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Effect of pH and incubation time against endoglucanase activity from Trichoderma reesei and Aspergillus niger with rice straw as substrat

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ABSTRACT

Endoglucanase is one important part of the enzyme cellulase, which works to solve bonding polymer β-1,4 which is a part of the amorphous cellulose, and produce cello-oligosaccharides. Analysing Carboxy Methyl Cellulase enzyme activity can reflect the Endoglucanase activity contained in cellulase enzyme. This study aimed to determine the effect of pH and incubation time on the activity of the enzyme cellulase Endoglucanase from Trichoderma reesei and aspergillus niger. The research divided into two phases, each phases was done with 2 variables namely pH and incubation time. pH 4, 5 and 6 for the 1st phase and pH X, pH X.2, pH X.4, pH X.5, pH X.6, and pH X.8 for the 2nd phase. The value of X in the second phase of treatment is the pH value of the first phase which produced the highest Endoglucanase activity. Observation was done for 10 days and were made every 24 hours. Each treatment performed with 3 replicates and treatments carried out with 2 different types of fungus.

The pH level and incubation time affects the activity of endo-β1,4-glucanase on the enzyme cellulase. The optimum pH condition of cellulose enzyme production with a high endo-β1,4-glucanase produced by Trichoderma reesei is at range of pH 4.5 to pH 6 with an optimum incubation time of 3 to 10 days. While the optimum pH condition of celluloase enzyme production with high endo-β1,4-glucanase produced by Aspergillus niger is at range of pH 5 to pH 5.6 with an optimum incubation time of 8 to 10 days.

KEYWORDS

Endoglucanase;
Trichoderma reesei;
Aspergillus niger;
pH;
Incubation time.

INTRODUCTION

Multi-enzyme cellulase is formed by several proteins. Converting cellulose to glucose in the enzymatic hydrolysis for ethanol production process[14]. Cellulase enzyme itself has a very important role in the hydrolysis of cellulose to produce glucose, which is sold in the market and are needed for various purposes both for the manufacture of chemicals which other higher economic value such as ethanol, acetone and organic acids, as well as used as a carbon source for the production of microbial utilization of enzymes and antibiotics[12,13,24].

The main obstacle in the development of industrial-scale use of enzymes is the high cost of production. To that end, the use of rice straw as a substrate fermenta-

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tion media which contains cellulose for growth of microorganisms has a bright prospect in the future, as it provides a lower cost alternative when compared to the manufacture of enzymes using synthetic chemicals as growth media microorganisme. Production of cellulase enzymes by using rice straw substrates containing cellulose will also produce other products that are useful to humans such as glucose, ethanol, single cell protein, and others[8].

Enzymes sellulase can convert cellulose become glucose, three components who has been identified in the cellulase are Endoglucanase (endo-β-1,4-D-glucans-4-glucanohidrolase, EC3.2.1.4) which breaking bonds of β-1,4 on cellulose chain at random, Exo-glucanase (β-1,4-D-glucans-selobiohidrolase, EC 3.2.1.91) which solves unit of cellobiose from the tip of the chain and β-glucosidase (EC 3.2.1.21) which solves cellobiose to become glucose[9]. According to Miyamoto[18], Endoglucanase attack the amorphous cellulose or cellulose hollow fibers that become cello-oligosaccharides, where the cello-oligosaccharides are short-chain cellulose. Analysing enzyme activity by Carboxy Methyl Cellulase (CMC-ase) may reflect Endoglucanase enzyme activity that attack cellulose.

Endoglucanase enzyme is one part of the enzyme cellulase, fungus used in the production of the cellulase enzyme to hydrolyze lignocellulosic materials are fungus Trichoderma reesei and aspergillus niger. Trichoderma Reesei has been known to produce Endoglucanase and Exo-glucanase to 80%[19], but lower β-glucosidase[20]. This problem can be overcome by adding β-glucosidase from the outside[23] or producing cellulase enzyme by combining a strong ability of microorganisms to produce Endoglucanase and Exo-glucanase as Trichoderma reesei with a strong ability of microorganisms to produce β-glucosidase strong such as Aspergillus niger[23]. Aspergillus niger can produce a high β-glucosidase, but produce a lower endo-β-1.4-glucanase and exo-β-1.4-glucanase.

To produce cellulase enzymes with high Endoglucanase activity with a shorter fermentation time, it is needed to add urea in the growth media[21]. During growth and to produce enzymes cellulase, almost all fungi Trichoderma sp. requires Mg^{++}, Ca^{++}, Fe^{++} dan Zn^{++}, but using Fe^{++} and Mn^{++} minerals can serve as inducers. The addition of a combination of mineral Fe^{++} or Mn^{++} with Zn^{++} or Co^{++} will increase the activity of cellulase which produced[15].

While Aspergillus than requiring major elements such as C, N-phosphorus and S also require trace elements such as Fe^{++}, Cu^{++}, Zn^{++}, Mn^{++}, Mg^{++}, Li^{++}, Na^{+}, K^{+} and Rb^{+}. In addition to requiring major minerals and elements, according to Mandels et al.[17] also takes organic nitrogen sources and commonly used is peptone. Andreotti et al.[2] suggested that the use of peptone is best for cellulase production was one-tenth of the numbers of sources C (Carbon) are used.

According to the type of medium, the fermentation process is divided into two categories, namely fermented solid medium and liquid medium. Fermentation solid medium is a fermentation process where the solid substrate is insoluble and contains no free water, but it contains enough water for the microorganisms. Instead fermentation liquid medium is fermentation process where the substrate is dissolved or suspended in a liquid phase[7].

Naturally fermented solid medium generally takes place in a medium with a water content ranging from 60% to 80% because the state of the medium containing enough water for microbial growth[1]. On solid fermentation, fermentation substrate is mixed with liquid water, or water with some mineral content, usually up to 50% in order to obtain a semi-solid substrate. On solid fermentation substrate cannot all be achieved by microbes. Generally microbes that grow in certain areas such as the substrate surface, so that the below area and the middle area of substrate of no overgrown microbes[22]. According to Frost and Moss[12], solid fermentation has some advantages over liquid fermentation, namely:

- Medium used relatively simple
- The space required is relatively small compared to the yield generated
- Extraction of enzymes easier, by adding a solvent directly
- Condition of fungi grow approach that is common in the state of nature
- Low water levels make it less likely for the growth of unwanted bacteria

In addition to these advantages, solid fermentation also has some shortcomings[11], namely:

- Only limited to fungi growth
- Not easy to measure several parameters of the process because it is less homogeneous culture

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- Several types of substrates require pre-treatment (example: delignification)

According Tangnu et al.[21], highly influential fermentation conditions are pH and temperature of fermentation. The optimum pH value for enzyme production varies depending on the type of fungi, and the kinds of fermentation substrates used. Necessary for fungi growth with different pH enzyme production. In general, fungi needs to grow a higher pH for example above pH 4.0, for the production of enzymes needed lower pH for example below pH 4.0. The use of low pH causes the maximum activity of enzymes Endoglucanase and Exoglucanase more quickly achieved. The highest activity of the enzyme Endoglucanase and Exo-glucanase achieved at pH 4.0, whereas β-glucosidase enzyme is achieved at pH 6.0.

Temperature for fungi growth and for the production of enzymes are also different, the temperature for fungi growth is generally higher than the temperature for enzyme production. The optimum temperature for growth ranges from 32 °C to 35 °C while the temperature for cellulase enzyme production ranges between 25 °C to 28 °C[10].

Busto et al.[5] reported that induces the synthesis of amorphous cellulose in Trichoderma Reesei Endoglucanase better than cellobiose, lactose, sucrose and cellulose. Conversely, not all carbohydrates can induce endo-b-1,4-glucanase in Aspergillus niger significantly. Used at CMC substrate, maximum activity obtained at pH range 4.5-5.5 and optimum temperature of 50 °C - 70 °C.

In the study conducted by Nadiem Anwar et al.[4], the production of enzymes sellulase performed at pH 5 and room temperature by CMC-ase analyzing produced Endoglucanase activity of 1.66 IU/ml on day 6 of the fungus Trichoderma reesei and amounted to 1.69 IU/ml on day 8 of Aspergillus niger using powdered rice straw as substrate.

The purpose of this study was to determine the effect of pH and incubation time on the Endoglucanase activity of the enzyme cellulase produced by the fungus Trichoderma reesei and Aspergillus niger by using rice straw as substrate in solid state fermentation (SSF).

MATERIALS AND METHODS

The study was conducted from July 2012 to March 2013. The study was conducted in the Mechatronics laboratory of Brawijaya University - Malang, Central Laboratory of Biological Sciences of Brawijaya University - Malang and Biomolecular and Genetics Laboratory of Biology Faculty of the Islamic State University of Maulana Malik Ibrahim – Malang, Indonesia.

In this study, there are several materials used, such as Trichoderma reesei and Aspergillus niger obtained from Microbiology laboratory PAU Food and Nutrition Gadjah Mada University Indonesia, nutrient solution (aquades, yeast extract, Bacteriological peptone, $(NH_4)_2SO_4$, KH_2PO_4, $FeSO_4 \cdot 7\ H_2O$, CMC 1 %), tween 80, NaOH and HCl. This study is divided into two phases, namely preparation of materials (substrates) and cellulose enzyme production. The sections include:

Preparation of materials (substrates)

Rice straw used in this study is the variety of Ciherang. That is because Ciherang an easy varieties found in the region of Java. Rice straw used in the study obtained from the Pakis - Malang, Indonesia.

Rice straw that had been obtained, then cleaned up until sticks rice straw obtained. Then dried in the sun to dry. Once dried, rice straw sticks then cut ± 2 cm, and milled using disk mill. Having obtained the milled and then sieved with a 100 mesh sieve. Straw powder was then used for the production of cellulose enzymes.

Cellulase enzyme production

5 gr of powdered rice straw inserted into 250 ml erlenmeyer and 25 ml of nutrient solution[3] with pH conditions in accordance with the treatment (Phase 1: pH 4, pH 5 and pH 6. Phase 2: pH X, pH X.2, pH X.4, pH X.5, pH X.6, and pH X.8, the value of X is the pH value which obtained the highest Endoglucanase activity at 1st phase). pH was measured again when the substrate was mixed with a nutrient solution so that pH changes are known to occur. Then the sludge of rice straw covered with cotton, aluminum foil and rubber which is then sterilized using autoclave for 15 minutes. Inoculum fungi Trichoderma reesei and Aspergillus niger put as much as 2% at a density of $2x10^8$ cfu/ml to $2.5x10^8$ cfu/ml into the mud of rice straw and covered with cotton, aluminum foil and rubber. Incubation was performed at 30°C with pH according to treatment conditions, harvesting is done by using a 1% tween 80. Incubation was performed for 10 days, and observations of enzyme activity is done every 24 hours with CMC-ase DNS method. Data were collected for en-

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zyme activity and an increase in the pH condition of the solution.

Research design

Research carried out by simple randomized block design. The research is divided into two phases, the first phase is the production of cellulase enzymes with 2 factors, namely pH treatment with 3 levels treatments which are pH 4, pH 5 and pH 6 and 10 days observations, to obtain 30 observations with three times repetition and being done to 2 different kind of fungus in order to obtain 180 data. Then the second phase is the production of cellulase enzymes with 2 factors, namely pH X, pH X.2, pH X.4, pH X.5, pH X.6, and pH X.8 where X is the pH value of the pH in the first phase where the highest Endoglucanase enzyme activity obtained, and observations made during the 10 days, up to 60 observations obtained with 3 repetitions and being done to 2 different kind of fungus in order to obtain 360 data.

Activities enzyme assay

CMCase activity in the culture filtrate was determined by incubating the 0.5 ml of crude enzyme sample with 0.5 ml of 1% CMC (0.05M Citrate buffer pH 5) at 50°C for 30 min. After incubation, the reaction was stopped by the addition of 1.5 ml of DNS and then boiled for 5 min in boiling waterbath. The reaction mixture was allowed to cool and the reducing sugars released were estimated by Miller's method (1959).

RESULT AND DISCUSSION

Materials preparation

Rice straw is dried and cut into pieces along approximately 2 cm in order to facilitate the work of grinding, then ground and sieved with a 100 mesh sieve size. The entire series of treatments in order to obtain rice straw powder 100 mesh. Then tested levels of lignin, hemicellulose and cellulose. Thus obtained:

Cllulase enzyme production

Cellulase enzyme produced in this study is the enzyme cellulase from fungi Trichoderma reesei and Aspergillus niger with rice straw as the substrate. The phase of the cellulase enzyme production begins with the selection of microbes that being used which is Trichoderma reesei with the consideration that the type

TABLE 1 : Content of rice straw

Component	Percentage (%)
Hemiselulosa	18.495
Selulosa	30.38
Lignin	7.935

Source: Analysys certificate from UGM (2012)

of microbes capable of producing endo-β-1.4-glucanase and exo-β-1.4-glucanase up to 80% and Aspergillus Niger to produce higher β-glucosidase. Furthermore microbes are cultured on PDA (Potato Dextrose Agar) slant in a zig-zag and incubated at a temperature of ± 30°C for 7 days. Subsequently the culture inoculated in inoculums solution for 3 days and then suspended into the media in the form of rice straw fermentation and nutrient solution, which where the sludge was sterilized first by using autoclave. Enzyme extracting process is done by adding 1% of tween 80 and mix using waterbath shaker, then sludge and liquid fermentation separated using centrifuge with a speed of 4000 rpm for 30 min at 4°C to obtain the enzyme liquid (supernatant). Cellulase enzyme production is then performed with the Endoglucanase activity measured using CMCase method and the Endoglucanase enzyme activity obtained in accordance the data below:

From the data above it can be seen that the Trichoderma reesei produced cellulase enzyme with Endoglucanase activity of 2.00 IU/ml at pH 5 to 6 days of incubation time, while Aspergillus niger cellulase enzymes can generate with an enzyme activity of 2.042 IU/ml at pH 5 with 8 days of incubation time. TABLE 2 shows that the pH conditions which produced the highest sellulase enzyme activity was pH 5, then the pH conditions used in the second phase of the study.

TABLE 2 : Endoglucanase activity in 1st phase

Day	Trichoderma Reesei			Aspergillus Niger		
	pH 4	pH 5	pH 6	pH 4	pH 5	pH 6
1	0.502	0.833	0.886	0.437	0.487	0.491
2	1.023	1.263	1.122	0.593	0.666	0.658
3	1.255	1.575	1.392	0.711	0.776	0.78
4	1.655	1.688	1.621	0.86	0.958	0.879
5	1.795	1.833	1.711	0.939	1.008	0.985
6	1.636	2.000	1.848	1.016	1.08	1.031
7	1.553	1.777	1.735	1.365	1.613	1.232
8	1.442	1.556	1.616	1.852	2.042	1.495
9	1.297	1.415	1.506	1.86	1.989	1.468
10	0.765	1.323	1.373	1.552	1.852	1.278

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Referring to the results of the research which was done by Nadiem Anwar et al[4], the Endoglucanase activity of Aspergillus niger is 2,042 IU/ml higher than the Endoglucanase activity of Trichoderma reesei which is 2,000 IU/ml on the first phase.

Figure 1.a shows that the area of cellulase enzyme production by Trichoderma reesei, conditions of pH and incubation time which gained high endo-β 1,4-glucanase activity in range of pH 4.5 to pH 6.5 with incubation period of 3 to 10 days. While the Figure 1.b shows that the longer the incubation time, the higher endo-β 1,4-glucanase activity obtained, but the pH condition of cellulose enzyme production with a high endo-β 1,4-glucanase activity found in range of pH 3.5 to pH 6.

Based on the analysis of variance, the endo-β 1,4-glucanase activity significantly different in 1st phase between pH conditions, both in the endo-β 1,4-glucanase activity between pH of treatment or even between fungus Trichoderma reesei and Aspergillus niger. On the endo-β 1,4-glucanase activity produced by Trichoderma reesei phase 1, significantly different pH conditions both on the 1st, 3rd, 6th, 7th, 8th, 9th and 10th day, then carried LSD 5% and 1%. Whereas in the endo-β 1,4-glucanase activity produced by Aspergillus niger in the 1st phase, significantly different pH conditions both on the 1st, 2nd, 3rd, 4th, 7th, 8th, 9th and 10th day, then made LSD 5% and 1 %.

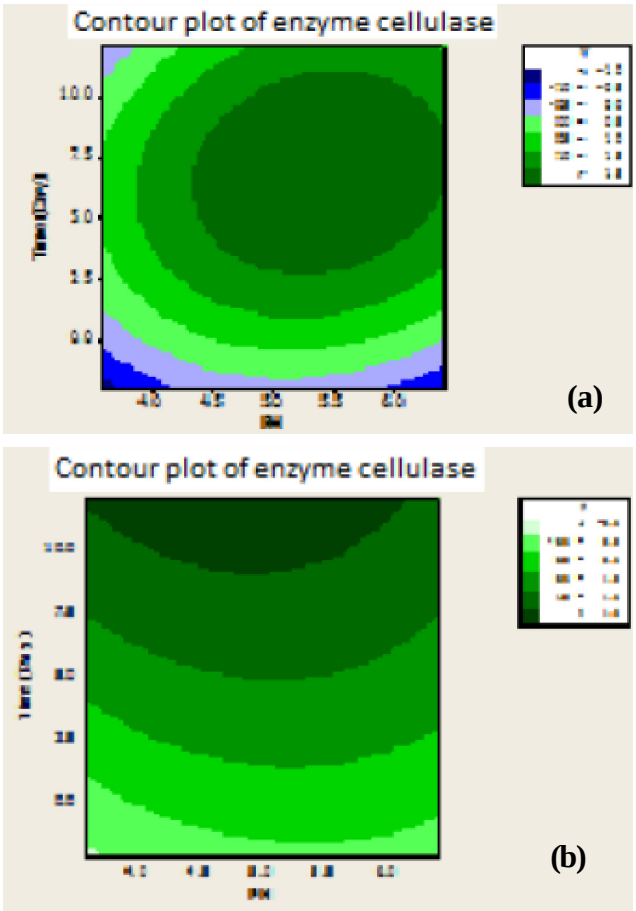

Figure 1: Contour plot of endo-β 1,4-glucanase activity in 1st phase. (a) Obtained from fungus Trichoderma reesei based on pH and incubation time; (b) Obtained from fungus Aspergillus niger based on pH and incubation time.

While on the second phase, the pH condition was set at pH 5, pH 5.2, pH 5.4, pH 5.5, pH 5.6 and pH 5.8 with 10 days of incubation, the observation was done every 24 hours. From the second phase, obtained the result of endo-β 1,4-glucanase activity as below :

In the second phase can be seen that the highest endo-β 1,4-glucanase activity of the cellulose enzyme produced by fungi Trichoderma reesei was 1.993 IU/ml at pH 5.5 with a long incubation of 6 days, while the highest endo-β 1,4-glucanase activity of the cellulose enzyme produced by fungi Aspergillus niger was 2.103 IU/ml at pH 5.2 with a long incubation of 8 days.

This study according to a statement issued by Busto et al.[9], that the conditions of enzyme production sellulase optimum pH ranges from pH 4.5 to pH 5.5. Optimum incubation time obtained in this study correspond well with Nadiem Anwar et al.[4] that the optimum time to obtain the production of the cellulase enzyme with the highest endo-β 1,4-glucanase activity using fungi Trichoderma reesei was on day 6 while the optimum time to obtain the production of the cellulase enzyme with the highest endo-β 1,4-glucanase activity using Aspergillus niger is on day 8.

Figure 2.a shows that the cellulase enzyme production of Trichoderma reesei, pH and time of incubation conditions which obtained high endo-β 1,4- activity was in the range of pH 4.5 to pH 6 with an incubation period of 3 to 10 days, in accordance with the contour plot in phase 1. While Figure 2.b shows that the longer the incubation time, the higher the endo-β 1,4-glucanase activity obtained, in accordance with the stage 1, but the pH condition where produce a high endo-β 1,4-glucanase activity is in range of pH 5 to pH 5.6.

Based on the analysis of variance, there are no real differences that occur in the study of endo-β 1,4-glucanase activity of sellulase enzyme production using fungus Trichoderma reesei or by using Aspergillus niger in phase 2.

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TABLE 3 : Endo-β 1,4-glucanase activity in 2nd phase

Day	Trichoderma Reesei						Aspergillus Niger					
	pH 5	pH 5.2	pH 5.4	pH 5.5	pH 5.6	pH 5.8	pH 5	pH 5.2	pH 5.4	pH 5.5	pH 5.6	pH 5.8
1	0.738	0.730	0.784	0.825	0.822	0.822	0.456	0.498	0.483	0.483	0.468	0.472
2	1.156	1.198	1.286	1.324	1.327	1.346	0.658	0.681	0.650	0.669	0.650	0.654
3	1.533	1.552	1.556	1.575	1.578	1.575	0.738	0.780	0.715	0.719	0.742	0.734
4	1.704	1.708	1.719	1.727	1.746	1.750	0.844	0.939	0.829	0.844	0.856	0.882
5	1.803	1.803	1.837	1.860	1.848	1.871	0.932	1.000	0.989	0.978	1.000	0.989
6	1.917	1.944	1.959	1.993	1.982	1.985	1.023	1.084	1.061	1.050	1.061	1.054
7	1.761	1.772	1.784	1.788	1.780	1.780	1.605	1.616	1.597	1.613	1.609	1.616
8	1.529	1.540	1.540	1.567	1.567	1.578	2.058	2.103	2.084	2.061	2.061	2.065
9	1.445	1.430	1.426	1.438	1.438	1.430	2.012	2.042	2.016	1.997	1.985	1.982
10	1.320	1.350	1.331	1.350	1.350	1.335	1.871	1.955	1.906	1.890	1.852	1.845

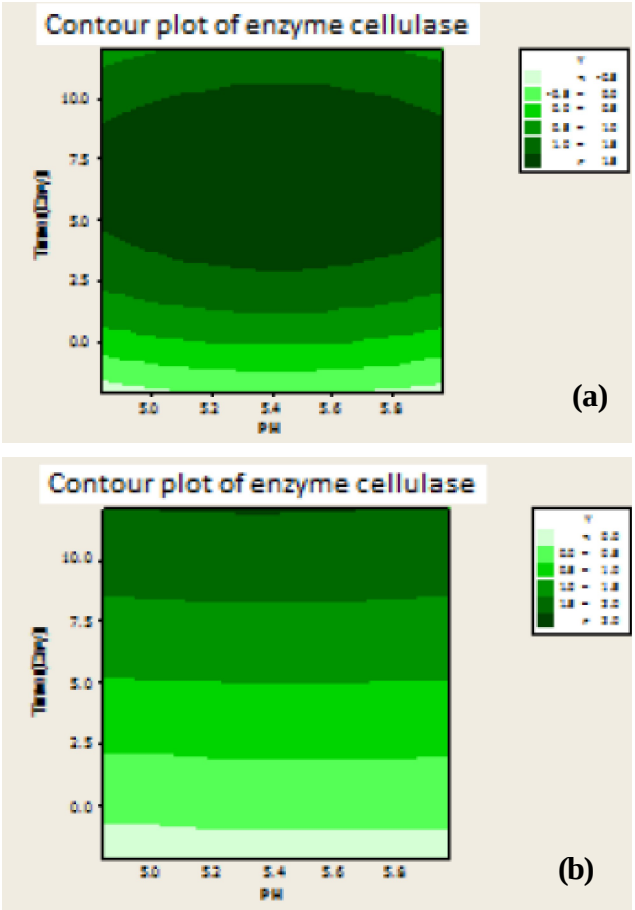

Figure 2 : Contour plot of enzyme cellulose activity in 2nd phase. (a) Obtained from fungus Trichoderma reesei based on pH and incubation time; (b) Obtained from fungus Aspergillus niger based on pH and incubation time.

CONCLUSION

The pH level and incubation time affects the activity of endo-β 1,4-glucanase on the enzyme cellulase. For the first phase of cellulase enzyme production by Trichoderma reesei shows the conditions of pH and incubation time which gained high endo-β 1,4-glucanase activity in range of pH 4.5 to pH 6.5 with incubation period of 3 to 10 days. While in the cellulase enzyme production by Aspergillus niger shows that the longer the incubation time, the higher endo-β 1,4-glucanase activity obtained, but the pH condition of cellulose enzyme production with a high endo-β 1,4-glucanase activity found in range of pH 3.5 to pH 6. The highest endoglucanase activity was at day 8, as the longer the incubation time, the higher the endo-β 1,4-glucanase activity obtained. The Second phase of cellulase enzyme production of Trichoderma reesei shows the conditions of pH and time of incubation which obtained high endo-β 1,4-glucanase activity was in the range of pH 4.5 to pH 6 with an incubation period of 3 to 10 days. While in the cellulase enzyme production by Aspergillus niger shows that the longer the incubation time, the higher the endo-β 1,4-glucanase activity obtained, but the pH condition where produce a high endo-β 1,4-glucanase activity is in range of pH 5 to pH 5.6.

The highest level of Endoglucanase activity which produced by Trichoderma reesei was obtained at pH 5.5 with long incubation time of 6 days. While the highest level of Endoglucanase activity which produced by Aspergillus niger was obtained at pH 5.2 with long incubation time of 8 days.

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