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A Yeast Isolated from Cashew Apple Juice and Its Ability to Produce First- and Second-Generation Ethanol

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Abstract The aim of this study was to isolate and identify an indigenous yeast from cashew apple juice (CAJ) and then use it in the production of first- and second-generation ethanol, using CAJ and the enzymatic hydrolysate of cashew apple bagasse (MCAB-OH), respectively. The isolated yeast was identified as belonging to the genus *Hanseniaspora*. Afterward, the effect of the medium initial pH on the production of ethanol from CAJ was evaluated in the range of 3.0 to 5.5, with its maximum ethanol production of 42 g L^{-1} and $Y_{P/S}$ of 0.44 g g^{-1} and 96 % efficiency. The effect of temperature (28–38 °C) on ethanol production was evaluated in a synthetic medium, and no difference in ethanol production in the temperature range evaluated (28–36 °C) was observed. At 32 °C, the yield, concentration, efficiency, and productivity of ethanol when using the CAJ medium were higher when compared to the results achieved for the synthetic medium. Regarding second-generation ethanol, the results showed that the yeast produced 24.37 g L^{-1} of ethanol with an efficiency of 80.23 % and a productivity of 4.87 g L^{-1} h^{-1} at 5 h. Therefore, *Hanseniaspora* sp., isolated from CAJ, is a promising microorganism for the production of first- and second-generation ethanol.

Keywords *Hanseniaspora* · Ethanol · Process integration · Cashew apple juice · Cashew apple bagasse

Abbreviations

CAJ	Cashew apple juice
CAB	Cashew apple bagasse
MCAB-OH	Enzymatic hydrolysate of cashew apple bagasse
P_{max}	Maximum ethanol concentration (g L^{-1})
NS	non-*Saccharomyces* yeasts

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η	Efficiency of sugar conversion to ethanol (%)
Q_P	Ethanol volumetric productivity (g L h^{-1})
θ_S	Substrate conversion (%)
$Y_{P/X}$	Yield of product based on cell growth (g g^{-1})
$Y_{X/S}$	Yield of cell growth based on substrate consumption (g g^{-1})
$Y_{P/S}$	Yield of product based on substrate consumption (g g^{-1})
μ_{max}	Maximum specific growth rate of cells (h^{-1})
FPU	Filter paper unit

Introduction

Nowadays, Brazil is one of the largest producers of ethanol, and it is also the country with the highest potential for expanding its production, due to its privileged weather and availability of soil for the large-scale production of the feedstock used in the production of this biofuel [1]. For these reasons, large companies of energy and food have invested in the sector, as well as in the research and development of new technologies for the production of ethanol [2]. Second-generation ethanol, produced from lignocellulosic materials, has been seen as the biofuel with a great potential to replace fossil fuels, presenting lower environmental impacts than the first-generation ethanol [1, 3]. Besides being abundant and having a low cost, the lignocellulosic materials do not compete for land use with food crops [4].

One of the reasons that should make the production of ethanol increase and the costs to decrease is the use of alternative substrates of low cost. In fact, substrate preparation is responsible for 60–70 % of the cost to produce ethanol [5]. With this in mind, cashew apple appears as a promising substrate, since juice and bagasse may be used to ethanol production [6, 7].

Several studies have highlighted the feasibility of using cashew apples for ethanol production with promising results [6–10]. It is important to mention that the production of ethanol from cashew apple cannot be considered as a substitute to the actual feedstock (sugarcane) but as a new activity to enlarge and diversify the agribusiness. In the state of Ceará, Northeast Brazil, the cashew (*Anacardium occidentale* L.) agribusiness has an important part in the local economy based primarily on the cashew nut market [11]. On the other hand, approximately only 12 % of the cashew apples are industrially exploited for juice production, so the largest part of the apple production is wasted in the field [12]. Therefore, the use of the cashew apple for the production of ethanol will not only bring economic benefits, since it is a low-cost feedstock, but will also solve a discard problem, avoiding cashew apples to rot in the field and adding value to the cashew market [6, 10]. A small distillery, for example, can use cashew apple as feedstock, both cashew apple juice (CAJ) and cashew apple bagasse (CAB—a lignocellulosic material), for ethanol production. Therefore, the production of second-generation ethanol can share part of the infrastructure where the production of the first-generation occurs (e.g., concentration, fermentation, distillation, storage, and cogeneration facilities).

The microorganisms most commonly used in the production of ethanol come from the *Saccharomyces* genus, due to their high ethanol tolerance [13, 14]. However, other microorganisms are present on the surfaces of some fruits, such as grapes, mainly non-*Saccharomyces* yeasts (NS), which predominate during the early stages of fermentation [14, 15]. Furthermore, some authors observed the potential of those NS for ethanol production [16], showing competitive values of yield and titer. One of the prevailing genera of NS yeasts that show this potential is *Hanseniaspora* [16, 17]. Since the idea is to produce ethanol in small facilities in the countryside of Brazil, the use of a strain isolated from the cashew apple itself may be an interesting strategy to achieve a low-cost and simple process. Therefore, it is interesting to

study the production of ethanol using this other genera of yeast in order to evaluate its efficiency.

Apart from the medium and microorganisms, operating conditions, such as temperature and pH, are factors that influence the effectiveness of the alcoholic fermentation. Evaluating the effect of temperature on the kinetic parameters of fermentation is important for developing more efficient control strategies in ethanol production industries. Although isothermal processes are the most common in the literature [18], in industrial processes, there is no constancy in temperature. pH is another factor that has an impact on the enzymatic activity of cells and can modify the chemical pathways of biological reactions, as well as the kinetics of alcoholic fermentation [19]. It is industrially important that fermentation occurs in pH range in which the kinetics of ethanol production is favored and, also, that the ambient resists to change in pH to maintain the stability of the process.

In this context, the aim of this work was to isolate and identify a new yeast strain from cashew apple peduncle and then study its potential for the production of first-generation and second-generation ethanol. The effects of temperature and pH on the kinetics of first-generation ethanol production were investigated using CAJ as carbon source. Last but not least, the efficiency of the selected yeast strain in the production of second-generation ethanol was evaluated using CAB as the lignocellulosic material.

Materials and Methods

CAJ

The CAJ was kindly donated by the Brazilian Agricultural Research Corporation, Embrapa, CE. Initially, CAJ was centrifuged at 10,000*g* for 15 min, and the initial concentration of reducing sugars (glucose and fructose) was adjusted to 90 g L^{-1} with the addition of distilled water. The pH of the medium was adjusted to the desired value, and subsequently, it was sterilized by autoclaving at 110 °C for 10 min.

CAB Pretreatment

CAB was donated by Jandaia Sucos do Brazil (Ceará, Brazil), and it was pretreated according to Rocha et al. [7]. First, a pretreatment of CAB with diluted sulfuric acid was conducted, and then the residue was treated with 1.0 M NaOH, using a solid concentration of 7.5 % (*w*/*v*), at 121 °C for 30 min. The resulting solid was designated as hydrolysate of cashew apple bagasse (CAB-OH).

Enzymatic Hydrolysis of CAB

CAB-OH (with particles ranging from 0.25 to 0.84 mm) was used for enzymatic hydrolysis. The enzymatic hydrolysis was performed using a commercial enzyme Celluclast 1.5L (Novozyme, Bagsvaerd, Denmark), with 16 % *m*/*v* CAB-OH (11.5 % *w*/*v* cellulose) and 60 FPU $g_{cellulose}^{-1}$ enzyme activity at 45 °C, 150 rpm for 72 h. After the enzymatic hydrolysis of CAB-OH, the solid was separated by centrifugation (10,000*g* for 15 min), followed by filtration. The supernatant was diluted with distilled water to the concentration of 50 g L^{-1} glucose and supplemented with 5 g L^{-1} yeast extract and 1 g L^{-1} $(NH_4)_2SO_4$. After this, it was sterilized at 110 °C for 10 min to be used as fermentation medium for ethanol production, coded enzymatic hydrolysate of CAB (MCAB-OH). MCAB-OH was characterized and

contained 85.79 g L^{-1}±3.11 g L^{-1} of glucose and 30.34 g L^{-1}±0.79 g L^{-1} of cellobiose, as well as traces of xylose (2.28 g L^{-1}±0.07 g L^{-1}). No inhibitors (formic acid, acetic acid, furfural, and hydroxymethylfurfural) were detected.

Isolation of an Ethanol-Producing Yeast from CAJ

One hundred milliliters of CAJ was added to a 250-mL sterile Erlenmeyer flask and allowed to ferment naturally. Thereafter, 30 mL of the fermented juice was added to 120-mL yeast extract peptone dextrose (YEPD) medium in 250-mL Erlenmeyer flask and incubated in an orbital shaker (Tecnal TE-420, Piracicaba, Brazil) at 30 °C under agitation of 150 rpm for 24 h. After this period, the fermented medium was centrifuged at 10 °C and 6,000*g*, and the precipitate was inoculated in Sabouraud agar plates and incubated at 30 °C for 48 h. Then, the preponderant colonies were picked and re-isolated in the Sabouraud agar to obtain pure cultures.

Afterward, YEPD complex medium, containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 20 g L^{-1} glucose, was used for the propagation of the cells. The pH of the media was adjusted to 4.5 using 6 N HCl, and both were sterilized in an autoclave (Phoenix, Araraquara, SP, Brazil) at 110 °C for 10 min. For the maintenance of cells, YEPD medium was solidified with 20 g L^{-1} agar.

Molecular Identification of the Ethanol-Producing Yeast

The selected yeast strain was grown on YEPD at 25 °C and 150 rpm for 24 h. Then, 4 mL of medium was centrifuged at 10,000*g* for 5 min, and the pellet of cells was washed three times with distillated water under centrifugation. The genomic DNA was obtained by extraction of the pellet by cetyltrimethylammonium bromide (CTAB) (Fisher Scientific, Fair Lawn, New Jersey, USA), in accordance with the protocol described by Warner [20]. The ribosomal RNA (rRNA) operon encompassing the 5.8S rRNA gene and the flanking internal transcribed spacers ITS1 and ITS2 was amplified by polymerase chain reaction (PCR) using the set of primers ITS1 (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TATGC-3′) [21]. Amplification reactions were performed in a final volume of 25 μL containing 50 ng of genomic DNA (template), 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 3.0 mM $MgCl_2$, 200 μM of each dNTP (Thermo Scientific, Lithuania, EU), 20.0 pmoles of each primer, and 1.0 U of Taq DNA Polymerase (Thermo Scientific, Lithuania, EU). The PCR cycles were composed by denaturing for 1 min at 94 °C, annealing for 1 min at 52 °C, and extension for 2 min at 72 °C for a total of 30 cycles, followed by a final extension at 72 °C for 8 min. The amplified fragments were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, US). The sequencing reactions were carried out with primers ITS1 and ITS4 using the ABI PRISM Bigdye™ terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) following the protocol supplied by the manufacturer. The fluorescently labeled fragments were purified by ethanol precipitation, suspended in water, and subjected to electrophoresis in an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). The partial sequences were used to generate a consensus sequence. The obtained consensus sequence was compared to sequences within the NCBI database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST).

Ethanol Production by the Isolated Yeast

The selected yeast was inoculated on Agar YEPD and incubated at 30 °C for 48 h. Inoculum preparation was performed in 500-mL Erlenmeyer flask with a medium volume of 300 mL of YEPD. The growth was carried out at 30 °C and 150 rpm on an orbital shaker for 24 h. After

that, cells were centrifuged at 10,000*g* for 10 min to obtain the initial biomass used in fermentation assays (5.00 g L^{-1}±1 g L^{-1}).

The effect of temperature on ethanol production was studied using a synthetic medium, composed of 45 g L^{-1} glucose, 45 g L^{-1} fructose, 2.5 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} KH_2PO_4, 0.65 g L^{-1} $MgSO_4 \cdot 7H_2O$, and 0.65 g L^{-1} $ZnSO_4$, with an initial medium pH of 4.5 adjusted with 1 M HCl or 1 M NaOH. The concentration of glucose and fructose in the synthetic medium was the same to those found in the CAJ. Fermentations were conducted in 500-mL Erlenmeyer flasks with 250-mL medium at different temperatures (28, 30, 32, 34, 36, and 38 °C) and 150 rpm in an orbital shaker (Tecnal TE-420, Piracicaba, SP, Brazil).

CAJ and MCAB-OH were fermented at 500-mL Erlenmeyer flasks, containing 250-mL medium at 32 °C and 150 rpm in an orbital shaker (Tecnal TE-420, Piracicaba, SP, Brazil). When the influence of the initial pH medium was evaluated, CAJ was used and the initial pH values studied were 3.0, 4.5, 5.5, and 6.3, adjusted with 1 M HCl and 1 M NaOH.

During fermentation, samples were withdrawn at predefined intervals and analyzed for cell growth, pH, substrate concentration, and ethanol concentration. All assays were conducted in triplicate.

Analytical Methods

Cell concentration was determined by a turbidimetric method at 660 nm in a Spectronic 20 Genesys spectrophotometer. The concentration (g L^{-1}) was calculated from the calibration curve constructed for the selected yeast. The concentrations of sugars (glucose, fructose, and xylose) and ethanol were measured by high-performance liquid chromatography (HPLC; Waters, Milford, MA, USA) equipped with refractive index detector (Waters 2414) and an Aminex column HPX-87H (Bio-Rad, Hercules, CA, USA). Five millimoles per liter sulfuric acid was used as the mobile phase at a flow rate of 0.5 mL min^{-1}, and the analysis was conducted at 65 °C. The injection volume of samples was 20 μL. The samples were identified by comparing retention times to the retention time of the standard samples.

Fermentation Parameters

Fermentation parameters, such as maximum specific growth rate (μ_{max}), conversion of substrate to product ($Y_{P/S}$), conversion of substrate to cells ($Y_{X/S}$), the yield of product based on cell growth ($Y_{P/X}$), ethanol volumetric productivity (Q_P), sugar conversion (θ_S), and efficiency of sugar conversion to ethanol (η), were estimated at the end of the fermentation process, as defined by Pacheco et al. [22], using Eqs. 1–6, respectively.

$$\mu_{max}\left(h^{-1}\right) = \frac{\ln(X - X_i)}{t} \tag{1}$$

$$Y_{P/S}\left({}^{g}/_{g}\right) = \frac{P}{S_i - S} \tag{2}$$

$$Y_{X/S}\left({}^{g}/_{g}\right) = \frac{X - X_i}{S_i - S} \tag{3}$$

$$Y_{P/X}\left({}^{g}/_{g}\right) = \frac{P}{X - X_i} \tag{4}$$

$$Q_P\left(g.L^{-1}.h^{-1}\right) = \frac{P}{t} \tag{5}$$

$$\theta_S\ (\%) = \frac{P}{0,511.(S_i - S)} \tag{6}$$

$$\eta\,(\%) = \frac{P}{0.511.(S_i - S)} \tag{7}$$

where X is the cell concentration (g L^{-1}), X_i is the initial cell concentration (g L^{-1}), S is the glucose concentration (g L^{-1}), S_i is the initial glucose concentration (g L^{-1}), P is the ethanol concentration (g L^{-1}), and μ_{max} is the maximum specific growth rate. In Eq. 7, the value 0.511 corresponds at $Y_{P/S}$ theoretical.

The yields ($Y_{P/S}$) of first- and second-generation ethanol production were determined using the same equation (Eq. 2). These yields were based on the concentration of carbohydrate present in the medium (synthetic medium, CAJ, or CAB hydrolysate).

Ethanol volume-to-volume percentage (alc/vol, %) was determined as the ratio of the volume of alcohol per volume of broth at the end of the assay, multiplied by 100. For comparative analysis between the fermentative processes, fermentation parameters were subjected to the ANOVA statistical test performed with Origin Pro 8.0 (Microcal Origin Pro 8.0) software to evaluate the deviation of the variances of a group of the experimental data in order to predict, with a confidence level of 95 %, if the population of experimental data has any significant difference.

Results and Discussion

Isolation and Identification of an Ethanol-Producing Yeast from CAJ

During the fermentation of CAJ, an increase in turbidity after 24 h was observed suggesting microbial growth. The cultivation of the fermented CAJ in Sabouraud agar led to the isolation of a predominant translucent colony of yeast coded, GPBIO03. The molecular identification of the rRNA operon encompassing the 5.8S rRNA gene and the flanking internal transcribed spacers ITS1 and ITS2 was determined, and it had a length of 665 bp. Similarity searches on public nucleotide databases using that sequence revealed 100 % identity with *Hanseniaspora opuntiae* and *Hanseniaspora uvarum*. Therefore, the yeast strain was classified as *Hanseniaspora* sp. GPBIO03. The sequence was deposited in GenBank under the accession number KF791566.

Several authors have used different *Hanseniaspora* strains in the fermentation processes. Escalante et al. [23] evaluated the fermentative activity of *H. uvarum* using grape juice to produce fermented beverages. Andorra et al. [13] tested *Hanseniaspora guilliermondii* for ethanol production. Pina et al. [14] studied the tolerance of non-*Saccharomyces* strains to produce ethanol, in which two belonged to the genus *Hanseniaspora*. Thus, yeast strains belonging to the genus *Hanseniaspora* have been used in various fermentation processes.

Therefore, the yeast isolated from CAJ and identified as *Hanseniaspora* sp. GPBIO03 was evaluated for the production of ethanol (first- and second-generation). First of all, the effects of temperature and pH on first-generation ethanol production were determined.

Influence of Temperature on Ethanol Production

Figure 1 shows the influence of temperature on ethanol production by *Hanseniaspora* sp. GPBIO03 using a synthetic medium. It is possible to see that from 28 to 36 °C, no significant difference is observed at ethanol concentration during the assay. The same behavior was observed for glucose and fructose consumption, as well as biomass production. On the other hand, when the fermentation was conducted at 38 °C, lower values of ethanol and biomass

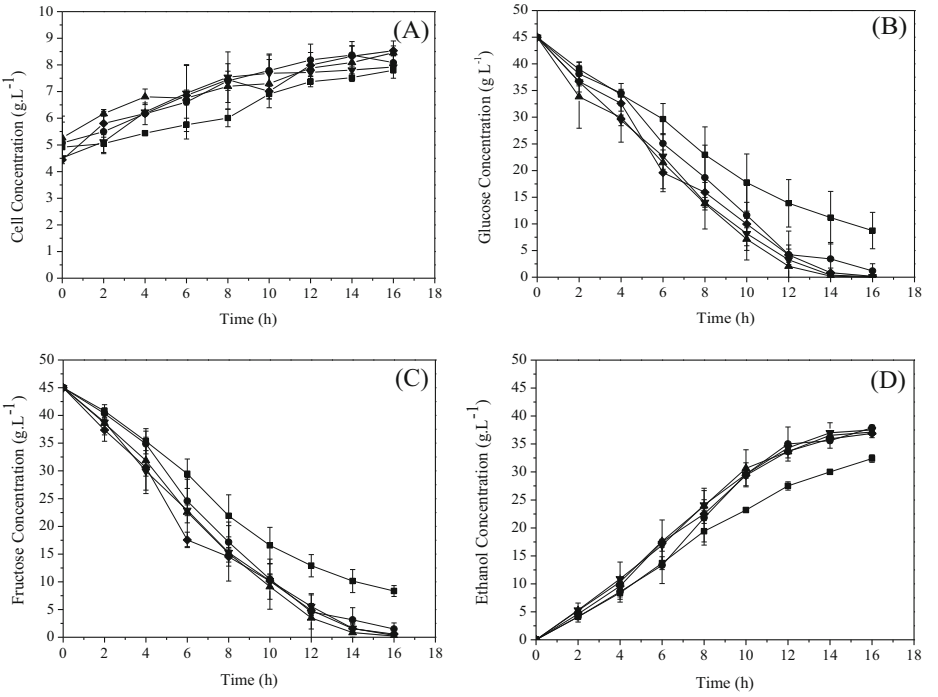

Fig. 1 Effect of temperature on substrate (glucose and fructose) consumption, biomass, and ethanol production by *Hanseniaspora* sp. GPBio03 at 150 rpm using a synthetic medium: 28 °C (*inverted triangle*), 30 °C (*triangle*), 34 °C (*diamond*), 36 °C (*circle*), and 38 °C (*square*)

were obtained. Table 1 resumes the effect of temperature on the kinetic parameters at the range evaluated. No significant ($p<0.05$) difference was observed in the conversion of the substrate into product ($Y_{P/S}$) in all tested temperatures. Likewise, efficiency was not significantly affected by the temperature. The yield of product ($Y_{P/S}$) was similar at temperatures of 28,

Table 1 Influence of the temperature on ethanol production by *Hanseniaspora* sp. GPBIO03 using synthetic medium

Parameter	Temperature					
	28 °C	30 °C	32 °C	34 °C	36 °C	38 °C
μ_{max} (h^{-1})	0.050±0.00	0.054±0.00	0.064±0.00	0.063±0.00	0.069±0.00	0.054±0.00
$Y_{P/S}$ (g g^{-1})	0.44±0.00	0.44±0.02	0.44±0.02	0.43±0.00	0.45±0.02	0.43±0.01
$Y_{P/X}$ (g g^{-1})	11.88±1.93	10.99±0.19	11.19±0.43	11.43±0.12	10.87±2.47	9.34±0.75
$Y_{X/S}$ (g g^{-1})	0.03±0.01	0.03±0.01	0.04±0.00	0.04±0.00	0.04±0.00	0.05±0.01
P_{max} (g L^{-1})	37.98±0.89	37.14±1.02	37.07±0.37	36.92±0.20	37.60±0.52	32.40±0.57
alc/vol (%)	4.81±0.11	4.71±0.13	4.69±0.03	4.67±0.12	4.80±0.14	4.11±0.08
Q_P (g L^{-1} h^{-1})[a]	2.37±0.06	2.32±0.06	2.31±0.02	2.24±0.12	2.35±0.03	1.95±0.13
θ_S (%)	99.53±0.17	99.72±0.00	97.77±1.35	99.12±0.43	98.57±0.56	83.66±1.76
η (%)	86.56±0.79	86.59±4.41	86.98±3.69	84.15±0.68	86.11±1.20	84.92±2.06

[a] Productivity determined at 12 h of fermentation

30, 32, 34, and 36 °C but was significantly different at 38 °C, reaching its lowest value (9.34 g g^{-1}±0.75 g g^{-1}). The substrate yield on cells ($Y_{X/S}$) was similar in the range of 28 to 36 °C, reaching its highest value at the temperature of 38 °C. On the other hand, lower ethanol production, productivity, and yield of product based on cells ($Y_{P/X}$) were achieved at this temperature. Therefore, the most suitable range for the operating process is at 28 to 36 °C.

The yeast consumed all the glucose and fructose (total sugar) during 16 to 18 h when grown in the temperature range from 28 to 36 °C, but when cultured at 38 °C, about 5 g L^{-1} of total reducing sugars was still present in the fermentation medium (Fig. 1b, c). The cell growth profile (Fig. 1a) was similar at all tested temperatures, reaching an average of 18 g L^{-1} of cells in the range of 28 °C to 36 °C and a lowest concentration at 38 °C (15 g L^{-1} cells). The higher ethanol concentrations obtained were similar in the assays conducted at 28, 30, 32, 34, and 36 °C and were approximately 37 g L^{-1} of ethanol. However, there was a decrease in the maximum production at 38 °C, reaching 32 g L^{-1} ethanol. These results indicate that the strain GPBIO03 is able to metabolize glucose and fructose for the production of ethanol at temperatures lower than 38 °C.

The results obtained in this study were different from those obtained by Torija et al. [24], which evaluated the effect of temperature on the kinetics of *Saccharomyces cerevisiae*, and stated that alcoholic fermentation is affected by temperature, with the highest yield at lower temperatures of 15–20 °C. In their study, when the bioreactor temperature was in the range of 25–30 °C, the initial fermentation rate was higher, and above 35 °C, a decrease in cell viability occurs, whereas in the present study, loss of viability occurred only when the yeast *Hanseniaspora* sp. GPBIO03 was grown at 38 °C.

Several studies with *Hanseniaspora* strains using grape or apple juice for alcoholic fermentation were conducted at temperatures between 25 and 28 °C [13, 17, 23, 25, 26].

The results of the fermentation parameters obtained in this work are higher than those obtained by Escalante et al. [23]. These authors used apple juice as culture medium with an initial substrate concentration of 127.2 g L^{-1} at 28 °C, which obtained the following parameters: specific cell growth rate, $Y_{P/S}$, and $Y_{X/S}$ of 0.05 h^{-1}, 0.15 g g^{-1}, and 0.062 g g^{-1}, respectively, after 22 h of fermentation.

Based on the obtained results, the average temperature of 32 °C was selected to conduct the next steps, using synthetic media, CAJ, or MCAB-OH.

First- and Second-Generation Ethanol Production by *Hanseniaspora* sp. GPBIO03

Figure 2a, b, c shows the profiles of biomass, substrate, and ethanol obtained of the fermentation with *Hanseniaspora* sp. GPBIO03 using synthetic medium, CAJ, and MCAB-OH, respectively.

The profile of cell growth was similar in all medium evaluated, being produced an average of 10 g L^{-1} cells. In general, *Hanseniaspora*, which is usually present in the early stages of wine production by natural fermentation of grape juice, shows limited growth time, due to its low tolerance to high concentrations of ethanol. Species such as *H. guilliermondii* and *H. uvarum*, for example, grow between 4 and 6 days of fermentation and then die largely as a result of increased ethanol concentration produced by strains of *S. cerevisiae*, also present in the broth, which has greater tolerance to alcohol. Generally, species of *Candida*, *Pichia*, *Metschnikowia*, and *Hanseniaspora* found in grape juice are not tolerant to higher concentrations of ethanol, superior to 4–7 % [17, 27–29].

On the other hand, Pina et al. [14] showed that *H. guilliermondii* and *S. cerevisiae* have similar tolerance to ethanol, presenting cell growth in an ethanol concentration of 25 % (*v*/*v*), and this yeast is more tolerant than other apiculate yeasts, for example, *H. uvarum*. In the

present work, the cell growth of *Hanseniaspora* sp. GPBIO03 was observed until the end of fermentation in all evaluated media, when the concentration of ethanol was 38 and 40 g L^{-1} for the synthetic media and CAJ (Fig. 2a, b, respectively) and 21.56 g L^{-1} ethanol for MCAB-OH (Fig. 2c).

The yeast consumed all the glucose and fructose (total sugars) in the period 16–18 h when cultivated in synthetic medium (Fig. 2a), but in the CAJ medium at 10 h of fermentation, total reducing sugar concentration was about 10 g L^{-1} (Fig. 2b). The maximum ethanol production was similar when using synthetic medium or CAJ medium, but the highest concentration was obtained at CAJ. These results indicate that the yeast *Hanseniaspora* sp. GPBIO03 is able to metabolize glucose and fructose to produce first-generation ethanol.

When grown in MCAB-OH (Fig. 2c), the yeast *Hanseniaspora* sp. GPBIO03 produced the highest ethanol concentration (24.37 g L^{-1}±0.68 g L^{-1}) at 5 h, and all glucose was metabolized by the yeast during this period. These results indicate that the yeast isolated is also able to produce second-generation ethanol.

Table 2 shows a comparison of the ethanol production by the yeast in the three fermentation media evaluated. The highest maximum specific cell growth rate (0.06 h^{-1}±0.01 h^{-1}), conversion of substrate to product (0.48 g g^{-1}±0.02 g g^{-1}), productivity of ethanol (4.40 g L^{-1} h^{-1} ±0.72 g L^{-1} h^{-1}), and efficiency (95.96 %±1.64 %) were obtained using CAJ as a carbon source, and thereby the juice is the best substrate for ethanol production by this strain. These results are probably due to the superior nutritional value of CAJ, which contains amino acids, vitamins, and minerals, according to Rocha et al. [30].

When MCAB-OH was used, ethanol productivity was 4.87 g L^{-1} h^{-1}, with an efficiency of 80.23 % and $Y_{P/S}$ of 0.41 g g^{-1} (Table 2), which was equivalent to 2 g of ethanol per 100 g of

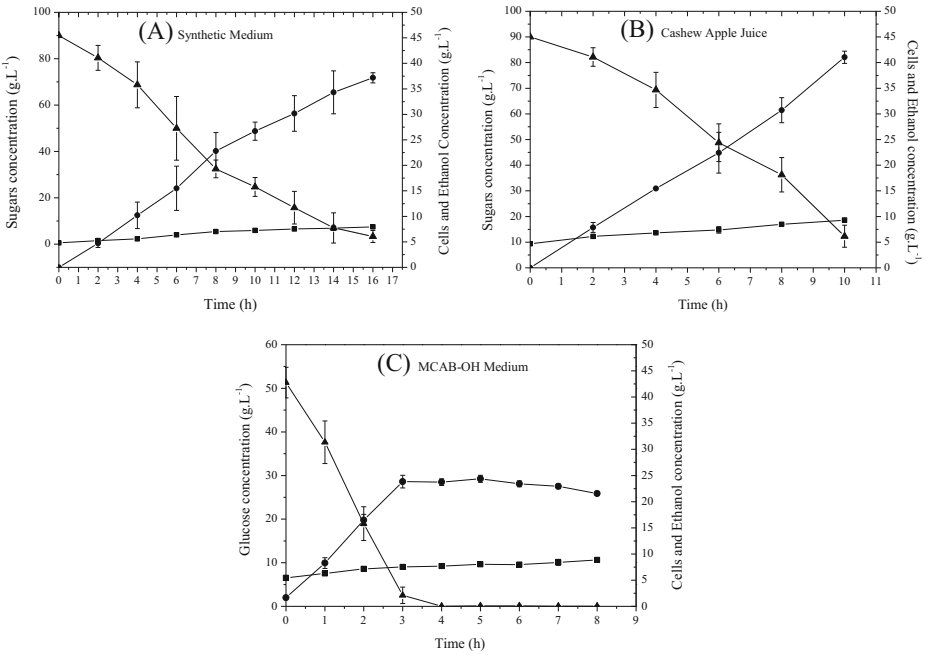

Fig. 2 Ethanol production by *Hanseniaspora* sp. GPBio03 at 32 °C and 150 rpm: synthetic medium (**a**), CAJ (**b**), and MCAB-OH (**c**). The experimental data are cell concentration (g L^{-1}) (*square*), ethanol concentration (g L^{-1}) (*circle*), and sugar (glucose + fructose) concentration (g L^{-1}) (*triangle*). Line of tendency (—)

Table 2 Parameters of ethanol production by *Hanseniaspora* sp. GPBIO03 at 32 °C using synthetic medium, cashew apple juice (CAJ), and cashew apple bagasse hydrolysate (MCAB-OH) as carbon sources

Parameter	Fermentation medium		
	Synthetic	CAJ	MCAB-OH
μ_{max} (h^{-1})	0.05±0.00	0.10±0.00	0.08±0.00
$Y_{P/S}$ (g g^{-1})	0.44±0.02	0.48±0.02	0.41±0.01
$Y_{P/X}$ (g g^{-1})	11.19±0.43	9.16±0.44	6.86±0.01
$Y_{X/S}$ (g g^{-1})	0.04±0.00	0.06±0.00	0.08±0.00
P_{max} (g L^{-1})	38.00±0.20	41.30±1.00	24.37±0.68
alc/vol (%)	4.69±0.03	5.07±0.19	3.04±0.08
Q_P (g L^{-1} h^{-1})	2.31±0.02[a]	4.40±0.72[a]	4.87±0.03[b]
θ_S (%)	97.77±1.35	95.22±0.16	100±0.00
η (%)	86.98±3.69	95.96±1.64	80.23±0.02

[a] Productivity determined at 12 h of bioprocess

[b] Productivity determined at 5 h of bioprocess

initial raw material. It is noteworthy that the enzymatic hydrolysate of CAB (MCAB-OH) has a low concentration of substrate compared to synthetic and CAJ media. However, these results indicate that yeast can also be applied in the production of second-generation ethanol, which was the objective of this work.

Several studies have investigated the production of second-generation ethanol using different lignocellulosic materials such as CAB [7, 9, 31], sugarcane bagasse [32], wheat straw [33], and corn stover [34] using the yeast *S. cerevisiae*, and the results of $Y_{P/S}$ are similar or lower than to those obtained in the present work.

Figure 3 shows a flowchart and the mass balance of the process to represent the integration of first- and second-generation ethanol production using cashew apple as feedstock. The cashew apple juice (CAJ) was used as carbon source and it contains 90 g L^{-1} of carbohydrate (glucose and fructose). The results obtained for this process were 41.3 g L^{-1} ethanol, with a yield and efficiency of 0.48 g g^{-1} and 96 %, respectively. Considering 100-g cashew apple bagasse as basis, a schematic diagram for second-generation ethanol production was carried out. Initially, CAB was pretreated using H_2SO_4 (0.6 mol L^{-1}), solid concentration of 30 % (*w*/*v*) at 121 °C for 15 min. The solid and liquid fractions were separated, and 61-g solid fraction was recovered and nominated of CAB hydrolysate (CAB-H). Afterward, the solid fraction (CAB-H) was treated using NaOH (4 % *w*/*v*), at a solid concentration of 7.5 % (*w*/*v*) at 121 °C for 30 min. It can be seen that 9.3 g of the solid fraction is recovered. Next, enzymatic hydrolysis was conducted using CAB-OH, and the hydrolysate was separated from the remaining solid fraction prior to ethanol production using *Hanseniaspora* sp. In this step, the sugar yield was equivalent to 5 g of glucose per 100 g of initial raw material, thus reaching 85.6 g L^{-1} of concentration in the liquid hydrolysate, which was adjusted for 50 g L^{-1} with distillated water. Finally, the yield of ethanol was 0.41 g g^{-1}, which represents approximately 24.4 g L^{-1} ethanol, 80 % of efficiency that could be theoretically obtained and a yield of 20 mg ethanol g^{-1} CAB (20 kg t^{-1} CAB).

Influence of Initial pH of CAJ on Ethanol Production

The profile of cell growth, consumption of sugars (glucose and fructose), and ethanol production was similar at all values of pH evaluated, see Fig. 4. However, the highest ethanol

production was achieved when initial pH was 3.0 (approximately 44 g L^{-1}). At pH 4.4, a concentration of ethanol of 42 g L^{-1} was produced with a lower consumption of substrate (Fig. 4b, c). Cell growth was lower in the initial pH 3.0 in which 7.5 g L^{-1} of biomass concentration was obtained (Fig. 4a). Figure 5 shows the evolution of pH throughout the fermentation with *Hanseniaspora* sp. GPBIO03 in the CAJ medium. In the media with initial pH 5.5 or 6.3, there was a decrease on the pH value at the beginning of the fermentation, which remained constant and equal to 4.5 at the end of each process. When the initial pH of the CAJ medium was adjusted to 3.0 or 4.5, it remained constant, thus demonstrating the buffering capacity of the CAJ. This capacity was also verified by Assad et al. [35], which studied the buffering capacity of 15 fruit juices by adding 0.1 N NaOH to 10 mL of a sample and found that 2.5 mL of the concentrated NaOH solution is needed to raise the cashew juice pH to 7.0.

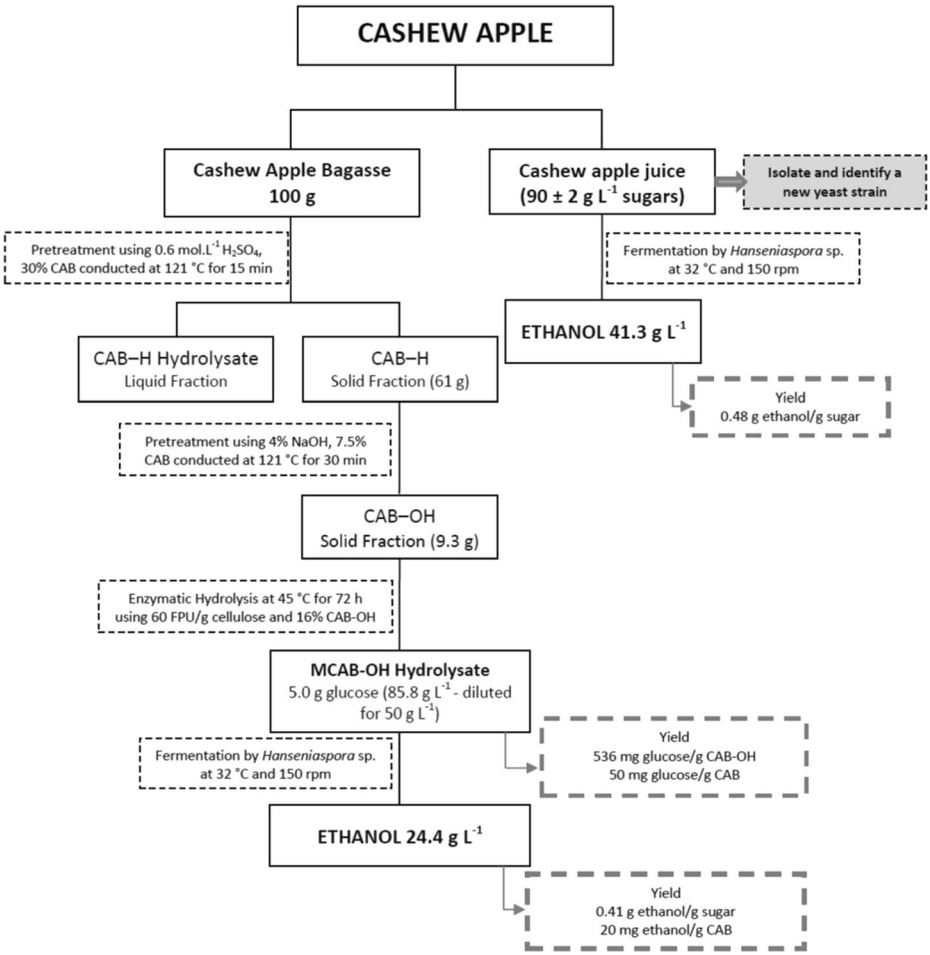

Fig. 3 Schematic flowchart and mass balance of the process to represent the integration of first- and second-generation ethanol production using cashew apple as feedstock

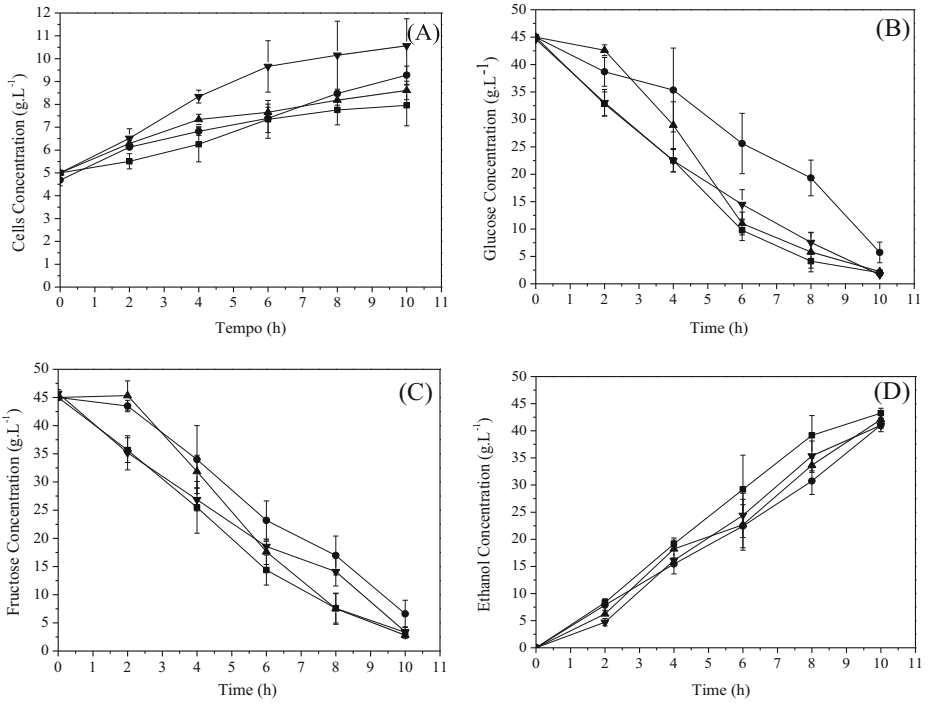

Fig. 4 Effect of initial pH on substrate (glucose and fructose) consumption, biomass, and ethanol production by *Hanseniaspora* sp. GPBio03 grown on cashew apple juice at 32 °C and 150 rpm: 3.0 (*square*), 4.5 (*circle*), 5.5 (*triangle*), and 6.3 (*inverted triangle*)

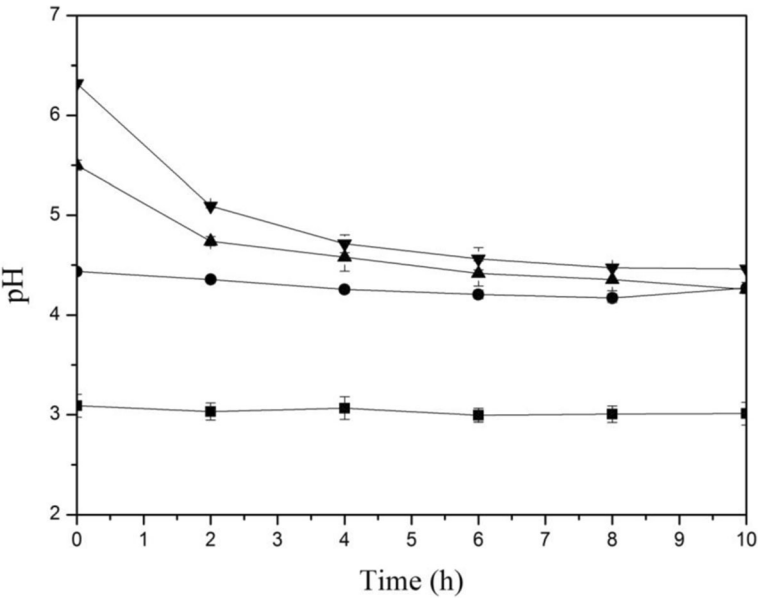

Fig. 5 pH values along time during ethanol production by *Hanseniaspora* sp. GPBIO03 using cashew apple juice (CAJ) at 32 °C and 150 rpm. Initial pH at 3.0 (*square*), 4.5 (*circle*), 5.5 (*triangle*), and 6.3 (*inverted triangle*)

The effect of pH on the maximum specific growth rate and in the $Y_{P/X}$, $Y_{X/S}$, and $Y_{P/S}$ yields, productivity, efficiency, and maximum production of ethanol was also evaluated, with the results shown in Table 3.

The pH did not influence the yield of substrate to product ($Y_{P/S}$), since the values were similar with a confidence level of 95 %. The cell yield in product ($Y_{P/X}$) decreased with increasing initial pH, reaching the maximum value (12.43 g g^{-1}) at pH 6.3 and the minimum value (6.06 g g^{-1}) at pH 3.0. The lowest value of $Y_{X/S}$ was observed at pH 3.0 (0.04 g g^{-1}), demonstrating that at this pH (3.0), the cells were subjected to metabolic stress [36].

The yields, the maximum ethanol production, and efficiency were similar at all pH values, demonstrating that the yeast was adapted to the environment in all conditions of pH.

Pina et al. [14], studying the ethanol tolerance of five non-*Saccharomyces* yeast strains (among which two were from the genus *Hanseniaspora*), used an initial pH of 3.8, 4.5, or 3.5 in their trials, and the results obtained demonstrated growth of *Hanseniaspora* strains at pH 3.5 to 4.5. These data corroborate the results obtained in this study.

Dorta [37] cited that pH 4.5 was sufficient to minimize the harmful effects of sulfite and ethanol on the cells. It is noteworthy that the fermentations conducted in more acidic media resulted in higher yields of ethanol due to low production of glycerol. This condition also helps to control infection as it reduces the growth of contaminating bacteria.

Therefore, the range of pH 3.0 to 5.5 is the more suitable to the process and is mostly used for yeasts of the genus *Hanseniaspora*, leading to a higher yield of ethanol, but also enables better control of infection and reduces the possibility of contamination of the process on an industrial scale where the conditions of perfect sterilization are more difficult to maintain.

According to Tables 1, 2, and 3, the maximum ethanol volume-to-volume percentage (alc/vol) is between 4 and 6 %. These results are similar to those of other authors [26] that studied the properties of oenological yeast non-*Saccharomyces* on fermentation of grape and using initial concentration of 270 g L^{-1} of total sugars and stated that the yeast produced 4–6 % ethanol, with a maximum of 6.07 % achieved by *H. uvarum*. However, the results obtained from the fermentation using *Hanseniaspora* sp. GPBIO03 reached the same range of ethanol production with a threefold lower concentration of total sugars, when compared to the experiments presented by Ciani et al. [26].

Table 3 Influence of pH on the ethanol production by *Hanseniaspora* sp. GPBIO03 at 32 °C using cashew apple juice (CAJ) as carbon source

Parameter	pH			
	3.0	4.5	5.5	6.3
μ_{max} (h^{-1})	0.104±0.00	0.100±0.00	0.106±0.00	0.140±0.01
$Y_{P/S}$ (g g^{-1})	0.48±0.02	0.48±0.01	0.49±0.01	0.48±0.00
$Y_{P/X}$ (g g^{-1})	12.43±0.25	9.16±0.12	8.45±0.15	6.06±0.20
$Y_{X/S}$ (g g^{-1})	0.04±0.01	0.06±0.00	0.05±0.01	0.07±0.01
P_{max} (g L^{-1})	43.26±0.89	41.05±1.10	42.10±1.11	40.03±0.59
v/*v* (%)	5.48±0.11	5.07±0.19	5.34±0.14	5.20±0.07
Q_P (g L^{-1} h^{-1})[a]	4.33±0.09	4.10±0.05	4.17±0.11	4.10±0.06
θ_S (%)	93.74±1.74	92.32±1.48	94.00±0.82	93.80±0.47
η (%)	94.83±3.05	93.23±3.64	95.89±2.77	93.71±1.21

[a] Productivity at 10 h of bioprocess

Conclusions

The yeast *Hanseniaspora* sp. GPBIO03, isolated from CAJ, has shown to be a promising strain for first- and second-generation ethanol production using CAJ and enzymatic hydrolysate of CAB as carbon sources. The optimum conditions of temperature and pH of the process range from 28 to 36 °C and pH 3.0 to 5.5.

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