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Long-Term Production of Ergot Peptides by Immobilized *Claviceps purpurea* in Semicontinuous and Continuous Culture

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The semicontinuous and continuous production of pharmaceutically useful ergot peptides with immobilized *Claviceps purpurea* could be demonstrated. A key aspect was the presence of high concentrations of $CaCl_2$ (96.9 mM) to give marked prolongation of the productive phase, and cultivation in a bubble column reactor became possible. Restriction of the phosphate supply avoided an otherwise problematic massive increase of outgrowing hyphae.

To meet the requirements of long-term alkaloid production by cells of *Claviceps purpurea*, some crucial properties of saprophytically growing mycelia have to be taken into consideration. Suspended cells of *C. purpurea* rapidly decrease in productivity (9, 13), and successful cultivation in a semicontinuous manner is difficult.

Claviceps mycelia immobilized in calcium alginate beads were demonstrated to have long-term stability during semicontinuous cultivation (7, 9–12, 18, 21, 22). Unfortunately, only precursors or simple derivatives of lysergic acid, such as clavines or ergometrine, were produced by these processes. Long-term production of pharmaceutically relevant ergot peptides as well as continuous cultivation of *C. purpurea* have remained elusive until now.

C. purpurea 1029/N5, the strain used in this study, excretes the ergot peptides ergotamine and ergocryptine in considerable amounts. According to the classification of Pazoutova et al. (16), this strain belongs to the group of so-called P1-type producers, forming alkaloids exclusively during growth phase. P2-type producers are productive during stationary phase. To extend the production phase of a P1-type strain, the growth phase has to be prolonged, for example by regulating the phosphate supply.

This report describes prolongation of growth of the ergotamine-producing P1-type strain *C. purpurea* 1029/N5 by means of semicontinuous and continuous cultivation using immobilized mycelia.

MATERIALS AND METHODS

Organism and cultivation conditions. *C. purpurea* 1029/N5 is a mutant of strain 1029, producing mainly ergotamine and ergocryptine (5, 13). For growth in solid or liquid phase, a modified T2 medium (2) [100 g of sucrose, 10 g of L-asparagine-1-hydrate, 1 g of $Ca(NO_3)_2 \cdot 4H_2O$, 62.5 g of KH_2PO_4 (if not otherwise stated), 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.12 g of KCl, 0.02 g of $FeSO_4 \cdot 7H_2O$, 0.015 g of $ZnSO_4 \cdot 7H_2O$, and 0.75 mg of nicotinamide per liter of tap water] was used. The inoculation medium (1) contained 100 g of sucrose, 10 g of citric acid, 1 g of $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.12 g of KCl, 0.007 g of $FeSO_4 \cdot 7H_2O$, 0.006 g of $ZnSO_4 \cdot 7H_2O$, and 0.75 mg of nicotinamide per liter of tap water. In all media, yeast extract was replaced with nicotinamide. Both media had a Ca^{2+} content of 4.2 mM. Inoculum preparation was carried out as described by Lohmeyer et al. (13). Mycelia were shredded to homogeneity with an Ultra-Turrax (Jahnke & Kunkel, Staufen i.Br., Germany).

Immobilization. The methods described by Lohmeyer et al. (13) were used to form calcium alginate beads of 3-mm diameter with 3.5% alginate and 0.05 g (wet weight) of mycelium per ml.

Alkaloid production. Shake cultures with immobilized cells were carried out as described by Lohmeyer et al. (13). If not otherwise stated, the phosphate content of the T2 medium was that optimized for immobilized cells (62.5 mg of KH_2PO_4 per liter). For medium exchange in semicontinuous culture, the beads were washed twice in demineralized water and resuspended in fresh medium under sterile conditions. For reincubation of outgrown cells, the supernatant of a semicontinuous culture was separated after the fourth period, centrifuged (5,800 × *g*, 4°C), washed (demineralized water), and resuspended in fresh T2 medium containing $CaCl_2$ (96.5 mM) under sterile conditions.

The fermentor used for semicontinuous and continuous cultivation was a conical bubble column reactor (BCR) (Fig. 1). The total reactor volume of the glass vessel was 500 ml, and the medium volume was 250 ml. The volume of the alginate beads was 50 ml. The reactor was aerated from the bottom with 8 vol/vol/min. For continuous cultivation, new medium was added and fermentation broth was withdrawn with the same flow rate by a peristaltic pump. If necessary, sunflower oil (*Oleum helianthi* DAC 1986; Henry Lamotte, Bremen, Germany) was added as antifoam (at an average of 0.1 ml/day). All fermentations were carried out in the dark at 24°C. Generally, alkaloid production was defined as the amount of alkaloids excreted.

Determination of alkaloids. The colorimetric method described by Michelon and Kelleher (15) was used as modified by Lohmeyer et al. (13).

Determination of sugars. Sucrose was determined via the glucose portion by means of high-pressure liquid chromatography (series 655; Merck Hitachi, Darmstadt, Germany) after hydrolysis, using an Aminex HPX-87H column (Bio-Rad, Richmond, Calif.) with H_2SO_4 (6.5 mM in double-distilled water) as eluant and a flow rate of 0.6 ml/min at 65°C (19) and fitted with a differential refractometer detector (no.

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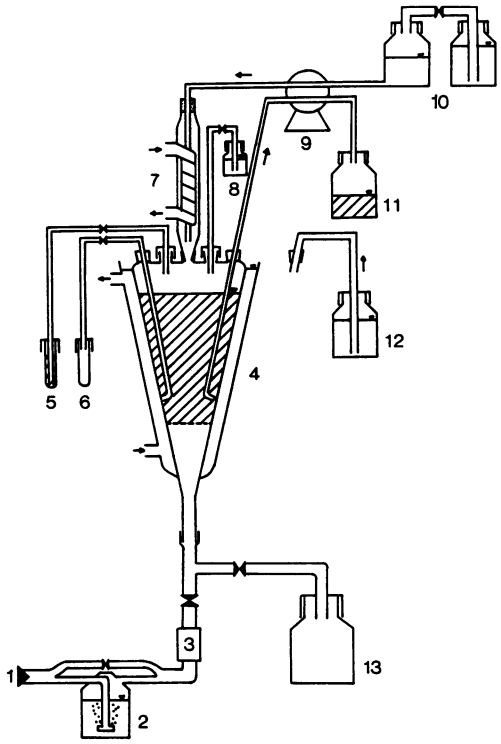

FIG. 1. Fermentation in a BCR. 1, air access; 2, air humidifier with shunt for regulation; 3, air inlet filter; 4, conical bubble column with glass sieve (diameter, 5 cm) and temperation jacket; 5, antifoam reservoir; 6, sample vial; 7, reflux condenser with medium inlet. Special devices for continuous cultivation: 8, phosphate solution reservoir; 9, peristaltic pump; 10, medium reservoir; 11, outlet of fermentation broth. Special devices for semicontinuous cultivation: 12, medium inlet; 13, outlet of fermentation broth.

98.00; Knauer, Berlin, Germany). For hydrolysis, 0.1 ml of sample was mixed with 0.9 ml of H_2SO_4 (0.4% in double-distilled water) and incubated (3 h, 70°C).

Determination of dry weight. The dry weight of the medium filtrate (supernatant of the alginate beads) was determined as described by Lohmeyer et al. (13). The dry weight of immobilized cells was determined (13) after resolving the alginate by gentle shaking (24 h, 45°C) in sodium hexametaphosphate (0.1 M) and washing the mycelium three times with warm tap water.

RESULTS

Fermentation course of semicontinuous culture. With each consecutive period in semicontinuous culture, immobilized cells of *C. purpurea* 1029/N5 showed decreased alkaloid formation, increased sugar consumption, and an increasing amount of hyphae growing out into the medium (Fig. 2). After six exchanges of the medium, alkaloid production was only one-third of that during the first period, while the sugar consumption had nearly doubled.

Influence of $CaCl_2$ addition on alkaloid production. By using medium with a higher $CaCl_2$ content (96.9 mM), the alkaloid production phase during semicontinuous cultivation of immobilized cells was significantly extended (Fig. 3). Under the influence of $CaCl_2$, the immobilized mycelium produced alkaloids over a time period of 70 days with a rate higher than 20 mg/liter/day.

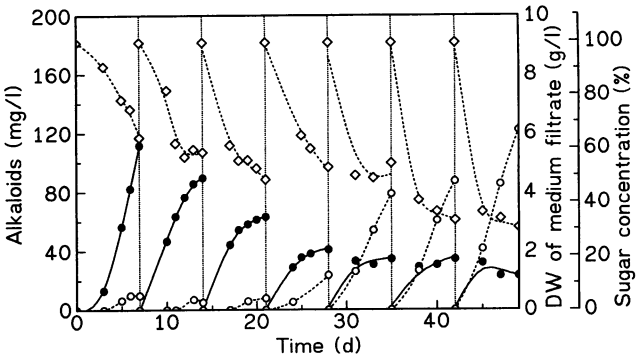

FIG. 2. Time course of semicontinuous cultivation. Symbols: ●, alkaloid concentration; ○, dry weight (DW) of medium filtrate; ◇, carbon source (100% equivalent to 83 g/liter, determined as the glucose portion of sucrose) in shake culture. Dotted lines mark medium exchange.

In contrast to the corresponding immobilized cells (Fig. 3), outgrown cells were obviously unproductive. Only about 10 mg of alkaloids per liter was found 10 days after reincubation of these cells, separated after the fourth period, while their dry weight increased from 3.8 to 8.5 g/liter (data not shown).

Influence of period length on alkaloid production. The period length could significantly affect the cumulative alkaloid production of a semicontinuous cultivation. Without $CaCl_2$ addition, a reduction of the period length from usually 14 to 7 days resulted in a nearly doubled cumulative alkaloid production (up to about 700 mg/liter) after 70 days. In contrast, the influence of period length in cultures with $CaCl_2$ (96.9 mM) was negligible (about 1,400 mg/liter under both conditions).

Fermentation course of semicontinuous culture in a BCR. Without additional calcium, a semicontinuous culture in the BCR yielded only low alkaloid concentrations: 70 mg/liter in the first period and decreasing values in the following periods. Moreover, the mechanical stability of the alginate beads was poor; after three periods, further cultivation was impossible because of bead disintegration (data not shown).

In presence of 96.9 mM $CaCl_2$, however, the stability of beads was markedly improved. Alkaloid production increased, reaching a maximum in the fifth period, and then decreased (Fig. 4); i.e., maximum productivity was reached

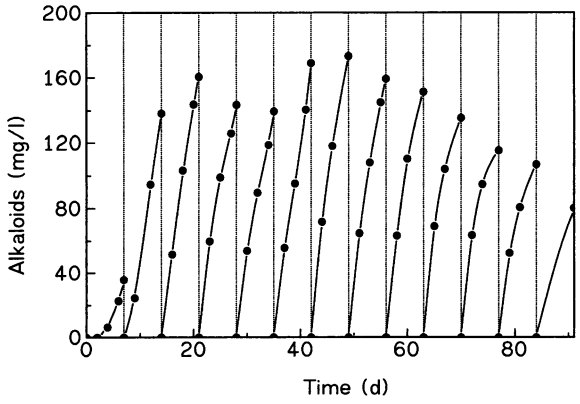

FIG. 3. Influence of increased $CaCl_2$ concentration (96.9 mM) on alkaloid formation (●) in semicontinuous shake culture. Dotted lines mark medium exchange.

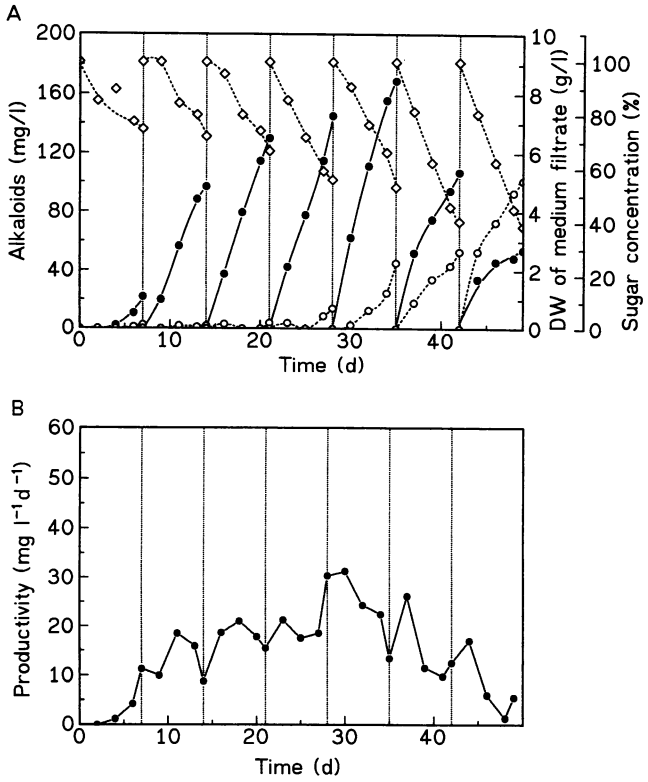

FIG. 4. Time course of semicontinuous cultivation in a BCR with 96.9 mM $CaCl_2$. (A) Alkaloid concentration in column (●); dry weight (DW; ○) of medium filtrate; carbon source (◇). (B) Productivity (●). Dotted lines mark medium exchange.

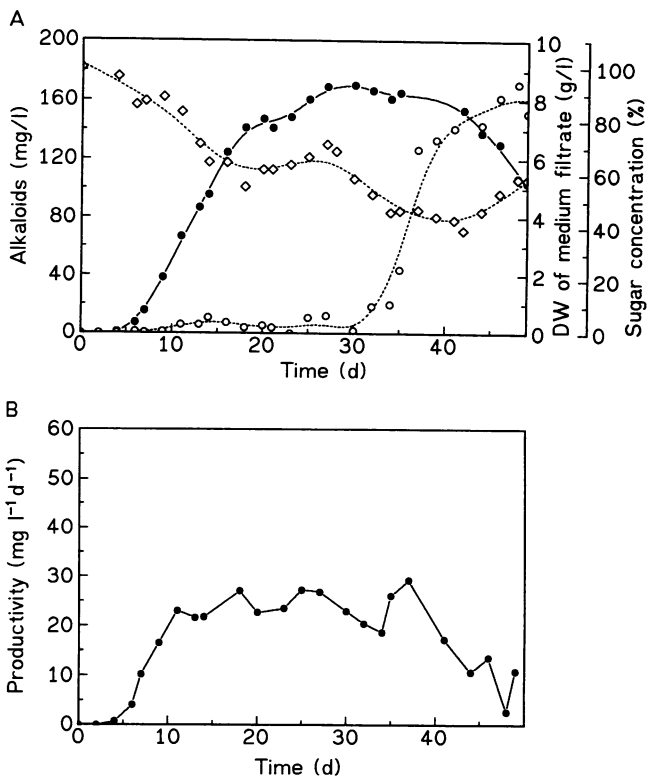

FIG. 5. Time course of continuous cultivation in the presence of 96.9 mM $CaCl_2$ at a dilution rate of 0.13 day^{-1}. (A) Alkaloid concentration in column (●); dry weight (DW; ○) of medium filtrate; carbon source (◇). (B) Productivity (●).

after about 30 days. By this time, the amount of outgrowing hyphae increased strongly with each period. Sugar consumption increased continuously throughout cultivation.

Fermentation course of continuous culture in a BCR. Continuous cultures were carried out in the presence of a higher $CaCl_2$ concentration (96.9 mM). At a dilution rate of 0.13 day^{-1}, a nearly constant alkaloid productivity was reached after 18 days, lasting for about 24 days. The productivity showed values of about 20 mg/liter/day during a period of 25 days (Fig. 5). The later decrease of productivity was preceded by a massive increase of free hyphae in the filtrate. The sugar concentration decreased in two steps; the first paralleled the increase in alkaloid concentration, and the second followed the stimulated outgrowth of hyphae.

Influence of phosphate dosage on alkaloid production and growth of a continuous culture. By plugging the fermentor outlet, even smaller amounts of free mycelia could severely hamper continuous fermentation or cause its interruption. When phosphate-free medium was used during the continuous phase, extensive outgrowth was actually prevented, but productivity decreased steadily (data not shown).

To preserve productivity, phosphate was added continuously but in a very low concentration (15.6 mg/liter) (Fig. 6). Therefore, cells received only 3.9 mg of KH_2PO_4 per liter per day. Under these conditions, alkaloid production was approximately constant (about 25 mg/liter/day) immediately after the switch to continuous conditions (Fig. 6). This state lasted about 30 days, and then the alkaloid concentration decreased. The amount of outgrowing hyphae was negligible, while the hyphae inside the beads were growing with increasing velocity up to a value of 18 g (dry weight) per liter after 33 days (data not shown).

DISCUSSION

For the first time, stable long-term production of ergot peptides has been demonstrated in saprophytic culture. With mycelia of *C. purpurea* 1029/N5, this was successfully performed under conditions of (i) stabilization by means of immobilization, (ii) increased $CaCl_2$ concentration, and (iii) restricted phosphate supply. These preconditions are determinated by the specific demands of *C. purpurea* in general and of strain 1029/N5 in particular. In semicontinuous cultivation, immobilized cells of this strain usually do not show any long-term stability; productivity sharply decreases during the second reincubation under standard conditions.

Obviously, a high frequency of medium exchange has a positive influence on alkaloid production, as demonstrated previously for semicontinuous clavine production with immobilized *Claviceps paspali* (22). While new substrate is supplied with each medium replacement, the resulting growth is coupled with a temporary increase of alkaloid formation, as is typical for a P1-type alkaloid producer. Nevertheless, the positive effect of medium exchange on alkaloid production of strain 1029/N5 fades with each period.

The mechanical stability of alginate beads in semicontinuous cultivation decreases with time. It is in a BCR that instability becomes a serious problem. A high concentration of Ca^{2+} ions markedly increases the stability of the ionotropic calcium alginate gel. This stability can affect the physi-

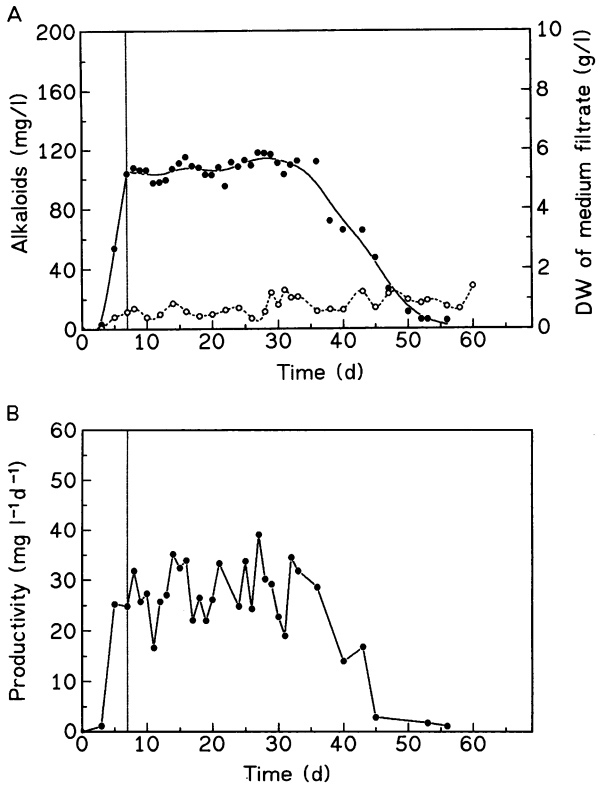

FIG. 6. Influence of continuous cultivation with restricted phosphate supply (dilution rate, 0.25 day^{-1}). The vertical line marks the shift from batch (with 62.5 mg of KH_2PO_4 per liter) to continuous cultivation with an inlet concentration of 15.6 mg of KH_2PO_4 per liter. For symbols, see the legend to Fig. 5.

ology of entrapped cells. Therefore, mechanical hindrance was discussed as an important factor for alkaloid formation by *C. purpurea* (8). Moreover, the calcium ions may directly affect the immobilized organism (4, 20) or decrease the effective phosphate concentration by precipitation (3). Kren et al. (11) used $CaCl_2$ as a medium component for immobilized *Claviceps fusiformis*. The effect on immobilized *C. purpurea* 1029/N5 yields prolonged alkaloid formation coupled with increased growth.

Ergot alkaloid synthesis is very sensitive even to small alterations in cultivation conditions (23) and generally to mechanical stress (6) as caused by agitation in bioreactors. In the presence of $CaCl_2$, however, the alkaloid concentrations achieved in a BCR were comparable to those in shake culture, whereas the total productive time was shorter.

Phosphate supply is of crucial importance during long-term cultivation, since phosphate may accumulate within the cells, stimulating excessive growth. In phosphate-free (11) or strongly reduced media, successful semicontinuous and continuous production of clavines as precursors of the ergot peptides has been shown (14). In the case of *C. purpurea* 1029/N5, exaggerated phosphate restriction led to poor ergot peptide production in continuous culture. Obviously, alkaloid production is strictly dependent on growth in this case, whereas the converse does not always follow. Growth, however, is regulated by phosphate. Higher concentrations of phosphate resulted in increased productivity but also favored the undesirable outgrowth of cells. Outgrown cells are unproductive but still grow at the expense of productive, i.e., immobilized, cells.

With cautious phosphate restriction (3.9 mg/liter/day), cells grow preferentially inside alginate beads during continuous cultivation. Obviously, protected growth inside this matrix favors alkaloid production, as discussed previously for batch cultivation (13). This growth and therefore alkaloid formation may end when the beads are fully packed with mycelia. In contrast to the suggestions of Pazoutova et al. (17), who recommended the use of resting immobilized cells, continuous production of ergot peptides by immobilized cells of *C. purpurea* 1029/N5 is apparently coupled to slow growth inside an alginate matrix. Regulation of this growth seems to be the key to successful ergot peptide production.

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