Jurnal Presipitasi
Media Komunikasi dan Pengembangan Teknik Lingkungan
e-ISSN : 2550-0023

Vol 18, No 1, 2021, 73-80
Article available at homepage presipitasi

Research Article

Optimization of Fermentable Sugar Production from Pineapple Leaf Waste (*Ananas comosus [L.] Merr*) by Enzymatic Hydrolysis

Yohanita Restu Widihastuty[1], Sutini[1], Aida Nur Ramadhani[1*]

[1,2,3] Chemical Engineering Department, Universitas Sebelas Maret, Surakarta, Indonesia
* Corresponding Author, e-mail: aidaramadhani@staff.uns.ac.id

Abstract

Pineapple leaf waste is one agricultural waste that has high cellulose content. Pineapple leaf waste's complex structure contains a bundle of packed fiber that makes it hard to remove lignin and hemicellulose structure, so challenging to produce reducing sugar. Dried pineapple leaf waste pretreated with a grinder to break its complex structure. Delignification process using 2% w/v NaOH solution at 87°C for 60 minutes has been carried out to remove lignin and hemicellulose structure so reducing sugar could be produced. Delignified pineapple leaf waste has been enzymatic hydrolyzed using cellulase enzyme (6 mL, 7 mL, and 8 mL) to produce reducing sugar. The sample was incubated in an incubator shaker at 155 rpm at 45, 55, and 60°C for 72 hours. Determination of reducing sugar yield had been carried out using the Dubois method and HPLC. The model indicated that the optimum operating condition of enzymatic hydrolysis is 7 mL of cellulase enzyme at 55°C to produce 96,673 mg/L reducing sugar. This result indicated that the enzymatic hydrolysis operating condition improved the reducing sugar yield from pineapple leaf waste. The optimum reducing sugar yield can produce biofuel by the saccharification process.

Keywords: cellulase enzyme, pineapple leaves, reducing sugar, agricultural waste

1. Introduction

Based on data from the Central Statistics Agency (Badan Pusat Statistik, 2012), Indonesia is in the third position of fresh pineapple producing countries and ninth globally with a production volume of 1.3 million tons per year which has exported to various countries. This certainly would be proportional to pineapple leaf waste because, at harvest time, the plants must be replaced with new pineapple plants. Thus the leaves are only disposed of as waste (Setiawan *et al.*, 2017). Pineapple plantations produce pineapple leaf waste as much as 9 tons/hectare each year (Ministry of Industry, 2004). Every single tree of pineapple produces waste between 2-3 kg, if the spacing of pineapples is 1 m, then every 100 m of land planted with pineapples can produce 200-300 kg of waste, and 1 hectare of land will reach 3 tons of waste (Irianti and Supadmi, 2019). Current practices of processing the wastes are mainly in situ burning of pineapple waste, decomposition of pineapple residues, and removal before planting. The in situ burning method is quick and easy to carry out but not environmentally sound since burning can lead to groundwater and air pollution and also cause a fire (Chen *et al.*, 2020). Excellent waste management must be practiced to minimize pollution to environments and convert the leaves into practical and economical products.

Reducing sugars are a class of carbohydrates that can reduce electron-accepting compounds. All monosaccharides (glucose, fructose, galactose) and disaccharides (lactose, maltose), except sucrose and starch (polysaccharides), are included as reducing sugars. To obtain reducing sugars, it is usually obtained from the hydrolysis of carbohydrates. However, in this study, reducing sugar produced from the hydrolysis of cellulose contained in pineapple leaves was studied.

Pineapple leaves are agricultural waste with a higher cellulose content than others, that is cellulose-α, hemicellulose, lignin, ash, extractives, and other materials such as pectin as 73,4%; 7,1%; 10,5%; 2,0%; 5,5%; 1,5% respectively (Tsoumis, 1991). High levels of cellulose-α make pineapple potential to be used as a more helpful cellulose derivative product and the conversion this gihar into ethanol by the fermentation process. Cellulose derivative product could be used to produce fuels (i.e., alcohol) and value-added product (i.e., enzymes, organic acids, and proteins). Nevertheless, this waste also has lignin as one of the components retarding the sugar production performance with conventional technology, the enzymatic activity. To solve this problem, the delignification treatment is applied to this process to breakdown the lignin structure in the pineapple waste and allow the enzymatic activity to convert more cellulose effectively. Therefore, this study aims to investigate the effect of the enzymatic treatment on reducing sugar production (Banerjee *et al.*, 2019).

Various research was carried out on pineapple leaf waste hydrolysis by chemical processes using sulfuric acid. In this study, the hydrolysis process was carried out using a biological process by enzymes. Biological processes by enzymes are chosen considering that chemical processes with the use of acids in the chemical industry have a worse environmental impact than the waste produced. The biological process uses xylanase enzymes that are known to have the ability to hydrolyze xylan in hemicellulose (Richana N., 2002) and cellulase enzymes that can hydrolyze cellulose to reducing sugar (Sadhu, 2013).

Cellulose-α is the highest quality cellulose (Sinaga, 2011; Tarmansyah, 2007), resulted in high-quality derivative. Reducing sugar is sugar that can reduce due to aldehyde or ketone groups. Reducing sugar as an intermediate product can be processed again through fermentation with various microbes, for example, bioethanol production with *Sacharomyces cerevisiae* or *Zymomonas mobilis*, glutamic acid with *Brevibacterium flavum*, and alcohol bio solvent with *Sacharomyces cerevisiae*. One gram of pineapple leaves can reduce sugar at about 0.502 grams (Kumar *et al.*, 2017). Every year, pineapple plantations produce pineapple leaf waste at about 9 tons per hectare (Kementerian Perindustrian, 2019). Therefore, it can be concluded that if the pineapple leaf waste per year is not utilized optimally, about 4.518.000 grams of reducing sugar is wasted. This research was conducted to determine the best enzymatic hydrolysis process of pineapple leaf waste into fermentable sugar with various cellulase enzyme temperatures and volumes.

2. Methods

2.1. Pretreatment

The pineapple leaves were from pineapple smooth cayenne variety from Subang, Indonesia. The leaves were washed with water and dried in an oven (Memmert Universal Oven Lab Type UN110) at 80^{o}C for 24 hours to remove water content up to 84% w/w. Dried leaves were then cut down into good size by 16 mesh sieves to obtain the same size and maximize the surface area. Dry samples were stored in sealed container for further use.

2.2. Delignification

0,1 g/mL delicate dried leaves were immersed in the 2% (w/v) NaOH solution followed by heat treatment using an electric stove (Maspion S-300) at 87^{o}C for 60 minutes in the three-neck flask (Pyrex 500 mL). All the delignified samples were intensively washed with distilled water until neutral pH (pH = 7) to remove impurities. The residue dried in an oven (Memmert Universal Oven Lab Type UN110) at 60^{o}C for 24 hours (Nashiruddin *et al.*, 2020).

2.3. Enzymatic Hydrolysis

Hydrolysis was conducted in a 250 mL Erlenmeyer flask. A 0,01 g/mL of sample was loaded into a mixture that contained 6 mL, 7 mL, and 8 mL diluted cellulase enzyme. Sodium acetate buffer 0,05 M pH 4,8 was used to maintain the acidity. The mixtures were sterilized in an autoclave (Hirayama HICLAVE HVE-50) at 121^{o}C for 15 minutes. The mixtures were then incubated in a shaker incubator (DAIHAN Labtech LSI-3016A) at 155 rpm at various temperatures; 45^{o}C, 55^{o}C, and 60^{o}C for 72 hours. The supernatant liquid was taken for total reducing sugar analysis.

2.4. Analytical Methods

Dubois method was used to analyze the reducing and total sugar concentrations. The sample was diluted until 200 times and mixed with 100 µL of 5% phenol. A 5 mL sulfuric acid 98% was added to the sample, mixed with vortex, and kept for 10 min. The absorbance was analyzed by spectrophotometer at 490 nm because the reducing sugar (hexoses and their methylated derivatives) has an absorption maximum at 485 to 490 nm (Dubois *et al.*, 1956). The calibration curve was prepared with range from 0,001 mg/mL to 0,01 mg/mL of glucose (Mariano *et al.*, 2020). The reducing sugars were calculated using calibration curve equation.

$$y = 0{,}0509\ x - 0{,}0028 \quad (1)$$

with, y = sugar concentration (mg/mL)
x = absorbance at 490 nm.

An HPLC (Shimadzu, model Prominence UFLC- Nexera XR, Japan) is equipped with a refractive index detector (RID 10A, Shimadzu, Japan) was used. It was obtained by the correlation between the areas of the chromatograms and calibration curves previously determined by standards of components (D-glucose). The calibration curves were prepared in concentrations ranging until 100 g/L for all sugars. The samples were analyzed once for collected samples of each experimental condition (Vedovatto *et al.*, 2021).

3. Result and Discussion

Pretreatment was first conducted by drying the pineapple leaves in an oven (Memmert Universal Oven Lab Type UN110) at 60^{o}C for 24 hours. The material's water content was removed to 84% w/w since the refining process is easier to conduct in dry conditions. The following pretreatment process was mechanical pretreatment. The grinding and sieving approaches, which were applied to break the materials down to 0,1-2 mm and reduce the lignin materials' crystallinity—grinding produced a maximum size of 0,1-2,0 mm. Sieving was used to divide ground samples into fractions of different sizes (Akhlisah *et al.*, 2021). In biomass conversion, physical pretreatment only increases surface area. Delicate sized leaves could expand contact between the substrate and the enzyme, thus accelerating the rate of a hydrolysis reaction.

The second process was delignification with NaOH 2% at 87^{o}C for 60 minutes, which reduced 56% of the pineapple leaves' weight. The delignification treatment was used to breakdown its lignin structure. After the treatment, the structure can be more exposed to the environment and have more surface areas. Without the delignification process, the lignin structure can not be broken down and decrease the effectiveness of enzymatic hydrolisis (Maneeintr *et al.*, 2018). The final water content was 52,5% after the delignification process. Thus, the cellulose was readily converted into reducing sugar

The hydrolysis process is a reaction to break down large molecules into smaller parts, which are the compound itself's monomer components through water addition. The hydrolysis process needs a catalyst to conduct it and the catalysts that can be used in this hydrolysis process are acids (for example, sulfuric acid) and enzymes. Processing pineapple waste by acid hydrolysis yields 51.4% glucose (Faria *et al.*, 2020) and hydrolysis using an enzyme can produce 63.75% glucose (Conesa *et al.*, 2016). It can be concluded that the enzyme catalyst produces more glucose than the acid catalyst. Besides, enzyme catalysts are more environmentally friendly than enzyme catalysts because acid catalysts can pollute the soil environment.

In this study, the catalyst used was cellulase enzyme. The cellulase enzyme can break the cellulose polymer's molecule into its monomer, reducing sugar (such as hexoses). The hydrolysis process of pineapple leaves was carried out in 2 testing processes, the Dubois analysis, and the HPLC analysis. The following results were obtained:

3.1. Dubois Test (Spectrophotometric Analysis)

Dubois method was used to analyze the reducing sugar concentrations. The sample was diluted until 200 times and mixed with 100 µL of 5% phenol. A 5 mL sulfuric acid 98% was added to the sample and mixed with vortex and kept for 10 min. Phenol in the presence of sulfuric acid can be for the quantitative calorimetric micro determination of sugars and their methyl derivatives. This method helps determine small quantities of sugars, simple, sensitives, and gives reproducible results and only requires one standard curve for each sugars (Dubois *et al.*, 1956). Then, the absorbance was analyzed by a spectrophotometer at 490 nm.

The working principle of spectrophotometry is based on light absorption measurement by shooting white light then broken down into a spectrum of light through a prism mirror. The light is monochromatic by a rate of color solution in a specific wavelength using a prism-shaped monochromator. UV-Vis spectrophotometry analysis was conducted by 490 nm wavelength. We obtained the graphical equation of the absorbance value for each standard glucose level, so each sample's glucose level can be calculated (figure 1).

Table 1. Spectrophotometry analysis result

No	Temperature (°C)	Volume Cellulase Enzyme (mL)	Absorbance	Total Glucose (mg/mL)
1.	45	6	0,767	7,24806
2.	45	7	1,083	10,46494
3.	45	8	1,074	10,37332
4.	55	6	0,947	9,08046
5.	55	7	1,907	18,85326
6.	55	8	1,739	17,14302
7.	60	6	0,882	8,41876
8.	60	7	1,741	17,16338
9.	60	8	1,714	16,88852

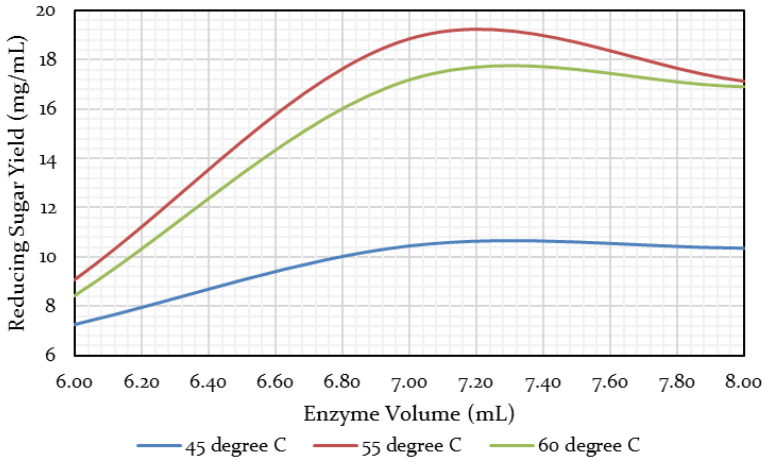

Figure 1. Relationship between enzyme volume and reducing sugar yield using dubois analysis

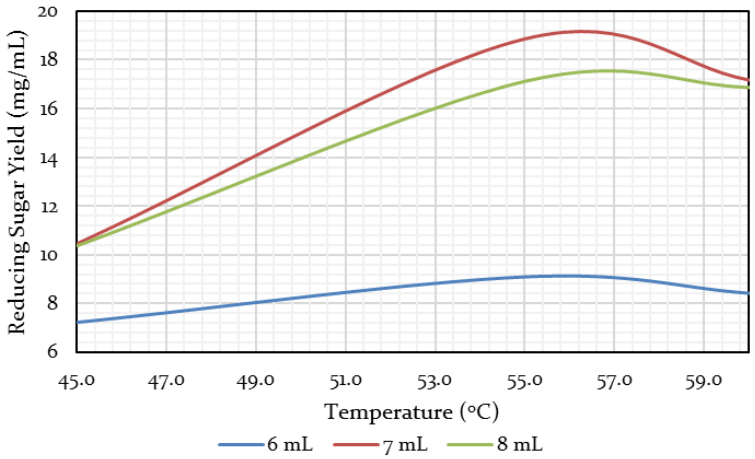

Figure 2. Relationship between temperature and reducing sugar yield using dubois analysis

Table 1 and Figure 2 show the reducing sugar content under the influence of hydrolysis temperature from 45°C to 60°C. The sugar yield trend climbed as the temperature increased from 45°C to 56°C, then declined slightly at about 57°C to 60°C. It predicted that at about 57°C the hydrolysis process already crossed the maximum temperature limit. This shows the same results as previous studies, which stated that cellulase enzyme activity would be maximum at a temperature of 55°C (Hamzah, 2018). Cellulase enzyme was isolated from some microbes, thus had a range of the optimum temperature, such as *Bacillus licheniformis* at 50°C (Dhillon *et al.*, 1985), *Bacillus subtilis* at 50°C (Robson and Chambliss, 1984), and *Cellulomonas uda* at 45-50°C (Nakamura and Kitamura, 1988), also Bacillus M-9 at 60°C (Bajaj *et al.*, 2009).

Also, the influences of enzyme concentrations on the reducing sugar content were presented in Table 1 and Figure 1. The graphics increased along the cellulase enzyme volume from 6 ml to about 7,2 ml then decreased at about 7,2 ml to 8 ml enzyme. It also predicted that at about 7,2 ml hydrolysis process already crossed the maximum concentration limit. So it can be concluded that the hydrolysis process will run optimally if in the right temperature conditions adjusted to the enzyme used because the enzyme can work optimally at that temperature.

3.2. HPLC Test (High-Performance Liquid Chromatography)

Four samples with high glucose yield (sample 5, 6, 8, and 9) were then tested using the HPLC test to confirm the samples with the highest reducing sugar levels. HPLC is a chromatography technique for high-pressure liquid substances, where the liquid a mobile phase, the dissolved sample can be separated and the concentration of the specific substance contained in it can be calculated. The HPLC test results in this research showed that the highest reducing sugar content was at 55°C with an enzyme concentration of 7 mL. It worked optimally by producing reducing sugar in yield 96.673% (Table 2).

As mention before, the cellulase enzyme had a range of optimum temperature as the microbial agent. At the optimum temperature, the collision between the enzyme and the substrate is very effective, so that the formation of enzyme complexes, the substrate is more accessible and the products formed increase. Increasing the temperature beyond the optimum temperature causes the enzyme to run into denaturation and the substrate to undergo a conformational change (denaturation) so that the active side of the substrate can no longer or experience obstacles in entering the active side of the enzyme and causes a decrease in enzyme activation (Kosim and Putra, 2010).

The performance of enzymes is influenced by several factors, especially the substrate, temperature, and pH (acidity level). However, this study focused only on the temperature and volume of cellulase enzyme in the hydrolysis process. Increasing the enzyme concentration to the optimum limit will increase the reaction rate as the enzymes are biomolecules proteins acted as catalysts that

speed up the reaction process without reacting in a chemical reaction. The speed of a reaction using an enzyme depends on the addition of the enzyme. At a specific substrate concentration, the reaction speed increases by increasing the enzyme concentration. But after reaching its maximum activity, increasing the enzyme concentration will not change the enzyme activity. Table 2 showed the relationship between enzyme volume and reducing sugar levels that the optimum enzyme volume was 7 ml. The optimum enzyme addition obtained in this study was not much different from the optimum enzyme addition in the previous study, 7.2 mL (Hamzah, 2018). At a specific substrate concentration, increasing the enzyme concentration will increase the enzymatic reaction's speed until the maximum reaction rate was achieved. After reaching it, the enzyme will be saturated by the substrate. Thus the increased enzyme concentration will not increase the reaction rate anymore (figure 3). A large number of substrates considers the use of enzymes; too few enzymes will cause the substrate hydrolysis process is not optimal or the substrate is hydrolyzed only a little, but if excess enzymes are used, the enzyme will become an inert compound and will not increase the conversion of the reaction.

Table 2. HPLC analysis result

No	Temperature (^{o}C)	Volume Cellulase Enzyme (mL)	Total Reducing Sugar (mg/L)
1.	55	7	96,673
2.	55	8	82,835
3.	60	7	89,676
4.	60	8	76,517

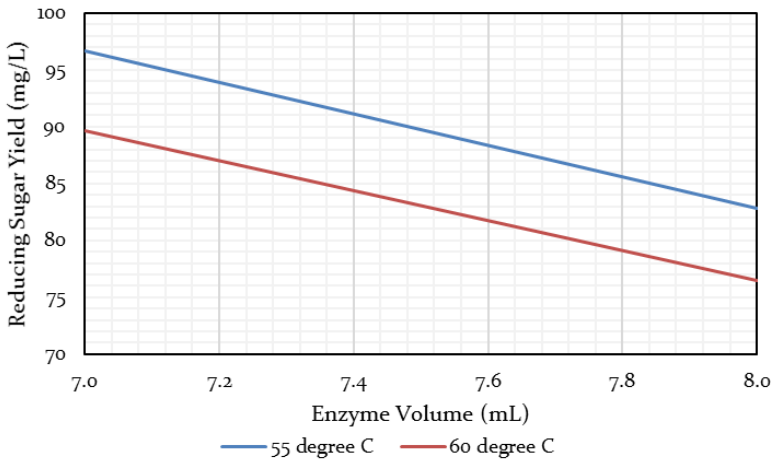

Figure 3. Relationship between enzyme volume and reducing sugar yield using HPLC analysis

3.3. Predict The Optimum Value

In the research of the enzymatic hydrolysis of pineapple leaves, after carrying out the stages including pretreatment, delignification, hydrolysis, and testing, the optimal reducing sugar content was 96.673% at 55^{o}C with 7 mL cellulase enzyme. The calculation was then carried out using a mathematical model to the graphical equations for the value of reducing sugar, resulting in the optimum hydrolysis conditions obtained at 55.2^{o}C and an enzyme volume of 7.2 mL. Therefore, it can be neglected that the prediction of the optimum conditions for the enzymatic hydrolysis process of pineapple leaf waste is around $\pm$ 55^{o}C and the enzyme volume is $\pm$ 7 mL. This prediction is carried out based on the results, so it depends on optimizing each stage of the process carried out and the conditions of the substrate and enzymes used.

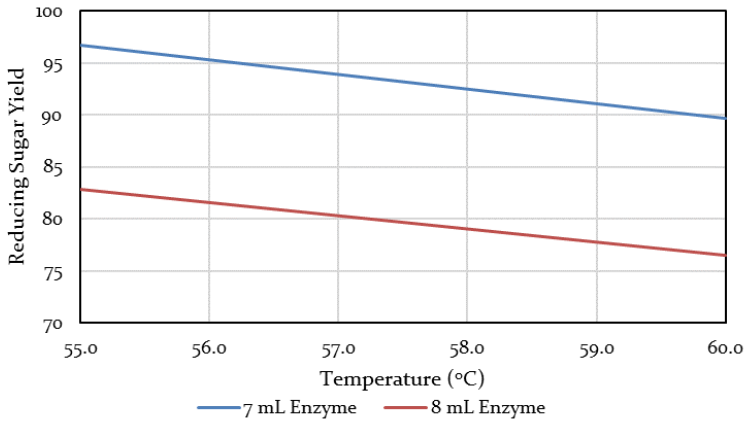

Figure 4. Relationship between temperature and reducing sugar yield using HPLC analysis

From the graphical equation for calculating the resulting reducing sugar, it can be seen that the temperature (^{o}C) and volume of the enzyme (mL) are proportional to the value of the resulting reducing sugar (mg / mL). The equation for calculating the value of reducing sugar obtained is as follows:

$$y = 0{,}2397x_1 + 3{,}2763x_2 - 23{,}2713 \quad (2)$$

with, y = reducing sugar yield (mg/mL)
x_1 = enzymatic hydrolysis operating temperature (^{o}C)
x_2 = cellulase enzyme volume (mL)

4. Conclusions

Reducing sugar could be produced from pineapple leaf wastes. In this study, the optimum operating condition of enzymatic hydrolysis is 7 mL of cellulase enzyme at 55^{o}C to produce 96,673 mg/L reducing sugar, followed by 6 mL of cellulase enzyme at 60^{o}C produces 89,676 mg/L reducing sugar. This result indicated that the enzymatic hydrolysis operating condition improved the reducing sugar yield from pineapple leaf waste. The optimum reducing sugar yield can produce biofuel by the saccharification process. Besides that, this article is expected to trigger other researchers to continue developing the hydrolysis of pineapple leaves or other agricultural wastes, so the problem of agricultural waste in Indonesia can be resolved and make waste that provides more benefits.

References

Akhlisah, Z.N., Yunus, R., Abidin, Z.Z., Lim, B.Y., Kania, D., 2021. Pretreatment methods for an effective conversion of oil palm biomass into sugars and high-value chemicals. Biomass and Bioenergy 144, 105-901.

Badan Pusat Statistik, 2012. Statistik Volume Ekspor Buah-Buahan Indonesia.

Bajaj, B.K., Pangotra, H., Wani, M., Sharma, P., Sharma, A., 2009. Partial purification and characterization of a highly thermostable and pH stable endoglucanase from a newly isolated Bacillus strain M-9. *Indian Journal* of *Chemical Technology*. 16, 382–387.

Banerjee, R., Chintagunta, A.D., Ray, S., 2019. Laccase mediated delignification of pineapple leaf waste: An ecofriendly sustainable attempt towards valorization. BioMed Central Chemical. 13, 1–11.

Chen, A., Guan, Y.J., Bustamante, M., Uribe, L., Uribe-Lorío, L., Roos, M.M., Liu, Y., 2020. Production of renewable fuel and value-added bioproducts using pineapple leaves in Costa Rica. Biomass and Bioenergy 141, 105675.

Conesa, C., Seguí, L., Laguarda-Miró, N., Fito, P., 2016. Microwaves as a pretreatment for enhancing enzymatic hydrolysis of pineapple industrial waste for bioethanol production. *Food* and *Bioproducts Processing*. 100, 203–213.

Dhillon, N., Chhibber, S., Saxena, M., Pajni, S., Vadehra, D. V, 1985. A constitutive endoglucanase (CMCase) from Bacillus licheniformis-1. Biotechnology Letters. 7, 695–697.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T., Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. Analytical Chemistry. 28, 350–356.

Faria, L.U.S., Pacheco, B.J.S., Oliveira, G.C., Silva, J.L., 2020. Production of cellulose nanocrystals from pineapple crown fibers through alkaline pretreatment and acid hydrolysis under different conditions. J. Mater. *Research and* Technology. 9, 12346–12353.

Hamzah, H., 2018. Isolasi dan Karakterisasi Enzim Selulase dari Keong Sawah Pila ampullaceal Menggunakan Substrat Serbuk Gergaji Kayu. Pharmauho J. Farm. Sains, dan Kesehat. 4, 16–21.

Irianti, Supadmi, A.H., 2019. Efektifivitas Proses Pengambilan Serat Daun Nanas (Ananas comosus [L.] Merr) dengan Metode Pengeratan.

Kementerian Perindustrian, 2019. Pemanfaatan Serat Nanas (Ananas comosus).

Kosim, M., Putra, R.S., 2010. Pengaruh Suhu pada Protease dari Bacillus subtilis. Pros. Skripsi Semester Gen.

Kumar, M., B, S.J., Banerjee, R., 2017. Biomethanation of Pineapple Wastes Using Potent Anaerobic Consortia Substituting Cow Manure. *Department* of *Food* Science and Technology.

Maneeintr, K., Leewisuttikul, T., Kerdsuk, S., Charinpanitkul, T., 2018. Hydrothermal and enzymatic treatments of pineapple waste for energy production. Energy Procedia 152, 1260–1265.

Mariano, A.P.B., Unpaprom, Y., Ramaraj, R., 2020. Hydrothermal pretreatment and acid hydrolysis of coconut pulp residue for fermentable sugar production. *Department* of *Food* Science and Technology. 122, 31–40.

Nakamura, K., Kitamura, K.B.T.-M. in E., 1988. Cellulases of Cellulomonas uda. In: Biomass Part A: Cellulose and Hemicellulose. Academic Press, pp. 211–216.

Nashiruddin, N.I., Mansor, A.F., Rahman, R.A., Ilias, R.M., Yussof, H.W., 2020. Process parameter optimization of pretreated pineapple leaves fiber for enhancement of sugar recovery. Ind. Crops Prod. 152.

Richana N., 2002. Produksi dan Prospek Enzim Xilanase dalam Pengembangan Bioindustri di Indonesia. Bul. AgroBio 5, 29-36.

Robson, L.M., Chambliss, G.H., 1984. Characterization of the cellulolytic activity of a Bacillus isolate. Applied *and* Environmental Microbiology. 47, 1039–1046.

Sadhu, S., 2013. Cellulase Production by Bacteria: A Review. British *Microbiology Research Journal*. 3, 235–258.

Setiawan, A.A., Shofiyani, A., Syahbanu, I., 2017. Pemanfaatan Limbah Daun Nanas (Ananas comosus) Sebagai Bahan Dasar Arang Aktif Untuk Adsorpsi Fe (II) 6, 66–74.

Sinaga, 2011. Comparison on The Nature of Polymer Matrix Composite Cellulose Acetate Synthesis and Cellulose Acetate Commercial Varied With Polypropilene for Packaging. Univ. Sumatera Utara.

Tarmansyah, U.S., 2007. Pemanfaatan Serat Rami untuk Pembuatan Selulosa.

Tsoumis, G., 1991. Science and technology of wood. Structure, properties, utilization. Sci. Technol. wood. Struct. Prop. Util. xiii + 494.

Vedovatto, F., Ugalde, G., Bonatto, C., Bazoti, S.F., Treichel, H., Mazutti, M.A., Zabot, G.L., Tres, M. V., 2021. Subcritical water hydrolysis of soybean residues for obtaining fermentable sugars. J. Supercrit. Fluids 167, 105043.