Screening of proteolytic bacteria from *tauco* Surabaya based on pathogenicity and selectivity of its protease on milky fish (*Chanos chanos*) scales for healthy and halal collagen production

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**Abstract:** This study was reported our work on the advanced screening of proteolytic bacteria from *tauco* Surabaya based on pathogenicity of the isolates and the selectivity of its proteases on milky fish (*Chanos chanos*) scales. Determination of pathogenicity was carried out through hemolytic test on blood agar medium. Formation of clear zones around the growing bacterial colonies indicate the pathogenic bacteria, whereas no clear zones around the bacterial colonies are formed in the results of non-pathogenic bacteria test. Protease which has selectivity to milky fish scales can hydrolyse peptide bonds in milk fish scales to produce soluble collagen that can be measured as dissolved protein. The results showed that the order of pathogenicity qualitatively from the lowest to the highest were the isolate of HTcUM 7.1<HTcUM6.1.1<HTcUM2<HTcUM10<HTcUM 9.1<HTcUM6.2.2<HTcUM8. Crude extract of protease from HTcUM 7.1, HTcUM10 and HTcUM 6.2.2 successively have specific activity of protease were 6.044±3.390, 0.311±1.099 and 1.718±0.040 U/mg. Furthermore, it was able to produce the dissolve protein with concentration as much as 0.1015±0.040, 0.0930±0.044 and 0.0825±0.004 mg/mL. Overall results showed that HTcUM 7.1 was the most potential proteolytic bacteria as source of protease for production halal and healthy collagen from milky fish scale.

**Keywords:** *tauco*, proteolytic bacteria, collagen, milky fish scale

1. **Introduction**

Collagen can be isolated from all connective tissue found in the skin, bones, tendons and cartilages [1]. The stages of production of collagen generally consist of the selection of collagen source material, initial treatment, extraction and purification. The main source of collagen is the connective tissue of animals such as pigs, cattle, sheep and chickens [2]. Initial treatment is by removing lipids. The collagen from the animal's connective tissue can be produced chemically, enzymatically, or both modifications. The isolation of collagen is done chemically with soaking in acid or alkali, while enzymatically using proteases such as pepsin. Purification is done to separate other proteins [3]

The source of healthy and halal collagen which is currently being studied is from bone, skin and fish scales [4-10]. Collagen from cows, pigs and chickens is considered to be harmful to health because it is suspected to be the outbreak of bovine spongiform encephalopathy, the food-and-mouth disease,
transmissible spongiform encephalopathy, and avian influenza. Collagen from pigs is prohibited for consumption in Muslim-majority countries. [5, 11].

Fish waste is very abundant in Indonesia and has the potential to become an alternative source of healthy and halal collagen, one of which is milky fish scales waste. The production of milky fish through aquaculture cultivation is increase continuously in East Java, especially Sidoarjo. The total milky fish production in 2009 reached 16,030 tons and it increased to 19,839 tons in 2010. The total production of milky fish aquaculture in Sidoarjo reaches 70% of the entire production of milky fish aquaculture in East Java. The milky fish is generally processed into various kinds of food products such as otak-otak, bandeng asap, bandeng presto as typical souvenirs of Sidoarjo. Various processes of processed milky fish products produce waste in the form of scales, so researchers are interested in using milky fish scales as a source of collagen. This selection was supported by a variety of research stating that fish waste in the form of scales, skin and bones of fish is a good source of collagen in addition to cow skin and pigs [7, 8, 10, 12-14]. Also, fish scales contain high collagen levels, large surface area and lower lipid levels than fish skin and bones [6]. These can increase the efficiency of collagen production especially in the early stages of collagen production because it does not require a reduction in size before removing lipid step.

Enzymatic collagen production generally uses porcine pepsin rather than other protease because of its lower price and high protease selectivity at various sources of collagen. Its trigger the scepticism about the halalness of collagen. Protease from non-pathogenic proteolytic bacteria can overcome this problem.

Local proteolytic microbes can be isolated from typical Indonesian fermented foods. Fermented food products made from high protein raw materials are known to be sources of non-pathogenic protease-producing bacteria [15, 16]. Indonesia has several fermented foods made from high protein raw materials such as tauco. Bacteria produced various types of protease. Each type of protease has a characteristic recognition of certain substrates or specific cleavage site as trypsin, chemo trypsin and elastase. The type and amount of protease produced by proteolytic bacteria is influenced by the type of bacteria, the production media and the conditions of growth. The researchers from Biochemistry Group, Department of Chemistry, Universitas Negeri Malang has been isolated 21 proteolytic bacteria isolates with different morphological characteristics of the colony from the tauco suspension from Surabaya. A total of 8 potential proteolytic bacterial isolates, based on proteolytic index values, have been successfully purified such as HTcUM2, HTcUM6.2.1, HTcUM6.2.2, HTcUM6.1.0, HTcUM6.1.1, HTcUM10, HTcUM8, and HTcUM9.1 [17]. There is no information beforehand about their pathogenicity. The safety of bacteria which would be used for food industrial purposes has to be guaranteed for future application. Therefore, pathogenicity of those isolated needs to be investigated. Biochemical characteristics several isolates have been known. HTcUM6.2.2 isolate can be produced high activity protease in a production medium that utilizes 10 % tofu liquid waste at pH = 7 for 3 days, HTcUM5 isolate can be produced crude extract of protease in peptone medium was greater (0.347 ± 0.077 U/mL) than in tofu waste (0.167 ± 0.013 U/mL), but the crude extract of protease produced in peptone medium did not have selectivity on milky fish scales such as crude extract of protease produce in tofu waste medium [18]. Presumably, the other isolates are suspected to have different biochemical characteristics especially selectivity its protease on milky fish scale. This study reported our work on the screening of proteolytic bacteria from tauco Surabaya based on the pathogenicity and selectivity of its protease on milky fish scales to be used as source of protease for production of healthy and halal collagen.

2. Material and methods

2.1. Microorganism

The eight HTcUM isolates i.e. HTcUM2, HTcUM6.2.1, HTcUM6.2.2, HTcUM10, HTcUM6.1.1, HTcUM7.1, HTcUM8 and HTcUM9.1 were inoculated in milky skim agar (MSA) medium at room temperature for 24 hours to obtain a single colony bacterial culture with streak methods of inoculation refer [17].
2.2. Determination of pathogenicity
Determination of pathogenicity was carried out through hemolytic test. Hemolytic tests are carried out by modifying [19], inoculating bacterial cultures in blood agar media and incubated at 37°C for 48 hours. The presence of hemolytic activity was characterized by the presence of a hemolytic zone in the blood agar medium. Hemolytic test results are grouped into 3 groups, namely hemolytic-α (formed green zone around bacterial colonies), hemolytic-β (formed clear zones around bacterial colonies), or hemolytic-γ (no clear zones around bacterial colonies) in the blood agar. Pathogenic bacteria are characterized by a reaction that occurs between bacteria that grow with blood agar media. Formation of clear zones around the growing bacterial colonies indicate the pathogenic bacteria, whereas no clear zones around the bacterial colonies are formed in the results of non-pathogenic bacteria test.

2.3. The production of crude extract protease
The production of crude extract protease was carried out by means of one bacterial single colony inoculated into 100 mL of liquid medium containing 5.0 g/L glucose; tofu liquid waste 10% v/v; MgSO₄·7H₂O (5.0 g); FeSO₄·7H₂O (0.1 g); and KH₂PO₄ (5.0 g), at pH 7. The inoculum was incubated at 37°C with 100 rpm. The product was centrifuged at 3000 rpm for 30 minutes. Supernatant was a crude extract of protease enzyme which then tested for activity and concentration of protein.

2.4. Protease activity assay
A total of 0.5 mL of the casein (1% w/v) substrate in a pH 7 phosphate buffer was mixed with 0.2 mL of the crude extract enzyme in a test tube. The solution was incubated at 40°C for 20 minutes. The reaction was stopped by adding 1 mL of 10% trichloroacetic acid (TCA) solution and maintained at room temperature for 15 minutes. The solution was centrifuged at a speed of 2500 rpm at room temperature for 20 minutes, added 2.5 mL 0.4 M Na₂CO₃ and 1 mL of Folin-Ciocalteu reagent into the supernatant and incubated for 30 minutes at room temperature in a dark room. This reagent can detect tyrosine residues in peptides of the hydrolysis protease product because the phenolic group in tyrosine was able to reduce phosphotungstate and phosphomolibdate (the main component of Folin-Ciocalteu reagent) to blue tungsten and molybdenum. The results of this reduction showed a wide absorption peak in the visible light spectrum (600-800 nm). The absorbance product measured at λ = 660 nm with blank using a standard tyrosine solution. The control reaction tube follows the same method, but the addition of TCA solution was carried out before adding the substrate. Protease activity in this assay was expressed as Unit/mL which was equivalent to the amount of micromol tyrosine in hydrolysis products per minute under experimental conditions.

2.5. Determination of standard tyrosine curves
Determination of standard tyrosine curves was carried out by as much as 2 mL of standard solution containing 0 (blank), 10, 30, 50, 70, 90, and 110 μg/mL of tyrosine were added with 5 mL of 0.5 M Na₂CO₃ solution and 1 mL of Folin-Ciocalteu reagent. The solution was homogenized using vortex. The solution was incubated at 37°C for 30 minutes. The blue complex was measured at a wavelength of 660 nm.

2.6. The selectivity test of the ability of protease isolates HTcUM to extract collagen from milkfish scales
Milky fish scale was washed with water until clean and dry. As much as one gram’s milkfish scales were soaked with 20 mL of crude protease extract and 10 mL of phosphate buffer as A (sample) and with 30 mL of phosphate buffer as B (control without enzyme and scale), and C (control without scales) only contain 20 mL of crude enzyme extract and 10 mL of phosphate buffer. The three treatments were shaken for 72 hours at 37°C and 100 rpm. The resulting product was centrifuged at a speed of 2500 rpm for 20 minutes. The supernatant obtained was measured by the protein content of the Lowry method.
2.7. *Determination of Protein Concentration by Lowry Method*
A total of 0.5 mL of sample and 0.5 mL of each standard protein solution containing 0 (blank), 20, 40, 60, 80, 100 μg BSA was added 2.5 mL of Biuret reagent and stirred by vortex to be homogeneous. The mixture was incubated at the room temperature for exactly 10 minutes. It was added 0.25 mL of Folin-Cioucalteau 1 N and incubated at the room temperature for exactly 20 minutes. The absorbance value of the solution was measured at a wavelength of 750 nm.

3. *Results and discussion*

3.1. *Pathogenicity of HTcUM isolates*
The eight potential proteolytic bacterial isolates from Tauco Surabaya was cultivated. The results of the cultivation carried out in this study succeeded in inoculating seven isolates in skim milk agar medium (SSA) namely HTcUM2, HTcUM3.1.1, HTcUM3.2.2, HTcUM7.1, HTcUM8, HTcUM9.1, and HTcUM10, while HTcUM6.2.1 was died. The results of pathogenicity test on the seven isolates by hemolytic test method on blood agar showed that the seven isolates had different pathogenic properties, as shown in Fig. 1. HTcUM7.1 isolates (Fig. 1.g) showed positive hemolytic-α because it produced a green zone, while the other six isolates (Fig. 1.a-f) showed positive hemolytic-β because it produced a clear zone. The green zone formed in the hemolytic-α group indicates that the bacteria has a low pathogenicity because it is only able to tear the red blood cell wall, whereas the clear zone formed in the hemolytic-β group shows that the bacteria has strong pathogenic properties because these microbes are capable of lyse of red blood cells. The wider the clear zone that is formed, the stronger the pathogenic properties possessed. Therefore, the pathogenicity of HTcUM isolates quantitatively from the lowest can be sorted from HTcUM7.1<HTcUM6.1.1<HTcUM2<HTcUM10<HTcUM9.1<HTcUM3.2.2<HTcUM8.

[Figure 1. Observation of pathogenesis test on HTcUM isolates in blood agar media at 37°C for 11 hours: (a) HTcUM3.1.1, (b) HTcUM2, (c) HTcUM10, (d) HTcUM9.1, (e) HTcUM3.2.2, (f) HTcUM8, and (g) HTcUM7.1.]
Pathogenic bacteria produce toxic metabolites in their growth. This toxic metabolite affects growth and can even lyse red blood cells. The use of pathogenic bacteria is not recommended in a food production process. The toxic metabolite residue is feared to still be present in food products that are produced and have a negative impact on the health of consumers.

3.2. The specific activity and selectivity of protease from HTcUM isolates on milky fish scales

At this stage HTcUM8 was not tested considering that the isolate was the most pathogenic isolates. Each isolate was inoculated in the production media using tofu waste as nitrogen source, then the activity of crude extract of protease were determined using casein as substrate. The Specific activity of protease (U/mg) was obtained by dividing the value of protease activity by its protein concentration. The results at Table 1 showed that the ability to produce proteases in the medium containing tofu waste from the highest was HTcUM7.1>HTcUM6.2.2>HTcUM6.1.1>HTcUM2>HTcUM9.1>HTcUM10. This result is in line with the proteolytic index test in previous studies [17]. The proteolytic index value was a comparison of the diameter of the clear zone with the diameter of the bacteria. A clear zone was formed because casein in a white medium was hydrolysed to a colourless soluble peptide. The greater the proteolytic index value directly shows the better ability of these isolates to produce proteases. If further examined the high proteolytic index value can be caused by two things, namely because the number of proteases produced by these isolates is increasing or although the number of proteases is low but has a large turnover which make the catalytic velocity hydrolyses casein is higher. Both of these can be reflected through the value of specific activity of protease.

Table 1. Results of measurement of specific activity and selectivity of HTcUM isolates protease

| No | Isolate | Specific activity of protease (U/mg) | Dissolve protein (mg/mL) |
|----|---------|-----------------------------------|------------------------|
| 1  | HTcUM7.1| 6.044±3.390                       | 0.1015±0.040           |
| 2  | HTcUM6.2.2| 1.718±0.451                      | 0.0825±0.004           |
| 3  | HTcUM6.1.1| 1.516±0.487                      | 0.0125±0.012           |
| 4  | HTcUM2   | 1.211±0.004                       | 0.0315±0.001           |
| 5  | HTcUM9.1| 0.427±0.053                       | 0.0425±0.007           |
| 6  | HTcUM10  | 0.311±1.099                       | 0.0930±0.044           |

Therefore, proteases from each isolate were tested for selectivity of its protease on milky fish scales. Protease which has selectivity to milky fish scales can hydrolyse peptide bonds in milky fish to produce soluble collagen that can be measured as dissolved protein [12-14]. The amount of dissolved protein came from extraction of protein concentration in sample (A) minus control without enzyme and scale (B) and control without scale (C). The higher amount of dissolved protein was the more selective activity of the protease on milkfish scales. This means that the protease can be used for collagen production from milkfish scales. Based on the results of the experiments in Table 1, it was known that HTcUM10 and HTcUM9.1 isolates produce low proteases; HTcUM9; HTcUM8.1, and HTcUM9.2 isolates produced moderate proteases and HTcUM7.1 isolates produced the highest protease, but it was not always proportional to its ability on collagen production from milkfish scales. The HTcUM10 isolate produced the lowest protease but the ability on collagen production from fish scales was higher than other isolates, and HTcUM6.1.1 which was able to produce high amounts of protease had the lowest ability on collagen production from fish scales. Isolates HTcUM7.1, HTcUM9.1, and HTcUM9.2 showed high selectivity to collagen of milkfish scales, but only HTcUM7.1 shows a linear relationship between ability to produce proteases and selectivity its protease.
on milky fish scales. HTcUM7.1 gave specific activity of protease as much as 6.044±3.390 U/g and produce collagen as much as 0.1015±0.040 mg/mL. Also, have the lowest pathogenic properties. Therefore, HTcUM7.1 was selected that will be used as source protease for halal and healthy collagen production from milky fish scales.

4. Conclusions

HTcUM7.1 was hemolytic-α and the lowest pathogenicity bacteria among HTcUM2, HTcUM6.1.1, HTcUM62.2, HTcUM6.8, HTcUM6.9, and HTcUM10. The crude extract of protease from these isolate had specific activity of protease as 6.044±3.390 U/g, can hydrolysed the peptide bond on milky fish scales to produce soluble collagen as 0.1015±0.040 mg/mL. Overall results showed that HTcUM7.1 was the most potential proteolytic bacteria from *tauco* Surabaya that could be develop as source of protease for halal and healthy collagen production from milky fish scale.

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