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ORIGINAL RESEARCH PAPER

Tryptophan supplementation and pH adjustment for optimizing the sporulation of *Coniothyrium minitans*

Junling Shi · Yin Li · Yi Zheng · Yi Zhu · Xiaoping Zhang · Guocheng Du · Jian Chen

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Abstract To determine the effect of tryptophan and pH on sporulation of *Coniothyrium minitans*, the fungus was cultivated using a two-stage, agar plate method in which addition of tryptophan and pH were controlled at the sporulation stage. The spore yield was enhanced by 4 times with 0.1 g tryptophan/l addition after 72 h. The optimal pH values were 4 for mycelia growth and 5.8–6 for sporulation. Mycelia grown at pH 6 had a higher productivity of spore production than did those grown at pH 4.

J. Shi (✉) · X. Zhang
College of Food Science and Engineering, Northwest Agriculture and Forestry University, Xinong Road 28, Yangling, Shaanxi 712100, China
e-mail: sjlshi2004@yahoo.com.cn

Y. Li
Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China

Y. Zheng
Biological and Agricultural Engineering Department, University of California Davis, One Shields Ave, Davis, CA 95616, USA

Y. Zhu
Department of Food Science and Technology, University of California Davis, One Shields Ave, Davis, CA 95616, USA

G. Du · J. Chen
School of Biotechnology, Southern Yangtze University, Huihe Road 170, Wuxi 214036, China

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Introduction

Coniothyrium minitans is an efficient biological inhibitor for *Sclerotinia sclerotiorum*, a fungal pathogen which causes rot diseases and reduces the yields of various crops (Li et al. 2006; Partridge et al. 2006). The production of *C. minitans* spores has been extensively studied to meet the rapidly increasing demand to inhibit *S. sclerotiorum* in soil and the hyphal growth of *S. sclerotiorum* on foliage (Shi et al. 2004a). Research has focused on the conditions for the sporulation of *C. minitans* mainly in aspects of nitrogen and carbon nutrients and mineral ions (Ooijkaas et al. 1999, 2002; McQuilken et al. 1997; Jones et al. 2004). Tryptophan promotes sporulation and spore germination of some fungi (Morquer and Montant 1952; Binu et al. 2001) but it remains unclear whether tryptophan can affect the sporulation of *C. minitans*.

pH also influences the growth and sporulation of *C. minitans*. The optimum pH for sporulation of *C. minitans* was 4.5–6.2, which is different from that for mycelia extension (from 3.3 to 5.6) (McQuilken et al. 1997, 2002; Ooijkaas et al. 1999). Thus, maintaining the correct pH during growth might maximize the yield of *C. minitans* spores. The pH values for hyphal growth and sporulation of

C. minitans, however, have not been optimized separately in previous studies when the fungus was cultivated in a single medium throughout the entire process. The objective of this paper was to investigate the effect of tryptophan and media pH on sporulation and growth of *C. minitans*.

Materials and methods

Organism and growth

Coniothyrium minitans CBS 148.96 (McQuilken et al. 2002; Jones et al. 2003), was grown on potato/dextrose/agar (PDA), pH 6, for 14 days. Conidial suspensions were made by flooding the plate with sterile 2%(w/v) glycerol. The concentration of spores in the suspension was measured using a haemacytometer. The spore suspension was diluted with sterile 2%(w/v) glycerol to give 10^7 conidia/ml.

A membrane-aid cultivation system

A membrane-aid cultivation system, developed by Shi et al. (2004b), was used. The PDA medium was overlaid with a cellulose acetate membrane allowing the biomass of *C. minitans* to be recovered and measured directly. Thus, the medium composition can be easily changed at specific stages of cell growth and spore formation.

Cultivation methods

Two cultivation methods, one-stage and two-stage, were used. In the one-stage process, *C. minitans* was cultivated on PDA medium throughout the whole growth (192 h). In the two-stage cultivation, *C. minitans* was first cultivated on a PDA medium and then transferred to fresh PDA medium in which different levels of tryptophan or pH values were used. The total cultivation time in the two-stage process was 192 h.

Growth profiles were determined for the one-stage process. Spore yield was measured for all experiments after 192 h.

Analytical methods

Determination of biomass

During the cultivation, wet weight of the fungal culture, together with the membrane, was aseptically measured and put back for continuous cultivation every 24 h. The initial weight was assumed as the weight at 8 h.

Measurement of the spore yield

The culture of *C. minitans* was collected and grounded with quartz sand (0.2 mm diameter) for 2 min and then diluted with 100 ml 1 g Tween 80/l. The above suspension was stirred for 10 min at 500 rpm and filtered through two layers of muslin. Conidia in the filtration were counted within 10 min in a haemacytometer after being well stirred (Ooijkaas et al. 2000).

Statistical analysis

All tests were replicated three times and the mean value was reported. Data analysis was performed using Microsoft Excel 2003 and Origin 7.0. The difference between treatments was determined by ANOVA with a significance level of 0.05.

Results

Effect of tryptophan on spore yield

The effect of tryptophan on *C. minitans* spore yield is shown in Fig. 1. For the one-stage process, the highest spore yield was at 0.5 g tryptophan/l but with the two-stage process, the highest yield was with 0.1 g tryptophan/l in the second medium after cultivating in the first medium for 72 h (Fig. 1). The spore yield was enhanced by about 4-fold compared with the control. When the fungus is transferred to the second medium, sporulation was not enhanced unless the tryptophan concentration was higher than that in the first medium.

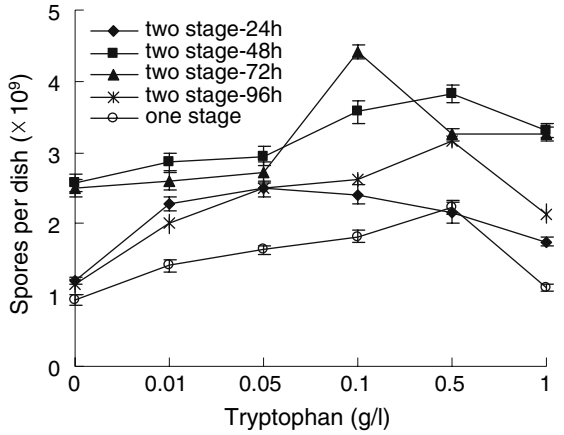

Fig. 1 Effect of tryptophan on spore yield. The standard deviation of three independent replicates with three measurements for each replicate is indicated for each point. (This applies to all other figures.) For the first stage in the two-stage process, tryptophan was at 0.05 g/l and the cultivation times were 24, 48, 72, and 96 h. The pH of all media was pH 6.0

Effect of pH on the biomass yield and growth profile

Figures 2 and 3 show the effect of pH on the biomass yield and growth profiles, respectively, at different pH values in the one-stage process. The highest biomass yield was using PDA at pH 2 for 192 h. However, at this pH the growth profile, color and morphology of the fungus were different from those with other pH values and no spores were harvested at this pH. For the other pH values, pH 4 produced the highest growth of *C. minitans*.

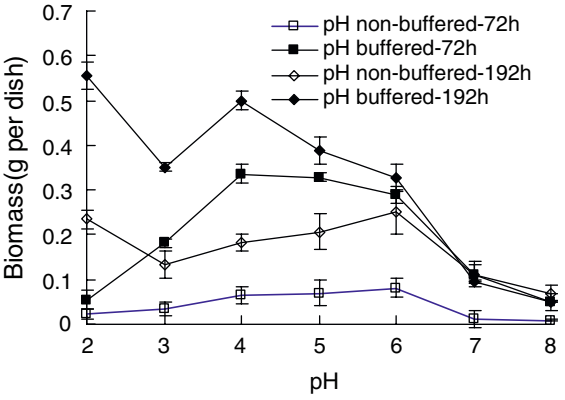

Fig. 2 Effect of pH on biomass production in the one-stage process. Results were measured at 72 and 192 h, as indicated. The pH was adjusted before autoclaving for pH non-buffered tests and for pH buffered tests, a buffer 0.2 M citric acid/0.2 M $Na_2HPO_4 \cdot 7H_2O$ was used

Effect of pH on the sporulation

Figure 4 shows the effect of pH on the sporulation of *C. minitans*. In the one-stage process, pH non-buffered media gave higher spore yield than did pH buffered media. In the two-stage process, pH 6 in the first stage showed higher spore yield than did pH 4. The optimal pH for sporulation was at pH 5.8–6 for cultivation at both pH 6 and 4 in the first stage during the two-stage process. In all processes, the highest spore yield was using the two-stage process with pH 6 at the first stage and pH values in the range 5–6 at the second stage.

Discussion

Tryptophan is important for the formation of fungal spores, especially black spores due to its role in the biosynthesis of melanin. Tryptophan promotes spore formation of *Trichothecium roseum* (Persoon) Link (Morquer and Montant 1952). In this study, tryptophan promoted the spore yield of *C. minitans*, by about 4-fold, especially when it was used after 48–72 h, the period for rapid induction of sporulation (Shi et al. 2004c).

Compared with a pH non-buffered process, a pH buffered process enhanced the hyphal growth but inhibited the sporulation of *C. minitans*. This is because the optimal pH for the two stages cannot be met well in one pH-buffered media since they are different. In pH non-buffered media, *C. minitans* could adjust the pH to a suitable level for sporulation (Shi et al. 2004c). In pH-buffered media, the variation in medium pH promoted by the fungi was counteracted. However, rapid hyphal growth does not necessarily mean high spore yield. In contrast, sporulation may be compromised if the cultivation environment is optimized for hyphal growth, such as at pH 2 and 4 as shown in this work. Therefore, we would recommend using growth media at a pH around 6.0 if the cultivation is aimed at obtaining high spore production.

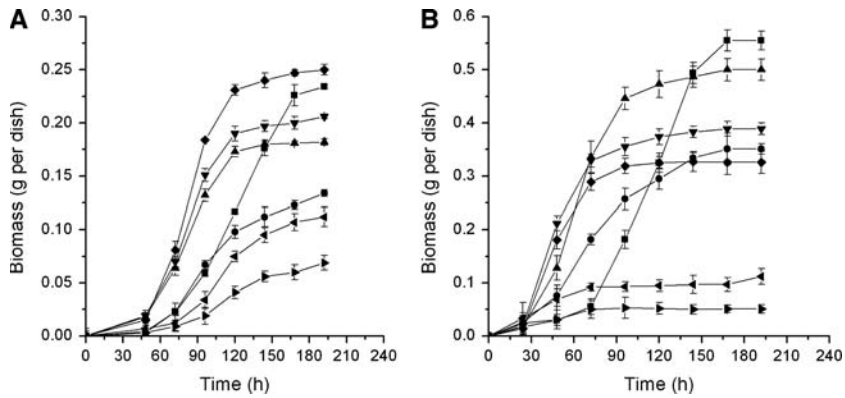

Fig. 3 Effect of pH on the growth profile in pH non-buffered media (**a**) and pH buffered media (**b**) in one-stage process. pHs of the media are 2 (■), 3 (•), 4 (▲), 5 (▼), 6 (♦), 7 (◀) and 8 (★)

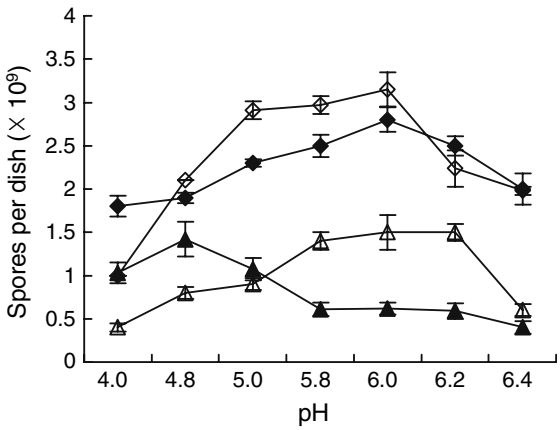

Fig. 4 Effect of pH on spore yield. In the one-stage process, both pH non-buffered media (♦) and pH buffered media (▲) were used. For the first stage in the two-stage process, two pH levels, 6 (◇) and 4 (Δ), were used and the cultivation time was 72 h

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