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PRODUCTION OF PROTEASE ENZYME FROM BACTERIA IN HOT SPRING OF SOUTH SULAWESI, *Bacillus licheniformis* HSA3-1a

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ABSTRACT

Bacillus licheniformis HSA3-1a is able to produce extracellular protease. The aim of this research was to find out its production optimum time, consentration of $MnCl_2$, temperature and optimum pH, also the cofactor influence on activity protease from *B. licheniformis* HSA3-1a. The protease activity was tested with a modified method of Walter. Protease production time was optimum for 36 hours with an activity value of 0,394 U/mL, protein content was 132,35 mg/mL. The Optimum condition of protease enzyme were pH 7,0 and temperature 45 ^{o}C with an activity value of 0,0463 U/mL. Protease activity could be inhibited by $MnCl_2$ concentration of 0,01 - 0,03 M.

INTRODUCTION

Today, the increasing of industrial users on enzyme was rapidly, especially hydrolyses enzymes group such as protease, amylase, lipase, chitinase, and xylanase. Several research of enzymes have been done, because the enzyme as one of the alternative agent to replace chemical processes both in industry and the field of biotechnology [6].

An enzyme is a group of protein that catalysts and regulate the chemical changes in biological system. The enzyme can be produced by animals, plants and microorganisms. In catalytic, the enzyme serves in many reactions such as hydrolysis, oxidation, reduc-tion, isomerization, addition, group transfers, and sometimes carbon chain termination [17].

Protease is one of hydrolysis enzyme group that break down protein becomes simpler molecules in the presence of water. Protease was instrumental in all living things because it is essential in the process of protein metabolism, among others, aid digestion of proteins in food, reusing the intracellular proteins, as enzyme, hormones, and neurotransmitters [13].

Various types of bacteria and fungi such as *Bacillus licheniformis, B. amylolique, B. subtilis, B. cereus, B. polymyxa, B. thermoproteolyticus, Mucor pucillus, M. miehei, Aspergillus oryzae, A. sojae,* and *A. Phoenicis* were capable in producing protease, some of which have been used on an industrial scale [15]. *B. licheniformis* is a bacterium that producing high extra-cellular protease enzyme so it is possible to be deve-loped as one as the protease producing bacteria [11].

Indonesia has a wealth of natural resources include microorganisms group. One type of high protease producing microorganisms was isolated by Natsir is the bacterium *Bacillus licheniformis* HSA3-1a [10]. This bacterium was thermo-philic bacteria which isolated from hot springs Sulili-Pinrang, South Sula-wesi, Indonesia. *Bacillus licheniformis* HSA3-1a has chitinase activity at optimum temperature and pH of 50 °C and 7 respectively, and increased in the presence of activator Ca^{2+}.

The metal ions have potentially increasing the activity of enzyme called as

an enzyme activator, whereas metal ions which inhibit the enzymatic activity called an enzyme inhibitor [17]. Several researches on the effect of cofactor on enzyme activity has been carried out, among others: protease activity isolated from *Staphylococus aureus* was reported that in adding of Fe^{3+} and Mn^{2+} on concentration of 1 mM and 5 mM could increase the activity of protease [1]. The activity of Protease from *Bacillus mojavensis* was also increased by the presence of Cu^{2+} and Mn^{2+} [4].

The research has been done that show the activity of protease from thermopile bacteria of *Bacillus licheniformis* HSA3-1a in the medium dense on the addition of the ion Ca^{2+} [7] and ion Mn^{2+}, therefore, the research about the effect of $MnCl_2$ has done in production of protease enzyme from these bacteria

The purposes of this research are determine the activity of protease enzyme from *B. licheniformis* HSA3-1a in the presence of $MnCl_2$, the optimum concentration of $MnCl_2$ on production the protease enzyme, determines the pH and optimum temperature of protease enzyme, and also determine the concentration of $MnCl_2$ as cofactor either increase or inhibit the activity of protease enzyme.

MATERIALS AND METHODS

Material

The materials used in this research work include bacteria culture from hot spring in south Sulawesi *Bacillus licheniformis* HSA3-1a, Biochemis-try Laboratory, Hasanuddin University, bacto agar, yeast extract, NaCl, bacto peptone, casein, KH_2PO_4, $(NH_4)_2SO_4.7H_2O$, $MgSO_4.7H_2O$, $MnCl_2.4H_2O$, buffer citrate ($C_6H_8O_7.H_2O$ and $C_6H_5O_7Na_3.2H_2O$), buffer phosphate (NaH_2PO_4 and Na_2HPO_4), buffer borax (borax and NaOH solution), Lowry reagent A, Lowry reagent B, BSA (Bovine Serum Albumin), TCA (Tri Chloroacetic Acid) pa, ammonium molybdate, H_2SO_4 concentrated, $Na_2HAsO_4.7H_2O$, and ethanol.

Apparatus

The apparatus used in this research work included glass tools which are generally used in the laboratory, analytical balance (Ohaus), stirrer magnetic (8000-DSE Napco Model), incubator (Memmert), shaker water bath incubator (Thermo Scientific MaxQ 7000 Benchtop Water Bath Shakers Model N0. SHKA 7000, oven (Memmert), waterbath (Memmert), micropipet 100-5000 µL (Finnipipette Campus), Thermo Spectronic 20D+, and centrifuse (Centrifuse Hettich Universal 320 R Zentrifugen)

Methods

Culture of Bacillus *licheniformis* HSA3-1a.

The isolate of *B. licheniformis* HSA3-1a bacterial were cultured in several mediums LA on a Petri dish for 2-3 days at temperature of 50°C. Indication that the microbes can degrade casein well could show through isolates who growing well and having a clear zone around the colony. The Isolate of culture results is used in the production of protease enzyme.

Preparation of Inoculums

The isolate of *B. licheniformis* HSA3-1a which had been grown on solid medium were taken 2-3 ose and planted into the inoculums medium. The cultures were put in a shaker incubator at temperature of 50°C, 180 rpm for 20-24 hours.

Determinate Optimum Production time and concentration of $MnCl_2$

Before enzyme production, the first treatment were determined the optimum concentration of $MnCl_2$ and the optimum production time of protease enzymes. Pure isolate that have been isolated was cultivated in fermentation medium for testing the activity of the protease enzyme. This process began with the manufacture of inoculums were followed by a

fermentation process (enzyme production). Inoculums which had been incubated for 20-24 hours at 50°C; 180 rpm was taken on 10% for inoculated into 130 ml on production medium. Every 12 hours, the sampling was done, measuring the OD (optical density) at 660 nm wavelength, the measu-rement of protease activity and analysis protein.

Production of Extracellular Protease Enzyme

The production of protease enzyme was done in large volume about 1.000 ml on optimum condition. The sample was centrifuged at 3500 rpm, 4°C for 30 minutes. The supernatant was analyzed on the protein content, Protease activity testing on the effect of pH and temperature.

Measurement of Protein Levels by Lowry method [16].

A total of 2 ml of the protease enzyme was added by 2.75 ml of Lowry B, incubated at 25°C for 15 minutes. Then it was added by 0.25 ml of Lowry A and incubated at 25°C for 30 minutes with occasional shaking, and then the absorbance was measured at λ maximum that has been determined by using UV-Vis spectrophotometer.

Protease activity test

The activity of protease was detected by using modification of Walter method [18]. There was three treatments should be done. There were Blanco, standards and samples. A total of 0.1 ml of enzyme solution was inserted into the test tube which containing 0.5 ml of casein 2% w/v and 0.5 ml of phosphate buffer pH 7. On the Blanco and standard treatment, the enzyme was replaced with distilled water and tyrosine 0.1 mM. The solution was incubated at 50°C for 10 minutes. Hydrolysis reaction was stopped by addition of 1 ml of TCA 0.1 M. Both Blanco and standard were added 0.1 ml of the enzyme, and the sample was added 0.1 ml of distilled water. Then, the solution was incubated again at 50°C for 10 minutes, followed by centrifugation at a speed of 10,000 rpm for 10 minutes.

A total of 0.75 ml of the supernatant was added to a test tube which containing 2.5 ml of Na_2CO_3 0.4M. Then, it was added by 0.5 ml of Folin-Ciocalteau reagent (1: 2) and incubated at 50^{0}C for 20 minutes. The results of incubation was measured with a spectrophotometer at λ = 645 nm (λ maximum). Protease enzyme activity can be calculated by the formula:

$$UA = \frac{(\text{Asp}-\text{Abl})}{(\text{Ast}-\text{Abl})} x \text{ p } x \text{ } 1/\text{T} \text{.....(1)}$$

Specification:
UA = Units of enzyme activity (U / ml)
Asp = Sample absorbance value
Ast = Standard absorbance value
Abl = Blanco absorbance value
p = Dilution factor
T = Time of incubation (min)

Determination of Optimum pH

The Protease activity was tested at various pH buffer at pH 5.0; 6.0; 7.0 and 8.0 by using 0.2 M phosphate buffer by the method of Walter [18] which has been modified at temperature of 50°C.

Determination of Optimum Temperature

The optimum temperature was tested with the protease activity tested at various temperatures of incubation at 40°C, 45°C, 50°C, 55°C and 60°C. The test was performed at the optimum pH.

The Cofactor Effect in Protease Enzyme Activity

The optimum pH of citrate phosphate and 0.1 ml of enzyme (sample) were added by metal ions from $MnCl_2$ compound with various concentrations of 0.01 M; 0,015M; 0,02M; 0,025M; and 0,03M then added by 0.5 ml of 2% casein. After that, it was incubated for 10 minutes with the

temperature of 45^{0}C. Protease activity was tested by the method of Walter [18].

RESULTS AND DISCUCCION

The Optimum Concentration of $MnCl_2$ and Production Ttime of Protease Enzyme from *Bacillus licheniformis* HSA3-1a

The first step of production the enzyme was rejuvenated of the isolate microbial of *B. licheniformis* HSA3-1a. These bacteria were grown in a liquid medium and solid medium using casein as substrate protease production. Furthermore, the isolate bacteria was grown in a fermentation medium with pH 7 in various concentrations of $MnCl_2$ respectively 0.005%, 0.010%, 0.015%, 0.020% and 0.025%.

In this medium the growth of microbes and the resulting product were monitored in optimal conditions during fermentation. Sampling during the fermentation process was done every 12 hours for 4 days to determine the optimum time of protease production, the enzyme activity testing and measurement of protein concentration.

Growth of bacteria was assessed based on the cell density absorbance value or optical density (OD). The bacteria were isolated by the addition of 0.015% $MnCl_2$ got the highest activity which measured by using spectrophotometer 20D+ at wavelength of 660 nm. The results (Figure 1) were shown the relative increase cell growth in fermentation time from 12 hours to 36 hours. The addition of Mn^{2+} metal ions on fermentation medium gave affects on the growth of bacterial cells. The increase of optical density absorbance value at the time of fermentation for $MnCl_2$ concentration of 0.005%; 0.010%; 0.015%; 0.020% and 0.025% indicates a metallic effect in inhibiting or accelerating the growth of bacterial cells. The optical density data on the addition of $MnCl_2$ was having the highest activity shown in Figure 1.

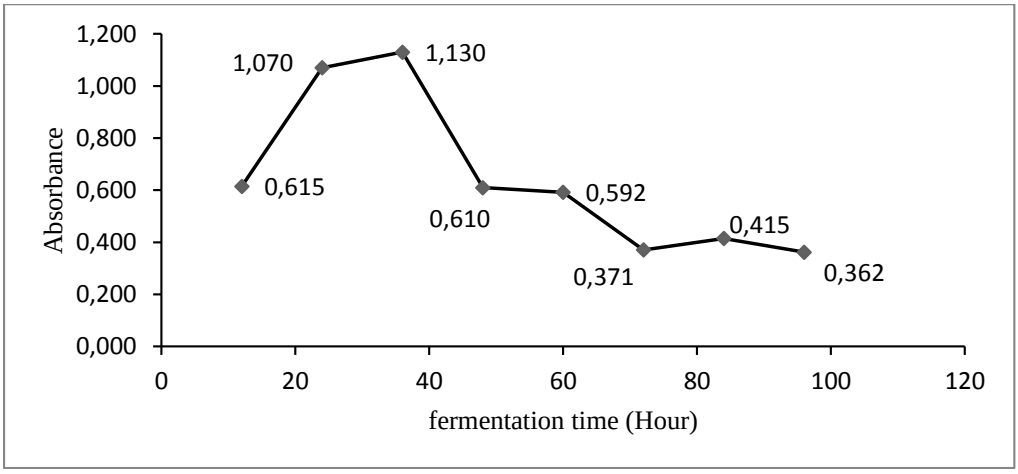

Figure 1. Effect of $MnCl_2$ concentration of 0.015% on the growth of bacterial cells *B. licheniformis* HSA3-1a

Cell and filtrate were separated by centrifugation at 3500 rpm, temperature of 4°C for 30 minutes. The filtrate was the extract enzyme crude. The enzyme protease from *B. licheniformis* HSA3-1a is an extracellular enzyme. The extra-cellular enzymes are synthesized in the cell, then secreted out of the cell through the cell wall and can function outside the cell. Thus extracellular enzymes are located in the fermentation medium and the biomass can be separated by centrifugation [10].

The results of cell separated enzyme were a crude extract, then testing protease activity and protein concentration. Tests performed protease activity using a tyrosine standard solution was measured at the maximum wavelength of 645 nm. The data of protease activity could be seen in Table 1.

Table 1. The optimum concentration of $MnCl_2$ and the optimum time of production on protease enzyme from *Bacillus licheniformis* HSA3-1a.

No.	Fermentation Time (Hours)	Protease Activity (U/mL)				
		[$MnCl_2$] 0,005	[$MnCl_2$] 0,010	[$MnCl_2$] 0,015	[$MnCl_2$] 0,020	[$MnCl_2$] 0,025
1	12	1.05×10^{-1}	2×10^{-3}	2×10^{-3}	1.04×10^{-1}	6.3×10^{-2}
2	24	1.2×10^{-1}	1×10^{-3}	2×10^{-3}	1.43×10^{-1}	2.99×10^{-1}
3	36	6×10^{-3}	5×10^{-3}	3.94×10^{-1}	7×10^{-3}	1.5×10^{-2}
4	48	5×10^{-3}	4.7×10^{-2}	1×10^{-3}	8×10^{-3}	5×10^{-3}
5	60	9×10^{-3}	7×10^{-3}	3.3×10^{-2}	1.2×10^{-2}	7×10^{-3}
6	72	3×10^{-3}	1×10^{-2}	1×10^{-3}	1.2×10^{-2}	7×10^{-3}
7	84	4×10^{-3}	4×10^{-3}	5.8×10^{-2}	2.8×10^{-1}	2×10^{-3}
8	96	7×10^{-3}	3×10^{-3}	6×10^{-3}	7×10^{-3}	1.2×10^{-2}

The data (Table 1) shows that the activity of protease with the greatest activity was concentration of 0.015% $MnCl_2$ by production time of 36 hours with the protease activity of 0.394 U/ml. This can be explained because the protease has a lot of negative charge on the surface of the enzyme derived from amino acids, which contained the acid groups in the side chain [12]. From the data (Table 1) protease activity was relatively low for all types of $MnCl_2$ concentrations ranging from fermentation time of 48 hours to 96 hours except for $MnCl_2$ concentration of 0.020% where activity tends to increase during the 84 hour fermentation. It can be caused by the presence of metal in the ability to neutralize the negative charge of enzymes group so the enzyme activity increased again. From the data, the optimum time was used to produce the protease enzyme in large quantities. A research worked by Kandolla using compound of $CaCl_2$ on production of protease enzyme which optimum production time at 60th hours with an activity value of 5.6×10^{-2} U/ml [7].

The Protein Content by Lowry Method

The measurement of protein content was using crude extract was done on the addition of $MnCl_2$ on concentration of 0.015%. The amount of protein was determined based on the absorbance of the solution at a wavelength of 715 nm to obtain a standard curve. From this standard curve, it made a straight line equation to calculate the levels of protein. Value of the protein content can be seen in Figure 2 where the highest levels of the protein values obtained on 12 hours at 140.60 mg/ml. While the protein content with the highest value of activity or production time on 36 hours was 132.35 mg/ml. This indicates that not all protein crude extract has the enzyme characters.

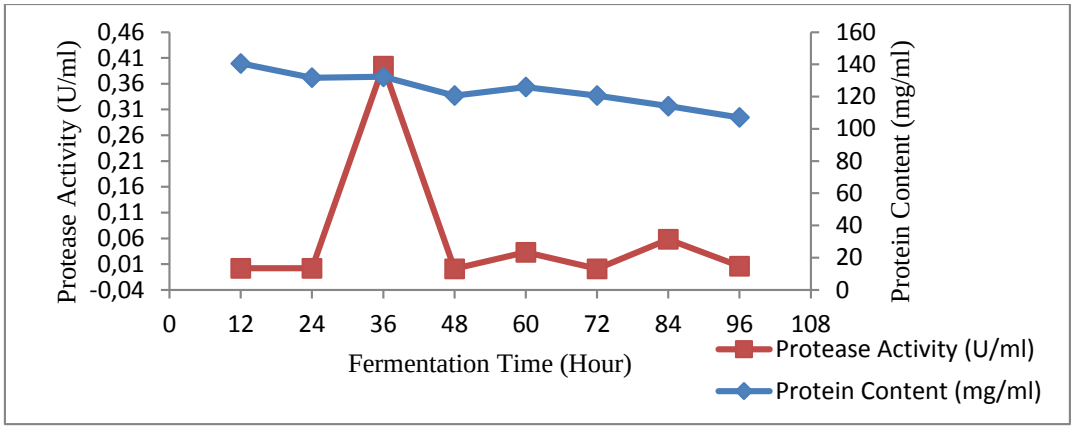

Figure 2. Levels of crude extracts protein of *B. licheniformis* HSA3-1a during fermentation at pH = 7.0; temperature of 50^0C.

Effect of pH on Protease Activity of *Bacillus licheniformis HSA3-1a*

The enzyme activity depends on the concentration of hydrogen ions. Each enzyme has its own optimum pH, is a pH where the enzyme can work well [17]. Enzymes are proteins, then the factors that affect the structure of protein also influence the stability of enzymes such as too acidic or alkaline, enzymes will be denatured [5].

The phosphate-citrate buffer at various pH were 5.0; 6.0; 7.0; 8.0 at temperature of 50°C. Based on the results (Figure 3), indicating that the protease enzyme activity reaches a maximum at pH 7.0 with the activity of 0.0394 U/ml.

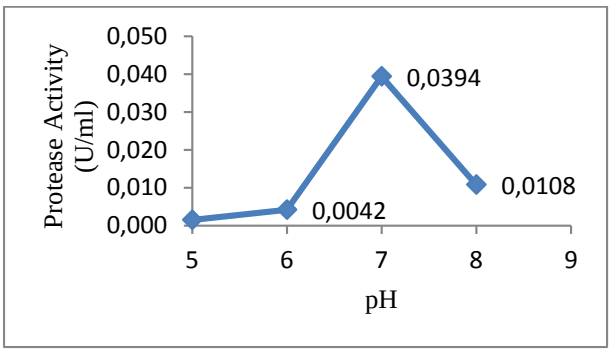

Figure 3. The effect of pH on protease activity from *B. licheniformis* HSA3-1a at [S] = 2.0%; tempera-ture = 50°C.

Kandolla was shown the protease from *B. licheniformis* HSA3-1a with the addition of 0.015% $CaCl_2$ was optimum at pH 7.0 and temperature of 50°C [7]. A protease from *B. licheniformis* Lbbl-11 was optimum at 60°C and pH 8.0 [11].

Effect of Temperature on Protease Activity of *Bacillus licheniformis* HSA3-1a

The optimum temperature of protease was done by measuring the protease activity at various incubation temperatures. The result obtained (Figure 4), indicating that the protease achieve optimum activity at temperature of 45°C at 0.0463 U/ml. Protease activity decreased over 45°C temperature. The temperature causes increased enzyme activity because it increases the kinetic energy that would add to the intensity of the collision between substrate and enzyme, but the protein at relatively high temperatures would cause denaturation.

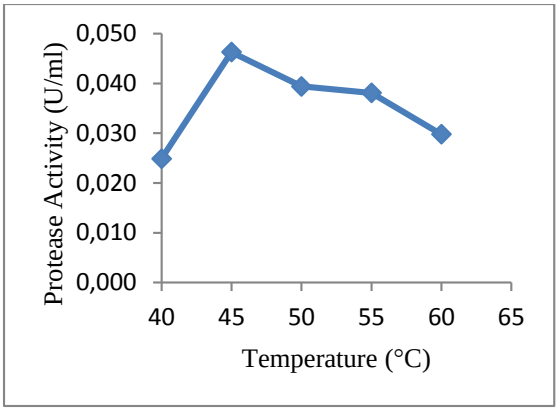

Figure 4. Effect of temperature on protease activity from *B. licheniformis* HSA3-1a at [S] = 2.0%; pH 7.

Effect of Cofactor on Enzyme Activity from *Bacillus licheniformis* HSA3-1a

One of the distinctive properties of the enzyme is in need of additional chemical components such as cofactor for its activity. This study used a metal cofactor of Mn^{2+} with a variety of $MnCl_2$ concentration such as 0.01 M; 0.015 M; 0.02 M; 0.025 M; and 0.03 M and without the addition of $MnCl_2$ as a control. From the data that the addition of $MnCl_2$ concentration of 0.01 M; 0,015M; 0,02M; 0,025M; and 0,03M gave the lower activity was shown in Figure 5.

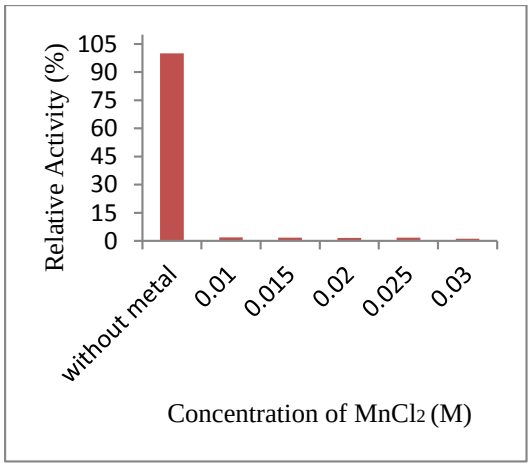

Figure 5. Effect of $MnCl_2$ on the protease activity from *B. licheniformis* HSA3-1a at [S] = 2.0%; pH 7.0; temperature of $45^{O.}$

The data was shown that the addition of $MnCl_2$ at various concentrations (0.01 M; 0.015 M; 0.02 M; 0.025 M; 0.03 M) led a relatively activity of protease decreased to 98.81%. It can be caused by the positive charges on Mn^{2+} bound to the active site resulted an unstable structure by reducing the activity of the enzyme. Inhibition of metal ions on protease activity at certain concentrations associated with the ionic strength, where the ionic strength influences either conformation or dimensional structure of the enzyme [14]. The $MnCl_2$ at concentrations greater than 0.01 M are inhibits the enzyme activity.

CONCLUSION

Bacillus licheniformis HSA3-1a has protease activity with optimum production time of 36 hours at $MnCl_2$ concentration of 0.015% with protease activity at 0.394 U/ml. The protease enzyme worked at temperature of $45^{O}C$ at pH 7.0 with the value of the activity of 0.0463 U/ml and protease activity can be inhibited by $MnCl_2$ concentrations of 0.01 to 0.03 M.

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