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# Degradation of phytate by *Pichia kudriavzevii* TY13 and *Hanseniaspora guilliermondii* TY14 in Tanzanian togwa

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ABSTRACT

The fermented cereal-based gruel togwa is used as weaning food for children in Tanzania. Togwa is rich in minerals but these are often not available for uptake in the human intestine due to natural inhibitors, such as phytate ($IP_6$). The yeasts *Pichia kudriavzevii* TY13, *Hanseniaspora guilliermondii* TY14 and TY20, isolated from Tanzanian togwa, and selected for high phytase activity in complex yeast medium YPD, were now studied regarding their ability to degrade $IP_6$ in maize-based model togwa. A modified constitutively high-phytase producing *Saccharomyces cerevisiae* BY80 and commercial *Aspergillus ficuum* phytase were included for comparison. In addition, a strain of *Lactobacillus plantarum* was included in the model-togwa set-up.
All yeasts in the study grew and reached final cell density 1.5–2 log units higher than the start value. *S. cerevisiae* BY80 degraded 85% of the $IP_6$ in 48 h; the same degradation level as with *A. ficuum* phytase (89%). Of the togwa-isolated yeasts, *P. kudriavzevii* TY13 and *H. guilliermondii* TY14 showed strong phytate degradation in the model-togwa; 95% or more of the initial $IP_6$ was degraded after 48 h. This corresponds to a remaining level of 0.4 and 0.3 μmol $IP_6$/g dw. Co-inoculation with *L. plantarum* did not increase $IP_6$ degradation. Moreover, fermentation with *P. kudriavzevii* TY13 yielded a successive increase in inorganic phosphate ($P_i$), from 0.7 to 5.4 mM, suggesting a phytase production in TY13 which is fairly insensitive to $P_i$ repression. The study shows that phytate in a model togwa is available for yeast phytase enzymes, and addresses the importance of strain selection for effectively degrading the phytate. Certain yeasts originating from togwa seem to have developed a natural high phytase production, and *P. kudriavzevii* TY13 and *H. guilliermondii* TY14 seem particularly well adapted to phytate degradation in togwa, and is our choice for further studies and strain improvement.


## 1. Introduction

Togwa is a fermented gruel prepared from cereals such as maize and sorghum or the root tuber of cassava. Togwa is a traditional food of Tanzania. The ground cereal is boiled to a thick porridge and flour made from germinated cereals, so called power flour, is added. Addition of power flour liquefies the porridge, mainly by amylase activity and contributes with a rich microflora of especially yeasts and lactic acid bacteria. The slurry is subsequently let to ferment over night. Togwa is used as a weaning food for the younger children or by adults as a refreshment drink (Lorri and Svanberg, 1995). Togwa is cheap to produce and contains, in addition to a high energy content, important minerals *e.g.* iron and zinc. Consumption of togwa may thus decrease the problems associated with mineral deficiencies, such as iron deficiency anemia (IDA) in developing countries. However, in spite of the fact that togwa is rich in minerals, the availability of these for uptake in the intestine may be low, mainly due to presence of different mineral binding compounds, especially phytate.

Phytate, *myo*-inositol hexa*kis*phosphate ($IP_6$) consists of a *myo*-inositol ring with one phosphate group esterified on each carbon atom. Phytate is used by plants as a storage molecule and is therefore present in high quantities in seeds. Phytate is negatively charged over a wide pH range and therefore readily chelates minerals such as $Fe^{2+}/Fe^{3+}$, $Zn^{2+}$ and $Ca^{2+}$. Enzymes degrading phytate – phytases – are not part of the human digestive system. Consequently, phytate in food reduces mineral uptake in the human intestine which for many population groups, especially when cereals dominate the food pattern, leads to mineral deficiencies (for a review see Cheryan (1980)). Endogenous phytases are however present in seeds of for instance cereals and may be activated during traditional processing methods such as soaking, germination and fermentation (for a review see Kumar et al. (2010)). This has however shown to often be insufficient for significantly improved mineral bioavailability. Since phytases are produced not only by plants but also by microorganisms, including yeasts (Sandberg and Andlid, 2002), the microorganisms present in togwa may contribute to phytate degradation during fermentation. A high-phytase yeast adapted to grow well in togwa would potentially improve the mineral bioavailability.

In a previous study (Hellström et al., 2010), yeasts were isolated from Tanzanian togwa. The isolated yeasts were thereafter screened for phytase activity and it was shown that a strain of *Pichia kudriavzevii*,

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TY13, and two strains of *Hanseniaspora guilliermondii*, TY14 and TY20, exhibited high phytate degrading capacity in complex yeast medium (YPD) supplemented with $IP_6$. The ability of the selected strains to degrade $IP_6$ in YPD was pronounced. In the more complex matrix of togwa, however, the ability of yeasts to degrade $IP_6$ may be low or absent. In cereal seeds $IP_6$ is known to be present in aggregates with minerals and proteins which may lead to low accessibility of the yeast phytases for $IP_6$. In addition, inorganic phosphate at various levels in togwa may repress production of yeast phytases (Mouillon and Persson, 2006; Oshima, 1997). A successful starter strain for improved mineral availability in togwa must perform well in the real food environment. In addition, real togwa is a mixed fermentation of lactic acid bacteria (LAB) and yeasts, implying that the yeast must perform well also in the changed environment caused by LAB activities, such as lowered pH and production of organic acid.

In the present work we investigate degradation of phytate by selected yeasts in a fermented complex food matrix — a maize-based model togwa. The yeasts had been selected based on their ability to degrade $IP_6$ in complex yeast medium (YPD supplemented with $IP_6$). A genetically modified high phytase yeast was used for comparison. We also studied the impact of lactic acid bacteria on the yeast fermentation and phytate degradation.

## 2. Material and methods

### 2.1. Strains and media

*Saccharomyces cerevisiae* BY80 (Euroscarf Acc. No. Y01692), previously shown to produce phytase independently of the medium phosphate concentration (Veide and Andlid, 2006), was used alone and in mixed fermentation with *Lactobacillus plantarum subsp. Argentoratensis* CCUG 50788 isolated from Nigerian fermented millet (baba), to establish if phytate is available for degradation by yeast in a maize-based model togwa.

The togwa-isolated yeasts *S. cerevisiae* TY12, *P. kudriavzevii* TY13 (previously named *Issatchenkia orientalis* TY13), *H. guilliermondii* TY14 and *H. guilliermondii* TY20 were used in a second fermentation experiment. These species have previously been identified by Hellström et al. (2010). In the mixed fermentations with yeast and lactic acid bacteria, *Lactobacillus plantarum subsp. Argentoratensis* CCUG 50788 was used in combination with *S. cerevisiae* TY12 and *P. kudriavzevii* TY13.

The yeast precultures were grown in Yeast Peptone Dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose), pH 5.8, over night at 220 rpm, 30 °C, and LAB precultures were grown in MRS medium (Difco), pH 6.2 over night at 37 °C and 220 rpm. All precultures were washed twice in 0.9% NaCl before being used.

### 2.2. Model-togwa fermentation with high-phytase S. cerevisiae BY80

The preparation of maize-based model togwa has previously been described by Hjortmo et al. (2008). Briefly, 10% milled maize flour was mixed with 90% tap water (w/w). The maize, (La plata variety) purchased from Lantmännen, Sweden, was milled using a Retsch ZM-1 mill with a 0.5 sieve. The slurry was cooked for 20 min resulting in a thick porridge, and the water loss was weighed and corrected by adding sterile tap water. To sterile Erlenmeyer flasks, 150 g of porridge was added and 4.5 mg α-amylase from *Bacillus subtilis* (Sigma, 110 U/mg solid) and 3 mg β-amylase from barley (Sigma, 19.9 U/mg solid) was used to compensate for the missing amylases known to be abundant in power flour. The amylase activity breaks the gelatinized starch and releases the water; the porridge becomes a liquid slurry again. Since *S. cerevisiae* BY80, the constitutive phytase producing strain used in some experiments, is auxotrophic for uracil, histidine, leucine and methionine, these growth requirements were added to all Erlenmayer flasks to a final concentration of 120 mg/L.

Five Erlenmeyer flasks in triplicates were inoculated to the model togwa with following inoculums: *Lactobacillus plantarum* ($OD_{600} = 0.1$), *L. plantarum* ($OD_{600} = 0.1$) plus *S. cerevisiae* strain BY80 ($OD_{600} = 0.25$), *S. cerevisiae* strain BY80 alone ($OD_{600} = 0.25$) at pH 3.8, commercial phytase derived from *Aspergillus ficuum* (21.4 mg; 3.5 U/mg solid), and an uninoculated control with chloramphenicol (250 mg/kg) and cycloheximide (200 mg/kg) to suppress yeast and bacteria, respectively. The model togwa was fermented during 48 h at 220 rpm, 30 °C and samples (10 mL) for $IP_6$ analysis, pH determination and CFU were withdrawn at time points 0 (immediately after inoculation), 24 and 48 h. Samples for $IP_6$ analysis were immediately put in the freezer at −80 °C. The pH was determined by using a pH meter (Radiometer analytical PHM210, Villeurban Cedex, France) with a Hamilton double pore electrode (Hamilton double pore, Bonaduz, Switzerland) and mean values were plotted with standard deviation. For CFU determinations, samples were plated from three different dilutions in duplicate on YPD agar plates supplemented with chloramphenicol (200 mg/L) and on MRS agar plates supplemented with cycloheximide (200 mg/L) for yeast and LAB enumeration respectively. Plates were incubated at 30 °C until colonies became visible. Colonies were counted and mean values plotted with standard deviation.

### 2.3. Model-togwa fermentation with yeasts isolated from Tanzanian togwa

The fermentation with yeasts isolated from Tanzanian togwa followed the same set-up as described in Section 2.2, with minor changes: To sterile Erlenmeyer flasks, 100 g of porridge was added together with 3 mg α-amylase from *Bacillus subtilis* (Sigma, 110 U/mg solid) and 1 mg amyloglucosidase from *Aspergillus niger* (Sigma, 57.7 U/mg solid). Yeast and LAB were inoculated in triplicates to the model togwa at level corresponding to $OD_{600}$ of 0.25 (yeasts) and to $OD_{600}$ 0.1 (LAB). Flasks inoculated with yeast only were supplemented with chloramphenicol (200 mg/kg), flasks for LAB only with cycloheximide (200 mg/kg). The uninoculated control flasks only containing gruel was supplemented with chloramphenicol (200 mg/kg) and cycloheximide (200 mg/kg). The experiment was carried out using two different batches of maize gruel: one for yeast fermentations (A) and a second for mixed yeast-LAB fermentations (B). From each batch an uninoculated control flask was included in parallel with the fermentation.

Samples (total volume of 10 mL) for $IP_6$ and $P_i$ were withdrawn at time points 0, 12, 24, 36, and 48 h. The samples were immediately put in the freezer at −80 °C. In mixed fermentations samples containing LAB and yeast, pH was measured at intervals.

### 2.4. $IP_6$ and $P_i$ extraction

Prior to extraction of phytic acid ($IP_6$) and $P_i$, deep frozen samples (−80 °C) from indigenous fermented togwa were lyophilized using a Heto Drywinner DW6-55. For $IP_6$ extraction, triplicates of ~0.25 g homogenized freeze dried togwa were extracted with 5 mL 0.5 M HCl for 3 h at 20 °C under magnetic stirring. For $P_i$ extraction, triplicates of ~0.25 g homogenized freeze dried togwa were extracted with 5 mL 0.1 M HCl for 1 h at 20 °C under magnetic stirring. The extracts were frozen in −20 °C until analysis. Before analysis the extracts were thawed and 1 mL was centrifuged at 16,000×g for 5 min using an Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany) to obtain clear supernatants. The clear supernatants were subsequently used for analysis.

### 2.5. $IP_6$ analysis

The method for $IP_6$ analysis has been described in detail by Carlsson et al. (2001). Briefly, the chromatograph consisted of a biocompatible (PEEK) HPLC pump (Waters model 626) equipped with a PA-100 guard column (Dionex Corp., Sunnyvale CA) and an HPIC CarboPac PA-100 analytical column (Dionex Corp.). The sample