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Optimization of enzymatic hydrolysis for bioethanol production by semi-simultaneous saccharification and fermentation using mature coconut fibre

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INFO

Keywords
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wheat bran,
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ABSTRACT

Alternative substrates to produce useful chemicals such as biofuel have been attractive, in particular, for cellulosic ethanol production. In this context, the objective of this work was optimized the synergistic mixture of enzymes and bioethanol production. The enzymes of *Trichoderma reesei* and crude enzyme extract from *Lichtheimia ramosa* were used in the hydrolysis of mature coconut fibre pretreated by sequential process of alkaline hydrogen peroxide (Alk-H_2O_2)-sodium hydroxide (NaOH). Furthermore, these enzymes and pretreated vegetable biomass were applied in the bioethanol production by *Saccharomyces cerevisiae* in semi-simultaneous saccharification and fermentation strategy (SSSF). Resulting in the yields and conversions of delignified mature coconut fibre into reducing sugars between 12.7-82.14% and 0.09-0.64 g reducing sugars/g dry biomass, respectively, with an initial hydrolysis rate at 12 h between 0.10-0.89 g/(L.h). Yields and conversions of delignified mature coconut fibre into glucose between 10.16-83.78% and 0.06-0.43 g glucose/g dry biomass, in that order, with an initial hydrolysis rate at 12 h between 0.03-0.35 g/(L.h). Bioethanol production by *S. cerevisiae* using delignified mature coconut fibre, enzymes from *T. reesei* and crude enzyme extract from *L. ramosa* resulted in the production of 4.62 g/L, yield of 0.41 g ethanol/g glucose and volumetric productivity of ethanol of 0.13 g/(L.h), respectively. The results showed synergistic effects between enzymes from *T. reesei* and crude enzyme extract from *L. ramosa*, without promoting inhibition in the alcoholic fermentation. Therefore, allowing to formulate an optimized enzymatic preparation aiming cellulosic ethanol production.

Palavras-chaves
Lichtheimia ramosa,
extrato de enzima bruta, *β*-glucosidase,
farelo de trigo,
SSSF

RESUMO

Otimização da hidrólise enzimática para produção de bioetanol por sacarificação e fermentação semi-simultâneas usando fibra de coco maduro

Substratos alternativos para produzir produtos químicos úteis, como biocombustível, têm sido atraentes, principalmente para a produção de etanol celulósico. Neste contexto, o objetivo deste trabalho foi otimizar a mistura sinérgica de enzimas de *Trichoderma reesei* e extrato de enzima bruta de *Lichtheimia ramosa* para a hidrólise da fibra de coco maduro pré-tratado com peróxido de hidrogênio alcalino (Alk-H_2O_2) - hidróxido de sódio (NaOH) e produção de bioetanol por *Saccharomyces cerevisiae* em estratégia de sacarificação e fermentação semi-simultânea (SSSF). Resultando nas conversões e rendimentos de fibra de coco maduro deslignificada em açúcares redutores entre 12,0-82,3% e 0,09-0,64 g de açúcares redutores/g de biomassa seca, respectivamente, com uma taxa de hidrólise inicial em 12 h entre 0,10-0,89 g/(Lh) . As conversões e rendimentos de fibra de coco maduro deslignificado em glicose entre 10,4-83,9% e 0,06-0,43 g de glicose/g de biomassa seca, nessa ordem, com uma taxa de hidrólise inicial em 12 h entre 0,03-0,35 g/(L.h). A produção de bioetanol por *S. cerevisiae* utilizando fibra de coco maduro deslignificado, enzimas de *T. reesei* e extrato de enzima bruta de *L. ramosa* resultou na produção, rendimento e produtividade volumétrica de etanol de 4,62 g/L, 0,41 g de etanol/g de glicose e 0,13 g/(Lh), respectivamente. Os resultados mostraram efeitos sinérgicos entre as enzimas de *T. reesei* e extrato bruto de *L. ramosa*, além de não promover inibição na fermentação alcoólica. Portanto, permitindo formular uma preparação enzimática otimizada visando a produção de etanol celulósico.

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INTRODUCTION

Climate change and the rising price of oil barrel, added to the energy production needs, motivate the fuels production from renewable sources. Thus, lignocellulosic materials are considered promising alternatives for biofuels production, enzymes and other useful chemicals, because of its abundance and renewable nature (Gonçalves et al., 2011, 2013a, 2013b; Billard et al., 2012; Saini et al., 2013; Pandit et al., 2013; Zimbardi et al., 2013; Gonçalves et al., 2015; Gonçalves et al., 2019). Lignocellulosic materials used in this work for bioethanol and enzymes production have ample abundance, for example, coconut trees have ideal growing conditions in a humid tropical climate and are distributed in over 200 countries (FAO, 2012), mainly derived from the Philippines, Indonesia, India and Sri Lanka. Brazil is the fifth largest producer of coconut, with production of approximately 1.5 million tons (IBGE, 2020). Due to this breakthrough, the production chain of coconut does not have the correct destination of their agro-industrial and urban waste. Furthermore, wheat bran used in this work is also a good carbon source for lignocellulolytic enzymes production by different microorganisms using in the solid state bioprocessing (SSB), even in the absence of any supplementary carbon or nitrogen sources (Leite et al., 2007; Gonçalves et al., 2013a; Zimbardi et al., 2013; Garcia et al., 2018).

SSB resembles the natural environment of the microorganisms and has tremendous potential for enzymes production (Pandit et al., 2013). Besides, the SSB has some advantages over submerged bioprocessing (SmB), including the superior productivity, simple technique, low capital investment, low energy requirement and less wastewater output and better product recovery (Pandey, 2003). Several types of microorganisms are used in SSB, highlighting of the filamentous fungi (Rocha et al., 2013).

Three categories of enzymes are necessary to convert cellulose into glucose. These include endoglucanase which hydrolyzes internal β-1,4-glucosidic bonds randomly in the cellulose chain, cellobiohydrolase which moves progressively along the cellulose chain and cleave off cellobiose units from the ends of the chain, β-glucosidase, which converts cellobiose and soluble cellodextrins into glucose (Zhou et al., 2009; Singh and Bishnoi, 2012; Zimbardi et al., 2013). But lacking in β-glucosidase production, cause accumulation of cellobiose, which produces repression and end-product inhibition during hydrolysis (Berlin et al., 2007; Singh and Bishnoi, 2012; Gupta and Lee, 2013; Singhania et al., 2013; Zimbardi et al., 2013).

Furthermore, application of cellulases for conversion of lignocellulosic material to ethanol has been investigated for a long time, and it is now well-known that a mixture of different cellulolytic activities is necessary for the hydrolysis of cellulose into glucose for fermentation into ethanol (Zhou et al., 2009; Gupta and Lee, 2013). An optimized enzyme mixture is crucial for reduced cost of enzymatic hydrolysis step in the bioethanol production process and its composition will depend on the substrate and the type of pretreatment used (Billard et al., 2012; Wang et al., 2013). Individual cellulases have very limited hydrolytic activity while a mixture of cellulases can exhibit a synergistic effect where the hydrolytic activity of the cellulase mixture is greater than the sum of the hydrolytic activities of the individual enzyme (Zhou et al., 2009; Rana et al., 2014). *Trichoderma reesei* is the major fungus used for industrial cellulase production (Zhou et al., 2009; Billard et al., 2012; Singh and Bishnoi, 2012; Rocha et al., 2013; Singhania et al., 2013), and the ratio of β-glucosidase in commercial enzymatic preparations used today from *T. reesei* for cellulosic ethanol production is often low (Wang et al., 2013) and once purity is not a prerequisite for various industrial applications, may then be directly employed crude enzyme extracts (Zimbardi et al., 2013), as in this work, to complement the ratio of β-glucosidase.

Therefore, in this work it will be produced enzymes from *Lichtheimia ramosa* that has been isolated by Gonçalves et al. (2013a). Although, initially described as belonging to a genus of pathogenic microorganism (Borrás et al., 2010; Schwartze et al., 2012; Bibashi et al., 2013), this microorganism has advantage of presenting rapid cell growth (Gonçalves et al., 2013a; Silva et al., 2014), as well as it presents potential for the bioproduct production, as described by Gonçalves et al. (2013a) that produced the enzymes CMCase, xylanase and β-glucosidase by the *L. ramosa* using sugarcane bagasse and wheat bran as substrate in SSB with emphasis on the β-glucosidase activity achieving 15.58 IU/mL when wheat bran was used as substrate. This recent discovery of cellulases productions and hemicellulases by *L. ramosa* arises the need of verifying the applicability of the enzyme complex produced by this microorganism, as well as the enzyme pool in order to perform efficient hydrolysis of the lignocellulosic materials. Furthermore, Silva et al. (2013) carried out amylases production, β-glucosidases, CMCase and xylanases in SSB utilizing wastes of the Brazilian savannah fruits. In addition, Ferreira et al. (2013) have reported invertase production by *L. ramosa*.

However, lignocellulosic materials cannot be easily converted to simple monomeric sugars due

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to the recalcitrant nature of these molecules. To make cellulose and hemicellulose available for the attack of cellulases and hemicellulases, pretreatments are generally used (Adsul et al., 2005). Pretreatment processes can be physical, chemical, biological or a combination of these methods (Gonçalves et al., 2013b; Gonçalves et al., 2015). That way, economics and environmental sustainability in the bioethanol production process is always affected by the cost of pretreatment (Singh et al., 2010) and commercial enzymatic preparation (Gupta and Lee, 2013; Zimbardi et al., 2013).

In the present work will be used an alkaline hydrogen peroxide pretreatment (Alk-H_2O_2) followed by sodium hydroxide pretreatment (NaOH) in mature coconut fibre. Hydrogen peroxide and sodium hydroxide react with lignin under certain conditions and has been widely used as bleaching in highly lignified wood pulps (Carvalheiro et al., 2008). Alk-H_2O_2 pretreatment leaves no residue in the biomass and the peroxide decomposes into oxygen and water (Carvalheiro et al., 2008). This is a great advantage, because water consumption is a critical factor for bioethanol production, as well as there are absence of waste treatment and effluent (Rabelo et al., 2011). The potential use of NaOH pretreatment was examined in some works, among which the most cases showed the greatest effect of this pretreatment under a variety of lignocellulosic materials, as sugarcane bagasse (Santos and Gouveia, 2009), coastal Bermuda grass (Wang et al., 2010) and coconut fibre mature (Gonçalves et al., 2016).

In general, after the pretreatment stage, the enzymatic hydrolysis and fermentation may be carried out in separate stages or simultaneously as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) are commonly used. However, arose the possibility of junction of the SHF and SSF creating semi-simultaneous saccharification and fermentation (SSSF) from blend the advantages afforded by SSF and SHF, which includes a presaccharification stage and SSF (Shen and Agblevor, 2010; Mesa et al., 2011; Gonçalves et al., 2014; Gonçalves et al., 2016).

In this context, the aim of this work was to optimize the synergistic mixture of enzymes from *T. reesei* and crude enzyme extract from *L. ramosa* in the hydrolysis of mature coconut fibre pretreated by Alk-H_2O_2/NaOH as well as the bioethanol production by *S. cerevisiae* using SSSF strategy.

MATERIAL AND METHODS

Enzyme production

Raw material for enzyme production

Wheat bran, a byproduct of flour production was obtained from Dias Branco Flour Mill, located in Natal, RN, Brazil. Wheat bran substrate was washed with distilled water and then oven-dried at 50 °C, during 48 h. According to Rostagno (2011), wheat bran has the following composition: 31% cellulose, 26% hemicellulose, 14% lignin, 15% protein, 4% lipids and 7% ash.

Microorganism and solid solid-state bioprocessing -state bioprocessing (SSB) for enzyme production

Microorganism used was *L. ramosa*, isolated by Gonçalves et al. (2013a) and maintained in the microbiological collection of Laboratory of Bioengineering at the Federal University of Grande Dourados, Dourados, MS, Brazil.

Preculture and inoculation

Microorganism was precultivated in 125 mL Erlenmeyer flasks containing 20 mL of culture medium of potato dextrose agar (PDA), stored in a BOD chamber by 120 h at 35 °C and then suspended in 25 mL saline solution (0.1% ammonium sulfate, 0.1% heptahydrate magnesium sulfate and 0.1% ammonium nitrate) constituting the microbial suspension. Inoculation was performed by transferring 10 mL of microbial suspension to the 250 mL Erlenmeyer flasks containing culture medium based on wheat bran, constituted by 5 g of wheat bran moistened with 5 mL of saline solution (moisture content was about 70%) (Leite et al., 2007), the SSB was maintained at 35 °C, during 120 h (Gonçalves et al., 2013a). The materials were previously autoclaved at 121 °C by 20 min.

Enzymatic extraction

For enzymatic extraction, 50 mL of distilled water were added to Erlenmeyer flasks containing the culture medium based on wheat bran. The culture medium was left during 1 h in an orbital shaker (60 rpm and 25 °C) and then filtered through synthetic filters, yielding the crude enzyme extract (Leite et al., 2007) and maintained at 4 °C.

Enzymes of *T. reesei*

The enzymes of *T. reesei* ATCC 26921 (Cellubrix) was obtained from Sigma A/S and it was used in the experimental design for hydrolysis of the delignified mature coconut fibre (Table 1).

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Enzymatic activities

The enzymatic activities of cellulases, β-glucosidase and xylanase were determined in the crude enzyme extract from *L. ramosa* and enzymes from *T. reesei*.

Cellulase activity

Total cellulase activity was analysed in accordance with the standard methodology established by Mandels et al. (1976). In a test tube were added 0.3 mL of diluted enzyme, 1.2 mL of sodium citrate buffer (0.5 µM at pH 4.8) and 50 mg Whatman filter paper No. 1 as substrate. The medium was incubated in a heater device at 50 °C, during 1 h and the glucose liberated was measured using the DNS method described by Ghose (1987). DNS reagent reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid, which absorbs light at 540 nm.

β-glucosidase activity

The β-glucosidase activity was measured by incubating the enzymatic solution with 15 µM of cellobiose and 50 mM sodium citrate buffer (pH 4.8) at 50 °C, during 30 min. The reaction was stopped, immersing the test tubes into boiling water during 5 min. Then, the glucose concentration was determined by the glucose oxidase (GOD) and peroxidase (POD) method (GOD-POD test kit, Accurex Biomedical Pvt., India), applied at 25 °C, during 10 min with the produced amount of glucose measured spectrophotometrically at 500 nm. Glucose oxidase converts glucose to gluconic acid and hydrogen peroxide, posteriorly the peroxide oxidatively couples with 4-aminoantipyrene and phenol in the presence of peroxidase to produce a red quinoeimine dye that absorbs light at 500 nm. One unit of enzyme activity (CBU/mL) was defined as the amount of enzyme that released 1 µmol of glucose per min under the assay conditions.

Xylanase activity

The ylanase activity was determined using a reaction mixture contained 0.1 mL enzyme and 0.5% (w/v) of oat spelts xylan solution (Sigma A/S) in acetate buffer (pH 5.0). The mixture was incubated at 50 °C, during 10 min. After predetermined period, the released reducing sugars were quantified by DNS method recorded spectrophotometrically at 540 nm (Ghose, 1987). One unit of xylanase activity (UI/mL) was defined as the amount of enzyme that released 1 µmol of xylose per min under the assay conditions.

Raw material for enzymatic hydrolysis and bioethanol production

Mature coconut fibre was obtained from the agroindustries located in the Northeast of Brazil. The assays in order to obtain the composition of the raw material was carried out as Gonçalves et al. (2014).

Pretreatment process

Preparation of raw material before the pretreatment

Mature coconut fibre was washed five times with distilled water at 70 °C for removal of residual compounds present in the material. After this procedure, the material was dried in an oven with air circulation at 40 °C, during 24 h. The material was milled using a knife mills and standardized into a particle size of 48 mesh (0.3 mm).

Alkaline hydrogen peroxide pretreatment (Alk-H_2O_2)

Alk-H_2O_2 pretreatment was carried out according to Rabelo et al. (2011). 0.4 g of mature coconut fibre was mixed with 31.75 mL of hydrogen peroxide (concentration of 7.35% (v/v)) in a flask at 25 °C, during 1 h with agitation at 150 rpm. The pH of hydrogen peroxide solution was adjusted . The material residual solid was separated via vacuum filtration and washed with distilled water.

Delignification process with sodium hydroxide (NaOH)

Mature coconut fibre pretreated by Alk-H_2O_2 was transferred to a flask with 4% (w/v) solution of sodium hydroxide. The mixture remained at 100 °C under agitation of 100 rpm, during 1 h. After the delignification process, the solid was separated from the liquor by filtration. The solid was submitted to seven washes with distilled water (Gouveia and Santos, 2009)

Enzymatic hydrolysis

Process of enzymatic hydrolysis

Mature coconut fibre pretreated by Alk-H_2O_2/NaOH was used as substrate in the enzymatic hydrolysis (EH). The EH were performed with 2%

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of delignified mature coconut fibre in 250 mL Erlenmeyer flask containing a volume of 48 mL at 50 °C, agitation at 150 rpm, during 96 h using crude enzyme extract from *L. ramosa* and enzymes from *T. reesei* (see Table 1) in 50 mM sodium citrate buffer with 0.02% (w/v) sodium azide to prevent microbial growth. The samples were taken at 6 h intervals for the first 12 h and every 12 h intervals until a total time of 96 h (Dowe and Mcmillan, 2001; Santos et al., 2010). All determinations were performed in duplicate. The total reducing sugars (TRS) released were determined using the DNS method (Ghose, 1987) and the glucose available was estimated by GOD-POD test kit. The hydrolysis yield were calculated for reducing sugars and glucose using Equations 1 and 2, respectively (Dowe and Mcmillan, 2001; Selig et al., 2008), in order to verify the synergistic actions of cellulases and β-glucosidase about delignified mature coconut fibre.

$$\text{Hydrolysis yield (\%)} = \frac{\text{total reducing sugars}}{\text{polysaccharide in the substrate}} \cdot 100$$

$$\text{Hydrolysis yield (\%)} = \frac{\text{glucose}}{1{,}111f \cdot \text{biomass}} \cdot 100$$

where:
total reducing sugars = reducing sugars concentration (g/L);
glucose = glucose concentration (g/L);
polysaccharide in the substrate = polysaccharide concentration (g/L);
biomass = concentration of dry biomass initial in the enzymatic hydrolysis (g/L);
f = constitutes of cellulose fraction of dry biomass (g cellulose/g dry biomass);
1.111 = consists in the conversion factor of cellulose to glucose equivalent.

Experimental design of enzymatic hydrolysis

In order to relate the influence of the independent variables of enzymes from *L. ramosa* (X1, mL) and enzymes from *T. reesei* (X2, FPU) on the dependent variable reducing sugars (reducing sugar, %) and glucose (glucose, %), a 23 central composite experimental design (CCD) was used, as shown in the Table 2.

The mathematical model (Equation 3) corresponding to the experimental design for reducing sugars and glucose is given in the form:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} + \beta_{22} + \beta_{12} X_1 X_2$$

where:
Yi = response function;
X1, X2 = values of the independent variables;
β0 = coefficient relating the interception of the plane with the axis of response;
β1, β2 = linear coefficients estimated by method of the least squares;
β11, β22 = coefficient of the quadratic variables;
β12 = coefficient of interaction between the independent variables.

The quality of the fit of the polynomial model equation was evaluated by the coefficient of determination R^2 and the statistical significance was evaluated by Fisher's F-test for analysis of variance (ANOVA) with a 95% confidence level. The effect of each independent variable and also their interaction effects were determined. ANOVA results generated the Pareto charts of interactions and effects. The experimental design package Statistica (Statsoft®, USA) was the software used for data analysis.

Bioethanol production

Microorganism

The microorganism *S. cerevisiae* was used for bioethanol production. *S. cerevisiae* PE2 strain was obtained from the Fementec Ltda Company (São Paulo, Brazil). Microorganisms were stored in (1) inclined culture medium immersed in mineral oil, (2) skim milk medium and (3) lyophilized.

Inoculum preparation

S. cerevisiae was maintained in Petri dishes containing PDA culture medium at 30 °C, during 24 h. Strain for inoculation was grown in 250 mL Erlenmeyer flask with 100 mL of sterile culture medium containing 50 g/L glucose, 1 g/L ammonium sulfate, 0.5 g/L potassium phosphate, 0.25 g/L magnesium sulfate, 10 g/L yeast extract, 10 g/L peptone at 30 °C and 200 rpm in an orbital shaker, during 12 h (Santos et al., 2010). Cell concentration was inoculated to an equivalent optical density of 2.0 at 600 nm in a spectrophotometer UV-VIS (de Souza et al., 2012).

Semi-simultaneous saccharification and fermentation (SSSF)

Semi-simultaneous saccharification and fermentation was carried out during 12 h of presaccharification + 36 h of SSF process using mature coconut fibre pretreated by Alk-H_2O_2/NaOH as substrate. SSSF was performed with 2% (w/v) of delignified pretreated solids in 48 mL of sodium citrate buffer 50 mM (pH = 5.0), enzymatic load of 24 mL of crude enzyme extract from *L. ramosa* and 25 FPU of enzymes from *T. reesei* per 2 g of delignified pretreated solid (see

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experiment 6 in the Table 1), selected in stage of enzymatic hydrolysis. In this presaccharification period, the medium temperature was maintained at 50 °C. After 12 h of presaccharification, the medium temperature was adjusted to 30 °C and supplemented with 1 g/L ammonium sulfate, 0.5 g/L potassium phosphate, 0.25 g/L magnesium sulfate, 2 g/L yeast extract and 1 g/L peptone. Then, the fermentative process was started by adding the strain, in an orbital shaker at 200 rpm. The samples were taken at 0, 6, 12, 24, 36 and 48 h. Concentrations of ethanol and sugars were determined by high performance liquid chromatography (HPLC) (see Analysis of samples in high performance liquid chromatography Section). All determinations were performed in duplicate. The ethanol yield (Equation 4) was calculated assuming that all the potential glucose in the pretreated delignified solids was available for fermentation and that 1 g of glucose yielded 0.511 g of ethanol and 1 g of cellulose gave 0.9 g of glucose (modified of Dowe and McMillan, 2001).

$$\text{Ethanol yield (\%)} = \frac{\text{ethanol}}{\text{glucose initial} \cdot 0.511} \cdot 100$$

where:
ethanol = final ethanol concentration (g/L);
glucose initial = initial glucose concentration (g/L);
0.511 = consists of the conversion factor of glucose to ethanol.

Analysis of samples in high performance liquid chromatography (HPLC)

All the samples were centrifuged, filtered through a 0.2 µm sterile membrane filter and used for analyses of glucose, xylose and ethanol. Chromatographic separation was performed using a Shim-pack SCR-101H column (Shimadzu, Japan) and mobile phase 0.005 mol/L sulfuric acid, flow of 0.6 mL.min^{-1} and column temperature 65 °C using a Shimadzu chromatograph (Shimadzu, Japan) equipped with refraction-index detector (Shimadzu, Japan).

RESULTS AND DISCUSSION

Analysis of the synergistic effects

The initial enzyme activities of enzymes from *T. reesei* were 70 FPU/mL of cellulase, 0.3 CBU/mL of β-glucosidase and 216 IU/mL of xylanase. The initial enzyme activities of crude enzyme extract from *L. ramosa* were 0.08 FPU/mL of cellulase, 3.7 CBU/mL of β-glucosidase and 2.41 IU/mL of xylanase.

Mature coconut fibre pretreated by Alk-H_2O_2/NaOH (biomass composition: 52.25 ± 0.60 of cellulose; 25.93 ± 0.46 of hemicellulose; 8.71 ± 0.15 of insoluble lignin; 0.10 ± 0.02 of soluble lignin; 0.07 ± 0.03 of extractable; 3.08 ± 0.12 of ash) was submitted to enzymatic hydrolysis using an experimental design with different concentrations of enzymes from *T. reesei* and crude enzyme extract from *L. ramosa* (see Table 1), resulted in the conversions and yields of delignified mature coconut fibre into reducing sugars between 12.7-82.14% and 0.09-0.64 g reducing sugars/g dry biomass, respectively (Figure 1A-B). Initial hydrolysis rate at 12 h between 0.10-0.89 g/(L.h) (Figure 1C). Conversions and yields of delignified mature coconut fibre into glucose ranged between 10.16-83.78% and 0.06-0.43 g glucose/g dry biomass, respectively (Figure 2A-B). Initial hydrolysis rate at 12 h were found between 0.03-0.35 g/(L.h) (Figure 2C).

Table 1 - Experimental design of the mixture of crude enzyme extract from *L. ramosa* and enzymes from *T. reesei* used in the hydrolysis of mature coconut fibre pretreated by Alk-H_2O_2/NaOH. Besides conversion of mature coconut fibre pretreated into total reducing sugars and glucose.

Experiment	Conditions				Results	
	Codified variable		Real value		Conversions (%)	
	Enzymes of *L. ramosa*	Enzymes of *T. reesei*	Enzymes of *L. ramosa* (mL)	Enzymes of *T. reesei* (FPU)	Reducing sugars	Glucose
1	-1	0	0	12.5	37.79 ± 0.99	10.16 ± 0.35
2	0	-1	24	0	12.07 ± 0.34	12.04 ± 0.39
3	1	-1	48	0	13.78 ± 0.37	13.25 ± 0.41
4	0	0	24	12.5	68.12 ± 0.87	57.07 ± 0.97
5	-1	1	0	25	44.55 ± 1.35	27.78 ± 0.24
6	0	1	24	25	76.58 ± 0.34	60.71 ± 0.92
7	1	0	48	12.5	68.17 ± 0.44	69.28 ± 0.72
8	0	0	24	12.5	66.43 ± 0.50	58.17 ± 0.62
9	1	1	48	25	82.14 ± 0.67	83.78 ± 1.00

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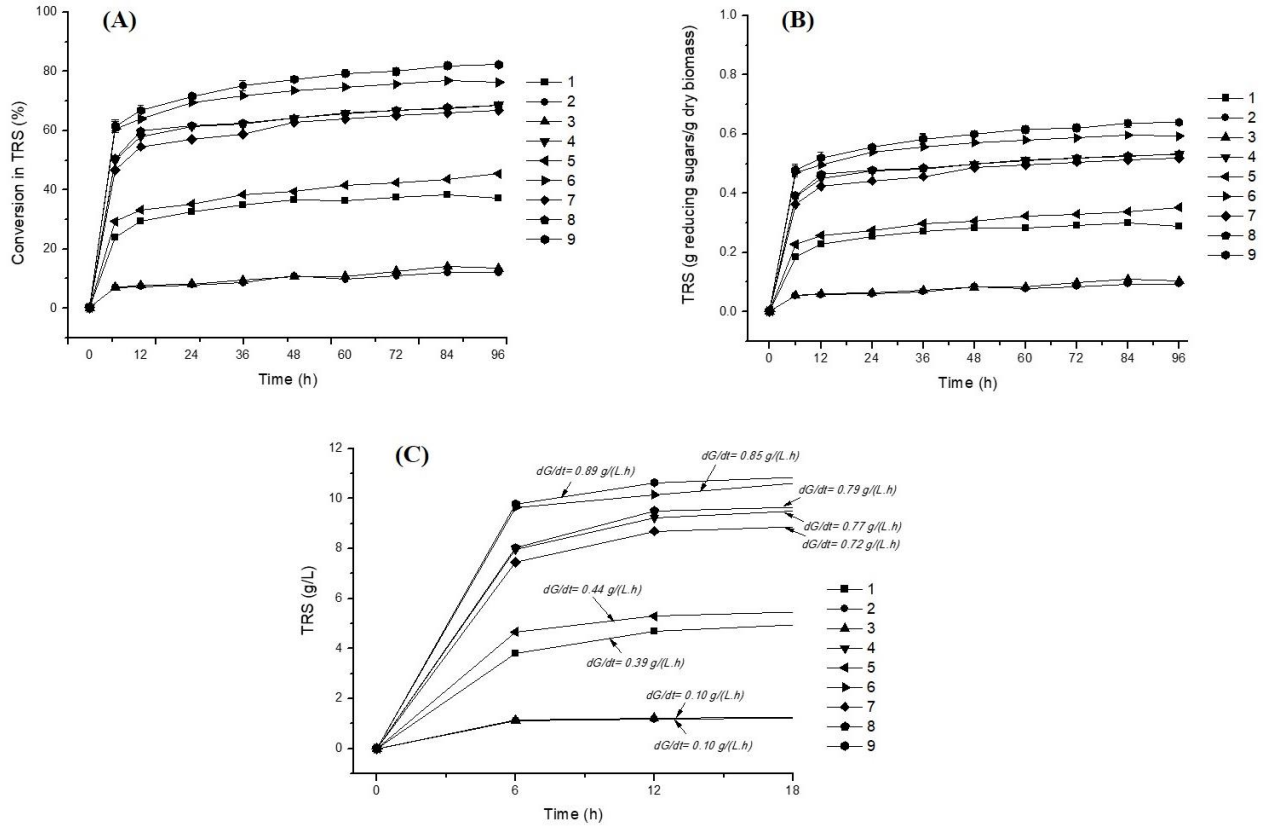

Figure 1 - Enzymatic hydrolysis of mature coconut fibre pretreated by Alk-H_2O_2/NaOH. A) Conversion of mature coconut fibre pretreated in total reducing sugars (TRS) (%); B) Digestibility of mature coconut fibre pretreated in TRS (g reducing sugars/g dry biomass); C) Initial hydrolysis rate at 12 h of mature coconut fibre pretreated. Symbol 1 to 9 in accordance with the experimental design (Table 1), being Experiment 1: enzymes of *L. ramosa* (0 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 2: enzymes of *L. ramosa* (24 mL) and enzymes of *T. reesei* (0 FPU); Experiment 3: enzymes of *L. ramosa* (48 mL) and enzymes of *T. reesei* (0 FPU); Experiment 4: enzymes of *L. ramosa* (24 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 5: enzymes of *L. ramosa* (0 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 6: enzymes of *L. ramosa* (24 mL) and enzymes of *T. reesei* (25 FPU); Experiment 7: enzymes of *L. ramosa* (48 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 8: enzymes of *L. ramosa* (24 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 9: enzymes of *L. ramosa* (48 mL) and enzymes of *T. reesei* (25 FPU).

The optimal point for enzyme mixture was enzymatic load of 24 mL of the crude enzyme extract from *L. ramosa* and 25 FPU enzymes from *T. reesei* per 2 g of delignified mature coconut fibre (see Table 1, experiment 6). The results obtained showed the synergistic effects between enzymes from *T. reesei* and crude enzyme extract from *L. ramosa*. These results corroborate with the results found by Gonçalves et al. (2013c) and Gonçalves et al. (2014) using commercial enzymes (30 FPU, 75 CBU and 130 IU xylanase per g of solid) and mature coconut fibre submitted to the hydrothermic pretreatment catalysed with sodium hydroxide reached conversion into glucose of 84.49% (Gonçalves et al., 2013c). Mature coconut fibre pretreated by Alk-H_2O_2/NaOH resulted in conversion into glucose of 76.21% (Gonçalves et al., 2014). Thus, the results obtained in this work can evidence the potential of this enzymatic cocktail.

Furthermore, the individual analysis of the experiments showed crude enzyme extract from *L. ramosa* enabled lower conversions of reducing sugars and glucose (see Table 1, experiments 1 and 5) derived from hydrolysis of cellulose and hemicellulose. This low concentration of β-glucosidase suggests an accumulation of cellobiose, inhibiting endoglucanase and cellobiohydrolase, consequently produces inhibition and repression of the final product during hydrolysis (Singhand Bishnoi, 2012; Gupta and Lee, 2013; Singhania et al., 2013; Zimbardi et al., 2013; Garcia et al., 2018). That way, the ratio of β-glucosidase is low, like in the commercial enzyme preparations of *T. reesei* (Wang et al., 2013), promoting lower yield of

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delignified mature coconut fibre into fermentable sugars. Furthermore, significant differences between conversion of delignified mature coconut fibre into reducing sugars and to glucose, possibly reflect the action of the xylanases in the solid, generating xylose.

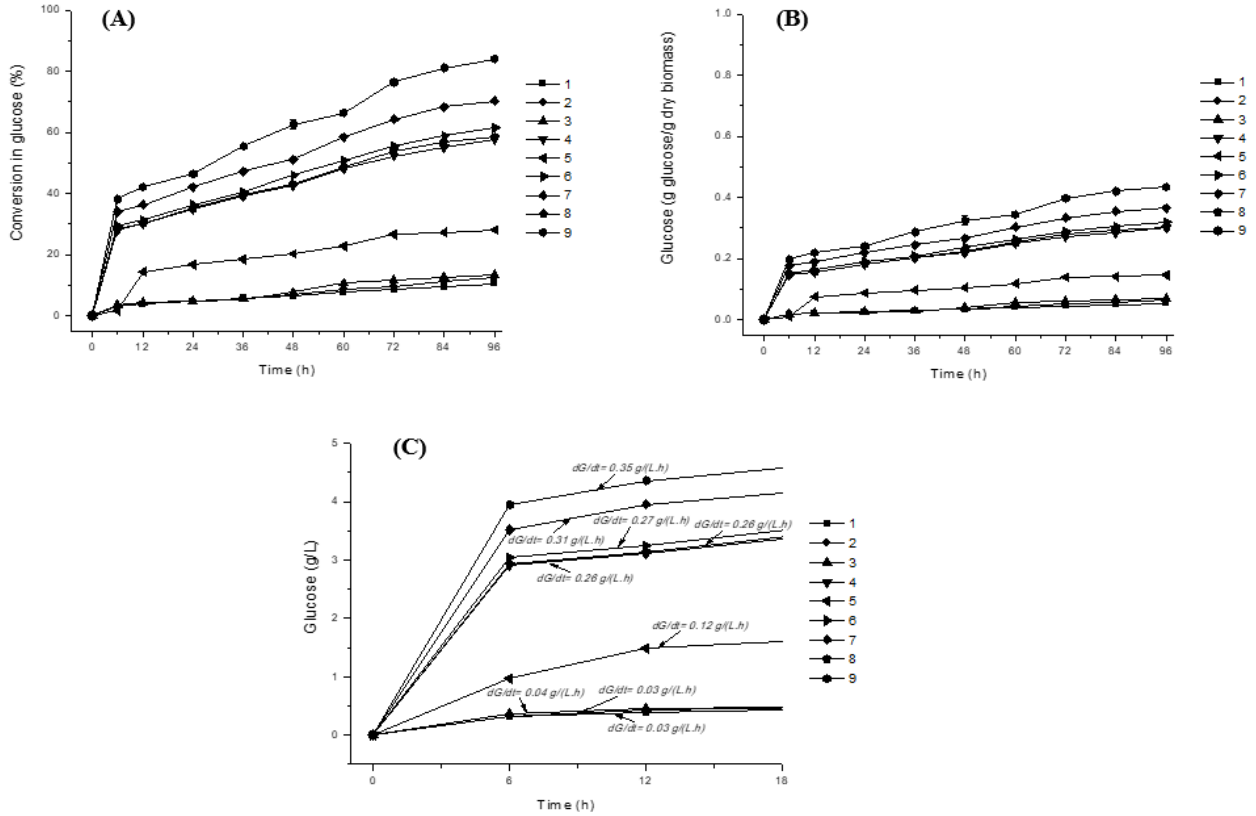

Figure 2 - Enzymatic hydrolysis of mature coconut fibre pretreated by Alk-H_2O_2/NaOH. A) Conversion of mature coconut fibre pretreated in glucose (%); B) Digestibility of mature coconut fibre pretreated in glucose (g glucose/g dry biomass); C) Initial hydrolysis rate at 12 h of mature coconut fibre pretreated. Symbol 1 to 9 in accordance with the experimental design (Table 1), being Experiment 1: enzymes of *L. ramosa* (0 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 2: enzymes of *L. ramosa* (24 mL) and enzymes of *T. reesei* (0 FPU); Experiment 3: enzymes of *L. ramosa* (48 mL) and enzymes of *T. reesei* (0 FPU); Experiment 4: enzymes of *L. ramosa* (24 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 5: enzymes of *L. ramosa* (0 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 6: enzymes of *L. ramosa* (24 mL) and enzymes of *T. reesei* (25 FPU); Experiment 7: enzymes of *L. ramosa* (48 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 8: enzymes of *L. ramosa* (24 mL) and enzymes of *T. reesei* (12.5 FPU); Experiment 9: enzymes of *L. ramosa* (48 mL) and enzymes of *T. reesei* (25 FPU)

The effects of the absence of enzymes from *T. reesei* have resulted in smaller conversions of delignified mature coconut fibre into reducing sugars and glucose (see Table 1, experiments 2 and 3), reflecting its low levels of cellulases. Therefore, inefficient hydrolysis of cellulose results in the low amount of cellobiose released and, consequently, low amount of glucose released by β-glucosidases.

However, when analysing the presence of both enzymes in the delignified mature coconut fibre, it was observed a significant increase in the conversion into reducing sugars and glucose (see Table 1, experiments 4-9). These facts demonstrate the need for cellulases and β-glucosidase during enzymatic hydrolysis. Besides these results confirmed the importance of β-glucosidase in this process as already reported by Gupta and Lee (2013), Singhania et al. (2013), Wang et al. (2013), Rana et al. (2014) and Garcia et al. (2018). Thus, the synergy between β-glucosidase and cellulases improves enzymatic hydrolysis yield of lignocellulosic materials (Berlin et al., 2007; Singh and Bishnoi, 2012).

In addition, Zimbardi et al. (2013) carried out the hydrolysis using *Colletotrichum graminicola* extract resulting in maximal yields of 11.3% for reducing sugars and 4.4% for glucose, in 72 h. Yields obtained using *T. reesei* extract, achieved 18.3% for reducing sugars and 18.7% for glucose. However, when using a mixture of both extracts, a synergic effect was observed and yields of 24.6% for

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reducing sugars and 25.5% for glucose were attained in 24 h. Moreover, maximal yields reached 37.6% of reducing sugars and 33.1% of glucose in 48 h. Pandit et al. (2013) have reported the cellulases production using wheat straw as substrate and subsequent hydrolysis, resulting in yield greater than 72% of reducing sugars. Roslan et al. (2011) carried out pretreatment of rice straw by few cycles of wet disc milling prior saccharification using the crude cellulase produced by *Aspergillus sp*., obtaining a hydrolysis yield of 90%. Saini et al. (2013) have reported the enzymatic hydrolysis of sweet sorghum bagasse using indigenously-produced cellulases from a novel fungal consortium of *A. flavus* F-80 and *A. niger* MTCC-2425, showing a maximum hydrolysis yield of 51.21%. Rana et al. (2014) using cellulases from *T. reesei* RUT-C30 and β-glucosidase from *A. saccharolyticus* in the hydrolysis of wet-exploded corn stover and wet-exploded loblolly pine obtained glucose yields of 81% and 55%, respectively. These results reported by Zimbardi et al. (2013), Pandit et al. (2013), Roslan et al. (2011), Saini et al. (2013) and Rana et al. (2014) were compared to the results obtained in this work, showing the synergistic mixture between enzymes from *T. reesei* and crude enzyme extract from *L. ramosa* were efficient.

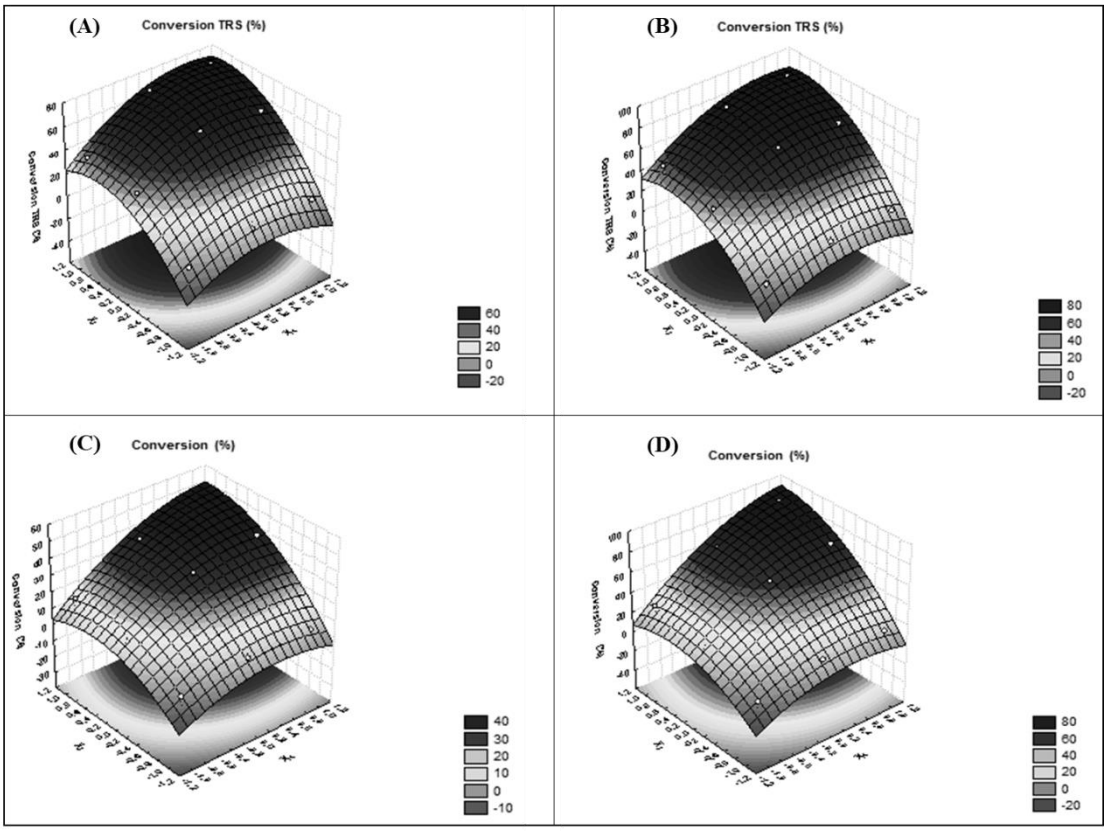

Figure 3 - Response surface and contour plot of mature coconut fibre pretreated by Alk-H_2O_2/NaOH converted into TRS and glucose (%). A) TRS variation in function of enzymes in 24 h. B) TRS variation in function of enzymes in 96 h. C) Glucose variation in function of enzymes in 24 h. D) Glucose variation in function of enzymes in 96 h.

Experimental design and statistical analysis of the effects of cellulase and β-glucosidase in the hydrolysis of mature coconut fibre pretreated by Alk-H_2O_2/NaOH

Optimization of hydrolysis process is one of the most important stages in the development of an efficient strategy as well as cost effective bioethanol fermentation process. Response surface methodology (RSM) is an effective optimization tool wherein many factors and interactions affecting the response can be identified with fewer experimental trials (Saini et al., 2013; Sindhu et al., 2014). The amount of protein components may greatly be decreased in a minimal, synthetic set of enzymes by optimizing the ratios of the necessary enzymes and by omitting unnecessary proteins (Wang et al., 2013). Singh and Bishnoi (2012) have reported the RSM using wheat straw pretreated by microwave alkali and enzyme production by *A. flavus* and *T.*

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reesei, resulting in the yield into glucose of 82%. Similar results were obtained in this work (Figure 3A-D) allowing optimization of enzymatic hydrolysis process, with optimal point for enzyme mixture with an enzymatic load of 24 mL for crude enzyme extract from *L. ramosa* and 25 FPU enzymes from *T. reesei* per 2 g of delignified mature coconut fibre (see Table 1, experiment 6), resulting in the yield into reducing sugars and glucose of 76.58% and 60.71%, in that order. Besides of Pareto charts show the importance of mixing between the enzymes from *T. reesei* and crude enzyme extract from *L. ramosa* (Figure 4A-D).

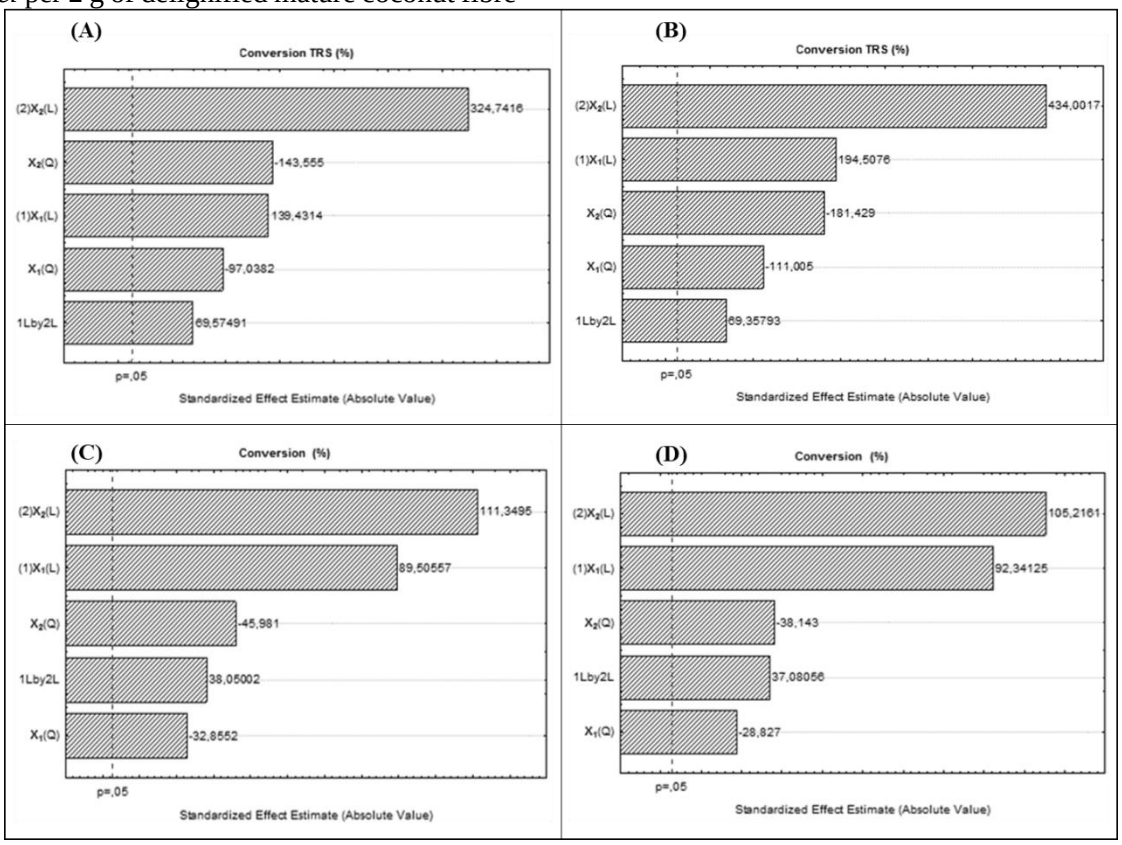

Figure 4 - Pareto charts for standardized effects of enzymes in mature coconut fibre pretreated by Alk-H_2O_2/NaOH. A) Conversion of mature coconut fibre pretreated into TRS in 24 h; B) Conversion of mature coconut fibre pretreated into TRS in 96 h; C) Conversion of mature coconut fibre pretreated into glucose in 24 h; D) Conversion of mature coconut fibre pretreated into glucose in 96 h.

According to ANOVA results for reducing sugars and glucose yields as function of crude enzyme extract from *L. ramosa* and enzymes from *T. reesei* in the delignified mature coconut fibre, the linear term for X_1, X_2, quadratic for X_1, X_2 and the interaction X_1X_2 have a significant effect on reducing sugars and glucose yields responses with *p*-value under a significance level of $\alpha = 0.05$. These effects can be visualized in the standardized Pareto charts (Figure 4A-D) and ANOVA (Table 2A-B). Observe that the variables for crude enzyme extract from *L. ramosa*, enzymes from *T. reesei* and crude enzyme extract from *L. ramosa* as well as from *T. reesei* interaction are important in a confidence level of 95% for the reducing sugars and glucose yields (Figure 4A-D and Table 2A-B). The effects of enzymes are positive, meaning that the higher the independent variables the higher the dependent ones (Figure 3A-D).

Multiple regression analysis and ANOVA of the experimental data were performed for the mathematical model fitting. The models in terms of codified values (see Table 2) expressed in Equations 5-8 represent the reducing sugars and glucose from delignified mature coconut fibre, in function of crude enzyme extract from *L. ramosa* (X_1) and enzymes from *T. reesei* (X_2).

$$\text{Sugar} = \text{Reducing sugars in 24 h} = 59.61 + 11.48*X_1 + 23.73*X_2 - 12.81* - 19.95* + 7.01*X_1X_2 \quad (R^2 = 0.989;\ R^2_{adj} = 0.975)$$

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Sugar = Reducing sugars in 96 h = 66.38 + 13.34*X_1 + 29.77*X_2 - 12.21* - 19.95* + 5.83*X_1X_2 (R^2 = 0.993; R^2_{adj} = 0.984)

Sugar = Glucose in 24 h = 32.73 + 12.01*X_1 + 14.94*X_2 - 7.07* - 9.89* + 6.25*X_1X_2 (R^2 = 0.940; R^2_{adj} = 0.926)

Sugar = Glucose in 96 h = 54.64 + 21.55*X_1 + 24.56*X_2 - 10.79* - 14.27* + 10.60*X_1X_2 (R^2 = 0.963; R^2_{adj} = 0.938)

Table 2 - Analysis of variance in the hydrolysis of mature coconut fibre pretreated by Alk-H_2O_2/NaOH converted into sugars in function of crude enzyme extract from *L. ramosa* (x1) and enzymes from *T. reesei* (x2). A) Total reducing sugars (TRS); B) Glucose.

A

Source	Sum of Squares	d.f.	Mean Square	*F*-value	*p*-value
x_1	805.81	1	805.81	199.14	0.001 *
x_1^2	376.33	1	376.33	93.0	0.002 *
x_2	3344.54	1	3344.54	826.54	0.002 *
x_2^2	906.22	1	906.22	223.96	0.001 *
x_1x_2	110.03	1	110.03	28.73	0.019 *
Residual Error	12.14	3	4.05		
Lack of Fit	12.14	2	6.07	5375.27	0.01 *
Pure Error	0.20	1	0.20		
Total	5567.21	8			

d.f., degree of freedom;
* significant;
Fcalculated: 261.42;
Ftabular: 6.26.

B

Source	Sum of Squares	d.f.	Mean Square	*F*-value	*p*-value
x_1	2256.80	1	2256.80	53.85	0.005 *
x_1^2	410.14	1	410.14	9.79	0.042 *
x_2	2822.81	1	2822.81	67.36	0.004 *
x_2^2	617.57	1	617.57	14.74	0.031 *
x_1x_2	214.63	1	211.87	9.52	0.042 *
Residual Error	125.72	3	41.91		
Lack of Fit	125.12	2	62.56	104.41	0.069
Pure Error	0.60	1	0.60		
Total	6572.79	8			

d.f., degree of freedom;
* significant;
Fcalculated: 30.33;
Ftabular: 6.26.

Response surfaces were drawn as three-dimensional plots of the second-order polynomial models (Equations 5-8) as a function of the two most strongly influencing variables. Reducing sugars and glucose yields were plotted in function of crude enzyme extract from *L. ramosa* and enzymes from *T. reesei* (see Figure 3A-D) and demonstrate that the increase in the reducing sugars and glucose yields are correlated with increases of crude enzyme extract from *L. ramosa* and enzymes from *T. reesei* in theexperiments (see Table 1).

Results of ANOVA listed in the Equations 5-8 revealed that the second-order polynomial models adequately represent the responses of sugars yield with coefficients of determination R^2, which indicates that 98.9%, 99.3%, 94.0% and 96.3% of the variability of response might be explained by the model. These values are in accordance with the adjusted coefficient of determination R^2_{adj} = 0.975, 0.984, 0.926 and 0.938.

An efficient hydrolysis of lignocellulosic materials into soluble sugars for biofuel production

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necessitates of the synergistic interaction of multiple enzymes (Zhou et al., 2009; Billard et al., 2012; Rana et al., 2014). Formulation of individual hydrolytic enzyme activities in an overall system can reduce total enzyme loadings and costs in a cellulosic ethanol production (Gupta and Lee, 2013; Wang et al., 2013; Rana et al., 2014). This way, a significant influence on bioconversion process in cost reduction of pretreatment and enzymatic hydrolysis is very important for influence bioethanol commercialization (Fang et al., 2010; Wang et al., 2013, Gonçalves et al., 2015). Therefore, the results obtained in this work allow to optimize the enzymatic load (crude enzyme extract from *L. ramosa* and enzymes from *T. reesei*) used in the hydrolysis of delignified mature coconut fibre.

In addition, enzyme should be produced *in situ* and used in crude form to reduce the cost (Fang et al., 2009; Gonçalves et al., 2013a). Thus, some works showed that wheat bran is a good carbon source for the cellulases production as well as hemicellulases by different fungi in SSB, even in the absence of any supplementary carbon and nitrogen source, as evidenced by Zimbardi et al. (2013) and Gonçalves et al. (2013a). In this work, enzymes were produced using as substrate the wheat bran, with subsequent use of this crude enzyme extract in the enzymatic hydrolysis. Thus, the crude enzyme extract did not provide negative effect on enzymatic hydrolysis and SSSF (see *Fermentation process for bioethanol production* Section), motivated by the residual compounds from SSB (secondary metabolites of the *L. ramosa* and residues of wheat bran).

The strategy of SSSF was carried out using *S. cerevisiae* PE2, mature coconut fibre pretreated by Alk-H_2O_2/NaOH, optimized enzymatic load of 24 mL crude enzyme extract from *L. ramosa* and 25 FPU enzymes from *T. reesei* per 2 g of solid (see Table 1, experiment 6). According to Shen and Agblevor (2010), the performance of fermentative strategy can be assessed by two indicators: conversion yield (g ethanol/g glucose) and volumetric productivity of ethanol g/(L.h).

The results of this work indicate that glucose from enzymatic hydrolysis of delignified mature coconut fibre could be fermented into ethanol by *S. cerevisiae* and the kinetic profile showed increases in the availability of glucose during the initial 12 h, with a gradual decrease of glucose after inoculation (Figure 5). Bioethanol production by the *S. cerevisiae* resulted in the production, yield and volumetric productivity of ethanol were 4.62 g/L, 0.41 g ethanol/g glucose (80.41% of value of theoretical ethanol) and 0.13 g/(L.h), in that order, in 36 h of SSSF. Therefore, showing the absence of inhibitors contained in the crude enzyme extract from *L. ramosa* used in the SSSF. Furthermore, Gonçalves et al. (2013c) carried out the SSSF using mature coconut fibre hydrothermally pretreated with sodium hydroxide, using commercial enzymes and *S. cerevisiae* PE2, obtaining for production, yield and productivity of ethanol values such as 26.18 g/L, 0.46 g ethanol/g glucose, 0.44 g/(L.h), respectively. Singh and Bishnoi (2012) carried out microwave alkali pretreatment in wheat straw and used enzymes produced by *A. flavus* and *T. reesei*. The concentrated enzymatic hydrolyzate was fermented for bioethanol production by *S. cerevisiae*, *Pichia stipitis* and co-culture of both. The ethanol yields were 0.48 g ethanol/g glucose, 0.43 g ethanol/g glucose and 0.40 g ethanol/g glucose, in that order. Roslan et al. (2011) carried out bioethanol production of rice straw pretreated by few cycles of wet disc milling prior saccharification. The crude cellulose was produced by *Aspergillus sp.* and fermentation was carried out by *S. cerevisiae*, resulting in 62.61% (value of theoretical ethanol). Rocha et al. (2013) carried out bioethanol production by *S. cerevisiae* Y904 using whey and rice byproduct as the substrate and the enzyme complex produced by *A. niger*, resulting 8.4 g/L of ethanol. Moreover, Gonçalves et al. (2016) carried out the SSSF using mature coconut fibre pretreated by hydrothermal pretreatment catalysed with sodium hydroxide and commercial enzymes, containing *P. stipitis* or *S. cerevisiae* or *Zymomonas mobilis*, obtaining yield of 90.18%, 91.17% and 91.03%, in that order. These results corroborate with the results obtained in this work, evidencing the biotechnological potentials of enzymatic cocktail and fermentative strategy tested.

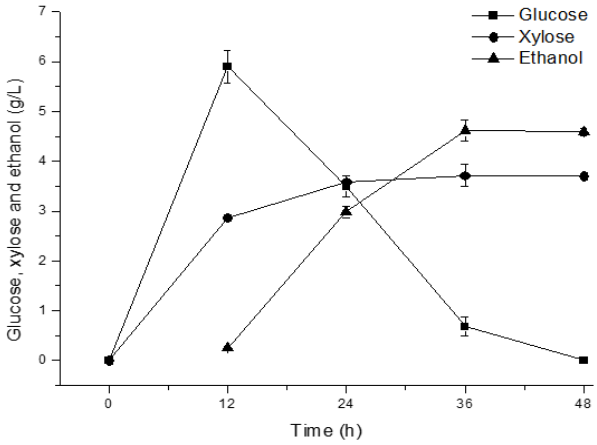

Figure 5 - Fermentation in SSSF by S. *cerevisiae* using mature coconut fibre pretreated by Alk-H_2O_2/NaOH, enzymes from T. *reesei* and crude enzyme extract from L. *ramosa*.

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CONCLUSION

Cellulosic ethanol production face bottlenecks, such as an example the enzymatic hydrolysis step to afford an economic viability of this productive chain. That way, in this work was shown a consistent alternative to alleviate these costs, based in the use of crude enzyme extract from *L. ramosa.* The mixtures of this extract and enzymes from *T. reesei* allowed significant results for enzymatic hydrolysis of mature coconut fibre pretreated by Alk-H_2O_2/NaOH. In addition, indicated absence of inhibitory effects of secondary metabolites from *L. ramosa* contained in the hydrolysed fermented by *S. cerevisiae*. In this sense, allowing to formulate an optimized enzymatic mixed for use in the cellulosic ethanol production. Also highlighted the efficient employments of agro-industrial residues — wheat bran and mature coconut fibre — in the enzymes and bioethanol productions.

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