

Optimisation of Phytate Degradation in Whole Grain Rice During Germination Processing Using Response Surface Methodology

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ABSTRACT

Phytic acid (IP6), stored in seeds as metal salts known as phytates, binds to micronutrients and prevents its absorption by the human body. The germination process could improve cereal nutritional values by stimulating endogenous phytase activity and promoting phytate degradation. This study evaluated the physicochemical changes of phytates in rice cultivars with different IP6 contents, followed by optimisation of phytate degradation using response surface modeling. The magnitude of changes in IP6 content and phytase activity differed among rice cultivars. This suggested that the efficiency of germination treatments relied on the amount of natural phytic acid and phytase activity present in the rice grains. The cultivar “*Tuan*” was then selected and studied for the germination effect on phytate degradation using a central composite design. The cultivar gave a lower IP6 content, enhanced phytase activity and improved minerals bioaccessibility under acidic conditions. Acidic germination facilitated the degradation of phytate complexes in whole grain rice by making phytate complexes more soluble, accelerating phytase activity and thus, releasing mineral micronutrients from phytate globoids. The optimum germination condition was identified at pH 2.7, 25 °C over 12 h. In conclusion, germination processing facilitated phytate degradation in whole grain rice to make value-added rice products with low phytic acid and good mineral bioaccessibility.

Keywords: Germination, phytase activity, phytic acid (IP6), whole grain rice

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INTRODUCTION

Whole grain foods generally provide more vitamins, minerals and fiber than their processed equivalent. Brown rice or whole grain rice has gained increasing attention from rice consumers due to its health-promoting properties (Champagne *et al.*, 2004; Sing *et al.*, 2015; Carcea, 2021). A positive association between whole grain rice consumption and lower incidence of many chronic diseases such as heart disease, obesity, cancer and Type 2 diabetes is often reported (Slavin, 2000; Panlasigui & Thompson, 2006; Dinesh Babu *et al.*, 2009; Zhao *et al.*, 2020). However, the abundance of phytates leads to antinutritive drawbacks from the consumption of whole grain rice.

Phytic acid is an anti-nutrient due to its strong ability to complex multi-charged metal ions (Raboy, 2009). It is a complex salt of Ca, Mg,

and K; in some cases, it is bound to proteins and starches (Graf, 1986; Thompson, 1986; Grases *et al.*, 2001). Whole grain rice contains 1.3 – 2.7 g phytates/kg sample at 14% moisture content (Juliano, 1985). The phytate complex can be broken down by the native phytase enzyme, which is accumulated during grain filling and activated or synthesized during germination. The phytase enzyme catalyzes the sequential phytate dephosphorylation, releasing phosphates and lowering inositol phosphates and chelated minerals (Bohn *et al.*, 2008; Kumar *et al.*, 2010). The enhancement of endogenous phytase activity for lowering phytic acid could improve the nutrient bioaccessibility of whole-grain rice.

The phytate removal or degradation processing methods are commonly applied to reduce the inhibitory effect of phytic acid. The removal process could be done either through mechanical (milling), diffusion (soaking) or

precipitation (metals addition) procedures, involving the removal of phytic acid molecules from the rice grain, accompanied by losses in micronutrients and dry matters (Liang *et al.*, 2008b). Phytate degradation involves the breaking down phytic acid molecules predominantly by enzymatic hydrolysis. This could happen with autolysis by endogenous phytase or hydrolysis by exogenous phytase. Soaking, germination and fermentation are methods that could stimulate endogenous phytase activity, which could promote phytate degradation.

Response surface methodology (RSM) is a collection of mathematical and statistical procedures to study the relationships between responses and factors for developing, improving and optimising processes (Murphy *et al.*, 2003; Myers *et al.*, 2016). RSM enables the determination of optimum conditions based on the interaction between variables with minimum numbers of experimental runs. These outdo the one-variable-at-a-time approach and full factorial design in saving cost and time. Rotatable central composite design (CCD) was among the popular RSM techniques due to its high model-fitting flexibility. The design is suitable for phytase activation or synthesis in the germination process involving interactions between variables to produce whole-grain rice with targeted quality.

Various approaches to the utilisation of endogenous phytase for lowering phytic acid were reported in brown rice, sorghum, whole grain cereals and sunflower seeds (Egli *et al.*, 2003; Liang *et al.*, 2008a; Agostini *et al.*, 2010; Afify *et al.*, 2011; Albarracín *et al.*, 2013). RSM of phytic acid and phytase activity in whole grain rice was only reported for the soaking process, which was accompanied by high nutrient losses (Liang *et al.*, 2008a; Albarracín *et al.*, 2013). Therefore, this study aimed to determine the effect of germination conditions on phytic acid and phytase activity and optimise the phytic acid content, endogenous phytase activity and mineral bioaccessibility in whole grain rice through rotatable CCD. This will enable the production of nutritionally enhanced rice products from local rice, increase their commercial value in the food industry and contribute to public health and nutrition.

MATERIALS AND METHODS

Rice Samples

Bario rice has unique soft eating quality and pleasant aroma from Sarawak, Malaysia. The rice is named after the location, a rural highland area with limited transportation access (Lee *et al.*, 2011). The rice cultivars for the germination study were selected from our prior research on the phytic acid in dehusked bario rice (Lee *et al.*, 2015). They were “Tuan” (TN), “Bario A” (BA), “Bario Pendek” (BP), “Bario Banjar” (BB), “Bario Hitam” (BH) and “Bario Merah” (BM). The whole grain rice was prepared freshly from paddy by passing through a rubber double roller dehulling machine.

Germination Method

Germination was conducted as described by Islam and Becerra (2012) with modifications. Whole grain rice (20 g) was sterilised with 0.01 percent sodium hypochlorite for 30 min and rinsed twice with distilled water. Whole grain rice was then steeped in various soaking solutions with a grain-to-solution ratio of 1:2 (w/v) for 6 h. The soaking solutions were prepared at different pH values and temperatures, as described in the experimental design section (initial screening and CCD). The soaking solution was drained after 6 h and wrapped with cheesecloth for dark germination at different time frames in a temperature-preset incubator. Treated rice samples were dried to <13% moisture content at 50 °C. Dried rice samples were blended into powder and sieved through a 425 µm sieve for further analysis.

Analytical and Characterisation Methods

Phytic acid and lower inositol phosphates were determined according to Lehrfeld (1989; 1994). Extraction with 0.5 M HCl was performed at room temperature for 2 h. The extract was centrifuged, and the supernatant portion was frozen overnight, thawed and centrifuged before analysis. The supernatant was diluted and passed through an anion exchange column, pre-washed with 0.05 M HCl for inorganic phosphates removal. Resin-bound inositol phosphates were then eluted with 2 M HCl, evaporated to dryness at 40 °C and reconstituted in 1 mL of mobile phase containing tetrabutylammonium hydroxide. Phytic acid sodium salt and

commercial phytic acid solutions (40%) were used for calibration. High-performance liquid chromatography (HPLC) was equipped with a Waters Symmetry C18 column (5 μm , 4.6 x 150 mm). The injection volume was 20 μL , and the mobile phase (methanol/0.035 N formic acid, 56:44 v/v, pH 4.3; 1% tetrabutylammonium hydroxide) flow rate was set at 1 mL/min for all the analysis. Peaks were detected using a refractive index detector.

Phytase activity was determined as described by Kim and Lei (2005) based on the inorganic phosphorus released during the incubation of the phytase enzyme for a specified time. The enzyme extract was prepared daily by shaking 500 mg of rice flour in 5 mL of citrate buffer (0.2 M, pH 5.5) for 30 min. The mixtures were centrifuged at $9000 \times g$ at 4 °C for 20 min. The supernatant was collected for analysis. The enzyme extract (200 μL) was activated by 5 min incubation in a 37 °C water bath. A sodium phytate solution of 9 mM in citrate buffer (200 μL) was added and further incubated for 15 min. The enzyme activity is terminated by adding 15% trichloroacetic acid solution (400 μL). The control for each sample was prepared by deactivation of enzyme extract on ice, followed by trichloroacetic acid and sodium phytate addition. Both samples and controls were centrifuged at $9000 \times g$ for 10 min. The supernatant was used for inorganic phosphorus analysis. One unit of phytase activity (U) is defined as the amount of enzyme required to liberate one micromole of inorganic phosphate per min from 5.0 mmol/L sodium phytate at pH 5.5 and 37 °C. Thus, the phytase activity of whole grain rice could be expressed as the total U per kg of the rice sample or U/kg.

In vitro mineral bioaccessibility was determined according to Hemalatha *et al.* (2007). Acid digestion was performed on 1 g of dried sample with nitric acid and perchloric acid at 10:3 to determine total mineral content. Bioaccessible minerals content was obtained through the dialysis of products from enzymatic digestion. Briefly, the 1 g rice sample suspension in water was acidified to pH 2. A new pepsin solution (300 μL) was added, and the suspension was up to 10 mL. The suspension was incubated at 37 °C shaking incubator for 2 h. A dialysis bag with 10 mL of NaHCO_3 equivalent to NaOH in titratable acidity was added, followed by 2 mL of pancreatin mixture after the suspension

reached approximately pH 5. The suspension with a dialysis bag was incubated at 37 °C shaking incubator for 2 h or longer until pH 7. The dialysate was acidified with 5% HNO_3 , centrifuged and filtered with Whatman No. 42 filter paper. The acid digest and treated dialysate were analysed for calcium, iron and zinc content using an atomic absorption spectrometer (AAS). The wavelengths monitored for these elements were 248.3 nm (Fe), 213.9 nm (Zn) and 422.7 nm (Ca). The amount of each element was quantified against a prepared standard calibration curve. *In vitro* minerals bioaccessibility was calculated as a percentage of bioaccessible minerals to total minerals content.

Experimental Design

The study involved two experimental stages: initial screening and response surface modeling. The initial screening of the phytic acid content of rice samples was conducted with germination treatments at a fixed temperature of 20 °C with acidic (pH 4) and alkaline (pH 10) conditions for 10 h. Subsequently, one cultivar was selected for further study using the response surface methodology based on central composite design (Table 1). A total of 20 experimental runs along with eight factorial points, six axial points and six center points were conducted to study the phytic acid content, phytase activity and minerals bioaccessibility in rice grain.

Data Analysis

Statistical differences between germination conditions were estimated from the ANOVA test, followed by Duncan New Multiple's Range Test (DNMRT). The alpha level used was $p < 0.05$ and the data was the mean of triplicates. Response surface analysis was performed using Minitab software (Minitab Inc., State College PA, USA). Surface and contour plots for each response were drawn as a function of two independent variables, while other variables were held constant in the middle setting. Optimum germination conditions were determined by superimposing the plots for all response variables. The region that fulfills the targeted requirements was considered. The optimum conditions were verified by analysing germinated rice flour experimentally and comparing the results statistically to predicted values. Statistical analysis was performed using SAS Version 9.0 (SAS Institute Inc., NC, USA).

Table 1. Coded levels for independent variables for developing experimental data in the response surface modeling

Variables	Symbol	Coded Variables				
		-1.682	-1	0	1	1.682
Temperature (°C)	A	13.2	20.0	30.0	40.0	46.8
pH	B	2.0	4.0	7.0	10.0	12.0
Time (h)	C	0.0	10.2	25.1	40.0	50.2

RESULTS AND DISCUSSION

Initial Screening

The screening experiment was conducted to identify the apparent effect of germination conditions on IP6 content in different rice cultivars at a fixed temperature (20 °C). Acidic pH significantly reduced the phytic acid content in all rice cultivars at an incubation period of 10 h (Figure 1). Prolonged incubation had no significant effect in lowering phytic acid content under acidic conditions but showed significant changes under alkaline conditions (Figure 2). The acidic germination condition effectively

reduced the phytic acid content, possibly due to better solubility of phytate complexes in low pH conditions. The high solubility of the phytate complexes under acidic conditions could accelerate the diffusion of soluble phytate and further enzymatic hydrolysis of phytate complexes by phytase enzyme (Liang *et al.*, 2008a; Albarracín *et al.*, 2013). However, prolonged germination time under an alkaline environment promoted lower phytic acid, possibly due to protein denaturation of phytate globoids. Cultivars “Tuan” was selected due to its popularity among local market demands (Nicholas *et al.*, 2014).

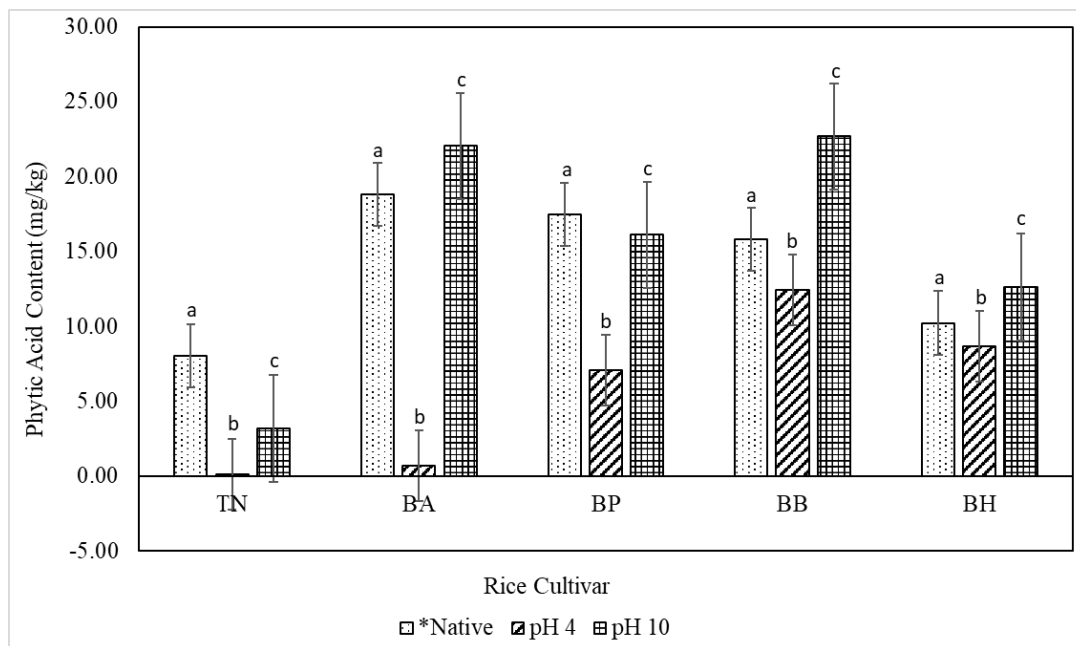


Figure 1. Comparison of phytic acid content (mg/kg) after incubation for 10 h at 20 °C in acidic and alkaline conditions. Native indicates control samples without any treatment. Bars with different letters in the same cultivars are significantly different (Duncan’s New Multiple Range Test, $p < 0.05$)

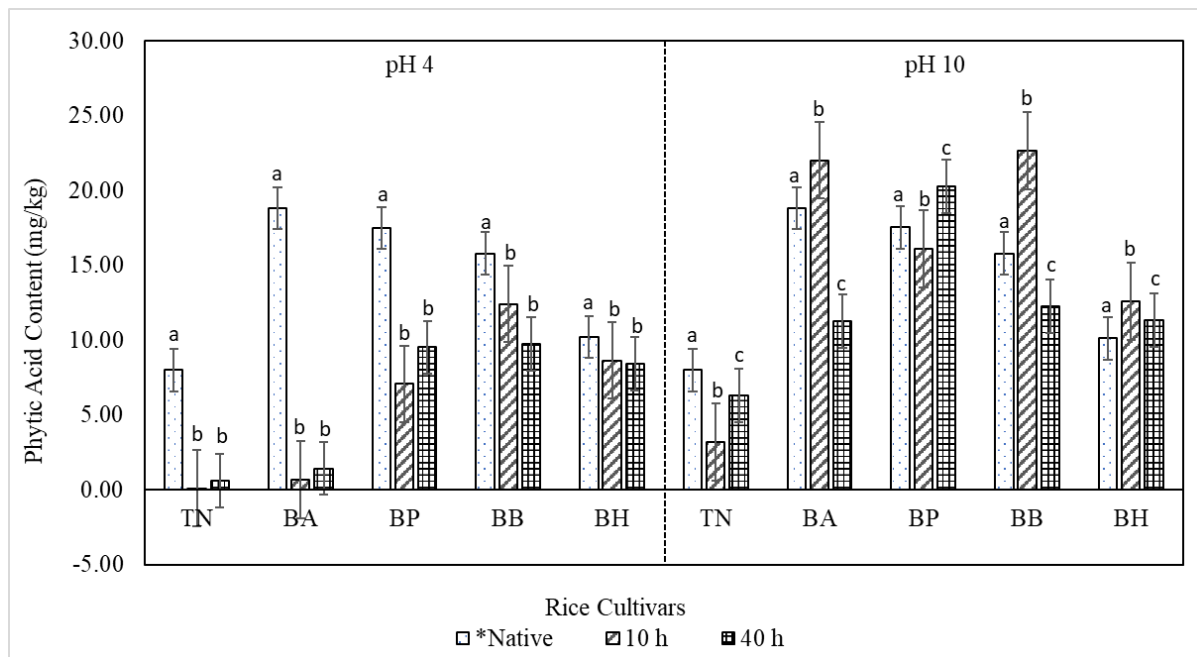


Figure 2. Comparison of phytic acid content (mg/kg) after incubation for 10 h and 40 h at 20 °C in acidic and alkaline conditions. Native designates control samples without any treatment. Bars with different letters in the same cultivars are significantly different (Duncan's New Multiple Range Test, $p < 0.05$)

Response Surface Analysis on Whole Grain Rice

Response surface analysis in central composite design was used to elucidate the influential factor or factors combination for phytic acid content, phytase activity, phytic acid to minerals moles ratio and *in vitro* minerals bioaccessibility in germinated whole grain rice. Hence, germination conditions could be optimised for the most favourable *in vitro* mineral bioaccessibility in whole-grain rice.

Phytic acid content and phytase activity in treated rice significantly differed between treatments (Table 2). Response surface analysis showed that the pH of the germination medium significantly influenced phytic acid content in a linear relationship (Figure 3). This was congruent to observations in the screening study, where phytic acid content was proportional to pH values. The pH-dependent solubility of phytate complexes could explain the lower phytic acid content under acidic germination. Phytase activity was influenced by two interacting factors of temperature-time and pH-time and a single time factor in a quadratic manner. These suggested germination period plays a critical role in the activity of the phytase enzyme, similarly reported by Azeke *et al.* (2011).

The mole ratios for calcium, zinc and iron were significantly reduced in most treatments due to the reduction of phytic acid content in whole-grain rice. Germination pH influenced the phytic acid content and the phytic acid to minerals mole ratios in a linear manner (Table 2). Several studies reported that acidic germination conditions could reduce phytic acid content and alter the phytic acid to minerals moles ratio in cereals and legumes (Liang *et al.*, 2008a; Liang *et al.*, 2009; Luo *et al.*, 2009; Albarracín *et al.*, 2013; Luo *et al.*, 2013). The linear relationship between pH and phytic acid to minerals mole ratios could be due to the higher solubility of phytate complexes under acidic conditions. In most circumstances, the mole ratio of zinc was reduced below the critical value of 15. Even so, treatments with acidic conditions could only significantly bring the calcium and iron moles ratio below these critical values. The calcium and iron, which are densely located at the bran layer, would be bounded tightly to phytic acid (Wang *et al.*, 2011; Iwai *et al.*, 2012; Lu *et al.*, 2013). Response surface analysis indicated that whole grain rice product with the lowest phytic acid level at an acceptable minerals mole ratio was achieved at pH 4 and 40 °C after 40 h treatment.

The *in vitro* bioaccessibility of calcium and iron were significantly affected by germination conditions at the $p < 0.05$ (Table 2), except for

zinc. *In vitro* calcium bioaccessibility was significantly influenced by temperature-pH interactions and the quadratic effect of pH at $p < 0.05$. In contrast, *in vitro* iron bioaccessibility was affected by pH-time interactions at $p < 0.10$ (Figure 4). *In vitro* mineral bioaccessibility increment under germination coincided with the lower mole ratio of phytic acid to minerals (Table 3). The whole grain rice products could provide the highest bioaccessibility of calcium, iron, and zinc at 18.03%, 9.57% and 7.33%, respectively. Some other studies showed similar improvement in iron and zinc bioaccessibility upon germination (Afify *et al.*, 2011; Albarracín *et al.*, 2013), but contradictory results with little

or no improvement were also reported (Liang *et al.*, 2008a; Luo *et al.*, 2009; 2013). These were probably due to the grain composition differences in cereals. The calcium bioaccessibility could be improved through acidic low temperatures treatments, while prolonged germination promoted a higher iron bioaccessibility. The changes in zinc bioaccessibility were insignificant, possibly due to massive zinc losses from whole-grain rice. These could lead to the localization of minerals in grains (Liang *et al.*, 2008a; Wang *et al.*, 2011; Iwai *et al.*, 2012; Lu *et al.*, 2013) and their solubility upon germination.

Table 2. Analysis of variance on the effect of germination conditions on response variables in cultivar “Tuan”

Source	Df	Sum of Squares							
		Phytic Acid Content (mg/g)	Phytase Activity (U/kg)	Phy/Ca ¹	Phy/Fe ¹	Phy/Zn ¹	IVB-Ca ² (%)	IVBFe ² (%)	IVBZn ² (%)
Temperature (A)	1	1.27	3.40	0.17	171.43	1058.84	61.85**	7.11	6.20
pH (B)	1	43.10**	8347.12***	4.91**	1497.99*	13.40**	181.95***	14.38	44.77
Time (C)	1	3.46	23465.67***	0.17	282.53	818.76	0.21	0.70*	28.02
A*B	1	1.77	3937.99**	0.11	70.36	15.61	53.99*	4.65	0.78
A*C	1	2.71	7721.10**	0.21	2.64	30.11	11.03	4.95	2.25
B*C	1	0.81	22446.57***	0.15	50.06	38.01	1.08	20.27*	0.12
A*A	1	0.79	8.11	0.016	3.06	36.22	25.20	1.14	2.80
B*B	1	7.21	1353.03	1.26	636.83	0.27	82.85*	0.065	0.089
C*C	1	0.0015	5000.83**	0.19	4.79	102.27	2.79	11.77	46.19
Residual Error	10	46.05	8063.81	6.50	3568.93	0.33	123.61	43.22	151.42
Lack of Fit	5	31.53	5464.18	3.40	1659.60	855.78	70.81	29.33	65.94
Pure Error	5	14.52	2599.63	3.10	1909.32	578.75	52.80	13.89	85.48
Total	19	106.87	79929.01	13.58	6281.41	277.03	553.74	109.26	285.91

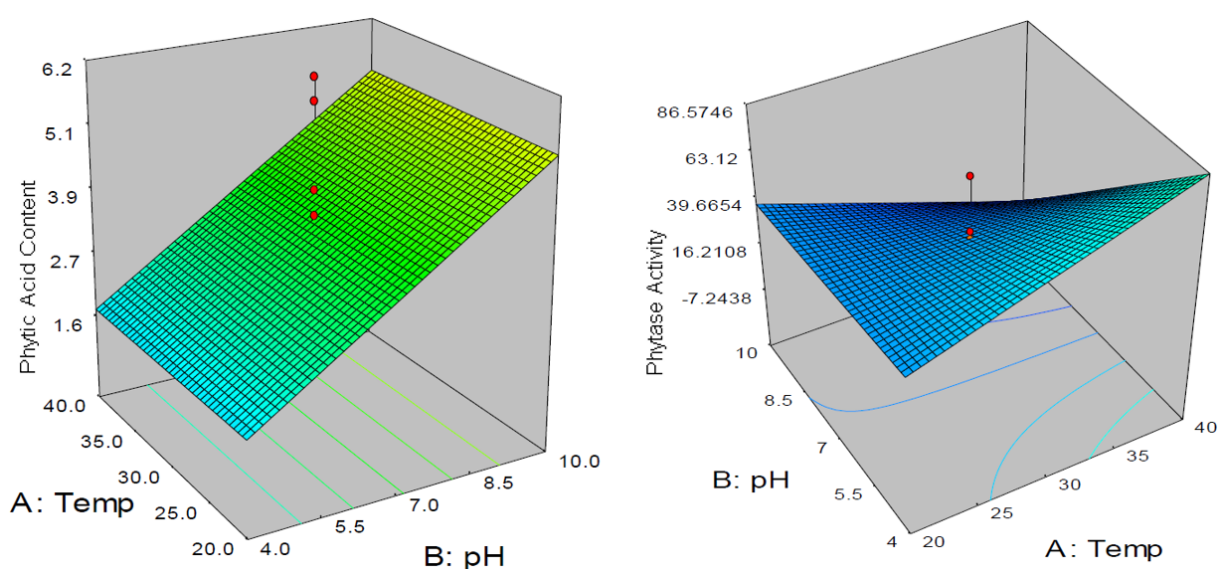


Figure 3. Response surface curve of phytic acid content (left) and phytase activity (right) after a continuous 25.1 h germination

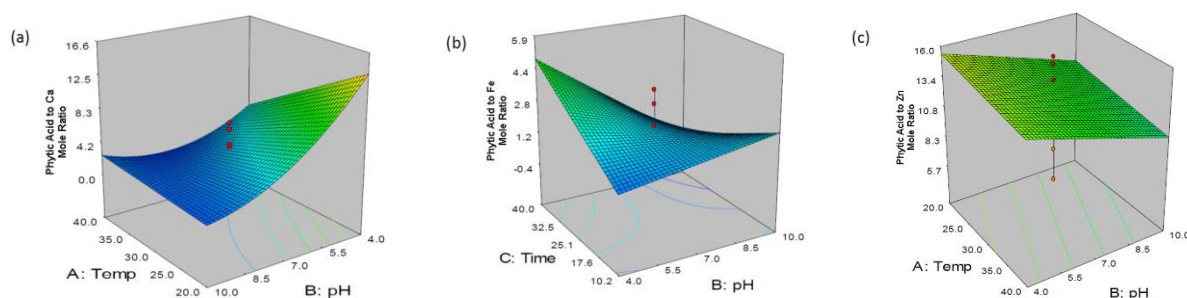


Figure 4. Response surface curve of phytic acid to minerals moles ratio (a) calcium after 10 h germination, (b) iron under constant germination temperature of 20 °C, and (c) zinc after 10 h germination

Table 3. Correlation between minerals content, moles ratio of phytic acid to minerals, and *in vitro* minerals bioaccessibility in germinated whole grain rice (“Tuan”)

	Calcium Content	Iron Content	Zinc Content	Phy/Ca ¹	Phy/Fe ¹	Phy/Zn ¹	IVBCa ²	IVBFe ²	IVBZn ²
Calcium Content	1.00	0.43**	0.59**	0.02	0.01	0.19	0.02	-0.59**	0.34**
Iron Content	0.43**	1.00	0.31*	-0.01	-0.25	0.13	-0.09	-0.45**	0.08
Zinc Content	0.59**	0.31*	1.00	0.17	0.13	0.18	-0.09	-0.17	0.16
Phy/Ca	0.02	-0.01	0.17	1.00	0.94**	0.96**	-0.38**	-0.07	-0.62**
Phy/Fe	0.01	-0.25	0.13	0.94**	1.00	0.89**	-0.27*	0.03	-0.53**
Phy/Zn	0.19	0.13	0.18	0.96**	0.89**	1.00	-0.42**	-0.13	-0.58**
IVB-Ca	0.02	-0.09	-0.09	-0.38**	-0.27*	-0.42**	1.00	0.04	0.26*
IVB-Fe	-0.59**	-0.45**	-0.17	-0.07	0.03	-0.13	0.04	1.00	-0.15
IVB-Zn	0.34**	0.08	0.16	-0.62**	-0.53**	-0.578**	0.26*	-0.15	1.00

Notes: Values with a single asterisk (*) and double asterisk (**) are significant at the $p < 0.05$ level and $p < 0.01$ level, respectively. ¹Phy/mineral indicates the mole ratio of phytic acid to respective minerals. ²IVB-mineral shows *in vitro* bioaccessibility of respective minerals.

Optimisation of Germination Conditions

Whole grain rice with a minimum phytic acid content, maximum phytase activity, *in vitro* minerals bioaccessibility, and acceptable phytic acid to minerals mole ratio was the targeted product quality for maximum minerals absorption. The targeted whole-grain rice could be achieved by optimising germination conditions using response surface models.

The germination conditions were optimised simultaneously for mineral bioaccessibility through the amendment of phytic acid content and phytase activity in grain. Iron and zinc molar ratios in whole grain rice dropped after 12 h of germination at 25 °C and pH 2.7. An acceptable *in vitro* bioaccessibility of calcium, iron, and zinc was achieved. Surprisingly, the bioaccessibility of these minerals became higher if the germination conditions were optimised by

each mineral individually, possibly due to the different behaviour of each mineral towards treatments. *In vitro* bioaccessibility of calcium and zinc was best obtained at 25 °C, pH 2, after 6 h of soaking. Meanwhile, an extended germination time (up to 50 h) gave the best *in vitro* iron bioaccessibility, in which phytic acid content could be predominantly suppressed.

The predicted germination conditions for all responses were verified experimentally (Table 4). A comparison between the products and native whole-grain rice showed improved mineral bioaccessibility with a reduced mole ratio of phytic acid to minerals. The optimised germination conditions were similar to previous studies (Liang *et al.*, 2008a; Albarracín *et al.*, 2013) in terms of acidity, but a shorter time and a lower temperature were needed to improve minerals (Ca, Fe, Zn) bioaccessibility.

Table 4. Comparison of phytic acid content, phytase activity, phytic acid to mineral mole ratio, and *in vitro* minerals bioaccessibility between native and germinated whole grain rice treated at optimised conditions

Treatments	Phytic Acid Content (g/kg)	Phytase Activity (U/kg)	Phytic acid to mineral mole ratio			<i>In vitro</i> bioaccessibility (%)		
			Ca	Fe	Zn	Ca	Fe	Zn
Native	5.54 ^a	112.36 ^a	2.61 ^a	26.26 ^a	17.27 ^a	18.84 ^{ab}	7.60 ^{ab}	19.56 ^b
Optimum 1: (0 h, pH 2.0, 25 °C)	3.30 ^b	376.61 ^{bc}	1.30 ^b	43.15 ^b	9.62 ^b	17.60 ^b	10.68 ^b	20.24 ^{ab}
Optimum 2: (12 h, pH 2.7, 25 °C)	2.98 ^c	563.89 ^c	1.39 ^b	18.77 ^a	15.07 ^c	25.04 ^c	5.35 ^a	28.24 ^c
Optimum 3: (50 h, pH 2.0, 25 °C)	0.00 ^d	249.68 ^{ab}	0.00 ^c	0.00 ^c	0.00 ^d	12.63 ^a	10.12 ^b	16.38 ^a

Notes: Means followed by different letters in the same column are significantly different (Duncan's New Multiple Range Test, $p < 0.05$)

CONCLUSION

In conclusion, acidity was the factor for phytate degradation in whole-grain rice during germination. Phytic acid content was significantly reduced with the facilitation of phytase activity during germination, leading to increments in calcium, iron and zinc bioaccessibility. This study suggested that germination at pH 2.7 and 25 °C for 12 h was necessary to improve the bioaccessibility of calcium, iron, and zinc in the Bario whole grain rice and provided an alternative to enhance the nutritional value of whole grain rice.

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