

Production Optimization and Characterization of Amylase Enzyme Isolated from Termofil Bacteria *Bacillus* sp RSAIL-1b from Lejja Hot Spring South Sulawesi

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Abstract: Thermostable amylase enzyme has a broad commercial value in its use in the processing of starch, sugar production, textile, paper, animal feed, pharmaceuticals and in the manufacture of detergents. This study aims to determine the optimum conditions of amylase production from the termofil bacteria *Bacillus* sp. RSAIL-1b isolated from a hot spring Lejja South Sulawesi and characterizing the amylase enzyme. The testing of amylase production was done with various concentration of starch and CaCl₂ in the production medium, then fermented to obtain maximum amylase activity, amylase enzyme was produced in optimum condition, and its characteristic was tested using 2% starch substrates in various pH, temperature and determining the compound cofactor which can act as activators or inhibitors of the amylase activity, the enzyme activity was tested using DNS method. Crude extract enzyme has the highest enzyme activity of the protein content determined by the method of Lawry. The results showed that the amylase enzyme from *Bacillus* sp RSAIL-1b isolates can be manufactured to a maximum at 33 hours of fermentation time with conditions: the concentration of substrate (starch) 1.5%, 0.08% CaCl₂, 55°C temperature, medium pH 7.0 and aeration speed 200rpm with the activity of 0.1323U/mL, amylase crude extract protein content of 1.86mg/mL, with spesifik activity 0.0711 U/mg protein. Crude extract amylase work optimally at pH 6.0; 55°C -60°C the amylase activity of 0.165U/mL, the specific activity of 0.089U/mg protein. Amylase enzyme is an enzyme that depends on metal because its catalytic activity can be activated by metal ions Ca²⁺, Mg²⁺, Cu²⁺, Ni²⁺ and Co²⁺ as activators whereas Zn²⁺ ions decrease the activity of enzymes as inhibitors. Amylase activity in the crude extract optimum conditions with the addition of 10 mm ions Ca²⁺ can increase amylase activity up to 32,89%, while the addition of ions Zn²⁺ can inhibit amylase activity up to 25%.

Keywords: Amylase, Hydrolytic, *Bacillus* sp, Isolate, Starch

1. Introduction

Starch molecules are polymers of alpha-D-glucopyranose which will be broken down by amylase enzyme at alpha-1,4 and alpha-1,6-glycosides bonds [1]. This polymer is very abundant after cellulose, found in many sago, cassava, maize, millet, etc. Amylase is a hydrolytic enzyme that can hydrolyze the compound starch or starch as its substrate, and an enzyme that is important and its existence is greatest in the field of food and biotechnology, these enzymes are traded as much as 25% of the total other enzyme [2].

Amylase is generally produced by plants, animals, humans and microbes. Enzymes derived from termofil microbe and hipertermofil are more widely used in industry, especially industries that use high temperatures in the process. This happens because an enzyme derived from the microbe has thermo stability and optimum activity remains at a high temperature [3]. Microbes producing enzymes that are widely used in industry, namely fungi and bacteria [4].

Amylase enzyme has a high economic value, because in the food industry, α -amylase enzyme functions provide sugar hydrolysis of starch that can be utilized for the production of glucose syrup or fructose syrup that has a high level of

sweetness to be used in the manufacture of bread, and baby food. In the textile industry α -amylase enzyme is used to assist in the removal of starch, which is used as an adhesive to protect the threads of woven in the bending moment. This process requires a temperature of around 70-80°C [5]. The usefulness of α -amylase under various conditions is strongly influenced by the stability of the enzyme [6]. α -amylase requires calcium ion (Ca^{2+}) to increase the activity and to maintain the structure and stability.

Several studies on thermophilic bacteria and thermo stable enzymes have been done. Raharjo et al. [7] reported that the hot springs of Sonai South East Sulawesi contains 26 isolates of amylase bacteria. They found that isolate of S60T3-4 bacteria had the highest activity at the optimum temperature of 80°C and pH 4.5 with specific activity of 3,54U/mg protein. In addition, Arfah et al. [8] reported that the hot springs of Lejja South Sulawesi contains 10 isolates of amylase bacteria. Isolate R-SAIL-1b has the largest clear zone of 5.6cm after 48 hours incubation at 50°C. The clear zone after the addition of iodine to the medium containing starch substrate indicates that these isolates have the ability to produce the amylase enzyme which can hydrolyze starch into glucose.

Based on those informations, research on the production of amylase from thermophilic bacteria *Bacillus sp* RSAIL-1b has been carried out. We report here the optimum condition of enzyme production and characterization of crude extract of amylase against the effect of pH, temperature and metal ions addition on the activity of the amylase enzyme.

2. Methods

2.1. Isolate Rejuvenation and Preparation of Inoculum

Rejuvenation of bacteria was done by taking 1 ose of bacteria isolates *Bacillus sp* RSAIL-1b and then inscribed in the medium Luria Agar (LA) then incubated for 24 hours at 50°C. Next step is the preparation of inoculum on medium with the production medium composition inoculum used by [6] were modified. Composition of the medium: yeast extract 0.2%; bactopectone 1%; NaCl 0.005%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%; CaCl_2 0.015% and 1.0% starch. Medium production and inoculum medium has the same composition but varying starch concentration of 0.5%-2.5%, and the CaCl_2 concentration of 0.01%-0.16%.

2.2. Optimization of Amylase Enzyme Production

2.2.1. Determination of the Optimum Soluble Starch Concentration

Bacterial culture isolates of *Bacillus sp* RSAIL-1b taken 1 loop of culture ages 24 hours put into the medium inoculum subsequently incubated in a shaker incubator at 200 rpm temperature of 55°C for 24 hours [7]. Furthermore, 10% of inoculum active included in the sterile production medium with varying concentrations of soluble starch 0.5%-2.5%. The next incubated in an incubator shaker at 200rpm temperature of 55°C for 24 hours. Furthermore, the separation of the cells from the medium by centrifugation at

3500rpm 30min, 4°C. Supernatant obtained a crude extract of the enzyme amylase, then tested its activity. Once known concentrations of certain starch which has the highest amylase enzyme activity.

2.2.2. Determination of the Concentration of CaCl_2

Bacterial culture isolates of *Bacillus sp* RSAIL-1b taken 1 loop of culture ages 24 hours put into the medium inoculum subsequently incubated in a shaker incubator at 200 rpm temperature of 55°C for 24 hours [7]. Furthermore, 10% of inoculum active included in the sterile production medium with contains optimum concentrations of soluble starch then performed the CaCl_2 concentration variation in production medium of 0.01%-0.16%, The next incubated in a shaker incubator at 200rpm temperature of 55°C for 24 hours, then the separation of the cells medium by centrifugation at 3500rpm 30minutes, 4°C. Supernatant obtained a crude extract of the enzyme amylase, then tested its activity.

2.2.3. Effect of Fermentation Time on Bacterial Growth (Optical Densit) and the Production of the Enzyme Amylase from *Bacillus sp* Isolates RSAIL-1b

Once known concentrations of CaCl_2 specific enzyme activity of amylase made amylase production on starch concentration, and the concentration of CaCl_2 optimum by sampling every 3 hours for the analysis of optical density (OD) at a wavelength (λ) 600nm [8], as well amylase activity is determined by the DNS method [9]. Results of testing the enzyme activity obtained data shows optimum amylase production on certain conditions.

2.3. Activity Test of Amylase Enzyme

Amylase enzyme activity measurement principle is based on the calculation of reducing sugars from the hydrolysis of starch with DNS method [9] mixture of 0.5mL of 2% soluble starch; 0.5mL 0.1M phosphate buffer pH 7.0; 0.5mL of the extract amylase enzyme, incubated at 55°C for 60minutes, after the incubation was added 1.5mL of DNS reagent (salicylnitrous acid) and then shaken and heated in boiling water for 10 minutes further cooled in ice water, and measuring absorbance by using a spectrophotometer at a wavelength of 430nm maximum.

2.4. Protein Assays

This test using the method of Lowry [10], the Lowry B composition is Na_2CO_3 2% in 0.1N NaOH; 1% CuSO_4 : sodium-potassium-tartrate (100:1:1) and Lowry A is phospho-tungstic acid-phospho-molybdic (Folin): distilled water (1:1). The protein content is measured by using BSA (bovine serum albumin) as a standard and was measured using a spectrophotometer at a wavelength of maximum [10].

2.5. Characterization of Amylase Enzymes

Amylase production is done in optimum condition and then conducted biochemical characterization of the nature of amylase about pH, temperature, and the influence of metal ions.

2.5.1. Determination of Optimum pH [11]

Enzymes, substrates, phosphate buffer 0.1M pH the range of 5.7 to pH 8.0 is mixed and incubated at 55°C for 60 minutes further testing amylase activity at each change in pH in order to obtain optimum amylase activity at a given pH.

2.5.2. Determination of the Optimum Temperature

Substrates, enzymes, buffer pH optimum (which has been achieved in part A) were mixed and incubated for 60 minutes at a temperature range of 35-65°C and then testing amylase activity at any temperature change in order to obtain optimum amylase activity at a certain temperature

2.5.3. Effect of Metal Ions on the Activity of Amylase [12]

To determine the effect of metal ions that act as activators or inhibitors used several types of metal ions ($MgCl_2$; $CaCl_2$; $CoCl_2$; $NiCl_2$; and $ZnCl_2$) with the same concentration as follows: 10mM. A mixture of enzymes, metalions, buffer and substrate respectively incubated for 60 minute further testing amylase activity in each of the ion species.

3. Results and Discussion

3.1. Amylase Enzyme Production Optimization

3.1.1. Determination of the Optimum Soluble Starch Concentration

Figure 1 shows that the production of amylase starch concentration increased to 1.5% (optimum) with amylase activity of 0.087U/mL with a maltose content of 1.890mg/mL. The increased concentration of soluble starch from 1.5% to 2.5% and maltose amylase production is relatively constant. This indicates that at concentrations of 1.5% soluble starch amylase production has reached the maximum.

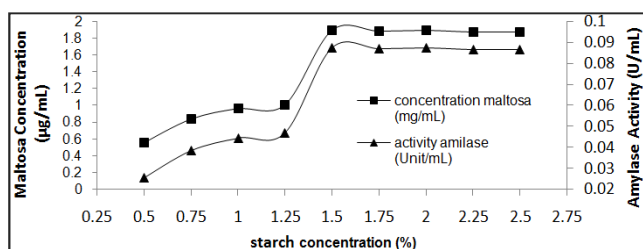


Figure 1. Effect of substrate concentration on the production of extracellular amylase from *Bacillus* sp. RSAIL-1b. Isolates termofil bacteria. In the fermentation conditions: pH 7.0; temperature 55°C; aeration speed 200rpm for 24 hours.

The production of amylase on starch substrate concentration effect seen in Figure 1, in contrast with the amylase produced from *Bacillus* species of land at 50°C with a concentration of 2.0% starch [6].

3.1.2. Determination of the Concentration of $CaCl_2$

Effect of $CaCl_2$ concentration on the production of amylase from isolates *Bacillus* sp. RSAIL-1b, $CaCl_2$ concentration optimum for producing amylase from isolates *Bacillus* sp RSAIL-1b $CaCl_2$ contained in a concentration of

0.08% (optimum) with amylase activity of 0.112U/mL with levels of maltose produced 2.430mg/mL. After passing the optimum concentration decreased amylase activity. This indicates that at a concentration of 0.08% $CaCl_2$ amylase production has reached the maximum (Figure 2).

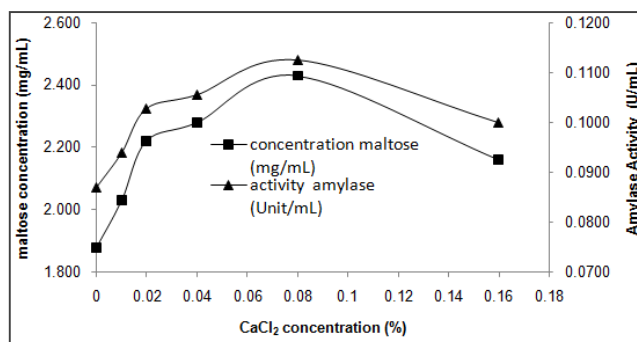


Figure 2. Effect of $CaCl_2$ concentration on the production of extracellular amylase from *Bacillus* sp RSAIL-1b isolates termofil bacteria. On the condition of fermentation: starch concentration of 1.5%; pH 7.0; temperature 55°C; 200rpm for 24 hours.

Figure 2 shows the compound $CaCl_2$ at a concentration of 0.08% amylase activity can increase as much as 29%. The production of amylase to the effect of $CaCl_2$ concentration seen in Figure 2 has in contrast with the amylase produced from *Bacillus* species At 50°C with a concentration of 0.02% $CaCl_2$ [6]. α -amylase requires calcium ion (Ca^{2+}) to increase the activity, maintaining the structure and stability. Ca^{2+} can enhance the activity of α -amylase in *Bacillus* sp. ANT-6 [14].

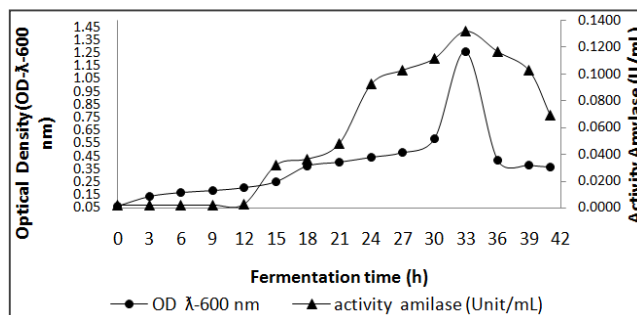


Figure 3. Influence of fermentation time on Optical Density (OD) Isolates termofil bacteria *Bacillus* sp. R-SAIL-1b and amylase enzyme activity in conditions of fermentation: starch concentration of 1.5%; pH 7.0; $CaCl_2$ 0.08% temperature 55°C; aeration speed 200 rpm.

3.1.3. Effect of Fermentation Time on Bacterial Growth (Optical Density) and the Production of the Enzyme Amylase from *Bacillus* sp Isolates RSAIL-1b

The growth of *Bacillus* sp. RSAIL-1b began to occur a phase of adaptation to the hours -0 until the 12th, and growth increased with increasing fermentation time until the 24th hour later rose sharply until the hour-33 in this case is still going on logarithmic phase, the clock phase to 36 deaths occurred. Amylase enzyme began secreted at the 15th hour of fermentation that 0.0319U/mL and increased to 0.0925U/mL at the 24th hour, then becomes 0.1323U/mL at hour-33 with a specific activity of 0.0711U/mg protein and the subsequent

decline in production of amylase (Figure 3). This shows that the optimum conditions of production of amylase from *Bacillus sp.* RSAII-1b is the 33rd hour. Amylase crude extract protein content of 1.8612mg/mL.

3.2. Characterization of Amylase

3.2.1. Effect of pH on Amylase Activity

pH is one of the factors that affect the activity of the enzyme, the results of research the effect of pH on the enzyme activity (Figure 4).

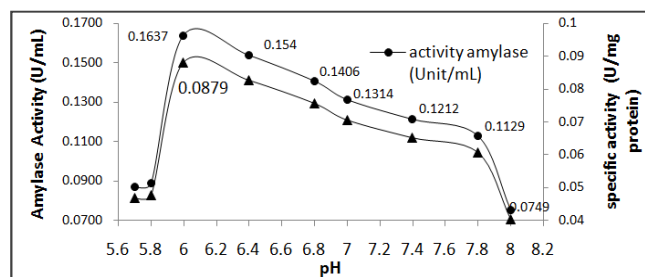


Figure 4. Effect of pH on the activity of *Bacillus sp.* RSAII-1B conditions: substrate 2% and a temperature of 55°C using 0.1M phosphate buffer.

Figure 4 shows that the amylase enzyme activity increased to pH 6.0 with amylase activity of 0.1637U/mL and specific activity of 0.0879U/mg protein, and then begin to decline at a pH of 6.4 with 0.1536U/mL activity and declined sharply at pH 8.0 with the activity of 0.0749U/mL. Based on this phenomenon, indicating that the conditions of pH 6.0 is the pH optimum, pH of the enzyme amylase which is an optimum condition to form a complex enzyme substrate precise and produce the maximum. Change the charge on the molecule of enzyme can affect the activity, either by changes in the structure and the charge on the amino acid residues that bind to the substrate can function. Suppose an enzyme negatively charged to react with the substrate positively charged form the enzyme-substrate complex, then the pH value higher substrate will be ionized and lose the positive, the same thing at low pH enzyme will be protonated and lose negative charge[13]. Changes in H⁺ ions present in the enzyme solution providing conformation effect on the enzyme so does the catalytic section. pH is too low or too high causes a conformational change in the enzyme that causes decreased activity. The optimum pH obtained in this study together with the optimum pH [12] from *Bacillus sp.* were isolated in a hot spring that is pH 6.0(optimum).

3.2.2. Effect of Temperature on Amylase Activity

Temperature is one of the factors that affect the activity of the enzyme. Changes in temperature can lead to protein folding or enzyme so that the enzyme active site is well positioned to catalyze substrate. In Figure 5 amylase activity at various temperatures showed that the amylase activity increases with increasing temperature. Enzyme activity of 0.0870U/mL at 35°C continued to increase to 0.0944U/mL at a temperature of 45°C and subsequently became 0.1471U/mL at 50°C, 0.1647U/mL with a specific activity of 0.089U/mg protein at a temperature of 55°C, 0.1656U/Ml

with a specific activity of 0,089U/mg protein at 60°C, then the activity decreased with increasing temperature becomes 65°C with activity value 0.1073U/mL and decreased continues to be 0.099U/mL at 70°C. Based on these data, it can be said that the maximum amylase activity at 55-60°C in hydrolyzing starch substrate. The optimum temperature obtained in this study is similar to that obtained the optimum temperature [12] from *Bacillus sp.* WA21 in the hot springs, to obtain maximum activity at 55°C.

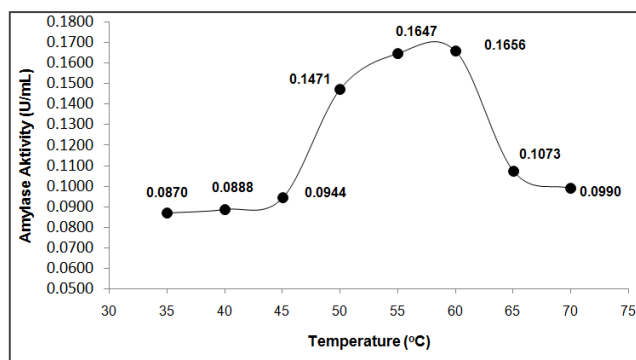


Figure 5. Effect of temperature on the activity of amylase of *Bacillus sp.* RSAII-1b conditions: substrate is 2% and pH 6.0 phosphate buffer.

The temperature is very influential in thermodynamics motion of molecules, as well as protein or enzyme molecules. A low temperature leads to a lack of collisions between molecules of the enzyme to the substrate, whereas at higher temperatures the thermodynamic motion of enzyme molecule large enough so that the collisions between the enzyme and substrate molecules will occur rapidly. At extreme temperatures high protein denaturation resulting in a change in the structure of the enzyme protein so that the enzyme active site changed. Thus, the enzyme becomes inactive because of a change in the active site [15].

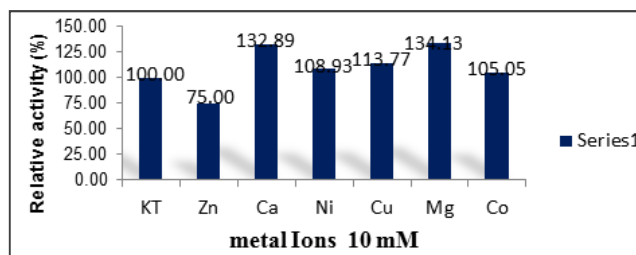


Figure 6. Effect of 10 mM metal ions on the activity of amylase crude extract of isolates of *Bacillus sp.* RSAII-1b at a temperature of 55°C and pH 6.0.

3.2.3. Effect of Metal Ions

Figure 6 shows that the enzyme amylase out the addition of a metal ion which control the activity of relaif 100%. The addition of CaCl₂, MgCl₂, CuCl₂, NiCl₂ and CoCl₂ with a concentration of 10mM may increase the activity of amylase respectively 32.89%, 34.13%, 13.77%, 8.93 and 5.05%, so that is an activator. For ZnCl₂ solution can reduce amylase activity by 25%, so that is the inhibitor, the results of research conducted by [13] obtained that the addition of

CaCl_2 , MgCl_2 , CuCl_2 , NiCl_2 and CoCl_2 with a concentration of 1.0mM and 10mM may increase the activity of amylase, while the ZnCl_2 solution is either a concentration of 1.0mM and 10mM may inhibit the activity of amylase. Similarly, a study conducted by [16] the addition of CaCl_2 solution at amylase enzyme isolated from bacteria termofilik SW2 can increase the activity of the enzyme α -amylase and the addition of ZnCl_2 metals decrease the activity of amylase.

4. Conclusion

Based on the research results, it can be concluded that: Isolates of *Bacillus sp.* RSAII-1b can produce maximum amylase: starch concentration of 1.5%, 0.08% CaCl_2 concentration and fermentation time 33 hours with medium pH 7.0 and incubation temperature 55°C, aeration speed of 200rpm with spesific activity 0.0711U/ mgprotein, crude extract amylase work optimally at pH 6.0; 55-60°C the amylase activity of 0.165U/mL, the specific activity of 0.089U/mg protein. Amylase is an enzyme that depends on the activity of the catalytic metal because metal ions can be activated by Ca^{2+} , Mg^{2+} , Cu^{2+} , Ni^{2+} and Co^{2+} that are activators whereas ions Zn^{2+} decrease the activity of enzymes that are inhibitors. Amylase activity in the crude extract optimum conditions with the addition of 10mM Ca^{2+} ions can increase amylase activity up to 32.89% while ions Zn^{2+} can reduce amylase activity by 25%.

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