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Water soluble collagen of *Oreochromis niloticus* skin as substrate for collagenase produced by *Bacillus subtilis* ATCC 6633

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Abstract
Collagen is the major insoluble fibrous protein in the extra cellular matrix and connective tissues. It has a wide range of application in cosmetics, biomedical, pharmaceutical, leather and food industries. Collagen can be extracted from fish scales and skin by enzymatic digestion methods. The aim of this research was to study the water soluble collagen isolated from *Oreochromis niloticus* skin with collagenase produced by *Bacillus subtilis* ATCC 6633 (a collection of Microbiology Laboratory, Faculty of Pharmacy, Universitas Padjadjaran, Indonesia). *Oreochromis niloticus* was selected as collagen source due to its high content of collagen. Results showed that water soluble collagen (WSC) substrate isolated from *Oreochromis niloticus* skin contained 1.978 mg/mL of protein, whilst collagenase extracted from *Bacillus subtilis* ATCC 6633 contained 0.326 mg/mL of protein. Furthermore, the highest collagenolytic activity was obtained at 50 °C (1.298 U/ml) and pH 8 (1.696 U/ml). Water soluble collagen of *Oreochromis niloticus* skin as substrate for collagenase extracted from *Bacillus subtilis* ATCC 6633 positively exerted collagenolytic activity. It can be concluded that the optimum condition for collagenolytic activity of the enzyme is at temperature 50 ºC and pH 8.

Keywords: Collagenolytic activity, eukaryotic enzyme, freshwater fish, Nile tilapia, tilapia

1. Introduction
Collagen is the major insoluble fibrous protein in the extra cellular matrix and connective tissues. It has a wide range of application in cosmetics, biomedical, pharmaceutical, leather and food industries [1], whilst collagenases are active proteinases that degrade collagen and proteoglycan. These enzymes are necessary to initiate collagen turnover in connective tissue at both normal and abnormal conditions [2].
Collagen could be extracted from fish scales and skin by enzymatic digestion methods [3-5]. *Oreochromis niloticus* was selected as collagen source due to its high content of collagen [6]. The work of Sujithra (2013) concluded that the fish wastes contained 22% of acid solubilized collagen (ASC) based on the lyophilized dry weight and 60% of pepsin solubilized collagen (PSC) on the same basis [7], while Potaros and colleagues calculated the dry weight yields of ASC using Noitup Method and Ogawa Method, were 38.84 and 20.70%, respectively. The same two methods were used to calculate the dry weight yields of PSC, and resulted 48.21 and 38.27%, respectively [4].
Baehaki and colleagues (2012) who studied about extracellular collagenase produced by *Bacillus licheniformis* F11.4, concluded that the enzyme could be purified using ammonium sulfate and DEAE Sephadex A-50 at 50 °C pH 7. According to their work, there were metal ions that decreased collagenase activity, e.g. Fe^{2+} (1 mM), Mg^{2+} (1 mM), Mn^{2+} (1 mM), Co^{2+} (1 mM), EDTA (1 mM), and β-mercaptoethanol (1 mM), whilst Ca^{2+} (1 mM) and Cu^{2+} (1 mM) were proven could enhance the enzyme's activity [8].

2. Materials and Methods
2.1 Materials
Materials used were pure strain of *Bacillus subtilis* ATCC 6633 (a collection of Microbiology Laboratory, Faculty of Pharmacy, Universitas Padjadjaran, West Java, Indonesia), 30 g of freshly prepared *Oreochromis niloticus* skin, 1.5% acetic acid solution, 20 g of Luria Agar (contains tryptone, yeast extract, sodium chloride and agar), 20 g of Luria Broth (microbial

growth medium), 10 mL of phosphate buffer, 10 mL of 5 mM leucin, 15 mL of 0.5% TCA solution, 15 mL of 0.1% ninhydrin, 30 mL of 50% isopropanol, 100 mg of Comassie Briliant Blue (CBB) G-250, 50 mL of 50% ethanol, 100 mL of 85% phosphoric acid, 100 mg BSA, distilled water, alcohol.
Glasswares and containers used in this work were wrapped in aluminium foil and sterilized priorly in an autoclave. Temperature was set at 121 ºC for 15 minutes.

2.2 Water soluble collagen extraction
In general, fish muscles and scales contains 0.2% to 10% of collagen, these are mainly type 1 collagen [7]. Extraction of collagen was carried out according to Yuniarti's method (2010) with minor modification. *Oreochromis niloticus* skin was washed and cleaned to discard the meat and fat, cut into small pieces (3x3 cm^2) using knife, and soaked in 1.5% acetic acid solution for 18 hours (ratio w/v of skin : acetic acid solution was 1 : 2). The sample was then rinsed repeatedly with distilled water until pH 6.5 was obtained. The neutral sample was further extracted using distilled water (ratio w/v of skin: distilled water was 2: 1) for 3 hours at 40 ºC. The extract was filtered and the filtrate was referred as water soluble collagen (WSC) [9].

2.3 Preparation of *Bacillus subtilis* ATCC 6633
Bacillus subtilis ATCC 6633 was cultured in Luria Agar medium (prepared by mixing 3.5 g Luria Agar in 100 mL of distilled water; the mixture was boiled and sterilized in autoclave 121 ºC for 15 minutes).

2.4 Collagenolytic activity assay of *Bacillus subtilis* ATCC 6633
Preparation of collagen substrate was carried out according to Baehaki (2012). 3.5 g of Luria Agar was mixed with 100 mL distilled water and 5 mL of WSC (II.2). The mixture was boiled and sterilized in autoclave 121 ºC for 15 minutes, and then it was poured onto a sterile petri dish.
Bacillus subtilis ATCC 6633 (II.3) was picked using ose needle, planted onto the collagen substrate (II.4). The dish was wrapped and incubated at 37 ºC for 24 hours. Collagenolytic activity was observed at the 2nd day.

2.5 Production of raw extract of collagenase
Production of raw extract of collagenase was carried out according to Baehaki (2012). First, *Bacillus subtilis* ATCC 6633 (II.3) was planted onto Luria Broth medium (prepared by mixing 2 g Luria Broth in 100 mL of distilled water; the mixture was boiled and sterilized in autoclave 121 ºC for 15 minutes) and incubated at 37 ºC with 120 rpm agitation. The optical density was measured at λ 600 nm until OD 0.8 was obtained. The mixture was added with 5% of WSC (II.2) and was incubated for 40 hours at pH 7, temperature 37 ºC, with 120 rpm agitation. The raw extract was separated using centrifugation at 7500 rpm for 15 minutes at 4 ºC.

2.6 Optimization of the reaction
The reaction of collagen of *Oreochromis niloticus* skin with collagenase of *Bacillus subtilis* ATCC 6633 was optimized by varying temperatures (30, 40, 50, 60, and 70 ºC) and pH (6-10). The activity of the enzyme was measured and calculated according to Moore dan Stein method (1954) as described in Yuniarti (2010).

2.7 Determination of protein content
Protein contained in WSC substrate isolated from *Oreochromis niloticus* skin and collagenase extracted from *Bacillus subtilis* ATCC 6633 were determined using Bradford method (1967) as described in Yuniarti (2010).

3. Results and Discussion
Of 51.95 g *Oreochromis niloticus* skin (Fig.1), a total volume of 320 mL of water soluble collagen (WSC) was obtained. Compared to the work of Lee and colleagues who isolated pepsin solubilized collagen (PSC) from the tissue of a starfish (*Asterias amurensis*) which is similar to calf skin type I collagen [1].

Fig 1: Freshly prepared *Oreochromis niloticus* skin

The fast growth of *Bacillus subtilis* ATCC 6633 cultured in Luria Agar medium is presented in Fig. 2a, whilst its collagenolytic activity, or its ability to hydrolyze collagen when reacted with collagen substrate, was indicated by the occurance of a clear zone (showed by white arrow) around the colony in Fig. 2b.

a) (b)

Fig 2: Colony (a) and collagenolytic activity (b) of *Bacillus subtilis* ATCC 6633

According to Waldvogel and Swartz (1969), when collagen lysis, it appears as a black circular area around the culture on a gray opalescent plate [10]. Chung *et al.* (2004) have stated that collagenase binds to and locally unwinds collagen before it cleaves the triple-helical interstitial collagen. Their studies had also revealed that collagenase cleaved the three α chains one by one [11].

The optimum temperature of the reaction between collagen of *Oreochromis niloticus* skin with collagenase of *Bacillus subtilis* ATCC 6633 was shown in Fig.3.

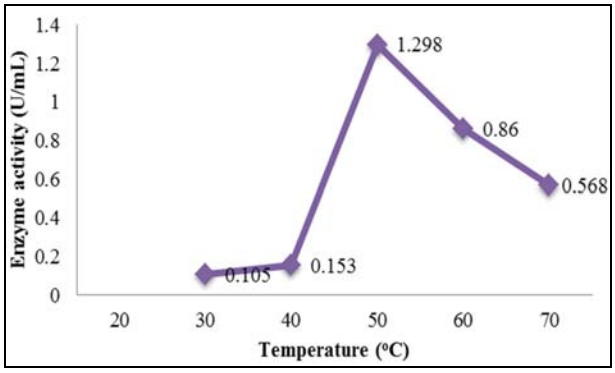

Fig 3: Optimum temperature of enzyme activity

Fig. 3 indicated that the highest enzyme activity (1.298 U/mL) occurred at 50 ºC. A work of Chung *et al*. (2004) proved that the critical aspects of the collagenolytic specificity rely on the structural changes in collagen, induced by interacting with collagenase. This is an evident of temperature-dependent collagenolysis. At 37 ºC the rate of cleavage of collagen was recorded to be faster, but at 10 ºC only a few or no collagenolytic activity was observed [11].

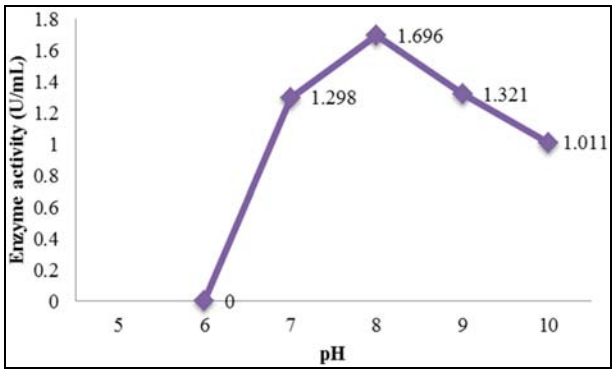

Fig 4: Optimum pH of enzyme activity

Furthermore, the enzyme activity was measured at various pH. The result was provided in Fig.4, which indicated that the highest enzyme activity (1.696 U/mL) occurred at pH 8. This result was compared to the works of Rahmayanti (2014) [12], who concluded that collagenase enzyme of *Bacillus licheniformis* F11.4 showed the highest activity at pH 8-9; Baehaki (2012) [8] proved that optimum pH of *Bacillus licheniformis* F11.4 collagenase was 7; whilst Nagano and To (1999) [13] who studied about collagenase of *Bacillus subtilis* FS-2 isolated from fish ketchup, concluded that its highest activity was at pH 9.

Protein content in WSC substrate isolated from *Oreochromis niloticus* skin and collagenase extracted from *Bacillus subtilis* ATCC 6633, were calculated using the linear regression equation in Fig.5.

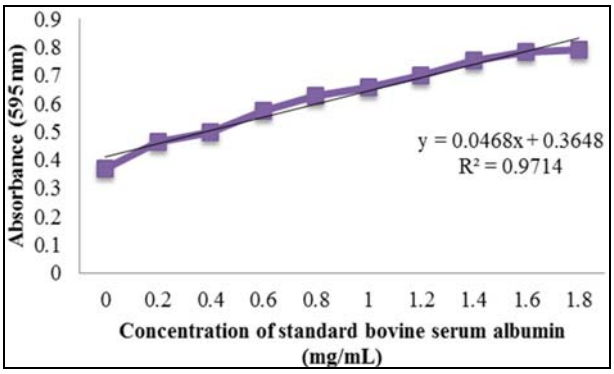

Fig 5: Bovine serum albumin standard curve

Water soluble collagen (WSC) substrate isolated from *Oreochromis niloticus* skin contained 1.978 mg/mL of protein, whilst collagenase extracted from *Bacillus subtilis* ATCC 6633 contained 0.326 mg/mL of protein. Similar study of Rahmayanti (2014) concluded that protein content in collagen of *Channa striata* skin was 1.323 mg/mL of protein, and extracting collagen using water would result less collagen than using acid [12].

4. Conclusion

Water soluble collagen of *Oreochromis niloticus* skin as substrate for collagenase extracted from *Bacillus subtilis* ATCC 6633 positively exerted collagenolytic activity. The optimum condition for collagenolytic activity of the enzyme is at temperature 50 ºC and pH 8.

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