Journal of Industrial Microbiology, 1 (1987) 295–301
Elsevier

SIM 00039

Phosphatase production by *Aspergillus ficuum*

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Received 13 June 1986
Revised 26 September 1986
Accepted 30 September 1986

Key words: Phytase; Phosphatase; Phytic acid; *Aspergillus ficuum*

SUMMARY

Effects of nutritional and cultural conditions on cell growth and phosphatase production by *Aspergillus ficuum* were studied. *A. ficuum* produced high levels of phosphatases when grown on a basal medium that contained a minimal amount (2 mg/100 ml) of phosphorus in an acidic growth medium. The organism produced a nonspecific acid phosphomonoesterase rather than phytin-specific phosphatase. The enzyme hydrolyzed a variety of phosphates and produced orthophosphate. The rate of phosphate hydrolysis was dependent on the pH of the reaction, where the pH optimum for acid phosphatase was 2.5 and that for phytase was 5.0. The organism slowly released the phosphatase, and the enzyme activity in the growth medium increased continually during a one-month growth period. For a high level of phosphatase production, low levels (1–5 mg%) of initial phosphorus were necessary and polyphosphates were the desired form rather than the monophosphate. The addition of surfactants, such as polyoxyethylene ethers and sodium oleate, to fungal culture medium markedly increased the level of phosphatase production.

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. The ability of phytic acid to bind metal ions is lost when the phosphate groups are hydrolyzed through the action of the enzyme phytase. Attempts have been made to reduce the phytic acid level in animal diets by application of microbial phytase [9]. Low yield and high cost of enzyme production, however, were cited as a limiting factor in use of the enzyme in animal diet [16]. *A. ficuum* produced two types of extracellular phosphatases: a nonspecific orthophosphoric monoester phosphohydrolyase and mesoinositol-hexaphosphate phosphohydrolyase [14]. Both enzymes hydrolyzed phytin. High phosphorus content in growth medium repressed the synthesis of phytase, but a certain level of phosphorus is needed for cell growth [13]. Thus, the medium composition and cultural conditions greatly affect the production of microbial phosphatases. Although several reports have been made on microbial phosphatase,

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particularly on phytase [2,4,6,13–15], few dealt with nutritional and cultural aspects of the enzyme production. In this study, efforts were made to elucidate the characteristics of phosphatase production by *A. ficuum* and to improve the enzyme yield by optimizing the nutritional and cultural conditions. Because of our particular interest in phytase, attention was given to phytase activity as a part of, and in relation to, the total phosphatase production. Although phytase is a kind of phosphatase, we have distinguished the two enzymes based on their activity toward substrates; *p*-nitrophenyl phosphate for phosphatase (EC 3.1.3.2) and sodium phytate for phytase (EC 3.1.3.8), respectively.

MATERIALS AND METHODS

Organism and growth conditions. Spores of *A. ficuum* (NRRL 3135) were formed and maintained on potato dextrose agar at pH 6.8 and 28°C. A portion of spore suspension (about 1% inoculim) was used in growing the mold in a basal medium containing (per liter): glucose 50.0 g; NH_4NO_3, 2.5 g; $Ca_2P_2O_7$, 0.02 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KCl, 0.5 g; $FeCl_3 \cdot 6H_2O$, 16.7 mg; $ZnSO_4 \cdot 7H_2O$, 0.18 mg; $CuSO_4 \cdot 5H_2O$, 1.6 mg; $CoCl_2 \cdot 6H_2O$, 0.18 mg; $MnSO_4$, 10 mg. Initial pH of the medium was 5.6. The cultures (50 ml per 125 ml flask) were incubated on a rotary shaker (200 rpm) at 30°C. Cell mass was determined by filtering cultures through Whatman No. 4 filter paper and drying overnight at 105°C. At least triplicate cultures were made for each treatment and the values reported were an average of the assays. All the chemicals used were reagent grade unless otherwise specified. The high amylose corn starch (Hylon V) was obtained from National Starch and Chemical Corp., Bridge Water, N.J.

Measurement of enzyme activity. Phytase activity was assayed by following the release of orthophosphate (Pi) from phytate. Liberated Pi was determined by the method of Fiske and SubbaRow [3]. In the study where high initial Pi interferes with the determination of Pi liberated by the enzyme, *p*-nitrophenylphosphate (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 ml of 0.1 M HCl-KCl buffer (pH 2.0), 0.5 ml of 15 mM PNPP. The reaction mixture was incubated for 10 min at 37°C. At the end of reaction, 1.0 ml of 0.2 M NaOH was added to terminate the reaction and to develop color. The yellow color developed was determined by reading the optical density

Table 1

Substrate specificity of *A. ficuum* phosphatase at pH 2.0 and pH 5.5

Substrate	pH 2.0		pH 5.5	
	Pi (μM)[a]	Relative activity[b]	Pi (μM)[a]	Relative activity[b]
Na-PNPP	1.055	100	0.792	75.1
Na-phytate	0.360	34.1	0.463	43.8
Ca-phytate	0.490	46.4	0.465	44.1
$Ca_2P_2O_7$	0.603	57.1	0.143	13.5
$Na_5P_3O_{10} \cdot 6H_2O$	0.953	90.3	0.334	31.6
$Na_3P_3O_9$	0.474	44.9	0.114	10.8
$(NH_4)_6P_4O_{13} \cdot 6H_2O$	0.958	90.8	0.176	16.7
Myoinositol 2-monophosphate	0.331	31.3	0.063	5.9

[a] Pi liberated from reaction mixtures containing 0.5 ml of substrate (10 mM Pi), 4 ml of buffer, and 0.5 ml enzyme during 30 min reaction time. Culture filtrater, 7–10 day old, was used as enzyme source.

[b] Relative activity on *p*-nitrophenyl phosphate (PNPP) at pH 2.0 is defined as 100%.

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. Protein was determined by the method of Lowry et al. [8] using bovine serum albumin as a standard. The following buffer systems were used throughout the studies: (pH 1.0–2.2), glycerin-HCl (pH 2.8–3.6), and citric acid-sodium citrate (pH 3.0–6.2).

RESULTS AND DISCUSSION

A. ficuum produced a nonspecific acid phosphomonoesterase, rather than phytin-specific phosphatase, which hydrolyzed a variety of phosphates (Table 1). The organism produced and slowly released the phosphatases in the growth menstruum and the enzyme activity increased continually during the one month growth period (Fig. 1). However,

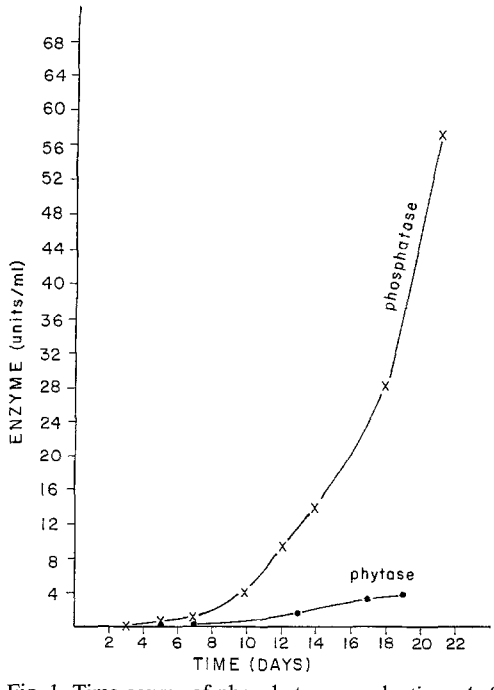

Fig. 1. Time course of phosphatases production. *A. ficuum* was grown on basal media and the enzyme activity was determined using PNPP (for phosphatase) and Na-phytate (for phytase) as a substrate.

the levels of enzyme activity toward PNPP (phosphatase) and Na-phytate (phytase) were different depending on the composition of the growth medium and cultural conditions. These activities showed different responses to the change in pH, nutrients, and sufactants. Of the substrates tested, phosphatase activity was highest on PNPP and lowest on myo-inositol 2-monophosphate. The rate of hydrolysis of the substrate was dependent on the pH of the reaction: the pH optimum for phosphatase was 2.5 whereas that for phytase was 5.0.

Phytase is commonly determined colorimetrically, measuring the liberation of inorganic phosphate (Pi) by the enzyme. The Fiske-SubbaRow method [3] and numerous other modified methods [5,7,10,11] are examples. However, because Pi is present in the growth media and the enzyme source is often the unpurified culture filtrate it is difficult for these methods to distinguish Pi present in the initial medium and Pi produced by the enzyme action. Thus, the Fiske-SubbaRow and other similar methods that determine Pi are of little value in a study where a high level of initial Pi is present. Because the organism produced a nonspecific phosphomonoesterase, the use of PNPP as a substrate for measurement of phytase activity was convenient and useful.

A. ficuum required more than 8 mg/100 ml of Pi for optimum cell growth, whereas 1–5 mg/100 ml of Pi was needed for maximum phytase production (Fig. 2). The presence of more than 10 mg/100 ml of Pi in growth medium severely repressed phytase synthesis. However, the level of protein in the growth medium was parallel to the increase in cell mass. The degree of repression was more apparent with monophosphates than with polyphosphate. Based on the same concentration of Pi, $Ca_2P_2O_7$ and phytic acid induced more enzyme synthesis than KH_2PO_4 (Fig. 3). Since both phytic acid and $Ca_2P_2O_7$ are sparingly soluble, the availability of Pi in the growth medium probably played a role. Contrary to the general belief [17] that the presence of phytin in the growth medium is indispensable for production of phytase, phytin was not necessary for phytase production as long as an adequate amount of slow-releasing phosphates were present. The

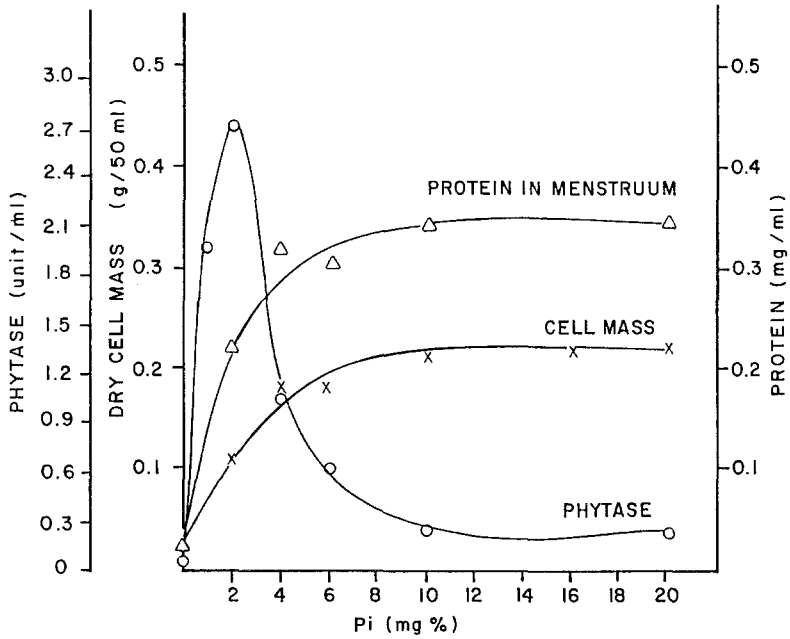

Fig.2. Effect of Pi on cell growth and phytase production. *A. ficuum* was grown for 7 days on basal media containing different levels of Pi.

levels of phosphatase activity induced by $Ca_2P_2O_7$ and sodium phytate were about 4-times higher than that induced by KH_2PO_4.

The organism utilized starch, glucose, cellobiose, maltose, sucrose and corn meal; galactose, lactose and carboxymethylcellulose (CMC) were not utilized. The level of phytase activity in sucrose, corn meal and corn starch was about 1.3 unit/ml after 10 days cultivation. The level of carbohydrate in the growth medium played an important role in phosphatase production. In early stages of growth, the use of corn starch resulted in a higher level of phosphatase but in later stages (after 10–15 day cultivation), glucose invariably surpassed the corn as a substrate for phosphatase production. Among the different forms of nitrogen, ammonium salts were readily utilized for cell growth while nitrite and urea were little or not utilized (Table 2). The pattern of phosphatase and phytase production on these nitrogenous compounds was different. For example, the cultures grown on $(NH_4)_2SO_4$ and NH_4Cl produced a high level of phosphatase activity but yielded almost negligible amounts of phytase activity. The organism grown on NH_4NO_3 produced high levels of phosphatase, while that grown on KNO_3 and NH_4NO_3 produced the most phytase. Cell growth was heavy on yeast extract and peptone, but very little phytase activity was found in these media.

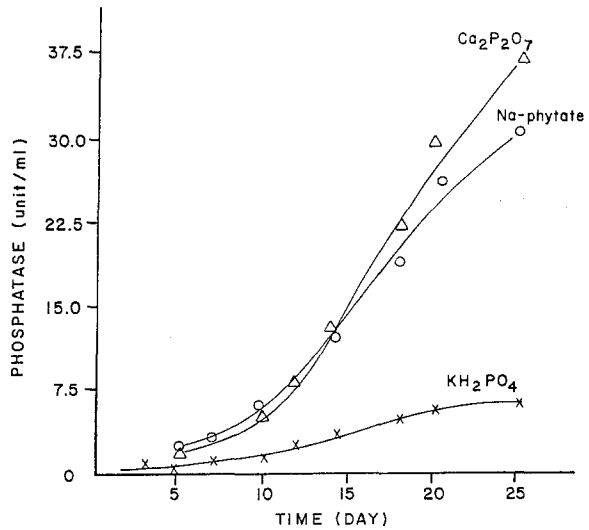

Fig. 3. Effect of different phosphates on phosphatase production. *A. ficuum* was grown on basal media containig 2 mg/100 ml Pi of different phosphates.

The effect of surfactants on enzyme production

Table 2

Effect of nitrogen source on cell mass, phytase and phosphatase production by *A. ficuum*

N Source (0.1% N)	Cell mass[a] (g/50 ml)	Phytase (unit/ml)	Phosphatase (unit/ml)
Control (no N)	0	0	0
$(NH_4)_2SO_4$	0.278	0.10	30.0
NH_4NO_3	0.194	2.57	22.6
NH_4Cl	0.225	0.56	26.3
KNO_3	0.302	2.39	15.9
$NaNO_3$	0.267	0.62	–
KNO_2	0	0	0
Urea	0.139	0	0.2
Peptone	0.284	1.16	22.0
Yeast extract	0.207	0.10	4.7

[a] *A. ficuum* was grown on a basal medium (50 ml per 125 ml flask) containing different nitrogen compounds for 10 days at 30°C on a rotary shaker.

was also different for the two enzymes. The addition of polyoxyethylene ether at the level of 0.5% to the culture medium markedly increased the phosphatase production by *A. ficuum*, whereas the activity of phytase was significantly increased by the addition of sodium oleate (Table 3). Tween 80 and dimethyl sulfoxide (DMSO) had little effect on enzyme production. The addition of cetyltrimethylammonium bromide (CTAB) and sodium lauryl sulfate at 0.1% level completely inhibited the cell growth. Various surfactants have been used in bacterial cultures to assist in cell growth and enzyme production [1,12]. It is generally known that surfactants play a role in enzyme production and secretion, but the explanation of how the surfactants act to increase enzyme yield is largely conjectural. Reese and Maguire [12] also observed that addition of surfactant in culture medium generally increased the enzyme yield, but the effects varied from organism to organism and enzyme to enzyme.

The level of phosphatase production by *A. ficuum* depended on the initial pH of the medium (Fig. 4). Higher levels of phosphatase were produced in the media with lower pH, whereas optimum pH for phytase production was between pH 4.0 and 5.6. The pH of unbuffered medium dropped (from pH 5.6 to 2.2) rapidly during the initial stage of growth and produced little phytase, although pH of the buffered media also dropped slightly during 14 day fermentation. When *A. ficuum* was grown on a rotary shaker the mycelia formed into small balls or clumps depending on the media composi-

Table 3

Effect of surfactant on phytase and phosphatase production by *A. ficuum*[a]

Surfactant	Phytase		Phosphatase	
	(unit/ml)	relative activity[b]	(unit/ml)	relative activity[b]
Control (no surfactant)	0.42	100	15.8	100
Triton X-100[c]	0.54	131	62.3	394
Tween 80	0.73	169	25.8	163
Na-oleate	2.01	482	20.4	129
Dimethyl sulfoxide	0.42	100	13.7	86
Cetyltrimethylammonium bromide	0	0	0	0
Na lauryl sulfate	0	0	0	0

[a] *A. ficuum* was grown on a basal medium containing 0.5% level of surfactants for 17 days on a rotary shaker at 30°C. Values are an average of triplicate samples.

[b] Relative activity of control which does not contain surfactant is defined as 100%

[c] Polyoxyethylene ether (Sigma Chemical Co., St. Louis, MO).

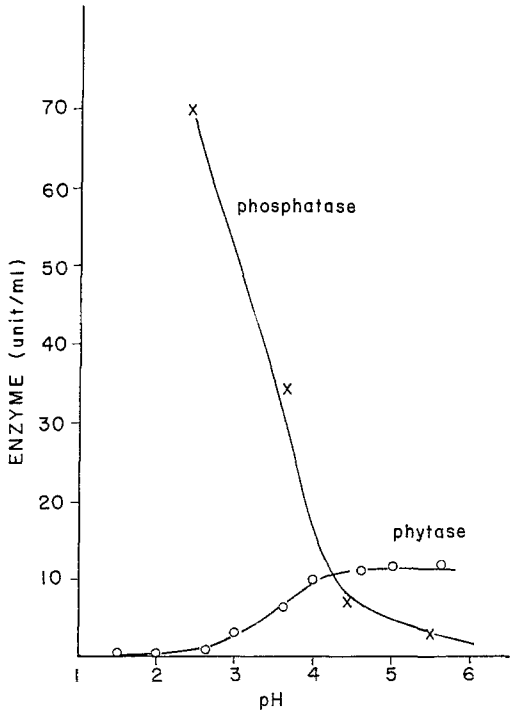

Fig. 4. Effect of pH on phosphatase production. *A. ficuum* was grown on basal media that were initially buffered at different pH.

tion and cultural conditions. In general, higher phytase activity was found in a culture where mycelia were dispersed or formed into small balls. The media with low pH, containing surfactants and a high level of carbohydrates, all produced dispersed mycelia and high levels of enzyme activities. Cultures grown without shaking produced almost no phosphatase activity in the growth menstruum although they yielded about the same level of cell mass as that on shake culture. Fungal growth in a submerged stirred tank fermentor resulted in good mycelial growth but the phosphatase activity declined after 10 days of steady increase. Phosphatase activity during the first 10 day period was about the same as that in the flask culture, but there was only a trace or no phytase activity in the submerged stirred tank fermentor.

A. ficuum produced phosphatase which hydrolyzed a variety of phosphates including phytin. Low levels of phosphorus in the growth medium were critical for the production of phosphatase. Addition of certain surfactants and low pH helped increase production of the enzymes. Responses of phosphatase and phytase activities toward nutritional and cultural variations were different.

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