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Survey of Microorganisms for the Production of Extracellular Phytase

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A culture enrichment technique was used to isolate phytase-producing microorganisms. Also, microorganisms from various culture collections were tested for their phytase-producing ability. A number of the *Aspergillus niger* group produced extracellular phytase which dephosphorylated calcium phytate in acidic solution. A soil isolate, *A. ficuum* NRRL 3135, produced the most active phytase in a cornstarch-based medium. Production of phytase was strongly repressed by inorganic phosphates and required a high carbon to phosphorus ratio in the medium.

Many species of plants contain appreciable amounts of phytate phosphorus (2). This organically bound form of phosphorus is poorly utilized by monogastric animals, such as poultry and swine (10, 14), because their simple digestive tract cannot hydrolyze substantial amounts of phytate. Selective enzymatic hydrolysis of phytate phosphorus should release the phosphorus in a form available to the animal. Enzymes of this type have been found in wheat bran (9), in various plant seeds (7, 8, 11), in plant storage organs such as potato (6), in tobacco leaves (13), and have been obtained from microorganisms (1, 3, 5, 12). This paper describes the screening of various microorganisms to determine which ones are producers of highly active extracellular acid phytase. Factors affecting production of this enzyme by a mold are also presented.

MATERIALS AND METHODS

Preparation of calcium phytate. Calcium phytate, free of orthophosphate, was prepared by a modification of the procedures of Common (2) and Casida (1). Crude sodium phytate (30 g; Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in 250 ml of 0.15 N HCl, and undissolved impurities were removed by filtration. The clear supernatant fluid was neutralized to phenolphthalein with 25% (w/v) NaOH solution, and 110 ml of 10% (w/v) $FeCl_3$ was added. Iron phytate was recovered by centrifugation and washed three times with 800 ml of 0.15 N HCl to remove ferric phosphate. The white iron phytate was resuspended in water, and portions of 5% (w/v) NaOH solution were added until no additional brown precipitate formed. The supernatant liquid was adjusted to *p*H 7.0, and portions of 20% (w/v) calcium acetate were added until no further precipitate formed. The calcium phytate was washed three times with 500 ml of methanol and air-dried to a white powder.

Organisms. A few of the microorganisms tested for extracellular phytase production were isolated from enrichment cultures. Other cultures tested were obtained from the American Type Culture Collection, Rockville, Md., and from the cultural collections of Purdue University and the University of Wisconsin.

Screening techniques for isolating phytase-producing organisms. Enrichment culture media containing calcium phytate as the sole phosphorus and carbon source were used for the primary screening of phytase producers. The method takes advantage of the insolubility of calcium phytate in aqueous media which gives a white turbidity in an agar plate. At least four different media containing 0.5% (w/v) calcium phytate were used for the plate screening method: (i) Czapek's medium with the omission of phosphate and sucrose was used as a phosphate- and carbon-deficient mineral medium; (ii) Czapek's medium without phosphate was used as a phosphate-deficient medium; (iii) malt-yeast extract medium was employed for fungi; and (iv) nutrient medium was used for bacteria. Every medium used was sterilized by autoclaving at 121 C for 15 min.

When diluted soil samples were seeded into the agar plate, any developing colony which produced a clear zone was considered a potential phytase producer. Any colony that developed in media (i) or (ii) and any colony that developed a clear zone in either media (iii) or (iv) was isolated. Flask tests were made in the following fermentation media. (i) Malt-yeast extract broth consisted of (per liter): malt extract (Difco), 3.0 g; yeast extract (Difco), 3.0 g; peptone (Difco), 0.5 g; and glucose (Difco), 10 g; *p*H 6.8. (ii) Cornstarch medium consisted of (per liter): cornstarch (Hubinger), 80 g; glucose, 30 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.1 g; $NaNO_3$, 8.6 g; K_2HPO_4, 0.2 g; *p*H 5.0.

Inoculum for the molds was prepared by transfer-

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ring 2×10^7 spores per ml from stock slants to 50 ml of cornstarch medium in 250-ml Erlenmeyer flasks which were incubated for 3 days at 28 C on a rotary shaker (270 rev/min). A 5-ml amount of 3-day cultures was used to inoculate 50 ml of fermentation medium. The molds were grown for 5 days on the rotary shaker. Bacteria and yeasts were tested for phytase production in the same manner, except that the inoculum was a 24-hr culture in malt-yeast extract broth. The cultures were grown for 3 days.

Phytase activity measurement. Phytase activity was determined by mixing 0.1 ml of suitably diluted culture filtrate and 0.9 ml of 0.2 M acetate buffer, *p*H 4.5, containing 0.5 mg of calcium phytate. The mixture was incubated for 15 to 30 min at 37.5 C. Liberated orthophosphate was determined by the method of Fiske and Subbarow (4). One phytase activity unit was defined as the amount of enzyme that liberates 1 mg of inorganic phosphate per hour.

RESULTS AND DISCUSSION

Survey of microorganisms for production of extracellular phytase. More than 2,000 cultures were isolated from 68 soil samples in enrichment culture medium. Extracellular phytase activity was found mainly among the various molds tested. Extracellular phytase activity was observed with 28 of 82 *Aspergillus* spp., 1 of 58 *Penicillium* spp., and 1 of 37 *Mucor* spp. None of the 13 *Rhizopus*, 4 *Cunninghamella*, 4 *Neurospora*, 140 yeasts represented by 17 genera, or 56 bacterial cultures represented by 18 genera produced extracellular phytase activity. It was demonstrated, however, that many of the above organisms possessed intracellular phytase activity. Of the 30 mold culture filtrates that attacked calcium phytate, 28 were from molds of the genus *Aspergillus*. The most active group was *A. niger* (Table 1). Many other species of *Aspergillus* produced considerable amounts of intracellular phytase activity but negligible amounts of phytase activity in the culture filtrate. The amounts of extracellular phytase produced by various *A. niger* strains varied (Table 2). Many of the soil isolates which cleared the calcium phytate agar plates were *A. niger* strains. *A. ficuum* NRRL 3135 was of special interest because it did not sporulate in most media and produced the highest phytase activity.

Effect of phosphate on the production of extracellular phytase by A. ficuum NRRL 3135. When simple sugars such as glucose and sucrose were used as a sole source of carbon, mycelial pellets were formed and low yields of phytase were observed. Certain types of cornmeal, however, gave dispersed mycelial growth and higher enzyme yield. The phytase yield in cornmeal medium, however, varied with the lot of cornmeal, depending on the phosphorus content of corns from various locations (Table 3). It became apparent that high total phosphorus content of corn grains suppressed phytase production by the mold. Feed grade cornstarch and dextrin were also used successfully by limiting the orthophosphate content of the medium. With limited

TABLE 1. *Survey of Aspergillus species for production of extracellular acid phytase*

Species	No. of cultures tested	No. producing acid phytase
A. fumigatus	6	0
A. flavus	10	2
A. flavipes	3	0
A. clavatus	4	0
A. glaucus	7	0
A. nidulans	10	0
A. ochraceus	2	0
A. versicolor	3	1
A. terreus	2	1
A. ustus	2	0
A. wentii	3	0
A. restrictus	1	0
A. niger	22	21
A. candidus	2	0
Aspergillus species[a]	3	3

[a] Soil isolates.

TABLE 2. *Survey of Aspergillus niger varieties and strains for production of extracellular acid phytase*

Varieties	Activity (units/ml)
A. niger ATCC 9142	2.1
A. niger ATCC 10864	0.8
A. niger van Tieghem	3.5
A. niger var. *cinnamoneum* NRRL 348	1.4
A. niger japonicus saito ATCC 1034	0.6
A. niger NRRL 372	1.0
A. niger NRRL 326	0.7
A. niger NRRL 330	0.8
A. niger NRRL 4361	0.7
A. niger NRRL 337	0.5
A. awamori ATCC 11382	2.3
A. awamori ATCC 11358	3.3
A. saitoi ATCC 11362	1.8
A. carbonarius NRRL 368	1.9
A. carbonarius PCC 104	1.5
A. tubingensis NRRL 4875	2.4
A. ficuum WB 4016	1.1
A. ficuum WB 320	1.0
A. ficuum WB 364	1.5
A. ficuum WB 4541	1.0
A. ficuum WB 4781	1.2
A. ficuum NRRL 3135 (soil isolate)	10.5
A. niger X (soil isolate)	5.0
A. niger K (soil isolate)	4.0

concentrations of phosphorus in the medium, mycelial growth of the mold was only suboptimal, but phytase production was maximal. The growth of mold increased with further increases in phosphate, but phytase synthesis decreased drastically when increasing amounts of phosphate were added to the medium (Fig. 1).

Inhibition of phytase synthesis by phosphate seems to be a general phenomenon, since it was observed in all species of molds and yeasts which were able to synthesize phytase.

TABLE 3. *Effect of phosphate content of various corn grains on phytase production by Aspergillus ficuum NRRL 3135*[a]

Sample no.	Total phosphate content (%)	Activity (units/ml)
15	0.10	6.4
14	0.17	6.0
5	0.24	1.5
8	0.28	0.52
1	0.29	0.52
9	0.30	0.40
6	0.31	0.48
7	0.32	0.40
4	0.44	0.16
3	0.69	0.15
2	0.70	0.15

[a] Basal medium consisted of (per liter): corn meal, 50 g; glucose, 30 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.1 g; $NaNO_3$, 8.6 g; K_2HPO_4, 0.2 g; *p*H 5.0.

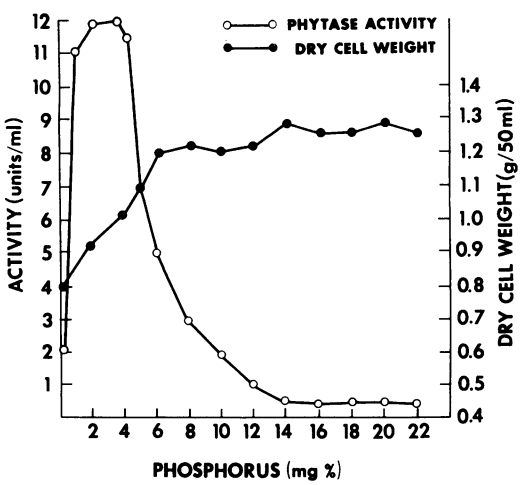

FIG. 1. *Effect of phosphorus content on growth and production of phytase by Aspergillus ficuum* NRRL 3135. *Basal medium contained (per liter): glucose, 30 g; cornstarch, 80 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.1 g; $NaNO_3$, 8.6 g; phosphorus was added as K_2HPO_4; pH 5.0.*

TABLE 4. *Effect of phosphorus and carbon content on phytase production by Aspergillus ficuum NRRL 3135*[a]

Phosphorus (mg/100 ml)	Cornstarch					
	2%		4%		8%	
	C/P[b]	Units/ml	C/P[b]	Units/ml	C/P[b]	Units/ml
6	148	0	296	1.1	532	10.8
4	222	0	444	1.9	882	11.4
3	296	0.10	592	1.9	1,184	12.7
2	444	0.65	888	2.3	1,776	9.7
1	888	1.30	1,976	1.9	3,552	10.8
0.4	2,220	1.10	4,440	2.2	8,880	12.7

[a] Basal medium contained (per liter): glucose, 30 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.1 g; $NaNO_3$, 8.6 g; *p*H 5.0.

[b] Carbon to phosphorus ratio.

The induction of phytase by limiting inorganic phosphate in the medium, however, was dependent upon the total carbon present in the medium. The production of phytase in pure cornstarch synthetic medium increased with increasing ratios of carbon to phosphorus at given low levels of phosphorus in the medium (Table 4).

A. ficuum NRRL 3135 produced the most active phytase when the inorganic phosphate concentration was less than 0.004% (w/v) in a medium containing 8% (w/v) cornstarch. Since phytase is produced when the inorganic phosphate concentration becomes limiting, the organisms are provided with a means of obtaining inorganic phosphate from organic phosphates when this becomes necessary.

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