

Production and Characterization of Crude Protease from RS1 Isolate from silage of Floating Bladderwort (*Utricularia gibba*)

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Abstract: The purpose of this research was to produce and characterizing crude protease from RS1 isolate of swamp plant silage. The optimum production time of RS1 isolate was 45 h. The optimum pH and temperature of protease from RS1 isolate were 10 and 45°C, respectively. Ion Mg³⁺ increased RS1 protease whereas ion of Na⁺, K⁺, Fe²⁺, and Zn²⁺ inhibited protease from RS1 isolate. Study on the effect of metals ion indicated that protease from RS1 isolate was metalloenzyme. Based analysis on SDS-PAGE, the molecular weight of RS1 protease had 12 bands with molecular weights ranging from 34.75 kDa to 263.53 kDa.

Keywords: Protease; RS1 Isolate ; Production; Characterization.

1. Introduction

Enzymes are proteins produced by living organisms which catalyze the chemical reaction in hugely efficient ways and are environment friendly. They have substantial advantages over chemical catalysts, in its specificity, high catalytic activity, its capability to work at moderate temperatures, and the ability to be produced in large amounts¹. Enzymes are diverse polymer molecules produced by living cells. Enzymes are biological catalysts (also known as biocatalysts) that speed up biochemical reactions in living organisms. They can also be extracted from cells and then used to catalyse a wide range of commercially important processes².

Protease enzymes are enzymes needed by all living things to hydrolyze peptide bonds in proteins to oligopeptides and amino acids. Proteolytic enzymes are capable of hydrolyzing peptide bonds in proteins³. Sources of protease enzymes are known to come from animals, microbes, and plants.

Microorganisms are the most widely used source of enzymes compared to plants and animals. As a source of enzymes, microorganisms are more beneficial because of their rapid growth, the results of which can be improved more easily through the regulation of growth conditions and genetic engineering. One source of enzyme-producing microbes is from silage of floating bladderwort (*Utricularia gibba*) with the addition of 5% fine bran. Screening of bacteria from the silage of swamp plants was done to select and obtain bacteria that produce protease enzymes.

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DOI: <http://dx.doi.org/10.13171/mjc02003251256ab>

Based on the results of preliminary research shows screening of protease-producing bacteria from silage of floating bladderwort (*Utricularia gibba*) was obtained RS1 isolate which had the highest proteolytic index which would then be used in the production and characterization of the resulting protease enzyme. Enzyme characterization includes the effect of pH, temperature and metal ions on enzyme activity, this protease characterization aims to determine the optimal conditions (pH, temperature and influence of metal ions) in enzyme application.

2. Material and Methods

2.1. Materials

The materials used in this study were Floating bladderwort (*Utricularia gibba*), RS1 isolate, trichloroacetic acid (Merck), NaOH (Merck), tryptone (Merck), yeast extract (Merck), NaCl (Merck). The tools used include pH meter, OHAUS analytical balance, incubator, micropipette (Single Channel Capp 10-100 U1, USA), autoclave (Hirayama, Japan), hotplate (Cimarec, United Kingdom) and a spectrophotometer.

2.2. Protease Production

The RS1 bacterial strain was inoculated for 1-2 loops on the Luria Bertani Broth (LB) media. The cell growth was monitored turbidimetrically through absorbance at $\lambda = 620$ nm. As much as 10 % of seedling culture with an optical density of 0.8 (at 620 nm) was sub-cultured into the same media for enzyme production.

Received January 8, 2020

Accepted March 3, 2020

Published March 25 11, 2020

2.3. Assay of Protease Activity

Protease activity was measured by the Bergmeyer⁴ method using a 2% (w/v) with casein Hammerstein as substrate. One unit of protease activity was defined as the amount of enzyme that can produce one μmol of tyrosine products per min under measurement conditions.

2.4. Protein Assay

Protein determination was carried out using the method of Bradford⁵. 5 ml of Bradford reagent was added to 0.1 ml sample in the test tube. The blank was made by mixing 0.1 ml of distilled water and reacted with 5 ml of Bradford reagent. After about 5 min, each reaction mixture was measured for absorbance at $\lambda = 595 \text{ nm}$. Bovine serum albumin was used as the protein standard.

2.5. Effects of pH on Enzyme Activity

The effect of pH on protease activity was carried out by reacting enzymes and substrates at pH 5-11 at 37°C for 60 min with universal buffer. Enzyme activity was analyzed quantitatively by the Bergmeyer method⁴.

2.6. Effect of Temperature on Enzyme Activity

The protease enzyme used in the characterization was crude extract protease. The effect of temperature on enzyme activity was carried out by reacting 50 μl protease with 250 μl casein solution and 250 μl 0.05 mM phosphate buffer with optimum pH and incubated at 35, 40, 45, 50, 55, 60, and 65°C for

60 min. Enzyme activity was analyzed quantitatively by Bergmeyer method⁴.

2.7. Effects of Metal Ions

The metal ions used are NaCl, KCl, FeCl_2 , ZnCl_2 and MgCl_2 . The test of the influence of metal ions is carried out when measuring enzyme activity. The reaction starts with the addition of a substrate, then the metal ions and buffer are added. The activity of the enzyme is compared with the activity of non-metal ion enzymes (100%). Enzyme activity was analyzed by Bergmeyer method⁴.

2.8. Determination of Molecular Weight with SDS-PAGE

The determination of molecular weight is done by electrophoresis under denaturing polyacrylamide-SDS (SDS-PAGE) with 8 % polyacrylamide gels using the Laemmli method⁶. The gel consists of two types, namely 8% separating gel and 4% holding gel.

3. Results and Discussion

3.1. Protease Production

Protease production was carried out on the Luria Bertani (LB) broth medium after being cultured in the same medium until it reaches OD = 0.8. Incubation was conducted at 37°C and samples were taken for analysis of enzyme activity, protein concentration and cell growth. The optimum production of protease from RS1 isolate can be seen in Figure 1.

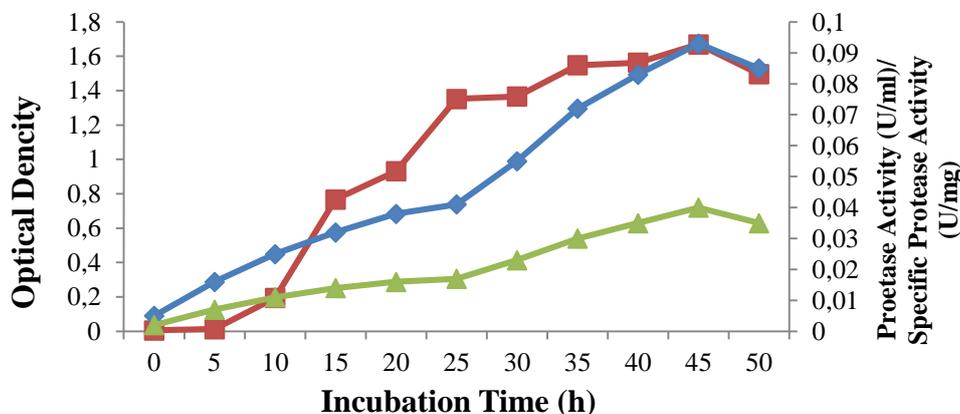


Figure 1. Protease production of RS1 isolate from silage of floating bladderwort (-■- optical density, -◆- protease activity, -▲- specific activity)

Incubation period affects the enzyme production significantly and it varies from 24 h to a week depending upon type of microorganism and other culture conditions such as source of media, inoculum size, metabolic state of cell pH and temperature⁷. Protease from RS1 isolate has optimum protease production at the 45 h with protease activity was 0.091 U/ml. Assay of specific activities was also carried out. To determine the specific activity of proteases, the enzyme protein concentration was measured. Protein

concentrations were analyzed concurrently when measuring protease activity. The optimum specific protease activity from RS1 isolate was 0.042 U/mg. *Bacillus subtilis* ATCC 633 and *Bacillus* sp. UFLA 817 CF have been reported to show maximum protease activity after 24 h growth⁸. Protease production by *Bacillus pumilus* UN-31-C-42 started 16 h after incubation⁹. Optimum incubation time for protease production by *B. licheniformis* and *Bacillus coagulans* has been reported as 96 h¹⁰.

3.2. Effect of pH on Enzyme Activity

All enzymatic reactions were influenced by pH, so a buffer was needed to control the pH of the reaction. In general, enzymes are ampholytic, which means that the enzyme has a dissociation constant in the acid group and its base group, especially in the carboxyl-terminal residue group and its amine terminal group.

It was estimated that changes in the activity of enzymes were caused by changes in ionization in the ionic enzyme groups, i.e. on the active side or another side which indirectly affects the active side. Figure 2 shows the effect of pH on the crude protease activity from RS1 isolate.

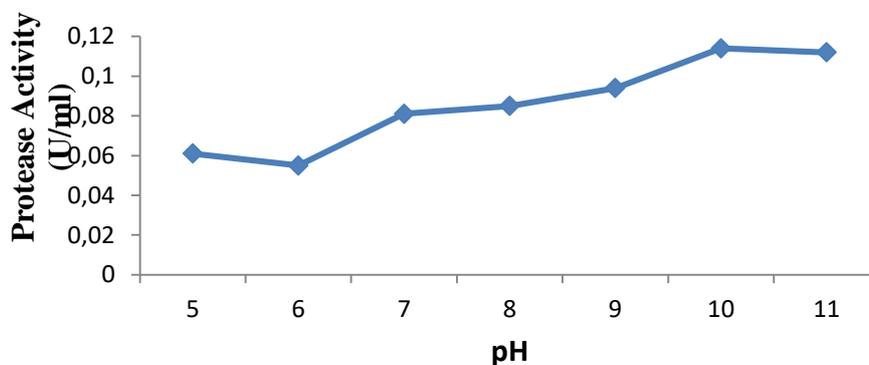


Figure 2. Effect of pH on Protease Activity from RS1 Isolate

The optimum protease activity from RS1 isolate was pH 10. The optimum pH value of protease from RS1 isolate was the same as the optimum pH of the protease of *Bacillus licheniformis* KBDL48¹¹. The optimum pH protease from RS1 isolate was greater than the protease from *Bacillus aryabhatai* K36 which has optimum activity at pH 8¹² and *Bacillus licheniformis* F11.4 protease which has optimum pH at pH 9¹³.

3.3. Effect of Temperature on Enzyme Activity

In general, each enzyme has a maximum activity at a specific temperature. Enzyme activity will increase with increasing temperature until the optimum temperature was reached. A further rise in temperature will cause the enzyme activity to decrease. From the results of the assay of enzyme activity at various temperatures can be seen in Figure 3.

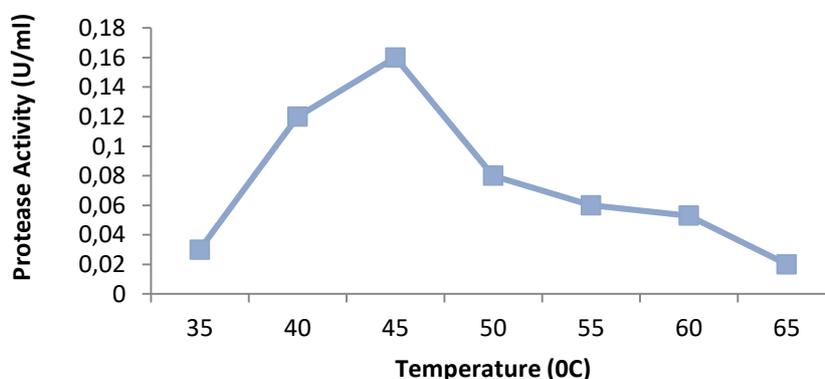


Figure 3. Effect of temperature on Protease Activity from RS1 isolate

The optimum temperature of the protease from RS1 isolate was 45°C. The optimum temperature in this study was the same as the protease from *Myceliophthora thermophila*¹⁴ and was greater than the optimum temperature for the protease from *Bacillus cereus* FT 1 that was 35°C¹⁵, but more compared to *Bacillus licheniformis* F11.4 protease which has an optimum temperature of 50°C¹³, *Bacillus licheniformis* UV-910 protease which has an

optimum temperature of 60°C¹⁶ and the *Bacillus HUTTBS62* protease with temperature the optimum was 80°C¹⁷.

3.4. Effects of Metal Ions on Protease Activity

Some enzymes need metal ions as a cofactor to support enzyme catalytic efficiency. The effect of metal ions on protease activity from RS1 isolate was shown in Table 1.

Table 1. Effects of Metal Ions on Protease Activity from RS1 Isolate.

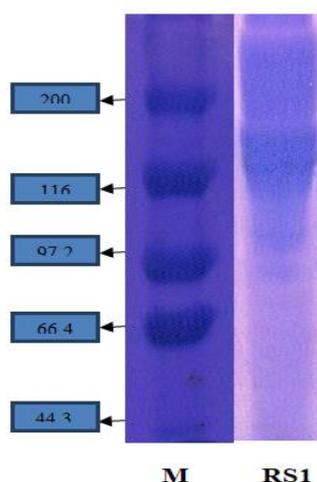
Treatment	Concentration (mM)	Activity (U/ml)
Control	-	0.074
NaCl	5	0.034
KCl	5	0.043
FeCl ₂	5	0.000
ZnCl ₂	5	0.016
MgCl ₃	5	0.101

Table 1 shows the Mg³⁺ ion as an activator of the protease from RS1 isolate because it can increase protease activity compared to controls. Metal ions that can increase enzyme activity are cofactors of these enzymes. Mn²⁺ activated enzyme activity of protease from *Pseudomonas thermaerum* GW1 by fivefold¹⁸. Ions of Na⁺, K⁺, Fe²⁺ and Zn²⁺ can reduce activity compared to control so that this ions as protease inhibitors of protease from RS1 isolate. Metal ions which can reduce the activity of enzymes may not be

cofactors for these enzymes. The effect of metal ions on protease activity indicates that protease from RS1 isolate was a metalloenzim.

3.5. Determination of Protease Molecular Weight

The results of SDS-PAGE protease from RS1 isolate can be seen in Figure 4. SDS-PAGE analysis of crude extract protease shows that protease from RS1 isolate has 12 bands with molecular weights ranging from 34.75 kDa to 263.53 kDa.

**Figure 4.** SDS-PAGE analysis of crude extracts of protease enzymes from RS1 isolate

Different molecular weight enzyme proteases will be obtained from different types of bacteria. Some protease from other bacteria were *Bacillus* sp. HS08A has a molecular weight of 30.9 kDa¹⁹ and *Bacillus* sp. S17110 with a molecular weight of 75 kDa²⁰.

4. Conclusions

The protease from RS1 isolate has the highest activity (0.091 U/ml) after incubating for 45 h in LB media. The characterization of protease activity from RS1 isolate was pH optimum at pH 10 and temperature at 45°C. Mg²⁺ ion as an activator while ions of Na⁺, K⁺, Fe²⁺ and Zn²⁺ as inhibitors. The effect of metal ions on protease activity indicates that protease from RS1 isolate was a metalloenzyme. SDS-PAGE analysis on crude extract protease showed that protease from RS1 isolate had 12 bands with molecular weights ranging from 34.75 kDa to 263.53 kDa.

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