Study of acid hydrolysis and submerged fermentation parameters in second generation ethanol production using cassava waste

Estudo de parâmetros de hidrólise ácida e fermentação submersa na produção de etanol de segunda geração a partir de resíduos da mandioca

Estudio de parámetros de hidrólisis ácida y fermentación sumergida en la producción de etanol de segunda generación a partir de residuos de yuca

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ABSTRACT
Biofuels are an alternative to reduce the environmental impacts caused by the use of petroleum-based fuels. Second generation ethanol comes from lignocellulosic materials that are little used or discarded in the environment, including agro-industrial waste. These residues are rich in sugars that can be converted by ethanol-producing microorganisms.
In this context, the present work uses cassava waste (*Manihot esculenta* Crantz) obtained during the processing of starch as a raw material for the production of cellulosic ethanol, as well as evaluating different parameters in acid hydrolysis and during submerged fermentation using the strain *Saccharomyces cerevisiae* ATCC 26602. Experiments of acid hydrolysis of these residues with sulfuric acid in concentrations of 0.5% to 5.0% (v/v) were carried out. The results demonstrated that 2.0% H$_2$SO$_4$ for 10 min. reaction at 121 °C/1.1 atm in an autoclave reached the highest release of reducing sugars present in the residue (131.09 g.L$^{-1}$). The ideal conditions for alcoholic fermentation using yeast were pH 6.5, temperature of 35 °C, without rotation and initial reducing sugar concentration of 50 g.L$^{-1}$, resulting in 21.23 g.L$^{-1}$ of ethanol with productivity of 1.86 g.L$^{-1}$.h$^{-1}$ and theoretical yield of 96.5% after 12 hours of fermentation. The results indicated that the cassava waste serve as a potential substrate for the production of second generation ethanol. Thus, this study provides data on the most suitable conditions for the use of these industrial wastes aiming at the generation of a renewable fuel, an increasingly attractive feature for the world, where the economic and environmental concern is growing.

**Keywords:** lignocellulosic material, cellulosic ethanol, biomass, bioenergy, biofuels, renewable energy.

**RESUMO**

Os biocombustíveis são uma alternativa para reduzir os impactos ambientais causados pela utilização de combustíveis derivados do petróleo. O etanol de segunda geração vem de materiais lignocelulósicos pouco usados ou descartados no ambiente, inclusive resíduos agroindustriais. Esses resíduos são ricos em açúcares que podem ser convertidos por microrganismos produtores de etanol. Nesse contexto, o trabalho atual utiliza resíduos de mandioca (*Manihot esculenta* Crantz) obtidos durante o processamento do amido como matéria-prima para a produção de etanol celulósico, além de avaliar diferentes parâmetros na hidrólise ácida e durante a fermentação submersa com a linhagem *Saccharomyces cerevisiae* ATCC 26602. Foram realizadas experiências de hidrólise ácida desses resíduos com ácido sulfúrico em concentrações de 0,5% a 5,0% (v/v). Os resultados demonstraram que 2,0% de H2SO4 por 10 min. de reação a 121°C/1,1 atm em uma autoclave atingiu a maior liberação de açúcares redutores presentes no resíduo (131,09 g.L$^{-1}$). As condições ideais para a fermentação alcoólica utilizando levedura foram pH 6,5, temperatura de 35 °C, sem rotação e concentração inicial de açúcares redutores de 50 g.L$^{-1}$, resultando em 21,23 g.L$^{-1}$ de etanol com produtividade de 1,86 g.L$^{-1}$.h$^{-1}$ e rendimento teórico de 96,5% após 12 horas de fermentação. Os resultados apontaram que os resíduos da mandioca servem de substrato potencial para a produção do etanol de segunda geração. Assim, este estudo fornece dados sobre as condições mais adequadas para a utilização desses resíduos industriais visando a geração de um combustível renovável, característica cada vez mais atraente para o mundo, onde a preocupação econômica e ambiental está crescendo.

**Palavras-chave:** material lignocelulósico, etanol celulósico, biomassa, bioenergia, biocombustíveis, energia renovável.

**RESUMEN**

Los biocombustibles son una alternativa para reducir los impactos ambientales causados por el uso de combustibles derivados del petróleo. El etanol de segunda generación proviene de materiales lignocelulósicos poco utilizados o desechados en el medio...
ampliamente, incluidos los residuos agroindustriales. Estos residuos son ricos en azúcares que pueden ser convertidos por microorganismos productores de etanol. En este contexto, el presente trabajo utiliza residuos de yuca (Manihot esculenta Crantz) obtenidos durante el procesamiento del almidón como materia prima para la producción de etanol celulósico, además de evaluar diferentes parámetros en la hidrólisis ácida y durante la fermentación sumergida con la cepa Saccharomyces cerevisiae. Los experimentos de hidrólisis ácida de estos residuos se llevaron a cabo con ácido sulfúrico en concentraciones de 0,5% a 5,0% (v/v). Los resultados demostraron que se utilizó H2SO4 al 2,0% durante 10 min. La reacción a 121°C/1,1 atm en autoclave logró la mayor liberación de azúcares reductores presentes en el residuo (131,09 g.L⁻¹). Las condiciones ideales para la fermentación alcohólica utilizando levadura fueron pH 6,5, temperatura de 35 ºC, sin rotación y concentración inicial de azúcares reductores de 50 g.L⁻¹, dando como resultado 21,23 g.L⁻¹ de etanol con una productividad de 1,86 g.L⁻¹.h⁻¹ y rendimiento teórico del 96,5% tras 12 horas de fermentación. Los resultados mostraron que los residuos de yuca sirven como sustrato potencial para la producción de etanol de segunda generación. Así, este estudio proporciona datos sobre las condiciones más adecuadas para utilizar estos residuos industriales para generar un combustible renovable, una característica cada vez más atractiva para el mundo, donde las preocupaciones económicas y ambientales son crecientes.

**Palabras clave:** material lignocelulósico, etanol celulósico, biomasa, bioenergía, biocombustibles, energías renovables.

### 1 INTRODUCTION

The growing world demand for energy is the result of accelerated population and economic development, which leads to the consumption of large quantities of fossil fuels. The incomplete combustion presented by these sources has a negative impact on the global climate due to the release of gases that contribute to the greenhouse effect and pollution of the environment [1].

Therefore, there is a need to implement renewable energy alternatives on a large scale to minimize the damage caused by fossil fuels. For this to be consolidated, it is essential to reduce the use of non-renewable and polluting sources. An alternative is the use of agro-energy crops and plant residues in the production of biofuels and energy cogeneration. These raw materials are promising, sustainable and low cost, called biomass [2].

Photosynthetic biomass presents itself as a promising alternative for the production of biofuels. Its use in the generation of renewable energy can, besides to avoid competition with food, stabilize the price of ethanol compared to gasoline and reduce environmental impacts [3]. In addition, the use of fuel ethanol in combustion engines
results in lower emissions of polluting gases and greenhouse gases (GHG) when compared to fossil fuels [4].

In Brazil, ethanol is the result of sugarcane fermentation, however, other available raw materials can be used, including lignocellulosic agroindustrial waste and those that have a large amount of starch [5].

Annually, large amounts of waste are generated from agro-industrial activities. Generally, these materials are disposed of directly in the environment without any type of treatment, leading to problems of environmental pollution, such as cassava waste [6].

The cassava waste are generated during the processing of the raw material (on average 0.47 t/t of processed cassava) and consist of 25.0% cellulose, 7.0% hemicellulose, 5.0% crude protein, 60.0% starch residual from the pulp, 20.0% fibers and 3.0% lignin [7]. Cassava processing industries face problems due to the exposure of waste in the open air without proper disposal and due to the excessive time it takes to decompose, the waste generated can be used for the production of second generation ethanol [8].

The global production of cassava shows a continuous growth, where, the highest rates occurred between the years 2012 and 2016 when it registered an increase of approximately 7% and went from 241.3 to 274.7 million tons. In 2017, world production reached 291.9 million tons, of which 18.8 million tons were produced in Brazil [9].

Based on this, the objective of this work is to contribute to the development of an integrated and diversified technology for the production of fuels using alternative materials that may arouse the interest of the industrial market. In addition, the use of renewable energy sources would promote the reduction of waste generation and emissions of gases harmful to the environment. Therefore, this process consisted of the acid hydrolysis of cassava waste discarded during the production of flour in order to release fermentable sugars for the production of ethanol (2G).

2 MATERIAL AND METHODS

2.1 RAW MATERIAL

Cassava waste (husks, intershells and tips) were supplied by the flour producing industry Moreá Alimentos Ltda., Located in the municipality of Monte Alegre de Minas – MG.
2.2 PROCESSING OF CASSAVA WASTE

The residues used were washed and dried in the sun for 48 hours. Then, crushed in an electric grinder adapted to increase the contact surface, and finally stored in plastic containers. The particle size was homogenized at ≤ 0.64 mm using a Produtest brand tamper. Then, they were packed in airtight bottles and stored at room temperature until use.

2.3 CASSAVA WASTE HYDROLYSIS AND DETOXIFICATION

The hydrolysis of cassava waste occurred in 250 mL Erlenmeyers, in the proportion of 10:50 (w/v) of cassava waste in sulfuric acid (H_2SO_4) with different concentrations (0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5 and 5.0%) to be evaluated. These were subjected to heating in an autoclave at 121 °C/1.1 atm for 5, 10 and 15 min. At the end of the hydrolysis, the Erlenmeyers were left at room temperature until cooling.

Other Erlenmeyers with different concentrations tested (1.5, 2.0 and 2.5%) did not undergo heating, therefore, they remained at room temperature (close to 28 ºC) for 24, 48, 72, 96 and 120 hours.

At the end of each hydrolysis, the pH of the hydrolyzed media was neutralized by 6.5 with the use of sodium hydroxide (NaOH) 50% (w / w). Each hydrolyzate was centrifuged at 3600 rpm for 20 minutes, to separate the remaining residues. After centrifugation, the hydrolyzate was filtered on Whatman No. 1 filter paper to completely remove the cassava residue (remaining cake). All filtrates were stored in flasks with a cap below 0 °C for further analysis.

The filtrate obtained was used as a substrate for ethanol production in two ways: crude acid hydrolyzate (CH) from the cassava waste, before being subjected to detoxification; and detoxified crude hydrolyzate (DH), which was subjected to a coal detoxification stage activated, to remove inhibiting agents from the fermenting microbiota, according to the methodology described by [10].

Before carrying out the fermentative tests with the substrates CH and DH, the levels of reducing sugars were determined by the cuproarsenate method [11] and [12]. In addition, the contents of phenolic compounds were analyzed by Folin-Ciocalteau modified by [13].
2.4 MICROORGANISM, MAINTENANCE, INOCULUM PREPARATION AND FERMENTATION MEDIUM

The yeast *Saccharomyces cerevisiae* ATCC 26602 was provided by the Department of Chemical Engineering at the University of Coimbra - Portugal. The yeast storage medium was composed of (g.L\(^{-1}\)): malt extract, 3; yeast extract, 3; meat peptone, 5; glucose, 10; and agar, 20. Stored at a temperature of 4 ºC.

The inoculum for yeast development was prepared in 250 mL Erlenmeyer flasks containing 100 mL of an enrichment medium with the same constituents as the maintenance medium, except for the use of agar, obtaining a pH broth. 5.0, where, the yeast previously cultivated in the maintenance medium was added for its development in a pre-fermentation incubated at 30 ºC for 24 h under agitation of 100 rpm. The inoculum was standardized by spectrophotometry at 0.600 absorbance with wavelength at 600 nm.

The ethanol production was carried out in a basal medium (pH 7) composed of yeast extract (5 g.L\(^{-1}\)); KH\(_2\)PO\(_4\) (1 g.L\(^{-1}\)); MgSO\(_4\).7H\(_2\)O (1 g.L\(^{-1}\)), (NH\(_4\))\(_2\)SO\(_4\) (1 g.L\(^{-1}\)) and the carbon source: crude hydrolyzate (CH) and detoxified crude hydrolyzate (DRH).

2.5 FERMENTATION PROCESS

For the fermentation process, 250 mL Erlenmeyer flasks containing 50 mL of crude hydrolyzate (CH) or detoxified crude hydrolyzate (DH) were used.

The conditions applied for the evaluation of yeast growth and production were: temperature of 25, 30, 35 and 40 ºC; initial substrate concentration of 25, 50 and 75 g.L\(^{-1}\); agitation 0, 75 and 150 rpm and pH 6.5, during 12 h of fermentation according to standardization carried out in previous experiments.

2.6 ANALYTICAL METHODS

The determination of the amount of glucose present in the hydrolysates was performed only for samples that did not undergo heat treatment, by means of an enzymatic reaction of β-glucosidase with the use of staining reagents in the reading of absorbances in a spectrophotometer (510 nm).

The final pH was determined directly in the fermented broth using the pH meter Digmed model DM20.

Cell concentration was determined by turbidimetry using a Biochrom spectrophotometer, model Libra S22.
Ethanol was determined by gas chromatography in cell-free fermented broth, using a Thermo Scientific Model Focus chromatograph with flame ionization detector (FID) and HP-FFAP column (25 m x 0.2 mm x 0.3 µm); oven temperature at 70 ºC (maintaining this temperature throughout the isothermal run); 5 min run time; injector temperature of 230 ºC; detector temperature of 270 ºC; injection of 200 µl of sample steam. The samples were left in a water bath at a temperature of 40 ºC (until reaching equilibrium).

2.7 CALCULATIONS

Productivity (P) was determined using equation 1, which lists the number in grams of bioethanol produced by the number in hours of the fermentation time.

\[ P = \frac{(P_f - P_0)}{(t_f - t_0)} \]  

(Equation 1)

Where:

\( P = \) productivity (g.L\(^{-1}\).h\(^{-1}\)); \( P_0 = \) initial mass in grams of product; \( P_f = \) final product mass in grams; \( t_0 = \) time in initial hours; \( t_f = \) time in final hours.

The metabolic yields were calculated based on the sugars consumed by subtracting the measured amounts of monomeric sugars in the liquid at the end of the fermentation process. The data used for these calculations were collected at the beginning of the experiment and at the end of the fermentation.

All statistical analysis were performed using the Minitab 16 software.

3 RESULTS AND DISCUSSION

3.1 STANDARDIZATION OF ACID HYDROLYSIS OF CASSAVA WASTE

Analyze of acid hydrolysis were carried out to determine which is the best contact time between sulfuric acid and biomass. For this, the total sugars, reducing sugars and phenolic compounds present were determined. Glucose quantification was also performed, only for hydrolysates without heating.

The choice of the best parameters for carrying out the hydrolysis was determined from the concentration of H\(_2\)SO\(_4\) that provided the largest amount of fermentable sugars.
under heating conditions in an autoclave at 121 °C/1.1 atm and also without heating. However, considering the heating time and the amount of reagent used.

The analysis of reducing sugars was performed to verify the presence of fermentable sugars in the hydrolyzed medium, the levels obtained for each acid concentration tested at different heating times are shown in Figure 1.

Figure 1 – Reducing sugars (RS) released during acid hydrolysis using different heating times (5, 10 and 15 min.) in an autoclave at 121 °C/1.1 atm and H$_2$SO$_4$ concentrations (0.5 to 5.0%).

![Reducing sugars (RS) released during acid hydrolysis](image)

The hydrolysis condition that favored the production of reducing sugars was in the time of contact of the acid with the biomass of 10 min. heating in an autoclave and using 2.0% (v / v) H$_2$SO$_4$. Where the highest release of 131.09 g.L$^{-1}$ of RS was observed (Figure 1).

In a research on the acid hydrolysis of cassava waste carried out by [14] 58.25 g.L$^{-1}$ of reducing sugars were obtained through the use of the following parameters: use of H$_2$SO$_4$ at 0.25 M, heating in an autoclave at 120 °C for 2 hours of treatment. In the present work, hydrolysis with 2.0% H$_2$SO$_4$ released 131.09 g.L$^{-1}$ of RS. These authors achieved a lower AR content with the use of a much longer process time (2 hours), consequently, the energy expenditure ends up making the process more expensive.

The hydrolysis that were carried out at room temperature used concentrations of 1.5; 2.0 and 2.5% H$_2$SO$_4$, in duplicate, these values are intermediate to the optimum obtained from the results of RS for the hydrolysis performed with heating in an autoclave.

Figure 2 shows the RS concentrations and the glucose concentration present in the samples hydrolyzed at room temperature. Most sugars have been identified as glucose and the rest have not been identified, however, during the hydrolysis of a lignocellulosic
material, a large part of the sugars from hemicellulose is xylose, which may be part of these remaining sugars.

From these results, it was found that the highest levels of total reducing sugars and glucose release occurred at a concentration of 2.5% sulfuric acid during 96 hours of treatment (5.20 g.L⁻¹ and 4.34 g.L⁻¹, respectively), being very low values when compared to those obtained where heat is applied to the hydrolysates. This shows the importance of heating, since the process without heating becomes completely unfeasible due to the low release of fermentable sugars and the long reaction time. Therefore, the energy costs with the autoclave to generate heat in the biomass (the basic raw material for obtaining hydrolysates for subsequent fermentation) end up being justified.

The use of low acid concentrations is important during the hydrolysis process, as lower levels reduce the risk of corrosion of the fermenters and also reduce costs due to the smaller amount of reagent to be used. Furthermore, lower temperatures also reduce energy costs, making the process more accessible in relation to the cost-benefit ratio [15].

Another factor analyzed for choosing the concentration of H₂SO₄ to be used in hydrolysis was the formation of inhibitor compounds, because when using lower concentrations of acid there is less release of toxic compounds from the degradation of lignin and sugars from the hemicellulose. The concentration of these compounds in the fermentation medium can be toxic to the cellular development of microorganisms and consequently inhibit the production of 2G ethanol [16].
Acid concentration, biomass contact time and fermentation temperature influence the formation of inhibitor compounds [17]. Figure 3 shows the contents of phenolic compounds obtained in the hydrolysates.

In Figure 3, it is possible to verify that all the levels of phenolic compounds (PC) obtained increased by increasing the concentration of H$_2$SO$_4$ and the biomass contact time at 121 ºC (in 15 min. of heating, the highest levels of PC were obtained). Therefore, the longer the contact time of acid and heat, the higher the index of phenolic compounds formed.

For heating times 5, 10 and 15 min. there was an increase of 48.5%, 52.63% and 57.5% for the times of 5, 10 and 15 min. of heating, respectively, when evaluated from the concentration of 0.5 to 5.0% of H$_2$SO$_4$. Therefore, it is visible that the greatest release of PC occurred during the time of 15 min. in autoclave heating (Figure 3).

The highest release was with the use of 5.0% acid concentration (0.58 g.g$^{-1}$ cassava residue) for 15 min. heating in an autoclave. The smallest release was 1.0 and 2.0% for the time of 5 min. of heating in an autoclave (0.27 and 0.31 g.g$^{-1}$ cassava residue, respectively).

At the concentration of 2.0% H$_2$SO$_4$ for 10 min. of heating, 0.35 g.g$^{-1}$ PC cassava residue was obtained and, after the detoxification process, this value was reduced, therefore, these parameters were used to standardize the acid hydrolysis (Figure 3).
3.2 DETOXIFICATION OF ACID HYDROLYSATE

The detoxification process aimed to reduce microbial growth inhibitory compounds that were generated during the acid hydrolysis step (Figure 3). This reduction is important, since the smaller the amount of these compounds in the medium, the better the development of microorganisms in the fermentation process and the higher the yield in ethanol production.

After carrying out the detoxification, the quantification of the resulting phenolic compounds in the hydrolysates was carried out. Table 1 shows the concentration of the phenolic compounds in the crude hydrolyzate (CH) and the detoxified hydrolyzate (DH).

<table>
<thead>
<tr>
<th>Hydrolyzated media</th>
<th>Phenolic compounds (g·g⁻¹ de residuo de mandioca)</th>
<th>Standard deviation (σ)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw hydrolyzated</td>
<td>0,39</td>
<td>0,05</td>
<td></td>
</tr>
<tr>
<td>Detoxification (1º cicle)</td>
<td>0,18</td>
<td>0,03</td>
<td></td>
</tr>
<tr>
<td>Detoxification (2º cicle)</td>
<td>0,05</td>
<td>0,02</td>
<td>0,002</td>
</tr>
</tbody>
</table>

Source: Self-authored.

In Table 1, it is possible to compare the amount of phenolic compounds for each hydrolyzate, the difference between them was significant (p=0.02), that is, in fact, the detoxification process was efficient.

The reduction of phenolic compounds in the cassava residue hydrolyzate showed a decrease of 87.17%. In a study of the detoxification of sugar beet bagasse hydrolyzate using commercial active charcoal, authors [18] quantified the removal of phenolic compounds in 71.10%, similar to the present work (87.17%).

From these results, the fermentation of crude hydrolysates (CH) and detoxified (DH) by the yeast *Saccharomyces cerevisiae* ATCC 26602 was carried out.

3.3 EVALUATION OF ETHANOL PRODUCTION BY *S. CEREVISIAE* ATCC 26602 FROM RAW AND DETOXIFIED CASSAVA RESIDUE HYDROLYSATES

A 24-hour fermentation was carried out using CH and DH media. The initial concentration of reducing sugars was 50 g·L⁻¹, without rotation (0 rpm) with different values of initial pH (5.5, 6.0 and 6.5) and 30 °C.

For both hydrolyzed media it was possible to verify a marked reduction in pH in the first 8 hours of fermentation, and after this decrease it was stable. At the end of 24 hours of fermentation, the media with an initial pH of 5.5 had a pH reduction to 4.6; media with an initial pH of 6.0 dropped to 4.8 and an initial pH of 6.5 reached 5.0.
These reductions were not sudden, which made possible the good adaptation of the yeast to the hydrolyzed medium and made it possible to convert the reducing sugars into 2G ethanol. This behavior is important, since pH plays a role against fermentation inhibition, due to the ability to dissociate acids inside the yeast, where intracellular acidification occurs, alteration in metabolism, thus inhibiting cell growth and formation of by-products, consequently, affecting ethanol production [19].

The 2G ethanol production results obtained by the fermentation of *S. cerevisiae* ATCC 26602 are shown in Figure 4.

![Figure 4 – Ethanol production by the yeast *Saccharomyces cerevisiae* ATCC 26602 in crude hydrolyzed medium and detoxified medium incubated at 30ºC for 24 h.](image)

Ethanol production was more accentuated in media containing crude hydrolysates (medium without undergoing detoxification process), where the maximum value obtained for ethanol was 23.65 g.L⁻¹ at pH 6.5 after 24 hours of fermentation. Thus, the detoxification process in cassava residue hydrolysates using activated charcoal is not a necessary step, since, even in the presence of phenolic compounds in the CH, there was greater production of 2G ethanol, indicating that there was no significant inhibition of microbial activity. Making the fermentation process more economical at an industrial level, since it does not require the detoxification step.

The highest productivity rate was 2.8 g.L⁻¹.h⁻¹ reached in 8 hours of fermentation and initial pH of 6.5. In 24 h of fermentation, productivity was 0.98 g.L⁻¹.h⁻¹. According to these results, the other fermentations were carried out by eliminating the detoxification stage of the media, with a fixed initial pH of 6.5 and the fermentation time was reduced to 12 hours.
Researchers [7] performed alkaline hydrolysis of cassava husks (50 g) with 200 mL of NaOH (0.5 M) and heating in an autoclave at 121 °C for 15 min., followed by enzymatic hydrolysis in a process of simultaneous saccharification and fermentation (SSF) (30 ± 2 °C at initial pH of 5.5) using the commercial yeast *Saccharomyces cerevisiae*. Where, the productivity obtained from the fermentation process was 1.3 g.L\(^{-1}\).h\(^{-1}\). In relation to the present work, it can be observed that the acid treatment for 10 min. of heating in an autoclave at 121 °C and subsequent fermentation of the cassava residue hydrolyzate in an initial concentration of reducing sugars of 50 g.L\(^{-1}\) kept without rotation at a temperature of 30 °C and initial pH of 6.5 achieved a productivity of 2.80 g.L\(^{-1}\).h\(^{-1}\) in just 8 h of fermentation (ethanol concentration of 22.47 g.L\(^{-1}\)). Therefore, the choice of waste, together with acid treatment can be advantageous for the production of bioethanol, since such results are promising and similar to those reported by [7].

3.4 FERMENTATION WITH CRUDE HYDROLYZED MEDIUM FOR EVALUATION OF TEMPERATURE PARAMETERS AND INITIAL SUBSTRATE CONCENTRATION

Submerged fermentation using the medium containing the crude acid hydrolyzate of cassava waste (CH) with the application of *S. cerevisiae* ATCC 26602 occurred in a 12-hour process with a fixed initial pH of 6.5 and change in the concentration of initial reducing sugars in: 25, 50 and 75 g.L\(^{-1}\). Incubated at different temperatures: 25, 30, 35 and 40 °C and shaking the culture medium: 0, 75 and 150 rpm. Aliquots were taken for analysis at fermentation times of 2, 4, 6, 8, 10 and 12 h.

The yeast showed cell development and there was ethanol production in all tested parameters. The best results obtained among the evaluations are presented in Table 2.

<table>
<thead>
<tr>
<th>Parameters evaluated</th>
<th>Crude acid hydrolyzate (CH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial concentration of sugars (g.L(^{-1}))</td>
<td>25.00  50.00  75.00</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>35.00  35.00  35.00</td>
</tr>
<tr>
<td>Cell growth (g)</td>
<td>3.11   3.80   4.90</td>
</tr>
<tr>
<td>Agitation (rpm)</td>
<td>0      0      75</td>
</tr>
<tr>
<td>Residual sugar (g.L(^{-1}))</td>
<td>1.70   2.60   4.50</td>
</tr>
<tr>
<td>Final pH</td>
<td>4.36   4.50   4.60</td>
</tr>
<tr>
<td>Ethanol 2G (g.L(^{-1}))</td>
<td>10.46  21.23  15.70</td>
</tr>
<tr>
<td>Productivity (g.L(^{-1}).h(^{-1}))</td>
<td>1.04   1.86   1.85</td>
</tr>
<tr>
<td>Yield (g.g(^{-1}) glicose)</td>
<td>0.47   0.49   0.45</td>
</tr>
<tr>
<td>Theoretical yield (%)</td>
<td>92.30  96.50  88.60</td>
</tr>
</tbody>
</table>

Source: Self-authored.
The greatest yeast cell growth occurred when using a temperature of 35 °C. The initial inoculum of each fermentation was 0.18 g of yeast cells. At the initial concentration of RS of 25 g.L⁻¹ and 0, 75 and 150 rpm, the greatest cell growth was 3.11; 3.20 and 3.90 g, respectively. For the initial concentration of RS of 50 g.L⁻¹ the best contents were 3.43; 3.73 and 3.80 g, for 0, 75 and 150 rpm, respectively. TS 75 g.L⁻¹, the maximum values obtained for cell growth were 2.50; 4.90 and 4.50 for rotations 0, 75 and 150 rpm, respectively.

The rotation of the medium favored cell growth, that is, as the Erlenmeyer’s agitation speed increases, the increase in yeast biomass in the medium becomes more accentuated. On the other hand, a process where there is the conversion of carbon sources aimed mainly at maintaining the cell growth of the microorganism may not be advantageous, because, in addition to the large amount of biomass formed, the concentration of ethanol in the media did not increase significantly proportional to growth. This can be explained by the fact that yeast not only produces ethanol as a product of microbial metabolism, but also another metabolite (glycerol), in which this pathway may be favored by agitation.

For the medium containing 75 g.L⁻¹ of initial AR, when the average agitation of 75 rpm was included, the best condition for ethanol production occurred at 35°C with the obtainment of 15.70 g.L⁻¹ of ethanol. However, when compared with the best condition for ethanol production (50 g.L⁻¹ and 0 rpm), which presented 21.23 g.L⁻¹, it shows that mechanical agitation is not necessary in the process. This would lower the cost when industrially applied (Table 2).

On the other hand, an initial concentration of glucose added to the medium in excess slows the mechanism of ethanol production in cells, due to the osmotic stress caused by the high concentration of the supplied substrate, as observed in recent research [17].

At 35 °C there was also the greatest assimilation of reducing sugars by yeast. These sugars quickly decreased during the first 6 hours of fermentation. At this temperature, the yeast used the reducing sugars more quickly and efficiently, since the concentration of residual sugars after fermentation was 1.70 g.L⁻¹ in the substrates where the initial concentration of AR was 25 g.L⁻¹, simultaneously to Ethanol production was higher. With the use of a temperature of 25 °C, there was a slow consumption of sugars and at the end of the fermentation there was still a good part of the carbon source offered to the yeast (9.28 g.L⁻¹) also for the initial concentration of AR of 25 g.L⁻¹.
Furthermore, after 10 hours of fermentation, glucose was almost depleted, and consequently, the scarcity of this carbon source led the yeast to use its own secondary metabolism product (ethanol), causing a slight decrease in ethanol levels. [20] evaluated two strains of *Saccharomyces* and found that diauxic change occurred after 8 to 12 hours of incubation. Yeast development is controlled in part by nutrient availability. When yeast cells are grown in liquid cultures in glucose-rich media, they metabolize this sugar predominantly via the glycolytic pathway, releasing ethanol into the culture medium. When glucose becomes limiting, there is a decrease in the rate of growth and metabolism by switching from aerobic glycolysis to the use of ethanol, and this change in growth is known as diauxic shift and explains the drop in the concentration of ethanol in the culture medium after 10 h of fermentation [20].

The pH at the end of the fermentations (after 12 h) for CH changed from 6.5 to 4.36 when using the initial concentration of 25 g.L\(^{-1}\) of RS; In 50 g.L\(^{-1}\) of initial RS it ended up at 4.5, and for 75 g.L\(^{-1}\) the pH at the end of fermentation was 4.6. Therefore, it was possible to verify that, even tripling the concentration of fermentable sugars offered, there were no sudden changes in the pH value of the medium.

In a research carried out by [21] for the production of ethanol from hydrolyzed sugars, also found that the pH of the culture medium is a very important factor to be controlled during fermentation. Once, yeast cells can efficiently adjust their metabolism in a moderately acidic environment. Confirming that *S. cerevisiae* ATCC 26602 cells could efficiently adjust their metabolism in the crude acid hydrolyzed medium from cassava waste (Table 2).

The best temperature for obtaining ethanol was also 35 °C after 10 h of fermentation. In the medium containing 25 g.L\(^{-1}\) of CH, 10.46 g.L\(^{-1}\) of ethanol were determined. In the medium containing 50 g.L\(^{-1}\) of initial RS, the highest value of ethanol was obtained, 21.23 g.L\(^{-1}\), and with 75 g.L\(^{-1}\) of initial RS, the yeast produced 15.70 g.L\(^{-1}\) of ethanol (Table 2).

In research using rice husk hydrolyzate, researchers [25] obtained 18.67 g/L of ethanol in 22 hours using the same yeast as the present research.

By using cassava pulp isolated and supplemented with cassava starch from wastewater as raw material for the production of bioethanol from enzymatic hydrolysis, [22], used a mix of enzymes (α-amylases, glucoamylases and β-glucanases) and obtained a maximum sugar release of 514.3 mg.g\(^{-1}\) of hydrolyzed starch. Fermentation of this cassava pulp hydrolyzate was carried out with *S. cerevisiae* (70 g.L\(^{-1}\) initial at pH 6.5)
and produced 12.9 g.L\(^{-1}\) of ethanol. These results are similar to those of the present study. Since, there is great similarity between the residues used, the RS concentrations (75 g.L\(^{-1}\)), and the ethanol production, which was higher than 15.70 g.L\(^{-1}\). These data indicate that the enzymatic hydrolysis applied in SSF to cassava waste has no advantage over acid hydrolysis, since the one used in the present research is faster and cheaper (10 min. against 24 hours of enzymatic hydrolysis used by the author), and in the end the ethanol production was equivalent for both processes.

In a recent survey, [23] evaluated the production of ethanol by separate enzymatic hydrolysis and fermentation (SHF) in cassava waste fermented by \(S. \text{cerevisiae}\) GIM2.213. The results obtained from hydrolysis were 27.29 and 30.17 g.L\(^{-1}\) of glucose and from fermentation they obtained a final ethanol titre of 13.74 and 15.09 g.L\(^{-1}\), respectively. Hydrolysis was performed using an enzymatic extract containing 20 and 40 mg of protein/g glucan cellulase, respectively. The levels of ethanol obtained were lower than those obtained in this research, where acid hydrolysis, a process with lower cost than enzymatic, released higher sugar content (131.09 g.L\(^{-1}\)) and higher conversion to ethanol (23.65 g.L\(^{-1}\)). These results also show that the use of enzymes is not necessary, considerably reducing the cost of the process.

A work carried out by [24], the production of ethanol was evaluated from primary sludge from the pulp and paper industry without prior treatment, from an SSF process with the same yeast (\(\text{Saccharomyces cerevisiae}\) ATCC 26602). The sludges were treated with cellulases at a temperature of 38 ºC, close to which the best results of this work were obtained (35 ºC). In that study, the authors started from a concentration of 150 g.L\(^{-1}\) of carbohydrates and the fermentation reached 41.7 g.L\(^{-1}\) of ethanol, however, a yield of 48.9% and productivity of 0.78 g.L\(^{-1}\).h\(^{-1}\). They also found that the sterilization step can be eliminated, reducing costs and time in the process. The ethanol yield achieved by the study cited was half the maximum yield obtained in our research (96.5%), possibly due to the type of fermentation performed (SSF) which contained high solids content (21.7%) without prior treatment. As a result, liquefaction of the initial mixture was hampered and ethanol production may have been limited. However, it reinforces our results regarding the elimination of the acid hydrolyzate detoxification step.

Low-cost agroindustrial residues are promising for the generation of biofuels, since in the present work an ethanol yield of up to 96.5% of the theoretical maximum described in the literature was obtained.
In general terms, the technology used in this research proved to be suitable for ethanol production, generating clean energy that uses residues that contaminate the environment.

4 CONCLUSIONS

The hydrolysis of cassava waste with 2% (v/v) sulfuric acid during 10 minutes of heating (121 °C) was the most effective parameter tested in the release of fermentable sugars to be used as a fermentative substrate (131.09 g.L⁻¹).

The hydrolyzed residue showed low concentrations of phenolic compounds (0.27 – 0.58 mg/g cassava residue) due to its chemical composition containing little lignin. Therefore, detoxification was not necessary for the production of bioethanol with Saccharomyces cerevisiae ATCC 26602, and it was observed that the presence of phenolic compounds did not exert microbial inhibition.

In alcoholic fermentation, the best conditions for bioethanol production in the present study were: medium containing the crude hydrolyzate (without previous detoxification), initial concentration of reducing sugar 50 g.L⁻¹, 10 hours of fermentation at 35 °C, no agitation, initial pH of 6.5. These parameters resulted in 21.23 g.L⁻¹ of ethanol with a productivity of 1.86 g.L⁻¹.h⁻¹ and theoretical yield of 96.5% (0.49 g/g fermentable sugar).

The development of appropriate technologies for the use of cheap raw materials (lignocellulosic) makes a significant contribution to reducing production costs and making the use of fuel ethanol more universal. In general terms, therefore, alcohol generates cleaner energy, an increasingly attractive feature for the world, where economic and environmental concern is growing.
REFERENCES


