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ALESSANDRA CAMELO

**POTENTIAL OF A NEW SEED PROPAGATED ELEPHANT GRASS CULTIVAR
FOR LIGNOCELLULOSIC BIOETHANOL PRODUCTION VIA ALKALINE
PRETREATMENT**

SÃO PAULO

2020

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PRETREATMENT**

Dissertation presented to the Post-graduation Program in Energy (PPGE) at the Institute of Energy and Environment, University of São Paulo, as partial requirement to obtain the title of Master in Science under Energy Analysis and Planning concentration area.

Advisor: Prof. Dr. Patricia Helena Lara dos Santos Matai

Co-advisor: Dr. Alfredo Eduardo Maiorano

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SÃO PAULO

2020

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“Aonde há vontade, há também um caminho.”

“Where there's a will, there's also a way.”

“Wo ein Wille ist, ist auch ein Weg.”

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ABSTRACT

CAMELO, Alessandra. **Potential of a new seed propagated elephant grass cultivar for lignocellulosic bioethanol production via alkaline pretreatment.** 2020. 97 p. Dissertation (Master in Sciences) – Graduate Program in Energy of the University of São Paulo, São Paulo, 2020.

The new elephant grass PCEA (*P. purpureum* x *P. purpureum*) was specifically developed to meet Brazilian tropical conditions and is the first seed propagated genotype worldwide to be launched by Embrapa Dairy Cattle (CNPGL). The advancement of lignocellulosic bioethanol in Brazil faces numerous challenges, including efficient pretreatments designed for each raw material. In this context, the effect of alkaline pretreatment on PCEA biomass was evaluated to infer about its potential for lignocellulosic bioethanol. The experimental design approached three factors: NaOH % w/v ranging from 1.0 to 3.0 varying on 0.5 % w/v, Temperature (T) from 80 to 120 varying on 20 °C and Reaction Time (RT) from 15 to 35 min varying on 5 min each. The effect of the factors was evaluated considering the response variables of biomass recovery, cellulose and hemicellulose yield, in addition to lignin removal. The untreated biomass and the 17 samples generated from assays were dried and characterized regarding its composition following NREL protocols. The untreated biomass of PCEA presented 31.79±1.55 %wt. of cellulose, 15.51±0.70 %wt. of hemicellulose and 18.23±0.45 %wt. lignin. The observed ash content of 3.94±0.07 %wt. is also important for bioenergy crops, it is desirable on lower values for energy conversion. In addition, the content of 20.54±0.11 %wt. extractives on PCEA indicates further potential to generate biofuels through thermochemical routes. After the alkaline pretreatment, the highest cellulose yield of 58.59 %wt. was achieved in assay 14 at 100 °C, 25 min of RT and NaOH 3% (w/v). Contrary to initial expectations, only alkali concentration statistically differed at 95% confidence level analysis for the full quadratic model at a coefficient of determination (R^2) of 0.78. In contrast, hemicellulose as response for linear with interactions model was significant at 95% confidence level (p-value 0.022), which suggests that the interaction of the three factors altogether had an effect on hemicellulose yield. The highest hemicellulose yield of 25.52 %wt. was reached in assay 9 at 80 °C, 25 min of RT and 2 % w/v concentration of NaOH. The alkali alone had a p-value of 0.000 (ANOVA at 95% confidence level), which suggests an effect of this factor on biomass recovery. The same was not true for the square and linear with interactions models that presented p-values of 0.231 and 0.353, respectively. The coefficient of determination (R^2) for the full quadratic model was 0.92 and the highest recovery of 67.92 %wt. was obtained at the 1.0 %w/v NaOH, at 100 °C and 25 min (assay 13). In conclusion, the optimal condition of the design was established considering efficiency in means of maximal lignin removal without degradation of cellulose, which was achieved at 100 °C, 25 RT and 3 % w/v NaOH with 99.03 %wt. recovery of cellulose and 73.76 %wt. of lignin removal. These results suggest an exciting potential of the new PCEA for lignocellulosic bioethanol production via alkaline pretreatment to be further explored by the Brazilian agroenergy sector. However, further studies should be carried out with enzymatic hydrolysis to confirm high yield of fermentable sugar and consequently, bioethanol.

Keywords: *Pennisetum purpureum*, biomass, biofuel, chemical pretreatment, cellulose

RESUMO

CAMELO, Alessandra. **Potencial de uma nova cultivar de capim-elefante propagada por sementes para produção de bioetanol lignocelulósico via pré-tratamento alcalino.** 2020. 97 p. Dissertação (Mestrado em Ciências) – Programa de Pós-Graduação em Energia da Universidade de São Paulo, São Paulo, 2020.

O novo capim elefante PCEA (*P. purpureum x P. purpureum*) foi desenvolvido para atender às condições de clima tropical do Brasil, sendo o primeiro genótipo propagado por semente no mundo a ser lançado pela Embrapa Gado de Leite (CNPGL). No Brasil, o avanço do bioetanol lignocelulósico enfrenta inúmeros desafios, incluindo pré-tratamentos eficientes designados para cada matéria-prima. Neste contexto, o efeito do pré-tratamento alcalino foi avaliado para biomassa de PCEA a fim de inferir sobre o seu potencial para bioetanol lignocelulósico. O delineamento experimental analisou três fatores: NaOH %w/v variando de 1,0 a 3,0 em 0,5 %w/v, Temperatura (T) de 80 a 120 variando em 20 °C e Tempo de Reação (RT) de 15 a 35 min variando em 5 min cada. O efeito dos fatores foi avaliado para resposta de recuperação da biomassa, rendimentos de celulose e hemicelulose, além da remoção da lignina. A biomassa não tratada e as 17 amostras geradas a partir dos ensaios foram secas e caracterizadas quanto à sua composição de acordo com protocolos NREL. A biomassa não tratada de PCEA apresentou 31,79±1,55 %wt. de celulose, 15,51±0,70 %wt. de hemicelulose e 18,23±0,45 %wt. de lignina. O teor de cinzas observado foi de 3,94 %wt. (±0,07) deve ser considerado para as culturas bioenergéticas, uma vez que é desejável em menor quantidade possível para usos energéticos. Além disso, o teor de 20,54±0,11 %wt. de extrativos no PCEA indica um potencial adicional de geração de biocombustíveis através de rotas termoquímicas. Após o pré-tratamento alcalino, o maior rendimento de celulose de 58,59 %wt. foi alcançado no ensaio 14 a 100 °C, 25 min de RT e NaOH 3% (p/v). Ao contrário das expectativas iniciais, apenas a concentração alcalina diferiu estatisticamente na ANOVA nível de confiança de 95% para o modelo quadrático completo com um coeficiente de determinação (R^2) de 0,78. Em contraste, a hemicelulose como resposta para o modelo linear com interações foi significativa ao nível de confiança de 95% (p-valor 0,022), o que sugere que a interação dos três fatores teve efeito sobre o rendimento da hemicelulose. O maior rendimento de hemicelulose de 25,52%wt. foi alcançado no ensaio 9 a 80 °C, 25 min de RT e 2%w/v de concentração de NaOH. Somente o álcali teve um p-valor de 0,000 (ANOVA a 95% de confiança), o que sugere um efeito deste fator na recuperação da biomassa. O mesmo não ocorreu com os modelos quadrático e linear com interações que apresentaram p-valores de 0,231 e 0,353, respectivamente. O coeficiente de determinação (R^2) para o modelo quadrático completo foi de 0,92 e a maior recuperação de 67,92 %wt. foi obtida a 1,0 %w/v NaOH, T of 100 °C e TR of 25 min (ensaio 13). Com isso, a melhor resposta do design considerando a eficiência quanto à máxima remoção de lignina sem degradar a celulose foi observada a 100 °C, 25 RT e 3 %w/v NaOH com 99,03 %wt. de recuperação da celulose e 73,76 %wt. de remoção da lignina. Estes resultados sugerem um potencial animador do novo PCEA para a produção de bioetanol lignocelulósico através de pré-tratamento alcalino a ser explorado pelo sector agroenergético brasileiro. No entanto, estudos futuros de hidrólise enzimática se fazem necessários, a fim de confirmar o elevado rendimento de açúcares fermentáveis e, conseqüentemente, de bioetanol.

Palavras-chave: *Pennisetum purpureum*, pré-tratamento químico, biomassa, biocombustível, energia renovável, celulose

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

db – dry basis
°C – Degrees Celsius
© – Copyright
DM – dry matter
g – grams
h – hours
ha – hectares
HPLC – high-performance liquid chromatography
kg – kilogram
MC – moisture content
MJ – megaJoule
mg – milligrams
min – minutes
ml – milliliter
mm – millimeters
NREL – National Renewable Energy Laboratory
PCEA – new cultivar of Elephant grass developed by Embrapa Dairy Cattle
® – Registered Trademark
RT – Retention Time, Residence Time, Reaction Time
STP – Standard Temperature and Pressure conditions
T - Temperature
™ – Trademark
ton – tons
%wt. – Weight percentage
% – Percentage

GENERAL SUMMARY

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1. INTRODUCTION

Fossil fuels have powered the world to the way we currently know it, enabling industrial and technological revolution (LISERRE, SAUTER & HUNG, 2010). However, the exploration of these energy sources in ancient times brought many environmental issues, such as emission of greenhouse gases to the atmosphere, which are known as GHGs. Such gases are recognized as accelerators and/or main cause of the climate change associated phenomena experienced nowadays. Adding to this scenario, crude oil will become scarce while its supplies will diminish with population growth and increasing demand of resources (ANDERSON et al., 2016; CAPAREDA, 2014). Moreover, the urgent need of energy transformation is reinforced by the rise of the petroleum barrel price, which recently unleashed a worldwide crisis and led to a strike of truck drivers and civilians in Brazil in the year of 2018. Nowadays, a sudden collapse of oil prices could be observed worldwide mainly due to the coronavirus (Covid-19) pandemic.

If the humanity returns to the previous pace and activities from before the pandemic, then a real paradigm shift is underway. Such shift is expected to transform societies that are highly dependent on fossil fuels into ones based on the use of renewable resources to generate power and biofuels, followed by a more sustainable economic model (CLARK et al., 2006; ZOU et al., 2016). In this context, Brazil is already facing a huge challenge that will extend to next coming decades regarding providence of solutions that meet energy and efficiency demands. At the same time, such solutions must satisfy or overcome sustainable criteria of economicity, supply, security, public health, guarantee of universal access and environmental protection (BRAZIL, 2016). The Brazilian energy sector urges for more attractive solutions regarding diversification of biofuels' offer nationwide that also provide decrease of greenhouse gases emissions, fulfilling the commitment with the Organization of United Nations (ONU) to fight climate change and global warming as established in the Paris Agreement (UFNCCC, 2016).

Hence, although there are no simple solutions to transform the world energy, renewable resources to generate bioenergy have become the most promising and attractive alternative to gradually displace traditional fossil fuels (IEA, 2019). Every renewable solution for energy, such as biomass converted into biofuels, must be profitable with potential for industrial production, sustainable for the environment, and accessible to the population (PATHAK, 2014). Despite the complexity associated with biomass as a feed for biofuels (JACOBSON, MAHERIA & DALAI, 2013), the use of biomass for bioenergy production is an outstanding

field towards the desire of clean energy prevention from alternative sources that are sustainable, affordable, and environmentally benign (HANIF et al., 2016).

According to Capareda (2014), biomass sources include all plants and living matter: agricultural residues such as sugar, starchy and oil crops; algae; animal manure; sewage; municipal solid waste (MSW); industrial waste; and fast-growing trees. Among many possibilities of vegetable agricultural feedstocks for lignocellulosic bioethanol production, the elephant grass biomass outstands combining high yields and resistance to hostile and extreme environments of tropical perennial grasses, as well as cultivation possible in marginal and nutrient-poor lands. The new seed cultivated PCEA (*P. purpureum* x *P. purpureum*) represents interesting potential to bioenergy, including biofuel generation, since they are well known as fast-growing, yield in the field, and as potential grasses for bioenergy conversion (Embrapa Agrobiology, unpublished data). In addition, another important advantage of *P. purpureum* is its responsiveness to Biological Nitrogen Fixation (BNF), where inoculation with N-fixing bacteria can dismiss with the use of nitrogen fertilizers, decreasing CO₂ input in the biomass production chain. Besides sustainability improvement of biomass plantations, there are several aspects of biofuel conversion that remain a huge R&D challenge.

The lignocellulosic bioethanol technologies have emerged as a promising way of generating biofuels adding to the already first-generation model. Bioethanol from perennial grasses, such as elephant grass, can reinforce Brazilian leadership on sustainable development goals and honor international agreements. Furthermore, bioethanol from PCEA could contribute with security and supply of this biofuel, complementing first-generation bioethanol from sugarcane residues and plant parts. Nowadays, the main lignocellulosic bioethanol R&D challenges are the identification of the best pretreatment strategies for specific biomass resources, in addition to the very expensive costs of enzymatic hydrolysis. Furthermore, elephant grass has been under investigation considering a few pretreatments aiming higher yields of lignocellulosic bioethanol generation, with the chemical pretreatment using alkali being considered the most promising one and therefore chosen to be tested to evaluate the potential of the new PCEA in the present study.

2. HYPOTHESIS

PCEA is an elephant grass genotype with potential for lignocellulosic bioethanol production, once this perennial biomass is usually compound by interesting amounts of

cellulose in the form of lignocellulose and hemicellulose. Considering the drawbacks of acid chemical pretreatments, the righteous chemical approach nowadays follows alkaline pathways in order to expose cellulose for enzymatic hydrolysis conversion aiming fermentable sugars. Analyzes such as compositional characterization of PCEA biomass prior and after alkali pretreatment, as well as efficiency of enzymatic hydrolysis and fermentation are important for validating feasibility and efficiency of the process performed for the first time for this specific newly developed crop.

Therefore, the hypothesis to be tested is:

- Is the alkaline route an effective pretreatment to expose cellulose of PCEA biomass?

3. JUSTIFICATION

The new elephant grass PCEA is the first genuine cultivar (*P. purpureum* x *P. purpureum*) in the world to be grown from seeds and offers many advantages, such as transportation from seed seller to the field and allows fully and more efficient mechanized operations, which was previously impossible to be achieved in cultivation systems via stems. More importantly, PCEA was developed considering Brazilian edaphoclimatic conditions and has been observed responsive to Biological Nitrogen Fixation (BNF) when inoculated with N-fixing bacteria, which dispenses with the use of artificial fertilizers and contributes with economic feasibility and lower or zero net of the GHGs emissions. Furthermore, the new genotype has been cultivated in the field and observed superior over a commercial genotype, offering a reliable option to contribute with the security of the Brazilian agroenergy sector.

The establishment of lignocellulosic bioethanol still faces technological and operational challenges. One of these challenges is the chemical pretreatment and the key role it plays in an integrated lignocellulosic biomass conversion process aiming bioethanol. The efficient design and performance of such pretreatment is particularly important, considering that planning and execution of subsequent steps of saccharification and fermentation rely strongly upon the components obtained and the overall recovery. An efficiently designed and adequate pretreatment have the potential to positively impact the profit at the end of the process. Adding to that scenario, different raw materials such as PCEA should be investigated to infer about the best approach possible, including alkali and optimal operational parameters.

4. OBJECTIVES

The main objective is:

Investigation of alkaline pretreatment applied to PCEA biomass for lignocellulosic bioethanol production.

The specific aims follow:

- (1) compositional characterization of the new elephant grass PCEA biomass;
- (2) effect of alkaline pretreatment using Surface Response Methodology (Central Composite Design) on:
 - (2.1) yield of components;
 - (2.3) biomass recovery; and
 - (2.4) water use.

5. LITERATURE REVIEW

5.1 Agricultural grasses feedstocks for bioenergy generation

Gramineae feedstocks for bioenergy conversion vary worldwide. Agricultural crops and wastes, including cotton gin trash (HANIF et al., 2016), rice straw (NAM et al., 2015) and rapeseed cake (SMETS et al., 2013) have been applied as energy sources. Given the variety of options, for specific regions of the globe, some crops offer more advantages than others in order to be converted into by-products and generate bio-based energy.

According to Campbell et al. (2008), the use of abandoned agricultural lands to grow bioenergy crops such as perennial grasses aims to avoid deforestation along with consequent carbon stored emissions in the atmosphere. In addition, C4 warm-season grasses and responsive to N-fixing can be high-yielding in marginal areas, benefiting these areas with nutrient leaching and minimizing soil erosion occurrence (SAMSON et al., 2005). For example, the perennial grass cropping systems possible on marginal lands are estimated to produce as many as 377 million tons of biomass in the U.S. alone (PERLACK et al., 2006). In Brazil such areas are nowadays considered along with permanent and protection areas at INPE database (Brazilian National Institute for Space Research), which makes it difficult to infer about marginal areas available for bioenergy plantations. In that sense, it is also hard to predict potential yield of biomass if such data on available hectares of marginal lands are unknown or unprecise.

However, there are still data dedicated on marginal lands obtained through the agroecological zoning for energy cane, which can be considered for elephant grass once the concept marginal land did not change and the grasses are very similar in means of yield and nutritional requirements. In a study developed by Violante (2012), approximately further 32.3 million hectares of marginal lands are suitable for the cultivation of grasses such as elephant grass and energy cane. Altogether, both use of marginal lands and abandoned agricultural areas beside particular advantages, imply less competition with arable lands, resulting in less food security threats.

Regarding responsible and smart choice of biomass, some characteristics and facts must be considered prior to defining which crops specific regions in the globe can rely on as bio sources for energy conversion. Sawasdee & Pisutpaisal (2014) imply that for choosing the ideal biomass it is crucial to consider the yield per hectare, facile agricultural operations and storage, resistance to hostile weather conditions, as well as soil of mediocre or poor quality regarding nutrient availability.

Furthermore, vegetal biomass plays a fundamental role on bioenergy generation in agricultural-based, countries under development. The annual Brazilian Energy Balance document of 2017 shows that 25% of the greatly diversified internal energy supply in Brazil was obtained exclusively from sugarcane, firewood, and charcoal. An outstanding feedstock known as elephant grass figured among few sources on the installed capacity of the electricity generation section, with 32 MW power capacity in 2018 (EPE, 2019). Besides difficulties reported, including low density and drying operations of available elephant grass cultivars to supply thermoelectric power plants in Brazil, the main challenge remains the lack of high yielding and adaptable elephant grass cultivars (EPE, 2016). In that sense, the new elephant grass PCEA is exactly the contribution of agronomic breeding groups (Embrapa Gado de Leite) to address the main issue of feedstock security experience at biomass fed power plants in Brazil.

5.2 Elephant grass (Napier grass)

Elephant or napier grass (*Pennisetum purpureum*) are monocotyledonous flowering plants that belong to the Poaceae family and the genus *Pennisetum* (BRUNKEN, 1977). This grass with fast-growing C4 metabolism is native from sub-Saharan Africa, currently cultivated along tropical and subtropical regions of the globe (NEGAWO et al., 2017). The introduction of the Napier grass in the Americas occurred in 1913. This grass received various names, including giant grass, grass-African-cane, and Napier in honor of its discoverer (JAUHAR, 1981).

According to Faria (1994), elephant grass was first introduced in Brazil in the State of Rio Grande do Sul. This first contact of Brazilians with this Poaceae occurred in the year of 1920 and the material provided was originated from the United States. In the following years, the Ministry of Agriculture worked on the development of a distribution plan for varieties of this grass within the entire Brazilian territory (OTERO, 1961). Since then, elephant grass has been used in Brazil mainly as a forage for dairy cattle, as well as bioenergy crop with increasing interest for biofuels (MARAFON et al., 2016).

Nowadays Brazil counts with several institutions that act with excellence in the investigation and development of elephant grass varieties and cultivars. Among these recognized institutions stand the Agronomic Institute of Pernambuco (IPA), the Agronomic Institute of Campinas (IAC), and Embrapa Dairy Cattle and Temperate Climate (Centro Nacional de Pesquisa em Gado de Leite – CNPGL – and Centro Nacional de Pesquisa em Clima Temperado – CNPCT, respectively). Initially, these institutes have developed genotypes aiming at animal feed to boost Brazilian meat sector for intern supply and exportation. The genotypes Pioneiro (BOTREL et al., 2000), Cameroon (ANDRADE et al., 1990), BRS Canará for Cerrado's region (JANK et al., 2014), BRS Kurumi and more recently BRS Capiaquê (PEREIRA, LÉDO & MACHADO, 2017) figure among famous elephant grass developed for Brazilian edaphoclimatic conditions. Such genotypes have occupied considerable hectares in plantations, with its high dry-matter yield provided extra biomass that was smartly applied for bioenergy generation by small and medium farmers.

Within time, the use of this multipurpose forage grass for bioenergy generation started to gain the attention of Brazilian breeding groups. However, the development of energy dedicated elephant grass genotypes by Brazilians institutes is quite new and very challenging, especially considering that the ideal composition is very different from vegetal materials to generate bioenergy and to be digested by cattle. The only desirable thing in common for both uses would be cultivation possible to be carried out by seeds materials instead of the current stem cultivation system. In addition, a few genotypes initially developed to meet animal feed have been evaluated as raw materials for conversion processes to guide further breeding studies.

In this context, biomass from various elephant grass genotypes have been evaluated regarding yield (FAVARE et al., 2019; MENEZES et al., 2014) and characteristics in specific plantations for bioenergy purposes (FLORES et al., 2013; FLORES et al., 2012). Furthermore, *P. purpureum* genotypes have been investigated for direct combustion (MORAIS et al., 2009; MORAIS et al., 2018; ROCHA et al., 2017), pyrolysis application (BRAGA et al., 2017;

SOUSA et al., 2016) and, more recently, lignocellulosic bioethanol (CARDONA et al., 2013; CARDONA et al., 2014; STANLEY et al., 2017; GODINHO et al., 2019).

Elephant grass is an efficient C4 Gramineae with high potential for biofuel generation (MORAIS et al., 2012). The majority of studies performed with elephant grass up to nowadays focus on its potential as solid fuel, including vegetal coal (QUESADA et al., 2004), pellets and briquettes (MARAFON et al., 2014), and by-products generated through thermochemical conversion process (CAMELO et al., 2018). The energy efficiency of elephant grass stalk cultivation system has been reported as a positive balance of 1:15, which means that for each unit of energy employed in the process of biomass growth including mechanical field operations, 15 units of energy are produced (MORAIS et al., 2018). In a study performed by Mazzarella and collaborators, this high yielding perennial was compared with eucalyptus as feedstock for ceramic industry by means of energy balance. Results showed an optimistic perspective on maximum balance ratio of up to 20.65 for elephant grass over the consolidated energy source eucalyptus, which reached a maximum ratio of 11.35.

More recently, several studies have been performed considering elephant grass as a potential raw material for lignocellulosic bioethanol production, including Cardona et al. (2013; 2014) and Phitsuwan et al. (2015; 2016). However, such important contributions do not crystal-clear feedstock source, and it is most likely that elephant grass is cultivated by stalks and imported hybrids with pearl millet in the case of importation. Furthermore, the feedstock biomass for bioethanol generation varies depending on availability, cost-effectiveness, and government incentives. Once high yielding in the field, the new hybrids and genotypes can play a key role on the agroenergy sector as reliable feedstocks with great potential for biofuels generation.

In addition, this perennial grass has been investigated regarding its potential to be better explored for sugar under different harvest regimes (LI et al., 2015), dry matter yield and N accumulation through BNF in the field (MORAIS et al., 2012). This C4 grass is responsive to BFN and has been reported to reach up to 40 tons ha⁻¹ of dry matter annually in tropical climate regions (MARAFON et al., 2014).

5.2.1 Biological Nitrogen Fixation (BNF) to improve sustainability of elephant grass biomass for bioenergy purposes

Several studies have concentrated efforts in developing and applying more sustainable operations in agricultural systems, such as bioenergy plantations, including Biological Nitrogen

Fixation (BNF). Among many known advantages, the BNF mainly allows sharp reduction on the use of synthetic nitrogen fertilizers, once it provides assimilable N through the nitrogen fixation/assimilation complex mechanisms during interaction of diazotrophic bacteria with BNF responsive plants (HENNECKE et al., 1985).

The discovery and elucidation of this remarkable mechanism is quite interesting. Already in the fifties Dr. Johanna Döbereiner and her research group from the National Center of Education and Agricultural Research (Ministry of Agriculture), Seropédica, RJ State, studied the occurrence of *Azotobacter* in acid soils of the "Baixada fluminense" (DÖBEREINER 1953). Baldani & Baldani (2005) imply that the research group started to gain visibility with the discovery of two specific nitrogen-fixing bacteria in association with grasses: *Beijerinckia fluminensis* with sugarcane (Döbereiner & Ruschel 1958) and *Azotobacter paspali* with *Paspalum notatum* cv. batatais (Döbereiner 1966). From then on, the research group approached several study lines focusing on sustainable agriculture always developed under the guidance and leadership of Dr. Johanna, including elucidation of diverse aspects of BNF and approach on association of various diazotrophic bacteria in endophytic symbiosis with non-leguminous plants. Interestingly, the broad work with diazotroph bacteria with Leguminosae and Poaceae and its potential to fight starvation worldwide have led to the indication of the Brazilian scientist Dr. Johanna Döbereiner to the 1997 Edition of Nobel Prize in Chemistry.

Besides environmental advantages, BNF has enormous economic potential with Poaceae to be explored as it achieved with Leguminosae. As of an example, Brazil has the main BNF successful case known worldwide: soybean inoculated with rhizobia resulting in an economy up to US\$ 14 billion dollars annually – depending on cultivated area (ha) and Real (R\$) to US Dollar (\$) exchange rate (EMBRAPA, 2018). Among many advantages depending on location, culture and purpose, BNF save mineral nitrogen in agricultural systems, provides reduction of production costs, and also allows reduction of GHG emissions. As an important drawback, the technology faces the inapt ability of farmers to handle precisely the inoculants, which usually causes inefficiency of the system such as low yield, affecting production costs and GHG emission.

As generally underestimated as merely sustainable N supply in assimilable form to crops, BNF involves way more already elucidated by science and also probably much more features to be investigated and discovered. The diazotroph bacteria known as Plant Growth Promoting Rhizobacteria (PGPR) and Bacteria (PGPB) can biosynthesize phytohormones such as auxins, including indole acetic acid – IAA (CASSÁN, VANDERLEYDEN & SPAEPEN,

2014), gibberellins and abscisic acid plant hormone – ABA (COHEN, BOTTINI & PICCOLI, 2008), cytokinin (EVSEEVA et al., 2011), among others. There are strains that present nitrogenase activity, indole production, and phosphate solubilization altogether. One of them is the LP343 (*Gluconacetobacter diazotrophicus*) first isolated from leaves of elephant grass (Videira et al. 2012). This strain was later re-inoculated in elephant grass on field for biomass aiming bioenergy application purposes, which happens to be the PCEA biomass investigated for lignocellulosic bioethanol production in this study.

5.2.2 PCEA (*Pennisetum purpureum* x *Pennisetum purpureum*): The first genuine seed propagated elephant grass genotype

PCEA is the first genotype of elephant grass in the world propagated by seeds. PCEA was obtained from the interbreeding of 37 different genotypes (interbreeding of *P. purpureum* x *P. purpureum*) which was subsequently submitted to several selection cycles by the breeding group at Embrapa Dairy Cattle. Therefore, this biomass has been specially developed for Brazilian edaphoclimatic conditions with the unique advantage of seed propagation. In that context, the PCEA offers advantage over other elephant grass genotypes, which have been exclusively cultivated by stalks and seedlings, by allowing full mechanized agricultural operations, lowering even more the GHG emissions in bioenergy plantations.

Elephant grass genotypes such as PCEA have a high potential in biomass production for biofuels. *Pennisetum purpureum* is widely cultivated in tropical climate regions as perennial grass capable of high dry biomass production (MARAFON et al., 2013) regarding its high photosynthesis efficiency. Grasses such as PCEA are mostly employed nowadays as raw material for lignocellulosic bioethanol production (SCHOLL et al., 2015a), silage for ruminants (GUERRA et al., 2016) or graze. *Pennisetum purpureum* was reported as potential grass to bioethanol production through a variety of combinations of the pretreatments with biological treatments (YASUDA, ISHII & OHTA, 2014).

New genotypes rich in protein to cattle feed are no longer of interest when the final purpose of plantations are of bioenergy generation. Instead, plants with high content in fiber, lignin and C:N ratio, combined with high dry matter yields and other desirable effects from biological nitrogen fixation (BNF), adding minimal consumption of fossil energy within field mechanized operations (QUESADA et al., 2004). Thus, older genotypes propagated by stalks will be gradually displaced by new seed propagated varieties that outstand as feedstock for

biofuel generation, such as the established intersectional hybrids with pearl millet and the genuine new genotype PCEA.

Additionally, another outstanding data for PCEA is that the production and quality (germination and purity) of the seeds are much higher than those obtained by commercial hybrids of elephant grass x millet (PMN hybrids). It also has similar persistence to that presented by the cultivars of *Pennisetum purpureum* of vegetative propagation. The genotype is expected to be launched as a new crop soon (EMBRAPA DAIRY CATTLE, 2017 – unpublished data). Furthermore, the new grass also meets at least two of the main goals of the Sectoral Plan of Mitigation and Adaptation to Climate Change for the Consolidation of a Low-Carbon Agriculture Economy - ABC Plan (Plano ABC de Agricultura de Baixo Carbono, in Portuguese). The grass increases atmospheric retention of CO₂, including negative net depending on local agriculture for specific application purposes, and also adoption of sustainable production systems to raise the income of producers through BNF (BRAZIL, 2016).

To contribute with Embrapa Dairy Cattle by providing additional data related to the cultivar, Embrapa Agrobiology has performed inoculation studies with PCEA in laboratory, greenhouse, and experimental fields. Such studies aimed to supply assimilable N to PCEA along with specific plant-bacteria association, dispensing with the use of nitrogen fertilizers and therefore CO₂ input in the biomass production chain. For PCEA inoculated with LP343 via leaf spraying, means of growth density 0.5 m between lines x 0.5 m between plants (CAMELO, et al., 2020). The PCEA cultivar of elephant grass presents potential to enhance biomass availability for lignocellulosic bioethanol production, contributing with the already successful sugarcane bioethanol industry in Brazil.

5.3 Lignocellulosic feedstocks for bioenergy generation

As a result of the photosynthesis reaction, the vegetal biomass growth depends on the solar energy (photons), the presence of water (H₂O) and carbon dioxide (CO₂), in addition to other very specific requirements dependable on every living plant specificity. The energy accumulated in biomass is then stored in chemical bonds of the vegetal structural components through complex photosynthetic processes (BNDES, 2008). Therefore, vegetal biomass feedstocks stand out with significant potential for energy generation, and even further, provide useful series of chemical products, materials, and fuels (CLARK et al., 2006; HANIF et al., 2016).

Environmental concern increases urgent demand for energy sources such as biomass that can provide lower or zero net balance of greenhouse gas emissions through its production and use chain (CAPAREDA, 2014), along with sharp reduction of sulfur and particulate emissions avoiding human health and nature externalities. As a matter of fact, the harmful respiratory effects of air pollutants have been investigated and determined to be caused by particulate matter (PM) components (RISTOVSKI et al., 2011). Levels of fine PM in the air have been associated with the risk of death from all causes including cardiovascular and respiratory illnesses (POPE III et al., 2002) and fine PM sulfur oxide-related pollution associated with lung cancer and cardiopulmonary mortality (COHEN et al., 2006).

Vegetal biomass has been widely applied worldwide as an energy source, more recently receiving attention due its potential as raw material for biofuels such as lignocellulosic bioethanol. According to Bridgwater et al. (1999), composition of lignocellulosic feedstock consists mainly of cellulose, hemicellulose, lignin and minor amounts of other organics, arranged in the vegetal kingdom. Such composition of lignocellulosic biomass plays a crucial role in the performance and efficiency of pretreatment and biodegradation stages (MOOD et al., 2013), once known composition ease definition of the best strategy for cellulose exposition and final bioethanol production.

5.4 Vegetal cell wall

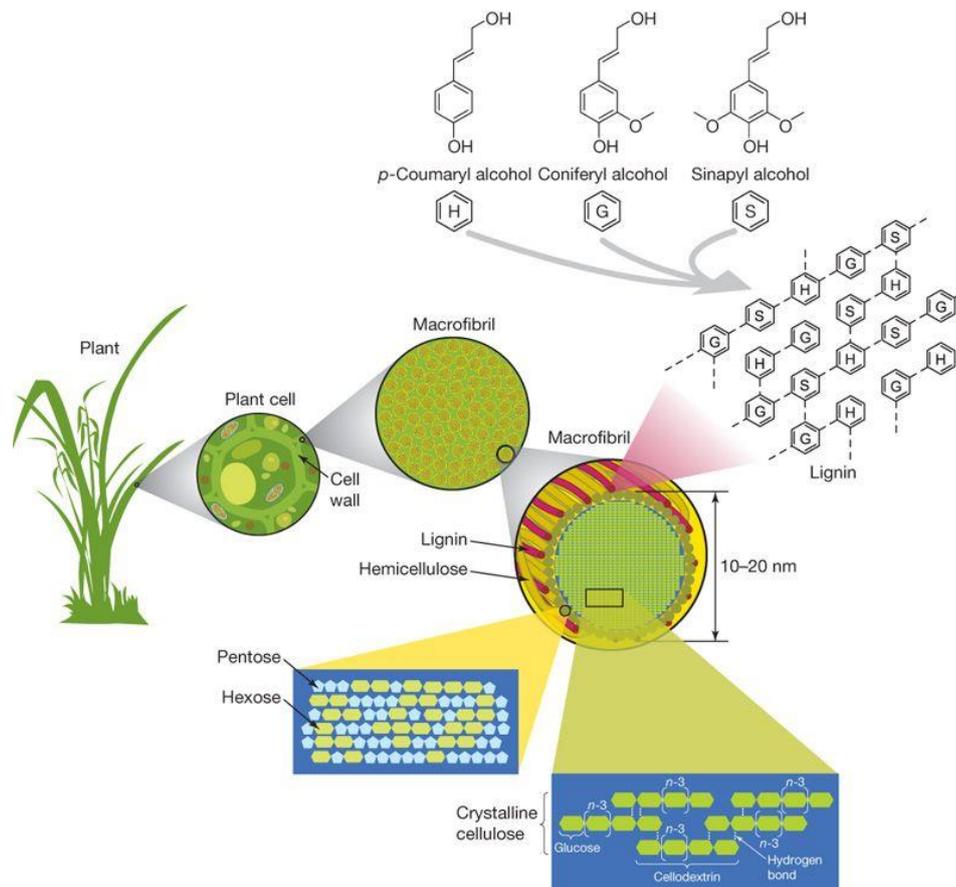
In grasses, the chemical structure of these walls differs from most flowering plant species. Nevertheless, it has been observed that in all cases conform under the influence of the same physical laws. The Poaceae (Gramineae) family of monocotyledons flowering plants, which includes elephant grass plants, presents specific cell wall composition (CARPITA & GIBEAUT, 1993). The vegetal cell walls of Poaceae plants are generally compound by primary and secondary cell wall structures, which differ regarding arrangement, mobility and structure of matrix polymers, organization of microfibrils, in addition to their roles in the life of the plant. Additionally, this composition can also greatly differ depending on the development stage of the plant (COSGROVE & JARVIS, 2012).

The primary cell walls of these monocotyledons' grasses consist mainly of cellulose, hemicelluloses, small amounts of pectic polysaccharides and xyloglucans compared with the presence of heteroxylans and (1,3;1,4)- β -D-glucans. Cellulose and lignin levels have been observed higher in secondary walls than in primary walls (BURTON, GIDLEY & FINCHER,

2010). In addition, the composition of the primary wall provides strength to withstands tensile forces generated from turgor pressure (HAMANT & TRAAS, J., 2009).

The second cell wall has hemicelluloses compound by polysaccharides of the xylan type with smaller amounts of a xyloglucan. Microfibrils also compose second walls and have been described as three highly structured layers (REID, 1997). Interestingly, cells with secondary wall-thickenings rich in lignin are very important in conferring rigidity on plant tissue, specifically those of the vascular tissues such as xylem vessels and phloem fibers (COSGROVE, 2005). Furthermore, a representation of vegetal cell wall architecture with macro arrangement of different compounds and microfibrils can be observed in Figure 5.1.

Figure 5.1 – Cellulose, hemicellulose and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall (dd)



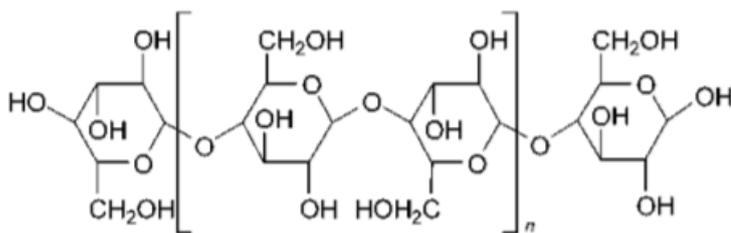
Source: RUBIN (2008).

5.4.1 Cellulose

Cellulose is the most abundant component of plant and vegetal biomasses, existing in nature almost exclusively within plant cell walls and also in minor extent produced by some animals and few bacteria (LYND et al., 2002). Cellulose can be further defined as the most

abundant and useful biopolymer on the Earth (COSGROVE, 2005) compound by a long chain of glucose molecules (d-glucose subunits) covalently linked by beta bonds (β -1,4 glycosidic bonds), with many of the neighboring chains typically linked together by hydrogen bonding in a crystal structure (HOLTZAPPLE, 2003a). Therefore, cellulose $(C_6H_{10}O_5)_n$ structure as shown in Figure 5.1 is insoluble in water and most organic solvents (SWATLOSKI et al., 2002).

Figure 5.2 – An example of cellulose polymer chain, where n can be found ranging from 400 to 1000



Source: SWATLOSKI et al. (2002).

The natural crystalline structure presented by cellulose in the environment can be rather defined as relatively unusual in the polysaccharide world. Such crystalline form is believed to be most probably regulated by associate hemicelluloses (ATALLA et al., 1993). This organic compound is synthesized in nature as individual molecules (linear chains of glucosyl residues), which undergo self-assembly at the site of biosynthesis (BROWN & SAXENA, 2000). Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils (protofibrils), which are packed into larger units called microfibrils, and these are in turn assembled into the familiar cellulose fibers. As everything in nature stands in the equilibrium position that requires less energy, or minimum conformal energy, hydrogen bonds and van der Waal forces can be found in these structures.

Moreover, Lynd and collaborators (1999) imply that the plant cell wall is very complex, and the disposition of cellulose within the wall varies across plant taxa and contributes to the complexity of this vegetal tissue. However, a unifying feature of the plant cell wall is its cellulose content, typically present in the range of 35 to 50% plant dry matter basis. Scientists were able to elucidate cellulose biosynthesis within time, including differences when compared with cellulose from microorganisms through investigation of a few bacteria, such as *Acetobacter xylinum*.

5.4.2 Hemicellulose

Hemicelluloses are polysaccharides that together with cellulose, pectin and lignin, constitute plant cell walls. This secondary vegetal cell wall with chemical formula $(C_5H_8O_4)_n$ is defined by Gírio and collaborators (2010) as heterogeneous branched biopolymers that consist of pentoses (β -d-xylose, α -l-arabinose), hexoses (β -d-mannose, β -d-glucose, α -d-galactose) and/or uronic acids (α -d-glucuronic, α -d-4-O-methylgalacturonic and α -d-galacturonic acids).

This heterogeneous class of polymers represents 15–35% of plant biomass (GÍRIO et al., 2010). Hemicelluloses are the most thermal-chemically sensitive molecule when compared with cellulose and lignin, being usually the first portion to react when thermochemically pretreated (BAUGH, LEVY & MCCARTY, 1988).

The solubility of hemicellulose polysaccharide contents increases with increasing temperature, although this logic cannot be simply applied for higher molecular polymers owing to the unknown melting points (GRAY, CONVERSE & WYMAN, 2003). In addition, further characteristics and parameters such as moisture content and pH have been also reported to have an effect on lignocellulosic materials solubilization, along with temperature (FENGEL & WEGENER, 1984).

5.4.3 Lignin

Lignins are complex structures of polymers of aromatic compounds. After cellulose, lignins are the second most abundant compound in nature (EMBRAPA, 2011a) and source of approximately 30% of the organic carbon on earth. Interestingly, the biosynthesis of lignin in ancient times has allowed evolution and successful adaptation of plants from aquatic to terrestrial environment (BOERJAN, RALPH & BAUCHER, 2003), providing needed strength for an erect habit (VANHOLME et al., 2010).

The presence of lignin in vegetal cell walls plays a fundamental role in providing structural strength, sealing of the water conducting system that links roots with leaves, in addition to protection against degradation by weathering and also biodegradation (GLASSER, 1985). Such important features are provided by lignin along with hemicellulose and pectin fulfilling spaces among cellulose fibers. Lignin bonds components together through covalent and hydrogenic linkages, which make the cell wall an extremely strong and recalcitrant structure (PONNUSAMY et al., 2019). Although very desirable in the plant kingdom, this

component must be removed along with its caused recalcitrance in biomass for bioethanol production.

5.5 Bioethanol: the fuel from vegetal biomass feedstocks

Bioethanol is the product from fermentative process of sugar and obtained not only from sucrose in sugar crops and starch presented in starchy crops, but also from carbohydrate fractions such as hemicelluloses and celluloses, after appropriate pretreatments, available in lignocellulosic raw materials (GALBE & ZACCHI, 2012). This biofuel represented by the chemical formula C_2H_6O outstands regarding its production from renewable fast-growing biomass. The lifecycle of this biofuel includes agricultural production of energy crops, bioethanol production, sugar and electricity co-production (LUO, VOET & HUPPES, 2009).

This biofuel has been employed since the 19th century, with the development of internal combustion engines first prototypes back in the 19th century by Samuel Morey (1826) and Otto Nicholas (1876). The first car developed by Henry Ford in 1896 could use pure ethanol as fuel, and in 1908 the Ford Model T (flexible vehicle) could be adjusted to ethanol and bioethanol as fuel in the same way as gasoline or any mixture of two (SOLOMON, BARNES & HALVORSEN, 2007).

The need for fuel during World War I increased demand for ethanol in the US, so the fuel was widely used in Europe and the United States in the beginning of the 20th century. After the war, there was a decrease in demand since it has become more expensive to produce ethanol than petroleum-based fuels, such as gasoline and diesel. However, there was interest (Ford Motor Company, General Motors Corporation and DuPont, for example) in ethanol as much as an antiknock agent (higher 29 octane), and as a possible substitute for oil (SOLOMON, BARNES & HALVORSEN, 2007; MUSSATTO et al., 2010).

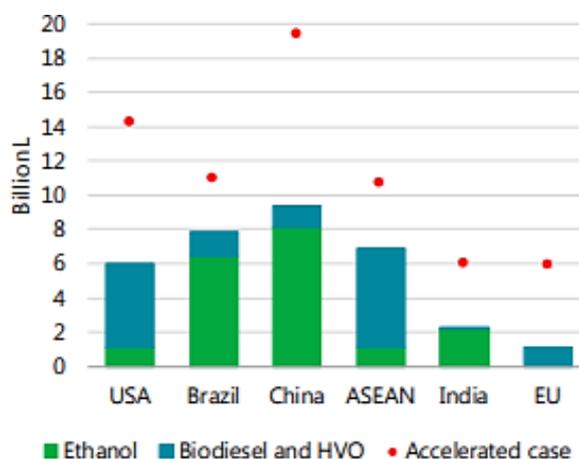
In Brazil, the first experience with bioethanol as biofuel dates back from the 1920s. The beginning and establishment of this biofuel was powered to relief crisis on the sugar sector and also to reduce the dependence on imported fuel, very unstable regarding price. In 1933, the Getúlio Vargas Administration passed the Law 737, which made mandatory a mixture of alcohol in gasoline, and created the *Instituto do Açúcar e do Alcool* – IAA (in English, Sugar and Alcohol Institute), responsible for boosting the bioenergy sector in the country (LEITE & CORTEZ).

Bioethanol properties and characteristics can promote cleaner combustion and better performance of engines, acting as an additive capable of improving the anti-knock quality of

gasoline (higher octane). In addition, its use reduces the emission of pollutants by replacing additives promoters of octane that have environmental restrictions, such as lead tetraethyl and MTBE (Methyl tert-butyl ether), which have been banned in several countries (BNDES, 2008). This biofuel figures among the best options for displacement of fossil fuels, perhaps a transition fuel to drive Brazilian fleet to the future cars powered by batteries and fuels cells. However, it is always reasonable to point out that renewable alternatives such as bioethanol have also drawbacks. In that context, despite the importance of comprehensive discussion of the attractiveness and backlashes of biofuels alternatives compared to petroleum and chemical industry, this analysis is way further the scope of this dissertation, which will only be approaching few issues of lignocellulosic production process.

Interestingly, in 2018 Brazil reached production records of ethanol production, representing 60% of the world expanded production increase of 6% y-o-y in 2018, to 110 billion. The projected growth of biofuels in means of billion liters for the next five years, from 2019 to 2024, is presented for key markets in Figure 3.5. In this projection, bioethanol outstands among high calorific biofuels, such as biodiesel and hydrated vegetable oil (HVO), with Brazil standing right next to China in 2nd position with roughly more than 6 billion liters. Furthermore, the International Energy Agency has given positive predictions for the sector. To that, IEA has considered continued output growth potential to improve production economics and the supportive environment for capacity investment provided by new policy would create at this launch in 2020 (IEA, 2019). It is important to highlight, that due to the Coronavirus Pandemic outbreak (Covid-19) the sector faces sharp reduction in demand, which several agencies and research institutes are trying to forecast impacts on the energy sector worldwide.

Figure 5.3 – Projected biofuels growth in key markets for the next 5 years of 2019 to 2024



Source: IEA (2019).

Two major concerns of the renewable bioethanol model in relation to its fossil fuel counterpart stand out and will be briefly addressed. First, the production of bioethanol demands growing biomass, which requires torque and heavy machines powered by fossil fuels. Only recently few technologies have addressed the issue, such as the biomethane model tractor powered by biogas released by New Holland (2017) and truck powered by biomethane and launched by Scania (2019) in partnership with ZEG. Besides, biomass growth from grasses require, even at low rates, available nutrients in soil that are usually provided according to particular necessities of every grass, where fertilizers production, transportation to the field and application of agrochemicals are expensive, highly pollutant and time consuming. This is an issue that perennial grasses responsive to biological nitrogen fixation try to overcome, and have successfully done it so far (MORAIS et al., 2012). The second concern involves food security assurance, once biomass grow fields take place in arable lands with great a deal of nutrients. Such issue has been currently addressed by employment of grasses that are not great demanders of nutrients, crops that are still capable to provide great yield of dry matter in abandoned agricultural areas (CAMPBELL et al., 2008) and marginal lands (PERLACK et al., 2005) as earlier discussed in section 3.1.

Moreover, the involvement of fossil fuels through bioethanol production chain are being addressed with biomass fields near industry placement, and both near fuel supply centers such as big metropolis, reducing transportation costs and use of fossils by trucks. Furthermore, agricultural machinery that have operated with diesel for production of biomass for renewable energy purposes are now being developed to be fed by biogas.

The competition with arable lands is another major backlash of bioethanol production. This important issue should be addressed by enhancing the employment of various residues and energy grass as feedstocks, once these provide good yields in poor land with lower fertilization rates in rainfed systems, which can also benefit from symbiosis with microorganisms. In addition, as previously stated for grasses such as PCEA, feedstock can be grown in marginal and abandoned agricultural lands and lower deforestation rates (CAMPBELL et al., 2008). In that context, bioethanol has enormous potential to meet the increasing demand in Brazilian pumps, generating new job opportunities (SOUZA et al., 2018) and contributing to meet Sustainable Development Goals – SDGs (ONU, 2020).

5.5.1 Bioethanol Scenario in Brazil: From Pro-Alcohol until Nowadays

With this biofuel acquiring extra-force since the major oil crises during the 70s, the Countries worldwide recurred to its production or importation. According to BERG (1999), the reassurance of biofuels was due possibility to address issues such as decrease dependency in petroleum, recognition and action regarding greenhouse gas and raise of environmental awareness, and improvement of air quality for better living.

Through the 70's decade the innovative National Alcohol Program called *Pró-Álcool* (in English, Pro-Alcohol) was launched in Brazil to overcome the second most expressive Petroleum crisis. The *Pró-Alcool* Program had sponsored construction, modernization and amplification of production units, meeting the needs of internal and external markets, automotive fuel policy, and striving economic growth. The bioethanol production from sugarcane, cassava or any other raw-material was encouraged in order to provide the biofuel and contribute with more sustainable development of the Country (BRAZIL, 1975).

In Brazil, sugarcane was chosen as the main crop for bioethanol production considering its higher energy balance over established crops countrywide. Silva and collaborators (1978) have early reported that from an energy balance perspective, sugarcane was the most efficient feedstock for industrial bioethanol production over sweet sorghum and cassava. The Petroleum crisis in the 70's triggered the launch of the program, which was designed to diversify the Brazilian energetic matrix and unleash the strong reliance of fuel imports at the time. Meanwhile, other countries have also embarked into massive conversion of their own available feedstocks into bioethanol (CAPAREDA, 2014). The production and use of bioethanol in particular have positioned Brazil in a favorable position worldwide regarding CO₂ emissions, an increasingly internationally valuable exchange currency.

As an unfortunate consequence of several reasons and scenarios including political, economic and cultural spheres at that time, the Program did not further develop, but it provided fundamental infrastructure and establishment of the technology countrywide. However, Brazil counts with more than 40 years of expertise in production and distribution of this biofuel, with established distribution net and integration of agricultural and industrial sectors with perspectives for expansion and improvement. The future seems to be pointing out to the co-existence of thermochemical process for energy co-generation and biochemical process for biofuels, depending on local necessity and feasibility to guide implementation.

Brazil has been a pioneer in the application of bioethanol for automobiles with pure bioethanol or blends of the biofuel with gasoline to produce “gasohol” E10 (10% of bioethanol)

and E85 (85% of bioethanol), and pure bioethanol. Moreover, the success of this first-generation biofuel relies on the energy auto sufficiency of bioethanol plants with sugarcane bagasse or straw use to generate electricity, in addition to the price of crude oil (LUO et al., 2009). Furthermore, Brazilian commitments in the Paris agreement with pledge to sharply reduce emissions is a boost for biofuels, encouraging the Country to maintain top position in the bioethanol production global ranking.

Bonomi, Maiorano & Rodrigues (2008) have described a relevant scenario in Brazil that applies until nowadays and can be explained by three main factors. The domestic increase of bioethanol demand would be the result of increased consumption in the country owing to flex-fuel cars, and figures as the first factor. In addition, the expansion of Brazilian's exports would be a second factor. The third and last one arises due to the increase of the bioethanol-gasoline mixing ratio and investments in biodiesel using bioethanol in the transesterification of oils and fats, both aiming to support the Country's efforts on mitigation of greenhouse gases emissions.

In that sense, Brazil counts with another Program which has had its directions approved in 2017 by the National Energy Policy Council (CNPE), under the Ministry of Mines and Energy. The *RenovaBio* (in English, RenewBio) opens up the possibility of integrating agroindustry and energy development policies, while at the same time meeting the objectives of industrial, environmental, and economic policies. The aims of the *RenovaBio* Program are mainly to provide an important contribution to the fulfillment of Brazil's Nationally Determined Commitments under the Paris Agreement and adequate expansion of biofuels in the energy matrix, with emphasis on the regularity of fuel supply. In addition, the Program urges to ensure predictability for the fuel market by inducing gains in energy efficiency and reduction of greenhouse gas emissions in the production, marketing, and use of biofuels (BRAZILIAN MINISTRY OF MINES AND ENERGY, 2018).

Therefore, the new *Renovabio* program should not only boost the biofuel sector in Brazil for first and second-generation bioethanol, but also biogas and biodiesel, favoring raw materials other than only sugarcane, as well as new process technologies (Table 5.1). In this sense, new lignocellulosic materials such genuine PCEA that can be cultivated as dedicated energy crops developed to meet Brazilian edaphoclimatic conditions and characteristics, can be high yielding in marginal and agricultural abandoned lands.

Furthermore, the main interest in the diversification of the raw materials to be used for the production of ethanol is to consolidate the new technological leap: the production of bioethanol from sugarcane. However, the biomass diversity available in Brazil including

residues and energy dedicated crops could improve the sector by allowing geographic decentralization of the production of sugar syrups (raw materials from bio-refineries) and fuel ethanol (BON, GÍRIO & PEREIRA, 2008). Overall, the saccharinic, amylaceous or lignocellulosic raw materials available in Brazil and worldwide require different approaches in order to be successfully converted into bioethanol, and the majority of advantages, disadvantages and bottlenecks of such technologies are already well acknowledged.

Finally, current development on the lignocellulosic bioethanol in Brazil supported the establishment of power plants for bioethanol that are biomass fed. Two main plants are *GranBio* (Piracicaba, SP State) and *Costa Pinto* (São Miguel dos Campos, AL State). The *GranBio* unit has been reported to use leaves as feedstock for the bioethanol production. On the other hand, the *Costa Pinto* plant seems to use bagasse to integrate first and second-generation process for bioethanol production (BUCKERIDGE et al., 2017).

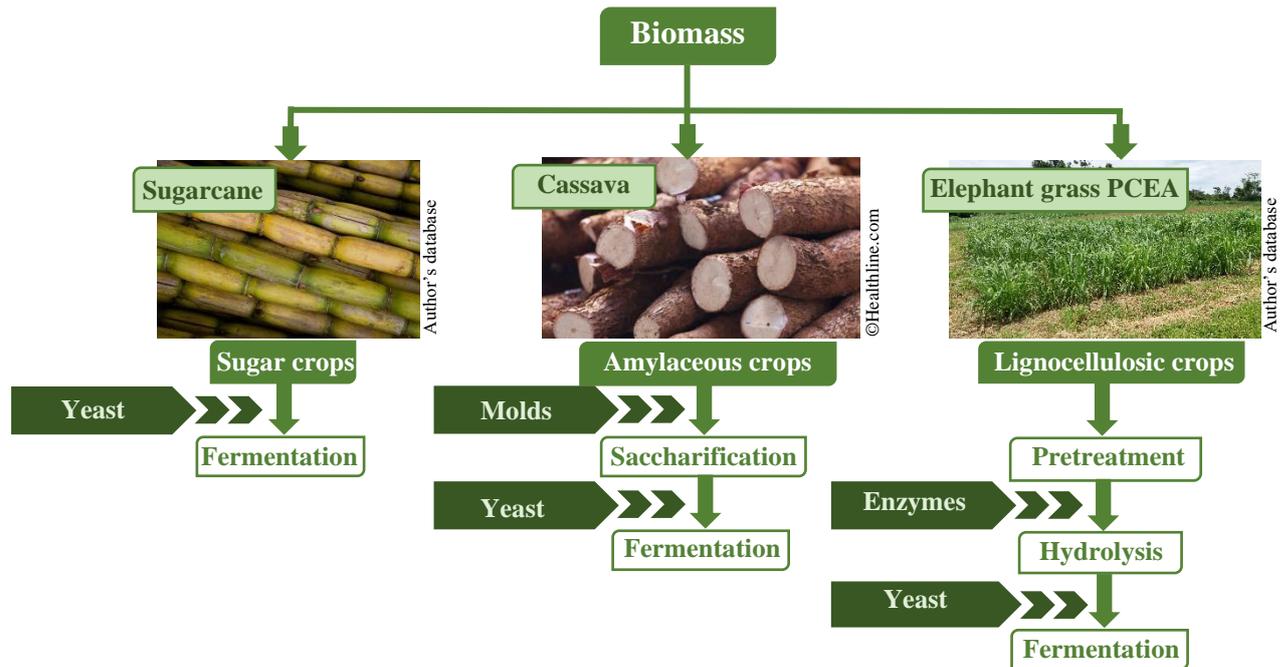
Table 5.1 – Classification of bioethanol regarding raw materials and summary of main advantages and disadvantages.

Raw Material	Definition ¹	Crops	Advantages/Opportunities ²	Disadvantages/Challenges ²
Saccharinic	materials that contain sucrose and contemplate sugars that can be directly consumed	Sugarcane Beet Sugar beet Sweet sorghum Molasses Fruits	Minimal preparation Co-products have value as feed or fuel	Human primary or indirect source of nutrients and food Fast decomposition Limited storage period Immediate conversion process after harvest and juice extraction Sterility of conversion process
Amylaceous	starch-rich materials, in which the polysaccharide must first be hydrolyzed to generate fermentable sugars	Cassava Corn Soybean Sorghum	Co-products have value as feed or fuel	Human/animal primary or secondary source of nutrients and food
Lignocellulosic	materials with available sugars in cellulose form that can be converted into bioethanol by different pretreatments applied aiming hydrolysis of the polysaccharides and the access of the microorganisms to the monosaccharides from these materials.	Wood Straw from crops Sugarcane bagasse Paper mill waste Cotton gin residue Grasses Agricultural residues Municipal Solid Waste (MSW)	Abundant resource Naturally available	Quite expensive process Cost-effective issues

Sources: ¹. SÁNCHEZ & CARDONA (2008) and CAPAREDA (2014)

Thus, the fermentable sugars to produce bioethanol such as sucrose, maltose, glucose, or fructose, may be derived from at least one of the three classes of raw materials and follow the production process as the chart in Figure 5.4 presents.

Figure 5.4 – Simplified flow chart of process steps for conversion of various biomass into fermentable sugars for fermentation



Source: elaborated by the author.

5.5.2 First-Generation or Bioethanol 1G

Bioethanol 1G can be generated worldwide with grasses rich in sugar content such as sugarcane in the tropical climate regions (GOLDEMBERG & GUARDABASI, 2010). Sugarcane is considered the most successful case known worldwide since the bioethanol industrial plants have the capacity to shift from sugar to bioethanol production (depending on price fluctuation). In addition, bagasse is recycled and applied for energy generation allowing bioethanol plants to be power self-sufficient. With that, instead of importing from the power network, sugarcane bioethanol plants end up injecting non-used power in the system adding to its profit. Furthermore, in 2017 the project ‘*Energia do Bem*’ (in English, *good will energy*) was conceived by Comerc Energia to support Cancer Specialized Hospitals and reinforced by donation of 4.674,800 MWh from the Chinese COFCO AGRI bioethanol plant located in the

surroundings. Each megawatt-hour donated represents a saving of R\$ 250 for the institution (COFCO AGRI, 2017).

During the 1975-1994 period, the Brazilian milling capacity was increased by 100%, the extraction process increased its efficiency from 93% to 97%, while the efficiency of the fermentation process was raised from 80% to 91%. On the other hand, the overall recovery of the bioethanol production process increased 30% and the thermal consumption (steam) in the distillation was reduced by around 44%. Such advances were achieved exclusively from incremental innovations, peripheral equipment and new operating procedures, which resulted in the expressive increase in industrial yield (ABARCA, 1999).

Considering all the advances acquired through recent past years, the bioethanol finds in Brazil extremely favorable conditions for its production and continued growth. The Country has already high technology of production, leadership in agriculture, bioenergy generation, and market for bioethanol without deforestation or competition with crops destined for food production. Although sugarcane is the Brazilian bioethanol industry's flagship with well-established processes, there are real possibilities for growth and cost reduction, especially in the development of new biomasses (PACHECO, 2011). In addition, more recently, another form of ethanol providing even higher yields from cultures with high lignin contents is lignocellulosic ethanol, widely known as second-generation bioethanol or Bioethanol 2G.

5.5.3 Second-Generation or Bioethanol 2G

Second-generation bioethanol has reached remarkable progress, although it still undergoes optimization and relies on policies to advance even more. The generation of this biofuel consists on a complex conversion of lignocellulosic and starch-rich materials, such as agricultural or forestry crops and residues (PACHECO, 2011; CAPAREDA, 2014). The most successful cases are the 2G bioethanol obtained from corn crop residues in the United States (CHUM et al., 2013; WANG et al., 2014) and the increasing use of elephant grass varieties (PACHECO, 2011) and sugarcane bagasse in Brazil (MARTINS, RABELO & COSTA, 2015; MILANEZ et al., 2015; NEVES, PITARELO & RAMOS, 2016).

Bioethanol from lignocellulosic biomass feedstock can be obtained following two-stages prior fermentation process: pretreatment and enzymatic hydrolysis. Pretreatment step can be defined as the process that eliminates lignin form and provides cellulosic intermediate for further enzymatic hydrolysis. In sequence, the obtained cellulosic intermediate component will be converted into fermentable sugars through enzymatic hydrolysis as the second and final

step prior fermentation (SINGH et al., 2014; MINMUNIN, LIMPITIPANICH & PROMWUNGKWA, 2015; NAGULA & PANDIT, A. B., 2016; TOSCANA et al., 2019). Counting only two reaction steps might appear trivial, yet several pretreatment technologies have been developed and investigated regarding feasibility and sustainable perspectives. Interestingly, an opportunity that arises in the bioconversion of lignocellulosic materials is the creation of biorefineries, producing value-added products in addition to the biofuel itself (BALAT, BALAT & ÖZ, 2008).

Plant cellulose is considered the most abundant resource in the world (CAPAREDA, 2014). As widely available in Brazil, the cellulose in native plants has to be investigated regarding its potential for complete hydrolysis and utilization under the proper and most promising chemical, microbial, and environmental conditions. The technology is still under development for several lignocellulosic raw materials, few treatments are used in the production of bioethanol in order to verify those with the best performance for the available raw material reality of each Country. According to Pacheco (2011), reduction of lignin content is required in order to allow the cellulases to access cellulose and the depolymerization of the chain occurs. Reach reduction of lignin is a barrier to such an event. Thus, lignocellulosic or 2G bioethanol has emerged as an attempt to maximize the yield of biofuels production.

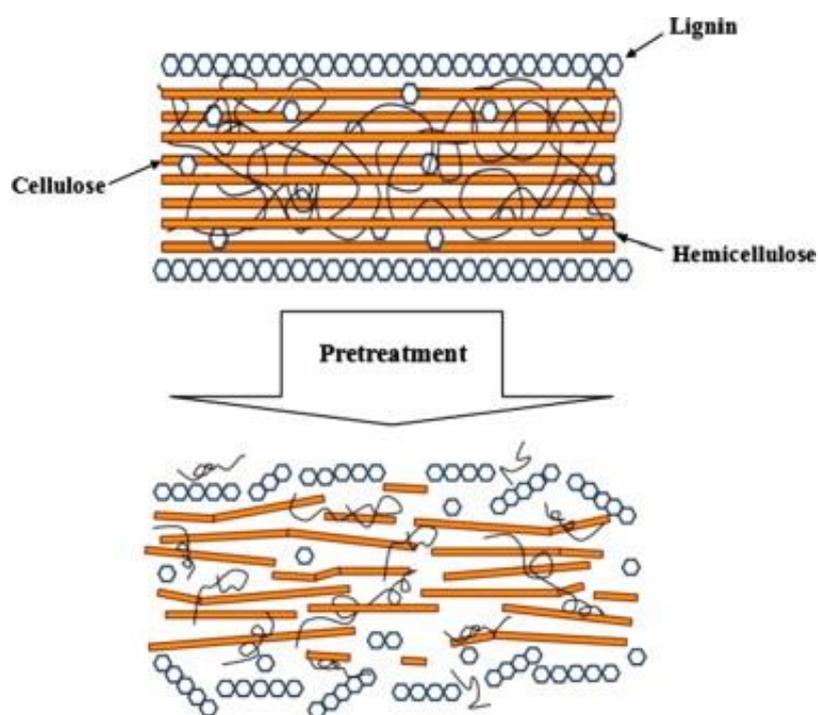
Nowadays, the high costs of cellulosic enzymes and high cost of pretreatment figure right in the center of technological impediments for second-generation bioethanol production (CAPAREDA, 2014). Cellulosic enzymes are not only expensive to afford, but also require massive investment in research and technological development (RTD) to achieve more efficient catalysts. In addition, a quite expensive step is the pretreatment, which can combine chemical and physical procedures and includes acidic, alkaline and steam explosion processes, and have been long acknowledged to account for approximately 20 % of industrial costs of lignocellulosic bioethanol (BANERJEE et al., 2010). Other than only financially expensive, chemical pretreatments also face serious drawbacks such as water use and also the effluents generated within the process to both treat the raw material and further washing process for neutral pH achievement. Therefore, pretreatment of lignocellulosic raw material is an important bottleneck that prevents this biofuel from reaching feasibility and viability.

5.6 Pretreatments for lignocellulosic bioethanol

The bioethanol production from lignocellulosic biomass is considered a complex process, where pretreatments play a key role. Such pretreatments are considered essential step

for this bioethanol production, since they overcome biomass recalcitrance and optimize cellulose access (TOQUERO & BOLADO, 2014). The treatments allow separation of structures that stiffen plant cells, such as lignin and hemicellulose, exposing the cellulose for conversion to fermentable sugars (MARTINS, RABELO & COSTA, 2015) as Figure 5.5 illustrates.

Figure 5.5 – Schematic pretreatment effect of lignocellulosic raw material

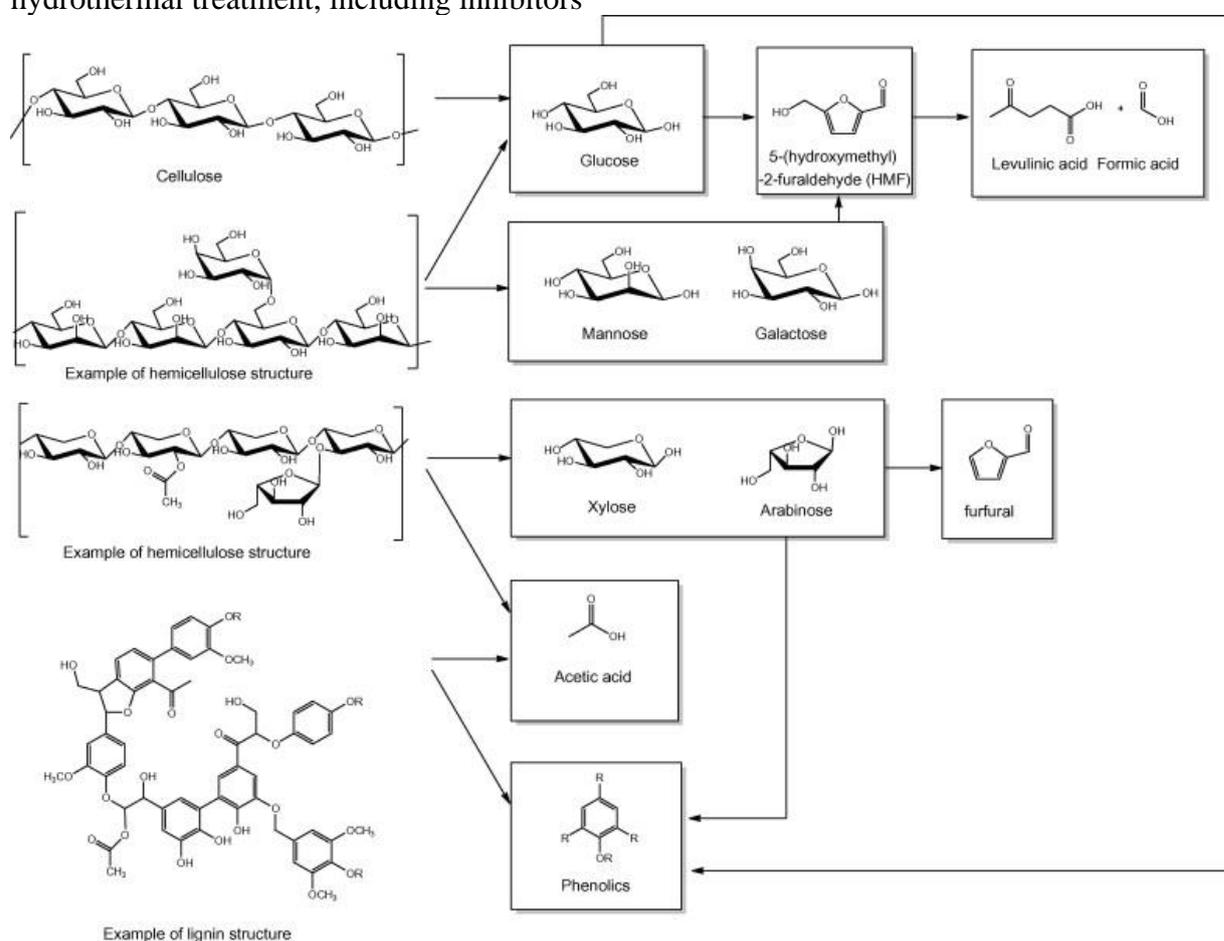


Source: MOOD et al. (2013).

The pretreatments currently comprehend four different categories including physical, chemical, physicochemical, and biological. Moreover, the several methods inside every category can be combined resulting in various approach possibilities. These types of pretreatments have been used to alter or remove lignin and hemicellulose, increase surface area and porosity, and decrease the degree of polymerization and crystallinity of the cellulose in order to facilitate further process (CANILHA *et al.*, 2010). Therefore, the chosen pretreatment approach must meet the following requirements: (1) improve the formation or enhance the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid degradation or loss of carbohydrates; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective (SUN & CHENG, 2002).

On the other hand, regardless of the pretreatment or combination of pretreatments applied, some inhibitory compounds will result from the process (Figure 5.6). These so-called inhibitory compounds are of relative concern, since their presence after pretreatment will negatively affect microbial activity in the hydrolysis step. According to Mood et al. (2013), inhibitors are generally classified into three groups, including weak acids (levulinic, acetic and formic acids), furan derivatives such as furfural and HMF (5-hydroxy-2-methyl furfural), and phenolic compounds.

Figure 5.6 – Suggested products and summary reaction routes for the degradation of lignocellulosic biomass saccharides (cellulose and hemicelluloses structures) and lignin during hydrothermal treatment, including inhibitors

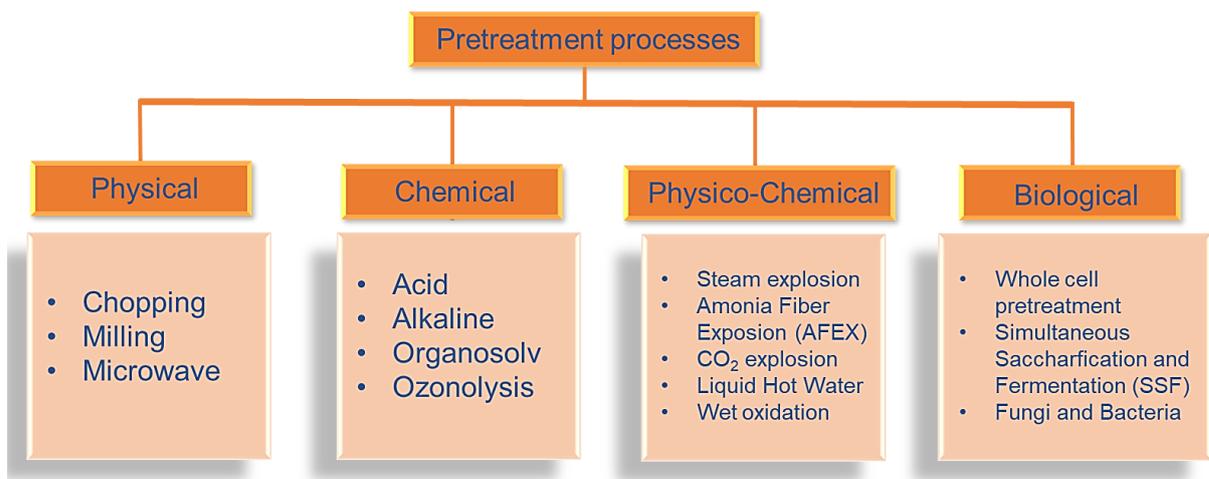


Source: RASMUSSEN, SØRENSEN & MEYER (2014).

Procedures involving inhibitors generated during lignocellulose pretreatments at high temperatures under acidic conditions lead to the formation and liberation of a range of compounds (Palmqvist & Hahn-Hägerdal, 2000). Moreover, depending on the reaction conditions glucose can be converted to or levulinic acid and/or 5-(hydroxymethyl)-2-

furaldehyde (HMF), formic acid, and different phenolics at elevated temperatures. At least four routes for the formation of HMF from glucose and three routes for furfural formation from xylose are possible, resulting in the formation of furan-2-carbaldehyde (furfural) and/or various C-1 and C-4 compounds as presented in Figure 5.6 (RASMUSSEN, SØRENSEN & MEYER, 2014). A summary of pretreatments currently most used for grasses is also presented in Figure 5.7.

Figure 5.7 - Schematic pretreatment categories and most used process for grasses, including elephant grass



Source: Elaborated by the author.

5.6.1 Physical

The physical pretreatments are employed to increase surface area by reducing particle size of lignocellulosic raw materials. According to Mood et al. (2013), the chopping, grinding, milling, and microwave are processes generally employed in this pretreatment category. Usually, the very first step after biomass harvest is size reduction by milling process combined with another pretreatment. Several types of milling methods can be found, although the most important drawback of this pretreatment is the high energy consumption.

More recently, a new eco-friendly approach freezes biomass providing good results on further biodegradation steps for bioethanol production. In particular, this approach benefits from density decrease and volume expansion when water content freezes breaking cell wall structure, which has been reported to increase the surface area and enzyme accessibility in the pretreated biomass (CHANG et al., 2011). However, only few studies have been performed including effect of this method on rice straw (CHANG et al., 2011) and combined with dilute-

acid pretreatment on Mongolian oak (SMICHI et al., 2016) on bioethanol production. Adding to this scarce investigation scenario, the overall high cost outstands as the main drawback of this technology (MOOD et al., 2013).

5.6.2 Physicochemical

The physicochemical pretreatments act on physical and chemical structural changes of the biomass and include steam explosion, liquid hot water, ammonia fiber explosion, wet oxidation, and CO₂ explosion and extrusion, which some authors classify as merely physical.

5.6.2.1 Steam explosion

Steam explosion has been extensively investigated and can be defined as a thermo-mechanochemical method that breaks structural components into minor parts by steam-heating (thermo), shearing (mechanic; owing to sudden decompression and evaporation of moisture content), and auto-hydrolysis (chemical) of glycosidic bonds. The hydrolysis of hemicellulose during steam explosion pretreatment is performed by organic acids, including acetic acids generated from hydrolysis of acetyl groups in contact with hemicellulose, formic and levulinic acids, derived from other groups (MOOD et al., 2013). However, owing to acid conditions, the degradation of sugars into inhibitors such as furfural and HMF might result from the process (PALMQVIST & HAHN-HÄGERDAL, 2000).

Local elephant grass (*P. purpureum*) has been investigated under steam explosion pretreatment aiming improvement of biomass conversion into bioethanol applying microorganism for enzymatic hydrolysis (*Penicillium echinulatum*) step. Severity degree of pretreatments varied regarding T, reaction time and pressure. The authors implied that the water washing process played a significant role in the study, where highest glucose yield was derived from water-washed steam-treated substrates that were produced after 10 min at 190 °C (Scholl et al., 2015a).

In the case of elephant grass genotypes, Scholl and collaborators (2015b) pretreated this biomass with steam explosion at different temperatures (180, 190 and 200 °C) and residence times (6, 8, 10 min). Among interesting fermentable sugar production reported, the study findings involved verification of biomass recovery of around 98% for elephant grass pretreated via steam explosion at 180 °C for 6 min, where biomass recovery was found to decrease with increased pretreatment severity.

5.6.2.2 Ammonia fiber explosion

In ammonia fiber explosion (AFEX) pretreatment, liquid ammonia is used applying steam explosion process concept improving the susceptibility of lignocellulosic biomass to enzymatic attack. AFEX technology includes biorefinery concept and has four main parameters, including ammonia and water loading, temperature and residence time, that should be varied in order to optimize this pretreatment for the available lignocellulosic material (BALS et al., 2011). The process involves rapid expansion of ammonia gas, which causes cleavage of lignin-carbohydrate complex (chemical), consequently resulting on physical disruption of fibers leading to biomass digestibility improvement (CHUNDAWAT, VENKATESH & DALE, 2007).

This process differs from steam explosion in some aspects such as production of solid material instead of slurry, which can be explained by low boiling point of the chemical (MOOD et al., 2013). Both bench scale and biorefinery concepts are expensive compared with other methods and have drawbacks such as uncertain sugar production from enzymatic hydrolysis, which result in fermentation yields that can greatly vary with changing of AFEX parameters (BALS et al., 2010; BALS et al., 2011).

5.6.2.3 CO₂ explosion

In CO₂ explosion technology a supercritical CO₂ explosion occurs with, working similarly as AFEX and steam explosion pretreatments, considering that CO₂ molecules have a similar size property to those of water and ammonia making them capable of penetrating into small pores of lignocellulosic material (ZHENG et al., 1995). In contrast with steam explosion, supercritical CO₂ explosion needs lower temperature and is also less costly in comparison with AFEX, making it an ideal choice among the explosion-type methods. Additionally, CO₂ explosion possesses other advantages such as non-toxicity and non-flammability (MOOD et al., 2013).

5.6.2.4 Liquid hot water

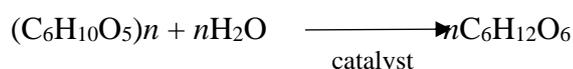
Liquid hot water (LHW) is a pretreatment method that employs water at high temperatures and pressure, to avoid evaporation. The LHW has been reported to increase solubility of biomass hemicellulose up to 80%, enhancing cellulose exposition and access for enzymatic digestibility (LASER et al., 2002).

5.6.2.5 Wet oxidation

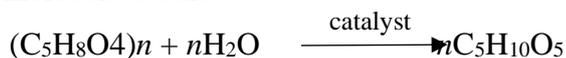
The wet oxidation is a technology that consists on water and air or oxygen employed at high temperature and pressure pretreatment of lignocellulosic raw material, where formation of inhibitors such as furfural and HMF is lower in this pretreatment when compared to steam explosion and LHW (MOOD et al., 2013). Despite advantages, economic feasibility faces constraint due to the high cost imposed by pressure equipment and high cost of large oxygen amounts used as catalyst (MERKLEIN, FONG & DENG, 2016).

5.6.3 Chemical

There are many chemical pretreatments, such as organosolv, ozonolysis, acid and alkali pretreatments. Special attention will be dedicated to the last two chemical methods, since they are the most employed and the alkali pretreatment strategy has been applied to elephant grass cellulose-to-bioethanol conversion in the present study. Chemical pretreatments can catalyze the reaction of water with the glucose molecules in these chains, resulting in simple molecules of glucose (monomers) by the following reaction:



In the hydrolysis of hemicellulose, the release of individual sugar chains contained in the longer hemicellulose molecule occurs.



Where $(C_5H_8O_4)_n$ represents a chain consisting of n arabinose or xylose (pentose) and $C_5H_{10}O_5$ molecules being one of the corresponding pentose sugars formed by the hydrolysis of hemicellulose (HOLTZAPPLE, 2003b).

5.6.3.1 Acid pretreatment

This chemical pretreatment type includes acid and diluted acid methods, with sulfuric acid outstanding as the most extensively method studied, especially considering elephant grass as raw material. However, pretreatments that involve acids for hydrolysis of biomass have been reported as not effective regarding lignin removal when compared with other pretreatments (CARDONA et al., 2014). Furthermore, several studies have investigated many compounds left after pretreatment that inhibit enzymatic hydrolysis when performed by microorganisms instead of commercial enzymes, and consequently affect negatively yeast fermentation. One of the

major compounds produced during pretreatments that may cause inefficient enzymatic hydrolysis is acetic acid, which some studies have removed using lime, adding more cost to the process.

The effects of organic acids pretreatments were evaluated on local elephant grass straw (*P. purpureum*) by using Box-behnken experimental design of Response Surface Methodology (RSM). The study approach included optimization of parameters for oxalic ($C_2H_2O_4$), citric ($C_6H_8O_7$), acetic (CH_3COOH) and hydrochloric ($HCl_{(aq)}$) acids. Research findings reported hydrochloric acid as the most effective among pretreatments at 0.7% (w/v), 150 °C for 60.18 min (AMNUAYCHEEWA et al., 2017).

Menegol and collaborators (2014) have investigated yield of reducing sugar of elephant grass biomass pretreated at 1:4 solid/liquid ratio with sulfuric acid (5% and 10% v/v) and two other alkaline solutions, autoclaved at 120 °C for 15 min. The yield of reducing sugars was 181 and 196 mg g⁻¹ for 5 and 10% (v/v) concentrations, respectively. However, the alkaline pretreatment presented the highest yield with low concentration compared with the acid pretreatments, and has been further reported in the following section.

Another interesting study has been performed by Li and collaborators (2015), where harvest regimes of elephant grass (*P. purpureum* cv. Hunan and Sumu 2 and three hybrids) were evaluated regarding cellulose yield after pretreatment. Dilute sulfuric acid solution 1% (solid base) was used for biomass presoaking with 1:10 rate solid/liquid at room temperature. The generated slurry was treated in batch tube reactors at 140 °C for 20 min.

More recently, Santos et al. (2018) studied elephant grass cultivated in the Experimental Station at Embrapa Agrobiologia (Centro Nacional de Pesquisa em Agrobiologia – CNPAB, Embrapa Agrobiologia) and reported, not surprisingly, that leaves had lower recalcitrance than stems under pretreatment with sulfuric acid (5, 10 and 20% mass acid/biomass). Although the biomass was locally cultivated, it was probably propagated by stalks with no further information regarding genotype.

5.6.3.2 Alkaline pretreatment

Alkaline pretreatment for lignocellulosic biomass feedstocks involves saponification of intermolecular bonds of esters of uronic acid associated with xylan chains. The esters form bridges of polymeric units that cross-link hemicelluloses and other components, where saponification breaks these links enhancing porosity of the lignocellulosic biomass and exposing cellulose (TARKOW & FEIST, 1969). Although an alkaline environment has been

reported to successfully solubilize hemicellulose (FENGEL & WEGENER, 1984), its efficiency as pretreatment depends on the substrate conditions and applied parameters.

Cardona and collaborators (2013; 2014) have applied the central composite design of the surface response methodology to alkaline pretreat elephant grass for bioethanol production purposes. The factors analyzed and levels employed in the experiment are summarized along with other alkaline pretreatment investigations in Table 5.2. Elephant grass biomass has been also investigated when alkali pretreated with aqueous ammonia (27% w/w, solid:liquid ratio of 1:12), where lignin removal reached 58.7% causing deconstruction of the fibrous matrix. Although the relative cellulose content increased, the degree of crystallinity of the pretreated fiber increased only marginally from 42.9 to 50.0%, which authors suggested as the driving force that caused partial disruption of the cellulose crystal structure (PHITSUWAN et al., 2015).

Table 5.2 – Literature summary of investigated plant tissues, factors and levels of alkaline pretreatments for elephant grass (*P. purpureum*)

Location	Plant Part	Size (mm)	Alkali	Alkali Concentration		Solid to Liquid ratio	Temperature (°C)	Residence Time (h)
				(% v/v)	(% w/v)			
Brazil ^[1]	leaves	2.0	NaOH	-	2.0, 5.0	1:10, 1:20	70	2
			NaOH+H ₂ O ₂	-	4.0+7.0, 2.0+2.6		121	0.66
Brazil ^[2]	leaves	-	H ₂ O ₂	1	-	1:50	80, 120	0.5
			NaOH	-	4.0	1:20		
Brazil ^[3]	aerial part	-	NH ₄ OH	10.0, 20.0	-	1:4	120	0.25
			NaOH	-	2.0, 3.0, 4.0, 6.0			
Colombia ^[4,5]	aerial part	3.0	NaOH	-	0.7, 1.0, 1.5, 2.0, 2.3	1:13.3, 1:15, 1:17.5, 1:20, 1:21.7	66.4, 80, 100, 120, 133.6	0.3, 1, 2, 3, 3.7
India ^[6]	aerial part	0.3-0.4	NaOH	-	1.0, 2.0, 3.0	1:10	121	0.5, 1.0, 1.5
Nigeria ^[7]	stem	≤0.45	NaOH	-	80.0	1:10	-	2, 4, 6, 8, 10
Uruguay ^[8]	aerial part	<0.5	NaOH	-	2.0	1:6	80	6
Venezuela ^[9]	leaves	1.0	NH ₄ OH	28.0	-	-	90	0.083
Taiwan ^[10]	aerial part	1.0, 5.0	NaOH	-	5.0	1:4	80	0.5
Taiwan ^[11]	stem	20.0	NaOH	-	10	1:20	90	1
Thailand ^[12]	stem	10.0-20.0	NaOH	-	2	1:6	120	1

^[1]. Nascimento & Rezende (2018) ^[2]. Godinho et al. (2019) ^[3]. Menegol et al. (2014) ^[4]. Cardona et al. (2013) ^[5]. Cardona et al. (2014) ^[6]. Haldar & Purkait (2020) ^[7]. Aiyejagbara et al. (2016) ^[8]. Camesasca et al. (2015) ^[9]. Urribarri et al. (2005) ^[10]. Chang et al. (2011) ^[11]. Tsai et al. (2018) ^[12]. Phitsuwan et al. (2016).

Phitsuwan and collaborators (2016) have investigated the effect of alkali pretreatment in local elephant grass approaching only stem fraction of the grass and applying varying concentrations for NaOH, Ca(OH)₂, NH₃, and H₂O₂. Research findings indicated NaOH as the best alkali reaching the most extensive removal of lignin (84%) among pretreatments, which agreed well with the high glucan conversion rate (94%) by enzymatic hydrolysis, while the conversion rates for fiber pretreated with other alkalis achieved at maximum 60%.

Menegol and collaborators (2014) have investigated yield of reducing sugar of elephant grass biomass pretreated at 1:4 solid/liquid ratio with solutions of sodium (2% to 6% w/v) and ammonium hydroxide (10% and 20% v/v) and an alkali solution. The generated samples were autoclaved at 120 °C for 15 min. Interestingly, the highest reducing sugars yield of 558.56 ± 41.18 mg g⁻¹ dry mass was observed with the hydrolysis of elephant grass (*P. purpureum*) pretreated with 3% NaOH.

5.6.4 Biological

This fourth category of pretreatment differs from the other three, especially regarding its non-chemical requirements and environment-friendly bioprocess, converting lignocellulosic raw materials into accessible components for further bioethanol production steps through action of microorganisms. In the biological pretreatment, conditions including moisture content, particle size, process time and temperature, affect the lignin degradation and further fermentable sugars yield (MOOD et al., 2013).

Normally, this type of lignocellulosic biomass pretreatment uses microorganisms, such as fungi and bacteria with ligninolytic potential to overcome recalcitrance and expose cellulose fraction of feedstock, enhancing digestibility of hydrolytic enzymes (VASCO-CORREA, GE & LI, 2016). Furthermore, there are environmental and cost advantages of biological when compared with chemical pretreatments. However, the drawbacks of this bioprocess limit industrial application, and include long process time associated and continuous monitoring of microorganism growth (TABIL, ADAPA & KASHANINEJAD, 2011).

5.6.5 Combined pretreatments

There are several method options that can be combined inside the four main categories. The combined pretreatments have been considered as a promising approach to overcome drawbacks of single pretreatments. According to Mood et al. (2013), this combined approach

has received attention regarding potential to increase efficiency of fermentable sugar yields, decrease inhibitors production and shorter processes time, which would collectively enhance bioethanol yield in economically feasible processes.

An elephant grass mixture of leaves and stems ground to particle size inferior to 1.5 mm has been investigated under high-pressure carbon dioxide (CO₂) and water pretreatments (TOSCAN et al., 2017). The high-pressure CO₂ and H₂O experiments were carried out in a stainless-steel high-pressure reactor, with a constant initial CO₂ pressure of 50 bar. The experiment investigated biomass pretreatment at temperatures of 180 °C, 200 °C and 220 °C and dry raw material mass ranging from 3 to 8 g and fixed 1:10 solid/liquid ratio. In conclusion, the authors have reported 46.4 wt.% xylose yield of elephant grass in this combined pretreatment strategy.

6. MATERIAL AND METHODS

6.1 Harvest and storage conditions of PCEA biomass

The unique *Pennisetum purpureum* PCEA genotype propagated by seeds was developed by the Breeding Group at Embrapa Dairy Cattle – Centro Nacional de Pesquisa de Gado de Leite (CNPGL) and cultivated under sustainable operations at Embrapa Agrobiology – Centro Nacional de Pesquisa de Agrobiologia, CNPAB (Seropédica, RJ State, Brazil) as shown in Figure 6.1. The PCEA biomass was obtained at the very first harvest 180 days after inoculation with the recently isolated diazotroph strain LP343 of *Gluconacetobacter diazotrophicus* (Embrapa Agrobiology, unpublished data), which was isolated from elephant grass leaves by Videira and collaborators (2012). This cutting regime was based on previous studies with elephant grass cultivation for bioenergy application purposes that achieved higher C:N relation and lower ash contents when compared with 120 and 150 days old cuts (FLORES, et al., 2013). Additionally, further studies considering the Brazilian edaphoclimatic conditions have shown that late cuts of 180 days provide feedstock ideal for bioenergy applications such as lignocellulosic bioethanol (FLORES et al., 2012).

Figure 6.1 – 180 days old PCEA in the Experimental Field Station at Embrapa Agrobiologia (Seropédica, RJ State, Brazil), reaching 1.80 m in 11/18/2017



Source: Author's database.

Plant samples were initially dried in an oven at 65 °C for 72 hours until constant weight was reached to determine total dry matter of the first harvest. Dried samples were then separated in paper bags, placed in hard plastic bags, transported to the Laboratory of Industrial Biotechnology at the Institute of Technological Research (São Paulo, SP State, Brazil) and stored in a refrigerated room at 8 °C.

6.2 Alkaline pretreatment

As presented in the review section, among the pretreatments and the most used up to date for elephant grass, the acid and alkali pretreatments outstand regarding the content of cellulose recovery and lignin removal. However, the production of inhibitors after acid pretreatments are of concern and undesirable for bioethanol production processes (Section 5.6). In addition, based on results obtained in the studies performed by Cardona and collaborators (2013; 2014), alkali pretreatment was considered the best pretreatment among several chosen processes, including pretreatment with acid (also approached in Section 5.6).

6.2.1 Experimental Design

Every scientific investigation based on practical experiments seeks for products and processes optimization, minimizing costs and time, and maximizing yield and quality of generated byproducts. Thereunto, experimental planning and performance was systematically studied prior development of this investigation. According to Rodrigues & Iemma (2005), the choice of the most adequate experimental planning and design is a direct function of number of independent variables involved in the investigation, availability of raw materials, replicates

possible, and process costs. Therefore, the experimental design with orthogonalized variables, similar to a Central Composite Design (CCD) of the Response Surface Methodology (RSM), was chosen as the experimental design to be employed in the present investigation.

The central composite design experiment, as well as the graphic surface for analyzed responses, were made using Action Stat[®] (Estatcamp^{Inc.}, São Carlos, Brazil) integrated within Excel software (Microsoft[®]) to investigate the significance of temperature (T), residence time (RT) and NaOH concentration effects on pretreated PCEA. The range of values chosen for the five levels of each one of the three parameters presented in Table 6.1 were based on previous results obtained for elephant grass investigations (Table 5.2) and the matrix generated is presented in Table 6.2. A visual representation of the cube is also presented (Figure 6.3). In Table 6.2 the experimental coded values (A, B and C columns) and respective levels (from -2 to +2) are presented, in addition to the corresponding parameters and analyzed real values for each level in the 17 runs.

Table 6.1 – Experimental range for alkaline pretreatment

Factor	Level				
	-2	-1	0	1	2
Temperature (°C)	80	90	100	110	120
Residence time (min)	15	20	25	30	35
NaOH (% w/v)	1	1.5	2	2.5	3

Figure 6.2 – 3D-sphere representation of experimental design by Action Stat[®] (Estatcamp^{Inc.}). Values in axis z, x, and y represent alkali, temperature and residence time, respectively

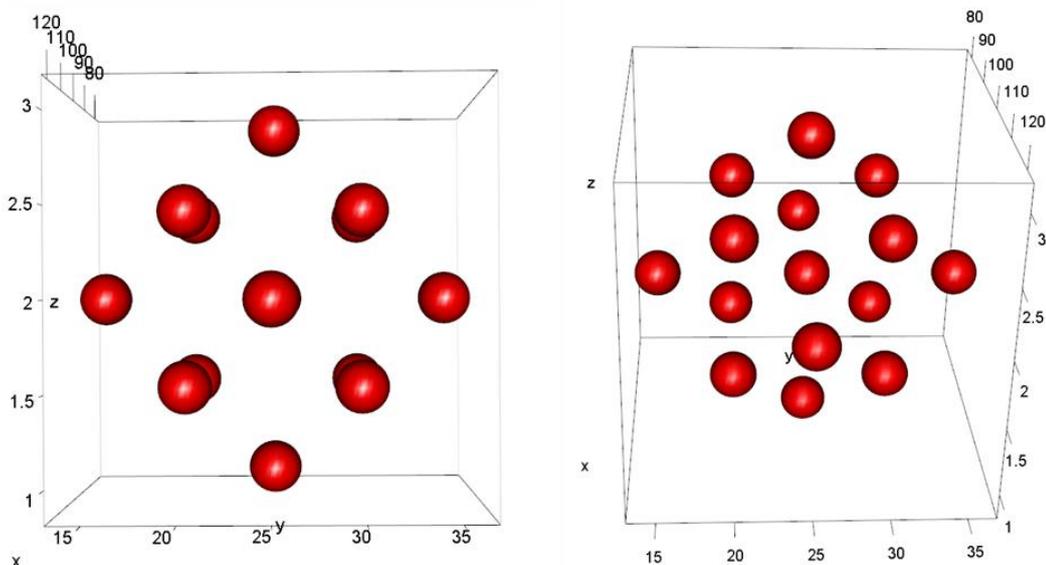


Table 6.2 - Assays for the alkaline pretreatment with coded and real parameters and levels analyzed. CP stands for Central Point and appears in triplicate

Assay	A	B	C	Temperature (°C)	Residence time (min)	NaOH (% w/v)
1	-1	-1	-1	90	20	1.5
2	1	-1	-1	110	20	1.5
3	-1	1	-1	90	30	1.5
4	1	1	-1	110	30	1.5
5	-1	-1	1	90	20	2.5
6	1	-1	1	110	20	2.5
7	-1	1	1	90	30	2.5
8	1	1	1	110	30	2.5
9	-2	0	0	80	25	2
10	2	0	0	120	25	2
11	0	-2	0	100	15	2
12	0	2	0	100	35	2
13	0	0	-2	100	25	1
14	0	0	2	100	25	3
15 (CP)	0	0	0	100	25	2
16 (CP)	0	0	0	100	25	2
17 (CP)	0	0	0	100	25	2

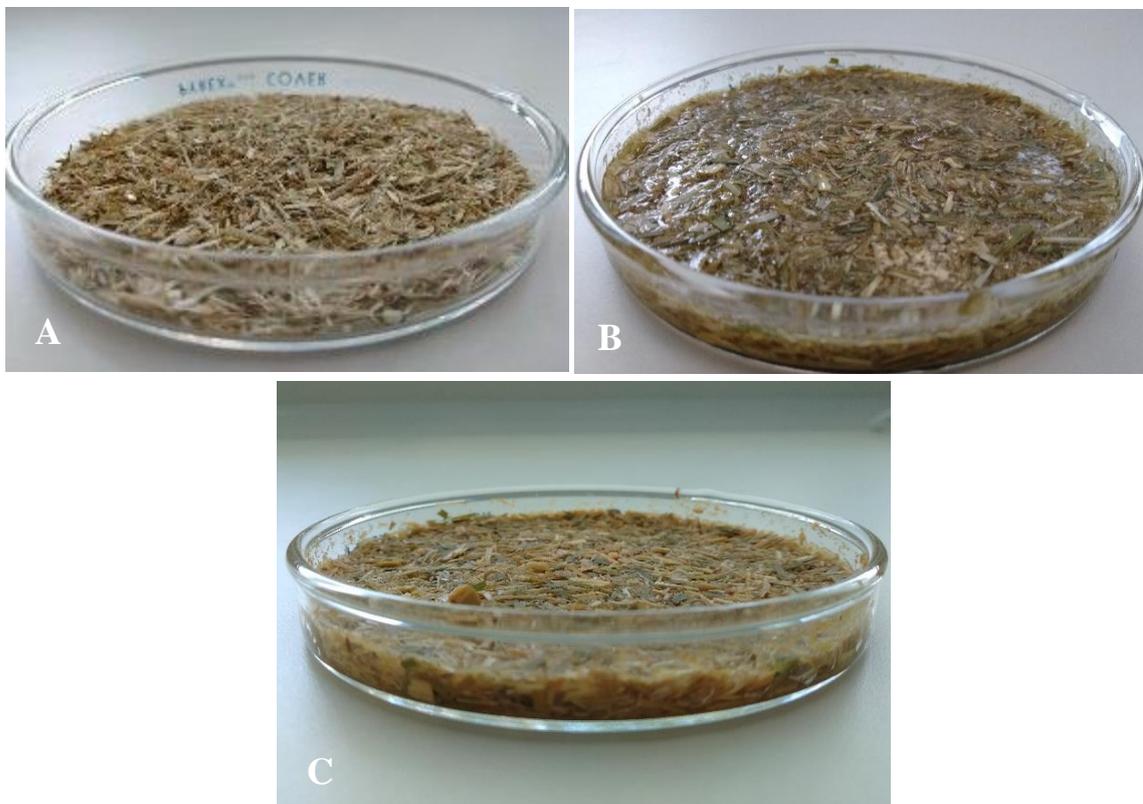
6.2.2 Experimental procedure

Initially, the PCEA biomass was ground using Knife mill (Residues Laboratory, LAREX, University of São Paulo) to reduce stem and leaves materials to ≤ 10 mm (1cm) for alkaline pretreatment. The ground feedstock was kept in hard plastic bag and stored in a refrigerated room at 8 °C at the Laboratory of Industrial Biotechnology at the Institute of Technological Research (São Paulo, SP State, Brazil).

The chemical pretreatment was performed to accomplish defined temperature, alkali concentration and residence time ranges established by the experimental design for each of the 17 runs. Each run and the three replicates performed at the central point were performed with 60 g of the PCEA immersed in 450 mL of the defined w/v% NaOH from Merck® (a solid to liquid ratio of 1:7.5) and homogenized with a rubber spoon in a 2 L capsule. The homogenized mixture of PCEA and NaOH solution was covered with plastic film and maintained at room temperature for 30 minutes, as this time interval was observed in prior tests to be ideal for the

alkali solution be absorbed by PECA biomass (Figure 6.3). Initially, the biomass was tested with different solid to liquid ratios to determine the minimal liquid use to allow complete soaking of biomass without water excess, as well as the absorption time. The untreated PCEA biomass is presented (Figure 6.3 – A), the trial and selected for experimental procedures solid to liquid ratio of 1:7.5 (1, 1.5, 2.0, 2.5, and 3.0 w/v% NaOH) is showed in Figure 6.3 – B, and the complete alkali absorption by PCEA biomass achieved after 30 minutes at room temperature is also presented (Figure 6.3 – C).

Figure 6.3 – A) untreated PCEA. B) PCEA soaked on 1 %wt. NaOH. C) PCEA after 30 min soaked on NaOH after completely alkali absorption



Source: Author's database.

After the 30 minutes, the mixture was transferred to two Erlenmeyers of 2 L and in the autoclave Fabbe[®] with Inova temperature and time controller coupled (Inova[®]) at temperatures and residence times adjusted to follow conditions established for each run. The 2 L Erlenmeyers were chosen to provide thicker layer of the mixture inside the autoclave, allowing better and homogenized heat exchange during the process. After performance of pretreatments, each pretreated solid was separated from the liquid fraction by filtration. The solid fractions were washed with deionized water until reaching a neutral pH. Subsequently, the solid samples were

dried in a 30 °C warm room for approximately 2 days and checked to assure moisture content between 11 to 13%. The 17 samples generated after the experiment were labeled, stored in sealed plastic bags and maintained in a refrigerated room at 8 °C for further compositional analysis characterization.

6.3 Biomass recovery

The mass recovery of lignocellulosic raw materials has been performed to access global biomass yield after alkaline pretreatment processes, allowing determination of cellulose and hemicellulose recoveries, in addition to lignin removal (CARDONA et al, 2013; CARDONA et al 2014). In this study, biomass recovery was calculated for each run meeting the established parameters by experimental design. To determine recovery, dried paper filters were previously weighed and used to retain the pretreated samples washed until neutral pH. Subsequently, the filters were dried in an oven at 105 °C for 24 hours to determine final weight of each sample. Moisture content was determined for the feedstock prior experimental runs and after oven drying process. The components, such as cellulose, and its respective yields for each assay from were adjusted with recovery (% wt.) to determine the final yields after alkaline pretreatment (Equation 6.1). The biomass yield of each assay was calculated as shown in Equation 6.2 and the cellulose yields were corrected as exposed in Equation 6.2 (adapted from BHANGE et al., 2015). Equation 6.3.

$$\text{Recovery (\%wt.)} = \frac{M_1 \times (1 - MC_1)}{(M_2 - FP) \times (1 - MC_2)} \times 100 \quad \text{Equation (6.1)}$$

Where,

M_1 = Biomass weight used (g)

MC_1 = Moisture Content before pretreatment (%)

M_2 = Dried weight of Filter Paper + pretreatment sample (g)

FP = Dried weight of the Filter Paper (g)

MC_2 = Moisture Content of dried pretreated sample (%)

$$\text{Recovery of component (\%wt.)} = \frac{C_{AP} \times R}{C_{UPCEA}} \quad \text{Equation (6.2)}$$

Where,

C_{AP} = Component content of specific assay after pretreatment (% wt.)

R = Recovery of specific assay (% wt.)

C_{UPCEA} = Content of specific component in the untreated PCEA biomass (% wt.)

$$\text{Lignin removal (\%wt.)} = 100\% - R_L \quad \text{Equation (6.3)}$$

Where,

R_L = Recovery content of lignin after pretreatment (% wt.)

6.4 Compositional characterization

PCEA biomass was characterized at the Laboratory of Industrial Biotechnology, Institute for Technological Research – IPT (São Paulo, SP State, Brazil), following standard Laboratory Analytical Procedures (LAP) protocols of the National Renewable Energy Laboratory – NREL (Golden, CO State, US). Although performance of these protocols is longstanding and time consuming, they provide comprehensive analysis of the feedstock such as the extractives in alcohol, as well as fibers, cellulose and hemicellulose contents. Such standard protocols allow acknowledgement of sugar contents in means of glucose, cellobiose, xylose and arabinose, in addition to minor organic contents including acetic acid, HMF and furfural. Therefore, the composition characterization is fundamental to evaluate the raw material and to be performed after alkaline pretreatment to infer about efficiency in means of lignin removal and enhancement of cellulose content, which is a reference to define active units (AU) of enzyme blend for enzymatic hydrolysis.

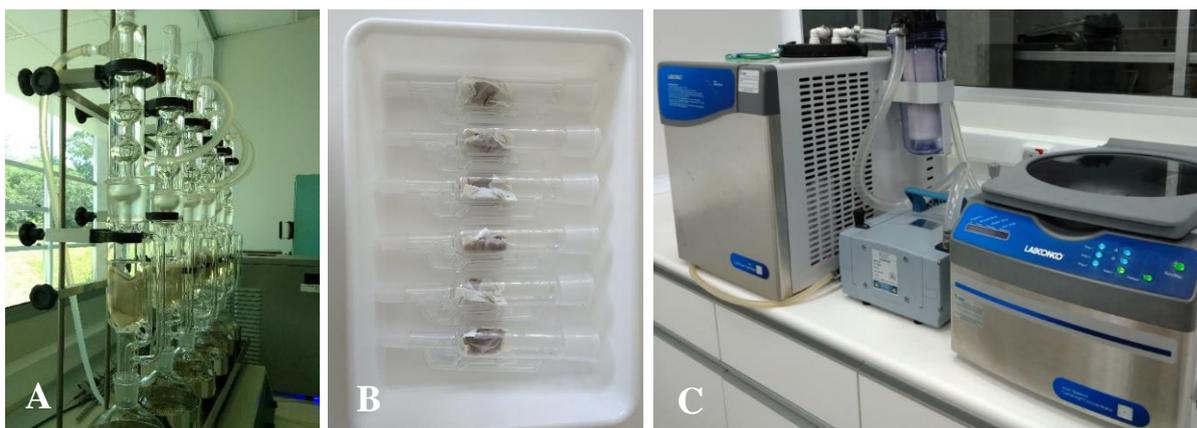
The compositional characterization was performed based on NREL LAPs at the Laboratory of Industrial Biotechnology: (1) Preparation of Samples for Compositional Analysis (NREL-42620); (2) Determination of Extractives in Biomass (NREL-42619); (3) Determination of Structural Carbohydrates and Lignin in Biomass (NREL-42618); and (4) Determination of Acid Soluble Lignin Concentration Curve by UV-Vis Spectroscopy (NREL-42617).

Untreated PCEA biomass and the 17 pretreated samples were reduced to 35 mesh, with the grinding process was preferentially performed during days with low relative humidity. The mill rotor was left open for 20 min between samples and knives and was completely cleaned with compressed air to prevent contamination. This interval of 20 min minimum was set to avoid exposing biomass samples at temperatures higher than 45 °C, since such temperature can degrade carbohydrates (NREL-42619; NREL-42618).

The determination of extractives (Figure 6.4 – A) was performed for untreated PCEA in a 6-positions Soxhlet apparatus for 24 h to be counted after each cycle's renewal in Soxhlet

was completed within 14 to 16 minutes (Figure 6.4 – A). Pre-weighed thimbles and 5 g of biomass with known moisture content were used for the protocol. After the extraction, the thimbles were left drying overnight on the bench (Figure 6.4 – B) and on the following day dried in Acid-Resistant CentriVap Vacuum Concentrator with heat control with a CentriVap Cold Trap -84 °C coupled system (Labonco®) at rotation 30.000 and temperature of 40 °C (Figure 6.4 – C). Since only untreated PCEA biomass had extractives content, its required mass for performance of NREL-42618 were extractive-free retrieved from the dried thimble. In the case of the 17 runs of the experimental design, the samples were extracted directly from pretreated biomass after particle size reduction. The moisture content was determined in all cases and before every step of the characterization process.

Figure 6.4 – A) Apparatus set-up for Soxhlet extraction method with PCEA. B) Thimbles left to evaporate the ethylic ethanol overnight. C) CentriVap Vacuum Concentrator set up with heat control and CentriVap Cold Trap -84 °C coupled system (Labonco®)



Source: Author's database.

The following steps of characterization would involve concentrated acid and very low pH (NREL-42618 and NREL-42617), therefore only glassware materials and flasks were used and handled with appropriate gloves and masks. Approximately 2 g of biomass samples and 10 ml of 72% H₂SO₄ were preheated separately for 5 minutes and mixed with a rod inside a 100 ml beaker for 7 minutes in a 45 °C water bath. The mixture was transferred to a 500 ml Erlenmeyer with help of 275 ml of deionized water and autoclaved for 20 min at 120 °C. The hydrolysate was cooled in a refrigerated room at 8 °C and sheltered from light to prevent degradation and interference in the soluble lignin determination. The hydrolysate of circa 275 ml was filtered in weighed quantitative paper filters in a 500 ml volumetric balloon, with deionized water added until the 500 ml meniscus was reached (Figure 6.5 – A). The insoluble

material retained in the filter was washed with 1.5 L of deionized water to prevent burning and losses when dried in the oven for 24 hours at 105 °C. The soluble lignin was determined from the 500 ml filtrate using quartz cuvettes and determined in the spectrophotometer (model Q-104, volume=3.5 mL; pathlength =10 mm; H×W×D=45×12.5×12.5 mm; AMSTAT Inc., Glenview, IL, USA). An aliquot of 20 ml 500 ml filtrate was transferred to a 25 ml volumetric balloon, its pH adjusted to 5.5 and 6.5 with NaOH for carbohydrate analysis and deionized water added until meniscus. The determination of the structural carbohydrates was performed in HPLC and detailed procedure and conditions are described in 6.5 Additional samples for carbohydrate analysis were labeled and stored in a freezer at -21 °C in case of needed re-injection in HPLC. Finally, the pre-weighed filter with retained mass from hydrolysate was calcined and burned in a muffle furnace for 4 hours at 800 °C for determination of insoluble lignin (Figure 6.5 – B).

Figure 6.5 – A) Sample filtration after acid hydrolysis for determination of structural carbohydrates and lignin contents. B) Muffle furnace with crucibles after 4 hours at 800°C for determination of insoluble lignin



Source: Author's database.

6.5 Determination of carbohydrates by high performance liquid chromatography (HPLC)

Samples from 25 ml volumetric balloons with pH adjusted to 5.5 and 6.5 were used for carbohydrate analysis. Initially, determination of sugars was carried in a Waters modular

equipment compound of a W515 isocratic pump, degasser, PCM, W2707 thermostatic automatic sampler, W2410 refractive index detector and Empower 3.2 data acquisition software. Aliquots of the hydrolyzed biomass samples were filtered through 0.45 μm pore size membranes for injection vials, and 10 μL were injected into a pre-column and Shodex SC 1011 column, previously balanced in mobile phase of 0.5 mM EDTACa (Na)₂ at 72 ° C and flow of 0.6 mL min⁻¹. Sample vials were maintained at 6 ° C in the sampler for the duration of the analysis. A standard cellobiose, glucose, xylose and arabinose curves were prepared in the concentration range of 0.1 to 10 g L⁻¹ from high purity PA reagents and used to quantify the sugars in the sample, identified by the coincidence of the retention time peaks in the sample and the standard curve.

In sequence, determination of furfural and 5-hydroxymethylfurfural (HMF) were performed on a Thermofisher Dionex Ultimate 3000 equipment compound of a quaternary pump, degasser, thermostatic automatic sampler, UV-VIS detector and Chromeleon Dionex data acquisition software. Aliquots of the hydrolyzed biomass samples were filtered through 0.45 μm pore size membranes for injection vials, and 20 μL were injected into a previously balanced Waters Novapak C18 60A 3.9x150 mm column in mobile phase of ACN: H₂O (1: 8) with 1% acetic acid at 60 ° C and flow of 0.8 mL min⁻¹. The detection was made at a wavelength of 276 nm. A standard furfural and HMF curve was prepared in the concentration range from 1 to 25 ppm from high purity PA reagents and used to quantify the components in the sample, identified by the coincidence of the peak retention time present in the sample and in the standard curve.

Finally, acetic acid was determined using a Waters modular equipment compound of a W515 isocratic pump, degasser, PCM, W2707 thermostatic automatic sampler, W2414 refractive index detector and Empower 3.2 data acquisition software. Aliquots of the hydrolyzed biomass samples with adjusted pH were filtered through 0.45 μm pore size membranes for injection vials, and 20 μL were injected into a pre-column and BioRad Aminex HPX-87H column, previously equilibrated in 0.05 N H₂SO₄ mobile phase at 30 ° C in the flow of 0.6 mL min⁻¹. A standard acetic acid curve was prepared in the concentration range of 0.1 to 10 g L⁻¹ from high purity PA reagent and used to quantify the analyte in the sample, identified by the coincidence of the retention time of the peaks present in the sample. and on the standard curve.

6.6 Statistical Analysis

The analysis of variance at 95% confidence level was carried out using Minitab® Statistical Software (Minitab Inc., 2019) to evaluate cellulose and hemicellulose yields, in addition to biomass recovery responses for experimental design with orthogonalized variables.

7. RESULTS AND DISCUSSION

7.1 Compositional characterization of the new elephant grass PCEA biomass

7.1.1 PCEA competitiveness among former Brazilian elephant grass genotypes

The compositional analysis of the new PCEA (*P. purpureum* x *P. purpureum*) preceded the alkaline pretreatment experiment and provided access the contents of cellulose, lignin, hemicellulose, extractives and ashes of the raw material (Table 7.1). The composition characterization of untreated PCEA allowed further comparison with cellulose contents obtained after the alkaline pretreatment, as well as comparison with other established Brazilian elephant grass genotypes at the same non-treated condition (Figure 7.2).

Table 7.1 – Compositional analysis of the new genotype of elephant grass PCEA propagated by seeds. Values are expressed as an average \pm standard deviation and on a dry weight basis

Composition of <i>P. purpureum</i> x <i>P. purpureum</i> (PCEA)			
Moisture content	9.85%		
Cellulose	31.79%	\pm	1.55%
Lignin	18.23%	\pm	0.45%
Hemicellulose	15.51%	\pm	0.70%
Extractives	20.54%	\pm	0.11%
Ashes	3.94%	\pm	0.07%
Total	99.86		

The initial access of the new genotype PCEA composition (Table 7.1) showed that this elephant grass had interesting amounts of cellulose for biofuels, which could be enhanced through pretreatment, such as the alkaline chosen to be applied in this present study. However, it is not reasonable to infer about its potential as raw material for lignocellulosic bioethanol generation by only analyzing its composition. Thereunto, PCEA has been compared with others successful genotypes in Brazil, previously established in plantations and the newest most

promising and high yielding genotype (Table 7.2). In that context, it is crucial to highlight the unique property of PCEA among the compared *P. purpureum* genotypes: its field cultivation with seeds, which has been limiting the plantations of elephant grass and represented the main challenge to be overcome by breeding groups (NEGAWO et al., 2017).

Table 7.2– Elephant grass genotypes compared regarding cellulose content on a dry weight basis

Elephant grass	Genotype	%Cellulose	DM* (ton ha ⁻¹ year ⁻¹)	Cellulose** (ton ha ⁻¹ year ⁻¹)
PCEA ^[1,2]	<i>P. purpureum</i> x <i>P. purpureum</i>	31.79	64.90	20.63
BRS Kurumi ^[3,4]	<i>P. purpureum</i>	30.40	29.25	8.89
Cameroon ^[5,6]	<i>P. purpureum</i> Schum.	41.88	31.22	13.07
Napier ^[5,7]	<i>P. purpureum</i>	43.38	23.89	10.36
Roxo ^[8]	<i>P. purpureum</i> Schum.	31.17	19.60	6.11

*DM – Dry Matter yield

**Calculated based on %cellulose and DM

^[1]. This study ^[2]. Embrapa Agrobiology, unpublished data (2020) ^[3]. Gomide et al. (2001) ^[4]. Pereira et al. (2014) ^[5]. Basso et al. (2014) ^[6]. Botrel et al. (2000) ^[7]. Andrade et al. (2003) ^[8]. Santos et al. (2003)

Overall, PCEA has been observed to be competitive with former and new genotypes of elephant grass, including Kurumi (PEREIRA et al., 2014), Roxo (SANTOS et al., 2001), Napier and Cameroon (BASSO et al., 2014). With exception of PCEA in this present study, the other genotypes were characterized by methodologies other than NREL LAPS. The highest yield of cellulose among these genotypes that are or have been widely cultivated in Brazilian soils is the genotype Napier (10.36 ton ha⁻¹ year⁻¹). In contrast, PCEA offers similar content of cellulose, but far more important than that, its annual dry matter yield of 64.90 ton ha⁻¹ year⁻¹ leaf inoculated (LP343 – *G. diazotrophicus*) outstand among the analyzed genotypes. With that, PCEA can reach exciting amount of 20.63 ton ha⁻¹ year⁻¹ of cellulose under sustainable agricultural operations, including mechanized sowing possible, rainfed system and BNF (Embrapa Agrobiology, unpublished data).

Comparisons among genotypes, such as presented in Table 7.2, must be performed to allow suggestions, technical support and further guidance on biomass breeding studies (NEGAWO et al., 2017) and also endorsement – or not – field cultivation of PCEA in Brazilian or edaphoclimatic alike tropical and subtropical regions around the globe. To the best of current literature knowledge, there is no available data on aerial part characterization of the widely cultivated and high yielding genotype BRS Capiaçú. Although composition of this genotype has been reported by Godinho and collaborators (2019) in an alkaline pretreatment investigation, the study reported only the composition of leaves, which unfortunately was not to be included in the Table 7.2 that compared stem and leaves (aerial part). Furthermore, a research project that investigated compositional contents of aerial part of several elephant genotypes, including BRS Capiaçú, has been recently reported by Andrade and collaborators (2018). In that context, cellulose content is expected to be known soon and consequently the cellulose yield ($\text{ton ha}^{-1} \text{ year}^{-1}$).

Besides cellulose content, advantages in agricultural mechanized operations and potential to reduce GHG emissions, the dry matter yield of the compared genotypes must be also taken into account to endorsing further cultivation of PCEA for biofuel generation purposes. Adding to that, for the practical issues regarding feasibility and further establishment of biomass, some authors including Mohapatra & Gadgil (2013) defend that environmental and technical advantages of biomass employment as raw material must be combined with governmental subsidy aiming to guarantee economic feasibility, technical viability, and social access.

7.1.2 PCEA in comparison with other Brazilian grasses employed for lignocellulosic bioethanol production

Fortunately, Brazil has many options to consider and employ as raw materials for lignocellulosic bioethanol production. A few grasses from Poaceae developed to meet Brazilian edaphoclimatic conditions were also compared with PCEA regarding cellulose content (Table 7.3). Furthermore, the yield in means of ton ha^{-1} have been also considered. The *Brachiaria brizantha* (Marandu), *Saccharum officinarum* (RB867515) and *Sorghum bicolor* (L.) Moench (BRS5073) genotypes were chosen regarding its novelty and widely application in plantations to be compared with the new elephant grass PCEA. Unfortunately, it is not possible to present the exact plantation area of such grasses and elephant grass itself. Such data is annually

provided by the National Supply Company (CONAB) only for sugarcane, since this grass is an important Brazilian commodity.

Table 7.3 – Comparison among PCEA and other established grass in Brazil with potential for lignocellulosic bioethanol generation

Grass	% Cellulose	% Hemicellulose	DM* (ton ha ⁻¹ year ⁻¹)	Cellulose yield** (ton ha ⁻¹ year ⁻¹)
PCEA ^[1,2] (elephant grass)	31.79 ± 1.55	15.51 ± 0.70	64.90	20.63
Marandu ^[3,4] (brachiaria)	30.88 ± 1.70	19.61 ± 2.10	56.86	17.56
RB867515 ^[5,6] (Sugarcane)	35.02 ± -	37.27 ± -	68.40	23.95
BRS716 ^[7,8] (Biomass sorghum)	35.05 ± -	25.26 ± -	35.00	12.27

*DM – Dry Matter yield

**Calculated based on %cellulose and DM

^[1]. This study ^[2]. Embrapa Agrobiology, unpublished data (2020) ^[3]. Carvalho et al. (2014) ^[4]. Souza et al. (2016) ^[5]. Pereira et al. (2014) ^[6]. Schultz et al. (2017) ^[7]. Almeida et al. (2019) ^[8]. Batista et al. (2017)

Besides potential for biofuels production, the grass Marandu in Table 7.3 are in majority applied for cattle feeding. In contrast, sorghum BRS7167 was developed aiming bioenergy purposes and has been recently launched with its high dry matter yield in field cultivation (ALMEIDA et al., 2019).

Furthermore, the most cultivated genotype BRS1503 (*Pennisetum glaucum*) in Brazil was not included in Table 7.3. This pearl millet has been launched quite recently, and to the best of the current knowledge, there were no investigations on compositional assessment of the biomass. Finally, it is worth to highlight that among the raw materials compared in Table 7.3, sugarcane is the only grass that still has the most expensive and higher GHG emissions involved with agricultural mechanized operations required for steam cultivation. Other than that, regarding yield, BNF responsiveness and cellulose yields, sugarcane is unbeatable. In that context, new high yielding genotypes contribute to the diversification of raw materials for lignocellulosic bioethanol production and security of the agroenergy sector.

7.1.3 PCEA and worldwide elephant grass genotypes at a glance

Elephant (Napier) grass is widely cultivated in African countries, although there is lack of data on aerial part characterization in literature. Such scarcity can be explained by expensive equipment and materials required to perform such analyses, which are not available in most countries. In addition, compositional characterization is generally performed focusing on nutrition and agricultural parameters that allow inference regarding the use of such grass for animal feed, including protein and dry matter yield (NYAMBATI et al., 2010; OKORUWA & OKUNLOLA, 2017). Furthermore, countries that have facilities and materials to perform such analyses are more interested in the characterization of obtained cellulosic fiber from elephant grass. To several African countries, power generation (MOHAMMED et al., 2015; DANQUAH, ROBERTS & APPIAH, 2018), by-products from thermochemical conversion (ADENIYI, IGHALO & ONIFADE, 2019) and cellulose fibers from *P. purpureum* employed in polymer industry seem to offer further interesting use of this grass other than biofuel generation (RIDZUAN et al., 2016). PCEA was compared with other *P. purpureum* genotypes cultivated worldwide (Table 7.4).

The new elephant grass PCEA has considered low ash content of 3.90 %wt in comparison with other genotypes worldwide (Table 7.4). Such low values are important since they represent key advantages over fossil fuels, considering that the sulfur commonly presented in ashes contributes significantly to PM air pollution and acid rain that leads to acceleration of soil acidification (GONZÁLEZ & ARISTIZÁBAL 2012; ZHAO et al., 2013). Besides potential to harvest higher yields as better genotypes grow in field cultivations, Brazil could also have a share on the global seed's market with unique genotypes propagated by seeds, such as PCEA. Countries located in tropical or subtropical regions, including Malaysia, Togo, Nigeria, where elephant grass is very important and widely cultivated through stems.

Table 7.4 – Compositional analysis of the new genotype of elephant grass PCEA propagated by seeds in comparison with elephant grass worldwide. Values are expressed as an average \pm standard deviation and on a dry weight basis

Name	Cultivation Site	Genotype	% Extractives	% Cellulose	% Hemicellulose	% Lignin	% Ashes
PCEA	Brazil	<i>P. purpureum</i> x <i>P. purpureum</i>	20.54 \pm 0.11	31.79 \pm 1.55	15.51 \pm 0.70	18.23 \pm 0.45	3.9 \pm 0.07
Hunan ^[1]	China	<i>P. purpureum</i> Schum.	7.28 \pm 0.17	33.35 \pm 0.17	16.08 \pm 0.16	16.42 \pm 0.08	6.85 \pm 0.05
Sumu 2 ^[1]	China	<i>P. purpureum</i> Schum.	5.81 \pm 0.33	32.82 \pm 0.19	16.43 \pm 0.14	13.44 \pm 0.08	9.07 \pm 0.04
Pasto Elefante ^[2]	Colombia	<i>P. purpureum</i>	9.90 \pm -	22.60 \pm -	20.90 \pm -	19.40 \pm -	11.10 \pm -
Napier grass ^[3]	Malasya	<i>P. purpureum</i>	9.26 \pm 0.27	34.21 \pm 2.17	20.44 \pm 1.7	24.34 \pm 1.34	6.34 \pm 0.01
Napier grass ^[4]	Uruguai	<i>P. purpureum</i> Schum.	14.30 \pm 0.40	27.00 \pm 4.00	20.20 \pm 2.60	20.60 \pm 1.30	7.80 \pm 0.10

^[1]. Li et al. (2015) ^[2]. Cardona et al. (2013) ^[3]. Mohammed et al. (2015) ^[4]. Camesaca et al. (2016)

7.2 Effects of the alkaline pretreatment on the new elephant grass genotype PCEA

7.2.1 Effect of the alkaline pretreatment on cellulose yield

The contents of cellulose in pretreated samples of PCEA obtained after each of the 17 assays of the experiment is shown in Table 7.5. The cellulose yields were observed to vary in a range comprehending from 46.30 up to 58.59 %wt. The lowest yield of 46.30 (%wt.) was observed in run 13 at 100 °C, 25 min residence time and NaOH 1% (w/v). The highest yield of cellulose (58.59%) was obtained in assay 14 at 100 °C, 25 min of RT and NaOH 3% (w/v). The only difference between these runs was observed to be the NaOH concentration, which strongly suggested an effect of alkali on the cellulose yield. Such effect has been statistically supported at 95% confidence level (p-value 0.007) and is presented in Table 7.6.

Table 7.5 – Cellulose yields (%wt.) obtained after the alkaline pretreatment. Values based on dry matter

Assay	Temperature (°C)	Residence time (min)	NaOH (%w/v)	Cellulose (%wt.)
1	90	20	1.5	48.89
2	110	20	1.5	48.62
3	90	30	1.5	48.03
4	110	30	1.5	51.61
5	90	20	2.5	51.31
6	110	20	2.5	53.35
7	90	30	2.5	50.52
8	110	30	2.5	54.86
9	80	25	2	52.10
10	120	25	2	48.44
11	100	15	2	48.81
12	100	35	2	53.35
13	100	25	1	46.30
14	100	25	3	58.59
15 (CP)	100	25	2	50.53
16 (CP)	100	25	2	50.25
17 (CP)	100	25	2	51.18

The analysis of variance was performed for the central composite design established for the alkaline pretreatment investigation (Table 7.6). The full quadratic model for the cellulose

yield analyzed as a response of the surface design provides a coefficient of determination R^2 of 0.78350, which implies that this model (Equation 7.1) explains around 78% of the cellulose yields obtained (Table 7.6).

$$\text{Cellulose: } 0.852 - 0.0031T - 0.0144RT - 0.083\text{Alkali} - 0.000010(T^2) + 0.000039x(RT^2) + 0.0176(\text{Alkali}^2) + 0.000154(T)(RT) + 0.00077(T)(\text{Alkali}) - 0.00070(RT)(\text{Alkali}) \quad \text{Equation (7.1)}$$

Where Cellulose is the yield (%wt.), T is Temperature ($^{\circ}\text{C}$), RT stands for Residence Time (min) and Alkali for NaOH (%w/v).

The linear model was statistically significant at 95% of confidence level (p-value of 0.013). The alkali alone had a p-value of 0.003 (ANOVA at 95% confidence level), which suggests an effect of this factor for cellulose yield. The same is not true for the square and linear with interactions models that presented p-values of 0.739 and 0.708, respectively.

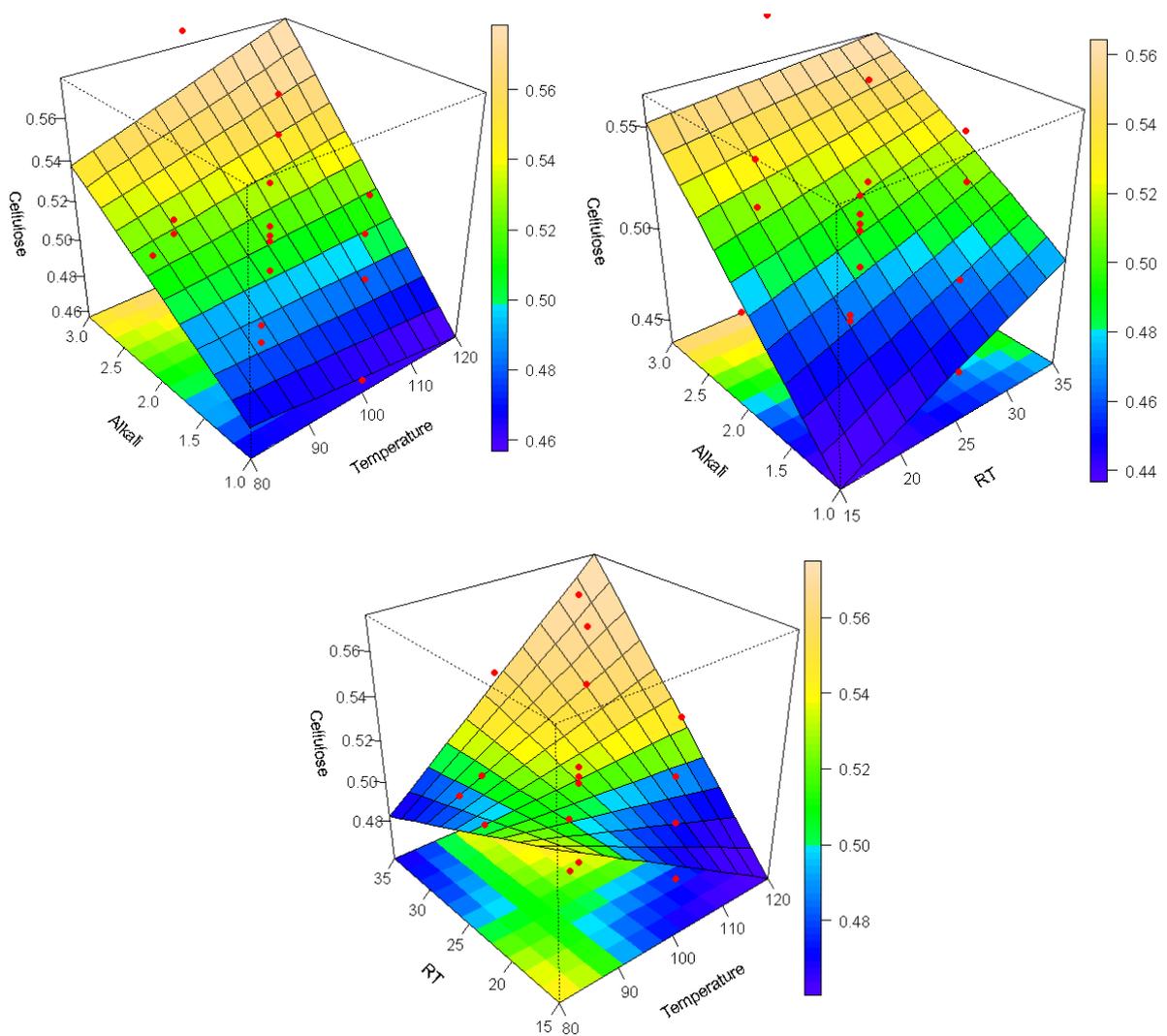
Table 7.6 – Analysis of variance for cellulose yield at 95% confidence level (Minitab[®])

Source	Sum of squares	Degrees of freedom	Mean square	F Value	p-value Prob>F
Model	0.01086	9	0.00121	2.82	0.093 ^b
<i>Linear</i>	0.00970	3	0.00323	7.54	0.013 ^a
Temperature	0.00004	1	0.00004	0.08	0.784 ^b
RT	0.00089	1	0.00089	2.07	0.193 ^b
Alkali	0.00877	1	0.00877	20.47	0.003 ^a
<i>Square</i>	0.00055	3	0.00018	0.43	0.739 ^b
Temperature ²	0.00002	1	0.00002	0.05	0.830 ^b
RT ²	0.00002	1	0.00002	0.04	0.843 ^b
Alkali ²	0.00037	1	0.00037	0.87	0.382 ^b
<i>Interactions</i>	0.00061	3	0.00021	0.48	0.708 ^b
Temperature*RT	0.00047	1	0.00047	1.1	0.329 ^b
Temperature*Alkali	0.00012	1	0.00012	0.27	0.617 ^b
RT*Alkali	0.00003	1	0.00003	0.06	0.818 ^b
Lack of fit	0.00296	5	0.00059	26.4	0.037 ^a
Error	0.00300	7	0.00043		0.093 ^b
Pure error	0.00005	2	0.00002		0.013 ^a
Total	0.01386	16			0.784 ^b
R ²	0.78350				0.193 ^b

a. Significant term ^b. Not significant term

Contrary to initial expectations, the interaction of the three factors altogether (temperature, reaction time and alkali) did not differ statistically at 95% confidence level analysis for the full quadratic model (Table 7.6). This result suggests that the combined three factors did not had an effect on cellulose yield, which can be explained through the range chosen for the residence time factor. Further investigation should be carried with a broader range of levels for this factor, varying more than 5 min among levels. The interactions of the independent factor, taken two at a time, are presented in response surface plots (Figure 7.1).

Figure 7.1 – Interaction plots of Alkali concentration (% w/v), Temperature and Reaction Time (RT) for cellulose yield (ActionStat®)



The central composite design of the surface response methodology has been employed by Cardona and collaborators (2013; 2014) aiming optimization of alkali pretreatment using aerial part of elephant grass as raw material. Although the composition characterization has

been performed for carried out assays of CCD, no statistical information has been provided on cellulose yield and other analyzed responses. Moreover, interesting investigations have also been performed for elephant grass, although no CCD was used and sometimes statistical analysis is not provided, offering a good idea regarding effects of temperature, alkali and residence time parameters in the final cellulose content of solid-fraction composition.

The highest cellulose yield obtained after pretreatment with NaOH on aerial part of elephant grass from various studies is presented in Table 7.7, along with the associated levels for each analyzed factor. The complete summary of conditions has been previously presented on the literature review section, more precisely on Table 5.2. In that context, PCEA achieved the considerable cellulose yield of 58.59 (% wt.), higher than the obtained yield of 51.32 (% wt.) by Menegol et al. (2014) on a lower solid to liquid ratio base, higher temperature and shorted residence time on the reactor. Moreover, even at the highest concentration of 6.0 % w/v NaOH, two times the highest concentration applied for PCEA, the cellulose yield was still around 2 (% wt.) inferior of the obtained 58.59 % wt. for PCEA. In addition, Cardona and collaborators (2014) have reported the cellulose yield of 31.20 (% wt.) from CCD design optimization, although in their work it is not specified which run has achieved such yield and only the levels employed for the analyzed factors are provided (Table 5.2).

Table 7.7 – Cellulose yields obtained for aerial part of elephant grass pretreated with NaOH

Location	Size (mm)	NaOH (% w/v)	Solid to Liquid ratio	Temperature (°C)	Residence Time (min)	Cellulose (% wt.)
Brazil ^[1]	≤10	3.0	1:7.5	100	25	58.59
Brazil ^[2]	-	3.0, 4.0, 6.0	1: 4.0	120	15	51.32, 53.37, 56.90
Colombia ^[3]	3.0	NE	NE	NE	NE	31.20
India ^[4]	0.3-0.4	3.0	1:10	121	30, 60, 90	70.40, 71.53, 72.30
Taiwan ^[5]	5.0	5.0	1:4.0	160*	30	69.00

*. Stem Explosion + Alkaline pretreatment

NE – Not Especificed

^[1]. This study ^[2]. Menegol et al. (2014) ^[3]. Cardona et al. (2014) ^[4]. Haldar & Purkait (2020)

^[5]. Chang et al. (2011)

Interestingly, the study performed for the smallest particle size of elephant grass (0.3-0.4 mm), although employing the longest residence time in the reactor has the highest cellulose yield among the compared studies (Table 7.7). In the work performed by Chang et al. (2011) at 3.0 %w/v at 121 °C the cellulose yields of 70.40, 71.53 and 72.30 (%wt.) were achieved at 30, 60 and 90 min of residence time, respectively. However, it is reasonable to assume that this process would be more expensive than what was performed with PCEA, once more energy is required to reduce the biomass to 0.3-0.4 mm than to achieve ≤ 10 mm of particle size. In addition, 20 °C for at least more 5 minutes also will increase energy costs to reach this temperature in the reactor and also maintain it for the specified residence time. Further analysis on energy expenditures is necessary to infer about the best cost-effective pretreatment strategy.

The last work presented on Table 7.7 describes alkaline pretreatment alone and combined with stem explosion, which has been included in Table 5.2, although it only provides results from after the alkaline pretreatment combined with stem explosion. Finally, Menegol and collaborators (2014) reported cellulose yields of 40.72 and 40.81 %wt. for elephant grass (aerial part) pretreated with 10 and 20 %v/v of NH_4OH , respectively. Such achievements were not included in Table 7.7 because they were inferior to the cellulose yields obtained with NaOH performed in their study. In addition, the liquid ammonia concentrations were at least three times higher than NaOH used in their investigation.

7.2.2 Effect of the alkaline pretreatment on hemicellulose yield

The hemicellulose contents in pretreated samples of PCEA were characterized after alkaline pretreatment for each of the 17 samples from the experimental design (Table 7.8). The hemicellulose contents were observed to vary from 14.98 to 23.52 (%wt.). The lowest yield of hemicellulose was achieved in run 4 at 110 °C, 30 min residence time and 1.5% w/v concentration of NaOH . This amount of hemicellulose was almost the same content accessed for the untreated PCEA feedstock considering the standard deviations (Table 7.1). In contrast, the highest yield of hemicellulose (25.52 %wt.) was obtained in assay 9 at 80 °C, 25 min of RT and 2 %w/v concentration of NaOH . The analysis of variance was performed for hemicellulose response of the central composite design of the alkaline pretreatment investigation (Table 7.9). The full quadratic model for the hemicellulose yield reported a coefficient of coefficient of determination (R^2) of 0.79730, which implies that this model (Equation 7.2) explains around 80% of the cellulose yields obtained (Table 7.9).

$$\text{Hemicellulose: } 0.475 - 0.00118T - 0.0147RT - 0.341\text{Alkali} - 0.000002(T^2) + 0.000039x(RT^2) + 0.0071(\text{Alkali}^2) + 0.000218(T)(RT) + 0.002733(T)(\text{Alkali}) - 0.00423(RT)(\text{Alkali}) \quad \text{Equation (7.2)}$$

Where Hemicellulose is the yield (% wt.), T is Temperature (°C), RT stands for Residence Time (min) and Alkali for NaOH (% w/v).

Table 7.8 – Hemicellulose yields (% wt.) obtained after the alkaline pretreatment. Values based on dry matter

Assay	Temperature (°C)	Residence time (min)	NaOH (w/v)	Hemicellulose (%wt.)
1	90	20	1.5	23.51
2	110	20	1.5	22.21
3	90	30	1.5	22.72
4	110	30	1.5	14.98
5	90	20	2.5	21.17
6	110	20	2.5	23.25
7	90	30	2.5	22.55
8	110	30	2.5	22.33
9	80	25	2	23.52
10	120	25	2	20.90
11	100	15	2	21.17
12	100	35	2	22.30
13	100	25	1	21.08
14	100	25	3	21.75
15 (CP)	100	25	2	22.89
16 (CP)	100	25	2	22.53
17 (CP)	100	25	2	20.82

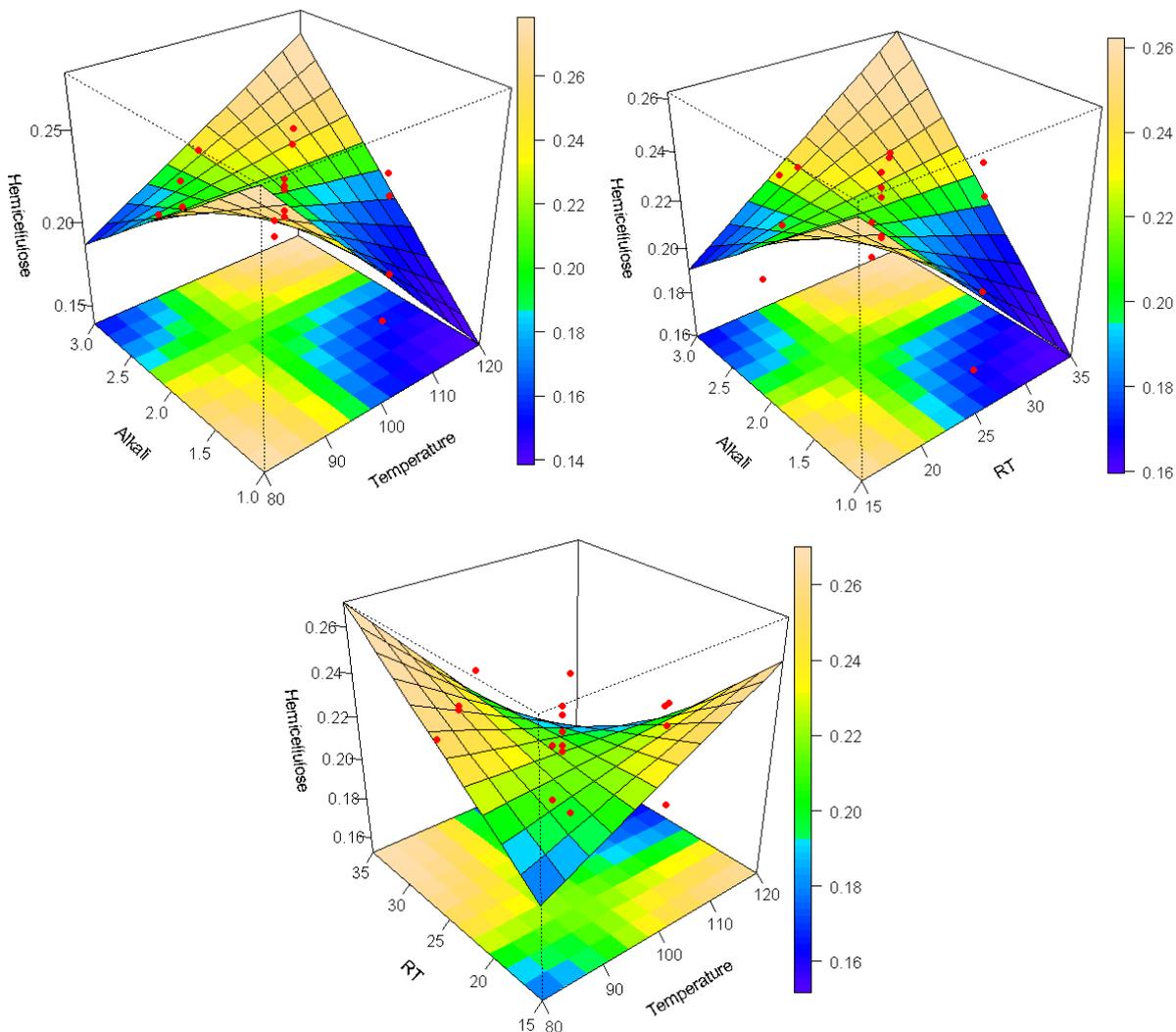
The linear and square models with p-values of 0.122 and 0.918 were not statistically significant at 95% of confidence level. However, the reaction time (RT) presented a p-value of 0.053 and was taken as significant (ANOVA at 95% confidence level), which suggests an effect of this factor on hemicellulose yield. The same is not true for the linear with interactions model, which was significant at 95% confidence level (p-value 0.022). This result suggests that the interaction of temperature with residence time and alkali had an effect on hemicellulose yield. The interaction of analyzed temperature, residence time and alkali factors, taken two at a time, are also presented (Figure 7.2).

Table 7.9 – Analysis of variance for hemicellulose yield at 95% confidence level (Minitab®)

Source	Sum of squares	Degrees of freedom	Mean square	F Value	p-value Prob>F
Model	0.00489	9	0.00054	3.06	0.077 ^b
<i>Linear</i>	0.00147	3	0.00049	2.75	0.122 ^b
Temperature	0.00096	1	0.00096	5.43	0.053 ^c
RT	0.00018	1	0.00018	0.99	0.352 ^b
Alkali	0.00033	1	0.00033	1.84	0.217 ^b
<i>Square</i>	0.00009	3	0.00003	0.16	0.918 ^b
Temperature ²	0.00000	1	0.00000	0.00	0.946 ^b
RT ²	0.00002	1	0.00002	0.10	0.758 ^b
Alkali ²	0.00006	1	0.00006	0.34	0.576 ^b
<i>Interactions</i>	0.00333	3	0.00111	6.26	0.022 ^a
Temperature*RT	0.00095	1	0.00095	5.38	0.053 ^c
Temperature*Alkali	0.00148	1	0.00148	8.36	0.023 ^a
RT*Alkali	0.00090	1	0.00090	5.05	0.060 ^b
Lack of fit	0.00100	5	0.00020	1.63	0.422 ^b
Error	0.00124	7	0.00018		
Pure error	0.00024	2	0.00012		
Total	0.00613	16			
R ²	0.79730				

^a. Significant term ^b. Not significant term ^c. Taken as significant term

Figure 7.2 – Interaction plots of Alkali concentration (% w/v), Temperature and Reaction Time (RT) for hemicellulose yield (ActionStat®)



Furthermore, the highest hemicellulose yields achieved after NaOH pretreatment on aerial part of elephant grass from various studies is presented (Table 7.7), along with the associated levels for each analyzed factor. The complete summary of conditions has been previously presented on the literature review section on Table 5.2. In that context, PCEA achieved the highest hemicellulose yield of 23.59 (% wt.) over other values reported for similar experimental conditions, except for Chang et al. (2011) that achieved 26.50 (% wt.) with steam explosion involved and did not provide information on the alkali pretreatment alone. The complete characterization of samples generated after experimental runs are presented in Table 7.11.

Table 7.10 - Hemicellulose yields obtained for aerial part of elephant grass pretreated with NaOH

Location	Size (mm)	NaOH (% w/v)	Solid to Liquid ratio	Temperature (°C)	Residence Time (min)	Hemicellulose (% wt.)
Brazil ^[1]	≤10	3.0	1:7.5	100	25	23.52
Brazil ^[2]	-	3.0, 4.0, 6.0	1: 4.0	120	15	21.99, 19.60, 20.37
Colombia ^[3]	3.0	NE	NE	NE	NE	21.90
India ^[4]	0.3-0.4	3.0	1:10	121	30, 60, 90	16.00, 15.90, 15.45
Taiwan ^[5]	5.0	5.0	1:4.0	160*	30	26.50

*. Stem Explosion + Alkaline pretreatment

^[1]. This study ^[2]. Menegol et al. (2014) ^[3]. Cardona et al. (2014) ^[4]. Haldar & Purkait (2020)

^[5]. Chang et al. (2011)

Table 7.11 – Composition characterization of experimental assays in comparison with untreated PCEA in weight percent (%wt.) based on dry matter. Contents presented as means of triplicates and respective standards deviation values (\pm)

Sample	Moisture	%Cellulose			%Lignin			%Hemicellulose			%Extractives		%Ashes		%Total
PCEA	9.85	31.79	\pm 1.55	18.23	\pm 0.45	15.51	\pm 0.70	20.54	\pm 0.11	3.94	\pm 0.07	99.86			
1	8.84	48.89	\pm 0.36	14.64	\pm 0.51	23.51	\pm 0.53	-	-	3.44	\pm 0.08	99.33			
2	9.00	48.62	\pm 1.49	16.01	\pm 0.29	22.21	\pm 0.83	-	-	3.57	\pm 0.00	99.41			
3	8.12	48.03	\pm 0.73	17.31	\pm 0.25	22.72	\pm 0.33	-	-	3.46	\pm 0.07	99.63			
4	7.03	51.61	\pm 0.35	14.98	\pm 1.78	14.98	\pm 2.13	-	-	3.30	\pm 0.03	99.93			
5	9.68	51.31	\pm 2.02	13.11	\pm 0.25	21.17	\pm 1.05	-	-	3.93	\pm 0.02	99.19			
6	8.44	53.35	\pm 0.64	10.52	\pm 0.12	23.25	\pm 1.40	-	-	4.30	\pm 0.03	99.87			
7	7.38	50.52	\pm 0.14	13.60	\pm 0.07	22.55	\pm 2.21	-	-	4.36	\pm 0.05	98.42			
8	7.05	54.86	\pm 2.29	10.97	\pm 1.82	22.33	\pm 1.05	-	-	4.35	\pm 0.06	99.55			
9	7.32	52.10	\pm 1.44	12.90	\pm 0.26	23.52	\pm 0.71	-	-	3.58	\pm 0.12	99.42			
10	9.97	48.44	\pm 0.22	14.18	\pm 0.74	20.90	\pm 0.22	-	-	3.65	\pm 0.19	97.14			
11	9.93	48.81	\pm 0.16	16.36	\pm 1.16	21.17	\pm 1.79	-	-	3.27	\pm 0.04	99.54			
12	7.01	53.35	\pm 2.07	13.39	\pm 0.42	22.30	\pm 2.07	-	-	3.89	\pm 0.05	99.94			
13	9.97	46.30	\pm 1.47	17.63	\pm 0.10	21.08	\pm 0.64	-	-	3.99	\pm 0.11	98.97			
14	7.00	58.59	\pm 0.96	8.90	\pm 0.11	21.75	\pm 2.38	-	-	3.65	\pm 0.08	99.89			
15*	7.54	50.53	\pm 1.05	15.01	\pm 0.46	22.89	\pm 0.45	-	-	3.95	\pm 0.09	99.92			
16*	8.26	50.25	\pm 0.47	15.03	\pm 0.55	22.53	\pm 1.00	-	-	3.90	\pm 0.05	99.97			
17*	8.01	51.18	\pm 0.73	15.10	\pm 1.14	20.82	\pm 1.42	-	-	3.99	\pm 0.05	99.09			

*. Central Point (CP)

7.2.3 Effect of the alkaline pretreatment on solid-fraction recovery

Various solid recovery yields of elephant grass biomass have been reported in the literature after alkaline pretreatment of elephant grass (*P. purpureum*) responding to different parameters and experimental conditions (CARDONA et al., 2013; CARDONA et al., 2014; MINMUNIN et al., 2015; PHITSUWAN et al., 2016; HALDAR & PURKAIT, 2020). In this study, the recovery of PCEA biomass was also accessed for each one of the experimental assays and the obtained weight yields (% wt.) based on dry matter are presented in Table 7.12.

Table 7.12 – Recovery of PCEA biomass for experimental assays in weight percentage on a dry matter basis

Assay	Temperature (°C)	Residence time (min)	NaOH (w/v)	Recovery (%wt.)
1	90	20	1.5	64.32
2	110	20	1.5	64.88
3	90	30	1.5	65.74
4	110	30	1.5	61.39
5	90	20	2.5	60.78
6	110	20	2.5	59.57
7	90	30	2.5	59.71
8	110	30	2.5	55.67
9	80	25	2	60.90
10	120	25	2	61.56
11	100	15	2	58.97
12	100	35	2	58.74
13	100	25	1	67.92
14	100	25	3	53.73
15 (CP)	100	25	2	62.19
16 (CP)	100	25	2	62.29
17 (CP)	100	25	2	62.02

The analysis of variance was also performed for the recoveries observed after the alkaline pretreatment. The full quadratic model for the recoveries observed presented a coefficient of determination (R^2) of 0.91740, which supports that this model (Equation 7.3) explains around 92% of the recoveries obtained (Table 7.13).

$$\text{Recovery: } -0.261 + 0.00913T + 0.0372RT + 0.060\text{Alkali} - 0.000020(T^2) + 0.000318x(RT^2) + 0.0121(\text{Alkali}^2) + 0.000194(T)(RT) + 0.00036(T)(\text{Alkali}) - 0.00145(RT)(\text{Alkali}) \quad \text{Equation (7.3)}$$

Where recovery is the yield (% wt.), T is Temperature (°C), RT stands for Residence Time (min) and Alkali for NaOH (% w/v).

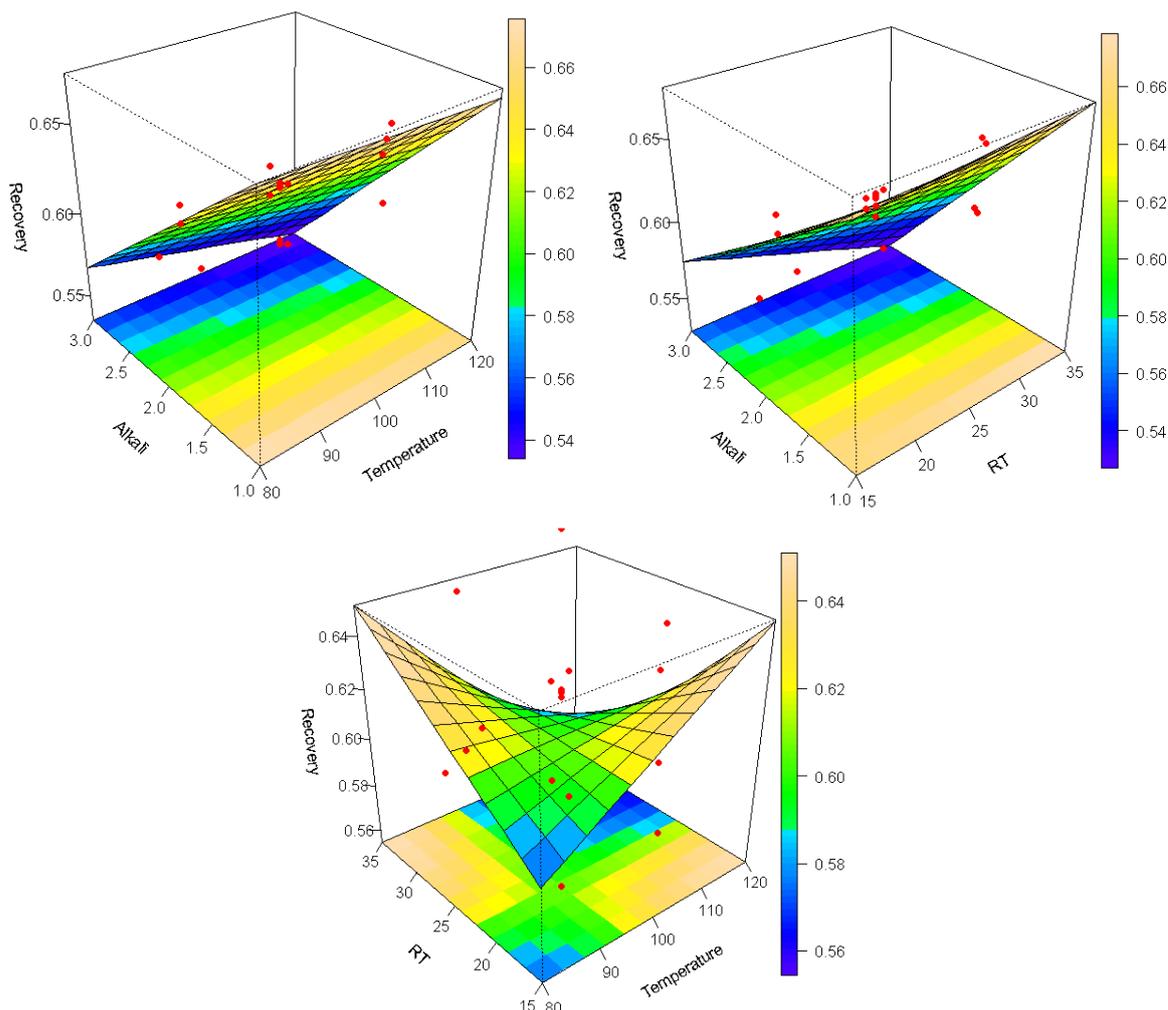
The linear model presented p-value of 0.001 and was statistically significant at 95% of confidence level analysis for the biomass recovery response. Similarly, to the observed for the cellulose response of the experimental design, the alkali alone had a p-value of 0.000 (ANOVA at 95% confidence level), which suggests an effect of this factor on biomass recovery. Once again repeating the observed for cellulose response of the experimental design with orthogonalized variables, the same is not true for the square and linear with interactions models that presented p-values of 0.231 and 0.353, respectively. Contrary to initial expectations, the interaction of the three factors altogether (temperature, reaction time and alkali) did not differ statistically at 95% confidence level of analysis of variance for the full quadratic model. This result suggests that the combined three factors interacting together did not had an effect on recovery yield, which can be explained through the range chosen for the residence time factor. Further investigation should be then carried out with a broader range of levels for the chosen factor. Finally, the interaction of analyzed temperature, residence time and alkali factors, taken two at a time, are presented in Figure 7.3.

Table 7.13 – Analysis of variance for the linear with interactions model for solid recovery

Source	Sum of squares	Degrees of freedom	Mean square	F Value	p-value Prob>F
Model	0.01785	9	0.00198	8.64	0.005 ^a
<i>Linear</i>	0.01571	3	0.00524	22.83	0.001 ^a
Temperature	0.00037	1	0.00037	1.61	0.244 ^b
RT	0.00035	1	0.00035	1.52	0.258 ^b
Alkali	0.01499	1	0.01499	65.35	0.000 ^a
<i>Square</i>	0.00126	3	0.00042	1.82	0.231 ^b
Temperature ²	0.00008	1	0.00008	0.34	0.576 ^b
RT ²	0.00123	1	0.00123	5.34	0.054 ^c
Alkali ²	0.00018	1	0.00018	0.77	0.409 ^b
<i>Interactions</i>	0.00088	3	0.00029	1.28	0.353 ^b
Temperature*RT	0.00075	1	0.00075	3.27	0.114 ^b
Temperature*Alkali	0.00003	1	0.00003	0.12	0.744 ^b
RT*Alkali	0.00011	1	0.00011	0.46	0.519 ^b
Lack of fit	0.00160	5	0.00032	171.41	0.006 ^a
Error	0.00161	7	0.00023		
Pure error	0.00000	2	0.00000		
Total	0.01946	16			
R ²	0.91740				

^a. Significant term ^b. Not significant term ^c. Taken as significant term

Figure 7.3 – Interaction plots of Alkali concentration (% w/v), Temperature and Reaction Time (RT) for biomass recovery (% wt.) (ActionStat®)



Moreover, a comparison of highest recovery (% wt.) achieved among PCEA and other elephant grass genotypes previously pretreated with alkali (NaOH) is presented on Table 7.14. The recoveries shown on this Table (7.14) are from studies that had its operational and experimental conditions summarized in the literature review section of this present work (Table 5.2). Unfortunately, not all of them presented a final recovery percentage alone, once the final removal of recovery of particular components were preferably reported.

Interestingly, the highest yields for aerial part of elephant grass pretreated with NaOH can be observed at lower alkali concentrations of experimental set ups (Table 7.14). Furthermore, the final recovery of 67.92 (% wt.) obtained at the 1.0 % w/v NaOH, at 100 °C and 25 min in the present study with PCEA was relatively high among other recovery responses of aerial plant pretreatment. Aerial part of elephant grass pretreated with 2 % w/v NaOH at 120 °C

and 15 min reached recovery of 59.41 (%wt.) In contrast, in this same study performed by Menegol and collaborators (2014), the recoveries of 69.40 and 69.90% were reported for NH₄OH concentrations of 10 and 20 %w/v, respectively. Furthermore, the recoveries of 53.90 and 53.60 (%wt.) for elephant grass pretreated with 0.7 and 1.0 %w/v NaOH, respectively, suggest that low alkali concentrations provide also interesting recovery yields. In that sense, it would be interesting that more studies performed with alkali pretreatment would also compare cost-benefits of parameters and conditions chosen. Unfortunately, the study performed by Chang and collaborators (2011) and previously analyzed regarding cellulose (Table 7.7) and hemicellulose (Table 7.10) did not report the recovery yield of pretreated elephant grass.

Table 7.14 – Recoveries obtained for aerial part of elephant grass pretreated with NaOH

Location	Size (mm)	NaOH (%w/v)	Solid to Liquid ratio	Temperature (°C)	Residence Time (min)	Recovery (%wt.)
Brazil ^[1]	≤10	1.0	1:7.5	100	25	67.92
Brazil ^[2]	-	2.0	1: 4.0	120	15	59.41
Colombia ^[3]	3.0	0.7	1:17.5	100	120	53.90
India ^[4]	0.3-0.4	1.0	1:10	121	30	53.60

^[1]. This study ^[2]. Menegol et al. (2014) ^[3]. Cardona et al. (2014) ^[4]. Haldar & Purkait (2020)

Furthermore, a comprehensive economic analysis including: energy cost of particle size reduction, different residence time and temperatures of reactors; how expensive are the highest solid to liquid ratio compared with the lowest ones; and finally, how expensive are the highest alkali concentration compared with recovery obtained for the lowest ones. In addition, several works on bench scale have pretreated either elephant grass leaves (URRIBARRÍ, FERRER & COLINA, 2005; NASCIMENTO & REZENDE, 2018; GODINHO et al., 2019) or stems (AIYEJAGBARA et al., 2016; PHITSUWAN et al., 2016; TSAI et al., 2018), which requires mechanical separation to be performed in the field and should definitely accounted as energetic expenditure. It is known that stem parts provide higher cellulose contents for bioethanol conversion, although this higher yield must be confronted with mechanical separation costs in order to infer if it is more cost-effective and less harmful to the chain (GHG emissions) than the aerial part use. Only after comprehensive and careful analysis it could be possible to infer about whether conditions are more sustainable for the alkaline pretreatment process of elephant grass raw material.

7.2.3.1 Recovery of desirable components and lignin removal

According to Kim, Lee & Kim (2016), alkaline pretreatments such as performed in this study with NaOH can remove lignin without degrading carbohydrates. This can be observed on Table (7.15), where cellulose had one of the highest recovery percentages (% wt.) at the same time it achieved the highest lignin removal of 73.76 (% wt.) corresponding to assay 14 of the design orthogonalized variables (T 100 °C, 25 min RT and 3 % w/v of NaOH). Several authors have claimed that an efficient pretreatment of biomass must remove lignin, including Martínez et al. (2016), Herbaut et al. (2018), Song et al. (2019) and Tsegaye, Balomajumder and Roy (2019).

Table 7.15 – Overview of components after pretreatment of PCEA biomass with NaOH. Values presented in weight percentage (% wt.) based on dry matter

Assay	%Solid recovery	%Cellulose recovery	%Hemicellulose recovery	%Lignin removal
1	64.32	98.92	97.46	48.33
2	64.88	99.22	92.87	43.03
3	65.74	99.32	96.27	37.58
4	61.39	99.67	59.30	49.54
5	60.78	98.09	82.94	56.31
6	59.57	99.98	89.29	65.64
7	59.71	94.90	86.79	55.44
8	55.67	96.07	80.12	66.49
9	60.90	99.81	92.32	56.92
10	61.56	93.81	82.93	52.12
11	58.97	90.54	80.48	47.09
12	58.74	98.58	84.44	56.86
13	67.92	98.94	92.28	34.32
14	53.73	99.03	75.33	73.76
15 (CP)	62.19	98.85	91.75	48.80
16 (CP)	62.29	98.47	90.44	48.64
17 (CP)	62.02	99.84	83.23	48.63

In contrast, some studies on alkaline pretreatment of lignocellulosic raw materials suggest that more important than the lignin removal is the cellulose retention and improvements on its surface area, allowing a better enzymatic access and providing higher sugar yields (SHIMIZU et al., 2020). In other words, delignification all alone does not guarantee that the

enzymes will be able to infiltrate lignocellulosic fibres to produce fermentable sugars (DONALDSON & VAIDYA, 2017).

Unfortunately, hemicellulose can hydrogen bond with cellulose and incorporate cellulose to its molecule, negatively interfering on saccharification (McCANN, & CARPITA, 2015). Although a crucial step to obtain lignocellulosic bioethanol, the enzymatic hydrolysis is out of scope in the present study. Only after such step performed, it would be possible to link cellulose and hemicellulose recoveries with yields of fermentable sugars. The highest recoveries of cellulose and hemicellulose (% wt.) components of PCEA pretreated samples, along with its lignin removal (% wt.), were compared with amounts obtained in various studies (Table 7.14).

7.2.4 Effect of the alkaline pretreatment on water employment for neutral pH

After the alkaline pretreatment of the new elephant grass genotype PCEA, the amount of water used to achieve neutral pH of the solid-phase samples was recorded and is reported on Table 7.16.

Table 7.16 – Deionized water volumes employed for pH neutralization after alkaline pretreatment

Run	Temperature (°C)	Residence time (min)	NaOH (w/v)	Water (mL)
1	90	20	1.5	7000
2	110	20	1.5	7000
3	90	30	1.5	7200
4	110	30	1.5	7100
5	90	20	2.5	8600
6	110	20	2.5	8400
7	90	30	2.5	8600
8	110	30	2.5	8200
9	80	25	2	8200
10	120	25	2	8000
11	100	15	2	8000
12	100	35	2	8100
13	100	25	1	6600
14	100	25	3	9200
15	100	25	2	8600
16	100	25	2	8500
17	100	25	2	8600

In the present study, the desirable lowest volume of water use was expected to meet the cellulose, hemicellulose and recovery yields discussed in the previous section (Table 7.5). However, the highest yields were achieved by the highest alkali use 3 %wt., which resulted in higher water expenditure to reach neutral pH. Furthermore, such water employment results are important to understand the impacts on water use and also to start to think on this effluent as an opportunity to improve the process, thinking on industrial scale. In addition, further characterization of such water should be performed to evaluate presence of cellulose, hemicellulose, salts formed during the alkaline pretreatment, acetic acid, and so on, to better explore and suggest recycling strategies.

8. CONCLUSION

In this study, the new elephant grass PCEA propagated by seeds was pretreated with alkali in a central composite design experiment with varying levels of temperature, retention time (RT) and alkali concentration, aiming cellulose exposure. Initially, the composition of PCEA was accessed and presented interesting contents of holocellulose 47.30 %wt. (db), but is also high yielding in the field outstanding as a competitive raw material to contribute with the already successful sugar-based Brazilian biofuel sector. In addition, the new seed propagated genotype, with its potential high yields in marginal and abandoned agricultural areas, can also contribute with agricultural and bioenergy sectors in tropical and subtropical edaphoclimatic alike locations worldwide. Moreover, it is not strategically secure in agricultural terms whatsoever depending on only one crop species for biofuel generation. The Country should always improve the acknowledgement and use of several residues from agroindustry and also incorporate the new crops developed within its plantations.

As initially expected, the alkaline pretreatment was effective in removing lignin without degrading cellulose. Although it did not provide the highest biomass recovery (%wt.), the best condition was achieved in assay 14, at 100 °C, 25 RT and 3 %wt, once it provided 99.03 %wt. recovery of cellulose and reached 73.76 %wt. of lignin removal. In that sense, the present study suggests exciting potential of the new elephant grass PCEA for lignocellulosic bioethanol production via alkaline pretreatment to be explored by the Brazilian agroenergy sector. However, further studies should be carried out approaching enzymatic hydrolysis, in special for

assay 14, to confirm the alkaline pretreatment as effective for the production of fermentable sugar and, consequently, lignocellulosic bioethanol.

9. FURTHER STUDIES

During the development of the present study, several process aspects outstand and questions were raised. However, these questions will be presented in the form of suggestions to encourage further investigation on biofuels from the new elephant grass PCEA:

(1) Alkaline pretreatment:

- Based on parameters and levels analyzed in the alkaline pretreatment, further experimental surface design investigating residence time, temperature and also alkali concentration should be performed. However, a new investigation should approach cellulose yield with range other than 1 to 3 wt varying in 0.5 to accomplish a better approximation of factors and reach the desired optimization response;
- Further enzymatic hydrolysis for the assay 14;
- Water use is of concern not only from an environmentally friendly perspective, but also from sustainability of economic and accessibility sides. Therefore, further studies should focus on the water recycling of the pretreatment process for alkaline pretreatment of lignocellulosic bioethanol; and
- Finally, but not least, cost-effective analysis of alkaline pretreatment process should be performed to infer about its sustainability. This analysis should at least comprehend 2 of its 3 areas, which are economic feasibility and environmental externalities. The third would be social impacts, but at the moment, this is a new field and there are no established methodologies to be used and infer about integrated impacts of raw material and chemical pretreatment choice.

(2) PCEA:

- Considering the extractives content of 20.54 ± 0.11 (% wt.) present in PCEA feedstock, it would be also interesting to thermochemically convert through pyrolysis for further liquid biofuels generation.

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