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Ethanol biosynthesis by fast hydrolysis of cassava bagasse using fungal amylases produced in optimized conditions

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ABSTRACT

The search for a renewable platform to produce high-value biochemicals and energy that are environmentally correct has been a current concern. A fast and inexpensive bioprocess for amylase production, able to hydrolyze complex residues in fermentable sugars to be used for ethanol production was developed. High titer amylase from *Rhizopus oligosporus* in solid state fermentation (SSF) was obtained by optimizing the medium supplementation using agro-industrial waste as substrate. Statistical experimental design and partial least square (PLS) regression were used to establish a relation between added chemical compounds and enzyme production, showing that urea was the most important nutrient. Crude amylase extract had competitive performance features giving higher productivities in starch hydrolysis than a commercial glucoamylase. The amylase produced was applied in the proportion of 15 U/g dry cassava bagasse to obtain cassava bagasse hydrolysate (CBH). More than 42% conversion in reducing sugars was achieved with an efficient 10 h single-step hydrolysis at 55 °C in a bioreactor. The concentrated CBH was subsequently used in fed batch process producing 89.2% ethanol yield. Furthermore, comparing just the cost of the raw materials sugarcane and CHB, the latter demonstrated to be a lower-cost feedstock for ethanol fermentation.

1. Introduction

Bio-ethanol can be produced from various renewable feedstock such as sugarcane (*Saccharum* spp.), corn (*Zea mays*), wheat (*Triticum* spp.), cassava (*Manihot* spp.), and cellulose biomass (Baeyens et al., 2015). In addition, starchy residues hydrolysis for ethanol production is a very promising technology that can become extensively adopted at large scale (Cinelli et al., 2015). In the US, world's largest ethanol producer (USDA, 2014), corn starch has been used as source of biofuel for decades, with the use of corn grain balanced for food and feed, and ethanol production (Mumm et al., 2014).

Cassava production has been increasing annually in developing countries like Nigeria, the largest producer worldwide (FAOSTAT, 2016), and for these countries is an important source of food (Anyanwu et al., 2015). This crop constitutes a staple food in Sub-Saharan Africa, so much that 29 million tons of the annual Nigerian production are demanded as food (Anyanwu et al., 2015). Cassava shows the highest yield of carbohydrates per hectare after sugarcane and sugar beet (*Beta* spp.), and one of the best water-footprints especially on relatively low fertility soils, in drought conditions, requiring low agrochemical input and adapts well in all ecological zones (Okudoh et al., 2014). Advances in the use of wastes from cassava industry can contribute to the development of these countries and supply of food and other compounds to the world. Between 2000 and 2013, Africa continent was the largest cassava producer with about 54% of the production, followed by Asian with 31% and Americas with 15%. The Brazilian production of cassava was the fourth largest in 2014 with 23.36 million tons (FAOSTAT, 2016), justifying the large amounts of cassava processing waste in the country.

For each 250–300 tons of cassava root processed, about 280 tons of bagasse with 85% moisture is produced (Pandey et al., 2000). Cassava bagasse is used as feed and has also been successfully assessed in the production of interest biomolecules demonstrating its fermentability as *n*-butanol (Lu et al., 2012), succinic acid (Shi et al., 2014), fatty acid, and neutral lipid (Chen et al., 2015). Currently, the majority of the cassava bagasse is discarded as waste, however, this residue can be

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considered as an excellent source of fermentation for higher-value biochemicals.

Currently, the starch hydrolysis processes employ two classes of enzymes, α-amylase, and glucoamylase, at hydrolysis times that typically exceed 24 h (Białas et al., 2010; Cinelli et al., 2015). Amylases include an important enzyme class with many industrial and biotechnological applications, representing 25% of the enzymes market (Kumar et al., 2012). They belong to the class of hydrolases and catalyze the hydrolysis of starch and its derivatives, releasing various products, including progressively smaller dextrins and glucose polymers (Gupta et al., 2003).

There is a wide range of applications for the hydrolysis of starch and ethanol production, such as in the food, detergent, paper, textiles, baking, chemical, and pharmaceutical industries. (Gupta et al., 2003; Castro et al., 2010; Baeyens et al., 2015). Due to increasing demand, there is a huge interest in the discovery of enzymes with improved properties on starch degradation, as well as developing techniques that reduce the cost of production of amylolytic derivatives. Potential of fungi, such as *Aspergillus* and *Rhizopus* among others, are known to produce enzymes including amylases (Vaidya et al., 2015). Fungi are especially valued as excellent producers of enzymes of industrial interest. *Rhizopus* has been highlighted for being a good producer of amylases and its ability to metabolize complex mixtures of organic compounds in most organic residues (Jin et al., 2002; Peixoto-Nogueira et al., 2008; Freitas et al., 2014). In addition, *Rhizopus oligosporus* is considered safe, which is traditionally used in the food industry (Bourdichon et al., 2012).

Solid-state fermentation (SSF) has gained attention over the past 20 years in the development of industrial bioprocesses, due to several advantages over the conventional method of submerged fermentation, such as the particular physiology presented by fungi in this mode of cultivation and particularly due to the lower energy requirement, higher product yields, and less wastewater production with lesser risk of contamination (Barrios-González, 2012; Thomas et al., 2013).

The search for economical bioprocesses for the production of enzymes able to hydrolyze complex carbohydrates, such as the combination of starch and lignocellulosic compounds, has been a current concern. This fact is due to the need for better use of waste as a renewable platform to produce chemicals, food, and biofuels, considering that oil and its derivatives are non-renewable, and expansion of agricultural land is limited. In this context, the objective of this study was to develop a rapid and low cost bioprocess using natural, robust, and safe microorganism for the production of enzymes able to use agro-industrial residues hydrolysis to produce fermentable sugars which can be used for ethanol production or another bioprocess.

2. Materials and methods

2.1. Microorganism and inoculum preparation

Rhizopus oligosporus (CCT 3762) was obtained from Fundação Tropical de Pesquisas e Tecnologia “André Tosello”, Campinas, SP, Brazil. The fungal strain was grown on potato dextrose agar (PDA) (Difco, USA) for 7 days at 30 °C. Viable spores were harvested from the culture by washing with 0.01% (v/v) Tween 80 water solution and the spore suspension was used as inoculum for SSF.

2.2. Solid state fermentation (SSF)

Tests for SSF were conducted in 250 mL Erlenmeyer flasks containing 10 g of wheat bran (Moinho Nacional, Assis, SP, Brazil). To determine the effect of nutrient supplementation on amylase production was carried out in a preliminary experiment to define the concentration range to be used in the central composite design (CCD). The standard solution comprised: 9.3% w/w $(NH_4)_2SO_4$; 4.75% w/w KH_2PO_4; 2.3% w/w urea (Soccol et al., 1994) was compared with trials without supplementation and intermediate concentrations. The substrates were prepared with 60% moisture content, sterilized at 121 °C for 20 min, inoculated with 1.10^6 spores per gram of dry substrate and incubated at 30 ± 0.5 °C (Fanem Ltda Mod.002 CB, São Paulo, Brazil) for 120 h (Escaramboni and Oliva-Neto, 2014). The amylase was extracted from the fermented substrates with 5 mL/g distilled water at 30 °C on an orbital shaker (Tecnal TE-421, São Paulo State, Brazil) at 180 rpm for 30 min. The enzymatic extract was separated by filtration and used for amylase activity assay.

Table 1
Experimental range and levels (including star points*) of each factor (nutrient concentration) in the CCD.

Nutrient[a] concentration (%, w/w)	Coded levels				
	−1.68*	−1	0	1	1.68*
X_1: ammonium sulfate	0	0.5	1.25	2	2.5
X_2: potassium phosphate	0	0.25	0.63	1	1.25
X_3: urea	0	0.25	0.63	1	1.25

[a] Ammonium sulfate – $(NH_4)_2SO_4$ Pure for analysis –American Chemical Society (P.A.-A.C.S.) (Catalog number: 01S1051.01.AH, Synth, Brazil); Monobasic potassium phosphate – KH_2PO_4 P.A. (Catalog number: 01F2002.01.AG, Synth, Brazil); Urea P.A. (Catalog number: 01U1001.01.AH, Synth, Brazil).

2.3. Central composite design (CCD)

Response surface methodology (RSM) was used to determine the mutual interactions among the selected variables and their corresponding optimum concentrations for maximizing the amylase production. Three variables (X_1: ammonium sulfate; X_2: monobasic potassium phosphate; and X_3: urea) were analyzed. A CCD having five coded levels (-1.68, −1, 0, +1, +1.68), eight points associated to 2^3 factorial design, six axial points and three replicates at the center point with a total number of 17 runs was formulated. The experimental range and the levels of each variable are summarized in Table 1.

The second-order polynomial model equation was fit for response variables, y (amylase activity, U/g), was given below:

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where y is the predicted response, β_0 is the offset term, β_i the linear coefficients, β_{ii} the quadratic coefficients and βij the interaction effect. The optimum levels of variables for maximal amylase production were obtained by analysis the response surface and contour plots.

2.4. Statistical analysis

The statistical software STATISTICA 8.0 trial version software (Statsoft Inc., Tulsa, OK, USA) was used to define CCD experimental design as well as analyze statistically the derived data and fit polynomial model for response variable under consideration (amylase activity). Means multiple comparisons in other experiments were performed by ANOVA and subsequently Tukey test (if necessary) in BioEstat 5.0 free software (Mamirauá Institute, Tefé, AM, Brazil).

2.5. Analytical methods

Amylase activity was determined using 0.5% (w/v) soluble starch (Sigma-Aldrich, USA) as substrate in 0.05 M sodium acetate buffer, pH 5.5 as a described method previously (Bernfeld, 1955) with some modifications. The reducing sugar concentration was estimated by the 3,5-dinitrosalicylic acid (DNS) method according to Miller (1959). The reaction mixture containing 100 µL of crude enzyme and 650 µL of substrate was incubated for 10 min at 60 ± 0.1 °C (Marconi MA 127, Piracicaba, SP, Brazil). The reaction was stopped by adding 500 µL of DNS solution followed by heating it in a boiling water bath for 5 min,

and cooling and diluting it with 3.75 mL of distilled water. The concentration of reducing sugar was measured at 540 nm using glucose as standard. One unit of amylase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugars per minute and expressed as units per gram of dry substrate (U/g). Detailed steps and equation performed to measure amylase activity are described by Barchi et al. (2016).

2.6. PLS data analysis

The PLS technique was used to define correlation levels between the chemical elements added in the fermentation medium, represented by a matrix X and the amylase activity, representing the response variable matrix Y. The independent variables matrix (X) included concentrations of total nitrogen (TN), organic nitrogen (ON), inorganic nitrogen (IN), total phosphorus (P), total potassium (K), and total sulfur (S), which were calculated based on different concentrations of nutrients used in experimental design to optimize the chemical composition of salt solution for wetting wheat bran. The organic nitrogen contribution provided by wheat bran was calculated considering that it contains 14% protein, according to the manufacturer's information ((Moinho Nacional, Assis, SP, Brazil)). The PLS modeling was done in SIMCA 14 demo version software (Umetrics, Umeå, Sweden).

2.7. Enzymatic hydrolysis

Soluble starch was mixed and gelatinized with 0.05 M sodium acetate buffer (pH 5.5) to obtain a 5% (w/v) concentration and amylolytic crude extract from *R. oligosporus* (1.5 U/mL starch solution) was added to the mixture and incubated at 55 or 60 ± 0.1 °C (Marconi MA 127, Piracicaba, SP, Brazil). In another experiment, glucoamylase NS22035 from the Novozymes' cellulosic ethanol enzyme kit was reacted with 5% (w/v) soluble starch in 0.05 M sodium acetate buffer (pH 5.5) at 60 °C (1.5 U/mL starch solution). In the hydrolysis for obtaining cassava bagasse hydrolysate (CBH), the amylolytic crude extract from *R. oligosporus* obtained by solid fermentation was reacted with 1.5 L of 10% (w/v) dried cassava bagasse in 0.05 M sodium acetate buffer (pH 5.5) at 55 ± 0.1 °C (1.5 U/mL starch solution) 3-L bioreactor (TEC-BIO-Plus, Tecnal, Piracicaba, SP, Brazil) with intermittent agitation in every 30 min. The cassava bagasse was previously gelatinized at 80 °C per 30 min. To study the kinetics of reducing sugar production, samples were taken at appropriate time intervals for 12 h. The yield and productivity of glucose were calculated based on Freitas et al. (2014).

2.8. Fermentation

For ethanol submerged fermentation, CBH was concentrated at 10.8% (w/v) of reducing sugars and supplemented with following composition of chemicals (Labsynth, Diadema SP, Brazil) according to Dorta et al. (2006) (% w/v): 0.5% yeast extract, 0.1% ammonium sulfate, 0.114% $K_2HPO_4{\cdot}3H_2O$, 0.024% $MgSO_4{\cdot}7H_2O$, 0.0028% $ZnSO_4{\cdot}7H_2O$ and 0.0017% $MnSO_4{\cdot}H_2O$, pH 5.0 and sterilized at 121 °C for 15 min. The microorganism was a commercial strain of *Saccharomyces cerevisiae* (Fleischmann®, Brazil). To start the fermentation 9 g (dry biomass) of yeasts was suspended in 75 mL distilled water in 500 mL Erlenmeyer flasks. The fed batch process was carried out by adding 15 mL of the must (semi-synthetic media with CBH) at 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 h of fermentation. The final volume of the fermentative cycle was 225 mL. The flasks were shaken at 80 rpm on an orbital shaker (Tecnal-TE421, São Paulo State, Brazil) at 32 ± 0.1 °C for 12 h. The yeasts were centrifuged (Excelsa-206 BL, Brazil) after the end of the fermentative cycle at 3000 rpm for 20 min. The concentration of ethanol was determined using distiller and digital densimeter (Anton Paar-DMA 4500 ME) technique. Ethanol yield was calculated using 0.6475 as the w/v conversion factor of consumed sugar to ethanol based on the theoretical maximum yield.

Table 2
Comparison of different chemical compositions of solution for wetting solid substrate on amylase production by *Rhizopus oligosporus* in solid state fermentation.

Runs	Variables (% w/w)			Amylase activity (U/g)
	$(NH_4)_2SO_4$	KH_2PO_4	Urea	
1–Standard	9.3	4.8	2.3	180.90 ± 10.07 a[a]
2	0.0	0.0	0.0	179.94 ± 11.81 a
3	5.0	2.5	2.5	367.61 ± 9.68 b
4	2.5	1.25	1.25	353.92 ± 4.26 b
5	2.5	1.25	2.5	361.86 ± 2.71 b

[a] Means followed by the same letter indicate no significant differences ($p > 0.05$).

3. Results and discussion

3.1. Optimization of the medium composition for amylase production in SSF

High fermentation cost limits the application of bioprocesses (Huang et al., 2013) and the potential of wastes for industrialization. About 30–40% of the production cost of the industrial enzymes is due to the growth medium (Joo and Chang, 2005). The development of an economically viable bioprocess seeks to maximum enzyme production with the lowest substrate cost. The use of agro-industrial residues as a substrate and minimum nutrients supplementation leads to a low cost bioprocess.

The amylase produced from SSF process depended on the level of salts concentration used. The intermediate level determined in this work showed better performances. The preliminary experiment to determine the optimal range of nutrient concentrations to be used in CCD indicated the differences in amylase production among standard solution (treatment 1) and other solutions under consideration (Table 2). The highest amylase production was 367 U/g (treatment 3) for intermediate values of concentration of salts $(NH_4)_2SO_4$, KH_2PO_4, and urea. This represents a 100% increase in enzyme activity compared with the tests using only wheat bran (180 U/g) as substrate (treatment 2) and those supplemented with the maximum concentration of salt (181 U/g) from the standard solution (treatment 1). As the treatments 3, 4, and 5 were statistically equal in enzyme activity, the treatment with the lowest concentration of nutrients (4) was selected to define the positive axial point (1.68) for each nutrient concentration in CCD (Table 1).

The experimental design and results are represented in Table 3. The analysis of variance (ANOVA) for the variables indicated that enzyme activity can be well described by a polynomial model with a high coefficient of determination ($r^2 = 0.9571$). This result showed an excellent correlation between independent variables and amylase activity and only 4.29% of the total variations could not be explained by the model. The statistical significance of the regression model was supported by the high F-value (29.01) with very low *p*-value (< 0.0001), as summarized in Tables 4 and 5.

The significance of regression coefficients, in the equation for amylases production, was defined considering significance level of 90% ($\alpha = 0.1$). As a result, a reduced quadratic model (Eq. (2)) in terms of coded factors was fitted:

$$y = 327.42 + 37.41X_3 - 10.25X_1^2 - 11.61X_2^2 - 13.70X_3^2 - 9.89X_2X_3 \quad (2)$$

where y represents amylases activity (U/g), and X_1, X_2, and X_3 were the coded values of the test variables ammonium sulfate, potassium phosphate, and urea concentrations, respectively. The significance of each coefficient determined by Student's *t*-test and *P*-values was demonstrated in Table 5. The statistical analysis of the model showed that each variable had a significant effect on amylase synthesis: linear term (X_3), the interaction term (X_2X_3), and all the quadratic terms (X_1^2, X_2^2 and X_3^2).

The amylase enzyme synthesized by *R. oligosporus* ranged from 227.7 U/g to 361.6 U/g in the experimental design. This variation

Table 3
Experimental design for CCD with observed and predicted amylase activity for defining nutrient supplementation influence on amylolytic enzyme production by *Rhizopus oligosporus* (CCT 3762) using wheat bran as substrate. The components assessed where $(NH_4)_2SO_4$ (X_1), KH_2PO_4 (X_2), and urea (X_3).

Runs	Independent variables			Amylase activity (U/g)		
	X_1	X_2	X_3	Experimental	Predicted	Absolut error[a]
1	−1	−1	−1	244.42	244.56	−0.14
2	1	−1	−1	240.58	264.34	−23.76
3	−1	1	−1	258.38	244.56	13.82
4	1	1	−1	264.67	264.34	0.33
5	−1	−1	1	328.19	339.16	−10.97
6	1	−1	1	339.14	319.38	19.76
7	−1	1	1	312.31	339.16	−26.85
8	1	1	1	313.95	319.38	−5.43
9	−1.68	0	0	287.94	298.49	−10.55
10	1.68	0	0	320.80	298.49	22.30
11	0	−1.68	0	318.88	294.65	24.23
12	0	1.68	0	282.19	294.65	−12.46
13	0	0	−1.68	227.72	225.90	1.81
14	0	0	1.68	361.59	351.60	9.98
15	0	0	0	323.81	327.42	−3.61
16	0	0	0	324.90	327.42	−2.52
17	0	0	0	331.47	327.42	4.05

[a] Difference between experimental and predicted values by the mathematical model. Uncertainty of amylase measurement was calculated in 2%.

Table 4
ANOVA analysis of the developed model for the amylase activity.

Source	Sum of squares	Degrees of freedom	Mean square
Model	22956.72	5	4591.34
Residual	1741.04	11	158.28
Total	24697.76	16	
% explained variation: 93.0%			

Table 5
Coefficients and test of statistical significance for the activity of amylase using coded values.

Term	Coefficient	Standard error	*t*-value	*p*-value
Constant	327.42	7.09	46.18	< 0.0001
X_1	5.15	3.33	1.55	0.1662
X_1^2	−10.25	3.67	−2.79	0.0268
X_2	−4.74	3.33	−1.42	0.1980
X_2^2	−11.61	3.67	−3.16	0.0158
X_3	37.41	3.33	11.23	< 0.0001
X_3^2	−13.70	3.67	−3.73	0.0073
$X_1 X_2$	0.10	4.35	0.02	0.9818
$X_1 X_3$	1.27	4.35	0.29	0.7795
$X_2 X_3$	−9.89	4.35	−2.27	0.0572

reflects the need of medium optimization to obtain higher yields. In order to gain a better understanding of the effects of the significant factors on amylase production, the three dimensional response surface, and contour graphs were generated to show the interaction of each pair of medium composition, which provided an interpretation of the optimum experimental conditions (Fig. 1).

There is a strong influence of urea concentration on the response since the lowest enzymatic activity was obtained without this nutrient, and higher activities were produced with the highest urea concentration (1.25%) (Fig. 1c and d). Therefore, the axial points of urea are the responses of maximum and minimum activity. These results are in accordance with other reports in which urea, as medium supplementation, was the best nitrogen source for the α-amylase production by *Rhizopus microsporus* on SSF using cassava peel (Vijayaraghavan et al., 2011) and glucoamylase by *Aspergillus* sp. on SSF with wheat bran (Ellaiah et al., 2002). In the last studies cited, the authors evaluated the effect of different nitrogen sources including urea, at a fixed concentration (1%), while in the present work was presented a design to model and optimize the concentration of urea for SSF using *R. oligosporus* in which 1.25% (w/w) urea was the appropriate level.

The results showed the concentrations of 1.25% and 0.63% (center point conditions) for ammonium sulfate and monobasic potassium phosphate, respectively, as the best for the production of amylolytic enzymes (Fig. 1a and b). In addition, higher levels of potassium phosphate decreased amylase production (Fig. 1e and f). The observed decrease in amylase production when a high level of potassium phosphate was used (Fig. 1e and f) match with previously described. Literature reports show that the production of enzymes may be limited by the high phosphorus concentration in the culture medium, such as the case of the manganese-dependent peroxidase from *Phanerochaete chrysosporium* in a medium of submerged culture (Liang et al., 2012). Accordingly, a high phosphate concentration was responsible for inhibition of amylases activity of *B. amyloliquefaciens* (Gupta et al., 2003).

3.2. Validation of optimal chemical composition of nutrient solution

Maximum amylase activity (361.6 U/g) was observed in experimental treatment 14 (0, 0, +1.68) with medium containing in w/w: 1.25% $(NH_4)_2SO_4$, 0.63% KH_2PO_4, and 1.25% urea. The adequacy of the model was examined by an additional experiment using the derived optimal conditions. The higher predicted response was 351.6 U/g and the experimental response was 346.8 ± 4 U/g. This revealed more than 98% of the predicted value, which indicated that the generated model gave an adequate prediction. The statistical optimization for amylase production was effective in predicting the fermentation profiles as a function of different salts content with a high coefficient of determination.

As the amylase activity increased with lower concentrations of potassium phosphate, and the bioprocesses requires an economical medium with the lowest concentrations of nutrients, an experiment was conducted comparing two solutions with the following concentrations of ammonium sulfate, potassium phosphate, and urea: (1) 1.25%, 0.63%, and 1.25% − coded values: (0, 0, +1.68) and (2) 1.25%, 0.25%, and 1.25% − coded values: (0, −1, +1.68), in four replications.

The activity of 386.36 ± 4.79 was obtained on the test with the salt solution 1 and 393.00 ± 3.47 with the salt solution 2. The analysis of variance showed there was no difference between the averages of salt solutions ($p > 0.01$), so the salt solution 2 with lower potassium phosphate was selected as standard. This experiment showed that is possible to obtain a large amount of amylase using a concentration range between 0.25 and 0.63% of potassium phosphate, giving versatility to the process.

Soccol et al. (1994) reported the combination of 9.3% (w/w) $(NH_4)_2SO_4$, 4.8% KH_2PO_4, and 2.3% urea as a nutrient solution to produce 108 U/g glucoamylase using *R. oryzae* 28627 in SSF and raw cassava as substrate. In this study, reduction in these nutrients concentration of 86.6, 94.8, and 45.7%, respectively, triples the enzyme activity of an extract produced by SSF with wheat bran. This fact demonstrates an inhibitory effect on the glucoamylase synthesis by *R. oligosporus* when relatively high concentrations of these salts were used, probably due to the increase in osmotic balance between the fungal cells and culture medium.

The amylase activities of crude aqueous extracts were significantly higher than the values from solid state fermentations (Peixoto-Nogueira et al., 2008; Vijayaraghavan et al., 2011) and submerged fermentations (Freitas et al., 2014; Hashemi et al., 2015) using agro-industrial wastes as substrate. These results demonstrate that the processes proposed in this work were superior to others published using bacteria or fungi as a biological agent.

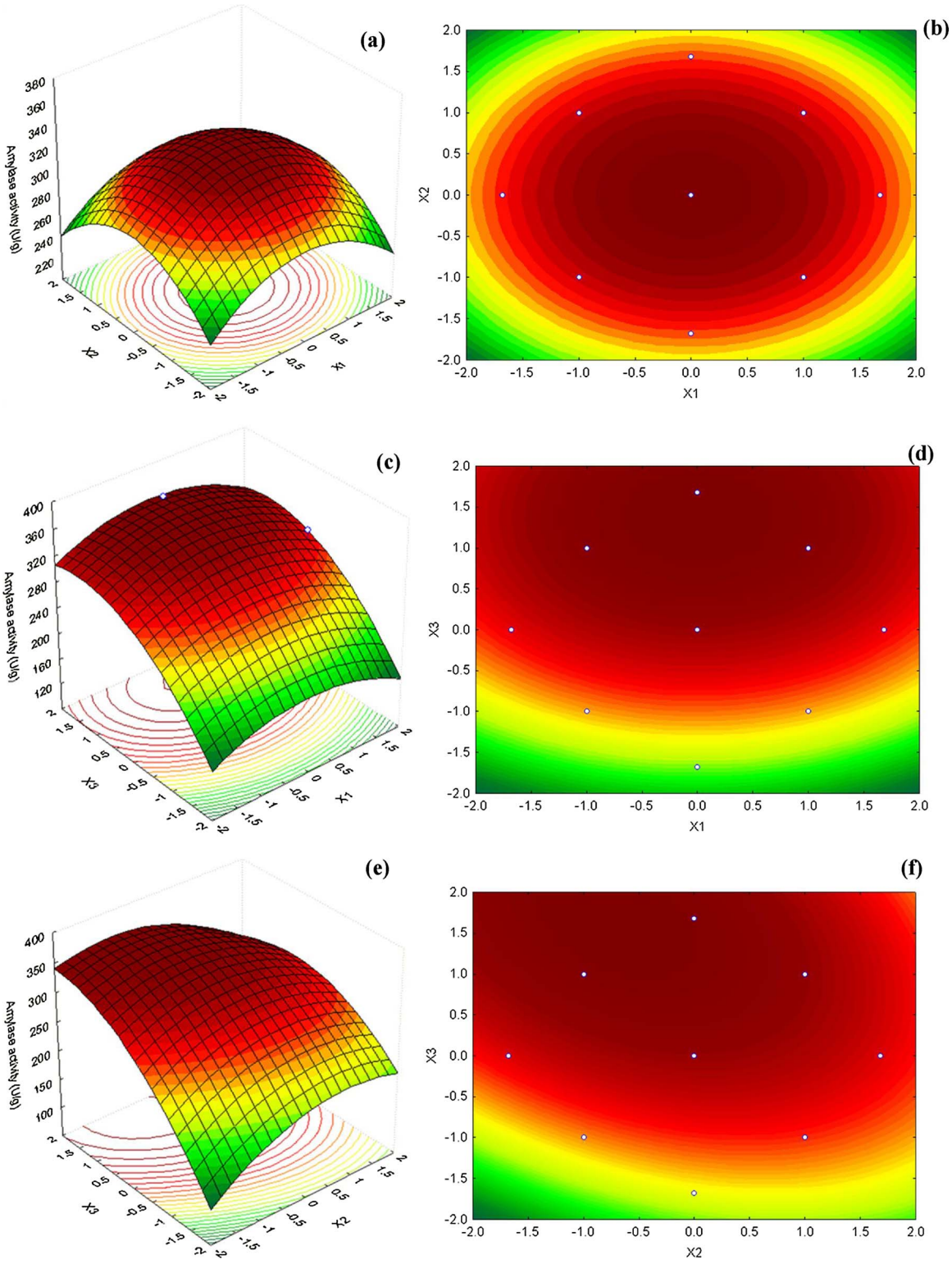

Fig. 1. Response surface plot and contour plot of interactions of variables on amylase activity (U/g): (a) and (b) X_1 and X_2; (c) and (d) X_1 and X_3; (e) and (f) X_2 and X_3. X_1: ammonium sulfate; X_2: potassium phosphate; X_3: urea.

3.3. PLS data analysis

Although partial least square (PLS) regression technique is one of the most published algorithms for multivariate calibration in different fields of knowledge, this is probably the first study which utilizes PLS to establish a relation between chemical elements in culture medium and amylase production in solid state fermentation by fungi. The choice was based on the suitability of using PLS as a tool to analyze multivariate datasets together with multiple targets (Procopio et al., 2013).

Two main components were required to satisfactorily perform modeling with a correlation coefficient of 0.89. Due to the natural variety of biological systems, models for protozoa and fungi allow approximate predictions ($0.8124 < r < 0.8944$) so that is considered suitable when the overall correlation coefficient is in the range of 0.89.

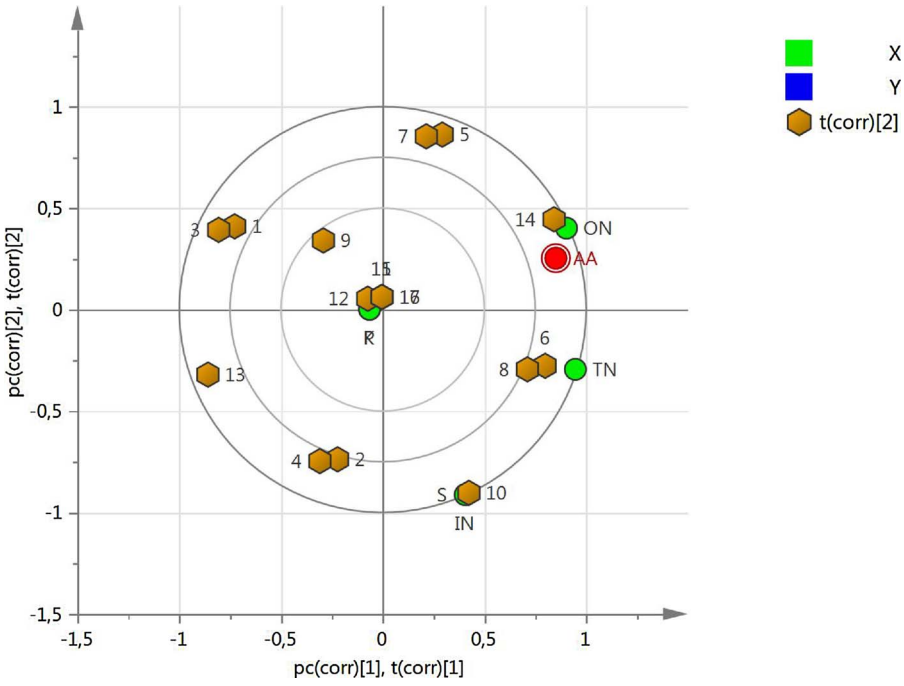

Fig. 2. Biplot graph for Nutrients-Amylase activity PLS model. Definition of model terms. *AA*: amylase activity; *TN*: total nitrogen; *ON*: organic nitrogen; *IN*: inorganic nitrogen; *P*: total phosphorus; *K*: total potassium; *S*: total sulfur. Numbers represent samples included in CCD.

(Reeves et al., 2000; Zornoza et al., 2008).

The amylase activity was directly influenced by the organic nitrogen content added to the SSF medium as represented in Fig. 2. The increased production of amylase shows a positive correlation with the highest amount of organic nitrogen added to the medium as urea, corresponding to the trial of number 14 from experimental design (Table 3).

The statistical importance of independent variables in the model in relation to dependent variables is corroborated by the variable importance in the projection (VIP) values. The effect analysis of each nutrient added in the formulation of culture medium for *R. oligosporus* showed that inorganic nitrogen, phosphorus, and sulfur were indifferent to the expression of amylase by the fungus. Whereas the organic nitrogen content positively influences the production of this enzyme. The amount of total nitrogen was also favorable since in this experiment the sum of the amounts of organic and inorganic nitrogen was regarded (Fig. 3).

In the present work, urea showed stronger influence, followed by $(NH_4)_2SO_4$ in this enzyme expression. Different nitrogen sources lead to higher amylase expression (Kammoun et al., 2008), according to the results obtained. This supports the use of these nitrogen sources to the formulation of SSF culture medium in this work, mainly urea, as mentioned in PLS data analysis, which is an organic nitrogen source.

3.4. Enzymatic hydrolysis and glucose production

Hydrolysis profile with crude amylolytic extract from *R. oligosporus* was similar in both tested temperatures. A gradual increase in conversion yield of substrates assessed for reducing sugars up to 7 h was observed, reaching 75.1 and 79.0% of pure starch conversion for hydrolysis at 55 and 60 °C, respectively. After 11 h of reaction, the yield was 81.1% at 55 °C and remained constant (78.8%) at 60 °C. In comparison, only in the first 2.5 h, the hydrolysis profile by commercial glucoamylase at 60 °C was higher than amylase from the studied fungi, evaluated at the same temperature. After this period, the commercial enzyme had lower yields than those of the enzyme of this study ($p < 0.01$), with a maximum yield of 75.0% after 11 h (Fig. 4). In addition, glucose productivity in hydrolysis using different protocols were compared and described in Fig. 5. The amylases from *R. oligosporus* showed higher productivity than commercial amylase. This fact suggests the amylases in this study are competitive with commercial amylases. The productivity of commercial glucoamylase (6.2 g L^{-1} h^{-1}) was lower than amylase of *R. oligosporus,* which was 6.5 and 7.1 g L^{-1} h^{-1} at 55 and 60 °C, respectively, in 6 h of reaction.

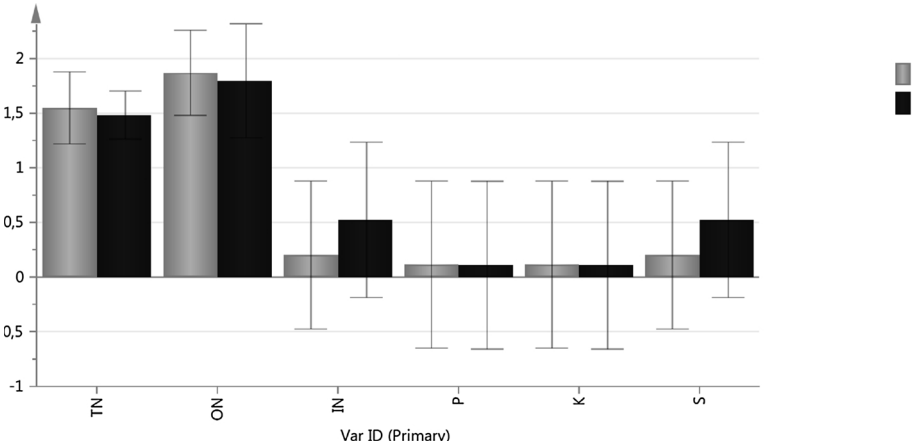

Fig. 3. Variable importance in the projection (VIP) values for Nutrients-Amylase activity PLS model in both principal components. Definition of model terms *TN*: total nitrogen; *ON*: organic nitrogen; *IN*: inorganic nitrogen; *P*: total phosphorus; *K*: total potassium; *S*: total sulfur.

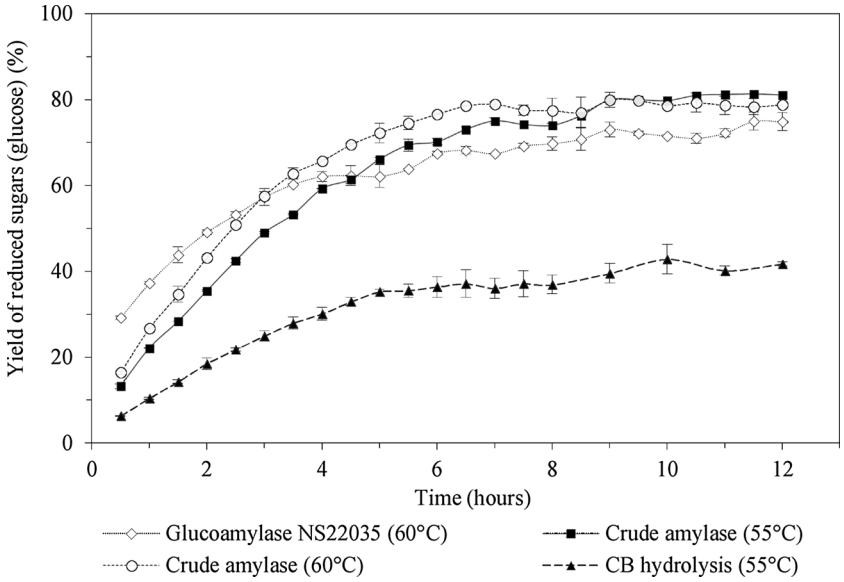

Fig. 4. Starch hydrolysis using commercial and produced amylases. 10% (w/v) cassava bagasse (CB) hydrolysis using crude enzyme extract from *Rhizopus oligosporus* (▲). 5% (w/v) soluble starch hydrolysis: a comparison of crude enzyme extract from *Rhizopus oligosporus* at 55 °C (■), 60 °C (○) and the commercial glucoamylase NS22035 60 °C (◊). The *error bars* represent the standard deviations (n = 3).

The efficiency of releasing sugars by mixing α-amylase and glucoamylase is known to be much higher than that using only one enzyme (Chen et al., 2015). *Rhizopus* species are also known to produce α-amylase and glucoamylase (Soccol et al., 1994; Han et al., 2003; Peixoto-Nogueira et al., 2008; Vijayaraghavan et al., 2011). The *R. oligosporus* strain used in this work produces glucoamylase (Del Re et al., 2003). Freitas et al. (2014) obtained just glucose as a final product during starch hydrolysis with glucoamylase produced by the same microrganism. In accordance, *R. oligosporus* has been reported as good glucoamylase producer (Jin et al., 1999; Jin et al., 2002; Freitas et al., 2014), which was purified and characterized by Kareem et al. (2011) and Kareem et al. (2014).

Freitas et al. (2014) have justified the application of *R. oligosporus* CCT 3762 amylase from submerged fermentation for the production of glucose from starch due to the high yield and product quality. The authors obtained the productivity of 9.7 g L^{-1} h^{-1} in 4 h of hydrolysis, similar to the one obtained in this work (9.1 g L^{-1} h^{-1}) with the same reaction time but with amylase from solid fermentation. In addition, this enzyme showed catalytic performance competitive with commercial glucoamylase. Therefore, the use of amylolytic extract from this study suggests a simple and widely applicable method for the hydrolysis of starch residues such as bagasse cassava. According to Cinelli et al. (2015), the costs of starch-based ethanol production can be reduced if starch hydrolysis is performed at temperatures below its gelatinization temperature. In order to reduce energy demand, the temperature selected for 10% cassava bagasse hydrolysis was 55 °C since the pure starch conversion was greater than 80% using the *R. oligosporus* amylase at this temperature.

The crude amylolytic extract was used for the cassava bagasse hydrolysis and obtaining CBH. After 6.5 h of reaction, 37% of the dry mass of the residue was converted into reducing sugar, reaching 42.82% after 10 h of reaction. Furthermore, after hydrolysis, 54.38% of the initial mass remained as solid waste from cassava bagasse. Hydrolysis yields may be different for each region since the starch content in the substrate can vary. Cassava bagasse contains about 30–50% of dry weight basis due to the processing techniques and crop varieties (Pandey et al., 2000). In spite of this, this work proposes a novel method for scaled hydrolysis of cassava bagasse in 3-L bioreactor, with which it is possible to obtain a yield of 42.82% of reducing sugars in 10 h of reaction using only the crude extract of *R. oligosporus* in the proportion of 15 U/g dry cassava bagasse.

The crude enzymes from *R. oligosporus* produced in the present work reached a rapid hydrolysis of cassava bagasse showing high starch conversion efficiency in glucose. The applicability of this method is supported by the absence of the need for investment in downstream steps, such as purification and concentration, and process robustness.

Among about 3000 species of plants, animals, microbes that contain cyanide, cassava is a food crop which produces cyanogenic glucosides that are stored in plant vacuoles (Gupta et al., 2010). Plants use cyanide as a defense mechanism to deter predators (Bradbury et al., 2011). High cyanide content is found in wastes as cassava peels (Ogbo, 2010) and wastewater (manipueira) (Maróstica and Pastore, 2007). Despite the

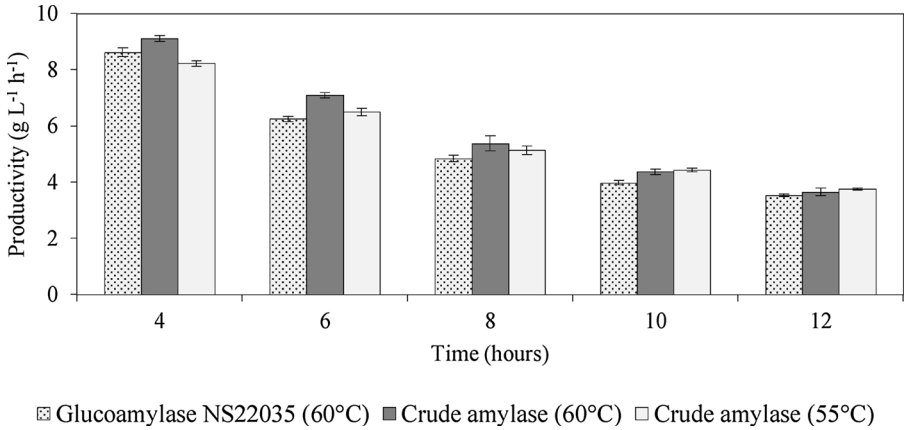

Fig. 5. Glucose productivity of starch hydrolysis: a comparison of crude enzyme extract from *Rhizopus oligosporus* at 55 °C or 60 °C and the commercial glucoamylase NS22035 at 60 °C. The *error bars* represent the standard deviations (n = 3).

cassava bagasse be considered free cyanide (Pandey et al., 2000) it is known that some cyanide may still remain in small proportions (Glanpracha and Annachhatre, 2016). Cyanides can be removed from industrial wastes by biodegradation, physical, and chemical methods, for example, evaporation, thermal treatment (Gupta et al., 2010), and wetting (Bradbury et al., 2011). The bioprocess proposed assures recovery and fermentability of a cassava residue even with a presence of residual cyanide. The steps of heating and evaporating the broth can eliminate by volatilization the possible presence of cyanide.

Shi et al. (2014) studied a cassava bagasse hydrolysis with three enzymes, α-amylase (15 U/g), glucoamylase (200 U/g), and cellulase, totalizing 28 h of reaction to achieve 45.7% of total sugars coming from the starch and cellulose. A cassava hydrolysis yield of only 16.2% expressed in percentage of reducing sugars released was obtained in 4.5 h of reaction using the steps of gelatinization, liquefaction with α-amylase and saccharification with glucoamylase, and yield of 33% when associated with cellulase in a total of 52.5 h (Chen et al., 2015). According to these results and considering that just one crude extract of amylases from *R. oligosporus* was used in this work, we can conclude these amylases of this strain is very efficient in starch residues hydrolysis.

3.5. Ethanol production

After cassava bagasse hydrolysis, CBH was concentrated to 10.8% (w/v) reducing sugars to be used for ethanol fermentation. Fermented must (wine) showed ethanol concentration of 3.95 ± 0.03% (v/v) obtained after 12 h of fermentation using *Saccharomyces cerevisiae* at 32 °C. Ethanol efficiency (calculated based on consumed sugars and theoretical yield) were 89.20 ± 0.68%. In this sense, this bioprocess represents a potential production of 247.32 L ethanol per ton of cassava bagasse on a dry basis.

The ethanol efficiency under consideration was significantly higher than the values derived from 72 h of simultaneous saccharification and fermentation process using corn and cassava flour, recently published (Białas et al., 2010; Nguyen et al., 2014). In addition, Cinelli et al. (2015) reported several studies on ethanol production by starch hydrolysis of raw material from cassava and corn. They obtained a conversion efficiency of 84.6–89.6% in more than 48 h. In the present study, there was more than 89% of the ethanol efficiency using a fast method of cassava bagasse hydrolysis in just 12 h fermentation, and a total period of 24 h for cassava bagasse hydrolysis and alcoholic fermentation. The amylolytic extract optimized in this work enables the rapid hydrolysis of starch with higher productivity than commercial glucoamylase, and conversion of complex wastes to fermentable sugars which can be used for an efficient production of ethanol.

Considering spending on raw materials, Table 6 shows the costs of producing ethanol from cassava bagasse hydrolyzed in comparison with the sugarcane. The sugarcane price (US$ 20.51/ton) was calculate according to UNICA (2015) data, and for cassava bagasse, the price considered in the present work was R$ 100.00 or almost US$ 31.25/ton (dry basis), estimated in accordance with the traded value of the producer's milk in the Paranapanema Valley region (São Paulo State, Brazil). The value of this by-product in dry basis could be cheaper (US$ 9.38/ton) if were not increased by the transportation value (DBO, 2017). Considering just the cost of feedstock, ethanol from cassava bagasse (US$ 0.13/L) is two times cheaper than ethanol from sugarcane (US$ 0.26/L). In studies using only commercial amylolytic enzymes for the cassava bagasse hydrolysis, the cost of ethanol, based on the raw material price and conversion yield, was similar or higher than ethanol from sugarcane. This confirms the superiority of the treatment with amylase produced by *R. oligosporus* compared to previous studies. Similar values to the present study were reported only in treatments with commercial cellulase, α-amylase and glucoamylase (Table 6).

Cassava root represents 83% of the costs in the tapioca starch industry (Chavalparit and Ongwandee, 2009). Therefore, the total use of this biomass received by the industry, including generated residues, is important for the industrial economy balance. In addition, the use of this agro-industrial waste as a carbon source for another process helps to solve the pollution problems. Each ton of cassava contain 211.2 kg of native starch and after industrial process 115.5–132.0 kg (Chavalparit and Ongwandee, 2009) or 150.0 kg (Pandey et al., 2000) is generated as solid waste. In addition, if considered the cassava bagasse generated in Brazil in 2014 (about 3.5 million tons, dry basis), it would have the potential to produce 866.6 million liters of additional ethanol, corresponding an increase of 3.04% of total ethanol production in Brazil during the same period, which was 28.48 billion liters, according to UNICA (2014).

Sugarcane is the most economical feedstock for ethanol production in the world (Baeyens et al., 2015). The ethanol price was US$ 0.39/L in the 2014/2015 harvest in Brazil (UNICA, 2015) and also achieved similar prices in the United States (US$ 0.37-0.45/L − New York and Chicago market) in 2015 (ANP, 2016). This value indicates that cost of feedstock accounts for 66.7% of the production cost of fuel ethanol. Cassava bagasse is a by-product of the agroindustry with low added value. Therefore, the use of this biomass as raw material to produce ethanol can be an economic alternative (Table 6). In addition, cassava was previously reported as a higher annual yield of bioethanol when compared with any other crop including sugarcane, sweet sorghum, and maize (Okudoh et al., 2014). Additionally, the processing of sugarcane results in 30% of bagasse with 50% moisture, which is used in the generation of electricity (Yadav and Solomon, 2006). In the present work, after the hydrolysis of cassava bagasse, the amount of the initial mass remained as solid waste (54,38%), which can be used for electricity or other purposes, was a higher value than sugarcane residue.

4. Conclusions

This study reported a fast and economical bioprocess for the optimized production of amylases from a natural and safe microorganism

Table 6
Comparison of ethanol production costs from cassava bagasse (CB − dry basis) and sugarcane considering spending on raw materials.

Source	Hydrolysis treatment [a]	Sugar[b] Kg/ton	Ethanol[c] L/ton	Source price US$/ton	Ethanol cost US$/L	Reference
Cassava bagasse	Fungal amylase (crude extract)	428.20	247.32	31.25	0.13	This study
Cassava bagasse	α-amylase + glucoamylase	162	93.57	31.25[d]	0.33[e]	Chen et al. (2015)
Cassava bagasse	Cellulase + α-amylase + glucoamylase	330	190.59	31.25[d]	0.16[e]	Chen et al. (2015)
Cassava bagasse	Cellulase + α-amylase + glucoamylase	457	263.94	31.25[d]	0.12[e]	Shi et al. (2014)
Cassava bagasse	Glucoamylase	206	118.99	31.25[d]	0.26[e]	Rattanachomsri et al. (2009)
Sugarcane	–	136.58	79.59	20.51	0.26	UNICA (2015)

[a] Amylase produced by *R. oligosporus* in optimized conditions for this study and commercial enzymes for the other References.
[b] Calculated as the total reducing sugar for CB and total recoverable sugar for sugarcane.
[c] Calculated based on the conversion of sugar into ethanol using the coefficient 0.6475 with 89.2% yield for CB and 90% yield for sugarcane.
[d] The same price of CB used in this study was adopted for the calculation with reference data.
[e] Calculated in this study.

using wheat bran supplemented with 1.25 (% w/w) $(NH_4)_2SO_4$, 0.25 (% w/w) KH_2PO_4, and 1.25 (% w/w) urea. The amylolytic complex containing glucoamylase presented competitive performance based on commercial glucoamylase. This crude extract was able to hydrolyze cassava bagasse to fermentable sugars in a fast and efficient hydrolysis. A cassava bagasse hydrolysed with high glucose content was obtained and was used as only carbon source for more economical ethanol production than sugarcane ethanol.

This work proposes a new alternative for the low cost ethanol production intercropped with food production, without any competition between them. Developing countries could work in this direction in order that, besides the starch and their derivatives to meet the demand in the food sector, the solid residue could be used to improve the fuel ethanol production, contributing to supply the demand for renewable fuels in the world.

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