CHEMICAL ENGINEERING TRANSACTIONS
VOL. 110, 2024
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Marco Bravi, Antonio Marzocchella, Giuseppe Caputo

ISBN 979-12-81206-10-6; **ISSN** 2283-9216
DOI: 10.3303/CET24110003

Use of Waste Generated in Agro-Industrial Processes in the Mezcal Industry

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Mezcal is a traditional Mexican alcoholic beverage made through the fermentation of agave cenizo (Agave durangensis), for which an annual demand of 1,200,000 tons is estimated in Mexico, generating approximately 480,000 tons of bagasse waste per year. This poses an environmental problem. The recovery of fermentable reducing carbohydrates from the bagasse to obtain ethanol is an innovative option. In this research, three types of hydrolysis (acidic, enzymatic, and acid-enzymatic) were developed on the residual bagasse from mezcal production, aiming for the highest possible concentration of reducing sugars. Acid hydrolysis was carried out with HCl at three different pH values and three different times, yielding a maximum concentration of 13.03 g/L. In enzymatic hydrolysis, cellulase enzymes were used with three different bagasse-enzyme ratios, keeping time and temperature constant, showing a maximum concentration of reducing carbohydrates of 8.46 g/L. Acid-enzymatic hydrolysis used the acid hydrolysis with lower carbohydrate production (2.2 g/L), and three different enzyme concentrations were added while maintaining constant temperature and time, resulting in a concentration of 4.8 g/L. Finally, the highest concentration of reducing carbohydrates was fermented for 7 days using baker's yeast, and after distillation, 96% v/v ethanol was obtained. The results of this research suggest that agave bagasse hydrolysis could be considered an alternative for obtaining a usable energy vector on an industrial scale.

1. Introduction

The state of Durango, Mexico, is one of the main producers of mezcal in the country (Chavez-Parga et al., 2016), which is a representative product obtained through the fermentation of agave (Martínez Jiménez et al., 2019). Its production differs from tequila in terms of raw materials, considering that tequila is exclusively produced from "*Agave tequilana weber*" or blue agave, while mezcal can be made from a variety of agave species (Trejo et al., 2018).
By 2022, Mexican mezcal production exceeded 14 million liters (Avendaño et al., 2023). Producing one liter of mezcal requires around 20 liters of water and approximately 12.7 kg of agave (Jaramillo-Villanueva, 2018), of which about 5 kg becomes bagasse after extracting the juice, used in the fermentation process. This process generates two types of residues: bagasse and vinasse. Agave bagasse is a solid lignocellulosic residue consisting of 43% cellulose, 19% hemicellulose, and 15% lignin, making it a potential material for various industrial uses (Abreu Sherrer & Alatriste Mondragón, 2013).
The pineapple head of agave cenizo (*Agave durangensis)* is the raw material for mezcal production in the state of Durango, with a national demand in Mexico of around 1,200,000 tons annually. In its processing, approximately 40% of bagasse is generated annually (Martínez et al., 2016). This residue is currently not utilized, posing an environmental problem and economic challenge for mezcal producers who have not found a financially feasible alternative for the use of this agro-industrial waste.

Paper Received: 09 February 2024; Revised: 19 April 2024; Accepted: 05 June 2024
Please cite this article as: Garzón E.C., Rodriguez M.C., Ordaz L.A., Milquez-Sanabria H.A., Sotelo D.L., 2024, Use of waste generated in agro-industrial processes in the mezcal industry, Chemical Engineering Transactions, 110, 13-18 DOI:10.3303/CET24110003

However, it is important to note that there is no regulation governing the handling and disposal of agave bagasse, leading to issues such as burning of the residues and discharge into nearby rivers by mezcal producers, as well as piling in plots and grasslands for natural degradation, causing negative environmental impact (Rodríguez Contreras et al., 2016).
According to the above, there is a clear need to explore new alternatives for the use of this residue to benefit production, reduce negative environmental impact, and properly valorize it (Santiago-Mateo et al., 2021). Therefore, there is an opportunity to propose the recovery of reducing sugars from the waste, aiming to obtain products from the biorefinery, such as ethanol, through acid and enzymatic hydrolysis, followed by fermentation and ethanol distillation using a rotary evaporator to achieve ethanol concentrations between 90% and 95%.

2. Material and Methods

2.1 Agave bagasse and reagents

The **Cuero Viejo** distillery, located at 34850 Nixtalpan, Durango, Mexico, cultivates and processes *Agave durangensis* for artisanal mezcal production. In May 2023, Cuero Viejo donated a 1 kg sample of agave waste to the **Universidad Politécnica de Durango**. Hydrolysis processes involved hydrochloric acid and sodium hydroxide (Sigma Aldric®), with glucose used as a standard for quantifying reducing carbohydrates. The acid 3,5-dinitrosalicylic (DNS) reagent and Rochelle salt were used in the quantification process. Enzymatic processes utilized citric acid and sodium citrate (Merck), along with commercial laundry enzymes. Bovine serum albumin served as a standard for quantifying the content of commercial enzymes, along with the necessary salts to prepare the Biuret reagent (Panreac), and carboxymethyl cellulose (CMC) to measure enzymatic activity. Finally, the fermentation of the hydrolysis product employed bakery yeast (TradiPa®) and glycerol (Carl Roth).

2.2 Hydrolysis

All hydrolysis processes were conducted using reflux setups, utilizing 10 g masses of fresh bagasse and carried out in duplicate. The results of the hydrolyses were quantified using the DNS technique, with glucose serving as the standard (Hoa Hung et al., 2021).

2.2.1 Acid: Nine different conditions were planned, considering three pH values (2, 3, and 4) prepared with diluted HCl and three different times (15, 30, and 40 minutes). The temperature was maintained constant at 80°C.

2.2.2 Enzymatic: They were prepared by adding 150 mL of citrate buffer solution with a pH of 5.0 to the bagasse, followed by different volumes of commercial enzymes (10, 50, and 100 mL) in various preparations. The processes were carried out at 50°C for 24 hours after determining the protein concentration (Zheng et al., 2017) in the commercial enzyme solution once its activity was determined (Suesca-Díaz, 2012) using carboxymethyl cellulose (CMC) as a standard.

2.2.3 Enzymatic with pretreatment acid: Three replicated samples under the conditions that produced the lowest concentration of reducing carbohydrates in acid hydrolysis were adjusted to pH 5.0 using citrate buffer. Subsequently, 10, 50, and 100 mL of commercial enzymes were added to each respective sample. The time and temperature of the process remained the same as in the previous section.

2.3 Fermentation

The hydrolysis that yielded the highest concentration of reducing carbohydrates was selected for fermentation. Two preparations (each of 300 mL) from this process were neutralized to pH 7.0. Subsequently, 2 g of previously activated yeast (Moreno-García et al., 2018) was added for every 100 mL of solution.

2.4 Destillation

The fermented samples were filtered to remove suspended and sedimentable solids, and subsequently tri-distilled using a Heidolph rotary evaporator at 50 rpm and a vacuum of -0.35 atm. The first distillation was carried out directly on the filtrate, while the next two required the addition of glycerol to perform extractive distillation.

3. Results and discussion

3.1 Acid hydrolysis

Table 1 shows the concentrations of carbohydrates obtained in the acid hydrolyses described in section 2.2, based on the established pH values and times. The highest RSD value is 8.52% (not shown), a value that falls within the Horwitz limit for RSD (Rivera Orozco & Rodríguez Baez, 2010).

Table 1: Sugars obtained from acid hydrolysis shown depending on pH and time (values shown in mg/L).

	Hydrolysis time		
pH	**15 min**	**30 min**	**40 min**
2.0	5277.5	6467.9	7800.0
Rep 2.0	5337.8	6588.6	8800.0
3.0	2328.3	5721.9	12117.5
Rep 3.0	2172.7	5782.2	13038.1
4.0	6464.8	3753.7	4641.3
Rep 4.0	6661.6	3807.6	4800.0

With α=0.05, the analysis of variance (ANOVA) showed results of P<α, indicating statistically significant differences for both individually analyzed variables and the 9 interactions among them. This suggests considering the influence of pH in combination with the hydrolysis time. This is confirmed in Figure 1 by observing the increasing behavior of the concentration of carbohydrates obtained as a function of time-dependent pH, up to the pH 3.0 and 40-minute interaction, with the highest mean value being 12577.8 ppm in the acid hydrolyses and an RSD of 5.18% (data not shown).

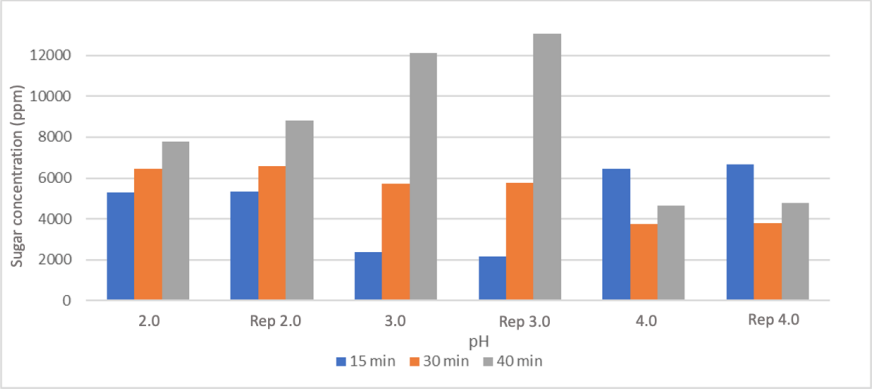

Figure 1: Sugars obtained from acid hydrolysis shown depending on pH and time.

The carbohydrate concentration values obtained with the combination of pH 4.0 and the three times employed do not exhibit the same behavior, showing oscillation in the data. In 2019, Velasco and colleagues reported conditions for acid hydrolysis with temperatures of 121 ºC, pH close to 0.01, and bagasse-to-acid solution ratios of 2:10; whereas in this research, a ratio of 1:10 was used (Velasco Rodríguez et al., 2019). The carbohydrate quantities reported by Velasco were 21150 mg/L, a value higher than the one obtained in this study due to the higher temperature and more acidic pH.
Similarly, Varela and colleagues reported optimal hydrolysis conditions with bagasse-to-acid solution ratios identical to this research, with times of 37 minutes but pH close to 0.15, differing in these values (Varela et al., 2014).

3.2 Enzymatic hydrolysis

Initially, the protein concentration in the commercial enzyme solution was determined as described in the methodological section using the Biuret reagent. The average protein concentration determined was 4.70% (data not shown). Subsequently, enzymatic activity was evaluated using carboxymethyl cellulose (CMC) as a

standard, following the procedure described by Suesca in 2012. This resulted in sugar production of 29616.6 mg/L from a 2% CMC solution (data not shown).

Table 2: Sugars obtained from enzymatic hydrolysis (all concentration values are shown in mg/L).

Enzyme volumen	Final enzymatic concentration	Final sugar concentration	Averange
10 mL	0.29%	6667.9	6603.8
Rep 10 mL		6539.7	
50 mL	1.17%	7744.9	8103.8
Rep 50 mL		8462.8	
100 mL	1.88%	5603.8	5219.2
Rep 100 mL		4834.6	

Table 2 presents the concentrations of carbohydrates produced during the acid hydrolyses carried out at 50°C for 24 hours and pH 5.0. The maximum sugar production (8103.8 ppm) is observed when using a protein concentration of 1.17%. These results align with the values reported by Velasco and colleagues in 2019, who conducted enzymatic hydrolyses with blue agave (Agave tequilana) bagasse, obtaining hydrolysates with 8140 mg/L of carbohydrates under similar pH, temperature, and time conditions (Velasco Rodríguez et al., 2019).

3.3 Enzymatic pretreatment with acid

Considering the results of the acid hydrolyses, the interaction between the pH and time variables that produced the lowest carbohydrate concentration was employed for this section, specifically the condition of pH 3.0 for 15 minutes. Table 3 presents the sugar concentrations from the acid hydrolyses and the respective concentrations after the enzymatic process.

Table 3: Sugars obtained using acid hydrolysis (pH 3.0 and 15 min) and later with enzyme solution on different concentrations (all values concentrations are shown in mg/L).

Sample	Sugar concentration after acid hydrolysis	Enzyme volumen	Final enzymatic concentration	Sugar concentration after enzymatic hydrolysis	Averange change
1	2026.9	10 mL	0.43%	4667.9	43.43%
Rep 1	2116.6	Rep 10 mL		4873.1	
2	2142.3	50 mL	1.56%	3667.9	54.75%
Rep 2	2129.5	Rep 50 mL		4167.9	
3	2116.6	100 mL	2.35%	2975.6	65.20%
Rep 3	2052.6	Rep 100 mL		3462.8	

The highest carbohydrate concentrations obtained in the acid-enzymatic hydrolysis process correspond to a protein concentration of 0.43% in the enzyme solution. However, the most significant concentration change occurs when using the highest percentage of enzymes, with an average variation of 65.20%.
These results align with the descriptions by Gutiérrez and Trujillo, who mentioned that enzymatic hydrolyses with acid pretreatment yield lower concentrations compared to hydrolyses using only acid (Gutiérrez Hernández & Trujillo-Roldán, 2011). Additionally, enzymatic hydrolyses show higher concentrations than when acid pretreatments are applied.

3.4 Fermentation and distillation

The fermentation process was carried out in duplicate, using the hydrolysates that exhibited the highest carbohydrate concentration, namely acid hydrolysis with pH 3.0 for 40 minutes. The ethanol degrees are presented in Table 4.

Table 4: Alcoholic degrees obtained after every single distillation.

	Ethanolic grades			
Sample	**After fermentation**	**1st destilation**	**2nd destilation**	**3rd destilation**
1	15	64	84	93
2	18	66	87	95

Taking into account the azeotropic point in the ethanol-water mixture, which is around 60% ethanol without the application of vacuum or the addition of substances (González Garzón & Linares Hernández, 2022), the inclusion of a reagent allowing extractive distillation is necessary to achieve ethanol concentrations exceeding 90%. This process was achieved by using glycerol as an additive, which is suitable for this process due to its properties such as boiling point, polarity, and miscibility with water (López Navarro, 2018). This allowed obtaining an average ethanol degree of 94º.

4. Conclusions

In this study, an alternative use for the waste generated by the mezcal industry was proposed by employing agave bagasse for carbohydrate production and subsequent fermentation to obtain ethanol, considered as an energy vector. Among the three hydrolysis processes developed, it was determined that acid hydrolyses yield higher concentrations of sugars, with the highest being 12577.8 ppm (mg/L) on average, compared to values obtained by enzymatic hydrolysis methods (maximum of 8103.8 ppm) and acid-enzymatic hydrolysis (maximum of 4770.5 ppm). However, the concentrations of the latter two processes could potentially increase by using specific enzymes for cellulose and lignocellulosic polysaccharide hydrolysis. In this work, laundry enzymes were used with the intention of employing everyday products that are readily available to mezcal producers, considering the future implementation of these results. This makes it necessary to conduct new experiments considering these variables.

Subsequently, the fermentation of the acid hydrolysates, previously neutralized and using bakery yeast, showed results ranging between 15 º and 18 º of ethanol. After the first distillation using a rotary evaporator, an average of 65 º was achieved. The addition of glycerol as an extractive agent allowed an increase after two more distillations, reaching 94 º. This confirms that it is possible to obtain industrial-grade ethanol from these wastes. This project is presented as a viable alternative to be studied for scaling up to pilot plant dimensions with the intention of determining its possible application in the state of Durango, Mexico, utilizing the waste from its mezcal industry.

Acknowledgments

This work was supported by the Universidad de America grant [grant numbers IIQ-004, 2022]. Likewise, the authors would like to express their gratitude to Vinata Cuero Viejo for their donations of raw materials for the development of this study, as well as to the professors of the Environmental Engineering Technology program at the Polytechnic University of Durango and the institution itself, considering their extensive collaboration in the experimental development of this research.

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