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Dephytinization of seed coat matter of finger millet (*Eleusine coracana*) by *Lactobacillus pentosus* CFR3 to improve zinc bioavailability

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ABSTRACT

Finger millet seed coat matter (SCM) is a rich source of dietary fiber, calcium, iron and zinc (Zn). However, the bioavailability of minerals especially Zn is limited as it occurs as insoluble complexes with phytate. This study envisages the potential of phytase-active *Lactobacillus pentosus* CFR3 to dephytinize SCM derived from native (non-processed), malted and hydrothermally treated finger millets with an expectation to improve its Zn bioavailability. After 24 h fermentation, the phytate in native, malted and hydrothermally treated SCM reduced to 56.70%, 66.65% and 87.85% respectively. Correspondingly, Phytate/Zn molar ratios decreased to 18.20, 19.20 and 22.32 indicating improved Zn bioavailability. Also, Zn bioaccessibility from native, malted and hydrothermally treated SCM increased to 28.40%, 34.57% and 12.10% as determined by *in vitro* dialyzability experiments following 24 h fermentation. Therefore, fermentation of SCM with *L. pentosus* CFR3 represents a safe and feasible solution to counteract the effects of phytate on Zn absorption.

1. Introduction

The seed coat matter (SCM) from native, malted and hydrothermally treated finger millet is a byproduct of the millet processing industry. It contains significant proportion of minerals, dietary fiber and phytochemicals. In recent years, SCM has been utilized as adjuncts in cereal foods to obtain products with high levels of dietary fiber, calcium (Ca), iron (Fe) and zinc (Zn) (Krishnan, Dharmaraj, & Malleshi, 2012; Krishnan, Dharmaraj, Manohar, & Malleshi, 2011). However, the bioavailability of minerals from SCM may be limited due to the presence of phytate (*myo*-inositol hexakisphosphate) (Poutanen, Flander, & Katina, 2009). The phosphate groups of phytate form stable complexes with dietary cations, thus hindering their bioavailability (Liang, Han, Nout, & Hamer, 2008).

Zinc bioavailability is most severely affected by dietary phytate (Lopez, Leenhardt, Coudray, & Remesy, 2002; Lönnerdal, 2002). Several studies have revealed a positive correlation between phytate-rich diets and Zn deficiency in humans (Lönnerdal, 2002). Clinical manifestations of Zn deficiency include growth retardation, hypogonadism in males, rough skin, impaired immunity, neurosensory disorder and cognitive impairment (Lönnerdal, 2002; Prasad, 2012). Therefore, optimization of Zn bioavailability is of significance.

Enzymatic phytate hydrolysis is a promising strategy to improve Zn absorption in humans (Fischer, Egli, Aeberli, Hurrell, & Meile, 2014). Food ingredients have been successfully dephytinized with phytate-degrading enzymes (phytases) (Haros, Rosell, & Benedito, 2001; Marklinder, Larsson, Fredlund, & Sandberg, 1995; Sandberg, Hulthen, & Turk, 1996). Several researchers have documented the presence of phytases in Lactic Acid Bacteria (LAB) (De Angelis et al., 2003; Palacios, Haros, Rosell, & Sanz, 2005; Raghavendra & Halami, 2009; Zamudio, González, & Medina, 2001). This opens up possibilities towards fermentative reduction of phytate with specific phytase-active LAB. The application of LAB to dephytinize food ingredients is safe due to the GRAS (Generally Regarded as Safe) status conferred on this bacterial group. Moreover, it is cheaper compared to the use of purified phytase.

Elimination of phytate from byproducts of millet milling with specific LAB is a relatively unexplored area of research. Therefore, the current investigation focused on the dephytinization of SCM from native, malted and hydrothermally treated finger millets by *Lactobacillus pentosus* CFR3 as a means to improve Zn bioacessibility.

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2. Materials and methods

2.1. Sample preparation

Finger millet (GPU 28 Variety) was procured from University of Agriculture Sciences, Bangalore, Karnataka, India. Native SCM (NSCM) was prepared as described by Chethan and Malleshi (2007). Malted SCM (MSCM) was obtained following the protocol given by Malleshi and Desikachar (1979), while hydrothermally treated SCM (HTSCM) was prepared as described by Krishnan et al. (2012). The SCM samples were finely powdered in a laboratory grinder, passed through a 60 BSS mesh and stored at 4 °C until analysis.

2.2. Materials

Dialysis membrane (10 kDa cut-off), hydrogen peroxide (H_2O_2; 30%), lanthanum chloride ($LaCl_3$), lysozyme, mineral standards (Ca and Zn), nitric acid (HNO_3; 90%), sodium phytate and wheat phytase were from Sigma (St Louis, MO, USA). Microbiological media components, pancreatin, pepsin and ox-bile were purchased from Hi Media (Mumbai, India). All other reagents of analytical grade were obtained from Sisco Research Laboratories (India). Deionized water from Millipore system and acid-washed glassware were used for the entire study.

2.3. Bacterial strain

Lactobacillus pentosus CFR3 which produced phytate-degrading enzyme was used in the study (Amritha, Halami, & Venkateswaran, 2017). The culture was maintained at −20 °C in MRS (De Man, Ragosa and Sharpe) broth with 40% (v/v) glycerol. The strain was activated from the glycerol stock by subculturing in MRS broth twice.

2.4. Preparation of crude phytase (CP) from L. pentosus CFR3

Lactobacillus pentosus CFR3 was propagated in modified MRS broth (pH 6.5 ± 0.2) under static condition for 8 h at 37 °C. Modified MRS broth was prepared by replacing glucose with maltose. Also, sodium di-hydrogen phosphate (KH_2PO_4) was replaced with sodium phytate (0.65 g/L) and 0.1 M 3-[N-morpholino] propanesulfonic acid (MOPS). The cell-associated phytase from the culture was extracted with lysozyme and NaCl (Amritha et al., 2017).

2.5. Dephytinization of SCM with CP of L. pentosus CFR3

The SCM samples (0.5 g) were suspended in 2 mL of CP (~8 mg protein) and incubated in a shaking water bath at 50 °C for 120 min. Similarly, samples suspended in 0.25% (w/v) wheat phytase (0.01–0.04 U/mg) in sodium acetate buffer (0.1 M, pH 5.5) and sodium acetate buffer constituted the positive and negative controls respectively. Post incubation; samples were freeze-dried (Freeze Zone Freeze dry System, Labconco, USA) and subjected to phytate extraction and estimation.

2.6. Inoculum preparation and fermentation of SCM with L. pentosus CFR3

Lactic fermentation of SCM were carried out based on the procedure given by Reale, Konietzny, Coppola, Sorrentino, and Greiner (2007). *Lactobacillus pentosus* CFR3 was propagated in MRS for 16 ± 2 h at 37 °C. Cells were harvested (7000 x g, 10 min, 4 °C), washed and resuspended in an equal volume of saline to obtain a cell suspension (10^8 CFU/mL). The SCM samples were autoclaved (15 psi, 121 °C, 30 min) and subsequently suspended in sterile water under aseptic conditions to obtain a 10% (w/v) suspension. The SCM-based suspensions were inoculated with saline suspension of the producer strain and incubated at 37 °C in a shaker incubator (New Brunswick, USA) at 150 rpm. Uninoculated control was run in parallel.

2.7. Determination of viable cell counts and pH

Microbial cell counts were obtained by pour plating on MRS agar. The plates were incubated at 37 °C for 24–48 h. Changes in pH of the fermenting SCM was noted with a pH meter (Eutech Instruments, Singapore).

2.8. Chemical assays

2.8.1. Phytic acid

Phytate was extracted and estimated from SCM samples as described by Gao et al. (2007) with sodium phytate as the standard.

2.8.2. Total zinc and calcium

Total minerals (Ca and Zn) were estimated according to Kumari and Platel (2016) with minor modifications. Briefly, samples (0.1 g) were wet digested for 24 h with 0.5 mL HNO_3 and 0.3 mL H_2O_2, followed by gradual refluxing in a hot plate. Subsequently, the volume was made up to 10 mL with 0.5% HNO_3. The samples were filtered through 0.45 μm syringe filters (Millex HV, Millipore) and appropriately diluted before estimation using Flame Atomic Absorption Spectrometry (FAAS; Shimadzu AAF-6701). For Ca estimation, the samples and standards were spiked with 0.1% (w/v) $LaCl_3$ to prevent phosphorous interference.

2.8.3. In vitro dialyzable Zn

In vitro dialyzable Zn was determined by equilibrium dialysis method as described by Luten et al. (1996) with modifications. In short, samples (10 g) were suspended in 80 mL water and pH was adjusted to 2.0 with 6.0 M HCl. Subsequently, pepsin solution (3 mL of 16 g/100 mL pepsin in 0.1 M HCl) was added to the mixture and the volume was raised to 100 mL. The mixture was incubated in a shaker at 37 °C for 2 h at 110 rpm to obtain gastric digests. The gastric digests were frozen for 30 min to stop the reaction. Titratable acidity (TTA) was measured in an aliquot (20 mL) of the gastric digest containing 5 mL of pancreatin-bile mix (5 g pancreatin +25 g ox-bile in 1 L of 0.1 M sodium bicarbonate ($NaHCO_3$)) by adjusting the pH to 7.5 with 0.2 M sodium hydroxide (NaOH). An aliquot of gastric digest (20 mL) was now subjected to intestinal digestion in a beaker containing dialysis membrane filled with 25 mL of $NaHCO_3$ equal in moles to the NaOH as determined by TTA. The beakers with the dialysis membranes were incubated in a shaker as described above until the pH of the digest changed to 5.0. Pancreatin-bile mix was added (5 mL) and the incubation was continued until the pH reached 7.0. After incubation, dialysis membranes were rinsed with distilled water and its volume was noted. Further, the dialysates (bioavailable fraction) were acidified with HNO_3 at 5% level, centrifuged (10,000 x g) and filtered before estimating the Zn content by AAS. Bioacessibility was calculated as follows

Bioaccessibility (%) = 100 × Y/Z where,

Y is the Zn content in bioavailable fraction (mg of mineral element/100 g sample) and Z is the total Zn (mg mineral element/100 g sample).

2.9. Statistical analysis

Results were expressed as mean of triplicates ± standard