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Phytase production by *Aspergillus ficuum* on semisolid substrate

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SUMMARY

Phytase production by *Aspergillus ficuum* was studied using solid state cultivation on several cereal grains and legume seeds. The microbial phytase was used to hydrolyze the phytate in soybean meal and cotton seed meal. Wheat bran, soybean meal, cottonseed meal and corn meal supported good fungal growth and yielded a high level of phytase when an adequate amount of moisture was present. The level of phytase production on solid substrate was higher than that obtained by submerged liquid fermentation. Higher levels of phosphorus (more than 10 mg Pi/100 g substrate) in the growth medium (static culture) inhibited phytase synthesis, and the degree of phosphorus inhibition was less apparent in semisolid medium than in liquid medium. A static cultivation on semisolid substrate produced a higher level of phytase (2–20-fold) than that obtained by agitated cultivation. The minimal amount of water required for growth and enzyme production on those substrates was about 15%, while the optimum level for phytase production was between 25 and 35% and that for cell growth was above 50%. Optimum pH for phytase production was between 4 and 6. *A ficuum* grew well on raw (unheated) substrate containing a minimal amount of water and produced as much phytase as on heated substrate. About half of the phytic acid in soybean meal and cottonseed meal was hydrolyzed by treatment with *A. ficuum* phytase.

INTRODUCTION

Phytic acid and its derivatives in cereals and legumes are known to bind essential dietary minerals, thus making them unavailable or only partially available for absorption by animals (Fig. 1). The ability of phytic acid to bind metal ions is lost when the phosphate groups are hydrolyzed through the action of the enzyme phytase. Thus, attempts have been made to reduce the phytic acid level in animal diets by application of microbial phytase [4,14,21]. However, low yield and high cost of enzyme production were cited as limiting factors in using the enzyme in animal diets.

Semisolid fermentation has been used for the production of enzymes, mycotoxins, mushrooms, fermented foods and feed [2,3,5,7,12,18,20,22]. In general, semisolid fermentation is simple, less expensive and yields higher amounts of product compared to liquid fermentation. The main difference between submerged liquid fermentation and semisolid cultivation is that the substrate in the former

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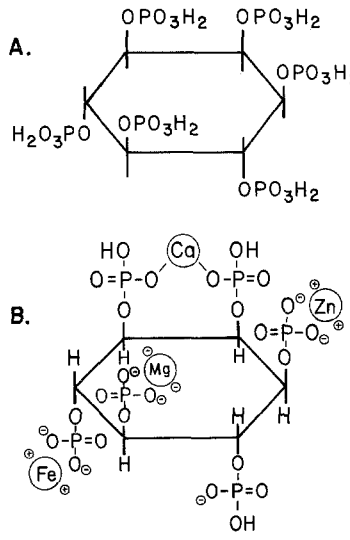

Fig. 1. Structure of phytic acid (A) and phytic acid-mineral chelates at neutral pH (B).

is completely dissolved and homogeneous whereas the latter employs insoluble substrate with relatively little liquid in the growth environment. In spite of its simplicity and ease of operation, due to heterogeneity of the fermentation mixture, the controls for semisolid fermentation are not as simple as those for homogeneous submerged cultures, especially in large-scale operations [1,7,9,12]. In this paper, we report the characteristics of microbial growth and phytase production by *Aspergillus ficuum* on semisolid substrates under various growth conditions.

MATERIALS AND METHODS

Organism and growth conditions. A ficuum (NRRL 3135) was grown and maintained on potato dextrose agar at pH 6.8 and 30°C. The conidia formed on the agar surface were scraped off and collected in distilled water. A portion of spore suspension was washed and resuspended in distilled water (about 10^7 spores/ml) and used as an inoculum. Water was added to cereal grains and legume seeds to obtain a final moisture content of 10–60%, and the mixtures were steam-sterilized before subjecting to fungal growth. Moisture content was determined by drying at 105°C to a constant weight and the materials were sterilized for 30 min at 121°C. The initial pH of both substrates was about 6.3. In a typical fermentation run, 50 g of substrate in a 400 ml glass container was inoculated with 0.5 ml of spore suspension and fermented at room temperature (about 25°C) for 1–2 weeks.The fermentation was carried out either in stationary culture or an agitated mode in which the fermentation mixture was agitated by rotating the jars on a plate rotating vertically at 1 rpm. Composition of the liquid medium and the method for cultivation in liquid medium were reported elsewhere [4]. Growth of cell mass in solid substrate was determined by measuring the level of glucosamine according to the method of Sakurai et al. [16].

Measurement of enzyme activity. Phytase activity was assayed by following the release of orthophosphate from phytate. The liberated inorganic phosphate (Pi) was determined by the method of Heinonen and Lahti [6]. In the study where high initial Pi interferes with the determination of Pi liberated by the enzyme, *p*-nitrophenyl phosphate (PNPP) was used as a substrate and the enzyme activity was reported as phosphatase activity. The enzyme reaction mixture contained 0.1 ml of suitably diluted culture filtrate, 3.0 ml of 0.1 M acetate buffer (pH 5.4), 0.5 ml of 15 mM PNPP or Na-phytate. The reaction mixture was incubated for 30 min at 37°C. At the end of the reaction, the color developed was measured by reading the optical density at 420 nm. Optical density was correlated to the unit of enzyme activity using commercial phytase (Sigma No. P-1259) and acid phosphatase (Sigma No. P-3627). One unit of enzyme is defined as the amount of enzyme required to liberate 1 μM of Pi per min under the assay condition.

Analytical methods. Soybean meal, corn meal, cottonseed meal and wheat bran were obtained from a local feed store. The moisture content in the air-dried substrate was about 11%. The water content in the substrates was determined by drying the material to a constant weight at 105°C. The phytic acid in soybean meal and cottonseed meal was hydrolysed by phytase produced by *A. ficuum* that was grown on semisolid soybean meal. The substrate

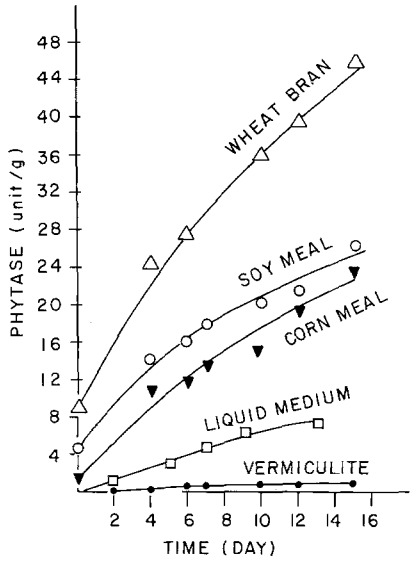

Fig. 2. Effect of different substrates on phytase production. *A. ficuum* was grown on cereal grains and legume seeds containing 30% initial moisture

was mixed with a phytase solution adjusted to pH 5.4 and the mixture was incubated at 50°C for 1 h. The phytate in the hydrolysate was extracted with 0.65 N HCl for 1 h at 28°C and passed through an anion exchange column (Dowex 1-X8, Bio-Rad Laboratory, Richmond, CA) using 0.7 M NaCl as an eluent. Phytic acid was determined according to the method of Latta and Eskin [11] and total phosphorus was determined by the X-ray fluorescence method of Knudsen et al. [10].

RESULTS AND DISCUSSION

A. ficuum grew well on steamed wheat bran, soybean meal and corn meal, without any added nutrients. The highest amount of phytase was produced on wheat bran, followed by soybean meal and corn meal (Fig. 2). Cell growth and enzyme production were poor on inert material (e.g., vermiculite) which was impregnated with the liquid growth medium, the composition of which is described elsewhere [4]. The levels of phytase production on semisolid substrates were higher than in the liquid medium. *A. ficuum* produced 45 units of phytase per g of wheat bran, whereas less than 10 units/ml of the enzyme were produced in liquid medium after a 2-week cultivation. Although vermiculite has been used successfully for production of amylase [13], it was not successful for phytase production by *A. ficuum*.

The water content of substrate plays an important role for both cell growth and enzyme production in a solid state fermentation [1,8]. Addition of water causes swelling of the substrate and facilitates its utilization by the microorganisms. However, the optimal amount of water required varies and it must be determined for each system. Our results show that the minimal amount of water required for cell growth and phytase production by *A. ficuum* on legume seeds and cereal grains is about 15%, whereas the optimal level for phytase production is between 25 and 35% and that for cell growth is above 50% (Fig. 3). The level of phytase produced was drastically reduced when the water content exceeded 40%, and the range of optimal water content for the enzyme production (20–50%) was much narrower than that for cell growth (over 50%). Kim et al. [8] also reported that the optimum water content for cellulase production was lower and the range was narrower than that for cell growth in the case of *Trichoderma reseii* and *Spo-*

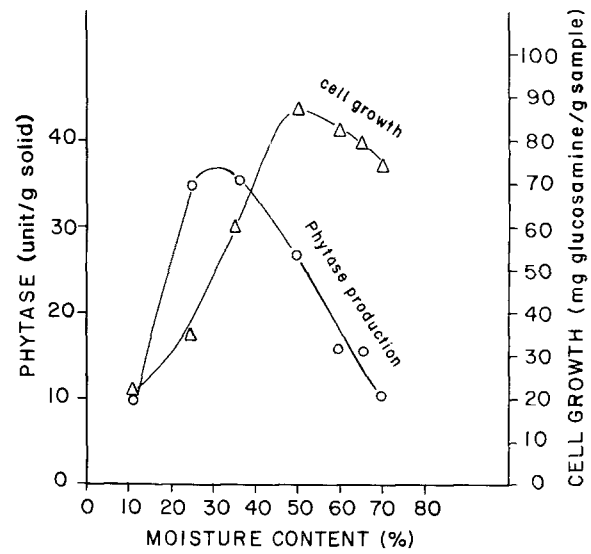

Fig. 3. Effect of moisture content on cell growth and phytase production. *A. ficuum* was grown for 7 days at room temperature on soybean meal containing different levels of moisture.

rotrichum cellulophilum grown on semisolid wheat bran. Apparently, excess of water is unfavorable for enzyme synthesis, while it is less detrimental for cell growth. Lower moisture resulted in a longer lag phase, a longer period to reach the production peak. Higher moisture not only yielded low phytase, it also resulted in substrate adhering to fermentor walls, and prevented the access of air to the substrate, rendering the substrate more vulnerable to bacterial contamination. As the fermentation proceeded, the moisture content in the substrate increased and the texture of the substrate became soggy due to the microbial metabolism.

The initial phosphate level in the growth medium is important for the production of phosphates. In general, a high level of inorganic phosphate (Pi) inhibits the synthesis of phosphatase, and the type and amount of phosphatase synthesized is dependent on the concentration of Pi present in the growth medium [15,17]. Therefore, the optimal amount of Pi in the growth medium should be carefully determined. The addition of 10–50 mg Pi to 100 g soybean meal produced the highest level of phytase by *A. ficuum* in a static solid substrate cultivation, whereas the minimal amount of Pi was difficult to measure because of the inherent phosphorus in the substrate. Corn meal, cotton seed meal and soybean meal contained 0.03%, 0.34% and 0.24% of total phosphorus, respectively. The optimum Pi concentration (10–100 mg Pi/100 g substrate) for phytase production on semisolid substrate was higher and the range broader than that found in liquid medium (1–5 mg Pi/100 g substrate) [4]. The mode of cultivation in semisolid fermentation also affected the optimal concentration of Pi. For instance, in a static culture where substrate was not disturbed during cultivation, optimal Pi concentration was 10–50 mg Pi/100 g substrate, whereas 50–100 mg/100 g substrate was found to be optimal in agitated culture (Table 1). Thus, the inhibitory effect of Pi in phytase synthesis was less apparent in semisolid culture, especially when the substrate was agitated during cultivation. The inhibitory effect of Pi was more apparent with monophosphate (e.g., K_2HPO_4) than with polyphosphate (e.g., $Ca_2P_2O_7$).

The level of enzyme synthesis was dependent on the initial pH of the substrate. The optimum pH for phytase production was between 4.0 and 6.0, whereas that for phosphatase was between 2.0 and 2.5. The pH profile for phytase production by *A. ficuum* on semisolid substrate was similar to that reported on liquid cultivation of the same organism [4].

A. ficuum grew well on raw (unheated) grains and produced about the same level of phytase as that on heated substrates (Table 2). Degradation of starch materials by amylolytic organisms generally

Table 1

Effect of inorganic phosphate (Pi) on production of phytase by *A. ficuum* on semisolid soybean meal

Pi[a] (mg/100 g substrate)	Phytase (unit/g substrate) Static growth[b]	Agitative growth[c]
0	8.0	4.0
1	8.0	4.0
10	82.5	4.0
50	56.0	12.0
100	17.5	9.0
500	15.0	6.0
1000	46.0	4.0

[a] Various amounts of K_2HPO_4 were added to soybean meal. The untreated soybean meal contained 0.24% total phosphorus.

[b] *A. ficuum* was grown on semisolid soybean meal for 10 days at room temperature without agitating the substrate.

[c] *A. ficuum* was grown on semisolid soybean meal for 10 days at room temperature with constant agitation on a rotating wheel as described in the Materials and Methods section.

Table 2

Effect of heating substrate on cell growth and phytase production by *A. ficuum*

Substrate	Phytase (unit/g)		Cell mass (mg/g)[a]	
	not heated[b]	heated[c]	not heated[b]	heated[c]
Corn meal	14.0	12.0	49.2	43.8
Soybean meal	27.0	25.0	38.3	38.9
Wheat bran	39.0	32.0	48.1	52.7

[a] Cell mass was measured by the level of glucosamine.
[b] Substrate was sterilized by propylene oxide (1 ml/100 g substrate) for 24 h at room temperature.
[c] Substrate was heated at 121°C for 30 min.

Table 3

Hydrolysis of phytic acid in soybean meal and cotton seed meal by *A. ficuum* phytase

Substrate	Treatment[a]	Phytic acid extracted[b] (%)	Phytic acid hydrolyzed (% of original)
Soybean meal	H_2O	2.3 ± 0.12	0
	Phytase	1.4 ± 0.33	38.5
Cotton seed meal	H_2O	4.1 ± 0.31	0
	Phytase	2.3 ± 0.20	43.4

[a] 2 g substrate was mixed with 30 ml phytase (10 units) solution adjusted to pH 5.4 and incubated at 50°C for 1 h. An equal portion of the substrate was mixed with H_2O and incubated under the same condition as a control.
[b] Phytic acid extracted with H_2O and 0.65 N HCl. Values are means and standard deviations of 10 replicated samples.

requires gelatinization of starch prior to amylase action. Because of the intensive use of energy in the cooking process, microorganisms that can attack raw starch have been sought [19]. Elimination of the heating process in enzyme production is also desirable.

Soybean meal and cotton seed meal contained about 2.3% and 4.1% phytic acid, respectively, and the phytate was readily hydrolyzed by microbial phytase (Table 3). About 38% of phytic acid in soybean meal and 43% in cotton seed meal was hydrolyzed by treating the substrates with phytase produced by *A. ficuum* grown on semisolid soybean meal. Further removal of phytate in the seeds may be possible by treatment with a higher enzyme activity, a longer reaction time and providing more favorable reaction conditions.

Higher levels of phytase were produced by *A. ficuum* when grown on semisolid substrates than on a liquid medium. The levels of moisture and phosphorus in the growth medium were important and should be carefully controlled in the production of phytase. The semisolid fermentation was simple and easy to operate, but its control for fermentation parameters (e.g., maintenance of pH, moisture, aeration and agitation) would be difficult in a large-scale operation.

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