PRODUCTION AND CHARACTERIZATION OF COLLAGENASE FROM *Bacillus* sp. 6-2 ISOLATED FROM FISH LIQUID WASTE

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Abstrak. Kolagenase merupakan enzim yang mampu menghidrolisis kolagen utuh menjadi fragmen peptida kolagen. Kolagenase dan produk hidrolisisnya telah mendapat perhatian yang luar biasa dalam aplikasi medis dan industri. Penelitian ini dilakukan untuk mengisolasi dan mengidentifikasi bakteri penghasil kolagenase, kemudian memproduksi dan mengkarakterisasi kolagenase. Sebanyak 7 isolat dari limbah cair ikan diskrining pada media selektif mengandung 2 % kolagen dan aktivitasnya dikonfirmasi melalui pembentukan zona bening. Isolat 6-2 positif sebagai penghasil kolagenase dan teridentifikasi sebagai *Bacillus* sp. 6-2 melalui karakteristik morfologi dan biokimia. Waktu fermentasi optimum enzim diselidiki. Ekstrak kasar kolagenase dikarakterisasi meliputi pengaruh pH, suhu, dan ion-ion logam. Isolat 6-2 secara optimal memproduksi enzim kolagenase setelah inkubasi 30 jam dengan aktivitas 0.072 U/mL dan kadar protein 3.768 mg/mL. pH dan suhu optimum adalah masing-masing 7.0 dan 40 ^{o}C. Enzim diaktifkan oleh Ca^{2+} and Mg^{2+} 1 mM, dan dihambat oleh Zn^{2+} dan Co^{2+} 1 mM . Kolagenase dari *Bacillus* sp. 6-2 mungkin memiliki potensi untuk aplikasi medis dan industri.

Kata Kunci : kolagenase, kolagen, limbah cair ikan, *Bacillus* sp. 6-2, aktivitas

Abstract. Collagenases are enzyme that are able to hydrolyze native collagen into fragment collagen peptides. Collagenases and its hydrolysis products have received tremendous attention in medical and industrial applications. The present study was conducted to isolate and identify new collagenase producing bacteria from fish liquid waste, then produce and characterize collagenase. A total of 7 isolate from fish liquid waste were screened on selective medium containg 2 % collagen and its activity was confirmed by the formation of clear zone. Isolate 6-2 was positif as collagenase producer and identified as *Bacillus* sp. 6-2 by morphological and biochemical characteristics. The optimum fermentation time of enzyme was investigated. Collagenase crude extract was characterized by the effect of pH, temperature, and metal ions. Isolate 6-2 optimally produced collagenase enzyme after 30 h of incubation with activity of 0.072 U/mL and protein content of 3.768 mg/mL. The optimum pH and temperature were 7.0 and 40 ^{o}C, respectively. The enzyme was activated by 1 mM Ca^{2+} and Mg^{2+}, and inhibited by 1 mM Zn^{2+} and Co^{2+}. Collagenase from *Bacillus* sp. 6-2 may have potentials for medical and industrial applications.

Keywords : collagenase, collagen, fish liquid waste, *Bacillus* sp. 6-2, activity

INTRODUCTION

Collagen is the most abundant structural protein in connective tissues and contributes about 30 % of total proteins in mammals (Song H, et al, 2017). Collagen consist of three α polypeptide chains that combine to form a triple helix structure in the ekstracellular matrix, Every chain contains hundreds of amino acids based on the Gly-X-Y residues (X and Y are often proline and hydroxyproline). Since collagen is triple helix, it is hard to digest and resistant to most proteases (Hashim P, et al, 2015; Zhang Z, et al, 2005).

Collagenases are the only protease enzymes that hydrolyze peptide bonds in native collagen under various physiological conditions and break them into small fragments (Howes J.M, et al, 2015). Collagenases have been successfully applied in the food, cosmetic, pharmaceutical, and leather industries. Recently, they are also used for medical investigation, as therapeutic agents in some diseases, production of collagen hydrolysate, and have promising potential as diagnostic tools for autoimmune diseases (Alipour, H, et al, 2016; Bousopha, S, et al, 2016; Wen-Jia, P, et al, 2012). Collagen peptides, the product of collagen hydrolysis exhibit various biological activities of interest, such as antioxidant, anti-inflammatory, antitumor and antihypertensive, which may contribute to the prevention and treatment of diseases. They are widely used as ingredient in food, drinks, dietary supplement, functional food, and cosmetic (Song, H, & Li, B, 20; Mohammad, A.W, 2014).

Collagenase have been isolated and characterized from bacterial cells and animal tissues. However, bacterial collagenases show high efficiency in hydrolizing collagen than mammals, can break down polypeptide chains at several sites in native and denatured collagen, producing small peptides (Alipour, H, et al, 2016). Bacterial collagenases have prospects for wider application in the future, so it is very interesting to study.

Fish liquid waste is a suitable habitat for growth of bacteria. The availability of protein substrates, mainly collagen offers a renewable sources of bacteria that can produce collagenase with novel characteristic for a broad application. Therefore, this study aims to isolate and identify collagenase producing bacteria from fish liquid waste, determine optimum production time and characterize collagenase.

MATERIAL AND METHOD

Instruments

The instrument used include analytical scales, autoclave, incubator, shaking waterbath, spectronic 20D+, pipette micro, petri dish, and Erlenmeyer flask.

Materials

Some of the materials used are fish liquid waste, collagen, yeast extract, bacto peptone, bacto agar, NaCl, K_2HPO_4, $MgSO_4.7H_2O$, Bovine Serum Albumin

(BSA), tyrosine, Trichloroacetic Acid (TCA), Na_2CO_3, folin ciocalteu, aquades, and aquabides.

Methods

1. Sample collection

Samples of fish liquid waste were collected aseptically from the sewer in a local market, Makassar, Indonesia. Samples were kept in sterile bottles and transferred to the laboratory, stored at 4 ^oC until further use.

2. Isolation and identification of collagenase producing bacteria

Collagenase producing bacteria were isolated by serial dilution and pour-plate method. Samples were serially diluted (10^{-1} - 10^{-6}) by sterile distilled water, and (10^{-4} -10^{-6}) dilutions were poured on LA medium, incubated at 37 °C for 24 h. Isolates were purified by the scratch method and screened on selective medium, then incubated at 37°C for 24 h. Collagenase activity was confirmed by the formation of clear zone around the colonies. The isolate with the highest activity was identified by morphological and biochemical characteristics following Bergey's Manual of Systematic Bacteriology (Boone, D.R, et al, 2001).

3. Preparation of inoculum

The isolate was inoculated in 250 mL Erlenmeyer flasks containing 50 mL of a inoculum medium and incubated at 37 ^oC for 18-24 h with shaking at 180 rpm.

4. Determination of the optimum production time of collagenase

The culture (10 % of inoculum) was transferred into 250 mL Erlenmeyer flasks a containing 100 mL of fermentation medium and incubated at 37 ^oC for 48 h with shaking at 180 rpm. Optical density (OD), collagenase activity and protein concentration were evaluated every 6 h to determine the optimum production time. OD was determined by measuring absorbance at 600 nm. The culture medium was centrifuged at 3500 rpm and 4 ^oC for 30 min, and the supernatants were collected as collagenase crude extracts.

5. Determination of collagenase activity

Collagenase activity was carried out according to the modified Bergmeyer's (1983) method with fish collagen as substrate. A reaction mixture, consisting of 0.5 mL of 1 % collagen solution, 0.5 mL of 0.2 M phosphate buffer (pH 7.0) and 0.1 mL of enzyme was incubated at 37 ^oC for 10 min. Reaction was stopped by the addition of 1mL of 0.1 M TCA and incubated again at 37 ^oC for 10 min, followed by centrifugation at 10.000 rpm and 4 ^oC for 10 min. The supernatant (0.75 mL) was mixed with 2.5 mL of 0.4 M Na_2CO_3 and 0.5 mL of Folin Ciocalteau. The mixture was incubated at 37°C for 20 min and measured at λ 578 nm by spectronic 20D+ (Thermo). Tyrosine (5 Mm) was used as standard. One unit (U) of enzyme activity was defined as enzyme which produces 1 μmol of tyrosine per min. The specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in U/mg.

6. Determination of proteint concentration

Protein concentration of collagenase crude extracts was determined by Lowry method, using bovine serum albumin (BSA) as the standard.

7. Characterization of collagenase

The effect of pH, temperature, and metal ion on enzyme activity was investigated by determining enzyme activity at various pHs (6.0, 6.5, 7.0, 7.5, and 8.0), temperatures (30, 35, 37, 40, 45, and 50 ^{o}C), and the presence of metal ions ($MgSO_4$, $CaCl_2$, $ZnCl_2$, and $CoCl_2$: 1 Mm and 5 mM). The pH was adjusted using 0.2 M phosphate buffer and the enzyme activity was determined as described in section 5.

RESULT AND DISCUSSION

1. Isolation and identification of collagenase producing bacteria

In the present study, a total of 7 isolate were successfully isolated from fish liquid waste collected from local market, Makassar, Indonesia. They were screened for collagenolitic activity on selective medium containing 2 % collagen, and the isolate 6-2 was found as the only collagenase producer. Collagenolitic activity was indicated by the presence clear zone around colonies (Figure 1). The clear zone was formed by secretion of collagenase enzyme to break down the collagen substrat into small fragments. Morphological and biochemical characteristics of the isolate 6-2 are presented in Table 1 and 2, respectively. According to Bergey's Manual of Systematic Bacteriology, isolate 6-2 had similarities with *Bacillus* genus. For next, the isolate was be *Bacillus* sp. 6-2. *Bacillus* had been reported as collagenase producers among them are *B. cereus MBL 13*, *B. pumilus* Col-J, *B. licheniformis* F11.4 and *B.* KM369985 (Liu, L, et al, 2010; Wu, Q, et al, 2010; Baehaki, A, et al, 2012; Savita, K & Arachana, P, 2015). This result proved that fish liquid waste are suitable habitat for growth of collagenase producing bacteria.

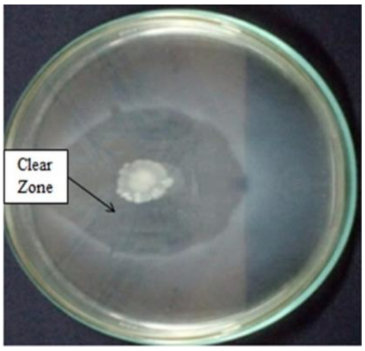

Figure 1. Collagenase activity of isolate 6-2 on selective medium

Table 1. Morphological Characteristics

Parameter Test		Remark
Macroscopic	Colony form	Circular
	The edge of the colony	Undulate
	Surface	Rude
	Elevation	Convex
	Color	White
Microscopic	Gram stain	Positive
	Cell form	Rod
	Spore	Spore forming

Table 2. Biochemical Characteristics

Parameter Test		Remark
TSIA	Acid formation	Positive
	Gas formation	Negative
	H_2S formation	Negative
SIM	Indol formation	Negative
	Motility	Negative
	H_2S formation	Negative
MR-VP	Metyl Red	Positive
	Voges Proskauer	Negative
Urease		Negative
Citrat		Negative
Fermentation of carbohydrate	Glucose	Negative
	Lactose	Negative
	Sucrose	Positive
	Mannitol	Negative

2. Determination of the optimum production time of collagenase

Collagenase enzymes was produced in fermentation medium containing 2 % collagen for 48 h. Figure 2 reports the time course of collagenase production by *Bacillus* sp. 6-2 .The activity of collagenase was detected in the culture medium since the beginning of adaptation phase, and significantly increased in the exponential phase until optimum after 30 h of incubation. Hence, the optimum production time was 30 h with collagenase activity of 0.072 U/mL and protein concentration of 3.768 mg/mL. This is similar to *Pseudomonas* sp, which optimally produce collagenase in the exponential phase (Gautam, M & Azmi, W, 2017). However, the optimum production time of collagenase by microorganisms can be different because it is influenced by several factors such as medium component, inoculum size, pH, and temperatures (Chauhan, A & Prabha, V, 2017).

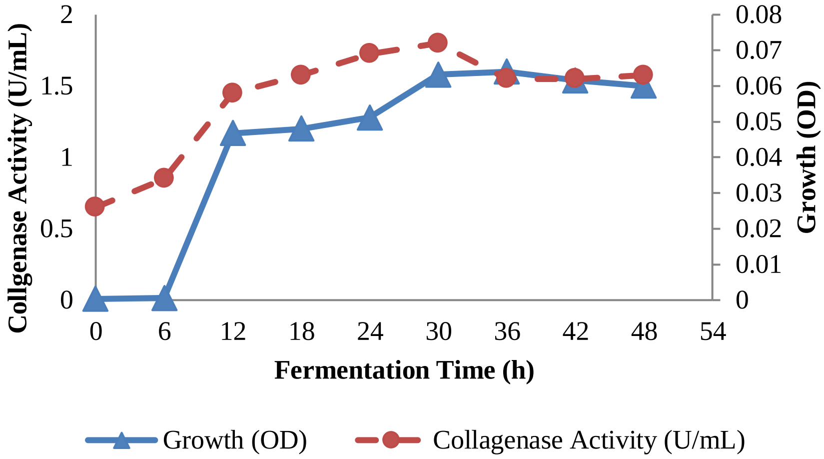

Figure 2. The time course of collagenase production by *Bacillus* sp. 6-2 in medium containing 2 % collagen, at pH 7.0 and temperature 40 ºC

3. Characterization of collagenase

3.1 The effect of pH

Enzyme activity was determined at various pHs (6.0, 6.5, 7.0, 7.5, and 8.0), at 37 ºC using 0.2 M phosphate buffer to adjusted pH. The results of the effect of pH on enzyme activity are shown in Figure 3. The optimum activity of the enzyme was observed at pH 7.0, with levels of 0.072 U/mL. Enzyme activity increases at pH 6.0-7.0, and decreases at pH 7.5-8.0. The pH of medium affects enzyme activity. The three-dimensional structure of enzyme depends on pH. A lower or higher pHs causes folding so that activity decreases. In addition, ionic groups in the active sites of the enzyme must be in a stable form. The variations in pH of the medium cause ionic shape changes which affect the reaction (Bhunia, B, et al, 2013). The optimum pH of collagenase produced by *Bacillus* sp. 6-2 is neutral, similar to collagenase from *B.licheniformis* F11.4, but lower than pH 8.0 of *B.cereus* MBL3, and pH 9.0 of *Penicillium* sp. UCP 1286 (Baehaki, A, et al, 2012; Liu, L, et al, 2010; Wanderleya, M.C.D.A, et al, 2017).

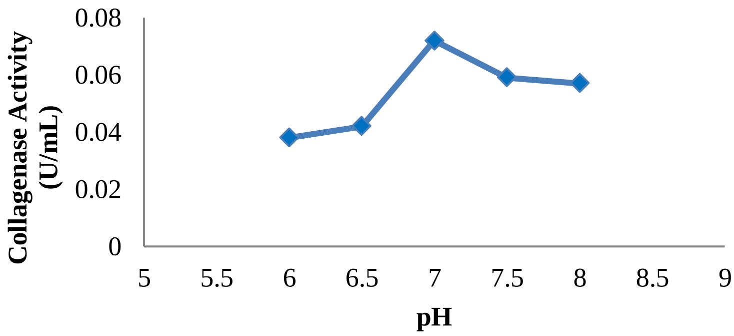

Figure 3. Effects of pH on collagenase activity at 37 °C using 0.2 M phosphate buffer

3.2 The effect of temperature

Enzyme activity was determined at various temperatures (30, 35, 37, 40, 45, and 50 °C), at pH 7.0 (using 0.2 M phoshate buffer). The results of the effect of temperatures on enzyme activity are shown in Figure 4. The optimum activity of the enzyme was observed at 40 °C, with levels of 0.092 U/mL. Enzyme activity significantly increased at 30-40 °C, then dramatically decreased at 45 °C. The rection is slow at low temperatures. But at higher temperatures, denaturation occurs causing decreased enzyme activity. Thus, the optimum temperatures of collagenase produced by *Bacillus* sp. 6-2 was reached at 40 °C. This result is similar to collagenase from *B. cereus* MBL3, but higher than most collagenase that have been reported (Wanderleya, M.C.D.A, et al, 2017; Liu, L, et al, 2010; Bhunia, B, et al, 2013; Kang, S.I, et al, 2005).

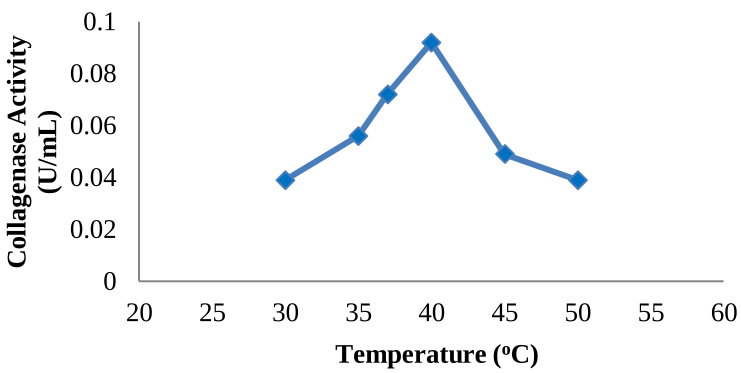

Figure 4. Effects of temperature on collagenase activity at PH 7 using 0.2 M phoshate buffer

3.3 The effect of metal ions

Enzyme activity was determined by the presence of metal ions ($MgSO_4$, $CaCl_2$, $ZnCl_2$, and $CoCl_2$: 1 Mm and 5 mM) at pH 7.0 and 40 °C, using 0.2 M phosphate buffer to adjusted pH. Enzyme activity without metal ions was considered as control (100%). The results of the effect metal ions on enzyme activity are shown in Figure 5.

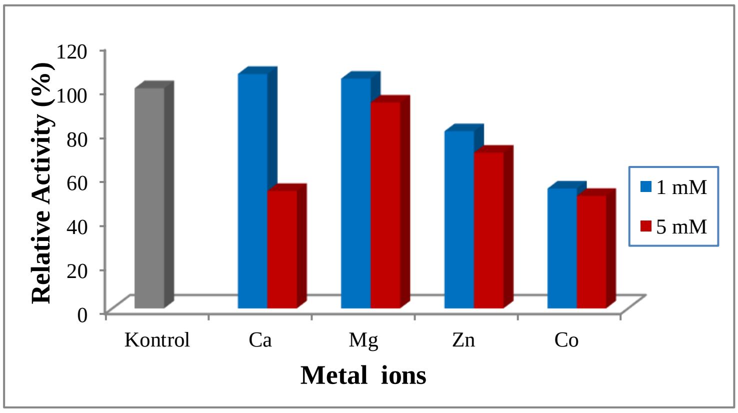

Figure 5. The effects of metal ions on collagenase activity at pH 7.0 and temperature (37 °C).

Some collagenases depend on metal ions for their catalytic activity, they are from the metalloprotease group. Metal ions are needed at active sites as activator or inhibitor in catalyzing substrate at the certain concentration [21,29]. The effect of metal ions on collagenase activity from *Bacillus* sp. 6-2 was studied using several divalent metals such as $MgSO_4$, $CaCl_2$, $ZnCl_2$, and $CoCl_2$. The results showed that the presence of Mg^{2+} and Ca^{2+} 1 mM increased enzyme activity by 6.52 % and 4.35 % respectively, but the presence of up to 5 mM caused a decrease in activity. Whereas Zn^{2+} and Co^{2+} 1 Mm and 5 mM strongly inhibited actvity. Mg^{2+} and Ca^{2+} with different concentrations give different effects, this can be explained by the concept that activator compounds up to a certain amount can improve the catalytic function of enzyme, but the excess amount of the activator can cause the competition of the free activator and the activator substrate complex to enzyme. The excess amount of the free activator causes the competitive inhibition (Natsir, H, et al, 2013). However, collagenase produced by *Bacillus* sp. 6-2 was activated by 1 mM Mg^{2+} and Ca^{2+}, and inhibited by 1 mM Zn^{2+} and Co^{2+}.

CONCLUSION

A total of 7 isolate were obtained from fish liquid waste collected from local market, Makassar, Indonesia. One Isolate was found as collagenase producer and identified as *Bacillus* sp. 6-2. The isolate optimally produced collagenase enzyme after 30 h of incubation with activity of 0.072 U/mL and protein concentration of 3.768 mg/mL. The optimum pH and temperature were 7.0 and 40 °C, respectively. The enzyme was activated by 1 mM Mg^{2+} and Ca^{2+}, and inhibited by 1 mM Zn^{2+} and Co^{2+}. Futher research is needed to find out the spesific application, but it may have potentials for medical and industrial applications.

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