

Use of *Aspergillus oryzae* during sorghum malting to enhance yield and quality of gluten free lager beers

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Abstract

Sorghum has been used for brewing European beers but its malt generally lower beer yields and alcohol contents. The aim of this research was to produce lager beers using worts from sorghum malted with and without *Aspergillus oryzae* inoculation. Worts adjusted to 15°Plato from the sorghum malt inoculated with 1% *A. oryzae* yielded 21.5% and 5 % more volume compared to sorghum malt and barley malt worts, respectively. The main fermentable carbohydrate in all worts was maltose. Glucose was present in higher amounts in both sorghum worts compared to barley malt worts. Sorghum-*A. oryzae* beer had similar specific gravity and alcohol compared to the barley malt beer. Sorghum-*A. oryzae* beer contained lower amounts of hydrogen sulphide, methanethiol, butanedione, and pentadione compared to barley malt beer. Sorghum-*A. oryzae* lager beer had similar yield and alcohol content compared to the barley malt beer but differed in color, key volatiles and aromatic compounds.

1. Introduction

Typically, barley has been the most relevant raw material for beer production. Malt production is considered one of the oldest and complex example of applied enzymology (Gupta et al. 2010). However, the growing interest for gluten-free products and need for efficient cultivars, especially in terms of water usage and drought resistance, have led to consider crops like sorghum. Sorghum is the fifth most important cereal worldwide with an annual production exceeding 57 million tons in 2017 (Food Agriculture Organization 2019). This cereal crop, widely adapted to arid and subtropical ecosystems around the globe, can play a dual role as refined brewing adjuncts or as a source of diastatic malt. For centuries, indigenous sorghum beers, also known as opaque or kaffir, have occupied a prominent place in the diets of many African people, and the industrialization of this kind of beer started more than 70 years ago (Bogdan and Kordialik-Bogacka 2017; Odibo et al. 2002). The main problems when brewing with sorghum are the lower diastatic power of its malt, especially deficient in β -amylase activity, and the comparatively higher gelatinization temperature of sorghum starch compared to barley starch (Serna-Saldívar 2010). In a previous research, Espinosa-Ramirez et al. (2013 a,b) successfully produced lager beers from different types of sorghum malts and gluten free adjuncts, supplemented with β -amylase or amyloglucosidase.

On the other hand, *Aspergillus oryzae*, commonly known as Koji, is a fungus important for the production of traditional fermented foods and beverages in Japan and China due to its ability to secrete large amounts of amylolytic, lipolytic and proteolytic enzymes. These features have facilitated the use of *A. oryzae* in modern biotechnology (Machida et al. 2005; Barbesgaard et al. 1992). In food fermentation, *A. oryzae* secretes significant amounts of amylases and proteases to breakdown complex starches to sugar and proteins to peptides, which are further fermented by yeast and lactic acid bacteria (Kobayashi et al. 2007). Both *A. oryzae* and its enzymes are accepted as constituents of foods (FAO/WHO,1988). A previous study by Heredia-Olea et al. (2017) has analyzed the use of *A. oryzae* with sorghum malt. The research evaluated the use of *A. oryzae* (Koji) as a supplement for sorghum malt in order to increase the enzyme content of the malt without the direct addition of exogenous enzymes. The results of this study

show that the sorghum malt originally inoculated with 1% of *A. oryzae* improved sorghum malt quality in terms of α -amylase and amyloglucosidase activities. Consequently, worts contained higher amounts of Free Amino Nitrogen (FAN) and fermentable carbohydrates. All this, without the generation of waste or additional byproducts that are generated with the addition of purified enzymes. To our knowledge there is no information about the production of lager beers using sorghum malt inoculated with *A. oryzae*. Thus, the main objective of this investigation was to produce and estimate yields of lager beers from optimally malted sorghum with and without *A. oryzae* inoculation and to assess properties of resulting beers in terms of extraction, alcohol content and physicochemical attributes.

2. Materials And Methods

2.1 Raw Materials

The white sorghum was donated by INIFAP Rio Bravo whereas the *A. oryzae* 22788 was acquired from the American Type Culture Collection (ATCC). Barley malt for the control mash, hops (*Humulus lupulus*) and fresh lager brewing yeast of the *Saccharomyces spp* strain for all experimental treatments were donated by Cuauhtémoc-Moctezuma-Heineken Brewery. Native cornstarch and yellow maize grits with a particle size of 40-60 United States (US) mesh were obtained from Industrias Mexstarch SAPI de CV and Agroindustrias Integradas del Norte SA de CV.

2.1.1 Sorghum Characterization

The thousand-sorghum kernel weight was determined by weighing 100 randomly selected whole kernels (Serna-Saldivar 2012). Test weight was measured with a Winchester Bushel Meter (Seedburo Equipment Company) according to official U.S. grain standard procedures (AACC 2000). Moisture (method 44-15), protein (method 46-13.01), ash (method 08-01) (AACC 2000) and starch (method 996.11) (AOAC 1980) were assayed based on the respectively methods.

2.1.2 *A. oryzae* Culture

A. oryzae was cultured in potato dextrose agar plates at 30°C for five days. Then spores were collected and counted in a Neubauer chamber and inoculated into potato dextrose broth and allowed to grow for five additional days (Kammoun et al. 2008). Media was centrifuged at 1000g for 10 minutes in sterile tubes to concentrate mycelium.

2.2 Malting Process

For steeping, sorghum grains were mixed with two parts of water (28°C) containing 0.01% formaldehyde for 30 hours in a cabinet set at 28°C. Steeping was conducted under aeration to enhance aerobiosis and respiration. Sorghum samples previously steeped were inoculated with 1% w/w *A. oryzae* mycelium and then placed in plastic trays in a germination cabinet set at 28°C with 90% relative humidity. Moisture losses due to evaporation were controlled by the spraying of water periodically. Samples were collected every 24 hours throughout the 96 hours germination time. Starch (method 996.11) and free amino

nitrogen or FAN (method 945.30) were measured daily according to the Association of Official Analytical Collaboration (AOAC) 1980. Malting losses were determined by comparing the weight of the dry malt to the dry weight of the original amount of sorghum kernels. The germinated grains were dehydrated for 24 hours in a forced convection oven set at 50°C. The malts were coarsely milled using the break rolls of the Chopin mill. Malt losses were obtained using equation 1 (see Supplementary Files)

2.3 Mashing Procedure

The grist formulation consisted of 60% malt (barley, sorghum or sorghum-*A. oryzae*), 20% native cornstarch and 20% maize grits. The brewing adjuncts were mixed with 10% of the total amount of malt, then mixed with deionized water in a 1:2.3 ratio. The double mashing was performed according to the procedure of Heredia-Olea et al. (2017). The °Plato and volume of resulting wort were measured to calculate the amount of water needed to adjust the wort to 15°P. The final adjusted volume was recorded as wort yield. Cascade hop pellets were added to the sweet wort adjusted to 15°Plato in a rate of 0.35 g/L. In a vessel, 90% of the hops were added to the sweet wort before heating to boiling for 50 minutes. The 10% remaining hops were added 10 minutes before discontinuing heat. The hopped wort was centrifuged at 1800g for 10 min in order to remove the spent hops and trub solids by decantation. The resulting hopped worts were readjusted with sterile water to 15°P.

2.4 Fermentation

Prior to the wort inoculation, the percent of yeast solids was determined using a Spin-Down Method (Bendiak 1997). The sample was homogenized by placing the yeast in a glass with agitation for 30 min in order to remove gas bubbles. The glass containing the sample was maintained in contact with iced water during the preparation. A yeast cell counter Nucleocounter® YC-100TM (Chemometec) was used to measure the total cell count and viability of cells in suspension. The system uses a cassette pre-coated with propidium iodide dye, which stains the cells' nuclei and is then measured by an automatic fluorescence microscope. This method was carried out before introducing the yeast to the fermentation tanks and after the yeast removal. For a lager analysis, the parameter of the Nucleocounter® YC-100TM was set to diploid. Worts from three different malt treatments were obtained from previously malted sorghum, barley and sorghum-*A. oryzae*. The wort placed in sterilized liter jugs was poured into a 6-liter flask inside a microbiological hood furnished with a gas-fired Bunsen burner to prevent cross-contamination. A previously sterilized oxygenator was introduced to the wort, transferring oxygen through a sterilized tube from a valve. The oxygenation time was set to 5 minutes. Inside the sterilized area, the oxygenated wort was then transferred to a sterilized 3 L fermentation laboratory-scale bioreactor (Applikon®). Once the wort temperature reached 12°C the yeast was pitched to the tank using a sterile pipette. Yeast was added at a rate of 10 mL/L wort equivalent to 6×10^6 yeast cells/ml. The pH of wort and beer were determined using a potentiometer sensor installed in the fermentation tank. Measurements were taken every minute during the programmed 120 h fermentation. The lager fermentation lasted 120 hours. The first 24 h was set at 12°C and afterward at 15°C for the remaining time. Samples were collected at 0, 12, 24, 48 and 120 h for analyses. After 120 h of fermentation, the maturation procedure

took place. The temperature of the bioreactors was set at 2°C for 24 hours. After the maturation step, the yeast sediment was removed from the fermentation tanks by opening the lower valve located in the cone structure. The yeast was recollected in a flask for further viability analysis. Once all of the yeast was removed, the beer was drained from the fermentation tanks into flasks for further yeast filtration. The remaining yeast was precipitated by centrifugation at 1800g during 10 min and the beer by decantation.

The total volume of the filtered beer was measured using a graduated cylinder. The filtered beer was stored in sterile glass jugs at 2°C with minimal headspace to prevent oxidation and deterioration until further analysis.

2.5 Analytical Assays

Sorghum grain germination capacity was determined according to Serna-Saldívar (2012). Briefly, a representative sample of 100 caryopses was taken randomly and soaked in excess water for 30 h with aeration. Then, the soaked kernels were placed on Petri dishes with soaked filter paper for controlled germination for 3 days in a cabinet set at 28°C. Germinated kernels were considered those that developed rootlets and/or acospores (plumulae). Values were expressed on percentage. Starch was measured according to the colorimetric Method 996.11 of the AOAC (1980) whereas FAN of wort and beer by the spectrophotometry methods 8.10 and 9.10 (EBC 2008) based on the ninhydrin reaction. Specific gravity was determined at 20°C with a digital density meter of the oscillation type using standard method 8.2.2 for worts and 9.43.2 for beers (EBC 2008). The extracts of worts were determined with a density meter following method 8.3 (EBC 2008). In beers, the calculations of original, real and apparent extracts were based on the original and specific gravities determinations following the method 9.4 (EBC, 2008). Color of hopped wort was measured by spectrophotometry according to the European Brewery Convention (EBC) and pH with a potentiometer previously calibrated with 4.0 and 7.0 pH buffers (EBC 2008). The yield was determined by measuring of wort volume (15°Brix) obtained per kilogram of dry materials (Cortés-Ceballos et al. 2015). Spent grains and solids lost after centrifugation were determined gravimetrically.

The contents of fermentable carbohydrates fructose, glucose, disaccharides and trisaccharides were determined using a High-Performance Liquid Chromatograph (HPLC) equipped with a Refractive Index detector following the method 8.7 for worts and 9.27 for beers (EBC, 2008). The ethanol in beer was determined using the Near Infrared Spectroscopy (NIR) rapid method EBC 9.2.6 (2008). The determination of lower boiling point volatile compounds in beer (alcohols, esters, acetaldehyde, and dimethyl sulphide) was measured by automatic headspace gas chromatography (method 9.39 of the EBC 2008) equipped with a chemically bonded fused silica capillary column and flame ionization detector. Volatile compounds were determined comparing with authentic international standards. Colors of worts and beers were determined according to methods 8.5 and 9.6 respectively (EBC 2008). The measurement of volatile sulfur compounds in beer was determined by Gas Chromatography equipped with a Sulfur Chemiluminescence Detector (ASBC 1994 Method Beer-44). The determination of vicinal diketones (VKD), 2,3-butandione (diacetyl), 2,3-pentanedione and their precursors in beers were measured by gas

chromatography coupled with an electron capture detector following the headspace method 9.24.2 (EBC 2008).

2.6 Statistical Analysis

The Minitab 16 statistical software (Minitab, State College, PA, U.S.A.) was employed to identify statistically significant differences among treatments and compare means with Tukey tests ($P \leq 0.05$).

3. Results

3.1 Sorghum Characterization

The white sorghum had a thousand-kernel weight of 24.42 ± 0.49 g, apparent bulk density of 79.09 ± 2.75 kg/HL and a subjective endosperm texture of 2.12 ± 0.37 (1 = totally corneous or vitreous, 2.5 = intermediate endosperm texture and 5 = totally floury or chalky). In terms of chemical composition, the sorghum contained $10.07 \pm 0.03\%$ moisture, $9.73 \pm 0.05\%$ protein, $69.27 \pm 0.52\%$ starch, $5.09 \pm 0.16\%$ crude fiber and $2.03 \pm 0.13\%$ ash expressed on dry matter basis. The physical and chemical properties fall within expected values for this cereal grain (Serna-Saldivar and Espinosa-Ramirez 2018; Serna-Saldivar 2010).

3.2 Malting Process

The addition of 1% (w/w) *A. oryzae* mycelium to sorghum kernels before malting did not affect the sorghum germination capacity, $98.33 \pm 1.03\%$ vs $98.75\% \pm 1.98\%$ for the control sorghum malt. After four days of germination, the regular and sorghum-*A. oryzae* malts lost 9.9% and 14.8% of their dry matter, respectively. The granulation of particle-size distribution is critically important because it affects lautering or filtration rate and the availability of malt reserve components to enzymes during mashing. Table 1 compares the particle size distributions of the three malts. There was a significant difference between the barley malt and both sorghum malts especially in terms of particles retained by the biggest sieve (US mesh No 20). This large sieve retained most of the glumes or husks of the barley malt. This is because the sorghum kernel is considered a naked caryopsis where the glumes usually detach during harvesting. A distinguished difference can also be noted between the barley malt and both the sorghum malts in the smallest mash (-100). This difference can be attributed to the difference in endosperm texture, which was harder for sorghum. The barley malt normally has a floury and friable endosperm, which is more prone to milling and therefore generate fine particles composed mostly of starch granules that pass the 100 mesh sieve. The barley malt contained less FAN (1.088 mg/g malt) compared to the sorghum malts. Figure 1 shows that the assayable starch and FAN contents of malts during germination. At the same day of malting, there were not differences between starch contents. Total starch content gradually diminished throughout germination in both treatments. The highest starch change was observed after the first 24 h of germination with a reduction of 4 units. The starch content of malts germinated for 96 h decreased 5 and 6 units in regular sorghum and sorghum-*A. oryzae* malts, respectively. The FAN values between malts were about the same between the original grains and malts germinated for only 24 h. However, the FAN contents greatly increased when malts were germinated for more than 48 h. FAN values gradually

increased with germination time so the regular sorghum and sorghum-*A. oryzae* malts germinated for 96 h contained at least 6 and 8 times more FAN compared to the unmalted sorghum kernels. The higher FAN content of the sorghum-*A. oryzae* malt is attributed to the presence of additional exogenous proteolytic enzymes synthesized by the mold.

Table 1. Particle size distributions of malts grinding using a Chopin mill. All data are expressed as percentage in weight*

US Mesh Sieve #	Barley Malt	Sorghum Malt	Sorghum and <i>A. oryzae</i> Malt
20	8.80±0.11 ^A	4.32±0.29 ^B	4.39±0.14 ^B
40	29.89±0.58 ^B	36.42±1.27 ^A	30.10±0.40 ^B
70	30.63±0.35 ^B	45.00±1.28 ^A	28.25±0.60 ^B
80	8.07±0.52 ^C	10.85±0.28 ^B	27.34±0.32 ^A
100	2.15±0.11 ^B	0.83±1.88 ^C	3.54±0.38 ^A
-100	20.59±0.49 ^A	1.42±0.23 ^C	6.43±0.23 ^B

* Means comparisons with different letter(s) within rows are statistically different ($P < 0.05$).

3.3 Wort Properties

Table 2 data showed that the worts obtained after the double mashing, lautering and the hop boil processes were within the specific gravity and dissolved solids contents expected for lager beers (Gialleli et al. 2017). The volume of 15° Plato wort was used as an indicator to quantify differences in wort yields from the various malts. The barley and sorghum worts yielded lower wort volumes compared to the wort obtained from the grist containing the sorghum-*A. oryzae* malt (Table 2). The regular sorghum malt yielded 17.2% less volume compared to the barley malt. Interestingly, the sorghum-*A. oryzae* treatment yielded 6.5% more wort volume and 27.6% compared with worts produced by the barley malt and sorghum malt, respectively. This relevant finding indicates that the proposed strategy to enhance enzymatic activity of the sorghum malt was effective and promising for the production of gluten-free beers. The amount of dry brewers spent grains obtained after lautering and centrifugation indicated that the regular sorghum yielded the highest amounts (Table 2). The amounts of spent grains generated by the barley malt and sorghum-*A. oryzae* malt were approximately 25 and 35% less compared to the regular sorghum. In terms of specific gravity, pH, original extract and fructose and maltotriose contents, there were no differences among the three worts. In all worts, maltose was the most abundant fermentable sugar followed by glucose. However, the malt treatment affected maltose and glucose concentrations in worts. The regular sorghum and barley worts contained the highest and lowest glucose values (Table 2). In case of maltose concentrations, the wort from barley contained approximately 35% and 29% more maltose compared to the regular sorghum and sorghum-*A. oryzae* worts, respectively. The wort produced from sorghum-*A. oryzae* malt contained 21.7 and 6.8% more FAN concentrations compared to the barley and regular sorghum worts. The color of worts (Table 2) was significantly affected by the sort of malt. Compared to the barley malt wort, both sorghum worts showed almost two-fold color values.

Table 2. Quality parameters, yields and carbohydrates profiles of worts (15° Plato) produced using malts from barley, and sorghum without or with *A. oryzae*.*

	Wort from Barley Malt	Wort from Sorghum Malt	Wort from Sorghum with <i>A. oryzae</i> Malt
Yield (15°Plato) (L/ kg)	4.02±0.03 ^B	3.33±0.05 ^C	4.25±0.04 ^A
Dry Brewer's Spent Grains After Lautering (g solids/kg grist)	151±3.20 ^B	175.92±2.62 ^A	181.3±0.27 ^A
Solids Lost After Wort Centrifugation (g solids/kg grist)	148±3.00 ^B	225±4.21 ^A	81±1.70 ^C
Specific Gravity @ 20°C	1.061±0.01	1.061±0.01	1.061±0.01
pH @ 20°C	5.81±0.08	5.50±0.16	5.73±0.13
Original Extract (%)	15	15	15
Carbohydrates:			
Fructose (%w/w)	0.039±0.001	0.037±0.001	0.036±0.001
Glucose (%w/w)	1.895±0.005 ^C	4.379±0.011 ^A	3.772±0.009 ^B
Maltose (%w/w)	7.661±0.039 ^A	5.009±0.022 ^C	5.435±0.028 ^B
Maltotriose (%w/w)	1.456±0.012 ^B	1.564±0.013 ^A	1.479±0.009 ^B
Total Fermentable Sugars (%w/w)	11.051	10.989	10.724
Free Amino Nitrogen (mg/L)	173.9±4.35 ^C	206.5±6.64 ^B	221.1±5.27 ^A
Color °(EBC)	8.65±0.012 ^A	4.62±0.012 ^B	4.11±0.012 ^C

* Means comparisons with different letter(s) within rows are statistically different ($P < 0.05$).

3.4 Fermentation Analyses

There were not differences between treatments in terms of yeast in suspension (10.55±0.33%) and dead cells (1.89±0.21%). Figure 2 depicts changes of fermentable sugars during 120 h fermentation of the different worts. The charts display that glucose and fructose were totally consumed after 50 h. On the other hand, maltose and maltotriose were gradually utilized throughout the whole fermentation process. Maltose was totally fermented in all worts after 120 h of fermentation whereas small amounts of maltotriose remained in the barley and sorghum-*A. oryzae* worts. For fructose, an increment in its concentration was visualized after 15 h of fermentation.

There were not differences between pH, FAN, superior alcohols, acetaldehyde, ethyl acetate, isoamyl acetate, dimethylsulfide and S-methyl acetate among treatments (Table 3). As expected there were close relationships among beer specific gravity, attenuation and ethanol concentrations (Table 3). The regular sorghum beer specific gravity were about 0.49% higher compared to the barley malt and *A. oryzae*-sorghum beers. The observed differences are attributed to the almost 11% higher ethanol concentrations observed in the barley and *A. oryzae*-sorghum beers. As a result, the apparent and true attenuation values of the regular sorghum treatment was about 11.5% and 7% lower compared to the barley beer and sorghum-*A. oryzae* beers, respectively (Table 3). Interestingly, the beer produced with sorghum contained 37% less ethyl hexanoate compared to the counterpart produced with barley malt. In terms of sulfur compounds, hydrogen sulphide content was highest for the barley beer and lowest for the sorghum-*A. oryzae* beer. On the other hand, methanethiol content was comparatively higher for barley malt beer than the sorghum-*A. oryzae* beer. Large differences between the concentration of VKD compounds 2,3 butandione and 2,3 pentandione were observed when both sorghum beers were compared with the barley beer. The amounts of these compounds were approximately 10% of the concentrations assayed in the

barley malt beer (Table 2). Likewise, the EBC color of the barley beer was significantly higher compared to the two experimental sorghum beers.

Table 3. Composition and characteristics of beers from barley, and sorghum without or with *A. oryzae**

	Beer from Barley malt	Beer from Sorghum malt	Beer from Sorghum with <i>A. oryzae</i> Malt
Specific Gravity @ 20°C	1.016±0.01 ^B	1.021±0.01 ^A	1.019±0.01 ^{AB}
pH	4.08±0.05 ^A	3.96±0.22 ^A	4.00±0.12 ^A
Apparent Attenuation (%)	72.76±0.09 ^A	64.35±0.01 ^B	68.05±0.01 ^B
Real Attenuation (%)	60.09±0.01 ^A	53.33±0.12 ^B	55.66±0.01 ^{AB}
Ethanol (%v/v)	5.25±0.07 ^A	4.68±0.05 ^B	5.28±0.02 ^A
FAN (mg/Lt)	56.18±3.45 ^A	59.72±6.13 ^A	66.23±3.64 ^A
Superior alcohols:			
Propanol (ppm)	13.78±0.01 ^A	14.40±2.02 ^A	16.31±0.45 ^A
Isobutanol (ppm)	13.14±0.20 ^A	18.52±8.77 ^A	15.21±0.45 ^A
Isoamyl alcohol (ppm)	85.55±1.86 ^A	86.34±9.10 ^A	99.16±1.22 ^A
Volatile and ester compounds:			
Acetaldehyde (ppm)	3.13±0.69 ^A	2.87±0.52 ^A	1.92±0.51 ^A
Ethyl acetate (ppm)	13.24±0.20 ^A	7.78±1.70 ^A	14.84±2.39 ^A
Isoamyl acetate (ppm)	0.977±0.001 ^A	0.648±0.130 ^A	1.28±0.25 ^A
Ethyl Hexanoate (ppm)	0.068±0.001 ^A	0.046±0.040 ^B	0.059±0.010 ^{AB}
Sulfur compounds:			
Hydrogen sulphide (ppb)	26.77±0.69 ^A	19.63±1.88 ^B	3.61±0.19 ^C
Methanethiol (ppb)	3.13±0.13 ^A	2.18±0.45 ^{AB}	1.73±0.01 ^B
Dimethylsulfide (ppb)	43.09±8.29 ^A	63.94±10.93 ^A	62.44±0.96 ^A
S-methyl acetate (ppb)	24.87±1.45 ^A	18.01±5.04 ^A	16.17±0.51 ^A
VDK compounds:			
2,3 Butanedione (ppm)	0.440±0.050 ^A	0.041±0.020 ^B	0.018±0.010 ^B
2,3 Pentanedione (ppm)	0.240±0.020 ^A	0.033±0.020 ^B	0.008±0.010 ^B
Color			
Color (°EBC)	6.36±0.09 ^A	2.91±0.29 ^B	3.37±0.14 ^B

* Means comparisons with different letter(s) within rows are statistically different ($P<0.05$).

4. Discussion

4.1 Malt Properties

The main chemical changes that grains undergo during malting are the reduction in assayable starch and hemicellulose contents. During this physiological process, the cell bound components weaken due to the synthesis of degrading enzymes like cellulases and arabinoxylanases. The hydrolyzed cell walls allow the entry of other relevant enzymes that will degrade protein bodies and matrix and starch granules (Boulton and Quain 2001). There are many investigations which clearly conclude that malted sorghum has significantly lower diastatic activity compared to barley malt especially in terms of maltose-producing β -amylase. These deficiencies have been counteracted by the addition of exogenous enzymes like α and β -amylases, glucoamylase or amyloglucosidase and even proteolytic enzymes that improve the

exposure of the starch granules to amylolytic enzymes (Serna-Saldivar and Rubio-Flores 2016). The idea of inoculating *A. oryzae* during the malting process of sorghum is based in the fact that this mold synthesizes relevant enzymes like amylases and proteases that enhance the conversion of starch and proteins into fermentable carbohydrates and simpler and soluble nitrogenous compounds, respectively. Heredia-Olea et al. (2017) observed that α -amylase activity of sorghum malt was positively affected by addition of *A. oryzae* but it did not affect β -amylase activity. The conversion of starch into linear and branched dextrans explain the significant reduction of starch content during germination. In addition, the proteases generated are distributed to the entire caryopsis and hydrolyze conjugated proteins associated with amylases in order to activate starch-degrading enzymes. The proteases also degrade germ and endosperm proteins, solubilizing approximately 30% of the total protein. The sorghum malt contained higher amounts of readily assimilable amino acids (FAN) compared to the barley malt and other cereals such as wheat (Hill and Stewart 2019) likely enhancing yeast nutrition during beer fermentation and production of relevant fusel alcohols.

4.2 Wort Properties

The regular sorghum malt generated less 15°P wort yield compared to the barley malt (Table 2). Similar difference was reported by Espinosa-Ramírez et al. (2013a) who observed that barley malt yielded was up to 24% more 12°P wort and similar FAN and pH values compared with their counterparts produced with white or red sorghum malts. According to Espinosa et al. (2014) and Heredia-Olea et al. (2017), the use of regular sorghum malt usually yields worts with lesser amounts of fermentable carbohydrates and FAN contents and their fermented beers with lower alcohol contents. These differences, especially in terms of generation of maltose and glucose, may be attributed to the higher β -amylase and amyloglucosidase activities of the barley and sorghum-*A. oryzae* malts. It is well known that malt and adjunct starches are hydrolyzed to dextrans and sugars during mashing (Serna-Saldivar and Espinosa Ramirez 2018). In terms of the preferred carbon source, Carlsen and Nielsen (2001) studied the influence of maltose and maltodextrans differing in chain length, glucose, fructose, galactose, sucrose, glycerol, mannitol and acetate on α -amylase production by *A. oryzae*. Productivity was found to be higher during growth on maltose and maltodextrans, which are present in relatively high amounts in typical worts. Maltose was the most abundant carbohydrate in the control and experimental worts (Table 2). This is due to the concerted enzymatic action of α and β -amylases. The sorghum worts contained more glucose and less maltose than the barley wort. This is related to the higher β -amylase activity reported in the barley malt. Even though the worts were adjusted to 15°Plato, the total amount of fermentable sugars did not total 15% due to the presence of dextrans. Dextrans commonly account for 90% of the residual carbohydrate of beer because regular yeast is not capable of fermenting these carbohydrates. Approximately, 40 to 50% of the dextrans are oligosaccharides containing 4 to 9 glucose units, and the remaining 50-60% are higher dextrans with 10 or more glucose units (Boulton and Quain 2001). Espinosa-Ramírez et al. (2013a) enhanced the amounts of fermentable sugars in the sorghum malt worts with the addition of β -amylase. When amyloglucosidase was added, the total sugar content increased 20% and consequently the glucose content was five times higher compared with worts without exogenous enzymes. It is well known that *A. oryzae* is a mold that synthesizes large amounts of amylolytic, proteolytic and lipolytic enzymes. In fact,

commercial mold cultures are used to produce and isolate enzymes widely used by the food industries. The main disadvantage of sorghum malt is its relatively low production of β -amylase, key enzyme in brewing operations because it complements the activity of α -amylase. Furthermore, the color of the beer is influenced by Maillard reactions that occur between sugars and amino-compounds (including the amino acids) during the hop-boil step, which gave rise to colored and flavored substances. The proportions of the flavored fermentation products made by yeast are dependent on the nitrogenous substances that are present. Nitrogenous components are present in the form of amino acids, small peptides, and proteins. Recommended FAN concentrations of wort range from 150 to 200 mg/L (Boulton and Quain 2001). Table 1 clearly depicts that the barley wort contained slightly lower FAN values compared to the sorghum with and without *A. oryzae* counterparts. In fact, the sorghum wort with *A. oryzae* contained 27% more FAN compared to the control barley counterpart. The significant difference is attributed to the synthesis and action of proteases (mainly carboxypeptidases) produced by the *Aspergillus*. Malt carboxypeptidases have optimum activity at temperatures between 40-60°C and are inactivated at 70°C. Likely the double mashing process gave these enzymes the opportunity to hydrolyze proteins into FAN components (Boulton and Quain 2001). The proteases generated by the mold facilitated the entrance of amylases associated to the sorghum malt and *A. oryzae*. This mold is known to express high amounts of amyloglucosidases that convert linear and branched dextrans into glucose (Heredia-Olea et al., 2017). The hydrolyzed peptides are converted to higher alcohols during fermentation (Barredo-Moguel et al. 2001). Briggs et al. (2004) indicate that 100 to 140mg/L is regarded as the minimum level of FAN needed for trouble-free fermentations. The soluble proteins and polypeptides that remain in the fermented wort contribute to the *body* and *mouth-feel* of the beer, its foaming properties, and its susceptibility to haze formation.

4.3 Fermentation and Beer Parameters

Glucose and fructose consumption profiles during fermentation followed the expected trend. According to Cason et al. (1987), glucose and fructose are taken up by the same membrane transport system which explains the similar consumption profiles. The maltose consumption profiles were the same for all treatments. Cason et al. (1987) observed that utilization rates of maltose were identical independently of the adjunct concentration. The rapid consumption of glucose and fructose exerted catabolite repression on the maltose membrane transport system or any of the subsequent metabolic steps of maltose catabolism to glucose. Therefore, maltose started to be utilized when glucose or fructose levels fall to a cut-off point below which catabolite repression did not occur (Cason et al. 1987). The metabolism of maltose and maltotriose is highly interconnected. Both sugars are α -glucosides transported by the activated α -glucoside-Hc symporter encoded by gen AGT1. This permease, which is maltose inducible, has the same affinity for maltose and maltotriose (Zastrow et al. 2000). These authors observed a single exponential growth phase of maltotriose fermented by *Sacharomyces cerevisiae* grown in medium containing glucose, maltose or maltotriose as carbon and energy sources indicating that the metabolism of this particular fermentable sugar was oxidative. In terms of sugar consumption, fermented beers for the three malt treatments did not show any significant differences.

Final specific gravity value in beer is closely related to the final ethanol content (Esslinger, 2009) and this relationship was observed in all beers. In case of attenuation, the values obtained herein were lower compared to the ones reported by Esslinger (2009) for regular barley and sorghum beers where a final attenuation of 82.1% and 79.7% were observed, respectively. In terms of beer specific gravity, pH, ethanol, and FAN contents, the use of sorghum-*A. oryzae* improved values compared to the use of only sorghum malt and similar to the barley malt beer. Espinosa-Ramírez et al. (2014) obtained similar ethanol contents when barley malt beers were compared with sorghum malt beers produced with exogenous amyloglucosidase. The superior alcohol yeast metabolism was not affected by the malt treatment despite dissimilarities in FAN concentrations. Esters are the products of the enzymatic catalysis of organic acids, ethanol and higher alcohols. Their formation is closely related to yeast propagation and lipid metabolism (Pires et al. 2014). Beer contains more than 50 different esters, from which three are of higher relevance because they greatly affect beer flavor: ethylacetate, iso-amylacetate, iso-butylacetate. Since the sorghum-*A. oryzae* treatment had six-fold and four fold less hydrogen sulfide than barley beer and sorghum beer, respectively, the difference can be attributed to its higher rate of catabolism mediated by the mold. In terms of hydrogen sulfide production, all beer treatments were statistically different, where only the sorghum-*A. oryzae* beer was under the threshold level of 5 ppb. It arises through yeast autolysis at the end of fermentation or during maturation. The best understood carbonyl flavor compounds associated with yeast fermentation are the VKD, which include diacetyl and 2,3 pentandione. This led to high levels of VKD, during fermentation owing to effects on the regulation of valine synthesis by the yeast. According to Esslinger (2009) the beer taste thresholds of diacetyl and 2,3 pentandione ranges from 0.08 to 0.2 ppm and from 0.5 to 0.6 ppm. Beer from barley contained significantly higher levels of both butandione and 2,3 pentandione whereas beer manufactured with the sorghum-*A. oryzae* malt contained about 96% lower content of VDK. This important difference in volatile compounds needs to be further researched especially in terms of beer stability and sensory analysis. According to the EBC scale, the sorghum beers are classified as pale whereas the slightly darker yellowish barley beer as Pilsner. The final beer color is influenced by the type of malt, color of brewing adjuncts, concentration and type of hops and pH (Esslinger 2009). The almost twice as high color score observed in the barley beer is attributed to the utilization of a malt rich in glumes that contain phenolic compounds that lixiviated into the wort and the production of Maillard type of compounds during boiling (Granato et al. 2011). It is worth mentioning, that both sorghum beers were produced from naked caryopses of white sorghum that do not contain tannins and were low in phenolic compounds (Serna-Saldivar and Espinosa-Ramirez, 2018). Differences of ethyl hexanoate, hydrogen sulphide, methanethiol and VKD compounds could have also affected beer color. According to Dack et al. (2017) Maillard reaction products inhibit the synthesis of esteres due to possible suppression of enzymes and/or gene expression linked to ester synthesis. The impact of FAN on formation of flavor and aroma compounds during fermentation has been previously studied. Initial wort FAN content and the amino acid and ammonium ion equilibrium in the medium impact the formation of esters, aldehydes, VKD, superior alcohols and acids, as well as sulfur compounds. Even small differences in wort composition can exert significant effect on the flavor of the resulting beer (Hill and Stewart 2019). According to Taylor et al. (2013), and Kobashi et al. (2008), there is some indication that the differences in the free amino acid profile of sorghum malt worts compared with

barley malt worts could influence beer flavor by affecting yeast metabolism. Sorghum malt worts were found to contain low levels of branched chain valine. The amino acid profiles of barley and sorghum differed because the first commonly contains 2.2 g of methionine and 1.8 g of cysteine per 100 g of protein whereas the second 1 g of methionine and 1.6 g of cysteine per 100 g of protein (Serna-Saldivar 2010).

5. Conclusion

Simultaneous solid state fermentation with 1% *A. oryzae* of sorghum undergoing germination produced a malt that increased wort and beer yield (12% more ethanol) compared to the regular sorghum malt. Beers produced with the *A. oryzae*-sorghum malt had similar yield and alcohol content compared to the barley malt beer but differed in color, key volatile and aromatic compounds. Further investigations into changes in protein solubility, amino acids profile changes during solid-state fermentation of sorghum malt with *A. oryzae* and its relation with changes in superior alcohols and other flavor beer components are needed.

Abbreviations

A. oryzae: *Aspergillus oryzae*; AAC: American Association of Cereal Chemists; ABSC: American Society of Brewing Chemists; AOAC: Association of Official Analytical Collaboration; ATCC: American Type Culture Collection; EBC: European Brewing Convention; FAN: Free Amino Nitrogen; FAO: Food Agriculture Organization; HPLC: High-Performance Liquid Chromatograph; NIR: Near Infrared Spectroscopy; US: United States; VKD: vicinal diketones; WHO: World Health Organization

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals

Consent for publication

I, Sergio O. Serna-Saldivar (SOSS), the corresponding author, hereby declare that it is my study and I developed the manuscript titled "Use of *Aspergillus oryzae* during sorghum malting to enhance yield and quality of gluten free lager beers"

Availability of data and materials

The data that support the findings of this study are available from the corresponding author Sergio O. Serna-Saldivar (SOSS), upon reasonable request.

Competing interests

All authors declared that they do not have competing interests.

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Authors' contributions

SOSS conceptualize the idea, designed the work. MRF and ARG performed experiments. All authors discussed the results. MRF, SOSS, EPC cowrote the paper. All authors read and approved the final manuscript

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Figures

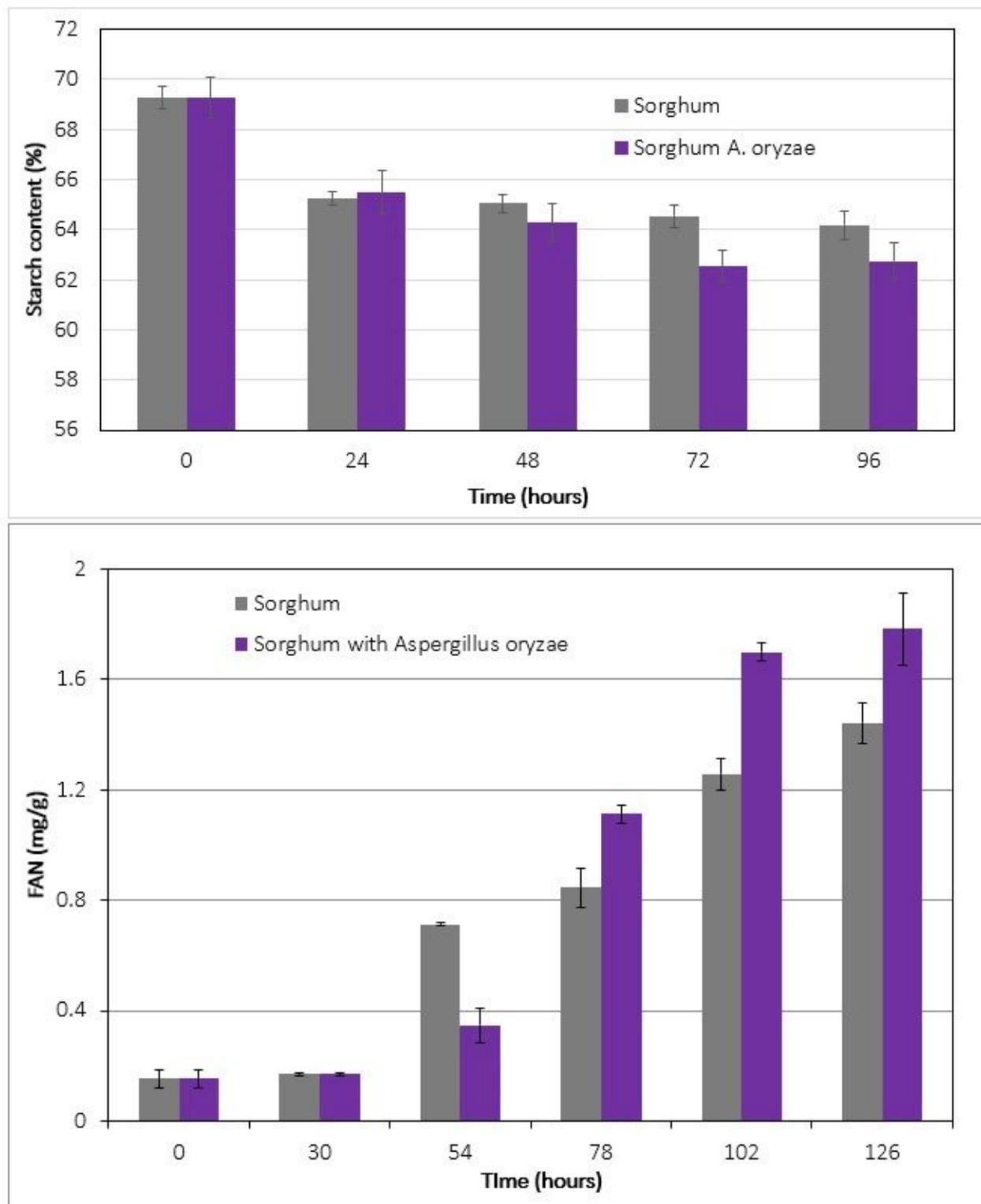


Figure 1

Changes in starch (dry basis) and Free Amino Nitrogen (mg/g) contents during germination of sorghum with or without *A. oryzae*.

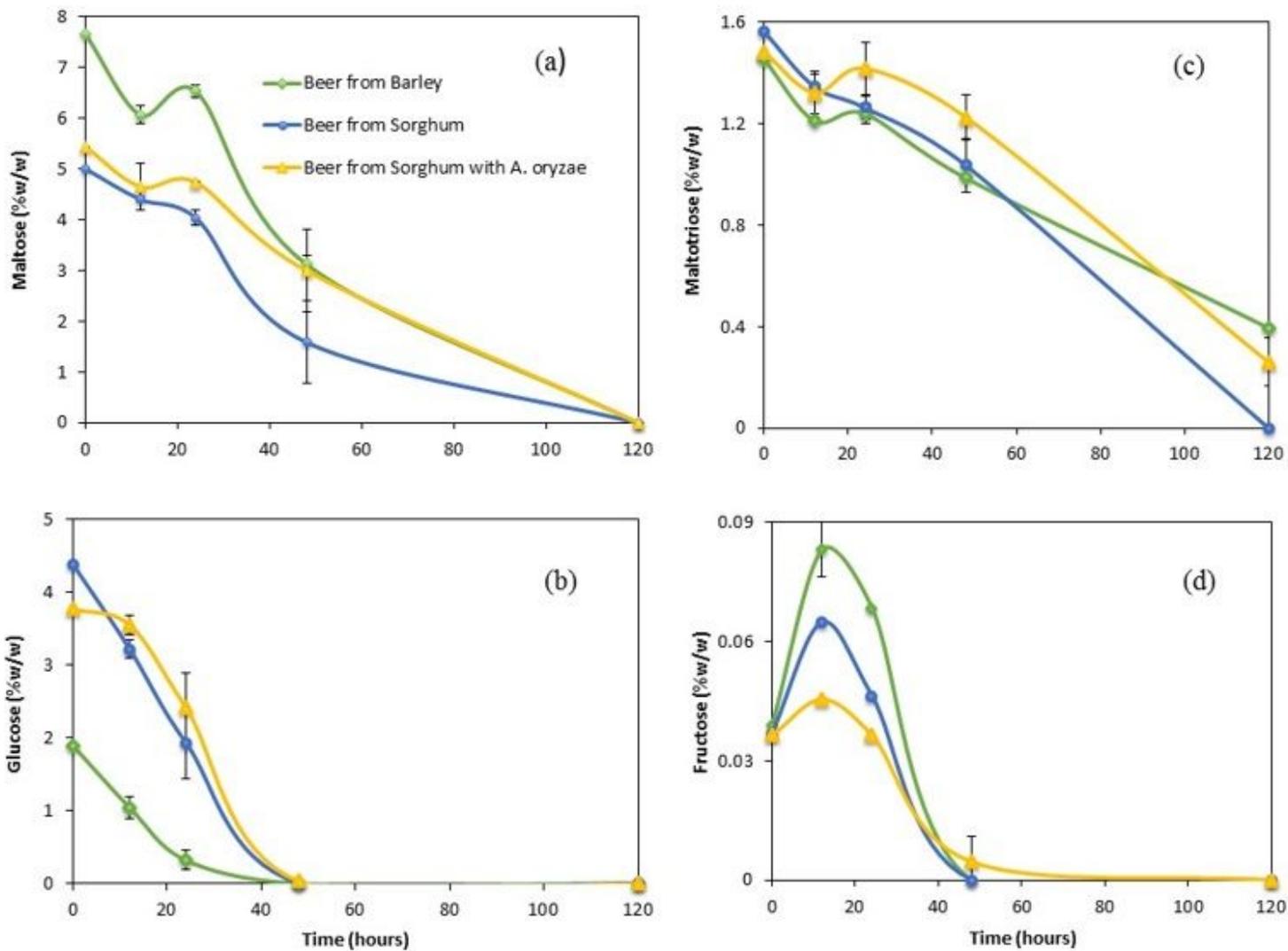


Figure 2

Fermentable sugar depletion in worts from barley, sorghum or sorghum with *A. oryzae* fermented during 120 hrs. a) Maltose (%); b) Glucose (%); c) Maltotriose (%); d) Fructose (%).

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