UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE PESQUISA EM BIOENERGIA

PROGRAMA INTEGRADO (UNESP, USP E UNICAMP) DE PÓS-GRADUAÇÃO EM BIOENERGIA

STRATEGIES TO IMPROVE THE CONVERSION OF SUGARCANE BAGASSE INTO SECOND GENERATION ETHANOL

LONGINUS IFEANYI IGBOJIONU

Rio Claro - SP
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Thesis submitted to Bioenergy Research Institute, São Paulo State University UNESP, Rio Claro - SP, Brazil, as part of the requirements for obtaining a Doctor of Science degree.

Advisor: Prof. Dr. Cecilia Laluce

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TÍTULO DA TESE: STRATEGIES TO IMPROVE THE CONVERSION OF SUGARCANE BAGASSE INTO SECOND GENERATION ETHANOL

AUTOR: LONGINUS IFEANYI IGBOJIONU

ORIENTADOR(A): *orientador não cadastrado*

Aprovado como parte das exigências para obtenção do Título de Doutor em BIOENERGIA, pela Comissão Examinadora:

Profa. Dra. CECILIA LALUCE (Participaçao Virtual)
Departamento de Bioquímica e Tecnologia Química / Instituto de Quimica - UNESP - Araraquara

Profa. Dra. FLAVIA VISCHI WINCK (Participaçao Virtual)
Departamento de Bioquímica / Universidade de São Paulo - USP

Prof. Dr. CARLOS ALBERTO LABATE (Participaçao Virtual)
ESALQ/USP / Piracicaba/SP

Prof. Dr. PEDRO DE OLIVA NETO (Participaçao Virtual)
Departamento de Biotecnologia / Faculdade de Ciências e Letras - UNESP - Assis

Prof. Dr. NELSON RAMOS STRADIOTTO (Participaçao Virtual)
Departamento de Química Analítica / Instituto de Quimica - UNESP - Araraquara

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DEDICATION

This thesis is dedicated to God Almighty and to the loving memory of my father.

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LIST OF ABBREVIATIONS AND SYMBOLS

CONAB - Companhia Nacional de Abastecimento (National Supply Company)

TVA - Tennessee Valley Authority

IQAr - Instituto de Química Araraquara

HS - Head Space

Q_P - Ethanol or Biomass productivity

$Y_{P/S}$ - Ethanol yield

$Y_{X/S}$ - Biomass yield

OECD - Organisation for Economic Co-operation and Development

FAO - Food and Agriculture Organization

GHG - Greenhouse gas

FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo

SB - Sugarcane Bagasse

5-HMF - 5-hydroxymethyl furfural

EMP - Embden Meyerhof Pathway

°C - Degree Celsius

SHF - Separate hydrolysis and fermentation

SSF - Simultaneous saccharification and fermentation

SSCF - Simultaneous saccharification and co-fermentation

CBP - Consolidated bioprocessing

CCD - Central composite design

BOD - Biochemical oxygen demand

FPU - Filter paper unit

IU - International unit

SRS - Soluble reducing sugar

DNS - 3,5-dinitrosalicylic acid

ASTM - American Society for Testing and Materials

AIR - Acid insoluble residue

AIL - Acid insoluble lignin

TRS - Total reducing sugar

UV - Ultraviolet

SP - São Paulo

ASL - Acid soluble lignin

GOD-PAP - Glucose oxidase-phenol and 4 aminophenazone

NIR - Near-infrared

nm - Nanometre

ODW - Oven-dry weight

cm - Centimetre

HPLC - High performance liquid chromatography

L - Liter

mL - Milliliter

g - Gram

mg - Milligram

μL - Microliter

CI - Crystallinity index

θ - Theta

pH - Potential of hydrogen

NREL - National Renewable Energy Laboratory

M - Molar

mM - Millimolar

mmol - Millimole

ANOVA - Analysis of variance

OD - Optical density

h - Hour

GC-MS - Gas chromatography-mass spectrometry

pKa - Acid dissociation constant

MA - Maleic acid

SA - Sulfuric acid

RT - Room temperature

wt% - Weight percentage

F-value - Fisher distribution value

p-value - Probability value

3D - Three dimensional

RESUMO

O etanol de segunda geração a partir da biomassa lignocelulósica é uma solução renovável e sustentável para os problemas ambientais decorrentes das grandes demandas de energia da sociedade moderna. Bagaço de cana-de-açúcar (SB), uma lignocelulose composta por dois grandes polímeros de açúcar (celulose e hemiceluloses) ligados à lignina. Devido à natureza recalcitrante do SB, o pré-tratamento é considerado uma etapa fundamental do processo antes de ser convertido em biomoléculas. SB bruto consiste em celulose (40,4% em peso), hemicelulose (23,8% em peso), lignina (27,2% em peso), cinzas (3,0% em peso) e extrativos (4,4% em peso). O pré-tratamento de SB com NaOH a 3% e NaOH a 8% em autoclave (121 ^{o}C) e à temperatura ambiente, respectivamente, levou a aumentos nas frações celulósicas (aproximadamente 62,0% em peso), enquanto o conteúdo de lignina diminuiu para valores inferiores a 11,0% em peso. O NaOH sequencial em duas etapas e o pré-tratamento com ácido resultaram em aumentos substanciais nas frações celulósicas (aproximadamente 80,0% em peso), que foi cerca de duas vezes o valor obtido a partir de SB não tratado (40,4% em peso). O conteúdo de hemicelulose de SB diminuiu substancialmente após o pré-tratamento da segunda etapa com ácido (ácido maleico e sulfúrico). No entanto, o índice de cristalinidade aumentou substancialmente após diferentes pré-tratamentos foram aplicados ao SB. A sacarificação enzimática do SB pré-tratado levou a rendimentos de glicose acima de 75% (g/g) em comparação com 27% (g/g) obtidos do SB não tratado após 72 h. A otimização da hidrólise enzimática resultou em rendimento máximo de glicose de 85,9% (g/g) pela aplicação de 1,8 FPU/g de celulose de celulose, 5,3% de carregamento de sólidos e 48 h de tempo de hidrólise. Por outro lado, a hidrólise ácida em duas etapas do SB pré-tratado resultou em um rendimento máximo de glicose de 56,8% (g/g). A otimização da hidrólise ácida em duas etapas aplicando ácido sulfúrico a 1,5%, $FeSO_4$ 40,0 mmol/L e 120 min na segunda etapa de hidrólise resultou em um rendimento máximo de glicose de 90,0% (g/g) e um valor de rendimento de glicose previsto de 88,9%. Após a validação, os valores experimentais e preditos de rendimento de glicose foram de 88,6% e 86,5%, respectivamente. Além disso, os hidrolisados ácidos obtidos continham baixa quantidade de compostos inibidores (furfural e 5-hidroximetilfurfural). Tanto o ácido quanto o hidrolisado enzimático foram eficientemente utilizados por Saccharomyces cerevisiae IQAr/45-1 para atingir rendimentos de etanol de 0,43 g/g e 0,46 g/g, respectivamente. No entanto, quantidades consideráveis de açúcar residual permaneceram no final da fermentação, indicando a incapacidade de S. cerevisiae IQAr/45-1 em metabolizar o açúcar pentose presente nos hidrolisados. Assim, o uso de levedura geneticamente modificada com a capacidade de utilizar simultaneamente os açúcares hexose e pentose melhorará ainda mais o processo de fermentação. Em conclusão, os processos que envolveram o uso de reagentes químicos baratos para superar a recalcitrância de SB e liberar açúcares fermentáveis poderiam potencialmente apoiar uma biorrefinaria e reduzir significativamente o custo do etanol de segunda geração.

Palavras-chave: Bagaço de cana-de-açúcar, Hidrólise ácida, Pré-tratamento, *Saccharomyces cerevisiae* IQAr/45-1, Fermentação de etanol

ABSTRACT

Second generation ethanol from lignocellulosic biomass is a renewable and sustainable solution to the environmental problems arising from the large energy demands of the modern society. Sugarcane bagasse (SB), a lignocellulose is made up of two major sugar polymers (cellulose and hemicelluloses) connected to lignin. Due to the recalcitrant nature of SB, pretreatment is considered a key process step before it can be converted to biomolecules. Raw SB consists of cellulose (40.4 wt%), hemicellulose (23.8 wt%), lignin (27.2 wt%), ash (3.0 wt%) and extractives (4.4 wt%). Pretreatment of SB with 3% NaOH and 8% NaOH in autoclave (121 ^{o}C) and at room temperature respectively led to increases in cellulosic fractions (approximately 62.0 wt%), while the lignin contents decreased to values less than 11.0 wt%. Two-step sequential 3% NaOH and acid pretreatment resulted to substantial increases in cellulosic fractions (approximately 80.0 wt%) which was about two-fold the value obtained from untreated SB (40.4 wt%). The hemicellulose content of SB decreased substantially after the second-step pretreatment with acid (maleic and sulfuric acid). However, crystallinity index increased substantially after different pretreatments were applied to SB. Enzymatic saccharification of pretreated SB led to glucose yields above 75% (g/g) compared to 27% (g/g) obtained from untreated SB after 72 h. The optimization of enzymatic hydrolysis resulted to maximum glucose yield of 85.9% (g/g) by applying 1.8 FPU/g cellulose of cellulase, 5.3% of solids loading and 48 h of hydrolysis time. On the other hand, two-step acid hydrolysis of pretreated SB resulted to a maximum glucose yield of 56.8% (g/g). The optimization of two-step acid hydrolysis by applying 1.5% sulfuric acid, 40.0 mmol/L $FeSO_4$ and 120 min in the second hydrolysis step resulted to maximum glucose yield of 90.0% (g/g) and predicted glucose yield value of 88.9%. After validation, the experimental and predicted values of glucose yield were 88.6% and 86.5% respectively. Besides, the acid hydrolysates obtained contained low amount of inhibitory compounds (furfural and 5-hydroxymethylfurfural). Both the acid and enzymatic hydrolysates were efficiently utilized by *Saccharomyces cerevisiae* IQAr/45-1 to achieve ethanol yield of 0.43 g/g and 0.46 g/g respectively. Nevertheless, considerable amounts of residual sugar remained at the end of fermentation, indicating the inability of *S. cerevisiae* IQAr/45-1 to metabolize pentose sugar present in the hydrolysates. Thus, the use of genetically engineered yeast with the ability to simultaneously utilize both hexose and pentose sugars will further improve the fermentation process. In conclusion, processes which involved the use of cheap chemical reagents to overcome SB recalcitrance and liberate fermentable sugars could potentially support a biorefinery and greatly reduce the cost of second generation ethanol.

Keywords: Sugarcane bagasse, Acid hydrolysis, Pretreatment, *Saccharomyces cerevisiae* IQAr/45-1, Ethanol fermentation

GENERAL INTRODUCTION

Second generation ethanol from lignocellulosic biomass is a renewable and sustainable solution to the environmental problems arising from the large energy demands of the modern society. Most importantly, second generation ethanol does not interfere with food security since they are based on non-food feedstock. Lignocellulosic biomass is an abundant renewable resource available mainly as residues from agricultural and forest industries across the globe. Sugarcane bagasse (SB) - a lignocellulosic biomass is a residual material derived after the extraction of cane juice. It is available in large quantity and consists of two major sugar polymers (cellulose and hemicelluloses), hence can be considered a promising raw material for the production of bioethanol. In addition, the use of SB as feedstock for ethanol production can possibly reduce sugarcane planting areas and consequently help to mitigate the impacts of climate change.

Lignocellulosic biomass can be converted into monomeric sugars through chemical and biochemical routes, while the sugars can be fermented into ethanol by *Saccharomyces cerevisiae*. But, the cost associated with the production of second generation ethanol still remains too high and unsustainable on a commercial-scale. Due to the recalcitrant nature of SB, pretreatment is required to deconstruct the cell wall structure and improve cellulose digestibility. Pretreatment cost which accounts for up to 40% of total ethanol production cost is a major challenge affecting the economic conversion of SB to ethanol. Several studies have been conducted on SB pretreatment, and each focusing mainly on improving cellulose digestibility, minimizing loss of sugars and formation of compounds toxic to yeast cells. Nevertheless, some of these goals are yet to be achieved, hence more research efforts are required with the view of reducing the high production cost of second generation ethanol.

Thus, technologies and strategies capable of reducing pretreatment cost with concomitant improvement in sugars yield are considered key steps for an economically viable biorefinery. Similarly, the current price of cellulase enzymes employed for second generation ethanol production is still considered too expensive and significantly raises the cost of production. To overcome these challenges, it is imperative to develop processes that make use of cheap chemicals, such as sulfuric acid or low enzyme loadings. In addition, process optimization is an important strategy that can be employed to improve the overall processes and maximize ethanol yield from SB.

1 LITERATURE REVIEWS

1.1 Bioethanol

Climate change and environmental degradation are major concerns for all sectors of society, from governmental agencies to industries and consumers, hence the quest for sustainable energy production technologies (FERRARI et al., 2019). The fossil fuels are likely to come at increasing environmental cost, so sustainable futures require energy conservation, increased efficiency, and alternatives to fossil fuels, including biofuels (DALE et al., 2014). Fossil fuels are non-renewable resources largely responsible for the increased carbon dioxide (CO_2) level in the environment and associated climate changes (BARRETO, 2018; SARAVANAN et al., 2018). Thus, the world's future energy needs should be based on renewable resources, since they are more sustainable and environmentally friendly (ZOGHLAMI and PAËS, 2019).

Biofuels are considered as a distinct type of alternative and viable renewable energy because of their non-toxic, biodegradable, and carbon neutral features (KOLEY et al., 2018; MATHIMANI and MALLICK, 2019). Various types of biofuels exist in the market as well as under development, for example, bioethanol, biogas, biodiesel, biohydrogen and biobutanol (LEE, 2016; SARAVANAN et al., 2018). So far, bioethanol has clearly maintained a dominant position as renewable energy (IBRAHIM et al., 2018). Bioethanol, as an alternative to the fossil fuels, is mainly produced by yeast fermentation from different feedstocks. It is a high octane number fuel and its physicochemical features are considerably different compared to the gasoline (BUŠIĆ et al., 2018).

The use of bioethanol started in 1826, but the first ethanol-driven car and engine were built by Henry Ford, Nicholas Otto and others in the last years of the 18th century (DEMIRBAS and KARSLIOGLU, 2007). The German government provided subsidies for ethanol during 1899 in order to intensify the use of ethanol and to reduce the difference in price between ethanol and gasoline. In 1908, Ford formed a Model T vehicle with carburettors having flexible fuel capacity which can utilize gasoline, alcohol or a mixture of 10% ethyl alcohol gasoline otherwise known as "gasohol" (AZHAR et al., 2017).

Similarly, the annual world production of bioethanol increased from 49.2 billion liters in 2007 to approximately 96.9 billion liters in 2015 (REN21, 2016). Despite these efforts, Brazil and the USA are the only countries that produce large quantities of bioethanol, 26.9 and 55.6 billion liters of ethanol per year, respectively (RFA, 2016). Besides, United States

and Brazil produce ethanol (first generation) mainly from food crops, corn and sugarcane respectively (KANG et al., 2010; ZHAO, 2015).

1.2 Lignocellulosic biomass

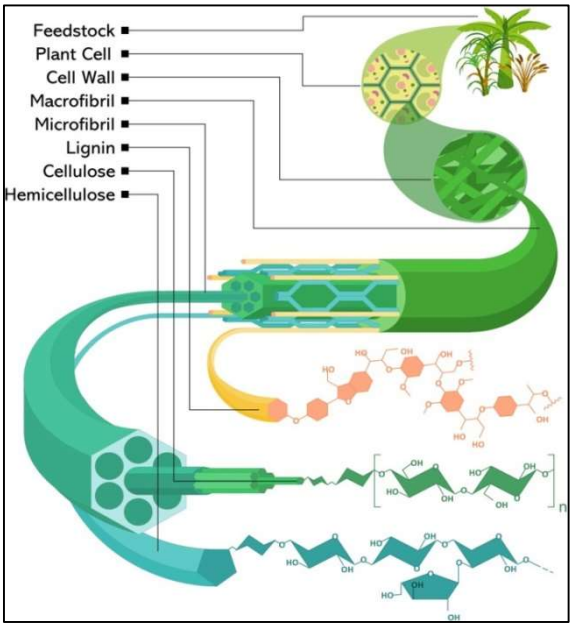
Lignocellulosic biomass has continued to attract global interest as a sustainable alternative to fossil carbon resources to produce second generation biofuels and biobased chemicals without affecting the global food security (MENON and RAO, 2012; DIAS et al., 2013; CHANDEL et al., 2018). The second generation bioethanol processes use sugars released from cellulose, which requires the additional cost of enzymes to hydrolyze cellulose (CARRILLO-NIEVES et al., 2019; ROCHA-MENESES et al., 2019). Therefore, to overcome the environmental problems associated with the large energy demands, the modern society needs to invest in sustainable biorefineries to convert lignocellulosic biomass into electricity, fuel, and chemicals (DIAS et al., 2013; DEMIRBAS, 2009). The various sources of lignocellulosic biomass include: agricultural wastes e.g. sugarcane bagasse, corn stover, wheat straw and sweet sorghum (YUAN et al., 2018; DIAS et al., 2009); dedicated energy crops e.g. switchgrass, miscanthus, energy cane, short rotation willow (LEWANDOWSKI et al., 2000; SCHMER et al., 2008; SANNIGRAHI et al., 2010; LYND et al., 2008); woody forest residues e.g. pine (COTANA et al., 2014).

Lignocellulosic biomass is a low cost renewable resource and is typically composed of ~30-50% cellulose, ~19-45% hemicellulose and ~15-35% lignin (SINGH et al., 2009; YANG et al., 2013; METZGER and HÜTTERMANN, 2009; MOOD et al., 2013). These lignocellulosic components are linked with each other in a heteromatrix to different degrees and composition depending on the type of biomass, species of plant, and even source of the biomass (HIMMEL et al., 2007). The structure of a typical lignocellulosic biomass and its three main components (cellulose, hemicellulose and lignin) is depicted in **Figure 1.**

Cellulose is the most abundant polymer, amounting to 40-60% in weight of lignocellulosic biomass (SHARMA et al., 2019), which is made up of β-D-glucopyranose units linked through β-(1,4) glycosidic bonds, with cellobiose as the primary repeating unit. The cellulose chains consisting of 500-1400 D-glucose units are arranged together to form microfibrils, which are packed together to form cellulose fibrils (MCKENDRY, 2002; ROBAK and BALCEREK, 2018). Cellulose fibrils are embedded in a lignocellulosic matrix thereby making it very resistant to enzymatic hydrolysis. In addition, the degree of polymerization (DP) of cellulose i.e. the number of glucose units in the polymer plays a

critical role on lignocellulose biomass recalcitrance (ZOGHLAMI and PAËS, 2019). Cellulose is an attractive raw material due to its low cost and abundance (LEE and LAVOIE, 2013).

Figure 1 - A schematic diagram of lignocellulosic biomass structure and its main components.



Source: Adapted from MAGALHÃES et al. (2019).

Hemicellulose consists of different heteropolysaccharides having different combinations of D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid and 4-O-methyl-D-glucuronic acid, representing 20-35% of the biomass weight (CHANDEL et al., 2018; ZHOU et al., 2018). The DP of hemicelluloses is in the range of 100-200 units (MOTA et al., 2018), which is considerably lower than that of cellulose, yet it can present a high degree of more or less complex substitutions. Hemicellulose is amorphous, with little physical strength hence it is readily hydrolysed by dilute acids or bases, and hemicellulase enzymes (ISIKGOR and BECER, 2015). Xylan is the most abundant hemicellulose in lignocellulosic biomass and contains mainly β-d-xylopyranosyl residues linked by β-1,4-glycosidic bonds (OCHOA-VILLARREAL et al., 2012).

Lignin is the second most abundant polymer after cellulose, corresponding to 15-40% of dry weight in lignocellulosic biomass (RAGAUSKAS et al., 2014). It is an aromatic polymer comprised of three mono-lignol monomers, methoxylated to various degrees: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (AGBOR et al., 2011). The three

primary units, namely phenylpropanoidsp-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), are randomly linked with aryl ether, ester or through carbon bonds and their ratio varies in grasses, hardwoods and soft wood (BALAN et al., 2011). Moreover, the complex formed as a result of the linkage between the sugar polymers (cellulose and hemicellulose) with lignin is responsible for the recalcitrant nature of lignocellulosic biomass (DING et al., 2012). Lignin can irreversibly adsorb cellulases and other enzymes during enzymatic hydrolysis due to its hydrophobic structural features comprising hydrogen bonding, methoxy groups, and polyaromatic structures (KUMAR and WYMAN, 2009; ZENG et al., 2014; QIN et al., 2016).

However, the amount of these three components in lignocellulose varies significantly depending on the type of biomass and harvest time (VAN DYK and PLETSCHKE, 2012; ROSALES-CALDERON et al. 2016).

1.3 Sugarcane

Sugarcane is a large grass belonging to the family Poaceae, tribe Andropogoneae, genus *Saccharum*. The currently cultivated sugarcane plants are hybrids derived from crossings mainly between plants of *S. officinarum* and *S. spontaneum* (DILLON et al. 2007). Sugarcane (*Saccharum* spp.) is a perennial crop which grows mainly in the tropical and sub-tropical regions, and serves as an important feedstock for sugar and ethanol production (DE AQUINO et al. 2017). It is possible to harvest same plants for several years, but at decreasing yields which makes them less replaceable compared to annual crops. Sugarcane is one of the most promising agricultural sources of biomass in the world. The sugarcane in natura is composed of 8% stem and green leaves, 20% sheath and dry leaves and 72% clean stalk (ALKOL BIOTECH, 2008). Sugarcane stem is the material removed before the milling of cane to obtain a juice which is later used for sugar or ethanol production. As shown in **Figure 2**, fresh leaves are green and yellow in color, tops are the part of cane plant between the top end and the last stalk node, and dry leaves are normally in brownish color (NETO, 2005).

The global production of sugarcane, estimated at 1.978 billion tons in 2019, is on the rise (STATISTA, 2019). According to OECD/FAO (2019), around 86% of the global sugar production is from sugarcane, with Brazil, India, Thailand and China responsible for 60% of the total production. Furthermore, sugarcane production as the main sugar crop is projected to grow by 1.3% per annum, slightly lower than the last decade. Brazil will continue to be a major producer of sugar and sugarcane-based ethanol, producing 37% of the world's sugarcane by 2028, which will be used for 18% of global sugar production and 88% of global

sugarcane-based ethanol production (OECD/FAO, 2019). Sugarcane is considered one of the main alternatives for the biofuels sector due to the great potential in the production of ethanol and its respective byproducts.

Figure 2 - Sugarcane crop (*Saccharum officinarum*).

Source: Adapted from AgricSite.com (2020)

1.4 Economical evaluation of sugarcane cultivation in Brazil, its products and future prospects

Brazil is world largest producer of sugarcane, it is estimated that during 2020/2021 harvest season ~ 630.7 million of tons of sugarcane will be harvested, representing a decline of 1.9% compared to 2019/2020 harvest season (CONAB 2020). According to Conab (2020), the estimated cultivated area, productivity and production, estimated sugar production, total re-coverable sugar, agricultural monitoring, harvesting system and export can be summarized as follows:

- **Estimated area, productivity and production**. In the 2020/21 harvest, the total area of sugarcane to be harvested is estimated **at 8,406.7 thousand hectares**, representing a 0.4% reduction compared to 2019/20 harvest season.
- **Estimated Sugar Production.** The world sugar supply framework in 2020 is expected to present a second balance sheet surplus. The increase in sugar production in important Asian producing countries, such as India and China, has contributed to the increase in world stocks and the reduction in international sugar prices. **São Paulo, the largest**

national producer, should produce 21,488.8 thousand tons, compared to the previous year and responsible for 61% of the sugar produced in the country.

- **Total Re-coverable Sugar.** The climate, the age of the crops and the form of harvest influence the total re-coverable sugar. Mechanized harvesting occurs most of the time, without the practice of burning the crops. Thus, a greater amount of vegetable impurities, such as straw, goes into the grinding process and ultimately reduces the efficiency of total re-coverable sugar extraction. In addition, straws create a microclimate favorable to the emergence of pests and diseases that impair total re-coverable sugar. **Estimated average total re-coverable sugar** for this current season **is 138.4 kg/t,** representing a reduction of 0.7% compared to last season.
- **Agricultural monitoring.** Agricultural monitoring aims to evaluate agro-meteorological conditions throughout the sugarcane cycle in the main producing states. The climatic conditions during the development and harvest period of the 2019/20 crop were analyzed. In Brazil, the development phase of the plant usually covers the period from May 2018 to March 2019, and the harvest phase from April 2019 to January 2020. The current location of the crop areas identified in the mapping by satellite imagery and agro-meteorological parameters, such as accumulated precipitation, precipitation deviation from historical average, maximum or minimum temperatures.
- **Harvesting system.** It occurs when sugarcane reaches the end of its growth period and begins maturation, reaching the maximum yield and accumulation of total re-coverable sugar. Harvesting is the stage of sugarcane production that undergoes the most changes due to new social and environmental requirements and the need to reduce costs. The type of sugarcane harvest can influence crop yield and longevity, physical, chemical and biological soil attributes, the environment and public health. One of the harvesting systems is the manual one, where the handy-man cuts with an appropriate tool and the entire sugarcane is loaded onto the trucks using mechanical winches. This type of harvest has been less frequent in the country. In this current season, the percentage of manual harvesting is estimated at 2.9% in the Center-South Region, where most of the production is concentrated.
- **Exports. A**t the end of the 2019/20 season, Brazil exported about 1.9 billion liters of ethanol. Besides, 2020/21 harvest presents many uncertainties about biofuel demand and prices, based on the perspective of reduced production and availability of ethanol for export. The sharp drop in prices of oil in the international market, as a result of

expanding supply in the context of the trade dispute between Russia and Saudi Arabia, or losing ethanol space for gasoline in the market and, lower consumption, the estimated prices of ethanol will be lower than those practiced in the previous season. Despite the prospect of a favorable exchange rate for ethanol exports in the 2020/21 harvest, the reduction of prices and less availability of biofuel limited ethanol export by Brazil. In the United States, main destination for ethanol exported by Brazil, the reduction in oil prices coupled with lower ethanol consumption in the country resulted to limited purchases of the Brazilian product.

However, the main destinations for ethanol exported by Brazil in the 2019/20 harvest were: United States (1.15 billion), South Korea (538.86 million liters), Holland (79.48 million liters), Japan (48.71 million liters) and Nigeria (14.9 million liters). With the arrival of the Covid-19 pandemic in Brazil, restrictive measures were adopted for circulation operations and reduction of commercial activity in general, with adverse effects on sales. This scenario of falling ethanol consumption was underscored by oil prices in the international market which made biofuel less competitive in relation to gasoline.

The Brazilian sugar and alcohol sector, unlike what happens in other countries, operates in a positive and sustainable manner. The Brazilian industrial segment produces environmentally friendly ethanol, which does not affect the ozone layer and is obtained from renewable sources (TSIROPOULOS et al., 2014). In addition to the production of ethanol and sugar, the production units have been seeking to increase their efficiency in electricity generation, helping to increase supply and reduce costs as well as contributing to increase the sustainability of the sector.

1.5 Carbon capture from sugarcane cultivation

Several studies have emphasized the environmental benefits of sugarcane ethanol as an effective option to mitigate greenhouse gases (GHG) emissions when compared to other biofuel feedstocks (SEABRA et al., 2011). Sugarcane is an efficient crop with respect to ethanol production, however it was reported that increased carbon dioxide (CO_2) concentration under controlled environment resulted to increased sugarcane photosynthesis, water use efficiency, biomass, and productivity (SINGELS et al. 2014; ZHAO and LI, 2015). Improved water use efficiency of sugarcane under elevated (CO_2) is mostly associated with the decreased stomatal conductance (VU and ALLEN, 2009; DE SOUZA et al., 2008). These

suggest that future increases in CO_2 concentration will have a resultant positive effect on sugarcane crop by increasing the diameter of cane and by reducing the use of water as well as improves sucrose production (MISRA et al., 2019). Nonetheless, these findings from the controlled environment are important for better understanding of physiological mechanisms of sugarcane plant response to increased (CO_2), they may not completely reveal the interactions of (CO_2) and other climatic factors under field conditions (ZHAO and LI, 2015).

However, agricultural soils are known to emit CO_2 mainly due to the association between soil attributes (physical, chemical and even biological) and the climate (TEIXEIRA et al., 2010; SILVA-OLAYA et al., 2013; BICALHO et al., 2014; BAHIA et al., 2015). The carbon sequestering ability of sugarcane (181 kg C is sequestered/ha year) implies that its cultivation at increasing CO_2 concentration will be beneficial in attaining higher productivity and yield of other crops, as well as in managing the effect of higher concentration of CO_2 (MISRA et al., 2019). Thus, higher sugarcane yields combined with large-scale production of biofuels could improve its GHG abatement potential (KERDAN et al., 2019).

1.6 Sugarcane bagasse

Sugarcane bagasse (**Figure 3**) is the fibrous residue of sugarcane after crushing and extraction of its juice, and it is considered one of the largest agriculture residues in the world (PANDEY et al., 2000; HERNÁNDEZ-SALAS et al., 2009). Only about one-third of the sugarcane by weight is turned into sugar and first generation ethanol, while the remaining (bagasse and straw) is a lignocellulosic biomass (PEREIRA et al., 2015). The global sugarcane bagasse generation is estimated to be ~ 600 million tons by 2024, which will serve as feedstock for bioenergy, biofuels, and other products (MARTINEZ-HERNANDEZ et al., 2018).

However, ~ 90 million tons of this byproduct is available annually in Brazil (CARPIO and SOUZA, 2017). The bulk of this amount is used to meet the steam and energy needs in industries. The steam feeds turbines to produce electricity, and the low-pressure steam leaving turbines meets the industry's thermal process needs (MAGALHÃES et al., 2019). The electric power generated is used to run the plant and the surplus is sold to regional electric distribution companies. Besides, the bagasse surplus could be diverted to other uses such as the production of single cell protein, ethanol, enzymes and food additives such as vanillin (MATTHEW and ABRAHAM, 2005) and xylitol (CARVALHO et al., 2005; SANTOS et al. 2003). Interestingly, more than 40 different applications of sugarcane bagasse have been

reported, which include animal feed, furfural, pulp and paper (MARTINEZ-HERNANDEZ et al., 2018).

Figure 3 - A stockpile of sugarcane bagasse.

Source: Adapted from Eduardo Cesar/FAPESP Research magazine (2019).

Sugarcane bagasse is mainly composed of cellulose (35-47.3%), hemicellulose (22.9-35.8%), lignin (14.1-30.6%), extractives (2.7-14.1%) and ash (0.8-8.8%) (BRIENZO et al., 2016). But, sugarcane bagasse composition may be affected by cane maturity, harvesting method, type of soil and efficiency of equipment used for the extraction process (BEZERRA and RAGAUSKAS, 2016).

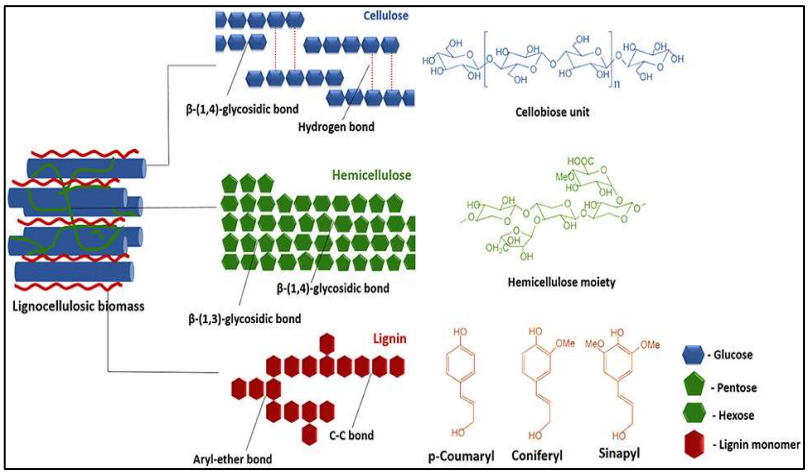
1.7 Pretreatments

Due to the recalcitrant nature of lignocellulosic biomass, pretreatment is considered as a very important process step before it can be converted to biomolecules (COSTA SOUSA, 2009; JEOH et al., 2007; YANG and WYMAN, 2008). Pretreatment removes the physical and chemical barriers that make native biomass recalcitrant and makes cellulose susceptible to enzymatic hydrolysis, which is a key step in biochemical processing of lignocellulose based on the sugar platform concept (JÖNSSON and MARTÍN, 2016). These pretreatments may be biological, chemical, and physical processes that are used separately, combined, and/or sequentially (GALBE and ZACCHI, 2012; SILVEIRA et al. 2015). As shown in **Figure 4**, pretreatment is applied to deconstruct the structure of lignocellulosic biomass into its individual components.

However, each pretreatment has a different effect on the cellulose, hemicellulose, and lignin fractions. Thus, it is imperative to choose suitable pretreatment methods for SB because

different lignocellulosic materials have different physico-chemical characteristics (ALVIRA et al. 2010). For a pretreatment to be considered effective, it should (1) improve the formation of fermentable sugars, (2) avoid the loss or degradation of carbohydrates, (3) avoid the formation of inhibitory by-products, and (4) be cost-effective (MARTÍN et al. 2007). In addition, it should be economically efficient, low energy consumption, and environmentally sustainable (MUSSATTO et al. 2016; PULIGUNDLA et al. 2016; FERRARI et al., 2019). The structural modifications of the lignocellulose are highly dependent on the type of pretreatment employed which consequently affect the enzymatic hydrolysis (KUMAR and SHARMA, 2017).

Figure 4 - Deconstruction of lignocellulose structure via pretreatment.



Source: Adapted from BARUAH et al. (2018).

1.7.1 Physical pretreatments

The first stage of pretreatment is the reduction of biomass particle size via physical processes which include: milling (e.g. two-roll milling, ball or hammer milling and colloid or vibroenergy milling), irradiation (e.g. gamma ray, electron beam, microwave) and others (e.g. hydrothermal, expansion, extrusion or pyrolysis) (BUŠIĆ et al., 2018; SANTIAGO and NETO, 2008). Physical pretreatment, decreases the particle size and crystallinity of lignocellulosic biomass, thereby increases the specific surface area and decreases the degree of polymerization (SUN and CHENG, 2002).

On the other hand, the particle size has to be optimized (very small particles are not desirable) due to high energy consumption during milling and subsequent negative impact on the pretreatment (TALEBNIA et al., 2010). Several of the size-reduction physical methods reported are not economically feasible as a result of very high energy demands. However,

extrusion is regarded as a new and prospective physical pretreatment method due to its capacity to ensure high shear rate, rapid heat transfer and effective mixing (KARUNANITHY and MUTHUKUMARAPPAN, 2010; YOO et al. 2011).

1.7.2 Chemical pretreatments

Chemical pretreatment involves the use of acid, alkali, ionic liquids and organosolv (BUŠIĆ et al. 2018). Acid pretreatment involves the use of different acids like sulfuric acid, phosphoric acid, nitric acid, hydrochloric acid, but sulfuric acid is the most commonly used for biomass pretreatment (TOMÁS-PEJÓ et al., 2011; MOSIER et al., 2005; SUN and CHENG, 2002). Besides, dilute acid pretreatment is the most widely adopted and has been considered to be one of the pretreatment methods with greater potential for wide-scale application (YANG and WYMAN, 2008; HU and RAGAUSKAS, 2012). A typical dilute pretreatment process follows the acid concentration range of 1-6%, temperature range of 100-200 °C, and reaction time varying from few minutes up to 5 hours min (TIMUNG et al., 2016; SABIHA-HANIM and HALIM, 2018). Pretreatments using dilute acid are normally used to degrade the hemicelluloses and increase the biomass porosity, hence improving the enzymatic hydrolysis of cellulose (KARP et al., 2013). Sulfuric acid is the most commonly used acid in the pretreatment of SB (LAVARACK et al., 2002) and this is mainly due to the cost effectiveness of this method (CHEN et al., 2011). Several reports have shown that multiple stages of acid pretreatment give greater efficiency and result to significant reduction in cellulases consumption during enzymatic hydrolysis (HAMELINCK et al. 2005).

However, during the hot acid pretreatment, some of the polysaccharides are hydrolyzed, mostly hemicelluloses. The resulting free sugars can be degraded to Furfural (from pentoses) and 5-Hydroxymethylfurfural (from hexoses). These compounds are inhibitory to the microorganisms, and their production signifies loss of fermentable sugars. But, organic acids such as maleic and fumaric acid have been suggested as alternatives to avoid inhibitory compounds formation (KOOTSTRA et al., 2009). Furthermore, pretreatment using Lewis acid such as $FeCl_3$ has been evaluated and found to be efficient in hemicelluloses solubilization, thereby resulting to increases in surface area, and improved enzymatic digestibilty (ZHANG et al., 2018).

Alkaline pretreatment is primarily a delignification process through which the cell wall of lignocellulosic biomass becomes disrupted by (1) dissolving hemicelluloses, lignin, and silica, (2) hydrolyzing uronic and acetic esters, and (3) swelling of cellulose under mild

conditions (SABIHA-HANIM and HALIM, 2018). This process results in two fractions, a liquid (hemicellulose oligomers and lignin) and a solid fraction (cellulose). The alkaline pretreatment can significantly degrade lignin without producing side products, eco-friendly and can be carried out at a lower temperature (60-100 °C) (ROCHA et al., 2012).

Pretreatments with sodium hydroxide (LALUCE et al., 2019; MARYANA et al., 2014; JU et al., 2011), potassium hydroxide (PAIXÃO et al., 2016), calcium hydroxide (GRIMALDI et al., 2015; RABELO et al., 2009; WYMAN et al., 2005), aqueous ammonia (HEDAYATKHAH et al., 2013) and combination of ammonium hydroxide and hydrogen peroxide (ZHU et al., 2012) have been applied to SB to remove lignin. Nonetheless, NaOH shows the greatest lignin degradation when compared to other alkalis, such as sodium carbonate, ammonium hydroxide, calcium hydroxide, and hydrogen peroxide (SABIHA-HANIM and HALIM, 2018). The effectiveness of alkaline pretreatment may vary with the types and characteristics of the studied substrates because of their distinct affinity to organic components.

Organosolv pretreatment method involves the use of organic or aqueous solvents (e.g. ethanol, methanol, acetone and ethylene glycol) with or without catalysts to extract lignin and to ensure more accessible cellulose (ZHOU et al., 2018). Recent studies have shown that organosolv pretreatment could lead to high delignification and hemicellulose degradation to value-added xylan oligomers (ZHANG et al., 2016). During organosolv pretreatment, solvents are mixed with water in different parts and added to the biomass followed by heating at temperature varying from 100 °C to 250 °C (PARK et al., 2010). Organosolv pretreatment of SB under optimized conditions (30 % by volume ethanol at 195 °C for 60 min) resulted in the production of 29.1 % by mass of fermentable sugars (MESA et al., 2011). Other authors reported the removal of 70 % hemicelluloses and 65 % lignin after organosolv (glycerol-based) pretreatment of wheat straw under optimized conditions at 220 °C for 3 h using liquid-solid ratio of 20 g/g (SUN and CHEN, 2008).

One major attraction of organosolv pretreatment method is that the solvents used can be recycled, thus reducing the operating costs. However, the use of low boiling point organic solvents necessitates high pressure conditions and expensive equipment, which is a major drawback of this pretreatment method (BORAND and KARAOSMANOGLU, 2018). To overcome this challenge, high-boiling point alcohols, such as ethylene glycol or glycerol, can also be used at low temperatures and pressure, but their recovery requires more energy (ZHANG et al., 2016).

Besides, ionic liquid pretreatments are labeled as green solvents, because they do not form toxic chemicals and nearly 100 % of solvents can be recovered (HEINZE et al., 2005). It involves the use of ionic liquids (ILs) such as 1-n-butyl-3-methylimidazolium, 1-allyl-3 methylimidazoliumchloride ([Amim]Cl), N-methyl morpholine N-oxide (NMMO), 1-buthyl-3-methylimidazolium acetate [BMIM][OAc], 1-ethyl-3-methylimidazolium acetate ([Emim]Ac) and 1-ethyl-3-methylimidazolium diethyl phosphate ([Emim]Dep) to disrupt the non-covalent interactions between lignocellulose components without leading to significant degradation (AKHTAR et al. 2016; JÖNSSON and MARTÍN, 2016). Currently, different ILs have been proposed for application in pretreatment due to their potential to dissolve a variety of lignocellulosic biomass such as corn stover, bagasse, wheat straw and woods of different hardness (ZHANG et al. 2015; MOHTAR et al. 2017). Kuo and Lee (2009) reported two times improvement in enzymatic hydrolysis using NMMO treated sugarcane bagasse at 130 C, 1 h.

However, the development of energy-efficient recycling methods, and the implementation of effective strategies for recovery of hemicelluloses and lignin from pretreatment liquids is required for the industrial application of ILs (JÖNSSON and MARTÍN, 2016). Despite the limited amount of inhibitors formed, the minor amounts of ILs remaining in the pretreated materials are potentially toxic to enzymes and fermentative microorganisms (YANG and WYMAN, 2008).

1.7.3 Biological Pretreatments

Biological pretreatment is attracting great attention due to its effectiveness, safety and environmental friendly approaches (SINDHU et al., 2016). Biological pretreatment process involves the use of microorganisms like brown, white, and soft-rot fungi to pretreat biomass (CAPOLUPO and FARACO, 2016). Microbes are known to effectively degrade lignin through lignin degrading enzymes such as peroxidases and laccases (SANCHEZ and CARDONA, 2008). The white-rot fungi such as *Phanerochaete chrysosporium*, *Clostridium butyricum*, *Trichoderma viride*, *Pycnoporus cinnarbarinus*, *Dichomitus squalens*, and *Phlebia radiate*, *Trametes versicolor*, and *Aspergillus oryza* have shown good delignification efficacy (SHI et al., 2008). Besides, the specie of white-rot fungi, *P. chrysosporium*, exhibits the highest efficiency in lignin degradation due to its high growth rate (PINTO et al., 2012). Also, brown-rot fungi like *Tyromyces balsemeus*, *Poria placenta*, and *Lentinus lepidius* can degrade cellulose and hemicellulose with slight modification of lignin structure (RASMUSSEN et

al., 2010). These microorganisms can completely degrade lignin to CO_2 and H_2O due to the action of lignin-degrading enzymes such as laccases, lignin peroxidase and manganese peroxidase which are regulated by carbon and nitrogen sources (SHI et al., 2008; HOWARD et al., 2004).

Biological pretreatments are mostly performed under mild conditions hence require low capital costs when compared to physical/chemical pretreatment that involves the use of expensive reactor systems (BALAN, 2014). However, biological process is a relatively slow process requiring several days to pretreat the biomass. In several cases, biological pretreatment followed by chemical pretreatment is considered effective and requires less severity pretreatment conditions for effectively hydrolyzing the biomass (BALAN et al. 2008).

1.8 Hydrolysis of lignocellulosic materials

The resistant nature of lignin to degradation, the inefficient breakdown of cellulose and hemicellulose as well as the variety of sugars released from the carbohydrate polymers are some the challenges associated with ethanol production from the lignocellulose (BALAT et al., 2008). Bioethanol production from lignocellulose typically consists of four major steps: (1) a pretreatment step to make polysaccharides more accessible, (2) an enzymatic hydrolysis process to break down polysaccharides to simple sugars, (3) a fermentation step where a microorganism ferments sugars into ethanol, and (4) a separation stage to obtain fuel grade ethanol (SCHWIETZKE et al., 2008).

1.8.1 Acid hydrolysis

Cellulose is a glucose-based polymer that can be hydrolyzed to liberate monosaccharides, which can subsequently be fermented to ethanol (CHANG et al., 2018). Acid hydrolysis of cellulose is a typical way to break down cellulose into glucose and can be done using either dilute acid or concentrated acid. The main advantage of cellulose hydrolysis using dilute acid is that the acid does not need to be recovered (NI et al., 2013), but its several disadvantages include the need of high temperature (at least 180 ºC) and pressure (around 10 atm) which still provides low glucose yields (IRANMAHBOOB et al., 2002). For example, cellulose hydrolyzed with 0.4 wt% acid at 215 ºC for 3 min after pre-treatment (hemicelluloses hydrolysis) with 0.7 wt% sulfuric acid at 190 ºC for 3 min gave a glucose yield of only 50% (HAMELINCK et al., 2005). Dussán et al. (2014) hydrolyzed cellulosic

fraction of SB with 1.0% sulfuric acid at 121 ºC for 20 min and obtained a maximun glucose extraction efficiency of 71%. Dilute acid hydrolysis is a more established method and the process generates less sugar degradation products such as 5-HMF or furfural, but more time and higher temperature are required to increase the reaction rate for sugar release (LUO et al., 2002).

On the other hand, cellulose hydrolysis using concentrated acid can take place at moderate temperature and pressure with concomittant high glucose yields of around 90% (HAMELINCK et al., 2005). But the process usually involves a longer reaction time (2-6 h) and economical separation of glucose and acid is also a major challenge. At high acid concentration, the hydrogen bonding of the cellulose chains gets disrupted thereby reducing the crystallinity of the cellulose to a relatively amorphous state (OROZCO et al., 2007). The concentrated acid hydrolysis of cellulose historically started in 1883 when the method of dissolving and hydrolyzing cotton cellulose with concentrated sulfuric acid was invented. From 1937 to 1948, three processes for concentrated acid hydrolysis of cellulose, namely the Bergius-Rheinau process (KENT, 2013; AMARASEKARA, 2013), the Peoria process, and the Hokkaido process (WENZL, 1970; CLAUSEN and GADDY, 1993) were deployed at large scale.

However, they were only successful at times of national crisis, when oil from the Middle East was not readily accessible, and fell out of use after World War II due to poor yields, significant waste streams and large quantities of unmarketable by-products (ARKENOL, 1999). Complicated process units, high energy consumption and difficulties to recycle acid were also mentioned as obstacles toward the commercialization of these technologies (FAN et al., 1987). In the mid-1970s, the Peoria process was improved at Purdue University and at the Tennessee Valley Authority (TVA) by recycling dilute acid, and became known as the TVA process. In 1989, Arkenol, an American company developed a two-stage concentrated acid hydrolysis process using a chromatographic method to separate sugar and acid (AMARASEKARA, 2014). Based on widely published work carried out since the 90's at the University of Sherbrooke, lignocellulosic biomass was first separated into four fractions, namely hemicelluloses, cellulose, lignin, and extractives (LAVOIE et al., 2011).

Furthermore, Chang et al. (2018) reported a two-step acid hydrolysis, which involved subjecting the cellulosic fraction to swelling and decrystallization by concentrated acid treatment step (72 wt% sulfuric acid, 2 h) followed by partial neutralization and a post-hydrolysis step (121 ºC, 10 min) to obtain high yield of glucose. Variations of this method

have the potential of being economically attractive if a minimum acid/cellulose ratio is used for the ionic liquefaction step and a high glucose yield is obtained after post-hydrolysis. However, additional requirements include recovering and recycling the used ions, and conditioning the final glucose solution for subsequent fermentation using established microbial systems.

1.8.2 Enzymatic hydrolysis

The key technical limitations on the practical application of enzymatic biotransformation technology for lignocellulosic biomass are the high enzyme production cost and low production efficiency (TOOR et al., 2020). Thus, the limited access of enzymes to the polysaccharides is a major obstacle to efficient enzymatic hydrolysis (ARANTES and SADDLER, 2010; DING et al. 2012). Besides, due to the heterogeneity and compositional variability of lignocellulosic biomass, the same enzyme may not be efficient for all and hence a cocktail of multifunctional enzymes can prove to be more efficient (BINOD et al., 2019).

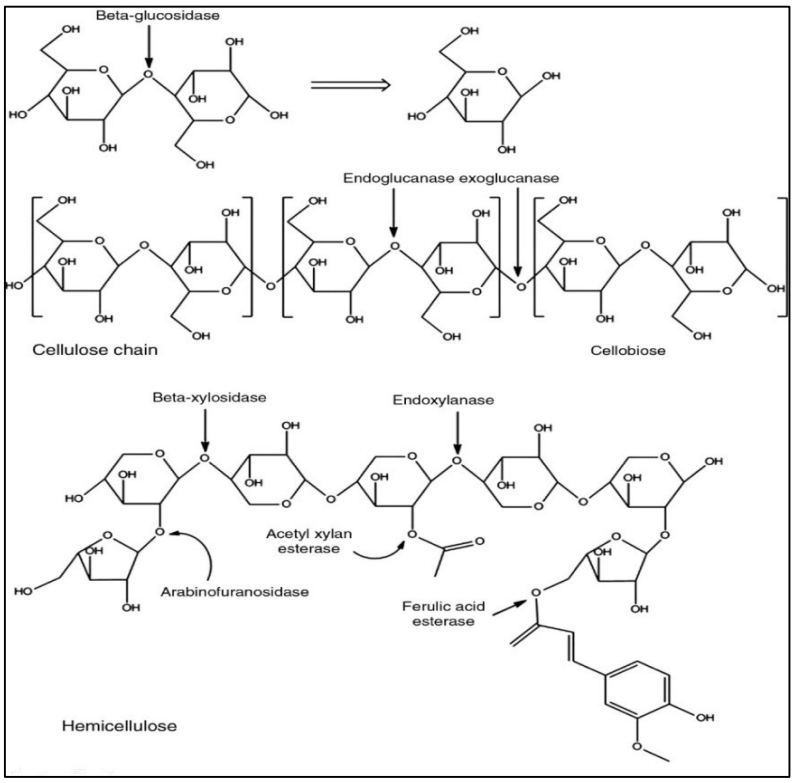
However, enzymatic hydrolysis is more advantageous than acid hydrolysis, the chemical alternative, since it requires less energy, milder operating conditions, and it is less corrosive and toxic (SUN and CHENG, 2002; TAHERZADEH and KARIMI, 2007). During enzymatic hydrolysis, cellulase and hemicellulase enzymes depolymerize cellulose and hemicellulose to hexoses (mannose, glucose, and galactose) and pentoses (xylose and arabinose) respectively (ROSALES-CALDERON and ARANTES, 2019). The most important cellulases and hemicellulases widely employed for cellulose and hemicellulose hydrolysis (ROSGAARD et al., 2007) includes the following:

- Endoglucanase I (EG I, GH family 7B),
- Cellobiohydrolase I (CBH I, GH family 7A),
- Cellobiohydrolase II (CBH II, GH family 6A),
- Beta-glucosidase (βG, GH family 3),
- Endoxylanase (EX, GH family 11),
- Beta-xylosidase (βX, GH family 3).

EG randomly hydrolyzes internal glycosidic bonds within cellulosic microfibrils (WOOD and MCCRAE, 1982), while CBH enzymes act progressively along cellulosic chains cleaving off cellobiose units from either end (CBH I acts at reducing ends and CBH II acts at non-reducing ends) (WOOD and MCCRAE, 1986) with βG finally hydrolyzing cellodextrins to glucose (SCHMID and WANDREY,1987). EX cleaves the xylan backbone at internal β-

1,4 xylosidic bonds, while βX hydrolyzes short xylo-oligomers to xylose (SHALLOM and SHOHAM, 2003). All these enzymes work synergistically with each other creating new accessible adsorption sites or active substrates for each other to act upon (GAO et al., 2010; SELIG et al., 2008; JØRGENSEN et al., 2007). Thus, preserving the enzyme activities during hydrolysis helps to maintain enzyme synergies that enable higher sugar conversion (VAN DYK and PLETSCHKE, 2012). **Figure 5** represents the enzyme system employed for bioconversion of lignocellulosic biomass to sugars.

Figure 5 - The cellulolytic enzyme system



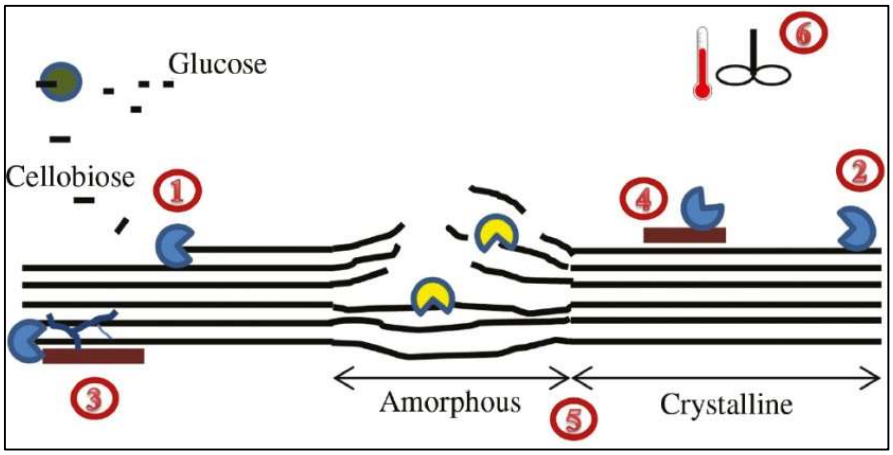
Source: Adapted from VOLYNETS et al. (2017).

The non-specific adsorption and inhibition of cellulases by lignin is an important factor that affects cellulose depolymerization to glucose (ROSALES-CALDERON et al. 2016; QI et al. 2011). However, limited studies have been carried out to isolate lignin from different pretreatment processes and evaluate their effects on cellulase and hemicellulase during hydrolysis (BERLIN et al., 2006; KUMAR et al., 2012; KIM et al., 2011), or by simply washing the biomass with water to evaluate their performance (QIN et al., 2013). Investigating the lignin inhibition on mono and multi-component cellulases and hemicellulases can help to reduce the enzyme mass loading during biomass hydrolysis,

thereby reducing the enzyme cost in industrial biorefinery (ZHOU et al., 2018). The factors affecting enzymatic hydrolysis of cellulose are depicted in **Figure 6**.

On the other hand, enzymatic hydrolysis yields can be increased through the addition of surfactants (Tween 20), bovine serum albumin, and polymers (PEG 6000), which prevent unproductive binding of enzymes to lignin (KUMAR and WYMAN, 2009). It was reported that enzyme adsorption to the substrate decreased from 90 to 80% by the addition of surfactant during enzymatic hydrolysis, with concomitant increase in glucose and xylose yields (ERIKSSON et al., 2002). However, the effectiveness of the surfactants depends on the type of pretreatment, while surfactant loading above 150 mg/g glucan insignificantly affects increases in sugar release (KUMAR and WYMAN, 2009).

Figure 6 - Factors affecting enzymatic hydrolysis of cellulose, (1) end-product inhibition by glucose and cellobiose; (2) unproductive binding of enzyme onto cellulose; (3) structural hindrance by lignin and hemicellulose; (4) unproductive binding of enzyme onto lignin; (5) cellulose crystallinity; and (6) shear- and thermal-induced denaturation.



Source: Adapted from VOLYNETS et al. (2017).

1.9 Fermentation

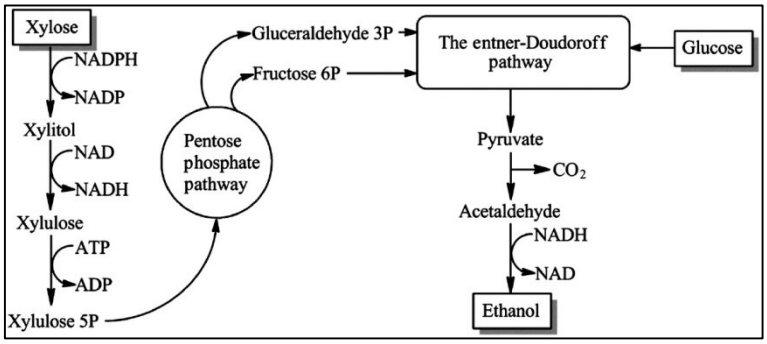
Fermentation is the process of converting the sugars generated from the pretreatment of lignocellulosic biomass with dilute acid and/or the saccharification of the pretreated solids with enzymes into ethanol using microbes such as yeast (LIU et al., 2019; XIA et al., 2019). Thus, microorganism selection is crucial and depends upon the conditions and types of substrates to be fermented into ethanol. *Saccharomyces cerevisiae* has been employed extensively in the brewery and wine industries for many years. In the batch fermentation

process, *S. cerevisiae* usually ferments hexose sugars (glucose) into ethanol in the big tank by way of Embden Meyerhof Pathway (EMP) under optimized temperatures and anaerobic environments (TOOR et al., 2020). It is possible to achieve high ethanol yield (equivalent of 90% of the theoretical) with *S. cerevisiae* using hexose sugars. During fermentation, the culture medium is often supplemented with nitrogen in order to boost the reaction and CO_2 is typically produced as the byproduct (GOMBERT and van MARIS, 2015; SULIEMAN et al., 2016; LIU et al., 2019; XIA et al.,2019).

S. cerevisiae has long been used by humankind for ethanol production in hexoses-based media, but *S. cerevisiae* is not capable of direct assimilation of cellulose and hemicellulose (CHU and LEE, 2007). However, to overcome this drawback, recombinant DNA technology or the pentose-fermenting microorganisms (e.g. *Pichia stipitis*, *Pachysolen tannophilus* and *Candida shehatae*) are employed (QUINTERO and CARDONA, 2011). These pentose-fermenting microorganisms have ethanol production rate of approximately five times lower than the ethanol production from glucose by *S. cerevisiae*. Besides, their oxygen and ethanol tolerance are 2-4 times lower than that of *S. cerevisiae* (CLAASSEN et al., 1999). Jeffries and coworker (1999*)* genetically engineered *S. cerevisiae* with arabinose-metabolizing ability using genes selected from yeasts such as *Candida aurigiensis*. Thus, in the recent times conscientious efforts have been directed towards obtaining an ideal microorganism that will be able to produce ethanol directly from any carbohydrate (KANG et al., 2014; BAEYENS et al. 2015). The metabolism pathway of glucose and xylose in yeast is shown in **Figure 7**.

The tolerance of yeast to its substrate (osmo-tolerance), fermentation product (ethanol-tolerance) and temperature (thermo-tolerance) has great potential to be used in industrial-scale fermentation (BALAKUMAR and ARASARATNAM, 2012). At the beginning of fermentation, cells are subjected to high substrate concentration and as the ethanol level increases, both the substrate and product causes stress to the organism (GUYOT at al., 2005). According to Balakumar and Arasaratnam (2012), strains of *Saccharomyces* showed viability and ethanol producing ability between 40 ºC to 50 ºC or 40 ºC to 45 ºC. Thus, cells given a heat shock prior to inoculation are able to perform better by exhibiting better tolerance to heat, ethanol and osmotic stresses by producing trehalose. Furthermore, Laluce and co-workers (2013) reported high tolerance to ethanol (64 g/L) and temperatures (42 ºC) by the yeast stain IQAr/45-1 during batch fermentation of sugarcane molasses.

Figure 7 - Glucose and xylose metabolism pathway in yeast.



Source: Adapted from VOLYNETS et al. (2017).

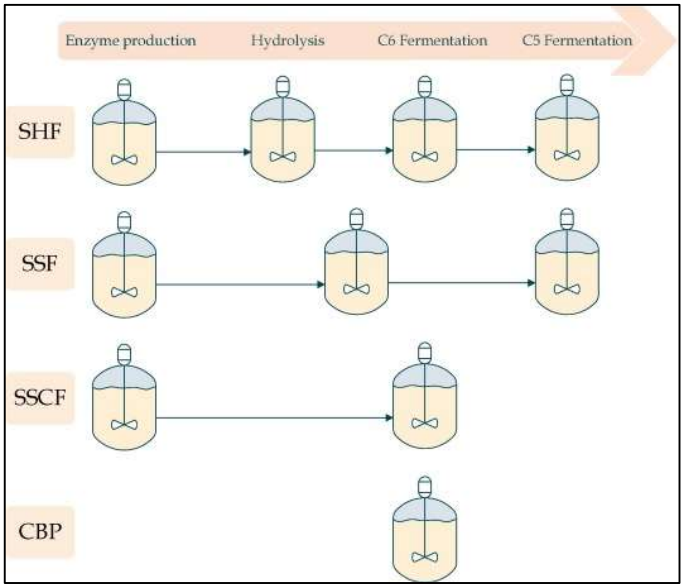
In the integrated bioprocess systems, the hydrolysis and fermentation often function as separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). Lately, new integrated bioprocesses such as simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP) have been developed (BUŠIĆ et al., 2018). **Figure 8** represents the key fermentation strategies for bioethanol production from lignocellulosic biomass.

In the SHF process, lignocellulosic material is decomposed into monomeric sugars through enzymatic saccharification, followed by fermentation to ethanol in separate bio-reactors (RASTOGI and SHRIVASTAVA, 2018). Optimal temperatures of 50 °C and 28-32 °C are employed for hydrolysis and fermentation respectively. In this case, suitable microorganisms perform the fermentation of hexoses and pentoses separately (IVANČIĆ et al., 2016). The main reason for separate fermentations is because pentose utilizing microorganisms metabolize pentoses and hexoses slower than microorganisms that only assimilate hexoses coupled with their high sensitive to ethanol and inhibitors (CARDONA and SÁNCHEZ, 2007).

On the other hand, in SSF process, hydrolysis and fermentation take place in a single bioreactor hence sugars released from the enzymatic hydrolysis are immediately utilized by the microorganism. Under these conditions, relatively low sugar concentrations are present in the broth, such that the inhibition of the fermentating microorganisms and cellulase by the released sugars is reduced (DA COSTA NOGUEIRA et al., 2019; SIRIWONG et al., 2019; PAVLEČIĆ et al. 2017). Consequently, optimal temperature for SSF (~38 °C) is balanced between the optimal hydrolysis (45-50 °C) and fermentation (30 °C) temperatures. Also, by selecting enhanced enzymes and yeast strains, it is possible to achieve further improvement in SSF (BRETHAUER and WYMAN, 2010). Thus, the overall ethanol production in SSF is

generally better than SHF. However, the major limitation of SSF is that the optimal temperature required for cellulose activity is usually higher than the suitable temperature required for yeast activity and numerous bacterial biofuel fermentation strains (BHALLA et al., 2013; WANG et al., 2015). In addition, SSF process does not allow the use of hexose and pentose sugars at the same time.

Figure 8 - Basic fermentation strategies for ethanol production from lignocellulosic biomass.



Source: Adapted from TOOR et al. (2020).

SSCF process is similar to SSF except for the fermentation of hexose and pentose sugars covered in a single step (TOOR et al., 2020). SSCF consists of a series of process, involving microbial assimilation of sugars released from the pretreatment process and simultaneous hydrolysis of lignocellulosic material (KOPPRAM et al., 2013). SSCF technology has a potential to lower the total cost of ethanol production as pentose sugars are also used during the process, hence the inhibitory effects of xylose are reduced (ZHANG and LYND, 2010). The main advantage of SSCF lies on the simultaneous fermentation of the released glucose which maintains the glucose concentration within the medium. Also, it minimizes the inhibition caused by the end product during enzymatic hydrolysis step, increases xylose to glucose concentration ratioas and diverts the fermenting microorganism to consume xylose (OLOFSSON et al., 2010).

CBP system is a promising fermentation strategy still in its premature stage that can potentially overcome the high cost associated with bioethanol production from lignocellulosic biomass (PARISUTHAM et al., 2014). In CBP, different actions comprising enzyme production, enzymatic saccharification/hydrolysis and subsequent fermentation of the resultant sugars to ethanol or any other valuable products are performed simultaneously (DANIEL et al., 2012; KUMAGAI et al., 2014; PARISUTHAM et al., 2014).

2 OBJECTIVES

The current study was aimed to establish optimal conditions for the economic conversion of SB into ethanol. To achieve the above objectives, different pretreatments were applied to SB using alkaline and acid solutions under autoclave and room temperature conditions. Then a two-step acid hydrolysis of pretreated SB was investigated and the process was optimized in the presence of $FeSO_4$ (co-catalyst) by applying the design of experiment. Moreover, enzymatic hydrolysis of the pretreated SB was evaluated and the process parameters were optimized at low enzyme loadings using a central composite design.

The acid and enzymatic hydrolysates which resulted from acid and enzymatic hydrolysis respectively were fermented using the thermo-ethanologenic *Saccharomyces cerevisiae* (IQAr/45-1) and ethanol yields were quantified. In addition, the effects of inhibitory compounds (mainly furfural and 5-hydroxymethylfurfural) generated during acid hydrolysis were evaluated concerning the cells viability and fermentation capacity.

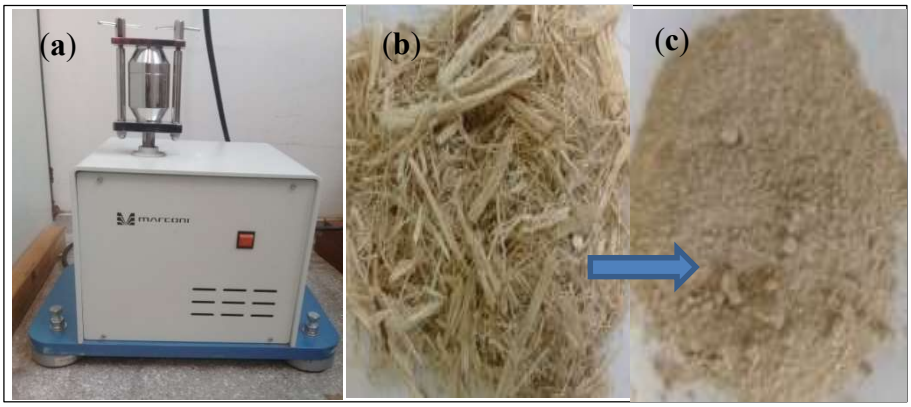
3 MATERIALS AND METHODS

3.1 Sample preparation and processing

Sugarcane bagasse (SB) was collected from a local sugar mill plant, Santa Cruz (member of São Martinho group) located in Américo Brasiliense, São Paulo, Brazil and transported at low temperature. Bagasse was stored in a laboratory freezer (Bosch, São Paulo, Brazil, model-GSD 32) at -20 ºC. Frozen samples were defrosted and dried at 60 ºC in a Biochemical Oxygen Demand (BOD) incubator (Marconi, São Paulo, Brazil, model-MA 415) until a constant weight was obtained (<10 % wt, moisture). Dry bagasse samples were stored in transparent plastic bags at room temperature before use.

Milling - a physical pretreatment method initiated the degradation of SB and conversion of the bagasse fibers into particles, thereby increasing its surface area (PALMOWSKI and MÜLLER, 2000). Thus, sugarcane bagasse was ground for 15 min/cycle in Marconi Ball Mill with Closed Chamber (Marconi, São Paulo, Brazil, model-MA 350/E) as shown in **Figure 9** to obtain particles of ≤ 0.5 mm after passing through a set of superposed sieves of different meshes (32, 35, and 150 mesh) as described by Miranda et al. (2016).

Figure 9 - (**a**) Marconi Ball Mill with Closed Chamber (model-MA 350/E), (**b**) Unground dry SB, (**c**) Ground dry SB (particle size ≤ 0.5 mm).

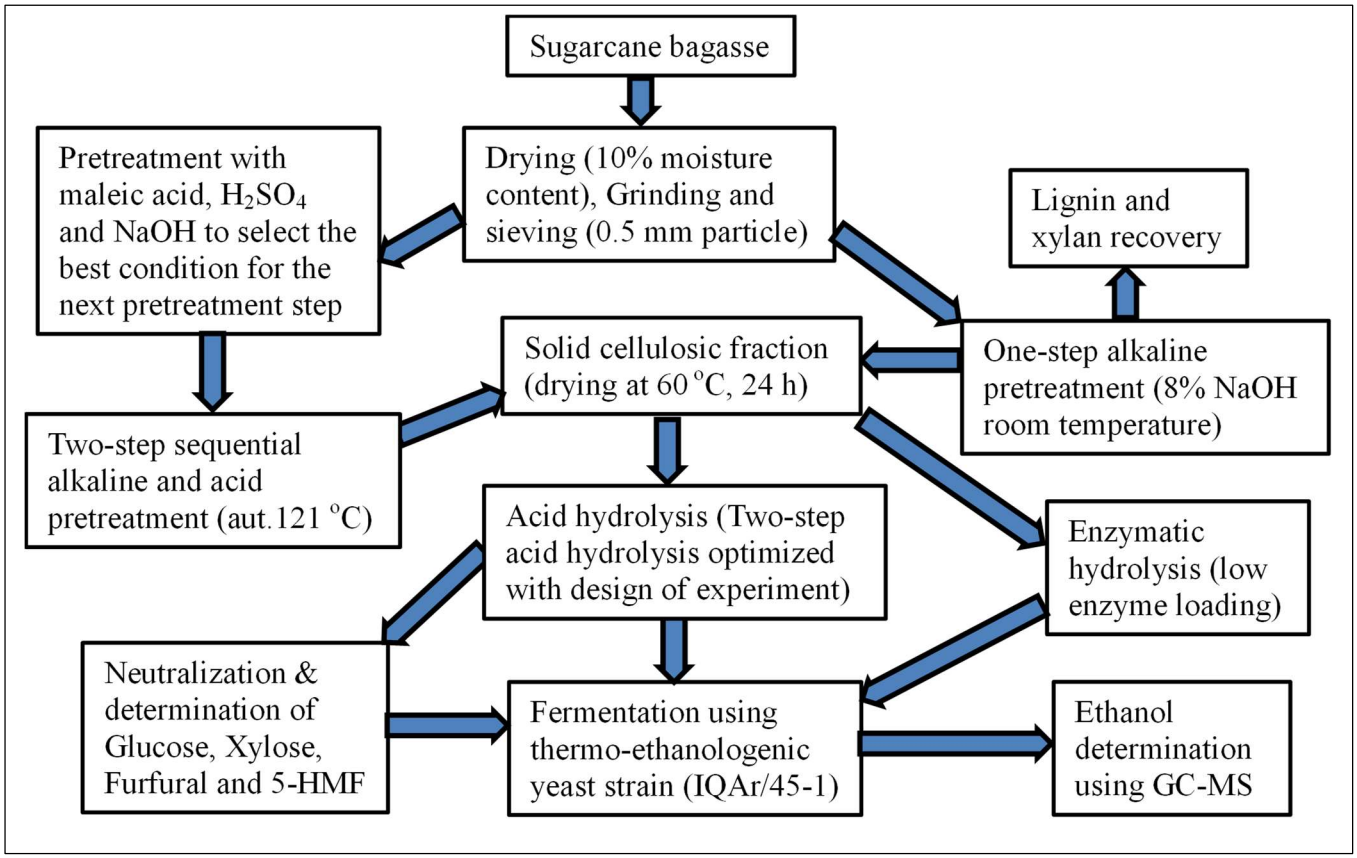


Dry sample of SB (particles of ≤ 0.5 mm) was repeatedly washed in cold water (room temperature) by filtration and then in warm water (60 °C) to extract soluble compounds (free sugars and other residues). The resulting SB was refluxed in a Soxhlet extraction apparatus containing a mixture of toluene:ethanol (2:1, v/v) (SUN et al., 2004; MIRANDA et al., 2016). The sample was refluxed in water (room temperature) for 30 min to remove the remaining solvents before drying in a Biochemical Oxygen Demand (BOD) incubator as recommended in the literature (BINOD et al., 2012) at 60°C to constant weight before being stored in a desiccator at room temperature until use. The schematic of steps proposed for the present work is depicted in **Figure 10**.

Two grams (2 g) of SB was weighed into the crucibles (W_2) and dried in the oven. The crucible and its contents were cooled to room temperature in a desiccator and reweighed. The procedure continued until a constant weight was obtained (W_3). The percentage moisture content was calculated as shown below.

$$\% \text{ Total Solids} = \frac{W_2 - W_3}{W_{2-W_1}} \times 100 \qquad (1)$$

Figure 10 - Process flow chart for ethanol production from sugarcane bagasse.



3.1.1 Chemicals and reagents

All the chemicals used were of analytical grade. Potassium dichromate, Maleic acid, Sulfuric acid, Iron (II) sulfate hydrate, Hydrochloric acid, Acetone, Ethanol, Acetic acid, Sodium hydroxide, Calcium carbonate, Calcium hydroxide, 3,5-Dinitrosalicylic acid, Phloroglucinol, Toulene and Sodium chlorite were purchased from Sigma-Aldrich® (Brazil). Chromatography standards used include D-cellobiose, D-glucose, D-xylose, Acetic acid, 5-Hydroxymethylfurfural (5-HMF), and Furfural and Sulfuric acid (99.999%) were purchased from Sigma-Aldrich® (Brazil). Commercial cellulase (Celluclast® 1.5L) and β-glucosidase (Novozyme 188) were purchased from Novozymes Latin America Ltd. Cellulase and β-glucosidase exhibited enzymatic activities of 83.53 FPU/mL (paper filter units by mL of enzymatic solution) and 544.37 IU/mL (international unit of enzymatic activity by mL of enzymatic solution) respectively.

3.2 Alkaline pretreatment

3.2.1 Alkaline pretreatment under autoclave condition

Two grams (2.00 g) of dry SB (particle size ≤ 0.5 mm) was suspended in different concentrations (0.0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0%) of NaOH solution (w/v) in 100 ml Erlenmeyer flasks at solid/liquid loading ratio of 1:20. Flasks were gently swirled to enable the solid to become completely soaked in the solution before been transferred into the autoclave (Phoenix, São Paulo, Brazil, model-AV-30) and heated for 30 minutes at 121ºC. Another set of samples, the same as above were heated for 60 minutes in autoclave at 121ºC. After heating, samples were removed from the autoclave and allowed to cool before been filtered using Whatman filter paper (Sigma-Aldrich, Darmstadt, Germany).

Filtrate was collected and used to assay for glucose, while the insoluble material (residues) on the filter paper was washed several times with deionized water to neutral pH. Residues were dried in BOD at 60 ºC to constant weight. 0.30 g of dry residues was used to assay for glucose using the method of Sluiter et al. (2008b), while 0. 50 g of the same dry residue was used for acid (0.1% sulfuric acid) extraction in autoclave.

3.2.2 Alkaline pretreatment at room temperature

15.0 g of dry SB ((particle size ≤ 0.5 mm) was suspended in NaOH solution (8%, w/v) in 100 ml Erlenmeyer flask at solid/liquid loading ratio of 1:15 (LALUCE et al.., 2019). Flasks were gently swirled to enable the solid to become completely soaked with the solution and kept at room temperature for 1 h, 4 h, 8 h, 12 h, 18h and 24 h respectively with stirring (magnetic stirrer) at 60 rpm. At the end of each residence time, sample was filtered using Whatman No. 1 filter paper. Filtrate collected was assayed for glucose, while the residues on the filter paper was washed severally with deionized water to neutral pH and dried in BOD at 50 ºC to constant weight (MIRANDA et al., 2016).

The loss in weight of dry biomass was calculated as mentioned below and expressed as % weight loss in biomass.

$$\%\ \text{weight loss in biomass} = \frac{\text{biomass obtained after drying (mg)}}{\text{biomass used for pretreatment (mg)}} \times 100 \qquad (2)$$

3.3 Acid pretreatment

3.3.1 One-step acid pretreatment

The method described by Dussán et al. (2014) was used to perform one-step acid pretreatment of SB. 0.5 g of dry SB was suspended in sulfuric acid solution ranging from

0.05% to 0.3% (v/v) in pressure glass tube with solid/liquid ratio of 1:20 at 100 ºC (water bath) or 121 ºC (autoclave) for 30 min and 60 min respectively. Similarly, 0.5 g of dry SB was suspended in solution of maleic acid ranging from 0.1% to 0.5% (w/v) in a pressure glass tube with solid/liquid ratio of 1:20 at 121 ºC (autoclave) for 30 min and 60 min respectively. Afterwards, sample was removed and allowed to cool on ice bath before filtration under vacuum using Whatman No. 1 filter paper.

Filtrate collected was assayed for soluble reducing sugar (SRS) by 3,5-dinitrosalicylic acid (DNS) method (MILLER, 1959). The lowest concentration of acid solution that yielded the highest amount of SRS was selected for the two-step sequential alkaline and acid pretreatment. The % SRS was calculated as mention below.

$$\%\ released\ SRS\ = \frac{\text{reducing sugar released in filtrate during pretreatment (mg)}}{\text{biomass used for pretreatment (mg)}} \times 100 \qquad (3)$$

3.3.2 Two-step sequential alkaline and acid pretreatment

1.0 g of dry residues obtained after pretreatment of SB with different concentrations of sodium hydroxide (NaOH) solution (0.0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%) was suspended in 0.1% sulfuric acid solution (v/v) or 0.3% maleic acid solution (w/v) in 100 ml Erlenmeyer flask at solid/liquid loading ratio of 1:20. Flasks were placed in autoclave and heated for 60 minutes at 121ºC. Thereafter, flasks were removed and allowed to cool before filtration using Whatman No. 1 filter paper. Filtrate collected was assayed for glucose, while the residues on the filter paper was washed several times with deionized water to neutral pH and dried in BOD at 60 ºC to a constant weight (CHANDEL et al., 2014).

3.4 Chemical characterization of raw and pretreated sugarcane bagasse

The chemical composition of raw and pretreated SB were determined by the method of Sluiter et al. (2008b) and standard procedures of American Society for Testing and Materials (ASTM). 0.3 g of dry raw SB or dry residues resulting from pretreatment was weighed into a pressure glass tube and 3.0 mL of 72% sulfuric acid was added, the mixture was stirred with glass rod and tube was place in water bath at 30ºC for 1 h. The mixture was stirred while in water bath every 10 min. After 1 h of hydrolysis, tube was removed from water bath and 84 mL of deionized water was added to bring the acid concentration to 4%. The tube was covered and inverted several times to allow sample to mix and autoclaved at 121°C for 1 h. In addition, a set of sugar recovery standards (D-(+)-glucose and D-(+)-xylose was prepared

by ensuring that the sugar concentrations chosen most closely resemble the concentrations of sugars in the test sample. The amounts of each sugar required were weighed to the nearest 0.1 mg, and 10.0 mL deionized water was added followed by the addition of 348 µL of 72% sulfuric acid. The sugar recovery standard was transferred to a pressure tube and capped tightly before it was autoclaved together with the test sample.

After autoclaving, all the pressure tubes were allowed to cooled down to room temperature and separated into solid and liquid fractions by vacuum filtration using glass filter crucibles. Solid fraction was then dried at 105°C in order to measure acid insoluble residue (AIR). Acid insoluble residue was burnt in muffle furnace at 600°C for about 6 h to measure acid insoluble lignin (AIL) content as the difference between AIR and ash as shown in Eq. 6. Before neutralization, the absorbance of liquid fraction at 240 nm was measured by UV-visible spectrophotometer (Femto, São Paulo, Brazil, model Cirrus 80 ST,) to determine acid soluble lignin (ASL). Liquid fraction was neutralized with calcium carbonate and the resulting filtrate was used to assay for glucose and xylose.

Glucose was assayed using a commercial enzymatic kit (GOD-PAP, Laborlab, São Paulo, Brazil) and xylose by the phloroglucinol method using xylose as standard (EBERT et al., 1979). Total furans were estimated by a spectrophotometric method based on the difference in absorbance at 284 and 320 nm (MARTINEZ et al., 2000) using a UV/Vis/NIR-spectrometer with a 3D WB Detector Module (Perkin Elmer, Inc., Shelton, CT USA).
Oven dry weight was calculated as mentioned below.

$$ODW = \frac{\text{Weight}_{\text{air dry sample}} \times \%\ \text{total solids}}{100} \quad (4)$$

The percent acid insoluble residue (AIR) and acid insoluble lignin (AIL) on an extractives free basis were calculated as mentioned below.

$$\%\ AIR = \frac{\text{Weight}_{\text{crucible+AIR}} - \text{Wei}_{\quad\text{crucible}}}{\text{ODW}_{\text{sample}}} \times 100 \quad (5)$$

$$\%\ AIL = \frac{(\text{Weigh}_{\ \text{crucible+AIR}} - \text{Weig}_{\quad\text{crucible}}) - (\text{Weight}_{\text{crucible+ash}} - \text{Weig}_{\quad\text{crucible}})}{\text{ODW}_{\text{sample}}} \times 100 \quad (6)$$

The percentage acid soluble lignin (ASL) on extractives free basis was determined as mentioned below.

$$\% \, ASL = \frac{\text{UVabs} \times \text{Volume}_{\text{filtrate}} \times \text{Dilution}}{\varepsilon \times \text{ODW}_{\text{sample}} \times \text{Pathlength}} \times 100 \quad (7)$$

Where: UVabs = average UV-Vis absorbance for the sample at appropriate wavelength (240 nm for SB).

$\text{Volume}_{\text{hydrolysis liquor}}$ = volume of filtrate, 86.73 mL

$$\text{Dilution} = \frac{\text{Volume}_{\text{sample}} - \text{Volu}_{\text{diluting solvent}}}{\text{Volume}_{\text{sample}}}$$

ε = Absorptivity of biomass at specific wavelength (25 L/g cm for SB),

$\text{ODW}_{\text{sample}}$ = weight of sample in milligrams,

Pathlength = pathlength of UV-Vis cell in cm,

Total amount of lignin (%) on an extractives free basis was calculation as mentioned below.

$$\% \, \text{Lignin}_{\text{ext free}} = \% \, \text{ASL} + \% \, \text{AIL} \quad (8)$$

For the sugar recovery standards, the amount of each component sugar recovered after dilute acid hydrolysis was calculated by accounting for any dilution made prior to HPLC analysis. The average values of the replicate ($\%R_{sugar}$) obtained for each individual sugar was calculated as mentioned below and reported as $R_{avg,\ sugar}$.

$$\% \, R_{\text{sugar}} = \frac{\text{conc.detected by HPLC,mg/mL}}{\text{known conc.of sugar before hydrolysis,mg/mL}} \times 100 \quad (9)$$

The percent of hydrolyzed sugar recovery values obtained was used to correct the corresponding sugar concentration values obtained by HPLC for each of the hydrolyzed samples ($C_{cor.\ sample}$), accounting for any dilution made prior to HPLC analysis as shown below.

$$C_x = \frac{C_{HPLC} \times \text{dilution factor}}{R_{avg.\ sugar}/100} \quad (10)$$

Where: C_{HPLC} = conc. of a sugar as determined by HPLC, mg/mL,

$\% \, R_{avg.\ sugar}$ = average recovery of a specific sugar recovery standard component,

$C_x = C_{cor.\ sample}$, concentration in mg/mL of a sugar in the hydrolyzed sample after correction for loss on 4% hydrolysis.

The concentration of the polymeric sugars was calculated from the concentration of the corresponding monomeric sugars, using an anhydro correction of 0.88 (or 132/150) for C-5 sugars (xylose and arabinose) and a correction of 0.90 (or162/180) for C-6 sugars (glucose, galactose, and mannose) as mentioned below.

$$C_{anhydro} = C_{corr} \times \text{Anhydro correction} \quad (11)$$

The percentage of each sugar on extractives free basis was calculated as mentioned below.

$$\%\ \text{Sugar}_{\text{ext free}} = \frac{C_{anhydro} \times V_{filtrate} \times \frac{1g}{1000mg}}{ODW_{sample}} \times 100 \quad (12)$$

Where: $V_{filtrate}$ = volume of filtrate, 86.73 mL

3.4.1. Holocellulose extraction

A standard procedure (ASTM D1104-56) was employed to extract holocelulose (hemicellulose and cellulose) from SB as follows: 3.0 g of extractive free SB was weighed into 250 mL Erlenmeyer flask and 48 mL of hot deionized water in a water bath at 70 ºC. Subsequently, 0.6 mL of Acetic acid was added followed by the addition of 1.2 g of Sodium chlorite and flask was shaken carefully. The addition of Acetic acid (0.6 mL) and Sodium chlorite (1.2 g) was repeated every 60 min for 5 h. The sample was left in water bath at 70 ºC for overnight. Thereafter, sample was cooled down and the residue was filtered using a tared glass filter crucible until the yellow colour became white and then finally washed with Acetone. The residue in glass filter crucible was dried at 105 ºC for 24 h, afterwards, it was removed and cooled in desiccator and later weighed. The amount of holocellulose was calculated as mentioned.

$$\text{Holocellulose (g)} = \text{oven dry residue} + \text{crucible (g)} - \text{crucible (g)} \quad (13)$$

3.4.2. Determination of α-Cellulose

The α-cellulose was determined by employing a standard procedure (ASTM D1103-55T). 2.0 g of oven dried holocellulose was weighed into 250 mL Erlenmeyer flask with a glass stopper and 10 ml of 17.5% NaOH at 20 ºC was added and flask was kept in water bath at 20 ºC. The holocelulose was gently mixed with NaOH solution using glass stirring rod and after 2 min, another 10 mL of 17.5% NaOH solution was added and the mixture was gently stirred. After 5 min interval, 5 mL of 17.5% NaOH solution was added and the mixture was stirred.

Thereafter, the mixture was allowed to stand at 20 ºC for 30 min followed by addition of 33 mL of deionized water at 20 ºC to the mixture. Finally, the mixture was thoroughly

mixed and allowed to stand for 1 h. Subsequently, sample was transferred to glass filter crucible and washed with 100 mL of 8.3% NaOH (wash solution) at 20 ºC. After the NaOH wash solution has passed through the residue in the crucible, washing was continued with deionized water at 20 ºC. The residue in the crucible was dried at 105 ºC for 24 h, afterwards it was removed and cooled in desiccator and later weighed. The weight of dry residue corresponds to amount of α-cellulose in the sample and hemicellulose was determined by substracting the weight of α-cellulose from the weight of holocellulose.

3.5 X-Ray Diffraction

The crystallinity of the cellulose fiber was evaluated by X-Ray Diffraction (Siemens, Germany, model D5000). Copper Kα radiation, 30.0 kV of voltage and 15 mA of electric current and a rate of 2.0 degrees per minute for a 2θ continuous scan from 5.0 to 50.0 degrees were applied. This analysis allowed the detection of the amorphous part of the lignocellulosic biomass, as well as the modification of the crystalline structure of the cellulose. The crystallinity index (CI) was obtained from the ratio of the maximum peak intensity 002 (I_{002}, 2θ = 22.5) and minimal depression (I_{am} 2θ = 18.5) between peaks 001 and 002 (RODRIGUES et al., 2007; RULAND, 1961; SEGAL et al., 1959) as stated below.

$$\text{CI}\ (\%) = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \qquad (14)$$

Where: I_{002} is the maximum intensity of the 002 peak and I_{am} the minimal depression of the amorphous structure.

3.6 Enzymatic hydrolysis

3.6.1 Preparation of 0.05 M Sodium citrate buffer (pH 4.8)

210.0 g of Citric acid monohydrate was weighed into 1.0 L Erlenmeyer flask containing 750 mL deionized water and 50-60 g of NaOH was added. The solution was stirred until all the NaOH has dissolved and pH (pH 4.5) was checked, otherwise NaOH was added to bring the pH to 4.5. Thereafter, the solution was made up to 1.0 L with deionized water and pH was checked and adjusted if necessary to pH 4.8.

3.6.2 Measurement of Cellulase Activities

The cellulase activity of cellulase (Celluclast® 1.5L) was determined using the methods of National Renewable Energy Laboratory (ADNEY and BAKER, 1996) and International Union of Pure and Applied Chemistry (GHOSE, 1987). The detection of glycosidic bond cleavage by these methods involves the parallel and identical treatment of three categories of experimental tubes (assay mixtures, blanks and controls, and glucose standards) prepared as detailed below.

Stock was prepared by mixing 1.0 mL of the original enzyme (Celluclast® 1.5L from *Trichoderma reesei*) with 9.0 mL of 0.05M Sodium citrate buffer (pH 4.8) in a 15.0 mL test tube. **Table 1** showed the different enzyme dilutions and the corresponding glucose concentrations after hydrolyses of filter paper strips.

Enzyme assay tubes:

I. A rolled Whatman filter paper strip (50.0 mg, 1.0 x 6.0 cm) was placed into each 13 x 100 test tube.
II. 1.0 mL 0.05 M sodium citrate (pH 4.8) was added to the tube; the buffer was allowed to saturate the filter paper strip.
III. Tubes were equilibrated with buffer and substrate to 50 °C.
IV. 0.5 mL enzyme appropriately diluted in Citrate buffer was added.

Note: At least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (absolute amount) and one slightly less than 2.0 mg of glucose. Thus, 2.1 mg and 1.9 mg glucose respectively were targeted for these two dilutions. Depending on the enzyme, these targets may be hard to achieve and additional dilutions must be run.

V. Tubes were incubated at 50 °C for exactly 60 min.
VI. At the end of the incubation period, each assay tube was removed from the 50 °C bath and the enzyme reaction was stopped immediately by adding 3.0 mL DNS reagent and mixed.

Blank and controls:

Reagent blank: 1.5 mL Citrate buffer.

Enzyme control: 1.0 mL Citrate buffer + 0.5 mL enzyme dilution (a separate control was prepared for each dilution tested).

Substrate control: 1.5 mL Citrate buffer + filter-paper strip.

Glucose standards:

A working stock solution of anhydrous glucose (10 mg/mL) was prepared. Aliquots of this working stock were stored in tightly sealed 1.5 mL Microcentrifuge tubes and frozen. Before use, the standard was thawed and vortexed to ensure adequate mixing.

Dilutions were made from the working stock in the following manner:

1.0 mL + 0.5 mL buffer = 1:1.5 = 6.7 mg/mL (3.35 mg/0.5 mL).

1.0 mL + 1.0 mL buffer = 1:2 = 5 mg/mL (2.5 mg/0.5 mL).

1.0 mL + 2.0 mL buffer = 1:3 = 3.3 mg/mL (1.65 mg/0.5 mL).

1.0 mL + 4.0 mL buffer = 1:5 = 2 mg/mL (1.0 mg/0.5 mL).

Glucose standard tubes were prepared by adding 0.5 mL of each of the above glucose dilutions to 1.0 mL of Citrate buffer in a 13 x 100 mm test tube.

Blanks, controls and glucose standards were incubated at 50 ^{o}C along with the enzyme assay tubes, and then "stopped" at the end of 60 minutes by addition of 3.0 mL of DNS reagent.

Color development (MILLER, 1959):

All tubes were boiled for exactly 5.0 minutes in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent. All samples, controls, blanks, and glucose standards were boiled together. After boiling, tubes were transferred to a cold ice-water bath. Tubes were allowed to stand until all the pulp has settled, or briefly centrifuged. All tubes (assays, blanks, standards and controls) were diluted in deionized water (0.2 mL of color-developed reaction mixture plus 2.5 mL of water in a spectrophotometer cuvette works well, pipettor was used to mix by drawing the mixture into the pipettor tip repeatedly). Color formation was determined by measuring the absorbance against the reagent blank at 540 nm.

A linear glucose standard curve was constructed using the absolute amounts of glucose (mg/0.5 mL) plotted against the absorbance at 540 nm. The data for the standard curve was closely fitted to a calculated straight line, with the correlation coefficient for this straight line fit being very near to one. The standard curve was verified by running a calibration verification standard, an independently prepared solution containing a known amount of glucose which falls about midpoint on the standard curve. This standard curve was used to determine the amount of glucose released for each sample tube after enzyme blank was subtracted.

The concentration of enzyme that released exactly 2.0 mg of glucose was estimate by means of a plot of glucose liberated against the logarithm of enzyme concentration. The required enzyme concentration was found by taken two data points that were very close to 2.0

mg and a straight line was drawn between them. This line was used to interpolate between the two points to find the enzyme dilution that would produce exactly 2.0 mg glucose equivalents of reducing sugar.

Table 1 - Determination of cellulase activity (Celluclast® 1.5L from *Trichoderma reesei*).

Dil nr.	Buffer (mL)	Enzyme* (mL)	Dilution factor	Enzyme conc.	Sample♀ (540 nm)	Enzyme (540 nm)	Actual♯ (540 nm)	Abs Glucose	Glucose (mg/0.5mL)
1	0.25	1.00	1.25	0.080	0.524	0.036	0.455	0.842	5.0
2	0.50	1.00	1.50	0.067	0.486	0.024	0.429	0.642	3.35
3	1.00	1.00	2.00	0.050	0.467	0.006	0.428	0.498	2.5
4	1.50	1.00	2.50	0.040	0.406	0.000	0.373	0.338	1.67
5	3.00	1.00	4.00	0.025	0.374	0.000	0.341	0.184	1.0

Absorbance of substrate control = 0.033; *Enzyme, stock enzyme; ♀Sample absorbance after reaction with DNSA reagent; ♯Actual Abs, Sample Abs - (Abs of substrate control + Abs of diluted enzyme).

Note: In this plot, and in the equation below for calculating FPU, the term "enzyme concentration" refers to the proportion of the original enzyme solution present in each enzyme dilution (i.e., the number of mL of the original solution present in each mL of the dilution).
The amount of enzyme that releases 2.0 mg glucose contains 0.37 unit, and therefore FPU was calculated as stated below.

$$\text{FPU (units/ml)} = \frac{0.37}{\text{Enzyme concentration required to release 2.0 mg glucose}} \quad (15)$$

Where enzyme concentration is the proportion of original enzyme solution present in the directly tested enzyme dilution (0.5 mL is added to the assay mixture). The derivation of FPU is described by GHOSE (1987)**.** Enzyme dilution of 0.043 was determined after the two points which released close to 2.0 mg glucose according to the standard curve of reducing sugar extrapolated against the logarithm of enzyme dilution.

3.6.3 Cellobiase assay

The cellobiase activity of β-glucosidase was determined by the method of Ghose, (1987) as mentioned below.
Substrate consisted of 15.0 mM Cellobiose in 0.05 M Sodium citrate buffer (pH 4.8). Stock enzyme (1.0 mg/mL) was prepared by mixing 10.0 mg of β-glucosidase with 10.0 mL of Sodium citrate buffer (pH 4.8) in a 15.0 mL centrifuge tube. The stock was diluted 10x with

Citrate buffer before use. D-cellobiose (10.0 mg/mL) was used as substrate and it was prepared by dissolving 100.0 mg of D-cellobiose in 10.0 mL of Sodium citrate buffer (pH 4.8). The dilutions and reaction steps used for the determination of β-glucosidase activity are as shown in **Table 2**. The procedure employed was the following:

I. 1.0 mL of enzyme, diluted in Citrate buffer was added to a small test tube. At least two dilutions were made of each enzyme sample investigated. One dilution should release slightly more and one slightly less than 1.0 mg (absolute amount) of glucose in the reaction conditions.
II. Tubes were equilibrated to 50°C.
III. 1.0 mL substrate solution was added and mixed.
IV. Tubes were incubated at 50°C for exactly 30 min.
V. The reaction was terminated by immersing the tube in boiling water for exactly 5 min.
VI. Tubes were transfer to a cold water bath and glucose produced was determined using a standard procedure (e.g. a kit based on the glucose oxidase reaction).

Cellobiose blank was treated as follows.

I. 1.0 mL Cellobiose substrate solution
II. 1.0 mL Citrate buffer
III. 30 min, 50 °C
IV. Boil 5 min, cool.

10.0 µL of sample was used in the glucose oxidase-phenol and 4 aminophenazone (GOD-PAP) reaction and absorbance at 505 nm was measured. Actual absorbance was determined by subtracting sample absorbance before GOD-PAP reaction from the absorbance after GOD-PAP reaction. Note that a single cellobiose blank can be used for a whole series of activity determinations for which an enzyme blank is not necessary. Enzyme blank was treated as follows:

I. 1.0 mL Citrate buffer
II. 1.0 mL enzyme dilution
III. 30 min, 50°C
IV. Boil, 5 min, cool.

10 µL was used in the GOD-PAP reaction and absorbance was measured at 505 nm. Actual absorbance was determined by subtracting sample absorbance before GOD-PAP reaction from the absorbance after GOD-PAP reaction. Enzyme blanks are necessary only when glucose is present in the enzyme preparation and/or when small dilutions are used.

Table 2 - Determination of cellobiase activity of beta-glucosidase.

Dilution nr.	Citrate buffer (mL)	Enzyme* (mL)	Dil factor (x)	Enzyme (mg/mL)	Enzyme† (505 nm)	Sample♀ (505 nm)
1	1	1	2	0.05	0.000	0.410
2	3	1	4	0.025	0.000	0.265
3	7	1	8	0.0125	0.000	0.170
4	15	1	16	0.00625	0.000	0.098
5	31	1	32	0.00313	0.000	0.025
6	63	1	64	0.00156	0.000	0.012

*Enzyme, stock enzyme (0.1 mg/mL); †Enzyme, absorbance before reaction with GOD-PAP; ♀Sample, absorbance after reaction with GOD-PAP

Unit Calculation:

I. The glucose concentrations (mg/mL) in the cellobiase reaction mixtures obtained were determined using at least two different enzyme dilutions.
II. Glucose concentrations were converted into absolute amounts (mg) by multiplying by 2.
III. Enzyme dilutions were translated into concentrations as follows:

$$\text{Concentration} = \frac{1}{\text{dilution}}\left(\frac{\text{volume of enzyme sample in dilution}}{\text{total volume of dilution}}\right) \quad (16)$$

IV. The concentration of enzyme that released exactly 1 .0 mg of glucose was estimated by plotting glucose liberated against the logarithm of enzyme concentrations.
V. Cellobiase activity was calculated as shown below.

$$\text{CB} = \frac{0.0926}{\text{enzyme concentration to release 1.0 mg glucose}}\text{unit/mL} \quad (17)$$

Enzyme concentration of 0.0165 mg/mL was determined after the enzyme concentration that released 1.0 mg glucose was extrapolated using the two points obtained from glucose standard curve (GOD-PAP method).

3.6.4 Experimental set up for enzymatic hydrolysis

Enzymatic hydrolysis was carried out according to NREL standard procedure (SELIG et al., 2008). Dry pretreated SB (3% NaOH, 3.0% NaOH + 0.1% SA and 3% NaOH + 0.3% MA) and untreated SB were each soaked in 50 mM Sodium citrate buffer (pH 4.8) at 5% total

solids (TS) loading (w/v) in 125 mL Erlenmeyer flask. Flask was placed in incubator with a shaker (150 rpm) and incubated for 2 h at 50 ºC. Thereafter, the soaked sample was supplemented with 8.6 FPU/g cellulose of Celluclast® 1.5L (Sigma Aldrich) and 5.6 IU/g cellulose of β-glucosidase (Sigma Aldrich). A dose of 0.005% sodium azide was introduced to avoid any microbial contamination and 1.0% (v/v) Tween 80 was added to facilitate the enzymatic action due to its capacity in swelling fibers, increasing surface area, and improving the cellulase accessibility to cellulose (KIM et al., 2006). Enzymatic hydrolysis was performed at 50 ºC with 150 rpm for 72 h. Samples were withdrawn at 6 h, 12 h, 24 h, 48 h and 72 h intervals and enzymes were inactivated by boiling at 100 ºC for 10 min after which samples were cooled on ice before subsequently analyzed for glucose released using a commercial enzymatic kit (GOD-PAP, Laborlab).

The glucan conversion rate was calculated as mentioned below.

$$\text{Conversion}\ (\%) = \frac{M_2}{M_1} \times 100 \qquad (18)$$

Where M_2 is the amount of glucose (g) present in the aqueous phase of the hydrolyzate, after enzymatic hydrolysis; M_1 is the amount of cellulose fraction (g) present in the dry sample.

3.6.5 Optimization of enzymatic hydrolysis at low enzyme loadings

A Central Composite Design (CCD) was used in the optimization of enzymatic hydrolysis of SB (pretreated with 3% NaOH in autoclave for 1 h) for maximal yield of glucose. The independent input variables chosen are; hydrolysis time (X_1), enzyme loadings (X_2), and solid loadings (X_3), while their levels varied from -1.682 to +1.682 (**Table 3**). A total of 17 experiments that included 8 cube points, 6 star points, and 3 replicas of the central points were performed to fit a second-order polynomial model. The values of test variables at

Table 3 - Coded values of the independent variables for the Central Composite Design.

Symbol	Independent variables	Levels				
		-1.68	-1	0	1	+1.68
X_1	Time (h)	8	24	48	72	88
X_2	Enzyme (FPU/g substrate)	1.06	1.60	2.40	3.20	3.74
X_3	Solid loadings (%, w/v)	3.32	5	7.50	10	11.68

different hydrolysis time (8h to 88 h), solids loadings (3.32% to 1.68%), and enzyme loadings (1.06 to 3.74 FPU/g cellulose) coded from -1.682 to +1.682 and their interaction according to CCD. The enzyme mixture consists of Celluclast® 1.5L and β-glucosidase. The glucose yield (%) was used as a dependent output variable. The ranges of variables used in this work were selected based on literature (KRISTENSEN et al. 2009).

3.7 Acid hydrolysis

3.7.1 One-step sulfuric acid hydrolysis

In the present work, pretreated sample which originated from the two-step sequential alkaline and acid pretreatment (3% NaOH + 0.1% SA) was hydrolyzed with sulfuric acid solution (2.5%, 5.0%, 7.5% and 10.0%, v/v) at solid/liquid ratio of 1:8. Hydrolysis was carried out in autoclave at 121 ^{o}C for 30 min, 60 min and 120 min respectively, time count started after reaching 121 ^{o}C. After heating, sample was allowed to cool on ice and filtered using Whatman No. 1 filter paper. The filtrate was collected and neutralized with calcium hydroxide and used for glucose assay.

3.7.2 Two-step sulfuric acid hydrolysis of pretreated sugarcane bagasse

In the present work, two-step sulfuric acid hydrolysis was performed on dry SB (10% moisture content) according to the method described by HEINONEN et al. (2012) with modifications as follows:

Step 1: De-crystallization.

Dry pretreated SB (8.0% NaOH at room temperature for 12 h) was suspended in sulfuric acid solutions (30%, 35% and 40%, v/v) at solid/liquid ratio of 1:5 in round bottom reflux glass. Samples were incubated for 1 h in oil bath at 45 ^{o}C with stirring (magnetic stirrer) at 40 rpm.

Step 2: Hydrolysis step.

Samples from the first step hydrolysis were diluted to final acid concentration of 1.5%, 3.0% and 4.5% (v/v) respectively with deionized water. Thereafter, temperature was increased to 90 ^{o}C and sample was allowed to stand for 2 h with stirring (magnetic stirrer) under reflux as shown in **Figure 11**. After hydrolysis, samples were allowed to cool on ice before filtration using filter papers (Whatman No. 1), the filtrates were collected and neutralized with calcium hydroxide.

Figure 11 - Reflux system used for two-step sulfuric acid hydrolysis of pretreated SB.

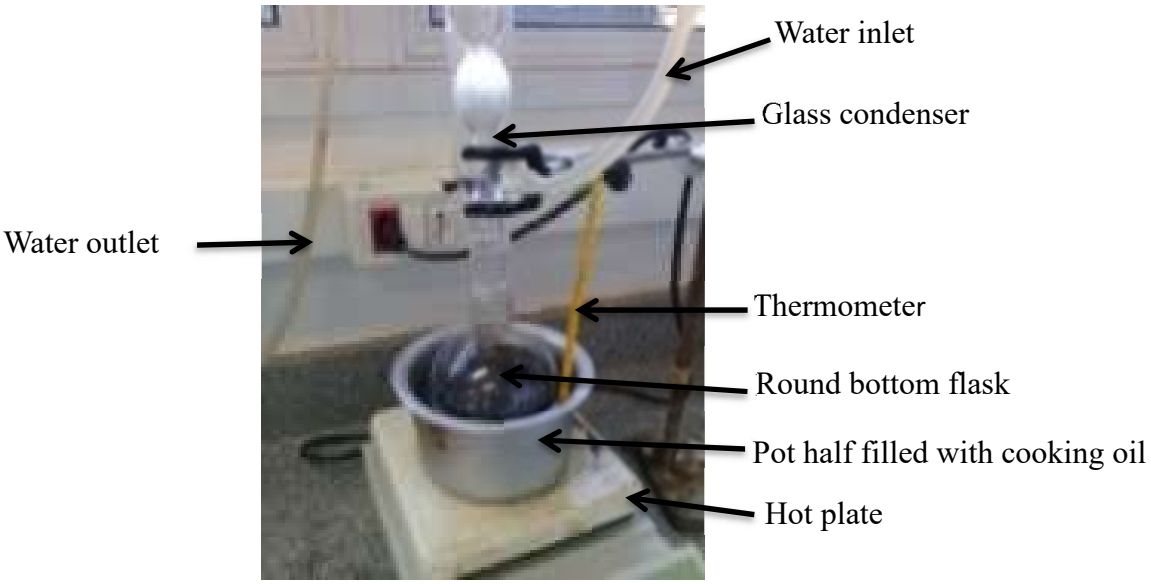
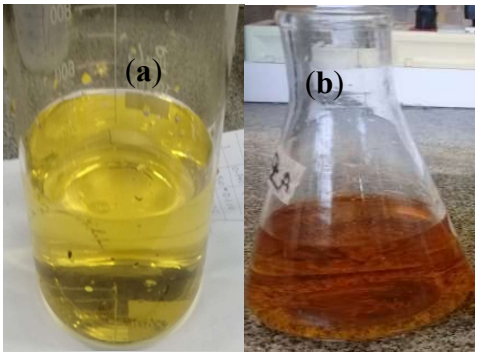


Figure 12 shows the acid hydrolysate obtained after filtration and neutralization, non-neutralized hydrolysate was light yellow in colour and after neutralization the colour changed to light brown. The samples were allowed to settle and the supernatants were used to assay for glucose. The insoluble residues on the filter paper were dried in BOD at 45 ºC for 16 h and later at 105 ºC for 3 h to determine yields.

Figure 12 - Acid hydrolysate of pretreated SB, (**a**) before neutralization, (**b**) after neutralization.



3.7.3 Optimization of acid hydrolysis using $FeSO_4$ as a co-catalyst

The influence of three independent variables, x_1 (sulfuric acid concentration), x_2 ($FeSO_4$ concentration) and x_3 (hydrolysis time), on the glucose yield was studied using a 2^3 full factorial design. Two experiments to each condition and three at the midpoint resulting to a total of 11 experimental runs were made to evaluate the effect of variables. The ranges of independent variable studied were sulfuric acid (1.5% to 4.5%), $FeSO_4$ (0.0 mmol/L to 40.0 mmol/L) and time (40 min to 120 min), while temperature was fixed at 90 ºC during the hydrolysis step. Experimental runs were randomized to reduce the effects of unaccountable lack of consistency in the actual responses due to outlying factors. Three experiments at midpoint of the design were carried out to provide an additional level for lack of fit testing (in case all coefficients were significant) and to provide degrees of freedom for pure error estimation, due to the replication of experiments at this point (REZENDE et al., 2018).

For statistical analysis, the variables were coded as stated below, where each independent variable is represented by x_i (coded value), X_i (real value), X_0 (real value at the midpoint), and ΔX_i (step change value). The ranges and levels of the variables investigated in this study are given in **Table 4**. Low and high factor levels were coded as -1 and +1; the midpoint was coded as 0.

$$x_i = \frac{X_i - X_0}{\Delta X_i} \tag{19}$$

The glucose yield (%) was taken as the dependent variable or response of the design experiments. The results were subjected to an analysis of variance (ANOVA), and the response and variables (in coded unit) were correlated by the response surface analysis to obtain the coefficients as mentioned below.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \tag{20}$$

Where y is the predicted response; β_0 is the constant term; β_1, β_2 and β_3 are the regression coefficients for the linear effect terms; β_{12}, β_{13} and β_{23} are the interaction effects; x_1, x_2 and x_3 are the coded levels of the independent variables (sulfuric acid, $FeSO_4$ and time).

The significance of each term for the response was also evaluated by observing the F-ratio, where the probability (p) is < 0.05. The adequacy of the model was determined using model analysis, a lack-of-fit test and coefficient of determination (R^2) analysis. For experimental design matrix, data analysis, fitting models, regression and surface plots, Statistica 10.0. (StatSoft Inc, Tulsa, OK, USA) software was used.

Table 4 - Experimental range and levels of variables.

		Range and level		
Independent variables	Symbol	-1	0	+1
Sulfuric acid (%, w/w)	x_1	1.5	3.0	4.5
$FeSO_4$ (mmol/L)	x_2	0	20	40
Time (min)	x_3	40	80	120

3.8 Fermentation

3.8.1 Yeast propagation

The *Saccharomyces cerevisiae* IQAr/45-1 is a thermotolerant ethanologenic yeast strain obtained from the hybridization between parental strains of *S. cerevisiae* and three Brazilian industrial strains (PE-2, CAT-1, SA-1) during fermentation of non-sterilized molasses (LALUCE et al., 2013). Revival of the yeast strain (IQAr/45-1) was carried out using a procedure established in our laboratory. Living cells of this yeast were isolated by plating the stock culture, maintained at pH 6.0 ± 0.2 and 30 ºC on solid medium containing (g/L): glucose, 30.0; Yeast extract, 3.0; Peptone, 5.0; Agar, 20.0 at pH 6.0 ± 0.2 and temperature 30 ºC.

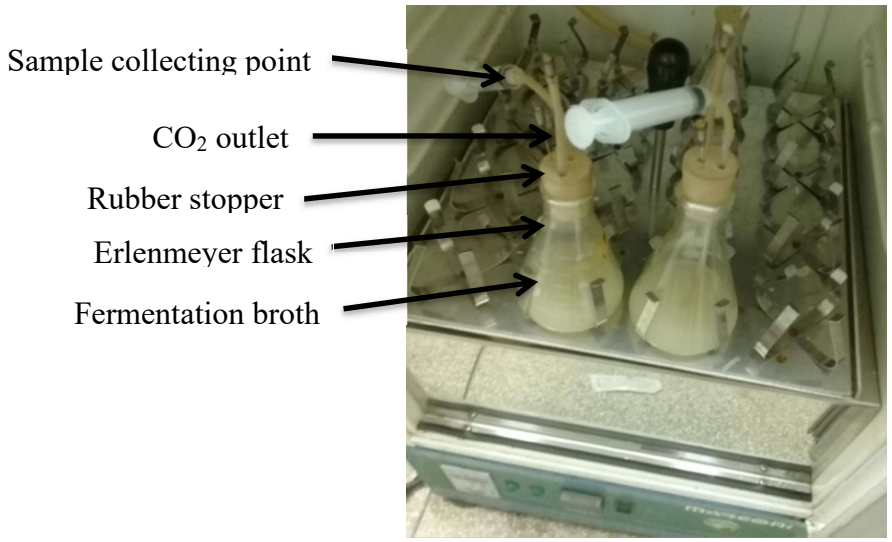
Starter culture was developed by growing the cells at 30 ºC for 16 h in a culture medium containing (g/L): glucose, 30.0; yeasts extract, 3.0; peptone, 5.0; pH 6.0 ± 0.2. After 16 h of propagation at 30 ºC in an orbital shaker-incubator operating at 150 rpm, cells are harvested by centrifugation at 5,000 x g for 2 min at 4°C. The harvested cells were re-suspended in sterilized water and the washed cell pellet was separated by centrifugation. In the next step, the washed cells were again re-suspended in sterilized water and diluted to obtain optical density (OD_{600}) of 0.6.

3.8.2 Batch fermentation set up using procedures established in our laboratory for stress tolerant yeasts

Acid hydrolysate (neutralized with calcium hydroxide) and enzymatic hydrolysate of pretreated SB was vacuum concentrated at 70°C in a concentrator. Fermentation of hydrolysate was performed in 125 mL Erlenmeyer flasks with 50 mL as working volume supplemented by the addition of yeast extract (3.0 g/L) and diammonium phosphate (0.25

g/L). It was inoculated with *S. cerevisiae* (10.0% v/v) at optical density (OD_{600}) of 0.6 followed by incubation for 24 h (acid hydrolysate) or 30 h (enzymatic hydrolysate) in an orbital shaking-incubator at 30°C, 150 rpm and the set-up is depicted in **Figure 13**. Samples were taken at 4 h interval, centrifuged at 10,000 x g for 15 min at 4 ºC and the cell free supernatant was used to determine the ethanol and residual sugar concentration.

Figure 13 - A batch fermentation set-up in incubator (Marconi, São Paulo, Brazil).



Calculations of fermentation parameters:

Ethanol yield and Productivity, Biomass yield and Productivity, Fermentation efficiency (SCHMIDELL et al., 2000) were calculated stated below.

$$Y_{P/S} = \frac{E_f - E_i}{(S_i - S_f)} \quad (21)$$

$Y_{P/S}$ is the ethanol yield, E_i and E_f are the ethanol concentration at the beginning of the fermentation and the end of the fermentation (g/L) respectively; while S_i and S_f are the total sugar concentration at the beginning of the fermentation and the end of the fermentation (g/L) respectively.

$$Q_{P_P} = \frac{E_f - E_i}{t_f - t_i} \quad (22)$$

Q_P is the volumetric ethanol productivity (g/L/h), E_i and E_f are the ethanol concentration at the beginning and end of fermentation (g/L) respectively; while t_0 and t_r are the fermentation time at the beginning of the fermentation and the end of the fermentation (g/L) respectively.

$$Y_{X/S} = \frac{x_f - x_i}{s_i - s_f} \quad (23)$$

$Y_{X/S}$ is the biomass yield, X_i and X_f are the biomass concentration at the beginning of the fermentation and the end of the fermentation (g/L), respectively; while S_i and S_f are the total sugar concentration at the beginning of the fermentation and the end of the fermentation (g/L), respectively.

$$Q_{P_X} = \frac{x_{f-x_i}}{t_{f-t_I}} \quad (24)$$

Q_P is the biomass productivity (g/L/h), X_i and X_f are the biomass concentration at the beginning and end of fermentation (g/L), respectively; while t_0 and t_r are the fermentation time at the beginning of the fermentation and the end of the fermentation (g/L), respectively.

$$\text{Fermentation Efficiency (\%)} = \frac{\text{Actual Yield}}{\text{Theoretical Yield}} \times 100 \quad (25)$$

3.8.3 Cell optical density and dry weight measurements

The cells concentration was determined by turbidimetry using spectrophotometer (Cirrus 80 ST, Femto) at wavelength of 600 nm and correlated with the dry weight of cells (g/L) through a calibration curve. The dry weight measurement was obtained by pipetting 5 mL of well mixed broth sample on to a pre-dried (24 hours in a drying oven at 90 ºC) Whatman GF/C, 1.2 mm "pore size", 47 mm diameter, membrane filter (Whatman, Maidstone, England), positioned in a Sartorious vacuum filtration apparatus (Sartorious, Belmont, England). The liquid component of the sample was significantly removed by applying a vacuum for 1-2 minutes, while the broth solids remained on the filter. The filter and the residual solids were washed in 10 mL of deionized water, and the vacuum was reapplied to remove excess liquid. The filter and solids were replaced in the drying oven, and dried to constant weight (90 ºC for 24 hours). After drying, the filters were allowed to cool to room temperature in a desiccator and then reweighed.

The dry weight of the residual solids was calculated as the difference between the weight of the filter before and after use. To convert the dry weight of residual solids into a concentration, the volume of the sample used to produce each dry weight measurement was required. Calibration curve was obtained by plotting OD_{600} (absorbance at 600 nm) measurements versus cell concentration (g/L).

3.9 Analytical methods

3.9.1 Glucose determination by GOD-PAP (glucose oxidase-phenol and 4 aminophenazone) method

The principle behind GOD-PAP method is described below.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{4-(p-bezoquinone-mono-imino)phenazone} + 4H_2O$$

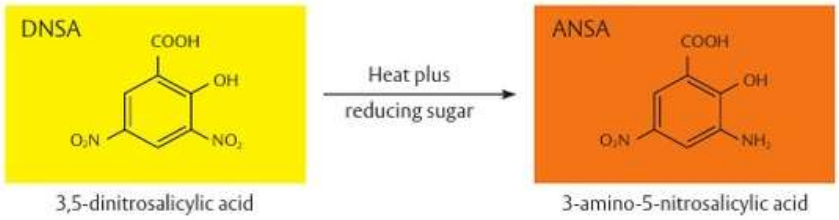
Glucose is oxidized by glucose oxidase to gluconate and hydrogen peroxide. In the presence of peroxidase, phenol reacts with 4-AAP and hydrogen peroxide to produce a Quinonimin dye, the intensity of the colour produced and measured at 505 nm, is proportional to the concentration of glucose in the sample. The reaction was performed according to the manufacturer's instruction. 1000 µL of reagent was pipetted into three 2 mL microcentrifuge tubes and 10 mL of test sample, distilled water and standard respectively was added into each tube. The mixture was vortexed for 10 seconds and later incubated at 37 ^{o}C for 5 min on water bath. Thereafter, tubes were quickly placed on ice and the blank (mixture of water and reagent) was transferred to cuvette and absorbance was read at 505 nm on a UV-visible spectrophotometer (Femto, São Paulo, Brazil, model-Cirrus 80 ST,) followed by the sample and standard. Sample concentration was determined through a glucose standard curve.

3.9.2 Reducing sugar determination by Dinitrosalicyclic acid method

The dinitrosalicyclic acid (DNSA) method is characterized by colour change of the reagent from yellow to orange (**Figure 14**), depending on the concentration of reducing sugar present. On heating with reducing sugars, the 3-nitro (NO_2) group of DNSA is reduced to an

amino (NH_2) group. DNSA test can detect glucose concentrations in the range of 0.5 mM (0.09% glucose w/v) to 40 mM (0.72% glucose w/v).

Figure 14 - A schematic of DNSA reaction



Source: www.ncbe.reading.ac.uk/MATERIALS/Enzymes/dnsareagent

DNSA reagent was prepared as mentioned below.

100 mL of DNSA reagent was prepared by adding 1.0 g of 3,5 Dinitrosalicyclic acid and 1.0 g sodium hydroxide into 250 mL beaker. Distilled water was added and the mixture was allowed to dissolve by stirring (magnetic stirrer). Thereafter, sodium potassium tartrate tetratehydrate (30.0 g), phenol crystals (0.2 g), and sodium metabisulfite (0.83 g) were added under stirring. The solution was made up to 100 mL with distilled water and thoroughly mixed before storing in a brown bottle.

Test was carried out by adding 0.3 mL of DNSA reagent to 0.3 mL of sample solution in 1.5 mL microcentrifuge tube and thoroughly mixed. The control contained 0.3 mL of DNSA reagent and 0.3 mL of distilled water. Tube was transferred to water bath at 100 ºC and allowed to stand for 5 min. Afterwards the tube was cooled on ice and sample solution was 10 times diluted with distilled water if required. Sample solution was placed in a glass cuvette and absorbance was read at 540 nm. Standard curve for reducing sugar was obtained using D-glucose of concentration in the range of 0.2 mg/mL to 2.0 mg/mL.

3.9.3 Xylose determination by phloroglucinol method

Colour reagent was prepared by weighing out 0.25 g of phloroglucinol into glass bottle and 50.0 mL glacial acetic acid was added followed by 5.0 mL concentrated hydrochloric acid. The mixture was thoroughly mixed by a magnetic stirrer until phloroglucinol was completely dissolved.

A stock solution of D-xylose was prepared by suspending 0.1 g of D-xylose in 50 mL of benzoic acid in 100 mL Erlenmeyer flask to obtain a concentration of 2.0 mg/mL. The standard curve was made using D-xylose concentration in the range of 0.2 mg/mL to 1.4

mg/mL. 50 µL of D-xylose sample was treated with 5.0 mL of colour reagent in 10 mL test tube and vortexed for 10 seconds. Sample was incubated at 100 ºC for 4 min, thereafter sample was quickly placed on ice to stop the reaction. Absorbance was measured at 540 nm after sample was appropriately diluted. A standard curve was obtained by plotting the absorbance (nm) against D-xylose concentration (mg/mL). For the test sample, the same procedure as described above was followed. The control contained 50 µL of distilled water and 5.0 mL of colour reagent.

3.9.4 Determination of glucose, furfural and 5-HMF by High-Performance Liquid Chromatography (HPLC)

Glucose, furfural and 5-HMF were determined by HPLC using Shimadzu chromatography equipment equipped with a pump (LC-10AD), a system controller (SCL-10A), a refractive index detector (RID-6A), a UV-vis detector (SPD-10A) set at 274 nm, and an oven (CTO-10A). Glucose was separated and quantified using a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) at 45 ºC with 0.005 mol/L H_2SO_4 as the eluent and a flow rate of 0.6 mL/min, while furfural and 5-HMF were separated and quantified using an RP 18 (C18) Hewlett-Packard column (200 mm) at 25 ºC with acetonitrile:water (1:8) with 1% acetic acid as the eluent and a flow rate of 0.8 mL/min. For these analyses, all samples were previously filtered in membrane Minisart 0.22 µm (Sartorius, Goettingen, Germany). The concentrations of glucose, furfural and 5-HMF were calculated using the standard calibration curves.

3.9.5 Ethanol determination by potassium dichromate method

Ethanol in the fermented broth was determined spectrophotometrically using the method described by Caputi et al. (1968). 1 liter Potassium dichromate solution was prepared by weighing out 34 g of reagent-grade Potassium dichromate into 1 liter volumetric flask containing about 500 mL deionized water. 325 mL of reagent-grade concentrated sulfuric acid was added and the solution was stirred using magnetic stirrer. The solution was finally made up to 1 liter with deionized water.

150 µL of sample (distillate) was mixed with 1.5 mL of Potassium dichromate solution in a 2 mL centrifuge tube and vortexed for 10 seconds. The tube was placed in water bath at 60 ºC for 20 min afterwards the mixture absorbance at 600 nm was read using

spectrophotometer. Ethanol concentration was calculated using ethanol standard calibration curve**.**

3.9.6. Ethanol determination by Gas chromatography - Mass spectrometry (GC-MS) using Head-space method

Ethanol in the broth samples was quantified by using GC-2010 Plus gas chromatograph coupled to a QP2010 mass spectrometer and equipped with an autosampler model AOC-6000 system (Shimadzu, Tokyo, Japan). The capillary column used was a Zebron ZB-WAX (15 m× 0.25 mm × 0.25 μm) (Phenomenex Torrance, CA, USA), maintained at 35 °C. Helium (purity 99.999%) was used as carrier gas at a flow rate of 1.2 $mLmin^{-1}$ and for injection of the samples a split injection port was used, operating at 220 °C and with a split ratio of 1:100. The MS, equipped with an electron impact (EI) source, was operated in selected ion monitoring (SIM) mode at 70 eV electron energy and the mass fragments used in the analysis were: m/z 46.0, 45.0 and 31.The temperatures of the ion source and transfer line were 230 °C and 220 °C, respectively. The solvent cut time was 1 min and the time of analysis was 3 min. The autosampler operating in a headspace mode used an incubation temperature of 65 °C and incubation time of 5 min. The sample volume used by sampler was 2500 μL and the headspace volume injected was 500 μL.

The quantification of ethanol was made by analytical curve method using standard solutions in the range of 1.03 to 50.50 $mmolL^{-1}$ (47.4 to 2369.0 ppm), made in triplicate. The last solution in the analytical curve range was used as stock solution to produce the less concentrated solutions, by dilution with deionized water. The stock solution was made by adding ethanol (Honeywell/Riedel-de-Haën, 99.9%) to deionized water. For samples solutions, 200 μL of solutions from fermentation broth was added to deionized water totaling a final volume of 2500 μL.

Statistical analyses were carried out using STATISTICA 10 (TIBCO Software Inc., CA, United States). All the graphs were created using the software OriginPro 8 (OriginLab Inc., MA, United States). Each data was expressed as a mean of standard deviation (SD) of triplicate measurements.

4. RESULTS AND DISCUSSION

4.1 Pretreatment

4.1.1 Mechanical pretreatment

The particle size of SB was reduced via mechanical grinding (milling) prior to subsequent pretreatment steps. Milling effectively reduced the particle size and cellulose crystallinity as a result of the shear forces generated during milling (KUMARI and SINGH, 2018; KUMAR and SHARMA, 2017). Besides, particle size reduction can significantly improve the efficiency of pretreatments due to high efficient mass and heat transfer resulting to high sugar recovery as well as low production cost (LIU et al., 2013). SB of particle size ≤ 0.5 mm was selected in the present study, because other studies have shown that further reduction of biomass particle below 0.4 mm had no significant effect on hydrolysis rate (CHANG et al. 1997). Lin et al. (2010) reported an optimum particle size of 0.5 mm for corn stover. Thus, the method applied for SB processing in the present study is consistent with those reported in the literature.

4.1.2 Alkaline pretreatment

As shown in **Figure 15**, retention time and NaOH concentration have significant effect on xylose extraction from SB in autoclave (121°C). Increase in the concentration of NaOH solutions from 0.5% to 3.0% yielded a corresponding increase in xylose extraction from 3.0% to 8.1% after 30 min, while xylose extraction increased from 6.5% to 12.5% after 60 min of reaction time. On the other hand, in the absence of NaOH, no significant increase in xylose extraction was obtained despite the increases in reaction time from 30 min to 60 min. This suggests that the presence of NaOH in the medium could be the main factor affecting xylose extraction from SB in autoclave at 121ºC and NaOH activity correlated with reaction time.

The action of alkaline solution leads to the degradation of side chains of esters and glycosides as well as the structural modification of lignin, cellulose swelling, cellulose decrystallization and hemicellulose solvation (CHENG et al., 2010; ŁUKAJTIS et al., 2018). This process generates two fractions, a liquid fraction -mainly consists of lignin and part of hemicellulose oligomers and a solid cellulosic fraction. However, the low amount of xylose extracted from SB could be attributed to very low solubility of hemicellulose during the alkali pretreatment (KUMAR and SHARMA, 2017). Maryana et al. (2014) reported a minor decrease in hemicellulose content from recovered dry SB biomass after NaOH pretreatment.

Similarly, pretreatment with 0.5% NaOH in autoclave at 120 ºC for 60 min resulted to ~18.8% of xylan removal from SB (ZHANG et al., 2018).

Figure 15 - Effect of reaction time on xylose extraction from SB in autoclave at 121ºC.

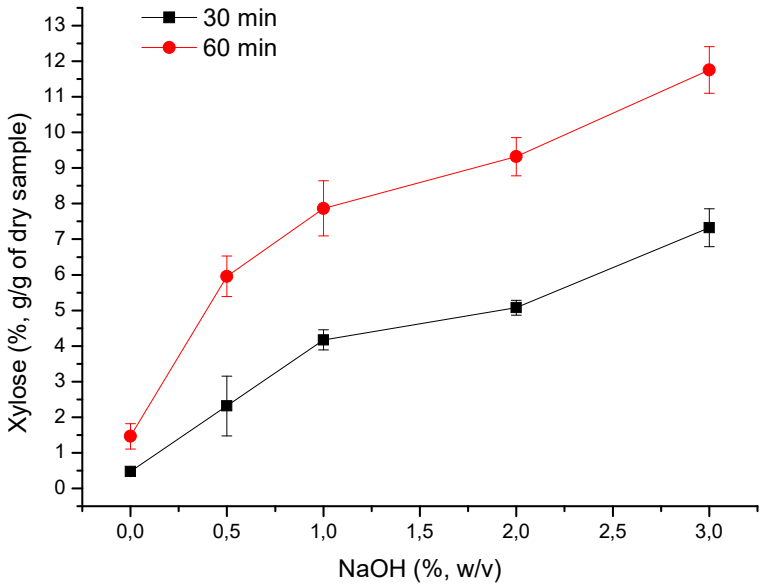
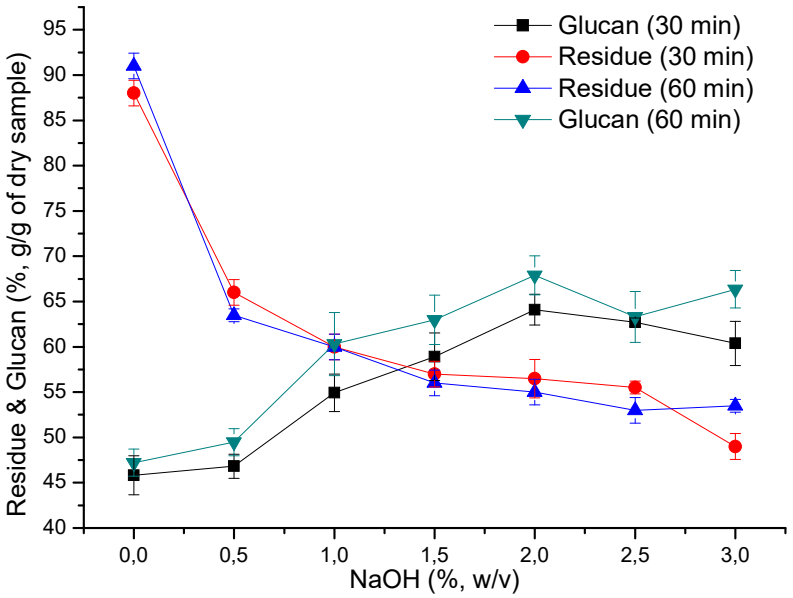


Figure 16 shows the effect of increases in NaOH concentration and reaction time on the yield of glucan and dry residues from SB at 121ºC in autoclave. Decreases in the yield of dry residue ranged from 100% to 50% and 100% to 55% after 30 min and 60 min respectively at increasing NaOH concentration (0.5% to 3.0%). The greatest decrease in the yield of dry residue (30%) occurred after 60 min with 0.5% NaOH solution. The yield of dry residue further decreased with increasing concentration of NaOH, before reaching the lowest value of 50%. Slight decreases in the yield of dry residue corresponding to 88% and 92% after 30 min and 60 min of pretreatment respectively were obtained in the absence of NaOH.

On the other hand, glucan content of SB increased from 47% to 65% after 30 min of pretreatment with increasing concentration of NaOH solution (0.5% to 3.0%). The highest amount of glucan content was obtained with 2.0% NaOH solution, while the lowest amount emanated from pretreatment with 0.5% NaOH. Similarly after 60 min of pretreatment with different concentrations of NaOH (0.5% to 3.0%), the glucan content of SB increased from 49% to 70%. The highest amount of glucan content (70%) was obtained with 2% NaOH, while the lowest amount of 49% was obtained with 0.5% NaOH. This shows that increases in

reaction time from 30 min to 60 min resulted to a slight increase in glucan content of SB. Besides, the effects of increases in NaOH concentrations had more significant effect on glucan content compared to increases in reaction time.

Figure 16 - Effects of reaction time and NaOH concentration on the yield of glucan and dry residue from SB pretreated at 121°C in autoclave.



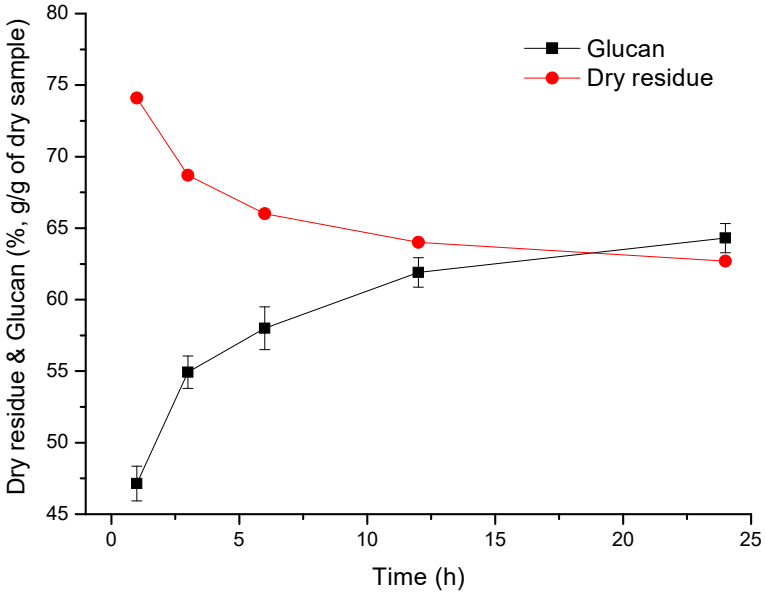
However, glucan contents and yield of dry residues were inversely correlated at increasing NaOH concentration and retention time. This indicates that simultaneous increases in NaOH concentration and reaction time contributed to the enhanced removal of lignin and part of hemicellulose from SB, with concomitant increase in the glucan content. According to the literature, pretreatment of lignocellulosic material using a strong base such as NaOH can effectively solubilize lignin and part of hemicellulose under certain conditions (KIM et al., 2016; KONDO et al., 2014; REZENDE et al. 2011). Haque et al. (2013) reported the maximum of 84.8% and 79.5% removal of lignin and hemicelluloses respectively from barley straw pretreated with 2% NaOH at boiling temperature.

Figure 17 shows the effects of reaction time on the yields of glucan and dry residues from SB pretreated with 8% NaOH at room temperature. The yield of dry residue ranged from 74% to 63% with increases in the reaction time. The highest amount (74%) of dry residues was obtained after 1 h of reaction time, while the lowest amount of 63% was obtained after

24h. Besides, the yield of dry residues was inversely related to reaction time. This may likely be connected with the higher rate of components removal from SB at increasing reaction time.

On the other hand, the yield of glucan increased from 47% to 64% as the reaction time was increased from 1 h to 24 h. The maximum glucan yield of 64% was obtained after 24 h, while the minimum yield of glucan was obtained after 1 h of reaction time. Also, the yield of glucan correlated with reaction time, indicating that reaction time significantly contributed to the increase in glucan content. By comparing glucan content of SB with the yield of dry residues, it was found that the yield of dry residues was inversely correlated with glucan content. This suggests that NaOH pretreatment resulted to the removal of components of SB except the cellulosic component which remained largely unaffected.

Figure 17 - Effects of reaction time on the yield of glucan and dry residues from SB pretreated with 8% NaOH at room temperature.

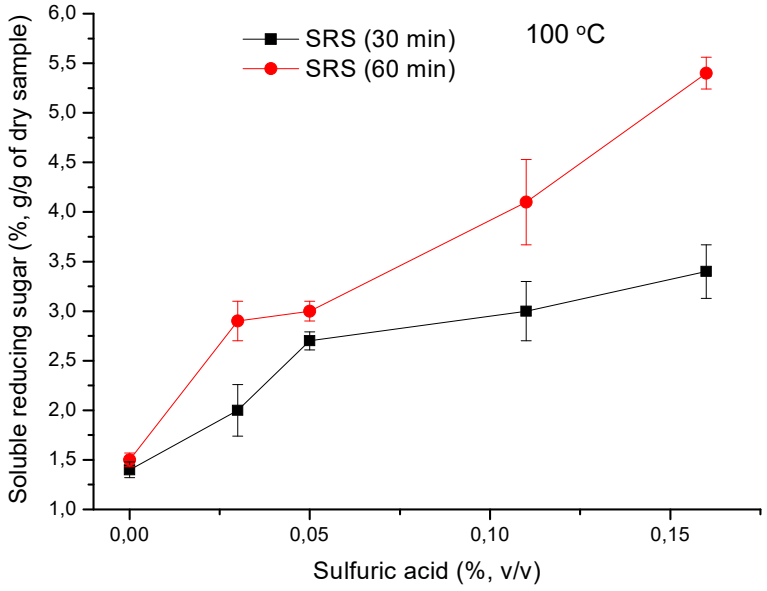


Alkaline pretreatment involves the solvation of lignocellulose particles and hydrolytic decomposition of lignocellulose via saponification (breaking) of intermolecular ester bonds between lignin, hemicellulose and cellulose (KUCHARSKA et al., 2018; GÁSPÁR et al., 2007). Thus, lignin and part of hemicelluloses can be separated from the cellulosic fraction through filtration with concomitant decreases in the yield of insoluble residues coupled with cellulose enrichment. Wang et al. (2015) reported 89.45% and 88.92% loss of hemicellulose and lignin respectively from rice straw pretreated with 4.0% NaOH at 60 ºC for 60 h.

4.1.3 Acid pretreatment

The effects of reaction time and concentrations of sulfuric acid on soluble reducing sugar (SRS) extraction from SB at 100 ºC are shown in **Figure 18**. The extraction of SRS from from SB varied from 2% to 3.4% after 30 min at different sulfuric acid concentrations. The highest amount of SRS (3.4%) was extracted with 0.16% sulfuric acid, while the least amount of SRS (2%) was obtained with 0.03% sulfuric acid. On the other hand, after 60 min of reaction time, the amount of SRS extracted increased from 2.9% to 5.4%. The maximum amount of SRS (5.4%) was obtained with the highest sulfuric acid concentration (0.16%).

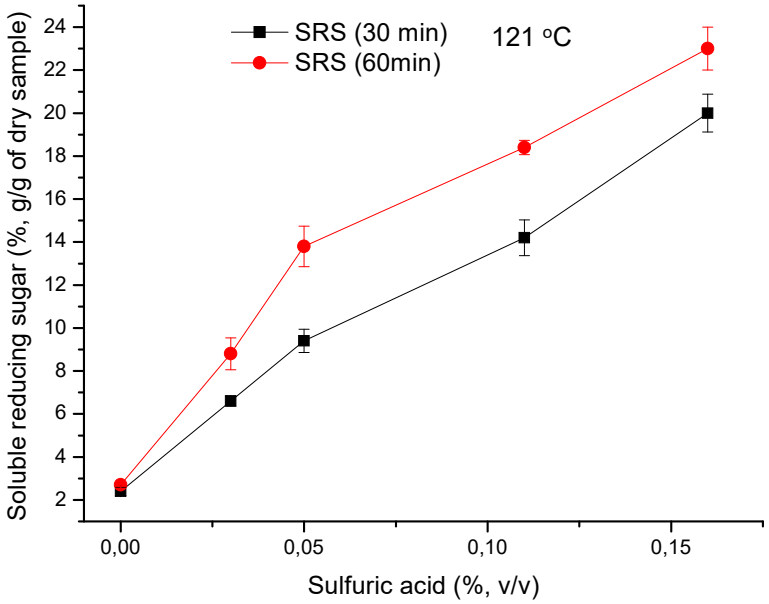
Figure 18 - Effect of reaction time (30 min and 60 min) and sulfuric acid concentration reducing on soluble sugar (SRS) extraction from SB at 100ºC (water bath).



However, the result of SRS extraction from SB at 100 ºC shows that at higher sulfuric acid concentration increase in reaction time led to significant improvement in SRS extraction. Nevertheless, the amount of SRS extracted in the presence of acid were relatively low, but were significantly higher than the amount of SRS extracted in the absence of acid. This indicates that sulfuric acid played an important role in the SRS extraction from SB. Timung et al. (2016) reported a maximum total reducing sugar yield of 15% and 16% from SB pretreated with 0.5% sulfuric acid at 100 ºC for 30 min and 60 min respectively.

The effects of sulfuric acid concentration and reaction time on SRS extraction from SB at 121ºC (autoclave) are shown in **Figure 19**. The amount of SRS extracted from SB during 30 min of reaction at increasing sulfuric acid concentration ranged from 6.6% to 20%. The highest amount of SRS (20%) was obtained with 0.16% sulfuric acid, while the least amount of 6.6% was obtained with the lowest acid concentration (0.03%). When the reaction time was raised to 60 min, the amount of SRS extracted increased from 8.8% to 23%. The highest amount of SRS (23%) was extracted with 0.16% of sulfuric acid.

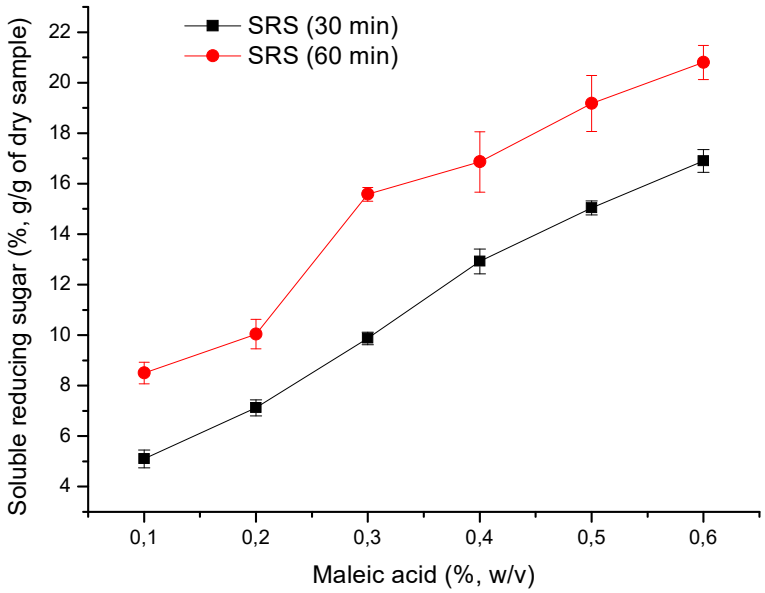
Figure 19 - Effect of reaction time (30 min and 60 min) and sulfuric acid concentration on soluble reducing sugar (SRS) extraction at 121ºC (autoclave).



However, higher amounts of SRS were obtained at 121ºC (autoclave) compared to 100 ºC (water bath). This suggests that the high pressure and temperature condition in the autoclave might have contributed to the enhanced SRS extraction from SB. Other authors reported SRS yield of 20% and 30% from SB after 30 min and 60 min of pretreatment respectively with 0.5% sulfuric acid at 120 ºC (TIMUNG et al., 2016). Dilute acid pretreatment can cause the dissolution of sugar in the hemicellulose and further improve the porosity of the plant cell wall for effective enzyme digestibility (JIANG et al., 2013). The activities of dilute acid solutions increase with increasing temperatures. Nonetheless, high temperatures may be accompanied by the generation of inhibitory compounds such as furan derivatives (CHANDEL et al., 2012).

Figure 20 shows the effects of maleic acid concentration and reaction time on the extraction of SRS from SB at 121ºC (autoclave). The amount of SRS extracted from SB varied from 5.1% to 16.9% after 30 min, while 60 min of reaction time resulted to SRS extraction in the range of 8.5% to 20.8%. The highest amount of SRS corresponding to 16.9% and 20.8% were obtained after 30 min and 60 min of reaction time respectively. However, the amounts of SRS extracted were found to correlate with both maleic acid concentration and reaction time. Furthermore, our findings collaborated with other studies reported in the literature on dilute acid pretreatment of SB at high temperature and pressure (SOARES et al., 2017; KUMAR and SHARMA, 2017).

Figure 20 - Effects of reaction time and Maleic acid concentration on the extraction of soluble reducing sugar (SRS) from SB at 121ºC (autoclave).



Interestingly, maleic and sulfuric acid showed similarities on SRS extraction from SB. Besides, the effects of sulfuric acid on SRS extraction were slightly greater than that of maleic acid, despite the higher maleic acid concentration. Maleic acid - a dicarboxylic organic acid exhibits two pKa values, hence a higher solution pH which supports cellulose hydrolysis to glucose over glucose degradation (LEE and JEFFRIES 2011; MOSIER et al., 2002). Furthermore, pretreatment with maleic acid solution is yet to be associated with inhibitors generation (KOOTSTRA et al., 2009). On the contrary, Cheng et al., (2014) reported degradation of xylan derived sugar (xylose) into furfural and other by-products in dilute sulfuric acid pretreated corncob.

4.1.4 Two-step sequential alkaline and acid pretreatment

The results obtained after two-step sequential alkaline and acid pretreatment was applied to SB are shown in **Table 5**. The glucan content of SB after two-step sequential NaOH and maleic acid pretreatment varied from 56.5% to 80.1%. The highest glucan content of 80.1% resulted from pretreatment combination of 3% NaOH in the first step and 0.3% maleic acid in the second step, while the lowest glucan content (56.5%) was obtained from the combination of 0.5% NaOH (first step) and 0.3% maleic acid (second step).

Table 5 - Glucan yields after two-step sequential alkaline (NaOH) and acid (maleic acid or sulfuric acid) pretreatment of SB in autoclave at 121ºC.

Pretreatment						
First-step			Second-step			
NaOH (%, w/v)	Temp (ºC)	Time (min)	Acid	Temp (ºC)	Time (min)	Glucan (wt%)
0.0	121	60	0.3% MA	121	60	46.74 ± 1.03
0.5	(“)	(“)	(“)	(“)	(“)	56.51 ± 2.07
1.0	(“)	(“)	(“)	(“)	(“)	63.02 ± 1.88
1.5	(“)	(“)	(“)	(“)	(“)	68.74 ± 2.63
2.0	(“)	(“)	(“)	(“)	(“)	73.85 ± 1.60
2.5	(“)	(“)	(“)	(“)	(“)	78.70 ± 1.32
3.0	(“)	(“)	(“)	(“)	(“)	80.10 ± 2.54
0.0	(“)	(“)	0.1% SA	(“)	(“)	47.67 ± 0.28
0.5	(“)	(“)	(“)	(“)	(“)	64.68 ± 1.03
1.0	(“)	(“)	(“)	(“)	(“)	71.00 ± 1.32
1.5	(“)	(“)	(“)	(“)	(“)	72.46 ± 1.88
2.0	(“)	(“)	(“)	(“)	(“)	74.32 ± 2.35
2.5	(“)	(“)	(“)	(“)	(“)	77.11 ± 2.44
3.0	(“)	(“)	(“)	(“)	(“)	80.63 ± 1.41

(“), same as the above; MA, maleic acid; SA, sulfuric acid

On the other hand, glucan content of SB after two-step sequential NaOH and sulfuric acid pretreatment ranged from 64.68% to 80.63%. The highest glucan content of 80.63% was obtained from pretreatment combination of 3% NaOH (first step) and 0.1% sulfuric acid (second step), while the lowest glucan content (64.68%) was obtained from pretreatment combination of 0.5% NaOH (first step) and 0.1% sulfuric acid (second step). Besides, glucan

contents of 46.74% and 47.67% were obtained from the combination of pretreatment without NaOH in the first step and second step pretreatment with 0.3% maleic acid and 0.1% sulfuric acid respectively. The values of glucan content obtained after SB was subjected to two-step sequential alkaline and acid pretreatment were comparable to the values reported in the literature (SANTOS et al., 2019; LÁINEZ et al., 2018). Guilherme et al., (2017) reported 68.3% increase in celulosic content after a two-step acid and alkaline pretreatment of SB with 2% sulfuric acid and 4% NaOH at 121 ºC for 30 min.

However, the similarities observed in glucan yield after second-step pretreatment with solutions of maleic and sulfuric acid indicate closeness in the acid strength at concentration used. Sulfuric acid - a strong inorganic acid is widely used for pretreatment of lignocellulosic biomass, but its use has been associated with inhibitors generation. On the contrary, maleic acid is a strong dicarboxylic acid and does not generate inhibitory compounds when applied for the pretreatment of lignocellulose biomass (KOOTSTRA et al., 2009).

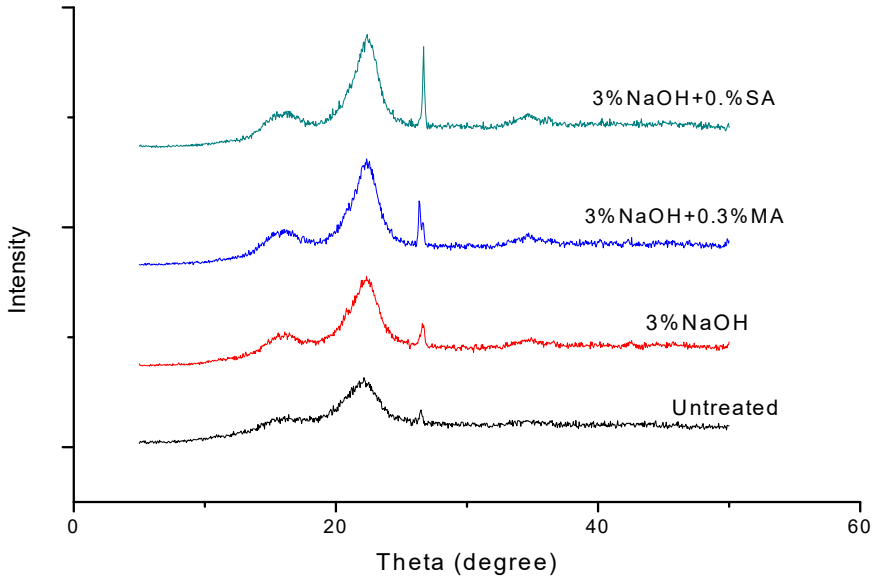
The correlation observed between the concentrations of NaOH solutions and glucan contents can be attributed to lignin removal at increasing NaOH concentration. In addition, reduced particle size contributed to increases in the available surface area for reaction to take place. Thus, increases in NaOH concentrations together with small particle size resulted to high lignin removal from SB. According to the literature, alkali can facilitate dissociation of entire cell wall polymers by breaking hydrogen and covalent bonds thereby enabling effective lignin removal (BALAT et al., 2008; LI et al., 2004). The second-step pretreatment with solutions of maleic acid and sulfuric acid led to about two-fold increase in glucan content compared with untreated SB and significantly higher than the glucan content obtained from one-step pretreatment with solutions of NaOH. In the literature, it was reported that dilute acid when combined with thermal treatment solubilize hemicellulose with concomitant cellulose enrichment (MOSIER et al., 2005).

4.1.5 X-ray diffraction studies

Figure 21 shows the comparisons of peak intensities at 002 crystallographic planes between untreated and pretreated SB. As can be observed, both untreated and pretreated SB displayed typical cellulose diffraction peaks, where the highest peak corresponds to the 002 crystallographic planes (CORRALES et al., 2012). The highest peak intensities were observed with 3%NaOH+0.1%SA and 3%NaOH+0.3%MA followed by 3%NaOH, while the least was with untreated SB. The peak intensities observed correlated with the glucan content of

pretreated and untreated SB and this finding is in agreement with the report of Rezende et al. (2011).

Figure 21 - Diffractograms of untreated SB (dewaxed only) and solid fractions from 3%NaOH pretreated SB, 3%NaOH+0.1%SA pretreated SB and 3%NaOH+0.3%MA pretreated SB, pretreatments were carried out at 121 °C (autoclave) for 1 h.



4.1.6 Chemical composition of raw and pretreated sugarcane bagasse

Table 6 shows the chemical composition and crystallinity index (CI) of raw and pretreated SB. Raw SB used in the present study consists of ~ 10% moisture content and particle size of ≤ 0.50 mm. Its chemical composition is comprised of cellulose (40.43 wt%), hemicellulose (23.82 wt%), lignin (27.22 wt%), ash (3.03 wt%) and extractives (4.42 ± 0.6). These values were similar to those reported by other authors (RABELO et al., 2009; SPORCK et al., 2017). Besides, chemical composition and productivity of raw SB are mostly dependent on sugarcane variety, climate, location, plant age and soil types (ZHAO and LI, 2015).

However, chemical composition of SB varied significantly after different pretreatments were applied. Based on the glucan contents obtained after pretreatment, one-step alkaline pretreatment of SB with 3% NaOH led to a significant increase in the cellulosic fraction (61.82 wt%) compared to raw SB (40.43 wt%), while the hemicellulose and lignin fractions decreased to 17.60 wt% and 3.04 wt% respectively. Similarly, pretreatment with 8%

NaOH at room temperature for 12 h resulted to an appreciable increase in cellulosic fraction of around 61.91 wt%, while the hemicellulose and lignin fractions decreased significantly to 17.12 wt% and 10.83 wt% respectively. Interestingly, the cellulose and hemicellulose contents of SB pretreated with 8% NaOH at room temperature for 12 h and 3% NaOH at 121 ^{o}C (autoclave) for 1 h were closely related, but the lignin contents were significantly different. This indicactes that high temperature and pressure condition in the autoclave could have facilitated a higher lignin removal from SB even at a much lower NaOH concentration.

Table 6 - Chemical composition, crystallinity index of solid fractions of raw and pretreated SB.

Pretreatment	Chemical composition of dry residues					
	Cellulose (wt%)	Hemicellulose (wt%)	Lignin (wt%)	Ash (wt%)	Extractives (wt%)	CI index (%)
Raw SB	40.43 ± 0.91	23.82 ± 0.53	27.20 ± 0.41	3.03 ± 0.74	4.42 ± 0.60	55.24
3% NaOH (Autoclave, 60 min)	61.82 ± 1.32	17.60 ± 0.53	3.04 ± 0.12	2.73 ± 0.10	na*	69.02
3%NaOH+0.1% SA (Autoclave, 60 min)	80.63 ± 1.04	6.61 ± 0.21	2.30 ± 0.11	2.24 ± 1.12	na*	79.80
3%NaOH+0.3% MA (Autoclave, 60 min)	80.10 ± 1.84	4.04 ± 0.53	3.71 ± 0.14	1.93 ± 0.22	na*	79.24
8%NaOH (RT$^{\sharp}$, 12 h)	61.91 ± 2.71	17.12 ± 0.34	10.83 ± 1.13	5.31 ± 0.84	na*	67.04

*na, not analyzed; $^{\sharp}$RT, room temperature

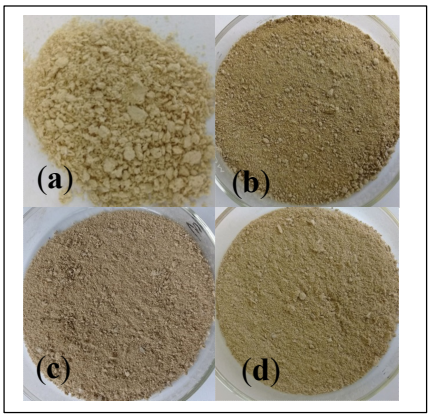
On the other hand, after the second step pretreatment of SB with 0.3% maleic acid a significant increase in cellulosic fraction (80.10 wt%) was obtained, while the hemicellulose and lignin fractions decreased significantly to 4.04 wt% and 3.71 wt% respectively. Similarly, second step pretreatment of SB with 0.1% sulfuric acid resulted to significant increase in cellulosic fractions (80.63 wt%), while the hemicellulose and lignin fractions decreased to 6.61 wt% and 2.30 wt% respectively. Concerning the ash content, one step pretreatment with 3% NaOH led to significant decrease in ash content (2.73 wt%), while no significant change on the ash contents occurred after second step pretreatment with 0.1% sulfuric acid and 0.3% maleic acid respectively.

The crystallinity index (CI) of SB increased after different pretreatments were applied. One step pretreatment of SB with 3% NaOH led to significant increase in CI (69.02%) compared to raw SB (55.24%), while CI of 67.04% was obtain from SB pretreated with 8% NaOH at room temperature for 12 h. The second step pretreatment of SB with 0.1% sulfuric

acid and 0.3% maleic acid further increased CI to 79.80% and 79.24% respectively. According to Chen et al. (2017), the crystallinity index increased after pretreatment was applied to SB which was mainly attributed to the removal of lignin and hemicellulose fractions. In addition, cellulose contents were positively correlated with CI as was previously reported by Rezende et al. (2011). Besides, the lowest value of CI obtained for raw SB could be attributed to its higher content of amorphous hemicellulose and lignin (XU et al., 2007; MAO and SCHMIDT-ROHR, 2004). Nonetheless, the relatively high CI of pretreated SB in relation to raw SB suggests that cellulose was not modified as a result of pretreatments. Sindhu et al. (2010) reported increased CI (67.83%) from SB samples pretreated with dilute sulfuric acid followed by formic acid pretreatment. Also, Velmurugan and Muthukumar (2011) reported CI of 66% from SB pretreated with NaOH, and subsequently increased to ~ 70.7% after sono-assisted pretreatment compared to 50% obtained from the native SB.

The dry solid fractions obtained after different pretreatments were applied to SB are presented in **Figure 22**. The dry solid fractions exhibited variation in colours and textures which might be attributed to different pretreatment conditions. Solid fractions from two-step

Figure 22 - Dry solid fractions obtained after different pretreatments were applied to SB, (**a**) 8% NaOH at room temperature for 12 h, (**b**) 3% NaOH in autoclave for 1 h, (**c**) 3% NaOH + 0.1% SA in autoclave for 1 h, (**d**) 3% NaOH + MA in autoclave for 1 h.



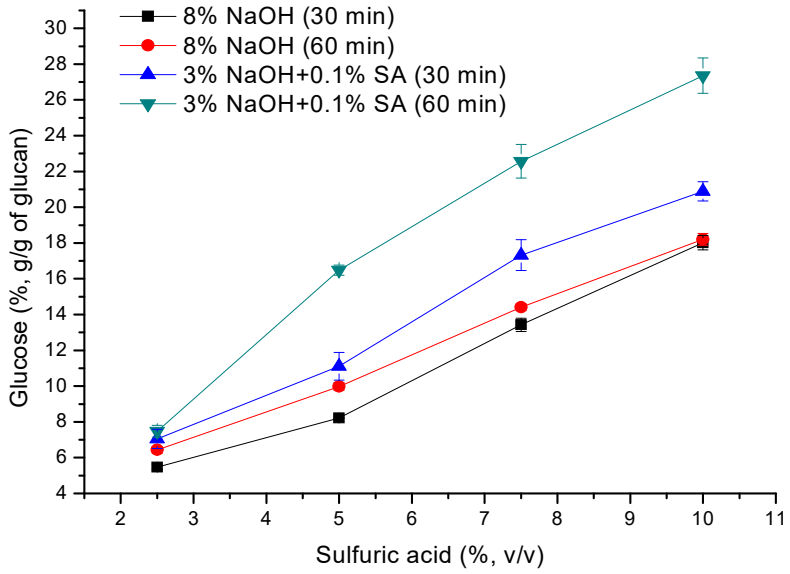
alkaline and acid pretreatment have fine texture but different colours - greyish brown (3% NaOH+SA) and brown (3% NaOH+MA), while solid fraction from 8% NaOH pretreated SB was light brown in colour with rough texture. Similarly, solid fraction from 3% NaOH pretreated SB exhibited brownish colour and granular in texture.

4.2. Acid hydrolysis

4.2.1. One-step sulfuric acid hydrolysis

Figure 23 shows the glucose yields obtained after one-step sulfuric acid hydrolysis of SB previously pretreated with 8% NaOH at room temperature for 12 h and SB pretreated with 3% NaOH at 121 ºC for 1 h followed by second step pretreatment with 0.1% sulfuric acid at 121 ºC for 1 h. After 30 min of hydrolysis time, glucose yields from SB pretreated with 8% NaOH increased from 5% to 18% as the sulfuric acid concentration increased from 2.5% to 10%, while glucose yields in the range of 7% to 21% were obtained

Figure 23 - Effect of reaction time and sulfuric acid concentration on glucose yields from pretreated SB subjected to one-step acid hydrolysis at 121 ºC.



from 3% NaOH + 0.1% sulfuric acid pretreated SB. On the other hand, after 60 min of hydrolysis, glucose yields in the range of 6% to 18% were obtained from 8% NaOH pretreated SB, while yields of glucose ranging from 7.5% to 28% were obtained from 3% NaOH + 0.1% sulfuric acid pretreated SB. The lowest glucose yield of ~ 4% was obtained from 8% NaOH pretreated SB after 30 min of hydrolysis with 2.5% sulfuric acid, while the highest glucose yield (~ 28%) was obtained from 3% NaOH + 0.1% sulfuric acid pretreated SB after 60 min of hydrolysis with 10% sulfuric acid.

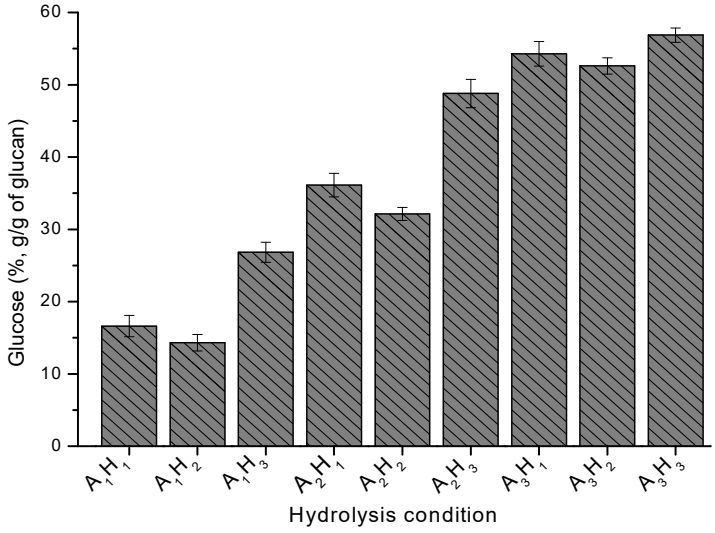
The effect of sulfuric acid concentration was apparently more significant on glucose yields than the effect of reaction time. Nevertheless, the yield of glucose was directly

proportional to the initial amount of glucan present in the substrate (pretreated SB), hence higher glucose yield was obtained from two-step sequential 3% NaOH + 0.1 %SA pretreated SB compared to 8% NaOH pretreated SB. Besides, the maximum glucose yield of about 30% obtained in the present study was significantly lower than the values reported in the literature at high temperatures ranging from 120-200ºC (LENIHAN et al., 2011; LAVARACK et al., 2002). This shows that the temperature and pressure condition (121 ºC, autoclave) applied during one-step acid hydrolysis is insufficient to cause significant degradation of glycosidic bonds present in cellulose.

4.2.2 Two-step sulfuric acid hydrolysis

Figure 24 shows the result of two-step sulfuric acid hydrolysis of SB (pretreated with 8% NaOH at room temperature for 12 h). The yields of glucose obtained ranged from about 14% to 57% after two step acid hydrolysis. The use of 30% sulfuric acid in step 1 resulted to glucose yields varying from 14% to 27%, while the use of 35% sulfuric acid led to glucose yields ranging from 32% to 49%. On the other hand, 40% sulfuric acid gave the highest glucose yields (52% to 57%). Besides, glucose yields were not significantly affected by the increase in sulfuric acid concentration in the second step as was the case of using 40% sulfuric acid in step 1.

Figure 24 - Effects of sulfuric acid concentration on two-step acid hydrolysis of SB (pretreated with 8% NaOH at room temperature for 12 h), A_1, A_2 and A_3 represent 30 %, 35 % and 40 % of sulfuric acid respectively in step 1, H_1, H_2 and H_3 represent 1.5 %, 3.0 % and 4.5 % of sulfuric acid respectively in step 2.



On the contrary, increases in sulfuric acid concentrations in the second-step resulted to appreciable increases in glucose yields especially when lower sulfuric acid concentrations (30% and 35%) were used in step 1. Nevertheless, the highest glucose yields were obtained mainly by using 40% sulfuric acid in step 1, which indicates that cellulose de-crystallization occurred at higher acid concentration.

According to Chang et al. (2018), acid hydrolysis of cellulosic material involves two steps in order to achieve high glucose yields. The first step involves concentrated acid treatment (low temperature) mainly to de-crystallize cellulose, while the second step with dilute acid (high temperature) converts amorphous cellulose to glucose. Thus, two- step acid hydrolysis is considered more efficient than one-step acid hydrolysis in terms of cellulose conversion to glucose. Nonetheless, the result obtained in this present work is similar to those reported by Heinonen and co-workers (2012) at relatively higher acid concentration using spruce and birch chips.

4.2.3 Optimization of two-step acid hydrolysis through 2^3 Full Factorial Designs

Table 7 shows the result of 2^3 full factorial designs for studying the effects of three independent variables (sulfuric acid concentration, $FeSO_4$ concentration and hydrolysis time) on glucose yields and inhibitory compounds (Furfural and 5-HMF) generation. The yields of glucose varied from 52.88% to 90.04% after $FeSO_4$ assisted-sulfuric acid hydrolysis of pretreated SB. The highest glucose yield (90.04%) was obtained at low level (-1) of sulfuric acid and high levels (+1) of $FeSO_4$ and hydrolysis time, while the lowest glucose yield (52.88%) was obtained at low levels (-1) of all the three factors (sulfuric acid, $FeSO_4$ and time) studied. However, when all the three factors were at high levels (+1) glucose yield of 78.52% was obtained, whereas at low levels (-1), glucose yield of 52.88% was obtained. Furthermore, at mid-point level (0) of all the three factors studied, glucose yields in the range of 71.62% to 74.06% were obtained. The high level (+1) of sulfuric acid appeared not to have any significant affect on glucose yield, while the yield of glucose seemed to be significantly affected by the high levels of $FeSO_4$ and hydrolysis time.

The experimental model was validated using a linear model equation (26) obtained from the experimental results and was used to explain the relationship between the independent variables (X_1-sulfuric acid, X_2-$FeSO_4$ and X_3-time) and dependent variable (Y-glucose yield) in the form of coded units. When the values of x_1, x_2 and x_3 were substituted in

the model equation below, the predicted values displayed strong agreement with the measured values of glucose yield derived from the experimental results.

$$y = 66.37 - 1.19x_1 + 8.40x_2 + 6.04x_3 - 3.9x_1x_2 - 0.22x_1x_3 + 3.47x_2x_3 \quad (26)$$

Table 7 - Glucose yields and inhibitors (furfural and 5-HMF) concentrations in the hydrolysates after $FeSO_4$ assisted-sulfuric acid hydrolysis of SB (pretreated with 8%NaOH at room temperature for 12 h).

	Independent variables			Glucose yield (%)		Inhibitory compound	
Run	Step 1 H_2SO_4 (wt%)	Step 2 $FeSO_4$ (mmol/L)	Time (min)	Measured	Predicted	5-HMF (mg/L)	Furfural (mg/L)
1	-1	-1	1	57.62 ± 1.57	58.28	1.96 ± 0.37	5.63 ± 0.53
2	-1	-1	-1	52.88 ± 2.76	52.83	2.08 ± 0.43	2.80 ± 0.64
3	-1	1	-1	69.70 ± 1.29	70.03	2.01 ± 0.46	5.35 ± 0.42
4	1	-1	-1	57.91 ± 2.71	58.56	1.37 ± 0.10	2.18 ± 0.56
5	1	-1	1	63.47 ± 2.11	63.13	2.18 ± 0.47	1.21 ± 0.35
6	0	0	0	71.47 ± 2.09	72.39	1.37 ± 0.16	3.12 ± 0.40
7	-1	1	1	90.04 ± 1.76	88.98	0.93 ± 0.13	1.03 ± 0.35
8	0	0	0	71.62 ± 3.71	72.39	4.60 ± 0.79	12.50 ± 1.80
9	1	1	1	78.52 ± 3.15	78.61	0.45 ± 0.13	1.20 ± 0.00
10	1	1	-1	60.80 ± 3.86	60.54	1.26 ± 0.27	1.21 ± 0.28
11	0	0	0	74.06 ± 3.47	72.39	2.50 ± 0.49	3.20 ± 0.42

The maximum glucose yield (90.04%) obtained in the present work is similar to those reported in the literature after a two-step hydrolysis of cellulose with 72% sulfuric acid, followed by neutralization with 40 wt% NaOH solution and subsequent post hydrolysis at 121 ^oC for 10 min (CHANG et al., 2018). The first step mainly de-crystallizes cellulose, while the second step breaks down cellulose to monomeric sugars. The first step treatment of SB with 40% sulfuric acid resulted in the disruption of hydrogen bond of cellulose chains thereby reducing the crystallinity of cellulose to a relatively amorphous state (OROZCO et al., 2007). On the other hand, the second hydrolysis step involving high levels (+1) of $FeSO_4$ and time resulted to conversion of amorphous cellulose to glucose. This suggests that the presence of Fe^{2+} ions might have contributed in the reduction of the activation energy required for cellulose hydrolysis. Li et al. (2014) reported improvement in the efficiency of $FeCl_3$ catalyzed acid hydrolysis of microcrystalline cellulose at low acid concentration. On the

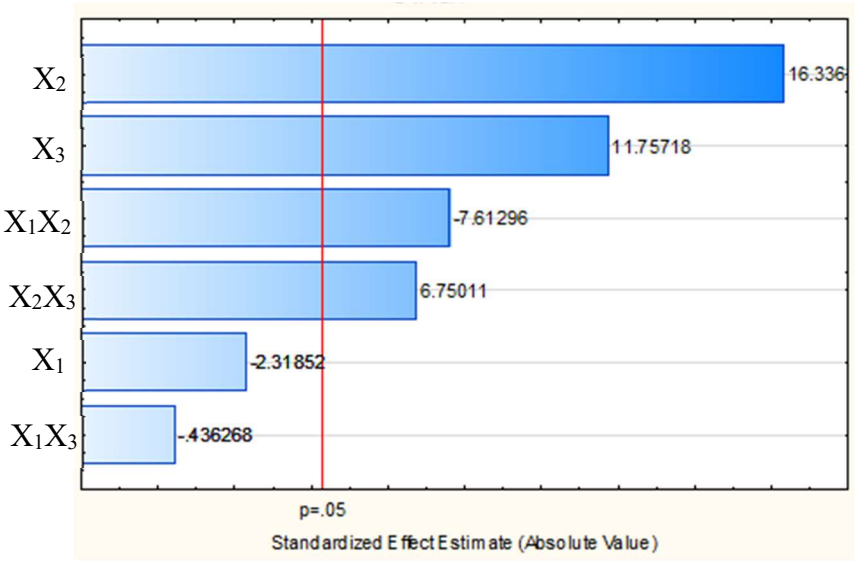
contrary, lower yields of glucose were obtained in the absence of $FeSO_4$, suggesting that only the amorphous part of cellulose was hydrolyzed.

The concentrations of 5-HMF in the acid hydrolysates varied from 0.45 mg/L to 4.60 mg/L, while furfural concentrations in the hydrolysates ranged from 1.03 mg/L to 12.50 mg/L. The highest concentrations of 5-HMF (4.60 mg/L) and furfural (12.50 mg/L) were obtained from the hydrolysate which resulted from hydrolysis with mid-levels (0) of all the three factors (sulfuric acid, $FeSO_4$ and time). On the other hand, the lowest 5-HMF concentration (0.45 mg/L) was obtained from the hydrolysate which resulted from the use of high levels (+1) of all the three factors, whereas the lowest concentration of furfural (1.03 mg/L) resulted from low level (-1) of sulfuric acid and high levels (+1) of $FeSO_4$ and time.

However, the low concentrations of 5-HMF and furfural in the acid hydrolysates may be attributed to the use of mild temperature condition in the present work. Stoffel et al. (2014) reported that temperature, acid concentration and time produced significant effects on the acid hydrolysis process. Furthermore, no direct relationship was observed between glucose yield and the concentration of degradation products (5-HMF and furfural). The degradation products of lignocellulose (furfural and 5-HMF) produced during acid hydrolysis can significantly inhibit fermentation process (RAN et al., 2014). Furfural and 5-HMF concentrations above 3.0 g/L and 3.2 g/L have been reported to inhibit fermentation by 20% and 50% respectively (XIROS et al., 2011). Therefore, 5-HMF and furfural concentrations in the range of 0.45 mg/L to 4.60 mg/L and 1.03 mg/L to 12.50 mg/L respectively obtained in the present work is well below the threshold that can inhibit fermenting yeasts.

Figure 25 shows the Pareto chart of absolute values of standardized effects from the largest to the smallest effect. X_2 ($FeSO_4$ concentration) showed the largest effect on glucose yield followed by X_3 (hydrolysis time), while the smallest effect was shown by X_1 (sulfuric acid). On the other hand, the interaction between X_1 and X_2 exhibited the largest effect followed by the effect of X_2 and X_3 interaction, while X_1 and X_3 interaction showed the smallest effect. This indicates that X_2, X_3 and their interactive effect as well as the interactive effect of X_1X_2 significantly influenced the yields of glucose during acid hydrolysis. According to Jabeen et al. (2015), any experimental process can be optimized as a function of its independent variables to maximize, minimize, or set the analyzed response to a desired target value.

Figure 25 - Pareto chart of main effects (sulfuric acid-X_1, $FeSO_4$-X_2 and time-X_3) and interactive effects (X_1X_2, X_2X_3 and X_1X_3) on the yield of glucose.



The analysis of variance (ANOVA) for the 2^3 full factorial designs is shown in **Table 8**. Independent factors (X_2-$FeSO_4$ and X_3-time) and interactions (X_1X_2 and X_2X_3) were significant (p-value <0.05), while independent factor (X_1-sulfuric acid) and interaction (X_1X_3) were not significant (p-value >0.05). However, the model was significant with a p-value of 0.002 (p<0.05), whereas the model Lack of Fit was insignificant (p >0.05). This indicates that the model adequately explained the relationship between the independent variables (X_1-sulfuric acid,

Table 8 - Analysis of variance (ANOVA) to identify significant factors and interactions on the yield of glucose. R-sq = 0.995, R-sq (adj) = 0.984.

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Model	964.566	6	160.761	84.421	0.002
x_1	11.365	1	11.365	5.968	0.092
x_2	564.223	1	564.223	296.291	0.000
x_3	292.246	1	292.246	153.467	0.001
x_1x_2	122.532	1	122.532	64.345	0.004
x_1x_3	0.402	1	0.402	0.211	0.677
x_{2x3}	96.330	1	96.331	50.586	0.006
Residual	5.713	3	1.904		
Lack of Fit	1.484	1	1.485	0.702	0.490
Pure Error	4.228	2	2.114		
Total SS	1171.756	9			

F-value, F-statistic; p-value, probability value

X_2-$FeSO_4$ and X_3-time) and dependent variable (y-glucose yield). Besides, the high R-square and R-square (adjusted) value of 0.9951 and 0.9838 respectively obtained indicates that the experimental design explained a lot of variation within the data. The plot of Predicted values versus Observed values (**Figure 26**) was used to make comparison between experimental values of the response and those predicted by the experiment. All points were clustered around the diagonal line, indicating good fitness of the experimental design.

Figure 26 - Plot of predicted and observed values for the two-step acid hydrolysis.

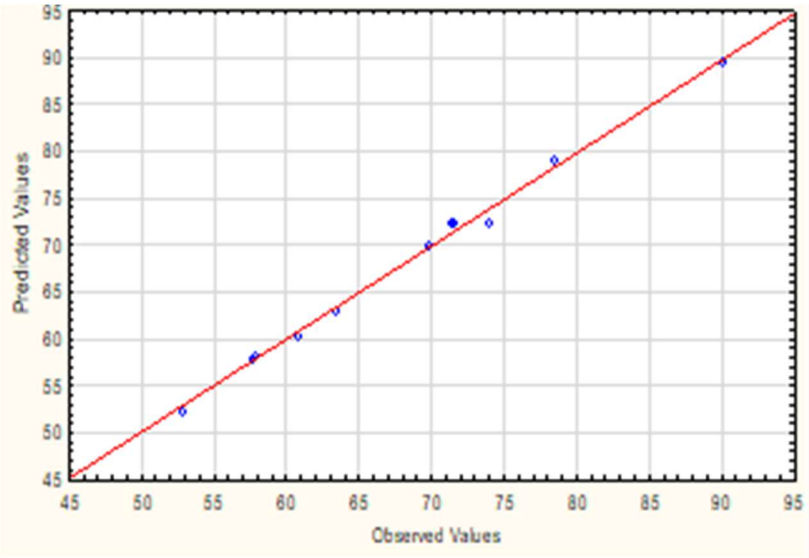
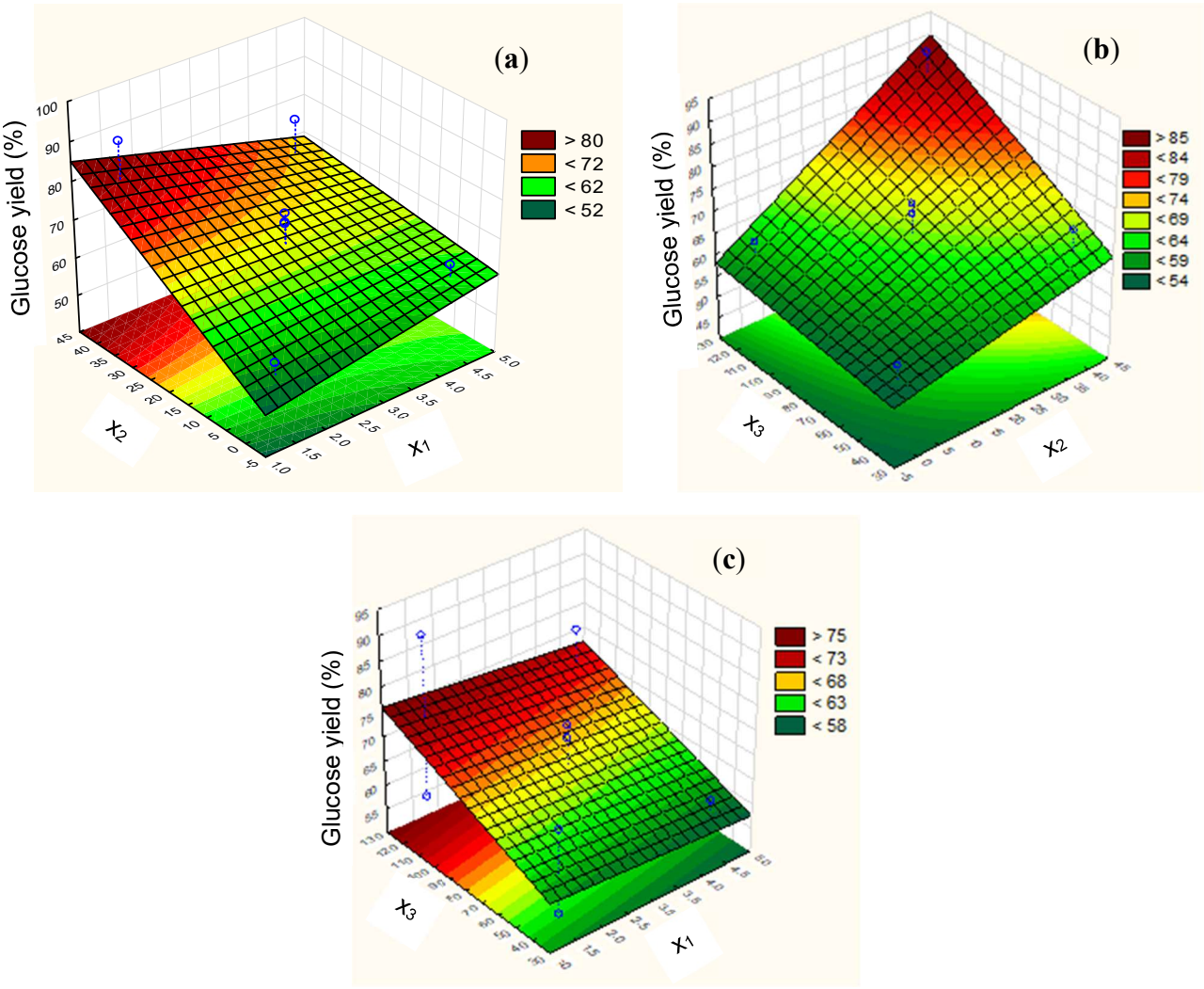


Figure 27 shows the three dimensional (3D) response surface plots to determine the optimum levels of the independent variables. The plots were generated by keeping one variable constant at the center point and varying the others within the experimental range. The resulting response surfaces showed the effects of sulfuric acid (X_1), $FeSO_4$ (X_2) and hydrolysis time (X_3) on glucose yield. The effects of sulfuric acid (X_1) and $FeSO_4$ (X_2) on glucose yield at fixed hydrolysis time are shown in **Figure 27a**. It was observed that decreases in sulfuric acid concentration (X_1) together with increases in $FeSO_4$ (X_2) resulted to glucose yield above 80%. Glucose yield of approximately 75% was obtained at 1.5% sulfuric acid and 40 mmol/L of $FeSO_4$.

Figure 27b showed that glucose yield above 85% can be reached by increasing both $FeSO_4$ (X_2) and hydrolysis time (X_3) at fixed sulfuric acid concentration (X_1). Glucose yield of approximately 85% was obtained at 40 mmol/L of $FeSO_4$ and 120 min of hydrolysis time. On the other hand, increasing hydrolysis time (X_3) and decreasing sulfuric acid (X_1) at fixed

$FeSO_4$ (X_2) resulted to glucose yield of above 75% (**Figure 27c**). Glucose yield of approximately 72% was obtained at 120 min of hydrolysis time (X_3) and 1.5% sulfuric acid (X_1). Thus, the optimum condition for the maximum yield of glucose during acid hydrolysis of pretreated SB was sulfuric acid (1.5%), $FeSO_4$ (40 mmol/L) and time (120 min).

Figure 27 - Surface plots of cellulose conversion (%) as a function of: (**a**) X_1, sulfuric acid concentration (%) and X_2, $FeSO_4$ catalyst (mmol/L); (**b**) X_2, $FeSO_4$ (mmol/L) and X_3, time (min); (**c**) X_1, sulfuric acid concentration (%) and X_3, time (min).



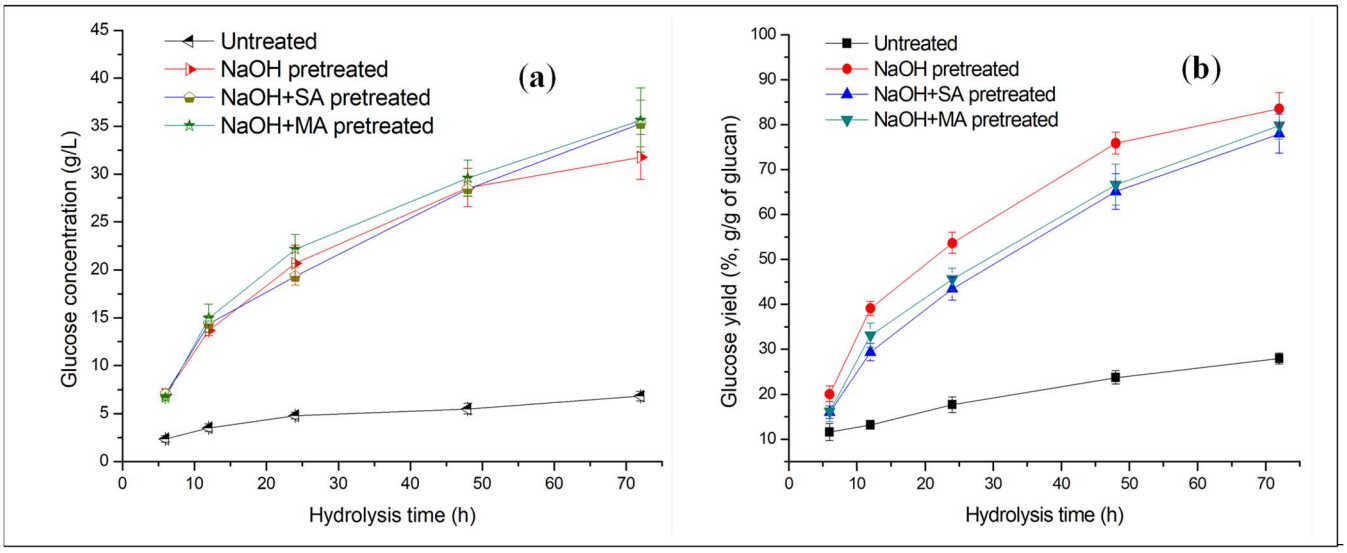
4.3 Enzymatic hydrolysis

Figure 28 shows glucose concentrations and glucan conversion rate (glucose yield) during enzymatic hydrolysis of untreated and pretreated SB. Glucose concentration increased slowly for untreated SB from 2.4 g/L to 6.8 g/L after 6 h and 72 h of hydrolysis respectively

(**Figure 28a**). For pretreated SB (3% NaOH), glucose concentration increased from about 7.0 g/L after 6 h of hydrolysis to 31.8 g/L after 72 h, while glucose concentration from pretreated SB (NaOH+MA) increased from 6.7 g/L after 6 h to 35.6 g/L after 72 h. Similarly, the glucose concentration from NaOH+SA pretreated SB increased from 7.1 g/L after 6 h to 35.3 g/L after 72 h. Furthermore, similarities in glucose concentrations were observed after 72 h of hydrolysis for the pretreated SB and these values were more than four times the value obtained for untreated SB. The highest glucose concentration (35.6 g/L) was obtained from NaOH+MA pretreated SB after 72 h, which was very close to the value of 35.3 g/L obtained from NaOH+SA pretreated SB after 72 h. Similarity in the glucose concentration shown by NaOH+MA and NaOH+SA pretreated SB might be connected to the closeness in their cellulose contents.

On the other hand, after 6 h of hydrolysis (**Figure 28b**), glucose yield (glucan conversion rate) of about 11.6% was obtained from untreated SB, while values of 20.1%, 16.2% and 16.0% were obtained from NaOH, NaOH+MA and NaOH+SA pretreated SB respectively. After 72 h, glucose yields obtained were 28.0% (untreated SB), 83.5% (NaOH pretreated SB), 79.8% (NaOH+MA pretreated SB) and 77.9% (NaOH+SA pretreated SB).

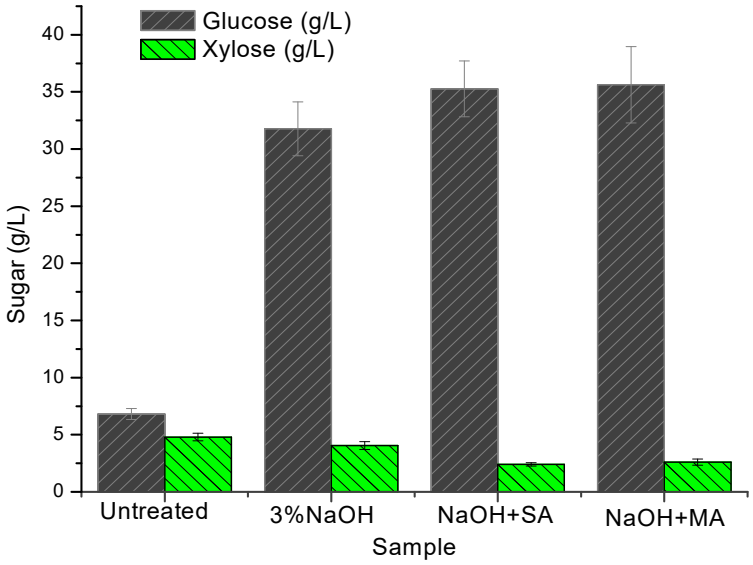
Figure 28 - (**a**) Glucose concentration, (**b**) Glucan conversion rate (glucose yield) during enzymatic hydrolysis of untreated SB (dewaxed only) and solid fractions which resulted from pretreatments with 3% NaOH, 3% NaOH + 0.3% maleic acid (NaOH+MA) and 3% NaOH + 0.1% sulfuric acid (NaOH+SA).



Interestingly, the glucose yields obtained from pretreated SB were over three times the amount obtained from untreated SB. Nonetheless, the highest glucose yield of 83.5% was obtained from NaOH pretreated SB despite its lower glucose concentration in relation to NaOH+MA and NaOH+SA pretreated SB. This difference might be connected to cellulose crystallinity, since low crystallinity suggests more accessibility to enzymes and improved digestibility of cellulosic material. Some authors mentioned cellulose crystallinity as an important factor influencing hydrolysis efficiency (PARK et al., 2010; PARIKH et al., 2007). This suggests that NaOH+MA and NaOH+SA pretreated SB consist of more crystalline cellulose than NaOH pretreated SB.

Figure 29 shows the sugars concentration (glucose and xylose) obtained after 72 h of hydrolysis of untreated SB and pretreated SB (NaOH, NaOH+MA and NaOH+SA). Relatively very low amount of xylose were obtained from the enzymatic hydrolysates of pretreated SB (NaOH+MA and NaOH+SA) compared to untreated SB and 3% NaOH pretreated SB. This is an indication that a greater part of hemicelulose was solubilized mainly during the acid pretreatment step. On the other hand, the presence of xylose in all the hydrolysates might be connected to the action of cellulases and β- glucosidase enzymes which cause the disruption of lignocellulosic matrix thereby releasing xylose (GUILHERME et al., 2015).

Figure 29 - Sugars concentration after 72 h of enzymatic hydrolysis of untreated SB and solid fractions which resulted from pretreatments with 3% NaOH, 3% NaOH + 0.3% maleic acid (NaOH+MA) and 3% NaOH + 0.1% sulfuric acid (NaOH+SA).



4.4 Optimization of enzymatic hydrolysis via Central Composite Design

Central Composite design (CCD) was used to investigate the effects of three variables: hydrolysis time (X_1), enzyme loadings (X_2) and solids loadings (X_3) under optimal conditions of enzymatic hydrolysis. The experimental (measured) and predicted values of glucose yield were presented in **Table 9**. A second order polynomial equation (27) was obtained from the analysis of the CCD data to evaluate the relationship between the independent variables (X_1, X_2 and X_3) and the response variable (Y). By substituting the values of x_1, x_2 and x_3 in the equation below, a strong agreement was observed between the predicted and measured values of glucose yield derived from experimental results.

$$Y = 61.69 + 11.17X_1 + 7.25X_2 - 11.65X_3 - 5.09X_1^2 - 0.40X_2^2 + 0.20X_3^2 - 0.03X_1X_2 - 1.15X_1X_3 + 1.06X_2X_3 \quad (27)$$

Table 9 - Central composite design and experimental results.

Run	Coded variables			Actual variables			Glucose yield (%)	
	Time	Enzyme loadings	Solids loadings	Time	Enzyme loading	Solids loadings	Measured	Predicted
1	-1	-1	-1	24	1.00	7	47.84 ± 3.61	49.35
2	-1	1	1	24	2.60	12	41.16 ± 2.83	43.09
3	1	-1	1	72	1.00	12	46.06 ± 0.19	46.43
4	1	1	-1	72	2.60	7	80.96 ± 2.41	86.55
5	0	0	0	48	1.80	9.50	59.49 ± 0.18	61.73
6	-1	-1	1	24	1.00	12	30.17 ± 0.24	26.29
7	-1	1	-1	24	2.60	7	60.60 ± 4.17	61.93
8	1	-1	-1	72	1.00	7	74.34 ± 1.03	74.11
9	1	1	1	72	2.60	12	62.91 ± 0.95	63.09
10	0	0	0	48	1.80	9.50	61.66 ± 4.78	61.73
11	-1.68	0	0	8	1.80	9.50	28.54 ± 0.87	28.82
12	1.68	0	0	88	1.80	9.50	68.86 ± 4.96	66.12
13	0	-1.68	0	48	0.46	9.50	46.49 ± 1.53	48.67
14	0	1.68	0	48	3.14	9.50	77.19 ± 2.21	72.43
15	0	0	-1.68	48	1.80	5.30	85.92 ± 1.64	81.85
16	0	0	1.68	48	1.80	13.70	41.12 ± 2.26	42.77
17	0	0	0	48	1.80	9.50	63.42 ± 2.11	61.73

According to **Table 10**, linear coefficients of hydrolysis time (β_1) and enzyme loadings (β_2) have high positive values. On the other hand, high negative values were obtained for the linear coefficient of solids loading (β_3) and quadratic coefficient of hydrolysis time (β_{11}).

Other coefficients (β_{22}, β_{33}, β_{12}, β_{13} and β_{23}) have low values indicating less significant effects on responses. A positive value indicates a synergistic effect, while a negative value indicates an antagonistic effect. Thus, increase in the effects of linear terms (hydrolysis time and enzyme loadings) tends to increase responses. On the contrary, increase in the effects of the linear term (solids loading) and quadratic term (enzyme loading) tends to decrease responses.

Table 10 - Estimated regression coefficients, t-values and p-values, R-squared (0.97602), Adj R-squared (0.94518).

	Reg. coeff.	Std.Err.	t-value (7)	p-value
Model constant	61.6909	2.315464	26.6430	0.000000
(1)X_1(L)	11.1744	1.090419	10.2478	0.000018
X_1(Q)	-5.0944	1.205479	-4.2260	0.003907
(2)X_2(L)	7.2488	1.090419	6.6477	0.000291
X_2(Q)	-0.4015	1.205479	-0.3331	0.748832
(3)X_3(L)	-11.6476	1.090419	-10.6817	0.000014
X_3(Q)	0.1990	1.205479	0.1651	0.873525
1L by 2L	-0.0344	1.421733	-0.0242	0.981348
1L by 3L	-1.1535	1.421733	-0.8113	0.443882
2L by 3L	1.0557	1.421733	0.7425	0.481943

Table 11 shows the ANOVA results for the second order polynomial model. The p-values (probability values) are used as tools to check the significance of each of the coefficients in the model, which in turn, may indicate the patterns of the interaction among the variables. The smaller the p-value, the more significant is the corresponding coefficient. It was noted that all the linear terms and quadratic term of hydrolysis time were significant (p-value <0.05), while all the interaction terms were insignificant (p-value >0.05).

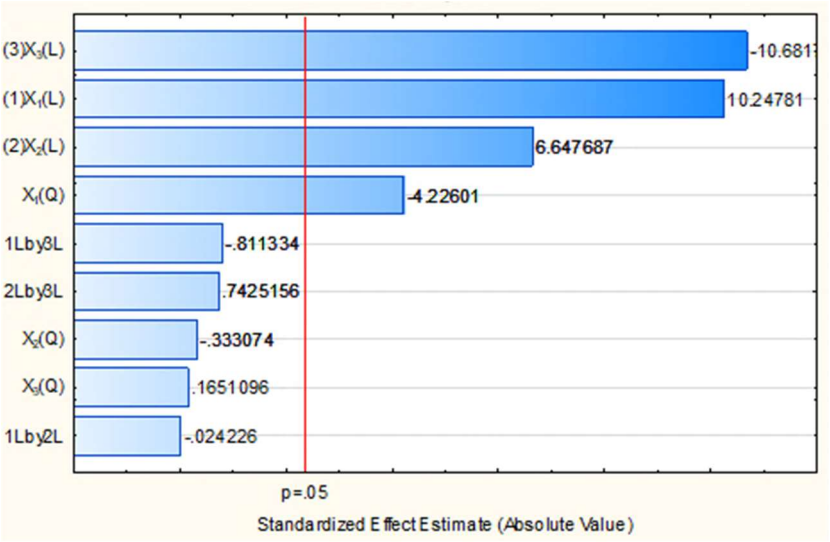
ANOVA of regression model demonstrated that the model was significant (p-value <0.05) with insignificant (p-value of >0.05) Lack of Fit indicating that the model fitted adequately to the experimental data. Also R-squared of 0.976 and adj. R-Squared of 0.945 showed that the responses were better predicted by model. A regression model having R-squared higher than 0.90 can be considered to have a very high correlation (HAALAND, 1989).

Table 11 - ANOVA of CCD for glucose yield from enzymatic hydrolysis of pretreated SB, R-squared (0.976), Adj R-squared (0.945).

Source	Sum of squares	Degree of freedom	Mean square	F-Value	P-Value
Model	4568.462	9	507.607	31.3899	0 .0000
(1)X_1(L)	1698.197	1	1698.197	105.0176	0.0000
X_1(Q)	288.794	1	288.794	17.8592	0.0039
(2)X_2(L)	714.607	1	714.607	44.1917	0.0003
X_2(Q)	1.794	1	1.794	0.1109	0.7488
(3)X_3(L)	1845.061	1	1845.061	114.0997	0.0000
X_3(Q)	0.441	1	0.441	0.0273	0.8735
1L by 2L	0.009	1	0.009	0.0006	0.9813
1L by 3L	10.644	1	10.644	0.6583	0.4439
2L by 3L	8.915	1	8.915	0.5513	0.4819
Residual	113.194	7	16.171		
Lack of Fit	105.455	5	21.091	5.4502	0.1623
Pure Error	7.739	2	3.870		
Total SS	4681.656	16			

The range of the effects on glucose yield is presented in **Figure 30**. The effect estimates divided by their corresponding standard errors were arranged from the largest absolute value to the smallest absolute value. The range of each effect was depicted by a column, and a line going across the columns indicates the extent an effect must reach to be considered statistically significant. It was observed that all the linear effects of independent variables were significant, while only the quadratic effect hydrolysis time was significant. On

Figure 30 - Pareto chart of standardized effects for the glucose yield (%).

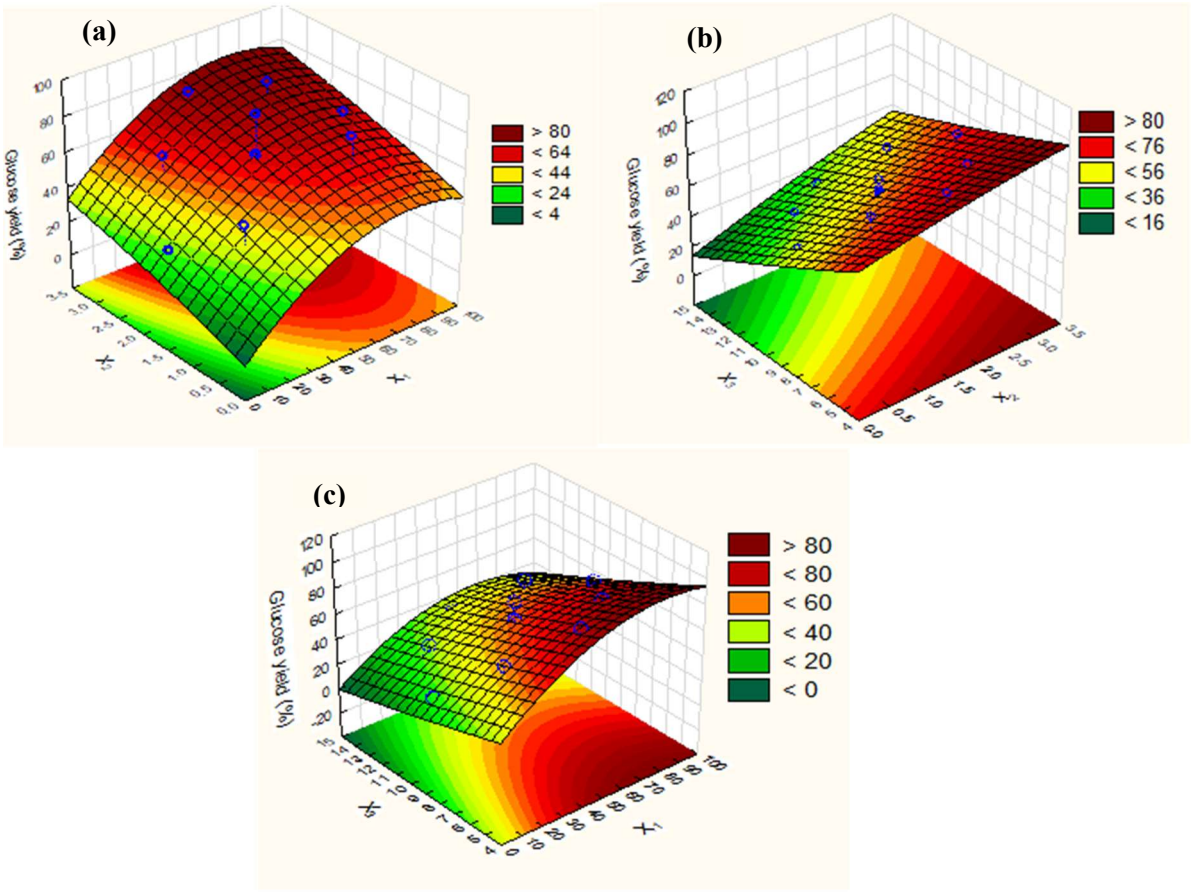


the other hand, all the interaction effects were insignificant. The largest effects on glucose yield were the linear effects of solids loading and hydrolysis time, whereas hydrolysis time exhibited the largest quadratic effects.

4.4.1 Effect of independent variables

Figure 31 shows the response surface plots with glucose yield (%) as a response variable. In each surface plot, two variables were altered, while the third variable was fixed at a constant value of zero coded level. Glucose yield (%) is represented as a function of hydrolysis time and enzyme loadings, or solids loading. **Figure 31a** shows the effect of the interaction of hydrolysis time and enzyme loadings on glucose yield at a fixed solids loading.

Figure 31 - Response surface plots of glucose yield from enzymatic hydrolysis of sugarcane bagasse on (**a**) Hydrolysis time (X_1) and enzyme loadings (X_2), (**b**) Enzyme loadings (X_2) and solids loading (X_3), (**c**) Hydrolysis time (X_1) and solids loading (X_3).



Increases in both reaction variables exhibited significant positive effect on the glucose yield. As the hydrolysis time and enzyme loading were gradually increased from low levels to high levels, glucose yield increased proportionately, and then began to flatten as both variables approached their high levels. This might be attributed to the product accumulation leading to the inhibition of enzyme activity. Consequently, a slight decline in glucose yield was observed when the hydrolysis time was increased beyond 75 h. Thus, a maximal glucose yield (above 80%) was reached at 75 h of hydrolysis time and enzyme loading of 3.5 FPU/g cellulose of cellulase.

Figure 31b shows the interactive effect of enzyme and solids loadings when the hydrolysis time is fixed. A sharp increase in glucose yield was observed at initial low levels of both reaction variables. Subsequently, a steady increase in glucose yields was observed at decreasing levels of solids loading and slightly increasing levels of enzyme loadings. Maximal glucose yield of above 90% was reached at enzyme loadings of 1.5 FPU/g cellulose and 4% solids loading. Thus, low levels of enzyme loadings in the range of 1.0-1.5 FPU/g cellulose and solids loading of 6-8% could be within the optimal conditions for the hydrolysis of pretreated SB. The high glucose yield suggests that the addition of a surfactant (Tween 80) might have played a role by reducing unproductive enzyme adsorption to lignin, thereby enhancing the conversion of cellulose into glucose (KRISTENSEN, 2009).

The effect of simultaneous variations of hydrolysis time and solids loading on glucose yields at a fixed enzyme loading is shown in **Figure 31c**. It was observed that at increasing levels of hydrolysis time coupled with decreasing levels of solids loading resulted to increased glucose yields. The effect of hydrolysis time was more significant at high levels, while the effect of solids loading was significant at low levels. Enzymatic hydrolysis process requires a longer time to hydrolyse the cellobiose to achieve glucose as the end-product (PHUMMALA et al., 2015). The maximal glucose yield (above 95%) was reached at 80 h of hydrolysis time and solids loading of 4%. Thus, based on the response surface plots, maximal glucose yield could possibly be achieved by increasing hydrolysis time, decreasing enzyme loadings and moderately increasing solids loading.

4.4.2 Model validation

To confirm the accuracy of the statistical model, a triplicate hydrolysis experiments were performed under the optimum condition (72 h of hydrolysis time, 1.34 FPU/g cellulose of enzyme loadings and 7.40% of solids loading) provided by the desirability function. The

predicted glucose yield determined based on the second order polynomial equation (27) was 75.04%, which was in good agreement with the experimental glucose yield of 80.95% (**Table 12**). This confirmed that the model successfully predicted the experimental data of glucose yield. Thus, CCD is an effective tool that can be employed to optimize enzymatic hydrolysis of pretreated SB.

Table 12 - Optimal values of the independent variables, experimental, and predicted yield of glucose.

			Glucose yield (%)	
Time (X_1) (h)	Enzyme (X_2) (FPU/g cellulose)	Solids (X_3) (%, w/w)	Measured	Predicted
72	1.34	7.40	80.95 ± 3.87	75.04

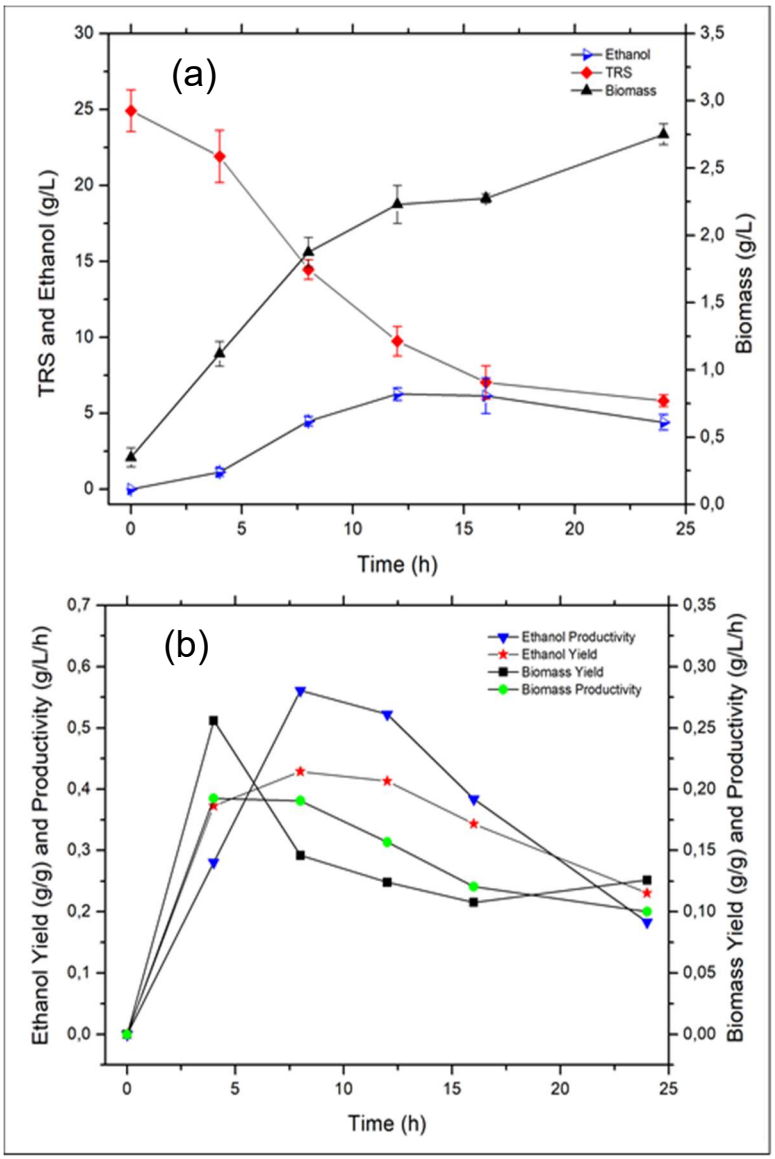
Alkaline pretreated SB presented relatively high cellulose content (62.8%) that was readily hydrolysed to high yield of fermentable sugars at low enzyme loadings. The yield of glucose obtained in this study was comparable to the values reported by other authors even at higher enzyme loadings ((MUKASEKURU et al., 2018; DE SOUZA et al., 2018; WANG et al., 2015; SUN et al., 2016).

4.5 Fermentation

4.5.1 Fermentation of acid hydrolysates

Figure 32 shows fermentation profile of the yeast strain (IQAr/45-1) on acid hydrolysate of 8% NaOH pretreated SB. Sugar consumption by the yeast cells increased steadily during 16 h of fermentation when most of the sugars were consumed. The highest amount of sugars was consumed at the time intervals between 4 h to 8 h (7.14 g/L) and 8 h to 16 h (4.71 g/L) respectively (**Figure 32a**). On the other hand, after 16 h of fermentation most of the sugars were consumed by the yeast cells leaving behind a significant amount of sugars presumably pentose sugars. The yeast strain -IQAr/45-1 cannot utilize xylose, hence significant amount of sugars (6.83 g/L) remained after 24 h of fermentation. According to Hector et al. (2011), *S. cerevisiae* lacks the genes required for the assimilation of pentose sugars. To overcome this challenge, different molecular strategies such as protoplast fusion or genetic recombination have been suggested for the future in order to induce xylose utilizing abilities in *S. cerevisiae* (HASHMI et al., 2017).

Figure 32 - (a) Concentrations of total reducing sugar (TRS), Ethanol, and Biomass, (b) Ethanol yield and Productivity, Biomass yield and Productivity during 24 h of fermentation of acid hydrolysate of SB (pretreated with 8% NaOH solution at RT for 12 h) by *S. cerevisiae* IQAr/ 45-1.



Ethanol concentration increased with the increases in fermentation time reaching the highest ethanol level (6.27 g/L) during 12 h of fermentation, while after 24 h, ethanol level decreased to 4.40 g/L. Similarly, the amount of biomass formed increased steadily throughout the period of fermentation reaching the highest amount (2.75 g/L) after 24 h. Biomass formation correlated with sugars consumption during 16 h of fermentation and afterwards this correlation disappeared. This suggests that in the absence of glucose or at low glucose levels,

yeast cells can utilize other carbon sources, hence the decrease in ethanol concentration obtained after 12 h of fermentation.

However, as shown in **Figure 32b**, ethanol yield reached the maximum level of 0.43 g/g after 8 h of fermentation and subsequently decreased to the lowest level of 0.23 g/g after 24 h. The highest ethanol productivity (0.56 g/L/h) was attained after 4 h which later decreased to 0.18 g/L/h after 24 h. On the other hand, the highest yield of biomass (0.26 g/g) was achieved after 4 h and thereafter a steady decrease was observed before reaching the lowest level (0.11 g/g) after 16 h. Similarly, the highest biomass productivity (0.19 g/L/h) was attained after 4 h and later decreased to the lowest level of 0.10 g/L/h after 24 h. Also, sharp decreases in ethanol yields and productivities occurred after 12 h which may be attributed to depletion of sugars (glucose) in the fermentation medium.

Thus, the relatively high ethanol yield which corresponds to fermentation efficiency of 84% based on the theoretical yield of ethanol suggests efficient utilization of glucose present in the acid hydrolysate by yeast strain -IQAr/45-1. On the other hand, the decreases in biomass yield and productivity after 4 h may suggest that the medium condition is more favourable towards ethanol formation.

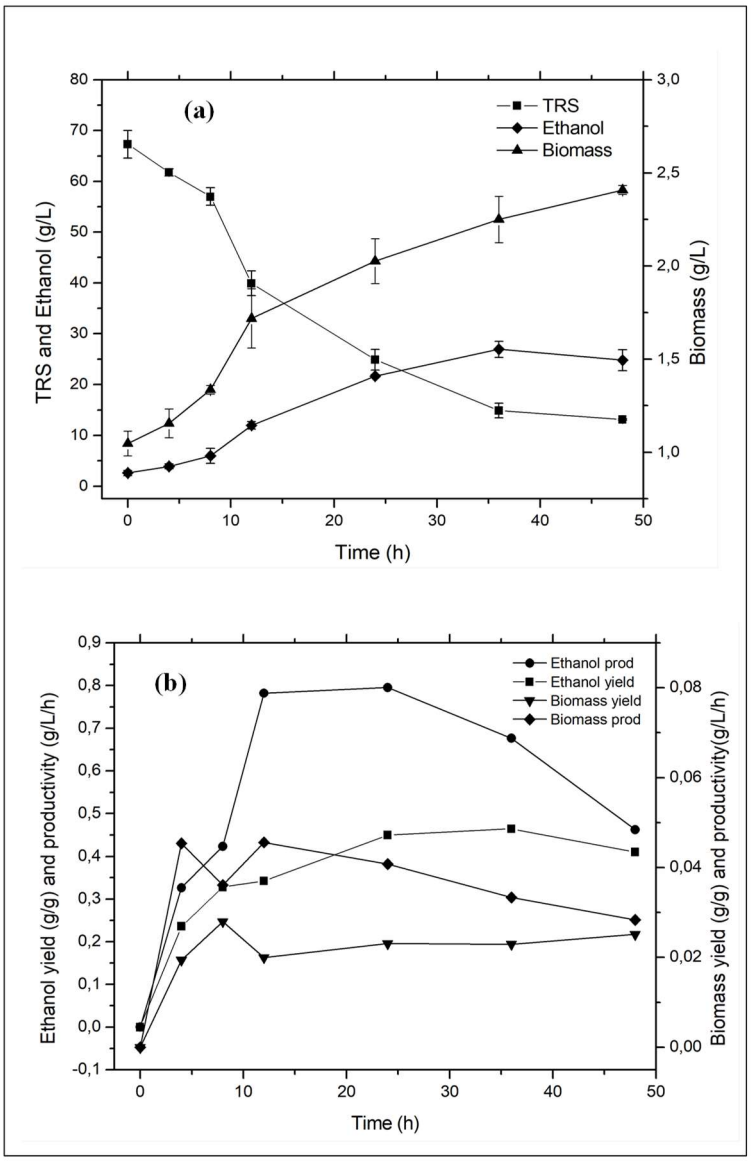
4.5.2 Fermentation of enzymatic hydrolysates

Figure 33 shows the fermentation profile of *S. cerevisiae* IQAr/45-1 on the enzymatic hydrolysate of alkaline pretreated SB. In **Figure 33a**, the slow growth rate observed during the initial 4 h of fermentation can be attributed to the adaptation of *S. cerevisiae* to medium environment. In this period, the cell synthesizes enzymes necessary for metabolism of the medium components. Thereafter, biomass concentration increased and continued throughout the course of fermentation, reaching a maximum concentration of ~2.5 g/L after 48 h.

However, sugars concentration decreased along the course of fermentation, reaching the lowest concentration of ~13 g/L after 48 h. This shows that the yeast cells were able to consume the sugars present in medium. The highest decrease in sugars concentration occurred between 8-24 h of fermentation, indicating that after the initial adaptation period, the yeast cells increased its sugar consumption to maintain growth and ethanol formation. On the other hand, ethanol levels increased slowly during 8 h of fermentation, afterwards a significant increase in ethanol levels was achieved up to a maximum level of ~26 g/L after 36 h. The levels of ethanol formation during 36 h of fermentation strongly correlated with sugar consumption. Thereafter, ethanol levels started to decrease as the concentration of sugars

remain largely unchanged. Thus, the decline in ethanol levels after 36 h could be attributed to the consumption of accumulated ethanol by the yeast cells in the absence of fermentable sugars. According to Kuhad et al. (2010), simultaneous consumption of sugar and ethanol by the adapted microbial population can occur in ethanol accumulated medium.

Figure 33 - (**a**) Concentrations of total reducing sugars, Ethanol, and Biomass, (**b**) Ethanol yield and Productivity, Biomass yield and Productivity during 48 h of fermentation of enzymatic hydrolysate of PSB by *S. cerevisiae* IQAr/ 45-1.



The yields of ethanol and biomass as well as productivities are depicted in **Figure 33b**. The highest ethanol yield of ~0.46 g/g was achieved after 36 h of fermentation corresponding

to a productivity of ~0.68 g/L/h, while the highest biomass yield (~0.028 g/g) was obtained after 8 h with productivity of ~0.04 g/L/h. On the other hand, the highest ethanol productivity of ~0.80 g/L/h was obtained after 24 h, while the highest biomass productivity of ~0.056 g/L/h was obtained after 12 h. Other authors reported 16.4 g/L of ethanol and productivity of 0.45 g/L/h from the enzymatic hydrolysate of microwave-assisted alkali pretreated wheat straw after 36 h of fermentation by *S. cerevisiae* (SINGH and BISHNOI, 2013). Also, experiments with *S. cerevisiae* UFPEDA 1238 using steam-exploded, enzyme hydrolysed SB showed ethanol yield of 0.39 g/g and productivity of 0.97 g/L/h after 24 h of fermentation (WANDERLEY et al., 2013).

The relatively high residual sugar after 48 h of fermentation suggests the presence of pentose sugar in the hydrolysate medium. This is mainly due to inability of *S. cerevisiae* to utilize pentose sugar (HECTOR et al., 2011). The Celluclast 1.5L preparations have a remarkable β-xylosidase activity (SCHMIDT et al., 1998), thus part of hemicelluloses could possibly be hydrolysed to pentose sugar during the enzymatic hydrolysis of pretreated SB.

5 CONCLUSIONS

One-step NaOH and two-step sequential NaOH and acid pretreatments were applied to SB in order to enrich the cellulosic fractions. Two-step sequential NaOH and acid pretreatments led to approximately two-fold increase in cellulosic fractions from SB. One-step pretreatment with NaOH solutions effectively removed lignin and part of hemicelluloses, while the second pretreatment step with acids removed significant part of hemicellulose fractions. The changes in the chemical composition of SB after pretreatments were revealed by XRD analysis indicating increases in crystallinity.

However, the cellulosic fractions resulting from one-step NaOH and two-step sequential NaOH and acids pretreatments were amenable to cellulolytic enzymes with glucose yields well above 70%. Furthermore, it was possible to achieve a glucose yield above 80% at low enzyme loadings through the use of Design of Experiment. Similarly, the optimization of two-step sulfuric acid hydrolysis with the addition of $FeSO_4$ signficantly improved the yield of glucose up to 90%. Thus, acid hydrolysis can be considered advantageous since it involves the use of cheap chemicals and short hydrolysis time, which can be beneficial to bio-refinery based on the current high cost of cellulolytic enzymes. Nonetheless, the main challenges with acid hydrolysis are associated with hydrolysate neutralization and the need for hydrolysate

concentration prior to fermentation. This post hydrolysis treatment process results to loss of fermentable sugars and the generation of large amount of calcium sulfate.

Interestingly, *S. cerevisiae* IQAr/45-1 efficiently utilized both the acid and enzymatic hydrolysates of pretreated SB to produce high yields of ethanol. Nevertheless, quite a high concentration of residual sugar remained indicating the inability of *S. cerevisiae* IQAr/45-1 to metabolize pentose sugar.

6 RECOMMENDATIONS FOR FUTURE WORKS

Furure perspectives based on the current study include the followings:

- Post hydrolysis treatment of acid hydrolysate needs further evaluation in order to increase sugars concentration and minimize the loss of sugars with overall reduction in processing cost;
- Enzymatic hydrolysis at a higher solids loading in the range of 15-20% with low enzyme loadings is required in order to reduce cost;
- Fermentation using initial high cell density is expected to decrease fermentation time and lower cost;
- The use of genetically engineered yeast with the ability to simultaneous utilizes hexose and pentose sugars are expected to further improve the fermentation process.

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APPENDICES

Appendix 1 - Calibration curves for reducing sugars.

Figure 34 - Standard calibration curve for D-glucose as a function of absorbance (505 nm) by GOD-PAP method using UV-visible spectrophotometer (Femto, Cirrus 80 ST).

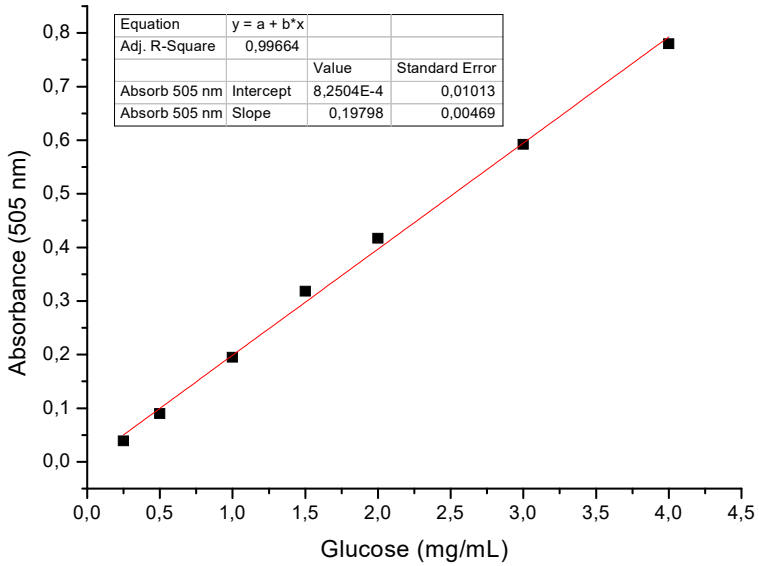


Figure 35. Standard calibration curve for D-glucose by Shimadzu chromatography equipped with a refractive index detector (RID-6A).

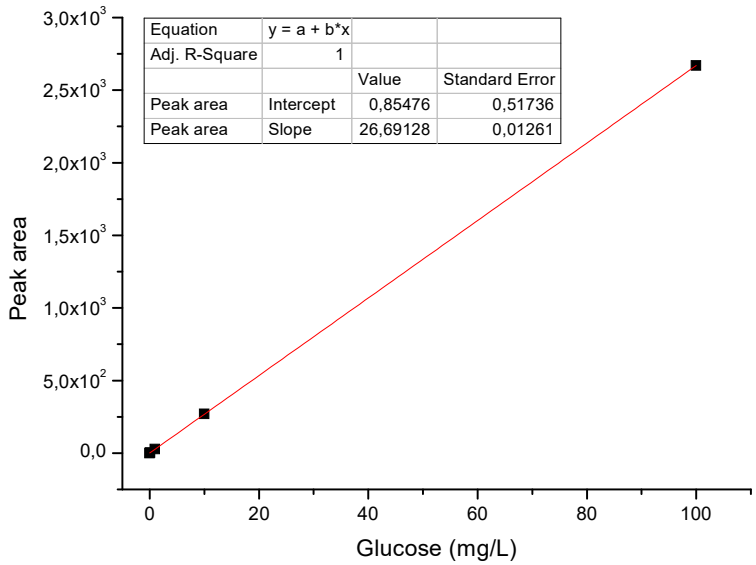


Figure 36 - Standard calibration curve for total reducing sugar (TRS) as a function of absorbance (540 nm) by DNS method using UV-visible spectrophotometer (Femto, Cirrus 80 ST).

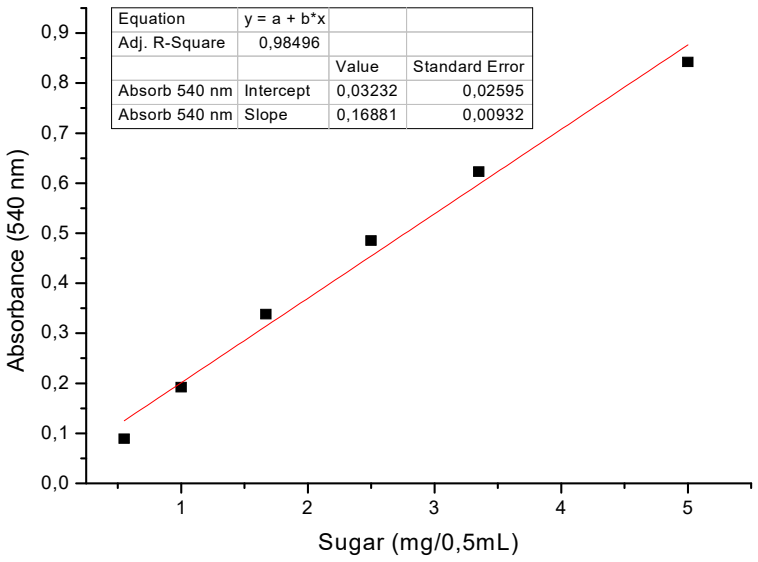
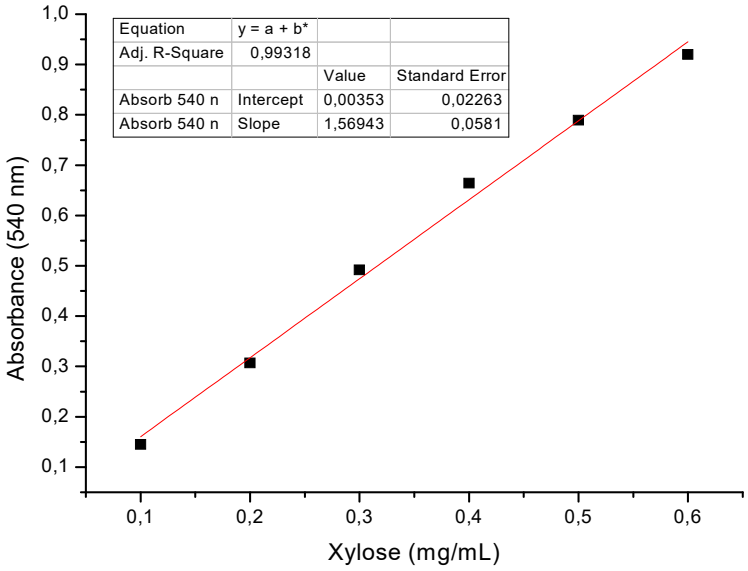
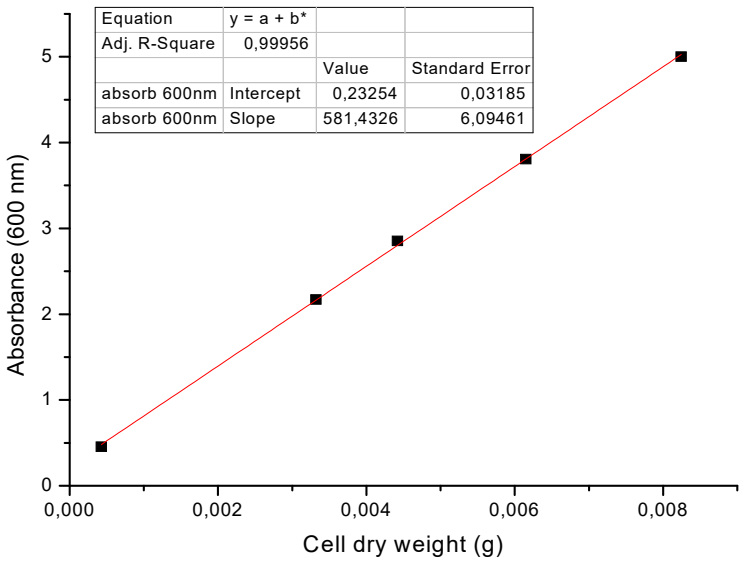


Figure 37 - Standard calibration curve for D- xylose as a function of absorbance (540 nm) by phloroglucinol method using UV-visible spectrophotometer (Femto, Cirrus 80 ST).



Appendix 2 - Calibration curve for *Saccharomyces cerevisiae* IQAr/45-1.

Figure 38 - Standard calibration curve for biomass (cell dry weight) of *Saccharomyces cerevisiae* IQAr/45-1 as a function of absorbance (600 nm) using UV-visible spectrophotometer (Femto, Cirrus 80 ST).



Appendix 3 - Calibration curves for 5-Hydroxymethylfurfural and furfural.

Figure 39 - Standard calibration curve for Furfural by Shimadzu chromatography equipped with a UV-vis detector (SPD-10A).

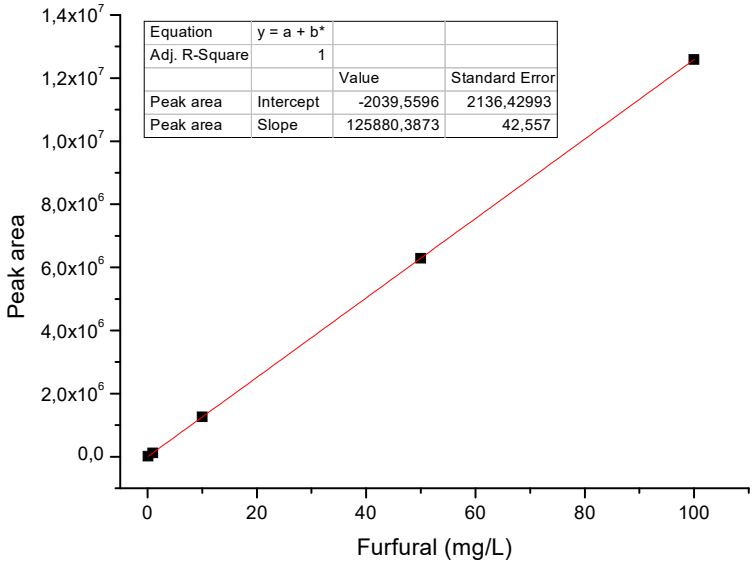
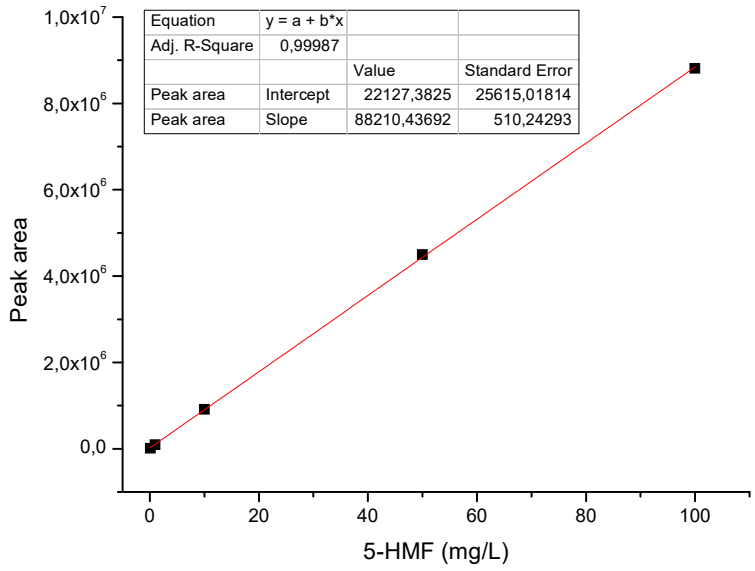


Figure 40 - Standard calibration curve for 5-Hydroxymethylfurfural by Shimadzu chromatography equipped with a UV-vis detector (SPD-10A).



Appendix 3 - Calibration curves for ethanol.

Figure 41. Standard calibration curve for ethanol as a function of absorbance (600 nm) by acidified Potassium dichromate method using UV-visible spectrophotometer (Femto, Cirrus 80 ST).

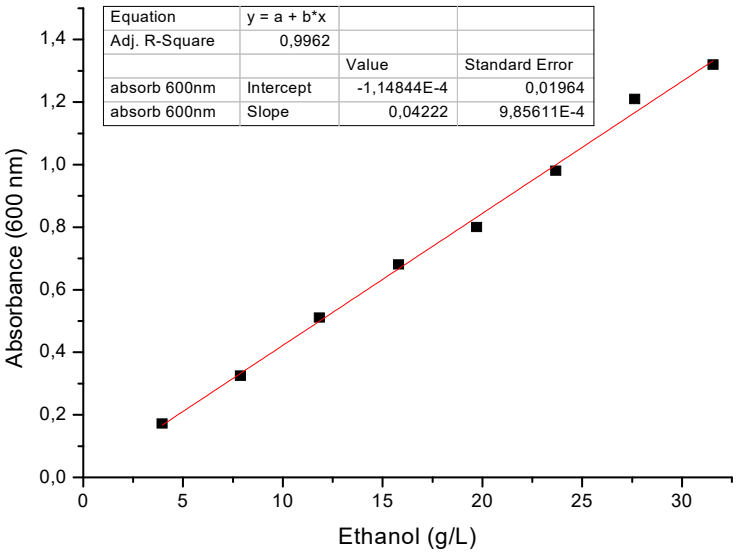
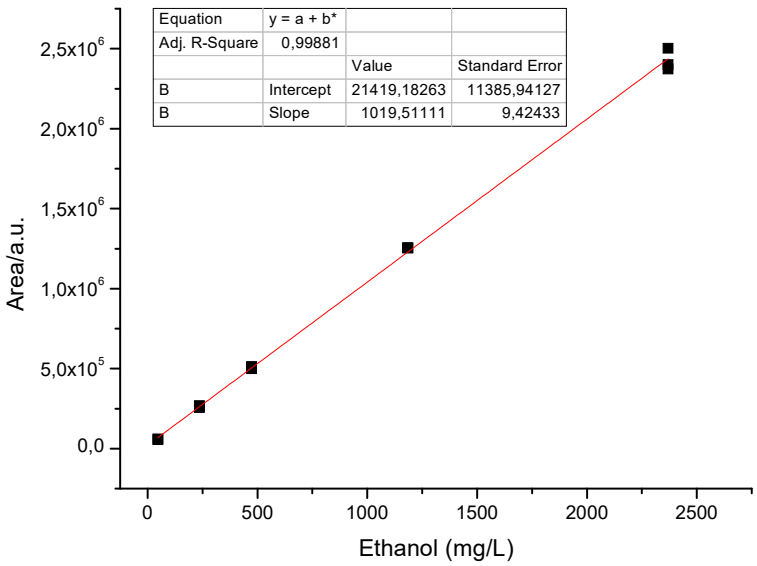


Figure 42 - Standard calibration curve for ethanol by GC-2010 Plus gas chromatograph coupled to a QP2010 mass spectrometer and equipped with an autosampler model AOC-6000 system (Shimadzu, Tokyo, Japan).



Appendix 4 - Raw data submitted to statistical analysis

Table 13 - Effect of reaction time on xylose extraction from SB at 121ºC in autoclave, reference to **Figure 15**.

	Pretreatment time	
	30 min	60 min
NaOH (%, w/v)	Xylose (%, g/g)	Xylose (%, g/g)
0.0	0.48 ± 0.07	1.46 ± 0.36
0.5	2.31 ± 0.84	5.96 ± 0.57
1.0	4.17 ± 0.28	7.86 ± 0.77
2.0	5.08 ± 0.21	9.32 ± 0.53
3.0	7.32 ± 0.53	11.76 ± 0.66

Table 14 - Effects of reaction time and NaOH concentration on the yield of glucan and dry residue from SB after pretreatment at 121°C in autoclave, reference to **Figure 16.**

	Pretreatment time			
	30 min		60 min	
NaOH (%, w/v)	Dry residues (%, g/g)	Glucan (%, g/g)	Dry residues (%, g/g)	Glucan (%, g/g)
0.0	88.00 ± 1.41	45.82 ± 2.16	91.00 ± 1.41	47.21 ± 1.50
0.5	66.00 ± 1.40	46.82 ± 1.32	63.50 ± 0.71	49.47 ± 1.49
1.0	60.00 ± 1.42	54.93 ± 2.06	60.00 ± 1.40	60.31 ± 3.48
1.5	57.00 ± 1.39	58.91 ± 2.63	56.00 ± 1.42	62.97 ± 2.73
2.0	56.50 ± 2.12	64.10 ± 1.69	55.00 ± 1.41	67.89 ± 2.16
2.5	55.50 ± 0.71	62.70 ± 0.47	53.00 ± 1.39	63.30 ± 2.82
3.0	49.00 ± 1.41	60.38 ± 2.44	53.50 ± 0.70	66.36 ± 2.07

Table 15 - Effects of reaction time on the yield of glucan and dry residue during pretreatment of SB with 8% NaOH at room temperature, reference to **Figure 17.**

Time (h)	Dry residue (%, g/g)	Glucan (%, g/g)
1	74.12 ± 1.90	47.15 ± 1.22
3	68.74 ± 0.92	54.93 ± 1.13
6	66.01 ± 1.41	57.98 ± 1.50
12	63.95 ± 1.06	61.90 ± 1.03
24	62.41 ± 1.98	64.30 ± 1.02

Table 16 - Effect of reaction time (30 min and 60 min) and sulfuric acid concentration on soluble reducing sugar (SRS) extraction from SB at 100°C (water bath), reference to **Figure 18.**

	Pretreatment time	
	30 min	60 min
Sulfuric acid (%, w/v)	SRS (%, g/g)	SRS (%, g/g)
0.00	1.40 ± 0.08	1.51 ± 0.07
0.03	1.97 ± 0.26	2.86 ± 0.20
0.05	2.65 ± 0.10	3.04 ± 0.09
0.11	2.91 ± 0.30	4.10 ± 0.43
0.16	3.42 ± 0.27	5.36 ± 0.16

Table 17 - Effect of reaction time (30 min and 60 min) and sulfuric acid concentration on soluble reducing sugar (SRS) extraction at 121ºC (autoclave), reference to **Figure 19.**

	Pretreatment time	
	30 min	60 min
Sulfuric acid (%, w/v)	SRS (%, g/g)	SRS (%, g/g)
0.00	2.39 ± 0.19	2.65 ± 0.06
0.03	6.59 ± 0.24	8.85 ± 0.74
0.05	9.43 ± 0.54	13.83 ± 0.94
0.11	14.19 ± 0.83	18.39 ± 0.33
0.16	20.00 ± 0.88	22.96 ± 1.02

Table 18 - Effects of reaction time and Maleic acid concentration on the extraction of soluble reducing sugar (SRS) from SB at 121ºC (autoclave), reference to **Figure 20.**

	Pretreatment time	
	30 min	60 min
Maleic acid (%, w/v)	SRS (%, g/g)	SRS (%, g/g)
0.1	5.10 ± 0.35	8.50 ± 0.43
0.2	7.12 ± 0.32	10.04 ± 0.58
0.3	9.87 ± 0.25	15.58 ± 0.27
0.4	12.92 ± 0.49	16.86 ± 1.20
0.5	15.04 ± 0.28	19.18 ± 1.11
0.6	16.90 ± 0.45	20.80 ± 0.68

Table 19 - Diffractograms of untreated SB (dewaxed only) and solid fractions from 3%NaOH pretreated SB, 3%NaOH+0.1%SA pretreated SB and 3%NaOH+0.3%MA pretreated SB, pretreatments were carried out at 121 ºC (autoclave) for 1 h, reference to **Figure 21.**

	Peak Intensity		
Sample	Theta (18.5)	Theta (22.5)	Theta (24.5)
Untreated	264	590	273
3% NaOH	245	810	251
3%NaOH+SA	209	1042	265
3%NaOH+MA	205	988	262

Table 20 - Effect of reaction time and sulfuric acid concentration on the yield of glucose from pretreated SB (3%NaOH+0.1%SA and 8% NaOH) during one-step acid hydrolysis in autoclave, reference to **Figure 23.**

	Hydrolysis			
	PSB (3%NaOH+0.1%SA)		PSB (8% NaOH)	
	Glucose (%, g/g of glucan)			
SA (%, w/v)	30 min	60 min	30 min	60 min
2.5	7.04 ± 0.54	7.48 ± 0.33	5.47 ± 0.14	6.43 ± 0.18
5.0	11.11 ± 0.77	16.48 ± 0.28	8.21 ± 0.03	9.97 ± 0.21
7.5	17.32 ± 0.86	22.57 ± 0.95	13.42 ± 0.37	14.42 ± 0.19
10.0	20.89 ± 0.54	27.36 ± 0.99	18.02 ± 0.40	18.21 ± 0.33

Table 21 - Effects of sulfuric acid concentration on two-step acid hydrolysis of SB (pretreated with 8% NaOH at room temperature for 12 h), A_1, A_2 and A_3 represent 30 %, 35 % and 40 % of sulfuric acid respectively in step 1, H_1, H_2 and H_3 represent 1.5 %, 3.0 % and 4.5 % of sulfuric acid respectively in step 2, reference to **Figure 24.**

Hydrolysis steps	Sulfuric acid (%, v/v)	Glucose yield (%,g/g)
$A_1 + H_1$	30% + 1.5%	16.63 ± 1.47
$A_1 + H_2$	30% + 3.0%	14.32 ± 1.14
$A_1 + H_3$	30% + 4.5%	26.83 ± 1.39
$A_2 + H_1$	35% + 1.5%	36.12 ± 1.63
$A_2 + H_2$	35% + 3.0%	32.14 ± 0.90
$A_2 + H_3$	35% + 4.5%	48.80 ± 1.96
$A_3 + H_1$	40% + 1.5%	54.28 ± 1.71
$A_3 + H_2$	40% + 3.0%	52.61 ± 1.14
$A_3 + H_3$	40% + 4.5%	56.87 ± 0.98

Table 22 - (a) Glucose concentration, (b) Glucan conversion (glucose yield) rate during enzymatic hydrolysis of untreated SB (dewaxed only) and solid fractions which resulted from pretreatments with 3% NaOH, 3% NaOH + 0.3% maleic acid (NaOH+MA) and 3% NaOH + 0.1% sulfuric acid (NaOH+SA), reference to **Figure 28.**

	Untreated SB		3% NaOH pretreated SB		NaOH+MA pretreated SB		NaOH+SA pretreated SB	
Time (h)	Glucose yield (%, g/g_{glucan})	Glucose (g/L)	Glucose yield (%, g/g_{glucan})	Glucose (g/L)	Glucose yield (%, g/g_{glucan})	Glucose (g/L)	Glucose yield (%, g/g_{glucan})	Glucose (g/L)
6	11.58 ± 1.85	2.35 ± 0.34	20.06 ± 1.71	7.00 ± 0.56	16.18 ± 2.26	6.65 ± 0.21	15.99 ± 1.44	7.05 ± 0.49
12	13.20 ± 1.00	3.51 ± 0.41	39.07 ± 1.53	13.68 ± 0.53	33.08 ± 2.69	14.94 ± 1.48	29.36 ± 1.92	14.36 ± 0.86
24	17.66 ± 1.73	4.78 ± 0.34	53.65 ± 2.36	20.68 ± 1.86	45.59 ± 2.42	22.14 ± 1.51	43.49 ± 2.55	19.34 ± 0.95
48	23.74 ± 1.47	5.50 ± 0.57	75.84 ± 2.45	28.57 ± 2.01	66.65 ± 4.56	29.58 ± 1.86	65.09 ± 3.97	28.45± 0.78
72	27.95 ± 1.20	6.80 ± 0.48	83.52 ± 3.56	31.78 ± 2.35	79.82 ± 3.07	35.62 ± 3.34	77.94 ± 4.31	35.26 ± 2.44

Table 23 - Sugars concentration after 72 h of enzymatic hydrolysis of untreated SB and solid fractions which resulted from pretreatments with 3% NaOH, 3% NaOH + 0.3% maleic acid (NaOH+MA) and 3% NaOH + 0.1% sulfuric acid (NaOH+SA, reference to **Figure 29**.

	Sugar concentration	
Sample	Glucose (g/L)	Xylose (g/L)
Untreated	6.80 ± 0.48	4.79 ± 0.33
3% NaOH	31.78 ± 2.35	4.04 ± 0.35
NaOH+MA	35.62 ± 3.34	2.59 ± 0.27
NaOH+SA	35.26 ± 2.43	2.40 ± 0.15

Table 24 - (**a**) Concentrations of total reducing sugar (TRS), Ethanol and Biomass, (**b**) Ethanol yield and Productivity, Biomass yield and Productivity during 24 h of fermentation of acid hydrolysate of pretreated SB (pretreated with 8% NaOH solution at RT for 12 h) by the *S. cerevisiae* IQAr/ 45-1, reference to **Figure 32**.

Time (h)	TRS (g/L)	Ethanol (g/L)	Biomass (g/L)	Ethanol (g/g)	Biomass (g/g)	Ethanol (g/L/h)	Biomass (g/L/h)
0	67.31 ± 2.75	2.58 ± 0.51	1.91 ± 0.12	0.00	0.00	0.00	0.00
4	61.79 ± 0.72	3.89 ± 0.48	2.10 ± 0.14	0.24	0.02	0.33	0.05
8	56.99 ± 1.73	5.97 ± 1.45	2.43 ± 0.04	0.33	0.03	0.42	0.04
12	39.92 ± 2.46	11.97 ± 0.75	3.13 ± 0.29	0.34	0.02	0.78	0.06
24	24.90 ± 2.02	21.68 ± 0.32	3.69 ± 0.22	0.45	0.02	0.80	0.04
36	14.88 ± 1.45	26.94 ± 1.59	4.09 ± 0.23	0.46	0.02	0.68	0.03
48	13.14 ± 0.14	24.79 ± 2.06	4.38 ± 0.04	0.41	0.02	0.46	0.03

Table 25 - (**a**) Concentrations of total reducing sugar (TRS), Ethanol, and Biomass, (**b**) Ethanol yield and Productivity, Biomass yield and Productivity during 48 h of fermentation of enzymatic hydrolysate of pretreated SB (pretreated with 3% NaOH at 121ºC for 60 min) by *S. cerevisiae* IQAr/ 45-1, reference to **Figure 33.**

Time (h)	TRS (g/L)	Ethanol (g/L)	Biomass (g/L)	Ethanol (g/g)	Biomass (g/g)	Ethanol (g/L/h)	Biomass (g/L/h)
0	24.92 ± 1.38	0.00 ± 0.00	0.35 ± 0.07	0.00	0.00	0.00	0.00
4	21.60 ± 1.72	1.12 ± 0.26	1.12 ± 0.09	0.37	0.26	0.28	0.19
8	14.46 ± 0.65	4.49 ± 0.31	1.88 ± 0.11	0.43	0.15	0.56	0.19
12	9.75 ± 0.97	6.27 ± 0.40	2.23 ± 0.14	0.41	0.12	0.52	0.16
16	7.04 ± 1.10	6.14 ± 1.17	2.28 ± 0.04	0.34	0.11	0.38	0.12
24	6.83 ± 0.38	4.40 ± 0.51	2.75 ± 0.08	0.23	0.13	0.18	0.10

CURRICULUM VITAE

Name: Longinus Ifeanyi Igbojionu

Name in bibliographic citations: IGBOJIONU, L. I.

Professional address: Institute of Chemistry - São Paulo State University (UNESP) - Bioenergy Research Institute (IPBEN) - Rua Prof. Francisco Degni 55, CEP -14800060, Araraquara - São Paulo, Brazil.

Academic Training

- MSc in Biomolecular Sciences: Vrije Universiteit Amsterdam, Netherlands (2009)
- Bachelor of Technology in Food and Industrial: Federal University of Technology Akure, Nigeria (2001)

Complementary Training

- Master's Internship - Swammerdam Institute of Life Sciences, University of Amsterdam, Netherlands (2009)
- Master's Internship - Royal Tropical Institute (KIT), Amsterdam, Netherlands (2008)
- Master's Intenship - Department of Molecular Genetics, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, Netherlands (2008)
- Students Industrial Work Experience Scheme (SIWES) - Central Public Health Laboratory, Yaba, Nigeria (2000)

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Professional experience

- Federal Polytechnic Ado-Ekiti, Nigeria - Lecturer (2013 - date)
- Unique Tutorial College, Abuja, Nigeria - Science Teacher (2003 - 2006)
- Garki Abattoir, Abuja, Nigeria - National Youth Service Corps (2001 - 2002)