UNIVERSIDADE FEDERAL DA FRONTEIRA SUL
CAMPUS ERECHIM
CURSO DE CIÊNCIAS BIOLÓGICAS

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UTILIZATION OF BANANA PEEL WASTE FOR THE PRODUCTION OF BIOETHANOL AND OTHER HIGH-VALUE-ADDED COMPOUNDS

ERECHIM
2024

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Trabalho de Conclusão de Curso apresentado ao Curso de Ciências Biológicas - Bacharelado da Universidade Federal da Fronteira Sul (UFFS), como requisito parcial para obtenção do título de Bacharel em Ciências Biológicas.

Orientador(a): Profª. Drª. Helen Treichel

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Bibliotecas da Universidade Federal da Fronteira Sul - UFFS

Klein, Gabriel Henrique
UTILIZATION OF BANANA PEEL WASTE FOR THE PRODUCTION OF BIOETHANOL AND OTHER HIGH-VALUE-ADDED COMPOUNDS / Gabriel Henrique Klein. -- 2024.
26 f.

Orientadora: Doutora Helen Treichel

Trabalho de Conclusão de Curso (Graduação) - Universidade Federal da Fronteira Sul, Curso de Bacharelado em Ciências Biológicas, Erechim,RS, 2024.

1. Fruit residue. 2. D-Limonene. 3. Second generation ethanol. 4. Alcoholic fermentation. 5. Soxhlet extraction. I. , Helen Treichel, orient. II. Universidade Federal da Fronteira Sul. III. Título.

Elaborada pelo sistema de Geração Automática de Ficha de Identificação da Obra pela UFFS com os dados fornecidos pelo(a) autor(a).

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Este trabalho foi defendido e aprovado pela banca em 31 de maio de 2024

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Prof.ª Dr.ª Helen Treichel – UFFS
Orientadora

AGRADECIMENTOS

Primeiramente, agradeço eternamente aos meus pais Moacir e Leufrida por não medirem esforços para que eu pudesse continuar fazendo aquilo que sempre almejei. Obrigado por sempre me incentivar, por sempre mostrar que eu era capaz de seguir em frente e por sempre mostrar que tudo que desejamos para um mundo melhor, só depende de nós mesmos. Obrigado Pai, por fazer o impossível para que eu pudesse começar a graduação em uma cidade longe de vocês. Obrigado Mãe, por sempre me apoiar e mostrar o quanto me ama, e o quanto somos uma família que se ama. Enfim, graças a vocês, hoje estou no término da minha graduação, essa conquista não é minha, é nossa!

Agradeço infinitamente à minha irmã Gabrieli, ao meu cunhado Rodrigo e ao meu sobrinho Anthony por todo amor entregue ao longo desta caminhada, por todo apoio que recebi e por tudo que fizeram por mim, com certeza vocês foram parte essencial para que eu pudesse continuar.

Também, devo agradecer à minha vó, Lídia, por toda ajuda que recebi! Muito obrigado por sempre me apoiar em todas as questões e por sempre me incentivar. Sou muito grato por tudo!

Em geral, agradeço à toda minha família, que de alguma forma me auxiliou ao longo desta jornada! Muito obrigado por todo apoio e por todo incentivo que recebi!

Agradeço de forma especial, à minha dinda Elisete e ao meu dindo Adilson, por sempre estarem abertos a discutir comigo sobre todos problemas, por todos momentos que precisei e vocês se dispuseram a ajudar. Dinda, obrigado por todas ligações atendidas nos momentos de maior dificuldade em que ajudamos um ao outro, e obrigado por confiar sempre em mim e no meu potencial, você fez com que eu sempre acreditasse na minha capacidade!

Um agradecimento enorme, tão grande quanto nós, vai para minha amiga e orientadora Helen Treichel. Agradeço com muito amor e admiração por tudo que me ensinou no período de graduação. Obrigado por confiar no meu trabalho desde o princípio, quando entrei como voluntário para o LAMIBI, até hoje, como bolsista e orientado. Obrigado por todo tempo que disponibilizou para me auxiliar em todas dúvidas que tive, sejam elas para meu crescimento pessoal ou acadêmico. Deixo claro que você é minha maior admiração, pois trabalha com tanto amor, coragem e profissionalismo. Espero continuar por um bom tempo compartilhando conhecimento e aqueles bolos de chocolate bem recheados no LAMIBI. Sou eternamente grato por tudo que vivenciei no laboratório graças à sua confiança em meu trabalho!

Obrigado a todos pesquisadores do LAMIBI, por todo conhecimento compartilhado e por toda ajuda que recebi para realizar este trabalho. Obrigado Charline, por me fazer evoluir no ambiente do laboratório, pois quando não conhecia nada da área, você fez muito para que eu conseguisse desenvolver a pesquisa. Em especial, agradeço a Aline Frumi por sempre se dispor a me ensinar tudo aquilo que estava inseguro para realizar, por todas as dicas e por todos momentos que compartilhamos no LAMIBI. Também, a todos bolsistas que compartilharam conhecimentos comigo ao longo do trabalho, Laura, Júlia, Vitória e Larissa, meu muito obrigado!

À todos meus amigos, em especial à Julia, à Lavinya e ao Lucas que sempre estiveram comigo, dando todo apoio que precisei para continuar tentando. Agradeço infinitamente por todos momentos que passamos juntos, por todas as viagens do curso que, além de aprender, nos divertimos muito, por todos os cafés da tarde em que pudemos descontrair e por saber que podemos confiar uns nos outros. Agradeço por tudo que compartilhamos!

À todos professores que fizeram parte da minha formação, desde o ensino básico até à minha graduação. Todos foram fundamentais para que eu chegasse onde estou hoje!

Por fim, agradeço a todos que acreditaram e depositaram confiança em mim, que me incentivaram e tornaram tudo isso possível. Muito obrigado a todos e que tudo seja retribuído!

RESUMO

A banana (*Musa sp.*) é uma das culturas mais produzidas em todo o mundo, ultrapassando 100 milhões de toneladas por ano, e considerando que aproximadamente 30% de seu peso consiste em cascas, a geração anual desses resíduos é significativa. Os resíduos de frutas, de forma geral, representam uma fonte alternativa potencial para a obtenção de açúcares fermentáveis que podem ser utilizados em um conceito de biorrefinaria e economia circular visando a reutilização de resíduos. Em busca da produção de bioetanol dentro de um quadro de produção sustentável, este estudo otimizou o pré-tratamento e a hidrólise enzimática usando um Delineamento Composto Central (DCC 2^3), com um nível de confiança de 95% ($p < 0,05$). As variáveis de planejamento incluíram a concentração de ácido sulfúrico (H_2SO_4), a massa de resíduos de casca de banana na etapa de pré-tratamento e a concentração de enzima celulase comercial (Sigma-Aldrich) na etapa de hidrólise enzimática. As respostas quantificadas foram açúcares redutores totais. O maior rendimento de açúcares redutores totais encontrado após a hidrólise enzimática foi de 11,88 g/L, enquanto o rendimento de etanol foi de 1,37 g/L. Na recuperação de D-Limoneno, o valor obtido foi de 0,56 mg/g. Assim, o uso de resíduos de casca de banana para a recuperação de D-Limoneno em um conceito de biorrefinaria integrada contribui para os princípios da economia circular e valorização de resíduos.

Palavras-chave: Resíduo de frutas; D-Limoneno; Etanol de segunda geração; Fermentação alcoólica; Extração Soxhlet.

ABSTRACT

Bananas (*Musa sp*.) are one of the most produced crops worldwide, surpassing 100 million tons per year, and considering that approximately 30% of their weight consists of peels, the annual generation of these residues is significant. Fruit residues, in general, represent a potential alternative source for obtaining fermentable sugars that can be used in a biorefinery concept and circular economy aiming at waste reuse. In pursuit of bioethanol production within a sustainable production framework, this study optimized pretreatment and enzymatic hydrolysis using a Central Composite Design (CCD 2^3) with a confidence level of 95% ($p < 0.05$). The planning variables included sulfuric acid (H_2SO_4) concentration, banana peel waste mass in the pretreatment stage, and commercial cellulase enzyme (Sigma-Aldrich) concentration in the enzymatic hydrolysis stage. The responses quantified were total reducing sugars. The highest yield of total reducing sugars found after enzymatic hydrolysis was 11.88 g/L, while the ethanol yield was 1.37 g/L. In the recovery of D-Limonene, the obtained value was 0.56 mg/g. Thus, using banana peel waste for D-Limonene recovery in an integrated biorefinery concept contributes to circular economy principles and waste valorization.

Keywords: Fruit residue; D-Limonene; Second generation ethanol; Alcoholic fermentation; Soxhlet extraction.

SUMÁRIO

UTILIZATION OF BANANA PEEL WASTE FOR THE PRODUCTION OF BIOETHANOL AND OTHER HIGH-VALUE-ADDED COMPOUNDS

1. INTRODUCTION

The population increase that occurred in the last 100 years, from 2.2 billion to 8 billion (JOHANNESSEN et al., 2022) (UN NEWS-UNITED NATIONS ORGANIZATION, 2023), instigated an increase in demand for food which, due to several factors like failure in distribution, it leads to waste. It is estimated that of all food produced for human consumption worldwide, approximately 33% is wasted. Consequently, this issue has become a research focus on valuing waste (PRAMANIK et al., 2019).

No matter how varied these residues are, as long as they contain fermentable sugars, they can be used as raw material for ethanol production. Analyzing ethanol production in different countries, we can see this wide variety of raw materials. For example, in the United States, corn is used. In Canada and China, the use of corn and wheat, while in Brazil, the use of sugarcane gains prominence. However, in addition to obtaining first-generation ethanol, which is produced so that the fermentative agent can metabolize the substrate without preparatory steps, to increase production and minimize environmental impacts, as well as using biomass that would otherwise be discarded, it increases the development of second generation ethanol production to use agroindustry byproducts (DE LIMA, 2017).

Banana (*Musa* sp.), for example, has approximately 35% of its total weight represented by the peels and, as it is one of the most produced crops in the world, around 100 million tons in 2019 (FAOSTAT, 2019), contributes significantly to the generation of agro-industrial waste that is discarded without any use.

In general, fruit waste products have significant amounts of fermentable sugars and, as they are abundant waste, they can be used as raw material in producing biofuels, such as second-generation ethanol, biogas, and butanol, among others, with high-value aggregate (HEDGE et al., 2018).

It is essential to highlight that plant biomasses are mainly composed of polysaccharides (cellulose and hemicellulose) and lignin, and polysaccharides, as they are sugar polymers, are a potential source of fermentable sugars (HASSAN et al., 2018) and, with the rotation of efficient production, good ethanol yield can be obtained.

Still, it is worth saying that, although the production of biofuels is an exciting and alternative option for the use of banana peel waste, specific methodologies that are efficient

and economical are permitted since this biomass has a lignocellulosic matrix and requires steps of optimized production capable of fractionating the structures of destruction (PEREIRA et al., 2021).

Biomass formed by banana peels has a lignocellulosic matrix composed mainly of lignin (31.6%), cellulose (20.7%), and hemicellulose (17.3%) (PEREIRA et al., 2021). Due to this matrix, pretreatment is allowed before the transition to transform the complex structure into sugar monomers for subsequent conversion of these compounds.

Using lignocellulosic residues for biomass valorization relies on various methodologies to convert polysaccharides into monomers that will subsequently be converted into ethanol through microbial action. Typically, for the production of second-generation ethanol from lignocellulosic biomass, pretreatment steps, enzymatic hydrolysis, and, finally, fermentation is conducted (TREICHEL et al., 2020; VENTURIN et al., 2018). Moreover, due to the scarcity of literature concerning the recovery of D-Limonene from banana peel residues, the present study aimed to quantify this compound, considering its high added value in pharmaceutical industries due to its antimicrobial and anti-inflammatory properties, as well as in food industries due to its aromatic nature or for use as a preservative compound (HOU et al., 2022; OZTURK et al., 2019). Generally, studies on this compound focus on citrus fruits due to biomass structures; however, in this case, D-Limonene values in banana peels tend to be lower, which may render the process unfeasible due to the low final yield (KHANDARE et al., 2021). Lastly, this study aims to optimize the second-generation ethanol production route and obtain high-value-added products using banana peels.

2. MATERIAL AND METHODS

2.1 RESIDUAL BIOMASS

Agro-industrial waste was collected at the UFFS – Campus Erechim, RS University restaurant. The banana peels, specifically *Musa sapientum* and *Musa acuminata Cavendish Subgroup*, were dried in a forced ventilation oven (40 °C), ground in a knife mill until they reached a particle size of 20 mesh and stored (-20 °C) until use.

2.2 CHEMICAL CHARACTERIZATION

The biomass characterization was performed by determining the structures of cellulose, hemicellulose, and lignin, as well as the ash content and total solids, following methodologies standardized by the National Renewable Energy Laboratory (NREL). The following methods were used: "Determination of structural carbohydrates and lignin in

biomass" (SLUITER et al., 2008b); "Determination of total solids in biomass and total dissolved solids in liquid process samples" (SLUITER et al., 2008a); "Determination of ash in biomass" (SLUITER et al., 2005a); "Determination of extractives in biomass" (SLUITER et al., 2005b).

2.3 SOLUBLE SUGAR EXTRACTION

To remove soluble sugars from the residues without altering the sugar content for subsequent steps, residual biomass consisting of 10% w/v of dried banana peels and deionized water was maintained in a Dubnoff bath at 30°C for 5 minutes. Subsequently, the biomass was filtered to separate the solid fraction, which was dried at 40°C and proceeded to the pretreatment step, from the liquid fraction, which was stored at -20°C for an alcoholic fermentation test (BONATTO et al., 2021).

2.4 STUDY OF DIFFERENT PRETREATMENTS

The study of pretreatments involved the application of different methodologies, namely acid pretreatment (5% (v/v) H_2SO_4), alkaline pretreatment (1% (w/v) NaOH), and ultrasonic bath treatment (132W) at a frequency of 40kHz, and (66W) at a frequency of 40kHz. After pretreatments, the results were quantified using a spectrophotometer based on the release of sugars after enzymatic hydrolysis (GABHANE et al., 2014).

2.5 ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis was performed using pretreated residual biomass, sodium citrate buffer, and commercial cellulase enzyme (Sigma-Aldrich). Process conditions were 1% w/v of pretreated solids (dry mass) at pH 4.8, adjusted using 0.05 mol L^{-1} sodium citrate buffer, and hydrolyzed with 50 FPU/g of the enzyme. They maintained at 50°C and 150 RPM for 120 hours (GABHANE et al., 2014).

2.6 ETHANOL PRODUCTION

For the production of ethanol, the yeast *Wickerhamomyces sp.* UFFS-CE-3.1.2 was utilized. The yeast was cultured in a YPD medium of 1% yeast extract, 2% peptone, and 2% glucose, supplemented with 2% agar until use (BAZOTI et al., 2017). For inoculation, cells were transferred to a liquid YPD medium (10 mL) and incubated for 24 hours at 30 °C in a BOD incubator (Solab Científica ® 200) (BONATTO et al., 2021).

The fermentation step was carried out using the resulting must from enzymatic hydrolysis, with 90 mL of fermentative medium placed in 250 mL Erlenmeyer flasks sterilized in an oven to prevent ethanol contamination. In each flask, the inoculum was added, containing a fraction of liquid YPD medium with cells repitched at a ratio of 10%. The Erlenmeyer flasks were placed in an orbital shaker at 30°C and 120 RPM for 48 hours (New Brunswick Scientific, Innova®, 42) (BONATTO et al., 2021). Samples were collected at 0h, 12h, 24h, and 48h during fermentation and subjected to compositional analysis using HPLC.

2.7 PLANNING: PRETREATMENT AND ENZYMATIC HYDROLYSIS

The experimental design was conducted using the Central Composite Design (CCD) 2^3, assessing the pretreatment and hydrolysis stages. The variables evaluated included the banana waste mass, H_2SO_4 concentration (used in the pretreatment of residual biomass), and enzyme concentration. Table 1 presents the variables and their actual and coded values.

As a response, total sugars were quantified after the hydrolysis step. The statistical analysis of CCD 2^3 was conducted using the Protimiza Experimental Design software, with a confidence level of 95% ($p < 0.05$). The results are expressed in g/L and were evaluated through analysis of variance (ANOVA) and the effects of the variables.

Table 1. Central Composite Design 2^3 for pretreatment and enzymatic hydrolysis (actual and coded values).

Essay	Mass (g)	H_2SO_4 Concentration (%)	Enzyme concentration (FPU/g)
1	5 (-1)	10 (-1)	5 (-1)
2	15 (1)	10 (-1)	5 (-1)
3	5 (-1)	30 (1)	5 (-1)
4	15 (1)	30 (1)	5 (-1)
5	5 (-1)	10 (-1)	50 (1)
6	15 (1)	10 (-1)	50 (1)
7	5 (-1)	30 (1)	50 (1)
8	15 (1)	30 (1)	50 (1)
9	10 (0)	20 (0)	27.5 (0)

10	10 (0)	20 (0)	27.5 (0)
11	10 (0)	20 (0)	27.5 (0)

2.8 RECOVERY OF D-LIMONENE

The recovery of D-Limonene was performed using the conventional Soxhlet extraction method, where 8 grams of banana peel waste were subjected to extraction with 200 ml of hexane (matrix/solvent ratio 1:25) over 4 hours at the boiling temperature of the solvent (68°C) (LOPRESTO et al., 2014). Subsequently, the extracted fraction underwent purification using a rotary evaporator at 120 RPM and 100°C. After complete solvent evaporation, the resulting fraction was resuspended in 50 ml of hexane and stored at -80°C until preparation for gas chromatography (GC) analysis. Sample preparation involved dilution with analytical grade hexane 20 times and subsequent filtration through a 0.45 μm Millipore® membrane.

2.9 ANALYTICAL METHODS

The quantification of carbohydrates was carried out using high-performance liquid chromatography (HPLC) equipped with an HPX-87P or 87H column, and the D-Limonene content was quantified using gas chromatography (GC) according to CHOI et al., 2015.

The samples collected during the enzymatic hydrolysis period were subjected to the quantification of total reducing sugars using the 3,5-dinitrosalicylic acid (DNS) method, as described by (MILLER, 1959).

For the quantification of sugars, ethanol, and acids, the samples were diluted using 0.005 M sulfuric acid solution previously vacuum-filtered through a 0.45 μm Millipore® membrane and degassed in an ultrasonic bath for 15 min (ZANIVAN et al., 2022). The system used was an HPLC equipped with a refractive index detector (RID-10A) and an Aminex® Biorad HPX-87H column, using 0.005 mol H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and a temperature of 45 °C (BAZOTI et al., 2017).

Analyses for D-Limonene content were performed on a gas chromatograph coupled to a mass spectrometer (GCMS – QP 2010 Ultra, Shimadzu). An NST 05ms 30m/0.25mm/0.25μm capillary column was used for analyte separation. The following experimental conditions were employed: Initial oven temperature at 40 ºC (3 min), oven heating ramp from 40 to 180 ºC (5 ºC/min), from 180 to 210 ºC (10 ºC/min), with an injection volume of 1 μL, injection temperature at 220 ºC, injection mode: Split (1/10), using helium gas as the carrier gas (Linde, purity = 99.9999%), the pressure of 63.9 kPa, total flow rate:

17.2 mL/min, the column flow rate of 1.2 mL/min, linear velocity of 39.5 cm/s, purge flow rate of 4.0 mL/min, detector voltage at 0.2 kV, interface temperature of the mass spectrometer at 250 ºC, ionization source temperature at 250 ºC, scan m/z ratio: 30 to 500, and limonene retention time at 13.425 min.

3. RESULTS AND DISCUSSION

3.1 CHEMICAL CHARACTERIZATION

Biomass composition can significantly affect the processes; thus, studying the structures that compose the residue is of utmost importance. Despite the limited studies on the lignocellulosic composition of banana peel biomass, in this study, the composition of raw biomass showed values of cellulose (16.13%), hemicellulose (10.51%), and lignin (36.34%) (Table 2), which are consistent with the values described by PEREIRA et al., 2021. Pectin is also a polysaccharide in banana peel biomass, with a value of 1.25%. Given the high percentage of lignin and cellulose, developing a route to deconstruct these structures, such as pretreatments, was necessary.

Table 2. Characterization of banana peel biomass

	Average	Standard deviation
Cellulose (%)	16.13	0.94
Hemicellulose (%)	10.51	0.82
Lignin (%)	36.34	0.58
Pectin (%)	1.25	0.41
Ashes (%)	11.53	0.12
Total solids (%)	89.14	1

3.2 PRETREATMENT STUDY

The studied pretreatments showed different biomass cleavage, confirmed by the release of total reducing sugars. Due to the varied effectiveness of the methodologies, the pretreatment carried out in an ultrasonic bath did not yield satisfactory results. Meanwhile,

pretreatments using H_2SO_4 and NaOH (Table 3) proved advantageous, with better results observed for the acid treatment.

The average sugar values found after enzymatic hydrolysis confirm that both acid and alkaline pretreatments, as well as enzymatic hydrolysis conducted with commercial cellulase enzyme (Sigma-Aldrich), were efficient in cleaving glycosidic bonds and consequent sugar release. The maximum sugar value found in biomass pretreated with H_2SO_4 was 11.57 g/L; for biomass pretreated with NaOH, it was 7.28 g/L, while for biomass pretreated in an ultrasonic bath, the maximum value was 1.5g/L. Considering the better efficiency of the acid pretreatment, it was selected for application in the Central Composite Design (CCD).

In general, acid pretreatments provide high sugar yields as they can solubilize cellulose and hemicellulose fractions. However, it is essential to analyze the possible formation of fermentation inhibitors such as citric acid, furfural, and others and to facilitate equipment corrosion processes (SAWARKAR et al., 2022).

Table 3. Quantification of hydrolyzed sugars after pretreatments and enzymatic hydrolysis with commercial cellulase.

Time (h)	Ultrasound pretreatment at 100% power (132W)	
	Sugars (g/L)	pH
72	1.50 ± 0.08	4.92 ± 0.03
120	1.43 ± 0.19	4.89 ± 0.03
Time (h)	Ultrasound pretreatment with 50% power (66W)	
	Sugars (g/L)	pH
72	1.40 ± 0.03	4.95 ± 0.02
120	1.22 ± 0.24	4.78 ± 0.15
Time (h)	H_2SO_4 pretreatment	
	Sugars (g/L)	pH
72	9.39 ± 0.54	5.09 ± 0.03
120	11.57 ± 0.03	5.15 ± 0.05
Time (h)	NaOH pretreatment	
	Sugars (g/L)	pH
72	7.28 ± 2.54	5.09 ± 0.43
120	5.86 ± 4.74	4.99 ± 0.21

3.3 FERMENTATION WITH BIOMASS WASHING BROTH

After the extraction of soluble sugars from the biomass, a sugar-rich juice was obtained, which underwent an alcoholic fermentation test conducted with the yeast *Wickerhamomyces sp.* (Table 4) for comparison with fermentations using pretreated biomass, aiming to assess ethanol yield due to the formation of elevated levels of inhibitors such as citric acid.

Table 4. Alcoholic fermentation using biomass washing broth conducted with the yeast *Wickerhamomyces sp*.

Time (h)	Average sugar (g/L)	Average ethanol (g/L)	Citric acid (g/L)

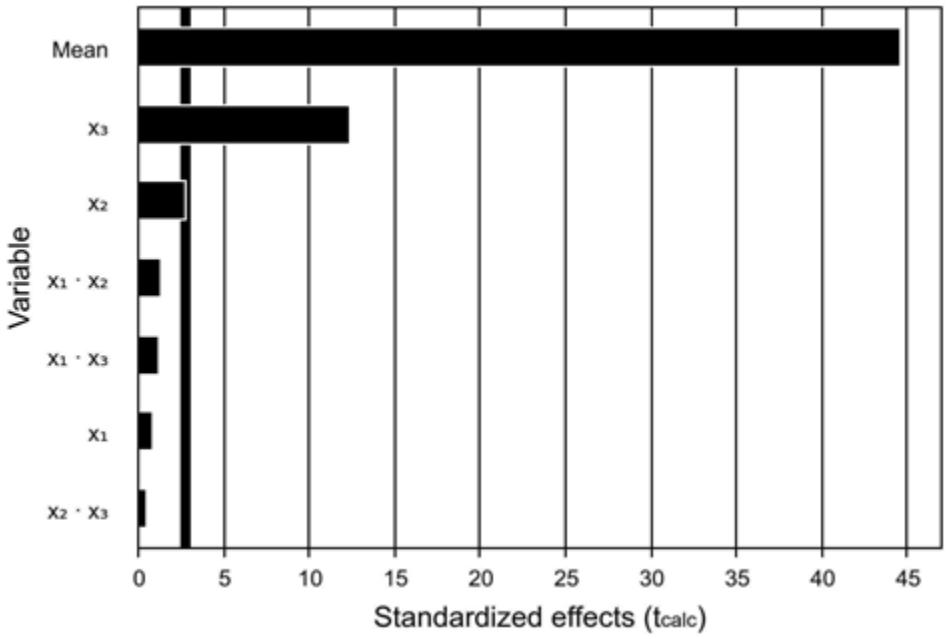
0	17.33 ± 1.23	0	2.30 ± 0.17
12	2.05 ± 0.04	7.92 ± 0.31	2.88 ± 0.04
24	1.87 ± 0.26	7.00 ± 1.13	2.63 ± 0.35
48	2.13 ± 0.15	7.75 ± 2.32	2.70 ± 0.28

Due to the high content of fermentable sugars in the biomass (17.33 g/L), significant ethanol values were obtained, with the optimal point observed at 12 hours (7.92 ± 0.31 g/L). Ethanol yield is directly associated with the high sugar concentration in the juice but also with the low presence of fermentation inhibitors, such as citric acid (2.88 g/L), possibly due to the route not undergoing chemical processes, as a large portion of inhibitors, such as acids are primarily generated after chemical pretreatment processes; in this regard, the yeast demonstrated efficient conversion of sugars into ethanol.

3.4 CENTRAL COMPOSITE DESIGN

For sugar release, it is observed that the only significant variable ($p < 0.05$) was the enzyme concentration (X3) (Figure 1). Nonetheless, using acid in pretreatment may interfere with the process due to forming fermentation-inhibitory byproducts (TANTAYOTAI et al., 2022). In this design, the highest total reducing sugar yield was achieved in trial 6, with a value of 11.88 g/L; however, it had a low ethanol yield, possibly due to fermentation inhibitors. Most likely, due to chemical pretreatment, a large amount of citric acid (9.05 g/L) was produced, resulting in a low ethanol yield at the end of the process, as shown in the table below (Table 5). The low amount of citric acid in stage 3.3 primarily supports this hypothesis since that stage was conducted without pretreatment. In contrast, acid values are almost four times higher in fermentation with pretreated biomass. Many discussions in the literature have also focused on studying inhibitors resulting from pretreatment stages, proving that routes involving chemical reagents and the presence of inhibitors in high concentrations resulting from the process lead to low ethanol yields (REZANIA et al., 2020).

Figure 1. Pareto chart for the effect of the studied variables on the release of total sugars

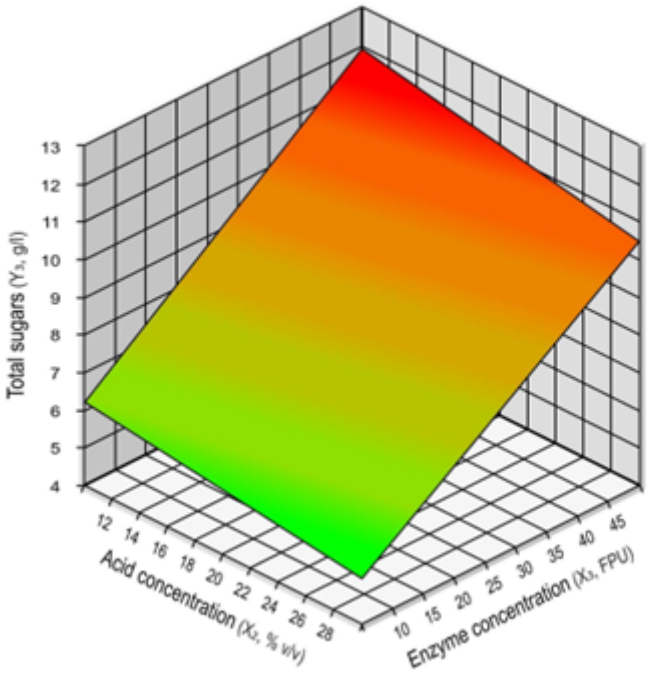


Due to the significance of only one variable, enzyme concentration, the process appears advantageous due to the possibility of low acid utilization (Figure 2). The use of low acid concentrations in pretreatment tends to favor the process economically due to the high cost of the reagent, as well as environmentally, considering reduced waste disposal. Using large enzyme concentrations may lead to increased process costs, but when combined with low acid quantities, it becomes viable due to the significant sugar release (KUSMIYATI et al., 2022). In previous studies with similar pretreatment and enzymatic hydrolysis conditions, sugar values were identical to those in this study, as described by GABHANE et al. (2014).

Table 5. Ethanol and citric acid concentrations at 12 hours of fermentation

Essay	Average sugar (g/L)	Ethanol g/L	Performance %	Citric acid g/L
1	7.09	0.28	0.19	8.49
2	5.00	0.00	0.00	8.73
3	4.89	0.00	0.00	6.87
4	5.19	0.32	0.34	6.12
5	11.63	01.03	0.16	7.52
6	11.88	0.96	0.14	8.8
7	10.24	1.20	0.23	5.92
8	10.39	1.37	0.40	7.33
9	8.61	0.66	0.28	6.9
10	8.79	0.53	0.17	8.66
11	9.12	0.18	0.06	9.05

Figure 2. Relationship of Acid Concentration Versus Enzyme Concentration to Sugar Results



3.5 RECOVERY OF D-LIMONENE

To date, the utilization of banana peel waste for D-Limonene recovery has not been reported, which strengthens the relevance of this step, which aims to achieve the integrated biorefinery concept by obtaining various biocompounds throughout the process. Thus, after compound extraction, the presence of D-Limonene in banana peel residues was quantified at average values of 0.56 mg/g.

4. CONCLUSIONS

Banana residues are rich in nutrients; nowadays, they are all disposed of in landfills. This work showed that it could be used for other things, such as alcohol, D-Limoneno, citric acid, etc. So, it is vital to bring this residue to a critical feedstock, aiming at products with aggregate value and some chance to employ people in entrepreneurship.

The values of fermentable sugars confirm banana peel waste as a potential biomass for biorefinery processes. Furthermore, the possibility of an integrated D-Limonene recovery is essential in obtaining bioproducts. In this sense, it can be concluded that utilizing these residues is promising in reducing environmental issues and valorizing food waste.

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