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Effect of Culture Conditions on the Production of PDM Phosphatase by *Fusarium moniliforme*

(phosphatase/culture condition/*Fusarium moniliforme*)

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Culture conditions suitable for production of PDM phosphatase by the fungus, *Fusarium moniliforme*, were investigated and established as follows. The medium contained 3% (w/v) glucose, 0.1% (w/v) ammonium sulfate and 0.1% (w/v) yeast extract in 50 mM sodium citrate buffer (pH 5.8) and the temperature was 25℃. In an early stage under these conditions, there was production of another unstable phosphatase presumably specific for phosphomonoesters. After the decline of this phosphatase activity at about 40 h of the culture time, PDM phosphatase began to increase and reached a plateau at about 100 h. This time course of production was slightly different from that observed under the same conditions for ribonuclease F1, another phosphoric acid hydrolyzing enzyme secreted by the fungus.

Introduction

PDM phosphatase is a unique phosphoric acid hydrolyzing enzyme secreted by a phytopathogenic fungus, *Fusarium moniliforme*. We isolated this enzyme from a commercial enzyme preparation, Toyocelase, which was dry powder of the filtrate of *F. moniliforme* culture (1). We studied properties of the enzyme and clarified various enzymatic and structural aspects. We are now trying to carry out protein engineering studies of the enzyme. To do so, it is indispensable to know under what conditions the fungus can produce the enzyme. In this paper, we investigated the effect of changing some factors of culture conditions on the production of the enzyme. This fungus is known to secrete another phosphoric acid hydrolyzing enzyme, ribonuclease F1 (2). The culture conditions suitable for the production of the ribonuclease were established by Hoshi and Yoshida (unpublished results). We started from the established conditions and investigated the effect of changing some factors. Finally, we found that ribonuclease F1 and PDM phosphatase were produced maximally under the same conditions, although the time courses of the production were slightly different.

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This paper is respectfully dedicated to the memory of the late Professor Isao Taguchi.

Materials and Methods

Reagents and Enzyme Assay —— *p*-Nitrophenyl phosphate and bis-*p*-nitrophenyl phosphate were purchased from Nacalai Tesque. *p*-Nitrophenyl phenylphosphonate was synthesized according to Yoshida *et al.* (3). Assay for phosphatase activity was carried out by the method described earlier (1). Briefly, the reaction mixture contained 5 mM *p*-nitrophenyl phosphate and an appropriate amount of enzyme in 2 ml of 50 mM sodium acetate buffer (pH 5.3). After incubation at 37℃ for 10 min, the reaction was stopped by addition of 1 ml of 0.5 M NaOH and absorbance of the mixture was measured at 400 nm against a control run in the same way except addition of the enzyme. For the control, the enzyme solution was added after addition of the alkali solution to compensate the color brought in by the enzyme solution. The liberated *p*-nitrophenol was determined using a molar absorbance of 18,500 $M^{-1}cm^{-1}$. The amount of phosphatase that liberates 1 μmol of *p*-nitrophenol under the

specified conditions was defined as 1 unit (U). When the substrate was bis-*p*-nitrophenyl phosphate or *p*-nitrophenyl phenylphosphonate, 0.5 M Na_2CO_3 was used instead of 0.5 M NaOH to stop the reaction.

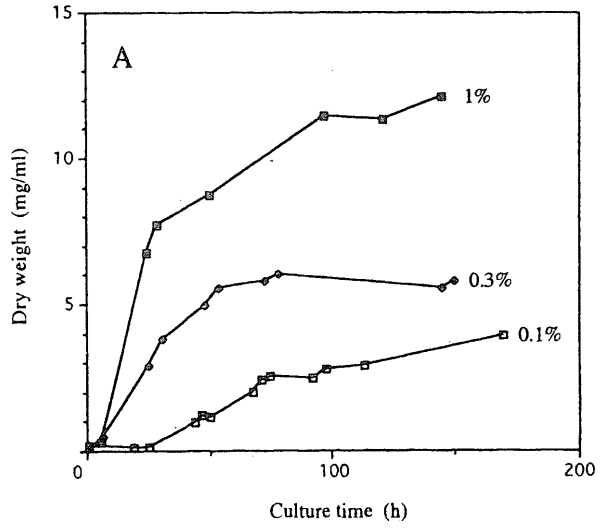
Strain and Medium —— A *Fusarium moniliforme* strain was donated by Toyo Brewery Co. in the form of lyophilized mycelia. They were put into the vegetative state by immersion in the standard liquid medium described below and maintained on potato-dextrose agar (Nissui) slants at 4℃.

Culture —— The standard liquid medium contained 3% (w/v) glucose, 0.1% (w/v) ammonium sulfate and 0.1% (w/v) yeast extract (Difco) in 50 mM sodium citrate buffer (pH 5.8). The fungus was inoculated from the slant into 3 ml of the medium and shaken in an incubator at room temperature (around 25℃) for 40 h. The whole preculture solution was put into 100 ml of the medium placed in 500 ml bottle and the incubation was continued under shaking. In summer when room temperature exceeded 30℃, care was taken so that the temperature of the culture was kept under 30℃. At various times, 5 ml-portion of the culture was withdrawn and filtered through a preweighed cellulose acetate filter (0.45 μm poresize, 47 mm diameter, Advantec). The material filtered off was washed with distilled water, then dried thoroughly under vacuum in a desiccator over NaOH and conc. H_2SO_4, and weighed. The filtrate was assayed for the phosphatase activity.

Results and Discussion

Hoshi and Yoshida observed that production of ribonuclease F1 by *F. moniliforme* was highly dependent on the yeast extract concentration but not very much so on glucose or ammonium sulfate concentration (unpublished results). Therefore, we started to investigate the effect of changing the yeast extract concentration. As shown in Fig. 1A, the higher was the yeast extract concentration, the better was the growth. On the contrary, the lower was the yeast extract concentration, the better the phosphatase production (Fig 1B). This phenomenon was exactly the same as what had been observed with ribonuclease F1. It seems that the fungus secretes phosphoric acid hydrolyzing enzymes under conditions rather unfavorable for growth. However, in the time course of production of phosphatase, a sharp peak was observed at time 25 — 50 h which did not exist in the case of ribonuclease F1.

To see whether this peak was due to PDM phosphatase, enzyme activity toward bis-*p*-nitrophenyl phosphate and *p*-nitrophenyl phenylphosphonate was examined. The most characteristic feature of PDM phosphatase is its broad specificity: it liberates *p*-nitrophenol at almost



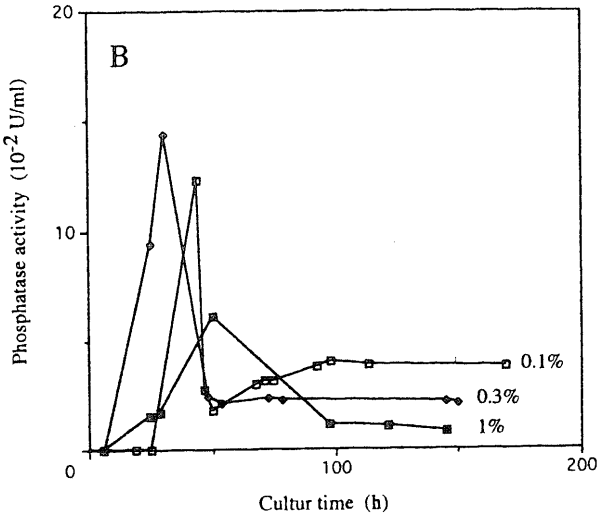


Fig. 1. The time courses of growth (A) and phosphatase production (B) at different yeast extract concentrations. The concentration indicated is in w/v.

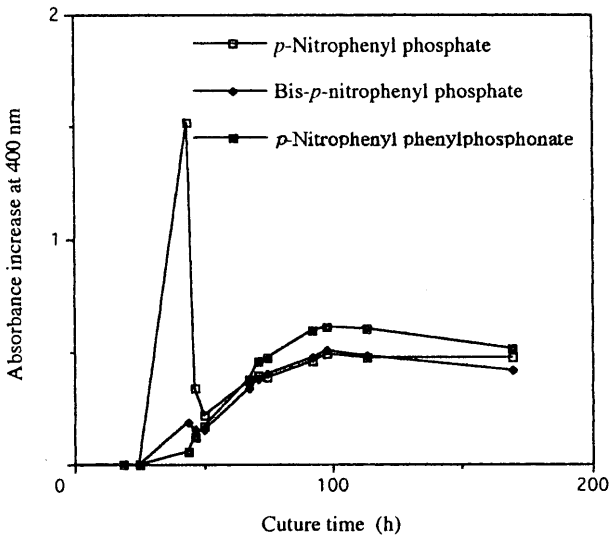


Fig. 2. Change of phosphatase activities toward three substrates as a function of time. *F. moniliforme* was cultured under the standard conditions.

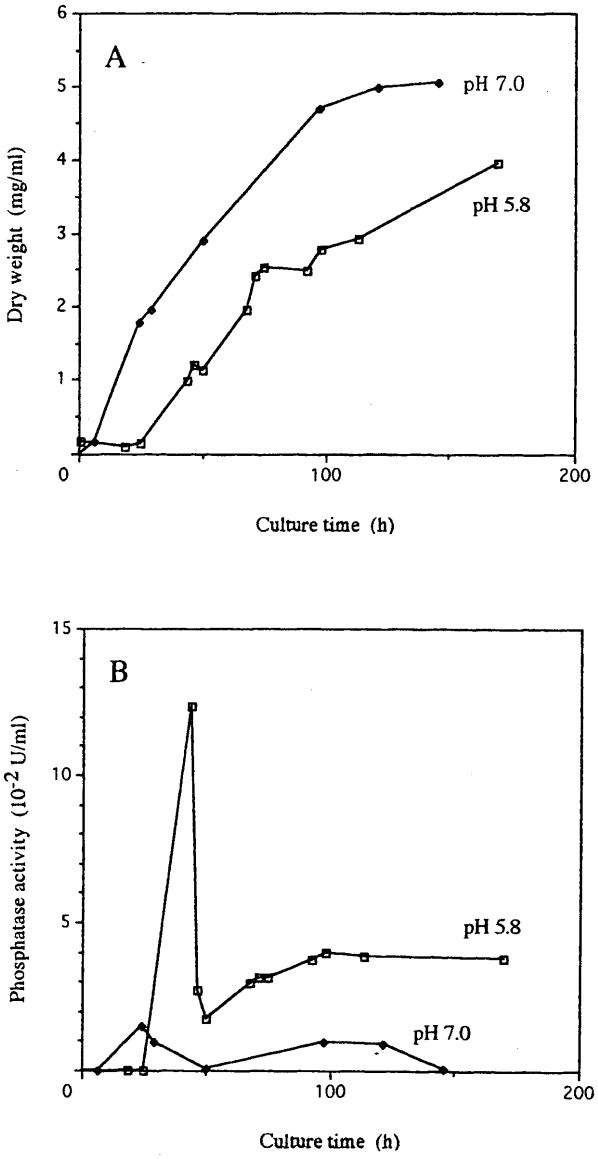


Fig. 3. Effects of initial pH of the culture medium on growth (A) and phosphatase production (B). Otherwise, the culture was carried out under the standard conditions.

equal rates from these substrates as well as from *p*-nitrophenyl phosphate (1, 3). Fig. 2 clearly shows that the sharp peak was only active toward *p*-nitrophenyl phosphate. Thus it must not have been due to PDM phosphatase. A phosphatase presumably specific for phosphomonoesters was produced at the early stage. This enzyme may have been unstable, which caused quick decline of its activity. The plateau reached at about 100 h must be due solely to PDM phosphatase, because 1) activities toward the three substrates are parallel and 2) ribonuclease F1 is known to be inactive toward these substrates.

Next, we investigated briefly the effects of pH and temperature. If 50 mM Tris-HCl buffer (pH 7.0) was used to prepare the medium instead of the citrate buffer (pH 5.8), the growth was somewhat better (Fig. 3A) but the production of phosphatase was much poorer (Fig. 3B).

When the incubation was carried out at 37℃ instead of 25℃, the growth was not very much affected (Fig. 4A), but again the production of phosphatase was poorer (Fig. 4B).

In conclusion, PDM phosphatase was well produced under the following conditions: medium, 3% glucose, 0.1% ammonium sulfate and 0.1% yeast extract dissolved in 50 mM sodium citrate buffer (pH 5.8); temperature, 25℃. Under these conditions, there was a rapid production of a phosphomonoesterase, whose activity declined quickly probably due to its instability. PDM phosphatase began to increase at about 40 h of the culture time and reached a plateau at about 100 h. Hoshi and Yoshida observed that ribonuclease F1 appeared at about 24 h, increased steadily and reached a plateau only after 120 h. Therefore, it seems that there is slight difference between the production time courses of these two enzymes. We are currently trying to isolate mRNA for PDM phosphatase from the mycelia of *F. moniliforme* cultured under the standard conditions for 50 — 60 h where the

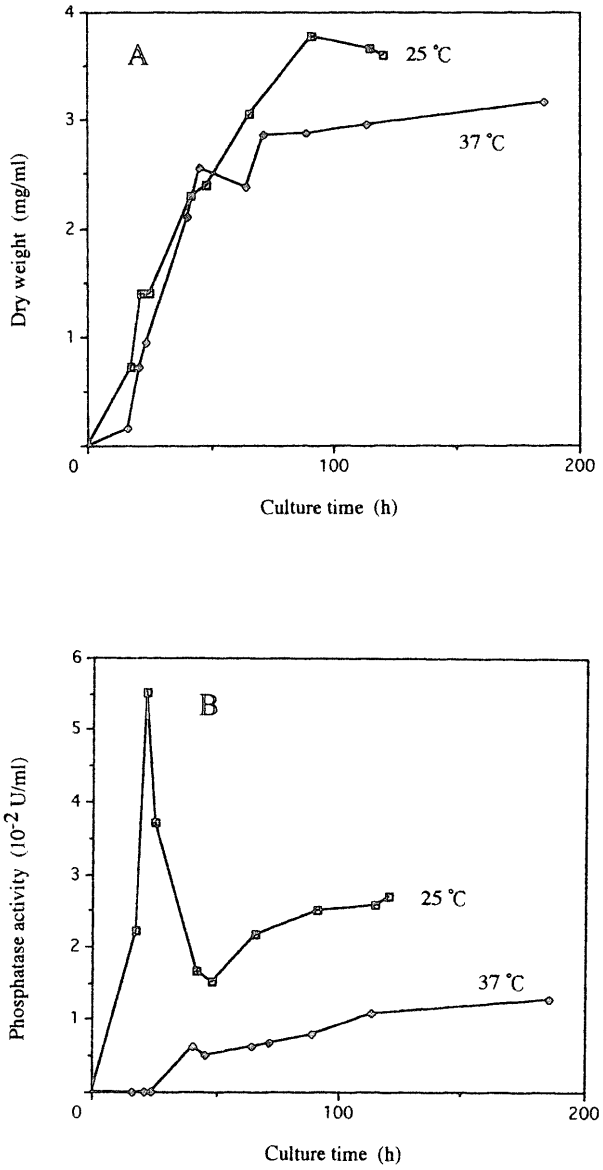


Fig. 4. Effects of temperature on growth (A) and phosphatase production (B). In these cases, the temperature was kept at the indicated value with a thermostat. The culture conditions were otherwise standard.

production rate is expected to be maximal.

References

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