The Effect of Carbon Source, Temperature and Aeration on the Production of Ascosteroside, a Novel Antifungal Agent, by *Ascotricha amphitricha*

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This paper describes the optimization of production of ascosteroside, a novel antifungal agent with an α-linked glycoside of a lanosterone-type triterpenoid structure. Glucose, sorbose and inositol were determined to be the best carbon sources for the production of ascosteroside. Temperature affected levels of ascosteroside, with production being highest at 16°C with 1% glucose, and lowest at 32°C. Dissolved oxygen levels were found to be critical in the production of ascosteroside in fermenter cultures. In order for production of ascosteroside to occur in fermenter cultures, the threshold level of dissolved oxygen was found to be above 26%.

In our continuing search for novel antifungal agents produced by microorganisms, a novel antifungal agent ascosteroside with a glycosylated lanostane type triterpenoid structure, was isolated from the fermentation of *Ascotricha amphitricha*[1,2]. Ascosteroside is active against several *Candida* species, *Saccharomyces cerevisiae* and filamentous fungi, and has little or no activity against bacteria[1]. The mode of action of ascosteroside is not known, however, it has been determined that it does not inhibit the biosynthesis of sterol, protein and nucleic acid, and has no effect on membrane integrity in *Candida albicans*[1].

In order to obtain sufficient ascosteroside for expanded biological activity testing and clinical evaluation, large scale fermentation was necessary. This paper describes the studies of the effect of carbon source, temperature and oxygen on the production of ascosteroside and successful scale-up production of this compound in laboratory fermenter.

Materials and Methods

Microorganism and Culture Conditions

Ascotricha amphitricha was isolated from a soil sample collected from Kenya[1], and has been deposited with the American Type Culture Collection as ATCC 74237. The culture was grown on a potato-dextrose agar slant for 7 days, and the surface growth of the slant was swabbed into 100 ml of medium F4 in a 500-ml flask. Medium F4 contained the following per litre of distilled water: tryptone 5 g, malt extract 3 g, glucose 10 g, and yeast extract 3 g. The pH of the medium was 6.5 and was not adjusted prior to sterilization. The medium was sterilized for 30 minutes. All shake flask studies were carried out using narrow mouth flasks. Frozen vegetative preparations were prepared by mixing a culture grown for 3 days in medium F4 with an equal volume of 20% (w/v) glycerol/10% (w/v) sucrose, and aliquots frozen in a dry ice-acetone bath, and stored at −80°C. From the frozen stock, 4 ml was used as an inoculum into 100 ml of medium F4. The culture was grown for 3 days and then 4% (v/v) of the culture was used to inoculate medium F4, which was also used as the production medium. All cultures were grown in liquid media at 28°C by shaking at 250 rpm unless stated otherwise.

Fermenter Conditions

Cultures were grown in a 15 litre Braun Biostat ED fermenter (B. Braun, Allentown, PA) containing 10 litre of medium F4. The pH of the medium was not adjusted prior to sterilization, and the medium was sterilized for 45 minutes. The fermenter cultures were grown at 28°C, with an agitation rate of 500 rpm, airflow of 1VVM and back pressure of 0.3 bar unless stated otherwise. The antifoam agent polypropylene glycol P-2000 was added to a final concentration of 0.01% (v/v) prior to sterilization. Each fermenter was equipped with an oxygen probe, and measurements of dissolved oxygen were taken throughout the fermentation. The fermenters were inoculated with 400 ml of a culture which had been

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grown in flasks by shaking for 3 days as described above.

Extraction

Samples from the fermentation were mixed with an equal volume of ethyl acetate for 1 hour. After centrifugation, 1 ml of the ethyl acetate layer was removed and dried under nitrogen, and stored at −20°C until use.

Analytical Methods

Growth of the organism was determined by the % sediment measured after centrifugation of 10 ml of culture broth at 3000 × *g* for 10 minutes. A correlation was established between % sediment and dry weight and this was used to calculate the fungal dry weight of each fermentation. Samples were taken from the supernatant for determination of glucose and ammonia. Glucose was measured as a reducing sugar with dinitrosalicylic acid reagent[3]. Ammonia was determined using the method of WEATHERBURN[4]. Crude extracts were examined by HPLC using a Rainin "Short-One" C18 (3 μ) 10 cm L column with a solvent system of acetonitrile-0.01 M potassium phosphate (monobasic) buffer pH 3.5 gradient with a flow rate of 1.2 ml/minute with UV detection at 230 nm[2].

Biological Activity

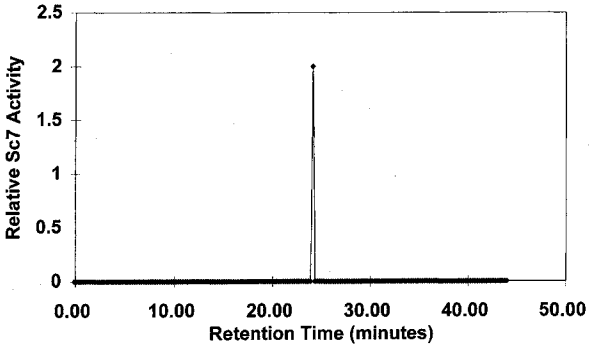
For antifungal activity, *S. cerevisiae* strain SC109 (Sc7) was used as the tester organism as described elsewhere[1].

Results

Effect of Carbon Source on Ascosteroside Production

By using flask cultures with various carbon sources the amount of ascosteroside produced was determined using a biological assay of antifungal activity against *S. cerevisiae* strain SC109 (Sc7). HPLC could not be used to accurately establish titers of ascosteroside in the crude extract due to the poor resolution of this compound. Ascosteroside was determined to be the only antifungal agent present in the crude extract as evidenced by microbiological assay of HPLC fractions (Fig. 1), and the retention time corresponded to the retention time of the pure compound[2]. As a good correlation existed between Sc7 activity and pure ascosteroside, biological activity was used to determine ascosteroside production. The pH during growth with the different carbon sources increased throughout the fermentation, ending between 7.8 and 8.8 (Table 1). The amount of biomass formed varied, depending on the carbon source. Highest levels of ascosteroside were found when *A. amphitricha* was grown on glucose, sorbose, glucosamine and inositol. Due to the cost of sorbose, glucosamine and inositol, further studies were carried out with glucose.

Fig. 1. Bioactivity of HPLC fractions of crude extract of *A. amphitricha* against *S. cerevisiae* (Sc7).



Effect of Glucose on Ascosteroside Production

The effect of 1%, 3% and 5% glucose on production of ascosteroside was studied (Fig. 2). The rate of glucose consumption was similar with all three concentrations of glucose, and the pH of the fermentation increased above 7.0 once the glucose had been consumed, with the exception of 5% glucose where the pH remained around 5.0. Highest levels of ascosteroside were found with 3% glucose, and with 3 and 5% glucose production occurred prior to exhaustion of the substrate.

Table 1. The effect of carbon source on production of ascosteroside by *A. amphitricha*[a].

Carbon source[b]	Final pH	Dry weight (mg/ml)	Sc7 (mm)
Glucose (control)	8.7	5.8	9.0
Mannose	8.5	6.1	6.5
Sorbose	8.4	3.2	10.0
Galactose	8.5	6.7	5.5
Glucosamine	8.2	3.2	9.0
Lactose	8.7	5.5	8.5
Inositol	8.6	4.1	10.5
Dulcitol	8.5	6.1	3.0
Mannitol	8.0	6.1	6.0
Sorbitol	8.2	6.1	8.5
Glycerol (0.5%)	8.5	7.9	4.0
Whey	8.8	4.1	8.0
Choline	8.6	2.0	0
Ethanolamine	7.8	2.0	0
Base medium[c]	8.2	2.0	0

[a] The culture was grown in flasks by shaking at 28°C and samples taken after 5, 6 and 7 days of incubation.
[b] All carbon sources were present at 1% (w/v) unless stated otherwise.
[c] The base medium contained tryptone, 5 g; malt extract, 3 g; yeast extract, 3 g.

Fig. 2. The effect of glucose concentration on ascosteroside production in shake flasks at 28°C.

Glucose concentration (open symbols) and Sc7 activity (closed symbols) at 1% (○, ●), 3% (△, ▲) and 5% glucose (□, ■).

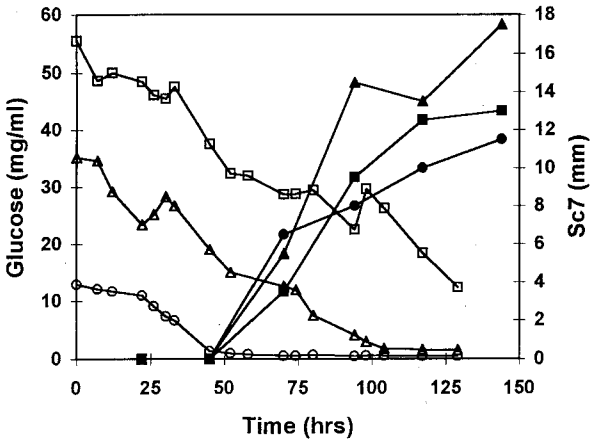
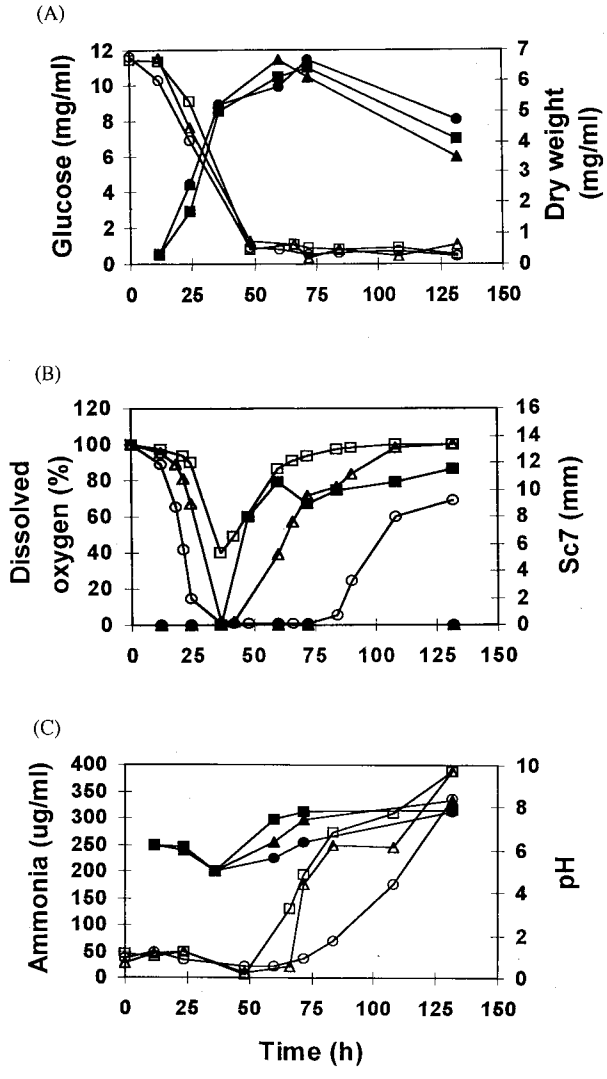


Fig. 3. The effect of stirring speed on ascosteroside production by *A. amphitricha* in the fermenter.

(A) Glucose concentration (open symbols) and biomass levels (closed symbols) at 200 rpm (○, ●), 350 rpm (△, ▲) and 500 rpm (□, ■). (B) Dissolved oxygen levels (open symbols) and Sc7 activity (closed symbols) at 200 rpm (○, ●), 350 rpm (△, ▲) and 500 rpm (□, ■). (C) Ammonia levels (open symbols) and pH (closed symbols) at 200 rpm (○, ●), 350 rpm (△, ▲) and 500 rpm (□, ■).



Production of Ascosteroside in a Fermenter

Using medium with 1% glucose and either 200 or 350 rpm with 1 VVM of aeration in the fermenters, the production of ascosteroside was not detected (Fig. 3). Increasing the agitation rate to 500 rpm resulted in similar levels of antifungal activity being detected, compared with the shake flasks. Growth of *A. amphitricha* under the different conditions did not show any significant differences with respect to glucose consumption and biomass formation, with the active phase of growth occurring between 20～40 hours. However, with the lowest agitation rate, ammonia production and consequently an increase in pH, was significantly slower compared to growth at higher agitation speeds. The most significant difference between the fermentations was the amount of dissolved oxygen present during growth. During the early part of the fermentation oxygen consumption was high resulting in significant depletion to around 1% with stirring speeds of 200 and 350 rpm, and no antifungal activity was detected. At 500 rpm the dissolved oxygen dropped to around 40% by 35 hours, before increasing back to 100%, indicating that cellular growth had ceased at 35 hours and ascosteroside production coincided with the increase in dissolved oxygen levels. In other experiments, if dissolved oxygen levels dropped below 26%, production of ascosteroside was not detected (Table 2). Ensuring that the minimum level of dissolved oxygen was above 40%, using increased agitation or increased back pressure did not result in improved production of ascosteroside, and higher levels of oxygen actually inhibited production.

Effect of Glucose on Ascosteroside Production in a Fermenter

With the fermenter being stirred at 500 rpm and an aeration rate of 1 VVM, the production of ascosteroside in media containing 1% glucose and 3% glucose was examined (Fig. 4). Similar levels of biomass were formed under these conditions. The pH of the fermenter with 1% glucose increased during stationary phase due to production of ammonia, and with 3% glucose the pH

Table 2. The effect of dissolved oxygen in the fermenter on production of ascosteroside by *A. amphitricha* during fermentation with 1% glucose at 28°C and 1 VVM.

Dissolved oxygen (%)[a]	Agitation rate (rpm)	Back pressure (bar)	Sc7 (mm)[b]
0	200	0.3	0
0	350	0.3	0
26	500	0.3	2.5
40	500	0.3	11.5
70	500	0.4	10.0
79	500	0.4	9.5

[a] The lowest level of dissolved oxygen detected during growth in the fermenter.
[b] Highest level of ascosteroside detected during the fermentation.

remained below 5.0, only increasing towards the end of the fermentation. Oxygen levels in the fermenter with 1% glucose dropped down to 70%, and this depletion in oxygen coincided with the onset of stationary phase and initiation of production of ascosteroside. In the fermenter with 3% glucose, the lowest level of oxygen (60%) occurred after the beginning of stationary phase. Similar levels of ascosteroside were produced under the two conditions.

Effect of Temperature on Ascosteroside Production

Incubation of *A. amphitricha* during growth in flask culture by shaking with 1% glucose at temperatures below 28°C resulted in an increase in the amount of ascosteroside produced (Table 3). Highest production of ascosteroside occurred when the organism was grown at 16°C, and levels were reduced with an increase in the incubation temperature, with lowest levels at 32°C. In the fermenter with 3% glucose, higher production of ascosteroside occurred at 25°C compared to 28°C.

Discussion

The optimal conditions for the production of ascosteroside in the fermenter included using a medium containing glucose as the main carbon source, maintaining dissolved oxygen levels above a critical threshold level, and running the fermentation at temperatures below 28°C.

The finding that ascosteroside production occurred during the non-active growth phase of the organism suggests that ascosteroside may be a metabolic sink product formed during the period of unbalanced growth in stationary phase. Biosynthesis of ascosteroside after

Fig. 4. The effect of glucose on ascosteroside production by *A. amphitricha* in the fermenter.

(A) Glucose concentration (open symbols) and biomass levels (closed symbols) with 3% glucose (○, ●) and 1% glucose (□, ■). (B) Dissolved oxygen levels (open symbols) and Sc7 activity (closed symbols) with 3% glucose (○, ●) and 1% glucose (□, ■). (C) Ammonia levels (open symbols) and pH (closed symbols) with 3% glucose (○, ●) and 1% glucose (□, ■).

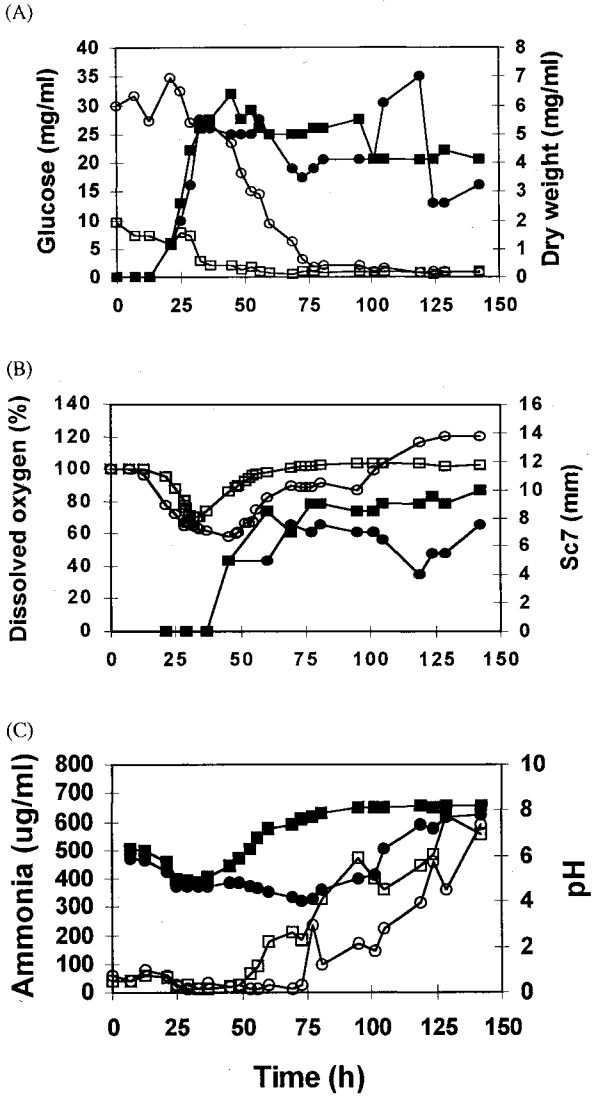


Table 3. The effect of temperature on production of ascosteroside by *A. amphitricha* during fermentation with 1% glucose in flasks.

Temperature (°C)	Sc7 (mm)
16	14.0
21	12.0
26	12.0
28	9.0
32	7.0

active growth has ceased is probably essential to the survival of *A. amphitricha*, as the antifungal activity of the compound could prove toxic to the producer. For most antibiotic producing organisms, resistance to the producing compound occurs only in stationary phase[5,6].

In many fermentations carbon source regulation influences antibiotic production, and glucose although an excellent carbon source for growth, often represses antibiotic synthesis[7]. This phenomenon does not apply to *A. amphitricha* when the organism was grown in shake flasks, as production increased at 28°C with increasing levels of glucose (up to 3%), and did not coincide with glucose limitation. Furthermore, the production of ascosteroside in the fermenter with 3% glucose occurred even though the residue glucose concentration was as high as 2.5%.

In the biosynthesis of a number of antibiotics oxygen has been found to play a crucial role. Specific penicillin production rates decreased sharply below 30% air saturation during growth of *Penicillium chrysogenum* in the fermenter, with no production at dissolved oxygen tension levels of 10%[8]. With *Streptomyces clavuligenes*, reduction in dissolved oxygen to almost zero delayed production of cephamycin C, and higher levels of dissolved oxygen resulted in increased titers[9]. The critical dissolved oxygen concentration of 20% was one of the most important factors in cephalosporin C production by *Cephalosporium acremonium*, and below this value repression of cephalosporin C production occurred and penicillin N concentrations increased[10]. For the optimal production of cephalosporin C, dissolved oxygen levels were maintained at no less than 30% of saturation[11]. Irreversible repression of ascosteroside synthesis resulted if dissolved oxygen levels dropped below 26%. A similar situation exists with *Bacillus subtilis* where oxygen levels during the first day of growth were critical in the production of the antibiotic iturin[12].

Due to the sterol nature of ascosteroside, the importance of oxygen in production could be attributed to its role in the biosynthetic pathway of the antibiotic. Oxygen is required for the synthesis of sterols, unsaturated fatty acids and various vitamins[13], and temperature has been shown to be important in the synthesis of a number of secondary metabolites[14]. Temperature has also been shown to effect ergosterol biosynthesis in yeast, with *de novo* synthesis at an elevated temperature of 40°C being only 32~35% of that at 20 or 30°C[15]. For production of ascosteroside a temperature of 25°C or lower was found to be optimal. Thus, the two most critical factors affecting sterol biosynthesis in fungi, namely oxygen and temperature, also influence the production of the sterol-like antibiotic ascosteroside.

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