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THE STRAIN IMPROVEMENT OF
ASPERGILLUS FICUUM NRRL 3135

BY

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B.S., University of Central Florida, 1984

THESIS
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ABSTRACT

A strain improvement program was initiated to increase the yield of extracellular phytase (EC 3.1.3.8) in *Aspergillus ficuum* NRRL 3135. A minimal plating medium containing 2.99 g liter\(^{-1}\) \(\alpha\)-ketoglutaric acid was developed to decrease and stabilize conidiogenesis time and facilitate the isolation of single colonies. Exposure of *A. ficuum* conidia to 0.5 mg ml\(^{-1}\) \(N\)-methyl-\(N'\)-nitro-\(N\)-nitrosoguanidine (NTG) for 15 min at pH 6.5 caused a one log kill and an increase in the mutation rate of 800-fold. Treatment of *A. ficuum* conidia with 46 ergs mm\(^{-2}\) sec\(^{-1}\) UV radiation for 30 sec resulted in a one log kill and a 100,000-fold increase in mutation rate. Mutants which overproduce extracellular acid phosphatase(s) were tentatively identified in a plate assay by histochemical staining with 0.1 g ml\(^{-1}\) \(\alpha\)-naphthyl acid phosphate and 0.04 g ml\(^{-1}\) fast black K-salt in 0.2 M acetate buffer pH 4.8. Confirmation of extracellular acid phosphatase overproduction was by analysis of shaker flask fermentation broths via colorimetric enzyme assays with 0.04 M p-nitrophenyl phosphate and 2.5 \(\mu\)M sodium phytate as substrates. A UV derived mutant had a two-fold decrease in conidiogenesis time and a 17% increase in acid phosphatase activity. Further characterization of this mutant by SDS-PAGE electrophoresis and inhibition studies using 1 mM fluoride showed that the apparent increase in enzyme activity was caused by hyperproduction or hypersecretion of a nonspecific acid phosphatase (EC 3.1.3.2).
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INTRODUCTION

Plant-derived feedstuffs are important sources of nutrition for humans and livestock. Recently, there has been increased emphasis placed on the use of plant proteins in human nutrition as a supplement or replacement for traditional animal protein sources for both economic and health reasons (33). It is important to produce high quality plant-derived feedstuffs, free from interfering and toxic substances (33,48,49).

Phytic acid (myo-inositol hexaphosphoric acid [Fig. 1]), a naturally occurring organophosphorus compound, is a primary source of inositol and phosphorus in plant seeds (19,33,40). Phytic acid constitutes 60-80% of the total phosphorus present in cereal grains (19). Cottonseed meal, wheat, corn, rice, oats, soybean meal, and other plant-derived feedstuffs contain variable amounts of this compound (1,2,3,4,14,19,40,48). In addition, phytic acid has been detected in chick erythrocytes (45).

Phytic acid and its corresponding salts, known as phytates, chelate and reduce the availability of nutritional elements to monogastric animals. Zinc, magnesium, iron, calcium, and trace minerals (manganese, copper, and molybdenum) are chelated by phytic acid (19,33,40,42,48). Nelson et al. (39) demonstrated that 1.0% phytic acid will chelate 0.36% of the available calcium in poultry feedstuffs. It is significant because chicks and other poultry require relatively
FIG. 1. Hydrolysis of phytic acid to inositol and phosphoric acid by *A. ficuum* NRRL 3135 phytase.
large amounts of calcium (40). Chicks have a limited ability to assimilate phytic acid phosphorus (42). Since phytate phosphorus is excreted by monogastrics in their wastes, it is a significant environmental pollutant (42). In human nutrition, phytic acid has been implicated in decreased bioavailability of zinc in some foods (33, 44). The binding of copper, zinc, and iron in fiber sources can be partially attributed to the concentration of phytic acid (59). Phytic acid has also been implicated in blocking protein absorption in monogastrics. Rojas and Scott (52) demonstrated that phytic acid reduced the protein availability of cottonseed meal fed to chicks. Also, phytic acid reduces the solubility of proteins in cereal grains (52). Calcium ions in association with phytic acid (calcium phytate) and protein reduces the solubility of protein (65).

Phytic acid and phytates are relatively heat stable. Autoclaving at 115°C for two h is required to hydrolyze 70% of the phytic acid in soybean meal (7). The degree of phytate destruction by heat treatment varies with the cereal or grain that is heated, soybean phytate being the most sensitive and rice phytate being one of the least sensitive to hydrolysis by heat (7, 60). Heat treatment to improve the nutritional value of feeds by removing phytates is impractical since amino acids are destroyed in the grain product. Acid leaching removes phytates, but it also causes protein destruction (19). Other methods which utilize pH and isoelectric point precipitation to remove phytates via ion exchangers have also been investigated. Hartman (25) extracted
water-soluble components from soy flakes containing 2-3% bound phytate. The extract was adjusted to pH 11.6, to precipitate the phytates, and vacuum dried. The final product contained 0.1% phytate. Ford et al. (21) treated full fat soy flour slurry with calcium chloride and acid after centrifugation and removed up to 90% of the phytic acid. The protein quality was not affected appreciably. The resultant product contained a relatively high residual calcium concentration.

Phytic acid can also be removed from cereal grain products by enzymatic treatment. Phytase (EC 3.1.3.8) is a specific acid phosphatase which liberates Pi from phytic acid (19,33). The enzyme varies in pH and temperature optima depending upon its source.

Phytase is present in many plant and animal tissues including: wheat bran (34,38), wheat germ (34,62), and vertebrates such as geese (51), turtles (50), and the intestine of albino rats (46). Phytase is produced by numerous microorganisms (11,66,67). Cosgrove and Irving (15) isolated phytase-producing Pseudomonas sp. from soil. Shieh and Ware (57) conducted a survey of microorganisms for the production of extracellular phytase. The highest extracellular phytase activity was produced by Aspergillus ficuum NRRL 3135. These results have been confirmed by other workers (27,28). A. ficuum is morphologically indistinguishable from A. niger. This organism belongs to the A. niger group (57,58). It is distinguished from A. niger by its rapid production of oxalic acid (58). Also, A. ficuum exhibits poor conidiogenesis on most media and has no known sexual cycle (58).
The properties of *A. ficuum* phytase have been investigated (27,28,29,56,57). Shieh et al. (56) demonstrated that *A. ficuum* NRRL 3135 produces two types of extracellular acid phosphatases: a nonspecific orthophosphate monoester phosphohydrolase (EC 3.1.3.2.) with an optimum pH of 2.0, and a more specific myo-inositol-hexaphosphate phosphohydrolase (EC 3.1.3.8) with pH optimum of 5.5, termed phytase. The synthesis of these enzymes is repressed by high Pi concentrations (greater than .004% total Pi) in the fermentation medium (56,57). The regulatory effects of high Pi on phytic acid synthesis was confirmed by Howson and Davis (27).

Wheat germ phytase hydrolyzes phytic acid (myo-inositol hexaphosphoric acid) to myo-inositol 1,2,3,4,5-pentaphosphate while *A. ficuum* phytase produces D-myo-inositol-1,2,4,5,6-pentaphosphate as the major initial hydrolysis product (29). *A. ficuum* phytase resembles *Pseudomonas sp.* phytase in its production of pentaphosphates from phytic acid (15,30). *A. ficuum* also produces an extracellular nonspecific acid phosphatase (orthophosphoric monoester phosphohydrolase: EC 3.1.3.2) that has a pH optimum of 2.0 (29). This enzyme hydrolyzes phytic acid to 1,2,3,4,6 pentaphosphate as the major hydrolysis product (28). The nonspecific acid phosphatase has a Km for sodium phytate of 127 µM; the Km of phytase is 12.9 µM (28). The two enzymes are structurally different. Pi competitively inhibits the nonspecific pH 2.0 enzyme but not the phytase (56). Fluoride ion competitively inhibits the nonspecific acid phosphatase but not phytase at pH 5.3 (28).
A. ficuum phytase has been purified to homogeneity and characterized by Ullah and Gibson (personal communication). It has a molecular weight of 85,000; pH optimum of 5.0 to 5.3; temperature optimum of 58°C; isoelectric point, 4.2; Km for sodium phytate, 0.04 µM; turnover number sec$^{-1}$ for sodium phytate of 216.

The use of A. ficuum phytase to hydrolyze phytic acid and phytates present in cereal grains has been investigated (39,41,42,63). Nelson et al. (39) reported that the addition of acetone dried preparations of A. ficuum phytase to chick diets containing natural phytate phosphorus resulted in the hydrolysis of phytate and the utilization of the liberated organic phosphorus by the chicks. Nelson et al. (41) demonstrated that feeding broilers a diet of A. ficuum phytase treated soybean meal and rice polishings not only made the phytin phosphorus available but that it also reduced the requirement for supplemental calcium. Rojas and Scott (52) demonstrated that the metabolizable energy values of several types of cottonseed meal were improved as well as the protein availability by treatment with A. ficuum phytase.

The hydrolysis of phytates by A. ficuum phytase in-vivo has also been investigated. Nelson et al. (39) fed an acetone dried preparation of A. ficuum phytase to chicks and measured its effect on phosphate assimilation by chicks fed cornmeal and soybean meal diets. Complete hydrolysis of the phytate phosphorus occurred when 3.0 g of the acetone dried solution containing 950 mg Pi h$^{-1}$ g$^{-1}$ phytase activity was added per kg diet. The enzyme was active in the digestive tract of the chicks. It was stable since enzyme activity was present in the feces.
Although the enzymatic approach is technically feasible, it is not economically feasible. In previous research, the yields of extracellular phytase by *A. ficuum* NRRL 3135 was increased ca: 500 times by optimizing the medium and fermentation conditions necessary for synthesis of the enzyme (57). Wodzinski (personal communication) estimated that the yield must be increased an additional 23-fold to make direct feeding of the enzyme competitive with the present practice of feeding supplemental Pi. The yield must be increased ca: 10-fold to make pre-treatment of soybean meal with the enzyme to release phytin phosphorus competitive with feeding supplemental Pi.

No previous attempts have been made to improve the enzyme yield of *A. ficuum* NRRL 3135 by strain improvement techniques.

The initiation of transcription, which involves the binding of RNA polymerase to the DNA at the promotor site, is the rate limiting step in protein synthesis (53). The maximum rate of expression for a given gene can be altered by mutation (54). Strain improvement uses mutagenic agents to increase the yield of gene product via alteration of the nucleotide sequence of the promotor site on the gene operon of interest.

Strain improvement of filamentous fungi utilizes radiation and/or chemicals as mutagenic agents (8,10,18,26,36,47). UV radiation, which induces pyrimidine dimer formation in DNA, has been used successfully in the strain improvement of *Aspergillus sp.* (32). Meyrath et al. used different irradiations with UV to induce mutants of *A. oryzae* which overproduce amylase (37). Best results were obtained using 120 sec exposure to 47.75 ergs mm$^{-2}$ sec$^{-1}$ UV. Ko et al. used UV irradiation of
A. *niger* conidia to obtain a mutant with a 1.5 to 1.8-fold increase in cellulase production (31).

The alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (NTG) has mutagenizing activity on replicating and nonreplicating DNA (12). *A. nidulans* conidia treated with 0.5 to 1.0 mg ml⁻¹ NTG at pH 6.8 and 37°C produced a high mutant per survivor ratio (12). Amylase and amylloglucosidase production by *A. awamori* was increased 2.5-fold by mutagenic treatment of fungal conidia with 0.30 mg ml⁻¹ NTG (43).

Certain enzymes present in the tricarboxylic acid and glyoxylate cycles are active during conidiogenesis in *A. niger* (5,6,22,23). α-Ketoglutaric acid stimulates conidiogenesis of *A. niger* in submerged cultures (23). Since *A. ficuum* displays poor and inconsistent conidiogenesis on common plating media, it would be advantageous to develop a media containing α-ketoglutaric acid which yields consistent conidiogenesis as well as facilitates the isolation of single colonies.

A rational approach to the strain improvement of microorganisms which employs selective methods yields the best results in the shortest time (15). The chromogenic substrate α-naphthyl acid phosphate has been used to detect mutants which overproduce acid phosphatases (16,24,35,55). The usual procedure is to spray a buffered solution of this substrate onto solid medium containing suspected acid phosphatase producers. In the presence of an acid phosphatase α-naphthyl acid phosphate is hydrolyzed to α-naphthol which combines with an aniline dye in solution to form a highly insoluble, colored azo-dye precipitate around a positive colony (24,35,55).
Since the synthesis of phytase in *A. ficuum* is under regulatory control and is inhibited by high Pi concentrations, it should be advantageous to select for deregulated mutants that are constitutive for phytase production.

The purpose of this research was to develop methodology necessary to increase the yields of extracellular phytase in *A. ficuum* NRRL 3135 via a rational strain improvement program.
MATERIALS AND METHODS

Organism. *Aspergillus ficuum* NRRL 3135 was provided by Dr. Wicklow, Northern Regional Research Center, United States Department of Agriculture, Peoria, Illinois. Stock cultures were maintained at 28°C on malt-yeast agar slants which contained per liter: yeast extract, 3.0 g; malt extract, 3.0 g; peptone, 0.5 g; glucose, 10 g; and agar, 15 g (Difco Laboratories, Detroit, Michigan). The medium was adjusted to pH 5.0 with 2.0 N NaOH or 2.0 N HCl prior to sterilization at 121°C for 20 min.

Ten-liter pilot scale fermentations. Ten-liter pilot scale fermentations were in a New Brunswick Scientific Co. 14-liter Microferm fermentor. The fermentation medium used was that developed by Shieh and Ware (57). The pH of the medium was adjusted to 5.0 with 2.0 N NaOH or 2.0 N HCl prior to sterilization at 121°C for 90 min. The fermentation conditions were: temperature 28°C; 10% (vol/vol) inoculum of a 3 day culture of *A. ficuum*; aeration at 0.18 liter liter⁻¹ min⁻¹ at 15 psi; agitation at 350 rpm. The fermentation was sampled by pressurizing the vessel to 40 psi before opening the sampling outlet.

Shaker flask fermentations were done in 250 ml Erlenmeyer flasks containing 50 ml cornstarch fermentation medium (57). All flasks were inoculated with approximately 10⁷ conidia. Cultures were propagated at 28°C for six days on a New Brunswick Scientific Co. orbital shaker at 276 rpm with a 5.1 cm displacement.
Sample processing. Fermentation samples were centrifuged at 15,000 X g for 20 min at 5°C. Cell-free broth was aspirated and stored at 5°C in dark sample bottles with an airtight seal until used.

Determination of phytase activity. Fermentation broths were assayed for extracellular phytase by measuring the release of Pi from sodium phytate. A reaction mixture containing 0.1 ml fermentation broth and 4.5 ml 2.5 µM sodium phytate (Sigma Chemical Company) in acetate buffer pH 4.5 was incubated at 37°C. The reaction was quenched by adding a 1.0 ml aliquot of reaction mixture to 4.0 ml 10% (wt/vol) trichloroacetic acid at 37°C. Liberated Pi was measured by the method of Fiske and Subbarow (20) as modified by Clark and Switzer (13). Absorbance was measured at 710 nm with a Bausch and Lomb Spectronic 20. One unit of phytase activity was defined as the amount of enzyme that liberates 1 µmol of Pi per min at 37°C.

Determination of acid phosphatase activity. Fermentation broths were assayed for acid phosphatase activity at pH 2.0 and 5.3 by measuring the release of p-nitrophenol from p-nitrophenyl phosphate at 37°C (61). A reaction mixture containing 0.1 ml of the fermentation broth and 3.0 ml of 0.04 M p-nitrophenyl phosphate in glycine HCl buffer pH 2.0 or acetate buffer pH 5.5 was incubated at 37°C. One ml aliquots were sampled at specified time intervals and added to 3.0 ml Tris buffer at pH 8.5 to develop color. Absorbance was read at 420 nm on a Bausch and Lomb Spectronic 20. One unit of acid phosphatase activity was defined
as the amount of enzyme that liberated 1 µmol p-nitrophenol per min at 37°C at pH 2.0 or 5.3.

**Determination of protein concentration.** Protein concentrations in fermentation broths were determined by the method of Bradford (9) with bovine serum albumin (Sigma Chemical Co.) as the standard.

**Determination of Pi in cornstarch preparations.** The levels of Pi in nine different cornstarch preparations were determined by two different methods. The autoclave method involved dissolving 4.0 g of the cornstarch sample into 50 ml basal medium (57) with no Pi added. The solution was adjusted to pH 5.0 before autoclaving at 121°C for 15 min. After cooling to 25°C, the sample was centrifuged at 12,000 x g for 10 min and the collected supernatant was assayed for Pi as previously described. The acid hydrolysis method involved dissolving 1.00 g cornstarch sample into 10 ml 2.0 N HCl and autoclaving at 121°C for 24 h. Samples were cooled to 25°C and assayed for Pi. Cornstarch samples were provided by: National Starch and Chemical Corporation, A.E. Staly Manufacturing Company, and Corn Products.

**Optimization and determination of phytase production by A. ficuum.** The fermentation medium developed by Shieh and Ware (57) was used, without the cornstarch, as a basal medium to test the effects of fermentation of nine different cornstarch preparations on phytase production by A. ficuum. Eighty g of each sample of cornstarch was added per liter of basal medium. Total Pi content of each medium was adjusted to 0.04 g liter⁻¹ by the addition of K₂HPO₄. The pH was adjusted to 5.0. Triplicate shaker flask fermentations were sampled three, four, five, and six days after inoculation and phytase activity was determined.
Media development for mutation studies. Thirty different media formulations using different carbon-nitrogen ratios and growth factors (22,23) were tested for their ability to yield rapid, consistent conidiogenesis and isolated fungal colonies of A. ficuum. A solid, semi-synthetic medium was developed which satisfied these criteria. The media, designated mutation medium, contained, per liter: yeast extract, 2.0 g; MgSO\(_4\)·7H\(_2\)O, 0.5 g; (NH\(_4\))\(_2\)SO\(_4\), 1.5 g; \(α\)-ketoglutaric acid, 2.99 g; K\(_2\)HPO\(_4\), 0.225 g; and agar, 17 g. The pH was adjusted to 6.8 with 2.0 N NaOH or 2.0 N HCl before addition of the agar and sterilization.

Determination of minimum inhibitory concentration (MIC) of amphotericin B. Resistance to amphotericin B (Gibco Co.) was used as a mutagenic marker for NTG and UV mutagenesis. The MIC was determined by plating dilutions of A. ficuum conidia in triplicate onto mutation agar medium containing concentrations of amphotericin B ranging from 0 to 25 µg ml\(^{-1}\). Triplicate plates were incubated at 28°C for 10 days. The mean number of colonies was used to determine the MIC.

Mutation conditions. The protocol for mutagenesis of A. ficuum using N-methyl-N' -nitro-N-nitrosoguanidine (NTG) or UV radiation is in Fig. 2. The UV radiation source was a 30 watt germicidal lamp (Sylvania) emitting radiation at 254 nm.

Mutation medium with and without 0.25 µg ml\(^{-1}\) amphotericin B was used to plate NTG or UV treated conidia in triplicate. The proportion of survivors that were amphotericin B resistant was determined for both mutagenic treatments and used as a basis to optimize the conditions for both treatments.
FIG. 2. Protocol for mutation of *A. ficuum* using $0.5 \text{ mg ml}^{-1}$ N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or $46 \text{ ergs mm}^{-2} \text{ sec}^{-1}$ UV radiation.
Stock culture on malt-yeast agar 96-120 h at 28°C

- Suspend conidia \(10^6\) to \(10^7\) ml\(^{-1}\) in 0.1% sodium lauryl sulfate
- Vortex for 2 min with fine glass beads to break up conidia chains
- Filter through a sterile 6 x 305 mm glass tube packed with 0.5 g of sterile glass wool
- Examine microscopically to determine if conidia exist as individuals. If chains are present, vortex again and refilter

- Suspend 1.0 ml conidia in 5.0 ml 0.10 M \(\text{PO}_4\) buffer pH 6.5 containing 0.5 mg ml\(^{-1}\) NTG
- Sample at 0, 5, 10, and 15 min
- Plate in triplicate onto each of the following media using dilutions of: \(10^{-3}\), \(10^{-3.5}\), \(10^{-4}\), \(10^{-4.5}\), \(10^{-5}\), \(10^{-5.5}\), \(10^{-6}\)

Mutation medium without amphotericin B or mutation medium with 0.25 µg ml\(^{-1}\) amphotericin B

- Sample at 0, 10, 20, 30, 40, 60, 90, and 120 sec
- Irradiate using 46 ergs mm\(^{-2}\) sec\(^{-1}\) UV radiation

- Incubate 10 days and check for growth and morphological changes. UV treated conidia must be incubated in the dark.
Mutant screening. Mutant cultures of *A. ficuum* that hyperproduce and/or hypersecrete phytase were tentatively identified using a histochemical staining plate assay (16,24,35,55). A solution containing α-naphthyl acid phosphate (1.0 mg ml\(^{-1}\)) and fast black K-salt (0.4 mg ml\(^{-1}\)) in 0.2 M acetate buffer pH 4.8 was prepared immediately before testing of the mutants. The solution was filter sterilized through a 0.22 µm membrane filter (Falcon Plastic Company). Each plate was flooded with the stain and incubated in the dark at room temperature (23-25 C) for 10 min. Colonies were observed for reddish-brown halos of precipitated α-naphthol-fast black complex indicating the presence of an acid phosphatase (35,55). Mutant colonies were compared with the wild type using color intensity, zone size, and relative rate of color change after 10 min exposure to the stain. Conidia of presumptive hyperproducers and/or hypersecreters were picked and transferred to slants of malt-yeast extract.

Confirmation of phytase hyperproduction and/or hypersecretion was tested in triplicate shaker flask fermentations on cornstarch fermentation medium containing 0.004% (wt/vol) Pi (56). Mutants constitutive for phytase production were tested and assayed for phytase activity using triplicate shaker flask fermentations on cornstarch fermentation medium containing 0.008% (wt/vol) Pi (56).

Histochemical stain toxicity. The effects of the α-naphthyl acid phosphate stain on *A. ficuum* wild type conidia were determined. Fungal conidia were suspended to a final concentration of ca: \(10^6\) ml\(^{-1}\) into 10 ml of the staining solution. Conidia were removed at regular time intervals, diluted in 0.9% (wt/vol) NaCl 0.1% (wt/vol) sodium lauryl
sulfate and plated in triplicate onto mutation medium with or without 0.25 µg ml\(^{-1}\) amphotericin B. Plates were incubated at 28°C and examined for growth daily for 10 days.

**Concentration of extracellular phytase.** Extracellular phytase in *A. ficuum* fermentation broths was concentrated by organic solvent precipitation with tert-butyl alcohol and acetone. One and a half volumes of tert-butyl alcohol were added to the fermentation broth with constant stirring. The precipitate was centrifuged at 10,000 X g for 10 min and resuspended in a minimum volume of tert-butyl alcohol.

Acetone (5°C) at half of the original sample volume was added with constant stirring. The precipitate was collected on Whatman No. 4 and washed twice with 200 ml cold acetone and dried overnight in a vacuum desiccator. Total protein concentrations were determined by the method of Bradford (9). Phytase activity in the precipitate was determined by resuspending the precipitated material in 4.0 ml of 0.2 M acetate buffer pH 4.5 and assaying for phytase activity.

**Partial characterization of mutants.** Mutants of *A. ficuum* which hyperproduce, hypersecrete, or are constitutive for production of extracellular acid phosphatase were partially characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (64) using fermentation broths concentrated as previously described. Polyacrylamide gels were stained for: proteins (64); acid phosphatase (55); and glycoprotein using periodic acid Schiff stain (Pharmacia Fine Chemicals).

**Statistical analysis.** Data was analyzed using one-way analysis of variance (ANOVA), two-way ANOVA, Schefee multiple comparison S-test and
Student t-test. Sample normality was determined using Lillefor test for normality. Data was transformed when necessary. Analysis was done on an Apple IIe computer using Statistics with Finesse software.
RESULTS

Total Pi in different cornstarch preparations. One-way analysis of variance (ANOVA) of total Pi content of different cornstarch preparations treated by autoclaving for 15 min indicated significant differences between at least two of the cornstarch preparations (Table 1). Corn Products 3005 cornstarch had the highest Pi concentration of 154.95 µg Pi g⁻¹ cornstarch. A.E. Staley Manufacturing Co. 7350 Waxy No. 1 cornstarch had the lowest Pi concentration of 96.75 µg Pi g⁻¹ cornstarch. No Pi was detected in the following cornstarches: National Starch and Chemical Corp. "Amioca", "Tapioca", "Flogel G", and "Tapon". One-way ANOVA of total Pi content (logarithmic transformed values) of the different cornstarch preparations treated by 24 h acid hydrolysis indicated a significant difference (p=0.0005) between at least two of the cornstarch preparations (Table 1). The highest Pi concentration was 196.59 µg Pi g⁻¹ cornstarch for Corn Products 3005 cornstarch. The lowest Pi concentration was 82.50 µg Pi g⁻¹ cornstarch for National Starch and Chemical Corp. "Amioca". Multiple comparisons using Schefee S-test (α=0.05) identified significant differences between: National Starch and Chemical Corp. "Amioca" and Corn Products "3005"; National Starch and Chemical Corp. "Amioca" and "Hylon V" (Table 1). Two-way analysis of variance between the two treatments indicated a significant difference (p=0.0001) in the levels of Pi detected in each cornstarch preparation.
TABLE 1. The Pi content of commercial cornstarch preparations treated at 121°C for 15 min in fermentation medium (A) or hydrolysis at 121°C for 24 h in 2 N HCl (B)

<table>
<thead>
<tr>
<th>Commercial Cornstarch Preparation</th>
<th>Total Pi Content (µg Pi g⁻¹ cornstarch)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>National Starch and Chemical Corp.:</td>
<td></td>
</tr>
<tr>
<td>&quot;Amioca&quot;</td>
<td>ND²</td>
</tr>
<tr>
<td>&quot;Tapioca&quot;</td>
<td>ND²</td>
</tr>
<tr>
<td>&quot;Flogel&quot;</td>
<td>ND²</td>
</tr>
<tr>
<td>&quot;Tapon&quot;</td>
<td>ND²</td>
</tr>
<tr>
<td>&quot;Melojel&quot;</td>
<td>135.45 +/- 0.00</td>
</tr>
<tr>
<td>&quot;Hylan V&quot;</td>
<td>100.65 +/- 12.87</td>
</tr>
<tr>
<td>A.E. Staley Manufacturing Corp.</td>
<td></td>
</tr>
<tr>
<td>&quot;7350 Waxy No. 1&quot;</td>
<td>96.75 +/- 0.90</td>
</tr>
<tr>
<td>Corn Products 3005</td>
<td>154.85 +/- 10.00</td>
</tr>
<tr>
<td>Total Pi Content</td>
<td>487.70</td>
</tr>
</tbody>
</table>

¹Significant difference (p=0.0001) between cornstarch Pi content dependent on treatment.
²ND denotes less than 0.1 µmol ml⁻¹ Pi detected.
preparation by the two treatments (Table 1). In all cases cornstarch preparations treated by 24 h acid hydrolysis showed higher total Pi concentrations than the same cornstarch preparations treated by autoclaving 15 min. The greatest difference in total Pi content between treatments was 175.44 µg Pi g\(^{-1}\) cornstarch for National Starch and Chemical Corp. "Flagel G" and the least difference of 2.88 µg Pi g\(^{-1}\) cornstarch for A.E. Staley Manufacturing Co. 7350 Waxy No. 1. The average difference in total Pi content for all cornstarch preparations between the two treatments was 120.47 ± 36.26 µg Pi g\(^{-1}\) cornstarch.

**Effect of cornstarch on production of phytase by A. ficuum.** One-way ANOVA indicated significant (p=0.0002) differences in the yields of phytase from five-day fermentations between at least two cornstarch preparations adjusted to 0.004% (wt/vol) total Pi within the two treatments (Table 2). Fermentation on media containing 80 g liter\(^{-1}\) Corn Products 3005 cornstarch produced significantly higher yields of phytase regardless of the treatment method used to adjust total Pi content of the media. These yields were 7.69 units ml\(^{-1}\) and 8.19 units ml\(^{-1}\) phytase respectively. Fermentation media containing 80 g liter\(^{-1}\) National Starch and Chemical Corp. "Tapon" cornstarch had the lowest yields of phytase regardless of the treatment used to adjust total Pi. These yields were 1.91 units ml\(^{-1}\) and 1.57 units ml\(^{-1}\) respectively. Multiple comparisons using Scheffé S-test (α=0.05) identified significant differences in specific phytase yields within the two
TABLE 2. The effect of commercial cornstarches (adjusted to 0.004% (wt/vol) total Pi) on phytase production.

<table>
<thead>
<tr>
<th>Commercial Cornstarch Preparation</th>
<th>Phytase Activity (units ml^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment I^a</td>
</tr>
<tr>
<td>National Starch and Chemical Corp.:</td>
<td></td>
</tr>
<tr>
<td>&quot;Amioca&quot;</td>
<td>4.35 +/- .29</td>
</tr>
<tr>
<td>&quot;Tapioca&quot;</td>
<td>4.69 +/- 1.71</td>
</tr>
<tr>
<td>&quot;Flogel G&quot;</td>
<td>2.48 +/- .52^c,e</td>
</tr>
<tr>
<td>&quot;Tapon&quot;</td>
<td>1.91 +/- .16^f</td>
</tr>
<tr>
<td>&quot;Melojel&quot;</td>
<td>5.84 +/- .61^e,f</td>
</tr>
<tr>
<td>&quot;Hylan V&quot;</td>
<td>3.36 +/- .06^d,g</td>
</tr>
<tr>
<td>A.E. Staley Manufacturing Corp.</td>
<td></td>
</tr>
<tr>
<td>&quot;7350 Waxy No. 1&quot;</td>
<td>5.09 +/- 1.12</td>
</tr>
<tr>
<td>Corn Products 3005</td>
<td>7.69 +/- .22^e,f,g</td>
</tr>
<tr>
<td>Total Phytase Activity</td>
<td>35.41</td>
</tr>
</tbody>
</table>

^aSupplemental Pi addition based on Pi content of cornstarch determined after treatment at 121°C in fermentation medium for 15 min.

^bSupplemental Pi addition based on Pi content of cornstarch determined after treatment at 121°C for 24 h in 2 N HCl.

^cSignificant difference (p=0.001) between treatments.

^dSignificant difference (p=0.0002) between treatments.

^eSignificant difference (p<.05) within treatment I.

^fSignificant difference (p<.05) within treatment I.

^gSignificant difference (p<.05) within treatment I.

^hSignificant difference (p<.05) within treatment II.
treatments (Table 2). The total yield of phytase for all cornstarch media formulations using Pi adjusted to 0.004% (wt/vol) as determined by treatment at 121°C for 15 min or 24 h acid hydrolysis was 35 and 43 units ml\(^{-1}\) respectively.

**Ten-liter pilot scale fermentation.** *Aspergillus ficuum* NRRL 3135 produced 8.74 units ml\(^{-1}\) phytase after 220 h in cornstarch medium containing 80 g liter\(^{-1}\) Corn Products 3005 cornstarch (Fig. 3). This compares with a mean phytase yield of 9.11 +/- .18 units ml\(^{-1}\) for triplicate six-day shaker flask fermentation on the same media.

**Mutation medium development.** The tricarboxylic acid cycle intermediates: glycine, glyoxylate, and \(\alpha\)-ketoglutaric acid and different carbon-nitrogen ratios were tested in agar based media for their ability to induce fast and consistent conidiogenesis and to facilitate the formation of isolated fungal colonies. The effect of light on these factors was also tested. A solid medium lacking glucose (low carbon-nitrogen ratio) and containing 2.99 g liter\(^{-1}\) \(\alpha\)-ketoglutaric acid satisfied these criteria. No differences in conidiogenesis characteristics were noted in media incubated under light or dark conditions.

**Minimum inhibitory concentration (MIC) of amphotericin B.** The MIC of amphotericin B for *A. ficuum* NRRL 3135 was 0.25 µg ml\(^{-1}\) in mutation medium (Fig. 4). The MIC was calculated as 0.225 µg ml\(^{-1}\) by regression analysis. The former value was used for mutagenesis optimization.

**Optimum conditions for mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (NTG).** A one log kill was obtained after a 15 min exposure of *A. ficuum* conidia to 0.5 mg ml\(^{-1}\) NTG (Fig. 5). Amphotericin
FIG. 3. Production of phytase by *A. ficuum* in a 14-liter vessel on cornstarch fermentation medium containing 80 g liter⁻¹ Corn Products 3005 cornstarch using 2.5 µM sodium phytate as substrate pH 4.5.
FIG. 4. The effect of amphotericin B on the growth of *A. ficuum* on mutation medium.
The diagram shows a plot of LOG CFU ml\(^{-1}\) against \(\mu g\) ml\(^{-1}\) AMPHOTERICIN B. The correlation coefficient, \(r\), is 0.9742.
FIG. 5. The effect of 0.5 mg ml\(^{-1}\) NTG on the production of amphotericin B resistant mutants of \textit{A. ficuun}.
B resistant mutants were generated at a rate 800-fold greater than the spontaneous rate indicating a high mutant per survivor ratio.

**Optimum conditions for mutagenesis using UV radiation.** A one log kill was obtained after exposure of *A. ficium* conidia to 46 ergs mm$^{-2}$ sec$^{-1}$ UV radiation for 30 sec. Amphotericin B resistant mutants were generated at a rate 100,000-fold greater than the spontaneous rate after 30 sec irradiation indicating a high mutant per survivor ratio (Fig. 6). Numbers of survivors and amphotericin B mutants decreased exponentially at a rate of 1.8 log min$^{-1}$.

**Effects of histochemical stain on *A. ficium* conidia.** During the first 15 min, $\alpha$-naphthyl-acid phosphate fast black K-salt exposed conidia displayed no significant ($p=0.001$) loss of viability (Fig. 7). Viability significantly ($p=0.01$) decreased at exposure times greater than 15 min. No significant ($p=0.001$) increase in mutation rates were detected between the treated and untreated conidia over a time interval of 30 min.

**Isolation of a mutant which hyperproduces phytase and/or acid phosphatase.** A mutant was isolated after 120 sec exposure to UV and presumptively identified as a possible hyperproducer of acid phosphatase using histochemical staining. This mutant displayed a two-fold shorter conidiogenesis time than the wild type. Analysis of fermentation broths from six-day shaker flask fermentations on cornstarch media containing 0.004% (wt/vol) total Pi indicated a significant ($p=0.001$) increase in acid phosphatase activity at pH 4.5 using sodium phytate as the substrate (Fig. 8). This mutant produces 10.95 units enzyme compared to the wild type which produces 9.11 units.
FIG. 6. The effect of 46 ergs mm$^{-2}$ sec$^{-1}$ UV irradiation on the production of amphotericin B mutants of A. ficuum.
TIME OF EXPOSURE TO 46 ergs mm$^{-2}$ sec$^{-1}$ UV RADIATION (sec)
FIG. 7. The effect of $\alpha$-naphthyl acid phosphate (1.0 mg ml$^{-1}$) and fast black K-salt (0.4 mg ml$^{-1}$) on $A. \text{ ficuum}$ wild type conidia.
untreated

•

treated

•

mutants

CFU ml⁻¹

TIME OF EXPOSURE TO STAIN REAGENT (min)
FIG. 8. Comparison of extracellular phytase production by mutant Apt and wild type after 6-day shaker flask fermentation on cornstarch medium containing 80 g liter$^{-1}$ Corn Products 3005 cornstarch (0.004% [wt/vol] total Pi) 2.5 µM sodium phytate as substrate pH 4.5.
When/if

- mutant Apt
- wild type

REACTION TIME (min)
enzyme. This corresponds to a 17% increase in acid phosphatase activity. This mutant also produces 41 units acid phosphatase per mg protein as compared to 10 units acid phosphatase per mg protein for the wild type. Analysis of fermentation broths of the mutant propagated on cornstarch medium containing 0.008% (wt/vol) Pi indicated that this mutant is not a constitutive producer of acid phosphatase(s). The yields of acid phosphatase were 0.037 units ml\(^{-1}\) when propagated on 0.008% (wt/vol) Pi-containing medium as compared to 0.52 units ml\(^{-1}\) for the wild type. Assay of fermentation broths using p-nitrophenyl phosphate and sodium phytate as substrate in the presence or absence of 1.0 mM fluoride indicated no significant difference (p=0.0001) in phosphatase activities of the mutant or the wild type at pH 2.0 and 5.3 in the absence of 1.0 mM fluoride. A significant difference (p=0.003) in phosphatase activities of the mutant and the wild type at pH 5.3 but not at pH 2.0 in the presence of 1.0 mM fluoride was detected when p-nitrophenyl phosphate was the substrate (Figs. 9,10).

The activities of fermentation broths of both the mutant and the wild type using sodium phytate as the substrate at pH 2.0 and 5.3 in the presence of 1 mM fluoride indicated a significant difference in the degree of inhibition at pH 5.3 between the mutant (p=0.002) and the wild type (p=0.0001) (Fig. 11). This mutant displayed a greater relative decrease in acid phosphatase activity in the presence of 1 mM fluoride at pH 5.3 than did the wild type. Relative fluoride inhibition of phosphatase activity was 26% for the mutant and 14% for the wild type at pH 5.3. This mutant was designated Apt.
FIG. 9. The effect of 1 mM fluoride ion on acid phosphatase (phytase) activities of mutant Apt and the wild type fermentation broths with 0.04 M p-nitrophenyl phosphate as substrate at pH 5.3.
ACID PHOSPHATASE ACTIVITY (μmol p-nitrophenol ml⁻¹)

REACTION TIME (sec)

- ● mutant Apt
- ○ mutant Apt + F⁻
- ▲ wild type
- △ wild type + F⁻
FIG. 10. The effect of 1 mM fluoride ion on acid phosphatase (phytase) activities of mutant Apt and the wild type fermentation broths with 0.04 M p-nitrophenyl phosphate as substrate at pH 2.0.
ACID PHOSPHATASE ACTIVITY
(μmol p-nitrophosphol ml⁻¹)

mutant Apt  
mutant Apt+F⁻  
wild type  
wild type +F⁻

REACTION TIME (sec)
FIG. 11. The effect of 1 mM fluoride ion on phytase activities of mutant Apt and the wild type fermentation broths using 2.5 μM sodium phytate as substrate at pH 5.3 and 2.0.
The graph shows the relationship between reaction time and phytase activity. The y-axis represents phytase activity in μmol Pi ml⁻¹, ranging from 0 to 100.0. The x-axis represents reaction time in minutes, ranging from 0 to 10.

- The black circle represents the mutant Apt.
- The orange circle represents the mutant Apt+F⁻.
- The red triangle represents the wild type.
- The blue triangle represents the wild type + F⁻.

Phosphate release is measured at different pH levels: 2.0, 5.3, and 7.5. The graph illustrates the increase in phytase activity over time under these conditions.
SDS-PAGE electrophoresis characterization of mutant Apt fermentation broths. SDS-PAGE electrophoresis of wild type fermentation broth concentrate (1.6 mg ml\(^{-1}\) protein) revealed 12 separate bands when stained for protein (Fig. 12). Three protein bands were in the 70,000 to 100,000 region. Wild type SDS-PAGE gels stained for acid phosphatase revealed one broad band in the 80,000 to 90,000 region and a sharp band in the 60,000 region (Fig. 12). SDS-PAGE electrophoresis of mutant Apt fermentation broth concentrate (1.8 mg ml\(^{-1}\) protein) revealed 10 separate bands when stained for protein (Fig. 12). Five protein bands were in the 70,000 to 100,000 region. SDS-PAGE gels stained for acid phosphatase revealed one broad band in the 80,000 to 90,000 region (Fig. 12).
FIG. 12. SDS-PAGE electrophoresis of broths from mutant Apt and wild type fermentations stained for total proteins and acid phosphatase activity. Lanes: A, mol wt standard (1, 205,000; 2, 116,000; 3, 97,400; 4, 66,000; 5, 45,000; 6, 29,000); B, wild-type proteins; C, wild-type acid phosphatase; D, mutant Apt proteins; E, mutant Apt acid phosphatase.
DISCUSSION

A program was initiated to develop the methodology necessary to utilize applied genetics to cause *Aspergillus ficum* NRRL 3135 to overproduce extracellular phytase.

A different commercial cornstarch was used in the fermentation medium than that used by Shieh and Ware (57). The medium had to be re-optimized for total Pi since commercial cornstarches vary in Pi content. Two treatment methods were examined for their effectiveness in releasing Pi from cornstarch. Cornstarch preparations were autoclaved at 121°C for 15 min in fermentation medium and hydrolyzed at 121°C for 24 h in 2 N HCl. Analysis of the hydrolysate from both methods showed that the cornstarches vary in total Pi content (Table 1). Significant differences (p=0.0001) were detected in the Pi content of the cornstarches between the two treatment methods. Hydrolysis at 121°C for 24 h in 2 N HCl released more Pi than did autoclaving at 121°C for 15 min in fermentation medium. The differences in Pi released by the two methods ranged from 2.88 to 175.44 µg Pi g⁻¹ cornstarch with a mean of 120.47 +/- 36.26 µg Pi g⁻¹ cornstarch. Less than 0.1 µg Pi g⁻¹ cornstarch was released by autoclaving at 121°C for 15 min in fermentation medium for National Starch and Chemical Corp.: "Amioca", "Tapioca", "Flogel", and "Tapon" cornstarch preparations. It is possible that hydrolysis at 121°C for 24 h in 2 N HCl releases Pi which is bound to the starch molecule or
present in a form that cannot be released by the 15 min heat treatment. Gibson (personal communication) demonstrated that covalently bound Pi is present in Hylan V cornstarch and in potato starch as glucose-6-phosphate and glucose-3-phosphate. These phosphodextrins are also effective substrates for the extracellular acid phosphatases produced by *A. ficuum*.

The Pi content of the different commercial cornstarches, calculated using 15 min heat treatment and 24 h hydrolysis, were used to adjust the total Pi of the fermentation medium to 0.004% (wt/vol) which is the optimum for synthesis of phytase (56). Analysis of five-day fermentation broths for phytase indicated significant differences (p=0.0002) in the yields of phytase between the different cornstarches for both the method of 15 min heat treatment and 24 h hydrolysis (Table 2). In all cases fermentation on a medium containing 80 g liter\(^{-1}\) Corn Products cornstarch gave significantly higher yields of phytase (7.69 and 8.19 units ml\(^{-1}\)) than did fermentation on media containing any of the other commercial cornstarches. Fermentation on a medium containing 80 g liter\(^{-1}\) National Starch and Chemical Corp. "Tapon" cornstarch consistently had the lowest yields of phytase (1.91 and 1.57 units ml\(^{-1}\)). Adjusting total Pi to 0.004% (wt/vol) for all cornstarch medium formulations did not result in similar yields of phytase. A factor other than Pi content may be present in the commercial cornstarches which affects phytase synthesis in *A. ficuum*. It is possible that some phosphoester linkages are hydrolyzed at a
faster rate than others which would increase the concentration of Pi in the medium and repress synthesis of acid phosphatases (Gibson personal communication). The isomeric configuration of phosphoester bonds of the substrate might also be a factor. Irving and Cosgrove (28) postulated that a pair of vicinal trans-equatorial phosphate groups on the substrate are required for the formation of an active complex with \textit{A. ficuum} phytase. The C3 and C4 phosphate groups of phytic acid have this configuration. It is possible that different commercial cornstarches contain different isomers of phytic acid and that these isomers are hydrolyzed at different rates. These isomers might also vary in ability to induce synthesis of phytase in \textit{A. ficuum}. Further experimentation is necessary to determine whether phytase synthesis is inducible in \textit{A. ficuum} and whether the variation in yields of the enzyme on the different cornstarches is caused by the amount and isomeric form of phytic acid present.

After the media was formulated for optimum total Pi concentration using Corn Products 3005 cornstarch, \textit{A. ficuum} produced 8.74 units ml$^{-1}$ phytase in a 10-liter pilot scale fermentation. This compares with a mean of 9.11 units ml$^{-1}$ phytase produced by \textit{A. ficuum} during a six-day shaker flask fermentation on the same media which is equivalent to the levels reported by Shieh et al. (56).

Histochemical staining, using $\alpha$-naphthyl acid phosphate and fast black K-salt, was useful in determining qualitatively the presence or absence of an extracellular acid phosphatase on fungal colonies. The stain did not affect conidia viability or increase mutation rate in the
10 min exposure used during screening. Histochemical staining did not indicate quantity or identify which acid phosphatase was produced.

A mutant was identified as a possible hyperproducer of an acid phosphatase via the plate assay. Analysis of mutant fermentation broths for acid phosphatase activities using sodium phytate as a substrate indicated a 17% increase in activity over the wild type.

The type of phosphatase produced by the mutant was characterized by using sodium phytate and p-nitrophenyl phosphate at pH 2.0 and 5.3 in the presence or absence of fluoride. Fluoride inhibits both nonspecific acid phosphatase (EC 3.1.3.2) and phytase (EC 3.1.3.8) at pH 2.0 but does not inhibit phytase at pH 5.3 (27,28). The mutant acid phosphatase activity was inhibited 26% by 1 mM fluoride at pH 5.3 and the wild type acid phosphatase activity was inhibited 14% when sodium phytate was the substrate. This demonstrates that the increase in the production/secreton of the nonspecific acid phosphatase (EC 3.1.3.2) and not the phytase (EC 3.1.3.8) was responsible for the apparent increase in phytase activity for the mutant relative to the wild type.

SDS-PAGE electrophoresis was used to further characterize the mutant. Differences in protein band patterns were detected in the 70,000 to 100,000 region when gels were stained for protein using coomassie blue. In this region, three protein bands were detected for the wild type broth and five protein bands for mutant Apt broth. Two protein bands corresponding to the 78,000 to 92,000 mol wt range are unique to mutant Apt. These additional protein bands may constitute additional protein subunits for an acid phosphatase or a protein which would enhance secretion of this enzyme (47). They may also account for
the higher acid phosphatase to protein ratio for mutant Apt (41 units ml$^{-1}$ mg$^{-1}$ protein) compared to the wild type (10 units ml$^{-1}$ mg$^{-1}$ protein). Since $\alpha$-naphthyl-acid phosphate stained the SDS-PAGE gel, at least one of the acid phosphatases produced by A. ficuum must be a monomeric protein because it retains activity against the chromogenic substrate. Ullah and Gibson (personal communication) reported that A. ficuum phytase is a monomeric protein.

It is evident that the methods employed did not result in the isolation of a mutant which overproduces phytase. This result was not unexpected due to the random nature of the mutation process and the fact that the preliminary screening process does not specifically select and detect extracellular phytase. The development of a specific method to select and detect phytase would be useful. The addition of 1 mM fluoride to the staining reagent and the use of replica plating techniques might increase the selectivity of the preliminary screening. Fluoride inhibition studies and SDS-PAGE electrophoresis provide evidence that mutant Apt is a hyperproducer or hypersecretor of the nonspecific acid phosphatase.

Although the $K_m$ of the nonspecific acid phosphatase for phytic acid is higher than that of phytase, it is still capable of hydrolyzing the phytic acid at a significant rate at pH 2.0.

A mutant which hyperproduces or hypersecretes the nonspecific acid phosphatase might be useful in hydrolyzing phytic acid in feedstuff by direct feeding. However, the activity would be in the stomach in which there is a shorter retention time and a lower pH rather than in the intestine which has a longer retention time and a higher pH.
If deletion mutations for acid phosphatase and phytase could be generated, then further work might be valuable in the genetic characterization of this organism. These types of mutants are useful for recombinant DNA studies.
LITERATURE CITED


