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Mechanochemical-assisted extraction and enzymatic hydrolysis of calcium phytate from defatted rice bran

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Abstract

Background Mechanochemical-assisted extraction (MCAE) and mechanochemical-assisted enzyme catalysis (MCAEC) methods were developed for extraction and enzymatic hydrolysis of calcium phytate from defatted rice bran, respectively.

Results The MCAE conditions were optimized by response surface methodology: 10% sodium carbonate as the solid reagent, 33 min grinding time, 18:1 ball–material ratio, 330 rpm ball milling speed, and pH 5.5 extraction. Under these conditions, the yield of calcium phytate was 97.0 mg/g. The MCAEC conditions were optimized as follows: 2% enzyme addition, MM400 milling time 36 min, PM400 milling speed 262 rpm, MM400 milling frequency 27 Hz, and the addition of pH 5.5 sodium acetate buffer 3 mL. Under these conditions, the hydrolysis rate of calcium phytate was 39.7%.

Conclusion Compared with traditional extraction and enzymatic hydrolysis methods, MCAE and MCAEC could obtain higher yields and hydrolysis rates, respectively.

Keywords Mechanochemistry, Extraction, Enzymatic hydrolysis, Calcium phytate, Rice bran

†Lei Zheng and XingYi Zhu contributed equally to this work.

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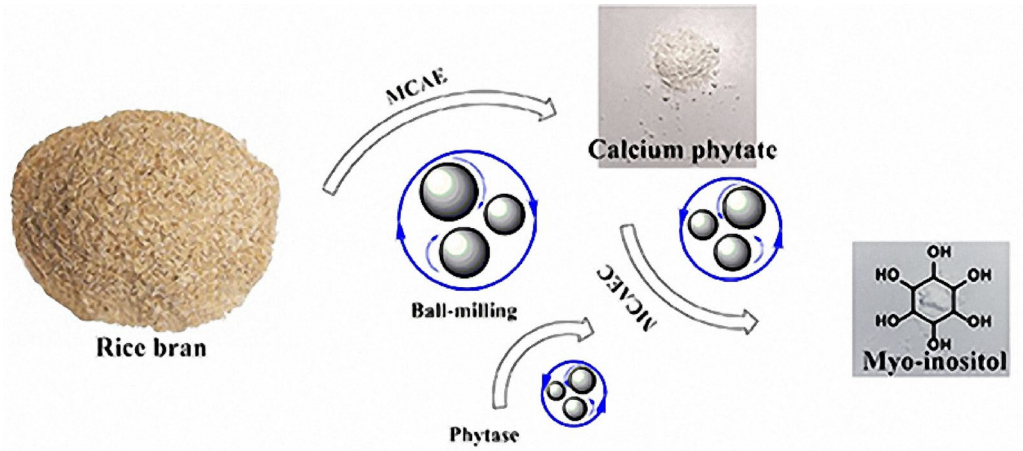
Graphical Abstract



Introduction

Rice bran, an agricultural waste product, has been ignored for a long time. However, rice bran contains many effective components. The defatted rice bran contains 10% phytate compounds [1, 2]. Phytate compounds can be hydrolysed by phytase to obtain *myo*-inositol, which has antiaging and antioxidation effects [3, 4]. Calcium phytate is the main form of phytate compound in rice bran. The conventional method of obtaining calcium phytate from rice bran usually uses large amounts of acid and alkali, which poses high pollution [5, 6]. A variety of enzymes, ion exchange resins, microwave extraction machines, and ultrasonic extraction instruments are greatly enriched with extraction methods, but these methods are not widely applied in actual production [7–9]. At present, the production of myo-inositol from phytate is implemented by using ion exchange resin, which is complicated and expensive [10].

Mechanochemistry research is the physical–chemical transformation generated by mechanical power, which is used to describe chemical reactions or physiochemistry changes that take place during mechanical energy [11, 12]. Because of the particularity of its principle, its application value in many fields is being explored by researchers, such as mechanochemical-assisted extraction, synthesis, and polymorphic transformation [13–15]. Mechanochemical-assisted extraction (MCAE) is a novel and promising extraction method compared to other methods. In this method, the plant material is pretreated with solid reagent by milling to obtain milling mixtures, extracted by solvent [16]. MCAE has been used to extract natural products such as flavonoids, terpene tri-lactones, kaempferol glycosides, and chondroitin sulfate from natural raw materials [17–20]. Recently, the application of mechanochemistry in enzyme catalysis has promoted enzyme catalysis to be more efficient and greener [21]. Mechanochemical-assisted enzyme catalysis (MCAEC) can be applied to the hydrolysis of cellulose and the decomposition of secondary alcohols [22, 23]. However, there have been no reports on the application of MCAE and MCAEC in the production of *myo*-inositol from rice bran.

In this study, we used MCAE and MCAEC methods to efficiently obtain *myo*-inositol from rice bran. The operating parameters of these two methods were optimized. These two methods were compared with traditional methods. The objective is to provide an efficient strategy for the preparation of *myo*-inositol from rice bran.

Materials and methods

Plant material and chemicals

The defatted rice bran used in this experiment was provided by Shijiazhuang Jinsui Agricultural Products Trading Co., Ltd (Shijiazhuang, China) and shattered to a uniform particle size (0.5 mm) in a DF-50-A pulverizer (Wenling Linda Machinery Co., Ltd, Zhejiang, China). Then they were stored in a dry environment at room temperature. Standard calcium phytate and *myo*-inositol were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). The analytical reagent sodium carbonate and acetic acid were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). BR phytase (*Aspergillus niger*) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). HPLC-grade mobile

phase acetonitrile, phosphoric acid, and methanol were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China).

MCAE procedure

Defatted rice bran (5.0 g) was added to the PM400 high-intensity planetary activator and a certain amount of solid reagent was added at the same time (Retsch Inc., Haan, Germany). After several minutes of milling, the milling mixture was dissolved in pure acetic acid. The mixture was then filtered by suction, and the filtrate was collected. The pH of the solution was adjusted to 7.0 with 10% NaOH solution, and then calcium chloride (1.0 g) was added to the solution. The calcium phytate precipitate was obtained by centrifugation at 5500 rpm for 10 min. After washing with 80 °C water and drying, the product calcium phytate was obtained. The calcium phytate yield was expressed as a percentage of the weight of calcium phytate obtained to the total weight of defatted rice bran inputted in the PM400 high-intensity planetary activator, as follows:

$$\text{Yield} = \frac{m_1}{m_2}, \tag{1}$$

where m_1 and m_2 represent the weight of calcium phytate obtained and the weight of defatted rice bran, respectively.

Traditional hydrochloric acid extraction [5]

According to the optimal conditions, the defatted rice bran (5.0 g) was put into a round-bottomed flask with 100 mL of 0.1 mol/L hydrochloric acid solution, and stirred for 3 h. The mixture was filtered by suction, and the filtrate was collected. The above process was repeated for two cycles. The pH of the filtrate was adjusted to 5.0 with calcium hydroxide, and then the pH of the solution was adjusted to 7.0 with 10% NaOH solution. The calcium phytate precipitate was obtained by centrifugation at 5500 rpm for 10 min. After washing at 80 °C and drying, the product calcium phytate was obtained.

MCAEC hydrolysis procedure

Calcium phytate (2.0 g) was added to 40 mg phytase which was pretreated by milling in a PM400 high-intensity planetary activator. The mixture was transferred to a milling vessel (stainless steel, volume: 25 mL) containing four balls (stainless steel, diameter: 10 mm). A certain amount of acetic acid–sodium acetate buffer (pH 5.5) was added. The mixture was milled in an MM400 high-intensity planetary activator (Retsch Inc., Haan, Germany). The milling mixture was then centrifuged at 5500 rpm for 10 min. The supernatant obtained from the above process was analysed using HPLC. The hydrolysis rate was expressed as the weight of hydrolysed calcium phytate to the total amount of calcium phytate input. The formula is shown as follows:

$$\text{Hydrolysis} = \frac{m_3 \frac{M_{\text{CP}}}{M_{\text{myo - inositol}}}}{m_4} \times 100\%, \tag{2}$$

where *Hydrolysis* is the hydrolysis rate of calcium phytate, m_3 is the content of *myo*-inositol in enzymolysis solution, m_4 is the total amount of calcium phytate, $M_{myo\text{-}inositol}$ is the *myo*-inositol molar mass, and M_{CP} is the calcium phytate molar mass.

Traditional hydrolysis procedure [24]

Calcium phytate (2.0 g) was added to 20 ml of sodium acetate buffer (pH 5.5). Forty milligrams of phytase solid was added to the solution. The mixture was reacted in a constant temperature water (50 °C) bath for 10 h. The mixture was then filtered by suction, and the filtrate was collected. The supernatant was analysed using HPLC.

Experimental design for calcium phytate extraction

Response surface methodology (RSM) is an empirical modelling technology. This method can be used to estimate the relationship between a set of control experimental factors and observation results. The optimum conditions for MCAE of calcium phytate were determined by Box–Behnken design (BBD). Taking the amount of solid reagents, the proportion of solvents to the solid, and ball milling time as the key variables, they are recorded as X_1, X_2, X_3, and X_4, respectively. The low, medium, and high levels of each variable were specified by "− 1, 0, and + 1", respectively (Table 1).

A total of 29 experiments were conducted with different combinations of 4 factors. The experimental design using response values as the yield of calcium phytate is shown in Table 2. The method of estimating the sum of squares of a pure error was repeated in 5 experiments at the design centre. This model used a complete quadratic equation or a simplified form of the equation, as follows:

$$Y = \beta_0 + \sum \beta_j X_j + \sum \beta_{jj} X_j^2 + \sum \sum \beta_{ij} X_i X_j, \tag{3}$$

Table 1 Code and level of factors chosen for the trials

Factor	Levels		
	− 1	0	1
Ball-to-material ratio (g/g)	10:1	18:1	26:1
Milling time (min)	10	30	50
Milling speed (rpm)	100	300	500
Extraction pH	4.0	5.5	7.0

Table 2 Experimental conditions for the Box–Behnken design and the corresponding responses measured

Exp. no.	X_1	X_2	X_3	X_4	Ball-to-material ratio (g/g)	Milling time (min)	Milling speed (rpm)	Extraction pH	Yield of calcium phytate (mg/g)
1	− 1	− 1	0	0	10:1	10	300	5.5	20.0
2	1	0	0	1	26:1	30	300	7.0	64.8
3	1	0	− 1	0	26:1	30	100	5.5	46.4
4	− 1	0	0	1	10:1	30	300	7.0	34.6
5	0	0	0	0	18:1	30	300	5.5	92.2
6	0	0	0	0	18:1	30	300	5.5	93.0
7	0	− 1	1	0	18:1	10	500	5.5	34.6
8	0	0	1	1	18:1	30	500	7.0	59.0
9	0	0	− 1	1	18:1	30	100	7.0	32.4
10	0	1	1	0	18:1	50	500	5.5	68.0
11	0	1	0	− 1	18:1	50	300	4.0	73.2
12	1	0	1	0	26:1	30	500	5.5	84.2
13	0	0	0	0	18:1	30	300	5.5	93.4
14	0	− 1	0	1	18:1	10	300	7.0	26.4
15	0	0	0	0	18:1	30	300	5.5	94.0
16	− 1	0	1	0	10:1	30	500	5.5	45.0
17	0	− 1	0	− 1	18:1	10	300	4.0	37.6
18	0	0	1	− 1	18:1	30	500	4.0	83.4
19	0	1	0	1	18:1	50	300	7.0	52.0
20	1	1	0	0	26:1	50	300	5.5	74.2
21	1	0	0	− 1	26:1	30	300	4.0	91.0
22	− 1	1	0	0	10:1	50	300	5.5	39.6
23	0	1	− 1	0	18:1	50	100	5.5	32.6
24	0	0	0	0	18:1	30	300	5.5	90.6
25	− 1	0	− 1	0	10:1	30	100	5.5	24.4
26	0	0	− 1	− 1	18:1	30	100	4.0	45.8
27	− 1	0	0	− 1	10:1	30	300	4.0	48.8
28	0	− 1	− 1	0	18:1	10	100	5.5	18.6
29	1	− 1	0	0	26:1	10	300	5.5	38.2

where Y = estimated response, β_0=intercept, β_j = linear coefficient, β_{jj} = quadratic-term coefficient, β_{ij} = cross-term coefficient, and X_i and X_j = independent encoding variables. The variables were encoded according to the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X}, \tag{4}$$

where x_i = dimensionless encoded value of X_i, X_0 = centre point X_i value, and ΔX = step change.

Determination of *myo*-inositol

Myo-inositol was quantified by an Agilent HPLC. *Myo*-inositol was detected by a refractive index detector. The mobile phase was methanol/H_2O (30:70, v/v) at a flow rate of 0.8 mL/min. The injection volume was 10 µL. An Athena C_{18} column (5 µm, 250 mm × 4.6 mm) was used.

Scanning electron microscopy (SEM)

The morphological alterations on the cross-section and surface of milled and raw rice bran samples were observed by SEM. The samples were gold-plated before SEM observation [25]. Electronic scanning images were obtained using a ZEISS Gemini 500 SEM (Carl Zeiss AG, Germany).

Experimental design for MCAEC hydrolysis

A BBD was used to determine the optimal conditions for MCAEC hydrolysis of calcium phytate. Taking the parameters including MM400 milling time, PM400 milling speed, and MM400 milling frequency as the key variables, X_5, X_6, and X_7, respectively, were recorded. The low, medium, and high levels of each variable were specified by "− 1, 0, and + 1", respectively (Table 3).

A total of 17 experiments were conducted with different combinations of 3 factors. The experimental design

Table 3 Code and level of factors chosen for the trials

Factor	Levels		
	− 1	0	1
MM400 milling time (min)	20	30	40
PM400 milling speed (rpm)	200	300	400
MM400 milling frequency (Hz)	18	24	30

using response values as the yield of calcium phytate is shown in Table 4. The method of estimating the sum of squares of a pure error was repeated in 5 experiments at the design centre. This model used a complete quadratic equation or a simplified form of the equation.

Results and discussion

Preliminary results

A preliminary extraction experiment was conducted to determine the main influencing factors and the appropriate experimental range of BBD. The parameters affecting the MCAE process include the types and doses of solid reagent, ball-to-material ratio, grinding time and speed, extraction time, extraction pH, and solution-to-solid ratio. Among the screened variables, the ball-to-material ratio, milling time, milling speed, and extraction pH was identified as the most significant variables with ranges of 10:1–26:1, 10–120 min, 50–500 rpm, and 4.0–7.0, respectively (Fig. 1). The changes in other parameters did not substantially affect the yield of calcium phytate obtained through the MCAE process. Therefore, these parameters of the MCAE process were selected as follows: solid reagent amount, 10%; extraction time, 30 min; extraction temperature, 25 °C; and solvent-to-solid ratio, 30:1 (mL:g). In addition, the effect of different solid reagent types ($NaHCO_3$, NaOH, Na_2CO_3) on the extraction yield was studied under the above conditions. The results showed that, when milling with Na_2CO_3, the yield of calcium phytate was higher than that with $NaHCO_3$ or NaOH. Therefore, Na_2CO_3 was selected as the solid reagent for subsequent optimization.

A preliminary experiment of the mechanochemical enzymatic hydrolysis process was carried out to determine the suitable BBD range. Various parameters, such as the ball-to-material ratio, MM400 milling time, PM400 milling speed, MM400 milling frequency, sodium acetate buffer pH, and the addition of sodium acetate buffer, potentially influenced the MCAEC process. The main factors affecting BBD were MM400 milling time, PM400 milling speed, and MM400 milling frequency, which ranged from 5 to 55 min, 50–500 rpm, and 10–30 Hz, respectively (Fig. 2).

RSM result analysis

The conditions of MCAE and MCAEC were further optimized by using BBD. Analysis of variance (ANOVA), regression coefficient, and regression equation were analysed by Design Expert 8.0.5 software. The polynomial equations, describing the yield of calcium phytate (Y_1) and the hydrolysis rate of calcium phytate (Y_2) as two functions are shown in Eqs. (3) and (4):

Table 4 Experimental conditions for the BBD and the corresponding responses measured

Exp. no.	X_5	X_6	X_7	MM400 milling time (min)	PM400 milling speed (rpm)	MM400 milling frequency (Hz)	Hydrolysis rate of calcium phytate (%)
1	− 1	1	0	20	400	24	13.8
2	0	− 1	− 1	30	200	18	16.3
3	1	0	− 1	40	300	18	18.5
4	− 1	0	1	20	300	30	20.9
5	0	0	0	30	300	24	34.8
6	− 1	− 1	0	20	200	24	19.3
7	− 1	0	− 1	20	300	18	9.79
8	0	− 1	1	30	200	30	32.2
9	0	1	1	30	400	30	23.0
10	1	1	0	40	400	24	24.2
11	1	− 1	0	40	200	24	33.8
12	0	1	− 1	30	400	18	11.7
13	1	0	1	40	300	30	36.5
14	0	0	0	30	300	24	37.2
15	0	0	0	30	300	24	35.7
16	0	0	0	30	300	24	31.4
17	0	0	0	30	300	24	36.3

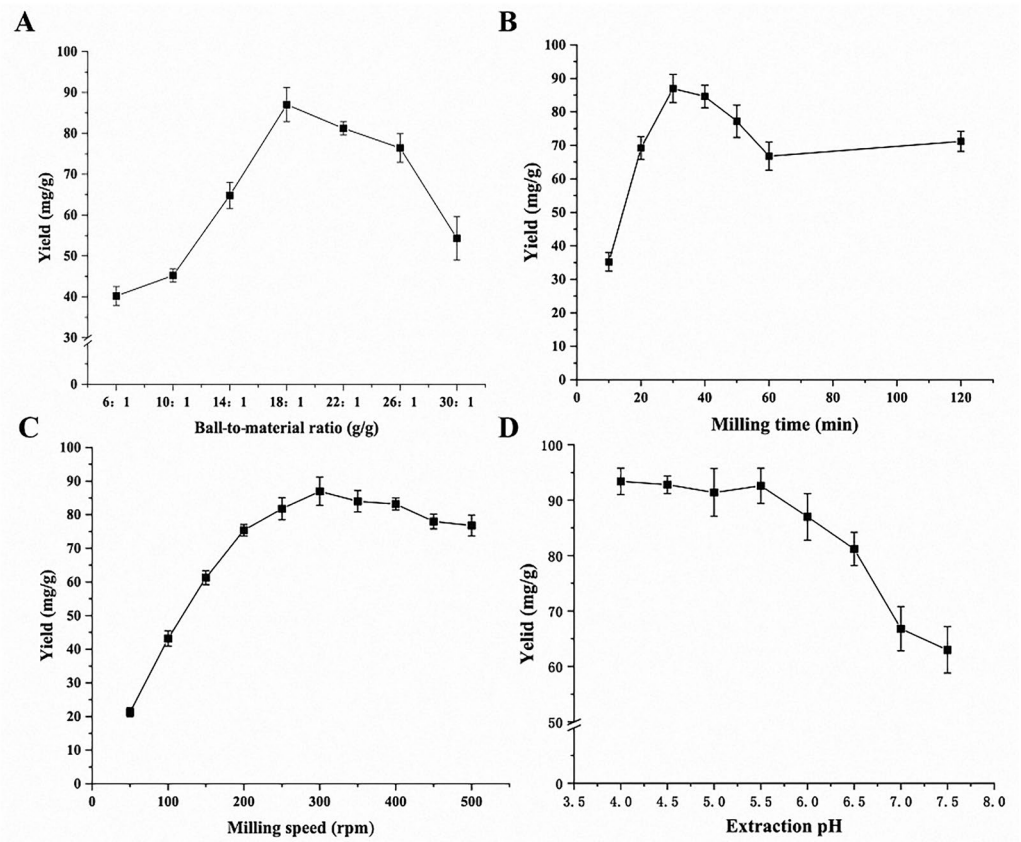


Fig. 1 Effect of different operating parameters on the yield of calcium phytate in preliminary experiments (**A**–**D**)

$$\begin{aligned} Y_1 = {} & 0.46 + 0.078\,X_1 + 0.068\,X_2 + 0.072\,X_3 \\ & - 0.046\,X_4 + 0.021\,X_1\,X_2 + 0.021\,X_1\,X_3 \\ & - 0.015\,X_1\,X_4 + 0.024\,X_2\,X_3 - 0.012\,X_2\,X_4 \\ & - 0.014\,X_3\,X_4 - 0.094\,X_1^2 - 0.15\,X_2^2 \\ & - 0.12\,X_3^2 - 0.071\,X_4^2, \end{aligned} \tag{5}$$

$$\begin{aligned} Y_2 = {} & 35.08 + 6.15\,X_5 - 3.61\,X_6 + 7.047\,X_7 \\ & - 1.02\,X_5\,X_6 + 1.72\,X_5\,X_7 - 1.15\,X_6\,X_7 \\ & - 5.84\,X_5^2 - 6.46\,X_6^2 - 7.82\,X_7^2\,. \end{aligned} \tag{6}$$

The variance analysis of the selected quadratic model is summarized in Table 5 and Table 6, and the experimental data fitted well with the quadratic models through ANOVA. The ANOVA of the RSM quadratic regression model showed that it was highly significant ($p < 0.0001$) with a very high F-value (165.88 for the yield of calcium phytate, 55.16 for the hydrolysis rate of calcium phytate). The regression analysis of the data also showed that two models were significant. The coefficient of determination (R^2) values for the yield of calcium phytate and the hydrolysis rate of calcium phytate were 0.9940 and 0.9861, respectively. The coefficients of determination (Adj $R^2 = 0.9880$ for the yield of calcium phytate and Adj $R^2 = 0.9682$ for the hydrolysis rate of calcium phytate) were also satisfactory after adjustment, which confirmed the significance of the models. These results indicated that Eqs. (3) and (4) were applicable for describing the response of the experiment pertaining to the yield and the hydrolysis rate of calcium phytate. At the same time, the lack-of-fit statistics, which were used to test the adequacy of the model, indicated that the p values for the yield and the hydrolysis rate of calcium phytate (0.0506 and 0.9844) were not very significant. In addition, no abnormality was found in the residual diagnosis results. In summary, it can be concluded that the models were statistically reasonable.

To determine the optimal levels of the test variables for the yield and hydrolysis of calcium phytate in defatted rice bran, the regression model described the 3D response surface that is presented in Fig. 3A–I. By solving the inverse matrix, the optimal values of the variables affecting the yields of calcium phytate given by the software were the amount of ball–material ratio 19.2:1, milling time 33 min, speed of ball milling 330 rpm, and

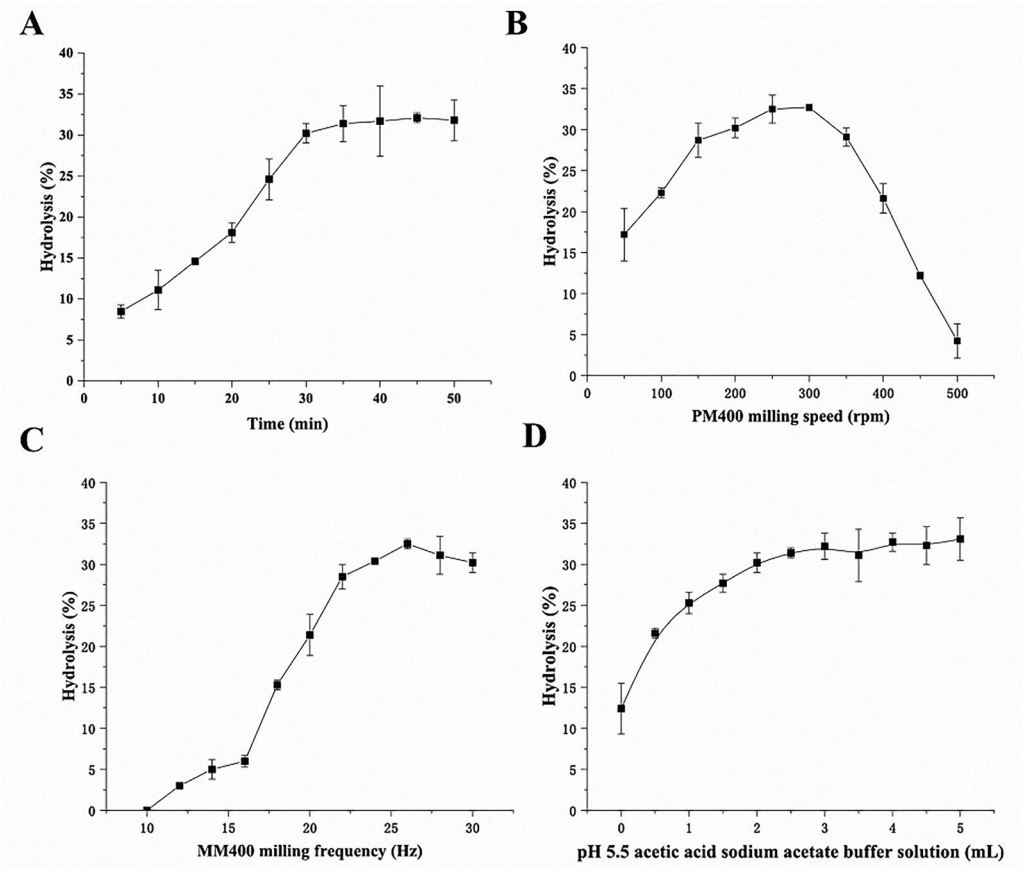


Fig. 2 Optimization of MCAEC hydrolysis procedure (**A**–**D**)

extraction pH 5.4. Under these optimal conditions, the maximum predicted value of phytase yield (98.5 mg/g) was given by the model. In view of the operating convenience, the optimal extraction parameters were determined to be an amount of ball–material ratio of 18:1, milling time of 33 min, speed of ball milling of 330 rpm, and extraction pH of 5.5. Moreover, through the software design expert to solve the regression equation, the optimal process conditions of mechanical chemistry-assisted extraction of calcium phytate could be obtained: MM400 milling time of 36 min, PM400 milling speed of 262 rpm, and MM400 milling frequency of 27 Hz. Under these optimal conditions, the maximum predicted hydrolysis rate of *myo*-inositol from calcium phytate hydrolysis (39.7%) was given by the model.

Under certain conditions, we conducted triplicate experiments. The yield and hydrolysis rate of calcium phytate (97.0 mg/g and 41.3%) were consistent with the predicted values (98.5 mg/g and 39.7%), indicating that the model was adequate for the calcium phytate extraction process.

Comparison of different methods

The extraction of calcium phytate by MCAE and traditional hydrochloric acid extraction were compared under the optimized conditions. As shown in Table 7, the yield of calcium phytate using MCAE was higher than that of traditional hydrochloric acid extraction. The extraction time of MCAE was only one-third of that of traditional hydrochloric acid extraction. In addition, the amount of acid, alkali, and solvent in MCAE was much less than that in traditional hydrochloric acid extraction. It is worth noting that MCAE had many advantages in the actual preparation of phytate calcium.

The hydrolysis of calcium phytate by MCAEC and traditional enzymatic hydrolysis were compared under the optimized conditions. Table 8 shows that the hydrolysis rate of mechanochemical enzymatic hydrolysis was higher than that of traditional enzymatic hydrolysis. The hydrolysis time of MCAEC was only 6% of that of traditional enzymatic hydrolysis. This method is relatively safe and environmentally friendly, because it is

Table 5 Results of the variance analysis of regression model for the extraction yield of calcium phytate

Source	df	Sum of squares	Mean square	*F*-value	Prob > *F*
Model	14	0.45	0.032	165.88	< 0.0001**
X_1	1	0.072	0.072	370.86	< 0.0001**
X_2	1	0.056	0.056	287.78	< 0.0001**
X_3	1	0.063	0.063	323.16	< 0.0001**
X_4	1	0.025	0.025	130.57	< 0.0001**
X_1X_2	1	1.600E-003	1.600E-003	8.61	0.0109*
X_1X_3	1	1.849E-003	1.849E-003	9.47	0.0082**
X_1X_4	1	9.000E-004	9.000E-004	4.61	0.0498*
X_2X_3	1	2.352E-003	8.100E-003	12.05	0.0037**
X_2X_4	1	6.250E-004	6.250E-004	3.20	0.0952
X_3X_4	1	7.562E-004	7.562E-004	3.87	0.0691
X_1^2	1	0.058	0.058	65.34	< 0.0001**
X_2^2	1	0.15	0.15	21.59	< 0.0001**
X_3^2	1	0.089	0.089	156.04	< 0.0001**
X_4^2	1	0.032	0.032	166.23	< 0.0001**
Residual	14	2.733E-003	1.952E-004		
Lack of fit	10	2.560E-003	2.560E-004	5.93	0.0506
Pure error	4	1.728E-004	4.320E-005		
Cor total	28	0.46	$R^2 = 0.9940$		

df, degree of freedom. *$p < 0.05$ significant; **$p < 0.01$ highly significant

Table 6 Results of the variance analysis of regression model for MCAEC hydrolysis

Source	df	Sum of squares	Mean square	*F*-value	Prob > *F*
Model	9	1468.06	163.12	55.16	< 0.0001**
X_5	1	302.70	307.70	102.37	< 0.0001**
X_6	1	104.40	104.40	35.31	0.0006**
X_7	1	396.35	396.35	134.04	< 0.0001**
X_5X_6	1	4.20	4.20	1.42	0.2720
X_5X_7	1	11.87	11.87	4.01	0.0852
X_6X_7	1	5.29	5.29	1.79	0.2229
X_5^2	1	143.66	143.66	48.58	< 0.0001**
X_6^2	1	175.92	175.92	59.49	< 0.0001**
X_7^2	1	257.24	257.24	86.99	< 0.0001**
Residual	7	20.70	2.96		
Lack of fit	3	0.71	0.24	0.047	0.9844
Pure error	4	19.99	5.00		
Cor total	16	1488.75	$R^2 = 0.9861$		

df, degree of freedom. **$p < 0.01$ highly significant

easy to operate, and does not require the use of strong acids and a large number of solvents. It should be noted that phytase pretreated by mechanochemistry showed higher activity than without pretreatment.

SEM observation

The chemical forms have important influences on the solubility of the substance, which affects the extraction efficiency [26]. The preliminary work of our research group has confirmed that the extraction yield can be improved by the previous mechanochemical reaction and mechanical treatment of the raw materials [27, 28]. In this study, the improvement in the extraction efficiency of calcium phytate from defatted rice bran was affected by many factors, such as the decrease in particle size, cell wall rupture, and chemical transformation of calcium phytate after mechanochemical treatment.

Through experimental verification, the particle size was considered to be one of the primary factors affecting the extraction efficiency of calcium phytate. In the process of mechanical activation, the impact displacement of defatted rice bran is not only accompanied by grinding, but also accompanied by the destruction of the cell shell. Based on electron microscopic observations, it is possible to estimate changes in mechanical treatment in PM400 high-strength planetary activators. Figure 4A shows a scanning electron microscope (SEM) image of defatted rice bran powder. It had a majority of regular blocky structures and highly rough surfaces. Figure 4B shows a detailed image of defatted rice bran milled with Na_2CO_3 for 30 min. The particle size of the defatted rice bran powder was significantly reduced, and most of the cell walls were almost destroyed. Through mechanical processing, the contents in the cells can be easily released and digested, because the cell wall is intensively distorted and finally ruptured under the action of strong squeezing force and shear force. In addition, the formation of new portions of soluble bioactive substances under mechanical force is also the main reason for the increase in the yield of bioactive substances. In the experiment, the phytate reacted with Na_2CO_3 to form water-soluble salt by milling. Strong acid acidification is not needed, so a pH 5.5 acetic acid solution was used as the extraction solvent.

The changes in phytase by mechanical treatment in the PM 400 high-intensity planetary activator can be estimated according to electron microscopic observations. SEM of phytase powder is shown in Fig. 4C and E. It has a complete crystal structure. A detailed image of phytase milled at 200 rpm for 30 min is shown in Fig. 4D and F. Most of the particle size of the obtained phytase powder was significantly reduced and tended to be amorphous. Through experimental results, phytase can withstand mechanochemical treatment and its particle size is significantly reduced, which may be the critical factor in improving the hydrolysis rate of calcium phytate.

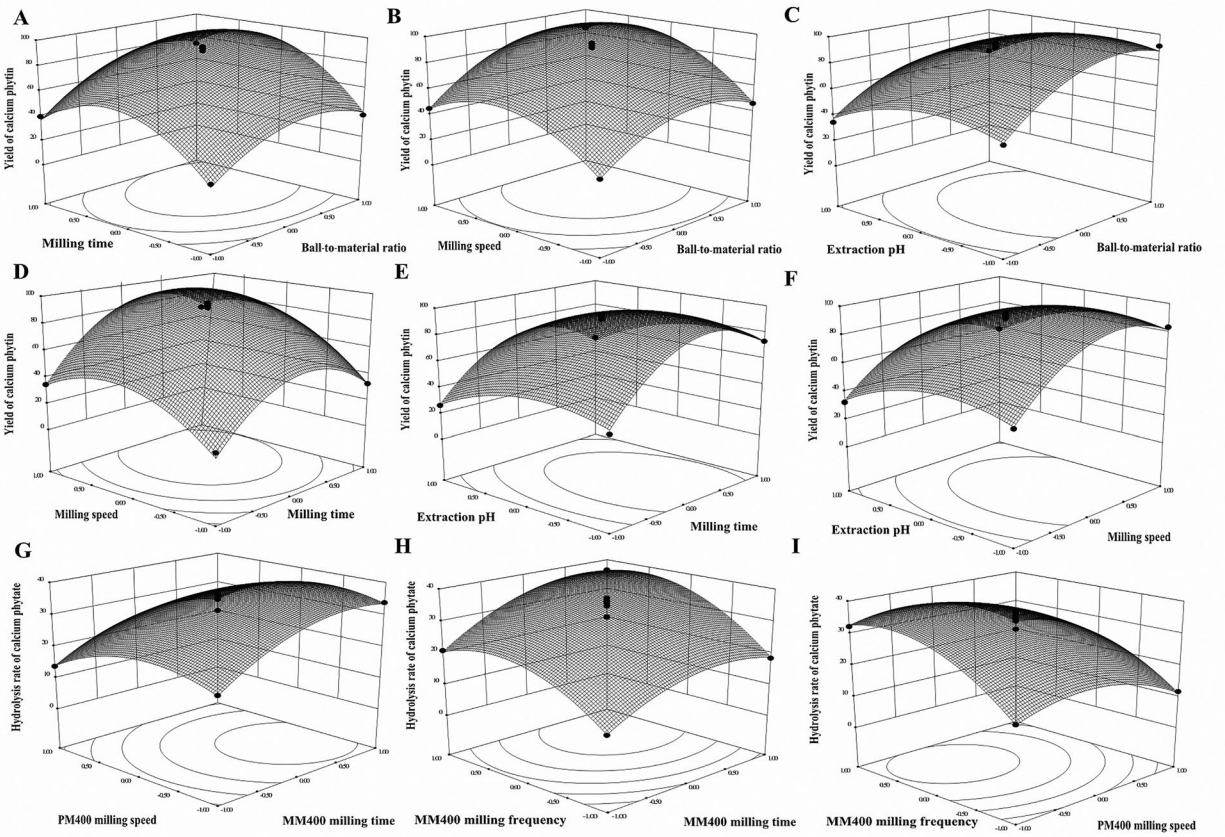


Fig. 3 Response surfaces representations for the yield of calcium phytate: **A** varying speed of ball milling and ball-to-material ratio; **B** varying milling speed and ball-to-material ratio; **C** varying extraction pH and ball-to-material ratio; **D** varying milling speed and milling time; **E** varying speed of extraction pH and milling time; **F** varying extraction pH and milling speed. Response surfaces representations for the hydrolysis rate of calcium phytate: **G** varying MM400 milling time and PM400 milling speed; **H** varying MM400 milling time and MM400 milling frequency; **I** varying PM400 milling speed and MM400 milling frequency

Table 7 Comparison of MCAE with traditional hydrochloric acid extraction

Extraction method	Extraction time	Solvent	Yield of calcium phytate (mg)[a]
MCAE	33 min	pH 5.5 NaOAc solvent	97.0 ± 1.0
Traditional hydrochloric acid extraction	2.5 h	pH 1.0 HCl solvent	74.4 ± 0.9

[a] Data are presented as means ± SD ($n = 3$)

Table 8 Comparison of MCAEC with traditional enzymatic hydrolysis

Extraction method	Enzymatic time	Buffer solvent (mL)	Hydrolysis of calcium phytate (%)[a]
MCAEC	36 min	3.0 mL	41.3 ± 0.1
Traditional enzymatic hydrolysis	10 h	20.0 mL	13.3 ± 0.2

[a] Data are presented as means ± SD ($n = 3$)

Activity of phytase

To evaluate the change in enzymatic hydrolysis activity after ball milling pretreatment, the enzymatic hydrolysis kinetics of calcium phytate were studied. Among them, the Michaelis–Menten equation is the most widely used. Using the Lineweaver–Burk method to plot phytase and

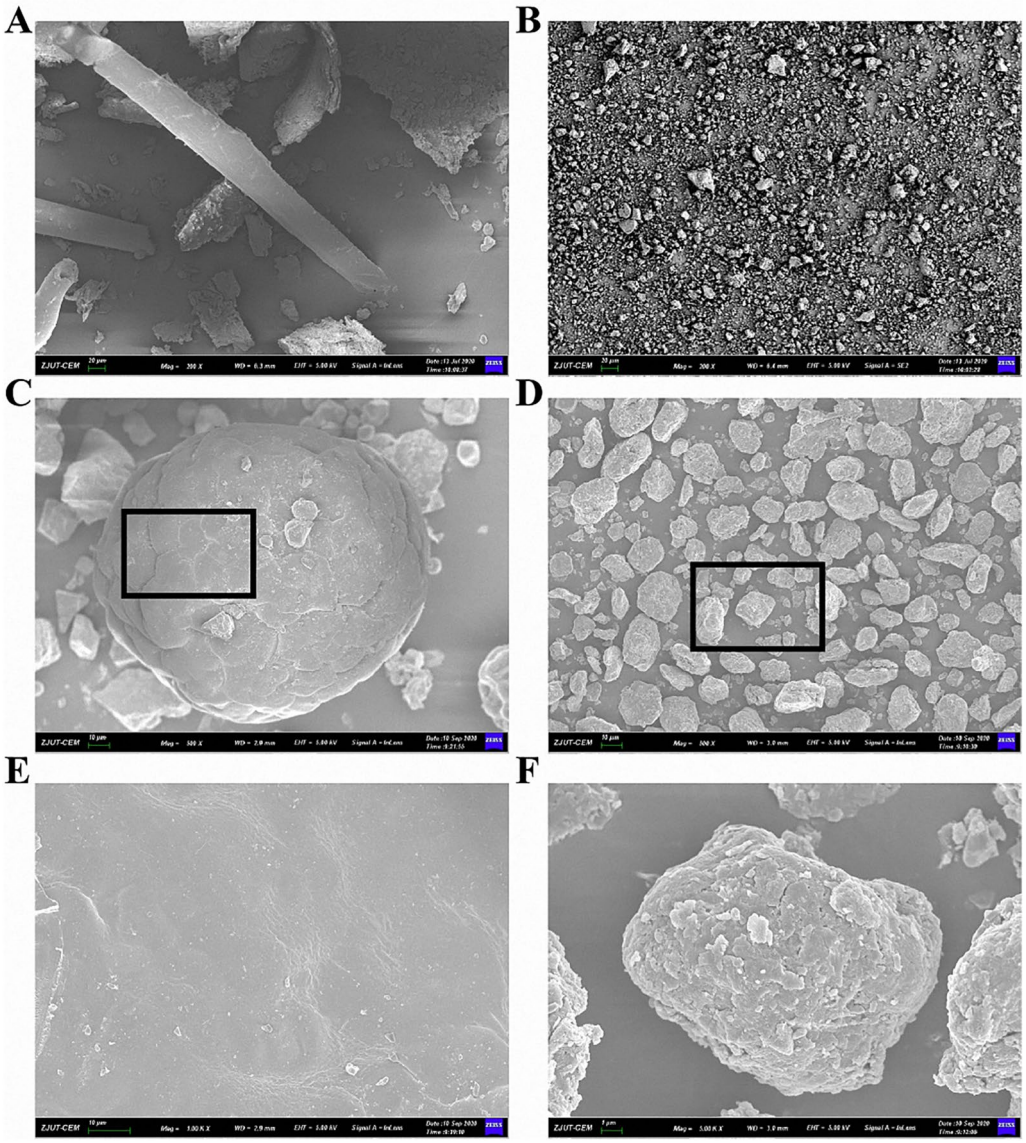


Fig. 4 SEM of defatted rice bran of raw material (**A**) and milled with Na_2CO_3 for 30 min (**B**); SEM of phytase of raw material (**C** and **E**) and milled for 20 min (**D** and **F**)

pretreated phytase at the same time, Fig. 5 was obtained. The K_m and V_{max} obtained from the Michaelis–Menten equation are shown in Table 9, which also shows that the V_{max} values of phytase hydrolysis after pretreatment is 5 times that of the original, but the K_m remains unchanged. This indicates that the protein structure of phytase will not change under ball milling pretreatment. After ball milling pretreatment, the increase in contact area between phytase and calcium phytate may be a critical factor in improving V_{max}, which improves the hydrolysis rate of calcium phytate.

Conclusion

In conclusion, MCAE has been used to extract calcium phytate from rice bran, and MCAEC has been used to hydrolyse calcium phytate to obtain *myo*-inositol. The optimum extraction and hydrolysis parameters were obtained. The results indicate that the MCAE has the advantages of reducing acid, alkali and solvent use, saving time, low temperature and high efficiency. It is a valuable method for preparing calcium phytate. Moreover, MCAEC can promote the hydrolysis of calcium phytate without the need to prepare phytic acid from

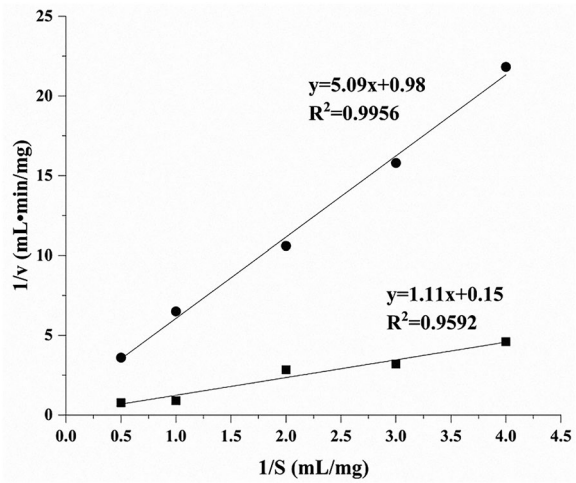


Fig. 5 L-B doubler-reciprocal plot

Table 9 Effect of pretreatment on enzyme kinetic characteristics

Method	Michaelis–Menten	V_{max} (mg/mL·min)	K_m (mg/mL)	R^2
Pre-mechanochemical phytase	$y = 1.11x + 0.15$	6.67	7.40	0.9592
Phytase	$y = 5.09x + 0.98$	1.02	5.19	0.9956

calcium phytate, which has economic value for the pharmaceutical industry.

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Author contributions
LZ: conceptualization, methodology, writing—original draft, data curation, software, investigation, formal analysis, writing—review and editing. XZ: validation, formal analysis, visualization, investigation. JW: resources, funding acquisition, project administration. WS: conceptualization, validation, investigation, formal analysis, resources, writing—review and editing, visualization, project administration, funding acquisition.

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Competing interests
The authors have declared that no competing of interest.

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