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Acquisition, Characterization, and Optimization of Distilled Bioethanol Generated from Fermented Carrot (*Daucus carota*) Residues

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Abstract: Bioethanol is a liquid biofuel produced from the digestion of biomass and usable waste of organic origin. The objective of this research was to obtain bioethanol from carrot (*Daucus carota*) residues of the Peruvian Chantenay variety, with a high content of lignocellulosic substances. The in-batch process method of enzymatic hydrolysis, with *Aspergillus niger* amyloglucosidase, and fermentation, with *Saccharomyces cerevisiae* yeast, was applied. The ferment was steam distilled and chemically characterized. The process was evaluated by controlling pH and enzyme/yeast mass ratio through the response surface optimization. The optimum conditions for the best values of TSS and % ethanol content for the distilled product were a time of 300 min, yeast/enzyme mass ratio of 24.0, and pH of 4.98. The results showed a significant decrease in sugars in the hydrolysis and fermentation stages, optimum alcohol content in the distilled product of 92.48% (*v*/*v*), lower organic compound content, and net calorific value of 23.82 MJ/kg, which is higher than those reported in the literature.

Keywords: bioethanol; *Daucus carota*; enzymatic hydrolysis; fermentation

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1. Introduction

Fossil fuels currently meet more than 80% of the world's energy demand, with a production of 55 million barrels (mb) per day and a projection of 25 mb per day by 2035 [1]. Their combustion directly affects human health, contributes to the release of large amounts of soot, and acts as the main driver of climate change [2].

By comparison, during recent decades, biofuels based on organic matter substitutes have been developed as cheap, renewable, safe, and cleaner energy sources [3]. For this purpose, organic wastes are used as feedstock in the production of bioethanol, biodiesel, or biohydrogen, through biomass fermentation and pyrolysis in solid state and liquid state [4,5].

Bioethanol (CH_3CH_2OH) is a clean, safe, and renewable biofuel [6], produced from lignocellulosic biomass due to its low cost and easy availability [7,8], in addition to its low consumption of water, chemical reagents, and yeast, which lowers the conversion cost [9,10]. Its addition to conventional gasoline (at a ratio of 5–25%) has been regularly employed in Europe and the United States [11,12].

Different sources of fermentable organic matter, such as agro-industrial waste, food waste [13], fruit and vegetable discards [14], and other substances with high cellulose, lignin, and/or hemicellulose content [15], have been used in their acquisition.

For bioethanol production, pretreatment is required to optimize sugar extraction, followed by enzymatic hydrolysis and subsequent alcoholic fermentation [6]. Hydrolysis consists of the chemical or enzymatic degradation of polysaccharides to fermentable sugars [16] (Equation (1)), while alcoholic fermentation is carried out by the action of

yeasts and bacteria to transform these sugars and other nutrients into ethanol and carbon dioxide [4,7] (Equation (2)). Both processes are exothermic, and the efficiency depends on the type of raw material used [10,17].

$$[C_6H_{10}O_6]_n + H_2O \overset{cat.}{\rightarrow} nC_6H_{12}O_6;\ \Delta H_h = -323.18\ KJ/mol \quad (1)$$

$$[C_6H_{10}O_6]_n \overset{cat.}{\rightarrow} CO_2 + C_2H_5OH;\ \Delta H_f - 70.9\ KJ/mol \quad (2)$$

The different modifications to bioethanol production processes aim to improve the chemical or biological activity of the participating organisms and increase the overall yield at low cost [10,18]. In the same way, optimization technologies based on multivariate analysis and the response surface have been applied [19,20]. However, the overall alcohol content of lignocellulose transformation into bioethanol does not exceed 50% [12].

Carrot (*Daucus carota*) pulp and pomace, being frequent residues in juice and food preparation, are important second-generation sources for bioethanol production due to their high content in weight of celluloses (20.8%), sugars (27.6%), and pectins (4.2%) [21]. Regarding carrots, hydrolysis optimization with different enzymes [22,23], nitrogen addition [24], and additional sugar sources [25] have been studied to improve fermentation, as well as modification of the fermenting agent [26]. Despite the significant number of studies performed, there is a considerable gap in the assessment of the optimization measures, characterization, and energy evaluation of products.

Therefore, the present study aims to obtain bioethanol from carrot residues, characterize the product, optimize the parameters of time, yeast/enzyme mass ratio, and pH in the re-reduction of sugar content during fermentation, and energetically evaluate biodiesel through net calorific value.

2. Materials and Methods

2.1. Biomass Collection and Analysis of Raw Material

Discarded carrot samples of the Chantenay variety were collected from a packing shed in the province of Chupaca (74°49′37.74″ W; 12°31′15.46″ S), Junín region, Peru, between April and July 2021. The samples were washed with water and placed in a shed at ambient conditions (5–20 °C; 5–10% relative moisture).

The moisture content was determined gravimetrically in the discarded carrot pulp to express all results on a dry weight basis (DWB). The composition of the dry pulp was determined according to the method of Ramos-Andres et al. [27] on discarded carrots. A 100.00 g sample of dried and pulverized carrot pulp was subjected to two consecutive Soxhlet extractions (the first with water and the second with hexane). Subsequently, the aqueous fraction was hydrolyzed with acid to fractionate it into insoluble material (lignin and ash) and soluble material (cellulose, hemicellulose, pectin, and lignin). The acid-soluble lignin was quantified by UV spectroscopy (UV2802SH-type, UNICO) at 250 nm and the acid-insoluble lignin was measured gravimetrically.

Polysaccharides were hydrolyzed in their monomers for analysis and quantified through HPLC (LC30 NEX-ERA, Shimadzu, Canby, OR, USA), as described by Ramos-Andres et al. [27].

First, 20.0 mL of ultrapure water, 5.0 mL of the liquid sample, and 1.0 mL of H_2SO_4 (72.1%) were mixed. The digestion process was carried out under stirring in a sealed vessel at 125 °C for 1 h. The hydrolyzed sample was cooled to room temperature in a desiccator and neutralized with Na_2CO_3. Impurities were separated with filter paper (pore size 0.22 mm, diameter 47 mm, mixed cellulose esters; Millipore, Burlington, MA, USA). The column of polystyrene divinylbenzene (PSDVB) SH1011 (6 um, 8.0 × 300 mm, Shimadzu, Kyoto, Japan) was maintained at 50 (±0.1) °C and the mobile phase had a flow of 0.95 mL/min of 0.01 N H_2SO_4.

Sugars were identified and quantified with a RID 10A detector, using sugar standards from Sigma-Aldrich: sucrose (99.0%), glucose (99.0%), fructose (99.0%), galacturonic acid

(97.0%), galactose (99.0%), and arabinose (99.0%). The concentration of hemicelluloses was corrected for galactose and arabinose at 0.90 and 0.88, respectively. For pectin, the factor of 0.90 was used for galacturonic acid.

2.2. Bioethanol Acquisition I: Enzymatic Hydrolysis

For the preparation of bioethanol, 5 kg of samples from the shed were selected, with individuals larger than 5 cm and presenting minimal areas contaminated by microorganisms. The selected samples were transferred to the laboratory and stored in areas without the presence of light to preserve them and ensure stable points of interest; α = 1.68179 [28,29].

For enzymatic hydrolysis, the initial acidity of the carrot should be conditioned by adding HCl diluted to a pH of 4.5 and controlled using a potentiometer (model B193528 096, SI Analytics), according to the measures suggested by Aimaretti et al. [30] and Yu et al. [26].

The acidified wort was heated to 70 °C for 2 min in a 2.5 L Pyrex beaker to hydrolyze the most complex polysaccharides. It was then cooled and poured into a batch reactor (BioFlo 510, Eppendorf, Hamburg, Germany), with automatic stirring and temperature control.

Then, 0.7 ± 0.1 mL of amyloglucosidase enzyme (Spirizime fuel, Sigma Aldrich, St. Louis, MO, USA) of Aspergillus niger origin was added at a dose of 0.25% v/v. The closed reactor was stirred at 150 rpm and maintained at a constant temperature of 30 °C for 1 h, as described by Aimaretti et al. [30]. Samples were taken every 30 min to evaluate the quantity of sugar, which was measured using a refractometer (HI96801, Hanna Instruments, Villafranca Padovana, Italy).

2.3. Bioethanol Acquisition II: Alcoholic Fermentation

Saccharomyces cerevisiae CCUB yeast cells were stored in a closed container for four days, at 4 °C and approximately 40.0% relative humidity, and without the addition of nutrients. A quantity of 9.0 g of yeast was added to the previously hydrolyzed wort in the batch reactor. The reactor was kept under constant agitation at 50 rpm and 55 ± 2.5 °C, with a time control of up to 5 h, based on the response surface model. Fermentation was controlled by monitoring CO_2 release, temperature, pH, and reducing sugar (RS) concentration, as described by Aimaretti et al. [30] and Demiray et al. [24], who used the same yeast. The sugars present in the ferment were quantified using HPLC, as described in Section 2.1.

2.4. Determination of TSS and Alcohol Content in Distilled Product

The fermented wort sample was settled and the supernatant was stored at −20 °C. The final concentration of total soluble solids (TSS) in °Brix was measured using a refractometer. Subsequently, the supernatant was separated and distilled at 75 °C with steam equipment. The distilled alcohol was then isolated for chemical characterization. In addition, a fraction of the broth was separated to determine the alcohol content. Statistical and comparison analyses were based on distilled alcohol.

2.5. Statistical Analysis and Response Surface

Before optimization, the variables were evaluated through a single factor, taking into account the publications of De Vrije et al. [21], Aimaretti et al. [23], Demiray et al. [24], and Khosko [25]. The relevance of the variables fermentation time, yeast masses, enzymatic mass, pH, and time were significant ($p < 0.05$), and the fermentation temperature was controlled at approximately 55 °C.

The relationships and effects of fermentation time (X_1), yeast mass/enzyme ratio (X_2) and pH (X_3) concerning response variables such as total suspended solids (Y_1) and percentage alcohol content (Y_2) were optimized by applying the response surface methodology.

The relationship between the variables was studied using the second-order polynomial model, as shown in Equation (3).

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i} \sum_{j}^{k} \beta_{ij} X_i X_j + e_i \quad (3)$$

where Y (as Y_1 and Y_2 responses) are the predicted answers; β_0 is the intercept; β_j, β_{jj}, and β_{ij} are the coefficients of the linear, quadratic, and interaction terms, respectively; X_i and X_j are the factors between $-\alpha$ and α; and e_i is the error relative to the measurements. This model was considered due to the presence of significant curvature in the usual optimization systems for biofuels [20].

The parameters were analyzed with the response surface technique using STATGRAPHICS 16 software, considering the R^2 coefficient values for the adjustment of coefficients. For the validation of the model, the significance test and analysis of variance (ANOVA), and curve fitting, with R^2 greater than 75% or $p < 0.05$ with a confidence level of 95%, consistent with Ngomade et al. [29], was used.

2.6. Chemical Analysis

The bioethanol that had the highest alcohol content after the experiments was used for chemical analysis. All chemical analyses were performed in triplicate, considering the ethanol sample distilled from the fermented must.

1. Relative density and viscosity were performed according to ASTM D4052-04 [31], which covers the determination of density, relative density, and API gravity of petroleum distillates and viscous oils, using a bulb density meter. Relative viscosity was measured in a U-type viscometer as the difference in flow time for water flow.
2. Aldehyde, carboxylic acid, and ketone analyses were performed using an 8860 GC gas chromatograph (Agilent, Wilmington, DE, USA), equipped with an FID Headspace detector and a 30 m × 0.25 mm packed column. The procedure was performed based on Medina et al. [32] for the determination of volatiles in alcoholic beverages. The conductive gas was hydrogen (flow rate of 1.5 mL/min). The temperature was programmed gradually from 50 °C (1 min), with a gradual increase of 4 °C/min up to 180 °C, and 8 °C/min up to 250 °C, with final maintenance for 5 min. Acetaldehyde, acetic acid, and acetone were used as standard. Aldehyde concentration was expressed as mg of acetaldehyde per 100 mL of anhydrous alcohol (AA). Carboxylic acid concentration was expressed as mg of acetic acid per 100 mL AA and ketones as parts per million (ppm).
3. Superior alcohol and methanol analyses were performed using the same chromatograph and column as in the previous section. The analysis was guided according to the European standard BS EN 1572:2013 [33] and Zhou et al. [34]. The conductive gas was helium of high purity (flow rate of 1.0 mL/min). The temperature was programmed gradually from 40 °C (2 min), with a gradual increase of 5 °C/min, up to 230 °C, with final holding for 3 min. The intensity of the individual peaks detected was related to the GC reference library to methanol, 1-propanol, 1-butanol, 2-butanol, isobutanol, 2-methylbutanol, and 3-methylbutanol.
4. Total phenol content was determined using the Folin−Ciocalteu spectrophotometric method compared with a standard gallic acid (GAE) curve as a reference. A quantity of 2 mL of the bioethanol was transferred to a flask and made up to 10 mL with a 10.75% (*m*/*v*) ethanolic solution of Na_2CO_3 and 1 mL of Folin−Ciocalteu reagent [35]. The sample was scanned in a UV/vis spectrophotometer at 760 nm; its absorbance was determined through the reference curve and reported as g/mL of solution.

2.7. Lower Heating Value (LHV)

LHV is the difference between the total energy released per unit of mass during the combustion process and that invested in the evaporation of the water it produced and

contained in the sample. Calorimetric equipment (BC-CMA-511, Malvern Panalytical, Oregon, Malvern, UK) was used according to the dynamic method of combustion in an adiabatic pump and oxygen atmosphere, considering the ASTM E711-87 standard [36] for waste-based fuels.

3. Results and Discussion

3.1. Raw Material Characterization

Discarded carrot pulp of the Chantenay variety used for bioethanol generation had a moisture content of 81.15%. For the characterization of the pulp on a dry basis, the average cellulose (12.65%), hemicellulose (5.85%), pectin (6.15%), and lignin (8.01%) contents were determined. The structural polysaccharide content is in the range reported by De Vrije et al. [21] and Khoshkho et al. [25]. Likewise, a high cellulose content is observed, which is the main input for ethanol generation.

3.2. Bioethanol Acquistion

The bioethanol acquisition process occurred through four steps: (i) breaking complex bonds in carrot must by the action of amyloglucosidase to produce cellulose and hemicellulose; (ii) depolymerization of carbohydrate chains into fermentable monosaccharides and disaccharides; (iii) controlling the conversion of pentoses and hexoses to pyruvate and similar molecules by glycolysis; and (iv) metabolization of pyruvate to acetaldehyde under anaerobic conditions and then reduction to ethanol by yeast dehydrogenase [15,37].

It was observed that the addition of a hydrolytic enzyme favors the degradation of sugars for ethanol production. In the case of yeast, it was found that, from 10.0 g of added agent, there is no more significant reduction in sugar content. It was determined that the final content of polysaccharides was made up of cellulose (1.38%), hemicellulose (0.52%), pectin (1.51%), and lignin (2.60%) of the original dry base. The fermentation broth presented a final alcohol concentration of 30.30% (23.90 g/L and 0.08 g alcohol/g yield sugar). This was higher than that found by Aimaretti et al. [30], who reported maximum alcohol content of 19.52% (concentration of 15.4 g/L and yield of 0.10 g alcohol/g sugar), and lower than the ethanol obtained by Aimaretti and Ybalo [23], with a maximum of 36.95% (concentration of 29.15 g/L and yield of 0.19 g alcohol/g sugar), using carrot discards and controlling only the enzymatic agent of the pretreatment. The comparison indicates that an efficient hydrolysis process was developed. Monitoring of yeast cell counts was not performed during the experiment.

At the end of distillation, bioethanol was obtained at a concentration between 68.15% and 92.48% (concentrations from 53.77 to 72.97 g/L, and yields from 0.34 to 0.47 g alcohol/g sugar), which indicates the feasibility of using carrot discards in bioethanol production, confirming the findings of Aimaretti and Ybalo [23]. These results are in the range determined for other lignocellulosic materials, with ethanol contents higher than 85% (concentration of 67.06 g/L, and 0.43 g alcohol/g sugar of yield) [8], and higher than those found by Yu et al. [26], with an alcohol content of 46% (concentration of 37.0 g/L, and yield of 0.23 g alcohol/g sugar), using carrot pulp and carrot peels, respectively.

The results also correspond to those found by Aimaretti et al. [22], for the production of bioethanol with an inoculum of 1011 cells/L of waste yeast and initial pH adjusted to 4.5, who determined an ethanol content of 80.0% (concentration of 63.12 g/L and 0.41 g alcohol/g sugar) and productivity of 10.40 g/L.h. Moreover, Khoshkho et al. [25] evaluated fermentation with the addition of beet molasses inoculum, finding a direct relationship between the alcohol concentration and additive, water, and dry residues used.

The results show that the programmed fermentation reaction reaches its optimum maximum at around 120−250 min, after which it declines rapidly. It is observed that the higher the value of the yeast/enzyme mass ratio, the faster the maximum TSS value is reached. The growth in TSS responds to the increase in monosaccharides after delignification, while the decrease corresponds to their transformation into ethanol [38].

The decreases in progressive TSS content are consistent with those described by Zabed et al. [15] and Tse et al. [37], which indicate that high substrate concentrations inhibit yeast growth and reduce fermentation for ethanol production as a result of high osmotic pressure producing glycerol as an osmoprotectant [4].

On the other hand, in the trials performed, there was no variation in fermentation yield with substrate concentrations, ensuring that sugar and ethanol inhibition did not limit the efficiency of the process, depending mainly on the variables of the amount of yeast and enzyme used in hydrolysis [23,39]. Since a differentiated treatment was employed for the same carbohydrate source, the substrate mass did not act as a differentiator. In this sense, the efficiency of enzymatic hydrolysis and the amount of yeast used in fermentation are considered important factors [4,38], since they are directly related to the availability of fermentable sugars transformed into ethanol, as highlighted by Manojkumar et al. [20] in different plant sources used to prepare bioethanol.

Regarding fermentation pH, as stated by Aimaretti and Ybalo [23] and Aimaretti et al. [30], values between 4 and 6 were used, with an optimum value of 5.5. Similar results were found by Clementz et al. [38] using nanometrically fixed yeasts. Unlike other vegetables and similar residues that work in basic media, carrot requires an acidified medium for its hydrolysis because of the density of lignocellulose in its cell walls, compared to other primary carbohydrate sources [4,40].

3.3. Statistical Analysis and Response Surface

The results of the TSS quantification (Y_1) and ethanol content (Y_2), relating to the variation in fermentation time (X_1), yeast/enzyme mass ratio (X_2), and pH (X_3), are shown in Table 1. A narrow variation was found in the range of 6.52 and 6.92 °Brix as TSS, while the alcohol content fluctuated between 82.26% and 88.73%. These ranges are consistent with those found in alcohols generated with second-generation carbohydrate sources [37] and significantly lower than those found by Vicente et al. [19], which were in the range of 86–100%, which modified the temperature and yeast/substrate mass ratio.

Table 1. Experimental design responses for TSS and ethanol content in the distilled product, by experiment conducted.

Exp.	Time (min), X_1	W_Y/W_E (g_{yeast}/g_{enzime}), X_2	pH, X_3	TSS (°Brix), Y_1	Ethanol Content (%), Y_2
1	180	6	5.0	6.73	82.26
2	120	12	5.5	6.87	82.89
3	120	12	4.5	6.86	82.92
4	180	15	6.0	6.85	83.37
5	180	15	4.0	6.83	83.44
6	240	12	5.5	6.67	83.48
7	240	12	4.5	6.66	83.51
8	60	15	5.0	6.92	83.82
9	180	15	5.0	6.72	84.41
10	180	15	5.0	6.71	84.42
11	180	15	5.0	6.72	84.43
12	300	15	5.0	6.52	84.99
13	120	18	5.5	6.83	85.05
14	120	18	4.5	6.82	85.08
15	240	18	5.5	6.63	85.63
16	240	18	4.5	6.62	85.67
17	180	24	5.0	6.60	88.73

It was found that, at the end of the designed experiments, the concentration of total soluble solids decreased significantly, while the percentage concentration of alcohol increased.

On the other hand, Table 2 shows the ANOVA analysis parameters for the set of variables analyzed in the second-order polynomial model. Significant variables in the regression ($p < 0.05$) were determined to be time, yeast/enzyme mass ratio, pH, and the quadratic relationships of the latter; the least significant relationships were then discarded.

In the correlation analysis of variables, a not very significant relationship (R^2 = 0.28) was found between the yeast/enzyme mass ratio and fermentation pH.

Table 2. ANOVA analysis for the response variables TSS and alcohol content (%) in the distilled product.

Variable	Sum of Squares	Df	Mean Squares	F-Relation	*p*-Value
Total Soluble Solids Content TSS (°Brix)					
Time (min)-X_1	0.16	1	0.16	86092.60	0.0000
W_Y/W_E (g/g)-X_2	0.00185548	1	0.00185548	998.40	0.0000
pH-X_3	0.02015460	1	0.02015460	10844.78	0.0000
X_2^2	0.00451098	1	0.00451098	2427.26	0.0000
X_3^2	0.02056800	1	0.02056800	11067.22	0.0000
Total error	0.00002044	11	0.00000186		
Total correlation	0.205494	16			
R^2 = 0.90		R^2 adjusted = 0.89			
Ethanol content (%)					
Time-X_1	0.05139200	1	0.0513920	1683.50	0.0000
W_Y/W_E (g/g)-X_2	0.01318220	1	0.0131822	431.82	0.0000
pH-X_3	1.22437000	1	1.2243700	40107.98	0.0000
X_1^2	0.00026785	1	0.0002679	8.77	0.0142
X_2^2	1.42777000	1	1.4277700	46770.84	0.0000
X_3^2	1.23625000	1	1.2362500	40497.20	0.0000
Total error	0.00030527	10	0.00003053		
Total correlation	36.0878	16			
R^2 = 0.95		R^2 adjusted = 0.90			

The polynomial functions presented in Equations (4) and (5), respectively, represent the regression for TSS and % content of alcohol. In both cases, coefficients of determination (R^2) higher than 90% are observed, indicating an excellent relationship of the studied variables in the range of the experimental results. The determination showed a curvature consistent with the optimizations for biofuel preparation by second-generation residues [20]. According to the analysis and values of $p < 0.05$, greater relevance is observed in the variables of pH and enzyme/substrate ratio on the fermentation time.

$$Y_1 = 9.92234 - 1.66667 \times 10^{-3} * X_1 + 0.0134409 * X_2 - 1.18987 * X_3 - 6.83072 \times 10^{-4} * X_2^2 + 0.119987 * X_3^2; R^2 = 0.90 \quad (4)$$

$$Y_2 = 55.8971 + 5.2589 \times 10^{-3} * X_1 - 0.03902 * X_2 + 10.1156 * X_3 - 1.03746 \times 10^{-6} * X_1^2 + 0.0132836 * X_2^2 - 1.01494 * X_3^2; R^2 = 0.95 \quad (5)$$

For the same experimental design, Ngomade et al. [29] determined the relevance of fermentation time and yeast mass applied in the regression equation for the optimization of response in ethanol content, with an R^2 between 0.83. Previously, Vicente et al. [19] determined the relationship of temperature and yeast/substrate mass ratio variables for the percentage ethanol concentration with an R^2 = 0.95.

It is important to mention that the variables studied have opposite effects on TSS and the ethanol content obtained. The present research shows a higher degree of correlation with both processes, highlighting the importance of time and pH in the 4.5–5.0 range in fermentation [23,38,40], as well as the improvement in the system response after the addi-

tion of a controlled amount of enzyme in the pretreatment. The importance of enzymatic pretreatment was previously exposed by Tse et al. [37] and Aimaretti and Ybalo [23] in the preparation of bioethanol from second-generation sources, due to the increase in the concentration of available fermentable sugars, longer yeast lifetime, and optimal control of pH, temperature, and oxygen variables in the reaction medium to preserve the hydrolytic properties of amylases [20].

The comparison of the theoretical and experimental results in obtaining bioethanol, applying Equations (4) and (5), as well as the optimized experimental values, are shown in Table 3. A general deviation in the range of 0.5–4% of the experimental data for the theoretical values was observed.

Table 3. Comparison of theoretical and experimental results in TSS and percentage ethanol content in distilled product.

Exp.	SST Theo. (°Brix)	SST Exp. (°Brix)	Relat. Deviation SST (%)	Alcohol Content Theo. (%)	Alcohol Content Exp. (%)	Relat. Deviation Alcohol Content (%)
1	6.93	6.73	2.89	81.96	82.26	0.37
2	7.12	6.87	3.51	82.76	82.89	0.16
3	7.02	6.86	2.28	82.79	82.92	0.16
4	7.15	6.85	4.20	83.06	83.37	0.37
5	6.95	6.83	1.73	83.13	83.44	0.37
6	6.92	6.67	3.61	82.94	83.48	0.65
7	6.82	6.66	2.35	82.97	83.51	0.65
8	7.12	6.92	2.81	83.78	83.82	0.05
9	6.92	6.72	2.89	84.11	84.41	0.36
10	6.92	6.71	3.03	84.11	84.42	0.37
11	6.92	6.72	2.89	84.11	84.43	0.38
12	6.72	6.52	2.98	84.15	84.99	1.00
13	7.07	6.83	3.39	84.91	85.05	0.16
14	6.97	6.82	2.15	84.94	85.08	0.16
15	6.87	6.63	3.49	85.09	85.63	0.63
16	6.77	6.62	2.22	85.13	85.67	0.63
17	6.80	6.60	2.94	88.42	88.73	0.35
Optimal	6.40	6.20	3.13	89.30	92.48	3.56

The optimum point was determined with X_1 = 300 min, X_2 = 24.0 g/g, and X_3 = 4.98. The experimental optimum result was better than that calculated through the equations, obtaining an ethanol content of 92.48% and a TSS content of 5.90 °Brix, due to an increase in the fermentation time, high enzyme/yeast ratio, and pH close to 5. It is important to mention that the effect of fermentation time is always constant and directly related to ethanol production, with the main yeast/enzyme mass ratio and pH being subject to optimization.

The small deviation found between the theoretical and experimental data is similar to that found by Ngomade et al. [29] and Del Aguila et al. [28] when applying surface response modeling using vegetable oil sources. In the case of other fermentable sugar sources, the percentage is similar to that found in seeds, which showed high cellulose content [20]. The optimized result found in the present study was similar to those reported by Aimaretti et al. [30], Aimaretti and Ybalo [23], and Khoshkho et al. [25], whose ranges vary from 95 to 100%, when using carrot discard, peels, and pulp, respectively.

Considering an average sugar concentration contained in the must of 93.1 g/L and the final ethanol content at 92.48%, the results of the experiment were comparable to those obtained by Aimaretti et al. [30], who used carrot discards for alcohol content of 35.6 g/L.day. They were also in agreement with the bioethanol concentrations obtained by Khoshkho et al. [25] at 51.5 mL/L (40.63 g/L), where mixtures of carrot pulp, sugar inoculum, and water were controlled.

While authors such as Aimaretti and Ybalo [23] focus on the enzyme preparation in the pretreatment, Khoshkho et al. [25], Yu et al. [26], and Demiray et al. [24], have optimized based on the fermentation conditions, independently. In the present study we address both

spectra together through the variable X_2 (yeast/enzyme mass ratio), since they are factors with a greater contribution to productivity than temperature [19].

3.4. Bioethanol Chemical Analysis

The result of the chemical analysis of the optimized bioethanol is shown in Table 4, compared to ASTM D4806 (and its complementary regulations) with characteristics required as an additive (the data were taken from Obeta et al. [41].

Table 4. Physicochemical analysis of bioethanol on distilled product compared to the ASTM standard for alcohol used as an additive.

Parameter	Bioethanol (This Study)	Standard Ethanol	ASTM
Ethanol content (% *v*/*v*) on distilled product	92.48	-	>92.00
Density at 15 °C (g/cm^3)	0.89	0.794	0.80
Specific gravity	0.90	--	--
Viscosity at 40 °C (mm^2/s)	1.65	1.30	1.34
Flashpoint (°C) of distilled product	14.5	12.5	18.60
Aldehydes (mg/100 mL)	1.30	--	--
Ketones (ppm)	0.10	--	--
Acidity (mg/100 mL acetic acid)	0.90	--	>0.70
Superior alcohols (mg/100 mL)	2.30	--	--
Methanol content (% m/m)	0.01	--	<0.50
Phenol−water ratio (g/mL)	0.20	--	--
Moisture content (%)	2.50	--	20.00
Sugar content (% m/v)	0.40	--	--

Traces of impurities from amino acids, minerals, and organic acids, as heating residues, were observed in the analysis. The determination of aldehydes, ketones, acetic acid, and higher alcohols in minimal quantities within the final product correspond to traces of enzymes, yeasts, and volatile compounds that could have contaminated the ferment before steam distillation [42].

Regarding the concentration of the bioethanol obtained, several studies with lignocellulosic sources have designed variants in the pretreatment and fermentation processes, with bioethanol contents between 75% and 95% after enzymatic pretreatment [4,15]. For the present study, the ethanol content (92.48%) showed higher percentages than those found in rice husks (82%), corn stover (90%), rapeseed husks (91%), Miscanthus (88%), and other forms of grasses (62–74%), pretreated with H_2SO_4, alkali, and enzymes [6,15]. The highest percentages of ethanol concentrations probably correspond to the enzymatic pretreatments (cellulases, accelerates, glucosidases, and *Aspergillus niger*), that were applied in the present study.

Regarding the other bioethanol properties, the characterization results were found to be within the ranges specified by the US Standard ASTM D4806 (and its complementary regulations) and the European Standard EN14214 for bioethanol and biofuels [29].

Density and viscosity are those variables that directly affect fuel distribution, transport, and storage processes. In addition, they affect the production of waste gases after combustion. In the case of biofuels, Tüccar et al. [43] found a direct relationship between CO_2 and NO_X production, while CO volume was reduced. The bioethanol obtained in our study had higher density and specific gravity (0.89 g/cm^3) than the standard ethanol (0.79 g/cm^3) and that stated in the ASTM standard for use as an additive (0.80 g/cm^3), as a result of the content of other oxygenated chemical species of higher molecular mass than that condensed during distillation. These indicators suggest sufficient content of chemical species with the capacity to burn completely through a stoichiometric relationship with the available air [44].

In the same sense, the viscosity of bioethanol at 1.65 mm^2/s is sufficient for pulverization and atomization, thus preventing carbonaceous deposits from forming after combustion [29].

The magnitude found is higher than that established in the D9751 standard for biodiesel in combustion engines (1.9–2.6 mm^2/s), which indicates its direct use should be avoided. Likewise, the density of the ethanol obtained was higher than the standard and that required according to D4806 for use as a gasoline additive (1.30–1.34 mm^2/s). The higher viscosity is probably due to the formation of dissolved esters and aldehydes of higher molecular weight that were generated during fermentation.

Of the other oxygenated compounds (higher alcohols, phenols, aldehydes, and ketones), their low concentration reflects good product quality, high homogeneity during combustion, and higher oxidation potential, unlike pure ethanol and other hydrocarbons present in fossil fuels [28].

The flash point at 14.5 °C was higher than that found in high-purity ethanol samples (12.5 °C) and lower than that recommended by the ASTM standard. In addition, a low methanol content ensures safe combustion and storage. The experimental inflation point found was lower than that reported using sugarcane bagasse at 19.20 °C [45], Abrus seed meal at 18.35 °C [41], corn cob at 16.50 °C [46], rice straw at 20.37 °C [47], and Dioscorea rotundata lentils at 17.5 °C [48], among others. These values indicate greater ease of incineration from other sources. The low phenolic index indicates that the distillation and storage of the product were sufficient to prevent its humidification and potential hydrolysis to generate esters and oxidizing fatty acids.

The intermediate characteristics described above demonstrate that the bioethanol obtained has the potential to be used as a gasoline additive in low proportions (10–20%) [11] due to its high alcohol content, low formation of residual carbonaceous compounds, and low proportion of oxidizing compounds [12].

3.5. Energy Assessment

The calorific potential found in distilled bioethanol was 23.82 MJ/kg, lower than that of standard ethanol of 28.58 MJ/kg, but within the range of 10.2–30.0 MJ/kg established in ASTM D4806 [41]. The calorific potential obtained compared to other secondary sources of lignocellulosic biomass is shown in Table 5.

Table 5. Comparison between the calorific potential of bioethanol obtained by different biomass sources.

Source	LHV (MJ/kg)	Reference
Rice husk	14.9	[49]
Wheat straw	17.0–18.9	[50,51]
Rice straw	14.5–15.5	[51,52]
Cotton stalk	17.3	[49]
Sorghum stalk	16.9	[49]
Corn stover	16.2–16.5	[51,53]
Mustard stalk	15.9	[49]
Sugarcane bagasse	18.61–18.73	[45,54]
Corn cob	15.5	[49]
Carrot residues	23.82	This study

The main differences between the sources compared lie mainly in the pretreatment medium applied before fermentation. According to Das et al. [39], the best yields in the hydrolysis of lignin, celluloses, and xyloses occur when enzymatic degradation is applied, followed by acid and basic hydrolysis. Combined degradation of acids and enzymes improves the degradation of complex sugars [4], which was corroborated in the present experiment with higher energy potential. This may also be due to the low content of oxidizing compounds identified in the chemical analysis, which increased the oxidation potential of the bioethanol generated [6].

The aforementioned characteristics of the bioethanol obtained give it the capacity to be used as an additive with regular gasoline in proportions close to 10% to obtain an expected combined calorific value of 40 MJ/kg [11].

For the energy balance, it was considered that the only product in the enzymatic hydrolysis of complex carbohydrates was glucose, disregarding the concentration of intermediate dextrins, as presented in Equation (1). On the other hand, in the fermentation, it was estimated that all the glucose was consumed by the bacteria for the generation of ethanol, as shown in Equation (2).

Since the process developed was biological and irreversible, there were significant thermodynamic limitations, limited only by pH and temperature. The overall balance of the process resulted in +1411 W, generating more energy than is consumed during ethanol production.

4. Conclusions

Bioethanol was obtained from carrot residues, considering a combined enzymatic pretreatment and acid degradation, fermentation with *Saccharomyces cerevisiae*, and distillation of the product. The process was optimized via the response surface based on the control of fermentation time, yeast/enzyme mass ratio, and fermentation pH in a central compound factorial design. The optimum conditions found were 300 min, yeast/enzyme mass ratio of 24.0, and pH of 4.98, obtaining an alcohol content of 92.48% and TSS content of 5.90 °Brix. The physicochemical and energetic characterization of the optimum product was found to be within the range accepted by ASTM standards for its use as a gasoline additive and of higher magnitudes than those found with other biomass sources reported in the literature.

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