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PROOF

Regulation of extracellular acid phosphatase biosynthesis by culture conditions in entomopathogenic fungus *Metarhizium anisopliae* strain CQMa102

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Abstract - *Metarhizium anisopliae* is an imperfect entomopathogenic fungus. Once invading into its host, *M. anisopliae* needs to absorb basic nutrients such as phosphorus from the host haemolymph. A large number of phosphorylated compounds in haemolymph cannot be directly utilised by the fungal cell and must be hydrolysed into available form by phosphatase before ingested. Aims of this paper were to investigate optimum fermentation conditions for production of acid phosphatase and phosphatase isoenzymes by *Metarhizium anisopliae*. The optimum fermentation conditions were: glucose, 20 g/l; $(NH_4)_2SO_4$, 2 g/l; casein, 4 g/l; $MgSO_4$, 0.5 g; KCl, 0.5 g; microelement salt solution, 10 ml; inoculum size, 1 x 10^7 spores per 100 ml medium; initial medium pH, 6.0. Under these conditions, the highest total acid phosphatase activity was 3.05 U/ml in 4 days at 27 °C and 160 rpm. Synthesis of the acid phosphatase was repressed by 0.01% inorganic phosphate in culture medium. The spectrum of isoenzymes produced by *M. anisopliae* varied depending on the phosphorus source employed in the culture. A specific isoform with pI 9.45 was induced by casein, and another isoform of pI 8.21 was induced by phytic acid and disodium phenyl phosphate.

Key words: acid phosphatase, biosynthesis, culture condition, *Metarhizium anisopliae.*

INTRODUCTION

The genus *Metarhizium anisopliae* is an imperfect entomopathogenic fungus found in soils throughout the world. It was first recognised as a biocontrol agent in the 1880s. The relative safety and selectivity of *M. anisopliae* make it an ideal candidate for integration into pest (such as locust, grasshopper) management programmes where its effects on other natural enemies will be less compared with conventional chemical insecticides (Goettel *et al.*, 1990; Ekesi *et al*., 1999). Up to now, *M. anisopliae* has been reported to infect more than one hundred insect species (Prior *et al.*, 1988; Zimmermann, 1993; Dromph *et al*., 2002; Lopez and Orduz, 2003). Today, *M. anisopliae* has been commercially developed for control of some pests, e.g., Bioblasts to control termites, Biostops and Meta-Guards to protect timber from termite infestation, and Green-Muscles to control locusts and grasshoppers. These products have been shown to be a good alternative to organophosphate pesticides and carries the benefits of specificity to insects, compatibility with conventional ultra low volume spray application and long term eject and persistence (Thomas *et al.*, 1996; Langewald *et al.*, 1999).

The first step of *M. anisopliae* kill host is invasion their hosts through the cuticle by mechanical pressure, via apressorium formation, and enzymatic degradation by synergistic action of hydrolases (Charnley *et al.*, 1991; Clarkson and Charnley, 1996; St. Leger *et al.*, 1996; Pinto *et al.*, 1997). Therefore, Particular attention has been paid to the extracellular enzymes involving in this process. Little is known about the interactions between insect and pathogen once the fungus has got into the haemolymph. In fact, once invading into its host, *M. anisopliae* depends largely on host's haemolymph for surviving, which is one of the reasons that the fungus kills the host. This dependence includes the need to extract basic nutrients such as phosphorus from the host organism. A large number of phosphorylated compounds have been identified in the haemolymph of insects, including glucose-1-phosphate and many phosphoproteins. However, those phosphorylated compounds cannot be directly utilised by the fungal cell and instead must be hydrolysed into available form before ingested. For surviving, the fungus must secrete corresponding hydrolytic enzyme. Xia *et al*. (2000) reports that the *M. anisopliae* (strain ME1) secretes acid phosphatase (AcP, EC 3.1.3.2) after invading into haemolymph of the desert locust *Schistocerca gregaria*. In order to investigate the characterisation and function of the AcP, it is necessary to induce and purify the enzyme *in vitro* from liquid medium. There are many reports about AcPs produced by fungi *in vitro* (Shieh *et al*., 1969; Yoshida and Tamiya, 1971; Nahas *et al*., 1982; Haas *et al*., 1991; Nozawa *et al*., 1998; Xia *et al*., 2000). However, the fungal AcPs reported previ-

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ously are almost induced in media containing an inorganic nitrogen source as $NaNO_3$ (Haas *et al*., 1991; Nozawa *et al*., 1998; Xia *et al*., 2000), $(NH_4)_2SO_4$ (Yoshida and Tamiya, 1971), or NH_4NO_3 (Nahas *et al*., 1982) and very low concentration of inorganic phosphate (Pi). Much less information is available on the production of AcPs by fungi using organophosphorus compounds as sole phosphorus source (Tarafdar *et al*., 1988). Although there are a few reports concerning the production of AcPs by fungi in cultures containing organic phosphorus compounds (Aleksieva and Micheva-Viteva, 2000; Micheva-Viteva *et al*., 2000), they investigate only the total AcP activity and do not distinguish between AcP isoenzymes. In the study, the effects of different phosphorus sources (casein, phytic acid, KH_2PO_4 and $C_6H_5Na_2PO_4{\cdot}2H_2O$) on AcP isoenzymes are investigated, and the results indicate that the activity and AcP isoenzymes are significantly difference between different phosphorylated matters as sole phosphorus source.

Aims of this paper were to investigate optimum fermentation conditions for production of acid phosphatase and phosphatase isoenzymes by *Metarhizium anisopliae*.

MATERIALS AND METHODS

Microorganism and basal medium. *Metarhizium anisopliae* var. *acridum* strain CQMa102, isolated originally from *Ceracris kiangsu* Tsai by the Genetic Engineering Center of Chongqing University, was used in this study. The strain is deposited in the China General Microbiological Culture Collection Center (CGMCC) under access No. 0877. Conidia were obtained from cultures in $^1/_4$-strength Sabouraud's Dextrose agar at 27 °C under constant light for 12 d.

The basal medium for AcP production contained (l^{-1}): glucose 20 g, $MgSO_4$ 0.5 g, KCl 0.5 g, $NaNO_3$ 2 g, Pi 0.01 g, microelement salt solution 10 ml, 2-(N-Morpholine) ethanesulfonic acid (Mes) 50 mM (pH 6.0). Microelement salt solution (l^{-1}) consisted of 2 g $FeSO_4{\cdot}7H_2O$, 10 g $ZnSO_4{\cdot}7H_2O$, 0.2 g $NaMoO_4{\cdot}2H_2O$, 0.2 g $CuSO_4{\cdot}5H_2O$ and 0.2 g $MnCl_2{\cdot}4H_2O$.

Effects of initial culture pH, inoculum density and aeration condition. To determinate the effect of initial culture pH on the AcP production by *M. anisopliae,* solutions of basal medium were prepared at 0.5 unit intervals from pH 4.0 to 8.0 (50 mM Mes buffer for pH 4.0-6.0, 50 mM Hepes buffer for pH 6.5-7.5, and 50 mM Tris buffer for pH 8.0), and each 100 ml medium in 250 ml Erlenmeyer flask was inoculated 1 x 10^7 spores.

The effect of inoculum density was examined by inoculating 1 ml of the spore suspension containing 1 x 10^6, 5 x 10^6, 1 x 10^7, 5 x 10^7 or 1 x 10^8 spores to 100 ml basal medium.

The effects of aeration conditions on AcP production were examined using a 50 ml, 100 ml, 150 ml or 200 ml basal medium, and each 100 ml medium was inoculated 1 x 10^7 spores. All disposals were cultured at 27 °C for 96 h and 160 rpm.

Effects of carbon, phosphorus and nitrogen source. In order to measure the effects of the carbon sources, to give 0.8 g carbon/100 ml medium, on AcP production, glucose was replaced by fructose, maltose, sucrose and starch respectively.

Different nitrogen sources to give 0.038 g nitrogen/100 ml medium (ammonium sulphate, sodium nitrate, yeast extract, peptone and beef extract) substituted the sodium nitrate source.

Similarly, different phosphorus sources including KH_2PO_4, phytic acid, $C_6H_5Na_2PO_4{\cdot}2H_2O$, and casein were used. The effect of different concentration (10 mg/l, 20 mg/l, 50 mg/l, 100 mg/l, 150 mg/l) of Pi on the AcP production by *M. anisopliae* was also determined. pH of the culture medium was adjusted to 6.0 before sterilisation. Spore suspension (1 ml) containing 1 x 10^7 spores was used to inoculate 100 ml of the media in 250 ml Erlenmeyer flasks. The cultures were incubated on a rotary shaker at 27 °C and 160 rpm.

Preparation of crude enzyme samples and measurement of fungal growth. Samples were taken out periodically, and the mycelia were filtered through Whatman N°·1 filter paper. The filtrates were centrifuged (10000 *x g*, 4 °C, 10 min). The supernatant was concentrated approximately 50-100 fold using PEG 20000 at 4 °C to produce crude enzyme samples used in the AcP isoenzymes assay. The mycelia were washed with distilled water three times and dried at 80 °C to a constant weight.

Determination of AcP activity. AcP activity against 4-Nitrophenyl Phosphate, disodium salt (*ρNPP,* Merck, Germany) was measured by the method of Andersch and Szcypinski (1947). Briefly, culture was filtrated through Whatman N°·1 filter paper to remove the mycelium then was centrifuged at 10000 *x g* for 10 min at 4 °C. The supernatant (40 µl) was added to 160 µl of 0.1 M sodium acetate buffer (pH 5.0) containing 8 mM *ρNPP*. In control treatment, culture supernatant was replaced with 40 µl of boiled (15 min) culture supernatant. The reaction mixture was incubated in a 37 °C water bath for 10 min. The reaction was terminated by the addition of 50 µl of 0.5 M NaOH. The absorbance of the reaction mixture was determined at 405 nm using M550 MICROPLATE READER (Bio-Rad). AcP activity was determined with reference to a calibration curve of standard 4-nitrophenol (Sigma, America). One unit (U) of AcP activity was defined as 1 µmol 4-nitrophenol released in 1 min at 37 °C and pH 5.0.

Protein determination. Protein concentration was determined using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories) with bovine serum albumin (BSA, Fluka) as the standard.

Vertical isoelectric focusing (IEF) gel and AcP isoenzymes assay. Isoelectric focusing was carried out in 5% (w/v) vertical IEF gel using the Mini-Protean apparatus (Bio-Rad), with a broad pH range (3-10). The cathode solution was 5 mmol/l NaOH, and the anode solution was 5 mmol/l acetic acid. Total AcP activities of the loading samples were equal. Following IEF, the gel was stained for AcP isoenzyme assay using the method described by Nagy *et al*. (1981). Briefly, gels were incubated for 20 min at room temperature in 100 ml solution of 150 mg 1-naphthyl phosphate (Sigma) and 50 mg fast blue RR salt (Fluka) in 50 mM MES buffer (pH 5.5), then transferred to a 7% acetic acid solution.

All experiments in the study were replicated four times, and the results expressed as mean ± standard deviation.

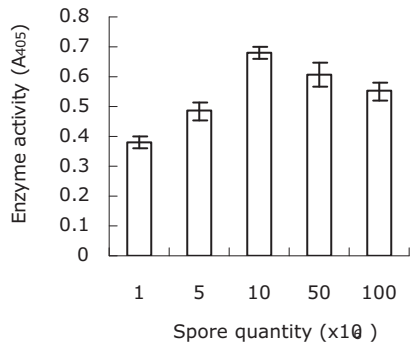

FIG. 1 - Effects of different inoculum density on acid phosphatase production by *Metarhizium anisopliae*

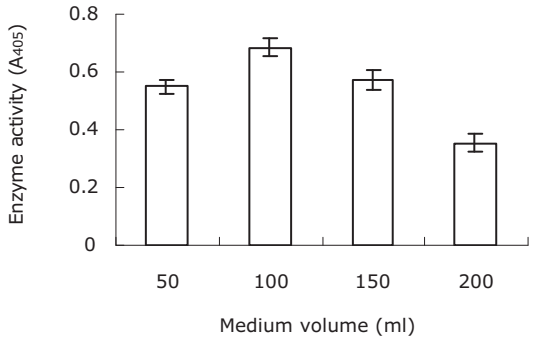

FIG. 3 - Effects of different aeration conditions on acid phosphatase production by *Metarhizium anisopliae.*

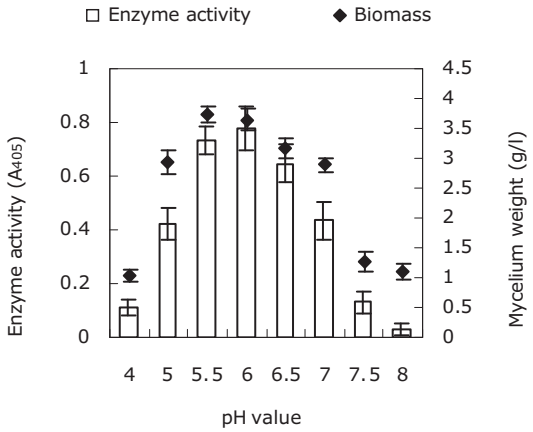

FIG. 2 - Effects of different initial medium pH on the fungi growth and acid phosphatase production of *Metarhizium anisopliae.*

RESULTS

Effects of inoculum density, initial pH and aeration condition on AcP production

The effect of inoculum density on AcP production was illustrated in Fig. 1. Five levels of inoculum concentration were tested. The inoculum level at 1 x 10^7 spores in 100 ml medium was found to be optimum for AcP production by *M. anisopliae*. Enzyme production and biomass of *M. anisopliae* were markedly influenced by the initial medium pH (Fig. 2). The maximum biomass and the highest of total AcP activities were obtained at initial pH values between 5.5 and 6.0. The fungus did not grow at initial pH below 4 or above 8. Figure 3 depicted the effect of aeration conditions on AcP production of *M. anisopliae*. The results indicated that optimum aeration condition was achieved when the medium occupied approximately 40% (2/5) of the total volume of the flask.

Effect of different carbon and nitrogen source

Different carbon and nitrogen sources were used to determinate the most effective carbon and nitrogen source for AcP production of *M. anisopliae* (Table 1). The results indicated glucose led to significantly greater AcP activity (0.365 ± 0.04, n = 4) after 144 h compared with other carbon sources, of which soluble starch was the least capable (0.186 ± 0.01, n = 4). And there was no obvious difference when sucrose, maltose or fructose as the carbon source.

Inorganic nitrogen sources had a dramatic effect on the AcP production of *M. anisopliae*. AcP production of *M. anisopliae* was far more effective using organic nitrogen than inorganic nitrogen. In general, the AcP production follows a peptone > beef extract > yeast extract > $(NH_4)_2SO_4$ > $NaNO_3$ sequence, but there was no evident difference between peptone, beef extract and yeast extract ($P < 0.05$, n = 4).

TABLE 1 - Influence of carbon and nitrogen source on the fungus growth and acid phosphatase activity (n=4)

Carbon source*	Enzyme activity (U/ml, Mean ± SD)	Biomass (g/l, Mean ± SD)	Nitrogen	Enzyme activity (U/ml, Mean ± SD)	Biomass (g/l, Mean ± SD)
Glucose	0.364 ± 0.04a	4.26 ± 0.29a	Peptone	2.690 ± 0.24a	13.29 ± 0.43a
Sucrose	0.260 ± 0.03b	3.95 ± 0.22a	Yeast extract	2.517 ± 0.19a	13.11 ± 0.28a
Maltose	0.235 ± 0.02b	3.28 ± 0.16b	Beef extract	2.454 ± 0.18a	10.87 ± 0.35b
Fructose	0.224 ± 0.02b	2.97 ± 0.17b	$(NH_4)_2SO_4$	0.491 ± 0.03b	5.89 ± 0.21c
Starch	0.185 ± 0.01c	2.18 ± 0.14c	$NaNO_3$	0.364 ± 0.02c	4.26 ± 0.29d

Means with different lowercase letters in each column differed significantly in Tukey's HSD test ($P < 0.05$); * $NaNO_3$ was the nitrogen source in the experiments done by changing carbon source; ** glucose was the carbon source in the experiments done by changing nitrogen source.

Regulation of AcP biosynthesis by Pi

The influences of Pi on biomass and AcP production of *M. anisopliae* were illustrated in Fig. 4. The biomass and the AcP activity increased with increasing of Pi concentration from 10 mg/l to 50 mg/l, and the highest total AcP activity was 0.38 U/ml at Pi concentration of 50 mg/l. Pi concentrations above 50 mg/l did not affect the fungus growth but strongly suppressed the AcP biosynthesis, and no activity was observed when Pi concentration was 100 mg/l or 150 mg/l.

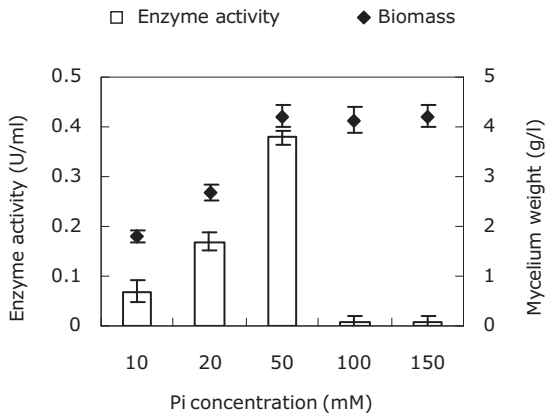

FIG. 4 - Effects of different Pi concentrations on the acid phosphatase production by *Metarhizium anisopliae.*

Effect of different phosphorus source on AcP production

Different phosphorus sources were used for determination of the most effective phosphorus source for total AcP produced by the strain (Table 2). The fungus showed maximal productivity when casein was used as phosphorus source and the augmentation compared to the other phosphorus sources was about 8-10 fold for the AcP activities, 3-4 fold for the biomass and 1.5-2 fold for the specific activity. The highest total activity and specific activity of AcPs produced in the casein medium after four days were approximately 3.052 U/ml and 45.17 U/mg proteins, respectively. The results suggested that the casein could be the optimal phosphorus source for the fungus growth and AcP production.

The results of the isoenzymes analysis were displayed in Fig. 5. The strain produced eight isoforms between pI 4.43 and pI 9.45. Isoforms between pI 4.70 and pI 8.01

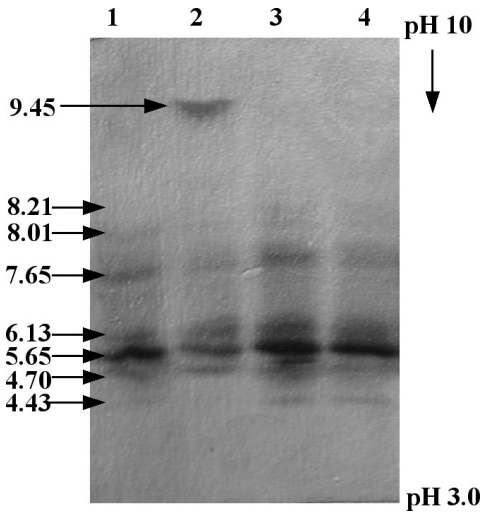

FIG. 5 - Acid phosphatase isozymes of *Metarhizium anisopliae* in different culture media analysed by IEF-PAGE Note: 1.pi medium; 2.casein medium; 3.phytate medium; 4. disodium phenyl phosphate medium.

were produced by *M. anisopliae* on all media. A specific isoform of pI 9.45 was produced by the strain when casein as the phosphorus source, while an isoform of pI 4.43 was produced when other phosphorus sources were used. In addition, an isoform of pI 8.21 was induced by the use of phytic acid or disodium phenyl phosphate as sole phosphorus source.

Time course of AcP production on the optimised medium

The time courses of growth (mycelium dry weight) and AcP production of *M. anisopliae* on the optimised culture condition (l^{-1}: glucose, 20 g; $(NH_4)_2SO_4$, 2 g; casein, 4 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; microelement salt solution, 10 ml; inoculum size, 1 x 10^7 spores per 100 ml liquid medium; aeration condition, 2/5 volume and initial medium pH 6.0) were illustrated in Fig. 6. The fungal biomass formation occurred very rapidly from 60 to 96 h of cultivation, after which the quantity of mycelium remained at an almost constant level. The total AcPs activities coincided with the fungus growth phase. The AcP activity rapidly increased during the exponential growth of the fungus, and remained at a steady level when the fungus entered the stationary stage.

TABLE 2 - Influence of phosphorus source on the fungus growth and AcP activity (n = 4)

Phosphorus source*	Enzyme activity (U/ml, mean ± SD)	Specific activity (U/ml, mean ± SD)	Biomass (g/l, mean ± SD)
Casein	3.050 ± 0.12a	45.16 ± 4.82a	13.11 ± 0.72a
Pi (KH_2PO_4)	0.365 ± 0.04b	30.87 ± 3.98b	4.26 ± 0.29b
Disodium phenyl phosphate	0.348 ± 0.06b	24.22 ± 4.21c	4.44 ± 0.37b
Phytate	0.261 ± 0.04c	32.43 ± 3.79b	3.27 ± 0.22c

Means with different lowercase letters in each column differed significantly in Tukey's HSD test ($P < 0.05$); * $NaNO_3$ and glucose were the nitrogen source and the carbon source respectively in the experiments done by changing phosphorus source.

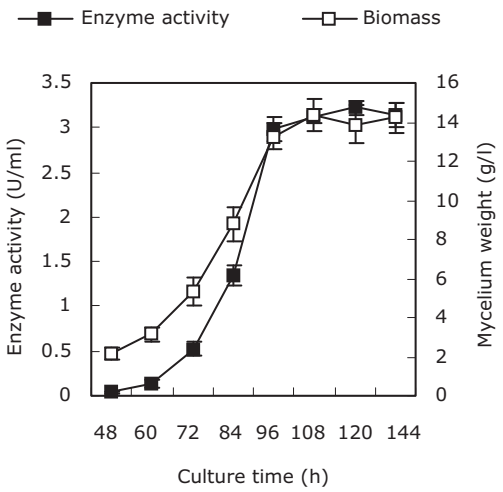

FIG. 6 - Time course of fungus growth and acid phosphatase production of *Metarhizium anisopliae* (CQMa102), grown on casein medium at 27 °C.

DISCUSSION

In fungi, acquisition of nutrients from the environment requires the secretion of an array of hydrolytic enzymes acting on specific resources (Nozawa *et al*., 1998). Extracellular AcPs have been reported in many fungi, such as *Aspergillus* (Nozawa *et al*., 1998), *Penicillium* (Haas *et al*., 1991), *Fusarium* (Yoshida and Tamiya, 1971) and *Neurospora* (Nahas *et al*., 1982). Generally, extracellular AcPs are a generic designation for nonspecific phosphoesterases, so previous reports discussed only the total AcP activity, and did not distinguished between the various isoenzymes characterised as AcPs. In this study, we found that different isoenzymes were produced by *M. anisopliae* when the fungus used different phosphorus sources. The strain produced a specific isoform with pI 9.45 when casein was the sole phosphorus source. This isoform probably dephosphorylated the casein from the medium ensuring orthophosphates for the fungal growth. An additional isoform of pI 8.21 appeared when phytic acid or disodium phenyl phosphate as phosphorus source, which probably dephosphorylated the cyclic hydrocarbon phosphate.

The total amount of AcP synthesized was dependent on the initial Pi concentration in the medium. The maximum activity of AcP was obtained when the concentration of Pi was 0.05 g/l, and there was no detectable activity when the Pi concentration was increased to 0.1 g/l. This suggested that Pi 0.05 g/l was the crucial concentration, which played an important role in the direction of the fungal metabolism towards enhanced AcP synthesis. The Pi-repression influence on the AcP formation was in agreement with the results reported by Shieh *et al* (1969) concerning the regulation of production of two kinds *Aspergillus. ficuum* AcPs by Pi.

The effects of inoculum density on the production of AcP indicated that the maximum enzyme production was achieved using a concentration of 1 x 10^7 spores in 100 ml liquid culture. An increase in the number of spores in the medium would ensure a rapid proliferation and biomass synthesis. However, beyond a certain limit, competition for the nutrients results in decreasing metabolic activity of the organism. With optimum inoculum density for the enzyme production, there is a balance between proliferating biomass and availability of nutrient that supports production of enzyme. For increasing AcP synthesis, we examined the effects of aeration conditions by changing the ratio of culture volume to flask volume. All flasks were agitated at the same rate. Therefore, the oxygen transfer rate to the medium decreased with increasing volumes of medium. The results indicated that 2/5 of flask volume should be the optimal aeration condition.

Our results indicated that AcP produced by *M. anisopliae* was far more effective using organic nitrogen than inorganic nitrogen. It could be possible that organic nitrogen contains all kinds of amino acids and these amino acids can be absorbed directly by mycelia. In contrast to organic nitrogen, *M. anisopliae* first synthesized inorganic nitrogen into amino acids. This additional synthetic requirement slows the growth rate, leading to reducing AcP production. However, high proteinase activities were measured in the culture supernatant when $(NH_4)_2SO_4$ was substituted by peptone, beef extract or yeast extract (data not shown), which was unfavourable for the storing of crude enzyme and purifying enzyme. Why there have high AcP activities in the experiments done with casein as phosphorus resource? It could be possible that casein is a protein, like other organic nitrogen, which contains all kinds of amino acids and these amino acids can be absorbed directly by mycelia after hydrolysed. On the other hand, phosphorus of casein needs hydrolyte before absorbed by mycelia, therefore, which could induce mycelia to secrete AcP, and not inhibit the production of AcP.

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