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Solid-State Fermentation of Phytase from Cassava Dregs

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Abstract

Phytases produced by numerous microorganisms and plants degrade phytic acid that has chelated with metal ions in food and feed. It is important to study phytase for the role of metal ions in nutrition of animals and humans as well as in the reduction of organic phosphate content of aqueous environment. This article reports on solid-state fermentation of phytase from a new substrate of cassava dregs. Large quantities of cassava dregs are produced in tropical areas as a byproduct of cassava starch processing. Protein and inorganic salts were found to be low in cassava dregs. Cassava dregs could be employed for phytase synthesis after the addition of a nitrogen source and mineral salts. Ammonium nitrate was the best nitrogen source among the nitrogen sources investigated, including beef extract, yeast extract, urea, ammonium nitrate, sodium nitrate, and ammonium sulfate. Sodium dodecyl sulfate promoted phytase production from cassava dregs. A maximum phytase yield of 6.73 U/g of dry mass was obtained. The obtained phytase was stable at feed-processing temperature, since 70% of initial enzyme activity was maintained after 30 min of treatment at 75°C.

Index Entries: Phytase; cassava dregs; nitrogen source; thermostability.

Introduction

Phytase, *myo*-inositol hexakisphosphate phosphohydrolase (EC 3.1.3.8), is an acid phosphatase capable of hydrolyzing phytic acid (*myo*-inositol hexakisphosphate) as well as a number of other organophosphate substrates *(1)*. Microbial and plant phytases are utilized to enhance the absorption of metal nutrients such as iron, calcium, and zinc from food and feed

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for humans and monogastric animals *(2,3)*, thereby decreasing the potential organic phosphate pollution of surface water. Since the first discovery of phytase from rice bran in 1907 and later from microorganisms, *Aspergillus spp.*'s phytases have been widely investigated and applied as feed additives. The most commonly used preparation of extracellular phytase and phosphatase was derived from *Aspergillus niger* (*ficuum*) NRRL 3135 and utilized for dephosphorylation of cottonseed meal, soya bean meal, and rapeseed meal, which are used as major protein supplements in poultry feeds *(4)*. Two phytases, PhyA (with a pH optimum of 5.0) and PhyB (with a pH optimum of 2.5) were isolated from the culture filtrate of *A. niger* NRRL 3135. These two enzymes were biochemically characterized, and partially sequenced, and the genes for these two enzymes were cloned *(1)*. A phytase with a high affinity for phytic acid was detected and purified to homogeneity in *Aspergillus niger* SK-57. A significant difference between a low-K_m phytase from *A. niger* SK-57 and a high-K_m phytase from *A. ficuum* was recognized *(5)*.

Phytase thermostability is of great interest because the enzyme should withstand the high temperature of feed processing when used as feed additive. Phytases produced from *Aspergillus fumigatus* and *A. niger* and a pH 2.5 acid phosphatase from *A. niger* were compared for their efficiency at high temperatures and their ability to withstand the heat generated during industrial processing. The phytases of *A. fumigatus* and *A. niger* were both denatured at temperatures between 50 and 70°C. In contrast to these two phytases, *A. niger* pH 2.5 acid phosphatase displayed considerably higher thermostability *(6)*. Pasamontes et al. *(7)* reported a heat-stable phytase able to withstand temperatures up to 100°C over a period of 20 min with a loss of only 10% of the initial enzymatic activity. The *phyA* gene encoding this heat-stable enzyme has been cloned from *A. fumigatus* and overexpressed in *A. niger*. The enzyme showed high activity with 4-nitrophenyl phosphate at a pH range of 3.0–5.0 and with phytic acid at a pH range of 2.5–7.5 *(7)*. A phytase gene was cloned from the thermophilic fungus *Thermomyces lanuginosus* and heterologously expressed in a *Fusarium oxysporum* strain. The *Thermomyces* phytase retained activity at a temperature up to 75°C *(8)*. A thermal-stable phytase was rapidly designed from DNA sequence using protein sequence comparisons to improve the phytase's stability *(9)*.

Nitrogen and phosphorus in media are two important factors in phytase production. Generally, inorganic nitrogen sources are more easily assimilated than organic nitrogen sources by fungi. Ammonium nitrate has found extensive application in a fungal nutrition source for a large number of imperfect fungi. Gibson *(10)* found that cornstarch was a good source of carbon and phosphorus for phytase production by *A. niger* NRRL 3135. Because the phosphorus that bound with the cornstarch was connected with C_3 of glucose in the starch and was released with difficulty, it had little effect on phytase production. On the other hand, phytase accumulation of *A. niger* NRRL 3135 was inhibited when the concentration of phosphorus was higher than 10 mg/100 g of feedstock using potato starch *(11)*.

Commercially, phytases are produced by aqueous fermentation. However, molds are naturally grown on solid-state substrates. Wheat bran and rice bran have been employed for phytase production *(12)*. Cassava is a starch crop planted in tropical and subtropical areas. Large quantities of dregs are produced annually in the cassava starch-processing factory. In China alone, >950,000 t of cassava dregs are produced each year. Apart from their partial use for ethanol production and feed, most of the dregs are discarded as landfill (13). In the present study, we report on phytase production from cassava dregs using *Aspergillus niger* PD by solid-state fermentation. We discuss nitrogen and surface tension reducing agents regarding their effect on phytase production from cassava dregs. We also note that the heat stability of the phytase obtained is suitable for feed processing.

Materials and Methods

A. niger PD is a strain stored in our laboratory. It was isolated in a rice field in Hainan, China, formerly for amylase production and now selected for phytase production. It was maintained at 4°C on potato dextrose agar (PDA) slants. Cassava dregs were obtained from a cassava starch–producing company in Qiongzhong County, Hainan, China. They were sieved to 40 meshes before using.

Inocula were prepared in 500-mL conical flasks containing 20 g of wheat bran and mineral salts solution containing 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.5 g/L of KCl, and 0.1 g/L of $FeSO_4 \cdot 7H_2O$ at 60–65% moisture content. Growth media were sterilized at 121°C for 30 min and then inoculated with spores grown on PDA slants. The cultures were grown for 3 d at 30°C.

Cassava dregs mixed with various nitrogen sources and optimized mineral salts solution were prepared with (g/kg of cassava dregs) K_2HPO_4 (0.1), $MgSO_4$ (0.5), KCl (0.5), and $FeSO_4$ (0.1), at 65% moisture content *(14)*. The media were used to produce phytase after being sterilized at 121°C for 30 min. Fermentation was carried out at 28–30°C.

Phytase activity was assayed in 1-mL samples extracted from the fermented cassava dregs with 2% $CaCl_2$ solution added, 1 mL of 0.2 *M* citric acid and sodium citrate buffer, and 5 mmol/L of phytate at pH 5.0. The mixture was incubated at 37°C for 15 min, and the reaction was terminated with 2 mL of trichloroacetic acid. The released phosphoric acid was estimated by adding 4 mL of freshly prepared solution containing 1 *M* H_2SO_4, 2.5% ammonium molybdate, and 10% ascorbic acid at a ratio of 3:1:1. The mixture was incubated at 50°C for 20 min and measured at 660 nm. A unit of phytase activity is defined as 1 µmol of phosphate/min under assay condition.

The fermented cassava dregs were dried at 42 ± 2°C for 8–10 h, comminuted, and stored at 4°C for the stability tests. We termed the enzyme obtained with the solid substances *koji*. Thermal stability at different temperatures was carried out as follows: The koji was treated at various tem-

peratures (25, 40, 50, 60, 70, 80, and 90°C for 30 min), and the phytase activities were analyzed after the koji had cooled. Thermal stability at 75°C was detected every 20 min after the koji was treated at 75°C for 30 min. The koji stored at 25°C was used as control during the analysis.

Results and Discussion

The composition of cassava dregs differed considerably from the conventionally used solid-state fermentation substrates. Compared to rice chaff and wheat bran, cassava dregs contain higher starch and cellulose but lower protein and minerals. Nitrogen sources and other necessary minerals should be added if these dregs are employed as phytase production substrates. The composition of three batches of cassava dregs varied in this study. No growth was observed using cassava dregs as a sole substrate, even though phytase was produced using rice chaff or wheat bran as sole substrates. On the other hand, phosphate content in cassava dregs was found to be very low compared to rice chaff and wheat bran (Table 1). This is a useful property for phytase production, since it was reported that high phosphate inhibited phytase accumulation *(10,11)*.

The nitrogen source is an important substrate that affects enzyme production. Various nitrogen sources were investigated for their effects on phytase accumulation. We found that higher phytase activities were produced when adding inorganic rather than organic nitrogen sources to cassava dregs. Urea gave the highest phytase accumulation among organic nitrogen sources, while NH_4NO_3 produced the highest accumulation among the inorganic nitrogen sources. The highest phytase activity was obtained at 1 to 2% NH_4NO_3 among the nitrogen sources tested (Fig. 1). It was suggested that when grown on cassava dregs, *A. niger* PD preferred ammonium (both from inorganic sources and organic sources) over other nitrogen sources, and ammonium nitrate was the most preferred salt among the ammonium salts used. Sodium nitrate was reported to be the most positive nitrogen for *A. niger* HZ-94 in phytase production on starch liquid culture, and no phytase activity was detected when using urea as the nitrogen source *(15)*. That finding clearly contradicts our result. Urea was found to promote α-galactosidase production by *A. niger* MRSS 234 in solid-state fermentation and its effect increased with the fermentation time, in contrast to the negative effect of all the ammonium salts used *(16)*. The effect of nitrogen sources on enzyme production by fungi is complicated. It is generally accepted that inorganic nitrogen sources are more easily assimilated than organic nitrogen sources by fungi; ammonium nitrate has been applied in a fungal nutrition source for many imperfect fungi. This may be owing to the fact that the principal metabolite of nitrogen metabolism is glutamic acid, and a nitrogen source assimilation that can easily lead to the formation of glutamic acid will be the most positive source. In our study, urea and ammonium nitrate may afford ammonium a more easily formed glutamic acid than other sources. When other mineral salts, including K_2HPO_4,

Table 1
Components of Cassava Dregs, Rice Chaff, and Wheat Bran and Phytase Activity Produced When Used as Sole Substrate

	Starch (%)	Protein (%)	Cellulose (%)	Minerals (%)	Fatty acids (%)	Total phosphorus (%)	Phytase activity (U/dry mass)[a]
Cassava dregs							
I	45.8	2.12	42.9	2.63	2.47	0.006	No growth
II	55.9	2.97	34.7	2.32	2.71	0.008	No growth
III	70.1	4.37	16.5	3.05	2.95	0.012	No growth
Rice chaff	39.1	12.1	15.6	13.6	14.7	1.21	1.63
Wheat bran	47.3	17.3	17.8	7.57	3.6	0.33	2.16

[a]Fermentations were carried out at 30°C for 7 d.

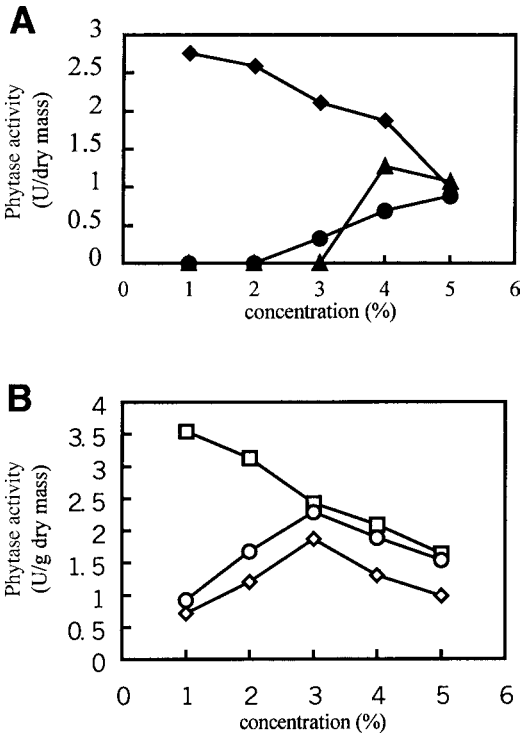

Fig. 1. Effect of **(A)** organic nitrogen sources of urea (◆), beef extract (●), and yeast extract (▲) and **(B)** inorganic nitrogen sources of NH_4NO_3 (□), $(NH_4)_2SO_4$ (○), and $NaNO_3$ (◇) on phytase synthesis from cassava dregs, with mineral salts of (g/kg of cassava dregs) K_2HPO_4 (0.1), $MgSO_4$ (0.5), KCl (0.5), and $FeSO_4$ (0.1) added at 65% moisture content and 30°C for 7 d.

$MgSO_4$, KCl, and $FeSO_4$, were investigated, together with ammonium nitrate for the purpose of optimizing their quantity mixed to cassava dregs, by $L_9(3)^4$ orthogonal experiments, the optimized concentration of ammonium nitrate was 2% *(14)*.

Surface tension–reducing agents at concentrations of 0–1.5% were investigated for their effect on phytase accumulation. Sodium dodecyl sulfate (SDS) promoted phytase accumulation by more than double, but Tween-80 and oleic acid had no effect (Fig. 2). Since SDS has a strong anionic surface tension–reducing activity, this activity may be owing to the effect of SDS on permeation of cell membranes. To prove this, further experiments will be necessary.

The maximum phytase activity was produced with the following added minerals (g/kg of cassava dregs): K_2HPO_4 (0.1), $MgSO_4$ (0.5), KCl (0.5), and $FeSO_4$ (0.1), at 65% moisture content. Under these optimized conditions, 6.73 U/g of dry mass phytase was obtained on d 8, and the maximum protein content presented on d 7. High total sugar content still remained at 31.9% on d 11 (Fig. 3). The high content of total sugar may owing to the unused starch in the dregs. Further investigation is in progress

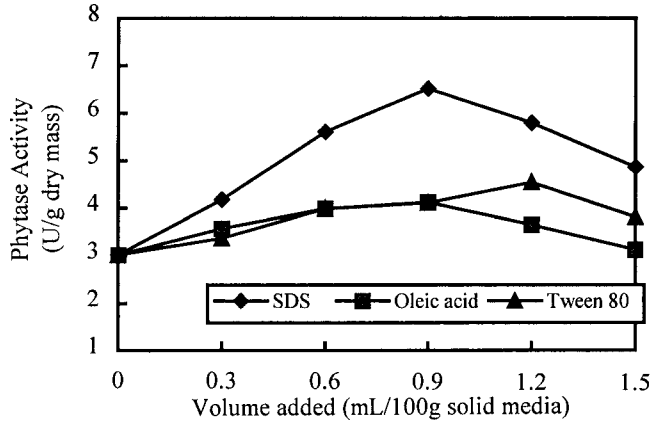

Fig. 2. Effect of surface tension reducers: SDS (◆), Tween-80 (▲), and oleic acid (■) on solid-state fermentation of phytase synthesis from cassava dregs, with mineral salts of (g/kg of cassava dregs) K_2HPO_4 (0.1), $MgSO_4$ (0.5), KCl (0.5), and $FeSO_4$ (0.1) added at 65% moisture content and cultured at 30°C for 7 d.

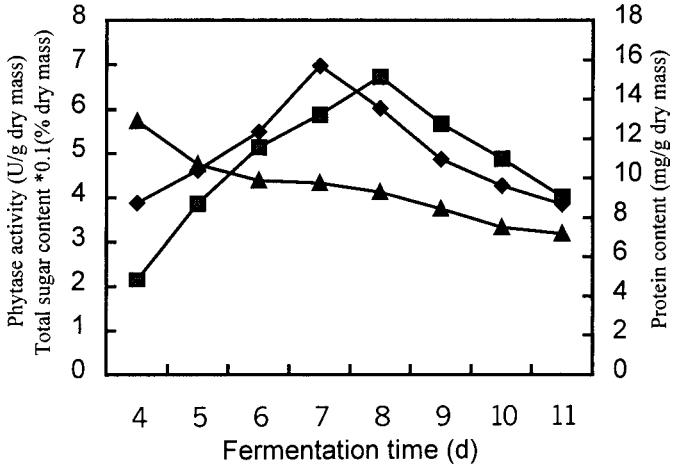

Fig. 3. Solid-state fermentation time course of phytase from cassava dregs, with mineral salts of (g/kg of cassava dregs) K_2HPO_4 (0.1), $MgSO_4$ (0.5), KCl (0.5), and $FeSO_4$ (0.1) added at 65% moisture content and 30°C for 7 d. Phytase activities (■), total sugar contents × 0.1 (▲), and protein contents (◆) vary with fermentation time.

using a mixed culture of *A. niger* PD with another amylase-producing strain that should improve phytase production by consuming more starch.

Phytase thermal stability was investigated to determine its utility as a feed additive. Enzymes used as animal feed supplements should be able to withstand temperatures as high as 60–90°C, which may be reached during the feed-pelleting process *(7–9)*. We treated the obtained solid phytase at temperatures of 25, 40, 50, 60, 70, 75, 80, and 90°C for 30 min and found that *A. niger* PD phytase maintained 50–80% activity between 60 and 90°C over

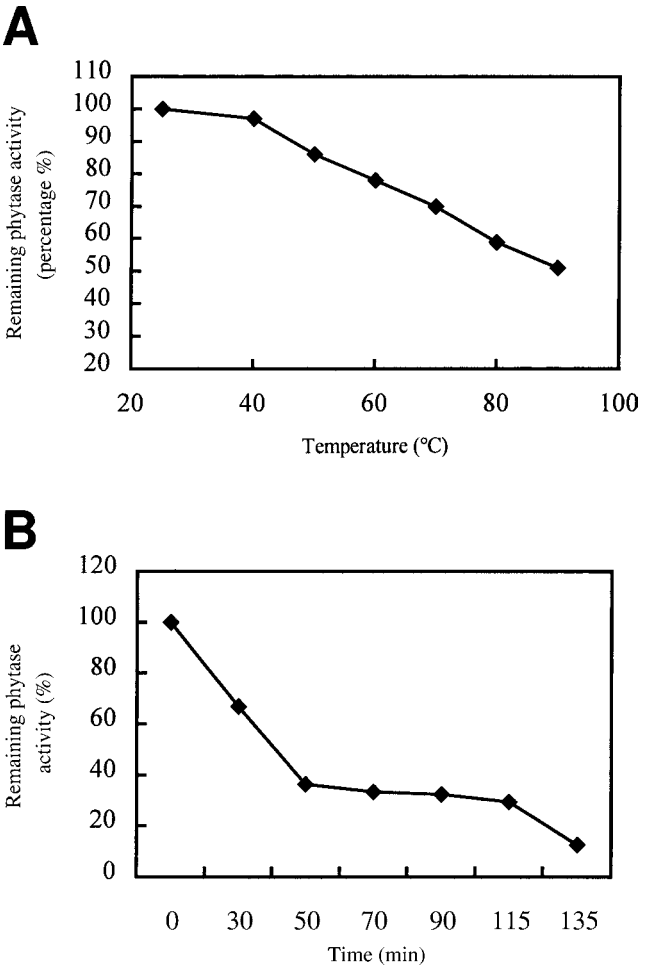

Fig. 4. **(A)** Thermal stability of phytase produced from cassava dregs. The obtained solid phytase was treated at various temperatures (25, 40, 50, 60, 70, 80, and 90°C for 30 min), and the phytase activities were analyzed after cooling. **(B)** Thermal stability at 75°C was detected every 20 min after the solid phytase was treated at 75°C for 30 min.

30 min, while 70% phytase activity was maintained at 75°C (the feed-pelleting temperature) over 30 min (Fig. 4). These findings suggest that this enzyme could be used in feed additives.

Conclusion

We have attempted to find a new application for cassava dregs by producing phytase from the dregs. The content of phosphorus in cassava dregs was lower compared to other conventionally used solid-state fermentation substrates, a feature useful for phytase production. Mineral salts, especially ammonium salt, should be added as a nitrogen source because

nitrogen content is low in cassava dregs. Ammonium nitrate was found to be the most effective nitrogen source among the nitrogen sources tested. SDS promoted phytase production. The thermal stability of the obtained phytase adapted to the temperature of feed processing. Further investigation will explore additional improvement in starch utilization of the dregs and the efficiency of application.

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