UNIVERSIDADE FEDERAL DO PARANÁ

JESUS DAVID CORAL MEDINA

EVALUATION OF THE PRODUCTION OF BIOFUELS AND CHEMICALS FROM OIL PALM EMPTY FRIT BUNCHES (OPEFB): A BIOREFINERY APPROACH

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Dissertação apresentada como requisito parcial para a obtenção do grau de Doutor em Engenharia de Bioprocessos e Biotecnologia, no curso de Pós-graduação em Engenharia de Bioprocessos e Biotecnologia, setor de Tecnologia, da Universidade Federal do Paraná

Orientadora: Profa. Dra. Adenise Lorenci Woiciechowski

Co-Orientador: Prof. Dr. Carlos Ricardo Soccol

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UNIVERSIDADE FEDERAL DO PARANÁ
Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia
Setor de Tecnologia

ATA DE DEFESA DE TESE DE DOUTORADO

Aos vinte e oito do mês de Julho de 2016, na Sala 3 – do Prédio de Bioprocessos, do Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pelo Profº. Drº. Júlio Cesar Carvalho - Coordenador do Programa de Pós – Graduação em Engenharia de Bioprocessos e Biotecnologia, a banca examinadora para a Centésima Décima quarta Defesa de Tese de Doutorado, Área de Concentração: Agroindústrias e Biocombustíveis. Estiveram presentes no Ato, além do coordenador do programa, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do Colegiado do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, ficou constituída pelos Professores Doutores, Adenise Lorenci Woiciechowski – orientadora da tese, UFPR, Carlos Ricardo Soccol – co-orientador da tese, – UFPR, Arion Zandoná Filho - UFPR, Susan Grace Karp - UP , Adriane Bianchi Pedroni Medeiros – UFPR, Cristine Rodrigues - UFPR.

Às 14h, a banca iniciou os trabalhos, convidando o candidato **Jesus David Coral Medina,** a fazer a apresentação da Tese intitulada: "EVALUATION OF THE PRODUCTION OF BIOFUELS AND CHEMICALS FROM OIL PALM EMPTY FRUIT BUNCHES (OPEFB): A BIOREFINERY APPROACH". Encerrada a apresentação, iniciou-se a fase de arguição pelos membros participantes.

Tendo em vista a tese e a arguição, a banca composta pelos professores doutores, Adenise Lorenci Woiciechowski, Carlos Ricardo Soccol, Arion Zandoná Filho, Susan Grace Karp, Adriane Bianchi Pedroni Medeiros, Cristine Rodrigues, declarou o candidato aprovado (de acordo com a determinação dos Artigos 59 a 68 da Resolução 65/09 de 30.10.09).

Curitiba, 28 de Julho de 2016.

Profª. Drª. Adenise Lorenci Woiciechowski

Profº. Drº. Carlos Ricardo Soccol

Profº. Drº. Arion ~~Zandoná Filho~~

Profª. Drª. Susan Grace Karp

Profª. Drª Adriane Bianchi Pedroni Medeiros

Profª Drª. Cristine Rodrigues

To my mother, who is the catalyst of my life, reducing the activation energy of all odds, moving my life to new equilibrium states and making my Gibbs energy lowest as is possible.

A mi madre, quien es el catalizador de mi vida, reduciendo la energía de activación de todas las dificultades, desplazando mi vida a nuevos estados de equilibro donde mi existir alcanza la tranquilidad del mínimo global correspondiente a la energía libre de Gibbs.

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The danger is that our power to harm or destroy the environment or our peers,
increases much faster than our wisdom in using that power

Stephen Hawking

Resumo

O excessivo incremento na população, tem repercutido na alta demanda de combustíveis, alimento e químicos, sendo atualmente as principais fontes de energia e químicos não renováveis; petróleo, gás natural e carvão. O consumo e queima desmesurada tem repercutido na liberação na atmosfera de grandes quantidades de material particulado, gases de efeito estufa CO_2, CH_4, NO_x e SO_x, até níveis nunca registrados, repercutindo em mudanças climatológicas e alterações nos ecossistemas (efeito estufa). Portanto, para diminuir os efeitos desses gases na atmosfera, o impacto ambiental e melhorar a sustentabilidade da terra, existe a necessidade de desenvolver processos e produtos que sejam ambientalmente amigáveis, eficientes e sustentáveis. Nesse contexto, os materiais lignocelulósicos têm sido objeto de estudo no últimos anos, para produção de biocombustíveis, líquidos, sólidos e gasosos, e químicos de alto valor agregado. A utilização de biomassa como fonte de combustíveis e químicos tem uma grande vantagem sobre os convencionais processos petroquímicos, a geração de créditos de carbono pelo balanço negativo na produção total de CO_2. No entanto, a viabilidade do uso de material lignocelulosico em processos industriais deve ser analisada mediante estudos técnico-econômicos e energéticos que permitam determinar o benefício econômico e a energia neta entregada pelo processo de biorefinamento de biomassa. A partir disso, neste trabalho é apresentado um estudo teórico-experimental da produção de etanol, xilitol e lignina usando os cachos vazios obtidos do processo de extração de óleo de palma *Eleaeis Guineensis*, cultivada no estado do Pará, Brasil. Foram desenvolvidos três pretratamentos: sequencial ácido/alcalino, explosão a vapor, explosão a vapor seguido de deslignificação alcalina. Dentro do conceito de biorrefinarias, foram caracterizadas e testadas diferentes propriedades biológicas da lignina obtida do processo ácido/alcalino, com a finalidade de propor usos alternativos da lignina fraccionada obtida nas etapas de pretratamento. Para determinar a efetividade dos pretratamentos foram desenvolvidos estudos experimentais de sacarificação enzimática analisada em função da digestibilidade enzimática dos cachos vazios tratados, no entanto, para determinar o tratamento mais adequado foi levado em consideração o balanço de energia e a energia neta entregada pela produção de etanol e lignina dentro do conceito de biorefineria.

Abstract

The global population is growing rapidly, therefore, the consumption of chemicals and fuels is largest, being the non-renewables the main sources of them. The consumption and burning of fossil fuels has had a significant impact on the environment and global climate, mainly because the presence of high levels in concentration of CO_2, CH_4, NO_x and SO_x in the atmosphere, producing changes in the environment, ecological disturbances and global warming. Consequently, at present it is necessary to develop new processes, using biotechnological and chemical tools, producing economical profits with environmentally friendly. The lignocellulosic biomass has been gaining special attention almost in the last two decades, because the uses of biomass as feedstock for biofuels and chemicals has a great advantage, which is the CO_2 credits, by the negative balance of CO_2 generation by the burning of biofuels. Nevertheless, the uses of lignocellulosic biomass as source of chemicals and biofuels must be analyzed through techno-economic and energetic evaluations, allowing to determine the economical profit and the net energy value given by the process. Based on this, this work presents the study of oil palm empty fruit bunches (OPEFB) as feedstock for ethanol, xylitol and lignin production, through theoretical and experimental approaches. Were made Three pretreatments; sequential acid/alkaline, steam explosion and steam explosion followed of alkaline delignification. The lignin obtained was characterized by conventional and modern techniques, to elucidate the presence of structures with industrial application, also, were tested different biological properties to re-valorize the lignin. The efficiency of pretreatment was measured in terms of enzymatic digestibility of OPEFB treated, however to determine the best pretreatment configuration, was carried out a techno-economic analysis, considering three possible configuration of biorefinery and four scenarios of production, producing twelve scenarios analyzed. The techno economic analysis was developed together with the energy balance of the process and determined the net energy value for all configuration and scenarios analyzed. Based on these results was established the most adequate configuration of biorefinery process in terms of economical profit and net energy value, for the biorefinery process of oil pam empty fruit bunches.

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Abbreviation list

Chapter 1	
MTOE	Millons of Tonnes of Oil Equivalent
OPEFB	Oil Palm Empty Fruit Bunches
IEA	International Energy Association
IPCC	Intergovernmental Panel on Climate Change
GHG	Greenhouse gas emissions
OVEG	Program of Vegetables Oil
Chapter 2	
DP	Polymerization degree
GX	Glucomannan
GM	Glucomannan
XG	Xyloglucan
GGM	Galactoglucomannans
AGX	Arabinoglucuronoxylan
AG	Arabinogalactan
Chapter 5	
NREL	National Renewable Energy Laboratory
FTIR	Fourier Transform Infrared
SEM	Scanning electronic microscopy
TPC	Total Phenolic Content
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
NMR	Nuclear Magnetic Resonance
HSQC	Heteronuclear single quantum coherence
MHB	Muller Hinton Broth
Chapter 6	
$K_{obs,o}$	Kinetic parameter
$C_{monomer}$	Concentration of glucose, xylose and arabinose
$C_{polymer,0}$	Initial concentration of cellulose, hemicellulose and lignin
NEV	Net energy value
EP	Economical profit
E_R	Energy requirements for each reactor
Cp_i	Heat capacity of the i component
LHV	Low heating value
Q_{rxn}	Heat of reaction for hydrolysis
$Q_{heating}$	Energy requirements for each reactor.
Δ_{rxn}^0	Enthalpy of reaction at standard conditions
υ_i	Stoichiometric coefficient

Summary

World energy matrix and Brazilian crops

Abstract

The high-rise population growth has caused a considerable increase in the energy and food requirements for the countries. Is estimated that by 2050 in the world dwell 10 billion people. This largest population will affect the quality of live mainly by the energy and food requirements. In terms of energy demand, is projected that by 2015 the world population requires 19,060 MTOE (Million of Tonnes of Oil Equivalent) and the fossil fuels as coal, oil and natural gas, does not be sufficient to supply these demand. Then, biofuels from lignocellulosic biomass are an environmental and social alternative, especially bioethanol and biodiesel, to energy supply without the problem of food competition. In this chapter is presented an overview of the biodiesel production in Brazil, the common raw material employed and the solid waste generation common called oil palm empty fruit bunches (OPEFB), as well as the common pretreatments reported in literature to produce a substrate cellulose enriched, with the aim to produce chemical building blocks and biofuels via chemical or biochemical.

Keywords: Oil palm empty fruit bunches (OPEFB); Biofuels; Biodiesel production

1.1. Background

The global population is growing and is likely to reach 10 billion by the end of the century, with potentially devastating consequences for our environment and living quality. It will be increasingly difficult to provide food and energy for such a dense population, especially since the both energy and food provision is projected to increase per capita [1]. In Figure 1is presented the estimated growing population until 2050, for develop and less develop countries. As is depicted in Figure 1, in the more developed countries, the population is almost constant and it is likely to remain so. While in less developed countries, like as the Central America, South America, middle orient, African countries and China, the growing is very fast, corresponding to approximately 88% of the total world population.

Together with the population growth the demand for food and energy supply increases, it is expected that in 2050, the energy demands reach the 19,064 MTOE (Millions of Tonnes of Oil Equivalent), being the more developed countries the highest consumers in terms of energy demands.

In Table 1 is presented the ten countries with highest energy demand. The more developed countries are the driving force behind the high demand of energy, being the world energy matrix dominated by fossil fuels. However, nowadays the global energy matrix has many sources of energy and many high quality and convenient energy carriers.

Is estimated that that the total world energy consumption, including renewables will increase 45% by 2030. The International Energy Association (IEA) suggest that crude oil will remain the dominant source of energy [2]. This scenario for high energy demand, will require more than $40 trillon in cumulative investment in energy supply over the period from 2014 to 2035, together with $8 trillion to improve end-use energy efficiency [3].

In Figure 2 is presented the world primary energy demand recorded since 1980 and the projections to 2030. As is displayed in Figure 2, it is showed that for the next decade, the fossil fuels oil, coal and gas are by far the most important contributors to the world's energy matrix. The nuclear energy is almost constant and their contribution to world energy matrix remain so. The rising of renewable energy sources as wind, solar, biomass, biofuels and hydrothermal is important because they are considered eco-friendly and the less countries

will have a key role in the disponibilization of this type of energy, especially biofuels from biomass.

Together with the energy demand increase, the big challenge for the energy industry and transport, during the next two decades is probably to keep the rise in global average temperatures below 2 °C, as was stablished in the 12th Conference of the Parties (COP21) – held in Paris in December 2015 [4]. The Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report highlighted that the burning of fossil fuels and the population growth are leading to the rapid increase in greenhouse gas emissions (GHG), in particular over the past 10 years, which contributes to climate change drastically. According to World Energy Council petroleum, natural gas and coal collectively contribute to nearly 82% of global energy needs and one fifth of the CO_2 emissions is due to 60% of petroleum based fossils fuels. Based on this, new challenges raise for biotechnology supplementation of biofuels with commodity products from renewable resources, instead of fossil based ones [5,6].

Currently, the biofuels production from biomass is well accepted as an environmental friendly solution for reduction dependence on fossil fuels, however, one of the most crucial problems with increasing biofuels production is that it competes for natural and agricultural resources with food and food related use, going to have an impact on world agricultural commodity prices and food security [7].

In Brazil, it is expected for the next 10 years, a 5.3% increase in the national energy demand per year, reaching 372 MTOE until 2020. In this context, from economic, environmental and social point of view, biofuels are seen as and attractive alternative [8]. The two major biofuels produced and commercialized actually in Brazil are the bioethanol and biodiesel.

The bioethanol was launched by the ProAlcool program, with the objective of slowing down the energy consumption, so as to maintain economic growth, by producing ethanol from biomass (sugarcane, cassava and sorghum) to substitute gasoline [9]. Nowadays, Brazil is the second largest bioethanol producer behind of USA. In 2013 were produced 25,530 million L of bioethanol, which approximately meant the 55% of sugarcane production used for bioethanol production [7].

In 1983 was conceived the Program of Vegetables oils (OVEG), it was a significant contributions to the use of vegetable oils in vehicles. In 2004, the Brazilian government established the National Program of Biodiesel Production (Programa Nacional de Produção de Biodiesel, PNPB), with the objective to introduce the small farmers withing the productive chain of biodiesel. According to the law no. 11.097/2005, of January 2008, the use of 2% biodiesel blended with petrodiesel (B2) is mandatory in Brazil and increasing amounts of biodiesel blended with petrodiesel will be required with time. Currently Brazil is using B5 diesel, which became mandatory before the scheduled date. Therefore, the demand for biodiesel tends to increase and the PNPB came as an important instrument of public policy in the sector [10].

In 2013 the production of biodiesel exceeded the 2.9 million m^3, being mainly produced from soybean followed by canola an oil palm *Elaeeis Guineensis*. The consumption of *E. guineensis* has increased over the last few years [11]. In Figure3(a) are presented the raw material to biodiesel production. As is depicted in Figure 3(a), the soybean represent more than 70% of raw material to biodiesel production, followed by beef fat with 20%. The 10% remaining are supplied by cotton, peanut, canola, oil palm, chicken fat and sunflower mainly.

The *E. guineensis* is a perennial crop of the Arecaceae family which originated in West Africa [10]. This is the highest yielding edible oil crop in the world, cultivated in 42 countries and in 11 million ha worldwide [12]. The global production of oil palm is of 58.4 million tons, of which Brazil produces 340,000 tons, representing only 0.58% of total production, which places it as the twelfth largest producer in the world and third in South America [13]. In Brazil the *E. guineensis* is grown in Pará and Bahia states, located in North and Northeast regions respectively. From the PNPB, it was established that the *E. guineensis* is an adequate candidate to reforest deforested areas, being Pará state a great region to develop cultivation. Currently, the state of Pará is the largest producer of oil palm in the country, corresponding to 80% of the production, nowadays there are 160,000 hectares with oil palm crops. The Pará state has nearly 13 million hectares considered suitable for the cultivation of palm oil, which represent 42% of the land indicated in the agro-ecological zoning and 10% of the total area of the State [14,15]. In Figure 3(b) is presented the political

map of Brazil divided in its five major regions and the main crops cultivated to biodiesel production in each one.

Despite the *E. guineensis* is actually not used as main raw material for biodiesel production, its potential is greater than of soybean mainly by the oil yield by hectare. In Table 2 are summarized main crops oils for biodiesel production in Brazil, their oil mass percentage and the oil yield per hectare. The *E. guineensis* oil yield is the highest varying between 2,000 to 8,000 kg of oil per hectare, which represents at least four times the yield of soybean, six times that of cotton, twenty times that of babassu and twice the physic nut yields.

Although *E. guineensis* present the largest yield in oil production per hectare, its cultivation and plantation has come under criticism, because it requires vast water supply, excessive land area and together with the oil extraction from oil from fruits, waste generation is considerable. The major solid by-product is a lignocellulosic material commonly called Oil Palm Empty Fruit Bunches (OPEFB) [16]. It is estimated that 1 ton of oil produced generates 1.1 tonnes of OPEFB [12]. In 2011, the production of OPEFB was approximately 14.5 million tons (dry base), where half of this amount was produced in Indonesia [17,18].

The mass composition of the lignocellulosic biomass OPEFB generally contains more than 60% polysaccharides (cellulose and hemicellulose), and is a low cost raw material for second generation ethanol production [19] and/or other chemical products of high-value, such as organic acids, polymers, resins, food and animal feed and fertilizers [5]. However, its direct use in a biorefinery is not possible due to its recalcitrance, making necessary pre-treatment stages to its conversion into chemical build blocks, via biological or chemical pathways.

1.2 Lignocellulosic biomass

The definition of biomass from the biological view is the biological material derived from living, or recently living organisms. From the energy approach, biomass is considerded as any material, excluding fossil fuel, which was a living organism that can be used as fuel either directly or after a conversion process [20].

Based on this definition, plants, woods and food crops as grasses, sugar cane, maize, corn, potato, beet etc., would be considered as biomass and source of energy, to the conversion to biofuels, ethanol, biogas or hydrogen mainly, through microbiological

transformation. To overcome the problem of biofuels and food competitionn, actually, solid agro-industrial residues, e.g., whet straw, corn stover, oil palm empty fruit bunches, rice straw, sugarcane bagasse, perennial crops plants, herbaceous (e.g., switchgrass) and woody (polar woods, softwoods and hardwoods), are considered as alternatives to biofuels production, especially ethanol, commonly called ethanol of second generation or biogas like methane or hydrogen [21,22]. Those residues are denominated as lignocellulosic materials and do not negatively affect the human food supply chain [23,24].

The lignocellulosic biomass is composed mostly by three biopolymers; cellulose, hemicellulose and lignin, with small amounts of tannins, chlorophyll and waxes [25]. The mass composition varies in each type of residue, and depends on the time of harvest, the final disposition of residue and the method of characterization.

In the Figure 4 is presented a schematic arrangement of cellulose, hemicellulose and lignin into lignocellulosic biomass matrix. The spatial disposition of the cellulose, hemicellulose and lignin, makes the lignocellulosic biomass difficult to convert directly to biofuels, its recalcitrance is mainly because of the presence of lignin and hemicelluloses, making the direct access to cellulose difficult. To overcome these problems, pretreatment stages are required, being a key step for using agricultural residues, with the aim to improve the accessibility of enzymes to hydrolysate cellulose [26,27].

1.3 Lignocellulosic biomass composition

1.3.1 Cellulose

Cellulose is the main constituent of lignocellulosic biomass. It is a polysaccharide with general chemical notation $(C_6H_{10}O_5)_x$ and is composed by D-glucose units linked by β-(1→4)-glycosidic bonds to conform a linear chain, which present intra and intermolecular hydrogen bonds, therefore cellulose is insoluble in water and most organic solvents [23].

1.3.2 Hemicellulose

Hemicelluloses are considered a group of heterogeneous polysaccharides that are formed through biosynthetic routes different from that of cellulose, being neither cellulose or pectin and by having β-(1→4) linked backbones of C_6 sugars (glucose, mannose and galactose) and C_5 sugars (xylose and arabinose) [28,29].

Due to the presence of C_6 and C_5 sugars, the hemicelluloses structure present a polymerization degree (DP) between 20 and 200 DP, also, the presence or lack of some sugars in hemicelluloses structure are function of the type of plant, wood and lignocellulosic material, i.e., in hardwood structures, are present glucuronoxylan (GX), glucomannan (GM) and xyloglucan (XG) mainly. In softwood are present galactoglucomannans (GGM), arabinoglucuronoxylan (AGX) and arabinogalactan (AG). In gramineae and lignocellulosic material as sugarcane bagasse and oil palm empty fruit bunches, are present arabinoxylan (AX) mainly [28,30].

1.3.3 Lignin

Lignin is the second most abundant natural polymer in the world [31]. Lignin is primarily a structural material to add strength and rigidity to cell walls, moreover, lignin is more resistant to most forms of biological attack than cellulose and other structural polysaccharides [32]. Lignin could be defined as a polymer, built up by the combination of three basic monomer types, as shown in Figure 5. These building blocks are often referred to as phenylpropane or C_9 units [33]. The link of these monomeric units conform an amorphous polymer which involves the cellulose and hemicelluloses structure, presenting also, interlinkage between hemicellulose constituents [34].

The structure is the result of biosynthetic pathway, which occurs via oxidative radicalization of monolignols (Figure 5). Despite the huge amount of studies, the definition of lignin and its exact chemical structure has never been clear as that of other bio-polymers as cellulose and protein [35]. The main reason is because the isolation method as alkaline oxidation, organosolve, acidolysis and kraft process, affect the lignin structure, generating a pull of molecules with different molecular weight, which affects directly the industrial use and applications [36,37].

1.4 Composition of lignocellulosic biomass

The chemical composition is a key factor and prerequisite for developing effective pretreatment technologies to deconstruct the rigid structure of lignocellulosic material, with the aim to make more accessible the cellulose to enzymatic hydrolysis [38]. Different types of biomass contain different amounts of cellulose, hemicellulose, lignin and extractives. The general mass composition of lignocellulosic biomass is presented in Figure 6.

The type of biomass, time of harvest and storage conditions, mostly, affect the composition in each type of lignocellulosic biomass, therefore, oscillations in mass composition are found in each type of biomass and between the same types of biomass, highlighting, that these variations would be detected using the same characterization method. In the Table 3 is presented the dry mass composition of common lignocellulosic biomasses in terms of cellulose, hemicellulose, lignin and the method of characterization reported in literature.

From the information summarized in Table 3, the characterization method reported by Sluiter et al. (2011) and the National Renewable Energy Laboratory (NREL) is the most common method for characterization of lignocellulosic biomass. The mass composition of cellulose present in lignocellulosic biomass is generally larger that 30 %wt, which is important from the industrial view, because biomass with high levels of cellulose concentration is suitable to fermentative process. However, the mass composition of hemicellulose generally does not exceed 35% which represents a source of pentoses for transformation to chemicals and biofuels. The mass composition of lignin varies from 17 % wt to 33 % wt. The presence of lignin affects drastically the pretreatment efficiency, nevertheless, it could be used as source of phenolic compounds, bio-gasoline, lignin polyolefin, vinyl and polyester blends and polyhydroxyalkanoates into others [32,36,40].

1.5 Pretreatment of biomass

Pretreatment of biomass is a very essential step in the biomass transformation to value added products. The pretreatment phase, in general, represents at least 20% of the total cost of production of biofuels, being considered by different authors as the most expensive process step [41]. Therefore, it has been well-investigated at least in the last two decades [42]. In theory, the ideal pretreatment breaks down the lignin structure, solubilize the hemicellulose components in their monomeric sugars, disrupt the crystalline structure of cellulose, increase the surface area and produces low concentration of degradation sugar compounds. In Figure 7 the reactions occurring during hydrolysis of lignocellulosic biomass are showed.

As is showed in Figure 7, the hydrolysis of biomass produce monomeric sugars in first instance, the concentration of glucose, galactose, mannose, xylose an arabinose, is

function of the type of biomass, type of pretreatment and conditions of process employed. However, if the temperature, reaction time and acidity of pretreatment are harsh, would be occurs the formation of degradation sugar products, furfural, HMF, levulinic acid and formic acid. The acetic acid appears into soluble hidrolizate, as function of the presence of acetyl group in the hemicelluloses structure. The lignin is fractionated to form phenolic compound, with different molecular weights, feluric acid, spirodienona and aromatic alcohols [43–45].

The pretreatments would be classified as conventional or non-conventional. Whiting conventional treatments are classified the chemical, thermal and thermochemical, as steam explosion, liquid hot water, ammonia fiber expansion (AFEX), alkali treatment, acid treatment, organosolve, ionic liquids, and ozonolysis. As non-conventional pretreatments are classified the microwave, gamma irradiation, electron irradiation and ultrasound. A biological treatment is an alternative technique in which white root fungi and brown root fungi, degrade the lignocellulosic material through enzymes as peroxidases and laccases [46,47]. An adequate pretreatment should present the following characteristics:

- ✓ Inexpensive with low capital and operational cost.
- ✓ Operation in reasonable size and moderate cost reactor.
- ✓ Effective on a wide range of lignocellulosic biomass.
- ✓ Considerable or moderated reaction time.
- ✓ Low or no load of catalyst.
- ✓ Enhance in almost 80 % of digestibility of cellulose.
- ✓ High concentration of monomeric sugars in liquid hydrolyzate.
- ✓ Low generation of fermentation inhibitors compounds.
- ✓ Easy lignin recovery
- ✓ Minimal solid waste generation.

Probably the temperature is one of the most important parameters in pretreatment stages. The increase in temperature has long been known to increase the severity of the treatment and improve the subsequent enzymatic cellulose degradation. Increasing temperature might result in losses, high-energy cost and formation of inhibitor for both the enzymes and fermenting microorganisms [48].

The temperature, reaction time and pH are commonly correlated into a single operational parameter called severity factor (Ro). The Ro was initially proposed by Overend et al., (1987), this equation correlated the temperature (T) and the reaction time (t) in a single equation that has showed on extensive research, a good fit with experimental data [44]. Other authors have reported the Ro modified including pH, in the work reported by Zhang and Chen (2012) [50] are presented a review the different correlations of Ro reported in literature. In Figure 8, a comparison of the treatment conditions of different pretreatments, in function of the temperature and pH are presented.

To compare the efficiency of pretreatment strategies is common used the concentration of monomeric sugars released after enzymatic hydrolysis (glucose and xylose mainly), in response to severity factor calculated is commonly used. However, comparison of different pretreatment strategies seems to be unreliable with the severity factor calculation currently in use, as is reported by Pedersen and Meyer (2010) [48]. The reason for the lack of correlation is because the pretreatment of lignocellulosic material depends on many other process variables, like type of biomass, mass composition, lignin moieties, moisture content, acetyl group concentration and particle size.

In Table 4 are presented the main features of common pretreatments techniques, in terms of energy demand, increase of surface area, removal of hemicelluloses and lignin and chemical peeling. The characteristic summarized in Table 4 were selected from literature reported [23,44,51–56]

1.6 Objectives

The lignocellulosic material selected to develop pretreatment studies was the oil palm empty fruit bunches (OPEFB). The following objectives were established

1.6.1 General

Develop pretreatment studies on oil palm empty fruit bunches (OPEFB) analyzing each fraction obtained, and the substrate produced in function of enzymatic digestibility.

1.6.2 Specifics

- Characterize the OPEFB in terms of cellulose, hemicellulose and lignin
- Develop a sequential acid/alkaline pretreatment
- Characterize the lignin extracted by alkaline methods
- Develop steam explosion pretreatment
- Develop enzymatic hydrolysis on OPEFB treated by sequential acid/alkaline and steam explosion pretreatment
- Analyze different configuration of biorefinery taking into account the economic profit and energy balance.

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Figure Caption

Figure 1. Global population growing. Date from [57]
Figure 2. World primary energy demand. Data from [2]
Figure 3. (a) Raw material used for biodiesel in Brazil. (b) Regions and crops growing
Figure 4. Schematic representation of biopolymers arrangement in biomass matrix
Figure 5. Lignin monomeric building blocks
Figure 6. General mass composition of lignocellulosic biomass
Figure 7. Scheme of hydrolysis reaction and inhibitors formation.
Figure 8. Comparison of temperature and pH of main pretreatment techniques. AH: Acid hydrolysis. DAH: diluted acid hydrolysis. ASE: acid steam explosion. 2-step: acid/alkaline treatment or vice versa. WO: wet oxidation. SE: steam explosion. BG: biological treatment. Lime: lime treatment. AFEX: ammonia fiber explosion.

Tables

Table 1. Highest ten countries in terms of energy demand

Country	Energy demand (MTOE)
China	2,747
United States	2,192
India	752
Russia	738
Japan	462
Germany	311
Brazil	270
South Korea	265
Canada	254
France	251

Table 2. Comparison of oil crops in Brazil. Data from [10]

Crop	Oil content (%)	Oil Yield ($kg \cdot ha^{-1}$)
Oil Palm *E. guineensis*	22	2,000-8,000
Physic nut	38	1,200-1,500
Babassu	60	120
Soybean	19.5	560
Cotton	19	361

Table 3. Mass composition of lignocellulosic biomass in dry basis

Biomass	Cellulose[a]	Hemicellulose[b]	Lignin	Method	Reference
Softwood	44	26	30	n.r	[1]
Softwood	42.68	24.82	32.50	ASTM D1108-80	[58]
Olive tree wood	34.4	20.30	20.40	NREL TP-510-42618	[59]
Miscanthus	47.4	26.7	25.9	NREL TP-510-42618	[60]
Corn cobs	31.7	34.7	23.9	n.r	[61]
Corn stover	35.00	28.00	19.5	NREL TP-510-42618	[61]
Rice straw	43.40	28.00	17.20	n.r.	[61]
Rapeesed straw	36.6	24.1	15.6	NREL TP-510-42618	[63]
Sugarcane bagasse	37.74	21.12	20.57	NREL TP-510-42618	[64]
Sugarcane bagasse	45.5	27.0	21.1	NREL TP-510-42618	[65]
Wheat straw	30.2	22.3	17.0	NREL TP-510-42618	[66]
Eucalyptus globulus	46	17.7	22.4	NREL TP-510-42618	[67]
Oil Palm Empty Fruit bunches	32.7	22.5	15.2	NREL TP-510-42618	[68]
Oil Palm Empty Fruit bunches	47.6	28.10	13.10	NREL TP-510-42618	[69]
Oil Palm Empty Fruit bunches	38.10	29.93	15.96	NREL TP-510-42618	[70]

Table 4. Effect of various pretreatments on chemical/physical structure of biomass

Pretreatment	High energy demand	Increase surface area	Decrystalize cellulose	Remove Hemicellulose	Remove lignin	Chemical peeling
Mechanical Comminution	X	X	X			
Acid hydrolysis	X**	X		X	X*	X
Alkali treatment		X	X	X	X	X
Wet Oxidation	X			X	X	
AFEX		X			X	
Steam explosion	X	X		X		X
Organosolv		X		X	X	
Liquid hot water		X		X		
Ionic liquids			X	X	X	
Biological		X		X	X	

Figures

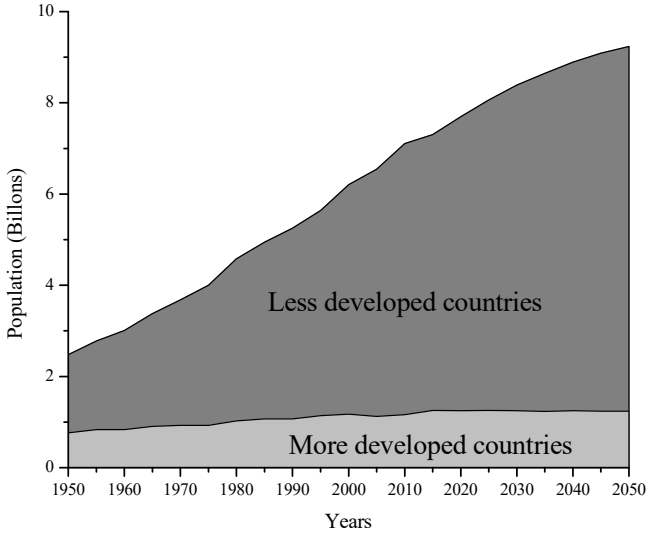

Figure 1.

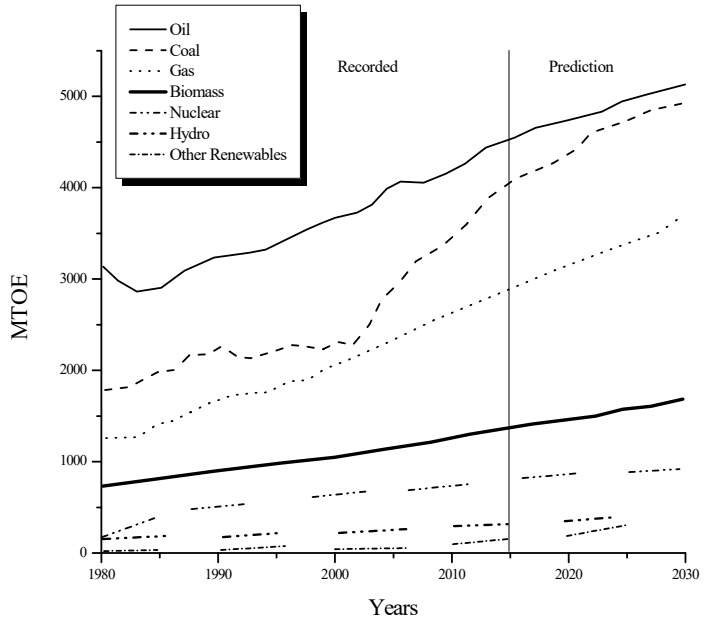
Oil
Coal
Gas
Biomass
Nuclear
Hydro
Other Renewables
Recorded
Prediction
MTOE
5000
4000
3000
2000
1000
0
1980
1990
2000
2010
2020
2030
Years

Figure 2.

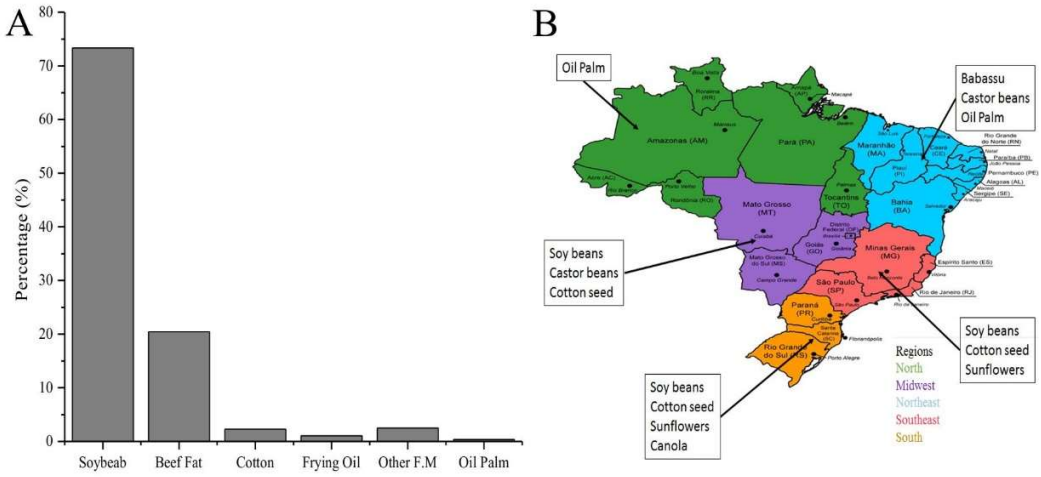
A
Percentage (%)
0
10
20
30
40
50
60
70
80
Soybeab
Beef Fat
Cotton
Frying Oil
Other F.M
Oil Palm
B
Oil Palm
Babassu
Castor beans
Oil Palm
Soy beans
Castor beans
Cotton seed
Soy beans
Cotton seed
Sunflowers
Soy beans
Cotton seed
Sunflowers
Canola
Regions
North
Midwest
Northeast
Southeast
South
Amazonas (AM)
Pará (PA)
Maranhão (MA)
Mato Grosso (MT)
Tocantins (TO)
Bahia (BA)
Minas Gerais (MG)
São Paulo (SP)
Paraná (PR)

Figure 3

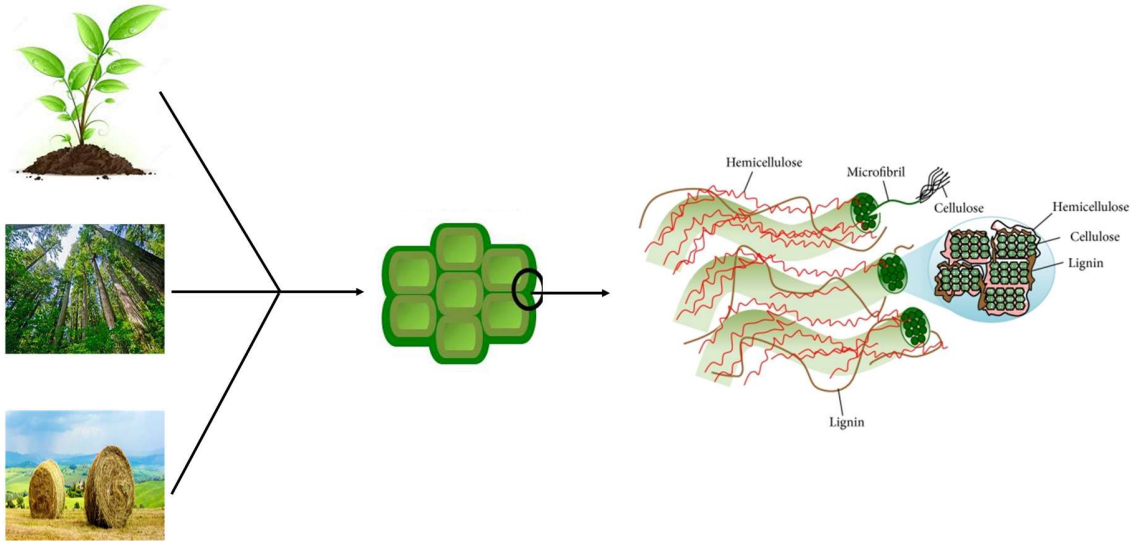
Hemicellulose
Microfibril
Cellulose
Hemicellulose
Cellulose
Lignin
Lignin

Figure 4.

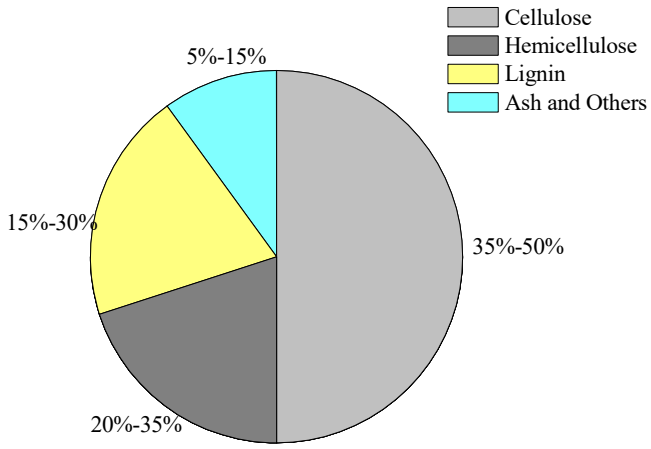
Cellulose
Hemicellulose
Lignin
Ash and Others
5%-15%
15%-30%
35%-50%
20%-35%

Figure 5

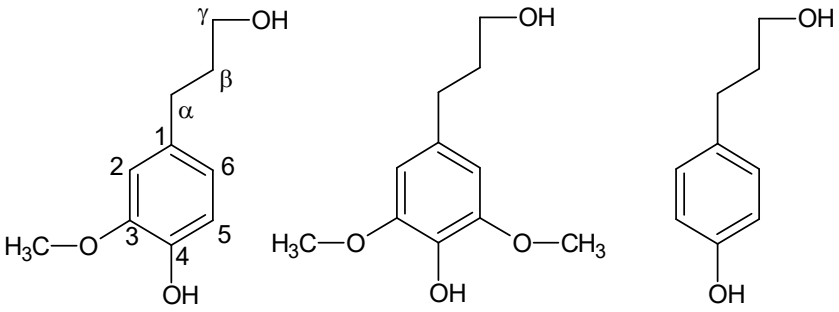
γ
OH
β
α
1
2
6
H3C—O
3
5
4
OH
Guaiacyl
OH
H3C—O
O—CH3
OH
Syringyl
OH
OH
p-Hydroxypheny

Figure 6

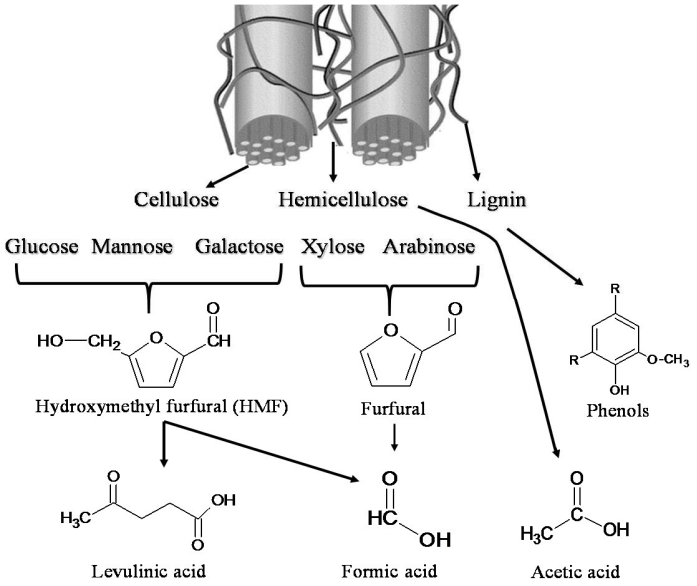
Cellulose
Hemicellulose
Lignin
Glucose Mannose Galactose Xylose Arabinose
HO—CH2
O
CH
O
Hydroxymethyl furfural (HMF)
O
O
Furfural
R
R
O-CH3
OH
Phenols
O
OH
H3C
O
Levulinic acid
O
HC
OH
Formic acid
O
H3C
C
OH
Acetic acid

Figure 7.

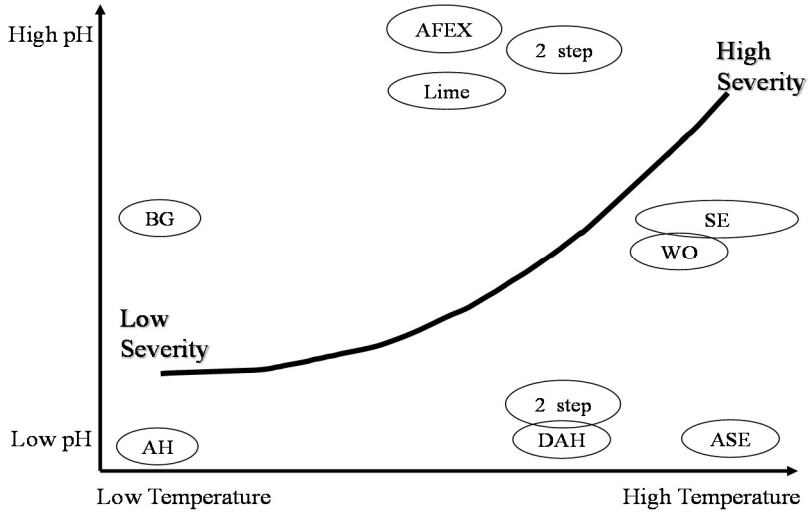
High pH
AFEX
2 step
Lime
High
Severity
BG
SE
WO
Low
Severity
2 step
Low pH
AH
DAH
ASE
Low Temperature
High Temperature

Figure 8

Physico-chemical characterization of solid residues from Oil Palm processing

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Abstract

Oil palm empty fruit bunches (OPEFB) are lignocellulosic biomass that gained special attention in the last decade. Their mass composition, generally more than 50% wt of polysaccharides, makes it an attractive feedstock for ethanol and chemicals production, in a biorefinery concept. Therefore, before developing any type of process, via chemical or biochemical pathway, for production of value added products, it is necessary to determine their mass composition in terms of cellulose and hemicellulose. In this work the moieties of cellulose, hemicellulose and lignin were determined by two methodologies widely reported in literature, also the correct wavelength for acid soluble lignin determination was determined. Analyses of FTIR and SEM were carried out with the aim to determine the structure of OPEFB and the presence of minerals and silica. The results obtained showed that the methodology reported by the National of Renewable Energy Laboratory (NREL) is the most accurate methodology for characterization of OPEFB, and 280 nm the correct wavelength for acid soluble lignin determination. Through SEM and element analysis was found that the OPEFB present a rigid structure with low porosity, with silica bodies embedded.

Keywords: Oil palm empty fruit bunches; Physicochemical Characterization; Fourier Transform Infrared spectroscopy; Scanning Electron Microscopy.

1. Introduction

Oil Palm *Elaeis guineensis, Jacq.* is a perennial plant of Arecaceae family which originated in West Africa (Bergmann et al., 2013). This is the highest yielding edible oil crop in the world, cultivated in 42 countries and in 11 million ha worldwide (Shinoj et al., 2011). The global production of oil palm is 58.4 million ton, of which Brazil produces 340,000 tons, representing only 0.58% of the total production. This places Brazil as the twelfth largest producer in the world and third in South America [1].

In 2004, the Brazilian government established the National Program of Biodiesel Production (Programa Nacional de Produção de Biodiesel, PNPB), with the objective to introduce the small farmers within the productive chain of biodiesel. According to the law no. 11.097/2005 of January 2008, the use of 2% biodiesel blended with petrodiesel (B2) is mandatory in Brazil and increasing amounts of biodiesel blended with petrodiesel will be required with time. Currently Brazil is using B5 diesel, which became mandatory before the scheduled date. Therefore, the demand for biodiesel tends to increase and the PNPB came as an important instrument of public policy in the sector [2].

In 2013 the production of biodiesel exceeded the 2.9 million m^3, being mainly produced from soybean followed by canola an oil palm *E. guineensis.* The consumption of *E. guineensis* has increased over the last few years [3], however, the oil palm has been used for food and cosmetics mainly, being les that 2% used for biodiesel production [4].

The *E. guineensis* is cultivated in Pará and Bahia states, located in Brazil's North and Northeast regions respectively. From the PNPB, it was established that this crop is an adequate candidate to reforest deforested areas, being Pará state a great region to develop cultivation. Currently, the state of Pará is the largest producer of oil palm in the country, corresponding to 80% of the production, nowadays there is 160,000 hectares with oil palm crops, the objective is increase the cultivate land to approximately 330,000 until the 2015, which is very little compared to the land available for the development of palm in the State. Pará has nearly 13 million hectares considered suitable for the cultivation of palm oil, which represent 42% of the land indicated in the agro-ecological zoning and 10% of the total area of the State [5].

Parallel to the increase of oil palm extraction, waste generation is also increased. The major solid by-product is a lignocellulosic material commonly called oil palm empty fruit bunches (OPEFB), it is estimated that for each 1 ton of oil palm produced 1.7 ton of OPEFB are generated of [6]. In 2010, in Indonesia, the largest country producer of oil palm, 55.73 million tons of these lignocellulosic material were generates. The mass composition of generally more than 60% in polysaccharides (cellulose and hemicellulose), makes this a low cost raw material for second generation ethanol production and/or other chemical products of high-value, such as organic acids, polymers, resins, food and animal feed and fertilizers [7,8].

The recalcitrance of OPEFB, mainly because of the lignin presence and hydrophobicity character of fibers, makes these lignocellulosic materials difficult to work, therefore, different types of pretreatment have been reported in literature [9,10]. The uses of OPEFB for different commercial purposes as bioethanol, biogas and chemicals of added value as organic acids and lignin, are directly related to their mass composition and moieties of polysaccharides. Therefore, it is necessary to establish adequately the composition before developing any type of academic study or industrial process.

In literature two methodologies have been widely reported in literature; the one reported by Sluiter et al. [11] and the one of Van Soest et al. [12] to determine the nonstarch polysaccharides content in fibers. The first one was developed for lignocellulosic biomass, especially corn stover, the other one was developed for polysaccharides determination relation to animal nutrition.

2. Material and methods

2.1. Raw material

Press-shredded OPEFB were obtained from a biodiesel palm factory in Pará State, republic of Brazil. These were sun dried for five days, then, were dried overnight in oven at 105°C. The material was grounded and sieved. The mesh particle selected for analysis was the equivalent to 0.35 mm.

89 2.2. Ash

90 The moieties of ash was determined following the process as reported by Sluiter et al.
91 [13].

92 2.3. Extractives determination

93 The procedure followed was reported by Sluiter et al. [13] with slight modifications,
94 briefly; extraction time of 8 h and continuous refluxing of solvent in the form of drip. The
95 solvents were used in function of the increase of the polarity, first was employed the
96 petroleum ether, then, was done the extraction with ethanol.

97 2.4. Carbohydrates and lignin determination

98 The determination of cellulose, hemicellulose and lignin was performed by two
99 methodologies. The one reported by the National Renewable Energy Laboratory (NREL)
100 [14] and by the method for dietary fiber, neutral detergent fiber and nonstarch
101 polysaccharides, reported by Van Soest et al. [12].

102 Using the NREL methodology, the cellulose and hemicellulose mass percentages
103 were correlated with the concentrations of glucose, xylose and arabinose, according with the
104 correlations reported by Tan et al. [6]. The presence of furfural (F) and 5-hydroxy-methyl-
105 furfural (HMF) was analyzed using Uv spectrophotometer following the procedure reported
106 by Scholl et al. [15].

107 The High-Performance Liquid Chromatography (HPLC) analyses were developed
108 using a Shimadzu Chromatograph equipped with an Aminex HPX-87H column, working in
109 a oven at 60°C. Mobile phase used was sulfuric acid (5 mM) at rate of 6mL/min with an IR
110 (Refractor index) detector.

111 The determination of acid soluble lignin (ASL) was carried out in a
112 spectrophotometer (SP – 2000 UV) using quartz cuvette and distilled water as blank, at 280
113 nm according to NREL procedure Sluiter et al. [11]

114 The neutral detergent fiber (NDF) and acid detergent fiber (ADF), were carried out
115 in an Ankom fiber analyzer 2000. The cellulose, hemicellulose and total lignin were
116 determined as is reported by Van Soest et al. [12] using the gravimetric technique.

2.4. Fourier transform infrared spectroscopy analysis

The modification in the structure of OPEFB was monitored using Fourier Transform Infrared Spectroscopy (FTIR). The measurements were carried out using BOMEM-Hartmann & Braun MB-series equipment with resolution of 4 cm-1, 32 scan per min and transmittance technique. Pellets of 0.1 g of OPEFB and 0.2 g of KBr were prepared for analysis. The range of wavenumber was from 400 to 4000 cm-1.

2.5. Scanning electron microscopy (SEM) and elements present in OPEFB

The morphology and elements present in OPEFB fibers was determined by SEM analysis, in a microscopy TESCAN VEGA 3 LMU, with acceleration voltage of 15 kV, resolution of 3 nm, under nitrogen atmosphere. The samples were vacuum dried and coated/metallized with gold in a sputter coater and dried overnight. The image zoom was 150x, 350x and 2500x, as reported by Medina et al. [16]. Together with the analysis of morphology was carried out the determination of elements present in OPEFB using the same equipment described above.

3. Results and discussion

3.1. Milling and sieving

The particle size distribution of biomass sieved is presented in Table 1. The smallest particle size, it means, fine particles and diameter 0.35 mm represents 62.65%, it influenced the global composition because the milling and crushing is considered a mechanical pretreatment Motte et al. [17], therefore the mass composition of cellulose and hemicellulose is slightly different.

The smallest particle size (fine particles) which are deposited on the bottom of the sieve represents 28.15% of the initial mass loaded. The particles with diameter of 0.35 mm compose a 34.50% of the total mass. The largest particles, 0.84 mm and 1.19 mm represent 18.94% and 18.41% respectively.

3.2. Mass composition of OPEFB

The mass composition for the fine particles and for the 0.35 mm particles, in function of cellulose, hemicellulose, total lignin and ashes, determined by the two methodologies, NREL and Van Soest's is presented in Figure 1.

In Figure 1 is presented the mass composition of cellulose, hemicellulose and lignin, for the fine particles (< 0.35 mm) and the 0.35 mm particles. The standard deviation of the Van Soest's procedure was larger than of the NREL methodology. It would be attributed to the fact that the determination of neutral fiber detergent (NFD) and acid fiber detergent (AFD) was carried out by gravimetric methodology, also by the washing of OPEFB, which could produce the mass variation. The determination of cellulose and hemicellulose by the NREL methodology employed the HPLC technique, therefore, this procedure presented small variation, being more accurate. On the other hand, the determination of acid soluble and insoluble lignin, by UV-Vis spectrometry, is more reliable than in Van Soest's methodology.

With the aim to compare the mass composition obtained in this work were done a review of the composition reported in literature and the method of characterization. The results are presented in Table 2.

As summarized in Table 2 the mass composition reported for OPEFB has been mainly reported using the NREL methodology. Confronting the results obtained in this work, it was found that the composition obtained is similar to the reported in literature.

3.3. UV-vis spectrometry for lignin determination

The UV spectra of acid soluble lignin (ASL) was confronted with the spectral of lignin from OPEFB obtained through alkaline oxidation and commercial lignin, with the aim to determine the adequate wavelength in which is presented a maximum point in absorbance. In Figure 2(a) is showed the UV-vis spectra of ASL, as is depicted, it presented a maximum peak at 280 nm, therefore this wavelength was considered as adequate for reading the percentage of ASL present in OPEFB. The result obtained is in agreement with the wavelength proposed by Sluiter et al. [14] for acid soluble lignin determination.

Figure 2(b) displays the spectrum profile of alkaline lignin extracted following the procedure reported by Minu et al. [18] and the commercial lignin sigma Aldrich, both lignin were solubilized in water at pH 12, after solubilization, the pH was set to 7. As is showed, the profile of alkaline lignin and commercial lignin are similar, the maximum absorbance for the two lignins are in the region between 210 – 220 nm. The difference in the behavior of absorbance with respect to ASL, would be explained by the difference in the non-

condensable groups present in lignin, its mean, the presence of ester-linked phenolic acids, such as p-hydroxybenzoic acid and ferulic acid as function of the extraction process [19].

3.4. Fourier transform infrared spectroscopy (FTIR)

FTRI spectroscopy was used to monitor modifications in the OPEFB structure after characterization. Figure 3 displays the FTIR of OPEFB after the NREL procedure, alkaline lignin extraction and raw OPEFB.

As is depicted in Figure 3 the differences between the OPEFB after NREL characterization technique and alkaline lignin extraction, whit respect to raw OPEFB, are slight. The bands were ascribed as follows: peak between 3245 and 3386 cm^{-1} was characteristic of OH hydroxyl from cellulose structure [20], this band remained in all samples, confirming the non-alteration in cellulose structure during characterization and lignin isolation process. The absorption bands at 2927, 2933 and 2852 cm^{-1} were attributed to C-H linkages in aliphatic groups of polysaccharides cellulose and hemicellulose [21].The OPEFB fibers after NREL procedure presented partially absence of these bands, mainly because of the partial hemicellulose hydrolysis.

The carboxyl acid group at 1735 and 1245 cm-1 has been attributed to hemicellulose components, and are only present in raw OPEFB [22]. The bands at 1608, 1514 and 1456 cm-1 have been reported for aromatic phenyl-propane skeleton vibrations in lignin structure [23]. The OPEFB fibers after alkaline extraction process did not present peaks of transmittance in these regions, only being present in raw OPEFB and after NREL procedure. These results corroborate that not all lignin components were removed.

The band at 1245 cm^{-1} represented aryl-alkyl ether linkage in lignin which was only present in raw EFB and after NREL process, confirming lignin extraction by the alkaline process. Transmittance peaks at 1164, 1103 and 1033 cm^{-1} in raw OPEFB and after NREL samples, were attributed to the C-H linkage of the lignin, which included other linkages of cellulose (C-O-C) as was reported by Baharuddin et al. [21].

Moreover, the spectrum also showed other signals that may be attributed to syringyl and guaiacyl groups, characteristic of lignin. Syringyl ring with C–O stretching at 1330 cm^{-1}, syringyl and guaiacyl ring with C–O stretching at 1218cm^{-1} which was not evident in

samples after alkaline process, and guaiacyl at 810 cm-1 only in samples after NREL procedure. These results are in agreement with those reported by [24].

3.5. SEM results and elements present in OPEFB

To gather information about the structure of OPEFB and presence of minerals in their external surface, SEM analysis was performed for particles with diameter 0.35 mm. The micrographs are displayed in Figure 4.

The structure of OPEFB fibers presented in Figure 4 showed a rigid structure with silica bodies (phytoliths) embedded in the OPEFB structure, similar to a micrograph shown by Baharuddin et al.[21] The silica bodies must be removed in pretreatment stages, because has been reported that its interferes in enzymatic saccharification, mainly by the physical impairment to penetrate the surface and access cellulose and hemicellulose [25].

The analysis of the percentage of elements present of raw OPEFB fibers is summarized in Table 3. The presence of minerals as potassium, calcium and magnesium and silica, are the most common minerals found on the surface of woody plants. The largest percentage of presence of atomic carbon is mainly because the presence of cellulose and hemicellulose

Conclusions

Oil palm empty fruit bunches (OPEFB) were characterized using two methodologies; NREL and Van Soest's, from the results obtained, was determined that the methodology reported by NREL is more accurately for the determination of mass composition of OPEFB, in terms of cellulose, hemicellulose and lignin. Also was determined that at 280 nm is the adequate wavelength for acid soluble lignin determination. Through SEM analysis, was corroborate the presence of silica bodies in OPEFB fibers, and by the atomic elements analysis was established the presence of K, Na, Mg and Si in the structure of OPEFB.

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312 Figure Caption

313 **Figure 1.** (a). Fine particles diameter < 0.35 mm. (b) Particles with diameter 0.35 mm.
314 **Figure 2.** Uv spectra of lignin. (a) Acid soluble lignin. (b) Alkaline and commercial lignin
315 **Figure 3**. FTIR spectra of OPEFB after pretreatment process
316 **Figure 4**. SEM of raw OPEFB. (a) 150X. (b) 350X. (c) 2500X

317 Tables

318 Table 1. Size distribution of OPEFB biomass after milling.

Size (mm)	Mass Percentage (%)
1.19	18.41
0.84	1894
0.35	34.50
Fine particles (<0.35 mm)	28.15

319

320 Table 2. Chemical Composition of OPEFB reported in recent literature in dry basis.

Cellulose	Hemicellulose	Total Lignin	Method	Reference
43.8	35	16.4	Updegraff	[18]
32.74	21.42	26.77	NREL TP51042618	[9]
23.7	21.6	29.2	Van Soest's	[19]
44.0	30.4	15.4	NREL TP51042618	[20]
32.7	22.5	15.2	NREL TP51042618	[21]
47.6	28.10	13.10	NREL TP51042618	[22]
38.10	29.93	15.96	NREL TP51042618	[23]
50.49	29.6	17.84	TAPPI 264	[24]
31.1	25.2	23.5	AOAC	[25]
42.85	24.01	11.70	NREL TP51042618	[26]
39.13	23.04	34.37	NREL TP51042618	[27]
36.10	22.38	26.55	NREL TP51042618	[28]

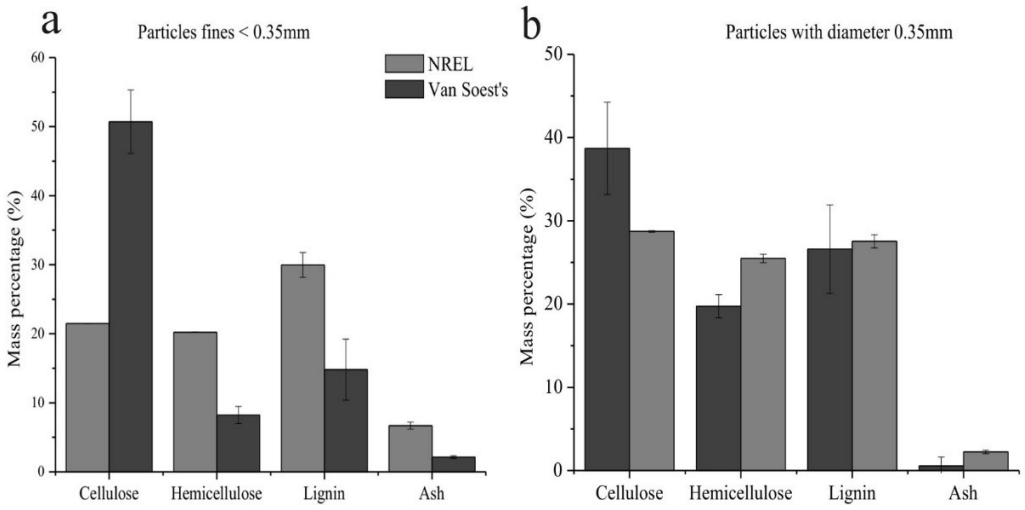
321

322

Table 3. Elemental analysis of raw OPEFB

Element	Percentage (%)
C	71.3
O	27.6
K	0.6
Ca	2
Si	0.2
Mg	0.1
Cl	0.1
S	0
Na	0

331 Figures



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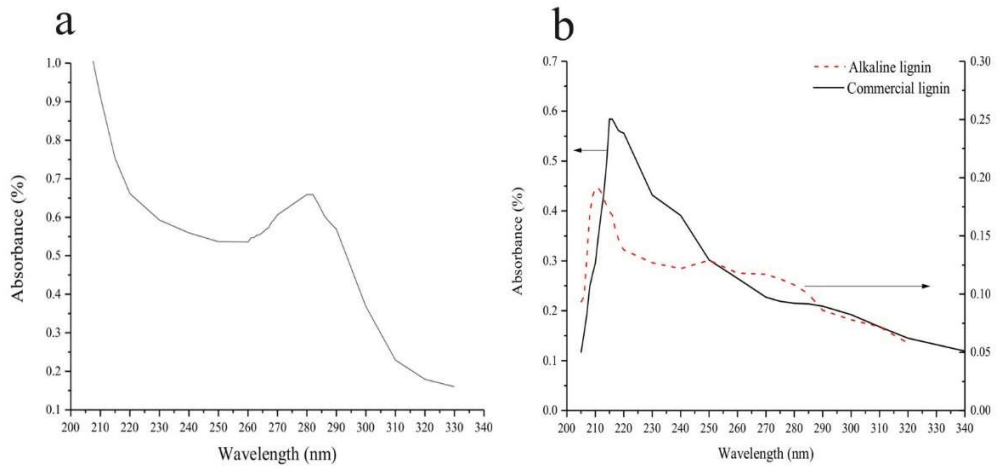
333 Figure 1



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335 Figure 2.

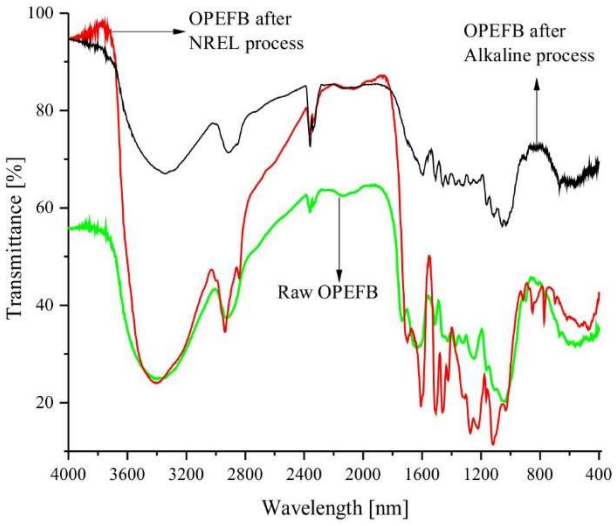
OPEFB after
NREL process
OPEFB after
Alkaline process
Raw OPEFB
Transmittance [%]
Wavelength [nm]
100
80
60
40
20
4000
3600
3200
2800
2400
2000
1600
1200
800
400

Figure 3

Lignin preparation from oil palm empty fruit bunches by sequential acid/alkaline treatment – A biorefinery approach

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Abstract

The lignin is an important raw material for the sustainable biorefineries and also the forerunner of high-value added products, such as phenolic chemical precursors, resin fillers, cement additives, pharmaceutical and cosmetics precursors. Therefore, the oil palm empty fruit bunches (OPEFB) were used for lignin preparation by successive treatment with 1% (w/w) at 121 °C for 80 min and 2.5% NaOH at 121 °C for 80 min resulting in the high lignin yield of 28.89 %, corresponding to 68.82% of the original lignin. The chemical quality and characteristic was analyzed determining the molecular weight distribution using gel permeation chromatography (GPC), while the characteristic groups present in lignin were studied through Fourier Transform Infrared Spectroscopy (FTIR) and nuclear magnetic resonance (NMR). The results indicated a lignin with molecular masses ramping from 4500 kDa to 12580 kDa. FTIR and NMR of these lignins showed more syringyl and *p*-hydroxyphenyl than guaiacyl units. Moderate acid/alkaline treatment provided lignin with high industrial potential and acid hydrolyzates rich in fermentable sugars and highly porous cellulosic fibers.

Steam explosion pretreatment of oil palm empty fruit bunches (EFB) using autocatalytic hydrolysis: A biorefinery approach

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Abstract

The oil palm empty fruit bunches (EFB) are an attractive source of carbon for the production of biochemical products, therefore, the aim of this work was to analyze the effect of the steam explosion (SE) pretreatment under autocatalytic conditions on EFB, in terms of lignin, hemicellulose and cellulose solubilization, including the modification of porosity of EFB fibers. The effect of temperature and reaction time were analyzed by a full experimental design. The EFB treated at 195 °C for 6 min showed an increase of 34.69% in glycan (mostly cellulose), and a reduction of 68.12% in hemicelluloses, with increased enzymatic digestibility to 33% producing 4.2 $g \cdot L^{-1}$ of glucose. Scanning electron micrographs of the steam treated EFB exhibited surface erosion and an increased fiber porosity. Fourier transform infrared spectroscopy showed the solubilization of hemicellulose and modification of cellulose in treated EFB.

Biological activities and thermal behavior of lignin from oil palm empty fruit bunches as potential source of chemicals of added value

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Abstract

The lignin is the most important renewable source of aromatic compounds on earth. It could represent up to 40% of dry matter in a biomass, however, its potential is underestimated being used as heat recovery source in industrial processes. Therefore, this work aimed to show the chemical feature of lignin isolated from sequential acid-alkaline pretreatment of oil palm empty fruit bunches. The extracted lignin was subjected to studies of this thermal behavior and biological properties as antioxidant, antimicrobial, and antidiabetic. The 2D HSQC spectroscopy analysis showed syringyl aromatic structure and presence of aromatic rings in lignin. The antioxidant assay showed that 2 mg of lignin were required to inhibit 50 wt.% DPPH, while the antimicrobial test inhibited the growth of *Escherichia coli, Salmonella enterica serovar thyphimurium*, *Bacillus subtilis* and *Staphyloccocus aureus*. The antidiabetic assay revealed inhibition of 20% of α-amylase activity. The thermogravimetric analysis gave out two peaks of decomposition at 230 °C and 350 °C and the glass transition temperature at 70 °C. These results showed the potential of lignin as precursor of chemicals of added value in a biorefinery process using as feedstock oil palm empty fruit bunches.

Keywords: Lignin extraction; Lignin valorization; Biological properties; Antimicrobial assay; Antioxidant capacity

1. Introduction

Oil palm empty fruit bunches (OPEFB) represents one of the solid residues in the palm oil mill industries (Kim and Kim, 2013). Is estimated that by 100 tons of oil palm fresh fruit bunches (OPFFB) processed to oil extraction are produced 22 tons of OPEFB (Hosseini and Wahid, 2014). The molecular structure of this solid residue is composed generally of 30 – 40 wt% of cellulose, 20 – 25 wt% hemicellulose and 20 – 35 wt% of lignin, containing also some quantities of pectin, protein, extractives (nonstructural sugars, nitrogenous material, chlorophyll and waxes) and ash (Shinoj et al., 2011). The mass composition of OPEFB makes it an attractive feedstock in a biorefinery process based on lignocellulosic material for chemicals and fuels production (Taylor, 2008). However, at present OPEFB residues have not been optimally used, because they are wet, bulky, and voluminous which are unfavorable properties for transportation and handling. Therefore, it has been used as organic fertilizer and as a source of heat and energy recovery in oil palm mills, and the remaining ashes are used as fertilizers (Arrieta et al., 2007; Chiew and Shimada, 2013; de Souza et al., 2010).

To make use of OPEFB, different approaches have been developed and reported in literature, including the production of ethanol, methane, hydrogen, briquette and organic acids as the levulinic acid (Chin et al., 2015; Ibrahim et al., 2015; Jung et al., 2013; Tan et al., 2013). However, the uses of lignin have been left in the background. The lignin represents 30 percent of all the non-fossil organic carbon and is the most important renewable source of aromatic compounds on Earth. Nevertheless, the valorization of residual lignin as a co-product or precursor of chemical value added products is considered a challenge and the technology is less developed than those for polysaccharides, being its amorphous structure the main cause (Cotana et al., 2014).

The lignin is composed by crosslinked phenylpropanoid monomer structures of sinapyl, coniferyl and *p*-coumaryl alcohols, with a molecular weight distribution between 1000–2000 $g \cdot mol^{-1}$. Depending on the type of biomass and the process of extraction, the lignin presents different chemical structures. It means that more or less syringyl, guaiacyl or *p*-hydroxycoumaryl units are present, which impacts in different molecular weight distribution (Yang et al., 2016).

The isolation process of lignin can be classified in two groups as it is reported by (Azadi et al., 2013). Processes in which lignin is degraded into soluble fragments, and processes that selectively hydrolyze polysaccharides and leave lignin along with some carbohydrates

deconstruction products as solid residue, being the lignin degradation processes the most commonly used.

Lignin residue has been traditionally burned in factories as a source of heat and energy, ignoring its potential as co-product (Mussatto et al., 2007). Because of recent growth of industries producing ethanol from lignocellulosic biomass, the availability of lignin as black liquor is huge. In 2010 the lignin production as a residue was 50 million tons, being only 2% of this amount used for low value-added products (Smolarski, 2012). Consequently, the valorization of lignin as a precursor of highly value-added products is an alternative to generate economic benefit and develop biorefineries from lignocellulosic biomass (Azadi et al., 2013; Doherty et al., 2011; Ghaffar and Fan, 2014).

Based on the fact that lignin would be a source of chemicals of added value, this work focused on the evaluation of biological activities and thermal performance of lignin extracted from OPEFB using sequential acid-alkaline treatment. The analyses of chemical structure were carried out using 2D HSQC NMR. The thermal behavior was studied through TGA and DSC analyses. The biological properties evaluated were antioxidant activity using DPPH and ABTS, antimicrobial activity against two Gram-negative bacteria: *Escherichia coli* and *Salmonella enterica serovar thyphimurium*, and two Gram-positive bacteria: *Bacillus subtilis* and *Staphyloccocus aureus*, one filamentous fungus: *Aspergillus Niger,* and one yeast: *Candida albicans*. The potential uses as antidiabetic agent were evaluated by the α-amylase inhibition, and the toxicity was determined by lethality assay on the wild-type *Caenorhabditis elegans*.

2. Material and methods

2.1. Lignocellulosic biomass OPEFB

Oil palm empty fruit bunches (OPEFB) were obtained from Biopalm Vale factory, located in Mojú, in the Pará state of Brazil. The OPEFB was dried in a cross flow stove at 65°C for 48 h, milled in hammer mill, sieved through a mesh 42 (0.350 mm) and used for all experiments. All experiments were carried out in triplicates.

2.2. Analysis of carbohydrates and total lignin

The composition of polysaccharides present in raw OPEFB was determined using the NREL analytical procedure reported by Sluiter et al. (2011), as was reported in our previous work (Coral Medina et al., 2015).

2.3. Lignin extraction from OPEFB

The residual lignin was obtained from OPEFB based on our previous work using sequential acid-alkaline pretreatment (Coral Medina et al., 2015). Briefly, the acid hydrolysis was carried out using 1 wt.% of H_2SO_4 at 121 °C by 60 min, following the procedure reported by Minu et al. (2012). Form the alkaline lignin extraction the time and temperature were fixed at 80 min and 121 °C respectively. The mass percentage of NaOH was varied from 0.5 to 5.5 wt.% in solution, whit increase of 1 wt.% in NaOH. The mass percentage of OPEFB in solution was 10 wt.%, and the balance of distilled water. Six fractions (L_1 to L_6) of lignin one by each concentration of NaOH used were obtained. After extraction, each fraction of lignin was washed three times with hot water at 70 °C to remove the residual sugars, then, vacuum dried at 50 °C by 8 h.

To perform analysis of biological properties as Total phenolic content (TPC), on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activity and antimicrobial assay, the lignin was solubilized using alkaline water at pH 11 using magnetic shuffler at 50 °C. After solubilization the pH of lignin solution was set at 6-7 using H_2SO_4 10% (v/v).

2.4. Two-dimensional 2D HSQC NMR spectroscopy.

2D-NMR spectra of underivatized lignin were carried out in a Bruker spectrometer at 400 MHz. The preparation of samples is described below. About 80 mg of solid lignin was dissolved in 0.5 mL of DMSO-d_6. The chemical shift for ^{13}C-NMR was calibrated with reference to DMSO-d_6, standard peak at 39.51 ppm. The spectrum was recorded keeping the following parameters constant: acquisition time of 0.68 s, frequency 100.6 MHz, receiver gain 182.08, sweep width 24,038.09 Hz and temperature 30 °C.

2.5. Determination of the total phenolic content (TPC)

The TPC of all lignin fractions were determined by Folin-Ciocalteu reagent method according to Singleton and Joseph (1965) and Rusaczonek et al. (2007) with modifications. Briefly, a lignin solution of 200 $\mu g \cdot mL^{-1}$ was prepared, an aliquot of 500 µl of each sample were added to 2.5 mL of (1:10 v/v) Folin-Ciocalteu solution, after 5 min, 2 mL of (7.5% w/v) Na_2CO_3 was added. The mixture was maintained at room temperature in a dark environment by 1 h. The absorbance was read at 740 nm in a spectrophotometer (SP – 2000 UV) using quartz cuvette and distilled water as blank. Gallic acid was employed as calibration standard (0 – 200

$\mu g \cdot mL^{-1}$) to determine the phenol quantity in milligrams of Gallic acid equivalent. All experiments were carried out in triplicates.

2.6. Antioxidant assay as DPPH and ABTS radical scavenging activity

The antioxidant capacity of lignin was studied by evaluating its free radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, following the procedure reported by (Dizhbite et al., 2004) with modifications. A solution of 0.004% (w/v) of DPPH-methanol was prepared. A set of lignin solutions was prepared from 5 to 200 $\mu g \cdot mL^{-1}$, aliquots of 1 mL of lignin solution were added to 3.9 mL of DPPH-methanol solution. The reaction time was 30 minutes at room temperature. The absorbance was recorded at 517 nm. Inhibition of free radicals by DPPH in percentage was calculated according to Eq. (1).

$$Scavening\ (DPPH\ or\ ABTS)\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} x100 \quad (1)$$

where $A_{control}$ is the absorbance of the control reaction. A_{sample} is the absorbance of the test compound. The values of inhibition were calculated according to ascorbic acid as control in the interval of 5 to 200 $\mu g \cdot mL^{-1}$.

The fraction of lignin which presented the best results of DPPH inhibitions, its mean the lowest concentration for DPPH inhibition, was used to determine the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activity. The ABTS assay was carried out based on the method reported by (Miller et al., 1993), with modifications. 2 mL of ABTS solution (7 mM) and 2mL of potassium persulfate solution (2.45 mM) were mixed to make the stock solution (ABTS+) and placed in the dark for 6 h. Then, 50 µl of ABTS+ solution was added to 200 µl of lignin solution (using the same concentration employed in DPPH assay), the mixture was stored in the dark for 30 min at room temperature. As a control a solution of 50 µl of ABTS+ and 200 µl of distilled water was used. The absorbance was recorded at 734 nm and the inhibition of ABTS+ as scavenging capability was determined using the Eq. (1).

2.7. Antimicrobial assay

The determination of antimicrobial properties of lignin was carried out using two Gram-negative strains, *E. coli* and *S. thyphimurium.* Two Gram-positive strains, *B. subtilis* and *S. aureus*, were also employed one yeast, *Candida albicans* and one filamentous fungi strain *Aspergillus niger LPB 12*. The pathogenic microorganisms were obtained from the Laboratory of Bioprocess Engineering and Biotechnology of Federal University of Paraná (UFPR), and maintained on brain heart infusion broth (BHIB) at 30°C, while the yeast and the fungi were

maintained in brain heart infusion agar (BHIA) at 37°C. The culture media used for the antimicrobial test in broth for Gram-positive and Gram-negative bacteria was Mueller Hinton broth (MHB), and Mueller Hinton Agar (MHA) for yeast and fungus strains, according to the Manual of Antimicrobial Susceptibility testing.

For the antimicrobial assay in broth four concentrations of lignin solution : 2000, 1000, 500 and 250 $\mu g \cdot mL^{-1}$ were evaluated. The pH of the solution was fixed between 6-7, to avoid interferences in the bacterial growth by the pH alteration. The total volume of each experiment in liquid incubation was 180 µL distributed as follows: 20 µL of inoculum, 80 µL of lignin solution and 80 µl of MHB medium. The bacteria growth was monitored by the absorbance of incubation solution, each 2 h during the first 12 h, then, was monitored each 12 h until 48 h. The blank incubation solution was composed by 20 µl of inoculum, 80 µL of distilled water and 80 µL of MHB medium. The growth inhibition was measured as the percentage of variation in the absorbance at 630 nm with the time, using the Eq (1).

For the inhibition growth assay using the technique of agar diffusion, plates containing MHA medium were inoculated with *C. albicans* and *A. niger*. The agar was drilled to form holes of about 0.1 cm, then 50 µL of lignin at 2000, 1000 and 500 $\mu g \cdot mL^{-1}$ were added. The time of incubation of *C. albicans* was 4 days and 7 days for *A. niger*. The antimicrobial activity was tested by the formation of a halo showing the inhibition zone.

2.8. Antidiabetic test

Antidiabetic test was developed by *in vitro* α-amylase activity inhibition based on the methodology reported by Quesille-Villalobos et al. (2013) as was established by Barapatre et al. (2015). Lignin concentrations were varied from 2000 to 62.5 $\mu g \cdot mL^{-1}$. 500 µL of each lignin solution were added to 500 µL of 0.02 M sodium phosphate buffer, pH 7.0, containing 0.5 $mg \cdot mL^{-1}$ of α-amylase solution. The incubation of solution was carried out at 25 °C for 10 min, the reaction was stopped with 1 mL of dinitrosalicylic acid color reagent. The reaction mixture was then diluted adding 15 mL of distilled water, and absorbance was measured at 540 nm. As control solution a sample of 500 µL de distilled water, 500 µL of buffer containing the same concentration of α-amylase was used. The inhibition of α-amylase was calculated according to the equation (1). All experiments were done in triplicate.

2.9. Cytotoxicity assay

The lignin cytotoxicity was evaluated as the lethality capacity on the wild-type *Caenorhabditis elegans*. The assay was carried out with complete S-medium in 96-well cell

culture plates. *C elegans* wild-type strain N2 (Bristol) and *E. coli* strain OP50 were kindly donated by Dr. Marcelo Mori (Federal University of Sao Paulo, Brazil). The stocks of *E. Coli* were maintained at 20 °C on OP50/NGM (nematode growth media). Synchronous populations were obtained by isolating embryos from gravid hermaphrodites using a bleaching solution according to standard procedures (Solis and Petrascheck, 2011).

Stock solutions of lignin were prepared in water on the day of experiments. Appropriate aliquots of lignin solutions were added to S-medium containing between 15 and 20 synchronized first-stage larvae. Final concentrations of lignin were added in the range of 5.0 – 40.0 $mg \cdot mL^{-1}$. As negative control experiment, nematodes were exposed to complete S-medium without lignin. The organisms were incubated for 24 h at 20 °C and the LC50 values, representing 50% lethality at a given concentration were calculated.

2.10. Thermal analysis

The thermogravimetric and differential scanning calorimetric analysis were carried out following the procedure reported by Toledano et al. (2010). The thermogravimetric analysis (TGA) was conducted from 30 °C to 800 °C with a constant heating rate of 10 $°C \cdot min^{-1}$, while the differential scanning calorimetric (DSC) was carried out from 25 °C to 500 °C using the same TGA heating rate under nitrogen atmosphere. In both experiments 5 mg of lignin were used. The analysis was developed in a NETZSCH STA 449 F3 Jupiter at the Chemistry Department at Federal University of Paraná (UFPR).

3. Results and discussion

3.1. Mass composition and lignin preparation from OPEFB

The mass composition of OPEFB used for lignin extraction and preparation is the following: ethanol extractives 7.14 ± 0.8 wt.%, water extractives 9.00 ± 1.0 wt.% , ash 2.26 ± 0.2 wt.%, cellulose 30.41 wt.% ± 0.4 wt.%, hemicellulose 19.44 ± 0.3 wt.%, AIL 32.17 ± 0.93 wt.% and ASL 1.17 ± 0.3 wt.%. From the mass composition, is possible to observe that the concentration of insoluble lignin is more than 30%, which makes it a very good feedstock for lignin preparation.

3.2. Lignin extraction

The lignin extraction under alkaline condition is accompanied with a OPEFB mass loss, mainly by the solubilization of hemicelluloses and cellulose components, because the alkali treatment is not selective, causing swelling and peeling in OPEFB fibers (Agbor et al., 2011).

In Figure 1 are presented the mass percentage of cellulose, hemicellulose, ASL and AIL after each lignin extraction conditions.

As is displayed in Figure 1, the mass percentage of cellulose in OPEFB increased gradually with the NaOH percentage, and reached 49 wt.%, at 2.5 wt.% NaOH, from this point, the mass composition of cellulose in OPEFB remained almost constant in 50 wt.%. The great decrease in hemicellulose mass composition with respect to raw OPEFB, was because the acid treatment with 1 wt.% of H_2SO_4 solubilized almost all hemicellulose components (xylose and arabinose mainly). Nevertheless, during the alkaline treatment, the mass percentage of hemicellulose in OPEFB fibers remained almost constant in 8 wt.%, because the alkaline treatment affects mainly the lignin structure.

The presence of lignin in OPEFB decayed vigorously at 0.5 wt.% NaOH, for the following condition, the solubilization of lignin was reduced progressively and reached 5.9 wt.% at 4.5 wt.% NaOH. The high solubility of lignin in alkaline conditions was attributed to the high amounts of ester-linked phenolic acids, such as *p*-hydroxybenzoic acid and ferulic acid. The mass composition of OPEFB after lignin preparation is important to enzyme production or fermentation process, with the aim to produce chemical value-added products as ethanol, itaconic acid, levulinic acid, propionic acid, aspartic acid, fumaric acid and succinic acid, improving the building blocks in the biorefinery concept (Cherubini, 2010).

The best condition of lignin preparation was chosen at 2.5 wt.% of NaOH, since presented largest yield of lignin extraction, reasonable solid recovery and enrichment in cellulose percentage. Moreover, the analysis of ^{13}C and ^{1}H NMR reported in our previous work (Coral Medina et al., 2015) showed that lignin (L_3) presented more representative signals (chemical shift) in this condition. Therefore, the analysis of 2D HSQC NMR, biological assay and thermal properties were focused mainly on the lignin obtained under this condition.

3.3. HSQC Spectrum

The analysis of 2D HSQC was carried out based on the results of yield of the lignin extraction in function of NaOH mass percentage and ^{1}H NMR and ^{13}C NMR reported in our previous work (Coral Medina et al., 2015). The lignin L_3 presented more signals of resonance, therefore was submitted to analysis of 2D HSQC, the results are displayed in Figure 2. The region between δ_c/δ_H 20/1 to 40/2.5 ppm corresponds to the fingerprint of lignin, in which the CH_3, CH_2 and H of the propyl chains and acetyl group present high resonance. The other regions are marked with a letter that corresponds to a particular structure fraction of lignin. The

correlation of each region of resonance with the type of bond and molecule fraction in lignin were assigned based on literature review about lignin extraction and characterization. The results are summarized in Table 1.

From the signals of resonance detected, we established that the lignin fraction L_3 presented high presence of aromatic groups, where the more intense signals were from syringyl against guaiacyl units. Those chemical characteristics influence the type of bonds and their abundance in lignin. Results revealed that lignin has the characteristic bond β-O-4, in the C(α), C(γ) and C(β), moreover a double bond typical of oleofinic structures. The presence of aromatic units in lignin L_3 is an important factor to study their properties as antioxidant, antimicrobial, antidiabetic and thermal properties in the possible uses as precursor of chemical building blocks.

3.4. TPC, DPPH and ABTS analyses

The TPC of all samples of lignin obtained were expressed in terms of milligram of gallic acid equivalent (GAE) per milligram of lignin, correlating to the absorbance and the standard curve of gallic acid. Antioxidant activity was established as the milligram of lignin required to inhibit the 50 wt% of the DPPH radical scavenging. The results of radical scavenger capacity of lignin were confronted with the ascorbic acid, which is recognized as one powerful antioxidant agent. In Table 2 are summarized the results of TPC, the standard curve of gallic acid, and the DPPH antioxidant assay. The TPC in lignin increased with the increase in the mass percentage of NaOH, using 2.5 wt.% of NaOH for lignin extraction the lignin (L_3)with the largest values of TPC was obtained, corresponding to 181.52 mg GAE by gram of lignin. From this point, a decrease in TPC for lignins L_4, L_5 and L_6 was evidenced, representing 145, 151 and 114 mg GAE·mg lignin^{-1}, respectively. Severe treatments (very long, at high temperature or at extremely basic conditions) promoted the dissolving of more components from the raw material as was reported by García et al. (2012). The lignin suffered degradation reactions, which affects the chemical structure of lignin, therefore the TPC was reduced, as was reported in our previous work (Coral Medina et al., 2015).

In terms of lignin yield extraction at 2.5 wt.% NaOH was the condition in which the largest value was obtained, moreover, in terms of chemical groups present in lignin, determined by the analysis of 2D HSQC NMR, the lignin L_3 presented more chemical signals of resonance, especially by the aromatic structures detected. Therefore, the largest TPC in lignin L_3 would be attributed to the presence of more condensed structures with respect to the other lignin fractions.

Form the results presented in Table 2, was evidenced a slightly relation between the TPC and the I50, its mean, with the increase with the TCP, the mass of lignin necessary to inhibit the 50% of DPPH was reduced. The mass of lignin necessary to reduce 50 wt.% of the DPPH (I50) varied between 2 and 3.8 mg lignin per gram of DPPH, highlighting the lack of direct relation between the TPC and the I50, however, the lignin L_3 presented the lowest concentration to attain the I50, it means 84.43 $\mu g \cdot mL^{-1}$. This represented approximately 4.4 times the equivalent concentration of ascorbic acid.

To confirm the radical scavenger capacity of lignin L_3, was developed the ABTS assay for these lignin fraction, the results were also summarized in Table 2. For all concentration of lignin L_3 tested, was found that the percentage of inhibition of ABTS was more than 50% for all concentrations, therefore in Table 2 is presented the inhibition percentage in function of the lignin concentration. The results obtained were in line with the antioxidant activity of lignin from black liquor of oil palm waste (Bhat et al., 2009). From the results obtained could be established that exist a direct relationship between the TCP content and antioxidant effect of lignin, represented by the DPPH and ABTS inhibition. The free radical scavenging and antioxidant activity of phenolics mainly depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules, but it is also affected by other factors, such as the presence of other proton donating groups that could reduce or increase their activity (García et al., 2012).

3.5. Antimicrobial assay

To develop the *in vitro* antimicrobial assay, the lignin L_3 was selected because it presented more chemical structures detected by 2D HSQC NMR, also presented the best performance in the evaluation of TPC, DPPH and ABTS radical scavenging. The results showed no antimicrobial effects against *C. albicans* and *A. niger*. In fungal strains, the ergosterol replace the cholesterol in cell walls, the biosynthesis of ergosterol is inhibited by heterocyclic compounds, generally with five carbons and three nitrogen atoms, therefore the lack of these structure in lignin L_3 could be the reason for no antimicrobial effect on *C. albicans* and *A. niger*. The non-antimicrobial effect of lignin against yeast and fungi was consistent with previous studies of lignin extracts of corn stover reported by Dong et al. (2011).

The results of growth inhibition of the Gram-negative and Gram-positive strains are summarized in Figure 3 at different concentrations of lignin during 48 h. The results showed that during the two first hours for all strains tested, all concentrations of lignin presented more than 80% of growth inhibition. For the two Gram-negative strains, *E. coli* and *S. thyphimurium*,

the inhibition effect of lignin was reduced gradually with the time, the effect of lignin concentration on inhibition growth showed that between 100 and 500 $\mu g \cdot mL^{-1}$ of lignin solution presented the largest inhibition percentage during 48 h.

The inhibition of the Gram-positive bacteria was greater that of Gram-negative bacteria, this would be explained by the lack of the second cell wall in Gram-positive bacteria. The *B. subtilis* was strongly inhibited during the first 8 h of assay using 2000 $\mu g \cdot mL^{-1}$,from this point the efficiency of lignin on microbial growth was reduced gradually. Comparable results were obtained in the assay of growth inhibition of *S. aureus*, which presented the maximal inhibition at 24 h of assay. For the Gram-negative strains tested, largest concentration of lignin solution (2000 $\mu g \cdot mL^{-1}$) presented strong inhibition during the antimicrobial assay.

The antimicrobial effect of lignin would be explained by the aromatic compounds detected by 2D HSQC NMR, which are similar to semisynthetic penicillins such as methicillin, carbenicilin and benzyl penicillin. Lignin would be permeable to cell wall of gram-negative bacteria and is resistant to *β*-lactamase, which converts lignin into a possible precursor of a broad spectrum of antibiotics (Madigan et al., 2015). Effect of different plant extracts on different Gram-negative, Gram-positive and fungal strains, and their possible mechanism of action was reported by Tekwu et al. (2012).

3.6. Cytotoxicity assay

The cytocixity of lignin L_3 was evaluated as the percentage of lethality on larvae of *C. elegans* after 24 h. Table 3 presents the initial and final average number of larvae of *C. elegans*, as well as the lethality percentage. For all lignin concentrations tested, there was no evidence of representative toxicity, with a lethality percentage lower than 10 %. To confirm the significant variation between the initial and final population of larvae, the ANOVA analysis was performed with a significance level of 95%, with p value < 0.05. From the results showed in Table 3, it was established that there is no statistically significant difference between the initial and final larvae number of *C. elegans*, therefore lignin would be considered non-toxic at all tested concentrations. These results open new possibilities for lignin application in cosmetics and pharmaceutical industries as it is reported by Ugartondo et al. (2008).

3.7. In *vitro* α-amylase assay

The α-amylase inhibitory activity was tested using different concentrations of lignin, the results are displayed in Figure 4. For all concentrations tested, the lignin had significant inhibitory effects on α-amylase activity, being almost constant between 25-30% for all

concentrations tested. Lignins have been recognized as inhibitors of cellulolytic enzymes and amylases by the phenolic characteristic, in which the hydrogen bonging and hydrophobic interaction between protein chain and phenolic structure of lignin would generate non-productive enzyme sites (Ximenes et al., 2010). The results obtained were similar to those of Barapatre et al. (2015) at low concentrations of lignin from biomodified lignin from *Acacia nilotica*.

3.8. Thermal behavior of lignin

The thermal performance of the lignin fraction L_3 was studied through TGA and DSC analyses, the results are displayed in Figure 5a and 5b. In Figure 5a is presented the TG and DTG curves for lignin. The initial mass loss below 100 °C was attributed to water evaporation. From 200 to 600°C occured the largest mass loss corresponding to 70 wt.%, the weigh lost was recorded up to 800 °C At this point remained 20 wt.% of total mass, which would be attributed to the ash present in lignin. The residual char at 800 °C would be due to the presence of complex and condensed structures increasing the C-C bonds. The first derivate of the TG curve showed a maximum peak (DTG_{max}) between 200 and 250 °C, in this interval of temperatures it was assumed that pyrolytic degradation reactions involves the fragmentation of inter-unit linkages β-O-4, releasing monomeric phenols, as is reported by Tejado et al. (2007).

Lignin glass transition temperature (Tg) is an important property, to evaluate the feasibility of lignin as precursor or filler in polymer industrial applications. Normally the Tg values of nonderivatized lignin range from 90 to 180 °C (Moynihan et al., 1974). In the DSC curve displayed in Figure 5b, the Tg was determined as the kink in the profile of heat versus temperature over the range between 50 to 90 °C, corresponding to 70 °C. Above the glass transition temperature the polymer chains have enough energy to form ordered arrangements and undergo crystallization, which is an exothermic process. In Figure 5b is showed the largest negative peak and the crystallization temperature (Tc), corresponding to 192 °C. After crystallization, the lignin was subjected to the melting process, corresponding to the biggest endothermic peak in Figure 5b, the melting temperature (Tm) was established at 420 °C.

Conclusions

In the present work the biological activities and thermal performance of lignin isolated from oil palm empty fruit bunches were demonstrated. The lignin fraction obtained using 2.5 wt.% of NaOH (L_3) presented five largest chemical shift signals in 2D HSQC NMR, corresponding to syringyl aromatic structures with *β*-O-4 bonds. The DPPH and ABTS assays

using lignin L_3 showed remarkable potential of lignin as antioxidant, while the α-amylase inhibition properties of lignin may be used as potential source of precursor drugs for the treatment of diabetes. The *in vitro* antimicrobial assays showed no inhibition growth against *C. albicans* and *A. niger*, while for Gram-negative *E. coli* and *S. thyphimurium*, and Gram-positive *B. subtilis* and *S. aureus* the effect of inhibition during 48 h at different concentration of lignin was evidenced. Through thermal analysis of lignin L_3 were determined the glass, crystallization and melting temperatures was 70, 192 and 420 °C respectively. The results obtained showed that lignin is a remarkable molecule, for the industries of cosmetic, pharmaceutics, polymers, resins and biofuels.

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Figure Caption

Tables

Table 1. Assignments of 2D HSQC of lignin L_3

δ_c/δ_H (ppm)	Assignments	Reference
55.5/3.70	C-H in methoxyls	(Wen et al., 2013)
59.5/3.73	Cγ-Hγ in β – O – 4	(Toledano et al., 2010)
71.8/4.9	Cα-Hα in β – O – 4	(Wen et al., 2013)
85.8/4.12	C_β-H_β in β – O – 4	(Wen et al., 2013)
104/6.70	$C_{2,6} – H_{2,6}$	(Wen et al., 2013)
114.5/6.70	$C_5 – H_5$	(Wen et al., 2013)
129/5.32	C=C in oleofinic structures	(Toledano et al., 2010)
129/7.2	$C_{2,6} – H_{2,6}$	(Toledano et al., 2010)

Table 2. Total phenol content and antioxidant activity of lignin obtained at 80 min, 121 °C and different NaOH mass percentage

$$Abs = 0.0177 \cdot CAC + 0.044; \quad R^2 = 0.997$$

Lignins	TPC (mg GAE mg sample^{-1})	DPPH
		I50 (mg sample·mg DPPH^{-1})
L_1 (0.5% NaOH)	73.06 ± 0.81	3.83 ± 2.02
L_2 (1.5% NaOH)	111.52 ± 0.24	3.46 ± 2.61
L_3 (2.5% NaOH)	181.21 ± 0.16	2.00 ± 0.44
L_4 (3.5% NaOH)	145.54 ± 0.44	4.24 ± 0.91
L_5 (4.5% NaOH)	151.22 ± 0.44	2.11 ± 0.96
L_6 (5.5% NaOH)	114.51 ± 0.31	2.93 ± 1.55
Ascorbic acid	n.d.	0.47 ± 3.4

ABTS assay	
L_3 (µg mL^{-1})	Inhibition percentage (%)
5	65.86 ± 1.28
50	85.91 ± 1.88
100	96.00 ±1.86
150	98.50 ± 1.11
200	98.25 ±1.08

n.d. Not determined
TPC: Total phenolic content
I50: Minimal concentration to inhibit 50% of DPPH or ABTS radical scavenging

Table 3. Larvae number of *C. elegans* before and after lignin addition at different concentrations

Lignin Concentration ($\mu g \cdot mL^{-1}$)	Initial (number)	Final (number)	Lethality (%)
200	23.33 ± 4.93	21.67 ± 5.69	7.49 ± 10.15
150	26.00 ± 6.00	24.00 ± 5.57	7.74 ± 3.39
100	27.67 ± 3.06	25.67 ± 3.21	7.22 ± 6.36
50	26.67 ± 2.52	25.33 ± 2.08	4.29 ± 1.72
40	22.67 ± 6.66	22.67 ± 6.66	0.00 ± 0.00
Control	26.00 ± 2.00	24.33 ± 2.08	6.34 ± 5.62
ANOVA analysis			
Square sum	8.33		
Degrees Freedom	1		
Average Square	8.33		
F critical	4.96		
F value obtained	2.98		
p value	0.11		

Figures

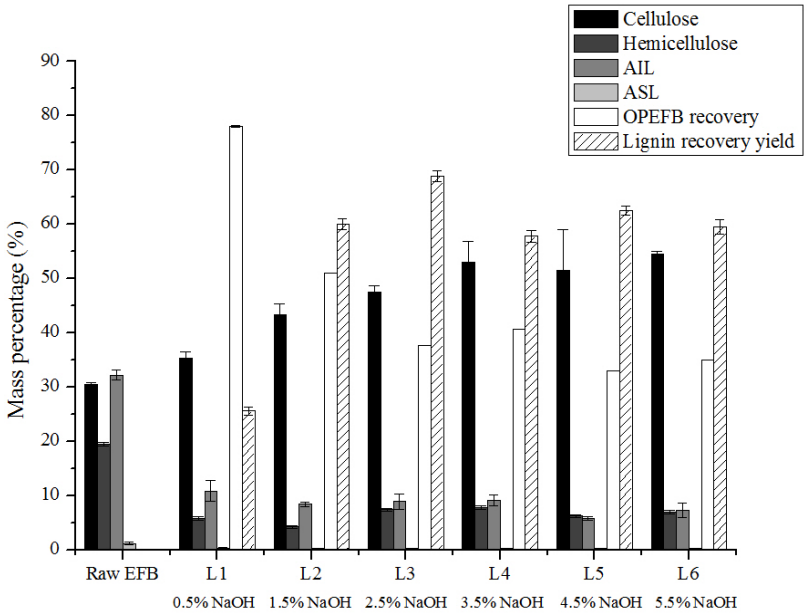

Figure 1.

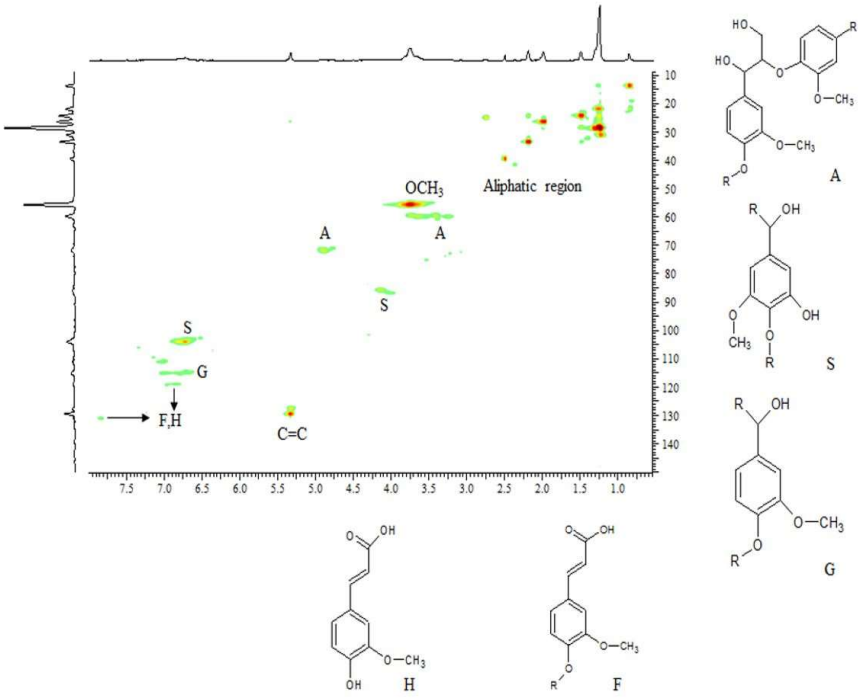

Figure 2.

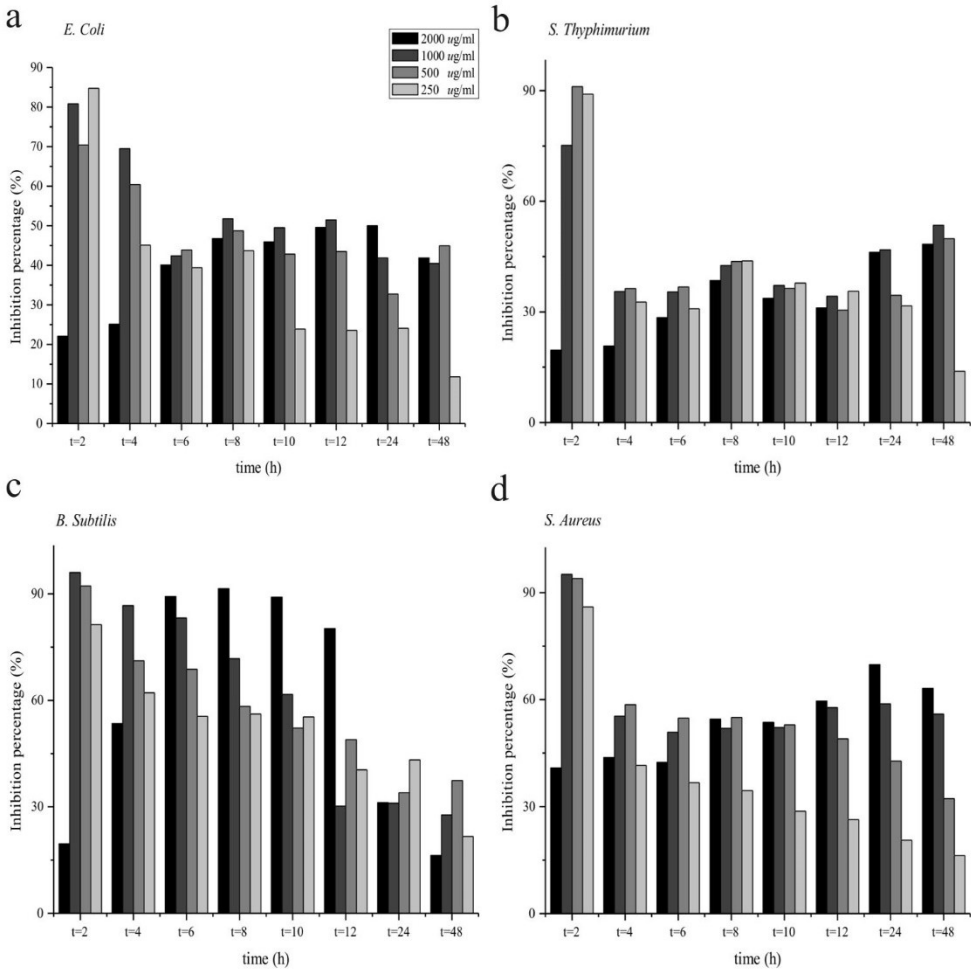
a
E. Coli
2000 ug/ml
1000 ug/ml
500 ug/ml
250 ug/ml
Inhibition percentage (%)
time (h)
t=2
t=4
t=6
t=8
t=10
t=12
t=24
t=48
b
S. Thyphimurium
c
B. Subtilis
d
S. Aureus

Figure 3.

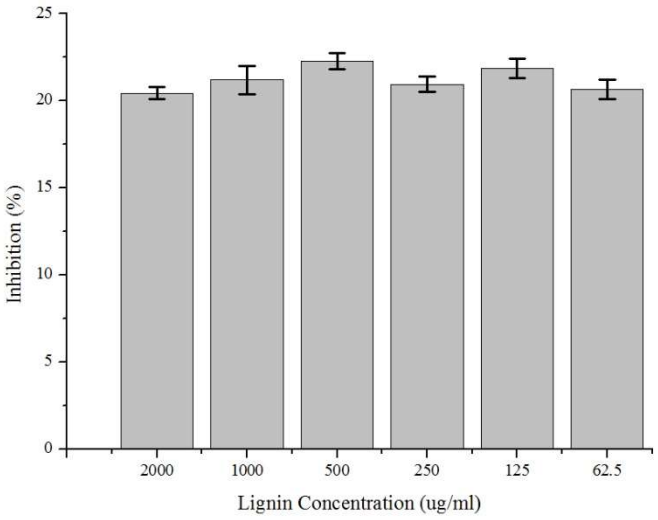
25
20
15
10
5
0
Inhibition (%)
2000
1000
500
250
125
62.5
Lignin Concentration (ug/ml)

Figure 4.

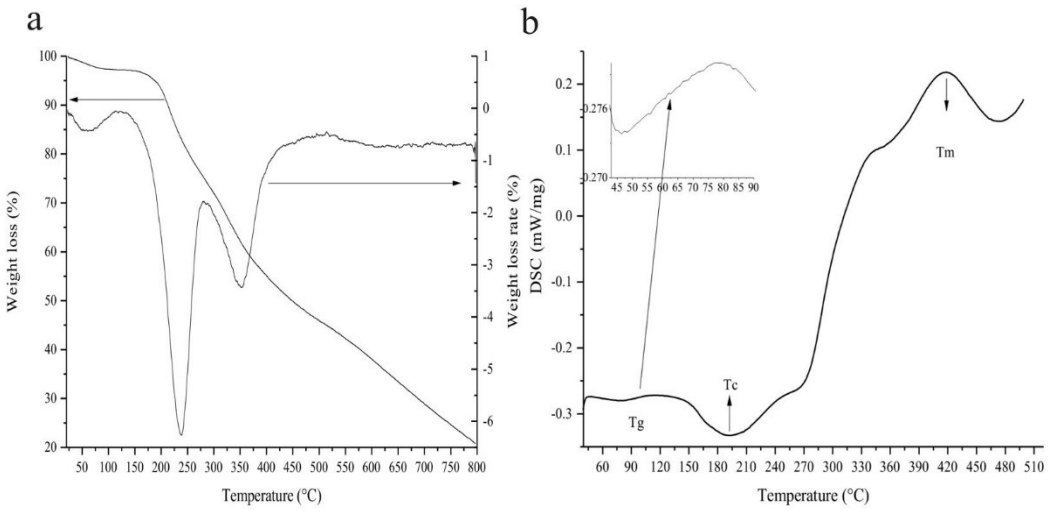
a
b
Weight loss (%)
Weight loss rate (%)
Temperature (°C)
DSC (mW/mg)
Tg
Tc
Tm
Temperature (°C)

Figure 5.

Techno-economic evaluation of pretreatment configurations for co-production of ethanol, xylitol and lignin from Oil Palm Empty Fruit Bunches

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Abstract

Oil palm empty fruit bunches (OPEFB) are lignocellulosic biomass that has drawn much attention in the last years for biorefinering, therefore, in this study three pretreatment configurations and four scenarios of biorefinery process for co-production of ethanol, xylitol and lignin from OPEFB were analyzed. The study involved the experimental determination of the cellulose digestibility as a function of three pretreatments carried out; sequential acid-alkaline, steam explosion and steam explosion followed by alkaline delignification. Optimal configurations of reactors for acid hydrolysis, alkaline oxidation and enzymatic saccharification were determined with the aim of analyzing the economical profit and the Net Energy Value (*NEV*). The sequential acid-alkaline pretreatment for co-production of ethanol from glucose, xylitol from hemicelluloses and lignin presented the largest economical profit corresponding to 1.1 USD·kg^{-1} OPEFB treated, while the steam explosion pretreatment accompanied of burning of the residual OPEFB presented the highest energy positive *NEV* of 9.9 MJ kg^{-1} OPEFB treated. The acid-alkaline treatment although attained the largest economical profit, presented a negative *NEV* of -2.8 MJ kg^{-1} OPEFB treated when lignin was not burned. The results showed that the use of OPEFB as potential feedstock for biorefinery process could be feasible if were valorized all fractions obtained in pretreatment stages.

Keywords: Biorefinery; Techno-economic analysis; Net Energy Value *NEV*; Ethanol; Xylitol; Lignin valorization; oil palm empty fruit bunches

1. Introduction

Currently, the global energy matrix is formed almost in 80% by non-renewable energy sources, such as coal, petroleum and natural gas, while the 90% of chemicals are from non-renewable sources [1]. The increment in energy and chemical demands, together with the gradual depletion of fossil fuels, instability in oil crude prices and the completion of crops for fuel production and food consumption, have motivated the research for exploitation of renewable resources to partially supply the dependence on fossil non-renewables sources [2].

The lignocellulosic biomass is considered as the largest renewable potential source of chemicals, fuels and materials that is not food-competitive. However, because of their recalcitrance, nowadays this feedstock is not cost-effective, due to different barriers that needs to be overcome, for feasible production of chemicals and fuels in large scale, inside a competitive scenario with respect to those traditionally produced from non-renewable sources [3].

The pretreatments stages are consider as one of the bottlenecks for the transformation of lignocellulosic biomass into value added products, which represents almost 20% of the total production cost. Therefore, the sustainability of a biorefinery process is depends on the valorization of each fraction obtained in pretreatment steps, it means five carbons sugars, lignin and cellulosic pulp [4]. The oil palm empty fruit bunches (OPEFB) are one of the most recent lignocellulosic materials considered as renewable source of chemicals and fuels. Brazil is currently the twelfth largest producer of oil palm from *Elaeis guineensis* with 340,000 ton per year, and is estimated to increase their production in the Pará state to almost 13 million hectares, representing 10% of the total area of the State [5]. The generation of OPEFB has been considered as an environmental problem, it is estimated that by 1 ton of oil palm fresh fruit bunches (OPFFB) processed are generated 220 kg of OPEFB [6], therefore, many researchers have proposing its lignocellulosic biomass for biofuels like ethanol and biogas, chemicals as xylitol and organic acids [7,8].

The relative largest cost and low yields of ethanol production from OPEFB at present, makes not feasible the ethanol production at industrial scale [9,10], consequently different researches through the simulation of different pretreatment configurations and co-production of molecules of value added have been reported alternatives to improve the economical profit and energy balance of a biorefinery process. The xylitol is one of the chemical molecule with special attention for production from biomass, because its consumption has been increasing in

the last decade. In 2013 the total consumption was approximately 160 thousands of metric tons and is expected to reach 242 thousands of metric tons in 2020, equivalent to 1 billion of USD [7]. Recent studies of biotechnological production of xylitol have reached important development as alternatives to the chemical process. The *Candida* strains have been reported as the most viable microorganism to transform the xylose into xylitol with good performance in terms of yield and productivity [11].

The black liquor rich in lignin fractions is mainly concentrated and burned for energy recovery, however, the fractionated lignin present in black liquor has been recognized as an promissory raw material for the production of low molar mass compounds, such as hydroxylated aromatics, quinines, aldehydes, aliphatic acids and many others chemical compounds, through different chemical or biological transformation processes [12].

Based on the biorefinery concept, the aim of this work was to analyze the economical and energetic viability of a biorefinery process based on OPEFB for ethanol production, and co-production of xylitol and lignin. In this paper the effect of three pretreatments, steam explosion, sequential acid-alkaline, and steam explosion followed by alkaline delignification, on the enzymatic digestibility of the OPEFB treated was investigated. The determination of the best performance of pretreatment configuration was conducted taking into consideration the economical profit and the net energy value given by the biorefinery process. In total three configurations of pretreatment and four scenarios of production were analyzed, considering the burning of residual OPEFB and lignin for energy recovery.

2. Materials and Methods

2.1. Lignocellulosic biomass

Oil palm empty fruit bunches (OPEFB) were obtained from Bio-Palma Vale Factory, located in Mojú, Pará, state of Brazil.

2.2. Biomass pretreatment

The OPEFB were submitted to three different pretreatments, sequential acid-alkaline (acid-alkaline), steam explosion (S.E.) and steam explosion followed by alkaline lignin extraction (S.E. + alkaline). The results of the first two pretreatments have been reported previously [13,14]. The sequential steam explosion and alkaline lignin extraction using NaOH, was carried out for this work. The best results obtained of the two first pretreatment were maintained to develop the sequential S.E + alkaline pretreatment.

2.3. Analysis of carbohydrates and lignin

The composition of polysaccharides in OPEFB before and after of pretreatment were determined using the NREL analytical procedure reported by Sluiter et al. [15]. The cellulose and hemicellulose mass composition were correlated through the concentration of glucose, xylose and arabinose. Analysis of acid hydrolysate was carried out by High-Performance Liquid Chromatography (HPLC), in a Shimadzu Chromatograph equipped with an Aminex HPX-87H column, working at 60 °C with sulfuric acid (5 mmol·L^{-1}) as mobile phase at flow rate of 6 mL·min^{-1}.

2.4. Enzymatic hydrolysis of OPEFB after pretreatment

For testing the enzymatic digestibility of the pretreated OPEFB, the cellulosic pulp was hydrolyzed by cellulose Celluclast® 1.5 L and Novozym 188. The enzyme sample used for activity measurements contained a 1:0.3 mass ration of Celluclast and Novozym 188 respectively, as was reported by Silveira et al. [16]. The enzymatic activity of the enzyme mixture loaded was 60 FPU·g $OPEFB^{-1}$, according to the procedure reported by Ghose [17]. The reaction was conducted in an air-bath shaker at 130 rpm for 5 days, at 55 °C and pH 4.8 (0.1 M sodium citrate buffer). The mass-volume relation of OPEFB was 2.5% (w/v), and the total volume of reaction was 50 ml. The enzymatic digestibility of the pulp was described as enzymatic sugar release during the enzymatic hydrolysis with respect to the initial mass percentage of cellulose; the digestibility calculation was carried out according to equation (1).

$$Digestibility\ (\%) = \frac{W_G}{Wc} x100 \qquad (1)$$

Where W_G is the weight of glucose released during enzymatic hydrolysis and W_c is the weight of cellulose in OPEFB pulp before enzymatic pretreatment. The experimental sugar concentration was monitored by HPLC analysis as was described in section 2.3. Aliquots of 1 ml were taken every 24 h during 120 h. The aliquots were centrifuged at 161 *x*g and the supernatant was collected for HPLC analysis. All experiments were made in triplicate.

2.5. Development of the kinetic model for enzymatic hydrolysis.

The enzymatic hydrolysis of OPEFB was simulated based on the kinetic model of Michaelis-Menten with a modification (the “impeded” Michaelis model), reported by Yang and Fang [18]. The reaction scheme is illustrated in equation (2)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E \cdot S \xrightarrow{k_2} E + P \quad (2)$$

Where *E*, *S*, *E·S* and *P*, are the enzyme, substrate, enzyme-substrate complex and product concentration, respectively. The equation to correlate the yield of glucose release with the time of reaction and the kinetic parameters $k_{obs,0}$ and α of the impeded model, was obtained through mass balance in batch system for *E*, *S*, *E·S* and *P*. The resultant equation is presented in equation (3).

$$\frac{t}{-ln(1-Y_{GR})} = \frac{1}{k_{obs,0}} + \frac{\alpha}{k_{obs,0}} t \quad (3)$$

Where $k_{obs,0}$, depends on the initial accessibility and activity of the enzyme linkage to the solid substrate. The impeded reaction gives the rate of transformation of active enzyme in ineffective enzyme through the parameter α. The equation (3) was used to correlate the experimental Y_{GR} with reaction time, to determine the kinetic parameters for all pretreatments developed, in function of the solid load in enzymatic hydrolysis.

2.6. Reaction of hydrolysis of biomass

The biomass hydrolysis was simulated using pseudo homogeneous irreversible first reaction models. The general scheme of degradation of polysaccharides (*P*), (cellulose and hemicellulose) to monosaccharides (*M*) and decomposition products (*DP*) was first proposed by Saeman [19] and presented in equation (4).

$$P \xrightarrow{k_p} M \xrightarrow{k_M} DP \quad (4)$$

Where k_p and k_M are the kinetic constant of production and degradation of monosaccharide respectively. Based on the equation (4), is possible develop a chemical equation for the hydrolysis of cellulose and hemicellulose to glucose and xylose, respectively. The reaction scheme are presented in equations (5) and (6).

$$Cellulose_{(s)} \xrightarrow{\kappa_1} Glu_{(ac)} \xrightarrow{\kappa_2} HMF_{(ac)} \quad (5)$$

$$Hemicellulose_{(S)} \xrightarrow{k_3} Xyl_{(ac)} \xrightarrow{k_4} F_{(ac)} \quad (6)$$

Where *Glu*, *Xyl*, *HMF*, *F* are glucose, xylose, 5-hydroxymethyl furfural and furfural, respectively. The lignin solubilization was also modelled based on equation (4), the reaction of solubilization is presented in equation (7)

$$L_{(s)} \overset{\kappa_5}{\rightarrow} AIL_{(ac)} \overset{k_6}{\rightarrow} DP_{(ac)} \quad (7)$$

Where L is the mass composition of lignin in OPEFB, AIL is the acid insoluble lignin, and DP, are the decomposition products (not measured here). The reaction model was used to determine the best operational condition of the acid and alkaline pretreatment reactors.

2.7. Determination of the best condition of operation in pretreatment reactors

To determine the best condition of operation for the reactor of acid hydrolysis and alkaline lignin extraction, the classical methodology of irreversible first-order reactions in series was adopted [20]. From the mass balance in CSTR for intermediate component in equations (5-7), the conversion was determined in terms of the specific constant reaction k_i and residence time τ for each reactor according to the equation (8).

$$\frac{C_{monomers}}{C_{polymer_0}} = \frac{k_P \cdot \tau}{(1+k_P \cdot \tau)(1+k_M \cdot \tau)} \quad (8)$$

Where $C_{monomers}$ are the concentration of monosaccharide at specific time, while the $C_{polymer_0}$ is the concentration of cellulose, hemicellulose and lignin in the biomass. The determination of optimal time (τ_p) was found by determining the $dC_{monomer}/d\tau = 0$. Thus

$$\tau_p = \frac{1}{\sqrt{k_p k_M}} \quad (9)$$

The optimal time would be replaced in equation (8) to determine the maximal concentration of monomer.

2.8. Techno-economic analysis

From the experimental study of pretreatment, three process configuration were projected and scaled, sequential acid-alkaline (C.1), steam explosion (C.2), steam explosion followed by alkaline delignification (C.3), these configurations are displayed in Figure 1. The reactor configuration was continuous stirred tank reactor (CSTR) for all pretreatments, except for steam explosion (batch operation). The capacity of processing was fixed in $2x10^5$ tons of OPEFB·per year, working 340 days. For each configuration of pretreatment, four scenarios were analyzed as are listed below:

1. Ethanol production from glucose and xylose, and lignin burning.
2. Ethanol production from glucose and xylose, and lignin production.

3. Ethanol production form glucose, xylitol production from xylose, and lignin production.
4. Ethanol production from glucose, xylitol form xylose, and lignin burning.

For all scenarios it was assumed that the residual OPEFB after pretreatment and saccharification process was burned to energy recovery. The modelling and simulation process was developed using MatLab® (The Mathworks, Natick, Massachusetts).

The production costs considered were: feedstock, high-steam pressure, water of process, enzyme price, chemicals (H_2SO_4 and NaOH) and additives for fermentation process. The production cost (P_c) and the economical profit (E_p) were determined according to equations (10) and (11).

$$P_C = C_F + C_s + C_w + C_A \quad (10)$$

$$E_P = P_S - P_C \quad (11)$$

In equation (10) P_C, C_F, C_S, C_W and C_A are the production cost of each molecule considered in USD·per kg of OPEFB processed, feedstock price, steam cost, liquid water and additives cost, respectively. In additive cost were included the cost of catalyst, enzyme, nutrients and yeast. The features considered for the simulation process are summarized in Table 1. In equation (11) P_S are the commercial price of sale of each molecule.

To be considered a viable alternative to fossil fuel, a fuel must have positive energy balance over the energy sources employed to produce it, beside of the environmental and economic benefits [21]. In this case, the Net Energy Value (*NEV*) was employed to determine the energy feasibility for all pretreatment configurations analyzed, and all scenarios proposed, the *NEV* was determined according to equation (12)

$$NEV = TEB - TEU \quad (12)$$

In equation (12), the term *TEB* represents the total energy derived from biofuel and *TEU* is the total energy used in biofuel production. The *TEB* was determined from the lower heating value of ethanol (LHV) corresponding to 21.1 $GJ \cdot m^{-3}$. The residual OPEFB after pretreatment and saccharification process was used also as heat source, assuming a LHV of 18 $MJ \cdot kg^{-1}$, while the LHV of lignin was assumed as 26 $MJ \cdot kg^{-1}$ [22]. The *TEU* was determined taking into account the energy necessary for drying, crushing, agitation, reactors pretreatment and

distillation of ethanol. The typical value of energy consumption for drying, crushing, agitation and distillation are also reported in Table 1.

2.9. Simple model for energy requirement in pretreatment process

The energy requirements (E_R) in the pretreatment and saccharification reactors were determined based on global energy balance without considering the agitation energy input for simplicity purposes. The E_R for each reactor were determined according to the equation (13)

$$E_R = Q_{heati} + Q_{rxn_j} \quad (13)$$

Where Q_{heati} was calculated only considering the biomass, water and catalyst. The heating energy for each one of these components was determined using the heat capacity as function of the temperature: Also only a ratio of the 5% of evaporation of water at reaction temperature (121°C) was considered [23]. The general expression for determination of heating value of each component Q_i is presented in equation (14)

$$Q_i = m_i \int_{T^{ref}}^{T^{rxn}} (a \cdot T^2 + b \cdot T + c) dT \quad (14)$$

Where m_i is the mass flow of each component, T^{ref} is the reference temperature of 25 °C, T^{rxn} is the reaction temperature of 121 °C. The heat reaction was determined by the heats of formation and conversion of polysaccharides to monosaccharides, based on the data reported by NREL [24]. The energy of reaction was determined according to equation (15).

$$Q_{rxn_j} = \sum_{i=1}^{6} \Delta H_{rxn_i}{}^{o} \cdot v_i + Cp_i \cdot W_i \cdot X_j \quad (15)$$

Where Q_{rxn_j} is the heat reaction energy for hydrolysis of cellulose (j=1) and hemicellulose (j=2), as well as lignin extraction (j=3); $\Delta H_{rxn_i}{}^{o}$ is the normal heat of formation of i component summarized in Table 2; v_i is the stoichiometric coefficient of i component in the reaction j; Cp_i and W_i, are the heat capacity and the mass composition of the i component in OPEFB, respectively; and X_j is the fractional conversion of polysaccharides to monosaccharides in the j reaction.

3. Results and discussion

3.1. Enzymatic hydrolysis

The mass composition of OPEFB after each pretreatments, in terms of cellulose, the initial mass employed in the enzymatic hydrolysis and the conditions used are presented in

Table 3. For each condition of pretreatment presented in Table 3 the enzymatic hydrolysis was performed, the results are displayed in Figure 2 in terms of digestibility percentage.

In Figure 2(a) is presented the digestibility of OPEFB pulp after sequential acid-alkaline pretreatment, the largest yield achieved was 72 wt.%, corresponding to pretreatment at 121 °C and 2.5wt. % of NaOH for lignin extraction. The peeling effect, the increase in surface area, and swelling of cellulose, would be the essential factors for improving the enzymatic hydrolysis after acid/alkaline treatment, as has been reported by other authors [25,26].

In Figure 2(b) is displayed the enzymatic digestibility of OPEFB after S.E. pretreatment, the best performance was obtained at condition 6, representing 14 wt.% of digestibility. The poor digestibility of OPEFB after S.E could be explained by the presence of lignin in the fibers and by the pore collapse of the biomass, leading to decrease of surface area available and accessibility [27]. The results obtained in this work are similar to those obtained by Duangwang et al. [28] and Shamsudin et al. [29], in enzymatic sacharification of OPEFB treated by steam explosion, using super heat steam and saturated steam respectively.

The effect of sequential S.E and alkaline lignin extraction on the digestibility is presented in Figure 2(c). For all experimental conditions, the enzymatic digestibility was remarkably increased with respect to S.E. Confronting the results of S.E at 195 ° and 6 min with the sequential S.E and alkaline lignin extraction, an increase of 66.7% of the digestibility was found. The increase in enzymatic digestibility was attributed to lignin removal. In Figure 2(d) the best results of the pretreatments tested and the untreated OPEFB in function of enzymatic digestibility are presented. As is displayed, the acid-alkaline pretreatment presented the best performance, followed by the sequential S.E. and alkaline lignin extraction, while the S.E. pretreatment presented the worst performance, increasing the digestibility in 8% in the best result with respect to untreated OPEFB.

According to equation (3), the enzymatic kinetic constant α and $k_{obs,0}$ for each pretreatment were determined, the results are summarized in Table 4. The results confirmed the performance of each pretreatment on OPEFB digestibility. The influence of solid load on the enzymatic yield conversion of cellulose to glucose was determined for all configurations of pretreatment. The results showed that the effect of solid load to volume of reaction ratio of OPEFB was similar for all cases analyzed (Supplementary S.1.), the reaction yield was increased with the solid load until 20 (w/v)%, after these point remain almost constant. From

the results obtained was found that is possible convert 80 % of cellulose to glucose at 20 (w/v)% solid load, according to the kinetic constant reported in Table 4.

3.2. Determination of best condition for reactors of acid hydrolysis and alkaline delignification

The simulation of reactors for acid hydrolysis (R.1) and alkaline delignification (R.2) was carried out based on the capacity of processing OPEFB assumed. The analysis of the best condition for both reactor operation were made as function of the hemicellulose conversion and the xylose yield for R.1, and conversion of lignin to acid soluble lignin for alkaline reactor R.2.

In Figure 3(a) is displayed the time-independent plot, relating the xylose yield with respect to hemicellulose conversion (X_H). Because the xylose yield was 40 wt% under laboratory conditions, different condition for the reactor operation (data not show) were analyzed to increase the xylose yield. Based on these analysis was selected the most viable reactor operation specified in Figure 3(a). The modification in the ratio k_4/k_3 would be made by the modification in the mass percentage of H_2SO_4, reaction time and/or temperature, affecting mainly the constant k_3. A correlation between k_3 and the catalyst mass percentage was reported by Rahman et al. [30], and used in this work. The selection of the suitable point of reactor operation must be done considering the time and volume of reaction, because these parameters affect directly the economical sustainability of the process. The reaction time, volume of reaction, concentration of xylose in effluent, and kinetic constant, determined from the laboratory data and by the simulation of reactor at industrial scale are summarized in Table 5. The optimization in operational conditions of the reactor for acid hydrolysis produced an increase in the xylose concentration from 9.2 to 16.9 $kg \cdot min^{-1}$, however the volume of reaction was increased to 60 m^3 and the time from 60 to 77 min.

The optimization of the reactor R.2, was carried out following the same procedure developed for reactor R.1. The results are also summarized in Figure 3(b) and in Table 5. The high concentration of lignin in the effluent of the reactor of lignin extraction after optimization was increased from 27.89 to 34.58 $kg \cdot min^{-1}$. In alkaline lignin extraction, the volume of reaction after optimization was reduced in 3 m^3, changing from 215 to 212 m^3, while the reaction time remained almost constant in 80 min.

3.3. Steam explosion

The optimization of the steam explosion process was carried out experimentally and reported in a previous work [13], and were maintained for the simulation in this work.

3.4. Techno-economical results

For all configurations of pretreatment and each scenario proposed, the mass and energy balance was developed. For determination of steam requirements, was assumed as energy source saturated steam at 22 atm, while the outlet temperature from reactors was fixed at 140 °C. This temperature is important for evaporation units and heat exchangers (not consider in this work). In the Table 6 are summarized the total requirements of utilities and additives, as well as the flux of residual OPEFB. The sequential acid alkaline pretreatment requires more quantities of utilities and additives, specifically water and steam, followed by the sequential steam explosion-alkaline lignin extraction, and steam explosion.

The productions of ethanol, xylitol and lignin for each configuration and scenario proposed are shown in Figure 4. The largest production of ethanol obtained was 21,500 ton·year^{-1} by the configuration 1 and the scenarios 1 and 2, these result was possible by the consideration of ethanol production from glucose and xylose. The lowest ethanol production under the configuration 3 was attributed to the poor recovery yield of OPEFB after S.E + alkali delignification, its mean 28 wt. % of initial OPEFB was available for enzymatic saccharification.

The production of xylitol was considered in scenarios 3 and 4. Through the configuration 1 was achieved the highest xylitol production of 17,550 ton·year^{-1}, while by the configuration 2 and 3 was only attained 940 ton·year^{-1}. The poor production of xylitol was attributed to low solubilization of hemicelluloses and formation of degradation components by steam explosion pretreatment [13]. The theoretical conversion of xylose to xylitol consider was 60%, this value was based on the experimental yield reported by Wei Wang et al. [31] for xylitol production from corn straw pretreated with steam explosion.

The lignin valorization was considered in scenarios 2 and 3. The results showed that the largest lignin production attained was 36,745 ton year^{-1} through the configuration 1, while the configuration 3 achieved 16,500 ton year^{-1}. The higher lignin production in configuration 1 was attributed to the larger OPEFB available to lignin extraction in configuration 1 than configuration 2. The re-valorization of lignin has been reported as a promissory alternative to

enhance the sustainability of the biorefinery process, as precursor of chemicals of high added value in cosmetic, pharmaceutical and food industries [32].

The economical profit for all configuration and scenarios proposed are summarized in Figure 5. The configuration 1 and scenarios 2 and 3 presented the largest economical profits of 0.88 and 1.5 USD·kg^{-1} OPEFB respectively, these values of economical profits were attained by the ethanol production, accompanied with lignin (scenario 2) and xylitol (scenario 3). From these results was established that only the production of ethanol is not feasible from the economical point view, mainly because ethanol is an inexpensive molecule (0.7 USD·L^{-1}), which should be produced in large volumes.

The configuration 2, presented negative economical profit for all scenarios analyzed. These results could be explained by the low production of ethanol, being not cost-effective in terms of sustainability for a bioprocess from OPEFB. These results are in accordance with the reported by Avellar and Wolfgang [33] as a general tendency in the processing of biomass using steam explosion.

The configuration 3 presented two scenarios with positive economical profits and 2 scenarios with negative values. The scenarios 2 and 3 presented an economical profit corresponding to 0.434 and 0.426 USD kg^{-1} OPEFB treated, while the scenarios 1 and 4 presented -0.048 and -0.037 USD kg^{-1} OPEFB treated. The positive economical profit was attained when was consider the production of xylitol and lignin, these results confirmed that only the ethanol production is not feasible from the economical view point in a biorefinery process using as feedstock OPEFB. The increase in the economical profit of steam explosion followed by alkaline delignification, could be explained by the increase in the ethanol production by the lignin extraction, which has a inhibitory effect on cellulase enzyme [26] and by the lignin production.

The energetic balance was made to determine the *NEV* of the process. In Figure 6(a) is displayed the *NEV* when only ethanol was considered as source of energy produced. The configuration 1 presented the largest negative *NEV* for scenarios 1 and 2 of -5.37 MJ·kg^{-1} OPEFB treated, while for scenarios 3 and 4 was obtained a *NEV* of -7.05 MJ·kg^{-1} OPEFB treated, the presence of 2 reactors, with heating and highest water requirements, the drying and milling process are the responsible for the largest energy requirements. The configuration 2 presented a *NEV* value of 1.47 Mj·kg^{-1} OPEFB treated for all scenarios proposed. The lowest energy requirements of steam explosion have been recognized as one of the advantages over

other type of pretreatments [34]. In configuration 3 the energy requirements was reduced considerably with respect to configuration 1, repercuting in a *NEV* of -1.71 MJ·kg^{-1} of OPEFB treated, the reduction in *NEV* was attained by the inclusion of steam explosion, however the energy requirements in alkaline delignification are higher than the energy saved using steam explosion.

The energy balance was improved by the burning of residual OPEFB and lignin (scenarios 1 and 4), the results are displayed in Figure 6(b). The configuration 1 and scenarios 1and 4 attained 3.93 and 2.25 MJ·kg^{-1} of OPEFB treated of *NEV* respectively, while the scenarios 2 and 3 presented a *NEV* of -1.13 and -2.25 MJ·kg^{-1} of OPEFB treated, respectively. The energy gain was achieved by uses of both residual OPEFB and lignin as energy sources, while for the scenarios 2 and 3 the energy balances remained negative, because the burning of residual OPEFB was not enough to offset the energy requirements. The *NEV* for the configuration 2 was considerably increased producing 9.9 MJ·kg^{-1} of OPEFB treated by burning residual OPEFB. The configuration 3 presented positives *NEV* balance for all scenarios analyzed, when was consider the residual OPEFB and lignin as energy source, when lignin is burned was obtained a *NEV* of 4.2 and 3.8 MJ·kg^{-1} of OPEFB treated for scenarios 1 and 4 respectively, while for scenarios 2 and 3 the *NEV* was almost constant in 1.9 MJ·kg^{-1} of OPEFB treated. The total energy involved in ethanol production *TEU*, for each pretreatment configuration proposed showed that were necessary 8.41, 0.86 and 2.2 MJ·kg^{-1} of OPEFB treated for configuration 1, 2 and 3 respectively. The results obtained showed that the acid-alkaline pretreatment is the most intensive treatment process in terms of energy consumption, with 8.41 MJ·kg^{-1} OPEFB, these energy consumption is very close to the energy consumption determined experimentally (8.1 Mj·kg^{-1} OPEFB treated) in a pilot plant of ethanol from OPEFB [9]. The steam explosion was the pretreatment process with lowest energy requirements, this is by the fact of the low requirements of steam, absence of crushing and the reactor of alkaline delignification, while the steam explosion followed by the alkaline treatment presented an intermediate behavior in terms of energy consumption.

Conclusion

Three pretreatments configurations were analyzed in function of economical profit and energy balance for four scenarios of biorefinery process using OPEFB as feedstock, for ethanol, xylitol and lignin co-production. These results showed that would be produced 21,500 ton·year^{-1} of ethanol, 17,550 ton·year^{-1} of xylitol and 36,745 ton·year^{-1} of lignin from 2x10^{5} ton of

OPEFB per year, generating economical profit of 1.1 USD·kg^{-1} OPEFB treated in the best of cases analyzed. Was also found negative economical and energy balance when only was considered the ethanol production, corresponding to -0.1 USD·kg^{-1} OPEFB and -5.4 *MEV*·kg^{-1} OPEFB treated, therefore, to increase the sustainability of the biorefinery process, the co-production of xylitol and lignin were considered as promissory molecules, generating economical profits, while the residual OPEFB and the eventual lignin burning contributed to improving the energy balance.

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Appendix A

Supplementary data related to this article can be found at:

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Figure caption

Figure 1. Process flow chart. (a) Configuration 1: acid-alkaline. (b) Configuration 2: Steam explosion. (c) Configuration 3: Steam explosion and alkaline delignification.

Figure 2. Enzymatic digestibility kinetic. (a) Acid-alkaline. (b) Steam explosion. (c) Steam explosion + alkaline delignification. (d) Best results confrontation

Figure 3. Reactor optimal configuration. (a) Acid hydrolysis. (b) Alkaline oxidation.

Figure 4. Production of ethanol, xylitol and lignin under different pretreatment configuration and biorefinery process scenarios.

Figure 5. Total economical profit

Figure 6. Net Energy Value (*NEV*) and energetic requirements.

Tables

Table 1. Cost of the considered features of the production process [35,36]

Variable	Cost (USD·kg^{-1})
Feedstock	
OPEFB	0.013
Utilities	
High-pressure steam	0.009
Water for process	0.002
Electricity (Kwh)	0.098
H_2SO_4	0.0094
NaOH	0.012
Enzyme	1.5
Yeast	7
Nutrients. Ethanol fermentation	0.3
Nutrients. Xylitol fermentation	13.95
Commercial price of molecules under study[a]	
Ethanol	0.7 (USD·L^{-1})
Xylitol	4 (USD·kg^{-1})
Lignin	5 (USD·kg^{-1})
Energy	
Energy for drying (Gj·kg^{-1})	3.6x10^{-5}
Energy for crushing (Gj·kg^{-1})	1.9x10^{-4}
Energy for agitation (Gj·h^{-1})	0.10
Energy for distillation (Gj·m;^{3}ethanol)	5.6
Process considerations	
Ethanol yield from glucose	0.9
Ethanol yield from xylose	0.8
Xylitol yield	0.6
Nutrients for alcoholic fermentation	1%[b]
Nutrients for xylitol fermentation	1%[b]
Inoculum for fermentation(g·L^{-1})	1%

[a]: Data obtained from the web site: www.alibaba.com accessed: 23-05-2016

[b]: mass percentage with respect to total mass of carbon source

Table 2. Constant of heat capacity (Cp)[a] in function of temperature

Component	a	b	C	Reference
Biomass	$4x10^{-5}$	$1.5x10^{-3}$	0.9325	[23]
Water (liquid)	$1x10^{-5}$	$1.3x10^{-3}$	4.2085	[23]
Water (steam)	$8x10^{-7}$	$2x10^{-4}$	1.8572	[23]
H_2SO_4 (solid)	0.0	0.0	1.34	[23]
NaOH (solid)	0.0	0.0	3.55	[37]
Standard Heat of formation				
Component	$\Delta H_{rxn}{}^{o}$ (kJ·kg^{-1})			[24]
1. Cellulose	0			[24]
2. Glucose	$-1.67x10^{4}$			[24]
3. Hemicellulose	0			[24]
4. Xylose	$-7.04x10^{3}$			[24]
5. HMF	$-2.21x10^{3}$			[24]
6. F	$-1.57x10^{3}$			[24]

[a]: heat capacity in kJ·kg^{-1}·K

Table 3. Pretreatment of OPEFB and Cellulose mass Composition (dry basis)

Pretreatment	Condition. T (°C)/ t (min)	Initial mass (g)	Cellulose (%)
Control	Untreated EFB	2.50	28.00
Steam Explosion	1. 175/6	2.51	23.50
	2. 175/10	5.52	23.46
	3. 185/6	2.51	32.45
	4. 185/10	2.50	31.47
	5. 195/6	2.50	34.89
	6. 195/10	2.49	25.44
	T (°C)/ (w/w) % NaOH		
Acid/alkaline	1. 121/ 0.5	2.51	35.45
	2. 121/ 2.5	2.51	47.47
	3. 121/ 4.5	2.51	51.54
	4. 121/ 5.5	2.50	54.52
	Condition. T (°C)/t (min)		
Steam explosion + alkaline lignin extraction (2.5% NaOH, 121°C and 80 min)	1. 175/ 6	2.51	28.26
	2. 175/ 10	2.50	28.21
	3. 185/ 6	2.03	39.02
	4. 185/ 10	2.35	37.84
	5. 195/ 6	2.50	41.96
	6. 195/ 10	2.51	30.59

Table 4. Enzymatic kinetic constant for each pretreatment developed

Pretreatment	α (min^{-1})	$k_{obs,0}$ (min^{-1})
Acid/Alkaline	0.0203	0.0151
Steam explosion	0.0140	0.0012
Steam explosion and alkaline	0.0132	0.0068

Table 5. Operation spot for acid hydrolysis and alkaline reactors.

Parameter	Acid hydrolysis reactor		Alkaline lignin extraction	
	Laboratory point stablished	Operational point selected	Laboratory point stablished	Operational point selected
τ (min)	60	77	80	78
k_f (min^{-1})[a]	0.012	0.045	0.118	0.005
k_r (min^{-1})[a]	6.0x10-4	3.5x10-3	0.118	0.001
C ($kg \cdot min^{-1}$)[b]	9.2	16.95	27.89	34.58
Y (%)[b]	40.92	77.7	64.59	68.78
Vr (m^3)	205	265	215	212

[a]: right kinetic constant, subscript f. Left kinetic rate constant, subscript r

[b]: concentration and yield form xylose and acid insoluble lignin

Table 6. Total requirements of utilities and additives for each pretreatment configuration

	Pretreatment		
Feature	Acid-Alkaline	S. E.	S. E. + alkaline
Water ($kg.s^{-1}$)	68.90	10.75	23.32
H_2SO_4 ($kg.s^{-1}$)	0.64	0.0	0.0
NaOH ($kg.s^{-1}$)	1.12	0.0	0.54
Steam ($kg.s^{-1}$)	92.6	5.47	19.50
Enzyme ($kg.s^{-1}$)	0.16	0.18	0.08
Yeast ($kg.s^{-1}$)	$5.0x10^{-3}$ [a]	$4.0x10^{-3}$ [a]	$4.5x10^{-3}$ [a]
	$4.9x10^{-3}$ [b]	$4.6x10^{-3}$ [b]	$5x10^{-6}$ [b]
Nutrients ($kg.s^{-1}$)	$7.1x10^{-4}$	$1.1x10^{-3}$	$2.0x10^{-4}$
	$9.8\ x10^{-4}$	$5.2x10^{-5}$	$2.0x10^{-4}$
Residual OPEFB ($kg{\cdot}s^{-1}$)	1.51	3.00	0.63

[a]: For ethanol fermentation
[b]: For xylitol fermentation

Figures

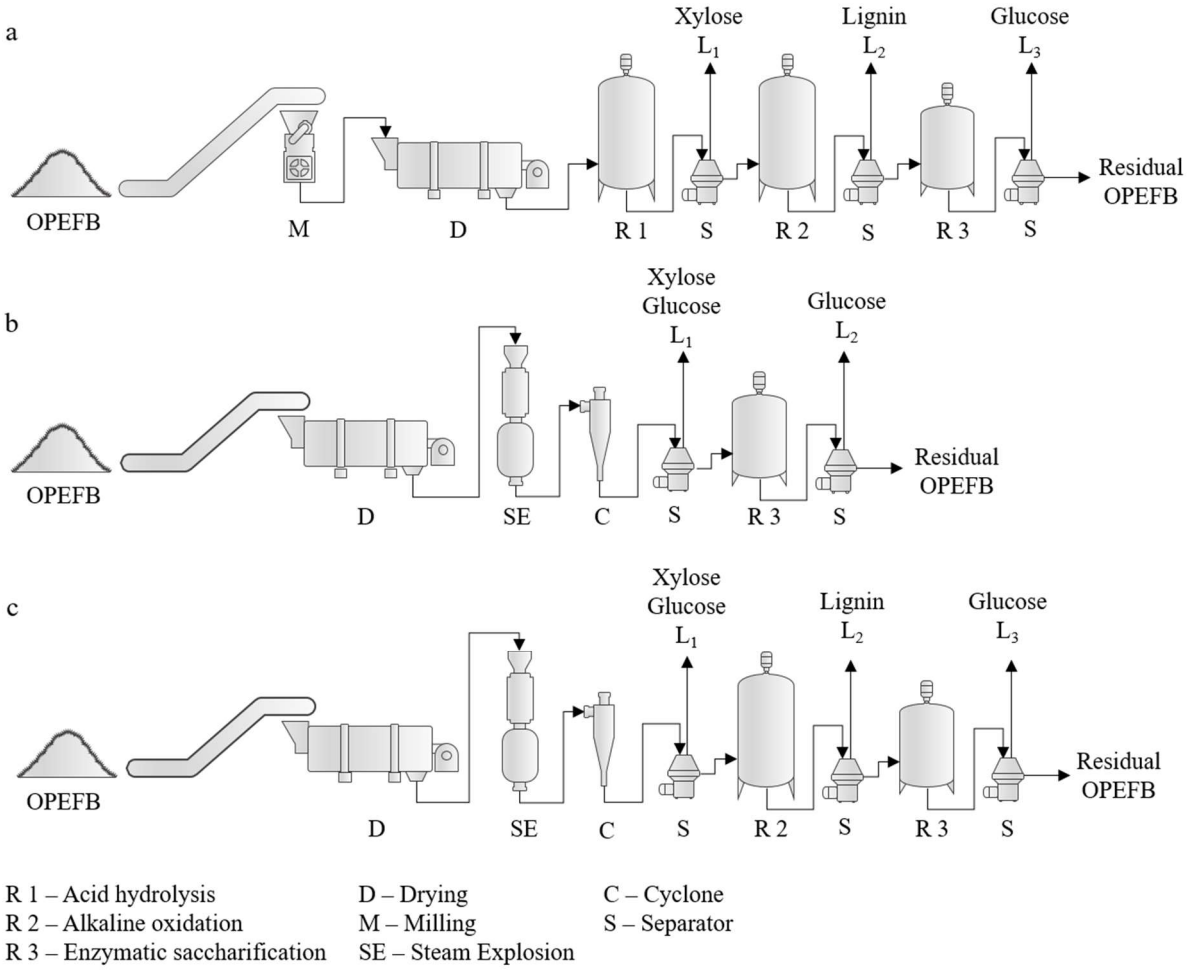


Figure 1.

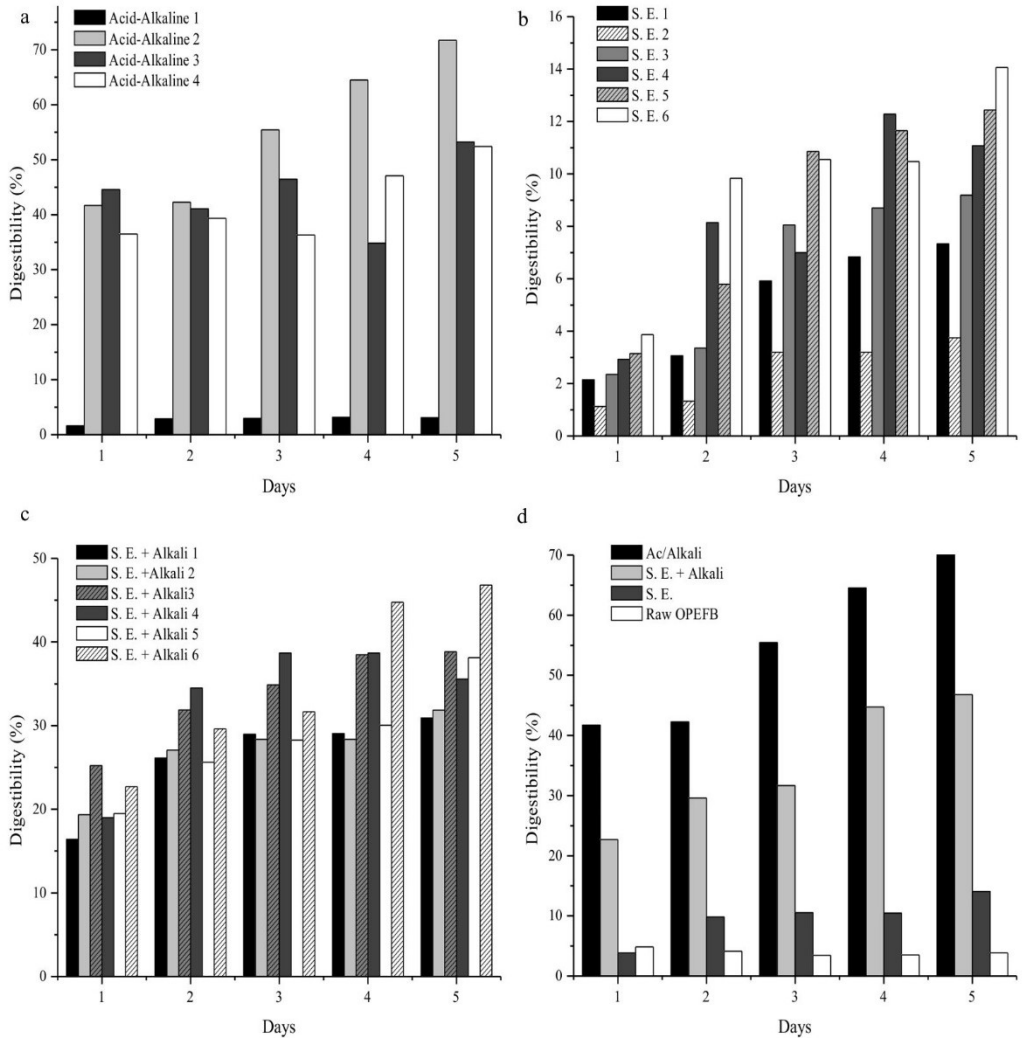

a
Acid-Alkaline 1
Acid-Alkaline 2
Acid-Alkaline 3
Acid-Alkaline 4
Digestibility (%)
Days
b
S. E. 1
S. E. 2
S. E. 3
S. E. 4
S. E. 5
S. E. 6
Digestibility (%)
Days
c
S. E. + Alkali 1
S. E. +Alkali 2
S. E. + Alkali3
S. E. + Alkali 4
S. E. + Alkali 5
S. E. + Alkali 6
Digestibility (%)
Days
d
Ac/Alkali
S. E. + Alkali
S. E.
Raw OPEFB
Digestibility (%)
Days

Figure 2.

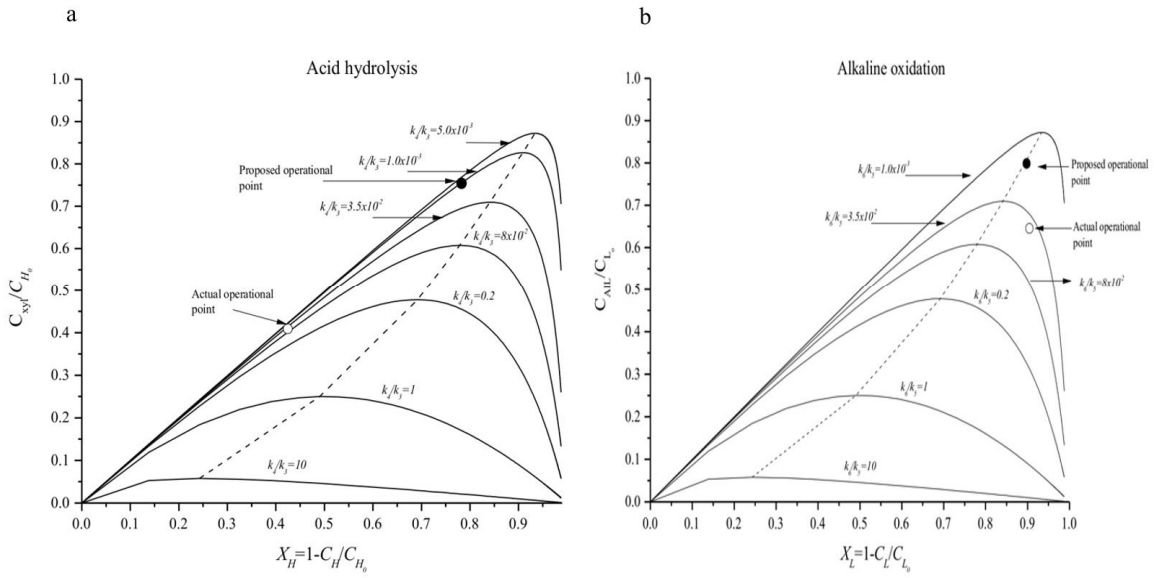

a
Acid hydrolysis
Proposed operational point
Actual operational point
$k_4/k_3=5.0x10^{-3}$
$k_4/k_3=1.0x10^{-3}$
$k_4/k_3=3.5x10^{-2}$
$k_4/k_3=8x10^{-2}$
$k_4/k_3=0.2$
$k_4/k_3=1$
$k_4/k_3=10$
C_{xyl}/C_{H_0}
$X_H=1-C_H/C_{H_0}$
b
Alkaline oxidation
Proposed operational point
Actual operational point
$k_4/k_3=1.0x10^{-1}$
$k_4/k_3=3.5x10^{-2}$
$k_4/k_3=8x10^{-2}$
$k_4/k_3=0.2$
$k_4/k_3=1$
$k_4/k_3=10$
C_{AIL}/C_{L_0}
$X_L=1-C_L/C_{L_0}$

Figure 3.

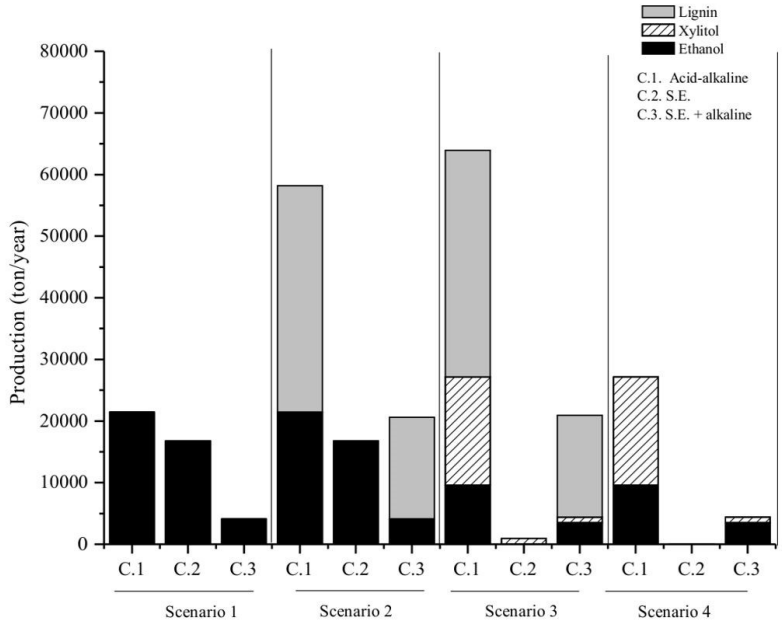

Lignin
Xylitol
Ethanol
C.1. Acid-alkaline
C.2. S.E.
C.3. S.E. + alkaline
Production (ton/year)
80000
70000
60000
50000
40000
30000
20000
10000
0
C.1
C.2
C.3
Scenario 1
Scenario 2
Scenario 3
Scenario 4

Figure 4.

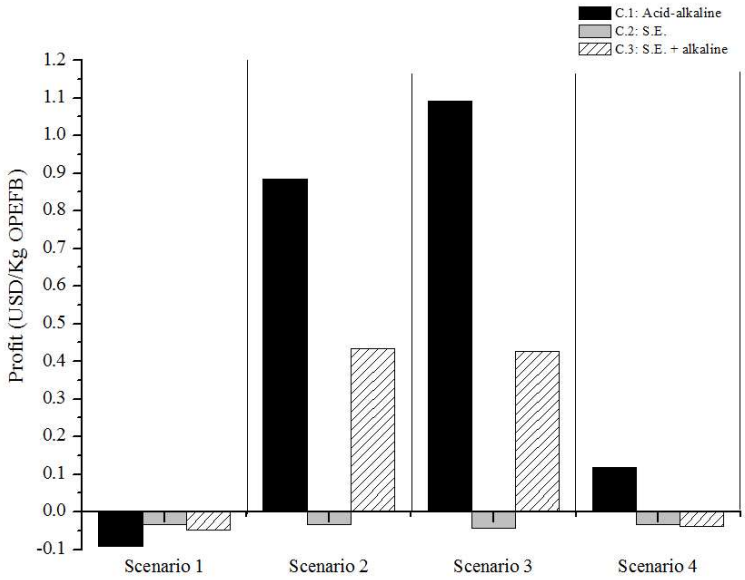

C.1: Acid-alkaline
C.2: S.E.
C.3: S.E. + alkaline
Profit (USD/Kg OPEFB)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
Scenario 1
Scenario 2
Scenario 3
Scenario 4

Figure 5.

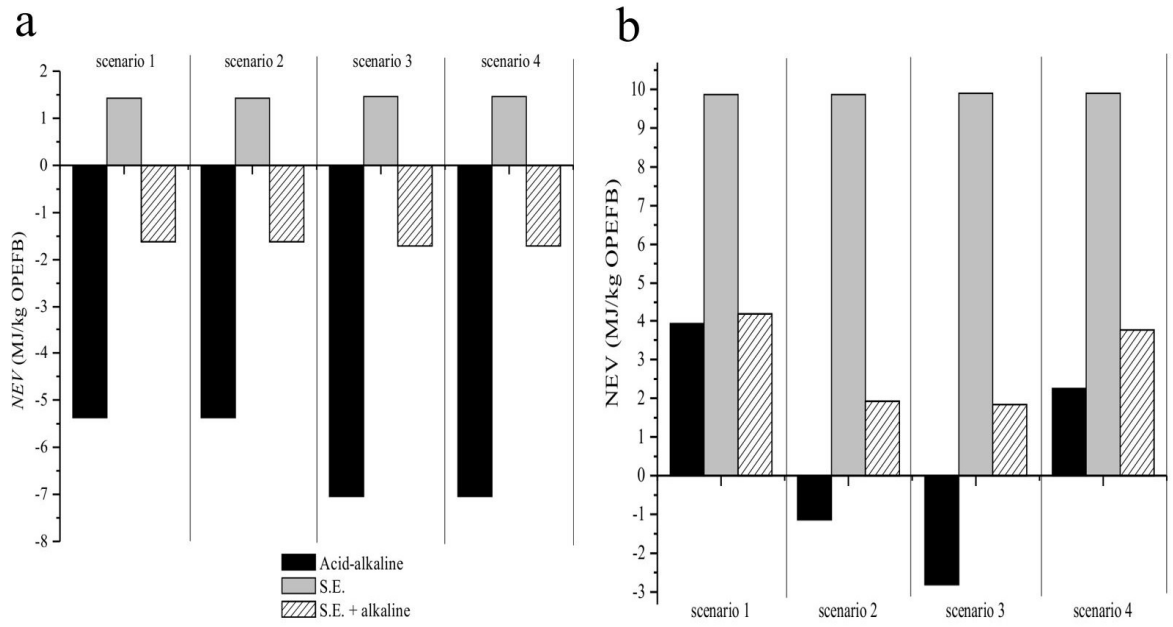
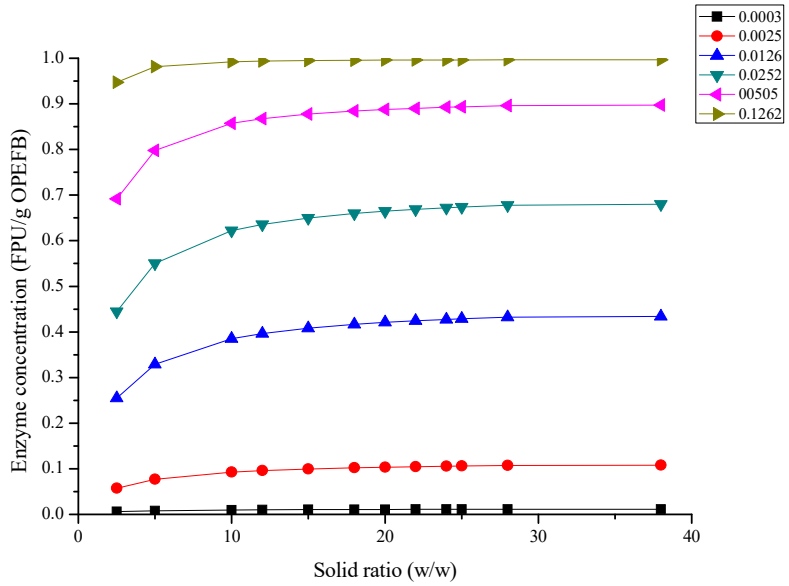
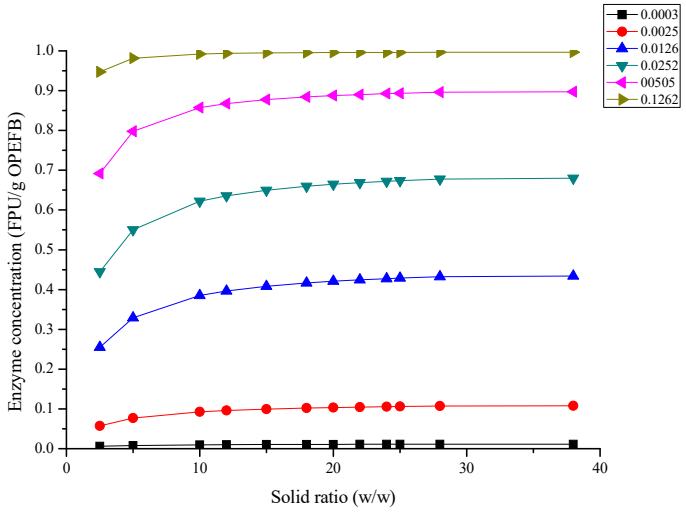

a
scenario 1
scenario 2
scenario 3
scenario 4
NEV (MJ/kg OPEFB)
Acid-alkaline
S.E.
S.E. + alkaline
b
NEV (MJ/kg OPEFB)
scenario 1
scenario 2
scenario 3
scenario 4

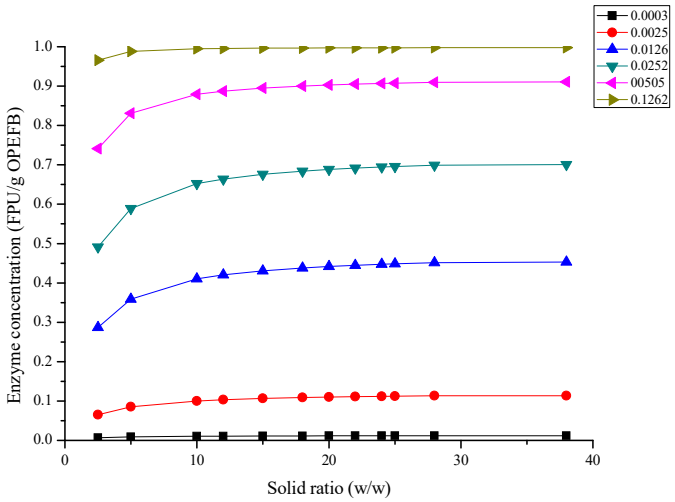
Figure 6.



Acid-Alkaline pretreatment



Steam explosion



Steam explosión + alkaline delignification

Paper I

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Steam explosion pretreatment of oil palm empty fruit bunches (EFB) using autocatalytic hydrolysis: A biorefinery approach

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HIGHLIGHTS

- Analysis of pretreatment step using steam explosion under autocatalytic conditions.
- Erosion in OPEFB structures after steam explosion, improving the porosity and reducing the crystallinity of cellulose.
- Solubilisation of hemicellulose in form of xylose and arabinose.
- Enrichment of 24% in cellulose per gram of OPEFB treated.
- Production of liquid hydrolysate rich fermentable sugars.

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ABSTRACT

The oil palm empty fruit bunches (EFB) are an attractive source of carbon for the production of biochemical products, therefore, the aim of this work is to analyze the effect of the steam explosion (SE) pretreatment under autocatalytic conditions on EFB using a full experimental design. Temperature and reaction time were the operational variables studied. The EFB treated at 195 °C for 6 min showed an increase of 34.69% in glycan (mostly cellulose), and a reduction of 68.12% in hemicelluloses, with increased enzymatic digestibility to 33% producing 4.2 g L^{-1} of glucose. Scanning electron micrographs of the steam treated EFB exhibited surface erosion and an increased fiber porosity. Fourier transform infrared spectroscopy showed the solubilization of hemicellulose and modification of cellulose in treated EFB.

1. Introduction

The production of oil palm from *Elaeis guineensis* in Brazil has increased in last decade, being the 10th producer in the world. However, 80% of oil palm in Brazil is used in the food industry. With the launching in January 2010 of the Brazilian National Program for the Production and Use of Biodiesel (PNPB), the inclusion of oil palm as feedstock for biodiesel production 31.8 million hectares (ha) of deforested area were identified in the Northeast, with good soil and climate conditions for palm oil cultivation. Moreover, the local installation and operation of almost 13 pilot plants improved the production capacity to 751 million of liters of oil per year.

Together with oil palm production, the generation of solid waste called oil palm empty fruit bunches, commonly referred as (EFB) is increasing. It is estimated that each 1 ton of oil palm produced generates 1.1 tons of EFB (Shinoj et al., 2011). This lignocellulosic biomass is mainly composed of cellulose (glucan), hemicellulose (xylan and arabinan mainly) and lignin. Being an attractive source for biofuels and value added chemicals it can be included in the energy matrix to improve the sustainability of palm oil biorefineries (Gnansounou and Dauriat, 2010; Noomtim and Cheirsilp, 2011).

Because of the recalcitrant nature of the lignocellulosic material found in EFB, a pretreatment stage is required to solubilize the hemicellulose and lignin and enhance the mass proportion of cellulose in the biomass. Pretreated material will have an increased porosity and improved accessibility toward the subsequent hydrolysis process, for the production of sugars useful for ethanol fermentation (Saikku et al., 2012).

Steam explosion (SE) has been proved to be effective for a variety of lignocellulosic biomass, including hardwoods, softwoods, herbaceous residues, sugarcane bagasse, and wheat straw, being the most widely employed physico-chemical pretreatment for

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0960-8524/

any lignocellulosic biomass (Fernandes et al., 2015; Ramos et al., 1992). During SE, the biomass is heated for a short time period with saturated steam at high pressure, followed by a sudden decompression. The high pressured steam provides an adequate vehicle to heat biomass, penetrating and modifying the cell wall structure, ensuring the effective hydrolysis of mainly hemicelluloses making cellulose more accessible. The sudden decompression causes an adiabatic expansion that makes the material to undergo a mechanical disruption of fibers modifying the plant cell wall (Hendriks and Zeeman, 2009; Mosier et al., 2005; Ramos, 2003).

In this study, the effect of steam explosion (SE) on the fractionation of EFB was evaluated through the application of a full experimental design (3^2), using temperature and reaction time as two main operational-variables. Changes in the mass composition of cellulose, hemicelluloses and acid insoluble lignin in solid fraction after SE were characterized and the liquid fraction was analyzed for the concentration of fermentable monomeric sugars and the generation of organic acids and/or inhibitory compounds. The effect of SE on EFB fiber was measured by the digestibility percentage, while the chemical modification of EFB was analyzed by Fourier Transform Infrared spectroscopy (FTIR) and the structure modification of EFB fibers was studied by scanning electron microscopy (SEM).

2. Methods

2.1. Raw material

Oil palm empty fruit bunches (OPEFB) were obtained from Biopalma Vale factory, located in Mojú, Pará, state of Brazil. The OPEFB was dried in a cross flow stove at 65 °C for 72 h and stored in polyurethane bags at room temperature to avoid biological degradation.

2.2. Characterization of the EFB pretreatment fractions

The composition of the raw EFB and the solid fractions obtained after SE pretreatment, was determined according to the NREL analytical procedures reported by Sluiter et al. (2011). The cellulose (Cel) and hemicellulose (Hem) mass composition were calculated using the equations reported by Tan et al. (2013), in which the concentration of glucose (Gl), xylose (Xyl) and arabinose (Ara) are correlated. Analysis of acid hyrolysate was carried out by High-Performance Liquid Chromatography (HPLC), in a Shimadzu Chromatograph equipped with an Aminex HPX-87H column, working at 60 °C with sulfuric acid (5 mmol L^{-1}) as mobile phase at flow rate of 6 mL min^{-1}.

Furfural (F), hydroxymethyl furfural (HMF), acetic, formic and levulinic acid were determined in the pretreatment hydrolyzates using a Shimadzu Chromatograph equipped with an Aminex HPX-87H and C18 columns at 60 °C. Mobile phase used was sulfuric acid (5 mmol L^{-1}) at rate of 6 mL min^{-1} with an IR detector. Detection was carried out by differential refractometry and the quantification was based on external calibration as described by Scholl et al. (2015a).

The acid soluble lignin (ASL) and acid insoluble lignin (AIL) were determined as suggested in the NREL procedure (Sluiter et al., 2011). The acid soluble lignin was measured by UV spectroscopy at 280 nm using a SP – 2000 UV spectrophotometer with dilution factor of 10, while the acid insoluble lignin corresponded to the ash free residue that was obtained after sulfuric acid hydrolysis of plant polysaccharides.

2.3. Ash determination

The ash determination was carried out following the procedure reported by Sluiter et al. (2008) with little modifications. Briefly, 0.3 g of dry EFB were placed into a crucible and the samples were calcined at 555 °C for 6 h.

2.4. Protein determination

The quantitative determination of soluble proteins was performed using the protocol reported by Hames et al. (2008).

2.5. Extractives in ethanol and water

The determination of extractives components was carried out according to the NREL procedure reported by Sluiter et al. (2005). All experiments were done in triplicate.

2.6. Steam explosion pretreatment (SE)

The SE pretreatment was carried out in a stainless steel reactor with a 10 L capacity. The process flow diagram is presented at Fig. 1. Pretreatment was performed with 300 g of almost dried EFB, containing 2.0% ± 0.7% moisture. It has been demonstrated that humidity has no influence on the recovery of solid and liquid fraction obtained after SE (Pitarelo et al., 2012).

The material was introduced in the reactor vessel and saturated steam was fed until the desired temperature was reached, the heating time in all cases was around 2 min. The reaction time was controlled after the temperature was reached. The sudden decompression released the material into a cyclone and the vapor was liberated to the atmosphere. The pretreated material was washed five times with water (1 L) and solids were recovered by centrifugation. The first two washings were collected for analysis. The solids were oven dried and a fraction was milled for carbohydrate and lignin analyses.

2.7. Screening of pretreatment conditions

The influence of temperature and time on EFB pretreatment was evaluated initially at 160 °C and 212 °C for a fixed reaction time of 8 min. The EFB fibers derived from pretreatment were characterized in terms of cellulose, hemicellulose and lignin. The morphological changes were followed by SEM, with the aim to check the influence of the severity of different pretreatments on EFB.

2.8. Experimental design and statistical analysis

From the screening study, a full factorial design was implemented, with three level and two operational variables (3^2) temperature and reaction time, with three replicates at the center point to evaluate the system error. The recovery of solids and their mass composition in terms of cellulose, hemicellulose and acid insoluble lignin were expressed as response variables. The resulting values and statistical analysis were processed using Statistica Version 7.0 (Minneapolis, USA). Analysis of variance (ANOVA) was employed to determine statistical significance of the model. The experimental response obtained was analyzed with a second-order polynomial as is presented in Eq. (1). The summary of experimental design is presented in Table 2.

$$y = \beta_o + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{i<j \neq 1}^{n} \beta_{ij} X_i X_j \quad (1)$$

where y is the response (cellulose, hemicellulose and acid insoluble lignin in fiber,%); Xi and Xj are the independent variables and β_o, β_i, β_{ii}, β_{ij} are the intercept, linear, quadratic and interaction coefficients, respectively.

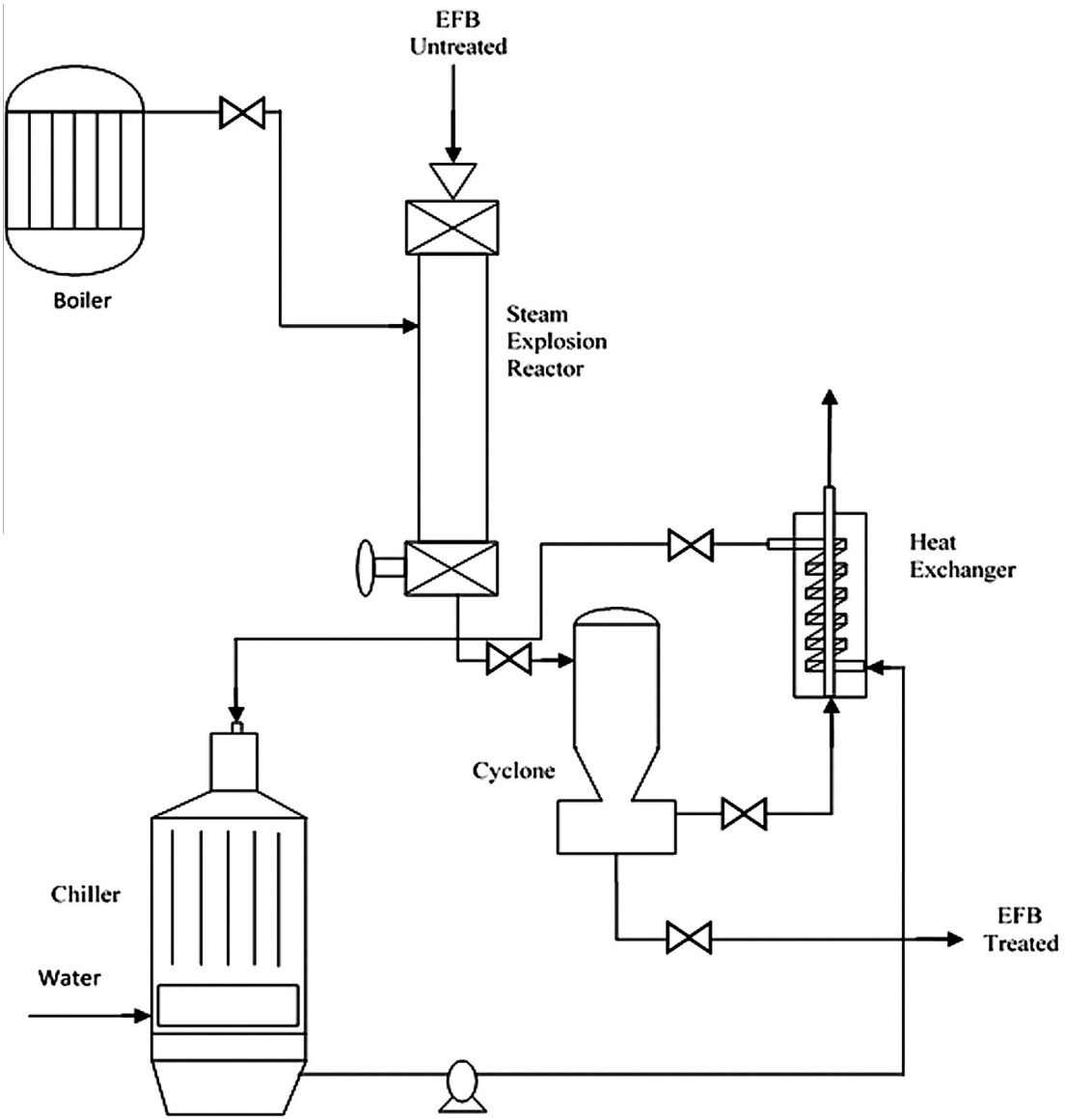

Fig. 1. Flow diagram of steam explosion process done on EFB at different temperature and reaction time.

The severity factor index for each condition of temperature and time, was computed using the equation reported by Overend et al. (1987), which is presented in Eq. (2).

$$R_o = \int_0^t \exp\left(\frac{T-100}{14.75}\right) \quad (2)$$

The liquid fraction of each run was analyzed in terms of glucose, xylose, arabinose, furfural, hydroxymethyl furfural, acetic, formic and levulinic acid contents, with the aim to establish the possible uses of these pretreatment hydrolyzates in a biorefinery concept.

2.9. Enzymatic hydrolysis of EFB treated

The efficiency of steam explosion was measured by the digestibility of EFB treated in enzymatic hydrolysis. The sacharification was developed in accordance with the LAP of NREL (Selig et al., 2008). The untreated and treated EFB at temperature of 175, 185 and 195 °C maintaining for 6 min were used. The commercial enzymes used were Celluclast 1.5L, produced by *Trichoderma reesei* and Novozymes 188 produced by *Aspergillus niger*. The enzyme sample used for activity measurements contained a 1:0.30 mass ratio of Celluclast 1.5L and Novozymes 188, respectively as reported by Silveira et al. (2014). The activity of the enzyme loaded was 10 FPU per g of cellulose added following the procedure reported by Ghose (1987). The reaction mixture was analyzed for the quantity of glucose released in 120 h of reaction by dinitro salicylic acid (DNS) assay. The digestibility was determined using the equation reported by Selig et al. (2008). All experiments were carried out using three replicates.

2.10. Fourier transformed infrared (FTIR) Spectroscopy

The FTIR analyses of the samples were carried out using a Varian equipment with a resolution of 4 cm^{-1} and 32 scans per minute. The biomass pellets were obtained by mixing and pressing it with KBr at 0.1% of biomass. The range of wave numbers was set from 400 cm^{-1} to 4000 cm^{-1}.

2.11. Scanning electron microscopy (SEM)

The morphology of treated EFB was monitored by SEM, under nitrogen atmosphere in a microscopy TESCAN VEGA 3 LMU, with

an acceleration voltage of 15 kV. The resolution of 3 nm, and the image magnification was 2500X. The samples were vacuum dried, coated/metallized with gold in a sputter coater and dried overnight before analysis.

3. Results and discussion

3.1. Biomass composition

The biomass composition of EFB on dry basis before steam explosion pretreatment was as following: ethanol extractives 4.79 ± 1.33%, water extractives 5.97 ± 1.55%, lipids 6.25 ± 1.11%, proteins 3.36 ± 1.23%, cellulose 28.00 ± 0.07%, hemicellulose 24.12 ± 0.07%, acid insoluble lignin (AIL) 17.84 ± 1.64%, acid soluble lignin (ASL) 2.12 ± 0.0%, acetyl group 5.20 ± 0.98% ash 3.19 ± 0.07%.

Based on the characterization done, correlating the glucose, xylose and arabinose as cellulose and hemicellulose, the total polysaccharide content present in EFB was 52.12 wt%, being glucose and xylose the main constituents of cellulose and hemicellulose, respectively. The total lignin fraction was 19.96 wt% with acid insoluble lignin being its main component.

3.2. Screening of pretreatment conditions

The influence of SE on EFB was analyzed at two distant severity factors (log R_o). The results have been summarized in Table 1. The recovery of solids decreased drastically with increasing pretreatment severity. However, the mass percentage of cellulose in the pretreated EFB fibers increased by at least 12% at 212 °C. The hydrolysis of hemicellulose was directly proportional to the increase in pretreatment temperature. At a severity of 4.2, 86% of the original hemicellulose was solubilized, generating EFB fiber residues with mass composition of only 3.93% of hemicelluloses. The results obtained in this work from the screening activity were compared with those reported by Baharuddin et al. (2013) for steam pretreatment at 230 °C and 10 min, the percentage of mass loss was 3.8% lower, whereas the increase in cellulose mass composition was 6% lower. These results must be considered important for a biorefinery pretreatment step, because fibers that are rich in cellulose and low in hemicellulose content are attractive for their subsequent biotransformation, in value added chemicals such as ethanol, butanol and organic acids.

On the basis of changes in the mass composition and the fiber morphology of the EFB after SE (See Supplementary data S1), a full factorial design was built with the aim of finding the ideal conditions to produce EFB fibers rich in cellulose, low in hemicellulose, with a higher cell wall porosity and with a noticeable reductions in their crystallinity.

Table 1
Mass balance of screening of SE on EFB fibers.

Operational conditions	Time: 8 min	
	Temperature: 160 °C R_o: 2.7	Temperature: 212 °C R_o: 4.2
Initial mass (g)	300.28	302.4
Final mass (g)	213.74	172.61
Solid recuperation (%)	71.25	57.08
Mass composition of EFB[a]		
Cellulose (%)	30.67 ± 2.19	40.42 ± 1.0
Hemicellulose (%)	24.66 ± 1.82	3.93 ± 0.9
Acid soluble lignin (%)	2.26 ± 0.04	1.46 ± 0.04
Acid insoluble lignin (%)	27.05 ± 3	46.84 ± 0.9

[a] Composition on dry basis.

3.3. Steam explosion experiments

The full experimental design and their respective global mass balance in both solid and liquid pretreatment fractions are presented at Table 2. The solid recovery decreased with increased temperature and time, being greater the effect of temperature than that of time, while the mass in hydrolyzate increased with temperature and time. These results are important from the industrial view point, because best treatment condition must provide a good relationship between the solid recovery and the cellulose content available in the pretreated biomass.

The mass composition of EFB after each run of SE was carried out in terms of cellulose, hemicellulose, acid soluble lignin (ASL) and insoluble lignin (AIL) as described in Section 2.3, the results are summarized in Table 3. The mass composition of cellulose in EFB peaked at 34.89 wt% at 195 °C for 6 min but higher severities led to substrate left with lower cellulose content. This is due to the progressive hydrolysis of plant polysaccharide to monomeric sugars and to the subsequent production of degradation compounds such as furfural, hydroxymethyl furfural, levulinic, acetic and formic acid.

The mass composition of hemicellulose in EFB decreased gradually with an increase in pretreatment severity, which was a desired effect in this pretreatment study. By contrast, the mass composition of AIL in steam treated substrates increased with pretreatment severity, mostly due to hemicellulose removal by acid hydrolysis. However the occurrence of condensation reactions involving lignin and other materials, such as tannins, furans and

Table 2
Experimental design and global mass balance[a] of SE in each experimental run.

Run	Temperature (°C)		Time (min)		Severity (R_o)	Initial mass (g)	Final mass (g)	Volume of filtrate (L)[b]	Mass in hydrolyzate[c] (g)	EFB Solid recovery (%)
	Real value	Code value	Real value	Code value						
1	175.00	−1	6.00	−1	2.99	302.60	241.30	3.45	8.24	79.74
2	175.00	−1	8.00	0	3.12	302.04	236.86	3.03	7.69	78.42
3	175.00	−1	10.00	1	3.22	302.55	235.25	3.14	8.48	77.75
4	185.00	0	6.00	−1	3.29	303.21	255.55	3.15	8.51	74.39
5	185.00	0	8.00	0	3.41	300.64	214.32	3.38	10.88	71.29
6	185.00	0	10.00	1	3.51	302.32	207.36	3.33	7.02	68.56
7	195.00	1	6.00	−1	3.58	302.35	200.23	3.07	13.80	66.22
8	195.00	1	8.00	0	3.71	300.18	194.16	3.3	13.66	64.68
9	195.00	1	10.00	1	3.81	304.29	195.46	3.84	18.68	64.23
10	185.00	0	8.00	0	3.41	303.90	221.25	3.11	9.87	72.80
11	185.00	0	8.00	0	3.41	300.17	211.59	3.08	10.53	70.49

[a] Bass balance determined on dry basis.
[b] Volume obtained as the sum of the first two washes.
[c] Mass corresponding to the sum of all compounds present in hydrolyzate fraction.

Table 3
EFB characterization after steam explosion – composition on dry weight basis.

Run	Cellulose (%)	Hemicellulose (%)	ASL (%)	AIL (%)
1	23.50	13.53	0.39	33.23
2	25.10	9.05	0.40	30.06
3	23.46	8.25	0.39	35.10
4	32.45	10.05	0.36	32.33
5	33.13	5.67	0.34	37.48
6	31.47	6.49	0.27	40.69
7	34.89	7.69	0.38	35.32
8	24.58	1.75	0.29	44.67
9	25.44	1.86	0.30	39.45
10	32.85	5.81	0.33	37.86
11	33.01	5.18	0.31	37.98

phenolic compounds cannot be ruled out as already reported by other authors (Haghighi Mood et al., 2013; Sun et al., 2012).

In general, SE caused an increase to 0.35 g of glucans per gram of EFB, which represents 24.6% more cellulose in relation to the untreated EFB. The hemicellulose quantity was reduced to 0.08 g per gram of EFB and this corresponds to 68.11% less hemicellulose compared to found in untreated EFB. These results are more promising than those reported by others using steam explosion for EFB pretreatment (Baharuddin et al., 2013; Shamsudin et al., 2012).

To confirm the effect of each operational variable on each response variable, analysis of variance ANOVA was developed with significance level of $p < 0.05$. The summary of the effects of operational variables on each response, the pure error and the correlation coefficient (R^2) are summarized in Table 4. The mathematical model adequately represented the solid recovery, cellulose and hemicellulose mass percentage in EFB after SE. The lack of fit in acid insoluble lignin would be explained by the increase in their mass composition as discussed above.

The linear effect of both temperature and time had a strong influence on the response of all variables. The quadratic effect of both temperature and time was more remarkable for solid recovery and hemicellulose mass composition. These effects and significance levels were important to generate a mathematical model that was able to describe the SE effect on EFB. The mathematical model to describe the effects of SE process on each response variable are presented in Eqs. (3)–(6) as function of the pretreatment temperature (T) and reaction time (t).

$$SR\ (\%) = 301.03 - 1.69 \cdot T + 2.70 \cdot 10^{-3} \cdot T^2 - 1.66 \cdot t + 0.05 \cdot t^2 \quad (3)$$

$$C\ (\%) = -2412.90 + 25.45 \cdot T - 0.07 \cdot T^2 + 18.65 \cdot t + 0.14 \cdot t^2 - 0.12 \cdot T \cdot t \quad (4)$$

$$H\ (\%) = 90.87 - 0.14 \cdot T + 3.6 \cdot 10^{-4} \cdot T^2 - 9.35 \cdot t + 0.58 \cdot t^2 - 6.91 \cdot 10^{-3} \cdot T \cdot t \quad (5)$$

$$AIL\ (\%) = -215.97 + 2.27 \cdot T - 6.00 \cdot 10^{-3} \cdot T^2 + 1.71 \cdot t - 0.36 \cdot t^2 + 0.02 \cdot T \cdot t \quad (6)$$

where SR, C, H, AIL, are the recovery of pretreatment solids and the cellulose, hemicellulose and acid insoluble lignin mass percentage of steam treated EFB. The temperature (T) and time (t) are given in Celsius degree and minutes, respectively. These equations are important for modeling and simulation of SE, in order to perform a scaling process or to develop a sensibility analysis of the applied biorefinery concept.

The response surfaces of these models are presented in Fig. 2. Fig. 2a shows the effect of increasing the reaction time and temperature on the recovery of solids pretreated, which decreased with the increase of temperature and reaction time. Fig. 2b, shows the response surface for the substrate cellulose content. This figure shows an optimal point, which corresponds to the greatest increase in cellulose content after SE at 195 °C for 6 min. Further increases in pretreatment temperature produced a decrease in the substrate cellulose content, mainly due to hydrolysis and degradation to products as furans, levulinic, formic and acetic acid (Larsson et al., 1999). Fig. 2c, shows the response surface for the substrate hemicellulose content in relation to changes in temperature and reaction time. The increase of both, time and temperature, decreased the mass percent of hemicellulose in EFB progressively, indicating that more severe pretreatment conditions caused a considerable mass loss of plant polysaccharides. Finally, the increase in the acid insoluble lignin (AIL) of EFB fibers is presented in Fig. 2d. This phenomenon is induced by hydrolysis and condensation reactions as discussed above. Therefore, the authors suggest a delignification process after steam explosion to improve the performance of EFB in a biorefinery factory.

3.4. Analysis of the pretreatment liquid hydrolyzate

The first two liquid fractions derived from water-washing of EFB were characterized by HPLC. In general, monomeric sugars were detected only in the first liquid fraction, while the 20% of the total concentration organic acids, as levulinic, formic and acetic acid, furfural, hydroxymethyl furfural (HMF) were recovered in filtrate of the second washing stage. The summary of this qualitative analysis is presented in Table 5. The results are aligned with the progressive mass loss that was observed at higher pretreatment severities. These losses were primarily associated with hemicellulose hydrolysis since the concentration of pentoses such as xylose and arabinose in washings increased together with an increase in temperature and reaction time. These results are in agreement with the pretreatment mass balance of sugarcane bagasse as presented by Rocha et al. (2012) and Scholl et al. (2015b).

The concentration of acetic acid in liquid hydrolyzate increased with the severity, as mainly effect of autocatalytic hydrolysis, together with the deacetylation of hemicellulose and lignin. The highest concentration of 5.2 g L^{-1} of acetic acid were detected at 195 °C for 6 min. These concentration represent the 61.9% of acetyl group present in untreated EFB. The values obtained in this work are similar those reported by Jung et al. (2013) in diluted acid pretreatment of EFB.

The concentration of fermentative inhibitory compounds, furfural and hydroxymethyl furfural (HMF) increased with the severity of pretreatment, the highest concentration were

Table 4
Summary of effects of each factor on each response with a significance level $p < 0.05$.

Response variable	T (L)	T (Q)	t (L)	t (Q)	T (L) by t (L)	P.E.	R^2
S.R.	4.28	6.57	−3.49	−0.55	−4.70	0.02	0.90
Cellulose	−13.59	−0.27	−3.25	−0.21	$-1.7 \cdot 10^{-3}$	1.38	0.97
Hemicellulose	−6.51	0.04	−5.22	−2.33	−0.30	0.11	0.98
Acid soluble lignin	2.27	$-6{\cdot}10^{-3}$	1.71	−0.36	0.03	0.07	0.70

S.R. Solid recovery L: Linear effect Q: Quadratic effect P.E. Pure error

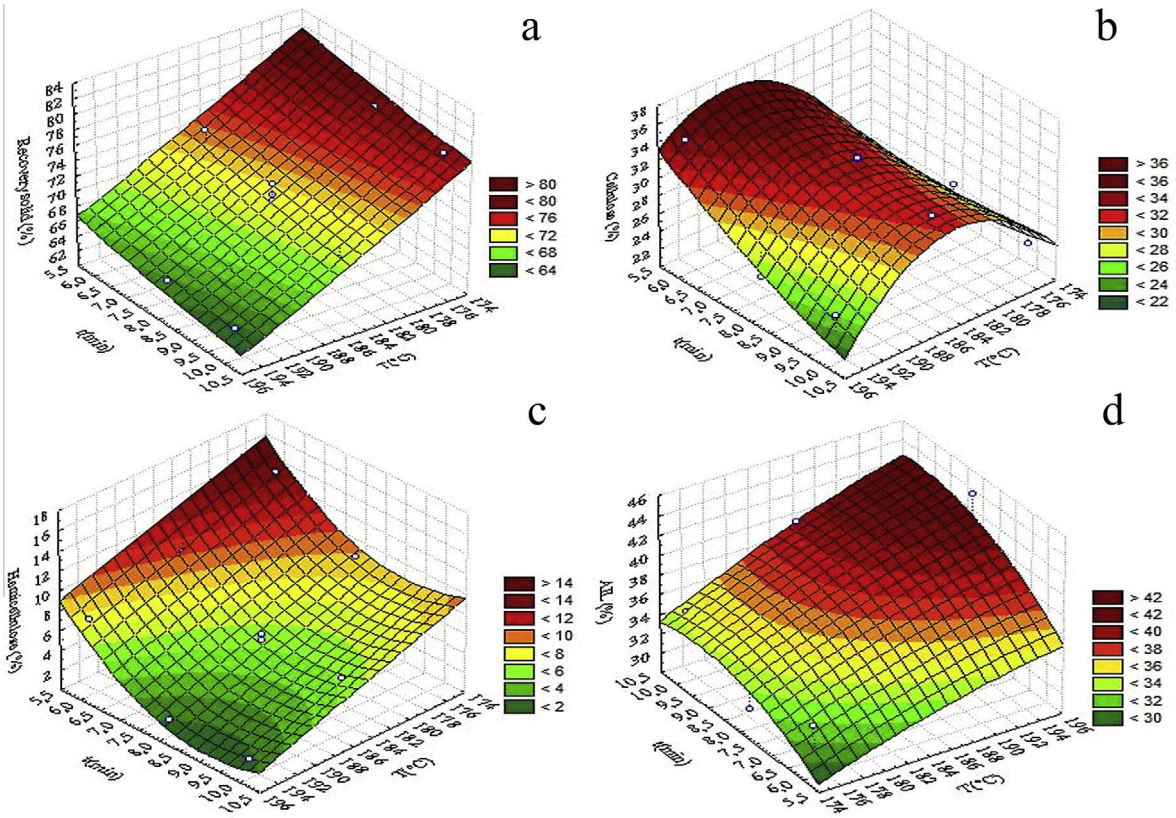

Fig. 2. Surface response of all variables analyzed. (a) Solid recovery response. (b) Cellulose response. (c) Hemicellulose response. (d) Acid insoluble lignin response.

Table 5
HPLC analysis of hydrolyzate from EFB fibers after steam explosion.

Run	Glucose (g/l)	Xylose (g/l)	Arabinose (g/l)	Acetic acid (g/l)	Furfural (g/l)	HMF (g/l)	Formic acid (g/l)	Levulinic acid (g/l)
R. 1.	0.1113	n.d.	0.4319	3.609	0.0089	0.0193	0.2915	n.d.
R. 2.	0.1915	n.d.	0.3943	3.1974	0.0277	0.0542	0.1359	n.d.
R. 3.	0.1255	n.d.	0.4396	3.3935	0.0372	0.0626	0.1456	n.d.
R. 4.	0.0916	n.d.	0.4308	3.6496	0.0897	0.0775	0.1433	0.0007
R. 5.	0.0926	0.1341	0.4419	4.0216	0.1727	0.0867	0.1487	0.0133
R. 6.	0.0852	0.1533	0.3198	2.7696	0.1604	0.0685	0.0809	n.d.
R. 7.	0.1801	0.5948	0.6764	5.219	0.4586	0.1925	0.1934	0.0855
R. 8.	0.1824	0.6339	0.5766	5.0042	0.1862	0.2348	0.2077	0.0898
R. 9.	0.192	0.6986	0.5899	4.9539	0.7122	0.2128	0.1783	0.1084
R. 10.	0.0926	0.1022	0.4884	4.0726	0.1659	0.0965	0.1353	0.0197
R. 11.	0.0934	0.1682	0.5795	4.1744	0.1741	0.0973	0.1244	0.0293

n.d. Not detected.

0.71 g L^{-1} and 0.23 g L^{-1} at 195 °C for 8 and 10 min, respectively. These concentrations are lower than that reported in literature as inhibitory for ethanol fermentation by *Saccharomyces cerevisiae* strain (Almeida et al., 2007; Palmqvist and Hähn-Hägerdal, 2000).

The presence of levulinic and formic acid in liquid hydrolyzate increased with the severity factor of pretreatment, indicating the hydration of the furfural and HMF, with the increase in the severity of treatment. The concentration of formic acid, was almost constant for all conditions of pretreatment, the highest concentration of 0.21 g L^{-1}. On the other hand, the highest concentration of 0.11 g·L^{-1} of levulinic acid was detected at treatment of 195 °C for 10 min. These concentrations are lower than the value commonly reported in biomass hydrolysis (Almeida et al., 2007).

3.5. Enzymatic digestibility of EFB treated by steam explosion

The percentage of digestibility was determined in g of cellulose in untreated EFB and SE-treated EFB at the runs 1, 4, 7 (Table 2). The results are summarized in Table 6. These results revealed that the digestibility was improved with the increase in the severity of pretreatment, 195 °C for 6 min being the best condition with a digestibility of 33.35% of glucans of EFB. The enzymatic hydrolysis of EFB at the same condition, also produced a syrup with 4.18 g L^{-1} of glucose, these results represent almost twice the glucose produced from enzymatic hydrolysis of untreated and treated EFB at 175 and 185 °C. The results obtained in this work are higher than reported by Shamsudin et al. (2012) using lowest enzyme concentration and reaction time.

The percentage of digestibility of untreated EFB was slightly higher that the treated EFB at 175 and 185 °C. These results could be explained by the presence of depolymerized low molecular weight lignin, tannins, saponificable extractives, part of hemicelluloses and other degradation products from EFB fiber surface arisen during the steam explosion pretreatment that can overcome the effect of availability of cellulose in treated EFB, with respect to untreated EFB (Fernandes et al., 2015).

Table 6
Enzymatic digestibility and glucose concentration in untreated and SE-treated EFB.

Condition	Digestibility (%)[a]	Glucose (g L^{-1})
Untreated EFB	19.63 ± 3.77	2.45 ± 0.47
Run 1	14.03 ± 1.05	1.76 ± 0.13
Run 4	15.62 ± 5.53	1.98 ± 0.70
Run 7	33.35 ± 1.40	4.18 ± 0.17

[a] Determined as g of cellulose (glucan) produced by per gram of cellulose in EFB.

3.6. FTIR analysis of the pretreated EFB

FTIR spectroscopy was used to analyze the effect of SE on the chemical structure of EFB (Supplementary data S2). For this, untreated samples of EFB were analyzed and compared with steam-treated materials that were obtained at 175 °C, 195 °C and 212 °C after 8 min of reaction time. The spectra is supplied in Supplementary data S2 and their description is presented below.

With the increase in SE temperature, the bands at 2869 cm^{-1} and 2792 cm^{-1} are reduced. These bands correspond to the C—H stretching vibrations of aliphatic moieties mainly in plant polysaccharides such as cellulose and hemicellulose. A stretching vibration at 1569 cm^{-1} was present in all samples and this was attributed to C=C in lignin structures as already reported by Nieves et al. (2011). The bands at 1461 cm^{-1} and 1415 cm^{-1}, are more remarkable with the increase of severity of pretreatment, these could be due to cellulose as proposed by Wang et al. (2009). The band at 1330 cm^{-1} represents the non-etherified OH in lignin structures specially in syringyl units, this band is modified with the temperature increase, confirming the modification in lignin structure as reported by others (Laurichesse and Avérous, 2013; Mansouri and Salvadó, 2006). The vibration at 1250 cm^{-1} disappeared with the temperature of SE, it was related with ring vibration of C—OH side groups and glycosidic bonds vibration typical of xylans. The band at 1163 cm^{-1} represent the C—O—C stretching in amorphous cellulose, its intensity increased with an increase in SE temperature with the modification of cellulose by SE. Finally the band at 1098 cm^{-1} decreased with the severity of pretreatment, it could be ascribed to β-glycosidic linkages as was mentioned by Alriols et al. (2009).

4. Conclusions

The EFB was treated by autocatalytic steam explosion. The best pretreatment performance was achieved at 195 °C for 6 min, with increase of 24% in cellulose and 68% reduction in hemicellulose. The treated EFB with higher porosity generated an enzymatic digestibility of 33% that produced a hydrolyzate with 4.2 g L^{-1} of glucose. The hydrolysates consisted of glucose, xylose and arabinose as fermentable sugars, with high acetic acid contents. This suggested the possibility of a subsequent separation/transformation process to obtain precursors for value-added products, enhancing the sustainability of EFB biorefineries.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.08.126.

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Paper II

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Lignin preparation from oil palm empty fruit bunches by sequential acid/alkaline treatment – A biorefinery approach

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HIGHLIGHTS

- OPEFB fibers has a good potential as raw material for lignin preparation.
- The lignin obtained present a high industrial potential as precursor of biocomposites.
- The acid pretreatment using 1% (w/w) of H_2SO_4 at 121 °C and 60 min hydrolyzed 80% of hemicellulose.
- The lignin extraction was 68.8% (w/w) of the original lignin in cell walls at 2.5% (w/w) NaOH, 80 min and 121 °C.
- Degradation products and excessive depolymerization by this method were detected using FTIR and NMR.

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ABSTRACT

Lignin is an important raw material for the sustainable biorefineries and also the forerunner of high-value added products, such as biocomposite for chemical, pharmaceutical and cement industries. Oil palm empty fruit bunches (OPEFB) were used for lignin preparation by successive treatment with 1% (w/w) H_2SO_4 at 121 °C for 60 min and 2.5% NaOH at 121 °C for 80 min resulting in the high lignin yield of 28.89%, corresponding to 68.82% of the original lignin. The lignin obtained was characterized by gel permeation chromatography (GPC), Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR). The results indicated a lignin with molecular masses ramping from 4500 kDa to 12,580 kDa. FTIR and NMR of these lignins showed more syringyl and *p*-hydroxyphenyl than guaiacyl units. Moderate acid/alkaline treatment provided lignin with high industrial potential and acid hydrolyzates rich in fermentable sugars and highly porous cellulosic fibers.

1. Introduction

Oil palm (*Elaeis guineensis*) is a dominant agricultural crop in many countries, Malaysia and Indonesia being the two largest growers and producers of oil palm. They altogether account for roughly 85% of the world oil palm production representing 53,500 million tonnes (Mohammed et al., 2012; Singh et al., 2013; Tan et al., 2013). In South America, Brazil is the third largest producer after Colombia and Ecuador, producing 340 million tonnes, representing 0.58% of the worldwide oil palm production (data from: 'www.indexmundi.com', 2015 accessed: 13-04-2015). However, Brazil has a potential area to cultivate oil palm in roughly 70 million of hectares, which may become one of the largest producer.

In tandem with oil production, the solid by-product oil palm empty fruit bunches (OPEFB) are generated. OPEFB are the fibrous mass left behind after separating the fruits from sterilized (steam treatment at 294 kPa for 1 h) fresh fruit bunches (FFB) (Shinoj et al., 2011). It is estimated that for each tonne of oil palm produced, about 1.1 tonnes of OPEFB are generated. In 2011, the world production was 14.5 million tonnes (dry basis), half of it being produced in Indonesia (Purwandari et al., 2013).

The OPEFB has been used traditionally as source of heat and power in incinerators of palm oil mills, causing environmental pollution (Cotana et al., 2014). Nevertheless, OPEFB can serve as a renewable source of sugar and lignin, available in large amounts and at relatively low costs for chemical and fuels, through the biorefinery approach (Wildschut et al., 2013).

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In a biorefinery, the lignin can be converted to high-value-added chemicals such as, resin precursors, antioxidants, antimicrobial agents, aromatics compounds, synthetics alcohols, Fisher–Tropsch liquid fuel, syngas, and molecules of high and low molecular weight, which can be profitable. Lignin is considered of utmost importance to achieve sustainable economy and to reduce carbon footprint (Azadi et al., 2013; Chew and Bhatia, 2008).

The extraction of lignin can be done using various methods such as, organosolv, acid-isolation, alkaline-isolation, Kraft process and ionic liquids. Nonetheless, these separation methods are generally expensive and/or require extreme temperature and pressure, which are not easy to apply at industrial scale production (except Kraft carried out in pulp and paper from wood). Therefore, an efficient and inexpensive method is necessary for extraction of lignin from lignocellulosic biomass.

In this work, the lignin extraction from oil palm empty fruit bunches was carried out through sequential acid/alkaline treatment. The acid pretreatment was carried out using a fixed concentration of H_2SO_4, whereas, the alkaline lignin extraction was performed by varying NaOH concentration and time of reaction. The black liquor resulting from alkaline treatment of OPEFB was processed lowering the pH to obtain lignin. The mass yield of lignin extraction was designed as the variable to determine the best conditions of lignin extraction from OPEFB. The chemical quality of lignin extracted was analyzed by comprehensive set of analytical methods, including gas permeation chromatography (GPC), Fourier transform infrared spectroscopy (FTIR), ^1H and ^{13}C nuclear magnetic resonance (NMR). The morphology of OPEFB fibers after each pretreatment was studied by scanning electron microscopy (SEM).

2. Lignin structure

The exact structure of lignin remains virtually unknown, however it is known and acceptable that basic chemical structure is composed of phenylpropane units, originating from three aromatic alcohol precursors (monolignols), *p*-coumaryl, coniferyl and sinapyl alcohols (Lin and Dence, 2007). These alcohols are converted to *p*-hydroxyphenyl, guaiacyl and syringyl respectively by enzymatic reactions. The presence of each monomer varies in every sort of biomass, for example, gymnosperm lignin is formed mostly by guaiacyl, angiosperm lignin by guaiacyl and syringyl units, while grass lignin is composed of guaiacyl, syringyl and *p*-hydroxyphenyl units. The polymerization of these monomers is catalyzed by laccase and/or peroxidase via radical coupling of their corresponding phenoxy radicals, forming the aliphatic and aryl ether linkages at C-α and C-γ, ester linkages at C-γ and β-O-C(4), resulting in a complex tree dimensional polymeric structure, being the two major ether bonds the β-O-C(4) and α-O-C(4) (Cyril et al., 2010).

3. Methods

3.1. Lignocellulosic biomass

Oil palm empty fruit bunches (OPEFB) were obtained from Biopalm Vale factory, located in Mojú, Pará state of Brazil. The OPEFB was dried in a cross flow stove at 65 °C by for 48 h, milled in mill knives and sieved, through a mesh 42 (0.350 mm) screen and used for all experiments. All experiments were carried out in triplicates.

3.2. Sequential acid/alkaline pretreatment for lignin extraction

Acid hydrolysis treatment was carried out according to the procedure reported by Minu et al. (2012). Briefly, about 6 g of dry OPEFB fibers were weighed into a flask with 53.4 ml of distilled water, and 452.9 μl (72% v/v) sulfuric acid was added as catalyst. This volume ratio represents in terms of mass percentage: 10%, 89% and 1% (w/w) of OPEFB, water and sulfuric acid, respectively. The temperature of autoclave was fixed at 121 °C and reaction time was 60 min.

The acid treated OPEFB was washed three times with distilled water and dried overnight at 80 °C. Later, alkaline lignin extraction was carried out. In a flask, 3 g of dry OPEFB were mixed with different volumes of 5 M NaOH and distilled water, obtaining solutions of 10% (w/w) OPEFB, 0.5% (w/w) to 5.5% (w/w) of NaOH and the volume made up by distilled water. The mixture was incubated at 121 °C in an autoclave, with the aim to analyze the effect of NaOH on lignin extraction. The reaction time was fixed at 60 min.

The yield of lignin extraction was determined as a function of NaOH mass percentage in solution. The global mass yield of lignin extraction (G_{Y_l}) and the specific lignin yield extraction (E_{Y_l}) are presented in Eqs. (1) and (2).

$$G_{Y_l} = \frac{M_l}{M_t} 100 \tag{1}$$

$$E_{Y_l} = \frac{M_l}{M_t^l} 100 \tag{2}$$

where M_l is the mass of lignin obtained, M_t is the total mass of OPEFB, M_t^l is the mass of acid insoluble lignin present in OPEFB after acid treatment.

The best condition, in terms of NaOH mass percentage in solution, was determined at the point in which the highest yield was achieved. This condition was selected to analyze the effect of reaction time, varying from 40 min to 120 min, while the temperature was maintained at 121 °C.

The precipitation of lignin was conducted overnight using (72% v/v) H_2SO_4 at pH 2.0. At higher pH values, precipitation was not detected, as reported by Minu et al. (2012). Whatman No. 4 filter paper was employed for vacuum filtration. The filtered liquid was analyzed for cellulose, hemicellulose and acid soluble lignin, as described in the Section 3.3.

The solid fraction so obtained, was washed three times with acid water for removal of carbohydrates and impurities. The excess acid was washed three times with hot water at 40 °C for 30 min. Finally, the solid lignin was vacuum dried for 8 h at 35 °C and stored at room temperature in a flask covered with foil.

3.3. Analysis of carbohydrates and total lignin

The composition of polysaccharides was determined using the NREL analytical procedure reported by Sluiter et al. (2011). The cellulose (Cel) and hemicellulose (Hem) mass composition were obtained using the equations reported by Tan et al. (2013), correlating the concentration of glucose (Gluc), xylose (Xyl) and arabinose (Arab), acquired from High-Performance Liquid Chromatography (HPLC), in a Shimadzu Chromatograph equipped with an Aminex HPX-87H column, working in a furnace at 60 °C. Mobile phase was sulfuric acid (5 mM) at rate of 6 ml min^{-1} with an IR detector.

The acid soluble lignin (ASL) and acid insoluble lignin (AIL) were determined following NREL procedure (Sluiter et al., 2011). The acid soluble lignin was measured by absorbance of liquid hydrolyzate at 280 nm with dilution factor of 10, while the acid insoluble lignin is defined as the Klason lignin ash free.

3.4. Gel permeation chromatography (GPC)

The determination of molecular weight and polydispersity of extracted fraction lignin was carried out by GPC analysis in a

Waters model 1515 liquid chromatograph. The samples were solubilized in tetrahydrofuran (THF 2.0 mg/ml) and filtered through a Teflon membrane with a pore size of 0.45 μm. Analyses were done using a series of one TSK-L guard column and two TSK GEL (G2000 HXL and G1000 HXL) columns at 45 °C, with exclusion limits ranging from $4{\cdot}10^3$ to 436 MM units. THF was used as elution solvent at a flow rate of 1 ml/min and the column eluent were monitored by refractive index and UV/VIS spectroscopy at 280 nm (Waters models 2414 and 2487, respectively).

Calibration curve was generated from the elution profile of eleven polystyrene standards. A typical dispersion of 1–2% was observed in the bimodal calibration curve as a result of quadratic fitting. Universal calibration was performed using coefficients available in the literature. The degree of polymerization (DP), the number average (M_n) and the mass average (M_w) molecular mass, were determined directly with the assistance of Waters HPLC software (Breeze).

3.5. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was carried out using BOMEM-Hartmann and Braun MB-series equipment with resolution of 4 cm^{-1}, 32 scan per minute and transmittance technique. The pellets of lignin were obtained by mixing and pressing lignin and KBr at 0.1% (w/w) of lignin. The range of wavenumber was set from 400 cm^{-1} to 4000 cm^{-1}. The data obtained were analyzed using the ACD/NMR processor free academic version.

3.6. Nuclear magnetic resonance (NMR)

The ^{13}C NMR spectrum was recorded in solution, using a Bruker spectrometer at 400 MHz, the preparation of samples is described below. About 80 mg of lignin was dissolved in 0.5 ml of DMSO-d_6. The chemical shift for ^{13}C NMR was calibrated with reference to DMSO-d_6, standard peak at 39.51 ppm. The spectrum was recorded for all the samples keeping the following parameters constant: acquisition time of 0.68 s, frequency 100.6 MHz, receiver gain 182.08, sweep width 24038.09 Hz and temperature 30 °C.

^1H NMR was conducted in solution in the same equipment as described earlier. The mass of dissolved lignin was 20 mg in 0.5 ml of DMSO-d_6. The chemical shifts of ^1H NMR spectra were calibrated with reference to DMSO-d_6, standard peak at 2.50 ppm; Acquisition time 5.12 s, frequency 600 MHz, receiver gain 17.78, sweep width 6393.76 Hz and temperature 30 °C.

3.7. Scanning electron microscopy (SEM)

The morphology of lignin and OPEFB fibers was monitored by SEM, in a microscopy TESCAN VEGA 3 LMU, with acceleration voltage of 15 kV and resolution of 3 nm, under nitrogen atmosphere. The samples were vacuum dried and coated/metallized with gold in a sputter coater and dried overnight. The image zoom was 150×, 350× and 2500×, however, in this study, only 350× image is analyzed, because this resolution permitted simultaneous analysis of all micrographs, keeping details of fiber modifications by pretreatment intact.

4. Results and discussion

4.1. Sequential acid/alkaline pretreatment for lignin extraction

The mass balance of acid treatment is presented in Table 1. As seen from Table 1, the acid treatment had a significant effect on hemicellulose hydrolysis, where almost 80% (w/w) was hydrolyzed. Acid treated OPEFB resulted in more than 90% (w/w) of initial acid insoluble, being a measure of the selectivity of acid hydrolysis treatment to hemicellulose hydrolyzation. The yield of recovery of OPEFB fibers was 69.83% (w/w), whereas, the cellulose hydrolyzation was only 3% (w/w). The poor hydrolysis of cellulose was assumed because of the cleavage of glycosidic intra-bonds, and the inter-bonds between cellulose, hemicellulose and lignin as reported by other authors (Ferrer et al., 2013).

The mass balance of each alkaline lignin extraction is presented at Table 2. In the liquid fraction from alkaline treatment, polysaccharides were not detected, probably their concentration in solution was lower than the detection limit.

The cellulose in OPEFB fibers increased with the increase of NaOH in solution from 27.21% (w/w) to 54.52% (w/w), however it also improved the loss of OPEFB fibers, swaying it from 22% (w/w) to 65% (w/w). The hemicellulose mass percentage was slightly constant from 7% (w/w) to 8% (w/w). The tendency to obtain constant hemicellulose percent in fibers after alkaline lignin extraction was also observed by Cara et al. (2006).

The acid soluble lignin remained constant at 0.3% for the entire NaOH percent analyzed, whereas the acid insoluble lignin decreased gradually with the increase of NaOH, it is a measure of the efficiency of the process. The solubilization of acid insoluble lignin is caused by the formation of alkali soluble fractions, created by the cleavage of C(4)-O-β and C(4)-O-α linkages and by demethoxilation of lignin (Cyril et al., 2010).

In the Fig. 1a are summarizes the results obtained in terms of global and specific yield. The standard deviation of G_{Y_l} and E_{Y_l} varying from 0.77 to 1.33, which were not reported.

Lignin yield increased simultaneously with NaOH percentage, reaching 2.5% (w/w) of NaOH, the highest global of 28.8%, which represents 68.8% of original lignin in cell wall. This is equivalent to 297.3 μg of lignin per gram of OPEFB treated. These results were higher than the values obtained by Guo et al. (2013) and Velmurugan and Muthukumar (2012), during delignification of steam-exploded cornstalk and ultrasound-assisted alkaline pretreatment of sugarcane bagasse. An increase of NaOH percentage in solution induced decrease in lignin yield. This was probably due to two factors; the formation of low molecular weight fragments, besides, the formation of acid soluble complex between lignin and hemicellulose, caused by the precipitation with sulfuric acid, forming lignosulfonate fragments with $pK_a < 2$ (Sun et al., 1999).

To analyze the yield of lignin extraction as a function of the time, the mass percentage of 2.5% (w/w) NaOH was selected, keeping the temperature at 121 °C. The reaction time was varied from 40 min to 120 min. The results are presented in Fig. 1b.

As seen in Fig. 1b, global yield of lignin production increased with the reaction time, attaining 28.89% (w/w) of global lignin yield at 80 min. From this point, the yield slightly increased by 0.42% and was almost constant. This result was similar to those obtained for pulp and paper process under bulk conditions, but

Table 1
Mass balance of acid pre-treatment on OPEFB.[a]

Component	Raw material (w/w)	Solid fraction (w/w)	Liquid fraction (w/w)	Percentage remaining in the fiber (w/w)
Cel (%)	30.5 ± 0.39	27.21 ± 4.019	0.49 ± 0.06	62.20
Hem (%)	19.5 ± 0.25	5.25 ± 0.71	13.24 ± 1.75	18.5
ASL (%)	1.17 ± 0.03	0.48 ± 0.01	0.04 ± 1^{-3}	34.40
AIL (%)	32.17 ± 0.93	43.19 ± 0.03		93.86
Mass (g)	6 ± 0.01	4.19 ± 0.04	1.81[b]	69.83

[a] The composition is not 100%, because were not characterized the others polysaccharides and the extractable compounds.
[b] Determined by mass balance.

Table 2
Mass balance of alkaline lignin extraction from OPEFB fibers.

Component	Initial	Final					
		NaOH 0.5%	NaOH 1.5%	NaOH 2.5%	NaOH 3.5%	NaOH 4.5%	NaOH 5.5%
Cel (% w/w)	27.21 ± 4.09	33.10 ± 1.14	43.40 ± 1.99	47.46 ± 1.22	52.96 ± 3.83	51.54 ± 1.41	54.52 ± 0.44
Hem (% w/w)	5.25 ± 0.71	8.52 ± 0.20	7.83 ± 0.72	7.4 ± 0.38	7.77 ± 3.4·10^{-3}	6.23 ± 0.21	6.93 ± 0.75
ASL (% w/w)	0.48 ± 1·10^{-3}	0.30 ± 0.04	0.23 ± 0.01	0.25 ± 0.014	0.28 ± 0.01	0.24 ± 8·10^{-3}	0.28 ± 0.02
AIL (% w/w)	43.19 ± 0.03	10.86 ± 1.9	8.38 ± 0.34	8.90 ± 1.43	9.14 ± 0.97	5.75 ± 0.34	7.22 ± 1.36
Total OPEFB mass (g)	3.00	2.34	1.53	1.13	1.22	0.99	1.05
EFB yield (%)		78	51	37.67	40.67	33.00	35.00
Polysaccharides mass (g)	2.28	1.17	0.80	0.72	0.86	0.63	0.72

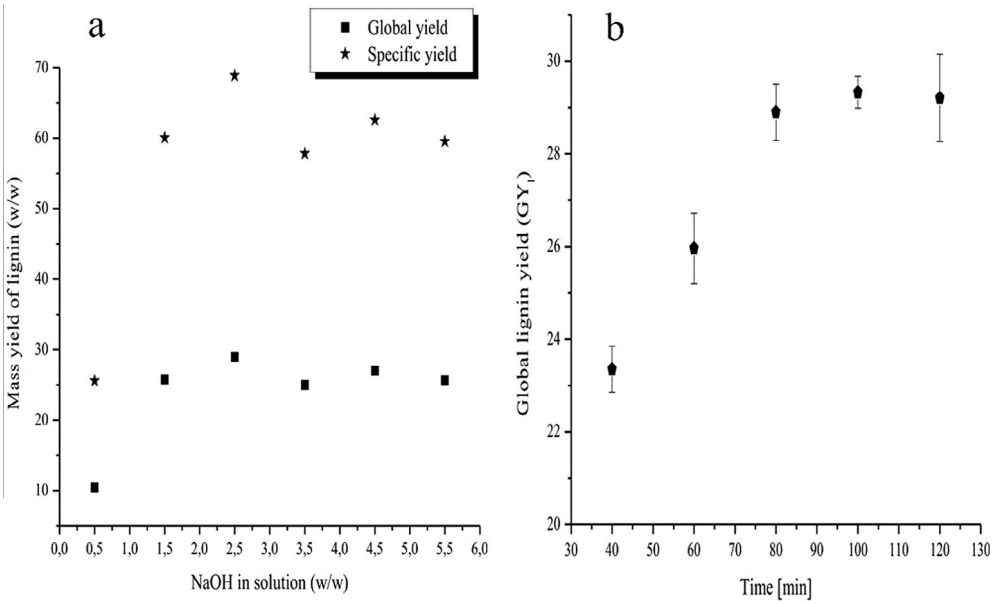

Fig. 1. (a) Yield of lignin extraction in function of NaOH percent in solution at 120 °C and 80 min. (b) Global yield of lignin extraction in function of time at 120 °C and NaOH 2.5% (w/w).

by employing milder conditions, its mean, 50 °C, 77% and 10% less of temperature, reaction time and NaOH mass percentage, respectively.

4.2. Determination of molecular weight of lignin

The molecular weight was analyzed as a function of NaOH percentage increase. The lignin fractions obtained at 0.5%, 2.5% and 5.5% of NaOH (w/w) were chosen. Henceforth, the fractions were called L_1, L_2 and L_3, respectively. The molecular weight (M_w), molecular number (M_n) and polydispersity (M_w/M_n) of these fractions are presented in Table 3.

L_1 presents the lowest average and number molecular weight and highest polydispersity, probably due to presence of several molecules with low and high molecular weight, with larger presence of small molecules. L_2 presents greater values for polydispersity and number and average molecular weight. These results are in line with the yield of lignin extraction, suggesting the presence of large molecules in greater proportion than for other lignins (L_1 and L_3). This result would be an indicator of cleavage of bonds β-O-C(4) and ester bonds of acetyl and coumaryl in lignin structure (Hage et al., 2009). The average molecular weight of L_3 and number molecular weight decreased with respect to L_2, which meant that probably at 5.5% (w/w) NaOH, lignin suffered excessive oxidation, forming degradation products and sulfonated molecules that were acid soluble, reducing the number and average molecular weight.

Table 3
Molecular weight and polydispersity of the lignin L_1, L_2 and L_3.

	L_1	L_2	L_3
M_n (kDa)	4368	12,580	12,013
M_w (kDa)	9702	19,037	16,756
DP ($M_w M_n^{-1}$)	2.22	1.51	1.38

L_1, L_2, L_3: Lignin obtained at 0.5%, 2.5% and 5.5% (w/w) of NaOH at 121 °C and 80 min.

4.3. FTIR analysis

FTIR analysis was based on earlier reported studies (Cyril et al., 2010; Lin and Dence, 2007). All assignments of the bands are reported in Table 4. Although there are many publications on FTIR analysis of biomass, this work cited lignocellulosic material that are not food competitive, which is the focus of this work. The FTIR spectra are presented in Supplementary data (Fig. S1).

The absorption bands nearly to 3400 cm^{-1} are assigned to OH group in aliphatic and aromatic structures. In the neighborhood of 3400 cm^{-1}, couple of peaks between 2920 cm^{-1} and 2850 cm^{-1} are common for all samples, representing the characteristic of C—H bonds of CH_2 and CH_3 of propyl side chain (Sahoo et al., 2011). The bands from 1450 cm^{-1} to 700 cm^{-1} correspond to the bonds of C—C and C—H in aromatic structures. The presence of syringyl units was detected by peaks from 1100 cm^{-1} to 1260 cm^{-1} (intensity of the peak due to oxygen electronegativity).

Table 4
Assignment of FTIR spectra of lignin from OPEFB alkaline pre-treated.

L_1	L_2	L_3	Description	References
Wavenumber (cm^{-1})				
3361.6	3301.1	3396.3	Aromatics and aliphatics OH	Sun et al. (1999)
2921.9	2923.8	2923.8	C—H in CH_2 and CH_3 and lignin interlinkages	Sahoo et al. (2011)
2852.5	2852.5	2852.5	C—H in CH_2 and CH_3 and lignin interlinkages	Sahoo et al. (2011)
1710.7	1708.8	1710.7	Carbonyl groups C=O unconjugated	Sun et al. (1999)
1654.8	1652.8		C=O in α and γ Carbon position	Minu et al. (2012)
		1596.9	Peaks for aromaticity	Sahoo et al. (2011)
1519.9	1508.2	1512	C—C aromatic skeleton	García et al. (2009)
1458	1461.9	1461.9	δ asymmetric C—H in CH_3 and CH_2	García et al. (2009)
1419.5	1423.3	1423.3	C—H deformation in guaiacyl	Minu et al. (2012)
1377	1363.5	1367.4	Syringyl and guaiacyl	García et al. (2009)
	1326.9	1326.9	Syringyl and guaiacyl	García et al. (2009)
	1269	1269	Syringyl and guaiacyl C—O bonds	Bhat et al. (2009)
1226.6	1220.8	1222.7	Syringyl l and guaiacyl C—O bonds	Bhat et al. (2009)
	1151.4	1153.3	Aromatic C—H in plane deformation typical in syringyl units	Cyril et al. (2010)
1116.7	1124.4	1122.4	C—H in plane deformation for aromatics in syringyl and guaiacyl	Cyril et al. (2010)
1033.7	1031.8	1031.8	Aromatic C—H in plane deformation	Cyril et al. (2010)
960.5	941.19		C—H deformation in aromatic rings	Cyril et al. (2010)
	831.59	835.1	C—H deformation out of plane, aromatic ring	Cyril et al. (2010)
721.8			Skeletal deformation of aromatic rings, substituent groups, side chains	Cyril et al. (2010)

L_1, L_2, L_3: Lignin obtained at 0.5%, 2.5% and 5.5% (w/w) of NaOH at 121 °C and 80 min.

These was notable for L_2, suggesting that L_2 presented syringyl units, while L_1 is mainly composed of small molecules and L_3 probably suffered excessive depolymerization, forming small molecules and acid soluble compounds. These results are in agreement with the low molecular mass of L_1 and the decrease in lignin yield at 5.5% (w/w) NaOH. The absorption bands at 850–720 cm^{-1} were attributed to the deformation of C—H bonds in the aromatic rings, which could be from guaiacyl group.

4.4. ^{13}C NMR spectrometry

The ^{13}C NMR spectrum were recorded to L_1, L_2 and L_3.The analyses were carried out without acetylation, as it was preferred for the study to avoid destructive methods and the small difference between the results obtained has also been reported in literature by Wen et al. (2013). The data were processed using ACD/NMR processor academic free edition. In all spectra, DMSO-d_6 exhibited strong signal at 39.51 ppm. The ^{13}C NMR spectra is provided in Supplementary data (Fig. S2).

The ^{13}C NMR analysis of all samples could be done taking into account that above 100 ppm are represented the ether aromatic rings of guaiacyl, syringyl, feluric and fatty acid (Sun et al., 2012). The signal at 129.68 ppm is present in all samples, characteristic of C-α and C-β bonds in Ar—CH=CH—CH_2OH, distinctive of guaiacyl, syringyl and *p*-hydroxycoumaril structures. The lignin L_3 has additional signal at 174.47 ppm originating from the double bond C=O in coumaric and feluric acid. Similar results were reported by Li et al. (2010) and Wang et al. (2012) in lignin extraction from *Neosinocalamus affinis* and *Lespedeza cyrtobotrya* stalks, respectively. In L_2, the signal at 152.06 ppm is attributed to C_3/C_5 in etherified syringyl units, produced by the cleavage of β-O-C(4). The absence of this signal in L_3 is probably due to the formation of degradation fragments and acid soluble lignosulfonates, it confirmed the results obtained by FTIR, GPC and the decrease in lignin yield. The C_2/C_6 in syringyl units has a characteristic peak at 104.36 ppm which is observed only in L_2 and L_3, confirming the results obtained by FTIR and GPC. The lignin L_2 present more signals of characteristic groups of lignin. This is important for industrial applications, such as resins, films, cement adhesives, antioxidant, antimicrobial and food additives (Bhat et al., 2009; Ghaffar and Fan, 2014; Ghatak, 2011).

Below 100 ppm, only L_2 and L_3 had a signal at 55.95–55.97 ppm characteristic of C in structures Ar-OCH_3. In L_2, a signal was observed at 60.15 ppm produced by C-γ in guaiacyl units, these results were similar to those obtained by Mohamad Ibrahim and Chuah (2004) using OPEFB for lignin production. In the region between 35 ppm and 10 ppm, the signals are commons in all samples, as this is the region known as the fingerprint of lignin, corresponding to aliphatic and propylic side chain. The signals from 34 ppm to 24 ppm represent CH_2 in aliphatic side chain. Between 22.09 ppm and 22.15 ppm, the moderate signal originated from CH_3 in acetyl groups present in OPEFB fibers. The signal at 13.97 ppm (region where the proton is highly protected) represents γ-methyl in *n*-propyl side chain. Similar results have been reported for different types of biomass, including wheat straw, *Miscanthus* and oil palm trunk fiber (Hage et al., 2009; Sun and Tomkinson, 2001; Xu et al., 2006).

To confirm the functional groups and the chemical quality of in lignin L_2 obtained from ^{13}C NMR, ^{1}H NMR was performed.

4.5. ^{1}H NMR spectroscopy

^{1}H NMR data was processed using ACD/NMR processor academic free edition. DMSO-d_6 and water exhibited signals at 2.51 ppm and 3.3 ppm, respectively. The spectra is provide in Supplementary data (Fig. S3). The analysis of ^{1}H NMRcan be done taking into account that above of 3.5 ppm are represented the aromatic rings, whereas below of 3.5 ppm are the aliphatic regions.

The signals at 6.78 ppm and 6.70 ppm were attributed to syringyl units, similar results were reported by García et al. (2009) during the characterization of lignin fractions from pulping of *Miscanthus sinensis*. Hydrogen linked to C(α) in phenylcoumaran was identified by the signals at 5.33–5.31 ppm. Similar results were reported by Wang et al. (2012) in lignin from *L. cyrtobotrya* pretreated by steam explosion, followed by alkaline extraction. The multiple signals between 3.37 ppm and 3.71 ppm originated from methoxyl groups (–OCH_3). The spectrum showed a triplet at 2.19 ppm, 2.18 ppm and 2.17 ppm identified the protons in aromatic acetates. Was detected a multiplet between 2.0 ppm and 1.97 ppm, which was attributed to aromatic and aliphatic acetyl groups. Protons in aliphatic groups and methylene saturated structures produced signals down to 1.5 ppm. The signal at 0.8 ppm was

characteristic of methyl protons in aliphatic saturated structure. These results were similar to those obtained by Sun and Tomkinson (2001).

4.6. SEM analysis of OPEFB

To gather information on the effect of sequential acid/alkaline pretreatment on OPEFB fibers, SEM analysis was performed for the six solid fractions: raw material, solid residue from acid hydrolysis, OPEFB fibers after alkaline treatment at 0.5% (w/w), 2.5% (w/w) and 5.5% (w/w) NaOH. The micrographs were analyzed with an amplification of 350× are depicted in Supplementary data (Fig. S4).

The raw material presented a compact, rigid and smooth surface, with low porosity. The external wall was characteristic of lignin structure and exhibited silica bodies (phytolis) embedded in OPEFB structure, these are the most common minerals found on the surface of woody plants, as also reported by Baharuddin et al. (2013). The OPEFB fiber from acid pretreatment showed partial internal disruption of cell wall, preserving external walls without silica bodies, the absence of this was important to enhance microbial attack or enzymatic saccharification of OPEFB fibers (Baharuddin et al., 2013).

The increment in NaOH removed the external walls of OPEFB fibers, attributed to lignin solubilization. The OPEFB fibers obtained had more porosity, homogeneity, uniformity and highest surface area. The holes present in OPEFB fibers were assumed to be effective in the swelling of the OPEFB structures, attracting the microbes and enzymatic reactions for the subsequent bioconversion as reported by Shamsudin et al. (2012). The lignin L_2 obtained is a polyhedral rigid structure with smooth surface.

5. Conclusions

The sequential acid/alkaline treatment of OPEFB was effective for lignin removal, extracting 68.86% (w/w) of lignin at 2.5% (w/w) NaOH, 121 °C and 80 min. The lignin obtained was analyzed by GPC, FTIR, ^{13}C NMR and ^{1}H NMR. Lignin L_2 was found has highest molecular weight and presence of characteristic groups of syringyl and guaiacyl units. OPEFB obtained had high porosity and are rich in cellulose. This provided the possibility of uses to ethanol production, paper production or biotransformation, integrating the possibilities of OPEFB and lignin valorization in biorefinery concept.

The spectra of FTIR, ^{13}C NMR, ^{1}H NMR and the SEM are presented in Supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.07.018.

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