International Jurnal Islamic Education, Research and Multiclturalism
(IJIERM)
Available online https://journal.yaspim.org/index.php/IJIERM/index

The utilization of Trichoderma Viride in Optimising Xylanase Production from Coffee Cherry Processing Waste

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Abstract: The use of enzymes in Indonesia is increasing rapidly and is used in various industries, especially FCMG. One of the enzymes that is widely used is xylanase, which is an extracellular enzyme that can hydrolyze xylan into xylose and xylo-oligosaccharides. This research aims to determine the optimum conditions for the xylanase production process from coffee cherry processing waste (CCPW) using Trichoderma viride through solid fermentation. The research method used was experimental using central composite design (CCD) in Design Expert 13.0 software. The treatment designs used were fermentation times of 24 hours, 48 hours, and 72 hours; and substrate concentrations of 25%, 50%, and 75%. The experimental response analysis design was enzyme activity, protein content, and specific activity. The results of the research show that the optimum conditions for xylanase production are at a substrate concentration of 75% and a fermentation time of 24 hours with a desirability value of 0.742, which produces an enzyme activity value of 36.388 U/mL and a dissolved protein content of 0.494 mg/mL with a specific activity of 73.660. U/mg. The verification results show conformity with the formula predicted by the Design Expert 13.0 software, namely enzyme activity of 36,292 U/mL and protein content of 0.488 mg/mL with a specific activity of 74,369 U/mg.

Keywords: natural sweetener, wood sugar, bioengineering process, sucrase

INTRODUCTION

Xylanase can be produced by microorganisms in the form of mold or bacteria through a solid-state fermentation (SSF) process. SSF has advantages including, fermentation media being more affordable, equipment and operation being simple but the amount of product produced is high [1], high fermentation productivity, high product concentration and stability [2], cost-effectiveness, and low consumption. low energy and simple technique [3]. The use of microorganisms also has advantages, namely, high speed of microbial growth, short production time, easy control, and relatively low production costs. The xylanase activity produced by mold was higher than bacteria on various substrates [4].

This research uses Trichoderma viride, which is one of the most frequently found molds among its types [5]. The advantages of Trichoderma viride are that it can grow quickly on a variety of simple substrates and mediums, does not use additional nutrients for growth, and has a pH range of 2.5-5 (acid). Based on research, it is known that Trichoderma viride produces cellulolytic and xylanolytic enzymes which can hydrolyze xylan very well [6].

Xylanase production by microorganisms requires a substrate to serve as a source of energy and nutrients for their growth. Generally, the substrate used in enzyme production is pure xylan, but its use is considered uneconomical because it is relatively expensive and must be imported. Abundant agro-industrial waste and its less-than-optimal utilization is an alternative to pure xylan because chemically it

[1] Mardawati, E., Sinurat, Y., & Yuliana, T. (2020, June). Production of crude xylanase from Trichoderma sp. using Reutealis trisperma exocarp substrate in solid state fermentation. In *IOP Conference Series: Earth and Environmental Science* (Vol. 515, No. 1, p. 012024). IOP Publishing.

[2] Maftukhah, S. (2020). Aplikasi Bacillus sp Pada Produksi Enzim Menggunakan Metode Fermentasi Padat-Review. *Jurnal Pendidikan dam Aplikasi Industri*, *7*(1), 6-9.

[3] Indriani, D. O., Syamsudin, L. N. I., Wardhani, A. K., & Wardani, A. K. (2015). Invertase dari Aspergillus niger Dengan Metode Solid State Fermentation dan Aplikasi Di Industri: Kajian Pustaka [IN PRESS SEPTEMBER 2015]. *Jurnal Pangan dan Agroindustri*, *3*(4).

[4] El-Gendi, H., Saleh, A. K., Badierah, R., Redwan, E. M., El-Maradny, Y. A., & El-Fakharany, E. M. (2021). A comprehensive insight into fungal enzymes: Structure, classification, and their role in mankind's challenges. *Journal of Fungi*, *8*(1), 23.

[5] Bhardwaj, N., Kumar, B., & Verma, P. (2019). A detailed overview of xylanases: an emerging biomolecule for current and future prospective. *Bioresources and Bioprocessing*, *6*(1), 1-36.

[6] Istiqomah, L., Cahyanto, M. N., & Zuprizal, Z. (2022). Xylanase production by Trichoderma virens MLT2J2 under solid-state fermentation using corn cob as a substrate. *Biodiversitas Journal of Biological Diversity*, *23*(12).

contains lignocellulose (cellulose, hemicellulose, and lignin) [7]. One of the agro-industrial wastes that contains lignocellulosic is coffee cherry processing waste (CCPW). The coffee waste contains high lignocellulosic materials. The high cellulose and hemicellulose content makes coffee bean shells potentially used as a substrate to produce xylanase. Apart from that, the use of coffee bean shells also aims to increase the added value of this waste [8].

Various factors influence enzyme production, namely substrate concentration, pH, temperature, aeration, and fermentation time. Several factors need to be combined to get the xylanase enzyme with maximum results [9]. Substrate concentration is the main factor determining enzyme production because the presence of substrate can be used as a source of energy needed by microorganisms, thereby influencing the activity of the enzyme produced [10]. Substrate concentration influences the contact of enzymes and highly specific substrates [11]. Apart from substrate concentration, fermentation time also influences xylanase production by microorganisms because it determines the harvest time of xylanase enzymes with high activity[4]. The growth period of mold varies greatly and has several growth phases in its metabolic activity. The effective time for enzyme production is in the exponential phase (24 to 48 hours) because in this phase mold is very effective in producing enzymes so that their life needs are met [12].

Several studies used substrate concentration and fermentation time in the production of xylanase enzymes. The xylanase production was conducted using Sunan candlenut shells and Trichoderma at varying substrate concentrations of 2%, 4%, 6%, and 8% with fermentation times of 12, 24, 36, 48, and 60 hours, producing the highest xylanase activity of 672,039 U/mL was produced at a substrate concentration of 8% and a fermentation time of 60 hours [13]. The conversion of corncob to produce

[7] Ravindran, R., Williams, G. A., & Jaiswal, A. K. (2019). Spent coffee waste as a potential media component for xylanase production and potential application in juice enrichment. *Foods*, *8*(11), 585.

[8] Cerino-Córdova, F. J., Dávila-Guzmán, N. E., León, A. M. G., Salazar-Rabago, J. J., & Soto-Regalado, E. (2020). Revalorization of coffee waste. *Coffee-production and research*, 1-26.

[9] Jnawali, P., Kumar, V., Tanwar, B., Hirdyani, H., & Gupta, P. (2018). Enzymatic production of xylooligosaccharides from brown coconut husk treated with sodium hydroxide. *Waste and Biomass Valorization*, *9*, 1757-1766.

[10] Salmanizadeh, H., Beheshti-Maal, K., Nayeri, H., & Torabi, L. R. (2023). Optimization of xylanase production by Pichia kudriavzevii and Candida tropicalis isolated from the wood product workshop. *Brazilian Journal of Microbiology*, 1-14.

[11] Bhardwaj, N., Kumar, B., & Verma, P. (2019). A detailed overview of xylanases: an emerging biomolecule for current and future prospective. *Bioresources and Bioprocessing*, *6*(1), 1-36.

[12] Wösten, H. A. (2019). Filamentous fungi for the production of enzymes, chemicals and materials. *Current opinion in biotechnology*, *59*, 65-70.

[13] Mardawati, E., Sinurat, Y., & Yuliana, T. (2020, June). Production of crude xylanase from Trichoderma sp. using Reutealis trisperma exocarp substrate in solid state fermentation. In *IOP Conference Series: Earth and Environmental Science* (Vol. 515, No. 1, p. 012024). IOP Publishing.

xylanase using Trichoderma longibrachiatum at varying substrate concentrations of 5%, 15%, and 25% with fermentation times of 24, 72, and 120 hours produced the highest xylanase activity of 62.43 U/mL [14]. achieved in treatment with a substrate concentration of 25% with 24 hours of fermentation time.

Based on the background explanation above, the xylanase enzyme can be produced at varying substrate concentrations and fermentation times depending on the type of substrate and microorganisms used. Therefore, this research needs to be carried out to determine the substrate concentration and optimum fermentation time in the production of xylanase using coffee bean shells and Trichoderma viride using the solid-state fermentation method. The optimum substrate concentration and fermentation time can be determined by optimizing using Response Surface Methodology (RSM). RSM is used because it can minimize experimental data and the number of samples to be tested, thereby shortening time and making research easier[15].

RESEARCH METHODS

The main material used was CCPW as raw material or substrate which was obtained from PDP Kahyangan Jember, East Java, Indonesia. It was first prepped by washing and then dried in an oven for 18 hours at 105^0C. After being dried, the material was processed in a disc mill and sieved through a mesh size of less than 80. The lignocellulosic and water contents of the raw material are next measured. The chemicals utilized in this study were acquired from Merck.

CCD research design for RSM optimization used two factors i.e. substrate concentration (%), and fermentation time (hours). The responses determined were xylanase enzyme activity (U/ml), and protein content (mg/ml). The RSM research design is shown in Table 1 and the level of each factor used after observed in preliminary research are as follows:

Substrate concentration

a) concentration of 25% ($X1_1$ =1)
b) concentration of 50% ($X1_2$ =1)
c) concentration of 75% ($X1_{13}$=1)

fermentation time

a) concentration of 24 hours ($X2_1$ =1)
b) concentration of 48 hours ($X2_2$ =1)

[14] Wösten, H. A. (2019). Filamentous fungi for the production of enzymes, chemicals and materials. *Current opinion in biotechnology*, *59*, 65-70.

[15] Mardawati, E., Sinurat, Y., & Yuliana, T. (2020, June). Production of crude xylanase from Trichoderma sp. using Reutealis trisperma exocarp substrate in solid state fermentation. In *IOP Conference Series: Earth and Environmental Science* (Vol. 515, No. 1, p. 012024). IOP Publishing.

c) concentration of 72 hours ($X2_3$ =1)

Table 1. Optimization treatment design

Run	Treatment Variable		Response	
	Substrate concentration (%)	Fermentation time (hours)	Xylanase enzyme activity (U/ml)	Protein Content (mg/ml)
1	14.6447	48	Y_1	Y_2
2	50	48	Y_1	Y_2
3	50	82	Y_1	Y_2
4	25	72	Y_1	Y_2
5	50	48	Y_1	Y_2
6	50	48	Y_1	Y_2
7	50	48	Y_1	Y_2
8	25	24	Y_1	Y_2
9	75	24	Y_1	Y_2
10	50	14	Y_1	Y_2
11	75	72	Y_1	Y_2
12	85.3553	48	Y_1	Y_2
13	50	48	Y_1	Y_2

The initial calculation data is processed in Microsoft Excel which will then be analyzed using Design Expert 13.0® software. The results of each variable calculation of enzyme activity and protein content were analyzed using Analysis of Variance (ANOVA) and the model with the highest R2 level was selected which provided significance in ANOVA and non-significance in Lack of Fit. The results of the calculation of enzyme activity and protein levels are then optimized using predetermined criteria and will produce recommendations for several optimal formulas. After obtaining recommendations for optimum process conditions, verification is then carried out to prove the accuracy of the recommended predicted values. The verification stage carried out production and analysis again 3 times following the best formulation resulting from previous recommendations. The results obtained at the verification stage are then compared with the values predicted by RSM so that the suitability of the results can be seen.

Rejuvenation of *Trichoderma viride* Isolate [16]

The mold isolate used in this research was Trichoderma viride. The Trichoderma viride isolate was rejuvenated in a test tube by taking it using a loop needle aseptically and streaking it in a zigzag manner on a slanted PDA medium then incubating at a temperature of 32.8°C for 72 hours.

[16] Mardawati, E., Sinurat, Y., & Yuliana, T. (2020, June). Production of crude xylanase from Trichoderma sp. using Reutealis trisperma exocarp substrate in solid state fermentation. In *IOP Conference Series: Earth and Environmental Science* (Vol. 515, No. 1, p. 012024). IOP Publishing.

Trichoderma viride was inoculated. The inoculum was prepared by suspending Trichoderma
viride spores in 5 mL of 5% physiological NaCl which was shaken until homogeneous and no spores remained in the test tube. The absorbance of the solution was then measured using a Uv-Vis spectrophotometer at 600 nm to produce a cell concentration of 0.616 or the equivalent of 10^6-10^8 spores/mL according to the McFarland standard. The inoculum is then ready to be added for enzyme production.

Xylanase enzyme production [17]

The enzyme production process begins with preparing cell cultivation media. It is a growth medium that provides the nutrients needed for the growth of microorganisms to increase enzyme production. Cultivation media is prepared by weighing coffee bean shell powder which is used as a source of carbon nutrition according to the treatment design in RSM. The composition of the moistening solution in 1000 mL of distilled water is 1.5 g $(NH_4)2SO_4$, 2 g KH_2PO_4, 0.35 g urea, 0.03 g $CaCl_2.2H_2O$, and 0.2 g $MgSO_4.7H_2O$.

The production of xylanase using the solid-state fermentation method begins by mixing each coffee bean shell powder with 30 mL of moistening solution, then homogenizing it in an Erlenmeyer flask. Before the media is used, it is sterilized using an autoclave at 121°C for 15 minutes to avoid contamination. After sterilization, the media was left at 20-25°C (room temperature). 3 mL of inoculum was added to each medium or the same as 10% of the medium used. Next, incubation was carried out in an incubator at a temperature of 32.8°C according to the RSM fermentation time plan. The enzymes produced from the fermentation process were harvested by adding 90 mL of 5% physiological NaCl (three times the initial solution) until the volume was 120 mL, then homogenized with an orbital shaker at a speed of 200 rpm for 1 hour at a temperature of 25°C. Next, centrifuged at 3,500 rpm for 15 minutes at 4°C until the supernatant and dregs were separated. The resulting supernatant was designated as an xylanase enzyme. The resulting xylanase enzyme was then analyzed for enzyme activity and protein content.

Xylanase enzyme activity [18]

Xylanase activity was determined by the DNS test which was carried out by preparing a blank solution by mixing 1 g of xylan with 100 mL of distilled water (1%

[17] Mardawati, E., Rialita, T., & Nurhadi, B. (2018, March). Optimization of moistening solution concentration on xylanase activity in solid state fermentation from oil palm empty fruit bunches. In *IOP Conference Series: Earth and Environmental Science* (Vol. 141, No. 1, p. 012018). IOP Publishing.

[18] Mardawati, E., Rialita, T., & Nurhadi, B. (2018, March). Optimization of moistening solution concentration on xylanase activity in solid state fermentation from oil palm empty fruit bunches. In *IOP Conference Series: Earth and Environmental Science* (Vol. 141, No. 1, p. 012018). IOP Publishing.

xylan). Take 0.5 mL of 1% xylan solution then mix it with 0.5 mL of distilled water and add 0.5 mL of pH 5 acetate buffer, then incubate in a water bath at 40°C for 15 minutes. The reaction was stopped by adding 1.5 mL of DNS and then boiling in a water bath for 5 minutes. The absorbance of the solution was measured using a UV-Vis spectrophotometer at 550 nm. The blank solution is a mixture of 1% xylan substrate, distilled water, and acetate buffer with an incubation treatment which is then added with DNS reagent without adding the xylanase enzyme extract.

Xylose analysis of the sample was carried out by dissolving 0.5 mL of xylanase enzyme in 0.5 mL of distilled water and mixing it with 0.5 mL of 1% xylan. The mixture was added with 0.5 mL of pH 5 acetate buffer and incubated in a water bath at 40°C for 15 minutes. The reaction was stopped by adding 1.5 mL of DNS and then boiling in a water bath for 5 minutes. The absorbance of the solution was measured using a UV-Vis spectrophotometer at 550 nm. The sample solution is a reaction between a mixture of 1% xylan substrate, distilled water, xylanase enzyme extract, and acetate buffer with incubation treatment to which DNS reagent is then added.

The activity of the xylanase enzyme was determined by substituting the absorbance value obtained in each measurement into the equation for the xylose standard curve. Xylanase enzyme activity was calculated based on Equation 1.

$$U = \frac{(Xylose\ concentration\ on\ sample - blank\ xylose\ concentration)\ x\ 100}{Xylose\ molecular\ weight\ x\ incubation\ time\ x\ volume\ of\ enzyme\ used\ for\ analysis}$$

…. (Eq 1)

Protein Content Evaluation [19]

Measurement of protein levels in samples was carried out using the Bradford method and using BSA (Bovine Serum Albumin) as a standard protein. Measurement of dissolved protein in the sample was carried out by mixing 0.5 mL of enzyme and 5 mL of Bradford reagent, then vortexing and incubating at room temperature for 30 minutes. Next, the absorbance was measured at 595 nm using a UV-Vis spectrophotometer. The absorbance results are then substituted into the regression equation on the BSA standard curve y = ax + b so that dissolved protein levels will be obtained.

[19] Mahardhika, B. P., Ridla, M., Mutia, R., & Adli, D. N. (2021, October). The evaluation of protease enzyme effectivenes in broiler chicken diet containing jack bean seed (Canavalia ensiformis) with different protein level toward internal organ size. In *IOP Conference Series: Earth and Environmental Science* (Vol. 883, No. 1, p. 012012). IOP Publishing.

Xylanase Specific Activity [20].

The results of measuring protein levels are then used to determine the specific activity of the xylanase enzyme. The specific activity of an enzyme is obtained from enzyme activity units divided by the protein content value, which is expressed in units per milligram. Specific activity can be calculated with equation 2.

$$Specific\ xylanase\ activity = \frac{Xylanase\ enzyme\ activity}{Protein\ content}$$

….. (eq 2)

RESULTS AND DISCUSSION

Robusta and Arabica coffee varietals were combined in a 1 to 1 ratio to create the coffee cherries processing waste (CCPW) used in this research. Based on the lignocellulose content analysis method by NREL (2007) in CCPW there is 38.5% cellulose, 15.8% hemicellulose, and 18.99%.

Xylanase Enzyme Activity

Table 2. Response Results of Xylanase Enzyme Activity of Substrate Concentration and Fermentation Time

Run	Treatment Variable		Xylanase Enzyme Activity (U/mL)
	Substrate Concentration (%)	Fermentation Time (hour)	
1	14.6447	48	4.934
2	50	48	17.927
3	50	82	14.802
4	25	72	33.551
5	50	48	9.210
6	50	48	11.184
7	50	48	7.894
8	25	24	6.579
9	75	24	34.867
10	50	14	11.842
11	75	72	38.814
12	85.3553	48	45.393
13	50	48	15.460

[20] Kamble, R. D., & Jadhav, A. R. (2012). Isolation, purification, and characterization of xylanase produced by a new species of Bacillus in solid state fermentation. *International Journal of Microbiology*, *2012*.

Based on Table 2, the highest enzyme activity occurred in the treatment with a substrate concentration of 85.35% and a fermentation time of 48 hours, namely 45,228 U/mL with a xylose concentration of 358,642 ppm. Meanwhile, the lowest enzyme activity occurred in the treatment with a substrate concentration of 14.64% and a fermentation time of 48 hours, namely 4,934 U/mL with a xylose concentration of 343,519 ppm. Xylanase activity is directly proportional to xylose content, the higher the enzyme activity, the higher the xylose produced. Apart from that, enzyme activity is also directly proportional to the substrate concentration, the more substrate, the higher the reaction rate catalyzed by the enzyme, so the enzyme activity is higher. This is caused by the higher substrate concentration in line with the high xylan content. The higher the xylan, the more substrate that will be hydrolyzed into xylose, so that the product (xylanase enzyme) that will be released will also be higher [21]. The high enzyme activity is due to the increasingly optimal performance of the enzyme in degrading the substrate, so that the product produced increases. The increase in xylanase production occurs due to a balance between microbial biomass and substrate as nutrients to produce optimal enzyme products [22].

The concentration of substrate used in fermentation must be balanced. Using a substrate with the right concentration can increase the reaction rate, but if the amount of substrate is too high it can reduce the enzymatic reaction rate because the active site of the enzyme is saturated. If the substrate concentration used is very low, the nutrients used by *Trichoderma viride* as energy for its growth will also be less, so enzyme production is less than optimal. The enzyme activity increases with higher substrate concentrations and increasing numbers of Trichoderma [23].

Fermentation time also affects enzyme activity, where each microorganism will need a different time to produce an enzyme. Fermentation time is the time required for *Trichoderma viride* to produce xylanase to hydrolyze the substrate into xylose and xylo-oligosaccharides. The optimal conditions for Trichoderma growth occur in the exponential phase, between 24 to 48 hours of fermentation [24]. If a small amount of fermentation time is used then a small amount of enzyme is produced, this is because the interaction time of *Trichoderma viride* with the substrate is very short and does not take place in its entirety [5]. To a certain extent, increasing fermentation

[21] Mardawati, E., Sinurat, Y., & Yuliana, T. (2020, June). Production of crude xylanase from Trichoderma sp. using Reutealis trisperma exocarp substrate in solid state fermentation. In *IOP Conference Series: Earth and Environmental Science* (Vol. 515, No. 1, p. 012024). IOP Publishing.

[22] Bhardwaj, N., Kumar, B., & Verma, P. (2019). A detailed overview of xylanases: an emerging biomolecule for current and future prospective. *Bioresources and Bioprocessing*, *6*(1), 1-36.

[23] El-Gendi, H., Saleh, A. K., Badierah, R., Redwan, E. M., El-Maradny, Y. A., & El-Fakharany, E. M. (2021). A comprehensive insight into fungal enzymes: Structure, classification, and their role in mankind's challenges. *Journal of Fungi*, *8*(1), 23.

[24] Mardawati, E., Rialita, T., & Nurhadi, B. (2018, March). Optimization of moistening solution concentration on xylanase activity in solid state fermentation from oil palm empty fruit bunches. In *IOP Conference Series: Earth and Environmental Science* (Vol. 141, No. 1, p. 012018). IOP Publishing.

time cannot increase the activity of the enzymes produced. This occurs at a fermentation time of 82 hours which has lower enzyme activity than a fermentation time of 72 hours. This is because the enzyme has been denatured so that no new product is formed. Denaturation will result in changes in the structure of the enzyme so that the amount of substrate that can be bound by the active site of the enzyme and its activity decreases.

Trichoderma viride can produce xylanase on soybean skin substrate with an activity of 18.71 U at a fermentation time of 60 hours [25]. The production of xylanase enzymes from sugarcane bagasse substrate resulted in the highest activity of 5.43 U/mL when treated with a substrate concentration of 2.5% for 14 hours of fermentation time with hemicellulose contained at 27.97% [26]. The production of xylanase from corncob substrate carried out resulted in the highest activity of 62.43 U/mL achieved in the treatment with a substrate concentration of 25% with 24 hours of fermentation time [27]. It can be concluded that the production of the xylanase enzyme will produce varying enzyme activity depending on the microorganism and substrate used.

The differences in enzyme activity values for each treatment indicate that molds are microorganisms that have varying performances in utilizing nutrients from substrates and producing their metabolites so that molds will produce high enzyme activity under appropriate environmental conditions. An increase or decrease in enzyme activity is caused by using too much substrate or an incubation time that is too long because enzyme activity is influenced by the concentration of available substrate [28].

Protein Contain

The calculating results of xylanase protein levels are presented in Table 3.

Table 3. Response Results of Protein Content of Substrate Concentration and Fermentation Time

Run	Treatment Variable		Protein Content Level (mg/ml)
	Substrate Concentration (%)	Fermentation Time (hour)	

[25] Mardawati, E., Sinurat, Y., & Yuliana, T. (2020, June). Production of crude xylanase from Trichoderma sp. using Reutealis trisperma exocarp substrate in solid state fermentation. In *IOP Conference Series: Earth and Environmental Science* (Vol. 515, No. 1, p. 012024). IOP Publishing.

[26] Wösten, H. A. (2019). Filamentous fungi for the production of enzymes, chemicals and materials. *Current opinion in biotechnology*, *59*, 65-70.

[27] *Ibid*

[28] Bhardwaj, N., Kumar, B., & Verma, P. (2019). A detailed overview of xylanases: an emerging biomolecule for current and future prospective. *Bioresources and Bioprocessing*, *6*(1), 1-36.

Run	Treatment Variable		Protein Content Level (mg/ml)
	Substrate Concentration (%)	Fermentation Time (hour)	
1	14.6447	48	0.447
2	50	48	0.436
3	50	82	0.559
4	25	72	0.455
5	50	48	0.520
6	50	48	0.455
7	50	48	0.460
8	25	24	0.427
9	75	24	0.507
10	50	14	0.486
11	75	72	0.519
12	85.3553	48	0.519
13	50	48	0.463

Based on Table 3, the protein content values range between 0.427–0.559 mg/mL. The highest protein content of 0.559 mg/mL was produced in the treatment with a substrate concentration of 50% with 82 hours of fermentation time. The higher the protein content, the higher the growth of microorganisms during the fermentation process. On the other hand, the protein did not entirely belong to the xylanase group. The sample used was an enzyme that had not been purified, so other enzyme proteins might have been also measured. Meanwhile, during the fermentation process, protein may be consumed by *Trichoderma viride* as a source of nitrogen needed for its growth [29]. The protein content measured in the xylanase enzyme from empty oil palm fruit bunches was 0.00923-0.00942 mg/mL, achieved when fermentation took place after 6 days of incubation [30]. It can be concluded that the choice of substrate type and substrate concentration influences the differences in protein content values in the xylanase enzyme.

Specific Activity of Xylanase Enzyme

The protein content levels are then used to determine the specific activity of the xylanase enzyme. The specific activity of an enzyme is defined as the number of enzyme units per milligram of protein. Specific activity shows the level of purity of

[29] Ramadhan, R. F., Montesqrit, M., PRODUKSI ENZIM SELULASE TERMOSTABIL DARI BAKTERI NG2 MENGGUNAKAN BERBAGAI SUMBER SELULOSA ASAL LIMBAH PERTANIAN DAN PERKEBUNAN. *JITP, 8*(2).

[30] Imanisa, T. W., Mardawati, E., & Masruchin, N. (2023). Xylanase Production from Aspergillus niger via Submerged Fermentation towards Oil Palm Empty Fruit Bunches (OPEFB) Valorization as Value-added Biorefinery Products. *Biomass, Biorefinery, and Bioeconomy*, *1*(1).

the xylanase enzyme produced, the higher the specific activity, the higher the level of purity of the enzyme. The higher the specific activity, the better the ability of xylanase to utilize the coffee bean shell substrate. The value of the specific activity of the xylanase enzyme is shown in Table 4.

Table 4. Response Results of Specific Activity of xylanase Enzyme of Substrate Concentration and Fermentation Time.

Run	Treatment Variable		The specific activity of xylanase enzyme (U/mg)
	Substrate Concentration (%)	Fermentation Time (hour)	
1	14.6447	48	11,038
2	50	48	41,117
3	50	82	26,479
4	25	72	73,378
5	50	48	17,712
6	50	48	24,580
7	50	48	17,161
8	25	24	15,021
9	75	24	68,446
10	50	14	24,366
11	75	72	74,786
12	85.3553	48	87,145
13	50	48	33,035

The Optimization Result of Xylanase Enzyme Production

Analysis of Variance, optimization result, and model suggested by RSM

Analysis of model selection in Design Expert 13.0 software shows the model significance value, lack of fit test, and coefficient of determination (R^2, customized R-squared, predicted R-squared). The influence of the independent variables on the attachment variable can be determined from the analysis of variance (ANOVA).

Table 5. Model Recommended by RSM

Source	Sequential p-value	Lack of Fit P-value	Adjusted R^2	Predicted R^2	Selection Model
Linear	**0.0240**	**0.0241**	**0.4307**	**0.0998**	**Suggested**
2FI	0.2919	0.0231	0.4448	-0.3728	
Quadratic	**0.0491**	**0.0561**	**0.6982**	**-0.0776**	**Suggested**
Cubic	0.2808	0.0370	0.7458	-3.8163	**Aliased**

Table 6. ANOVA Calculation Result Quadratic Model

Source	Sum of Square	Df	Mean Square	F value	P value	
Model	1856.06	5	371.21	6.55	0.0143	**significant**
A-Substrate cons	1028.63	1	1028.63	18.15	0.0037	
B-Fermentation Time	155.31	1	155.31	2.74	0.1418	
Residual	396.63	7	56.66			
Lack of Fit	325.79	3	108.60	6.13	0.0561	**Not significant**
Pure Error	70.84	4	17.71			
Cor Total	2252.69	12				
R^2	0.8239					
Adjusted R^2	0.6982					
Predicted R^2	-0.0776					
Adeq Precision	8.4886					

Based on the analysis results in Table 5, the model chosen for the enzyme activity response is quadratic, and the model for the protein content response is linear. The results of the ANOVA test show that enzyme activity has a p-value of 0.0143 and 0.0251 for protein content. This shows that the quadratic and linear models have a significant effect on enzyme activity and protein levels and have a chance of model error of >5%. The model that showed significance in ANOVA and non-significance in Lack of Fit was selected to analyze the variables. A significant model will have a greater influence on the response compared to other models. In addition, the selection is based on the model with the largest R-squared (R2) value compared to other models. A larger R^2 indicates the recommended model.

The lack of Fit (p-value) for enzyme activity is 0.0561 and protein content is 0.5731 which indicates a p-value> 0.05 so Lack of Fit shows an insignificant result. A Lack of Fit value that is not significant is a requirement for a good model because it shows the suitability of the model to the response of enzyme activity and protein levels [31]. The R-squared (R^2) value shows the data that can be described by the model, namely 82.39% in the enzyme activity response and 52.14% in the protein content response. The R-squared value of protein content is still low compared to enzyme activity which is close to 1. If R^2 is close to 1, the relationship between the variables is stronger, but if R^2 is smaller, the relationship between the variables is weaker.

The resulting protein level response model was declared to have met the criteria. These criteria are the difference between Adj R-squared and Pred R-squared

[31] Kumari, M., & Gupta, S. K. (2019). Response surface methodological (RSM) approach for optimizing the removal of trihalomethanes (THMs) and its precursor's by surfactant modified magnetic nanoadsorbents (sMNP)-An endeavor to diminish probable cancer risk. *Scientific Reports*, *9*(1), 18339.

<0.2 and Adeq Precision value >4 [32]. These test results show that the equation formed by RSM to predict protein levels can be accepted and used in the design space. This criterion is not met by the enzyme activity response, because Pred R-squared on enzyme activity is negative, namely -0.0776. A negative Pred R-squared value indicates that the resulting enzyme activity response analysis data is not appropriate. This was proven at a substrate concentration of 50% and a fermentation time of 48 hours with the same number of five treatments but the enzyme activity values were not uniform and had a difference between 1,316-7,401 U/mL. However, the Adeq Precision value for enzyme activity still shows that the model is adequate for the design space, namely 8.4886, which means >4. ANOVA results can produce a model in the form of a quadratic equation, but the model is not sufficient to predict the actual response value [33]. The resulting RSM model equation for the enzyme activity response model is shown in equation 3:

$$\text{Xylanase Activity} = 11{,}3321A + 4{,}40232B - 5{,}71525AB + 8{,}6497A^2 + 2{,}78001B^2 + 12{,}3129 \quad \ldots\ldots$$

The RSM model chosen by the Design Expert 13.0 software to describe the enzyme activity response is the quadratic model. This model describes the response of enzyme activity as influenced by substrate concentration, fermentation time, and the interaction of the two. The influence of substrate concentration is more dominant than the influence of fermentation time because the coefficient A value of 11.3321 is greater than the coefficient B value of 4.4023. Substrate concentration and fermentation time have a directly proportional influence on the enzyme activity response. This can be seen in the coefficients A and B and the positive constants.

[32] Kim, S. A., & Rhee, M. S. (2015). Predictive model and optimization of a combined treatment of caprylic acid and citric acid for the reduction of Escherichia coli O157: H7 using the response surface methodology. *International Journal of Food Microbiology*, *197*, 9-14.

[33] Zhang, B., & Hui, Y. (2017). Optimization of cryoprotectants for Streptococcus thermophilus during freeze-drying using Box-Behnken experimental design of response surface methodology. *Emirates Journal of Food and Agriculture*, 256-263.

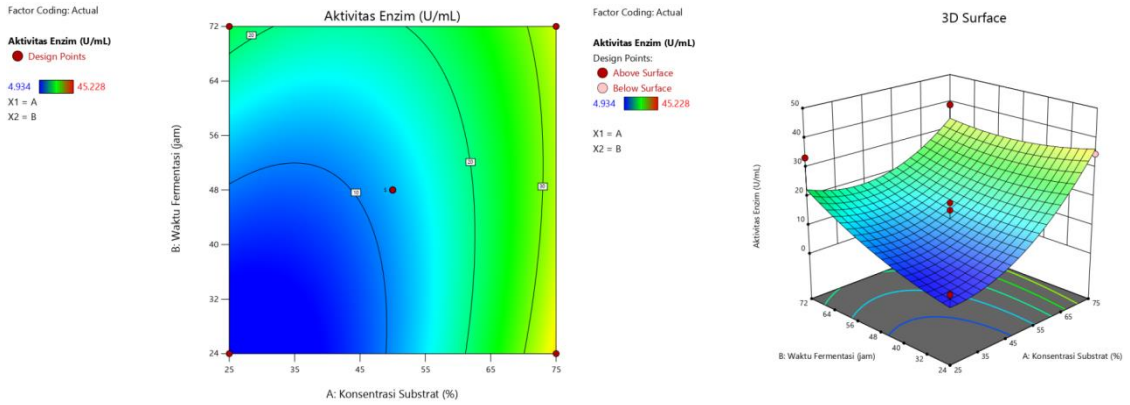

Figure 1. Contour Graph and 3D Graph of Enzyme Activity Response from Substrate Concentration and Fermentation Time

Figure 1 is a contour graph and 3D graph produced between the interaction of substrate concentration and fermentation time on enzyme activity. The contour graph depicts the relationship between substrate concentration and fermentation time on the enzyme activity response. The lines formed on the contour graph represent the height of the surface formed. The various colors on the graph indicate the value of each response. The color blue depicts the lowest response and the colors yellow to red depict the highest response [34]. However, in this study, the highest response to enzyme activity was shown in yellow with a value of 45.228 U/mL. The color change from yellow to blue in each treatment shows that the substrate concentration and fermentation time have a significant influence on enzyme activity. The enzyme activity response increased with increasing substrate concentration and fermentation time. This is shown in the 3D graph which forms a curvature pointing upwards. The quadratic model has an equation that shows that the regression formed will be a parabolic line, so in this case, variations in substrate concentration and fermentation time need to be added to produce a contour graph that forms a parabolic line [35].

The enzyme activity response is set with a maximum target, it is expected to obtain a value close to the upper limit of the criteria, which was 45.228 U/mL. Enzyme activity determines the amount of xylanase enzyme production produced by *Trichoderma viride*, for this reason, the maximum target for this response is determined. Based on these conditions, the optimization results based on the RSM model are presented in Table 7.

[34] Kumari, M., & Gupta, S. K. (2019). Response surface methodological (RSM) approach for optimizing the removal of trihalomethanes (THMs) and its precursor's by surfactant modified magnetic nanoadsorbents (sMNP)-An endeavor to diminish probable cancer risk. *Scientific Reports*, *9*(1), 18339.

[35] Zhang, B., & Hui, Y. (2017). Optimization of cryoprotectants for Streptococcus thermophilus during freeze-drying using Box-Behnken experimental design of response surface methodology. *Emirates Journal of Food and Agriculture*, 256-263.

Table 7. Optimum Formula Optimization Recommendations

No	Substrate Concentration (%)	Fermentation Time (hour)	Xylanase Enzyme Activity (U/ml)	Protein Content Level (%)	Desirability	
1	**75.000**	**24.000**	**36.388**	**0.494**	**0.742**	**Selected**
2	75.000	24.193	36.333	0.494	0.741	
3	75.000	24.870	36.142	0.494	0.738	
4	74.739	24.000	36.030	0.493	0.737	
5	75.000	32.747	34.252	0.500	0.698	
6	75.000	49.072	32.242	0.513	0.608	
7	75.000	54.610	32.144	0.517	0.571	
8	25.000	62.189	16.584	0.461	0.255	

The production on condition at 75% substrate concentration and 24 h fermentation time was selected as the optimal condition because this condition had the highest desirability value, which was 0.742. The desirability value is an optimization function value that shows Design Expert 13.0's ability to fulfill desires based on specified criteria. A desirability value close to 1 is the most desired value because it shows Design Expert 13.0's ability to produce the desired product more precisely [36]. However, the aim of optimization is not to obtain a desirability value of 1, but rather to find the best condition that meets all objective functions. The predicted response value was selected for enzyme activity of 36.388 U/mL and protein content of 0.494 mg/ml.

Model Validation

The verification aims to test the accuracy of predictions of optimum conditions produced based on selected variables from the RSM optimization results. Verification is carried out by testing in the laboratory to find enzyme activity values and protein levels with 3 repetitions. Enzyme activity resulting from validation was 36,292 U/mL, and the protein content was 0.488 mg/ml. Verification results must be in the range of 95% CI (Confident Interval) and 95% PI (Prediction Interval) to obtain a sufficiently accurate empirical model developed [37]. The CI value shows that 95% of

[36] Turuvekere Sadguruprasad, L., & Basavaraj, M. (2018). Statistical modelling for optimized lyophilization of Lactobacillus acidophilus strains for improved viability and stability using response surface methodology. *AMB Express*, *8*(1), 1-11.

[37] Qi, K., Chen, H., Wan, H., Hu, M., & Wu, Y. (2017). Response surface optimization of lyoprotectant from amino acids and salts for bifidobacterium bifidum during vacuum freeze-drying. *Acta Universitatis Cibiniensis. Series E: Food Technology*, *21*(2), 3-10.

the average response data measurement results are in that range, while the PI value shows that 95% of the measured sample response data is in that range. The prediction and response verification results are presented in Table 8.

Table 8. Model Validation

Response	Prediction	Verification	95% CI *low*	95% CI *high*	95% PI *low*	95% PI *high*
Enzyme activity (U/mL)	36,388	36,292	22,325	50,451	16,344	51,180
Protein level (mg/mL)	0,494	0,488	0,455	0,533	0,474	0,585

The accuracy of the optimization prediction model to predict actual values (verification) can be measured by the degree of reliability [38]. This is defined by the Design Expert 13.0 program as [39]:

a. Verification comparison > 0.90 means perfect reliability
b. Verification comparison 0.70 – 0.90 means high reliability
c. Verification comparison 0.50 – 0.70 means medium reliability
d. Verification comparison < 0.50 then reliability is low

Based on Table 8, the results of verifying the optimum conditions recommended by the Design Expert 13.0 program with the RSM CCD model, obtained the xylanase enzyme with an enzyme activity of 36.388 U/mL and a protein content of 0.494 mg/ml. The difference between the predicted value of the enzyme activity model and the verification results is 0.096 U/mL or has an accuracy of 99.74%, and the difference between the predicted value of the protein content model and the verification results is 0.006 mg/mL or has an accuracy of 98.79%. It can be said that the accuracy of the prediction model can predict the verification value with perfect reliability. This shows that the optimization process for substrate concentration and fermentation time is good enough to determine the optimum conditions for the response of enzyme activity and protein content.

[38] Mardawati, E., Herliansah, H., Matheus, A., Badruzaman, I., Wira, D. W., Hanidah, I. I., ... & Cahyana, Y. (2018). Acid Hydrolysis Optimization of Corn Cob As Raw Material for Xylitol Production. *Journal of Advanced Research in Materials Science*, *51*(1), 1-10.

[39] Zhu, Z., Wu, M., Cai, J., Li, S., Marszałek, K., Lorenzo, J. M., & Barba, F. J. (2019). Optimization of spray-drying process of Jerusalem artichoke extract for inulin production. *Molecules*, *24*(9), 1674.

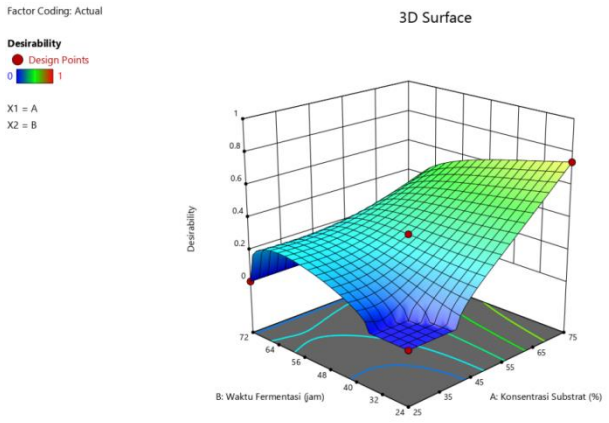

Figure 2. Three-dimensional (3D) Graph of the Combination of Substrate Concentration and Fermentation Time on Optimized Desirability Values

Figure 2 is a response surface for variations in substrate concentration and fermentation time which shows the achievement of optimum conditions for each response following the expected criteria. Three-dimensional (3D) graphics have low areas and high areas. The low area shows a low desirability value, namely 0.255, while the high area shows a high desirability value, namely 0.742. The model is said to be good because it has a desirability value of 0.742 which indicates that with the selected combination of substrate concentration and optimum fermentation time, the response can be achieved according to the expected criteria of 74.2%.

CONCLUSION

The optimum conditions for xylanase production are a substrate concentration of 75% and a fermentation time of 24 hours with a desirability value of 0.742. Production of the xylanase enzyme using optimization of substrate concentration and fermentation time obtained an enzyme activity value of 36.388 U/mL and a protein content of 0.494 mg/ml. The results of the verification carried out showed a balance of results with the prediction formula in the CCD-designed RSM, namely enzyme activity of 36.292 U/mL and protein content of 0.488 mg/ml.

This research was funded by LPDP (Education Fund Management Institution – Ministry of Finance of Indonesia Republic) through the RIIM (Research and Innovation for Advanced Indonesia) Phase 2 Research Scheme organized by BRIN (National Research and Innovation Agency) with the research title "Optimization of Xylose Sugar Crystallization Results of Enzymatic Hydrolysis of Coffee Waste Towards National Sugar Self-Sufficiency."

ACKNOWLEDGMENT

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