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Large-scale Production of Clavine Alkaloids by *Claviceps fusiformis*

By G. T. BANKS, P. G. MANTLE AND CHRISTINE A. SZCZYRBAK
Department of Biochemistry, Imperial College, London SW7 2AZ

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SUMMARY

A strain of *Claviceps fusiformis* produced clavine alkaloid (principally agroclavine) in yields of 4 mg/ml within 6 days when grown in a sucrose–ammonium sulphate–inorganic salts medium in 400 l stirred fermenters. Alkaloid accumulated rapidly during the growth phase, most of the synthesis coinciding with a mycelial growth form consisting of plectenchymatic hyphae, the cells of which resembled the constituent cells of naturally occurring *C. fusiformis* sclerotia. The broth was rendered low in glucan by the activity of a β-glucanase and this, together with increased fermenter shaft speed, ensured that oxygen supply was not limiting throughout the fermentation. Alkaloid was extracted efficiently from the glucan-free culture filtrate by a solvent extraction procedure involving sequential transfer of the product into *n*-butanol, aqueous tartaric acid and chloroform, followed by crystallization from acetone. Improved alkaloid yield (6 mg/ml) was obtained by using a modified medium containing increased concentrations of magnesium sulphate, zinc sulphate and potassium dihydrogen orthophosphate, in a 400 l multi-stage fermentation.

INTRODUCTION

Large-scale production of the ergot alkaloids in submerged culture has been confined solely to the commercially important lysergic acid types produced by *Claviceps paspali* (Arcamone *et al.* 1961) and *C. purpurea* (Arcamone *et al.* 1970). A characteristic feature of alkaloid-producing strains of both *C. paspali* and *C. purpurea* is their instability, whereby they revert to non-producing forms after only a few subcultures (Kobel, 1969; Mantle, 1969*a*). By contrast, the clavine alkaloid-producing strains of *C. fusiformis* are usually more stable in submerged culture. Thus strains of this species have been used mainly in biosynthetic investigations (Birch, McLoughlin & Smith, 1960; Groger, Wendt, Mothes & Weygand, 1961; Baxter, Kandel, Okany & Pyke, 1964). As a source of pharmaceuticals *C. fusiformis* has received much less attention than ergot fungi producing lysergic acid derivatives, since the clavine alkaloids have been reported to possess much weaker oxytocic and vasopressor properties (Hofmann, 1961). However, it has since been shown that agroclavine prevents implantation in mice when the drug is administered orally during the first 2 to 3 days of pregnancy (Mantle, 1969*b*). Lactation is also inhibited when agroclavine is administered to mice during late pregnancy (Mantle, 1968).

The need for large quantities of pure agroclavine for pharmacological studies stimulated the development of the relatively inexpensive large-scale fermentation process described in this paper.

METHODS

Origin of strains. A strain of *Claviceps fusiformis* (designated 139/2/1), producing high titres of clavine alkaloids (> 3·0 mg/ml) in surface culture on a sucrose–asparagine–mineral salts medium (medium A), was isolated from a sclerotium parasitic on bulrush millet (*Pennisetum typhoides* Staph & Hubbard) grown in Senegal. Agroclavine was the principal (> 90 %) alkaloid produced but traces of other clavines including elymoclavine, chanoclavine, setoclavine and penniclavine were also present.

Strain 139/2/1 produced moderate titres of alkaloid (1·2 to 1·4 mg/ml) in submerged culture but the fermentations were impeded by restricted aeration as a result of high culture viscosities after the accumulation of an extracellular glucan during the growth phase.

During a programme of strain selection a variant strain (139/2/1G) arose yielding more alkaloid, partly as a result of the low culture viscosity which was restored after autolysis of the extracellular glucan. Consequently this new strain was used for the development of a large-scale fermentation process.

Maintenance of cultures. Stock cultures were preserved in Pyrex ampoules under liquid nitrogen at −196 °C. Cultures for liquid nitrogen preservation were grown for 5 to 6 days in submerged culture (medium A). Sterile glycerol (10 %, v/v) was added, and the mixture dispensed (1 ml) into sterile ampoules. The ampoules were cooled slowly from room temperature to −45 °C by placing them at −60 °C overnight in an insulated, expanded polystyrene box. The ampoules were then quickly immersed in liquid nitrogen.

Cultures for use in experiments were obtained by removing an ampoule from liquid nitrogen, allowing it to attain laboratory temperature, and inoculating medium A agar slopes which were subsequently grown at 24 °C and subcultured every two weeks.

Media. Medium A, modified from Stoll, Brack, Hofmann & Kobel's (1957) medium, was used for surface cultures and seed-stage submerged cultures, and had the following composition (g/l distilled water): sucrose, 100; L-asparagine, 10; $Ca(NO_3)_2.4H_2O$, 1; KH_2PO_4, 0·25; $MgSO_4.7H_2O$, 0·25; KCl, 0·125; $FeSO_4.7H_2O$, 0·033; $ZnSO_4.7H_2O$, 0·027; L-cysteine hydrochloride, 0·01; yeast extract, 0·1; pH adjusted to 5·2 with concentrated NaOH. Solid medium contained, in addition, 2 % (w/v) Oxoid agar and was sterilized by autoclaving at 106 °C for 15 min.

Medium B was used for production-stage submerged cultures and had the following composition (g/l distilled water): sucrose, 200; $(NH_4)_2SO_4$, 11·8; KH_2PO_4, 0·25; $MgSO_4.7H_2O$, 0·25; $FeSO_4.7H_2O$, 0·033; $ZnSO_4.7H_2O$, 0·027; pH adjusted to 5·2 with concentrated NaOH. When used in shaken flasks, 2 % (w/v) $CaCO_3$ was added to the medium before sterilization, to avoid depression of pH during subsequent growth of the fungus.

The use and composition of medium C was the same as medium B, except for increased concentrations of the following inorganic salts (g/l): KH_2PO_4, 0·5; $MgSO_4.7H_2O$, 0·5; $ZnSO_4.7H_2O$, 0·108.

The conditions employed for sterilization of the different media were scale-dependent, and are fully described below for each scale of operation.

Shaken-flask fermentations. The rotary shakers employed for all submerged flask cultures operated at 200 rev./min with a 9 cm eccentric throw. Under these conditions, an oxygen transfer rate of 38 ml oxygen (at s.t.p.)/100 ml/h was recorded by means of the sulphite oxidation technique of Cooper, Fernstrom & Miller (1944).

For the seed stage, 100 ml quantities of medium A were dispensed into 500 ml Erlenmeyer flasks. The flasks were closed with cotton-wool plugs and sterilized by autoclaving at 106 °C for 15 min. The flasks containing medium A were inoculated with a spore suspension

derived from a 14-day agar slant culture of the organism, grown at 27 °C, to give a final spore concentration in the medium of 4×10^5 spores/ml. These seed-stage cultures were then incubated for 5 days at 27 °C on a rotary shaker.

The appropriate production-stage medium was dispensed and sterilized as described above. The flasks were inoculated with 10 ml fully-developed seed-stage culture and incubated at 27 °C on a rotary shaker for various periods of time (usually 10 to 14 days).

Pilot-scale fermentations. The procedure described below is that for carrying out production-stage fermentations on a 400 l scale.

1. Development of laboratory inoculum. The procedure employed in the primary seed-stage was identical to that described above for the seed-stage of shaken-flask fermentations. For the secondary seed-stage, 1 l portions of medium A were dispensed into 4 l conical flasks fitted with a side-arm. The flasks were closed with cotton-wool plugs and sterilized at 121 °C for 1 h. They were then inoculated with 100 ml of fully developed primary seed-stage culture and incubated for 4 days at 27 °C on a rotary shaker, as previously described.

2. Five-litre seed-stage fermentation. The vessels employed were glass/stainless steel New Brunswick 5 l laboratory fermenters (model FS-600, V. A. Howe, London). Temperature control was achieved by partial immersion of the fermenters in a thermostatically-controlled water bath. Culture aeration was provided by sparging air through a single-orifice sparger into the culture fluid, which was agitated by means of two open-turbine impellers.

Into each fermenter was dispensed 3·1 l medium A, containing 0·01 % (v/v) polypropylene glycol antifoam (Polyglycol P-2000, manufactured by Dow Chemical Co.); the vessel outlets were appropriately closed and the fermenters and medium sterilized by autoclaving at 121 °C for 1 h.

The fermenters were inoculated with 300 ml fully developed secondary seed-stage culture. Subsequent incubation proceeded at 27 °C for 4 days, culture aeration being facilitated by employing an agitator shaft speed of 400 rev./min, an air flow rate of 2 l/min and an air pressure of 7 to 14 kN/m^2. Sterile polypropylene glycol was added as necessary during the fermentation to control foaming.

3. Sixty-litre seed-stage fermentation. The vessels employed were stainless steel 60 l fermenters of conventional design. Temperature control was achieved by the automatically regulated flow of cooling water (approx. 15 °C) through the fermenter jacket. Culture aeration was provided by sparging air through a ring sparger into the culture fluid, which was agitated by means of a single disc turbine impeller.

Fifty-four litres medium A, containing 0·01 % (v/v) polypropylene glycol antifoam, were prepared *in situ*. Medium and fermenter were sterilized at 121 °C for 20 min by live-steam injection. During medium preparation allowance was made for condensate subsequently formed during sterilization.

The fermenters were inoculated with approx. 6 l of fully developed 5 l fermenter seed-stage culture (i.e. the contents of two such fermenters). Incubation proceeded at 27 °C for 4 days. An agitator shaft speed of 183 rev./min was employed during the first 48 h, being subsequently raised to 367 rev./min, together with an air flow rate of 60 l/min and an air pressure of 100 kN/m^2. Polypropylene glycol antifoam was added as necessary to control foaming.

4. Four hundred-litre production-stage fermentation. The vessels employed were stainless steel 400 l fermenters of conventional design. Provisions for temperature control and culture aeration were as previously described for 60 l fermenters, except that two disc turbine impellers were employed.

Three hundred litres of appropriately concentrated production-stage medium (medium

B or C), minus ammonium sulphate but containing 0·01 % (v/v) polypropylene glycol antifoam, were prepared *in situ*. Medium and fermenter were sterilized at 121 °C for 20 min by live-steam injection. The ammonium sulphate was separately sterilized in a 60 l fermenter (total volume of solution 60 l, adjusted to pH 3·5) and transferred aseptically to the concentrated bulk medium in the 400 l fermenter before inoculation. The pre-inoculation volume was thus 360 l, allowance having been made for condensate formed during sterilization.

The fermenters were inoculated with 40 l fully developed 60 l fermenter seed-stage culture. Subsequent incubation proceeded at 27 °C for about 10 days. An agitator shaft speed of 153 rev./min was employed initially; this was raised to 306 rev./min when the dissolved oxygen decreased to about 30 % of the saturation value, in order to avoid conditions of oxygen limitation. An air flow rate of 400 l/min was employed throughout, together with an air pressure of 100 kN/m^2. Automatic pH control was employed to maintain a constant pH of $5{\cdot}0 \pm 0{\cdot}1$ throughout the fermentation, by the addition of sterile 30 % (w/v) NaOH solution; a steam-sterilizible glass electrode (model GHQN 28; Electronic Instruments Ltd), together with a remote calomel reference electrode, were employed for pH measurement whilst control was achieved with a conventional on/off system. Polypropylene glycol antifoam was added as necessary to control foaming.

5. Four hundred-litre half-replacement multistage fermentation. A 400 l fermenter, containing medium C, was inoculated as described above and the fermentation allowed to proceed under normal conditions for 5 days. At this stage, 200 l of culture were run off, and 200 l of double-strength medium C added; the fermentation was again allowed to proceed under normal conditions. This procedure was repeated at 3 day intervals.

All the fermenters employed were of conventional design. Their characteristic features and relative internal dimensions are given in Table 1 and Fig. 1.

Pilot-scale extraction and purification of agroclavine. A solvent extraction procedure was devised for the isolation of agroclavine from culture fluids. Use was made of the facts that agroclavine is readily soluble in many organic solvents but relatively insoluble in water at alkaline pH values, whereas when protonated in acid conditions it is freely soluble in aqueous systems. Sequential partition between solvent and aqueous phases at suitably adjusted pH values, together with a progressive reduction in the volume of successive extracts, effected both a concentration and a purification of the agroclavine. *n*-Butanol was chosen for the initial solvent extraction step in view of its low cost. Since *n*-butanol and water are partially miscible (solubility of water in butanol at 10 °C, 19·7 % v/v; solubility of butanol in water at 10 °C, 8·9 % v/v), hexane was used during the procedure both to remove water from butanol extracts and to remove butanol from aqueous phases. Throughout the procedure, the product was maintained under chilled conditions (< 5 °C) unless otherwise specified. All liquid/liquid extractions were carried out as batch operations in stainless steel stirred, baffled and jacketed vessels.

The following procedure was employed for the isolation of agroclavine from approximately 400 l of glucan-free culture fluid. Mycelium was removed from the broth by filtration under pressure through a plate and frame filter press. The culture filtrate was adjusted to pH 8·5 with ammonium hydroxide solution, the product extracted into 0·3 vol. *n*-butanol and the butanol extract separated by centrifugation with an Alfa-Laval disc bowl continuous centrifuge. An equal volume of hexane was added to the butanol extract, and the aqueous phase which formed was separated by decantation and discarded. The product-rich butanol–hexane mixture was then extracted twice with 0·14 vol. 5 % (w/v) tartaric acid solution and the two tartaric acid extracts bulked after separation by decantation and/or centrifugation. Butanol was removed from the combined tartaric acid extracts by the addition of an equal

Table 1. *Dimensions of pilot plant fermenters*

Fermenter dimension or characteristic	Fermenter scale (l) 5	60	400
Liquid volume (l)	3·4	60	400
No. of baffles	4	4	4
No. of impellers	2	1	2
No. of blades/impeller	4	4	8
Type of impeller (turbine)	Open	Disc	Disc
Type of sparger	Single orifice	Ring	Ring
H/D ratio	1·52	1·20	1·43
d/D ratio	0·55	0·38	0·40
A/d ratio	1·15	2·14	1·14
B/d ratio	1·00	—	1·57
C/d ratio	0·64	1·07	0·85
W/D ratio	0·11	0·10	0·08

For explanation of symbols see Fig. 1.

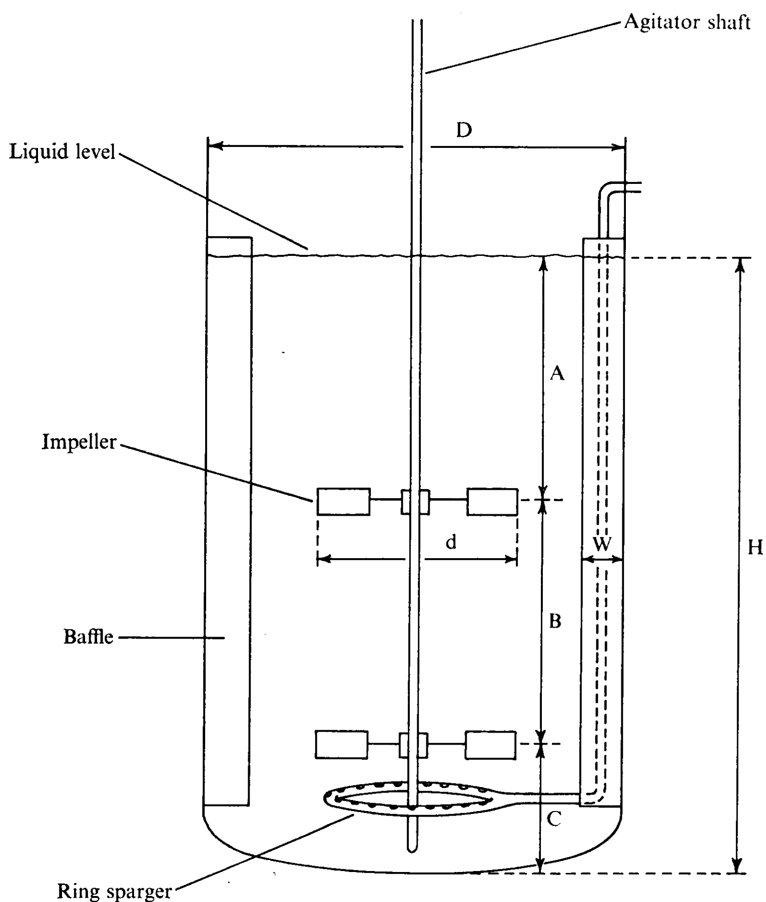
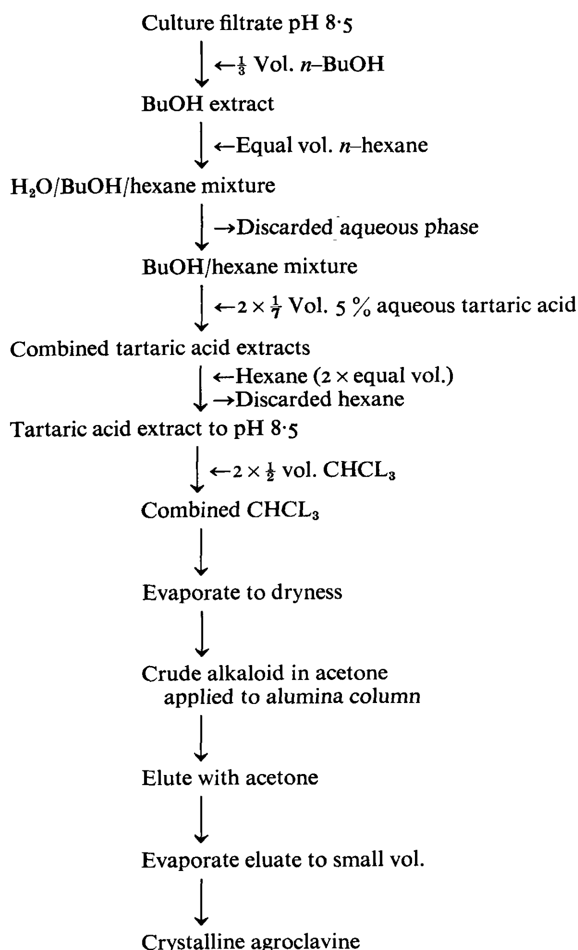


Fig. 1. Diagrammatic representation of internal design of fermenter.

Table 2. *Procedure for large-scale extraction of agroclavine*



volume of hexane and the subsequent removal of the hexane–butanol phase by decantation on two separate occasions. The pH of the combined tartaric acid extract was then adjusted to 8·5 with ammonium hydroxide solution and the agroclavine extracted from it by means of two half-volume extractions with chloroform; the chloroform extracts were separated from the aqueous phase by centrifugation and subsequently bulked. The combined chloroform extract was evaporated to dryness under reduced pressure to yield a crude, coloured (brown) agroclavine preparation initially by means of a climbing-film evaporator (Quickfit Jobling Laboratory Division, Stone, Staffordshire) and subsequently in an Apex Evaporator type 263A (Apex Construction Ltd, London).

Crystalline agroclavine was obtained from the crude preparation on a laboratory scale by dissolution in acetone, removal of coloured impurities on an alumina column, concentration of the acetone column eluate under reduced pressure in a rotary film evaporator and, finally, crystallization was induced by chilling the concentrate.

The extraction and purification procedures referred to above are illustrated in diagrammatic form in Table 2.

The overall efficiency of obtaining a crude alkaloid extract (after chloroform extract concentration) from culture filtrate was approximately 70 %. The principal loss occurred during concentration of the chloroform extract. Crystalline agroclavine was obtained from the crude alkaloid with an efficiency of 80 %.

Analytical procedures. Unless otherwise stated, the maximum absorbancies of coloured complexes were measured on a Unicam SP 600 spectrophotometer (Unicam Instruments, Cambridge) using a 1 cm light path.

1. Alkaloid. Suitably diluted culture filtrate (5 ml) was mixed with 10 ml of Van Urk Reagent (*p*-dimethylaminobenzaldehyde, 0·125 g; 65 % v/v H_2SO_4, 100 ml; 5 % w/v $FeCl_3.6H_2O$, 0·1 ml). The absorbance of the blue colour formed was measured after 10 min with an Eel Colorimeter (Evans Electroselenium Ltd, Halstead, Essex) at 570 nm, and compared with standard solutions of pure agroclavine (dissolved in 1 % tartaric acid) in the range 0 to 25 μg/ml, within which a linear relationship between absorbance and alkaloid concentration existed.

2. Mycelial dry weight. A sample (50 to 75 ml) of culture was filtered through dried weighed filter paper (Whatman no. 541), washed twice with distilled water, and dried to constant weight at 70 °C. Highly viscous cultures (containing glucan) were diluted at least 5 to 10 times before processing.

3. Glucan. The filtrate and washings from the mycelial dry weight determinations were stirred with an equal volume of ethanol. The precipitated glucan was collected on a glass rod, transferred to dried weighed filter paper and dried to constant weight at 70 °C.

4. Glucose. Free glucose in the culture filtrate was measured after diluting the culture filtrate to contain 10 to 100 μg glucose/ml. A 1·0 ml sample was mixed with glucose-oxidase reagent (3·0 ml) and the absorbance of the blue colour formed was measured after 10 min at 625 nm. The glucose-oxidase reagent had the following constitution: 'Fermcozyme' [Hughes & Hughes (Enzymes) Ltd, Romford, Essex], 0·5 ml; 0·5 M-acetate buffer pH 5·2, 93·5 ml; peroxidase solution (20 mg in 100 ml acetate buffer), 5·0 ml; 1 % (v/v) *O*-tolidine (in absolute ethanol), 1·0 ml. The reagent was stored in the dark at 3 to 4 °C.

5. Sucrose. This was determined by an auto-analyser method (Dickerson, Mantle & Szczyrbak, 1970), or manually as follows. Culture filtrate (10 ml) was incubated at 37 °C for 1 h with two drops of yeast invertase concentrate (BDH). After suitable dilution the glucose liberated by the enzymic hydrolysis was determined with glucose-oxidase reagent. A calibration graph was prepared for glucose by plotting absorbance units against glucose concentration in the range 10 to 100 μg/ml. The sucrose content was calculated as the difference between hydrolysed and unhydrolysed culture filtrate.

6. Reducing sugar. Reducing sugar in culture filtrate from which glucan had been precipitated was determined as follows. A 5·0 ml sample of the ethanolic culture filtrate was hydrolysed with 4 N-H_2SO_4 (5·0 ml) for 30 min in a boiling water bath. After rapid cooling and neutralization with 2 N-NaOH (10 ml), the volume was made up to 20 ml with distilled water. Fehling's solution A ($CuSO_4.5H_2O$, 6·93 % w/v; 5·0 ml), Fehling's solution B (potassium sodium tartrate, 3·4 % w/v; NaOH, 14·2 % w/v; 5·0 ml), and hydrolysed sample (20 ml) were placed in a 100 ml conical flask (together with 3 or 4 glass beads) and boiled for 3 min. The samples were cooled rapidly, 4 N-H_2SO_4 (10 ml) and 30 % w/v KI (5·0 ml) were added, and the liberated iodine titrated with 0·05 N-$Na_2S_2O_3$ using starch as the indicator. A blank was prepared using distilled water (20 ml) instead of the sample. A standard graph was constructed relating volume of sodium thiosulphate solution to reducing sugar concentration in the range 5–30 mg glucose/ml.

7. Cell-associated sucrase. In order to assess the result of sucrase activity, culture samples

were taken at intervals during fermentation and assayed chromatographically for sugars as follows. Culture filtrate (2·5 μl) was applied to Whatman no. 1 paper and developed by descending chromatography in *n*-propanol–ethylacetate–water (7:1:2) for 22 h at room temperature. After drying, the chromatograms were sprayed to saturation with a solution of the following composition: *o*-phthalic acid, 1·7 g; aniline (redistilled over Zn), 1·0 ml; 40 % (w/v) trichloroacetic acid, 5·0 ml; glacial acetic acid, 5·0 ml; made up to 100 ml with absolute ethanol. The sugars appeared as brown spots after heating the dried papers at 120 °C for 5 min.

8. Inorganic phosphate. Suitably diluted culture filtrate (1·0 ml), distilled water (25 ml), sodium molybdate solution (2·5 % w/v in 10 N-H_2SO_4; 5 ml) and 0·15 % (w/v) hydrazine (2·0 ml) were made up to 50 ml with distilled water and heated at 100 °C for 10 min. After cooling rapidly the volumes were readjusted to 50 ml and the absorbance of the blue colour determined at 830 nm. A calibration curve in the range 0 to 50 μg phosphorus/ml was constructed by using standard KH_2PO_4 solutions.

9. Ammonium nitrogen. Suitably diluted culture filtrate (0·6 ml) was mixed with 20 % (w/v) NaOH (3·4 ml). After 1 min, 0·8 ml alkaline phenol reagent (25 g phenol made up to 100 ml with 20 % w/v NaOH) was added and the solution allowed to stand for a further 2 min. Sodium hypochlorite solution (12 %, w/v, available chlorine; 0·42 ml) was then added and the absorbance of the blue colour determined at 630 nm within 8 to 10 min. A calibration curve was prepared of μg nitrogen/ml against absorbancy units using $(NH_4)_2SO_4$ solution in the concentration range 12 to 45 μg N/ml.

10. Culture-filtrate lipid. Glucan was first removed from the culture filtrate by the addition of an equal volume of ethanol. After evaporation to the original volume the treated filtrate was extracted with petroleum spirit (b.p. 40 to 60 °C; twice, with equal volumes). The combined solvent extract was evaporated and the residual oil weighed.

11. Mycelial lipid. Washed mycelium was dried *in vacuo* over P_2O_5 and $CaCl_2$ and a finely milled sample (0·5 g) was extracted with chloroform–methanol (2:1; 200 ml) at room temperature for 9 h, followed by further extraction at 37 °C overnight. The solvent was filtered through fat-free cellulose thimbles (Whatman) and shaken with 0·1 N-KCl (40 ml) to effect a separation of the chloroform. The chloroform was removed, and the aqueous phase washed with an equal volume of chloroform. The combined chloroform extract was evaporated and the residual oil taken to constant weight.

12. Dissolved-oxygen concentration. This measurement was made by using a Teflon coil containing oxygen-free argon as the reference gas (after Roberts & Shepherd, 1968). Recording was achieved by a Kent Recorder (Kent Cambridge Ltd, Cambridge) coupled to a Hersch meter.

13. Oxygen consumption. Input air and exhaust gas from fermenters was dried by passage through a chilled-water condenser followed by silica gel. The oxygen content of the dried gases was determined with a Servomex Paramagnetic Oxygen Analyser (model DCL 83; Servomex Controls Ltd, Crowborough, Sussex) connected to a Kent Recorder. The oxygen consumption of the culture was calculated from the difference between input and output measurements.

14. CO_2 evolution. Exhaust gas was dried by passage through a chilled-water condenser followed by anhydrous $CaSO_4$, and the CO_2 content determined by a Hilger and Watts Infra-red Gas Analyser (model SC/F; Hilger & Watts Ltd, London N.W.1), coupled to a Foster Micro-amp Recorder (Foster Cambridge Ltd, London N10 2NA). A correction for the CO_2 present in input air was applied.

15. Rheological measurements. In order to characterize the rheological properties of

samples of culture fluid, shear stress measurements were carried out over a range of shear rates by means of a Contraves Rheomat model 15 Couette viscometer; such measurements were performed at a minimum of ten different shear rates for each sample. The true shear rates were obtained by adjusting for non-Newtonian flow behaviour in the viscometer with the correction factors reported by Calderbank & Moo-Young (1959). All the culture fluids were found to obey the power law, $\tau = K\gamma^n$, where τ is the shear stress (dynes/cm^2) and γ the shear rate (s^{-1}); the values of the consistency coefficient (K, in g/cm/s) and flow-behaviour index (n) were calculated from this relationship. The values of the average apparent viscosity in the fermenter were derived in accordance with the procedures described by Metzner & Otto (1957); a value of 11·5 was assumed for the proportionality factor relating agitator shaft speed and average shear rate in the fermenter, this figure being the average of those reported by Metzner & Otto (1957) and Calderbank & Moo-Young (1959). Reynold's number was calculated from the normal equation for stirred baffled vessels, $N_{Re} = \rho N D^2/\mu a$, where μa is the average apparent viscosity in the fermenter (poise), N_{Re} is Reynold's number, ρ is the liquid density (gm/cm^3), N the agitator shaft speed (rev./s), and D the impeller diameter (cm).

RESULTS

Development of an optimum production medium

Preliminary studies (Szczyrbak, 1972) in shake flasks on the selection of suitable carbon sources showed that in a simple defined medium either sucrose or glucose (2 % w/v) supported maximum mycelial growth (8·5 mg/ml) and allowed alkaloid production, whereas mannitol and mannose were less well utilized. Fructose, maltose, lactose, galactose, glycerol, sorbitol and starch supported little growth. Thus sucrose was selected on economic grounds and was used initially at a concentration of 10 % w/v (medium A).

Neither nitrate nor tryptophan supported growth as sole nitrogen sources. Tests on a wide range of other sources of nitrogen (Table 3) showed that good growth and alkaloid production could occur if a suitable pH value was maintained during the fermentation. However, the best nitrogen source for alkaloid production was $(NH_4)_2SO_4$ provided that the pH value could be maintained above 5·0. This was adequately achieved in shake flasks by the addition of $CaCO_3$.

The optimum concentrations of sucrose and $(NH_4)_2SO_4$ for maximum alkaloid production were determined in a modification of medium A, from which $Ca(NO_3)_2$, KCl, cysteine hydrochloride and yeast extract were omitted. The assessment of twenty-five sucrose/ammonium sulphate combinations (Table 4) showed that 200 g/l sucrose and 11·8 g/l $(NH_4)_2SO_4$ (= 2·5 mg N/ml) gave a maximum alkaloid yield of 3·7 mg/ml within 9 days, at which stage no glucan remained in the culture filtrate. Thus this medium (medium B) was tested in a 400 l fermentation in which pH was controlled by automatic additions of NaOH.

400 l batch fermentation process

The course of a typical 400 l fermentation is illustrated in Fig. 2. High yields (4 mg/ml) of alkaloid were obtained, which slightly exceeded the yield in shake flasks (Table 4). Rapid alkaloid accumulation occurred during the mycelial growth phase, but ceased when the growth rate declined at 5 to 6 days.

Alkaloid production ceased although approximately 30 % of the batched nitrogen source remained unused. A relatively high concentration of reducing sugar remained at the end of the fermentation. Sugar chromatography showed that sucrose had disappeared within the

Table 3. *Effect of various nitrogen sources on growth and alkaloid production in medium A*

The nitrogen sources replaced asparagine in medium A on an equivalent N basis. Cultures were grown for 9 days in production stage shake flasks. Morphology was sclerotial except for those cultures grown in valine and serine which showed the sphacelial growth form.

Nitrogen source	Mycelial dry weight (mg/ml)	Alkaloid titre* (mg/ml)	Final pH	Estimated residual glucan (– to + + +)	Approximate cost of N-source in 1971 (£/kg)
L-Asparagine	13·60	1·49 ± 0·04	5·45	–	11·0
L-Aspartic acid	13·53	1·62 ± 0·04	6·00	–	9·0
L-Sodium glutamate	14·83	1·68 ± 0·17	5·75	–	1·5
L-Proline	13·48	1·64 ± 0·04	5·15	–	94·0
L-Phenylalanine	17·10	1·17 ± 0·04	6·40	–	36·0
L-Alanine	10·18	1·21 ± 0·00	5·40	–	35·0
L-Valine	17·60	1·36 ± 0·06	5·60	–	30·0
L-Serine	14·60	0·31 ± 0·01	5·30	+	113·0
Glycine	12·00	1·35 ± 0·01	5·10	–	2·0
Urea	1·75	0·03 ± 0·00	9·08	+	1·0
Ammonium citrate	14·60	1·40 ± 0·07	4·60	–	1·0‡
Ammonium tartrate	13·45	0·57 ± 0·01	3·40†	+ + +	1·6‡
Ammonium succinate	10·55	1·62 ± 0·02	5·50	–	2·0‡
Ammonium DL-malate	15·36	1·87 ± 0·04	5·52	–	2·4‡
$(NH_4)_2SO_4$	4·85	0·04 ± 0·00	2·70†	+	0·8
$(NH_4)_2SO_4$ + 2 % (w/v) $CaCO_3$	19·26	1·94 ± 0·01	6·50	–	0·8

* Mean of 3 flasks, ± standard error.
† Low pH value due to acidity following ammonia uptake.
‡ Cost of free acid.

first 5 days, leaving fructose and fructofuranoside polymers. Glucose, which remained after fructosyl transferase action on sucrose, was preferentially utilized by the fungus and was barely detectable after 4 days. Thus some other nutrients, such as inorganic phosphate, magnesium or zinc, may have been limiting for alkaloid production and fungal growth.

Inorganic phosphate was taken up rapidly within 2 or 3 days of inoculation and its rapid uptake coincided with high respiratory activities.

Although mycelial morphology throughout the fermentation was principally in the sclerotial form consisting of short branched hyphae, frequently septate with the cells full of lipid, some sporulation occurred during the lag and early exponential phase (about 2 to 3 days from inoculation) indicating a transient phase of sphacelial growth with its characteristically lower lipid content. This could account for the fall in the mycelial lipid at 1 to 3 days after inoculation. There was also a decrease in the mycelial lipid content towards the end of the fermentation. However, the free lipid contents (0·2 to 0·4 %, w/v) of the culture filtrate samples at 10 days were not sufficient for this decrease to be accounted for by the release of lipid from the mycelium by autolysis of the cells. Thus the dry weight of mycelium which accumulated after day 6 may have been due to the proliferation of some sphacelial cells together with the sclerotial cells.

Respiration measurements during fermentation showed that the O_2 consumption and CO_2 evolution rose rapidly to maximum values at the beginning of the exponential phase, but then decreased gradually as the growth rate declined. The high respiratory rates were transitory since the true exponential growth phase appeared to be relatively short (24 to 48 h).

In view of the viscous nature of *Claviceps fusiformis* cultures, rheological studies were

Table 4. *Effect of varying concentrations of sucrose and $(NH_4)_2SO_4$ in medium B on alkaloid production by C. fusiformis after 9 days growth in production-stage shake flasks*

Sucrose (g/l)	$(NH_4)_2SO_4$ (mg N/ml)	Alkaloid titre* (mg/ml)
50	3	1·24 ± 0·03
	5	0·81 ± 0·01
	7	0·39 ± 0·08
	10	0·06 ± 0·00
100	2·0	2·71 ± 0·03
	2·5	2·79 ± 0·03
	3·0	2·19 ± 0·08
	4·0	1·93 ± 0·02
	5	1·11 ± 0·06
	7	0·29 ± 0·00
	10	0·07 ± 0·00
150	2·0	3·25 ± 0·15
	2·5	3·19 ± 0·05
	3·0	3·05 ± 0·03
	4·0	1·86 ± 0·10
	5	0·58 ± 0·07
	7	0·19 ± 0·01
	10	0·07 ± 0·01
200	2·0	3·31 ± 0·03
	2·5	3·67 ± 0·03
	3·0	3·18 ± 0·00
	4·0	2·10 ± 0·21
	5	0·50 ± 0·05
	7	0·19 ± 0·01
	10	0·06 ± 0·00

* Standard error indicated.

carried out on whole broth samples from a typical 400 l scale batch fermentation (Table 5). The glucan content reached a maximum value on the fourth day of the fermentation. As expected at this stage, the culture fluid exhibited highly pseudoplastic non-Newtonian properties, as indicated by the low value of the flow behaviour index. The consistency coefficient of the fluid and the average apparent viscosity within the fermenter (as defined by Metzner & Otto, 1957) also reached maximum values at this time. Subsequent hydrolysis of the glucan by a constitutive β-1,3 glucanase restored the culture viscosity to its original low value. These results clearly indicate that the viscous nature of the culture fluid in the early stages of the fermentation was principally due to the glucan, since in the later stages, when the glucan content was low and the mycelial dry weight high, the average apparent viscosity values were extremely low. The small extent to which the mycelium affected the viscosity of the culture fluid is unusual and is thought to be associated with the sclerotial (i.e. non-filamentous) nature of the mycelium. The dilatant properties ($n > 1$) exhibited by the culture fluid in the absence of glucan, or in the presence of low concentrations of glucan, were unexpected.

The Reynold's number was also calculated at different stages throughout the fermentation and this reached a minimum value after 4 days because of the high culture viscosity. However, the Reynold's number was maintained at values above 10^4 throughout, indicating that the highly desirable conditions of turbulent flow existed at all stages of the fermentation.

The dissolved-oxygen concentration reached a minimum value at the fourth day, because of the limitation imposed upon the oxygen supply by the high culture viscosity. Dissolved

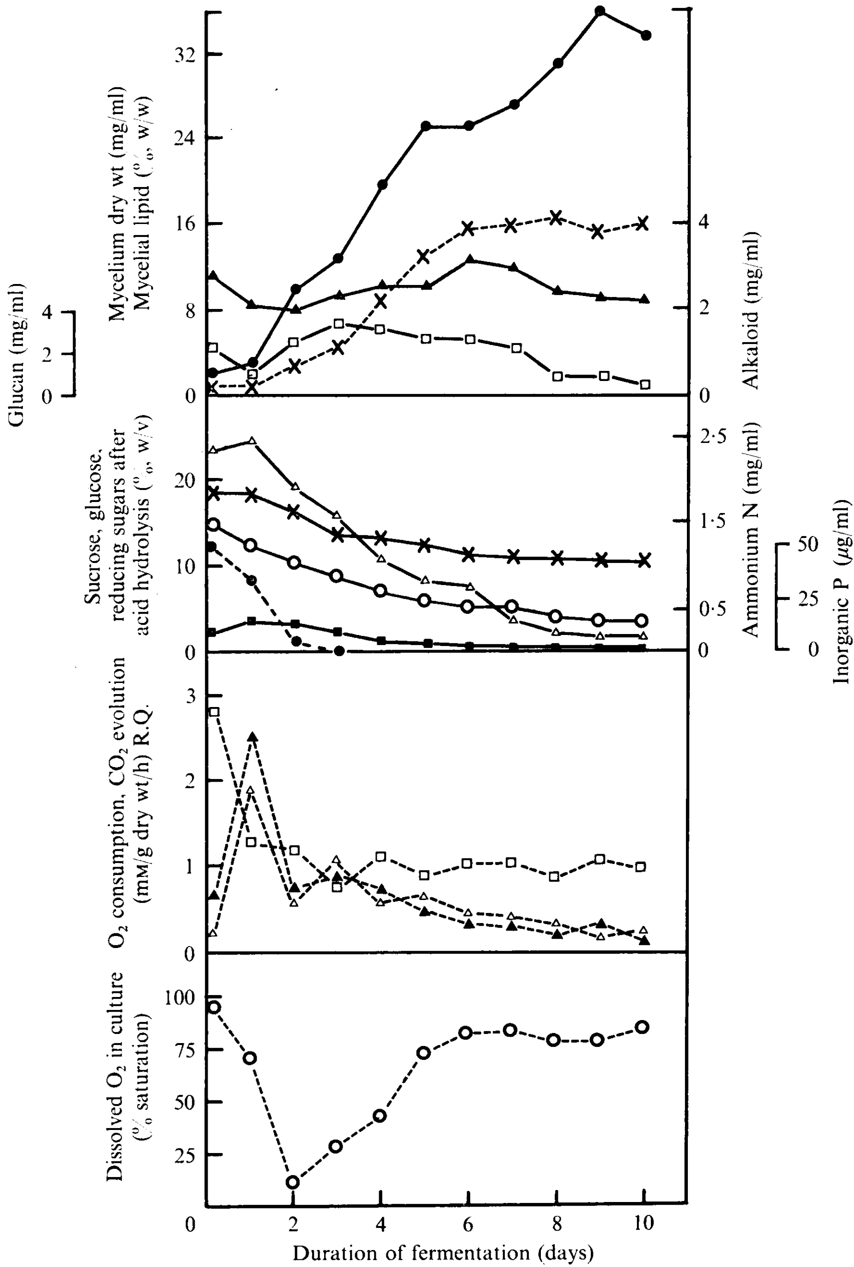


Fig. 2. Course of *Claviceps fusiformis* (strain 139/2/1G) fermentation in a 400 l fermenter. ●——●, Mycelium; ■——■, glucose; ×----×, alkaloid; ▲——▲, mycelial lipid; ○——○, sucrose; □——□, glucan; △——△, ammonium N; ●----●, inorganic P; ×——×, reducing sugar; □----□, R.Q.; ▲----▲, qCO_2; △----△, qO_2; ○----○, dissolved oxygen.

Table 5. *Rheological data for* 400 *l fermentation in medium B*

Days	Shaft speed	n	K (g/cm/s)	μa (centipoise)	N_{Re}*	Dissolved oxygen concn (% saturation)	Glucan (mg/ml)
0	153	—	—	—	—	—	1·26
1	153	1·59	$9·3 \times 10^{-4}$	0·69	$3·15 \times 10^{5}$	78	2·27
2	153	1·39	$4·2 \times 10^{-3}$	1·59	$1·37 \times 10^{5}$	64	1·97
3	306	0·38	6·8	53·25	$8·17 \times 10^{3}$	35	4·23
4	306	0·43	3·9	37·52	$1·16 \times 10^{4}$	42	3·53
5	306	0·88	$1·6 \times 10^{-1}$	10·24	$4·25 \times 10^{4}$	73	2·34
6	306	1·36	$6·7 \times 10^{-3}$	2·97	$1·46 \times 10^{5}$	89	2·36
7	306	1·61	$1·4 \times 10^{-3}$	1·71	$2·54 \times 10^{5}$	81	0·00
8	306	1·57	$1·3 \times 10^{-3}$	1·33	$3·27 \times 10^{5}$	78	0·00
9	306	1·87	$2·0 \times 10^{-4}$	0·70	$6·21 \times 10^{5}$	87	0·00
10	306	1·69	$6·1 \times 10^{-4}$	1·03	$4·22 \times 10^{5}$	90	0·00

* Calculated on the assumption that ρ (liquid density) = 1 g/cm³.

Table 6. *Effect of inorganic phosphate on alkaloid production*

Duplicate flasks assayed after 9 days growth in production stage shake flasks in medium B containing different inorganic phosphate concentrations.

Initial KH_2PO_4 concn (g/l)	Mean alkaloid titre* (µg/ml)	Mean final sucrose concn (mg/ml)	Mean final glucose concn (mg/ml)	Estimate of glucan concn (− to +++)	Mycelial morphology
0·050	0·27 ± 0·01	164·0	30·6	+	Sphacelial, few spores
0·075	1·35 ± 0·00	129·0	22·4	−	Sphacelial, no spores
0·100	2·22 ± 0·11	100·4	16·0	−	Sclerotial, no spores
0·150	3·32 ± 0·04	61·6	9·4	−	Sclerotial, no spores
0·200	4·09 ± 0·09	37·2	4·6	−	Sclerotial, no spores
0·250	4·18 ± 0·04	29·4	7·3	−	Sclerotial, no spores
0·300	4·27 ± 0·01	19·5	5·9	+	Sclerotial, few spores
0·400	4·87 ± 0·09	5·5	0·4	++	Sclerotial, few spores
0·500	5·90 ± 0·10	4·1	0·4	+++	Sclerotial, many spores
0·750	5·20 ± 0·09	3·0	0·4	+++	Sclerotial, many spores
1·000	5·20 ± 0·00	3·3	0·7	+++	Sclerotial, many spores

* Standard error indicated.

oxygen subsequently increased, partly owing to glucan hydrolysis and partly to the increase in agitator shaft speed at this time. The fact that the dissolved oxygen concentration never fell below 35 % of the saturation value suggests that the oxygen supply was not limiting at any stage.

Further medium development in shake flasks

Having obtained alkaloid titres in 400 l fermentation equivalent to those obtained in shake flasks, further investigations were made in shake flasks to determine the optimal concentrations of phosphate, magnesium, iron and zinc.

The phosphate concentration in medium B was varied in the range 0·05 to 1·00 g/l (Table 6). At all concentrations phosphate was totally absorbed from the medium within the 9 day fermentation period, but maximum alkaloid yield (6 mg/ml) occurred at about 0·5 g KH_2PO_4/l, coinciding with maximum depletion of sucrose. It was notable that the higher phosphate concentration caused purple mycelial pigmentation, increased sugar uptake, and also increased culture viscosity which was at least in part due to residual glucan.

Table 7. *The effect of different levels of magnesium sulphate in medium B on alkaloid production*

Triplicate flasks assayed after 9 days in production stage.

$MgSO_4.7H_2O$ (g/l)	Mean alkaloid titre* (mg/ml)
0·05	4·32 ± 0·15
0·10	4·86 ± 0·15
0·25	4·48 ± 0·08
0·50	5·34 ± 0·09

* Standard error indicated.

Table 8. *Effect on alkaloid production of concentrations of iron and zinc in medium B*

Triplicate flasks assayed after 9 days in production stage.

$ZnSO_4.7H_2O$ (g/l)	$FeSO_4.7H_2O$ (g/l)	Alkaloid* (mg/ml)
0·014	0·017	3·82 ± 0·14
	0·033	4·14 ± 0·05
	0·066	4·00 ± 0·13
0·027	0·017	4·48 ± 0·18
	0·033	4·40 ± 0·08
	0·066	4·36 ± 0·05
0·054	0·017	4·73 ± 0·12
	0·033	5·00 ± 0·08
	0·066	4·87 ± 0·12

* Standard error indicated.

Table 9. *The combined effect of different zinc, magnesium and inorganic phosphate concentrations on alkaloid production in medium B*

Triplicate flasks assayed after 9 days growth in production stage.

$MgSO_4.7H_2O$ (g/l)	$ZnSO_4.7H_2O$ (g/l)	KH_2PO_4 (g/l)	Mean alkaloid titre* (mg/ml)	Estimate of glucan concn (− to +++)
0·25	0·027	0·25	3·57 ± 0·15	−
0·50	0·054	0·50	5·37 ± 0·03	++
0·50†	0·108	0·50	5·99 ± 0·06	+++
0·50	0·054	0·75	5·34 ± 0·14	+++
0·10	0·054	0·50	5·69 ± 0·10	+++

* Standard error indicated.
† Medium C.

Medium B was therefore modified by doubling the phosphate concentration, after which the effect of different concentrations of magnesium, iron and zinc was investigated. Magnesium concentration did not appear to be critical (Table 7) and a change in iron concentration was of no advantage (Table 8). However, an increase in the zinc concentration raised alkaloid titres to 5 mg/ml (Table 8). Further assessment of the effect of magnesium, zinc and phosphate concentrations (Table 9) indicated that medium B could be modified to advantage, giving alkaloid yields of 6 mg/ml at 9 days, by doubling the concentration of $MgSO_4$ and KH_2PO_4 and quadrupling the concentration of $ZnSO_4$. This new medium was designated medium C.

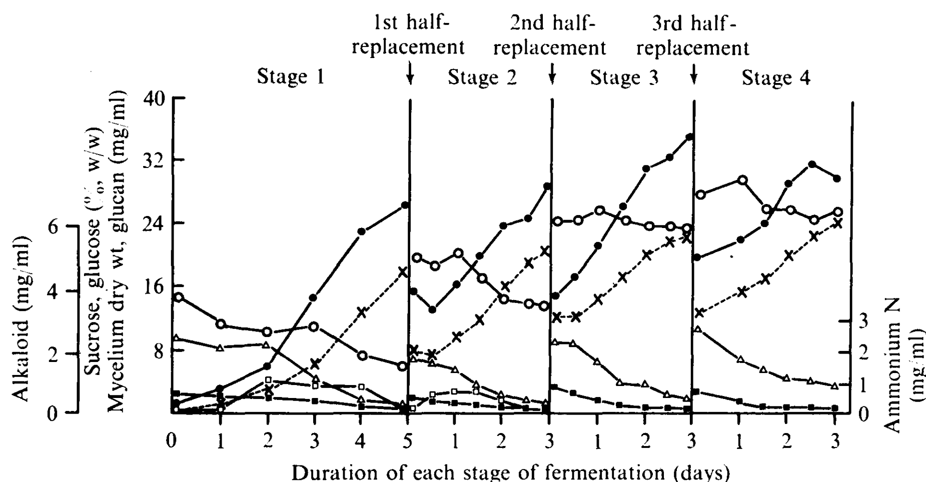


Fig. 3. Course of a 400 l multistage fermentation of *C. fusiformis* (strain 139/2/1G). ●——●, Mycelium; ■——■, glucose; □——□, glucan; ×----×, alkaloid; ○——○, sucrose; △——△, ammonium N.

Table 10. *Comparison of alkaloid production in medium C in seed and production-stage laboratory culture*

Group 1: triplicate flasks of medium C inoculated with spore suspension and incubated for 17 days (i.e. single-stage batch culture). Group 2: triplicate flasks of medium C inoculated with 10% (v/v) of 7-day-old Group 1 flasks and incubated for 17 days (i.e. medium C used for both seed and production stages).

	Alkaloid (mg/ml)	
	After 12 days*	After 17 days*
Group 1	4·63 ± 0·07	6·51 ± 0·09
Group 2	5·73 ± 0·18	6·55 ± 0·15

* Standard error indicated.

All these tests had been performed in production-stage shake-flask cultures with medium A for the seed stage. Thus the suitability of medium C as a seed medium and as a single-stage batch medium for shake flasks was assessed (Table 10). Alkaloid titres of 6·5 mg/ml occurred in both seed and production stage cultures within 17 days. The lower titres of seed cultures at 12 days can be attributed to the lag in establishing sclerotial mycelium in flasks inoculated with spores.

400 *l Half-replacement multi-stage process*

The superior nature of medium C for alkaloid production was confirmed in a multi-stage fermentation. The course of this fermentation is illustrated in Fig. 3. By maintaining the concentration of nutrients at a non-limiting value in half-replacement culture, progressively higher alkaloid titres (up to 6 mg/ml) were achieved in 400 l multi-stage fermentation. In all stages of this fermentation, alkaloid production proceeded at a high rate in linear fashion and, as in previous 400 l fermentations in medium B, coincided with the rapid growth phase. Glucan was entirely absent from the third and fourth stages of the fermentation, probably because of the accumulation of glucanase in the culture. During the third and fourth stages of the fermentation very little sucrose had been broken down, suggesting that sucrase activity was low.

DISCUSSION

This method for the large-scale production of agroclavine by batch or half-replacement fermentation has several important features. Utilizable nitrogen and carbon sources remained in abundance at the end of the fermentation. Through the action of a β-fructofuranosidase similar to that which operates in the utilization of sucrose by *Claviceps purpurea* (Bassett *et al.* 1972; Dickerson, 1972), sucrose was converted to a series of fructofuranoside polymers by fructosyl transfer and the residual glucose was preferentially utilized by the fungus. Some of the higher polymers were partly broken down later in the fermentation. β-Glucanase hydrolysed the glucan, which commonly accumulates during *C. fusiformis* growth on sucrose or glucose (Tonolo & Udvardy-Nagy, 1968; Buck, Chen, Dickerson & Chain, 1968; Dickerson *et al.* 1970), and later restored availability of free glucose for uptake by the fungus. This enzyme rendered the third and fourth stages of half-replacement fermentations free from glucan. The rapid uptake of phosphate was similar to that observed for other Claviceps species (Arcamone *et al.* 1970; Mantle, 1973). Zinc ions stimulated agroclavine production, a feature also observed by Rosazza, Kelleher & Schwarting (1967) in *Claviceps paspali* fermentations which yielded alkaloid. Optimum Zn^{++} concentration was found to be 25 times greater than that used by Tonolo & Udvardy-Nagy (1968).

Alkaloid synthesis closely followed growth, as expressed by dry weight accumulation. In high-alkaloid yielding fermentations the growth form of the cells, of which the short-branched septate hyphae are composed, resembled those comprising the medullary tissue of naturally occurring sclerotia of *Claviceps fusiformis* (Mantel & Szczyrbak, 1972). However, a certain amount of alkaloid can also be synthesized by sphacelial hyphae of *C. fusiformis*. Alkaloid synthesis by this species is not as closely restricted to sclerotial cells as has been demonstrated in *Claviceps purpurea* (Mantle & Tonolo, 1968; Bassett *et al.* 1972). The sclerotium-like growth form was also associated with the activity of the β-glucanase, by which glucan was eliminated thereby maintaining adequate dissolved oxygen concentration. This, in turn, facilitated both the production of high yields of alkaloid and its subsequent extraction from the culture filtrate. The β-glucanase activity was also reflected in the changing rheological properties of the cultures during fermentation.

The high viscosity and pseudoplastic nature ($n < 1$) of the whole fermentation culture, coinciding with the presence of high concentrations of glucan, was to be expected, since polysaccharide solutions generally exhibit pseudoplastic properties. Subsequent hydrolysis of the glucan restored the original low viscosity of the culture even in the presence of increased quantities of mycelium. Under these conditions the whole culture exhibited dilatant properties ($n > 1$). This unusual feature was undoubtedly associated with the sclerotial form of the hyphal fragments, which contrasted with the more filamentous form in which fungal hyphae usually grow in submerged culture and on which they confer pseudoplastic properties.

Values of the Reynold's number (N_{Re}) remained above 10^4 throughout the fermentation, indicating that conditions of turbulent flow existed. Such conditions are desirable if maximum aeration is to be provided under defined agitation conditions. Culture aeration was not limiting, since the values of the dissolved oxygen concentration did not fall below 35% saturation at any stage.

Thus maintenance of well-aerated non-viscous cultures, by the combined action of the β-glucanase and the persistence of the sclerotium-like growth form, allowed the production of high alkaloid titres to be supported in an inexpensive nutrient medium.

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