Literature study of production dry cellulase from *Trichoderma reesei*, *Aspergillus niger*, and *Bacillus subtilis*

Y Maryanty*, D R Wulan, M D Hidayati, D A Rizal and F Aliffandri

Chemical Engineering, State Polytechnic Malang, Malang, Indonesia

*yanty.maryanty@polinema.ac.id*

Abstract. Cellulase is an enzyme that can degrade cellulose through a catalytic process. The enzyme that works synergistically to release sugar (glucose). This review aims to determine cellulase enzymes’ activation from three different microorganisms, Aspergillus niger, Bacillus subtilis, and Trichoderma reesei. Enzymes’ activation using various kinds of lignocellulosic substrates and the effect of adding carrier agents on the freeze-drying process. The cellulase enzyme from Trichoderma reesei has an activity of 3.4 IU / mL. This enzyme is produced by pre-treatment 1% H2O2 in lignocellulosic media. Aspergillus niger has a cellulase enzyme activity of 0.229 IU / mL using a sugarcane bagasse substrate that has been pre-treated by shiitake mushrooms. The cellulase enzyme from Bacillus subtilis has an activity of 0.907 IU / mL, with the addition of CMC levels of 5% as an inducer. Cellulase enzymes in liquid form are susceptible to denaturation during storage. The production of cellulase enzymes in solid form is expected to maintain the stability of the resulting cellulase enzyme activity longer. The addition of non-reducing sugar as a carrier agent in the freeze-drying process is reported to preserve the biological activity. The addition of sucrose and trehalose, each with a concentration of 300 mM, maintained the amylase enzyme activity up to 90%. The addition of sucrose with an optimum concentration of 60 mM protects both total protein stability and good lysozyme activity. It is suspected that a carrier agent with an optimal concentration can maintain protein stability in cellulase enzyme activity in this review.

1. Introduction

Cellulase is a complex enzyme that cuts down the cellulose chain into glucose. At least three groups of enzymes are involved in the enzymatic hydrolysis process on cellulose. The enzymes are endoglucanase, exoglucanase or cellobiohydrolase, and β-glucosidase [1]. Microorganisms produce cellulase during its growth in cellulotic material. Although many organisms are capable of degraded cellulose, only a few of them produce large amounts of bioactive cell-free compounds that can fully hydrolyze cellulose crystals *in vitro* [2].

Type Trichoderma Fungi Reesei can produce endo-β 1.4-glucanase and Ekso-β-1.4-glucanase up to 80% [3]. Still, the β-Glucosidase is low, while the Aspergillus Niger type fungi can produce higher glucosidase when compared to Endo-β-1.4-glucanase and Ekso-β-1.4-glucanase. But according to Menendez *et al.* [4], the ability of cellulolytic bacteria has been widely utilized for a wide range of industrial purposes. Because the cost of production is cheap, short production time, producing complex multienzyme, and tend to be stable in extreme conditions. The difference in the ability to produce cellulase enzymes from these three microbes can have compared through the value of enzyme activity.
In general, enzymes are fluid-shaped and have a weakness in which enzymes become inactive due to changes in structures caused by denaturation. Denaturation has usually caused by external influences (stresses) such as heat, pH change, and chemical interactions. This stresses can have avoided if the proteins have converted to dry form in the presence of stabilizing substances that prevent protein denaturation and help preserve protein. Freeze drying can minimize thermal stress and reduce stress due to dehydration, making it an extraordinary tool to preserve enzymes. Therefore, it is necessary to produce enzymes in the form of solids, such as powders and granules. However, the step of the enzyme drying process often causes enzyme to lose its activity, and the end product is still vulnerable to inactivation. The proper design of the drying process should ensure the value of high enzyme activity.

The principle of this freeze-drying method is to freeze the sample rapidly with a vacuum condition to avoid forming crystals – the ice crystals that can damage the sample. Still, this process also often damages the structure of proteins that will eventually cause the denaturation and aggregation of proteins when reconstituted in water.

The protein conformation has essential to protection. The lyophilization is processing to maintain pharmacological effects and reduce the risk of product immunogenicity. Sucrose has the ability as stabilizers, can protect proteins during the lyophilization process and subsequent storage processes. The interaction between the hydroxyl groups of non-reducing sugars with protein molecules in powder form can prevent the release of hydrophobic sites, which can then lead to aggregation. The interaction between the hydroxyl group of non-reducing sugar with the protein molecule of the powder pad can prevent the discharge of hydrophobic sites, which can then lead to aggregation.

In many cases, a disaccharide or even a mixture of two different disaccharides. They seem most effective in stabilizing enzymes during the freeze-drying procedure. These cases include Chymotrypsinogen protection in the presence of 300 MM sucrose [7], complete protection of four restriction enzymes by 15% trehalose [8] and stabilization of recombinant human serum albumin [9]. The type and concentration of disaccharides influence protein stabilization during the Liofilisation [10]. If the freeze-Drying operation has appropriately done, the process will protect most of the initial biological activity of the substance in a dry state [11].

Study of this literature aims to compare the activity of the enzyme cellulase produced from Trichoderma reesei, Aspergillus niger, and Bacillus subtilis as well as knowing the influence of carrier agent (sucrose) in the process of freeze-drying of the activity of cellulase Enzyme.

2. Methods
This literature study method by conducting a study of experimental methods conducted by previous researchers related to the topic of research is qualitative descriptive and identified in each journal. The information to analyze the ability of Trichoderma reesei, Aspergillus niger, and Bacillus subtilis in the production of cellulase enzymes. The Freeze Drying method has used to acquire enzymes in the form of dry cellulose (powder). The addition of sucrose as a carrier agent has expected to maintain a stable enzyme that has remained a steady freeze-drying process. The methodology below is a result of studies and comparison of several previous journals that will be used as a reference when researching in the laboratory.

2.1. Pra-treatment media
Media that contains a mixture of wood powder sengon, bran, and lime are placed in a tray to be in the count to reduce the size of the media. The media that has minced has dried under the heat of the Sun for a full-day. After experiencing the drying process is expected Moisture content in the media can be less and the media has sifted on 80 mesh to expand the surface area, the enzyme has a substantial enough chance to degrades the cellulose in the media.
2.2. Analysis media
Media character analysis (NDF degradation analysis, ADF, cellulose, hemicelluloses, and lignin). Dry solids (residue) can have used to determine the content and degradation of NDF, ADF, cellulose, and hemicellulose using Van Soest method.

2.3. Inoculum production
Inoculum produces by taking spores from the microbe (aspergillus niger, Trichoderma reesei, and Bacillus subtilis). They have then resuspended in 8 mL of sterile aquades. Suspense has taken each containing 13 mL of Liquid sterile media. Subsequently, the medium has incubated in a shaker for 49 hours.

2.4. Media preparation
Pleurotus ostreatus Media in the form of a mixture is taken as much as 1.8 grams and added nutrients in the way of BHM (Basal Hard Medium) to 18 mL. Subsequently, the media was given an inoculum suspension for the samples. To be tested and blank without being given inoculum microorganisms.

2.5. Production of crude cellulase
Wahyuningtays Research [3] to produce cellulase started by mixing 5 grams of straw powder with 25 mL of nutrients into the Erlenmeyer 250 mL, then covered with sterile cotton and coated with aluminum foil, paper, and tied with yarn. A mixture of substrates and media has then sterilized using the autoclave at 121°C temperature for 15 minutes. The media is then cooled down to room temperature before the aseptic inoculation process has been performed. Spores grow in a single reaction tube. The spore has resuspended into 1ml of 0.1% tween 80 and then inoculated aseptically into the media. The results have subsequently incubated for 4.6 and 8 days at 27 °C, 30 °C, and 35 °C with pH variations in liquid media of 4.5 and 6, respectively.

2.6. Glucose standard curve
In the Julaeha research [12] has taken the methodology on the creation of the standard curve of glucose that is utilizing a standard stock glucose curve is made using glucose concentrations 0.25; 0.5; 0.75; 1; 1.25; 1.5 ppm. The standard curve produces by glucose solution. The solution has dissolved as much as 250 mg of glucose with aquades up to a volume of 50 mL. The reducing sugar test has done by taking 1 mL of the sample into the tube, then added 1 ml of the DNS reagent and 2 ml of the aquades, attached to each of the test tubes using a pipette. The Reaction tube is heated inside the water bath for 5 minutes to react to glucose and DNS. The absorption of each solution has measured at 540 nm [13]. Its absorbable absorption rate is linked to the standard curve to determine the concentration of glucose in the sample.

2.7. The activity test of cellulase enzyme
According to Rosyda Study [14], determining enzyme activity was tested using the CMCase method in units of International units (I.U.) with the reagent dinitro-salicylic Acid (DNS) [15]. Cellulase activity has determined by utilizing each liquid enzyme. The production taken an of 1.5 mL to has inserted into the microtube. Then The sample is centrifuged at a speed of 10000 rpm for 5 minutes at a temperature of 4 °C. The supernatant has taken as much as 0.5 mL. And inserted into the closed reaction tube and added 0.5 mL of the 1% CMC substrate was then homogenized and incubated for 30 minutes at a temperature of 50 °C. After that, it is added 3 mL of DNS and heated for 5 minutes on boiling water. Each solution in the tubes measured its absorption with a UV-Vis spectrophotometer at a wavelength of 550 nm. The calculation to get activity volume enzyme CMCase has based on one μ-mol glucose = 0.18 mg, and 1 unit of CMCase activity is one μ-mol glucose produced per minute.

2.8. Drying enzyme process using freeze-drying method
Shariat Research [11] uses the enzyme lactoperoxidase as a sample that will be freeze-drying. The first step is 15 mL of the enzyme solution that has mixed with a variation of the non-reducing sugar
concentration is inserted into the freeze-Drying tube and frozen at a temperature of -70°C for 24 hours. The frozen samples have transferred to the vacuum rubber at the freeze Dryer. The temperature has set at a temperature of -80 °C. A vacuum pressure of 0.001 bar has awaited until the sublimation process in freeze-drying was completed with the marked formation of solid Powder in the resulting sample.

3. Results and discussion

3.1. Fibre component values
The analysis of the media content using the Van Soest method has done by determining the rate of NDF, ADF, hemicellulose, cellulose, and lignin.

Table 1. Water content analysis, ADF, NDF, cellulose, and lignin.

| Analysis                  | Baglog Age       |
|---------------------------|------------------|
|                           | 0 months | Two months | Four-months |
| Water content average (%) | 8.59 ± 0.26 | 5.62 ± 0.58 | 3.98 ± 0.27 |
| ADF average               | 62.14 ± 15.36  | 70.05 ± 3.71 | 88.04 ± 1.29 |
| NDF average               | 69.34 ± 11.63  | 74.89 ± 0.19 | 97.93 ± 0.18 |
| Cellulose average (%)     | 46.41 ± 13.82  | 64.08 ± 4.21 | 85.25 ± 1.51 |
| Lignin average (%)        | 15.59 ± 1.59   | 5.52 ± 0.03  | 2.67 ± 0.12  |

*each data was report as average ± standard deviation obtained from two times measurement.

The results showed that *Pleurotus ostreatus* media analysis containing water content decreased as the age while percentage of cellulose increased. According to Setyowati [16] that the content of cellulose in the mixed Media of sengon powder and bran contains a high content of cellulose, as is the case in Bran as a diverse medium that has bacteria *acetobacter xylinium* which plays a role in converting carbohydrate waste in the media into cellulose.

ADF (acid Detergent Fiber) is a component of cell Wall that is soluble in acidic detergents. The ADF has used as a preparatory step to determinate lignin. That hemicellulose can have estimated from the difference in cell wall structures [17] NDF (Neutral Detergent Fiber) is a component of the cell wall that is soluble in neutral detergent [17]. From the data above, obtained NDF rate at the age of 0 and 2 months. The data decreased at a period of 4 months. The rate is rising. It is in line with Zenebe Research 2016 [18] stating that any *Pleurotus ostreatus* media treatment has followed by a decreased percentage of NDF, ADF, cellulose, and lignin content with the same pattern.

Table 2. Changes in fiber content (NDF, ADF, cellulose, lignin) on the various moisture content of substrates and inoculum doses of *Pleurotus ostreatus*.

| Treatment         | Response variables |
|-------------------|--------------------|
|                   | NDF    | ADF    | Cellulose | Lignin |
| **Substrate Moisture** |        |        |           |        |
| 62.50%            | -8.99<sup>a</sup>| -7.74<sup>a</sup>| -9.18<sup>a</sup>| -13.78<sup>a</sup>|
| 70.00%            | -13.19<sup>b</sup>| -11.75<sup>b</sup>| -14.65<sup>b</sup>| -15.76<sup>a</sup>|
| 77.50%            | -27.13<sup>c</sup>| -15.45<sup>c</sup>| -19.33<sup>c</sup>| -21.73<sup>b</sup>|
| 85.00%            | -26.64<sup>c</sup>| -15.88<sup>c</sup>| -19.88<sup>c</sup>| -21.98<sup>c</sup>|
| **Inoculum Dose** |        |        |           |        |
| 10g               | -15.58<sup>a</sup>| -7.95<sup>a</sup>| -9.5<sup>a</sup>| -14.98<sup>a</sup>|
| 15g               | -19.18<sup>bc</sup>| -12.73<sup>b</sup>| -15.66<sup>b</sup>| -17.4<sup>b</sup>|
| 20g               | 20.24<sup>c</sup>| -14.71<sup>c</sup>| -18.68<sup>c</sup>| -19.97<sup>c</sup>|
| 25g               | -21.15<sup>c</sup>| -15.42<sup>c</sup>| -19.2<sup>c</sup>| -20.97<sup>c</sup>|
At each dosing treatment inoculum, *Pleurotus ostreatus* has been able to lower the content of all Fiber Components (NDF, ADF, cellulose, and lignin) so that the content of each component decreases (The percentage of decline becomes higher).

### 3.2. Cellulase enzyme activity

The best result of the literature study, the highest value of cellulase activity gained from the *Trichoderma reesei*, amounted to 3.4 U/mL. The result has shown in research conducted by Zhao [19] at Optimum conditions incubation time for 139 hours using a lignocellulose substrate of 1% residual furfural, which has a content of cellulose 38.06% and lignin 40.23%. It’s quite a difference for *Aspergillus niger* 0.229 U/mL, 216 hours [14]. *Bacillus subtilis* 0.907 U/mL with 2% bagas sugarcane, 72 hours, and using CMC 5% [20].

| Microbes         | Source            | Substrate              | Operating conditions | Enzyme activity of cellulase (U/mL) |
|------------------|-------------------|------------------------|----------------------|------------------------------------|
| *Trichoderma reesei* | Zhao (2018)       | Residu Furfural 1%     | 50 °C                | 3.4                                |
| *Aspergillus niger* | Rosyida (2018)    | Sugar Cane Bagas 5%    | 50 °C                | 0.229                              |
| *Bacillus subtilis* | Yandri (2012)     | CMC 5%                 | 55 °C                | 0.907                              |

Based on the studies of the above literature compared to the variables we will use in the media waste baglog planting oyster mushroom with the utilization of *Pleurotus ostreatus*. The value of the enzyme activity generated in the research of Zhao [19] by using furfural waste Media 1% with *pre-treatment* using H₂O₂ 1% can produce enzyme Activity of 3.4 U/mL. It has also been influenced by incubation conditions of 50 °C and pH 6. The content of furfural waste that does not contain hemicelluloses. On Rosyida [14] using the media bagas sugarcane 5% and has cellulose content of 52.42% therein. The media has also undergone a delignification process assisted by shiitake mushroom. Other conditions that affect also use an optimal temperature of 50 °C. Next, the activity assay of cellulase enzyme Using *Bacillus subtilis* has the most substantial activity value produced at 0.907 U/mL [20]. It has been influenced by the absence of the addition of lignocellulose substrate instead of adding CMC Nutrients of 5 grams and contains cellulose content of 73.4%. Besides, the incubation time also affects Yandri Research [20], which uses the condition for 72 hours at 40 °C temperature, which can obtain optimum health to determine the activity of the cellulase enzyme. From the data collected on some sources in this literature, the study can be a reference for using optimum operating conditions on each cellulase-producing microorganisms.

### 3.3. Freeze drying and carrier agent

In Gianini research [21] compares the resulting proteolytic activity using a variety of Drying methods such as solar drying, cabinet drying, vacuum drying, and freeze-drying. Operating conditions in various types of drying methods have their variable differences. The best lyophilization is 4 °C for three months [22]. The stability of the protein in the same period 0.1, and 2 are 42,40 and 41 %, respectively. The lysozyme activity of 1140 U/mg, 1140 U/mg, and 950 U/mg were the most stable results when compared with other variables. Besides, the test for the water content lost during lyophilization or freeze-drying was the highest at 76.12%, with the addition of the concentration of the composition of the stabilizer or carrier agent CMC 0.3% and 25% sucrose conducted by Cahyadi et al. [23].
Figure 1. Proteolytic activity of papain coarse with various drying methods.

Research by De Jesus and Michael Fillio [24] shows that the drying process using freeze-drying can maintain better biological activity than Permata’s study [25]. In the freezing stage, the enzyme has carried out using liquid nitrogen so that it was able to reduce water activity to 0.2 for 4 hours, and the results we’re able to maintain enzyme activity by 98% after 16 hours of the process [24]. So, the fast cooling process, of course, also affects the freeze-drying process and the resulting activity because the quicker the water activity decreases, the higher the enzyme activity is maintained in the freeze-drying process.

Figure 2. Influence of drying time of the enzyme activity after the primary Cooling is carried out using: (Δ) liquid nitrogen; (○) Dry Ice and acetone; (□) Conventional freezer. (Source: De Jesus and Michael Fillio, 2014).

Table 4. A freeze drying literature study and carrier agent.

| Test         | Save time and the concentration of sucrose | Activity value (U/mg) |
|--------------|-------------------------------------------|-----------------------|
|              |                                            | At temperature 4°C    | At temperature Spaces |
| Kadar Total  | 0 months (60 MM)                           | 1140                  | 1140                   |
| Protein Serum| 1 months (60 mM)                           | 1140                  | 1000                   |
| Otologus     | 3 months (60 MM)                           | 950                   | 690                    |


Based on some literature, adding a carrier agent in the form of sucrose with various concentrations with storage at room temperature, there are differences in the operating conditions of the process. The addition of a stabilizer of sucrose (non-reducing saccharide) is a stabilizer that can protect protein and can replace the hydrogen bonds that occur between proteins (enzymes) and water. The OH-group present in sucrose can bind to proteins and protect chemical and physical degradation in aqueous solutions during the freeze-drying process and the storage process. At a later stage, the addition of a carrier agent can improve the performance of reducing water content during freeze-drying. Enzyme or protein storage at lower temperatures shows a very significant effect and can maintain enzyme activity longer than storage using room temperature or higher. High temperatures accelerate the physical aggregation of proteins in a dense and denatured state. Increasing the temperature affects protein indirectly. Heat can break the hydrogen bonds of proteins. Storage temperature has an essential factor. Temperatur influence the denaturation of the enzyme, a decrease in the stability of the enzyme activity or protein content. In the research of Shariat (2015) also confirmed that the use of optimum sucrose concentration and storage at low temperatures was able to maintain the enzyme activity produced by 90% after 40 days.

4. Conclusion
From the results of the literature study that we have compared, it can have to be concluded that Trichoderma reesei, Aspergillus niger, and Bacillus subtilis can produce cellulase enzymes with varying capabilities. The difference in ability can be seen from the activity value of the resulting cellulase enzyme and factor the factors that affect it involved. Substrate selection and optimal operating conditions can produce cellulase enzymes with high enzyme activity values—the use of carrier agents to maintain enzyme activity at an optimum concentration during the freeze-drying process.

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