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Optimization of Cellulase Production by *Aspergillus niger* InaCC F506 in Solid-State Fermentation of Tofu Dreg

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Abstract

Indonesia has a growing demand for cellulase enzymes; however, 99% of the enzymes are imported from other countries. *Aspergillus niger* is well recognized for using the widely accessible tofu by-product, often known as tofu dreg, as a growth medium for synthesizing cellulase enzymes. This study aims to optimize the production of cellulase enzymes by *Aspergillus niger* InaCC F506 using tofu dregs as a substrate through the Solid-State Fermentation (SSF) method by varying the additives. The results showed that the E fermentation system with the composition of urea 0.5%; CMC 0.5%; KH_2PO_4 0.2%; $MgSO_4.7H_2O$ 0.2% produced the highest cellulase enzymes from the tofu dregs substrate. The highest cellulase enzyme activity was at a fraction of ammonium sulfate saturation level of 40-60%. The optimum condition of enzyme activity was observed at pH 5 with an activity of 33 x 10^{-4} Units/mg protein and at 30°C with an activity of 31 x 10^{-4} Units/mg protein.

1. Introduction

According to information from the Ministry of Research, Technology, and Higher Education, about 99% of enzyme demands in Indonesia are still imported from countries like China, Japan, and some regions of Europe. Indonesia has a greater need for enzymes every year, and it is predicted that the global market demand for enzymes will increase by around 7% annually (2015-2020). Enzymes are becoming a growing biotechnology industry because of their energy efficiency, environmental friendliness, substrate specificity, and tendency to function at moderate temperatures and pH [1]. Enzyme production and trade are dominated by hydrolytic enzyme groups such as amylase, cellulase, protease, and lipase [2]. This research is used as a preliminary study for future enzyme production and encourages Indonesia's independence in producing enzymes at low production costs by utilizing by-products.

The cellulase enzyme is an enzyme that breaks the β-1,4 glycosidic bonds in cellulose, cellobiose, and other cellulose derivatives [3]. Cellulase is an enzyme widely used in several industries, such as the chemical industry, textiles, detergents, coal, organic fertilizers, and coffee processing [4]. Cellulase enzymes are also used as a substitute for chemicals in producing alcohol from materials containing cellulose [5]. With the many benefits and the high need for cellulase enzymes, it is necessary to produce them.

The development of enzyme utilization technology faces some difficulties, including the high cost of production on an industrial scale and the lack of public knowledge regarding the utilization of tofu dregs, which currently have primarily been used as animal feed. Many tofu-producing enterprises in Indonesia lack waste management knowledge, resulting in an abundance of improperly managed tofu dregs by-products. According to Tarmidi (2010), tofu waste contains 13.3% dry matter, 21% crude protein, 23.58% crude fiber, 10.49% crude fat, 51.93% NDF, 25.63% ADF, 2.96% ash, 0.53% calcium (Ca), 0.24% phosphorus (P) and 4,730 kcal/kg gross energy [6] can be considered as a substrate for enzyme production. According to the research of Li *et al.* [7], the content of crude fiber in tofu dregs is in the form of cellulose, hemicellulose, lignin, galactan, arabinan, arabinogalactan, rhamnogalacturonan, xylan, and xyloglucans. The study results increase the potential of tofu waste as a substrate for manufacturing cellulase

enzymes due to the high cellulose content. A study by Mizumoto *et al.* [8] also showed that tofu dregs are a good substrate for producing bioactive substances.

Cellulase enzyme production requires microorganisms, one of which is *Aspergillus niger*. *Aspergillus niger* is a cellulase enzyme-producing microorganism in the form of a filamentous fungus that is widely used in fermentation processes both on an industrial scale and on a laboratory scale [2]. This fungus is generally used to produce commercial enzymes, food ingredients, organic acids, and pharmaceutical products [9]. *Aspergillus niger* can produce hydrolytic enzymes, which can cause mold to grow on foods containing starch, cellulose, protein, and lipids [10]. *Aspergillus niger* is a harmless fungus that can easily adapt to various substrates and does not create mycotoxins [11].

Shu-bin *et al.* [12] researched the production of cellulase enzymes by *Bacillus* PA5 using tofu dregs as a substrate and additives $MgSO_4.7H_2O$, KH_2PO_4, $FeSO_4.7H_2O$, CMC to obtain an optimum temperature of 30°C. Aryani [13] reported that isolated and characterized cellulase enzymes by *Mucor* sp mold with rice straw and corn cob substrates using the SSF method and additives $MgSO_4.7H_2O$, KH_2PO_4, $FeSO_4.7H_2O$, $CaCl_2.2H_2O$ obtained an optimum pH of 5. Idiawati *et al.* [14] produced cellulase enzymes by *Aspergillus niger* with sago pulp substrate using the SSF method and the addition of $MgSO_4.7H_2O$, KH_2PO_4, $CaCl_2.2H_2O$, urea as an additive whose optimum results were achieved at 30°C and pH 5.

This research was conducted to optimize the production of cellulase enzymes from *Aspergillus niger* InaCC F506 with tofu dregs as a substrate using the Solid State Fermentation method with various additives content.

2. Methods

2.1. Materials

Tofu dregs were obtained from the local tofu industry, potato dextrose broth (PDB), nutrient agar, distilled water, urea, KH_2PO_4 (Merck), $MgSO_4.7H_2O$ (Merck), CMC (Carboxymethylcellulose) (Merck), DNS (3,5 dinitro salicylic acid) (Sigma Aldrich), NaOH (Merck), potassium sodium tartrate tetrahydrate (Merck), NaH_2PO_4 (Merck), $Na_2HPO_4.2H_2O$ (Merck), CH_3COONa (Merck), Whatman filter paper no. 1, Na_2CO_3 (Merck), $CuSO_4.5H_2O$ (Merck), Glucose (Merck), Folin–Ciocalteu reagent, bovine serum albumin (BSA), Ammonium sulfate (Merck), cellophane membrane (Serva MWCO), $BaCl_2$ (Merck).

2.2. Refreshment of *Aspergillus niger* InaCC F506

Pure *Aspergillus niger* isolates were refreshed in slanted agar media with a composition of 0.5 g PDB and 1 g agar using a wire loop which had been inoculated on the media and then incubated for five days at room temperature.

2.3. Production of *Aspergillus niger* Starter InaCC F506

GDP growth medium was prepared by mixing 0.5 g of PDB powder and 1 g of agar and adding 50 mL of distilled water. The refreshed *Aspergillus niger* was inoculated using wire loops in 50 mL of PDB growth medium in Erlenmeyer and then incubated for ± 5-7 days in the incubator (IN-30 Memmert). As a result of incubation, the number of spores was calculated using the counting plate method. The number of spores was counted using a counting cup, namely the dilution in each tube. Tube (A) was prepared by diluting 1 mL of starter to a concentration of 10^{-1} using 9 mL of distilled water. Tube (B) containing 10^{-2} concentration was filled with 1 mL of Tube (A) solution and diluted with 9 mL of distilled water. A total of 1 mL of Tube (B) solution was added to Tube (C) with a concentration of 10^{-3} and diluted with 9 mL of distilled water. A total of 0.1 mL of solution from each tube was added to each petri dish containing nutrient agar. The solution was leveled by gently shaking the petri dish and then allowed to stand until the nutrient became solid. After the agar solidified, the samples were incubated in an incubator at 30°C, and the number of spores that grew was counted after 24 hours.

2.4. Sample Preparation

The tofu dregs were dried for three days at around 45°C and then ground after drying. A total of 10 g of dried tofu dregs was put into an Erlenmeyer for further sterilization using a pressure cooker for approximately 45 minutes. Tofu dregs function as a substrate in the fermentation process.

2.5. Production of Cellulase Enzyme

Initially, the additive solutions were prepared by adding 10 g of additive each to 100 mL of distilled water. Various solutions were prepared with different additive compositions, which can be seen in Table 1. The additive compositions followed the method of Shu-bin *et al.* [12] with modifications.

Table 1. Additive compositions

Fermentation system	Urea (%)	CMC (%)	KH_2PO_4 (%)	$MgSO_4.7H_2O$ (%)
A	0	0	0	0
B	0	0	0.1	0.1
C	0	0.5	0.1	0.1
D	0.5	0	0.1	0.1
E	0.5	0.5	0.2	0.2

Cellulase enzyme production was started by adding an additive solution with a total volume of 25 mL and 5 mL of *Aspergillus niger* starter to tofu dregs in an Erlenmeyer, then stirring and incubating at room temperature. All samples were fermented for approximately three days which was the log phase or the maximum growth indicated by the appearance of white hyphae.

2.6. Extraction of Cellulase Enzyme

Each of the five fermentation products was added 100 mL of phosphate buffer (0.1 M, pH 6), shaken at 125 rpm for 2 hours in an incubating shaker (TS-330A), and filtered with Whatman paper no 1. The filtrate obtained was centrifuged at 6000 rpm for 30 minutes. The filtrate obtained is a crude extract of enzymes.

2.7. Determination of Cellulase Enzyme Activity

The cellulase enzyme solution from each fermented product (1 mL) was mixed with 1 mL of 1% CMC solution (in 0.1 M phosphate buffer, pH 6) and incubated for 30 minutes at room temperature. The incubation results were heated for 30 minutes and then allowed to stand for 5 minutes at room temperature. At the end of the incubation, 1 mL of DNS reagent was added to 1 mL of the cellulase mixture and incubated for 10 minutes, and 4 mL of distilled water was added. The DNS reagent was prepared by initially dissolving 1.25 g of DNS into 25 mL of 2 N NaOH under continuous stirring. Subsequently, 37.5 g of sodium potassium tartrate was dissolved with 62.5 mL of distilled water, and the remaining volume was added to distilled water until 125 mL of total volume. The absorbance was measured at 550 nm using a UV-Vis spectrophotometer (T60U Spectrometer PG Instruments Ltd.).

In order to determine the optimum conditions for cellulase enzyme activity, the cellulase enzyme solution from each fermented product that had been added with 1% CMC solution was given a variety of pH buffer treatments (4, 4.5, 5, 5.5, 6, 7, and 8) and incubation temperature (20, 25, 30, 35, and 40°C) for 30 minutes. The procedure for determining these optimum conditions was the same as for determining cellulase enzyme activity from each fermentation. The specific activity of the enzyme was calculated with the protein content at the highest ammonium sulfate saturation level.

2.8. Determination of Protein Content by Lowry Method

A total of 1 mL of each fermented cellulase enzyme solution was added to 8 mL of lowry C and incubated for 10 minutes at room temperature. The incubation solution was added with 1 mL of lowry D and incubated for 30 minutes at room temperature. The absorption of the solution was measured at a maximum wavelength of 725 nm.

2.9. Partial Pure Cellulase Activity

2.9.1. Ammonium Sulfate Fractionation from the Optimum Fermentation

Cellulase enzymes were initially produced and extracted using the optimum composition of the additives achieved in this study. The resulting crude enzyme extract was used for enzyme fractionation by ammonium sulfate.

The fractionation was initiated by determining the amount of ammonium sulfate salt that must be added to each saturation level by taking into account the initial volume of the crude extract of the enzyme obtained. The added ammonium sulfate, according to the ammonium sulfate saturation table, was grouped into 0-20% (F1), 20-40% (F2), 40-60% (F3), 60-80% (F4) and 80-100% (F5). The crude enzyme extract produced from the best additive composition was gradually added with ammonium sulfate salt under stirring using a magnetic stirrer and left for 6 hours. After standing, the mixture was centrifuged for 30 minutes at 6000 rpm [15]. The resulting precipitate was separated and suspended with phosphate buffer pH 6. The filtrate obtained was continued with the same treatment for the following fraction.

2.9.2. Dialysis of Cellulase Enzyme

The dialysis process began with preparing a cellophane membrane to be boiled with 0.001 M phosphate buffer pH 6 for 30 minutes and then washed with distilled water. The cellophane membrane was filled with enzyme solutions fractionated at various saturation levels, then tied at both ends. The cellophane membrane containing the enzyme was immersed in phosphate buffer (0.001 M, pH 6) and stirred with a magnetic stirrer for 2 hours. Phosphate buffer (0.001 M, pH 6) was replaced every 2 hours and tested for ammonium sulfate content by adding $BaCl_2$. The dialysis process was terminated when the white precipitate ceased to form.

3. Results and Discussion

3.1. Fermentation System Screening

Fermentation system screening is used to obtain the best fermentation system that produces the highest specific activity of the cellulase enzyme in the presence of various additive ingredients. Fermentation media is essential because all metabolic processes depend on the available medium. Fermentation medium provides all the nutrients microorganisms need to obtain energy, cell formation, and biosynthesis of metabolic products [16]. Additives are classified into macro-enrichments, including nitrogen, carbon, and micro-enrichers (magnesium, potassium, and vitamins) [17].

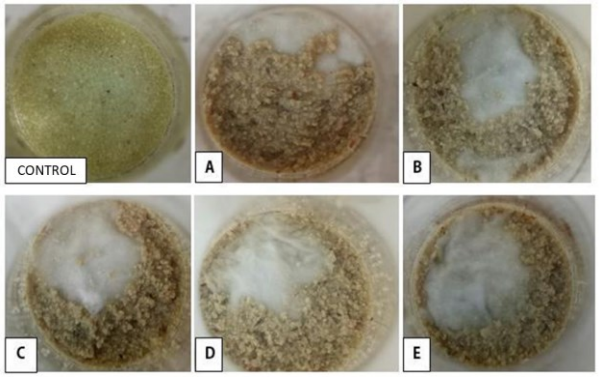

Figure 1. Fermented tofu dregs with various system fermentations on the third day

This study varied the additives (urea, CMC, KH_2PO_4, and $MgSO_4.7H_2O$) with the composition shown in Table 1 by following the method of Shu-bin *et al.* [12] with modifications. CMC is a specific carbon source that acts

as an inducer and can induce the production of cellulase enzymes in *Aspergillus niger*. Urea is a source of inorganic nitrogen used as a carbon companion energy source [18]. KH_2PO_4 and $MgSO_4.7H_2O$ additives are mineral sources. The results of tofu dregs fermentation with variations in additive levels on the third day are shown in Figure 1. The description of hyphal growth from tofu dregs fermentation with variations in additive levels on the third day is in Table 2.

Table 2. The description of hyphal growth of fermented tofu dregs with variations in additive levels on the third day

Fermentation system	Description of hyphae
Control	not growing
A	white hyphae grow on the edges. Hyphae grow the least
B	white hyphae grow unevenly on the surface of the substrate
C	white hyphae grow almost evenly and the same as fermentation D
D	white hyphae grow almost evenly and the same as fermentation C
E	white hyphae grow almost evenly on the surface of the substrate and become the most hyphae that grow on the surface of the substrate

Based on Table 2, the results of the physical appearance of hyphae from fermented tofu dregs with the system (E) showed the highest specific enzyme activity as indicated by the number of hyphae growing on the surface of the substrate. The composition of the additive contents in the fermentation (E), which tends to be higher, is a possible cause of *Aspergillus niger* can grow well because the source of nutrients is sufficient. Determination of the most optimum fermentation system is not only seen from the appearance of hyphal growth. However, it is determined by testing the specific activity of enzymes using the DNS and the Lowry method. The results of the specific activity of cellulase enzymes from each fermentation system with variations in additive levels are shown in Figure 2.

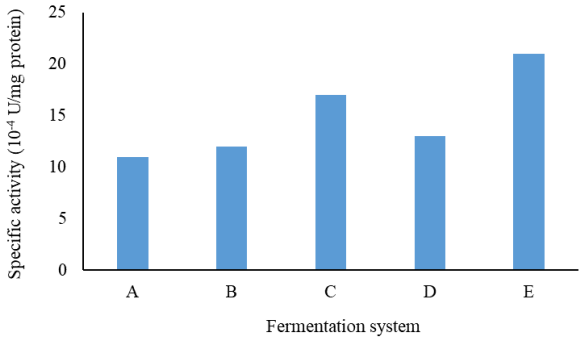

Figure 2. The specific activity of cellulase enzymes from each fermentation

Based on Figure 2, the fermentation system (E) obtained the highest specific enzyme activity, while the fermentation system (A) had the lowest specific enzyme activity. This is consistent with the appearance of hyphae growth with the fermentation system (E), which grows almost evenly on the substrate surface, while the fermentation system (A) makes the hyphae grow the least. The fermentation system produces each specific activity of the cellulase enzymes in the order from highest to lowest as follows: E, C, D, B, and A. The fermentation systems (E) and (C) have relatively high specific activities made possible due to the addition of CMC levels which functions as an inducer of *Aspergillus niger* to produce cellulase enzymes. CMC is the primary nutrient source, effectively broken down by *Aspergillus niger*. This shows that the addition of CMC has a significant impact on enzyme activity.

Fermentation system (E) has a higher composition of mineral salts KH_2PO_4 and $MgSO_4.7H_2O$ compared to other fermentations, resulting in hyphae growing more evenly and having the highest specific activity of enzymes. This is due to the presence of micronutrients that act as cofactors. Cofactors are metal ions that can be bound and easily released from enzymes, for example, Mg^{2+} and K^+ ions [19]. Mg^{2+} ions can bind to the substrate so that the substrate can attach to the enzyme to form an active structure [20]. K^+ ions can optimize the catalytic function in microorganism cells. According to research by Yin *et al.* [21], K^+ ions added to fermentation media can increase cellulase enzyme activity.

In the fermentation system (D), the addition of urea as a nitrogen source did not significantly increase the specific activity of the enzyme, unlike the addition of CMC in fermentation C and E. The addition of urea did not have a significant effect because the composition of tofu dregs contained sufficient protein for the growth of *Aspergillus niger*.

Urea and CMC were not added to the fermentation system (B), resulting in lower specific enzyme activity compared to (E), (C), and (D) because the need for nitrogen and carbon as the primary sources for the growth of microorganisms was not fulfilled. The addition of mineral sources to the fermentation system (B) resulted in a higher specific activity of the enzyme than (A). However, the increase in the specific activity of the enzyme was not significantly different. The fermentation system (A) produced the lowest specific activity of the enzyme because there was no additional additive which caused *Aspergillus niger* to lack carbon, nitrogen, and mineral sources. The fermentation system (E) is the most optimum system to be used to produce cellulase enzymes.

3.2. Partial Pure Cellulase Enzyme Activity

Partial purification of the cellulase enzyme was carried out by a precipitation process using the ammonium sulfate fractionation method and the dialysis process. Partial purification aims to obtain the pure form of enzymes from crude extracts. The stages of enzyme production use a fermentation system (E) because it has the highest specific activity of enzymes.

The results obtained in the partial purification process are then used to determine the specific activity of enzymes, including enzyme activity tests and protein content tests of various saturation levels. Table 3 shows the test results of enzyme activity, the protein content of

enzymes, levels of specific enzyme activity, and purity levels from various saturation levels.

Table 3 shows that the 40-60% saturation level fraction has the highest specific activity of cellulase enzyme. Processing the unit data of enzyme activity and protein content can determine the specific activity of the cellulase enzyme, which indicates the enzyme's purity level. The specific activity of cellulase enzymes is the number of units of enzyme activity per mg of protein (Units/mg protein) [22]. The results of the specific activity of the enzymes in each fraction of the saturation level are shown in Figure 3.

Table 3. Specific activity and purity level of cellulase enzymes

Saturation fraction (%)	Activity Units (Units/mL)	Protein Content (mg/mL)	Specific Activity (Unit/mg protein)	Purity Level
EK	0.420	436.7	0.00096	1
0-20	0.580	399.45	0.0014	1.4583
20-40	0.627	342.18	0.0018	1.8751
40-60	0.702	262.18	0.0026	2.7083
60-80	0.682	294.90	0.0023	2.3958
80-100	0.653	391.45	0.0016	1.6666

The specific activity of the enzyme is highest at the ammonium sulfate saturation level of 40-60% (Figure 3). The saturation level fraction with the highest specific activity of the enzyme was the fraction with the highest purity of cellulase enzymes compared to other fractions. The specific activity of the cellulase enzyme at each saturation level of ammonium sulfate salt is different because it depends on the unit of activity and protein content. The highest specific activity of the enzyme in the saturation level fraction of 40-60% indicates that the presence of cellulase enzymes is the most abundant compared to the other saturation level fractions. The saturation level fraction with many enzyme activity units does not necessarily have a high specific enzyme activity because of the protein contained therein.

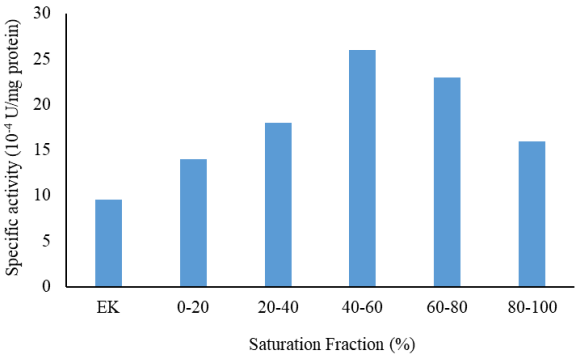

Figure 3. The specific activity of cellulase enzyme for each saturation level fraction

The results obtained in this study include the stages of partial purification using ammonium sulfate salt and dialysis. Fractionation is the initial stage of purification with precipitating proteins by adding electrolyte compounds such as ammonium sulfate salts. Fractionation in this study aims to separate the cellulase enzyme protein from other protein molecules. The addition of ammonium sulfate salt is used to reduce the solubility of the enzyme protein. Based on the principle of like dissolves like water, which is polar, it tends to interact with ammonium sulfate molecules compared to proteins because ammonium sulfate is more polar than protein [22]. For compounds with high ionic strength, the solubility of proteins will decrease to the point where the proteins will precipitate completely from the solution, and this effect is called "salting out". The use of ammonium sulfate salt because it has greater ionic strength than other monovalent salts so that the solubility in water becomes higher and does not affect protein structure [23]. In this research, multilevel fractionation was carried out based on the amount of different salt added to produce precipitated protein fractions, namely, 0-20%, 20-40%, 40-60%, 60-80%, and 80-100% saturations. The protein fraction precipitate was then suspended with 0.1 M phosphate buffer pH 6.

Dialysis is an advanced purification process for enzyme proteins, where cellulase enzyme proteins are released from ammonium sulfate. The principle of dialysis is a process of diffusion where a flow of high to low concentrations passes through a semipermeable membrane to separate large and small molecules. The buffer concentration outside the membrane is low (0.001 M phosphate buffer pH 6), while inside the membrane is higher (0.1 M phosphate buffer pH 6), so ammonium sulfate can diffuse out of the membrane and separate from the cellulase enzyme. The dialysis process causes small molecules, such as ammonium sulfate salt ions, to pass through the membrane. In contrast, large molecules, such as enzyme proteins, will be retained in the cellophane membrane [24]. The addition of $BaCl_2$ is used to identify the release of ammonium sulfate, characterized by the occurrence of a white precipitate which is $BaSO_4$. The dialysis process was stopped when no white precipitate was formed. The enzyme protein produced from the dialysis process is a pure partial enzyme protein. The cellulase enzyme produced from the dialysis process is an ammonium sulfate-free enzyme.

The results obtained from partial purification were then tested for enzyme activity and protein content. In this study, cellulase enzyme activity was measured using the DNS method. One unit of cellulase enzyme activity is the ability of the cellulase enzyme to produce one mmol/mL of glucose product per unit of time. The cellulase enzyme activity was measured by the amount of glucose formed from the substrate through the process of hydrolysis of cellulose by the cellulase enzyme, which turns a yellow color to produce a yellowish-orange compound when it reacts with DNS. The absorbance of the yellowish-orange solution was measured using a UV-Vis spectrophotometer at a maximum wavelength of 550 nm. The obtained absorbance values were then plotted with the standard glucose curve to determine the unit size of the enzyme activity of each fraction. The principle of testing using

the dinitro salicylic acid (DNS) method is that the aldehyde group in a monosaccharide is oxidized to a carboxyl group. In contrast, the aldehyde group in sugar will reduce 3,5-dinitro salicylic acid to become 3-amino-5-nitrosalicylic acid [25].

The principle of the Lowry method is that protein will react with Lowry reagent in the form of copper under alkaline conditions to produce Cu^+ tetradentate complexes and react with Folin–Ciocâlteu reagent to produce blue tungsten molybdenum. The absorbance of the blue solution was measured using a UV-Vis spectrophotometer at a maximum wavelength of 725 nm [26]. The absorbance values obtained were then plotted with the standard BSA curve to determine the protein content of each fraction. The use of BSA as a standard curve because BSA contains a relatively complete range of about 20 amino acids. By knowing the overall protein content, the amount of protein that functions as an enzyme is known through its ability to convert substrates into the desired product.

3.3. Characterization of Cellulase Enzyme

3.3.1. Optimum pH

Each enzyme will have the highest specific activity at its optimum pH conditions. Determining the optimum pH will affect changes in the enzyme's tertiary structure and changes in charge of the amino acid residues that bind the substrate. The optimum cellulase enzyme pH was determined by reacting enzymes with substrates at various pHs (4, 4.5, 5, 5.5, 6, 7, and 8). The results of determining the optimum pH of the cellulase enzyme are shown in Figure 4.

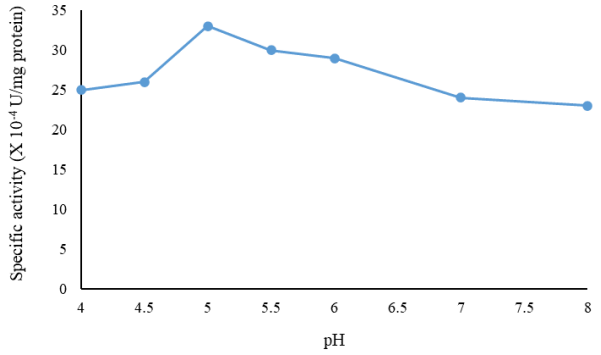

Figure 4. Graph of specific activity of cellulase enzyme with various pHs

Based on Figure 4, the optimum pH of the cellulase enzyme is pH 5. The cellulase enzyme has an optimum pH of 5 because it produces the highest concentration of glucose products at this pH condition. The optimum pH conditions will form the right H^+ and OH^- for enzymatic reactions to form products [27]. The optimum pH condition depends on the conformational structure of the amino acids found on the active site of cellulase in the form of Asp 255 and Glu 87 [28]. Aspartate 255 acts as a nucleophile, while Glu 87 acts as an electrophile. The enzyme protein will conformationally alter in an unsuitable position if the pH is above or below the ideal pH, which may cause impaired or poor enzyme performance. Positive and negative ions from added acids and bases react with enzymes in alternating electric charge processes [29]. The presence of excess H^+ and OH^- affects the overall conformational change, specifically in the R groups of amino acids [30].

Asp 255 and Glu 87 functioned as protonated nucleophiles due to an excess of H^+ at the optimum pH conditions. The ends of the protein structure in acidic pH conditions will be overloaded with proton H^+ pulled by NH_2, and protonation of amine NH_3^+ is formed. In contrast, in the carboxylic group COOH, H^+ ions will be challenging to escape. The large number of H^+ in the enzymatic environment will facilitate its interaction with the R group of amino acids, thereby disrupting enzymatic reactions This causes changes in the conformation of the enzyme protein to be in the wrong position, resulting in a decrease in specific activity of cellulase enzyme.

Asp 255 and Glu 87 act as electrophiles and undergo deprotonation at above the optimum pH conditions where there is an excess of OH. The ends of the protein structure at pH conditions in a basic charge will be overcharged with OH^-, which will attract H^+ ions in the COOH group, causing deprotonation. A large number of OH^- in the enzymatic environment will facilitate its interaction with the R group of amino acids, thereby disrupting enzymatic reactions [30]. This causes changes in the conformation of the enzyme protein to be in an inappropriate position so that the specific activity of the enzyme decreases.

This study obtained an optimum pH of 5 with specific enzyme activity of 33 x 10^{-4} Units/mg protein. Aryani [13] researched the cellulase enzyme's optimum pH on the isolation and characterization of the cellulase enzyme by the mold *Mucor* sp from straw and corncob substrates using the SSF method to obtain an optimum pH of 5. Another study was conducted by Dini and Munifah [31] regarding the production and characterization of enzymes cellulase by bacteria isolated from seaweed obtained optimum pH 5. Research conducted by Idiawati *et al.* [14] on cellulase enzyme production by *Aspergillus niger* from sago pulp substrate using the SSF method obtained an optimum pH of 5.

3.3.2. Optimum Incubation Temperature

Enzyme work systems are influenced by environmental conditions such as temperature, which can increase or decrease the interaction between the substrate and the enzyme. The optimum cellulase enzyme temperature was determined with variations in temperature (20, 25, 30, 35, 40°C). The results of determining the optimum temperature are shown in Figure 5.

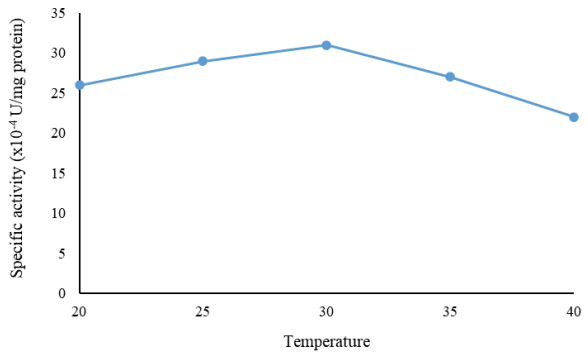

Figure 5. Graph of specific activity of cellulase enzyme with various temperatures

The highest specific activity of the cellulase enzyme was obtained by incubation at 30°C (Figure 5) because it produces the highest concentration of glucose products at this temperature. The conformation of the enzyme protein is in the proper position under the optimum temperature, allowing the enzyme and substrate to react and form a more stable enzyme-substrate complex. This increased the specific activity of the enzyme.

Enzyme kinetic energy tends to be low when the temperature is below the optimum temperature, causing only a small amount of substrate to bind to the enzyme's active site. The low kinetic energy is caused by the slow collision between the enzyme and the substrate, resulting in a decrease in the specific activity of the enzyme.

An increase in temperature will generally increase the speed of the enzyme reaction. However, an increase above the optimum temperature will cause stretching of the enzyme protein caused by the breaking of non-covalent interactions that change the conformation of the enzyme protein. The weakest non-covalent bonds, such as van der Waals bonds, will break first, followed by several other bonds so that less enzyme binds to the substrate [32], resulting in a decrease in the specific activity of the enzyme.

This study obtained an optimum temperature of 30°C with a specific enzyme activity of 31 x 10^{-4} Units/mg protein. Research on the optimum temperature of the cellulase enzyme was previously reported by Shu-bin *et al.* [12] from the tofu dregs substrate by *Bacillus* PA5; the optimum temperature was 30°C. Another study was conducted by Idiawati *et al.* [14], the production of cellulase enzymes by *Aspergillus niger* from sago pulp substrate using the SSF method obtained an optimum temperature of 30°C.

4. Conclusion

The highest cellulase enzyme produced by *Aspergillus niger* using tofu dregs as a substrate was obtained from fermentation system (E) with an additive composition of 0.5% urea: 0.5% CMC: 0.2% KH_2PO_4: 0.2% $MgSO_4.7H_2O$. The highest specific activity of the cellulase enzyme was obtained at the saturation level of the ammonium sulfate salt of 40-60%. The specific activity of cellulase enzymes has an optimum pH of 5 and an optimum temperature of 300C. The highest specific activity of the cellulase enzyme was achieved at pH 5 and an incubation temperature of 30°C.

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