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Effect of surfactants on the production of ergot-alkaloids by immobilized mycelia of *Claviceps paspali*

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In a semicontinuous process immobilized *Claviceps paspali* mycelia produced alkaloids over a period of 60 days (six reincubations). By addition of the surfactant Pluronik, a polyethoxypolypropoxy polymer, a considerable increase in alkaloid biosynthesis occurred. The maximum product concentration achieved was 8.35 g l^{-1}, and the overall productivity was 5.80 mg l^{-1} h^{-1}, which is half the productivity of the batch process. Maximum process productivity for a single reincubation (12.3 mg l^{-1} h^{-1}) was almost equal to the batch process productivity.

Key words: *Claviceps*, ergot alkaloids, immobilization, surfactant.

Recently, there have been attempts to improve the biosynthesis of ergot alkaloids in submerged cultures of *Claviceps purpurea* (Kopp & Rehm 1983; Dierkes *et al*. 1993), *C paspali* (Rozman *et al*. 1989; Matošić *et al*. 1992) and *C. fusiformis* (Kren *et al*. 1987; Rozman *et al*. 1987) by the introduction of processes using immobilized mycelia. These processes are also more interesting because of easier culture handling and more prolonged metabolic activity of the cells (Kopp *et al*. 1984). Furthermore, the results of Gil'manov *et al*. (1996) on the stimulatory influence of hydrocarbons on some *Claviceps* cultures are very promising. Although biosynthesis of ergot alkaloids is regulated genetically, temporary changes in this regulation can be induced by manipulation of the cultivation conditions and the composition of medium. Biosynthesis of alkaloids depends on the control mechanisms of tryptophan biosynthesis. Both the biosynthesis of alkaloids and the biosynthesis of tryptophan are inhibited in cultures of *C. purpurea* by inorganic phosphate at concentrations that do not inhibit growth (Pažoutova & Rehaček 1984). However, increased concentrations of inorganic phosphate do not inhibit alkaloid synthesis in cultures of *C. paspali* (Matošić *et al*. 1983).

Aeration of the cultivation liquid has an adverse effect on alkaloid production. A decrease in respiration is one of the possible ways of increasing alkaloid formation, although this view is not supported by Rehaček (1980). Desai *et al*. (1986); Mizrahi & Miller (1969) and Matošić *et al*. (1994) have doubled the production of alkaloids by adding Tweens and glycols to the cultivation medium in submerged cultures of *C. paspali*. Dimethylsulphoxide increases the alkaloid yield by 50%. These surfactants affect permeability of the cytoplasmic membrane; by disturbing the permeation barrier, the cells increase the liberation of alkaloids from the region of their synthesis and thus prevent possible feedback inhibition (Rehaček 1980). Surfactants do not affect the biosynthetic pathway (Mizrahi & Miller 1968).

The increase of alkaloid formation produced by the addition of Tween-80 is accompanied by a shift in the organic acid and amino acid level in the cell pool (Rehaček & Basappa 1971). From this it follows that alkaloid biosynthesis is regulated by the level of the intracellular precursors of the primary metabolites. An increased concentration of numerous metabolites in the cell pool can also be attained by increasing the osmolarity of the medium. High osmolarity may favour the production of alkaloids if the carbon source added serves both these functions and a nutritional role (Tonolo 1967; Rehaček 1991). In this respect, NaCl may reduce the sugar requirement (Puc & Sočić 1977).

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Here, a semicontinuous process for the biosynthesis of ergot alkaloid with immobilized cells of *C. paspali* was studied. Particular emphasis was placed on the effect on process productivity of surfactants added to the medium.

Materials and Methods

Microorganism

The strain of *C. paspali* F-2057 from the Culture Collection of Department of Biochemical Engineering Faculty of Food Technology and Biotechnology, University of Zagreb, was used in this study.

Culture Media

Medium for maintenance of the culture was potato infusion–glucose agar. Seed cultures were prepared in the medium (g l^{-1} tap water): succinic acid neutralized with ammonia to pH 5.2, 10; mannitol, 40; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.3. The production medium contained (g l^{-1} tap water): succinic acid neutralized with ammonia to pH 5.2, 50; mannitol, 60; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.3. (Matošić *et al*. 1984). Media were sterilized for 30 min at 110 °C. Surfactants added to the media were commercial antifoaming agents for fermentation processes, and are known commercially as Tween-80 (0.50%), Span-80 (0.75%), Pluronik L61SCU (0.25%). Optimum concentrations based on the results of Matošić *et al*. (1994) were used.

Immobilization

According to the target of each particular experiment, different concentrations of *C. paspali* mycelium (g 1^{-1} dry weight: 3.20; 4.30; 5.30) and different concentrations of sodium alginate (Type IV. Sigma; 2%, 4%, 6%) were chosen for immobilization.

Twenty milliliters of sodium alginate solution was autoclaved (121 °C/25 min), mixed with appropriate concentration of washed homogenized (15000 min/15 sec) 6-day-old seed-stage mycelia and dropped aseptically into 2% $CaCl_2$ solution. The resulting beads (2–4 mm, diameter) were hardened for at least 1 h, washed with 0.9% NaCl solution and incubated in 100 ml of the production medium.

Cultivation

Culture was maintained on agar plates, and a 2-week-old single colony was used for inoculating the medium in each seed flask. Batch fermentation experiments were carried out in two stages: a seed-stage fermentation for 6 days, and a production-stage fermentation for 14 days; inoculated with 10% (v/v) of the seed culture homogenized in Waring blender (15000 min^{-1}/15 sec). Cotton-wool plugged 500 ml Erlenmeyer flasks containing 100 ml of culture media were incubated at 24 °C on a rotary shaker with a 6 cm stroke. A semicontinous process was also performed in the shake-flask cultures with aseptic washing of beads and a change of nutrient medium every 10 days. This was also carried out separately with diluted basal medium (two, four and 10 times).

Analytical Determinations

Alkaloids were determined spectrophotometrically, with reference to a standard solution of ergometrine base, by van'Urk reagent (Banks *et al*. 1974). Biomass dry weight was determined by filtration, washing and drying at 105 °C. Mannitol was estimated by the polarimetric method (Arcamone *et al*. 1961).

Results and Discussion

Some commercial surfactants were used in an attempt to improve the biosynthesis of ergot alkaloids in batch cultivation and semicontinuous production by immobilized cells of *C. paspali*. Increase of alkaloid accumulation range from 70 to 100% was observed if the surfactant Pluronik (polyethoxypolypropoxy polymer) was added to the medium in batch process or semicontinuous production by immobilized cell (Figures 1 and 2).

The duration of the biosynthesis process of ergot alkaloids with immobilized *C. paspali* cells, was 60 days or six replacements of nutritive medium (Figure 2). The semicontinuous process with non-immobilized free cells of *C. paspali* was possible only during two successive

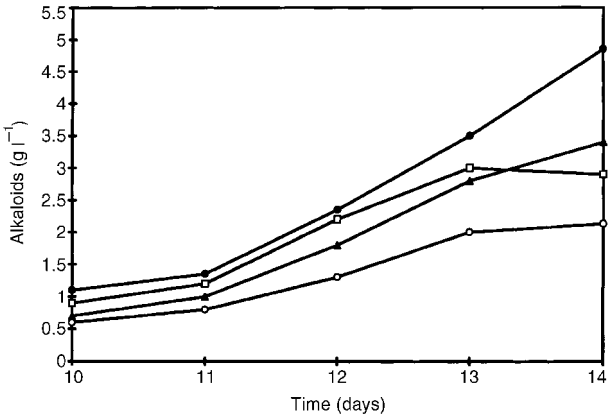

Figure 1. Batch alkaloid production with free cells of *C. paspali*. Effect of surfactants added to the medium: no addition, ○; Pluronik L61SCU (0.25%), ●; Span-80 (0.75%), ▲; Tween-80 (0.5%), □.

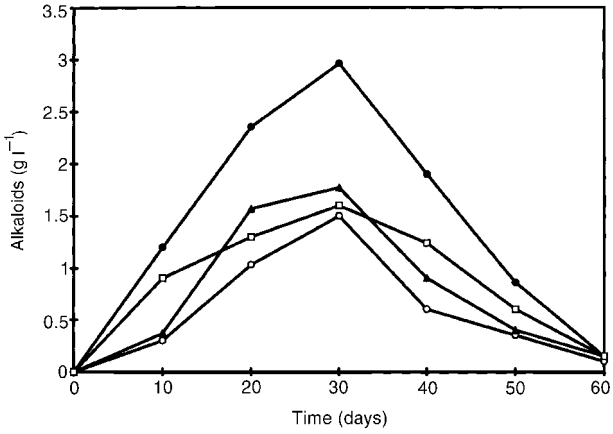

Figure 2. Semicontinous alkaloid production by immobilized cells of *C. paspali* with different surfactants added to the medium: no addition, ○; Pluronik L61SCU (0.25%), ●; Span-80 (0.75%), ▲; Tween-80 (0.5%) □. (Biomass dry weight 4.25 g l^{-1}.)

Table 1. Productivity of semicontinous process of alkaloid biosynthesis by immobilized mycelium of *C. paspali* as a function of media concentration and surfactant addition [cultivation 60 days; Pluronik L61SCU 0.25% (v/v) added; biomass dry weight 4.25 g l^{-1}].

Basal medium dilution	Surfactant addition	Total alkaloids produced (g l^{-1})	Mannitol consumed (g l^{-1})	Process productivity (mg l^{-1} h^{-1})	Substrate* yield coefficient (mg g^{-1})
0	–	5.00	147	3.47	34
	+	8.35	145	5.80	58
1:2	–	4.03	101	2.80	40
	+	6.68	99	4.64	67
1:4	–	2.52	59	1.75	43
	+	4.31	59	2.99	73
1:10	–	1.59	38	1.10	42
	+	2.76	37	1.92	74

*Calculated on the concentration of consumed mannitol.

reincubations with a serious decrease of process productivity (Matošić *et al.* 1992).

By addition of surfactant Pluronik (0.25%) there was a considerable increase in ergot alkaloid biosynthesis. The maximum product concentration achieved was 8.35 g l^{-1} of total alkaloids in 60 days of cultivation (Figure 2, Table 1). Productivity in the semicontinuous process of cultivation reached 5.80 mg l^{-1} h^{-1}, 50% of the batch process productivity. During the first 30 days of cultivation productivity amounted to 9.0 mg l^{-1} h^{-1} which is 70% of batch process productivity. Maximum productivity of a single reincubation (12.3 mg l^{-1} h^{-1}) between 20 and 30 days of cultivation was almost equal to the batch (Pluronik-supplemented) process productivity (Figures 1 and 2).

The results show that a 4% concentration of alginic acid, polymerized with Ca^{2+} ions, proved to be optimal for biosynthesis of ergot alkaloids, with immobilized cells in the Pluronik-supplemented culture (Figure 3). Pluronik also made it possible to carry out the process using larger quantities of immobilized cells, e.g. 5.31 g of biomass dry matter per liter (Figure 4). The decreasing concentration of nutrients reached by simple dilution of basal medium did not influence process productivity or alkaloid accumulation proportionally (Table 1). For instance by dilution of the basal medium by a factor of 10 process productivity decreased only by a factor of three but the substrate yield coefficient increased up to 27.6% (Table 1).

There are some possible explanations of the effect of antifoaming agents (surfactants) on the biosynthesis of ergot alkaloids. Surfactants, probably by wetting and lowering surface tension of the medium, affect the permeability of the cytoplasmic membrane. By disturbing the permeation barrier, the cells increase the liberation of alkaloids from the region of their synthesis and thus prevent possible feedback inhibition. Surfactants do not affect the biosynthetic pathway but promote the utilization

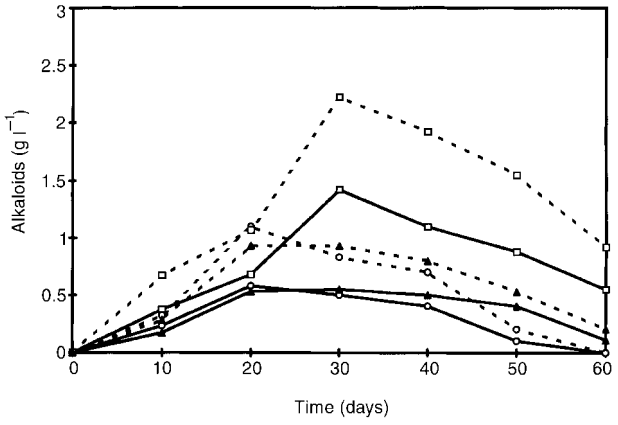

Figure 3. Production of alkaloids by cells of *C. paspali* immobilized in different alginate concentration: 2% alginate, ○; 4% alginate, □; 6% alginate, ▲; solid line, surfactant not added; broken line, Pluronik L61SCU (0.25%) added to the medium. (Biomass dry weight 4.25 g l^{-1}.)

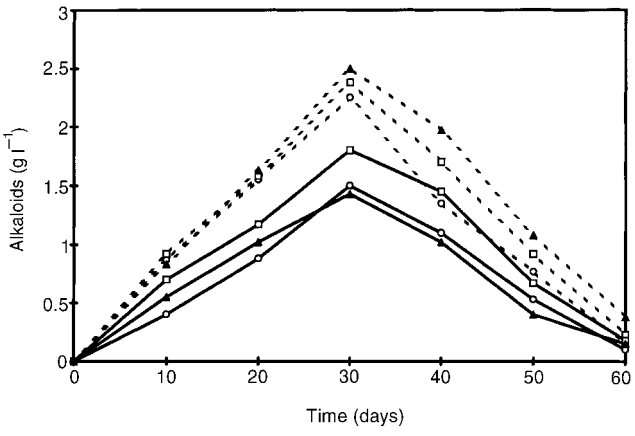

Figure 4. Effect of quantity of immobilized mycelium (dry weight per litre of medium) on the productivity of a semicontinuous process of alkaloid biosynthesis. Mycelium dry weight per litre of medium (g l^{-1}): 3.2, ○; 4.25, □; 5.3, ▲; solid line, surfactant not added; broken line, Pluronik L61SCU (0.25%) added to the medium.

of metabolites (Mizrahi & Miller 1969). Alkaloid formation with the addition of surfactants is also accompanied by a shift in precursor organic acid and amino acid levels in the cell pool (Rehaček & Basappa 1971; Rehaček 1980, 1991).

In addition, the non-ionic surfactant Tween-80 has been reported to increase the yield of a number of extracellular enzymes, bacteriocins and other microbial metabolites. It appeared to affect cell permeability in certain microorganisms to promote both uptake and exit of compounds from cell through modification of plasma membrane permeability (Huot *et al*. 1996).

The results obtained show that immobilization of mycelia-forming microorganisms might also be effective in the biotechnological production of complex metabolites such as ergot alkaloids. These effects should be considered in commercial production of pharmaceutically interesting ergot alkaloids.

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