



Evaluation of Bioethanol Production from a Mixed Fruit Waste by *Wickerhamomyces* sp. UFFS-CE-3.1.2

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Abstract

The use of fruit residues from domestic consumption for energy purposes is a perspective of innovation in the search for a more sustainable fuel, due to the low cost of this biomass, combined with its large amounts of generation, which has sugars readily available in its composition and does not require process pretreatment hydrolysis complexes of the material. This work evaluated residues of melon, pineapple, banana, apple, and mango, to compose a mixture of fruit waste (MFW), simulating a household waste for bioethanol production by a non-conventional yeast *Wickerhamomyces* sp. UFFS-CE-3.1.2. Sugar extraction was optimized by experimental design (MFW: 12% g dry mass v^{-1} ; 25 °C; 5 min) and resulted in the liberation of 36.32 ± 0.72 g L^{-1} fermentable sugar. MFW was used for alcoholic fermentation with and without nitrogen supplementation (urea and yeast extract). The results demonstrated that it is not necessary for the supplementation, making the process more economically viable. The maximum ethanol productivity (2.50 ± 0.06 g $L^{-1} h^{-1}$) was achieved in 9 h of the operation. The MFW extracted is an alternative for the bioethanol process as low cost and straightforward, adapted for different fruit residues, and used as a unique or diluent medium in the biorefinery context. Moreover, non-conventional yeast demonstrated the more new one in this study that explores the potential of yeast recently isolated.

Keywords Biofuel · Sugar extraction · Nitrogen source · Sustainability · Bioprocess

Introduction

The use of fruit residues on bioethanol production can be a promising alternative to face the energy crisis and improve environmental quality due to the characteristics presented by these biomasses, such as low cost, generation in large quantities, and high concentration of free sugars [1–3], which dispenses complex pretreatment and hydrolysis processes of the material, resembling first-generation bioethanol systems.

It is estimated that the final consumption is responsible for the waste of up to 20% of fruits and vegetables sold in the world due to their high perishability and also due to the natural

residue or inedible portion of the fruits, which includes mainly seeds, peel, and bagasse [4, 5]. In Brazil, the consumption of fruits is 57 kg per inhabitant per year [6]. Among the most consumed fruits in the country, banana, apple, and pineapple generate, on average, 37% of residues considering only the inedible parts of the fruit [7–9]. This information suggests that large quantities of domestic fruit waste generated daily could be used as raw material for bioethanol production. This presents itself as one of the novelties of this study. Based on concerns with the destination of domestic fruit waste for recovery in the bioethanol production chain, without pretreatment and hydrolysis, in an eco-friendly, simple, and inexpensive process.

The increase in the energy matrix by residual biomass is possible with optimized fermentation systems, high yields, and adapted microorganisms, whose potential economic interest can reach industrial scale [10]. In this sense, fermenting microorganisms are the key to highly efficient systems. In the bioethanol industry, strains of *Saccharomyces cerevisiae* are traditionally used. Yet, the exploration and characterization of different yeast species are essential for biotechnological development and are still a non-explored resource [11, 12]. This

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study addresses the application of a recently isolated yeast from decomposing wood that showed tolerance to inhibitory compounds, with a still non-explored potential that requires behavioral evaluations on different substrates [13, 14]. Especially considering that a medium rich in free sugars, such as the fermentative broth extracted from fruit residues, can be used as a single fermentation medium or as a diluent medium for fermentative broths with a high concentration of microbiological inhibitors. Therefore, we evaluated a non-explored fermentation medium and a recently isolated unconventional yeast capable of tolerating inhibitory compounds.

In this sense, this study evaluated the broth's use from a mixture of fruit waste from domestic consumption for bioethanol production using *Wickerhamomyces* sp. UFFS-CE-3.1.2 yeast. For this purpose, the ideal conditions for sugar extraction were investigated, besides the influence of adding yeast extract and urea as nitrogen sources to the fermentation medium for bioethanol production.

Material and Methods

Microorganism and Inoculum

The yeast used in the fermentation process is a new *Wickerhamomyces* strain (GenBank access number MF538579 and MF538580) that was denominated *Wickerhamomyces* sp. UFFS-CE-3.1.2 [14].

The yeast growth medium was prepared using yeast extract, peptone, and dextrose medium (YPD) (1% yeast extract, 2% peptone, 2% glucose, and 2% agar). Yeast maintenance was performed in test tubes containing 10 mL of the YPD in solid inclined-plane, and maintained for 72 h in a BOD. Refrigerated Incubator (SOLAB© 200) at 30 °C. After, the strain was transferred to tubes containing 10 mL of liquid YPD (without agar) and incubated for 24 h at 30 °C. Then, the liquid medium with the yeast cells was inoculated into the fermentation broth in a proportion of approximately 10% (v v⁻¹).

Fruit Waste

Fruit waste of banana, apple, pineapple, mango, and melon, including seeds, peel, and parts of the fruit inappropriate for consumption was collected individually in household waste and used as a substrate source for bioethanol production. These fruits were selected because of their tropical fruits, produced on a large scale in Brazil, and a high disposal percentage (Table 1).

After the collection, the wastes were crushed in an industrial blender (Colombo© Premium), dried in an oven with air circulation at 60 °C for 48 h, and subsequently ground in a 2-mm mesh knife mill (STAR FT50, Fortinox©, Brazil). This

Table 1 Fruits that were selected for MFW composition, amount consumed, and percentage of residues in each fruit

Fruit	Consume (ton year ⁻¹)	Waste (%)	Reference
Pineapple	92,991.2	50	[9]
Apple	139,692.1	25	[7]
Banana	68,075.4	35	[8]
Mango	95,871.4	20	[15]
Melon	79,730.9	23	[16]

procedure was conducted with each waste separately and aimed to reduce the particle that improved homogenization and increased the superficial area facilitating contact with water after sugar extraction.

The five fruit wastes (after grinding) were a mixture to simulate the residual fruit composition present in household waste. The mixing was carried out in different percentages, in a calculation that considered the amount consumed and the generation of residues of the respective fruits. These data used in the analysis are shown in Table 1.

Considering the fruit consumption and the amount of waste generated (Table 1), each fruit waste percentage to compose MFW was calculated (Eq. 1), where MFW_x (%) is the portion of each fruit used in MFW; Consume_x (ton year⁻¹) the amount of each fruit consumed (Table 1, column “Consume”); Waste_x (%) the percentage of waste for each fruit (Table 1, column “Waste”).

$$\text{MFW}_x(\%) = \frac{\text{Consume}_x(\text{ton year}^{-1}) \times \text{Waste}_x(\%)}{\sum(\text{Consume}_x(\text{ton year}^{-1}) \times \text{Waste}_x(\%))} \quad (1)$$

Thus, MFW was constituted by 32.9% pineapple, 24.7% apple, 16.8% banana, 13.6% mango, and 12.0% melon.

MFW Sugar Extraction

The free sugars present in MFW were extracted through solid/liquid extraction with mechanical agitation. For that, the residues were mixed, according to proportions determined in the previous section, resulting in the MFW that was maintained in contact with water in different proportions (solid/liquid ratio) under mechanical agitation [13].

Central composite rotatable design (CCRD) 2² was performed, assessing the influence of the solid/liquid ratio (0.69% to 23.31% m_{dry} v⁻¹) and temperature (14.64 to 85.36 °C), resulting in an experimental matrix (Table 2) with eight assays (combination levels -1;+1, and axial levels -1.41;+1.41 plus central points) and three central points (level 0) that represent the reproducibility of the process and possibility to estimate the standard error [17]. The matrix results were evaluated based on the liberation of free sugar (g L⁻¹) and broth volume extracted.

Table 2 Experimental planning of sugar extraction (real and coded values) and responses regarding sugar concentration, and additional evaluation of the broth volume

Assay	S/L (% m_{dry} v $^{-1}$)	Temperature (°C)	Sugars (g L $^{-1}$)	Broth volume (mL)
1	4 (-1)	25 (-1)	12.53	78.0
2	20 (1)	25 (-1)	61.63	11.5
3	4 (-1)	75 (1)	16.52	76.0
4	20 (1)	75 (1)	65.27	18.0
5	0.69 (-1.41)	50 (0)	2.18	90.0
6	23.31 (1.41)	50 (0)	76.78	4.5
7	12 (0)	14.64 (-1.41)	28.98	49.0
8	12 (0)	85.36 (1.41)	25.34	41.0
9	12 (0)	50 (0)	39.32	40.0
10	12 (0)	50 (0)	35.11	41.0
11	12 (0)	50 (0)	42.17	35.0

The experimental procedure was performed in Erlenmeyer flasks of 250 mL with a working volume of 100 mL, and the MFW was weighed according to the assay outlined in the experimental matrix. The assays were incubated in a water bath with agitation at 100 rpm for 10 min. The assays were filtered in a Whatman filter paper no. 1. The results were evaluated in the supernatant in terms of sugar-free. The determination of sugars-free was conducted based on methodology using 3,5-dinitrosalicylic acid (DNS) methodology [18].

An additional experiment was carried out aimed to evaluate the time to extract sugar in MFW. Therefore, after determining the best conditions (S:L and temperature) in the experimental matrix, the extraction was conducted in 5, 10, and 15 min, and the results evaluated in terms of sugar-free using DNS methodology [18].

Bioethanol Production Using MFW Broth

The alcoholic fermentation was conducted using the broth of sugars extraction from MFW optimized based on CCRD 2². The experiment was performed in 250-mL Erlenmeyer flask using 90 mL of fermentation broth [14] previously sterilized in autoclave (1 atm, 120 °C, 15 min) and 10 mL of inoculum of *Wickerhamomyces* sp. UFFS-CE-3.1. The samples were maintained in an orbital agitator (New Brunswick Scientific, Innova® 42) at 30 °C and 80 rpm [10]. Also, the experiments were conducted to evaluate the need to add nitrogen sources to the medium to improve fermentation parameters. The experiments with nitrogen source were conducted using the broth extracted from MFW supplemented with 15 g L $^{-1}$ urea and yeast extract, separately [19]. Fermentation conditions were the same described for a process without supplementation.

Aliquots were collected in 0, 3, 6, 9, 12, and 24 h of fermentation to analyze sugar consumption and bioethanol production using high-efficiency liquid chromatography (HPLC). The experiments were conducted in triplicate and presented in

sugar and bioethanol concentrations. The statistical analysis was carried out, as described in “Statistical analysis.”

Volumetric productivity of bioethanol (g L $^{-1}$ h $^{-1}$) was determined by the quotient between the maximum bioethanol concentration (g L $^{-1}$) and fermentation time (h). The bioethanol yield (g g $^{-1}$) was determined by the quotient between bioethanol concentration and total substrate consumed, as described by Shuler and Michael [20]

Analytical Methodology

Glucose, fructose, and ethanol concentrations were determined by HPLC using the Shimadzu Chromatograph, equipped with RID-10A detector, and operated with AMINEX® BIORAD HPX87H column. The samples were adequately diluted for the analysis in the mobile phase of 0.005 M sulfuric acid (H₂SO₄) (Sigma Aldrich©) and filtered in a 45- μ m cellulose acetate filter. The samples were injected at 45 °C using H₂SO₄ 0.005 M as eluent. H₂SO₄ solution was prepared adequately diluted (0.005 M) and vacuum filtered using a 0.45- μ m Millipore® membrane and degassed in an ultrasonic bath for 15 min. The eluent flow rate used was 0.6 mL min $^{-1}$. The concentration of all the analyzed compounds was determined using calibration curves, using specific HPLC standards (Sigma-Aldrich©) [14].

Statistical Analysis

Statistical analysis of CCRD 2² was performed using the software Protimiza Experimental Design, with 95% confidence level ($p < 0.05$). The results of the alcoholic fermentation procedures are expressed mean \pm standard deviation. Variance analysis (ANOVA), effects, and means comparison test (Tukey) were performed using the software Statistica 8 (Statsoft, Tulsa, USA) with a 95% confidence level ($p < 0.05$).

Results and Discussion

MFW Sugar Extraction

The sugar extraction was performed under conditions established in the CCRD 2² methodology, with two independent variables (temperature and S/L) and one response variable (sugar concentration). Table 2 shows the experimental matrix with the real and coded values and the result obtained to reduce sugar concentration. Broth volume recovered in each test after filtration was considered for evaluating the results obtained, given the importance of this parameter for the fermentation process conduction.

The extracted sugar concentration increased with the S/L ratio. The lowest sugar concentration was obtained from S/L of 0.69% ($m_{\text{dry}} v^{-1}$) at 50 °C (2.18 g L^{-1}), and the highest sugar concentration was achieved when an S/L of 23.31% ($m_{\text{dry}} v^{-1}$) at 50 °C (76.78 g L^{-1}) was employed (Table 2). These data show that S/L is an essential parameter in extracting sugars from fruit waste [21].

The variable effects show that only S/L is significant ($p < 0.05$) in the sugar extraction process, with 95% confidence.

Statistical analysis is shown in Table 3, and the empiric coded model for sugar extraction as a function of S/L ratio and temperature was generated (Eq. 4). The S/L rate is represented by X_1 and the temperature by X_2 , with S_{MFW} the concentration of sugars extracted from MFW:

$$S_{\text{MFW}} = 36.89 + (25.42 * X_1) + (0.31 * X_2) \quad (4)$$

As shown in Table 3, the resulting F value was 72.54, which demonstrated that the model is significant. Also, the lack of fit F value of 3.4329 compared to the regression F value of 72.54 (21.15 times larger) showed that the empiric model proposed is satisfactory to represent the sugar extraction process. The empiric coded model for sugar extract was validated by an ANOVA, with R^2 (correlation coefficient) value (0.947) and the F value for regression explaining the coded model (Eq. 4) for sugar extraction as a function of the S/L ratio and temperature.

Consequently, the CCRD 2² is reasonable; therefore, the adoption of temperatures that tend to ambient

temperature reduces energy consumption. Based on this result, the extraction of sugars from MFW in the other stages of the work was conducted at 25 °C. The higher the S/L, the greater the sugar extraction favoring is. However, in practice, a higher S/L implies a lower volume of extracted broth, as verified in Table 2. This occurs due to the capacity of water adsorption by the solid particles of the waste mixture. The adsorption of water in carbonaceous materials has been extensively studied due, among other aspects, to the impact on many industrial processes [22].

Thus, although the addition of a higher concentration of solids results in a higher concentration of extracted sugars, the adsorption of water by the particles leads to a significant reduction in the volume of filtered liquid, and it is necessary to carry out the process repeatedly to achieve sufficient volume of broth to conduct the fermentation process. Although the concentration of sugars is essential for bioethanol production, using an appropriate amount of substrate implies reducing water, biomass, time, and energy waste. Thus, 12% ($m_{\text{dry}} v^{-1}$) was the most appropriate S/L ratio from the sugar concentration released into the liquid medium and final volume of the filtered broth.

No difference in the extracted sugars concentration was verified in the three process times tested (5, 10, and 15 min). The extraction occurs by the contact equilibrium process; once the equilibrium is reached, the diffusion of soluble solids in the liquid mass is stopped [21].

The concentration of reducing sugars extracted in the optimal condition (12% S/L, 25 °C, 100 rpm, 5 min) was $36.32 \pm 0.72 \text{ g L}^{-1}$.

Batch Bioethanol Production Using MFW Broth

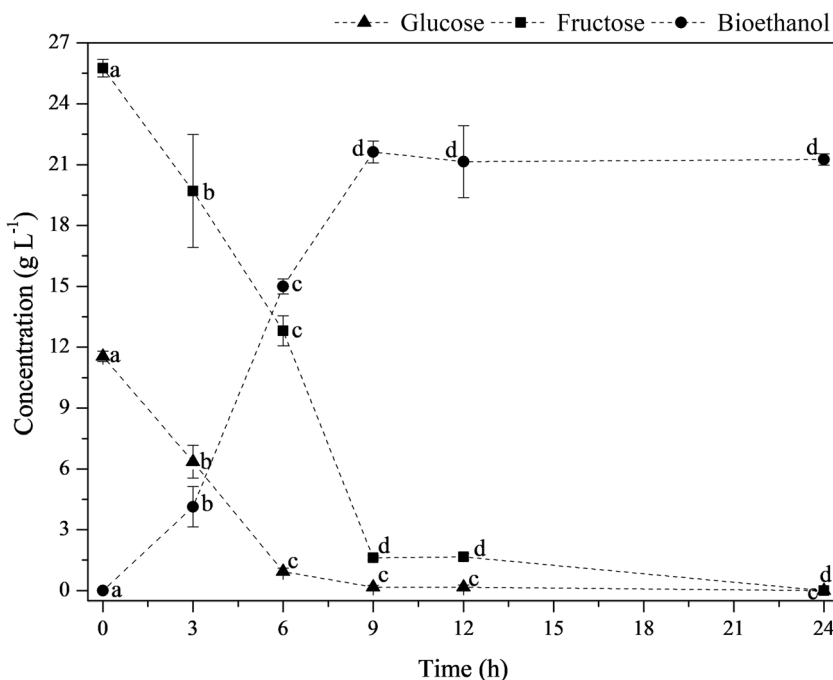
The performance of bioethanol production by *Wickerhamomyces* sp. UFFS-CE-3.1.2 yeast grown in MFW broth is shown in Fig. 1. A bioethanol concentration of $21.63 \pm 0.54 \text{ g L}^{-1}$ obtained from $11.55 \pm 0.25 \text{ g L}^{-1}$ of glucose and $25.75 \pm 0.43 \text{ g L}^{-1}$ of fructose was reached after 9 h of fermentation, remaining constant until the end of the process ($p > 0.05$).

Table 3 ANOVA for sugar extraction as the response for the CCRD 2²

Variation source	Sum of squares	Degrees of freedom	Mean squared	F value	p value
Regression	5169.69	2	2584.84	72.54	<0.01
Residual	285.07	8	35.63		
Lack of fit	259.84	6	43.31	3.43	0.24
Pure error	25.23	2	12.62		
Total	5454.76	10			

Ftab. 2; 8; 95%=4.46

Fig 1 Sugar consumption and bioethanol production by *Wickerhamomyces* sp. UFFS-CE-3.1.2 using MFW broth. ^aDotted lines were used to facilitate the visualization of the sampled points. Equal lowercase letters do not show the statistical difference by Tukey’s test with 95 % ($p < 0.05$)



Bioethanol production is slower during the first 3h of fermentation, due to the latency phase of yeast, a characteristic of recently inoculated culture. An important observation can be highlighted when observing that even with slower consumption (latency phase and immediately after), this occurs shortly (< 3 h). There is a tendency for rapid adaptation and beginning of consumption substrate and product formation. This factor results from a fermentation medium easily assimilated to yeast, with high concentrations of sugars and low concentration of inhibitors, which is favorable for the development of cells [20]. The short latency phase (where the substrate consumption is zero) is advantageous for industrial processes and deserves to be highlighted at this stage of the study.

The accelerated substrate consumption between 3 and 9 h of fermentation reaches bioethanol volumetric productivity of $2.50 \pm 0.06 \text{ g L}^{-1} \text{ h}^{-1}$. The bioethanol yield was 0.60 g g^{-1} , considering the sugars detected in this study (glucose and

fructose). This extrapolation in the yield data was compared to the maximum theoretical yield (0.511 g g^{-1}). However, given the biomass used, the presence of sucrose in these substrates is recognized (an extensive review of this characteristic is in Scapini et al. [1]). This factor may have resulted in a yield above the theoretical maximum expected, considering that this sugar was not evaluated in this study due to experimental limitations. Therefore, it is not considered a sugar that can be fermented even with a yield of up to 0.54 g g^{-1} [23].

The results are comparable with the research by other authors (Table 4), who also investigated the use of waste without applying pretreatment in bioethanol production. This study demonstrated that the MFW broth has potential as a substrate for bioethanol fermentation due to the high yield and efficiency of fermentation compared to other raw materials and a robust and straightforward process and environmentally safe.

Table 4 Comparison of bioethanol production using different substrates without pretreatment and results obtained by this study

Biomass	Microorganism	Bioethanol (g L ⁻¹)	Fermentation time (h)	Productivity (g L ⁻¹ h ⁻¹)	Reference
MFW	<i>Wickerhamomyces</i> sp. UFFS-CE-3.1.2	21.63 ± 0.54	9	2.50 ± 0.06	This study
Fruit and citrus peel waste	<i>S. cerevisiae</i>	29.5	9	3.8	[24]
Banana peel	<i>Enterobacter</i> sp. EtK3	3.07	48	0.06	[3]
Carob extract	<i>S. cerevisiae</i>	34.24	12	2.85	[21]
Banana frond	<i>S. cerevisiae</i>	45.75	15	3.05	[19]
Fresh oil palm frond	<i>S. cerevisiae</i>	18.67	24	0.78	[25]

Effects of MFW Broth Supplementation

MFW broth supplementation with yeast extract (Fig. 2a) returned a maximum bioethanol production of $19.82 \pm 1.66 \text{ g L}^{-1}$ from $8.82 \pm 1.17 \text{ g L}^{-1}$ glucose and $22.22 \pm 0.89 \text{ g L}^{-1}$ fructose after 6 h of fermentation, without significant difference in production in 9 h, 12 h, and 24 h ($p > 0.05$). The medium supplemented with urea (Fig. 2b) initially had $8.85 \pm 0.28 \text{ g L}^{-1}$ of glucose and $19.75 \pm 0.20 \text{ g L}^{-1}$ of fructose, returning maximum bioethanol production of $15.39 \pm 0.42 \text{ g L}^{-1}$ in 9 h of fermentation, maintaining the bioethanol concentration constant in the next hours of the fermentation process ($p > 0.05$).

From the results achieved, it can be seen that the two sources of nitrogen added to the MFW broth affect the fermentation process in different ways, as also observed by Zhao et al. [26]. Although higher bioethanol production has not been achieved in supplementing the medium with yeast extract, 3 h of fermentation process time is a promising result.

Tan et al. [19] found an increase in the growth of the *S. cerevisiae* strain and bioethanol production when higher concentrations of yeast extract were applied to the process. This increase in bioethanol production was associated with essential cofactors present in yeast extracts such as biotin and riboflavin. Thus, in this study, the reduction of fermentation time may be related to the stimulation of energetic metabolism of the strains provided by supplementation with a more assimilable type of nitrogen and vitamins besides other growth factors that may better satisfy the metabolic needs of the *Wickerhamomyces* sp. UFFS-CE-3.1.2 strain makes fermentation occur more quickly and results in better bioethanol volumetric productivity ($3.30 \pm 0.28 \text{ g L}^{-1} \text{ h}^{-1}$). The study conducted by Li et al. [27] also showed that yeast extract returned higher bioethanol yields, and higher process efficiency, by

supplementing the high-gravity corn starch fermentation medium with several nitrogen sources for bioethanol production using a strain of *S. cerevisiae*.

The fermentation conducted with the medium supplemented by urea returned a lower production of bioethanol. Although sugars were fully assimilated by yeast, there was no conversion to bioethanol as in the other two fermentations carried out, evidencing the occurrence of a metabolic deviation for the formation of other products, which can also be noticed by the reduction in bioethanol volumetric productivity ($1.71 \pm 0.05 \text{ g L}^{-1} \text{ h}^{-1}$). Glycerol production, a byproduct of alcoholic fermentation, is a significant parameter of bioethanol production and in the cells is responsible for osmotic regulation. When it occurs unbalanced, the carbon sources are deviated to glycerol production [28]. Moreover, urea and bioethanol may be acting as possible precursors in the formation of ethyl carbamate during the alcoholic fermentation process, as observed by Kim et al. [29]. The results corroborated with a recent study where the urea was used as a nitrogen source in broth obtained from papaya (*Carica papaya* L.) in alcoholic fermentation with *Wickerhamomyces* sp. yeast and showed negative effect under the system [13].

Therefore, this stage of the study demonstrated that the yeast extract was a potential source of nitrogen in MFW medium using the yeast *Wickerhamomyces* sp. as a fermenting organism, resulting in a reduction of 3 h of process and an increase of 1.37-fold the bioethanol volumetric productivity. These data are a novelty of this study for the scientific community, considering that this system can be coupled with other fermentative broths rich in sugars. In the future, interaction systems with nitrogen-rich media can be evaluated that can provide the necessary contribution to the MFW medium, exempting from the addition of external sources.

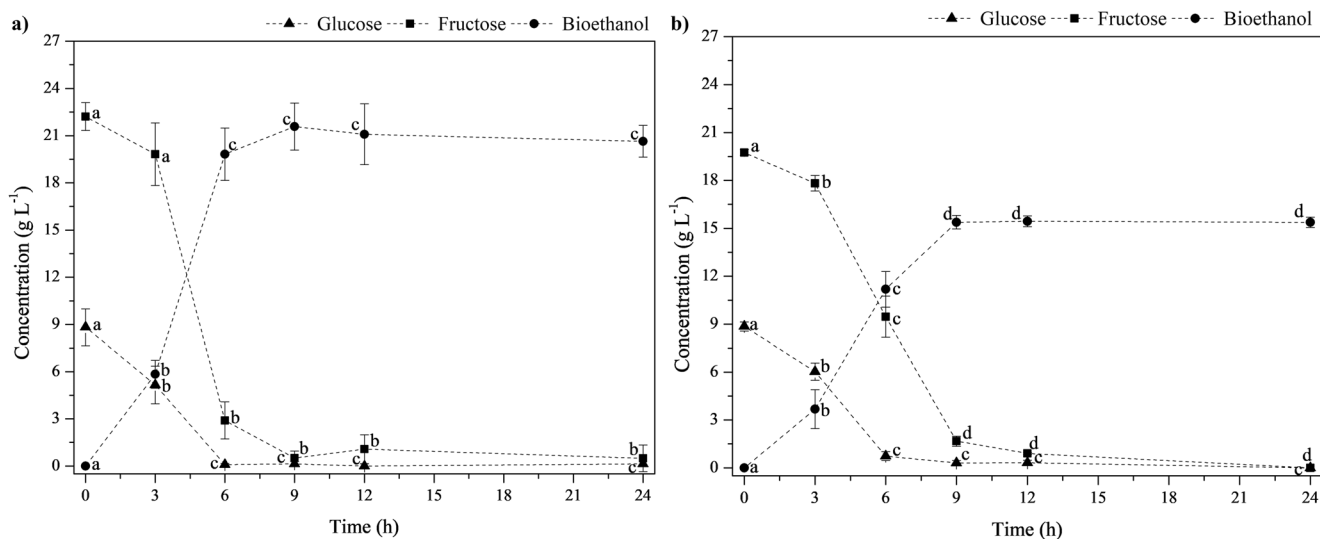


Fig 2 Sugar consumption and bioethanol production in broth supplemented with yeast extract (a) or urea (b). ^aDotted lines were used to facilitate the visualization of the sampled points. Equal small letters do not show the statistical difference by Tukey's test with 95 % ($p < 0.05$)

Conclusions

Fruit waste is a potential and alternative biomass for bioethanol conversion, mainly high sugar concentration. The non-exploration and discarding of fruit waste results in loss of an essential source of energy production. In this scenario, the presented study demonstrated that using an isolated yeast and employing the MFW as the substrate in bioethanol production is a viable alternative. The extracted broth was used for the alcoholic fermentation. Results showed high bioethanol yield, with concentrations of 21.63 g L⁻¹ in 9 h. The addition of yeast extract to the fermentation improved the performance, reducing fermentation time by 3 h and increased by 1.37-fold the volumetric productivity of bioethanol. The results indicated that household waste could be applied in bioethanol chain production, contributing to this process through single use or combined with other substrate sources. Besides, the potential of recently isolated yeast for alcoholic fermentation that has proved efficient contributes to the development of new technologies based on the application of non-conventional yeasts. From future perspectives, studies evaluating the behavior of the efficiency of the sugar extraction through the flexibility of biomasses, applying residues with more significant heterogeneity, have been observed as a great and important advance of this study.

Author's Contribution Jessica Zanivan: conceptualization, investigation, data curation, writing—original draft

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Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate All the authors consent to participate.

Consent for Publication All the authors consent to publication.

Conflict of Interest The authors declare no competing interests.

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