Effect of pH on physiology of *Metarhizium anisopliae* for production of swainsonine

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Abstract

The effect of pH on the production of swainsonine and fungal morphology at different stages of fermentation of *Metarhizium anisopliae* was investigated. When no control was applied, the pH of the culture dropped from 6.5 to 3.8 within the first 72 hours and the concentration of swainsonine reached 43.3 mg l\(^{-1}\). When the pH was held constant either at the beginning or throughout the fermentation, the maximum recorded swainsonine level was only 8.4 mg l\(^{-1}\) corresponding with an increase in the formation of pellets. A late pH control applied after 72 hours, resulted in a swainsonine titer of 45.5 mg l\(^{-1}\). © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The effect of pH on different aspects of microbial activity, including biomass concentration, product formation and cell morphology has been investigated by various groups. A regulatory influence of pH shift on the production of both primary [1] and secondary metabolites [2] has been demonstrated. The pH of the culture broth is one of several parameters which affects morphology and productivity. Dramatic changes in cell size or shape are a consequence of the responses of microbes to changes in pH. For example *Penicillium chrysogenum* varies its hyphal length according to the pH of its environment [3]. Secondary metabolite production in filamentous fermentations varies with physiology and morphology [4]. In submerged fungal cultures, various morphological forms ranging from hyphae to pellets are observed. The maximum product yield is usually achieved with a particular form.

The manipulation of microbial cultures using pH control for improved production of both primary and secondary metabolites has been exploited in the fermentation industry. Optimal pH requirements can be different for each stage of microbial growth and sudden pH changes can cause temporary or permanent perturbations in the associated metabolism. However to date, little work has been published on the stage-wise control of pH during microbial fermentations. In our previous papers, we have reported some of the factors affecting the microbial
physiology of the fungus *Metarhizium anisopliae* for production of swainsonine [5–7]. Swainsonine is an indolizidine alkaloid with anti-viral and anti-tumor properties [8,9] and it has a promising future as a chemotherapeutic drug. In our earlier studies, pH was not controlled during fermentation and its influence on the culture was not established.

In this report we investigate the effect of controlling medium pH at various stages of fermentation on the morphology of *M. anisopliae* and the production of swainsonine in stirred-tank bioreactors.

### 2. Materials and methods

#### 2.1. Organism and medium

A swainsonine producing strain of *M. anisopliae*, IMI 152487, was used in this study. The slopes were maintained on oatmeal agar. Oatmeal extract (OMX) was soaked in distilled water for 30 min before filtering through a muslin bag. The filtrate was autoclaved for 15 min at 121°C and 1.02 atm. and used as liquid or solid agar medium.

#### 2.2. Inoculation and culture conditions

Spores were scraped off agar slopes using sterile 2 mm glass beads in sterilized distilled water and vortexed for 3–4 min to disperse spore chains. The number of spores was counted using a Neubauer hemocytometer. Shake flasks containing 50% (v/v) of sterile OMX were inoculated with spore suspension (approximately 5×10⁶ spores ml⁻¹). The culture was incubated at 26°C in an orbital shaker at 200 rpm for 72 h. A 1% (v/v) of this culture was aseptically added to a fermenter containing sterile OMX for growth of *M. anisopliae* and swainsonine production.

#### 2.3. Fermenter culture

A series of batch fermentations were carried out in a 20 l (with 14 l working volume) stirred-tank reactor (STR) (Inceltech, Reading). Adequate mixing of the culture was achieved at a stirrer speed of 400 rpm. The culture was aerated at 0.6 vvm and the temperature of each fermentation was kept at 26°C. Dissolved oxygen tension (% DOT air saturation), pH, temperature and air flow-rate were all monitored throughout the fermentations. Antifoam (1 ml l⁻¹ Foamaster TDB-1) was added to OMX medium in the fermenter prior to sterilization in situ at 121°C and 1.02 atm. for 30 min. A 72 h old inoculum, grown in shaken flasks, was added aseptically to the fermenters and samples were removed aseptically at intervals for analyses.

Solutions (2 M) of either ammonium or sodium hydroxide were added incrementally to the cultures to keep the pH between 6.5–6.8 for the runs when pH control was applied.

#### 2.4. Assays

##### 2.4.1. Cell dry weight

Duplicate samples (10 ml) of the cultures were filtered through preweighed Whatman No. 1 filter paper and washed with distilled water. Biomass was measured as cell dry weight (CDW) after the filters were dried to a constant weight.

##### 2.4.2. Morphology

Small amounts of broth samples were placed on glass microscope slides and stained with lactophenol cotton blue, before viewed under a light microscope at ×40, ×100 and ×400 magnifications. Photographs were taken using black and white camera films (Kodak-FP4).

##### 2.4.3. Total carbohydrate assay

Total carbohydrate concentrations in the broth were measured using the phenol sulfuric acid assay described by Chaplin [10].

##### 2.4.4. Swainsonine assay

Swainsonine titers in broth samples were measured using the high-pressure ion-exchange chromatographic (HPIC) method described by Donaldson et al. [11].

### 3. Results and discussion

The pH and swainsonine concentration profiles of a series of reproducible fermentations (168 h duration) in the 20 l STR are shown in Figs. 1–3. In a
Fig. 1. The effect of full (from 0 to 168 h) pH control on swainsonine production. ●, swainsonine concentration vs. time for NaOH controlled run at pH 6.5; ■, swainsonine concentration vs. time for NH₄OH controlled run at pH 6.5; ▲, swainsonine concentration vs. time for no pH controlled (standard) run; ▪, swainsonine concentration vs. time for NH₄OH controlled run at pH 5.5; ○, pH vs. time, controlled by addition of NaOH and NH₄OH at pH 6.5; Δ, pH vs. time, no pH control; □, pH vs. time, controlled by addition of NH₄OH at pH 5.5.

Fig. 2. The effect of initial (from 0 to 72 h) pH control on swainsonine production. ●, swainsonine concentration vs. time for NaOH controlled run at pH 6.5; ■, swainsonine concentration vs. time for NH₄OH controlled run at pH 6.5; ▲, swainsonine concentration vs. time for no pH controlled (standard) run; ○, pH vs. time, controlled by addition of NaOH at pH 6.5; △, pH vs. time, no pH control; □, pH vs. time, controlled by addition of NH₄OH at pH 6.5; Δ, pH vs. time, no pH control.
fermentation without pH control (standard fermentation) swainsonine production started after the mid-exponential phase of growth and reached to final concentration of 43.3 mg l\(^{-1}\) after 168 h. The morphology of the culture gradually changed from free to entangled hyphae and eventually to pellets (Fig. 4A).

The pH controlled fermentations were carried out by addition of solutions of either NaOH or NH\(_4\)OH at different stages of growth, (a) 0 to 168 h; (b) 72 to 168 h; (c) 0 to 72 h. A common observation in all these runs was that specific growth rate, swainsonine yield (based on CDW), swainsonine production rate and culture morphology were all affected to different extent by application of pH control (Table 1, Fig. 4B).

When compared with the standard run, lower swainsonine titers were obtained in all pH controlled runs except when NH\(_4\)OH was used at a later stage. When pH was controlled at the early stage of growth, especially when NaOH was used rather than NH\(_4\)OH, the reductions in swainsonine concentrations were much more pronounced (Figs. 1–3). The early control of pH, irrespective of the alkali used, delayed the onset of swainsonine production, and reduced both the specific growth rate and the swainsonine production rate (Table 1). In contrast to the standard run, pellets were formed at the start of the fermentations and with time the morphology gradually changed into ‘hairy’ structures [12] (Fig. 4B). The total carbohydrate consumption rate was almost the same in all fermentations (0.11–0.12 g l\(^{-1}\)).

The addition of NaOH in all fermentations markedly reduced the maximum recorded swainsonine concentration particularly when it was introduced at the early stage of the run (3.8 g l\(^{-1}\)). The addition of NH\(_4\)OH, at the beginning of fermentation, also impaired swainsonine production (8.4 g l\(^{-1}\)). The detrimental effect of both NaOH and NH\(_4\)OH is caused by an interference with the metabolic activity of \(M.\ \text{anisopliae}\) during its growth phase. A late addition of NH\(_4\)OH results in only slight improvement over that obtained in the standard run (45.5 and 43.3 g l\(^{-1}\), respectively) and is not significant. However the ammonium ion can be assimilated by the fungus which is able to utilize it as a nitrogen source for swainsonine production. The biosynthesis of swainsonine, a nitrogen containing bicyclic sugar analogue, involves the conversion of the precursor lysine.

Fig. 3. The effect of late (from 72 to 168 h) pH control on swainsonine production. ●, swainsonine concentration vs. time for NaOH controlled run at pH 6.5; ■, swainsonine concentration vs. time for NH\(_4\)OH controlled run at pH 6.5; ▲, swainsonine concentration vs. time for no pH controlled (standard) run; ○, pH vs. time, controlled by addition of NaOH at pH 6.5; □, pH vs. time, controlled by addition of NH\(_4\)OH at pH 6.5; △, pH vs. time, no pH control.
via a number of nitrogen containing intermediates [5].

Many biological systems involve acid-base equilibria and therefore depend critically on the pH of the
solution. Cell membranes are not completely permeable to hydrogen ions so the intracellular and extracellular pH may not be the same. However any enzyme mediated reaction in the external medium, whether truly external or in the periplasm, will be influenced by the culture pH, resulting in possible changes in productivity. Structures such as membranes in contact with the external environment are also subject to chemical changes in response to pH. Microorganisms may need to adapt their function in order to cope with a change in hydrogen ion concentration. If this change is too abrupt, the response of microbes might lag behind or overshoot. Apart from affecting cell membrane permeability, pH may also determine the solubility of some components of the medium. Thus a modification in the pH might also cause some micronutrients to precipitate and become impossible to be assimilated [13]. One or more of the mechanisms described above could account for the observed responses of *M. anisopliae* to the change of pH in the external medium.

Our study suggests that in order to enhance swainsonine production no pH control should be exerted during the period of rapid growth when the pH drops due to organic acid secretion. If the pH is maintained at a neutral value during this period, swainsonine production is adversely affected. Late control of pH, using NH₄OH provides little advantage in swainsonine yield when compared to the uncontrolled fermentation.

In view of the importance of swainsonine as an anti-tumor/anti-viral agent, its microbial production at large scale is envisaged. In this regard, pH control of the culture is not essential.

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### References


