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ORIGINAL ARTICLE

Evaluation of a process for producing fermentable sugars from porva corn by *Rhizopus oryzae*

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Abstract

This work aimed to assess the effectiveness of utilizing *Rhizopus oryzae* for starch hydrolysis from Porva corn (*Zea mays*) by evaluating different conditions during the fungus growth process in solid-state fermentation and enzymatic hydrolysis. The goal is to propose a potential methodology to produce commodities like beer, bypassing the traditional grain malting phase. Solid-state fermentation (SSF) was employed to analyze fungal growth on cereal cooked at distinct time intervals (0 min, 30 min, and 60 min) over 72 hours, maintaining an incubation temperature of 32 ºC. Subsequent enzymatic hydrolysis, conducted in the liquid phase at different temperatures (32 ºC, 44 ºC, and 56 ºC) for 5 hours, aimed to extract maximum fermentable sugars. Parameters such as cell concentration, reducing sugars, and dissolved solids (ºBrix) were measured. Furthermore, solid samples obtained at each stage underwent Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy analysis to identify changes in the composition of maize that occurred in each phase. The results indicate that the growth phase exhibited a significantly higher cell concentration of *R. oryzae* when corn was subjected to a 60-minute cooking pre-treatment. Conversely, the most favorable conditions for glucose concentration were observed during the hydrolysis phase at a temperature of 56 ºC, reaching up to 307 $mg_{glucose}/g_{corn}$ (51.23% of hydrolyzed starch). Subsequently, the obtained hydrolysate underwent fermentation for 84 hours using commercial yeast (*Saccharomyces cerevisiae*), resulting in a corn-based ferment with an alcohol content of 12% ABV, demonstrating that the process has good application potential in the manufacture of alcoholic beverages.

Keywords: Solid state fermentation; Enzymatic hydrolysis; Porva corn; *Rhizopus oryzae*.

Highlights

- *Rhizopus oryzae* augmented starch hydrolysis when employing 60-minute cooked Porva corn
- Enzymatic hydrolysis of corn koji at 56 °C yielded high starch breakdown (51.23%)
- Corn-koji hydrolysates produced 12% alcohol, suitable for cereal malting enhancement

1 Introduction

Corn holds a significant position in Colombia, standing as one of the primary and widely consumed food staples. It ranks as the third-largest crop concerning cultivated area, following coffee and rice, with a production that has exceeded 600 tons over the past three years (Centro Internacional de Mejoramiento de Maíz y Trigo, 2019). The grains of this cereal, renowned for their storage of starches, proteins, and essential micronutrients, demonstrate a proximate composition spanning from 44% to 70% of carbohydrates, 11% to 20% of moisture, 4% to 10% of protein, 2% to 5% of fat, 2% to 5% of fiber, and 1% to 3% of ash (Langyan et al., 2022). Owing to its chemical and physical properties, corn has been a focal point in research studies exploring the production of fermentable derivatives, such as alcohol or organic acids. Additionally, it serves as a fundamental element across diverse industries, including energy, cosmetics, medicine, and various other sectors (Chakraborty et al., 2022).

Various microorganisms exhibit a remarkable capability to produce a diverse range of highly sought-after bioproducts, driving substantial market demand (Vivas et al., 2021). The production of industrially significant compounds has been successfully achieved through diverse fermentation strategies, notably Solid-State Fermentation (SSF), which involves a heterogeneous triphasic fermentation (solid-liquid-gas), wherein microorganisms grow on the surface of a porous solid substrate with sufficient moisture to sustain microbial growth and metabolism. The solid particles represent the main phase, and the process occurs in the absence or near absence of visible water between particles (Borrás et al., 2020). It has attracted significant scientific and technological attention due to its efficacy in generating a spectrum of enzymes and other valuable compounds, including phenols, vitamins, and flavor compounds, among others (Londoño-Hernández et al., 2017). The process facilitates the growth and development of bacteria and fungus, within insoluble materials with limited or no free water content and bears historical significance, due to its integral role in food production (Borrás et al., 2020). This methodology finds application in Western countries for producing bread and cheese and in Eastern nations for Koji production. Moreover, solid-state fermentation does not solely rely on traditional sources but also harnesses plant-based substrates like cereals, legumes, and specific agro-industrial residues (Bhanja Dey & Kuhad, 2014; Mejía et al., 2021).

Within the realm of food fermentations, bacteria, yeasts, and filamentous fungi or molds play essential roles. However, in most cases, filamentous fungi are preferred for SSF processes due to their unique ability to colonize the interparticle spaces of solid matrices and to secrete various enzymes to hydrolyze the solid substrate. Furthermore, they are renowned for their enzymatic prowess in breaking down plant cell walls and elevating the chemical composition and bioactivity of substrates, and stand as primary candidates in this domain (Abd Razak et al., 2017). Notably, the genera *Rhizopus*, *Penicillium*, and *Aspergillus* stand out, recognized for their secretion of a myriad of enzymes, including proteolytic, amylolytic, and lipolytic enzymes, among others (Venturini Copetti, 2019). These organisms thrive in solid-state fermentation, closely mimicking their natural habitat (González, 2020). *Rhizopus oryzae,* a prominent microorganism employed in SSF, produces a vital array of enzymes crucial in starch hydrolysis processes and various chemical reactions. These processes result in the production of lactic acid, ethanol, and other compounds that significantly contribute to food preservation (Wang et al., 2013).

Enzymatic hydrolysis finds extensive industrial use owing to the availability of diverse commercial enzymes, including multiple isoforms such as β-amylase, α-amylase, starch phosphorylase, glucosidases, and other debranching enzymes (Miguel et al., 2022). These enzymes play a pivotal role in degrading starch, yielding smaller compounds like glucose, maltose, dextrin, and various oligosaccharides (Bernal & Martínez Barajas, 2006). Enzymatic hydrolysis offers numerous advantages, enabling better control over undesired product formation and increased production of targeted compounds. Nonetheless, the duration of enzyme exposure to distinct parts of the starch substrate significantly influences the degree of hydrolysis (Román et al., 2019).

Furthermore, while corn (*Zea mays* L.) exhibits promising characteristics, such as its capacity, adaptability, and high content of easily hydrolysable carbohydrates, making it an auspicious avenue for

fermented beverage production, studies addressing the impact of SSF of cereal grains with *R. oryzae* fungi on starch, proteins, and total sugars are limited in existing literature (Bolívar et al., 2018). It is important to determine whether factors such as the initial substrate preparation, typically steam cooking before SSF, or the hydrolysis temperatures of corn inoculated with the fungus, present differences compared to those already reported by other authors regarding the standard conditions established from cultures in rice or sorghum (Bhanja Dey & Kuhad, 2014; Bolívar et al., 2018; Zhao et al., 2023).

Hence, this research project aimed to assess the potential application of Koji with *R. oryzae*, utilizing corn grains for fermentable sugar production via enzymatic and microbial processes. The study also aimed to gauge the effectiveness of *R. oryzae* application in corn starch hydrolysis by evaluating parameters such as cell concentration, reducing sugars, and dissolved solids (DS) concerning variables including the cooking time of the substrate (corn) during the fungal growth phase in solid-state, and behavior during enzymatic hydrolysis at different temperatures. Additionally, Fourier Transform Infrared Spectroscopy (FTIR) analysis will identify the primary chemical species and possible structural alterations occurring in each phase. Finally, during the fermentation phase with *Saccharomyces cerevisiae* (commercial yeast), the process will be monitored by evaluating parameters like reducing sugars, optical density (OD), acidity, and pH. The alcohol content of the resulting ferment will also be determined.

2 Materials and methods

2.1 Raw material

A total of 4.50 kg of Porva corn (*Z. mays*) grains from the "Central de Víveres" distributor in Tunja, Boyacá, underwent milling. Table 1 displays the values corresponding to the composition through a proximate analysis.

Table 1. Proximal analysis and starch content of Porva Corn (*Zea mays).*

PARAMETERS	VALUES (%)	METHODS
Moisture	12.30	Gravimetric
Dry material	87.70	Gravimetric
Ash	1.18	Gravimetric
Crude fiber	8.55	Gravimetric
Fat	3.81	Gravimetric
Protein	6.54	Kjeldahl
Carbohydrates	67.60	Gravimetric
Starch	62.70	Gravimetric

2.2 *Rhizopus oryzae*

To produce corn koji (corn inoculated with fungus), commercially acquired *R. oryzae*, known as "rice yeast", from the Chinese brand Angel Yeast, series "Sweet Rice Leaven," was used.

2.3 Solid phase growth

Porva corn samples underwent autoclave cooking at 120 ºC using an All American 75X unit, with two distinct durations assessed: 30 and 60 minutes, alongside a control test involving the raw cereal. In triplicate for each test, aluminum trays lined with canvas-type fabric were utilized, containing 250 g of corn per tray.

Following this, the corn was inoculated with 0.5 grams of yeast rice (1.2 x 10^{13} spores/g) directly from its original packaging. The inoculation process involved uniform stirring to ensure comprehensive distribution.

Subsequently, the inoculated mixture was covered with canvas material moistened with distilled water. The entire culture was then maintained in a Memmert incubator UN75 at a constant temperature of 32 ºC, with atmospheric humidity controlled within the range of 80% to 90%, for 72 hours. Throughout this incubation period, corn koji samples were extracted at 12-hour intervals, accompanied by corresponding assessments of temperature, dissolved solids, spore concentration, and reducing sugars. These procedures are explained in Section 2.6.

2.4 Hydrolysis with the enzymatic extract of the fungus

Following the growth and sporulation of the fungus within the corn, the mixtures obtained for each triplicate were transferred separately to 500 mL Erlenmeyer flasks. Then, suspensions were prepared adding distilled water at a 1:2 ratio to hydrolyze the starches and other carbohydrates present in the corn koji. This process was evaluated at three different temperatures in the Memmert incubator UN75: 32 ºC, 44 ºC, and 56 ºC, for 5 hours. For each hydrolysis temperature, the concentration of reducing sugars was measured hourly. Finally, a larger quantity of hydrolysate was prepared under the conditions that yielded the highest sugar value, to be used in the fermentation phase.

2.5 Fermentation

For the fermentation phase, 1600 mL of hydrolysate were prepared in two 1000 mL Erlenmeyer flasks, each containing 800 mL. Additionally, one gram of active dry yeast (*S. cerevisiae*) from the Colombian brand Levapan® was added to each flask ($OD_0 = 0.5$). This process was controlled in the Memmert incubator UN75 to ensure fermentation, where temperature maintained between 20 ºC and 23 ºC. Throughout the process, parameters such as pH, acidity, optical density, dissolved solids, and reducing sugars were assessed every 12 hours for 4 days. At the end of the process, the alcohol content was determined following NTC 5113-2003.

2.6 Chemical analysis

2.6.1 Turbidity analysis

The spore concentration was determined using the Mc Farland turbidimetric method (Izquierdo Poma & Ramírez Camarena, 2019), which measures the growth of a microorganism suspension through a standard, considering that absorbance is directly proportional to the spores/mL present. A photometer calibration curve was used for each assay, employing theoretical cell concentrations ranging from 1.5 to 30 x 10^8 spores/mL; (spores/mL = (18.138·absorbance + 1.2212) x dilution factor x 10^8; $R^2 = 0.9957$). For this analysis, distilled water was initially established as the standard. During the inoculation stage, the samples were collected in triplicate by taking 0.5 grams of koji corn, which were dissolved in 5 mL of distilled water. Subsequently, the sample was vortexed for 1 minute and then it was left to decant for 15 minutes. The suspensions obtained were analyzed using a Hanna Instruments HI 801 iris photometer based on the absorbance at the selected optical density. Equation 1 was used to determine spores in the solid phase growth (X, spores/g), where S is the spores/mL in suspension, V_D is the volume of distilled water used (mL), M_D is the grams of sample used in the dilution (g), and H is the humidity of koji corn.

$$X = \frac{S * V_D}{M_D} \quad (1)$$

2.6.2 Reducing sugars

The glucose concentration was determined using the 3,5-dinitrosalicylic acid (DNS) method. For each assay, a calibration curve was constructed using glucose as a standard at concentrations ranging from 0.2 to 1.0 mg/mL; (glucose = (1.2095·absorbance + 0.1246) x dilution factor; R^2 = 0.9948). For the analysis, in triplicate, 1 mL of the sample was placed in a test tube along with the addition of 1 mL of DNS reagent. The tubes were then placed in a water bath at a temperature close to 89 ºC for 5 minutes. After this time, the tubes were removed, and the volume was adjusted to 10 mL with distilled water. The solution was allowed to cool for 15 minutes until it reached room temperature, then shaken to homogenize, and finally, the corresponding absorbance was measured using a Hanna Instruments HI 801 Iris photometer at a wavelength of 540 nm (Cortés-Sánchez et al., 2014). Equation 2 was used to determine glucose extracted from the corn in the solid phase growth and hydrolysis (mg/g), where A is the concentration of reducing sugars in liquid (mg/mL), V_D is the volume of distilled water used (mL), M_D is the grams of koji corn sample (g), and H is the humidity of koji corn.

$$\text{Glucose} = \frac{A*V}{M_D} \quad (2)$$

2.6.3 Dissolved solids (ºBrix)

The concentration of dissolved solids (DS) was determined based on the principle of Brix degrees, which measure the percentage of dissolved solids in a liquid through the refraction of light. Measurements were made using a Soonda digital refractometer. For the triplicate analyses, grams of the sample were taken for each phase and diluted in a volume of distilled water. Three drops of the sample were placed on the refractometer, and Brix degree values were obtained. Equation 3 was used to determine DS, where ºBrix is the value given by the refractometer, V_D is the volume of distilled water used (mL), and M_D is the grams of sample used in the dilution (g).

$$\text{DS}\left(\frac{\text{mg}}{\text{g}}\right) = \frac{ºBrix*10*V_D}{M_D} \quad (3)$$

2.6.4 Optical density

Yeast (*S. cerevisiae*) growth during fermentation was controlled by measuring optical density. For each measurement in triplicate, 3 mL of the sample were taken and measured in the Hanna HI 801 Iris photometer, obtaining absorbance values at a wavelength of 600 nm.

2.6.5 Acidity

Following NTC 4978 of 2001, acidity in the fermentation was determined through titration. 1 mL of the sample was taken and titrated with 0.1 N NaOH, using phenolphthalein as an indicator. The percentage of acidity was calculated by using Equation 4, where N_{NaOH} is the concentration of the NaOH solution used in the titration, V_{NaOH} is the volume consumed (mL), and PM_{eq} is the equivalent weight of the base.

$$\%\text{Acidity} = \frac{N_{NaOH}*V_{NaOH}*PM_{eq}*100}{V_{sample}} \quad (4)$$

2.6.6 pH

pH values were taken every 12 hours during the fermentation process. According to the NTC 3651 of 1994 standard, measurements were made directly in the fermented liquid phase using an Orion 8107UWMMD Ross Ultra pH/ATC triode electrode from Thermo Scientific.

2.6.7 FTIR Analysis

Spectral analysis of the samples obtained at each stage was conducted using the Fourier Transform Infrared Spectroscopy (FTIR) technique. For this analysis, a PerkinElmer Spectrum Two FTIR spectrometer was used, employing the uATR mode with 32 scans in a range from 400 cm^{-1} to 4000 cm^{-1}.

To prepare the samples, raw corn, cooked corn, corn koji, and corn koji hydrolysate obtained with a cooking time of 60 minutes at 56 ºC in the hydrolysis stage were dried in an oven for 12 hours at 50 ºC. They were then ground for 5 minutes using an electric grain pulverizer. The powder obtained from each sample was measured in the FTIR spectrophotometer. Data processing and spectral analysis were conducted using the Origin 2019b software.

3 Results and discussion

3.1 Solid phase growth of *Rhizopus oryzae*

Figure 1 illustrates the spore growth of the *R. oryzae* strain over time when exposed to Porva corn (*Zea mays*), considering various cooking times (0, 30, and 60 minutes). Cooked corn samples exhibited a latency phase within the initial 12 hours post-inoculation, followed by exponential growth in the subsequent 12 hours. This growth then plateaued, entering a stationary phase that lasted until the 72-hour mark. Conversely, uncooked corn showed minimal growth within the first 12 hours, maintaining a relatively constant trend over the following 60 hours. The spore concentration notably increased more in corn cooked for 60 minutes, followed by that cooked for 30 minutes, while the lowest concentration was observed in the uncooked corn incubation.

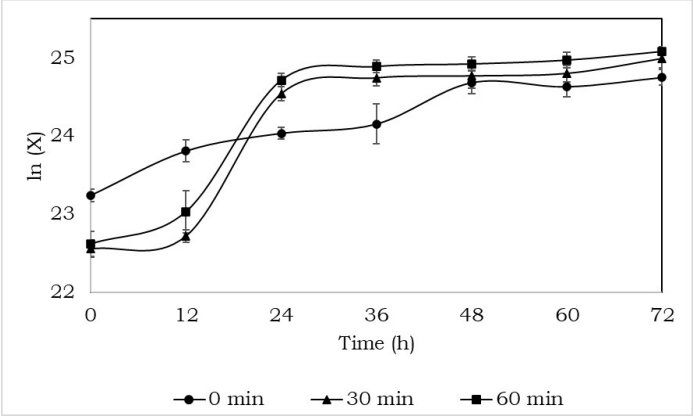

Figure 1. Growth of biomass vs. time for the three different corn cooking times in the incubation process with *Rhizopus oryzae.*

The pre-cooking of maize significantly influenced the growth patterns, resulting in the metabolism-derived formation of enzymatic extracts necessary for starch hydrolysis (Figueroa Ceballos et al., 2019). Among the various cooking times evaluated (0, 30, and 60 minutes), the most optimal performance was observed after cooking the corn for 60 minutes. This is in alignment with previous works, indicating that for traditional Koji preparation, pre-steaming the rice, the primary cereal used, for a minimum of two hours is crucial. This process ensures adequate moisture and porosity, facilitating fungus penetration and growth while enabling effective gas exchange for both CO_2 and oxygen. It serves to prevent anaerobic organism growth, thereby reducing the risk of substrate contamination (Manrique, 2019).

The pre-chopping of corn before the steam cooking process enhanced contact with the fungus. This, combined with its hydrophilic nature, provided the necessary moisture for optimal growth when cooked for 60 minutes. Abd Razak et al. (2017) highlighted the significance of homogenizing particle sizes in fostering microorganism development. Smaller particle sizes offer an expanded surface area for microorganism attachment, but excessively small particles can agglomerate, hindering oxygen transfer and delaying microorganism growth. Conversely, larger particle sizes facilitate better aeration but limit the available surface area for microorganism attachment (Ceballos-González et al., 2016).

Figure 2 illustrates the glucose formation resulting from *R. oryzae* activity in corn. Uncooked corn exhibited negligible changes, maintaining a consistent level over the 72 hours. Conversely, corn cooked for 30 minutes displayed an increasing trend starting from the 12th hour of incubation, achieving glucose concentrations of 69.43 mg/g at 60 hours. Similarly, corn cooked for 60 minutes exhibited an increase in concentration at the same time, reaching a peak concentration of 90.10 mg/g at 48 hours. Subsequently, for both treatments (30 and 60 minutes), the concentration gradually declined by the 72nd hour post-inoculation.

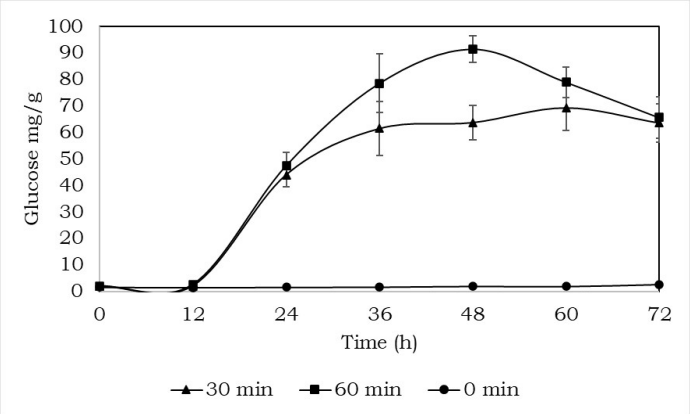

Figure 2. Glucose behavior vs. time for the three different corn cooking times in the incubation process with *Rhizopus oryzae*.

Within the initial 48 hours post-inoculation, carbohydrates were the primary carbon source consumed for the growth and sporulation of *R. oryzae*. This consumption led to the production of glucose monomers, crucial for developing functions essential for growth and the production of enzymes involved in starch hydrolysis. Some authors emphasized the capability of *R. oryzae* to utilize plant compounds and polysaccharides as sources of energy and carbon. Moreover, it was also found that this fungus primarily metabolizes glucose as its carbon source and urea as a nitrogen source to fulfill growth and production functions (Londoño-Hernández et al., 2017).

Figure 3 demonstrates the concentration of dissolved solids over time for each cooking duration. Uncooked corn displays no variation in dissolved solids concentration. In contrast, for corn cooked for 30 and 60 minutes, the concentration of dissolved solids increased up to 48 hours, reaching concentrations of 130.00 mg/g and 160.00 mg/g, respectively. Subsequently, the concentration decreased, reaching levels of 106.67 mg/g and 116.67 mg/g of dissolved solids at the 72-hour mark for the 30 and 60-minute cooking times, respectively.

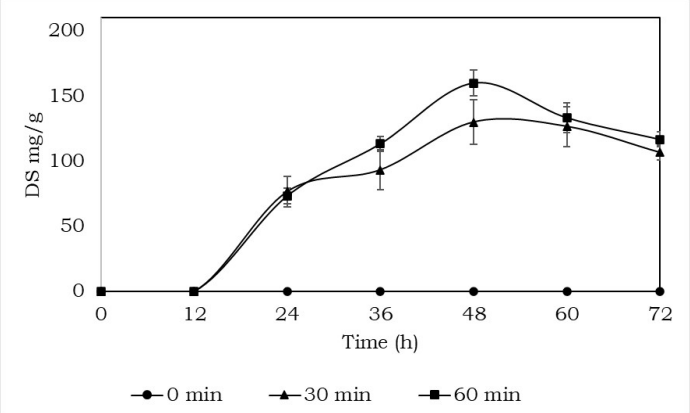

Figure 3. Concentration of dissolved solids vs. time for the three different corn cooking times in the incubation process with *Rhizopus oryzae*.

The evaluation of dissolved solids using Brix degrees provided insight into corn's suitability as a raw material for fermentative processes involving *R. oryzae*. It offered an assessment of the substrate's capability to serve as a medium for substances, including glucose, salts, and acids. Throughout the solid growth phase, there was an escalating trend, reaching the peak concentration of dissolved solids at 48 hours post-inoculation, followed by a subsequent decline. These concentrations indicate the approximate percentage of solids per 100 grams of the sample and the ratio of sugars per gram of water present in the medium, a parameter critical to this research's development. Notably, the inoculation of corn cooked for 60 minutes exhibited the highest production of dissolved solids, reaching 160 mg/g, surpassing concentrations obtained in other cooking variations.

Considering these findings, the best conditions for the initial growth phase of *R. oryzae* in corn entail cooking the corn for a minimum of 60 minutes at 32 °C, with an incubation period of 72 hours. These conditions foster an environment conducive to the fungus's growth and subsequent production of necessary enzymes for starch hydrolysis in corn. This aligns with the exploration of optimal conditions for α-amylase and glucoamylase production in SSF of rice bran with *R. oryzae* in Erlenmeyer flasks. They found that the optimal incubation conditions were at 30 °C, pH 5.5, and initial humidity of 67%, with an incubation time of around 5 days (Londoño-Hernández et al., 2017). Throughout its growth, various substances are produced as part of its secondary metabolism, including enzymes beneficial for degrading diverse substrates (Figueroa Ceballos et al., 2019).

Starch, a predominant component in corn, is a key dietary macromolecule and macromolecular reserve found in most plants and is one of the most abundant renewable resources on Earth. Chemically, starch comprises amylose (a linear polysaccharide of glucose units linked by insoluble α-1-4 bonds) and amylopectin (a branched polysaccharide with soluble α-1-6 bonds).

3.2 Hydrolysis

Figures 4, 5, and 6 illustrate the glucose behavior following the addition of the obtained biomass (inoculated corn) to a water suspension at different temperatures: 32 °C, 44 °C, and 56 °C, respectively.

In Figure 4, at 32 °C, the glucose behavior varied for different corn preparations. Uncooked corn displayed an increase in glucose concentration, reaching a concentration of 63.35 mg/g at 5 hours. For corn cooked for 30 and 60 minutes, both exhibited similar patterns of gradual increase in the first two hours, with concentrations of 239.09 mg/g and 242.93 mg/g, respectively, by the end of the 5-hour hydrolysis period.

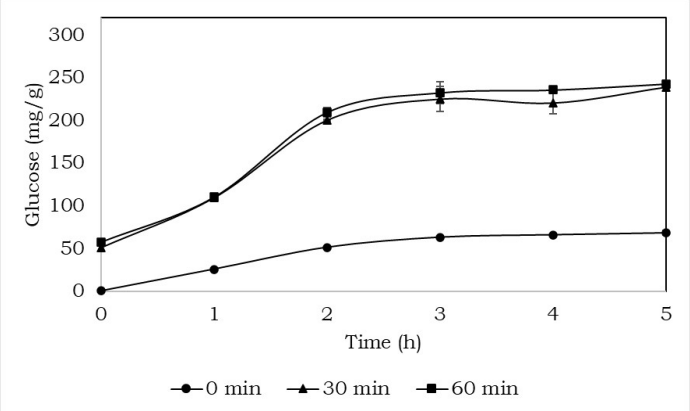

Figure 4. Glucose behavior in the hydrolysis process at 32 ºC for the three different corn cooking times.

In Figure 5, at 44 °C, the glucose concentration for uncooked corn displayed a progressive increase, reaching concentrations of 86.69 mg/g after the 5-hour hydrolysis. Similarly, for corn cooked for 30 and 60 minutes, there was a substantial increase in glucose concentration within the initial 2 hours, followed by a consistent trend, resulting in concentrations of 239.74 mg/g for corn cooked for 30 minutes and 243.36 mg/g for corn cooked for 60 minutes after the 5-hour hydrolysis.

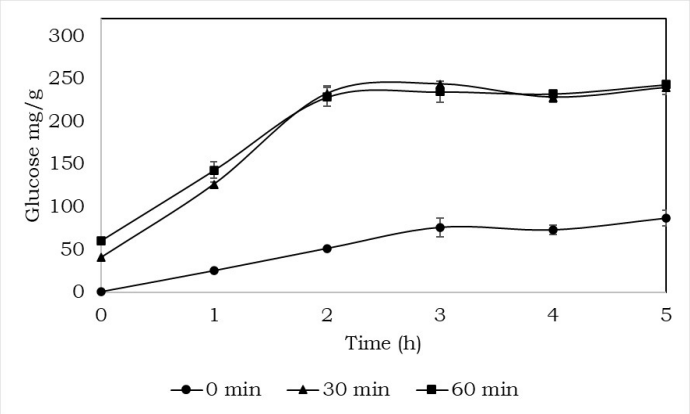

Figure 5. Glucose behavior in the hydrolysis process at 44 °C for three different corn cooking times.

In Figure 6, at 56 °C, all three corn cooking times exhibited an increase in glucose concentration. Specifically, uncooked corn attained a concentration of 120.15 mg/g. There was a notable upward trend, culminating in glucose concentrations of 294.41 mg/g for corn cooked for 30 minutes and 307.70 mg/g for corn cooked for 60 minutes after 5 hours of hydrolysis. Notably, the latter treatment showed the highest glucose concentration among the three hydrolysis temperatures evaluated.

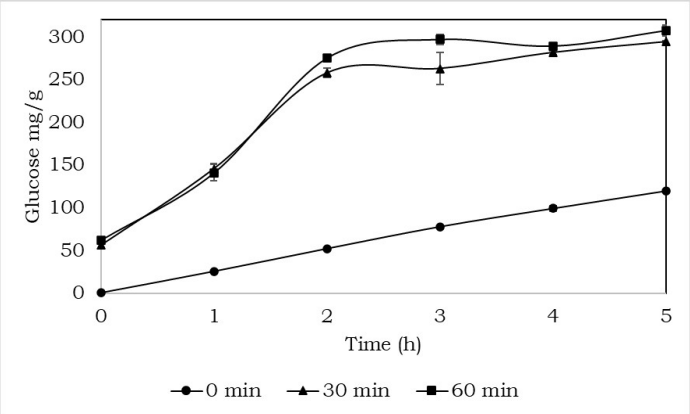

Figure 6. Glucose behavior in the hydrolysis process at 56 °C for three different corn cooking times.

Considering that the starch content of the corn (*Z. mays*) sample was 62.75%, the hydrolysis stage, which involved 50 grams of corn cooked for 60 minutes at 56°C, achieved a maximum hydrolysis percentage of 51.23%. This behavior can be explained, in part, by the structure of starch. Starch granules are practically insoluble in chilly water, but they start to retain water and swell as the temperature increases in an aqueous solution.

In 2012, Cruz explained that when a certain temperature is reached, starch granules reach their maximum volume. Further heating causes the swollen granules to partially rupture, releasing amylose and amylopectin into the solution, increasing viscosity. However, as the granules break, viscosity decreases and stabilizes, leading to the formation of a distinct gel with unique physical and chemical characteristics for each type of starch (Mendoza et al., 2020).

The conversion of starch to glucose and other products requires prior gelatinization of the granules, initiating the hydrolysis process. This process demands a significant input of energy and can be affected by the composition and organization of starch granules, making some more resistant than others (Compart et al., 2023).

Enzymes play a crucial role in initiating hydrolysis as temperature increases. *R. oryzae* produces various enzymes, including amylases, lipases, cellulases, proteases, and tannases, all classified as hydrolases

(Londoño-Hernández et al., 2017). Fungal amylases are particularly important, with alpha-amylase being widely applicable in various bioprocesses.

The hydrolysis of corn starch in aqueous media consists of two stages: liquefaction and saccharification. The action of amylase in the first stage prevents gel formation, while the second stage, saccharification, involves glucoamylase converting starch into glucose (Larroque et al., 2021). Alpha-amylase acts on internal regions of starch polymer chains, leading to a decrease in viscosity (Far et al, 2020). It breaks α-(1→4) bonds, producing maltodextrins and glucose. This process results in various maltodextrins with low molecular weight, which depend on the microorganism producing the enzyme.

The behavior of glucose formation during hydrolysis, as seen in Figures 4, 5, and 6, typically shows a higher rate of glucose formation in the first two hours compared to subsequent hours. This behavior is due to the initial availability of active enzyme sites, which react with the substrate, leading to a rapid increase in the rate of product formation.

At 56 °C, hydrolysis exhibits the highest amylase activity, resulting in a higher concentration of fermentable glucose. However, it is important to note that various factors, including temperature, time, substrate, strain, enzyme availability, and starch composition, significantly influence the degree of starch hydrolysis for sugar production (Mendoza, et al., 2020).

In summary, the complex interplay of factors in starch hydrolysis, along with the characteristics of the enzymes involved, contributes to the dynamics of glucose formation during the hydrolysis process.

3.3 FTIR analysis

Figure 7 displays the FTIR analyses for the solid samples of each phase of the process, showing bands in the range of 4000 to 400 cm^{-1} and Table 2 summarizes the types of bonds observed in the spectrums.

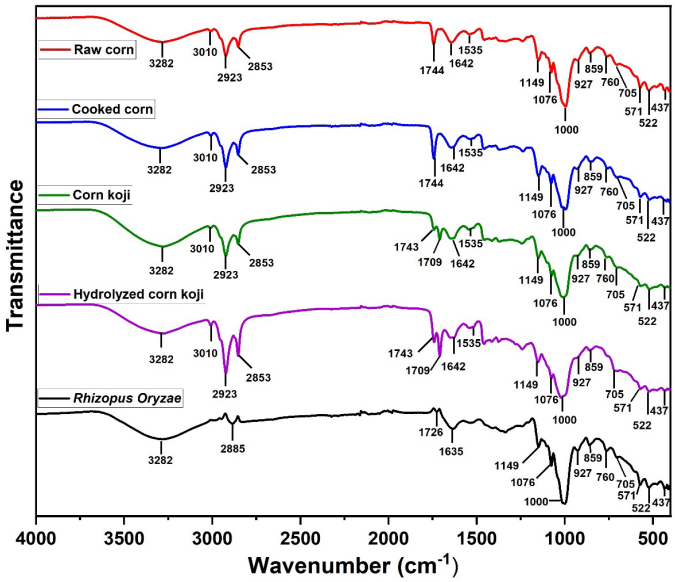

Figure 7. FTIR spectrum of raw corn, cooked corn, corn koji, hydrolyzed corn koji, and commercial *Rhizopus oryzae.*

Common to all spectra is a band at 3282 cm^{-1}, corresponding to a stretching vibration characteristic of the O-H bond. Due to hydrogen bonding associations in water molecules, a broad band is evident. In the case of raw corn, cooked corn, corn koji, and hydrolyzed corn koji, stretching vibrations in the range of 3000 cm^{-1} to 2900 cm^{-1} correspond to the asymmetric stretching of C-H bonds, primarily from carbohydrates. Notably, the hydrolyzed koji exhibits a much more intense signal in this range compared to the others (Daza Orsini & Parra Aparicio, 2023).

Table 2. Types of bonds observed in the FTIR spectrum of raw corn, cooked corn, corn koji, hydrolyzed corn koji, and commercial rice yeast (*Rhizopus oryzae*).

Wavenumber (cm^{-1})	Assignment	Samples
1000	C-O (from starch)	All corn samples and rice yeast
1535	N-H and C-N (from proteins)	All corn samples
1635- 1642	C=O (from proteins)	All corn samples and rice yeast
1726	C=N (primary amide)	Rice yeast
1743-1744	C-O (glycosidic linkages of starch)	All corn samples
2885	C-H (from carbohydrates)	Rice yeast
3000-2900	C-H (from carbohydrates)	All corn samples
3282	O-H (water)	All corn samples and rice yeast

At 1744 cm^{-1} (for raw corn and cooked corn) and 1743 cm^{-1} (for corn koji and hydrolyzed corn koji), a low-intensity band is present, likely corresponding to stretching vibrations of the C-O bond in the glycosidic linkages of starch. The absorption at 1642 cm^{-1} in all four cases possibly corresponds to a vibration of the C=O double bond in proteins, which may overlap with a broad band representing the stretching of the H-O-H bond, characteristic of water in corn. A low-intensity band at 1535 cm^{-1} is associated with bending vibrations of the N-H bond and stretching of the C-N bond, both belonging to the protein groups present in the cereal. At 1744 cm^{-1} (for raw corn and cooked corn) and 1743 cm^{-1} (for corn koji and hydrolyzed corn koji), a low-intensity band is present, likely corresponding to stretching vibrations of the C-O bond in the peptide linkages of starch.

The absorption at 1642 cm^{-1} in all four cases possibly corresponds to a vibration of the C=O double bond in proteins, which may overlap with a broad band representing the stretching of the H-O-H bond, characteristic of water in corn. A low-intensity band at 1535 cm^{-1} is associated with bending vibrations of the N-H bond and stretching of the C-N bond, both belonging to the protein groups present in the cereal (Daza Orsini & Parra Aparicio, 2023).

Moving to the fingerprint region to the right of 1400 cm^{-1}, for all samples between the range of 859 to 1149 cm^{-1}, there are bands associated with stretching vibrations of C-O bonds, rocking vibrations of C-H bonds, and stretching and rocking bands for C-O-C bonds. These correspond to lipid biomolecules and glycosidic linkages in carbohydrates. Within this region, a prominent band at 1000 cm^{-1} is observed, which is related to the stretching vibration of the C-O bond and is a consequence of the significant starch concentration present in corn. In terms of intensity, this band is much stronger in the spectra of raw and cooked corn, while the intensity of this signal decreases for corn koji and hydrolyzed corn koji, respectively (Aragón-López et al., 2020).

Finally, in the infrared spectrum of the commercial *R. oryzae*, certain bands differentiate it from the spectra described earlier. Among them is a band at 2885 cm^{-1} corresponding to stretching vibrations of the C-H bond. There is also a very low-intensity band at 1726 cm^{-1}, possibly associated with the vibration of the C=N bond in a primary amide, which, together with a band at 1635 cm^{-1} related to the C=O bond vibration, indicates the presence of proteins (Cortés-Sánchez et al., 2014).

Observable spectral changes were identified in each phase of the analysis. Initially, stretching vibrations of the C-O species in the peptide bonds exhibited increased intensity for cooked corn but decreased during the inoculation phase with *R. oryzae*. This reduction could be attributed to the consumption of carbohydrates by the fungus for its growth. Notably, the koji spectrum revealed a signal indicative of protein structures, likely associated with the enzymes produced by the fungus. Furthermore, C-H vibrations corresponding to carbohydrates increased in intensity for the hydrolyzed koji, suggesting alterations in the composition of corn starch and other carbohydrates. This, coupled with intensified C-O bands, could imply the formation of glucose.

In all samples, a discernible signal signifying starch presence consistently decreased in intensity in each phase. This decline in signal intensity denotes the conversion of starch into glucose molecules.

Starch molecules possess two significant functional groups: the -OH groups, prone to substitution reactions, and the C-O-C linkages, susceptible to chain cleavage. These reactions lead to starch modifications. Cross-linkages and bridges of the -OH type alter the chain structure and elevate viscosity (Kaur et al., 2022). Additionally, the potential for hydrogen bond formation arises from intermolecular interactions in amylose. This interaction is influenced by the C-OH groups present at every 2nd, 3rd, and 6th carbon atom of the glucose residue in amylose, facilitating diverse reactions throughout each process.

3.4 Fermentation of hydrolysates

Considering the results obtained in the previous experiments, it was determined that there was higher cell concentration in terms of the growth of *R. oryzae* in corn cooked for 60 minutes, making it the most suitable condition as it allowed for easier conditioning of the cereal for the entry and sporulation of *R. oryzae*. Likewise, by observing the hydrolysis behavior, it was determined that a higher degree of hydrolysis occurs when the substrate is at 56 °C.

In Figure 8, microbial growth of the yeast (*S. cerevisiae*) can be observed through optical density. A latency phase of three hours was followed by exponential growth, which occurred approximately between three and twenty-four hours of fermentation. From there until 36 hours, the stationary phase was observed, where finally, due to substrate exhaustion, the death phase occurred, marking the end of the fermentation process.

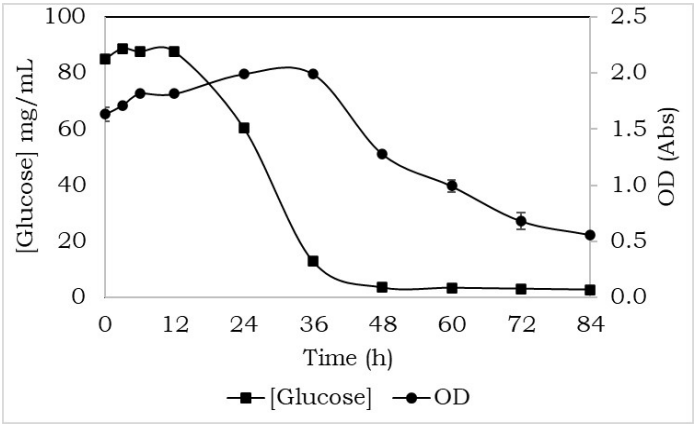

Figure 8. Glucose behavior and optical density of *Saccharomyces cerevisiae* during fermentation.

Within the fermentation process, the behavior of glucose concentration was evaluated, as shown in Figure 8. Initially, upon adding the yeast to the hydrolysate medium, it had a glucose concentration of 87.07 mg/mL, which remained constant for the first 12 hours. Subsequently, there was a decrease in glucose concentration from 12 hours to 48 hours of fermentation, resulting in a final concentration of only 3.58 mg/mL of glucose. From that point on, the concentration remained constant until the end of the 84-hour fermentation period.

The pH parameters and acidity (Figure 9) were also monitored during the fermentation process. Regarding their behavior, it can be observed that the pH values remained constant throughout fermentation, fluctuating between pH values of 4.4 and 4.5, respectively. Similarly, the acidity percentage of the fermentation medium remained between 0.18% and 0.20% over the 84 hours.

Finally, the fermented corn was found to contain 12% of alcohol by volume (ABV).

In a general sense, koji functions as a source of enzymes (amylase, protease, lipase, among others) that hydrolyze solid materials into soluble products, providing fermentable substrates for yeast and bacteria in the subsequent fermentation process to produce miso, soy sauce, sake, amazake, and other fermented products

(Londoño-Hernández et al., 2017). The hydrolysate obtained was used in the third phase, where the sugars from corn were fermented in the absence of oxygen with *S. cerevisiae* (yeast) at 22 °C for 84 hours. During the process, monitoring was conducted regarding the yeast's behavior and fermentation conditions. Optical density values (Figure 8) demonstrated that yeast growth occurred during the first 36 hours of fermentation, leading to the formation of ethanol and CO_2. However, beyond the initial 36 hours, yeast activity declined, indicating reduced fermentation activity. Simultaneously, the concentration of glucose during fermentation was monitored (Figure 8), showing insignificant changes until the 12-hour mark, followed by a decrease up to 36 hours. From that point onwards, and up to 84 hours, the concentration remained stable.

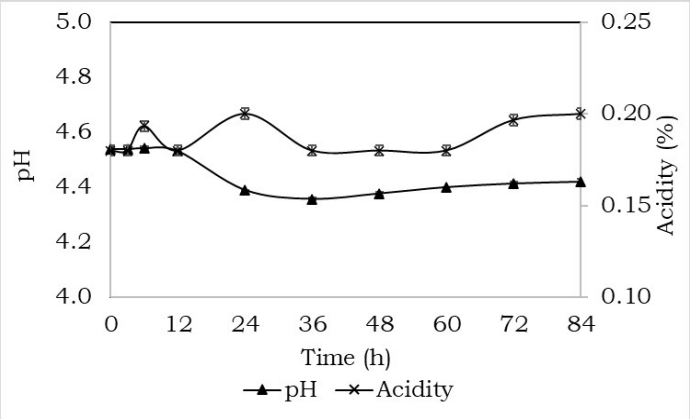

Figure 9. pH and acidity behavior in fermentation with *Saccharomyces cerevisiae*.

This behavior indicates that the yeast, *S. cerevisiae*, utilizes the available glucose as an energy source during the initial 36 hours to support growth and ethanol production. Additionally, the decrease in glucose concentration is attributed to the transformation of sugars into ethanol and carbon dioxide. Beyond the 36-hour mark, in the absence of glucose, yeast activity decreases, leading to a reduction in fermentation functions. Regarding its growth and development, some authors mention that yeast produces enzymes that act upon sugar and the compounds that subsequently form; this process culminates in the production of primary products, including alcohol and carbon dioxide, along with secondary products such as glycerin, aldehyde, acetic acid, succinic acid, butylene glycol, and acetone (Maicas, 2020).

Within the fermentation process, pH monitoring (Figure 9) revealed values ranging between 4.3 and 4.5 over 84 hours. This range of acidity or alkalinity in the medium containing yeast influences alcohol production, substrate consumption, and fermentation rate. Debarati et al. (2023) have found that yeast activity decreases in low pH conditions, suggesting that the optimal pH facilitates proper and satisfactory metabolism to promote substrate consumption. Some research reports recommend a pH range of 3 to 5 for yeast growth in alcoholic fermentation (Debarati et al., 2023). However, Robles et al. (2023) indicated that most yeast strains can tolerate a pH range between 3 and 10, although they tend to thrive in a slightly acidic environment with a pH between 4.5 and 6.5 (Robles et al., 2023).

In addition to pH measurements, acidity levels (Figure 9) were recorded, resulting in a consistent percentage between 0.18% and 0.20% during the fermentation. This indicates the production of acids formed throughout the phases, imparting specific flavor and odor characteristics to the fermentation. Yeasts and lactic acid bacteria participate in the fermentation, contributing certain characteristics through the products generated in this process. Among these products is the production of lactic acid and volatile compounds such as acetoin, acetone, and butyric acid, many of which are associated with the aromas of various foods, including wine, cocoa, coffee, and fermented starches like cassava or corn-derived starch (Debarati et al., 2023).

Furthermore, various researchers suggest that lactic acid produced through SSF is a process defined by the growth of microorganisms (mainly fungi) within a moist solid material in the absence of water flow, resulting

in the formation of various acids during fermentation. Similarly, in 2006, Pandey and Ramachandran conducted a comparative study of the *R. oryzae* strain, evaluating lactic acid production in submerged fermentation (SmF) and SSF. They found that SSF yielded higher lactic acid production than SmF based on the alcohol content obtained from the corn hydrolysate fermentation, it can be determined that it falls above the upper limits of ranges typically found in cereal-based processes (Oluranti and Smidt, 2023).

Based on the aforementioned results, the process of corn koji production using *R. oryzae* could be incorporated into the making of one of the most well-known fermented beverages, such as beer. SSF could replace the traditional cereal malting process (humidification, germination, and drying), potentially reducing the preparation time by up to 300% before wort preparation and fermentation (Segobia et al., 2021). Additionally, this alternative aligns with current craft brewery standards, which have continually refined production methods over time, influencing the organoleptic characteristics of the product. Advances in science and technology have facilitated improvements, resulting in diverse products in today's market (Solórzano et al., 2024).

4 Conclusions

For the growth of *R. oryzae* on cooked maize (*Z. mays*) through SSF, a prior preparation of raw materials is necessary as it influences the microbial cell concentration. During the growth phase, *R. oryzae* demonstrated a higher cell concentration when the cooked maize was pre-treated for 60 minutes, resulting in increased glucose formation under these conditions. Cooking times below 60 minutes or raw corn limited the interaction between *R. oryzae* and the cereal, resulting in lower biomass concentrations.

The temperature's effect on hydrolysis plays a crucial role in starch hydrolysis and sugar production. Among the evaluated temperatures, it was concluded that in an aqueous medium at 56 °C, the starch breakdown was primarily facilitated due to the temperature's effect, causing modifications in the starch granule structure. This provided an ideal environment for the enzymatic extracts to hydrolyze starches, resulting in a degradation percentage of 51.23% and reaching glucose concentrations up to 307 mg/g.

During the fermentation with commercial yeast, ethanol, and CO_2 production resulted in fermented corn with a 12% alcohol content. This demonstrates that through microbial and enzymatic processes, *R. oryzae* can produce fermentable sugars as an alternative to malting, using only Porva corn as the substrate. These results provided valuable insights into the production of fermented beverages using fungi and enzymatic productions, and they encourage further research on this and other microorganisms.

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