl_2$ and $CuSO_4$ lowered its activity by 60% [6]. This paper reports on the determination of the optimum condition of pectinase production and also characterization of the produced pectinase including optimum condition of pectinase activity and the influence of some metal ions.

MATERIALS AND METHODS

Determination of Optimum condition of pectinase production from *Bacilus firmus*

Bacillus firmus was isolated from local cow (Blitar, East Java), and then it was maintained in nutrient agar containing 1% of pectin. Pectinase production was carried out using medium consist of yeast extract (5 g/L), $MgSO_4.7H_2O$ (0.2 g/L), KH_2PO_4 (1 g/L) and pectin (1 g/L). Medium was added 5 mL starter of *Bacillus firmus* and incubated with rotary shaking at 125 rpm with different pH values (5, 6, 7, 8, 9, and 10), temperatures (30, 35, 40, 45, and 50 ^{o}C) and fermentation times (6, 12, 18, 24, 30, and 36 h). Then, the fermented medium was centrifuged at 5 ^{o}C, 3000 rpm for 20 min. The resulted supernatants were crude pectinase extract and further assayed its activity.

Pectinase assay

One unit pectinase activity is defined as the number of reducing sugar (galacturonic acid) obtained from 1 mL of pectinase in 1 min. The resulted galacturonic acid was analyzed using modified Miller method. Measurement of pectinase activity was carried out using 1 mL of pectin 1%, 1 mL phosphate buffer pH 6.5 and 1 mL pectinase. This mixture was incubated at 37 ^{0}C for 30 min. Reagent of dinitro salicylic acid was added to the mixture and warmed in water bath 100 ^{o}C for 15 min. This mixture was measured its absorbance using visible spectrophotometer at 540 nm.

Pectinase characterization

Crude extract of pectinase was precipitated by addition of saturated ammonium sulphate 20-60%, and the precipitate was re-suspended in 10 mL of phosphate citrate buffer (0.2 M)pH 7, and dialysed against 100 mL of phosphate citrate buffer (0.07 M) pH 7 for 24 h. Determination of optimum conditions was conducted by measuring pectinase activity in various pH conditions (4, 6, 7, 8, and 10), temperatures (30, 35, 40, 45, and 50 ^{o}C), and reaction times (10, 20, 30, 40, and 50 min), respectively.

Determination of Michaelis–Menten Kinetic Parameter

Enzymatic reaction was conducted at the optimum conditions of pectinase activity, but using a constant amount of 1 mL of pectinase concentrations and with different initial pectin concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 % w/v). The influence of metal ions (Zn^{2+}, Mg^{2+}, K^{+}) was carried out at various concentrations (0.2, 4.6, 8.10 mM) and 0.7% w/v pectin, while kinetic parameter of inhibition was conducted at 0.2 mM Zn^{2+}, 0.2 mM K^{+} and 0.2 M of K^{+} metal ions.

RESULTS AND DISCUSSION

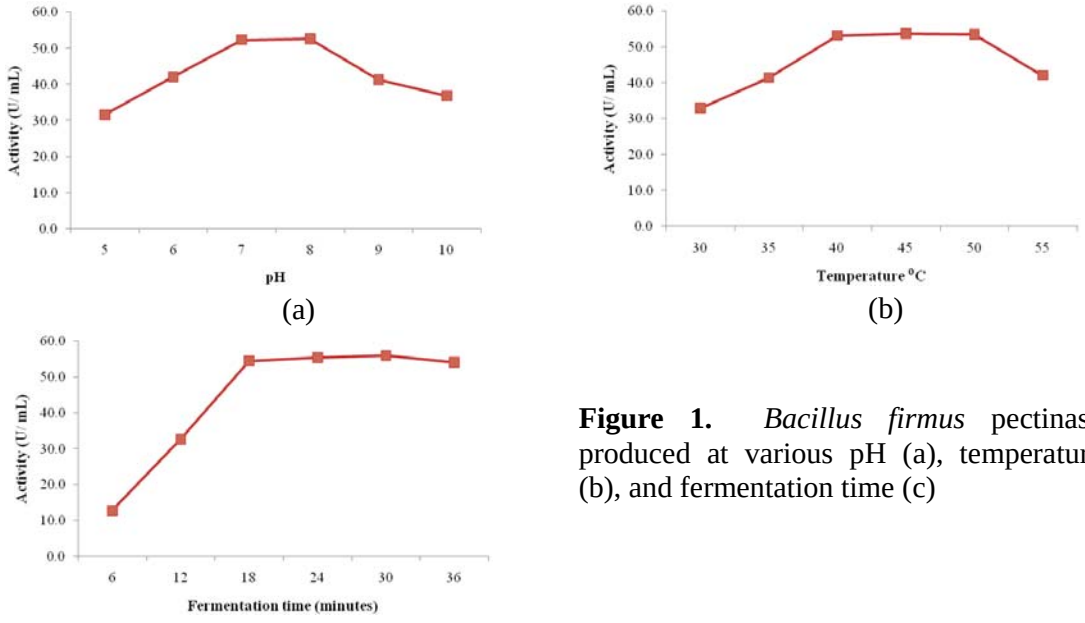

Figure 1. *Bacillus firmus* pectinase produced at various pH (a), temperature (b), and fermentation time (c)

Production of Pectinase

Pectinase is extracellular enzyme produced from various microorganisms such as fungi, mold and bacteria. Production of pectinase from bacteria is affected by environmental conditions, pH, temperature, fermentation time and inducer. The conditions correlated to pectinase production from *Bacillus firmus* in basal medium at various pH, temperature and fermentation time was presented at Figure 1a-c. Pectinase activity is assumed as pectinase concentration, because the high activity means the high concentration. The optimum

conditions of pectinase production from *Bacillus firmus* recorded at pH 7-8, temperature 40-50 °C and fementation time for 18 h. In a similar report, *Bacillus firmus* isolated from soil produced pectinase maximum at pH 6, temperature 37 °C and fermentation time 96 h [4]. While pectinase isolated from *Bacillus subtilis* WSHB04-02 could produce alkaline pectinase maximum at fermentation time 25 h [7]. Pectinase is produced concomitantly with growth curve of bacteria, and this result indicated that *Bacillus* life in different environment has different characters.

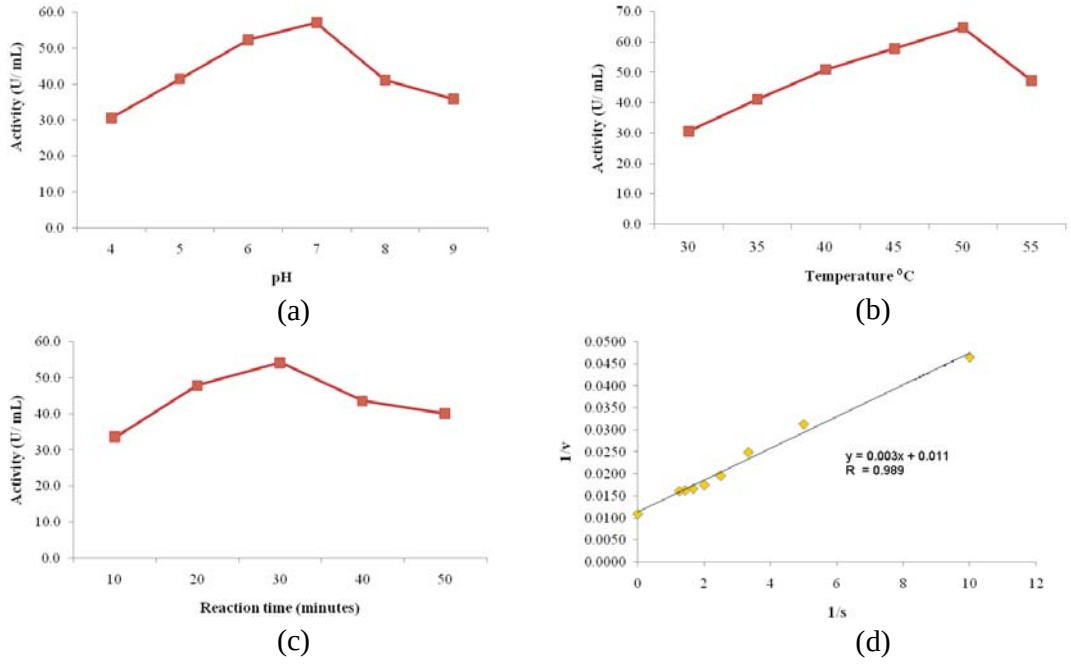

Figure 2. Pectinase activity : (a) various pH value (37 ^{o}C, reaction for 30 min), (b) various reaction temperature (pH 7, reaction for 30 min), and (c) various reaction time (pH 7, temperature 50 ^{o}C). (d) Lineweaver-Burk plot between 1/S and 1/v

Characterization of pectinase

In spite of pectinase production, pectinase activity is influenced by environmental condition. Pectinase has active site containing His_{223} and Asp_{201}. His_{223} acts as proton donor, while Asp_{201} acts as a nucleophilic site. Mechanism of catalysis of pectinase initiates by binding of substrate on side of susceptible glycosidic bond through multiple hydrogen bondings and provide suitable strain and distortion in order to locate the substrates sitting on the enzyme active site. Catalytic site of His_{223} transfers a proton to susceptible glycosidic bond and lead to the glycosidic bond breaking, and release first product and also forms a new covalent bond between substrate and active site from nucleophile Asp_{201}. While, the other Asp_{202} residue locates a water molecule for nucleophilic attacking, to result and release the second product and restore again the enzyme active site [8]. Therefore, pH condition affects in ionization of His and Asp.

The influence of pH condition toward pectinase activity was presented in Figure 2a. Pectinase activity increased from pH 4.0 to 7.0. At this pH range, ionization of active site of pectinase (-COOH) can gradually increase following the increase of pH condition. This will lead to a higher number of ionized of carboxyl group (COO^-), and much easier for the enzyme to bind substrates. As a result, complex of enzyme-substrates were formed, and yielded a higher value of pectinase activity. The optimum pH was recorded at pH 7.0, and it was predicted that the number of H^+ derived from ionization of carboxylic group was maximum to form enzyme –substrates complex. On the other hand, at pH above 7, pectinase activity decreased, and it was predicted due to not many of carboxylic groups were able to protonize the substrates.

The influence of temperature to pectinase activity was displayed in Figure 2b. Pectinase activity increased between temperature 30 and 50 ^oC. This was predicted correspond to increasing of the kinetic energy. The increasing of kinetic energy can lead to increasing of collisions between enzyme and substrates to form a complex of enzyme-substrates (ES) and finally can increase the product. However, at temperature above 50 ^oC, pectinase activity decreased and it was predicted due to partial denaturation of pectinase. This can change conformational of the enzymes and as an effect, substrate difficult to enter the active site and enzymatic reaction did not occur easily. In addition to pH and temperature, reaction time also affects pectinase activity. The influence of various reaction time to pectinase activity was shown in Figure 2c. At reaction time from 10 to 30 min, pectinase activity was proportional to reaction time. The longer reaction time, the more complex enzyme–substrates was formed and also the product. At 30 min reaction times, pectinase activity reached maximum product, and the enzyme was saturated with substrates.

In enzymatic reaction, the kinetic parameter is also important, which describes enzyme efficiency. Michaelis-Menten Kinetic parameter is determined by measuring pectinase velocity (v) with various substrate concentrations (S). Then, it was plotted in Lineweaver Burk graph (Figure 2d), to provide an linear equation y=0.003+0.011 and correlates to equation $\frac{1}{V_0}=\frac{K_M}{V_m}\bullet\frac{1}{[S]}+\frac{1}{V_m}$. The kinetic parameter resulted in V_m and K_M value of 90.090 U/mL and 0.27%, respectively.

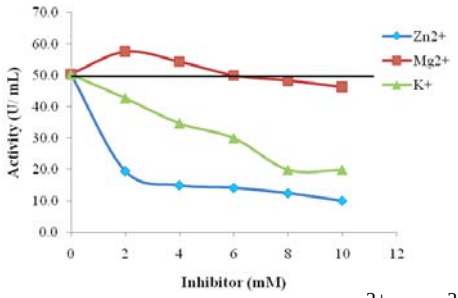

Figure 3. The influence of Zn^{2+}, Mg^{2+}, K^+ ions to pectinase activity

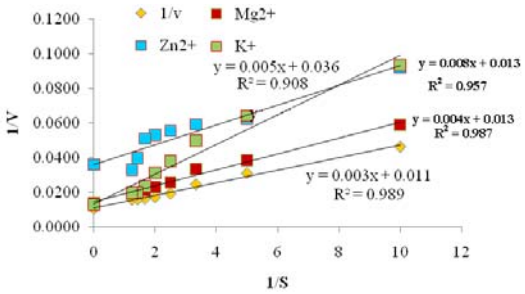

Figure 4. Lineweaver-Burk plot of the influence of metal ions to pectinase activity

Pectinase produced by *Aspergillus niger* for certain strain has optimum condition of activity at temperature 35 ^oC, pH 4.5, Vm 0.0046 %(w/v) pectin/s and K_M 1.137 %w/v pectin [2]. This result indicated that *Bacillus firmus* pectinase was better than that isolate from *Aspergillus niger*. A lower K_M value indicates a higher binding of enzymes and substrates.

Pectinase activity is also affected by metal ions. Measurement of pectinase activity was carried out using metal concentrations from 0 to 10 mM of Zn^{2+}, Mg^{2+}, K^{+}. This concentration mimics the metal content in fruits. The results showed Zn^{2+} and K^{+} could act as inhibitor at all range concentrations, whereas Mg^{2+} ion started to inhibit at concentration above 6 mM (Figure 3). Therefore, in order to determine inhibition types, pectinase activity was measured in various substrate concentrations within addition of Zn^{2+} (2 mM), Mg^{2+} (8 mM), and K^{+} (2 mM). Lineweaver-Burk plot of the effect of metal ions toward pectinase activity was showed in Figure 4, and inhibition kinetic parameter in Table 1.

Table 1. Kinetic parameter of pectinase and inhibition

Treatment	Vm (U/mL)	K_M	K_I
Without inhibitor	90.090	0,27%	-
Zn^{2+}	27.778	0.14%	0,88
Mg^{2+}	71.428	0.28%	22
K^{+}	76.923	0.61%	11

The presence of Zn^{2+} and K^{+} altered the Vm and K_M value, while Mg^{2+} ion affected significantly on Vm. In this case, Zn^{2+} and K^{+} inhibited pectinase but uncompetitively, while Zn^{2+} and K^{+} were bound to the enzymes-substrates complex to hinder forming of the product. In the other hand, Mg^{2+} bound to both free enzyme and enzymes-substrates complex as well. This was predicted since the size of Mg^{2+} is smaller than Zn^{2+} and K^{+}.

CONCLUSION

Bacillus firmus pectinase production and its activity were affected by the environment conditions. Pectinase produced by *Bacillus firmus* at pH 7, temperatures of 40-50 ^{o}C and fermentation time 18 h within activity of 56 U/mL. While optimum conditions of enzyme activity were achieved at pH 7, temperature 50 ^{o}C and incubation time 30 min. Michaelis-Menten kinetic parameter was recorded with Vm and K_M value of 90.090 U/mL and 0.27%, respectively. Pectinase activity was significantly affected by the presence of Zn^{2+}, K^{+} and Mg^{2+}, and for K^{+} and Zn^{2+} acted as uncompetitive inhibitor, while Mg^{2+} acted as non competitive inhibitor. The investigation of the immobilization process of pectinase isolated from *Bacillus firmus* in order to be reusable and to be stable in use is recommended.

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