Optimization of Agricultural Waste Substrate as an Alternative Medium for Xylan in Producing Xylanase Enzymes by Thermophilic Bacteria

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Abstract. Xylanase enzyme is a thermostable enzyme produced by thermophilic bacteria which in this study used Aneurinibacillus thermoauerophilus SSA2 isolates. Xylanase enzyme is able to hydrolyze xylan into xylose and xylooligosaccharides. The use of xylan in large quantities was very ineffective because of the high price and low production at this time, therefore it was necessary to find a cheaper substrate, derived from agricultural waste and potentially as an alternative to replace xylan. The purpose of this research was to find a replacable substrate of xylan from agricultural waste containing hemicellulose. Xylan of extraction results from some agricultural waste are mixed into the fermentation medium. Extraction results that produce the most optimum enzyme activity will be treated to find the optimum concentration of the substrate. This research is an experimental study using RAL, where each treatment has 5 replications. Data on the results of enzyme activity were further analyzed by ANOVA test and continued with DMRT test at a significant level of 0.05. The results showed that the the results showed that the administration of agricultural waste substrate as a substitute for xylan affected the xylanase enzyme activity, were straw substrate had the highest average of enzyme activity at 6,033 Unit/mL and the husk substrate had the lowest average of enzyme activity at 5,667 Unit/mL. Substrate concentration had no significant effect on xylanase enzyme activity.

1. Introduction
The enzyme industry has developed rapidly and has occupied an important position in the industrial field. As biotechnology advances, enzymes are applied to catalyze chemical reactions outside the cell. Enzymes are used in various industrial sectors such as textiles, food, detergents, paper and cosmetics, and biofuels [1]. One type of enzyme that is widely used in industry is the xylanase enzyme [2].

Xylanase is a group of enzymes that have the ability to hydrolyze hemicellulose in this case is xylan or polymer of xylose and xylooligosaccharides. Xylanase can be classified based on hydrolyzed substrate, namely β-xylosidase, exoxylanase, and endoxylanase. β-xylosidase, which is xylanase which
is able to hydrolyze short chain xylooligosaccharides to xylose. Enzyme activity will decrease with increasing xylooligosaccharide chain [3].

Xylan is a major component of hemicellulose. Hemicellulose is the second largest polysaccharide in nature after cellulose [4]. Xylan is a substrate of the xylanase enzyme found in Beechwood Xylan. The use of xylan in the production of large-scale xylanase enzymes is too expensive, so research is needed to find substrates as alternative media [5]. Utilization of hemicellulose waste is one solution, because the xylan component is also found in agricultural wastes such as rice straw, corn cobs, wheat bran, bagasse and rice husk [6].

Rice straw is lignocellulosic waste which has the potential as an alternative to xylan because the xylan content in rice straw is quite high at 20% [7] and can be used as a source of carbon, substrate and as an inducer in the growth media of microorganisms [8]. Utilization of lignocellulosic waste by using the services of microorganisms in producing extracellular enzymes capable of degrading lignocellulosic material into its constituent fractions [9].

Corn cobs are also one of agricultural waste containing lignocellulose and its availability is abundant in nature [10]. Agricultural wastes such as corn cobs have a hemicellulose content of 40% which is a xylanase substrate as a carbon source [11]. Corn cob is a lignocellulosic material containing 12.4% xylan [6].

Another lignocellulosic waste which has the potential as an alternative to xylan is rice husk because the xylan component is found to be 12.1% in rice husk [6]. Rice husk has main components such as cellulose (31.4–36.3%), hemicellulose (2.9–11.8%), and lignin (9.2–18.4%) [12].

Xylan can be used by microorganisms as a source of carbon, substrate, and inducer in growth media to produce xylanase enzymes [13]. The production of xylanase enzymes for industrial needs is extracted from various types of living things, such as bacteria, fungi, yeast and plants. Enzyme-producing microorganisms that can be utilized by humans are available in varying amounts and types, are easy to cultivate and have the potential and are suitable for industry [14]. Thermophilic microorganisms are one of the right choices for use in industrial processes, because they are thermostable [15].

One of the thermophilic microorganisms that can produce thermostable enzymes is thermophilic bacteria, where thermophilic bacteria can grow optimally in the temperature range of 60-1080°C [16]. Indonesia is a region that has quite a lot of hot water sources. One of the hot springs is Sapan Sungai Aro hot spring in South Solok Regency. Sapan Sungai Aro hot springs also have a temperature of 75°C and a pH of 8 or are alkaline [17]. The type of isolates obtained at the Sapan River Aro Solok Selatan hot spring selected was SSA2 isolates because Aneurinibacillus thermoaerophilus SSA2 isolates had good ability to produce xylanase enzymes to the maximum. This can be seen from the high xylanolytic index of Aneurinibacillus thermoaerophilus SSA2 compared to other isolates which is 0.74 [18]. Therefore, the aim of this study is to find xylan substrate from agricultural waste.

2. Methods

2.1. Manufacture of Agricultural Waste Flour Powder Substrate

Straw, corn cobs and wet husks are sorted, then washed and dried. After it is half dry, it is then ovened with a temperature of 50°C for 7 days. Once dry and can be broken, then blended and obtained powder which will be used as a substrate.

2.2. Xylan Extraction from Agricultural Waste

Flour powder from straw, corn cobs and husks of 50 g each which has been made soaked in NaOCl solution for 5 hours at 28 °C. After that, it is rinsed and filtered and then immersed in 10% NaOH solution at 28 °C for 24 hours. The filtrate obtained was then centrifuged at 4000 rpm for 30 minutes. Then the centrifugation liquid (supernatant) is neutralized with 6N HCl to be centrifuged again at 4000 rpm for 30 minutes. The supernatant produced already contains xylan, to separate the dissolved xylan is done by adding 95% Ethanol and centrifuged at 4000 rpm for 30 minutes [19].
2.3. Optimization of Agricultural Waste Substrate
The optimum substrate is determined by the following working procedures: providing 250 mL Erlenmeyer containing 50 mL of fermentation medium with a composition of 0.5% polipeptin, yeast extract 0.1%, K2HPO4 0.1%, MgSO4.7H2O 0.02% and 0.1% xylan was extracted from agricultural waste (rice straw, rice husk, and corncobs) and beechwood xylan as a control. A total of 5 mL of inoculum were inoculated into the enzyme production medium above. Then incubated at 60°C at a 150 rpm shaker for 6 hours. The production medium was centrifuged at 5000 rpm at room temperature for 15 minutes until an enzyme solution was obtained and then the enzyme activity was determined. The optimum substrate optimization results are used as a substrate for the treatment of variations in substrate concentrations\(^5\) (Modifications).

2.4. Optimization of Optimum Substrate Concentration
The optimum concentration of the optimum substrate is determined by the following working procedures: providing a 250 mL Erlenmeyer containing 50 mL of fermentation medium with a composition of 0.5% polipeptin, 0.1% yeast extract, K2HPO4 0.1%, MgSO4.7H2O 0.02%. Then xylan was extracted from the optimum substrate according to the treatment, namely 0.1%, 0.2%, 0.3%, 0.4% and 0.5%. A total of 5 mL of inoculum was inoculated into the enzyme production medium above. Then incubated at 60 °C at a 150 rpm shaker for 6 hours. The production medium is centrifuged at 5000 rpm at room temperature for 15 minutes until an enzyme solution is obtained and then the enzyme activity is determined.

2.5. Xylanase Enzyme Activity Test
The xylanase enzyme activity was tested using the method of Bailey et al. (1992) 1% (w / v) xylan in phosphate buffer (pH 8) for the reaction. The test mixture consisted of 1 mL of substrate solution and 0.5 mL of crude enzyme solution then incubated at 60asiC for 10 minutes. After that the reaction is stopped by adding 1 mL of dinitrosalicylic acid then incubated at 90°C for 25 minutes. To determine the enzyme activity absorbance measurements were carried out at a wavelength of 540 nm. The absorbance measurement results are used to determine the xylose content using the linear regression equation as follows:

\[
y = a + bx
\]

Information:
\[y\] = absorbance value at wavelength \(\lambda = 540\) nm
\[a\] and \[b\] = calculation of xylose standard sugar
\[x\] = xylose content

The amount of reducing sugar released is determined using a xylose standard curve \(^{21}\). One unit of xylanase activity is defined as the amount of enzyme needed to release 1 µmol xylose per minute under test conditions. Blank when testing using Aquadest. The substrate is deactivated by heating at 100°C for 30 minutes.

Standard xylose curves were made in the range of 20, 40, 60, 80, and 100 µg / mL. each standard solution of 0.5 mL is mixed with 5 mL aquadest, then 1 mL of DNS reagent is added. The tube is put into a boiling water bath for 15 minutes, then cooled and the absorbance measured at a wavelength of 540 nm \(^{21}\).

2.6. Data analysis
The obtained data were analyzed statistically using ANOVA. If there is a real difference then a DMRT test is performed, with \(\alpha = 5\%\).

3. Results and Discussion
3.1. Optimization of Agricultural Waste Substrate
The results showed that the treatment with several different substrates instead of xylan had an effect on xylanase enzyme activity. Where the results of the analysis of variance indicate that agricultural
waste has the potential to be an alternative substitute for xylan in producing xylanase enzymes. DMRT further test results showed enzyme activity in the treatment of corncobs, rice straw and control did not have significant differences, namely 5.667 Units / mL, 6.033 Units / mL and 6.142 Units / mL, the results of enzyme activity tests on the average value of enzyme activity of rice straw substrate had average tendency is higher. The lowest enzyme activity was found in rice husk substrate which was 5.667 Unit / mL. Comparison of xylanase enzyme activity on different substrates can be seen in Table 1.

Table 1. Activity of xylanase enzymes on different substrates

| No. | Treatment     | Average of Enzyme Activity (Unit/mL) |
|-----|---------------|-------------------------------------|
| 1   | Rice Husk     | 5.667a                              |
| 2   | Corncob       | 5.785ab                             |
| 3   | Rice Straw    | 6.033b                              |
| 4   | Control       | 6.142b                              |

Note: Numbers followed by the same letter are not significantly different at α = 5% according to the DMRT test.

The results of the analysis showed that the administration of xylan extracts from some agricultural wastes significantly affected the xylanase enzyme activity. Where the highest tendency of the highest enzyme activity was found out of control was found in the xylan from the extraction of rice straw which was 6.033 Unit / mL. This is supported by research Richana et al. [6] that the content of xylan in rice straw is quite a lot, which is around 20%.

In this study the control treatment showed higher enzyme activity results, but because the aim was to find the substrate that could produce the most optimal xylan extracts and when compared to the three substrates used, rice straw was the most optimal because it showed the highest enzyme activity results, after control. Based on previous research by Richana et al. [6], indeed rice straw is the most optimal substrate for xylan extraction as a substitute for pure xylan because of its higher xylan content compared to corncobs or rice husks. The results of enzyme activity are strongly influenced by the selection of substrates for the fermentation process. In choosing the substrate to be used to replace pure xylan, the hemicellulose content must be considered because hemicellulose is a major component of xylan. In several studies mentioned that agricultural waste contains a lot of hemicellulose, such as research conducted by Jacobsen et al. [22] that rice straw contained 24.5% hemicellulose.

In Ardiansyah's research [8] using Bacillus subtilis bacteria with the addition of rice straw substrate, the result of xylanase enzyme activity was 5.178 Units/mL. Wahyuningtyas research [23] showed the results of enzyme activity of 1.0313 units/mL using Trichoderma reesei.

Meanwhile, rice husk is a substrate whose extraction results show the lowest enzyme activity which is also in accordance with the research of Richana et al. [19] which showed that the levels of xylan contained in rice husks were only 6.3% and in corncobs as much as 12.9%. The content of hemicellulose contained in corn cobs based on the results of the study of Septiningrum [24] was 30.91% and according to Champagne [12], rice husks contained hemicellulose of around 2.9% - 11.8%.

3.2. Optimization of Optimum Substrate Concentration

The treatment by giving xylan extract from the optimum substrate (straw) with different concentrations shows that there is no real effect on the xylanase enzyme activity, it can be seen from the results of the analysis of F count (3,618) <F table (3,24). Where from the data in Table 2, the highest average tendency of xylanase enzyme activity was shown by substrate concentration of 0.3%.
9.291 Units/mL and the lowest average tendency was indicated by substrate concentration of 0.5%, which was 6.735 Units/mL.

Table 2. Xylanase Enzyme Activity at Different Substrate Concentrations

| No. | Treatment | Average of Enzyme Activity (Unit/mL) |
|-----|-----------|-------------------------------------|
| 1   | 0.5%      | 6,735                               |
| 2   | 0.4%      | 6,918                               |
| 3   | Control   | 7,579                               |
| 4   | 0.2%      | 7,951                               |
| 5   | 0.1%      | 9,016                               |
| 6   | 0.3%      | 9,291                               |

The results of the analysis showed that the administration of different substrates at optimum concentration optimization had no significant effect or difference on the xylanase enzyme activity. However, in this study there were 3 treatments (substrate concentrations of 0.1%, 0.2% and 0.3%) which showed an average result of xylanase enzyme activity better than control treatments and 2 treatments (substrate concentration of 0.4% and 0.5%) which is less good than the control. The optimum concentration of straw substrate is 0.3% where the average enzyme activity obtained is 9,291 Units/mL, which tends to have a higher enzyme activity value than other treatments.

The results of this study indicate that if the concentration of the given substrate is too high, the enzyme activity will be low. This is also proven by Naomi's research [25] which uses xylan substrate from corncob extraction as much as 0.5%, 1% and 1.5% where the highest enzyme activity results are obtained at 1% substrate concentration. In this study the activity of xylanase enzymes at 0.4% and 0.5% substrate concentrations decreased due to increased media density which inhibits the interaction of enzymes with the substrate where the excess substrate can be an inhibitor of the enzyme work [26].

Giving the right substrate concentration is very influential on the production of enzymes and enzyme activity, therefore it is necessary to have a proper estimate to give the substrate with the appropriate concentration so that the enzyme produced is optimal and the enzyme activity produced is also high. Enzymes with a high degree of purity, within certain limits have a linear relationship between concentration and enzyme activity [25]. If the substrate concentration is fixed and the enzyme concentration decreases, the enzyme catalyzed reaction rate will decrease because there are not enough enzymes available to react with the substrate [28].

Xylan is a long chain of monosaccharides that are bonded together by a chemical bond that when hydrolyzed by xylanase enzymes can produce simple sugars in the form of xylooligosaccharides, xylobiose and xylose. Xylan with xylopyranosil β-D unit homopolymer bound by (1→4)-β-glycosidic linkages are heteropolymers connected by side chains of other sugars, generally single chains of (4-O-methyl)-α-D glucuronic acid (in dicots and gymnosperms) or in 1 or more α-L-arabinofuranosil in grass [29].

4. Conclusion
From this research that has been done, it can be concluded as follows:
1. The best agricultural waste substrate as a substitute for xylan is rice straw substrate with an average enzyme activity of 6,033 Unit/mL.
2. The optimum concentration of straw substrate (optimum substrate) in producing xylanase enzyme is 0.3% with an average enzyme activity of 9,291 Unit/mL.

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References

[1] Kosim M & Putra RS. 2010. Pengaruh Suhu Pada Protease Dari Bacillus subtilis. Prosiding Skripsi Semester Genap.

[2] Thakur VV, Jain RK & Mathur RM. 2012. Studies on xylanase and laccase enzymatic prebleaching to reduce chlorine-based chemicals during CEH and ECF bleaching. BioResources.

[3] Reilly PJ. 1991. Xylanase: Structure and Function. In Hollander, A. (Ed.). Proceeding of A Symposium on Trend in Biotechnology of Fermentation for Fuels and Chemicals. New York: Lenum Press.

[4] Da Silva TM, Maller A, De Lima Damásio AR, Michelin M, Ward RJ, Hirata IY, De Polizeli MLTM. 2009. Properties of a purified thermostable glucoamylase from Aspergillus nivens. Journal of Industrial Microbiology and Biotechnology.

[5] Richana N. 2002. Produksi dan Prospek Enzim Xilanase dalam Pengembangan Bioindustri di Indonesia. Buletin AgroBio.

[6] Richana N, Lestina P & Irawadi TT. 2004. Karakterisasi Lignoselulosa dari Limbah Tanaman Pangan dan Pemanfaatannya untuk Pertumbuhan Bakteri RXA III-5 Penghasil Xilanase. Balai Besar Penelitian dan Pengembangan Pascapanen. Institut Pertanian Bogor. Jawa Barat, 23, 112.

[7] Roberto IC, Mussatto SI & Rodrigues RCLB. 2003. Dilute-acid hydrolysis for optimization of xyllose recovery from rice straw in a semi-pilot reactor. Industrial Crops and Products.

[8] Ardiansyah YT, Mulyani NS & Sarjono PR. 2014. Isolasi dan Karakterisasi Enzim Xilanase dari Bacillus subtilis pada Media Nutrien Broth dengan Penambahan Xilan Hasil Isolasi Jerami Padi. Jurnal Kimia Sains dan Aplikasi. 95-99.

[9] Stanbury PF, A Whitaker & SJ Hall. 2003. Principles of Fermentation Technology. 2nd Ed. Great Britain: Elsevier Sci. Ltd.

[10] Fachry AR, Astuti P & Puspitasari TG. 2013. Pembuatan Bioetanol dari Limbah Tongkol Jagung dengan Variasi Konsentrasi Asam Klorida dan Waktu Fermentasi. Jurnal Teknik Kimia.

[11] Setyawati I. 2006. Produksi Dan Karakterisasi Xilanase Mikroba Yang Diisolasi Dari Tongkol Jagung. Skripsi. Tidak Diterbitkan.

[12] Champagne ET. 2004. Rice: Chemistry and Technology 3rd Edition. Minnesota, USA: American Association of Cereal Chemist, Inc. St. Paul.

[13] Patong AR. 2013. Analisis Kimia Pangan. Makassar: Dua Satu Press.

[14] Palmer T. 1991. Understanding Enzymes 3rd Ed. Chichester: Ellis Horwood.

[15] Vikram S, Vinod C, Dinesh CP & Sanjeev A. 2012. Purification and characterization of Laceyella sacchari strain B42 xylanase and its potential for pulp biobleaching. African Journal of Microbiology Research.

[16] Kumar S & Nussinov R. 2001. How do thermophilic proteins deal with heat?. Cellular and Molecular Life Sciences.

[17] Irdawati, Syamsuardi, Agustien A & Rilda Y. 2016. Xylanase Enzyme Stability and Biochemical Characteristics thermoXylanolytic Bacteria from Mudiax Sapan Hot Springs at Solok Selatan District. Der Pharmacia Lettre.

[18] Irdawati, Syamsuardi, Agustien A & Rilda Y. 2018. Screening of Thermophilic Bacteria Produce Xylanase from Sapan Sungai Aro Hot Spring South Solok. IOP Conference Series: Materials Science and Engineering.

[19] Richana N, Tedja Irawadi T, Anwar Nur M, Sailah I & Khaswar K. 2007. The Process of Xylanase Production from Bacillus pumilus RXAIII-5. Microbiology Indonesia.

[20] Bailey MJ, P Biely & K Poutanen. 1992. Interlaboratory Testing of Methods for Assay of Xylanase Activity. Journal of Biotechnology. 23: 257-270.

[21] Miller IG. 1959. Use of Dinitrosalicylic Acid Reagent for Determination Reducing Sugar. Analytical Chemistry. 31: 426-428.
[22] Jacobsen SE, & Wyman CE. 2000. Cellulose and hemicellulose hydrolysis models for application to current and novel pretreatment processes. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. https://doi.org/10.1385/ABAB:84-86:1-9:81.

[23] Wahyuningtyas P, Argo BD & Nugroho WA. 2013. Studi Pembuatan Enzim Selulase dari Miktofungi Trichoderma reesi dengan Substrat Jerami Padi sebagai Katalis Hidrolisis Enzimatik pada Produksi Bioetanol. Jurnal Bioproses Komoditas Tropis 1 (1): 21-25.

[24] Septiningrum K & Apriana C. 2011. Produksi Xilanase dari Tongkol Jagung dengan Sistem Bioproses Menggunakan Bacillus circulans untuk Pra-Pemutih Pulp. Jurnal Riset Industri 5 (1): 87-97.

[25] Naomi A. 2006. Produksi Xilooligosakarida dari Xilan Tongkol Jagung dengan Menggunakan Xilanase Streptomyces sp. Asal Indonesia. Skripsi. Tidak Diterbitkan. Bogor: Departemen Biologi: Institut Pertanian Bogor.

[26] Irawadi TT. 1999. Kajian Hidrolisis Enzimatik Limbah Lignoselulosa dari Industri Pertanian. Tek Ind Pert 3: 20-25.

[27] Pelczar, Michael J & Chan ECS. 2008. Dasar-Dasar Mikrobiologi Jilid I. Jakarta: UI Press.

[28] Murray RK, dkk. 2003. Biokimia Harper ed 25. Jakarta: EGC.

[29] Collins T, Gerday C & Feller G. 2005. Xylanases, Xylanase Families and Extremophilic Xylanases. FEMS Microbiology Reviews.