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Degradation of Phytate in Composite Bread by Addition of Phytase Releasing Yeast *Pichia kudriavzevii* TY13

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Abstract

Whole sorghum flour is rich in phytate, which strongly binds minerals preventing their absorption by humans. Diets based on whole sorghum has therefore been associated with high prevalence of iron and zinc deficiencies in low-income countries. To improve iron absorption, the phytate content needs to be degraded to a phytate:iron molar ratio <1, and for an improved zinc absorption, a phytate:zinc molar ratio <15. Our study aims at finding conditions which effectively reduce the phytate content in whole flour composite bread. The means that was applied included activation of endogenous sorghum phytase and addition of high-phytase secreting yeast *Pichia kudriavzevii* TY13 at optimal pH in the baking process as well as in a pre-soaking step of the whole sorghum flour. The phytate content was analysed using high performance ion chromatography (HPIC). The initial phytate content in the composite flour was 5.3 µmol/g, that was reduced to 0.85 µmol/g in the composite bread after fermentation for 2 hours at pH 4.0. Adding *P. kudriavzevii* TY13 at the dough mixing stage further reduced the phytate content in the composite bread to 0.30 µmol/g. The optimal pH for the phytate degradation by *P. kudriavzevii* TY13 was between 3.5 and 4.0. Pre-soaking the whole sorghum flour prior to baking, resulted in a composite bread with a phytate content of 0.33 µmol/g that was further reduced with addition of *P. kudriavzevii* TY13 in the soaking step to 0.02 µmol/g, corresponding to 99.6% degradation of the initial phytate content. The phytate:iron molar ratio of the composite bread was then 0.10 and the phytate:zinc molar ratio 0.2, ratios low enough to significantly improve Fe and Zn absorption in humans.

Keywords: Bioprocessing; Bread; Iron; Nutrition; Phytate; Sorghum;

Introduction

Iron deficiency is the most common and widespread nutritional disorders in the world. More than four billion people are estimated to suffer from anaemia and iron deficiency is a major cause mainly affecting children and women in low-income countries [1]. In Mozambique, three-quarters of preschool aged children are anaemic [2] which to a large extent is related to diets of rural populations based on cereals and legumes rich in phytate, and thus have a low iron bioavailability [3]. Due to its high negative net charge (-9 at neutral pH) phytate (*myo*-inositol hexakisphosphate; IP6) has a strong tendency to chelate positive ions such as iron, zinc and calcium. Since humans lack digestive enzymes to degrade phytate, the minerals in such a complex are largely unavailable for uptake in the gut. The phytate-mineral complexes have low solubility at gut conditions, and dissolved IP6 (still chelating minerals) reduces mineral uptake since there is no uptake protein in human intestinal epithelia for IP6 [4,5].

Bread is a non-Mozambican staple food but has become popular and is now widely consumed in Mozambique. However, due to climatic conditions, there is not enough production of wheat to satisfy national food industrial needs and substantial quantities must be imported at a high cost. For this reason, the use of composite flour in bread making has recently been promoted by the Mozambican Government in collaboration with local research institutions. Such composite flours, which consists of wheat flour in combination with locally grown crops such as starchy tubers

(cassava, yam or sweet potato) or other cereals (maize, sorghum or millet) are important sources of minerals such as iron and zinc. However, the mineral availability from these flours are limited due to the presence of phytate [6,7]. In order to significantly improve the iron absorption from cereal based foods, the phytate needs to be almost completely removed or degraded [8,9]. It has been suggested that a phytate:Fe molar ratio of 1:1 and preferably below a 0.4:1 molar ratio needs to be achieved for a 2-fold increase in iron absorption [10]. To achieve an improved zinc bioavailability from cereal based foods the phytate:zinc molar ratio needs to be lower than 15 [11, 12]. A long term and sustainable approach to combat iron deficiency anaemia could therefore be to efficiently apply phytate reducing techniques in the bread making process. Lopez et al. [13] have shown that the phytate content was reduced in wheat sourdough bread by about 62% compared with about 38% in control yeast fermented wheat bread. A prolonged sourdough fermentation of whole rye bread (~12 h) resulted in a 99% degradation of phytate due to a high phytase activity in the rye flour [14].

The use of industrial phytase has been shown to be the most efficient phytate reducing method in several products including, animal feeds [15], bread [16,17], porridge [7], and legume/cereal based foods [18]. There is however still no industrially produced food grade phytase but many different for feed. It would be an advantage if the phytase can come with the yeast especially if the yeast becomes approved for food.

The use of industrial phytase is however expensive for a low-income country like Mozambique. A yeast that could synthesize and release the phytase during bread making would therefore be an alternative to reduce the phytate content of whole sorghum flour, one of the bread alternative flours in the present study. Such a yeast, *Pichia kudriavzevii* TY13, was recently isolated from the Tanzanian fermented cereal gruel togwa [19]. TY13 was in a screening of many togwa-strains selected for high-phytase degrading capacity and low sensitivity for inorganic phosphate (Pi) repression (Pi normally reduces phytase production in yeasts, even at low levels). In addition, TY13 was shown to secret phytase, not only through the cell membrane but also through the cell wall, resulting in non-cell bound free phytase in the surrounding environment [20,21].

To achieve the goal of sufficient phytate degradation without adding industrial phytase (expensive, not yet food grade and allowed for human consumption), the present study addresses the impact of adding the high-phytase yeast *Pichia kudriavzevii* TY13 to either whole sorghum during soaking prior to baking or to a dough of composite flour at mixing stage. The assumption is that the phytate content will be degraded to levels where minerals in the composite bread will be more available for absorption by humans.

Material and methods

Composite flour

The ingredients used for the composite flour were wheat (*Triticum aestivum*) flour with an extraction rate of 72% (Frebago 1050 Bagerivetemjöl, Sweden), cassava (*Manihot esculenta Crantz*) flour and non-tannin white whole sorghum (*Sorghum bicolor*) flour from Inhambane province, respectively Inharrime and Massinga districts in Mozambique (harvested in 2014). Cassava roots were peeled, washed, cut in pieces and sun dried for 4 days. During the drying period the cassava pieces were flipped from time to time to ensure no mould contamination and then milled, packed and stored. The sorghum grains were harvested, and then washed and damaged grains sorted out. After sun drying for 2 days, the grains were milled at 100% extraction rate, and finally packed and stored.

Preparation of yeast culture

The yeast *Pichia kudriavzevii* TY13, isolated from Tanzanian togwa by Hellström et al. [19], was long term stored in 15% glycerol at -80°C and short term stored during experimental periods on YPD plates (yeast extract 10 g, peptone 20 g, glucose 20 g and agar 20 g in 1 L H_2O at 4°C). As precultures, 5 mL YPD in Falcon tubes were inoculated with TY13 from fresh YPD plates and incubated in a rotating carousel for 24 hours at 30°C. The precultures were inoculated into yeast biomass production flasks: a set of 250 mL shake flasks containing 200 mL YPD which were incubated for 24 hours at 30°C under rotary shaking. To collect the yeast biomass, cultures were centrifuged at 4500 x g using a Hereaus Multifuge (Kendro, Osterode, Germany) for 10 minutes, the supernatants were discarded, and the compressed yeast pellets were stored in cold room (+4°C) until used within a few days.

A baker's yeast strain of *Saccharomyces cerevisiae* (Kronjäst, Jästbolaget AB, Sweden) was purchased and used fresh as described in baking procedures below.

Baking procedures

Soaking of whole sorghum flour and phytase induction

White whole sorghum flour was soaked by mixing 50 g of flour with 75 mL of tap water. In this step, also fresh yeast biomass of *Pichia kudriavzevii* TY13 (1 to 3 g pelleted fresh weight) was added to induce phytase production by the yeast. The pH was adjusted within a pH range of 3.5 to 5.0 with added lactic acid solution (20%) and the soaking/time was 2 hours at ambient room temperature.

Bread composition

The bread dough formula consisted of wheat flour (100 g), cassava flour (50 g), whole sorghum flour (50 g), sugar (4 g), salt (2 g), baker`s yeast (4 g *Saccharomyces cerevisiae* from Kronjäst, Jästbolaget AB, Sweden), margarine (6 g), ascorbic acid (0.1 g) and compressed *Pichia kudriavzevii* TY13 yeast biomass from 1 g to 3 g.

Bread making procedure

Bread were prepared by mixing the dry ingredients for 1 minute in a kitchen aid (Artisan, Model 5KSM 150, USA), then mixed with 130 mL of water and the rest of the ingredients, blended for 2 minutes at speed level 2 and 3 minutes at speed level 4. The compressed yeast *Pichia kudriavzevii* TY13 was added during either the mixing stage or during the soaking pre-treatment. After

the mixing stage the dough was covered and left to ferment for 1 h at room temperature. The dough was then weighed, scaled and allowed to ferment for another 1 or 2 hours and thereafter baked for 8 minutes at 250°C in a kitchen oven. During the baking process samples were taken at all stages; after mixing, after 1 to 3 hours of fermentation and at 30 minutes after baking and cooling. After each stage the samples were placed into a flask tube and frozen at -20°C and then lyophilized for three days.

Phytate extraction and analysis

The phytate content was determined using an HPIC method developed by Carlsson et al. [22]. A milled freeze-dried sample of 0.5 g was extracted with 10 mL of 0.5 M HCl for 3 h at room temperature (22°C) under magnetic stirring. The extracts were frozen overnight, thawed and centrifuged at 12000 rpm corresponding to 13400 g for 5 min, and the supernatants were decanted and 50 µL injected and analysed by HPIC with an Omni Pac PAX-100 (4 mm x 250 mm) analytical column and a PAX-100 (4 mm x 50 mm) guard-column (Dionex Corp., Sunnyvale, CA, USA). The phytate was detected and quantified after a post-column reaction with $Fe(NO_3)3x9H_2O$ by measuring the absorption at 290 nm using UV detection (Waters 486, tunable absorbance detector, Massachusetts, USA). All the reagents were of analytical grade (Sigma-Aldrich Co, St. Louis, MO, USA), and de-ionized water was used. The concentrations are presented on dry weight (DW) basis as the mean ± SD µmol/g.

Mineral extraction and analysis

Minerals (Fe and Zn) were determined according to the method by Fredrikson et al. [23] using ion chromatography coupled with UV-vis detection (Waters, Milford, MA). Approximately 250 mg of freeze dried and ground sample was digested to a transparent solution with concentrated nitric and hydrochloric acid in Teflon vessels in an Ethos Plus microwave oven (model Multiwave PRO, Anton Paar Co., USA). After digestion, samples were diluted to 10 mL with de-ionized water and 50 µL was injected on an analytical column CS5A from Dionex Corp. (Sunnyvale, CA). The mobile phase was composed of a pyridine-2.6-dicarboxylic acid (PDCA) acetate solution with a pH 4.3±0.1. After a post column reaction with a 4-(2-pyridylazo)resorcinol solution at pH 10.2 the peaks were detected by UV-vis absorbance at 500 nm.

Determination of dry matter

The dry matter was determined by using a balance device (Torrviktsvåg, Precisa 310M Precisa Gravimetrics AG, Switzerland). Approximately 0.5 g of food material was weighed into the device and heated to a temperature of 80°C until constant weight.

Determination of pH

The pH was measured using, a Mettler Toledo MA 235 pH/Ion Analyser. A 16 g dough piece was weighed and put into a flask tube containing a magnetic stirrer and then 8 g of water was added. The content was stirred for 5 minutes using an electromagnetic plate (Retsch, Rühra mag type Mo12). Finally, the pH was read using the pH-meter probe.

Chemicals

Hydrochloric acid, nitric acid and lactic acid were purchased from Scharlau (Scharlab S.L., Spain), de-ionized water, ethanol 70%, iron nitrate and ascorbic acid from Sigma-Aldrich (Stockholm, Sweden). For yeast culture preparation, peptone was purchased from BactoTM (Thermo Fisher Scientific, UK), glucose from Sigma-Aldrich, yeast extract from Scharlau, and agar from Oxoid AB (Thermo Fisher Scientific, UK).

Statistical analysis

Results are expressed as mean values of at least three replications. Multiple sample comparison of the means and Fisher's least significances (LSD) was used to establish significant differences between treatments. All statistical analysis was carried out with SPSS version 15 software and differences were considered significant at $p<0.05$.

Results

Phytate and minerals content in the composite flours

The phytate, minerals and moisture content of the flours are shown in Table 1. The highest phytate concentration was obtained in whole sorghum flour, with a mean value of 12.0 µmol/g, and the lowest mean value was for cassava flour with 2.4 µmol/g. The highest iron content was found in whole sorghum flour with a mean value of 25.2 µg/g, and the cassava and wheat flours had a similar mean value of 7.7 µg/g. The zinc content varied between 17.3 µg/g in sorghum and 4.7 µg/g in wheat. The phytate molar ratio for iron varied from 17. 0 to 26.7 and for zinc from 26.8 to 46.3.

The moisture content of the flours ranged from 9.9% for whole sorghum flour to 13.0% for cassava flour.

Table 1: Phytate, water, mineral (Fe and Zn) content and phytate:mineral ratios of flours used in the composite bread

Flour type	Moisture (g/g)	Phytate (µmol/g)	Fe (µg/g)	Phytate:Fe molar ratio	Zn (µg/g)	Phytate:Zn molar ratio
Cassava	0.15±0.03	2.4±0.18	7.7±0.17	17	6.0±0.12	26.8
Whole sorghum	0.11±0.03	12.0±0.32	25.2±0.15	26.7	17.3±0.19	45.2
Wheat flour	0.12±0.02	3.3±0.20	7.7±0.09	23.8	4.7±0.17	46.3

The effect of adding *P. kudriavzevii* TY13 during bread making

The yeast *Pichia kudriavzevii* TY13, isolated from fermented togwa and selected for exceptional properties with respect to phytate degradation, was added to the soaking step at pH 4.0 of whole sorghum flour. Phytate concentration was assessed at 30 minutes intervals (Figure 1). The yeast contribution to phytate degradation was pronounced and rapid. Already after 30 minutes, the phytate content was in the presence of TY13 significantly lower ($p<0.05$) than in the control; 6.4 µmol/g and 8.9 µmol/g, respectively. This corresponds to 46% (TY13) and 25% (control) phytate reduction. Following the early degradation during the first 30 minutes, the phytate level continued to decrease during soaking with TY13, however, at a slower rate, whereas in the control soaking (no yeast), no further degradation took place. After 2 hours soaking, only 33% of the initial sorghum phytate remained with added TY13 (3.7 µmol/g) as compared with 76% in the control (8.6 µmol/g).

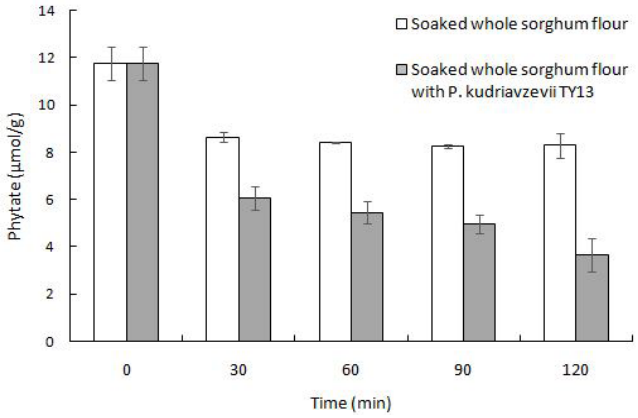

Figure 1: Phytate degradation in whole sorghum flour soaked for 2 hours at pH 4.0 with and without 2.5 g of *P. kudriavzevii* TY13

After mixing the soaked whole sorghum flour into a composite flour dough, which also includes common commercial baker`s yeast, the phytate degradation during a 2 hours fermentation stage was assessed every 30 minutes (Figure 2). In the dough with added *P. kudriavzevii* TY13 the final phytate content was 0.08 µmol/g, as compared with 0.53 µmol/g in the control dough without TY13. This means, that an almost complete degradation of the initial phytate content (99.3%) was obtained with added TY13 in the soaking step, compared with 95% degradation in the control. If looking at the fermentation phase only, it was again clear that TY13 contributed to an increased phytate degradation, from 2.1 µmol/g to 0.08 µmol/g.

Phytate degradation as a function of pH, amount of *Pichia kudriavzevii* TY13 and fermentation time

Soaking whole sorghum flour at different pH values from 3.5 to pH 5.0 (Figure 3) with added *P. kudriavzevii* TY13 prior to mixing into composite flour dough showed a slightly higher phytate degradation in the bread at pH 3.5 and 4.0. As a result, the phytate content was almost completely degraded compared with about 96% degradation in the composite bread with whole sorghum soaked at pH 4.5 and 5.0. The effect of adding different amounts of *P. kudriavzevii* TY13 during the soaking of whole sorghum flour at pH 4.0 resulted in a significantly ($p<0.05$) lower phytate content in the composite flour bread (0.04 µmol/g) when higher amounts of *P. kudriavzevii* TY13 (3 g) was added, compared with 0.31 µmol/g when 1 g was added, see Figure 4.

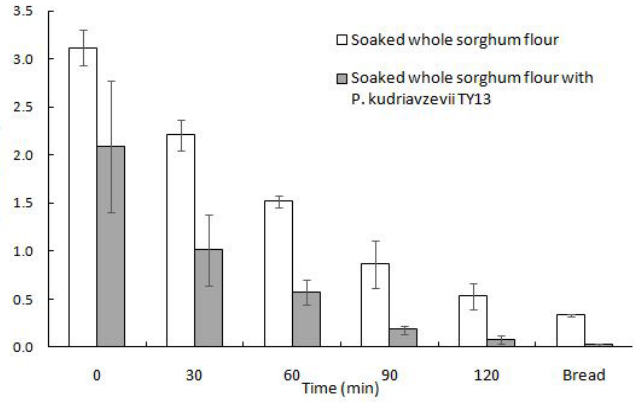

Figure 2: Phytate degradation during fermentation stage of composite flour dough with whole sorghum flour soaked for 2 hours at pH 4.0 with and without 2.5 g of *P. kudriavzevii* TY13

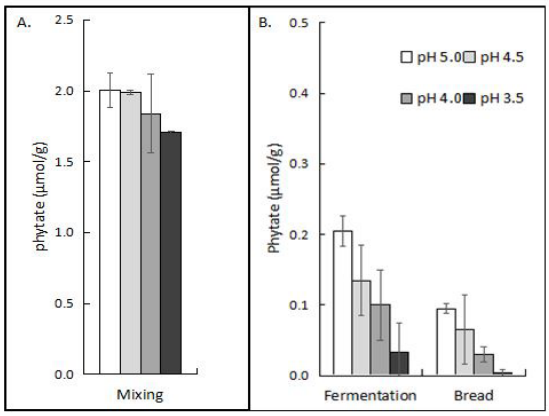

Figure 3-A and B: Phytate degradation during baking of composite flour bread with whole sorghum flour soaked at different pH values for 2 hours with 2.5 g of *P. kudriavzevii* TY13 and fermented for 2 hours

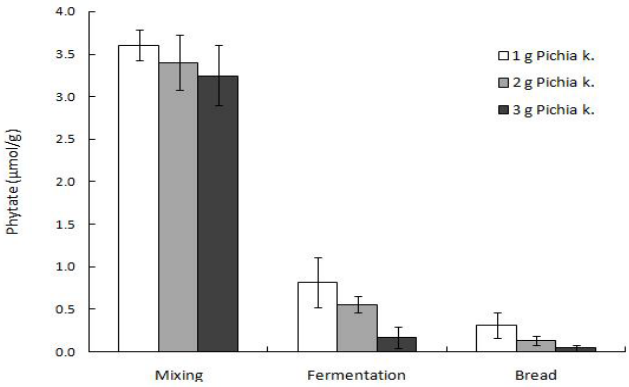

Figure 4: Phytate degradation during baking of composite flour bread with soaked whole sorghum flour for 2 hours at pH 4.0 with different amounts of *P. kudriavzevii* TY13and fermented for 2 hours

Table 2 shows that the phytate content was significantly reduced in the composite bread with pH 4.0 adjusted (0.85 µmol/g) compared with the initial amount in the composite flour (5.3 µmol/g). Adding 2.5 g of *P. kudriavzevii* TY13 at the mixing stage and pH adjustment to 4.0 resulted in a further degradation of the phytate content to 0.30 µmol/g corresponding to a 94% degradation of the initial phytate content. Soaking whole sorghum flour for two hours at pH 4.0 with added *P. kudriavzevii* TY13 prior to mixing the dough promoted an almost complete reduction of the phytate content to 0.02 µmol/g in the composite bread. A consequence of the extensive phytate degradation is a lowered ratio of phytate (IP6) to Fe, and Zn, respectively. The IP6:Fe ratio decreased from 30 to 0.1 and the IP6:Zn ratio from 41 to 0.2 (Table 2).

Table 3 shows the effect of adding *P. kudriavzevii* TY13 during the mixing stage at either pH 3.5 or 4.0, and dough fermentation for 1 to 3 hours. Increasing the fermentation time to 3 hours resulted in significantly lower phytate content of 0.09 µmol/g in the composite bread at pH 4.0, compared with 1.5 µmol/g in the corresponding bread at pH 3.5.

Table 2: Phytate (IP_6) and mineral (Fe and Zn) content, and phytate:mineral molar ratios of composite flour breads baked with soaked (2 hrs at ambient temperature and pH 4.0) or non-soaked whole sorghum flour prior to baking, and in the presence or absence of the yeast *P. kudriavzevii* TY13 (*P.k*). The non-baked initial composite flour mix is included as reference before treatments.

Composite bread type	Phytate content (µmol/g)	Fe content (µmol/g)	Phytate:Fe molar ratio	Zn content (µmol/g)	Phytate:Zn molar ratio
Composite flour[1)]	5.26±0.20	0.24±0.02	29.8	0.13	40.8
Composite flour bread, pH 4.0 at mixing	0.85±0.18	0.23±0.02	3.7	0.12	7.1
Composite flour bread, pH 4.0 at mixing+ 2.5 g yeast *P.k*	0.30±0.01	0.23±0.01	1.3	0.12	2.5
Composite flour bread with soaked sorghum, pH 4.0	0.33±0.01	0.20±0.02	1.7	0.11	3.0
Composite flour bread with soaked sorghum, pH 4.0 + 2.5 g yeast *P.k*.	0.02±0.02	0.20±0.01	0.1	0.11	0.18

[1)]100 g wheat flour, 50 g cassava flour and 50 g whole sorghum flour.

Table 3: Phytate content in composite flour bread after 1 to 3 hours fermentation with 2.5 g of *P. kudriavzevii* TY13 added at mixing stage and pH adjusted to 3.5 or 4.0

Time (hours)	pH 3.5 (µmol/g)			pH 4.0 (µmol/g)		
	Mixing	Fermentation	C. bread	Mixing	Fermentation	C. bread
1	4.2±0.22	3.2±0.13	2.9±0.04	2.5±0.35	1.2±0.07	0.80±0.25
2	3.7±0.18	1.8±0.22	1.8±0.15	3.2±0.15	0.6±0.11	0.30±0.03
3	3.9±0.41	1.5±0.25	1.5±0.27	2.6±0.23	0.1±0.03	0.09±0.01

Discussion

Phytate and minerals in the composite flours

Due to the inclusion of the bran fraction in the whole sorghum flour, the phytate content was high (12.0 µmol/g). Since the main function of phytate in plant seeds is storage of phosphate and chelated minerals needed by the germinating seed, it follows that high phytate coincides with high mineral content. The sorghum flour in our study contained more than three times higher iron content as compared with wheat and cassava flours. A practical consequence of the high phytate-high mineral connection is that in a raw material for food fermentation, it may be an advantage with a high phytate content, provided an efficient phytase producing strain is present which degrades the phytate and thereby releases the minerals. To investigate whether a combination of high phytate flour and high phytase strain would lead to high level of accessible unbound Fe and Zn, was the main target of the present work.

The wheat and cassava flours used in this study had low iron and zinc content as a result of a low extraction rate for the wheat flour (not fortified) and non-inclusion of peels for the cassava roots during flour preparation. Similar phytate content has been reported for wheat flour (4.1 µmol/g) and cassava flour (2.9 µmol/g) by Lazarte et al. [24] and for non-tannin sorghum flour (12.4 µmol/g) by Svanberg et al. [25].

The effect of adding *P. kudriavzevii* TY13 during bread making

The addition of *P. kudriavzevii* TY13 during the mixing stage, defined as the five minutes period with mechanical blending of dry and liquid ingredients to form the dough, had a significant positive effect on phytate degradation, about 47% of the phytate content in the composite flour. The hydration of the flours and energy input during the mixing stage to provide gluten structure causes phytate to come into contact with the enzyme phytase leading to phytate degradation. Further degradation up to 89% was obtained during the fermentation stage (2 hours) and to a final degradation of 94% in the composite bread. The fermentation stage thus provided suitable conditions for the phytase from *P. kudriavzevii* TY13 as well as the endogenous phytase from the composite flour, resulting in high phytate degradation. The addition of *P. kudriavzevii* TY13 at the mixing stage resulted in a significantly lower phytate content in the composite bread (0.3 μmol/g) compared with that of the control composite bread (0.85 μmol/g). However, still the phytate:iron molar ratio was above 1 (1.3) implying a low iron bioavailability [10]. An increased phytate degradation (89%) in wheat flour bread with 5% wheat bran was also observed by Haros et al. [14] after the addition of fungal phytase from *A. niger. Porres* et al. [26] obtained a similar high phytate reduction of 85% in whole wheat bread. However, these studies differed in one important way: free phytase enzyme was added in the Haros and Porres studies whereas in our present work, a live phytase producing organism was added. The remarkable degradation when including TY13 shows that the yeast phytase enzyme, produced inside the yeast cells, reached the substrate in the dough, demonstrating the strong phytase secretion capacity by TY13.

Soaking the whole sorghum at pH 4.0 prior baking showed to be an efficient way of reducing phytate content, due to the activity of endogenous phytase present in the sorghum flour. Egli et al. [27] used the same method of reducing the phytate content in complementary foods for children by soaking at optimal pH and temperature with addition of whole cereal grains as the phytase source. Soaking whole sorghum flour without *P. kudriavzevii* TY13 for 2 hours at pH 4.0 had the same effect as adding *P. kudriavzevii* TY13 during the mixing stage at pH 4.0 and fermentation for 2 hours. Soaking whole sorghum flour at pH 4.0 with 2.5 g of *P. kudriavzevii* TY13 was tested with the aim to further degrade the phytate content prior to the baking of the composite bread. The phytate content was then about 60% lower after 2 hours soaking compared to the whole sorghum soaked at the same conditions without *P. kudriavzevii* TY13, see Figure 1. Moreover, the phytate degradation during soaking with TY13 present continued from time 30 min at a steady rate throughout the whole soaking step which lasted for 120 minutes. If the soaking step was prolonged, and we assume degradation at unchanged rate, the phytate level would approach zero after another 60 minutes soaking. Hence, three hours soaking with T13 should be tried. The control soaking (no TY13) on the other hand, resulted in initial degradation, but after 30 minutes no further degradation was detected.

The composite flour bread prepared with soaked sorghum flour with added *P. kudriavzevii* TY13 resulted in a bread with a phytate:iron molar ratio of 0.1 and a phytate:zinc ratio of 0.18, which is by far below the suggested threshold ratios for improved iron and zinc absorption in humans [10, 11, 12]. The composite flour bread (Table 2) prepared with soaked sorghum flour without TY13 had about 94% phytate reduction. However, soaking whole sorghum flour in the presence of *P. kudriavzevii* TY13 resulted in an almost complete degradation of the phytate content in the composite bread. This shows that the yeast was actively synthesizing and secreting phytase during the soaking stage.

Phytate degradation as a function of pH, amount of Pichia and fermentation time

The adjustment of the pH between 3.5 and 5.0 during the soaking pre-treatment with addition of lactic acid was included to determine the optimal pH for phytate degradation by the exogenous phytase from *P. kudriavzevii* TY13. The phytate degradation was slightly higher in doughs with pH adjusted to 4.0 and 3.5, respectively, compared with doughs with higher pH. Most likely the phytase protein has a stable conformation throughout the entire pH range and with optimal activity at pH 4.0 and 3.5.

The composite bread with whole sorghum flour soaked for 2 hours with different amounts of *P. kudriavzevii* TY13 had a lower phytate content with higher amount of yeast added. With 3 g *P. kudriavzevii* TY13 the phytate content was almost zero, with a degradation of >99%, due to the increased amount of enzyme for phytate degradation. The almost complete phytate degradation resulted in a phytate to iron molar ratio less than 0.2 implying a high iron absorption from the composite bread [10].

The effect of increasing the fermentation time from 1 to 3 hours gave a similar result. At both pH 4.0 and pH 3.5 the degradation of phytate was increased with longer fermentation time in line with the results reported by [28]. Since TY13 in our prior work showed outstanding properties, it was further improved by non-GMO UV-mutagenesis and selection [29]. This yielded additionally increased phytase production as compared with the parent wild-type TY13. The natural mutant is expected to reduce the fermentation time and amount of yeast (discussed above) needed to reach full phytate degradation, as will be assessed in coming studies.

Conclusions

Our study demonstrates that careful selection of strain and process for production of bread can result in almost complete degradation of phytate. By combining conventional baker's yeast with non-conventional *Pichia kudriavzevii* TY13 (originally found in fermented cereal-based togwa) gas-production was maintained and exceptional phytase activity introduced. Addition of TY13 during the mixing stage increased the phytate degradation in the composite bread, but still the molar ratio phytate:iron was above 1 (1.3) implying a low iron bioavailability. This degradation was approximately the same as by soaking whole sorghum flour (without yeast) at pH 4.0 prior to mixing. However, the composite bread prepared with soaked sorghum flour with added *Pichia kudriavzevii* TY13, resulted in remarkable phytate degradation, and

a bread with a phytate:iron molar ratio of 0.1 and a phytate:zinc molar ratio less than 0.2 implying both improved iron and zinc absorption in humans. An increased fermentation time as well as higher amounts of added *Pichia kudriavzevii* TY13 resulted in additionally improved degradation of phytate in the composite bread.

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Conflicts of Interest

The authors do not have any conflicting interests.

Ethical Statements

This study does not involve any human or animal testing.

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