World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
Vol:16, No:9, 2022

Use of Corn Stover for the Production of 2G Bioethanol, Enzymes and Xylitol under a Biorefinery Concept

Astorga-Trejo Rebeca, Fonseca-Peralta Héctor Manuel, Beltrán-Arredondo Laura Ivonne, Castro-Martínez Claudia

***Abstract*—**The use of biomass as feedstock for the production of fuels and other chemicals of interest is an ever growing accepted option in the way to the development of biorefinery complexes. In the Mexican state of Sinaloa, a significant amount of residues from corn crops are produced every year, most of which can be converted to bioethanol and other products through biotechnological conversion using yeast and other microorganisms. Therefore, the objective of this work was to take advantage of corn stover and evaluate its potential as a substrate for the production of second generation bioethanol (2G), enzymes and xylitol. To produce bioethanol 2G, an acid-alkaline pretreatment was carried out prior to saccharification and fermentation. The microorganisms used for the production of enzymes, as well as for the production of xylitol, were isolated and characterized in our work group. Statistical analysis was performed using Design Expert version 11.0. The results showed that it is possible to obtain 2G bioethanol employing corn stover as a carbon source and *Saccharomyces cerevisiae* ItVer01 and *Candida intermedia* CBE002 with yields of 0.42 g and 0.31 g, respectively. It was also shown that *C. intermedia* has the ability to produce xylitol with a good yield (0.46 g/g). On the other hand, qualitative and quantitative studies showed that the native strains of *Fusarium equiseti* (0.4 IU/mL - xylanase), *Bacillus velezensis* (1.2 IU/mL – xylanase and 0.4 UI/mL - amylase) and *Penicillium funiculosum* (1.5 IU/mL - cellulases) have the capacity to produce xylanases, amylases or cellulases using corn stover as raw material. This study allowed us to demonstrate that it is possible to use corn stover as a carbon source, a low-cost raw material with high availability in our country, to obtain bioproducts of industrial interest, using processes that are more environmentally friendly and sustainable. It is necessary to continue the optimization of each bioprocess.

***Keywords*—**Biomass, corn stover, biorefinery, bioethanol 2G, enzymes, xylitol.

I. Introduction

CORN stover (CS) is an abundantly available lignocellulosic agricultural biomass around the world. Nowadays, CS is either incorporated into agricultural soils or burned, causing environmental pollution problems. This lignocellulosic biomass is not yet used for the production of products of biotechnological interest. CS is mainly composed of: lignin (15-25%), hemicellulose (20-40%), cellulose (30-50%) [1]-[3]. Due to its abundance and low cost, CS is a promising source of raw material for the production of bioenergy, enzymes, feedstock, etc.

In order to give value to agricultural residues and reduce environmental pollution, it is necessary to carry out a pretreatment since lignocellulosic biomass is a complex, rigid and difficult to hydrolyze structure. This important process helps to reduce the recalcitrance of the molecule and increases the availability of cellulose for subsequent enzymatic hydrolysis [4], [5]. Moreover, pretreatment allows the release of simple sugars from cellulose and hemicellulose, which can be used to obtain products of commercial importance.

Obtaining products such as: chemicals, food, enzymes and biofuels from lignocellulosic biomass can be done by extraction, chemical extraction or degradation. In 1990 the term biorefinery appeared, and it was defined as "a facility that integrates biomass conversion processes and equipment to produce fuels, energy and chemicals from biomass" [6]. In this sense, it is important to study and evaluate raw materials with high availability for their potential transformation into products of commercial interest.

There are many studies that indicate that 2G bioethanol is considered a potential alternative biofuel to gasoline of fossil origin, and it can be produced from agricultural residues such as CS [3], [7].

Over the last decades, the study of the application of enzymes at an industrial level has increased. In particular, in the area of biofuels (second generation bioethanol production: from agro-industrial waste), enzymes are essential during the saccharification process, that is, during the obtaining of fermentable sugars for their subsequent transformation into bioethanol, as well as in the production of xylitol, which can be obtained as a co-product of obtaining 2G bioethanol, by using the hemicellulosic fraction of the process. In the aquaculture area, the use of enzymes such as: amylases, xylanases, proteases and cellulases is of utmost importance to improve animal nutrition, facilitating their digestion and favoring the growth and quality of the organism [8]. Enzymes can be obtained from any living being. However, the use of enzymes produced by microorganisms via fermentation is preferred, because they have certain advantages: high yields, possibility

Astorga-Trejo Rebeca is with the Instituto Politécnico Nacional-Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional. Guasave, Sin. 81101 MX (phone: 687-134-4888; e-mail: rastorgat2000@alumno.ipn.mx).

Fonseca-Peralta Héctor Manuel is with Universidad Autonoma de Sinaloa, Facultad de Ciencias Biológicas, Culiacán, Sin. MX (e-mail: hectormanuelfonseca@outlook.com)

Beltrán-Arredondo Laura Ivonne was with the Instituto Politécnico Nacional-Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional. Guasave, Sin. 81101 MX (e-mail: laura_beltran_a@hotmail.com).

Castro-Martínez Claudia is with the Instituto Politecnico Nacional-Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (IPN CIIDIR Sinaloa). Guasave, Sin. 81101 MX (corresponding author, phone: 687-872-9626 ext. 87661; e-mail: clcastro@ipn.mx).

of modification and optimization, low cost of culture media, rapid growth of microorganisms, etc. [9].

Cellulases are a group of three enzymes (endo-glucanases, exo-glucanases and β-glucosidases) that act synergistically to hydrolyze cellulose, the main component of lignocellulosic biomass, and obtain second generation bioethanol [2], [10]. Xylanases are a class of hemicellulolytic enzymes that are necessary for the hydrolysis of the β-1,4 xylan present in lignocellulosic material. Xylanases are enzymes that have taken great interest due to their potential application in industry, as well as in the use of agro-industrial waste, improvement of feeds made from lignocellulosic material, clarification of juices and wines and bleaching in the paper industry [11]. Amylases are important in biotechnological applications, such as in the food, fermentation, detergent, pharmaceutical, textile and beverage industries [12]. Due to an extensive distribution of lignocellulosic matter, there is a great variety of microorganisms capable of producing enzymes, among which the genuses of: Trichoderma, Penicillium, Aspergillus and Bacillus, amongst others stand out [13]. On the other hand, xylitol is a five-carbon sugar with applications in the pharmaceutical, nutraceutical and food industries [14]. The biotechnological conversion of xylose to xylitol offers a more accessible alternative in terms of energy and process costs, since it is carried out at lower temperatures and pressures than in production by chemical synthesis [15]. In addition, it is possible to use lignocellulosic hydrolyzed substrates for its production, acid hydrolysates obtained during the 2G bioethanol production process being an example.

For the above, the objective of this work was to evaluate the potential of corn residues as raw material for the production of 2G bioethanol, xylitol and enzymes using isolated microorganisms in the work group.

II. Materials and Methods

A. Material

CS was obtained from the northern region of Sinaloa, Mexico. It was collected from the remnants in the soil during the grain harvest. The material was washed with distilled water to remove dust, dried at 60 °C for 72 h, crushed and sieved to a particle size of 1 mm using a blender and lastly, stored in an airtight bag at room temperature for later analysis.

B. Pretreatment of Biomass

Sulfuric acid (H_2SO_4) was used for acid pretreatment. Glass flasks containing 10 g of dry material (10% w/v) and 1% H_2SO_4 were autoclaved at 121 °C for 60 min. After the hydrolysis, the reaction mixture was cooled to room temperature. The recovered samples were centrifuged and filtered. The supernatant was subjected to a total reducing sugars analysis using dinitrosalicylic acid (DNS) method [16].

C. Preculture and Culture Conditions

Pre-inoculums were made in a 250 mL Erlenmeyer flask containing 50 mL of mineral medium and glucose. After inoculation, they were incubated at 30 ºC for 12 h, at a shaking speed of 150 rpm. After the incubation time, cells were transferred to a growth media made using 5% glucose and mineral salts as substrate: $(NH_4)_2SO_4$ - 0.2%, KH_2PO_4 - 0.5%, $MgSO_4.7H_2O$ - 0.04% and Yeast extract 0.1% [17]. The culture conditions were 30 ºC, 150 rpm and pH 4.5. The inoculum was 3 X 10^6 cells/mL. All experiments were done in duplicate.

D. Ethanol and Xylitol Production

To evaluate the ethanol production using CS as raw material, the yeasts *Saccharomyces cerevisiae* Itver-01 [18] and *Candida intermedia* CBE002 [19] isolated by our working group were used. The fermentations were performed in a minimal medium containing mineral sales and yeast extract [17] and supplemented with pretreated CS in Erlenmeyer flasks (50 mL of culture medium). The inoculum was 3 X 10^6 cells/mL, at a shaking speed of 150 rpm and a pH of 4.5. Samples were taken periodically.

To evaluate the production of xylitol, the acid hydrolysates of CS were used as raw material as well as the yeast *C. intermedia* CBE002. The procedure followed was the same as for ethanol production.

E. Enzyme Production

For the production of enzymes, strains from the collection of microorganisms of the Laboratory of Bioenergetics of the IPN CIIDIR Sinaloa were used. The strains evaluated were: *Penicillium funiculosum, Bacillus velezensis* and *Fusarium equiseti*.

Enzyme production was carried out in submerged fermentation (SF) and CS was used as the sole carbon source (1%). SF was performed in 250 mL Erlenmeyer flakes containing 50 mL of basal medium, with pH adjusted to 5 prior to sterilization (15 min at 121 °C). The inoculated flasks were then incubated for 5 days at 30 °C and 200 rpm.

Cellulase activity: Extracellular cellulase activity of microorganisms was evaluated on Congo red plates [20]. The agar plate was prepared with carboxymethyl cellulose (1% w/v) and agar (2% w/v). After solidification, wells were formed aseptically. The wells were filled with supernatant sample and incubated at 30 °C for 24 h. Then plates were stained with 1% w/v Congo red solution for 15 min and discolored with 1M NaCl for 15 min.

CMCase activity: CMCase activity was determined according to [21] with some modification by incubating 0.25 mL of the assay mixture containing 0.20 mL of 1% CMC in acetate buffer, pH 5.0 and 0.05 mL of enzyme extract at 50 °C for 50 min. Enzyme and substrate blanks were simultaneously incubated with the samples. The reaction was stopped by adding 0.250 mL of DNS reagent, followed by 5 min boiling. After cooling, the absorbance was read at 540 nm using glucose as a standard, following, the protocol reported by [16].

Xylanase activity: The xylanolytic activity was determined by the hydrolysis of 1% beechwood xylan (w/v) at 50 ºC in 0.05 M sodium citrate buffer at pH 5. The reaction mixture was incubated at 50 ºC in a thermoblock for 10 min. Subsequently, 250 µl DNS were added and incubated at 100 °C for 10 min, the reaction was put on ice and read in a spectrophotometer at 550 nm. A xylose standard curve was used [22].

Amylase activity: The activity of α-amylase was determined by the DNS method [16]. The reaction contained the following: 200 µl of 0.05 mM sodium acetate buffer at pH 5 plus 0.5% (w/v) of hydrolysis of 1% starch (w/v) as substrate, 50 µL of enzyme extract from a dilution 1: 4, the reaction mixture was incubated at 50 °C for 20 min. Subsequently, 250 µL DNS were added and incubated at 100 °C for 10 min, the reaction was put on ice and read in a spectrophotometer at 540 nm. A D-glucose standard curve was used.

One unit of enzyme activity was defined as the amount of micromoles of glucose liberated per milliliter of enzyme solution in one minute.

F. Analytical Techniques

The supernantant was stored at -20 ºC until analysis. Glucose, xylose, ethanol and xylitol were analyzed using a High Performance Liquid Chromatography (HPLC) System (Waters, Milford, MA, USA) using a Biorad Aminex HPX-87H column (BioRad Laboratories, Inc., Hercules, CA, USA) [17].

III. Results and Discussion

A. Production of Bioethanol 2G

The production of second generation bioethanol involves three fundamental processes: pretreatment, enzymatic hydrolysis and fermentation. In this work, bioethanol 2G was obtained using corn stubble pretreated with 1% H_2SO_4 and 4% hydrogen peroxide, commercial cellulases (Cellitec-3) were used for saccharification and the *S. cerevisiae* ItVer01 strain was used for fermentation. The results obtained show that it is possible to produce bioethanol from CS as raw material. Yields of 0.42 g/g were obtained and with a metabolic efficiency greater than 80%. Regarding the production of bioethanol using yeast *C. intermedia* CBE002, yields of 0.31 g/g and a process efficiency of 60% were obtained (Table I).

Various studies indicate that CS is a widely studied raw material for the production of bioethanol. Different concentrations and types of acids and alkalis during the pretreatment stage have been evaluated, obtaining process efficiencies between 83% and 91% [23], [24].

TABLE I
Bioethanol Production by *S. cerevisiae* Itver01 and *C. intermedia* CBE002 Using CS as Raw Material

Parameters	*S. cerevisiae* Itver01	*C. intermedia* CBE002
Sugar consumed (g/L)	15.29	18.52
Production (g/L)	8.24	6.21
Yield (g ethanol/biomass)	0.42	0.31
Conversion efficiency (%)	82.11	60.0

B. Production of Enzymes

The integral production of enzymes within a biorefinery could contribute to reduce the production costs of a bioprocess. Enzymes such as cellulases, xylanases and amylases are widely used commercially, in food, wine, beverages, textiles, and biofuels industries to mention some [8]. A large number of microorganisms including bacteria and fungi have been reported as enzyme producers using fermentations [25].

The production of enzymes using CS as substrate took place in 250 mL flasks (50 mL culture media) inoculated with the cell or spore suspension followed by incubation at 30 °C at 150 rpm. The microorganisms evaluated were: *B. velezensis, F. equiseti* and *P. funiculosum*. These strains were previously isolated and characterized in synthetic media in our working group. First, enzymatic assays were performed at a qualitative level. Fig. 1 shows the presence of enzymatic activities in the evaluated microorganisms.

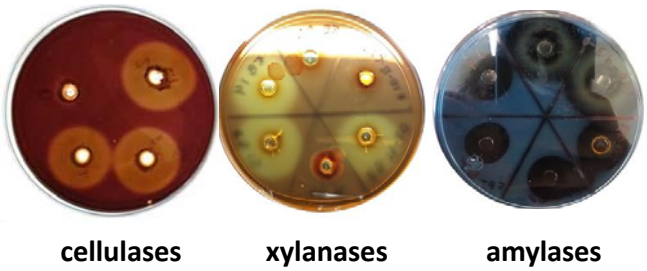

Fig. 1 Qualitative determination of enzyme production by three microorganisms from the IPN-CIIDIR Sinaloa collection. Cellulases (*P. funiculosum*), Xylanases (*F. equiseti* and *B. velezensis*), amylases *(B. velezensis)*

Secondly, the enzymatic activities of cellulases, amylases and xylanases were determined, following the previously mentioned methodologies. Fig. 2 shows that the *B. velezensis* strain is capable of producing xylanases and amylases (1.2 and 0.4 IU/mL). While the *F. equiseti* strain has the capacity to produce 0.4 IU/mL and *P. funiculosum* (1.5 IU/mL). The results show that the production of enzymes using the same carbon source is different and depends on the growth conditions of the microorganism, the culture medium used and the type of fermentation, among others [26].

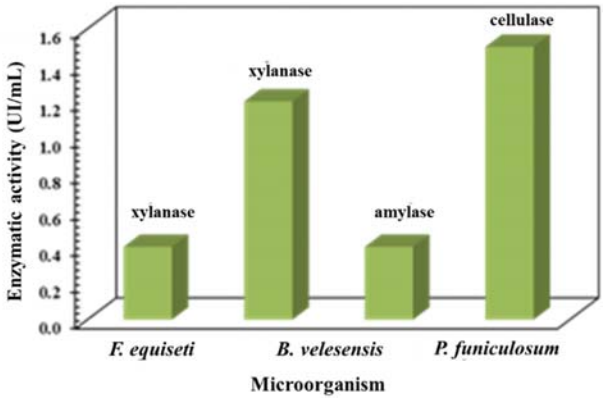

Fig. 2 Production of enzymes in SF using CS as the only carbon source

In general, cellulase production can be induced and affected by the nature of the substrate used in fermentation. That is why the choice of substrate is important [26]. Differences in enzyme concentrations in various studies can be attributed to the use of different materials, cultural practices, microorganisms and toxic products obtained from the hydrolysis process [27].

C. Xylitol Production

Xylitol is a sugar alcohol naturally present in fruits and vegetables in small concentrations, and therefore its extraction is not commercially scalable. It is a product of interest in the food and pharmaceutical industry mainly [28]. Pentoses fermentation by microorganisms for the production of xylitol is an environmentally friendly alternative that can be carried out in less severe conditions than those used in the chemical process [29].

The production of xylitol was carried out using the acid hydrolysates from the CS pretreatment process and the yeast *C. intermedia CBE002*. The results are shown in Fig. 3. The strain CBE002 reached a xylose consumption of 99% with respect to the initial substrate at 36 h of fermentation, producing 9.04 g/L of xylitol in the same time, this corresponds to a process yield of 0.46 g/g. These results are similar to those reported by Bedö et al. [30].

IV. Conclusion

Sinaloa is the largest corn producer in Mexico, its residues are not used in a sustainable way; therefore, this work showed that it is possible to use lignocellulosic residues of corn for the production of 2G ethanol, xylitol and enzymes. Future studies of optimization of production technologies are required to improve the yields and productivity of each process.

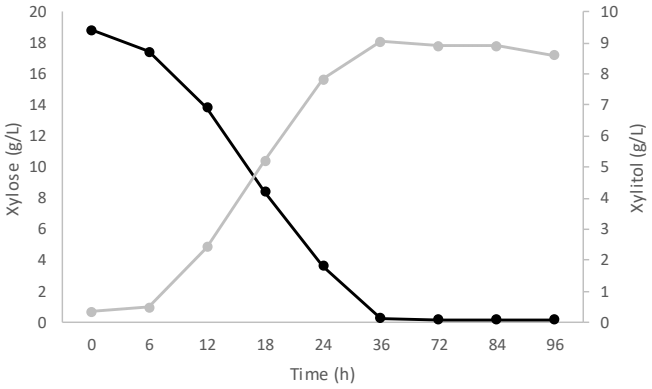

Fig. 3 Sugar consumption from CS acid hydrolysates and xylitol production by *C. intermedia* CBE002

Acknowledgment

RAT acknowledges CONACYT, México for Ms. Cs fellowships. Authors thank funding granted for this work by the Instituto Politécnico Nacional (IPN) SIP 20201422-20210227.

References

[1] Chandel, A.K., Garlapati, V.K., Singh, O.V., Fernandes-Atunes, F.A., da Silva, S., (2018). The path forward for lignocellulose biorefineries: Bottlenecks, solutions, and perspective on commercialization. Bioresource Technology. 264, 370-381.

[2] Champreda, V., Mhuantong, W., Lekakarn, H., Bunterngsook, B., Kanokratana, P., Zhao, X., Zhang, F., Inoue, H., Fujii, T., and Eurwilaichitr, L. (2019). Designing cellulolytic enzyme systems for biorefinery: From natura to application. Journal of Bioscience and Bioengineering. 6, 637-654.

[3] Suriyachai, N., Weerasai, K., Upajak, S., Khongchamnan, P., Wanmolee, W., Laosiripojana, N., Champreda, V., Suwannahong, K., and Imman, S., (2020). Efficiency of catalytic liquid hot water pretreatment for conversion of corn stover to bioethanol. ACS Omega. 5, 29872-29881.

[4] Feng, Y., Yu, Y., Wang, X., Qu, Y., Li, D., He, W., and Hong, B. (2011). Degradation of raw corn stover powder (RCSP) by an enriched microbial consortium and its community structure. Bioresource Technology. 102, 724-747.

[5] Behera, S., Arora, R., Nandhagopal, N., Kumar, S., (2014). Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. Renewable Sustainable Energy Rev. 36, 91-106.

[6] Kamm, B., Kamm, M., Gruber, P.R., Kromus, S., Biorefineries – Industrial Processes and Products. Eds. Wiley: Hoboken, NJ, USA. ISBN: 9783527310272.

[7] Zhao, Y., Damgaard, A., Christensen, T.H., (2018). Bioethanol from corn stover – a review and technical assessment of alternative biotechnologies. Progress in Energy and Combustion Science. 1-17.

[8] Shahryari, Z., Fazaelipoor M.H., Ghasemi, Y., Lennartsson, P.R., Taherzadeh, M.J., (2019). Amylase and xylanase from edible fungus *Neurospora intermedia:* production and characterization. Molecules. 24, 721-734.

[9] Fath, M., and Fazaelipoor, M.H., (2015) Production of proteases in a novel trickling tray bioreactor. Waste Biomass and Valorization. 6, 475-480.

[10] Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. Microbiology Molecular Biology Rev. 66, 506-577.

[11] Kumar, D., Kumar, S.S., Kumar, J., Kumar, O. Mishra, S.V., Malyan, S., Kumar, R., (2017) Xylanase and their industrial applications: A review. Biochemical Cell. Arch. 17. 353-360.

[12] Saxena, R. and Sing, R. (2011). Amylase production by solid state fermentation of agro-industrial wastes using *Bacillus* sp. Brazilian Journal of Microbiology. 42, 1334-1342.

[13] Sukumaran, R. Rani, R., Pandey, A. (2005). Microbial cellulases: production, applications and challenges. Journal of Scientific and Industrial Research. 64, 832-844.

[14] Dasgupta, D., Bandhu, S., Adhikari, D.K. and Ghosh, D. (2017). Challenges and prospects of xylitol production with whole cell bio-catalysis: A review. Microbiological Research. 197, 9-21.

[15] Rao, L.V., Goli, J.K., Gentela, J., and Koti, S. (2016). Bioconversion of lignocellulosic biomass to xylitol: an overview. Bioresource Technology. 213, 299-310.

[16] Miller, G.L, (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426–428

[17] Ortíz-Muñoz, B., Carvajal-Zarrabal, O., Torrestiana-Sánchez, B., Aguilar-Uscanga, M.G. (2010). Kinetic study on ethanol production using Saccharomyces cerevisiae ITV-01 yeast isolated from sugar cane molasses. Journal of Chemical Technology and Biotechnology. 85: 1361-1367.

[18] Ortíz-Zamora, O., Cortés-García, R., Ramírez-Lepe, M., Gómez-Rodríguez, J., Aguilar-Uscanga, M.G., (2009). Isolation and selection of ethanol-resistant and osmotolerant yeasts from regional agricultural sources in Mexico. Journal of Food Process Engineering. 32, 775-786.

[19] Beltrán-Arredondo, L.I., Hernández-Leyva, S.R., Maldonado-Mendoza, I.E., Reyes-Moreno, C., Contreras-Andrade, I., Castro-Martínez, C., (2020). Valorisation of agroindustrial residues acid hydrolysates as carbón sources for ethanol production by native yeast strains with different fermentative capabilities. Biotecnia. 22, 78-87.

[20] Carrasco, M., Villreal, P., Barahona, S., Alcíno, J., Cifuenes, V., Baeza, M., (2016). Screening and characterization of amylase and cellulase activities in psychrotolerant yeasts. BMC Microbiology. 16, 21

[21] Chiranjeevi, T., Baby Rani, G., Anuj, K., Chandel, Prakasham, R.S., Uma, A., (2012) Optimization of holocellulolytic enzymes production by *Cladosporium cladosporioides* using Taguchi-L_{16} orthogonal array. J. Biobased Mater Bioenergy. 6, 1-10

[22] Bailey, M.J., Biely, P., Poutanen, K. Interlaboratory testing of methods for assays of xylanase activity. Journal of Biotechnology. 23, 257-270.

[23] Papa, G., Rodríguez, S., George, A., Schievano, A., Orzi, V., Sale, K.L., Singh, S., Adani, F., Simmons, B.A., (2015). Comparison of different pretreatments for the production of bioethanol and biomethane fromo corn stover and switchgrass. Bioresources Technology. 183, 101-110.

[24] Chen, S., Xu, Z., Li, X., Yu, J., Cai, M. and Jin, M. (2018). Integrated bioethanol production from mixtures of corn and corn stover. Bioresource Technology. 258, 18-25.

[25] Gupta, R., Gigras, P., Mohapatra, H., Goswani , V.K., Chauhan, B., (2003). Microbial – amylases: a biotechnological perspective. Process Biochemical. 8, 1599-1616.

[26] Kang, S.W., Park, Y.S., Lee, J.S., Hong, S.I., Kim, S.W., (2004). Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresource Technology. 91, 153–156.
[27] Hattaka, A.I., (1983) Biological pretreatment of lignocellulose. Applied Microbiol. Biotechnol. 18,350-357.
[28] Prakasham, R., Rao, R. S., Hobbs, P. J., (2009). Current trends in biotechnological production of xylitol and future prospects. Current Trends in Biotechnology and Pharmacy. 3, 8-36.
[29] Antunes, F. A. F., dos Santos, J. C., da Cunha, M. A. A., Brumano, L. P., dos Santos Milessi, T. S., Terán-Hilares, R., ... Dalli, S. S., (2017). Biotechnological production of xylitol from biomass Production of Platform Chemicals from Sustainable Resources (pp. 311-342): Springer.
[30] Bedő, S., Fehér, A., Khunnonkwao, P., Jantama, K., Fehér, C., (2021). Optimized Bioconversion of Xylose Derived from Pre-Treated Crop Residues into Xylitol by Using *Candida boidinii*. Agronomy. 11, 79.