Ozonation Pre-treatment Evaluation for Xylanase Crude Extract Production from Corncob under Solid-State Fermentation

Efri Mardawati [1] *, Surya Martha Pratiwi [1], Robi Andoyo [1], Tita Rialita [1], M Djali [1], Yana Cahyana [1], Een Sukarminah [1], In-In Hanidah [1], Imas Siti Setiasih [1]

[1]Faculty of Agro- Industrial Technology
Universitas Padjadjaran Jl. Raya Bandung Sumedang KM.21, Sumedang, 45363, Indonesia

Email: efri.mardawati@unpad.ac.id*

Abstract

Xylanases are a highly exploited enzyme in industries, including food and chemical industry. Xylanases can be utilized in catalyzing the endohydrolysis of 1,4-β-xylosidic linkages in xylan, lignocellulosic component to produce xylose-monomer. This research aims to optimize xylanase production from an alternative substrate, corncob. Corncob contains 41.17% of hemicellulose, the polymer of xylan. Xylanases are produced through solid state fermentation by Trichoderma viride. The ratio between substrate and moistening solution was 0.63 g/mL with fermentation temperature 32,8OC. Variables varied include incubation time and pre-treatment (using autoclave, ozonation, a combination of ozonation and autoclave, also without pre-treatment). Xylanase activity was measured by DNS method using 1% of xylan as substrate standard. The result showed that the best incubation time is 36 h with 14403.8707 U/mg protein for specific xylanase activity by using autoclave as pre-treatment. Ozonation pre-treatment process can increase the enzyme activity of xylanase.

Keywords: *Corncob, Pre-treatment, Solid-state fermentation, Xylanase.*

1. INTRODUCTION

The use of enzymes in Indonesia reached 2500 tons with the import value of 187.5 billion in 2015. Almost all enzymes for industrial needs in Indonesia are imported from other countries. One of the enzymes needed is xylanase. Xylanase in the food industry is used to hydrolyze xylan into xylose. Xylanase is often used in bread industry to make a soft dough and to increase the volume. Juice and wine industry also need xylanase on the process of extraction, purification, and stabilization (1). Therefore, there must be an independent production of xylanase using a widely available raw materials such as agricultural waste.

Waster is a major source of environmental pollution and has a negative impact on society. Corncobs are usually used only for animal feed or fertilizer. Chemical analysis of the corncobs indicates that there are cellulose, hemicellulose, and lignin. Hemicellulose main chain can consist one polymer, for example, xylan, that is useful in the production of xylanase (2).

Maize (*Zea mays L.*) is the second staple food after rice in Indonesia. Indonesia's maize production in 2015 reached 19.6 million tonnes spread over 3.8 million hectares of plantation area. The corn production wasted corncob as much as 1 ton per hectare or approximately 3.8 million tonnes.

Almost all xylanases are produced by using fungi. The enzyme activity produced by the fungi is higher than that of bacteria. Production of the enzyme can be made by submerged fermentation or solid-state fermentation. Research of xylanase production from agricultural waste such as rice, wheat, barley, corn, and soybeans usually use solid-state fermentation methods. Solid-state fermentation is made from agricultural waste

with the help of fungi because bacteria and yeast are not resistant to low humidity levels. Affecting factors of solid-state fermentation include incubation time, inoculum concentration, as well as physiological and nutritional parameters.

Results of xylanase production by using solid-state fermentation method can be maximized by pre-treatment. Pre-treatment in the production of xylanase aims to eliminate lignin that can inhibit lignin xylan extraction (2). If the lignin is not degraded, the accessibility of fungi on the xylan cannot be maximized and affect the final result. Pre-treatment is usually done physically by autoclave. Ozone pre-treatment is able to do the same. Lignin is specifically degraded by ozone (up 95%), leaving a solid fraction rich in sugar (3).

Ozonation has various advantages. It is simple and relatively fast in high dry concentration (45-60%), low temperature (less than 40°C), and without the production of fermentation inhibitors such as furfural or 5-HMF. Most of the carboxylic acid and phenolic compounds arising from the degradation of lignin can be removed only with distilled water (at room temperature). All of cellulose and hemicellulose are basically found in the fibers after plasma pre-treatment without any degradation (3).

Further investigation is required for the influence of many pre-treatments on the xylanase production from the corncob under a solid-state fermentation process by using fungi. The production of the xylanase by using pre-treatment also needs to be compared with those which is not given. These things need to be done because the pre-treatment of the lignocellulosic material has a different effect on the xylanase.

2. MATERIALS AND METHODS

2.1 Material

Raw materials used in the experiment were corncobs from Universitas Padjadjaran. Before used, the materials were sun dried, cleaned, oven dried at 105 ˚C overnight and ground. The particle size of materials 40-60 mesh were used Other materials used in the production of xylanase were a microbial culture (*Trichoderma viride*), PDA, $CaCO_3$, $MgSO_4$, distilled water, $(NH_4)_2SO_4$, KH_2PO_4, urea, $CaCl_2$, $MgSO_4.7H_2O$, 0.45 µm porous filter paper, H_2SO_4, bovine serum albumin, ethanol, xylose, xylan, acetate buffer at pH 5, dinitrosalicylic acid, and oxygen.

2.2. Ozonation process

The ozone was produced from pure oxygen using an ozone generator (LUSO, OZ-5G). Ozone is formed by a high energy input splitting the O_2 (oxygen) molecule. Single O rapidly combines with available O_2 to form the very reactive O_3. At the equipment outlet, a hose with a porous surface made of sand was connected with oxygen flow rate of 0.25 L/min (Fig. 1). This system was used to distribute the generated ozone as micro-bubbles in the water, improving the ozone mass transfer and homogeneity. The ozonation was carried out in a stainless steel rectangular tank of 0.10 m diameter and 0.3 m height. The 0.8 L of water/sample was placed in the tank, the ozone generation system was turned on and applied to the sample for 10 min. These processes were carried out in duplicate. Ozone concentration at exposing time of 10 min is approximately 4.195 ppm.

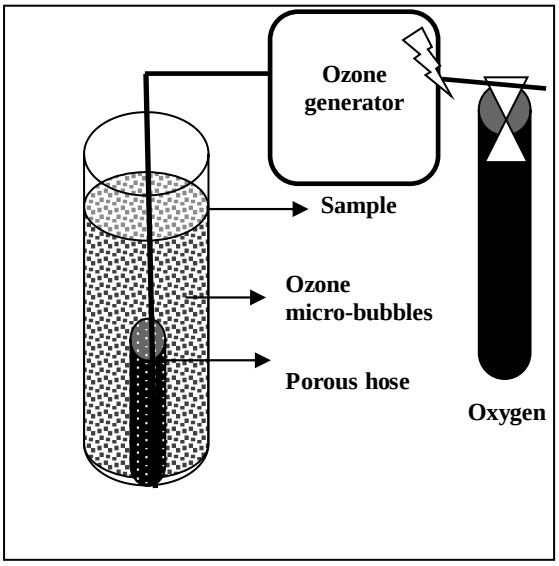

Figure 1. Schematic diagram of ozonation system

2.3. Production of crude xylanase

Crude xylanase was produced from solid state cultivation of *T.viride* ITBCC L67 on Corn Cob. The cultivations of *T.viride* ITB CCL.67 was carried out in 300 mL shake flasks on the media that composed of corncob and mineral solution. The inoculum was prepared by suspending the spores in sterile physiological solution to give a final spore count of 1×10^6 spores/mL. Enzymes were extracted from the fungal cultivation by adding distilled water (four-time volume of liquid medium) to the cultivation solution. Subsequently, the solution was stirred with a sterile glass stick and then shook at 100 rpm for 1 h at room temperature. The crude enzyme was obtained by separating the solid (leftover corncob and fungal) from the solution by centrifugation. The supernatant was then analyzed for enzymatic activity following the DNS method using Larchwood xylan (Sigma Co., USA) as substrate.

2.4 Analysis

The xylanase activity was determined using Oat spelts xylan (Sigma Co., USA) as the substrate. The reducing sugars produced were quantified by the dinitrosalicylic acid method using D-xylose as standard.

2.5 Research Method

The method used was experimental descriptive which described the effects of various types of pre-treatment (using an autoclave, ozonation, ozonation combined with an autoclave, and without pre-treatment) of the xylanase production on corncobs. The hypothesis test was using two parties. The independent variable (X) in this study was the type of pre-treatment given. The dependent variable (Y) was the value of enzyme activity and specific activity of the enzyme produced due to the provision of various pre-treatments. The study consisted of 4 treatments (using an autoclave, ozonation, ozonation combined with the autoclave, and without pre-treatment) with two replicates.

3. RESULTS AND DISCUSSIONS

3.1 Raw Material Analysis

Proximate analysis of corncobs consists of content analysis such as hemicellulose, cellulose, lignin, water, and ash. Results of the compound biomass analysis of corncobs can be seen in Table 1.

Table 1. Corncobs Proximate Analysis.

No	Component	Content (%)
1	Hemicellulose	41,17
2	Cellulose	20,89
3	Lignin	15,90
4	Water	5,96
5	Ash	7,40

Hemicellulose content needs to be analyzed to determine how potential the corncobs are to be used as raw materials for xylanase production. The required main component, xylan, is contained in the hemicellulose. Hemicellulose content of the corncob was 41.17%, which is higher compared with other agricultural waste.

Lignin content needs to be analyzed to determine how many components can inhibit the enzymatic hydrolysis process. Lignin prevents the entry of enzymes from breaking down polysaccharides into monosaccharides. Hemicellulose and cellulose are united by lignin and form a layer as an inhibitor of the solution penetration. The lignin content in corncob was 15.90%. This suggests that the corncob was potential to be used because it contained ≤ 25% lignin (4).

The water content needs to be analyzed to determine whether the corncobs have a long shelf life or not. High water content causes the corncob to be quickly damaged by the growth of microorganisms. If the water content is known then customized storage conditions can be made for raw materials to minimize the possibility of growing microorganisms. Used corncobs had 5.96% of water content. This shows that the corncob was very dry, so it could be stored for a long period of time and did not require special treatment. Storing at room temperature in a covered

place could be made to avoid mechanical damage and absorption of moisture from the surrounding environment. The corncob was potential because it had ≥ 85% amount of dry matter (4).

The ash content indicates the content of organic substances or mineral elements of a material. The ash content is influenced by the type, age, and others. Based on the analysis, the corncob had the ash content of 7.40%. The ash content of corncobs ranged from 1.36 to 1.76%. The differences could be caused by the difference on the addition of fertilizer and soil conditions where the corn grows.

3.2 Enzyme Activity of Xylanase Crude Extract

The incubation time search was performed to determine the best time for the growth of microorganisms in the production of xylanase. The incubation time search process applied to *Trichoderma viride* with 32.8°C temperature and 0.63 ratios of corncobs substrate with moistening solution. Sampling was done every 12 hours for 72 hours and enzyme activity test performed on each sample. Results of the enzyme activity test are shown in Figure 1. The testing process was done in duplicate enzyme activity.

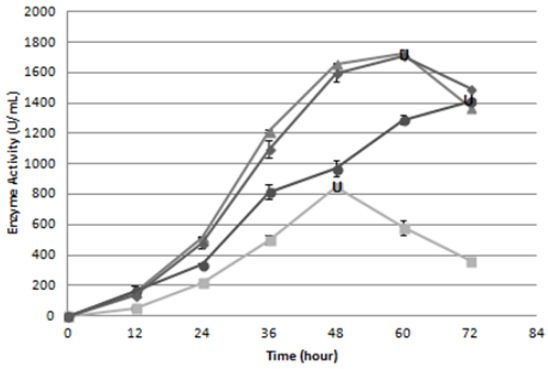

Figure 1. Effect the pre-treatment method on Enzyme Activity during incubation time.

= without *pre-treatment*; = autoclave; = ozonation; = ozonation combined with autoclave; U = maximum enzyme activity

The relationship between the enzyme activities was almost equal to the growth curve of microorganisms. The growth curve of microorganisms consists of four phases: adaptation, logarithmic phase, stationary phase, and death phase (5).

Trichoderma viride grown on corncobs will have a process of adaptation. The adaptation process includes the synthesis of new enzymes in accordance with the medium and the recovery of the metabolites that are toxic to the previous media.

Xylanase was formed because of the growth of *Trichoderma viride* assisted with the number of substrates in the form of xylan on corncobs. There was no increase in the number of cells in the adaptation phase so the enzyme activity did not increase significantly. This was shown on 0 to 12 h in all treatments. It only increases the volume because cells in static phase usually do downsizing.

The cell divides after obtaining ideal conditions. Ideal conditions had been achieved by *Trichoderma viride* with the nutrients contained in the mixture of corncobs and moistening solution. Microorganisms performed nutrient consumption and other physiological processes in this phase. Compound products desired by human are formed.

The number of microorganisms rapidly increased and was comparable with the high activity of the xylanase produced. The value of enzyme activity produced by using autoclave as pre-treatment, as well as the combination of ozonation and the autoclave, were at a logarithmic phase in 12 to 48 hours. The xylanase produced without pre-treatment was also the same. The only anomaly of the result was xylanase produced using ozonation pre-treatment which continued to rise during the observation. This occurred because ozonation as a significator splitting strong carbon-carbon bonds of aromatic ring was slower than the weak carbon-carbon bonds of chain aliphatic (6). This slower process caused the logarithmic phase to last longer. It was not certain whether the fermentation time was longer, the value of enzyme activity by using ozonation as pre-treatment would be higher or not. Further research is needed to look into that matter. The multiplication of the number of cells occurred to a certain extent, then entered the stationary phase.

Some cells are dead in the stationary

phase while others grow and divide so that the number of alive cells is fixed. Enzyme activity value tended to be static at 48-60 h on corncob with pre-treatment using an autoclave as well as a combination of ozonation and autoclave. Corncobs with ozonation pre-treatment did not reach stationary phase yet.

This study showed that *Trichoderma viride* died within few days, making the value of the enzyme activity decrease. The value of enzyme activity began to decline from the 60th hour on the cob corn by thermal pre-treatments, such as using the autoclave as well as a combination of ozonation and autoclave. Ozonation pre-treatment had not shown any activity since continued to rise. The value of enzyme activity from corncob without pre-treatment decreased from 48 h.

Based on Figure 1, the activity of the enzyme at each pre-treatment reached a maximum at different times. Corncob without pre-treatment reached maximum enzyme activity after incubation for 48 hours, the fastest of the others. This happened because the delignification process did not happen. Lignin delignification is an acquisition process of a complex compound. The delignification process is important because lignin may inhibit penetration. Without pre-treatment, the penetration of *Trichoderma viride* was inhibited.

The xylanase produced by pre-treatment reached maximum activity with longer incubation than without pre-treatment. This happened because of the delignification process. Lignin could be free and penetration of *Trichoderma viride* was going well. Autoclave pre-treatment as well as a combination of ozonation and autoclave had a maximum enzyme activity by incubation for 60 hours. *Trichoderma viride* could produce xylanase with longer maximum value because lignin had been damaged so that the fungi could optimally use xylan.

The value of enzyme activity by using ozonation as pre-treatment continued to rise for 72 h. The dynamics of the early stages of fermentation were due to the corncob treated by ozonation contained more dissolved oxygen. Oxygen is required for the propagation of cells, *Trichoderma viride,* in this study. The results showed that with ozonation process for 5 min, the concentration of dissolved oxygen in the wort had doubled (7). Oxygen dissolved in corncobs created suitable conditions for the early stages of fermentation so that the number of microorganisms continued to rise, and so did the value of the enzyme activity.

Corncob without pre-treatment had a maximum value of enzyme activity to 847.0223 U/mL. The value was the lowest of other treatments because of the absence of delignification process. Maximum enzyme activity was obtained by autoclave pre-treatment with a value of 1727.6694 U/mL. The combination of ozonation and autoclave processes also had high maximum enzyme activity, 1711.6576 U/mL. Xylan is soluble in hot water. This led xylan easier to be enzymatically synthesized (8). Higher temperature (50°C) facilitates the release process of hemicellulose and lignin (9).

Ozonation process produced maximum enzyme activity to 1411.7039 U/mL, lower than the autoclave pre-treatment. There are two phases in the delignification process using ozonation as a pre-treatment. As explained previously, ozone splits carbon-carbon bonds of aromatic ring slower than the weak carbon-carbon bonds of chain aliphatic (6). Lignin as an aromatic polymer subunits is usually derived from phenylalanine (10). Therefore, the value of enzyme activity with ozonation pre-treatment was lower than autoclave. Aromatic rings in lignin were possibly splitting less than by using a thermal treatment.

3.3 Specific Activity of Xylanase Crude Extract

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 a better ability of xylanase to utilize the substrate, i.e. corncob. The calculation of specific activity was done by comparing the value of enzyme activity with protein content.

Enzymes are the most common group of proteins in living cells and have an important

function such as catalysts of biochemical reactions that collectively form metabolisms-intermediates from cells. The protein content was used as one of the tests because the solid-state fermentation caused the cells to mix with the substrate, making it difficult to calculate the number of dry weight cells. The protein testing process, similar to enzyme activity, was applied to *Trichoderma viride* with a temperature of 32.8°C and a corncob substrate ratio with moistening solution of 0.63. Samplings were performed every 12 hours for 72 hours and protein testings were performed on each sample.

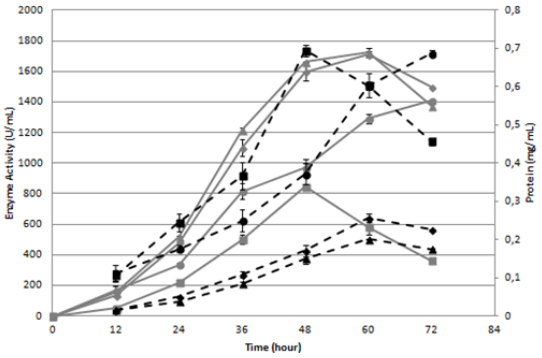

Figure 2. Effect the pre-treatment method on Enzyme Activity and protein content during incubation time.

= enzyme activity (without pre-treatment); = enzyme activity (autoclave); = enzyme activity (ozonation); = enzyme activity (ozonation-autoclave); = protein (without pre-treatment); = protein (autoclave); = protein (ozonation); = protein (ozonation-autoclave)

The relationship between time and protein, as well as the relationship between time and enzyme activity, were almost the same as the growth curve of microorganisms. This was in line with the literature which states that: The higher the protein content, the higher the growth of microorganisms during the fermentation process. Protein at each pre-treatment reached a maximum at different times. Maximum protein was reached after fermentation process for 48 hours without pre-treatment. The protein produced by pre-treatment reached the optimum point longer than without pre-treatment. Ozonation caused the protein content to get higher and higher for 72 hours. Autoclave produced maximum protein at the 60th hour, as well as the combination of ozonation and autoclave.

The protein produced in the absence of pre-treatment had a value of 0.6942 mg/mL, the highest of other treatments. This happened because there was still protein content in the corncob that was not damaged in the absence of additional pre-treatment. The protein contained in the crude extract of xylanase was not the only enzyme, but there were also sources of protein from the growth medium.

Ozonation produced 0.6875 mg/mL of protein, which was not much difference with protein without pre-treatment. There is a study with wheat grain material, which also contained many hemicelluloses (11). The study showed that there is no change in water content, ash, protein, and color after grinding wheat grains with those extracted using water with ozone content in it (12). Peptide nitrogen is relatively inert for reaction with ozone (13). The side carbon chains found in peptides are still available for oxidation reactions. Oxidation of the side chain without breaking the amide bond will result in complex, partial oxidation formation, with a high molecular weight of the compound.

The combination of ozonation and autoclave, especially autoclave alone, has a low protein. The combined pre-treatment produced 0.2577 mg/mL of protein. The protein produced by autoclave was 0.2007 mg/mL. Zhao, et al. (2007) conducted a study on wheat seeds that also contained lots of hemicellulose such as corncobs (15). The study stated that high temperature is a major determinant of seed growth and yield formation in wheat. High temperatures will reduce the levels of protein in whole grains (14).

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. The causes of denaturation are heat, pH, pressure, electric current, and the presence of chemicals such as urea, alcohol, and soap. Temperature is the midpoint of the denaturation process, which is called melting temperature (Tm). In general, protein has a Tm value less than 100ºC. If the temperature

is above Tm, then the protein will experience denaturation.

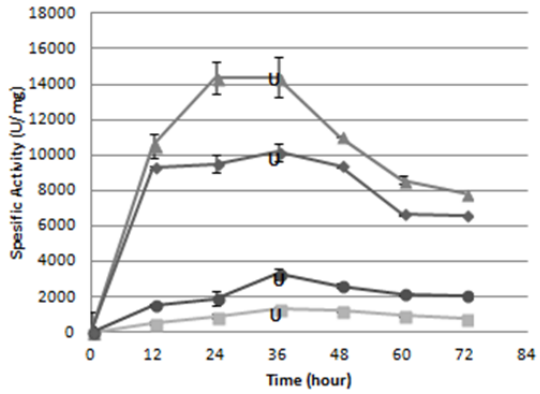

Figure 3. Effect the pre-treatment method o n Enzyme Specific Activity during incubatio n time.
= without pre-treatment; = autoclave; = ozonation; = ozonation combined with autoclave; U = maximum specific activity

The specific activity of the enzyme raised over a period of time, then decreased, almost the same as the relationship between time and enzyme activity. The value of the specific activity of the enzyme at all pre-treatments is maximum at 36 hours. It showed that *Trichoderma viride* is most active to produce xylanase crude extract by using corncob within one minute after a 36-hour fermentation process.

The specific enzyme activity from the lowest to the highest was the same as the value of enzyme activity, i.e. without pre-treatment, ozonation, ozonation combined with the autoclave, and autoclave. The maximum specific activity of xylanase was obtained by pre-treatment of the autoclave. The value of the specific activity was 14403.8707 U/mg. The combination of ozonation and autoclave had the highest specific activity value of 10169.5048 U/mg. The ozonation treatment produced xylanase with a specific activity value of 3304.5499 U/mg after 36 hours. The lowest specific activity value was corncob without pre-treatment with a value of 1369.1277 U/mg. There should be a t-test to know whether pre-treatment on corncob is significant or not.

The result of the t-test between pre-treatment and without pre-treatment showed that the specific activity of the enzyme without and with pre-treatment (such as an autoclave, ozonation, and combination between ozonation and autoclave) was significantly different. Pre-treatment in xylanase production could significantly increase the value of the specific activity.

The t-test was also performed between ozonation and autoclave. This suggested that the specific activity of the enzyme in the ozonation and autoclave treatment was significantly different. The real difference occurred because the ozone molecules that divide the strong carbon bonds of lignin were slower than the autoclave.

CONCLUSIONS

The pre-treatment process can increase the enzyme activity and the specific activity enzyme produced when compared with no pre-treatment. Pre-treatment using autoclave produced the highest enzyme activity and specific activity. Ozonation and a combination of ozonation and autoclave also increase the value of enzyme activity and the specific activity, but still lower than using the autoclave.

REFERENCES

[1]. Polizeli, M L T M, et al., et al. 2005 *Xylanases from Fungi: Properties and Industrial Applications.* Appl. Microbiol. Biotechnol, pp. 577-591

[2]. Bajaj, B K and Singh, N P. 2010. *Production Xylanase from an Alkali Tolerant Streptomycess sp. 7b under Solid-State Fermentation, Its Purification, and Characterization*, Appl. Biochem. Biotechnol., pp. 1804-1818

[3]. Vazquez, M J, et al. 2000. *Xylooligosaccharides: Manufacture and Applications.*. Trends in Food Science and Technology

[4]. Schultz-Jensen, N, et al., et al. 2011. *Plasma-Assisted Pre-treatment of Wheat Straw for Ethanol Production*, Appl. Biochem. Biotechnol, pp. 1010-1023.

[5]. Cirakovic, J and Diner, B A. 2010. *Ozone Treatment of Biomass to Enhance Enzymatic Saccharification.* US20100159521 A1.

[6]. Willey, J M, Sherwood, L and Woolverton, C J. 2008. *Prescott, Harley, and Klein's Microbiology*, McGraw-Hill Higher Education.

[7]. Souza-Corrêa, J A, et al., et al. 2013, *Atmospheric Pressure Plasma Pre-treatment of Sugarcane Bagasse: The Influence of Moisture in the Ozonation Process.* Appl. Biochem. Biotechnol, pp. 104-116.

[8]. Dziugan, P, et al., et al. 2016. *Ozonation as an Effective Way to Stabilize New Kinds of Fermentation Media Used in Biotechnological Production of Liquid Fuel Additives*, Biotechnol. Biofuels.

[9]. Whistler, R L. 1950. *Xylan, in: Cantor, C.S.H. and S.M. (Ed.), Advances in Carbohydrate Chemistry*, Academic Press, pp. 269-290.

[10]. Wu, L, et al., et al. 2011. *Low Temperature Alkali Pre-treatment for Improving Enzymatic Digestibility of Sweet Sorghum Bagasse for Ethanol Production*, Bioresour. Technol., pp. 4793-4799.

[11]. Whetten, R and Sederoff, R. 1995. *Lignin Biosynthesis*, Plant Cell, pp. 1001–1013.

[12]. İbanoğlu, S. 2001. *Influence of Tempering with Ozonated Water on The Selected Properties of Wheat Flour*, Research Gate, pp. 345-350.

[13]. O'Donnell, C, et al., et al. 2012. *Ozone in Food Processing.*

[14]. Lacheur, R M Le and Glaze, W H. 1996. *Reactions of Ozone and Hydroxyl Radicals with Serine*, Env. Sci Technol, pp. 1072-1080.

[15]. Zhao, H, et al., et al. 2007. *Leaf Senescence and Grain Filling Affected by Post-anthesis High Temperatures in Two Different Wheat Cultivars*, Research Gate, pp. 149-158.

[16]. 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*, Curr. Trends Biotechnol. Pharm, pp. 1434-1444.

[17]. Wolfrom, M L and Tipson, R S. 2014. *Advances in Carbohydrate Chemistry*, Academic Press.