Pre-treatments applied to rice husk enzymatic hydrolysis: effect on structure, lignocellulosic components, and glucose production kinetics

Fernando Aredo, Ing.[1], Meliza Lindsay Rojas, Dr.[2], Sandra Pagador, M.Sc.[3], Leslie Lescano, M.Sc.[4], Jesús Sanchez-Gonzalez, M.Sc.[4], Guillermo Linares, Dr.[4,*]
[1]Escuela Profesional de Ingeniería Agroindustrial, Universidad César Vallejo, Perú, antonio.ao.1997@gmail.com
[2]Dirección de Investigación y Desarrollo, Universidad Privada del Norte, Perú, meliza.rojas@upn.edu.pe
[3]Instituto de Investigación en Ciencias y Tecnología, Universidad César Vallejo, Perú, spagador@ucv.edu.pe
[4]Departamento de Ciencias Agroindustriales, Universidad Nacional de Trujillo, Perú, llescano@unitru.edu.pe, jsanchezg@unitru.edu.pe, glinares@unitru.edu.pe
Corresponding author

Abstract*– Freezing (-20 °C, 12h) and alkaline (NaOH 8%) pre-treatments were applied individually and combined in rice husk (Oryza sativa L.) before their hydrolysis with cellulase (EC: 3.2.1.4.). The effects on structural modifications, lignin content, cellulose, hemicellulose content and glucose production were evaluated. In addition, the glucose production kinetics were described by using the Peleg model. The homogenous rice husk (1 g) with and without pre-treatments was hydrolysed with 150 U of Cellulase in 10 ml of acetate for 60 h (37 °C, pH 5.5, 100 rpm). As results, the SEM images evidenced porous microstructures with less agglomeration generated by all pre-treatments, which were intensified by the combined pre-treatment. This pre-treatment allowed to obtain higher cellulose (62.51 ± 0.3 %) content. Besides, the glucose content after pre-treatments increased. The Peleg model parameters from glucose production kinetics during enzymatic hydrolysis were related to initial glucose content (G0), glucose production rate (1/k1) and maximum glucose yield (1/k2). After enzymatic hydrolysis process, compared to control glucose yield (0.359 ± 0.002 g G/g rice husk), this was 27%, 71% and 88% higher for freezing, alkaline and combined pre-treatments respectively.

Keywords*-- rice husk; freezing; alkaline pre-treatment; cellulase; enzymatic hydrolysis.

I. Introduction

To face the growing global demand for energy together with the gradual shortage of energy resources, biotechnology offers technological alternatives that, from the production of lignocellulosic biomass, green energies such as bioethanol, biodiesel and biogas can be obtained. Second generation biomass can come from lignocellulosic residues which are renewable, accessible, abundant and do not disturb human food [1-3]. The second-generation lignocellulosic biomass is constituted by lignin, hemicellulose and cellulose in a network of microfibrils, this causes difficult access of enzymes for their transformation into fermentable sugars [4]. For this reason, these lignocellulosic residues demand a pre-treatment (biological, mechanical, chemical, etc.) that allows modifying the structure and its accessibility.

Digital Object Identifier (DOI):
http://dx.doi.org/10.18687/LACCEI2020.1.1.42
ISBN: 978-958-52071-4-1 ISSN: 2414-6390

In this study, rice husk has been considered as a source of lignocellulosic biomass since, according to the MINAGRI [5], rice is the second most consumed cereal in Peru, and the third most widely grown cereal crop in the world after maize and wheat [6].Therefore, the lignocellulosic rice residue generated is high, and so far, there has been no adequate used or disposed of. The rice husk, due to its composition has a high potential for monosaccharides (glucose) production which could be used in the manufacture of biofuels [6, 7]. Depending on rice variety, the rice husk is composed by lignin (18.20% - 24.60%), hemicellulose (18.10% - 27.06%) and cellulose (25.89% - 39.05%) [8].

Pre-treatments are aimed at improving the availability of cellulose to maximize enzymatic hydrolysis and generate greater yield in fermentable sugars. Some pret-reatments have been studied to modify the structure and composition of lignocellulosic sources from residues. Using rice husk, it was studied freezing pre-treatment [9] and alkaline pre-treatment [10], pre-treatments with laccase from Lentinus polychrous [11], in all cases the enzymatic digestibility was increased. On the other hand, mechanical fractionation pre-treatments at different levels were applied in corn cobs [12]; cellobiose, cellulase and NaOH in pineapple stubble [13]; H2O2, H2SO4 and NaOH in cassava residues [14]; steam explosion, acid and alkaline pre-treatment in pine sawdust [15].

This study proposed to take advantage of an easily accessible lignocellulosic source such as rice husk in Peru to obtain usable substrates as an alternative to producing biofuels. For this, the effect of the application of physical (freezing), chemical (alkaline, using NaOH solution) and physicochemical (alkaline and freezing) pre-treatments on the microstructure, lignocellulosic compounds and the subsequent kinetics of glucose production by enzymatic hydrolysis was evaluated.

II. Material and Methods

A. Rice husk conditioning

Rice husk free of other particles and contaminants such as fungi was used. It was obtained from NIR-I rice variety provided by the "San Francisco" rice industry (Huanchaco, La

Libertad, Perú). To promote a high contact area, the dry rice husk was ground and sieved (sieve n° 60, Standard Test Sieve, EE.UU.), obtaining particles of 0.25 mm. Homogenized rice husk presented the following characteristics of moisture (4.44%), ash (14.14%) and pH (6.08).

B. *Pre-treatments*

Homogenized rice husk particles were chemically and physically pre-treated. In addition, the combined application of these two pre-treatments was evaluated. For this, the samples were exposed to conditions described in Table 1, after each pre-treatment the samples were dried at 60 °C for 12h.

TABLE I
PERFORMED PRE-TREATMENTS DESCRIPTION AND CODE USED ALONG THIS STUDY TO IDENTIFY EACH ONE.

Pre-treatment	Description	Code
None	Rice husk without pre-treatment	Control
Physical (freezing)	Freezing at -20 °C for 12 h	F
Chemical (alkaline)	Alkaline treatment with NaOH 8%, at 121 °C for 85 min using 1:10 (g of rice husk/mL of alkaline solution)	A
Physico-chemical (alkaline + freezing)	Alkaline treatment followed by freezing treatment	A+F

C. *Rice husk structure images by Scanning Electron Microscopy (SEM)*

The samples, with and without pre-treatments, were previously dehydrated and sputtered with a thin gold layer of 0.150 nm by plasma deposition (PVD) using a DC Sputtering-SPI. Then, they were observed in a scanning electronic microscope operated at an acceleration voltage of 10 kV (TESCAN VEGA 3 LMU, Czech Republic) using 1.00kx magnification.

D. *Determination of lignocellulosic components content*

The % of lignin was determined according to the TAPPI 222 method [16]. The ANSI/ASTM standard [17] was used to determine the % of cellulose. To determine the % of hemicellulose, the % of holocellulose was first determined as described by Browning [18], and subsequently, by difference between the %cellulose and %holocellulose, the % of hemicellulose was obtained.

E. *Enzymatic hydrolysis*

To perform the hydrolysis, 150 U of Aspergillus niger cellulase (Sigma-Aldrich, USA) was used for each g of rice husk in 10 ml of sodium acetate (50mM), at pH: 5.5 and 37 °C. The dispersion was stirred with an orbital shaker (Orbital Shaker, BS-GS-30) at 100 rpm for 60 hours [9, 19]. After that, the dispersion was filtered, and the supernatants were centrifuged at a speed of 4800 rpm for 15 minutes.

F. *Total reducing sugars determination*

Before and during enzymatic hydrolysis, the content of reducing sugars was quantified using the method of 3.5 Dinitrosalicylic acid (DNS) described by Miller [20]. For that, 0.1 mL of sample and 0.9 mL of DNS (previously prepared) were mixed, heated at 100 °C for 10 min, cooled and diluted to 10 mL using distilled water. The absorbance was read at 540 nm using a spectrophotometer (Unico Spectro Quest 4802E, United States) A calibration curve was constructed using different glucose concentrations (0.5-2.1 g/L) mixed with DNS as previously described. Then, the total reducing sugars were expressed in g of glucose per g of rice husk (g G/g rice husk).

G. *Glucose production kinetics*

To construct the glucose production kinetics, during enzymatic hydrolysis process were taken samples each 12 h for 60 h. The glucose production kinetics was fitted using the Peleg model (Eq. 1) [21] where $G(t)$ is the glucose content (g glucose/g of rice husk) at time t (h), G_0 is the initial glucose content (after pre-treatments), k_1 is the rate constant (h·(g glucose/g of rice husk)$^{-1}$) and k_2 is the constant of the asymptotic level ((g glucose/g of rice husk)$^{-1}$). The reciprocal of k_1 represents the glucose production rate and the reciprocal of k_2 represents the glucose production in the equilibrium.

$$G(t) = G_o + \frac{t}{k_1 + k_2.t} \quad (1)$$

The parameters of the Peleg model (Eq. 1) were iteratively adjusted to the experimental data by minimizing the sum of squared errors (SSE in Eq. 2). The Generalized Reduced Gradient algorithm (GRG Nonlinear Solving method), implemented in the 'Solver' tool of software Excel 2016 (Microsoft, USA) was used. The regressions were conducted for each replicate.

$$SSE = \sum_{i=1}^{x}((predicted) - (experiemental))_i^2 \quad (2)$$

H. *Statistical analyses*

A completely randomised design (CRD) was conducted. All processes and analyses were performed at least 3 times. The analysis of variance (one-way ANOVA), followed by Tukey post-hoc test were carried out with a significance level of 5%. Statistical analyses were performed using the IBM SPSS Statistics 23 software (IBM SPSS, USA).

III. RESULTS AND DISCUSSION

Effect of pre-treatments on rice husk microstructure

The morphological changes of the rice husk by applying the different pre-treatments were evidenced in Figure 1. The structure of the rice husk without any pre-treatment (Figure 1, Control) shows the rigid and orderly epidermal tissue due to the presence of amorphous hemicelluloses in its area [13], also had none pores showing the appearance of interrupted cells. It is due to the crossing of their components [22]. The same rigid structure without pores was observed in raw rice husk surface by Ebrahimi, Caparanga [23].

When the pre-treatment by freezing was performed (Figure 1, F), the cracking of the structure was observed due to freezing effects. During the freezing of the fluid (water), the crystals formed fill a greater volume inside the tissue, so that, when thawed, the spaces occupied by the ice crystals are free observing the cracking of the structure [24, 25]. The same effects on rice husk structure after freezing was reported by Chang, Thitikorn-amorn [9].On the other hand, alkaline pre-treatment with 8% NaOH (Figure 1, A) caused erosion in the structure. In fact, Kahar, Taku [26] affirmed that pre-treatment with NaOH could fragment the structural rigidity of the fibre lignocellulose matrices and make many micropores inside. Finally, the combination of pre-treatments (Figure 1, A + F) originated a less agglomerated and highly partitioned structure with larger pores, observing a distribution in the form of an irregular crystalline network. This occurred due to the suppression of hemicellulose and the dispersion of the lignocellulosic structure fibres, primarily due to pre-treatment with NaOH and in addition to the increase in pore thickness due to freeze pre-treatment.

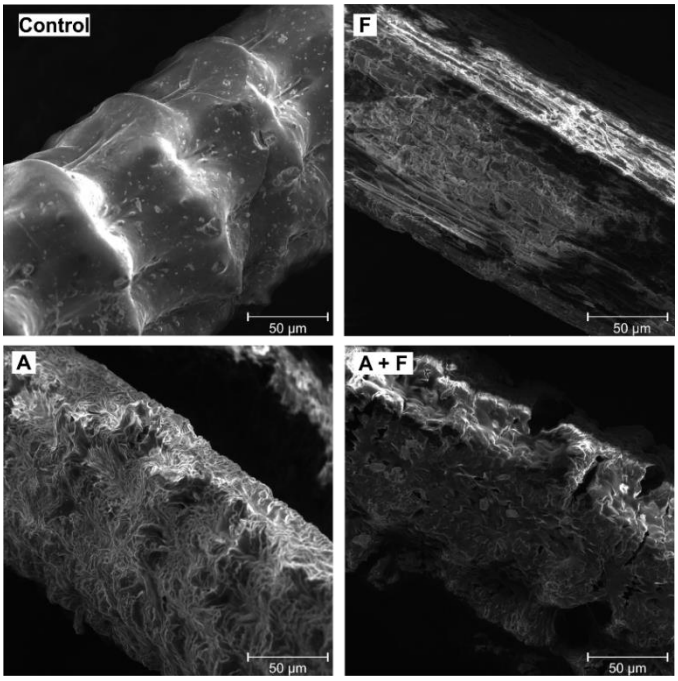

Fig. 1. SEM images (10 kV, magnification= 1.00kx) showing the effects on rice husk microstructure without pre-treatments (Control) and after F (freezing), A (alkaline) and A+F (alkaline + freezing) pre-treatments.

It was demonstrated that all pre-treatments impact on microstructure in different levels, which increased the contact area and probably better exposed their compounds by making them more accessible.

Effect of pre-treatments on lignocellulosic components content

The rice husk used for all pre-treatments presented 37.28 ± 0.3729 of cellulose, 28.28 ± 0.2279 of hemicellulose, and 14.69 ± 0.147% of lignin. These values were like the reported by Chang, Thitikorn-amorn [9], Valverde, Sarria [27], Arias Ortiz and Meneses Cruz [28].

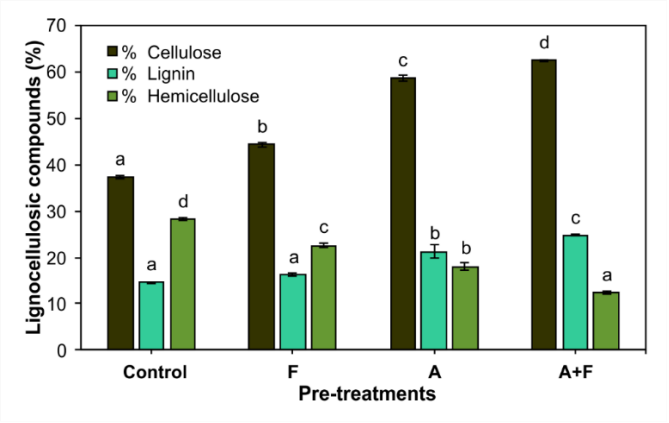

Fig. 2. Lignocellulosic compounds (%) after pre-treatments. Different letters indicate significative differences among pre-treatments ($p<0.05$).

Figure 2 shows the content of the lignocellulosic component after performed pre-treatments. It was observed that for all pre-treatments, the cellulose and lignin content increased. After the combined pre-treatment application, the highest cellulase (62.51 ± 0.06%) and lignin (24.72 ± 0.16%) were reached. Pre-treatment effects on lignocellulosic content could be explained by the structural modifications evidenced in Figure 1. In addition, there was also chemical modifications, such as the condensation and fusion of lignin or the grouping of pseudo-lignin by the mixture of carbohydrates formed in the fragmentation of hemicellulose [29]. These effects could explain the observed increase of lignin content and the decrease of hemicellulose content. In fact, the hemicellulose content trends to decrease with pre-treatments application remaining 12.44 ± 0.23% after the physicochemical pre-treatment.

Effect of pre-treatments on glucose production kinetics during enzymatic hydrolysis

After pre-treatments, the glucose content increased (Figure 3. A). Compared to control, the F, A and A+F pre-treatments increased the glucose content in 17, 37, and 103 times respectively. That is, as evidenced in Figure 2, when enzymatic hydrolysis start, the glucose content for each pre-treated rice husk samples were different.

The Peleg model was used to describe the glucose production behaviour during enzymatic hydrolysis (Figure 3. B). The glucose production behaviour for each pre-treatment was reflected in the Peleg model parameters (Table 2). The G_0 values were related to the initial glucose content that was according to the showed in Figure 1.A. The k_1 was related to the glucose production rate, at lower k_1 value, the higher rate. Therefore, the pre-treatment that showed the highest rate (lowest k_1 value) was the freezing pre-treatment with 0.058 ± 0.003 (g G/g rice husk)/h. On the contrary, the pre-treatment

that showed the lowest rate (highest k_1 value) was the combined pre-treatment with 0.011 ± 0.000 (g G/g rice husk)/h.

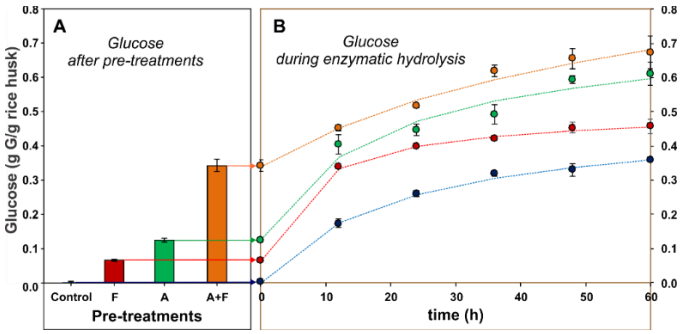

Fig. 3. Glucose content after pre-treatments (A) and the produced during enzymatic hydrolysis (B), dotted lines are the modelled data (Eq. 1).

On the other hand, it was determined that after 60 h of enzymatic hydrolysis, compared to control (0.359 ± 0.002 (g G/g rice husk)), the use of pre-treatments allowed to increase the glucose yield in 27% for freezing, 71% for alkaline and 88% for combined pre-treatment (A+F). Similar results were reported after freezing pre-treatments that increased the rice husk from 48% to 84% Chang, Thitikorn-amorn [9], León, German [10]. However, the obtained glucose yield was higher compared to the obtained after rice husk pre-treatments with Laccase from *Lentinus polychrous* [11] or after high-pressure microwave [30].

The k2 values were related with the glucose yield at the equilibrium, that is, the maximum glucose content reached by enzymatic hydrolysis under the studied conditions (available substrate, temperature and pH). The lowest k_2 value means the highest glucose yield. Therefore, as observed in Table 2, the highest glucose yield could be achieved by the alkaline and the combined pre-treatment application reaching predicted glucose yield values of 0.616 ± 0.023 and 0.726 ± 0.180 (g G/g rice husk) respectively. On the contrary, the Control and Freezing pre-treatment could reach predicted glucose yield values of 0.482 ± 0.011 and 0.442 ± 0.008 (g G/g rice husk) respectively.

TABLE 2
PELEG MODEL (EQ.1) PARAMETERS OBTAINED FROM MODELLING GLUCOSE PRODUCTION KINETICS DATA. DIFFERENT LETTERS SHOW SIGNIFICATIVE DIFFERENCES AMONG PRE-TREATMENTS (P<0.05).

Peleg Model parameters	Pre-treatments			
	Control	F	A	A+F
G_0 [g G/g rice husk]	0.002±0.002^{a}	0.066±0.003^{b}	0.13±0.001^{c}	0.34±0.011^{d}
k_1 [h·(g G/g rice husk)$^{-1}$]	43.339±2.671^{c}	17.311±1.177^{a}	30.798±4.397^{b}	88.421±1.167^{d}
k_2 [(g G/g rice husk)$^{-1}$]	2.076±0.048^{bc}	2.264±0.043^{c}	1.624±0.062^{ab}	1.447±0.419^{a}

Therefore, the effects of pre-treatments in making available the cellulose as a substrate to enzymatic hydrolysis was very important, being the cellulose content, the glucose yield-limiting during enzymatic reaction. As described above, the effects of freezing pre-treatment caused and increase in the rate of glucose production during enzymatic hydrolysis, therefore, the equilibrium glucose yield is reached at short times. However, probably the available substrate (cellulose content, Figure 2) was not enough to reach a higher glucose yield. The same could have occurred with the Control samples. In fact, the Figure 4 shows the % of cellulose conversion after 60 h of enzymatic hydrolysis. As was observed, no significant differences were obtained for %cellulose conversion among pre-treatments after the enzymatic hydrolysis process.

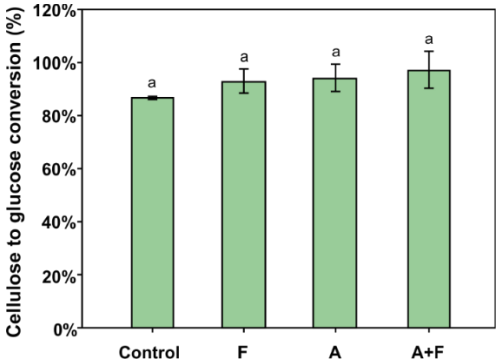

Fig. 4. % of cellulose conversion into glucose after 60 h of enzymatic hydrolysis for each pre-treated sample. Different letters show significant differences (p<0.05) among pre-treatments.

IV. CONCLUSIONS

The pre-treatments applied to the rice husk modified its microstructure. Compared to the control sample, pre-treatments caused cracked, porous structures with smaller particle sizes. On the other hand, the combination of pre-treatments (Freezing + Alkaline) has a greater impact on lignocellulosic components. Obtaining an increase in %cellulose (62.51 ± 0.06%), decrease in %hemicellulose (12.44 ± 0.28%) and an increase in %lignin (24.72 ± 0.16%). Total reducing sugars were expressed in glucose, the amount of which was evaluated after pre-treatments and during enzymatic hydrolysis. Compared to the control, all pre-treatments significantly increased the amount of glucose, being higher in the combined pre-treatment. On the other hand, there was no evidence of an improvement in the rate of glucose production (kinetic parameter, k) with the application of pre-treatments. However, there were significant differences in the amount of glucose generated after hydrolysis, being the highest when the combined pre-treatment was used.

REFERENCES

[1] Almarsdottir, A.R., M.A. Sigurbjornsdottir, and J. Orlygsson, Effect of various factors on ethanol yields from lignocellulosic biomass by Thermoanaerobacterium AK17. *Biotechnology and bioengineering*, 2012. 109(3): p. 686-694.

[2] Alvira, P., et al., Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresource Technology*, 2010. 101(13): p. 4851-4861.

[3] Kootstra, A.M.J., et al., Differential effects of mineral and organic acids on the kinetics of arabinose degradation under lignocellulose pretreatment conditions. *Biochemical Engineering Journal*, 2009. 43(1): p. 92-97.
[4] Echeverria, C., et al., Pre-treatment by Acidification and Freezing on Corncob Polymers and its Enzymatic Hydrolysis. *Asian Journal of Scientific Research*, 2018. 11: p. 222-231.
[5] MINAGRI, Informe del arroz, D.d.e.e.e.i. Agraria, Editor. 2017, MINAGRI: Lima.
[6] Wood, I.P., et al., Comparison of saccharification and fermentation of steam exploded rice straw and rice husk. *Biotechnology for Biofuels*, 2016. 9(1): p. 193.
[7] Montipó, S., et al., Bioprocessing of rice husk into monosaccharides and the fermentative production of bioethanol and lactate. *Cellulose*, 2019. 26(12): p. 7309-7322.
[8] Choi, H. and C. Yi, Diseño de un gasificador de 25 kW para aplicaciones domésticas usando como combustible cascarilla de arroz. 2013.
[9] Chang, K.-L., et al., Enhanced enzymatic conversion with freeze pretreatment of rice straw. *Biomass and Bioenergy*, 2011. 35(1): p. 90-95.
[10] León, C., H. German, and D.A. Sandoval Rojas, Determinación de las concentraciones de enzimas celulasa y ß-glucosidasa para la obtención de azúcares fermentables a partir de cascarilla de arroz. 2015.
[11] Sarnthima, R. and S. Khammuang, Laccase from Lentinus Polychrous Increases the Efficiency of Cellulose Hydrolysis of Rice Straw to Glucose. *Oriental Journal of Chemistry*, 2018. 34(4): p. 1961.
[12] Ji, G., et al., Mechanical fragmentation of corncob at different plant scales: impact and mechanism on microstructure features and enzymatic hydrolysis. *Bioresource Technology*, 2016. 205: p. 159-165.
[13] Córdoba, M. and M. Molina, Determinación del efecto de la concentración de la celulasa, celobiasa y de NaOH en la hidrólisis para la producción de etanol a partir de rastrojo de la piña. *Revista Ingeniería UCR*, 2014. 24(2): p. 19-25.
[14] Niño López, L.C., A. Acosta Cárdenas, and G.R. Gelves Zambrano, Evaluación de pretratamientos químicos para la hidrólisis enzimática de residuos lignocelulósicos de yuca (Manihot esculenta Crantz). 2013.
[15] López-Miranda, J., et al., Optimización del proceso de obtención enzimática de azúcares fermentables a partir de aserrín de pino. *Revista internacional de contaminación ambiental*, 2009. 25(2): p. 95-102.
[16] TAPPI, TAPPI Test Methods T222. Technical Association of the Pulp and Paper Industry. 1997, Tappy Press: Atlanta.
[17] ANSI/ASTM, American National Standards Institute (ANSI) and American Society for Testing and Materials (ASTM). Standard test methods for alpha-cellulose in wood D 1103-60. 1977, American National Standards Institute: Washington DC.
[18] Browning, B.L., Methods of wood chemistry. Volumes I & II. *Methods of wood chemistry. Volumes I & II.*, 1967.
[19] Baadhe, R.R., R. Potumarthi, and N.K. Mekala, Influence of dilute acid and alkali pretreatment on reducing sugar production from corncobs by crude enzymatic method: a comparative study. *Bioresource Technology*, 2014. 162: p. 213-217.
[20] Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, 1959. 31(3): p. 426-428.
[21] Peleg, M., An Empirical Model for the Description of Moisture Sorption Curves. *Journal of Food Science*, 1988. 53(4): p. 1216-1217.
[22] Mood, S.H., et al., Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renewable and Sustainable Energy Reviews*, 2013. 27: p. 77-93.
[23] Ebrahimi, M., et al., Effect of ammonium carbonate pretreatment on the enzymatic digestibility, structural characteristics of rice husk and bioethanol production via simultaneous saccharification and fermentation process with Saccharomyces cerevisiae Hansen 2055. *Industrial Crops and Products*, 2017. 101: p. 84-91.
[24] Noh, E., et al., Freezing of soybeans influences the hydrophobicity of soy protein. *Food Chemistry*, 2006. 97(2): p. 212-216.
[25] Hong, H., et al., Effects of different freezing treatments on the biogenic amine and quality changes of bighead carp (Aristichthys nobilis) heads during ice storage. *Food Chemistry*, 2013. 138(2-3): p. 1476-1482.
[26] Kahar, P., K. Taku, and S. Tanaka, Enzymatic digestion of corncobs pretreated with low strength of sulfuric acid for bioethanol production. *Journal of bioscience and bioengineering*, 2010. 110(4): p. 453-458.
[27] Valverde, A., B. Sarria, and J.P. Monteagudo, Análisis comparativo de las características fisicoquímicas de la cascarilla de arroz. *Scientia et technica*, 2007. 1(37).
[28] Arias Ortiz, R.A. and J.D. Meneses Cruz, Caracterización físico-química de residuos agroindustriales (cascarilla de arroz y cascarilla de café), como materia prima potencial para la obtención de bioetanol, Laboratorios de Química UNAN-Managua I-II semestre 2016. 2016, Universidad Nacional Autónoma de Nicaragua, Managua.
[29] J. Zheng, K. Choo, and L. Rehmann, "The effects of screw elements on enzymatic digestibility of corncobs after pretreatment in a twin-screw extruder," *biomass and bioenergy*, vol. 74, pp. 224-232, 2015.
[30] Wu, J., et al., Optimising conditions for bioethanol production from rice husk and rice straw: effects of pre-treatment on liquor composition and fermentation inhibitors. *Biotechnology for Biofuels*, 2018. 11(1): p. 62.