The study of xylanase immobilize enzyme using sol-gel method

S Hadiantoro, Y Maryanty*, D R Wulan, S A Putri, E M Putra and N S Achmadin

Chemical Engineering Department, State Polytechnic of Malang, Jalan Soekarno-Hatta No. 9 Malang, Indonesia

*yanty.maryanty@polinema.ac.id

Abstract. Xylanase has many benefits, but many obstacles to produce it. Common microorganisms produce fungi and bacteria such as Aspergillus niger, Trichoderma viride, and Bacillus subtilis. Various methods can do enzyme immobilization; one of them is entrapment in the form of sol-gel. This study discusses the xylanase activity. The enzymes have produced from Aspergillus niger, Trichoderma viride, and Bacillus subtilis. After that, the xylanase enzyme has immobilization using the sol-gel method. This study is doing by lab work and study literature. Lab work is for knowing fiber amount by van Soest method with amount of % hemyselulosa 4.8% 7.33%, 9.1% for media 0 months, two months, and four-month. The study literature doing with compare xylanase amount by Aspergillus niger, Trichoderma viride, and Bacillus subtilis the highest enzyme activity by Trichoderma viride with optimum condition temperature 25°C, pH five and incubation time 36 hours with enzyme activity 26.67 U/mL. The immobilization obtained xylanase activity of 28.88 U/mL. The matrix composition of TEOS, 5% xylan, and 5% calcium alginate during fermentation for 168 hours. This value is higher than the fermentation process without immobilization carried out for 72 hours with an activity value of 22.04 U/mL.

1. Introduction

Second of the most significant plant constituent components, in addition to cellulose, is xylan, which covers cell wall material of annual plants accounts for 30, 15–30% of hardwoods and 7–10% of softwoods. Xylan is a polymer from xylose, which has founded in hemicellulose. Hemicellulose, lignin, and cellulose are components that make up the wooden structure.

Xylanase (EC 3.2.1.8) is an enzyme that plays a role in the hydrolysis of xylan (hemicellulose) into xylooligosaccharides and xylose. The application of this enzyme is quite widespread, among them are used in the pulp industry for the eco-friendly process of deinking and bleaching [1]; in livestock feed industry; in food and beverages industry for dough handling improvement, for the extraction of coffee, plant oils, for clarification of fruit juices [2] and starch assisted in the pre-treatment of biomass in the bioenergy industry [3].

Xylanase has various benefits, but many obstacles have been an encounter in the production process. One of them is the lack of availability of superior xylanase-producing microbial cultures. The use of microbes as enzyme-producing agents has several advantages, including high speed of microbial growth so that it can be produced in a short time, easy to control, and relatively cheap production costs [4]. Xylanase can have been delivering from bacteria, fungi, and yeast, including Aspergillus, Trichoderma, Streptomycetes, Phanerochaetes, Chytridiomycetes, Ruminococci, Fibrobacteres, Clostridia, and Bacilli.
Xylanase producing organisms require xylan as a carbon source. Because xylan has a relatively high price, the use of pure compounds directly in the production medium is quite expensive. Xylan is an essential component in the agricultural biomass that contains hemicellulose on the cell wall of the plant. Xylan components are also abundant in agrarian waste such as wheat bran 12.3%, Bagas sugarcane 9.6% and rice husk 12.1% [3]. Based on the xylan content in the trunk is quite high, then in this research used wood powder from Sengon tree as a source of carbon. Wood powder from the Sengon tree that has utilized as media grows oyster mushroom contains an amount of cellulose about 45.42%, and the rest is hemicellulose 21%, lignin 26.50% and ash 7.08% [9]. Hemicellulose contained in wood powders can produce xylan after the process of de lignification by white weathered mushrooms.

Enzyme immobilization is a process whereby the movement of enzyme molecules in the chamber where the reaction has held in such a way that the enzyme system forms active and insoluble water [10]. Enzyme immobilization synthesis with various methods. The selection of enzyme immobilization methods depends on how technology affects the activity of enzyme catalysts. There are several methods of immobilization of enzymes with multiple types of support, namely: entrapment, carrier-binding, adsorption, and cross-linking. Among these three methods, one new approach to development in the mobilization of enzymes is the method of encapsulation/entrapping. The encapsulation method is the packing of a protein molecule in the sol-gel matrix that is not covalent, but the particle has physically trapped in the tissue of the gel that has grown around it.

2. Material and methods
This literary study was the information to analyze the ability of *Trichoderma reesei*, *Aspergillus niger*, and *Bacillus subtilis* in the production of cellulase enzymes. The Freeze-Drying method use 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. 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. 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 1 blank without being given inoculum microorganisms.

2.4. Enzyme production
Three 250 ml Erlenmeyer has prepared, each containing 150 ml of liquid media, then sterilized in an autoclave at a temperature of 121 °C with a pressure of 15psi for 15 minutes. After cooling, add 15 ml of inoculum aseptically. Furthermore, the media has incubated in a shaker. The speed of 150 rpm at room temperature for 98 hours, after which the enzyme isolation has to carry out. The enzyme filtrate was centrifuged at 2000 rpm for 20 minutes at 4 °C. The supernatant obtained was a crude extract of the xylanase enzyme. The xylanase enzyme crude extract has then tested for enzyme activity.
2.5. Immobilized with sol-gel method
In general, the sol-gel method process can be summarized as follows: preparation of precursors solution, hydrolysis and partial condensation of alkoxides forming “sol”, polycondensation of hydrolyzed precursors forming “gel”, drying (solvent evaporation) forming a dense “xerogel” via collapse of the porous network [11].

3. Results and discussion
Based on several journals used in analyzing differences in xylanase enzyme activity against Aspergillus niger, Trichoderma viride, and Bacillus subtilis with various substrates from various kinds of literature. Knowing how the immobilization process with the sol-gel method from some journals.

3.1. Trichoderma viride

Table 1. Literature study of the activity of the enzyme xylanases from Trichoderma viride.

| Media          | Temperature (°C) | pH | Incubation time (Hours) | Enzyme activity (U/ml) | Source |
|----------------|-----------------|----|------------------------|------------------------|--------|
| Corn Klobot    | 25              | 5  | 60                     | 14.53                  | [12]   |
| Corn Klobot    | 30              | 5  | 36                     | 17.01                  | [13]   |
| CORN COB       | 25              | 5  | 36                     | 26.67                  | [10]   |
| Corn Klobbot   | 40              | 5  | 60                     | 2.97                   | [14]   |

Research conducted Trichoderma viride [12]. Cornhusk used as a substrate with Trichoderma viride inoculum. Incubation time 60 hours at room temperature ± 25°C. Immobilization has carried out with a bentonite matrix with a shaking time of 1, 2, 3, 4, and 5 hours. The optimum time for shaking was 3 hours, with the enzyme activity of 10.245 units/ml. A study related to xylanase immobilization using a Ca-Alginate-chitosan. Matrix capable of producing enzymes with an activity value of 17.008 U/ml, with optimum conditions at pH 5, temperature 30 °C, and an incubation time of 36 hours [13].

Trichoderma viride can produce endo-1,4-β-xylanase enzymes that can degrade xylan [10]. Corn Cob can have utilized as the media production of the enzyme xylanases from Trichoderma viride. The increase xylanases enzyme activity of Trichoderma viride needs to have done optimizing by combining several variables that can affect the activity of enzymes such as temperature, pH, the concentration of enzymes, substrate concentration, activator, and inhibitors. This study aims to determine the effect of temperature and pH on the xylanase enzyme activity of Trichoderma viride. Growing media in the form of corn cobs. The media contains lignocellulose. The highest xylanase enzyme activity has obtained at a temperature of 25 °C at pH 5 and an incubation time of 36 hours. The value of xylanase enzyme activity was 26.67 units.

At the influence of temperature, enzyme activity at 60 °C storage has the highest activity. Based on the graph produced at 60 °C is the optimum temperature. At a temperature of 70 °C, the enzyme activity is the lowest because it has undergone denaturation. Denaturation can cause conformational changes in the enzyme. The amount of substrate can have bounded by the active site of the enzyme decreases, and the enzyme activity decreases. Enzyme activity at 40°C at storage time of 15-25 hours has lower activity when compared to storage at 30 °C. This condition can have cause by the presence of the protease enzyme, which has an optimum temperature of 35°C. In this condition, the xylanase enzyme has degraded more. The stability of the xylanase enzyme can have seen from the activity of the remaining enzymes. The enzyme is said to be stable when the remaining enzyme activity is more than 50% of the initial enzyme activity. The activity of the residual enzymes decreases with the more extended the xylanase is stored. The highest residual enzyme activity is at a storage temperature of 60°C.

Meanwhile, the lowest residual enzyme activity has stored at 70 °C. In general, and xylanase is still stable up to 25 hours of storage. However, for storage of 40 °C, xylanase is stable for up to 20 hours,
with the residual enzyme activity of 56.95% (2.973 units). Permanent xylanase enzyme storage at 70 °C, for up to 15 hours with residual enzyme activity 54.88% (2.865 units).

From several studies discuss the incubation time. The xylanase enzyme activity was 26.67 U / ml with an incubation time of 36 hours, 14.528 U / ml with an incubation time of 60 hours [12]. This difference in incubation time affects the value of enzyme activity. The longer the incubation time, the lower the enzyme activity value. From the data, conducted with an incubation time of 36 hours when adjusted for the variables that future researcher can do at 40, 45, 50, 55, and 60 minutes, it is very different. The different incubation times will produce different values so that in the experiment, and the incubation time will have extended so that future researcher can find out the amount of the enzyme activity. From 0 hours, the value of enzyme activity will be seen whether the amount increases with increasing incubation time.

3.2. Aspergillus niger

Fungi growth is very related to breeding that affects the increase in the number or volume of cells [4]. The reproduction of fungi is asexual in general is through the formation of spores, to know the growth can have done the calculation of spore density. Spore density is measured using hemacytometer based on the way, which is done every consecutive day until the densest spores to has used as a working culture. The relationship between the number of spores Aspergillus niger with the time of spore calculation has shown in Figure 1, seen increasing the number of spores a. Niger until the sixth day. Based on Figure 1, the number of spores A. Niger increased from the first day, i.e., 1, 12x10^7 cells/ML until the second day entered the exponential phase of the cell multiplication phase.

Moreover, reached the peak of stationary on the third day, with the number of spores from the highest A. Niger that is 1, 65x10^8 cells after the fourth day the number of spores A. Niger starts to decrease but relatively still stable until the sixth day with the number of spores 1, 46x10^8 cells/ML. On the fourth day, it begins the death phase. Thus, to obtain the optimum production of the enzyme is done harvesting at incubation time 2-3 days because, in that phase, the number of Spore A. Niger is at the peak point. With some of the spores, Aspergillus niger has expected its enzymatic production is also high.

Table 2. Literature Study of xylanase enzyme activity of Aspergillus niger.

| Media      | Temperature (°C) | pH | Incubation Time (Hours) | Enzyme (U/ml) activity | Source |
|------------|------------------|----|-------------------------|------------------------|--------|
| Bran       | 30               | 6  | 120                     | 1.4                    | [15]   |
| Rice straw | 37               | 6  | 56                      | 0.055                  | [4]    |

The fermentation process was carried out in a Shaker incubator at 37 °C, stirring at 200 rpm and pH 6. The reduction test of sugar content used the DNS method to obtain enzyme activity. The results showed that the addition of molasses to rice straw media could increase the growth of A. niger but did not significantly increase the xylanase enzyme activity and required a longer incubation time. The most optimal concentration of molasses for xylanase enzyme production is 1%, with the highest enzyme activity of 0.055 U / ml and an incubation time of 56 hours.

Based on the research in Table 2, the optimum condition gained; differently 56 hours, and 120 hours for the optimum condition incubation time. At the optimum state of pH obtained at pH 6. The optimum temperature varies from 37 °C [4] and 30°C [15]. The difference quite far lies in the value of enzyme activity of 0.055 U/ml and 1.4 U/ml.

The difference between the two studies affects the value of its enzymatic activity. The longer the incubation time than the smaller the value of the enzymatic activity. However, if the incubation time equation does further research, then the value of the resulting enzyme activity will not differ considerably. It has expected to conduct experiments using the conditions made by Kamimozi and Nagalakshmi [15] at pH 6; temperature 30 °C and the incubation time 120 with the test data that future
research can have obtained optimum condition. The variables we use are in the range of pH 5, 6, 7, 8, 9; Temperatures (40, 45, 50, 55, 60) °C and incubation times 40, 45, 50, 55, and 60 minutes. If based on Wahyu et al. [4] research, future researcher need to adjust from the incubation time and the temperature.

3.3. Bacillus subtilis

Based on research conducted by Erika et al. [16] in the production of xylanases using the Inoculum Bacillus subtilis which is grown in 50 ml of sterile media with the composition: xylan beechwood 0.5%; Yeast extract 0.35%; Tryptophan 0.35%; NaCl 0.2%; KH₂PO₄ 0.245%; MgSO₄ 0.035%; (NH₄)₂SO₄ 0.175%) and Corn Cob. The determination of xylanases activity has done by the method of 3.5-dinitrosalicylic acid (DNS). The enzyme activity is measured based on the absorption rate of the sample solution at 575 nm wavelength. As a blank, the addition of enzymes into the reaction tube has performed after the incubation process. One unit of xylanase activity (U/ml) has been defining as the number of enzymes that release the µmol of xylose per minute. The value of Xylanase activity generated is 0.2 U/ml.

One unit of xylanase activity is the number of enzymes needed to break the xylan into 1 µmol of reducing sugar per minute in test conditions. This enzyme, when manufactured using xylan broth Media in temperature conditions of 30°C, 150 rpm with starter containing total bacteria (2, 43, 3) x 10⁸ CFU/ML or OD600 0.715 as much as 10% takes two days. The resulting xylanases enzyme works optimum at PH 8 and temperature 70 °C with the activity value of 5.17 U/ml.

Based on some studies conducted by Erika et al. [16] and Ardiansyah et al. [17]. There are different outcomes due to differing work on each researcher, and the substrate they use the optimum condition of the enzyme obtained 12 hours, while in the research of Fawzya et al. [18] received 24 hours, pH 8 is the optimum pH achieved by Fawzya et al. [19] and Ardiansyah et al. [17], while Erika gets pH six as the optimum pH of the enzyme. In the optimum temperature test, the enzyme got the result that the temperature 40°C is the optimum temperature of the enzyme [16,17], while Fawzya et al. [19] got at 70°C as the optimum temperature. Highest activity value obtained with activity value 6.088 U/ml with optimum conditions incubation time 12 hours pH eight and temperature 40°C [17].

In this experiment the range of pH 5, 6, 7, 8, 9, temperature (40, 45, 50, 55, 60) °C and incubation time 40, 45, 50, 55, and 60 minutes. When it has based on the optimum condition of Ardiansyah et al. [17]. At the incubation time, 12 Hours, PH 8, and temperature 40oC are quite the same. The optimum condition of the enzyme that we do can get optimum value because the type of media used is different from the content of the xylan in the media is also different so that each media also has a specific optimum condition that results in the difference in the value of the enzyme activity itself.

| Media       | Temperature (°C) | pH | Incubation time (Hours) | Enzyme (U/ml) activity | Source |
|-------------|-----------------|----|-------------------------|------------------------|--------|
| CORN COB    | 40              | 6  | 12                      | 0.2                    | [16]   |
| Xylan broth | 70              | 8  | 48                      | 5.17                   | [18]   |
| Rice husk   | 40              | 8  | 12                      | 6.088                  | [17]   |

3.4. Immobilization techniques

The activity of xylanase of 28.88 U/ML received from a culture that has mobilized in sol-gel with a matrix composition of TEOS, 5% Xylan, and 5% calcium alginate at the time of fermentation for 168 hours [20]. The value is higher than that of the fermentation process, with no immobilization conducted for 72 hours with activity value 22.04 U/ML (Figure 1).
Figure 1. Production of free xylanase and the mobilization of the culture *Aspergillus* on hybrid matrices with TEOS composition, 5% xylan, and 5% Calcium alginate [20].

Calcium alginate provides a positive effect on the production of the immobilization of xylanase. Mobilized cultures can maintain biosynthetic capabilities over a long period during the fermentation process. The *sol-gel* matrix used in research studies provides high porosity for the diffusion of nutrients and better products. However, a hybrid matrix determination analysis is composed of TEOS, 5% Xylan, and 5% calcium alginate as a suitable ingredient as a *sol-gel* immobilization. The mobilized culture can increase the production capacity of xylanases. Furthermore, maintain biosynthetic capability during the development process versus free culture. The use of calcium alginate as an organic component in the matrix composition also solves the problem of diffusion in the Support section.

Immobilized by using the *Sol-gel* method as an increase in enzyme activity, the research enzyme that has immobilized with *Sol-gel* matrix containing 10% PEO get the value of activity 1297.44 U/ml [20]. The amount of the activity is higher than the enzyme value that has not been immobilizing with the activity value of 542.76 U/ml. From these results, the mobilized culture can maintain the biosynthetic capability for a long time during the fermentation process. The use of calcium alginate in the hybrid composition of the *sol-gel* matrix proves that calcium alginate gives a better effect on nutrient absorption, which causes the increased value of enzyme activity and reduces fermentation time.

The main goal in the experiments conducted by Peralta-Pérez et al. [21] was to immobilize xylanases from *Aspergillus niger* in the *sol-gel* matrix. The decreased activity caused by the gel pores formed. Optimization-based of immobilization techniques and the subsequent work that is to increase the value of the activity of the mobilization enzyme. Although there is a decrease in the amount of enzyme activity, it can has used periodically.

Research conducted by Pal and Khanum [22] shows that the immobilized enzyme has increased activity value from 7092 U/ml to 8000 U/ml with Optimum pH condition of 5 to 5.5 and the temperature 40 to 45°C. Immobilized enzymes can also be used repeatedly five times and can withstand a total of 85% of the first activity value. The Immobilization method can also address the problem of decreasing xylan loss.

Enzyme immobilization Process using the *Sol-gel* method when comparing between various journals can be very different. So, if to immobilize using the *Sol-gel* method, it is good to do the research first from previous research. Which *sol-gel* method fits and corresponds to the research time.

An increase in the value of the activity of free enzymes with enzymes held immobilization, i.e., from 3333.33 U/ml to 5000 U/ml. In this study, pH stability and temperature could increase as well as the immobilized enzyme can have used up to 10 times with the remaining 60% of the initial activity. The maximum activity of free xylanases and xylanase mobilization is at pH 7. However, the activity of the generally mobilized enzymes is higher when compared to free enzymes. The pH stability indicates that the immobilized enzyme shows excellent stability when compared to free enzymes [23].

The process of enzyme immobilization is an increased process of enzyme activity where the value of enzyme activity has expected to stabilize within a given time. However, this process requires additional costs because the enzyme immobilization requires a matrix. Many types of matrices also affect the stability of the enzyme activity itself. In the experiments, we will do that is using hydrogel.
The enzymes that have produced will be adding to the hydrogel. In hopes of increasing the time, the enzymes in the enzyme hydrogel will have trapped in the hydrogel.

4. Conclusion
The best microorganisms capable of producing the activity value of the highest xylanase enzyme are from *Trichoderma viride* with corn cob media with the optimum condition at 25°C, pH 5, and incubation time 36 hours with activity value of 26.67 u/ml. An enzyme that has immobilized with a sol-gel matrix containing 10% PEO gets the amount of 1297.44 u/ml activity. The value of the activity is higher than the enzyme value that has not been immobilizing with an activity value of 542.76 u/ml. From these results, the mobilized culture can maintain the biosynthetic capability for a long time during the fermentation process. Although there is a decrease in the value of enzyme activity, it has advantages that have use periodically.

Acknowledgments
The authors would like to acknowledge The Ministry of Education and Culture of Republic Indonesia for financial research support through “Penelitian DIPA Riset Terapan 2020” State Polytechnic of Malang.

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