Potential for improvement in yeast nutrition in raw whole grain sorghum and maize lager brewing and bioethanol production through grain genetic modification and phytase treatment

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ABSTRACT

Brewing and bioethanol production with raw grain and exogenous enzymes produces wort with satisfactory hot water extract (HWE). However, the free amino nitrogen (FAN) and mineral content can be too low, due to low protein digestibility (PD) and phytate-mineral chelation, respectively. This study evaluated the potential for improvement in yeast nutrition in raw whole sorghum and maize brewing and bioethanol production by genetic modification (GM) of sorghum to improve PD and reduce phytate content and by treatment with exogenous phytase. While phytase addition decreased sorghum spent grain phytate content (88%) and content of minerals (17 to 59%) (i.e. increased wort mineral content), it did not affect maize phytate spent grain mineral content or HWE significantly. However, phytase addition did increase maize wort FAN (20%), and sorghum HWE (2.8 percentage points) and wort FAN (23%). GM sorghum gave reduced spent grain mineral contents (11 to 38%), increased HWE (5.5 percentage points) and wort FAN (71%). Hence, genetic modification of sorghum to improve PD and reduce phytate content has considerable potential in raw grain brewing and bioethanol production to improve yeast nutrition.

Key words: bioethanol, genetic modification, maize, minerals, phytase, protein digestibility

INTRODUCTION

Lager beer brewing and bioethanol production using sorghum and maize are growing rapidly due to the fact that these grains are readily available and also because they represent a gluten-free option for brewing. Sorghum is used for lager brewing extensively in Nigeria and also in East and Southern Africa and USA[36] where it is also used for bioethanol production[39].

Brewing with malted sorghum presents some problems including insufficient β-amylase, limited protein modification, high malting losses, lack of malting capacity, high malting costs and also the need to supplement mashes with exogenous enzymes[18]. This has led to the development of mashing procedures using raw grain and commercial enzymes[5,24]. These enzymes include amylases, proteases, β-glucanase, cellulases and hemicellulases.

With raw sorghum brewing[5,24] and maize[31] and sorghum[31,40] bioethanol production the wort free amino nitrogen (FAN) content can be too low. Brewing with malted sorghum provides much higher levels of FAN[27]. The low level of FAN in raw sorghum worts is directly related to the poor protein digestibility (PD) of sorghum, especially following wet cooking[10,28]. FAN has long been regarded as a general index for prediction of healthy yeast growth, viability, vitality and fermentation efficiency[22]. The sources of FAN in wort are individual amino acids (approximately 70%), small peptides and ammonium ions formed during malting and/or mashing[22].

When mashing using raw whole grain sorghum or maize, the bran is present, which contains substantial levels of phytate (myo-inositol hexaphosphate)[30]. Phytate is a chelating agent which, through multiple bonds, forms insoluble, complex molecules with some proteins and particularly divalent metal ions. Metals important for yeast fermentation performance, which may be limited through phytate chelating include iron, zinc, magnesium, phosphorus and calcium[38]. These minerals play an important role in yeast fermentation performance[32] as

during fermentation yeast cells take up minerals for growth, cell division, energy transduction, and survival in the face of stress[38]. Endogenous phytase activity in raw sorghum and maize is absent or very low[11]. When brewing with malted grains the intrinsic phytase increases during germination, which could decrease the phytate content during mashing[34]. While the addition exogenous phytase on fermentation performance has been investigated[21,], as both exogenous phytase and increased α-amylase enzymes were used, the improvement in yeast fermentation performance could not specifically attributed the effect of phytase. Further, no research could be found on the effect of a phytate reduction through breeding or GM on wort nutritional quality or yeast fermentation performance.

The objective of this study was therefore to evaluate the potential for improvement in yeast nutrition in raw whole grain sorghum and maize brewing and bioethanol production through genetic modification of sorghum to improve PD and reduce phytate and by treatment with exogenous phytase.

MATERIALS AND METHODS

Materials

Grains. Genetically modified sorghum, line ABS 032 grown in 2009 at Johnston, Iowa, USA in a summer, confined field trail was used for this study (ex. Pioneer Hi-Bred, Johnston, Iowa). Genetic modification included kafirin synthesis suppression, lysine ketoglutarate reductase and myo-inositol kinase synthesis suppression. The parent line used for the modifications was P898012 a white type II tannin sorghum (grown 2008), which was then backcrossed into Macia, a white tan-plant sorghum. Three independent genetically modified non-tannin sorghums (GM 1-3), two non-tannin null controls (NC1 and 2) samples and a non-tannin wild type control (WTC) were analysed. The relevant modifications for this study were

the suppression of myo-inositol kinase synthesis, which decreases the phytic acid synthetic capacity of the plant during seed development[25] and the kafirin synthesis suppression, which results in improved protein digestibility[8]. The maize grain used was a white hybrid PAN 6Q-521 R, grown in 2009 at the South African Agricultural Research Council, Grain Crops Institute, Potchefstroom.

Enzymes. Cerezyme® Sorghum 2X and Fungamyl® 4000 BG (both kindly donated by Novozymes SA, Marlboro, South Africa) and Natuphos® 10 000 G (phytase) containing 10 000 FTU/g (FTU is the quantity of enzyme which liberates 1 micromole of inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at pH 5.5, 37°C) (kindly donated by Advit, Johannesburg, South Africa).

Small scale mashing

Due to limited sample sizes, small scale mashing was used on the GM sorghums and their controls. Mashing was carried out in a shaking water bath. This mashing was used to compare the effect of the genetic modifications (GM1-3) on the FAN and hot water extract (HWE) of the wort to their null controls (NC1 and 2) and the wild type control (WTC). Whole grain flour (10 g, db) and distilled water (34 ml) were heated to 55°C in a 125 ml Erlenmeyer flask, and Cerezyme® Sorghum 2X was added at a concentration of 1 g enzyme/kg flour. The mashing mixture was rested at this temperature for 30 min. The temperature was increased to 85°C at a rate of 1°C/min and the mash was rested at this temperature for 45 min. The mash was cooled to room temperature and the contents of the beaker adjusted to exactly 108 g by the addition of distilled water. The mash was centrifuged at 10 000 g, 22°C and the supernatant was stored at 4°C for not more than 24 h before analyses.

Laboratory scale mashing

One GM sorghum (GM 3) was selected to compare to the WTC, WTC + phytase, maize and maize + phytase at a larger scale. Laboratory scale mashing was carried out in a BRF mashing bath (Brewing Research Foundation, Nutfield, United Kingdom). Whole grain sorghum/maize flour (100 g, db) and distilled water was mixed at a grist:liquor ratio of 1:3 and heated to 50ºC. As required, the pH of the mash was adjusted to 5.6 with orthophosphoric acid. Cerezyme® Sorghum 2X (1.5 g enzyme/kg raw grain) and phytase (1 g enzyme/kg raw grain) were added and the mash rested at 50ºC for 30 min. The temperature of the mash was then increased to 85ºC at a rate of 1ºC/min and rested for 45 min. The temperature of the mash was then reduced to 58ºC and if necessary, the pH was adjusted to 5.5 with orthophosphoric acid. Freshly prepared Fungamyl® 4000 BG (0.3 g enzyme/kg raw grain) and phytase (1 g enzyme/kg raw grain) were added and the mash was rested at 58ºC for 10 min. The temperature was increased to 63ºC and rested for 40 min. The temperature was then increased to 72ºC followed by another rest for 15 min. The temperature was then increased to 78ºC. The mash was filtered through cheese cloth twice, to separate the wort and spent grain. The wort was then clarified by centrifugation at 10 000 g for 10 min at 4^{o}C and treated as described.

Analyses

Phytate. By anion exchange chromatography as described[17].

Protein. Protein content (N x 6.25) determined by a Dumas combustion method[2].

Protein digestibility (PD). The method of Mertz et al.[26] was used, as modified[4]. Accurately weighed samples (approximately 200 mg) were digested with 35 ml 105 mg/100 ml P7000 pepsin (Sigma, Johannesburg, South Africa), (activity 863 units/mg protein) for 2 h at 37°C.

PD was calculated by the difference between the total protein and the residual protein after pepsin digestion, and expressed as a percentage of total protein.

FAN. Wort FAN was determined by the European Brewery Convention ninhydrin assay[13] using glycine as standard and expressed as mg FAN/L wort.

HWE. The specific gravity of the wort was measured using the American Society of Brewing Chemists approved method WORT to 2[3] using a Reishauer pycnometer. HWE (%) was calculated from the specific gravity[9].

Minerals. Nitric-perchloric acid digestion of the raw flour and spent grain samples was performed as described[41]. The iron, zinc, magnesium, calcium and phosphorus contents of the digested flour, digested spent grain and wort were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 5300 DV, PerkinElmer, Johannesburg, South Africa).

Statistical analyses

All the mashings were performed four times. Data were analysed by one way analysis of variance (ANOVA) at a confidence level of $p \leq 0.05$.

RESULTS AND DISCUSSION

Grain composition

The phytate contents (1188-1245 mg/100 g whole grain) of the NC and WTC sorghums did not vary significantly ($p > 0.05$), while the maize had the highest phytate content (1366 mg/100 g whole grain) (Table I). The GM sorghums had significantly ($p \leq 0.05$) lower phytate contents compared to their NCs and the WTC (27 to 47% less). The sorghum phytate contents fell within the range previously reported of 300 to 2000 mg/100 g whole grain flour[30]. The phytate content of the maize was higher than the average reported phytate content of

610 to 988 mg/100 g whole grain flour[19], but not unseen before. Maize with a phytate content of 1443 mg/100 g whole grain flour has been reported[1].

The protein contents of all the sorghum samples were similar (10.9 to 11.5 g/100 g whole grain), but that of the maize (7.6 g/100 g whole grain) substantially lower (Table I). The GM sorghums had significantly higher ($p \leq 0.05$) raw (13-23 percentage points) and cooked (21-29 percentage points) PD compared to the controls. This was due to the reduced kafirin content of these grains[8,35]. All the grains' PDs were reduced upon cooking. When sorghum is wet cooked the kafirin proteins became more disulphide bonded, which decreases the PD[10]. The reduction in PD after cooking in the GM sorghums and maize was not as much as the NC and WTC sorghums. The GM sorghum grains contained less undigestible kafirin proteins[8] and the maize contained zein proteins which are more digestible than kafirin[12].

All the mineral contents of these grains fell within or were close to previously reported ranges for sorghum[16] and maize[15]. There was no trend that the GM substantially increased or decreased any of these minerals.

Effect of genetic modification and phytase treatment on HWE

The HWE of the GM sorghums were 3.7 to 5.5 percentage points higher than that of the WTC (Tables II and III and 1.7 to 3.0 percentage points higher than that of their NCs (Table II), while the WTC+phytase had a HWE 2.8 percentage points higher than that of the WTC (Table III). There was no difference in the HWE between the maize control and the maize+phytase (Table II).

It has been found that phytate can form complexes with proteins, which are resistant to the enzymatic attack from the proteolytic enzymes[33]. It has been found that sprouting increased the PD of pearl millet and that the increase was dependant on the reduction of the

phytate content of the sprout[20]. In the present work, as the phytase was added with the Cerezyme® Sorghum 2X at the start of the mashing, it could have hydrolysed the phytate, reducing the phytate to protein complexes and increasing the PD. It is also possible that there was a side activity of protease in the phytase enzyme preparation, which could have resulted in increased protein hydrolysis.

In sorghum and maize, starch granules are surrounded by a protein matrix, containing prolamin protein bodies[10]. It has been found that the protein matrix of sorghum prevented full starch granule expansion, by physically restricting swelling of the starch granule[7]. Increased PD results in increased hydrolysis of this protein matrix, which increases the access by α-amylase to the starch, which in turn increases enzymatic starch hydrolysis[14]. Thus, the increased PD by GM and phytate reduction probably caused the increased HWE in the GM sorghums and phytase added sorghum worts.

Effect of genetic modification and phytase treatment on wort FAN

The wort FAN content of the GM sorghums was significantly higher than that of the WTC (61 to 72% increase) (Tables II and III) and their NCs (67 to 78% increase) (Table II). The FAN of the sorghum+phytase wort was 23% higher than that of the WTC and the FAN of the maize+phytase wort 20% higher than that of the control maize (Table III). Notably, the increase in FAN due to GM was much higher than that by phytase addition.

The improved PD (Table I) of the GM sorghums and possibly improved PD due to the addition of phytase presumably resulted in the proteolytic enzymes in the Cerezyme® Sorghum 2X preparation more effectively hydrolysing the proteins into FAN. Also, possible protease side activity in the phytase enzyme could have resulted in increased protein hydrolysis. The effect of adding a reducing agent during sorghum mashing on wort FAN has

been studied[28]. The reducing agent broke the disulphide bonds in the kafirin, increasing wort FAN by approx. 15%. The GM sorghums used in this present study contained reduced levels of the γ-kafirin sub-class, which is responsible for the disulphide cross linking during wet cooking[10]. While the methods differed, both a genetic reduction in kafirin and the addition of a reducing agent would have decreased the disulphide cross-linking, resulting in more digestible protein. It has also been found that mashing non-GM high PD sorghum increased wort FAN by approximately 22% and also that the addition of protease increased the FAN of normal and high PD sorghums worts 5 and 6 fold, respectively[27]. Together, these findings seem to confirm that improving the PD of sorghum grain can substantially improve the FAN content of wort.

Effect of genetic modification and phytase treatment on spent grain phytate content

The phytase reduced the phytate content of the sorghum spent grain substantially (Table III). To determine the full potential of phytase addition, a high level of phytase was added (2 g/kg whole grain). However, while very low, a small amount of phytate still remained in the spent grain of the WTC sorghum+phytase. This may have been because mashing is not the ideal environment for the phytase. Heat and proteases could inactivate it. According to the manufacturer's specification sheet, the phytase should be thermally stable up to 85°C, however, there was no information as to its proteolytic stability.

Notably, the phytase treated maize spent grain did not have significantly lower phytate content compared to the control maize (Table III). This may be due to the fact that the phytate salts in maize are more soluble than in sorghum. It has been found that soaking substantially reduced the phytate content of whole grain maize, but not that of whole grain sorghum[23]. The difference may be related to the fact that in sorghum, phytate is mainly localized in the

aleurone layer, whereas in maize it is mainly in the germ[29]. It has been found that the combination of soaking and boiling reduced the phytate content of sorghum by approximately 23%[37], which may explain the phytate reduction in the WTC and GM3.

Recalculating the phytate contents of the spent grain to the original weight of the grain mashed, revealed large reductions in phytate content (Table III, {}). The WTC+phytase, maize control and maize+phytase all showed a ≥97% reduction in phytate. The phytate content of the WTC and GM 3 were both reduced by 72% despite the fact that the starting phytate contents differed significantly. This suggests that the starting phytate content could have an influence on the phytate reduction during mashing and consequently the final phytate content in the spent grain.

Effect of genetic modification and phytase treatment on wort mineral content

Preliminary results indicated that measuring the mineral content of the wort directly was unreliable. It was therefore decided to measure the mineral content in the dried spent grain to avoid possible mineral removal from the wort during mashing and wort clarification, having an effect on the wort mineral content data. With sorghum, the reduction in phytate content both through genetic modification and phytase addition, resulted in substantial reductions in all minerals in the spent grain, except for zinc (Table III). As the mineral contents of the WTC and GM 3 differed (Table I), the reduction in the mineral contents of the spent grain, expressed as a percentage of the total minerals are considered. The reductions were 33 to 43, 16 to 26, 23 to 30 and 5 to 14 percentage points for Mg, P, Fe and Ca, respectively. The high amounts of zinc in the spent grain were probably due to the fact that the phytase enzyme contained between 0.3 and 0.7% zinc sulphate, as it is added to stabilise the enzyme during storage and processing[6]. In Table III the square brackets show the

percentage of minerals solubilised into the wort. This was calculated by subtracting the mineral content of the spent grain from the total mineral of the grain mashed. With phytase treated and GM sorghum there would have been a substantial increase all the proportion of all the minerals solubilised into the wort. However, there did not seem to be any consistent difference in wort minerals between the maize+phytase and the maize+inactivated phytase, which agrees with the absence of phytase effect on maize phytate contents.

CONCLUSIONS

Addition of exogenous phytase has some potential to increase yeast mineral nutrition in raw sorghum brewing and bioethanol production but not with raw maize. Genetic modification of sorghum to improve PD and reduce phytate content has considerable potential in raw grain brewing and bioethanol production to improve wort quality, especially with regard to yeast FAN and mineral nutrition.

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Table I: Phytate and protein contents, raw and cooked protein digestibility (PD), free amino nitrogen (FAN) and mineral (Fe, Zn, Mg, P) contents (db) of whole grain genetically modified sorghums (GM1 to 3), their null (NC1 to 2) and wild type control (WTC) and maize.

	WTC	NC1	NC2	GM1	GM2	GM3	Maize
*Phytate (mg/100 g)	1245^{C} (69) {12}	1227^{C} (53) {12}	1188^{C} (70) {11}	664^{A} (48) {6}	911^{B} (38) {9}	749^{A} (40) {7}	1366^{D} (128) {14}
*Protein (g/100 g)	10.9^{B} (0.1)	10.9^{B} (0.1)	11.2^{C} (0.1)	10.9^{B} (0.1)	11.5^{C} (0.2)	11.2^{C} (0.1)	7.6^{A} (0.1)
*Raw PD (%)	69.7^{A} (1.3)	77.2^{C} (1.7)	73.8^{B} (0.6)	90.9^{D} (1.5)	90.4^{D} (2.2)	93.0^{D} (0.4)	76.2^{C} (0.0)
*Cooked PD (%)	51.8^{A} (0.4)	57.6^{C} (2.0)	55.1^{B} (0.4)	78.4^{E} (1.7)	80.2^{E} (0.3)	80.8^{E} (0.0)	65.1^{D} (0.1)
*FAN (mg/100 g)	21.5^{A} (2.2)	23.5^{A} (1.5)	23.7^{A} (2.4)	72.6^{B} (6.1)	81.9^{C} (3.6)	91.9^{D} (4.8)	21.8^{A} (1.6)
**Mg (mg/kg)	1452^{C} (15)	1510^{C} (60)	1239^{B} (40)	1621^{D} (60)	1690^{D} (41)	1462^{C} (29)	806^{A} (7)
**P (mg/kg)	3357^{C} (14)	3143^{B} (64)	3370^{C} (83)	3232^{BC} (119)	3142^{B} (126)	3375^{C} (54)	1570^{A} (39)
**Fe (mg/kg)	89^{C} (2)	58^{A} (0)	70^{B} (1)	68^{B} (3)	93^{C} (5)	70^{B} (1)	62^{A} (1)
**Zn (mg/kg)	22^{C} (0)	29^{F} (0)	24^{D} (1)	26^{E} (1)	29^{F} (0)	20^{B} (1)	18^{A} (1)
**Ca (mg/kg)	132^{D} (4)	123^{C} (4)	135^{D} (0)	139^{D} (6)	105^{AB} (4)	108^{B} (4)	98^{A} (4)

^{ABC} - Values with different superscripts in the same row differ significantly ($p \leq 0.05$)

() - Values in parentheses are ±1SD of *n=4 and **n=2

{} - Approximate inorganic phosphorus bound by phytate (1 mole phytate = 6 mole inorganic phosphorus) (µmol /100 g whole grain).

Table II: Effect of genetic modification (improved protein digestibility and reduced phytate content) of sorghum on wort free amino nitrogen (FAN) and hot water extract, obtained by small scale mashing of raw whole grain sorghum.

	WTC	NC1	NC2	GM1	GM2	GM3
FAN (mg/L)	23.2^{A} (3.3)	19.8^{A} (3.3)	18.2^{A} (3.5)	59.9^{B} (8.0)	71.7^{C} (7.1)	82.1^{D} (8.3)
HWE (%)	71.3^{A} (0.3)	72.5^{B} (0.3)	73.3^{B} (0.4)	75.2^{C} (0.8)	75.5^{C} (0.3)	75.0^{C} (0.9)

^{ABC} - Values with different superscripts in the same row differ significantly ($p \leq 0.05$)
() - Values in parentheses are ±1SD of n=4

Table III: Effects of genetic modification (improved protein digestibility and reduced phytate content) of sorghum and of phytase addition, on the spent grain phytate content (db), wort free amino nitrogen (FAN), hot water extract (HWE) and spent grain mineral content (Mg, P, Fe, Zn, Ca) (db), obtained by laboratory scale mashing of raw whole grain sorghum and maize.

	WTC+inactivated phytase	WTC+phytase	GM3	Maize+inactivated phytase	Maize+phytase
Phytate (mg/100 g)	1152^{D} (118) {72}	139^{B} (25) {97}	700^{C} (58) {72}	58^{A} (15) {99}	80^{A} (33) {98}
FAN (mg/L wort)	29.4^{B} (1.4)	38.4^{C} (1.3)	102.3^{D} (6.2)	22.2^{A} (1.1)	27.9^{B} (0.2)
HWE (%)	71.0^{A} (1.1)	73.8^{B} (1.0)	76.5^{C} (1.8)	76.4^{a} (5.4)	73.6^{a} (7.5)
Mg (mg/kg)	3142^{D} (307)[33]	1302^{B} (307)[76]	1947^{C} (324)[66]	1058^{A} (201)[62]	878^{A} (28)[69]
P (mg/kg)	4756^{E} (463)[56]	2163^{C} (211)[82]	3617^{D} (310)[72]	1663^{B} (643)[70]	1169^{A} (9)[78]
Fe (mg/kg)	191^{C} (32)[33]	124^{B} (10)[63]	122^{B} (28)[56]	93^{A} (21)[58]	103^{AB} (19)[53]
Zn (mg/kg)	68^{B} (12)[6]	80^{B} (4)[3]	49^{A} (16)[38]	46^{A} (3)[28]	63^{B} (15)[1]
Ca (mg/kg)	198^{D} (9)[53]	163^{B} (5)[67]	177^{C} (9)[58]	140^{A} (7)[60]	137^{A} (2)[59]

^{ABC} - Values with different superscripts in the same row differ significantly ($p \leq 0.05$)

^{abc} - Values with different superscripts in the same row differ significantly ($p \leq 0.05$), but lower case and upper case in the same row indicate maize and sorghum were analysed separately

() - Values in parentheses are ±1SD of n=4, {} - % reduction in phytate content from total phytate content of originally added grain (spent grain was approximately 30% of original weight of sorghum or maize added) [] - % of total mineral which was solubilised in the wort