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A N I N T E R N A T I O N A L J O U R N A L

Cassava Pulp Enzymatic Hydrolysate as a Promising Feedstock for Ethanol Production

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ABSTRACT

The aim of this study was to produce bioethanol from enzymatic hydrolysates of cassava pulp, a by-product of cassava flour manufacturing, using an alcohol-tolerant Saccharomyces cerevisiae strain. First, the best operational condition of the starch hydrolysis process was determined through a complete factorial design (2^4), with triplicates at the central point. The independent variables evaluated were: the concentrations of α-amylase (Termamyl 2X) and glucoamylase (AMG 300L) and both liquefaction and saccharification times. The most favorable hydrolysis condition in the assay was achieved using 0.517 mL AMG.g starch^{-1} and 0.270 mL Termamyl.g starch^{-1}, with liquefaction and saccharification times of 1 and 2 h, respectively. The broth obtained at this hydrolysis condition contained a high glucose concentration (160 g.L^{-1}). Once the best reaction conditions were determined, fermentation tests were carried out in a 3 L bioreactor, in a batch system, at 30 °C, 100 rpm and pH 5.5, using 3 g.L^{-1} (dry biomass) of yeast as inoculum. After 24 h of fermentation, an ethanol concentration of 68 g.L^{-1} was obtained, with 0.48 ethanol yield and 2.83 g.L^{-1}.h^{-1} productivity. These results indicate the potential use of cassava pulp, a by-product of cassava flour industries in Brazil, as a raw material for bioethanol production.

Keywords: Starch waste, Enzymatic hydrolysis, Alcoholic fermentation

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INTRODUCTION

In Brazil, majority of the fuel ethanol is produced by the fermentation of sugarcane. However, other raw materials such as agro-industrial waste rich in cellulosic fibers and starch might be used as well. The use of waste for ethanol generation can stabilize the price of biofuels compared to gasoline and reduce the potential environmental impacts associated with them. An agro-industrial waste, quite abundant and little explored to this end is the solid by-product of cassava starch and flour processing (cassava bagasse or pulp), which has a large amount of starch. Cassava (*Manihot esculenta* spp. *esculenta*) is a root crop with a high starch content of up to 90% (dry weight) and grows well on infertile land with minimal input of chemicals, such as fertilizers, herbicides and insecticides, making it one of the cheapest and most sustainable agro-based feedstocks[1]. Cassava is native to South America and has been probably taken to other continents by Europeans after the colonization of America[2]. Cassava shows a high tolerance to drought and infertile soils and presents the highest production of starch per hectare in the world, because of which cassava cultivation is practiced in the less developed countries[3]. It is mainly cultivated by smallscale farmers in Africa, Latin America, and Asia, with a total farming area of over 18 million hectares[1]. Among the countries that cultivate cassava, Brazil ranks the second place (with 7.7% of world production) after Nigeria (19.5%)[3]. Cassava can be classified into bitter or *brava* (toxic) and sweet or *mansa* (cyanide free), according to the concentration of its cyanide content, a harmful substance to the human health. In general, the bitter is used industrially whereas the sweet is used in the homemade food. Generally, for each ton of processed cassava, an average of 150 kg of cassava pulp (dry basis) and 6 kg of bark are produced. These solid wastes are often discarded at landfill sites without any form of treatment, which raises concerns about its disposal in the environment[4]. Cassava pulp contains approximately 40-70% starch that cannot be physically extracted and 11.5% fibers[5]. Thus, at a time of sustainable development, the use of agro-industrial waste rich in polysaccharides, which are not fermentable directly, has been a potential alternative to produce ethanol from an abundant and low commercial value biomass[6]. To recycle the high concentration of starch in the cassava pulp, the hydrolysate of this waste has been used as a substrate in microbial processes to manufacture higher value-added products[4]. Since there are starch manufacturers near the alcohol distillery, cassava pulp can also be used for ethanol production, mainly fine alcohol which has a specific market[7]. Moreover, this alcohol might be utilized to generate hydrogen for fuel cells, which in turn generates electricity. Hydrogen may become a promising energy source of the global energy matrix within a few decades, after overcoming certain technological barriers. Starch is a macromolecule which is non-fermentable by yeast, thus requires to be hydrolyzed through chemical or enzymatic processes. Enzymatic hydrolysis is the most widely used process owing to its low energy consumption, high yield, selectivity and high operating flexibility[8]. However, there are some limitations regarding the industrial use of enzymes, including high initial investment, enzyme cost, need of specialized professionals and sophisticated laboratories[9, 10]. Several publications have focused on the synergic action of enzymes in the enzymatic hydrolysis processes, mainly on those involving endo- and exoamylases, such as α-amylases and glucoamilases, α-amylases and glucosidases, α-amylases and maltases, amyloglucosidases and pullulanases, β-amylases and pullulanases, and other blends of commercial enzymes. Acting together, each endocatalytic event increases the number of substrate sites for exoamylases, leading to an enhanced conversion rate[10]. In the enzymatic conversion of starch where α-amylase and amyloglucosidase act synergically, the key steps are liquefaction and saccharification.

During liquefaction at 90-100 °C, α-amylase hydrolyzes the α-(1,4) glycosidic bonds in the starch, leading to the rapid reduction of the gel viscosity and the production of

maltodextrins which are further saccharified by amyloglucosidase, generating a variety of sugars[11]. *Saccharomyces cerevisiae* does not produce α-amylase and glucoamylase, the necessary enzymes to hydrolyze starch, thus hydrolysis of the substrate is required before the alcoholic fermentation[9]. Production of ethanol from starch can be carried out by means of two processes, namely, separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF)[12]. In SSF, the simultaneous addition of amylolytic enzymes and microorganisms hinders the establishment of ideal conditions for the alcohol production. Therefore, the main advantage of a separate process (SHF) is the possibility of working with optimized conditions in the both enzymatic hydrolysis and the fermentation steps[12]. The aim of this study was to establish the appropriate conditions for the enzymatic hydrolysis of starch from cassava pulp and the production of ethanol from this hydrolysate using an alcohol-tolerant strain of yeast *Saccharomyces cerevisiae* Hansen BY4741, in order to determine a condition that provides higher yields.

MATERIALS AND METHODS

Materials

Cassava pulp (CP) was provided by Halotek-Fadel Industrial Ltda, Palmital, Brazil and was used after being crushed in a hammer mill. The analysis of two batches of cassava pulp with 10.1% moisture content showed the following average composition: 54.4% ($w.w^{-1}$) starch, 1.65% ($w.w^{-1}$) ash, 1.55% ($w.w^{-1}$) protein and 0.16% ($w.w^{-1}$) fatty matter. The two enzymes used in this study were kindly provided by Novozymes. The Termamyl 2X used in the liquefaction is a thermostable α-amylase produced by *Bacillus licheniformis* with optimum pH and temperature of 6-8 and 90-105 °C, respectively. In the saccharification process, AMG 300L (Amyloglucosidase) was used which was produced by a strain of *Aspergillus niger* with an optimum pH and temperature of 4-4.5 and 58-60 °C, respectively. The microorganism used was an alcohol-tolerant strain of *Saccharomyces cerevisiae* Hansen BY4741, obtained from the Chemistry Institute of the Federal University of Rio de Janeiro (Universidade Federal do Rio de Janeiro - UFRJ).

Enzymatic hydrolysis experiments using complete factorial design

The starch hydrolysis process was determined through a complete factorial design (2^4), with triplicates at the central point. The selected independent variables were the concentration of the enzymes α-amylase (Termamyl 2X) and glucoamylase (AMG 300L) and the liquefaction and saccharification times. Each variable was evaluated at two different levels listed in Table 1. Statistical analysis of the experimental data was performed by the *Statistica software*, with 95% confidence[13].

Table 1 Actual and coded values used in complete factorial design (2^4) of hydrolysis reactions

Factor	1st phase-Liquefaction (100 °C)		2nd phase-Saccharification (60 °C)	
Level	A: [Termamyl] (mL.g starch^{-1})	B: Liquefaction time (h)	C: [AMG] (mL.g starch^{-1})	D: Saccharification time (h)
-1	0.270	1	0.110	2
0	0.477	2	0.313	3
1	0.683	3	0.517	4

The hydrolysis experiments were performed in 250 mL Erlenmeyer flasks containing 30 g CP with the solid:liquid ratio of 1:3. The flasks were heated for 15 minutes at 120 °C to improve the availability of the starch macromolecules (amylose and amylopectin) for the enzymatic action. In the liquefaction phase, the pretreated CP was mixed with an enzyme solution of Termamyl 2x (0.270 to 0.683 mL.g starch^{-1}) and maintained at 100 ºC for 1, 2 and 3 h. This mixture was then acclimatized at 60 °C and saccharified by AMG 300L (0.110 to 0.517 mL.g starch^{-1}) for 2, 3 and 4 h. The CP hydrolysates were filtered to remove unhydrolyzed starch and other fibrous materials.

Ethanol fermentation in bioreactor

The strain was cultivated on YEDP (Yeast Extract Dextrose Peptone) agar slants and incubated for 72 h at 32 ºC. The slants were then kept in a refrigerator at 4 °C. The inoculum was grown in Erlenmeyer flask containing 100 mL YEDP medium at 30 °C with continuous shaking at 100 rpm for 12 h, centrifuged (3000 rpm for 15 min) and finally standardized to 3 g.L^{-1}. Cassava pulp hydrolysate was frozen to avoid gelatinization of the starch and the possible contamination. The fermentation broth was prepared using 1 L of hydrolysate enriched with yeast extract (1%) and meat peptone (1%), added with Tween 80 as an antifoaming agent at a concentration of 0.05%. Fermentation studies were carried out in a bioreactor (Biostat A Plus-Sartorius) using a 3.0 L vessel (working volume of 1.0 L) equipped with pH, temperature, and agitation controls. The bioreactor was operated in a batch system under agitation rate of 100 rpm, at 30 °C and pH of 5.5 controlled by addition of 2 M NaOH or 1 M H_2SO_4. After inoculation, fermentation process lasted up to 24 h and samples were withdrawn every two hours to measure cell, glucose, and ethanol concentrations.

Determination of concentration of cells, substrate, and product

Cell growth was estimated by measuring optical density of the culture at 570 nm (OD_{570}). The results were used to generate yeast growth curves, from which dry cell weight (DCW) was estimated in the culture solution. A good linear relationship was obtained between DCW and OD_{570}. The DNS (dinitrosalicylic acid) method[14] was used for the determination of total reducing sugar (TRS) and starch content after acid hydrolysis[12]. Glucose and ethanol were analyzed by High-Performance Liquid Chromatography (HPLC) using *Waters Chromatographic* system equipped with W600 pump and Biorad HPX 87C column (300 mm x 7.8 mm), and were detected based on 2014 refractive index. The column was maintained at 80 °C and samples were eluted by Milli-Q water at a flow rate of 0.6 mL.min^{-1}. All samples were filtered through a membrane (pore size = 0.22 µm) connected to a vacuum filtration system. Quantification of ethanol and glucose was done by comparison with external standards.

RESULTS AND DISCUSSIONS

Hydrolysis of Cassava Pulp

The starch content obtained in this study was 54.4% w.w^{-1}, a value similar to that (50% w.w^{-1}) reported by Kosugi et al.[15]. Based on this value, these authors considered cassava pulp to be a competitive and potential source of raw material for ethanol production compared to the dried cassava root. Moreover, these values are similar to those reported by Saito and Cabello[16] who analyzed the same material used in this study. The best experimental conditions for CP enzymatic hydrolysis were selected through the

complete factorial experimental design (2^4), using the concentration of total reducing sugars as the dependent variable (Table 2).

Table 2 Complete factorial design (2^4) of four independent variables with three center points showing the actual and coded values along with the dependent variable (total reducing sugars-TRS).

Essay	Codified variable				Actual variable				Dependent variable
	A	B	C	D	[Termamyl] ($mL.g^{-1}$ starch)	Liquefaction time (h)	[AMG] ($mL.g^{-1}$ starch)	Saccharification time (h)	TRS ($g.L^{-1}$)
1	-1	-1	-1	-1	0.270	1	0.110	2	165.7
2	1	-1	-1	-1	0.683	1	0.110	2	169.4
3	-1	1	-1	-1	0.270	3	0.110	2	146.0
4	1	1	-1	-1	0.683	3	0.110	2	165.3
5	-1	-1	1	-1	0.270	1	0.517	2	186.5
6	1	-1	1	-1	0.683	1	0.517	2	192.4
7	-1	1	1	-1	0.270	3	0.517	2	156.0
8	1	1	1	-1	0.683	3	0.517	2	182.0
9	-1	-1	-1	1	0.270	1	0.110	4	167.2
10	1	-1	-1	1	0.683	1	0.110	4	183.5
11	-1	1	-1	1	0.270	3	0.110	4	152.7
12	1	1	-1	1	0.683	3	0.110	4	170.1
13	-1	-1	1	1	0.270	1	0.517	4	182.0
14	1	-1	1	1	0.683	1	0.517	4	183.5
15	-1	1	1	1	0.270	3	0.517	4	177.2
16	1	1	1	1	0.683	3	0.517	4	192.1
17	0	0	0	0	0.477	2	0.313	3	179.8
18	0	0	0	0	0.477	2	0.313	3	173.5
19	0	0	0	0	0.477	2	0.313	3	170.1

The lowest sugar content was obtained in the tests 3, 7 and 11 which have a lower enzyme concentration in common in the liquefaction step. Indeed, the estimation of the effects of the independent variables on the concentration of total reducing sugars indicated that TRS was significantly influenced by the concentration of the enzymes α-amylase (Termamyl 2X) and glucoamylase (AMG 300L) as well as the liquefaction time, as shown in the Pareto chart (Fig. 1a). The surface response showed that TRS increased linearly with the concentration of enzymes (Fig. 1b) in the selected experimental conditions. The ANOVA data with 95% confidence interval ($p<0.05$) are presented in table 3. The statistical parameters $F_{calculated}/F_{tabulated}$ ratio was higher than 1, thus, the mathematical model with significant coefficients ($p<0.5$) can be described by the fitted model.

Table 3 ANOVA for enzymatic hydrolysis evaluation.

Source of variation	Sum of Squares	df	Mean Square	$F_{calculated}$	$F_{tabulated}$	p
$C_{Termamyl}$	689.1	1	689.1	18.77	4.41	0.0025
$t_{liquefation}$	492.8	1	492.8	13.43		0.0064
$C_{Amyloglucosidase}$	1085.7	1	1085.7	29.58		0.0006
Residual	293.6	8	36.7			
Total SS	2972.8	18				

df- degree of freedom

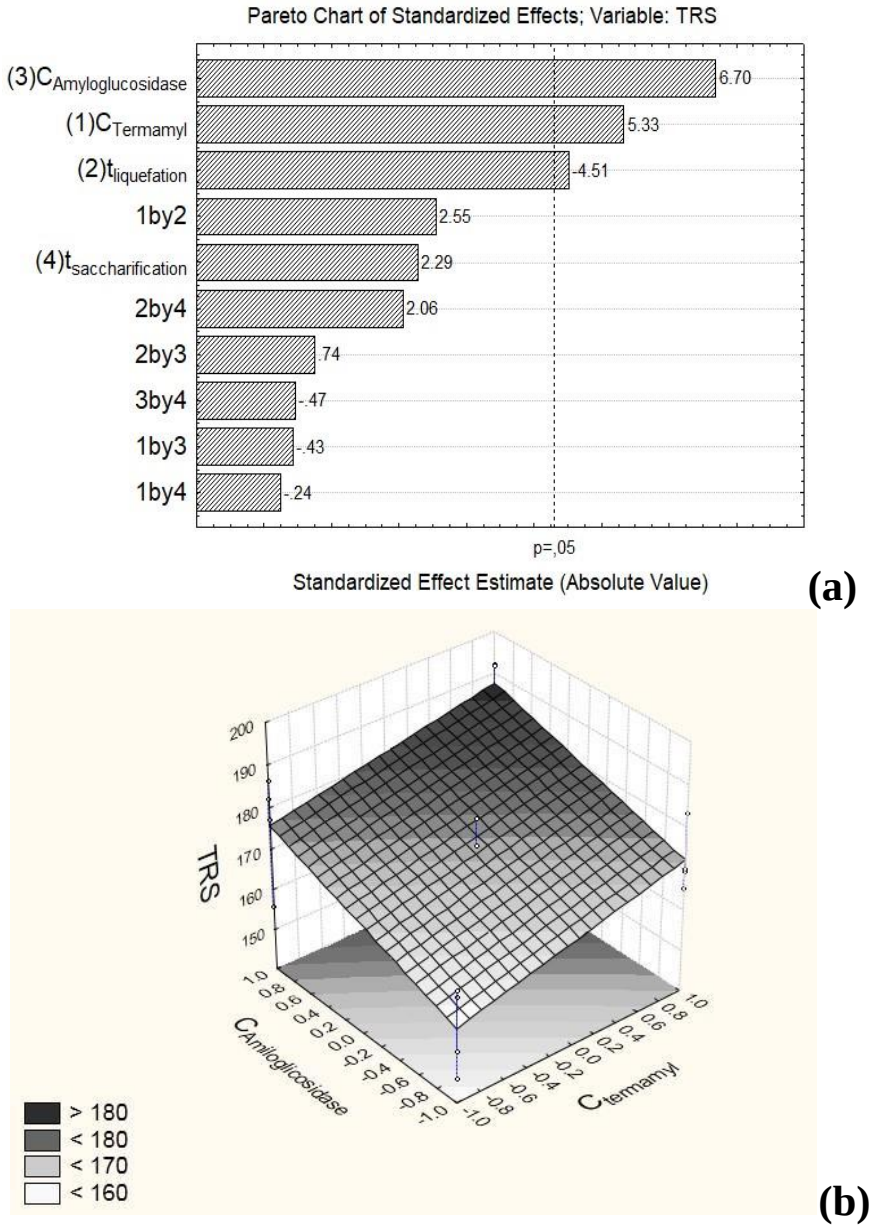

Figure 1 (a) Pareto chart of standardized effects for cassava pulp enzymatic hydrolysis for the 2^4 full factorial design. The point at which the effect estimates were statistically significant (at $p \leq 0.05$) is indicated by the broken vertical line. **(b)** Response surface showing the effect of enzyme concentrations. Dependent variable: concentration of TRS ($g.L^{-1}$).

The highest concentration of reducing sugars (192.4 TRS $g.L^{-1}$**)** was obtained when the enzyme concentrations were highest (assay 6). However, by taking into account the high cost of enzymes, the assay condition 5 which yielded 186.5 $g.L^{-1}$ TRS was selected. The concentration of AMG was maintained at its maximum level (0.517 mL AMG.g^{-1} starch) whereas the other parameters were set at their lowest levels (0.270 mL Termamyl.g^{-1} starch, 1 h of liquefaction and 2 h of saccharification). In the enzymatic hydrolysis, besides the high cost of enzyme, a high amount of energy can be consumed when the reaction takes several hours or days. The lowest liquefaction and saccharification times adopted in this study (1 and 2 h respectively) decreased the incubation time at high temperatures. Under assay condition 5, the yield of total reducing sugar obtained was 560 mg.g^{-1} of cassava pulp and 1029 mg.g^{-1} of starch. On the other hand, Virunanon et al.[17] tried to reduce the hydrolysis process reaction time of cassava pulp and obtained

the highest level of released reducing sugar (514.3 mg.g^{-1} starch) by simultaneous liquefaction and saccharification with a mixture of Liquozyme-SC DS, Spirizyme Fuel and Novozyme NS 50012 enzyme, incubated at pH 4.5, 50 °C for 24 h. The yield obtained in this study (1029 mg.g^{-1} starch) was far greater than that obtained by Virunamon et al.[17], however, in this study, two-step CP liquefaction and saccharification process was performed using two other enzymes (Termamyl 2X and AMG 300L). Once the essay 5 was selected, cassava pulp (600 g) was hydrolyzed producing 170 g.L^{-1} TRS, the equivalent of 160 g.L^{-1} glucose (HPLC). Moreover, according to Curvelo-Santana et al.[18], an enzymatic hydrolysis efficiency of 79.4% was estimated, which was achieved merely within 1 h of liquefaction and 2 h of saccharification.

Alcoholic fermentation

Figure 2 shows the mean of four experiments for ethanol production, cell growth and substrate consumption during 24 hours of fermentation.

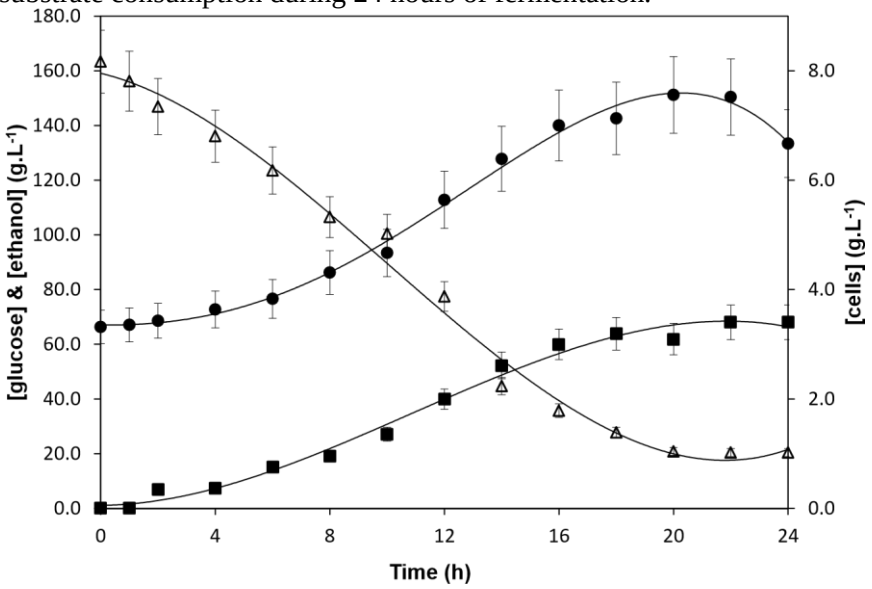

Figure 2 Kinetics of alcoholic fermentation using cassava pulp hydrolysates containing 16% glucose. Ethanol production (■), cell growth (●) and substrate consumption (Δ). The bar represents the percentage error.

As shown in the cellular growth curve (Fig. 2), the adaptation phase of yeast to the cassava pulp hydrolysates was about 3 to 4 h. Moreover, in the exponential growth phase, the maximum specific growth rate (μ_{max} = 0.07 h^{-1}) was achieved within the range of 8 to 16 h. This value was lower than the ones reported by Kasavi et al.[19] for starch molasses using different industrial strains of *S. cerevisiae* ranging from 0.33 a 0.39 h^{-1}. These results remark the need of yeast adaptation to fermentation broth containing high sugar concentration (18%), after hydrolysis of cassava pulp. In addition, figure 2 shows that the glucose was not completely consumed (S_f=20.4 g.L^{-1}) after 24 h of fermentation and ethanol production reached 68.0 g.L^{-1}, which is 8.6 % (v.v^{-1}). Therefore, it was not possible to observe the yeast inhibition by ethanol. However, assuming that inhibition of Hansen BY4741 yeast is attained only by alcohol concentrations above 10% (v.v^{-1}), initial glucose concentrations in the medium should be above 180 g.L^{-1}, and the fermentation should be carried out for at least 45 h. According to this argument, the fermentation of cassava pulp hydrolysates by *S. cerevisiae* Hansen BY4741 has similarities with the fermentation of corn, in which fermentation time of 48-72 h is normally required to obtain 10-12% (v.v^{-1}) ethanol[20, 21]. However, this approximate 2 to

2.5 fold increase in fermentation time does not promote an increased ethanol production at the same ratio. Table 4 compares the results of this study to those from other three studies carried out under similar conditions using agro-industrial waste hydrolysates of cassava and corn meal. In all cases fermentation was carried out as a batch process, using media containing fermentable sugars and strains of *S. cerevisiae*.

Table 4 Comparison of the fermentation results of this study with those of the previous reports.

Parameters	This study	Shah et al.[22]	Curvelo-Santana et al.[18]	Nikolic et al.[23]
Material	cassava pulp hydrolysates	starch industry waste hydrolysates	cassava root hydrolysates	corn meal hydrolysates
Starch content (%)	54.40 ($w.w^{-1}$)	-	71.65 (dry weight)	76.75 ($w.w^{-1}$)
Substrate	16% glucose	15% RS	~6.5% RS + 10 $g.L^{-1}$ sucrose	15% glucose
Microorganism	*S. cerevisiae* Hansen BY4741	Thermo-tolerant *S. cerevisiae*	*Saccharomyces*	*S. cerevisiae* var. *ellipsoideus*
Inoculum	$3g.L^{-1}$ (~1.5% $v.v^{-1}$)	10% ($v.v^{-1}$)	-	2% ($v.v^{-1}$)
t_f (h)	24	72	120	26
Ethanol ($g.L^{-1}$)	68	74	29.3**	52.8**
$Y_{P/S}$	0.48	0.47	0.45	0.35
Q_P ($g.L^{-1}.h^{-1}$)*	2.83	1.03*	0.24*	2.03

RS=reducing sugar *calculated by authors of this study ($Qp=[ethanol].t_f^{-1}$) **recalculated for g.L-1.

The values of kinetic parameters obtained in this study were higher than those reported in the previous studies (Table 4)[18, 22, 23], and ethanol yield ($Y_{P/S}$) and productivity (Q_p) were 0.48 and 2.83 $g.L^{-1}.h^{-1}$, respectively, after 24 h. However, in the fermentation of starch industry waste hydrolysates containing 15% reducing sugars[22] gave rise to slightly higher alcohol concentration, probably because of the amount of the inoculum used (10% $v.v^{-1}$). It is noteworthy that the fermentation time in this study was the lowest among all. Therefore, the results of this investigation indicate that cassava pulp is a potential feedstock for ethanol production like other classic starchy sources. In Brazil, the greatest starch manufacturers that have the potential to produce this waste on a large scale are concentrated in the South-Central and Southeastern regions. Owing to this situation, micro-distilleries can be constructed near these sources of residual raw materials in order to reduce their cost of transportation.

CONCLUSION

This study clearly showed that a high yield ethanol production from cassava pulp can be carried out in two stages, namely separate hydrolysis and fermentation (SHF). In the hydrolysis of cassava pulp, the AMG concentration was the most significant factor. A high yield of total reducing sugars (TRS) were obtained with reduced liquefaction and saccharification times, decreasing the requirement of high incubation temperatures. Suitable conditions for obtaining enzymatic hydrolysates of cassava pulp were 0.517 mL $AMG.g^{-1}$ starch, 0.270 mL $Termamyl.g^{-1}$ starch, 1 h of liquefaction and 2 h of saccharification. In batch culture studies of cassava pulp hydrolysate, *Saccharomyces cerevisiae* Hansen BY4741 showed tolerance to an ethanol concentration of 68 $g.L^{-1}$ (8.6% $v.v^{-1}$) obtained after 24 h of fermentation. The kinetic parameter values of 0.48

ethanol yield and 2.83 $g.L^{-1}.h^{-1}$ ethanol productivity showed that this process can be a good alternative for alcohol production. Furthermore, the use of solid waste from flour processing (cassava pulp) may reduce the potential environmental impacts associated to it.

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