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(RESEARCH ARTICLE)

Characteristics of amylase enzyme produced by *Bacillus thuringiensis* on cassava pulp substrate

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Abstract

This study aims to analyze the effect of incubation duration on the activity of crude amylase produced by *Bacillus thuringiensis* using cassava pulp as the substrate. The research employed an exploratory method, observing incubation durations of 12, 18, 24, 30, and 36 hours. The crude amylase activity was characterized, and its protein content was analyzed. The findings indicate that incubation duration significantly influenced crude amylase activity. The highest enzyme activity was observed at 30 hours, reaching 126.67 U/mL. The optimum conditions for crude amylase activity were determined to be 40°C and pH 6, with a protein content of 15.08 mg/mL.

Keywords: Crude amylase; *Bacillus thuringiensis*; Cassava Pulp; Enzyme Activity; Incubation duration

1. Introduction

Enzymes play a crucial role in industrial applications, with amylase, protease, and lipase being the most widely utilized. Amylase, in particular, is essential in the food and biotechnology sectors due to its ability to hydrolyze glycosidic bonds in starch polymers. The demand for amylase continues to rise, comprising approximately 25% of the global enzyme market [1]. Given this growing demand, cost-effective production methods using microorganisms, particularly *Bacillus* species, have gained prominence [2].

The genus of bacteria known to be the largest producer of amylase is *Bacillus*. *Bacillus* species have been widely known to be productive in producing amylase, and hydrolyzing starch into monosaccharides such as glucose and maltose, including *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus cereus* [3], [4], [5]. *Bacillus* is a gram-positive, rod-shaped bacterium, has the ability to produce protein crystals during its sporulation period and is a bacterium capable of producing amylase and cellulase enzymes. The field of biotechnology of *Bacillus* species can be applied widely in various environmental growth conditions [6]. Furthermore, *Bacillus thuringiensis* has shown great potential in producing enzymes including amylase, chitinase and protease [7].

Several studies related to amylase production from *Bacillus thuringiensis*, including the optimum time of *Bacillus thuringiensis* for amylase production is for 24 hours isolated from tapioca liquid waste [4]. Meanwhile [8], reported that *Bacillus thuringiensis* J2, isolated from hot springs in Shimla district, was able to produce amylase with apple pomace substrate with an optimum time of 72 hours.

Bacillus thuringiensis is well known for its ability to produce various enzymes, including amylase. However, optimal production conditions, including substrate composition and incubation time, must be determined to enhance enzyme yield. Amylase production is influenced by the composition of the growth medium, especially the availability of carbon and nitrogen. Among the organic substrates that are widely used and proven to increase amylase production, such as

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starch, malt extract, and various agricultural wastes [4]. Catabolic repression occurs due to the presence of substrates in the growth medium that are easily metabolized, but amylase production is inhibited in favor of the utilization of simple sugars such as glucose. So optimizing the composition of the media is very important in this process [3].The substrate used in this study was cassava pulp. Cassava pulp, a by-product of the starch industry containing 51.8% residual starch [9].

Different incubation times will show the level of enzyme activity, the highest enzyme activity at a certain incubation time, being the optimum incubation time for producing enzymes [6]. The increase in metabolism of a microorganism causes an increase in cell division, this activity results in the enzyme activity produced by the microorganism increasing, and the nutrient content of the substrate decreases. The metabolic activity can be illustrated through the growth curve. In the process of formation, amylase is generally produced from the late adaptation phase and reaches its peak in the late exponential phase [4]. This study investigates the optimal incubation period for amylase production by *Bacillus thuringiensis* on cassava pulp substrate. The best results will be observed optimum conditions of amylase and amylase protein content.

2. Materials and Methods

2.1. Research Design

This study employed an exploratory research design, analyzing amylase production from *Bacillus thuringiensis* using cassava pulp as a substrate. Observations were conducted on enzyme activity, characterization, and protein content across various incubation periods (12, 18, 24, 30, and 36 hours).

2.2. Preparation of Cassava Pulp Substrate

Cassava was washed, peeled, and shredded to obtain pulp, which was repeatedly pressed until the liquid was clear. The solid pulp was then dried at 40°C for 24 hours and sieved to 80 mesh particle size.

2.3. *Bacillus thuringiensis* Culture and Inoculation [10]

The bacterial culture was rejuvenated on nutrient agar and incubated at 37°C for 24 hours. A liquid inoculum was prepared in a nutrient broth medium and incubated for 18 hours at 30°C.

2.4. Amylase Fermentation [4], [11]

Fermentation was carried out in a medium containing cassava pulp and a nutrient solution consisting of essential minerals and yeast extract. The culture was incubated at 27-30°C under different time treatments (12-36 hours).

2.5. Enzyme Extraction and Analysis [12]

Crude amylase was extracted using acetate buffer (pH 4.6) and centrifuged at 4500 rpm for 15 minutes. The enzyme activity was determined using the dinitrosalicylic acid (DNS) method, and protein content was analyzed using the Bradford assay.

3. Results and Discussion

3.1. Crude Amylase Activity

Amylase activity is defined as the rate of product formation at optimum conditions, where one unit of amylase activity is the amount of enzyme that produces one micromole of reducing sugar (glucose) every minute (Lehninger (1993) *cit* [13]. The ability to break down amylum is related to the bacteria used, where the release of amylase by microbes will break down amylum in the medium so that starch becomes a product in the form of reduced sugar molecules that can be used by these microbes for growth. The activity of crude amylase produced in cassava pulp medium by *Bacillus thuringiensis* based on the length of incubation can be seen in Figure 1.

Amylase activity increased during the incubation period. Amylase experienced the highest increase in activity at 30 hours incubation time with an enzyme activity of 126.67 U/ml. The enzyme activity is due to the formation of amylase produced by *Bacillus thuringiensis* bacterial cells.

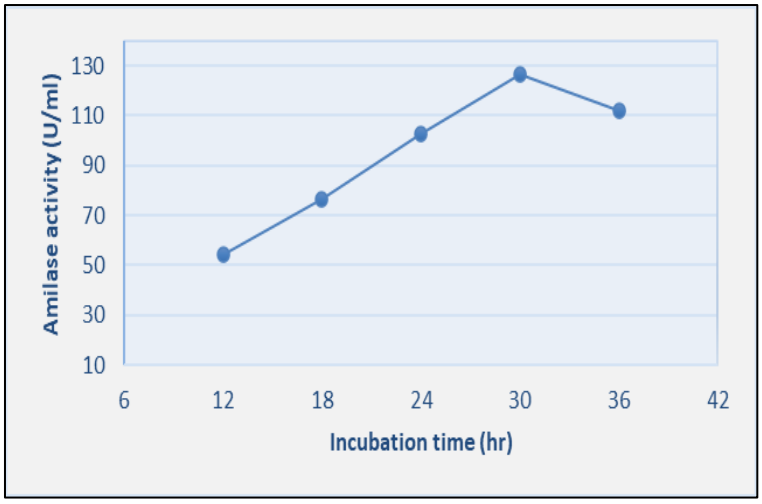

Figure 1 Effect of incubation time on crude amylase activity

Bacillus thuringiensis is known as a bacterium that produces protein crystals (δ-endotoxin) that are insecticidal. The formation of protein crystals by bacterial cells occurs in the stationary phase of the growth curve. It is said that bacteria that produce amylase depend on the type of bacteria, medium composition, cell growth, nutrients needed, temperature, incubation time, pH and are protected from contamination during fermentation [4]. If microbes are transferred into a medium, at first the bacteria will undergo an adaptation phase to adjust to the surrounding environmental conditions [14]. *Bacillus thuringiensis* undergo an adaptation phase starting at the incubation time of 0 hours to 12 hours. In the adaptation phase, *Bacillus thuringiensis* that are newly inoculated into cassava pulp media need time to adjust after being transferred from the old media.

After being able to pass the adaptation phase, the cells enter the second phase of the growth curve, the exponential phase. The initial exponential phase was characterized by amylase activity of 54.44 U/ml at the 12th hour of incubation. Cell division began to increase and was accompanied by the formation of metabolite products, namely amylase by *Bacillus thuringiensis*. According to [15], the increase in enzyme activity occurs due to increased bacterial cell division after being able to adapt to the new environment and adequate nutrition in bacterial growth media.

At the incubation time of 18 hours to 24 hours, the number of *Bacillus thuringiensis* cells experienced a high increase, where the enzyme activity increased by 76.67 U/ml and 102.59 U/ml. Until the incubation time of 30 hours, *Bacillus thuringiensis* entered the final exponential phase characterised by the highest number of cells and metabolite products produced. The highest amylase activity was obtained at 126.67 U/ml. [16] stated that the amylase activity produced generally reaches its highest peak during the late exponential phase, and afterwards the amylase activity will decrease slowly due to the amount of nutrients in the media that begin to decrease so that cells do not get enough nutrients to reproduce themselves and the toxicity of cell metabolites that begin to accumulate.

After cell division and the formation of high metabolite products, the composition in the medium slowly begins to change. This change triggers a constant cell division of *Bacillus thuringiensis*, which indicates that *Bacillus thuringiensis* enter the stationary phase. According to [4], the number of cell populations in the stationary phase is fixed because the number of cells that divide is equal to the number of cells that die. The stationary phase began at 36 hours incubation time with the resulting amylase activity of 111.85 U/ml. After experiencing a longer incubation time, there will be a struggle for space to develop in the medium which triggers cell death and enters the last phase of the growth curve, namely the death phase.

The death phase is the last phase in the growth curve where there is a reduction in the number of cell populations in a medium. In the death phase, *Bacillus thuringiensis* cells consume almost all the nutrients in the medium so that cell division no longer occurs.

Amylase activity produced by *Bacillus thuringiensis* in this study showed the highest activity at the 30th hour incubation time of 126.67 U/ml. In the research of [17], the growth conditions of amylase produced by *Bacillus thuringiensis* with rice flour medium produced amylase activity of 104.79 U/ml. In research by [4] reported the results of isolation of microorganisms from tapioca liquid waste is *Bacillus thuringiensis* to produce amylase obtained total amylase enzyme activity of 5804,94 U.

3.2. Determination of optimum themperature

The optimum themperature of the enzyme has the highest speed to carry out the substrate hydrolysis process [17]. Figure 2 shows that the optimum temperature of amylase produced by *Bacillus thuringiensis* is 40°C, incubation time of 30 hours and enzyme activity of 122.96 U/ml. According to [18] the optimum temperature of the enzyme is the highest activity of the enzyme, where more collisions between the enzyme and the substrate form an enzyme-substrate complex. [8] reported amylase production by *Bacillus thuringiensis* J2 using apple pomace medium with the highest amylase activity at an optimum temperature of 45°C.

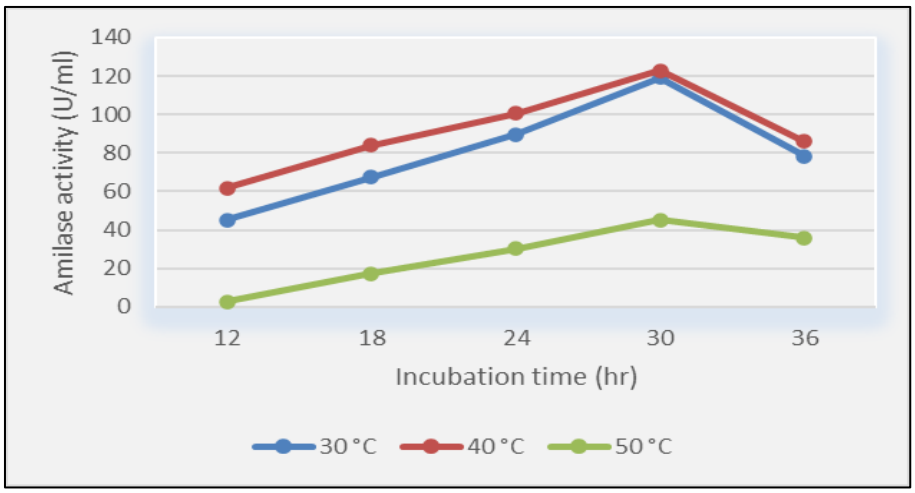

Figure 2 Optimum amylase temperature determination

The highest amylase activity at 30 occurred at an incubation time of 30 hours at 119.26 U/ml. And at 50°C the highest amylase activity was produced at 30 hours incubation time of 45.19 U/ml. Temperature that exceeds the optimum temperature will cause the enzyme protein molecules to denature so that the enzyme structure changes gradually. This makes it difficult for the substrate to bind to the active side of the enzyme so that less substrate enzyme complex (ES) is formed.

The optimum themperature for optimal amylase activity was reported to be at high temperature (50°C for *Bacillus* strains), so temperature plays an important role in this regard [3], [6]. The amylase activity of *Bacillus amyloliquefaciens* with thermostable and industrially beneficial properties is significant at temperatures between 50°C until 60°C [6]. However, on the contrary, excessive temperatures cause a decrease in microbial growth and enzyme production, so it is necessary to optimize fermentation conditions [3], [6].

3.3. Determination of optimum pH

Acidity (pH) is a parameter that affects the formation of products because proteins have groups that can be ionised, so changes in pH will affect the catalytic enzyme. Amylase plays a role in the process of breaking down amylum or starch into simple sugars can be active at an optimum pH interval. Enzymes have maximum activity in a certain pH range called the optimum pH, so the determination of the optimum pH is needed to determine at what pH the amylase enzyme works optimally. For the determination of the optimum pH is done with 3 degrees of acidity, namely pH 6, 7 and 8. Amylase activity in determining the optimum pH can be seen in Figure 3.

The pH of the growth medium is another important factor that affects amylase production. Many studies have shown that the optimal amylase activity of *Bacillus* species is at neutral to slightly alkaline pH levels, usually around pH 6 to 7. For example, amylase production by *Bacillus subtilis* has been reported to be effective at pH 6, [5], [19].

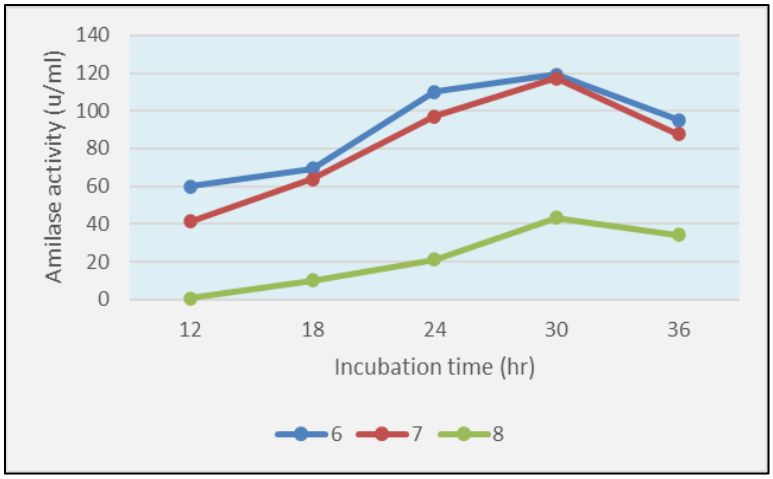

Figure 3 Optimum pH determination of amylase

The optimum pH of amylase produced by *Bacillus thuringiensis* occurred at pH 6 with an enzyme activity of 119.26 U/ml at 30 hours incubation time. According to [20], enzyme activity will be optimum if there is an equilibrium between the two charges and the enzyme will be denatured if it is at a pH far from its optimum pH, due to changes in the structure of the enzyme that will affect enzyme activity. Furthermore, at pH 7, the highest amylase activity was obtained at 30 hours incubation time with an activity of 117.41 U/ml. While at pH 8 the highest amylase activity was at 30 hours incubation time with an activity of 43.33 U/ml. Based on the research of [21], regarding the purification and characterisation of amylase by *Bacillus thuringiensis* optimum at pH 6. The optimum pH degree is the same as the optimum pH value of this study which is optimum at pH 6.

3.4. Amylase protein content

Enzyme is a protein, therefore it is necessary to measure the protein content of the enzyme produced by substituting the absorbance value obtained into the protein standard curve [22]. To calculate the protein content of the enzyme, a standard curve of bovine serum albumin (BSA) was used. From the standard curve, a straight line equation was made to calculate the protein content of amylase.

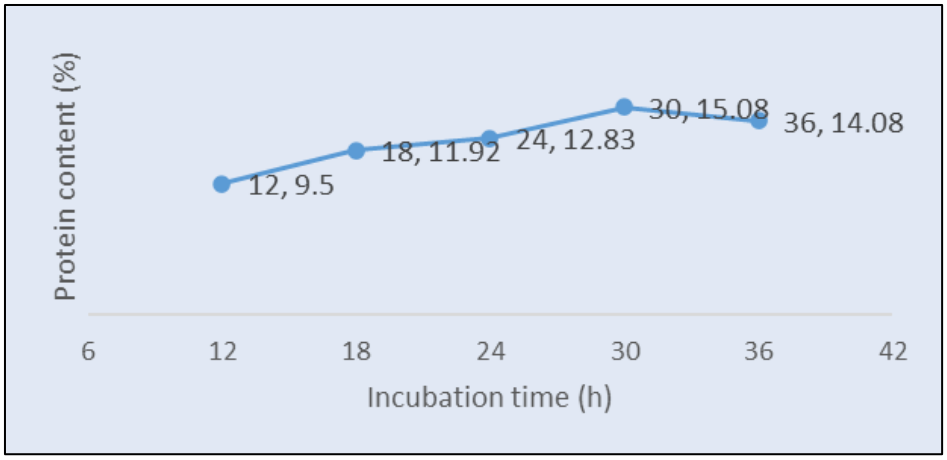

Figure 4 Amylase protein content

The protein content of amylase increased in line with the incubation time, the highest protein content at 30 hours incubation time of 15.08%. This is in line with the highest amylase activity with 30 hours of incubation. The increase in protein content started from the 12th to 24th hour of incubation, when *Bacillus thuringiensis* was in the exponential phase. When *Bacillus thuringiensis* in this phase, the catalytic power of the enzyme becomes high and the amino acids (products) produced increase, where the enzyme itself is composed of amino acids [18]. According to Pierce (2005) *cit* [18], the protein content in the enzyme greatly affects the catalytic power of the enzyme. An increase in protein content in an enzyme, the catalytic power will also increase. At 36 hours of incubation, there was a decrease in protein content in amylase, this occurred because *Bacillus thuringiensis* bacteria were in the stationary phase. According to Kresnamurti

(2001) cit [18], the lower the enzyme activity, the lower the protein content produced, due to the accumulation of sulfur as a by-product of cell metabolism.

4. Conclusion

Crude amylase produced by *Bacillus thuringiensis* in cassava pulp media produced the highest enzyme activity at 30 hours incubation time. Characterization of crude amylase produced by *Bacillus thuringiensis* reached optimum at 40 °C and pH 6. Protein content of amylase, showed the highest protein content at 30 hours incubation time.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Author Contributions

All authors contributed to the production of this article. The first author contributed to the idea, method, data processing and discussion. The second author contributed to the method, data processing and discussion. The third author contributed to data collection and discussion

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