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Addition of protease during starch liquefaction affects free amino nitrogen, fusel alcohols and ethanol production of fermented maize and whole and decorticated sorghum mashes

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ABSTRACT

The aim of this research was to study the effect of the dual treatment of sorghum decortication and protease addition during liquefaction with α-amylase, on the concentration of free amino nitrogen (FAN), fusel alcohols and ethanol during yeast fermentation. A bifactorial experiment was designed to revise the differences among grains (maize, whole and 9.7% decorticated sorghum) and the effectiveness of protease addition during liquefaction. The decorticated sorghum was more susceptible to protein hydrolysis compared to the whole kernel sorghum due to its lower fiber content. The protease improved the levels of FAN approximately to 60% and 30% in the maize and sorghum mashes, respectively. The maize mash contained the highest amount of FAN followed by the decorticated and whole sorghum mashes. The protease treatment improved the fusel alcohol concentration in both sorghum beers and did not affect ethanol concentration in the maize mash. Both sorghum decortication and protease addition during liquefaction are therefore recommended treatments to obtain mashes with a higher FAN level. The decorticated protease-treated sorghum mashes yielded higher amounts of ethanol compared to the maize treatments and produced the maximum ethanol after only 20 h of fermentation. This research proved that a positive synergistic effect on FAN concentration, fusel alcohols and bioethanol yields can be achieved through the proposed proceeding of sorghum decortication and protease addition.

1. Introduction

Sorghum (*Sorghum bicolor* L. 'Moench') is considered as an alternative energy crop for the bioethanol production. However, limited research has been conducted on the performance of sorghum cultivars for bioethanol. The main reason for the restricted use of sorghum is its relatively lower ethanol yield compared to maize and its lower susceptibility to hydrolysis, especially after heat–moisture treatments. Disulfide crosslinking, enhanced by cooking, is thought to be the main cause of the reduced protein digestibility [1] and ethanol conversion rates. Pretreatment technologies have been developed to improve ethanol yields including mechanical methods such as size reduction through milling, decortication, and extrusion cooking; physical methods such as steaming, radiation, and sonication; chemical methods such as alkaline and acid hydrolysis; biological methods such as microbial and enzyme degradation; and a combination of these [2–5].

Abrasive decortication operates on the principle of progressively rubbing off the outer layers of the kernels [6]. MacLean et al. [7] after studying the effect of decortication on the apparent protein quality and on the digestibility of sorghum concluded that the use of decorticated kernels improves markedly the apparent protein digestibility. Higiro et al. [26] compared the quality and yield of starch extracted from sorghum grits that were obtained by decortication/degermination and roller milling processes. The first process resulting grits presented a higher starch recovery (61–70%) in comparison to the grits obtained after roller milling (51–68%).

It has been reported that the glucose consumption rate and the ethanol production efficiency can be increased and the fermentation time might be reduced supplying a source of nitrogen, either at the beginning or at early stages of yeast fermentation. Nitrogen limitation is usually considered as the main reason for a sluggish or stuck fermentation [8]. The process of fermentation for the bioethanol production can be conducted by the yeast *Saccharomyces*, which converts sugars into ethanol and carbon dioxide. The nitrogenous constituents of worth include amino acids, peptides, proteins, nucleic acids and their degradation products. Of particular importance is the status evaluation of available nitrogen prior to the conduction of the fermentation. Sub-optimal concentrations of available nitrogen are associated with lagging and

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incomplete fermentation and sulfide evolution [9]. As observed in Kłosowski et al. [10], the availability of free amino acids results in a lower requirement for amino acid synthesis by yeasts and a higher biomass yield per mole of glucose consumed. Moreover, it has been found that the glycerol production can be reduced and the ethanol yield increased when a mixture of amino acids as the nitrogen source is used. During alcoholic fermentation, yeast produces small amounts of higher aliphatic alcohols. This mixture of alcohols, often referred to as fusel oils, is composed principally of *n*-propanol (1-pronanol), isobutanol (2-methyl-1-pronanol), active pentanol/2-methyl-1-butanol), and isopentanol [11]. The levels of produced esters and higher alcohols have a significant effect on the flavor of alcoholic beverages. Two pathways exist for the production of higher alcohols, both of which involve amino acids [14,15]. Higher chain alcohols, (C3–C5) in particular, are attractive due to their increased energy density and low hygroscopic capacity [12].

This research was undertaken to study the effects of sorghum decortication and protease treatment during liquefaction with thermoresistant α-amylase on the production of FAN, the various types of fusel alcohols and bioethanol throughout conventional yeast fermentation.

2. Materials and methods

2.1. Grain sources

A commercial regular yellow dent maize (*Zea mays*) (YMZ) with a relatively soft endosperm imported from the USA Corn Belt by Agroinsa (Guadalupe, N.L. México) and an experimental regular white sorghum (RWS) (ATX 631*TX 436) with an intermediate endosperm texture donated by Texas A&M University were used. The physical properties of the grains were previously described by Perez-Carrillo and Serna-Saldivar [3].

2.2. Sorghum decortication

Sorghum was decorticated in order to remove 9.7% of the grain weight according to the process described by Perez-Carrillo and Serna-Saldivar [3]. Briefly, cleaned sorghum kernels (4 kg) were decorticated in an IDRC mill equipped with a set of abrasive disks (Nutana Machine, Saskatoon, Canada). The finely ground abraded pericarp-rich material and the decorticated kernels were separated using a screen with triangular orifices (3 mm each side).

2.3. Milling

Whole and decorticated kernels were milled into flour in a Wiley Mill (Arthur Thomas Co. Philadelphia, PA, USA) equipped with a 2 mm screen. The particle size distributions of the resulting grindings were described by Perez-Carrillo and Serna-Saldivar [3].

2.4. Liquefaction with α-amylase

Foremost ground samples of each grain in triplicate (150 g dry weight) were mixed with water (25 °C) in 500 mL plastic containers in order to obtain mashes with 35% solids for the hydrolysis with thermoresistant α-amylase (Termamyl® 120L, Novozyme Mexico, Mexico City, Mexico), as described by Perez-Carrillo and Serna-Saldivar [3]. Termamyl® 120L is a heat-stable α-amylase produced by a genetically modified strain of *B. licheniformis*. This enzyme is an endoamylase which hydrolyzes α-1,4 glycosidic linkages in both amylose and amylopectin chains. Its optimal activity is reached by pH 7 and 90 °C.

2.5. Proteolytic dry-grind process

Ground grains (150 g dry weight) were mixed with water to obtain mashes with 35% solids. The pH value of the mash was adjusted to 6.5 with 0.1 N HCl. Subsequently 0.5 mL of Neutrase 0.5L (Novozyme Mexico, Mexico City, Mexico) with a declared activity of 0.5 Anson units/g was added and the temperature of the slurry was gradually increased from 25 to 60 ± 1 °C in a shaking water bath (Hot Shaker, BellCo. Glass, Inc., Vineland, NJ, USA) during 30 min. Neutrase 0.5L is a bacterial protease produced by a selected strain of *Bacillus amyloliquefaciens*. It is a neutral protease with optimum activity at pH 5.5 ≈ 7.5 and 45–55 °C. It is classified as a metallo-proteinase, which requires Zn^{2+} and Ca^{2+} ions for its activity. Consequently it is stabilized by the presence of Ca^{2+} ions. Moreover it can be inhibited by the presence of EDTA and can be inactivated by a heat treatment of 85 °C for 2 min. As the proteolytic stage was concluded, the hydrolyzates were treated with thermoresistant α-amylase (Termamyl® 120L) as described by Perez-Carrillo and Serna-Saldivar [3].

2.6. Saccharification

The hydrolyzates were allowed to cool down to 60 °C, thereafter the pH value was adjusted to 5.5 with 0.1 N HCl. Afterwards 3000 U/mL amyloglucosidase (Dextrozyme, Novozyme Mexico, Mexico City, Mexico) were dosed at a ratio of 1 mL/500 mL hydrolyzate. The enzymatic reaction was carried out in vessels that were placed in an incubator-shaker (Lab-Line 3526 Model) set to 60 °C and a rotation speed of 125 rpm for 16 h. As the saccharification step was completed, the resulting mashes were cooled down and the distilled grains (DG) were separated by filtration using a plastic sieve (1 mm) and afterwards washed with distilled water (200 mL). The pH of the filtrate was adjusted to 5.0 and the total soluble sugars concentration to 13°Plato.

2.7. Fermentation

Saccharomyces cerevisiae (ATCC # 24860) was used for the ethanol fermentation process. Yeast cells were maintained and cultured on YPD Yeast extract/Peptone/Dextrose agar (20 g of yeast extract/L, 5 g of peptone/L, 5 g de dextrose/L, and 20 g of agar/L) in an Omniplus bioreactor at 200 rpm and 32 °C for 48 h (pH 5.5) and counted using a Neubauer-counting chamber [13]. The mashes (500–600 mL), previously adjusted to 13°Plato, were placed in Erlenmeyer flasks and inoculated with a 5% yeast suspension (15×10^6 cells/mL). The flasks were sealed and incubated in a shaker (Lab-Line 3526 Model), which was set to 30 °C and a rotation speed of 125 rpm for 72 h.

2.8. Analytical methods

FAN was determined according to approved the AOAC method 945.30L [14]. Glucose was measured using a commercially available glucose oxidase:peroxidase enzymatic kit (K-Glu 04/06) by Megazyme® (Bray, Ireland). Ethanol and fusel alcohols were quantified in a gas chromatograph with a flame ionization detector (FID 6850 Agilent) equipped with a HP-1 Column (30 m × 0.32 mm × 0.25 μm). A glass liner packed with fiberglass was used to retain nonvolatile compounds. A calibration curve was constructed using an ethanol standard with >99.5% purity. One microliter of the sample was injected into the column. Helium was utilized as the carrier gas at a flow rate of 1.5 mL/min, and samples were injected using the split mode with the ratio of 1:10. The temperature was initially set to 35 °C and was gradually increased to 45 °C at a rate of 5 °C/min and held for 1 min. Subsequently, the temperature was raised to 47 °C at a rate of 0.5 °C/min and finally