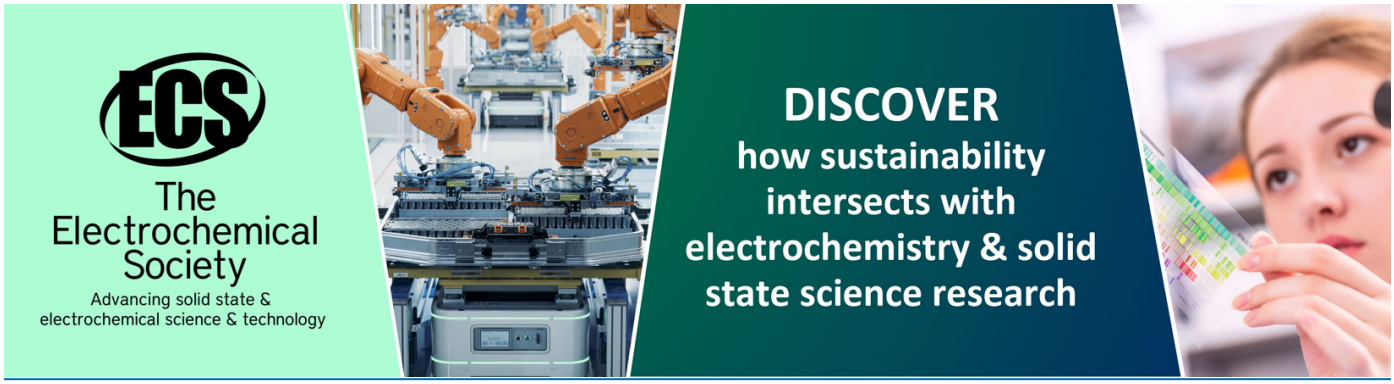
Production of Crude Xylanase From *Trichoderma sp.* Using *Reutealis trisperma* Exocarp Substrate in Solid State Fermentation



Production of Crude Xylanase From *Trichoderma sp*. Using *Reutealis trisperma* Exocarp Substrate in Solid State Fermentation

E Mardawati[1], Y Sinurat[1], and T Yuliana[1]

[1] Department of Agroindustrial Technology, Padjadjaran University

Corresponding author's emamil address: efri.mardawati@unpad.ac.id

Abstract. *Reutealis trisperma* exocarp is a waste containing hemicellulose, cellulose, and lignin which can be used to produce xylanase enzymes. *Reutealis trisperma* exocarp contains 44.48% of hemicellulose, a polymer of xylan. The xylanase is an enzyme that can be utilized for hydrolyzing hemicellulose (xylan) to xylose. Xylanase can be produced by solid-state fermentation using isolate *Trichoderma* sp and *Reutealis trisperma* exocarp as substrate. This research aims to determine the fermentation time and substrate concentration that produce the highest xylanase activity using *Trichoderma sp.* In this study, the crude extract enzyme was evaluated on xylanase activity, protein content and specific activity at various fermentation times of 12, 24, 36, 48, 60 h using substrate concentrations of 2%, 4%, 6%, and 8%. The highest xylanase activity was observed with the substrate concentration of 8% at the 60 hour fermentation time. In this process, 672.039 U/mL of xylanase activity, protein content around 0.590 mg / mL and 1137.638 U/mg of the specific activity were produced.
Keywords: Enzyme, Fermentation, Solid-State Fermentation, Substrate, Xylanase

1. Introduction

The enzyme is a compound used in the agricultural product processing industry. Enzymes are widely used in the food and non-food industries. One of the enzymes that have high economic value in the industry is xylanase that can be used for bioleaching in the paper industry, improving the quality of bread and animal feed, cleansing and improving the flavor of juice and wine, waste processing and composting [1].

Indonesia imports enzymes reach 2500 tons with an import value of 200 billion in 2017. It increases with an average volume growth rate of 5 percent to 7 percent per year. The increasing use of this enzyme requires extensive and sustainable production. The utilization of lignocellulosic waste is one way to produce xylanase. The lignocellulosic waste consists of cellulose, hemicellulose, and lignin. Xylan is one of the main polymers of hemicellulose which is found and the largest polysaccharide component after cellulose found in the cell wall of plants [2]. Xylanase is a group of enzymes that have the ability to hydrolyzed hemicellulose, in this case, is xylene or polymer of xylose and xylooligosaccharides. Xylan which is a substrate for xylanase enzymes is found in many annual crops and especially agricultural wastes such as *Reutealis trisperma* exocarp.

The high content of hemicellulose in agricultural wastes has the potential to produce xylanase by microorganisms that produce xylanase by utilizing xylan. Xylanase can be produced from microorganisms such as fungi and bacteria [3]. Almost all xylanases are produced by using fungi. The

enzyme activity produced by the fungi is higher than that of bacteria. Enzymes from fungi such as the *Trichoderma* sp. deserve the most attention. *Trichoderma* sp. including *T. reesei, T. harzianum* and *T. viride* are well known as excellent producers of both xylanolytic and cellulolytic enzymes[4].

Research of xylanase production from agricultural waste such as rice, wheat, barley, corn, and soybeans usually uses solid-state fermentation methods. Solid-state fermentation is made from agricultural waste. Agricultural waste that can be developed as raw material for enzyme production is *Reutealis trisperma* exocarp. Several factors including incubation time, pH, carbon and nitrogen, temperature, and substrate concentration significantly influence the production of fungal enzymes during fermentation [5]. Therefore this research was conducted to find the fermentation conditions that would produce high xylanase enzyme activity by combining several variables that could influence the enzyme activity. Substrate concentration is a factor that determines the growth of microorganisms that affect to the activity of the enzymes produced because of the substrate as a source of xylan needed by microorganisms. In addition to substrate concentration, fermentation time also affects xylanase activity to determine the harvest time of xylanase enzymes with high activity. Therefore, research is needed to find out the substrate concentration and fermentation time which produce the highest xylanase enzyme activity from *Trichoderma sp*.

2. Methods

2.1 Raw Material Preparation

Reutealis trisperma exocarp as substrate and the raw material was collected from Manoko Experimental Garden Research Institute, West Bandung Regency, West Java. *Reutealis trisperma* exocarp was washed using tap water and then sun-dried for 12 hours and it was ground using a disc mill. Then, the oven-dried at 105 ° C for 4 hours and sieved manually using a 100 mesh.

2.2 Fungal Preservation

The fungal strain is *Trichoderma sp*. was preserved in potato dextrose agar (PDA) slants. The spore of *Trichoderma sp*. were preserved as immobilized spores on dried rice pellets. The inoculum was prepared by suspending the fungal spores in sterile physiological sodium chloride give a final spore count of 1×10^7 spores/mL.

2.3 Preparation of Cultivation Medium

Media for solid-state fungal cultivation was prepared by mixing dried substrate *Reutealis trisperma* exocarp with concentration of 2%, 4%, 6 %, and 8 %. The substrate as the carbon source for the fungal cultivation with liquid moistening solutions[6]. The moistening solution contained $(NH_4)2SO_4$, KH_2PO_4, urea, $CaCl_2.2H_2O$, and $MgSO_4.7H_2O$. The media was first sterilized in an autoclave at 121^0C for 15 minutes before use.

2.4 Solid-State Fermentation

In the process of determining the substrate concentration and fermentation time that produced the highest enzyme activity, each sample with a concentration of 2%, 4%, 6%, and 8% mixed with 10 mL of mineral medium (moistening solution). The fungal cultivation of 1×10^7 spores/mL was carried out in 250 mL shake flasks at 32.8^0C and was performed throughout 60 h. Sampling was done 12, 24, 36, 48, and 60 h incubation and the obtained samples were used for the analysis of xylanase activity, protein content and xylanase activity-specific [10, 19]. Each run was performed in triple.

2.5 Harvesting of Enzyme

Enzymes were extracted from the fungal cultivation by adding 40 mL distilled water (four times the volume of liquid medium) to the cultivation solution. Subsequently, the solution was stirred with a sterile glass stick and then shaker at 100 rpm for 1 hour at room temperature (25^0C). Then filtered

vacuum to remove solids and particulates using whatmann filter paper. The filtered solution was further centrifuged at 10,000 rpm for 12 minutes at 4 ° C. The obtained supernatant (the crude enzyme) could be used for enzyme activity analysis.

2.6 Analytical Methods

2.6.1 Lignocellulosic material composition

Analysis of chemical components of raw materials aims to determine the lignocellulosic levels contained in the raw material with the Chesson method [7].

2.6.2 Enzyme Activity

Xylanase activity was determined using the DNS method [8]. The analysis was carried out by taking 0.5 mL of crude enzymes and 0.5 mL of 1% xylan mixed with 0.5 acetate buffer ph 5 incubated in a water bath at 40 ° C for 15 minutes. The reaction is stopped by adding 1.5 mL of dinitro salicylic acid (DNS) solution. After incubation in a boiling bath for 5 min, the liberated reducing sugars were measured with a spectrophotometer at 540 nm wavelength. The reducing sugars produced were quantified by the dinitro salicylic acid method using D-xylose as standard. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 μmol of xylose per minute under the assay conditions.

2.6.3 Protein Content

The measurement of protein content in the sample was determined by the Lowry method with Bovine Serum Albumin (BSA) as the standard [9]. The analysis was carried out by taking 0,1-1 mL of crude enzymes added with distilled water until the volume was 4 mL. Then the Lowry reagent is added until the volume was 10 mL, dissolved and leave it for 30 minutes until it forms a blue color. Measure the absorbance at 650 nm.

2.6.4 Enzyme Specific Activity

The specific activity of crude extract of xylanase enzyme states several μmol substrates which can be converted into products within a minute by 1 milligram of the enzyme under optimum conditions. The higher the value of the specific activity of the enzyme, the better the ability of xylanase to utilize the substrate. Calculation of specific activity of crude extract of xylanase enzyme was done by comparing the value of the activity of crude extract of xylanase enzyme with protein content [10].

3. Results and Discussion

3.1 Characterization of Reutealis trisperma exocarp

Characterization of *Reutealis trisperma* exocarp carried out includes testing the levels of cellulose, hemicellulose, and lignin. The results are presented in Table 1.

Tabel 1 Component of Lignocellulostic

Component	Percentage
Hemicellulose	44.48 ±7.83
Cellulose	27.38 ±2.06
Lignin	28.13 ±9.87

Reutealis trisperma exocarp lignocellulosic levels using the Chesson method. Lignocellulose content in each plant is different depending on the type. Hemicellulose levels need to be analyzed to find out how big the potential of *Reutealis trisperma* exocarp as raw materials in the production of xylanase enzymes. The hemicellulose level in *Reutealis trisperma* exocarp used was 44.48 %, which is higher compared with other agricultural waste. Hemicellulose for xylanase production in the hope that xylanase production will increase.

Xylan is a hemicellulose polymer composed of xylose monomers. Hemicellulose is a name to indicate a class of substances including pentoses, hexoses, xylan. The xylan content in hemicellulose

is used for xylanase enzyme production. Also, *Reutealis trisperma* exocarp contained quite high carbon because they contain cellulose of 27.38 %. The component of cellulose as a carbon source for microorganisms. Lignin levels need to be analyzed to determine the large components that can inhibit the enzymatic hydrolysis process. Lignin prevents the entry of enzymes in breaking down polysaccharides into monosaccharides [11]. Lignin, which protects cellulose, is resistant to hydrolysis because it has arylalkyl bonds and ether bonds. The level of lignin is 28.13%. Therefore, before enzyme production is carried out, delignification is carried out.

3.2 Concentrations of Trichoderma sp cell

Trichoderma sp cell concentrations were performed by calculating cell growth using the TPC method in each sample. The growth curve of *Trichoderma sp.* can be seen in Figure 1. Based on the results of the analysis, the concentration of *Trichoderma sp* showed an increase during fermentation at a higher concentration according to the time of its growth. The highest number of *Trichoderma sp* growth at 48 hours was seen from the graph increase at each concentration, and the concentration that produced the highest cell growth was 8%. The cells concentrations produced at each hour and this concentration is influenced by cell growth. Cell growth is an increase in the number and size of cells. In this study the calculation of cells in each sample that has been fermented. The growth curve of microorganisms consists of 4 phases, namely the adaptation phase, the logarithmic phase, the stationary phase, and the death phase [12].

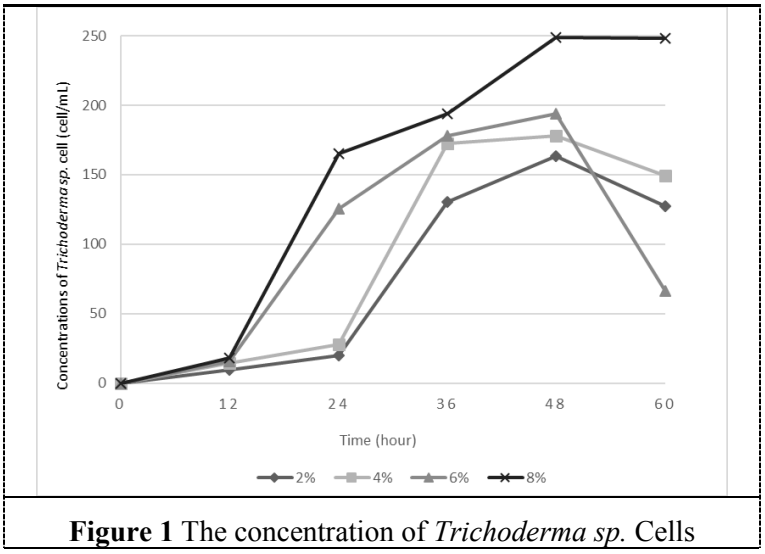


Figure 1 The concentration of *Trichoderma sp.* Cells

Cell growth begins by showing a lag phase at 12-hour fermentation. The lag phase is the adjustment period in the environment. If the microbes are transferred into a medium, they will first undergo an adaptation phase to adapt to the surrounding environmental conditions. Based on the results of the study, at 12 hours the cells have increased in concentration even though the increasing are small. The lag phase of the growth curve takes place rapidly at each concentration. This is evidenced by the curve that goes up directly with cell growth. This proves the cell is going into an exponential growth phase which is seen at 24 hours until 48 hours. The decline occurs very quickly due to the depletion of nutrient sources found in cell growth media and enters the death phase because cell growth begins to stop and deplete reserve energy for respiration, so cells dead. The stationary phase in this study was on average not visible, only at 8% concentration, namely 48 and 60 hours. At 48 hours was 248.6 x 10^8 and 60 hours 248.1 x 10^8 cells/mL. The decrease of concentration of cells only slightly. At this concentration, the cell undergoes a stationary phase. While the concentration of 2-6% to 60 hours is the death phase. Based on the results of the study, the concentration of 8% with 48 hours of fermentation time has a nutrient content and a high growth time.

3.3 The Activity of Xylanase Crude Extract

An incubation time search is performed to determine the growth time of microorganisms that produce the highest xylanase activity in xylanase production. The process of finding the incubation time was applied to *Trichoderma sp*. with a temperature of 32.8 ° C. Sampling was carried out every 12 hours for 60 hours and the enzyme activity test was carried out on each sample. The results of the enzyme activity evaluation are shown in Figure 2.

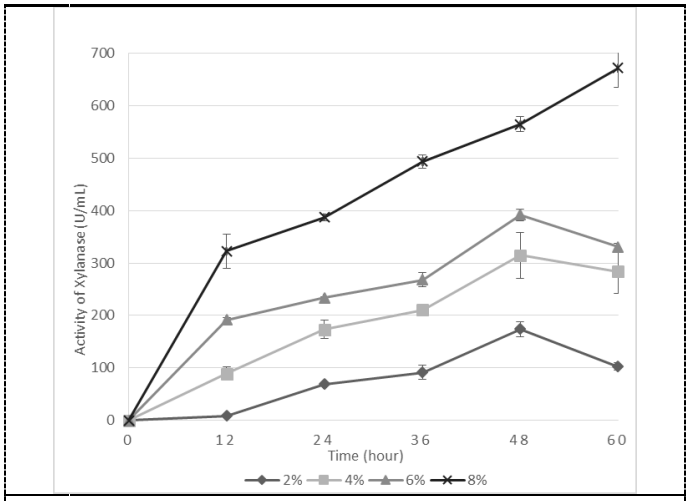
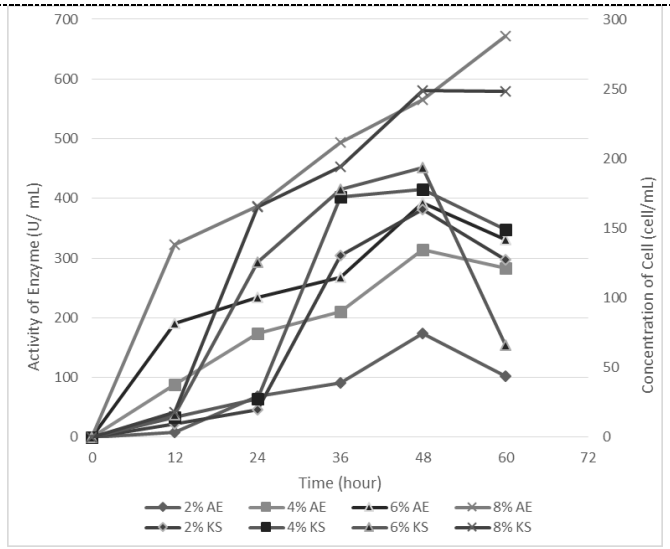


Figure 2 Activity of Xylanase

Based on the picture above, it can be seen that each substrate concentration within a certain time produces different enzyme activities. The highest enzyme activity is at a concentration of 8%, at the 60 hours with a value of 672.039 U / mL and the lowest is a concentration of 2% at the 12 hours, which is 8.389 U / mL. Substrate fermentation at 2-6% concentration results in low enzyme activity, and 8% substrate is the highest enzyme activity. This is caused by the higher concentration causing high xylan content also for the production of xylanase enzymes by *Trichoderma sp*. If the xylan is getting higher, the substrate that will be converted to xylose is more and more, so that the xylanase enzyme as the desired product released by *Trichoderma sp* is higher.

Enzyme activity is still low at the beginning of the 12-hour fermentation, and activity increases with increasing fermentation time up to the 48 hours and tends to decrease at the 60 hours, except at a concentration of 8% it still experiences an increase at the 60 hours. Enzyme activity increases with an increasing number of *Trichoderma sp*. It means that the enzyme activity is influenced by the growth of *Trichoderma sp.* Further increase of fermentation period beyond this resulted in a decline in enzyme production which might be due to the production of toxic metabolites during microbial growth which inhibits enzyme synthesis.

The relationship between time and enzyme activity is almost the same as the growth curve of microorganisms. Cell growth can be seen in the previous cell growth curve. The curve shows an increase that is in tune with the activity of the enzyme. The number of microorganisms rapidly increased and was comparable with the high activity of the xylanase produced. High cell growth, causes many cells of microorganisms that degrade xylan substrates to xylose. To change the xylan, microorganisms release the xylanase enzyme. The relationship between enzyme activity and cell concentration can be seen in Figure 3.



AE = Activity of Enzyme, KS = Concentration of Cell

Figure 3 The activity of Enzyme and Concentration of cell Relationship

Trichoderma sp. secreting endoxylanases with an activity of 2836 U/g (dry weight) in solid fermentation [13]. Xylanase production by *Bacillus sp*. reported that maximum xylanase production was observed in 48 h and 72 h using wheat bran and corn cob as a substrate respectively [14]. In another study, some strains of *Bacillus* showed maximum xylanase production after 24 h using digested bran and 48 h of fermentation using sawdust as a substrate respectively[15]. Production of xylanase by *Trichoderma sp*. using sugarcane bagasse reported than the highest xylanase enzyme activity was 380 U/g [16]. So it can be concluded that the production of xylanase enzymes produces xylanase activity that varies depending on microorganisms and the substrate used.

3.4 Protein Content

Enzymes are the most common group of proteins in living cells and have an important function such as catalysts of biochemical re-actions that collectively form metabolisms-intermediates from cells. The protein content was used as one of the measurement due to the solid-state fermentation caused the cells to mix with the substrate, making it difficult to calculate the dry weight cells. The protein testing process, similar to enzyme activity, was applied to *Trichoderma viride* with a temperature of 32.8°C. Samplings were performed every 12 hours for 60 hours and protein testings were performed on each sample.

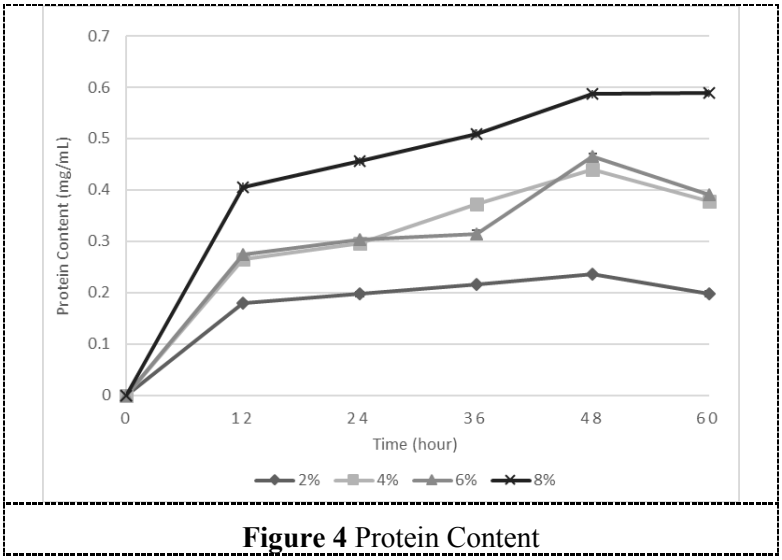


Figure 4 Protein Content

Protein at each concentration reaches its highest value at different times. The protein produced is 8% the highest compared to other concentrations. At a concentration of 4% specific activity fluctuates at 24 and 36 hours, and at a concentration of 6%, specific activity decreases from 48 to 60 hours. The highest protein content was produced in this study at 48 hours of 8% concentration with a protein content of 0.585 mg/mL. While low protein levels are produced by a concentration of 2% at 12 hours. This result shows the activity and protein content is directly similar trend. Protein levels continue to increase with optimal fermentation time (up to 48 hours). Then a decrease in protein content occurs. *Trichoderma* sp in the substrate overhaul is increasingly so that the resulting protein will be used as a source of nitrogen for the nutrition of *Trichoderma sp.*

Compare with other research, xylanase was produced by *Aspergillus terreus* cultivated on finely ground wheat straw in solid-state fermentation produced 333.3 U/mg protein [17]. Production of xylanase by *Trichoderma viride* reported that protein content produced 0.6875 mg/mL using corncobs as a substrate [18]. Protein is very sensitive to the physical effects of chemicals and is easy to change shape. Changes or modifications to the structure of a protein molecule are called denaturation. Denaturation caused by heat, pH, pressure, electric current, and the presence of chemicals such as urea, alcohol, and soap. Temperature is the midpoint of the denaturation process. If the temperature is less than 100ºC, then the protein will experience denaturation.

3.5 Specific Activity of Xylanase Enzyme

The specific activity of xylanase crude extract states a number of µmol substrates which can be converted into products within a minute by 1 milligram of the enzyme under optimum conditions. The higher the value of the specific activity means the better the ability of xylanase to utilize the substrate. The calculation of specific activity was done by comparing the value of enzyme activity with protein content.

Enzyme specific activity, in general, has increased until a certain time. The 2% concentration had high protein content at 48 hours. The highest value of enzyme specific activity at a concentration of 6% and 8% was at 60 hours. While the concentration of 4% at 36 hours. It shows that *Trichoderma sp*. most active in using *R. trisperma* exocarp to produce a crude extract of the xylanase enzyme within one minute after the fermentation process is done during that time.

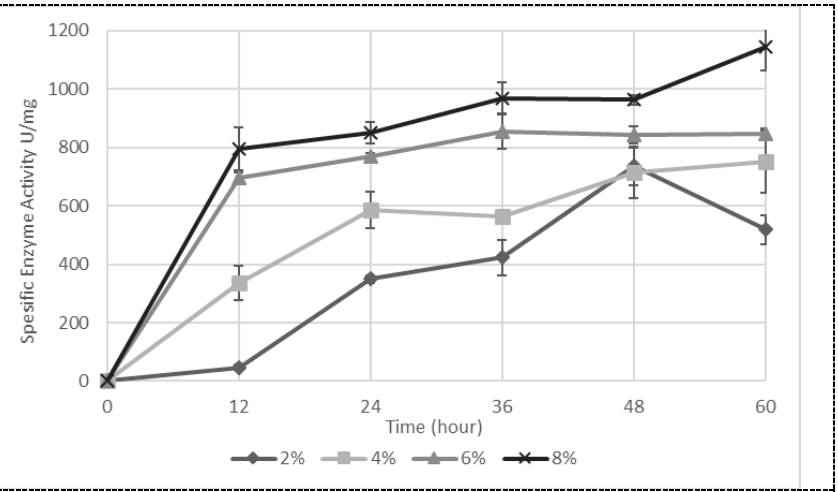


Figure 5 Specific Enzyme Activity

High enzyme activity is not always followed by a high specific activity. The highest specific activity of crude xylanase enzyme extract was obtained with a concentration of 8% at 60 h. The enzyme specific activity value obtained was 1137.638 U/mg. This is the same as the highest enzyme activity produced by the same concentration and fermentation time Concentration of 8 % is always products high enzym activities, high proteins, and high enzyme specific activities.

A research reported that xylanase was produced from Corncob under Solid-State Fermentation produce that the best incubation time is 36 h with 1727.669 U/mL for specific xylanase activity [18]. It shows that enzyme specific activities were obtained early at a different time fermentation depends on the treatment and conditions of the process.

4. Conclusions

The production of crude xylanase from *Reutealis trisperma* exocarp as substrate using *Trichoderma sp*. in solid-state fermentation was obtained. The highest enzyme activity, protein content and cell growth have been achieved under condition of 8% substrate concentration with 60 hours fermentation time. Substrate concentrations of 2%, 4%, and 6% produce high enzyme activity at 48 hours and have decreased at 60 hours. Cell concentration has increased until 48 hours fermentation and decreased at 60 hours.

References

[1] Polizeli M L T M, Rizzatti A C S, Monti R, Terenzi H F, Jorge J A, and Amorim D S 2005 Xylanases from fungi: properties and industrial applications *Applied Microbiology and Biotechnology* **67**(5) 577–591.

[2] Knob A and Carmona E C 2010 *Purification and Characterization of Two Extracellular Xylanases from Penicillium sclerotiorum: A Novel Acidophilic Xylanase* 429–443.

[3] Lakshmi G S, Bhargavi P L, and Prakasham R S 2011 Sustainable Bioprocess Evaluation for Xylanase Production by Isolated Aspergillus terreus and Aspergillus fumigatus Under Solid - State Fermentation Using Oil Palm Empty Fruit Bunch Fiber *Current Trends in Biotechnology and Pharmacy* **5**(4) 1434–1444.

[4] Wong K K, Tan L U, and Saddler J N 1988 The multiplicity of beta-1,4-xylanase in microorganisms: functions and applications *Microbiological Reviews* **52**(3) 305–317.

[5] Xiong H, Nyyssölä A, Jänis J, Pastinen O, Von Weymarn N, Leisola M, and Turunen O 2004 Characterization of the xylanase produced by submerged cultivation of Thermomyces lanuginosus DSM 10635 *Enzyme and Microbial Technology* **35**(1) 93–99

[6] Alves-Prado H F, Pavezzi F C, Leite R S R, de Oliveira V M, Sette L D, and Dasilva R 2010 Screening and Production Study of Microbial Xylanase Producers from Brazilian Cerrado.

Applied Biochemistry and Biotechnology **161**(1–8) 333–346
[7] Datta S K De (n.d.) *Principles and Practices of Rice Production* Philippines: Int. Rice Res. Inst.
[8] Miller G L 1959 Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar *Analytical Chemistry* **31**(3) 426–428
[9] Lowry O H, Rosebrough N J, Lewis A, and Randall R J 1951 *The folin by oliver*.
[10] Mardawati E, Parlan, Rialita T, and Nurhadi B 2018 Optimization of moistening solution concentration on xylanase activity in solid state fermentation from oil palm empty fruit bunches *IOP Conference Series: Earth and Environmental Science*, *141*(1)
[11] Schultz-Jensen N, Kádár Z, Thomsen A B, Bindslev H, and Leipold F 2011 Plasma-assisted pretreatment of wheat straw for ethanol production *Applied Biochemistry and Biotechnology*, **165**(3–4), 1010–1023.
[12] Willey J M, Sherwood L, and Woolverton C J 2008 *Prescott, Harley, and Klein's Microbiology* McGraw-Hill Higher Education
[13] Chen L L, Zhang M, Zhang D H, Chen X L, Sun C Y, Zhou B C, and Zhang Y Z 2009 Purification and enzymatic characterization of two β-endoxylanases from Trichoderma sp. K9301 and their actions in xylooligosaccharide production *Bioresource Technology* **100**(21) 5230–5236
[14] Bain R V S, De Alwis M C L, Carter G R, and B K G 1982 *Haoemorrhagic Septicaemia* Rome: FAO of the United Nations
[15] Simphiwe P B, Ademola O O, and Balakrishna P 2011 Sawdust and digestive bran as cheap alternate substrates for xylanase production *African Journal of Microbiology Research* **5**(7) 742–752.
[16] Norazlina I, Meenalosani N, Selangor U, Campus B J, Tambahan J T, Selangor U, Mara U T 2013 Production of Xylanase by Trichoderma sp. Via Solid State Culture Using Sugarcane Bagasse *International Journal of Energy Science* **3**(2) 99–105.
[17] Ghanem N B, Yusef H H, and Mahrouse H K 2000 Production of Aspergillus terreus xylanase in solid-state cultures: Application of the Plackett-Burman experimental design to evaluate nutritional requirements *Bioresource Technology* **73**(2) 113–121.
[18] Mardawati E, Pratiwi S M, Andoyo R, Rialita T, Djali M, Cahyana Y, and Setiasih I S 2017 Ozonation Pretreatment Evaluation for Xylanase Crude Extract Production from Corncob under Solid-State Fermentation *Journal of Industrial and Information Technology in Agriculture* **1**(2) 27.
[19] Mardawati E, Kresnowati M T A P, Purwadi R, Bindar Y, Setiadi T 2018 Fungal Production of Xylanase from Oil Palm Empty Fruit Bunch via Solid State Fermentation *International Journal on Advance Science Engineering and Information Technology* **8**(6) 2539-2547.