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Production of lysergic acid derivatives with immobilized *Claviceps paspali* mycelium

Damjana Rozman[1], Elizabeta Pertot[2], Radovan Komel[1], and Miro Prošek[2]

[1] Institute of Biochemistry, Medical Faculty of the E. Kardelj University, Vrazov trg 2, 61000 Ljubljana, Yugoslavia
[2] "Boris Kidrič" Institute of Chemistry, 61000 Ljubljana, Hajdrihova 19, Yugoslavia

Summary. A semicontinuous fermentation with immobilized mycelium of *Claviceps paspali*, producing mainly methylcarbinolamide of lysergic acid, ergometrine and the amide of lysergic acid, was carried out. It was found that immobilization itself improved ergometrine production. The type and concentration of alginate used dit not influence the alkaloid spectrum. The age and quantity of inoculum as well as the length of a particular cycle affected total alkaloid production but did not seem to affect the changes in relative amounts of particular alkaloids. The best results were obtained with 1000 mg dry mycelium weight of 6-day-old fungus from the vegetative phase in 100 ml production medium immobilized in 4% alginate of medium or low viscosity and by recycling every 10 days. When no fungus was found outside the gel beads it was possible to improve the yield of ergometrine from 52% in the first cycle to 85% in the fourth cycle of the semicontinuous procedure.

Introduction

Production of clavine ergot alkaloids in long-term semicontinuous fermentations with immobilized *Claviceps* sp. cells is well documented. Kopp and Rehm (1983, 1984) have described the biosynthesis of agroclavine and elymoclavine with immobilized *C. purpurea* cells, while Kren et al. (1987) have documented the production of these clavine alkaloids with immobilized *C. fusiformis* mycelia. The cyclol-type ergot alkaloids have the broadest spectrum of pharmacological applications. However, simple lysergic acid amides (Vining et al. 1979; Kobel and Sanglier 1986) and clavines (Stadler and Giger 1984) also play a role in acting as medicaments. Since cyclol-type ergot alkaloids accumulate in the mycelia, their production in a semicontinuous procedure with immobilized cells is not promising, but the production of ergotamine with immobilized protoplasts of *C. purpurea* has been reported (Komel et al. 1985).

The bulk of simple lysergic acid derivatives are synthesized by different *C. paspali* strains. In a previous paper (Pertot et al. 1988) we reported the morphological differentiation of immobilized *C. paspali* cells that showed prolonged metabolic activity during semicontinuous fermentation. The most interesting biosynthetic product of *C. paspali* is ergometrine, since it has a particularly prominent uterocontractant activity and is widely used for treatment of postpartum haemorrhage (Floss 1976; Vining and Taber 1979). Kopp and Rehm (1983, 1984) have already reported increased ergometrine production with *C. purpurea* immobilized in 4% calcium alginate.

Our present work describes some parameters which affect alkaloid biosynthesis in a long-term semicontinuous procedure with immobilized *C. paspali* cells. The total alkaloid synthesis as well as ergometrine production under different conditions constituted our main interest.

Materials and methods

Microorganism and cultivation conditions. A selected strain of *C. paspali* ATCC 13892, designated as L-52, producing 1.5 g/l of simple LA derivatives, was employed. Colonies taken from glucose-potato agar were grown for 6 days in mannitol-suc-

Offprint requests to: R. Komel

Abbreviations. LA, lysergic acid; ALA, amide of lysergic acid; MCLA, methylcarbinolamide or α-hydroxyethylamide of lysergic acid; EMT, ergometrine; DMW, dry mycelium weight; PM, production medium

cinic acid growth medium (Pertot et al. 1988). By fermentation with free cells, 10% (v/v) of the vegetative broth was inoculated into mannitol-succinic acid production medium (mannitol, 100 g; succinic acid, 30 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; per litre of H_2O). Fermentation was stopped after 10 days.

Determiantion of dry mycelium weight. The fungus broth (100 ml in two parallels) was filtered and dried at 80° C for 1 h. The volume aliquot which represented the determined dry weight was filtered and taken for immobilization. The difference between the mycelium weight after 1 h or 24 h of drying was 5%–10%.

Immobilization conditions. According to the target of each particular experiment different amounts and ages of *C. paspali* mycelium were chosen for immobilization in different types of Na-alginate gel (Sigma, St. Louis, USA). All amounts are defined for 100 ml PM.

Six-day old mycelium (500 mg DMW) of the vegetative phase was mixed with 20 ml of 2%–4% alginate of different viscosities: (a) high viscosity (older designation Type IV); (b) medium viscosity (older designation Type VI) and (c) low viscosity (older designation Type VII).

Six-day-old mycelium (350 mg DMW) of the vegetative phase, 6-day-old mycelium (500 mg DMW) of the production phase and 11-day-old mycelium (750 mg DMW) of the production phase were mixed with 20 ml of 4% alginate of low viscosity.

Six-day-old mycelium (750 mg, 1000 mg or 1500 mg DMW) of the vegetative phase were immobilized in different amounts of 4% alginate of medium viscosity, so that the concentration of the fungus inside the gel beads remained constant (25 mg DMW/ml alginate).

Sterilization of the alginate took place at 120° C for 20 min. After cooling, the mycelium was mixed with alginate solution. The suspension was, with the help of a sterile pump system, added dropwise to 2% $CaCl_2$ solution and allowed to stand for 1 h. Low viscosity alginates (2% and 4%) formed spherical beads with an average diameter of 5 mm. The 2% alginates of medium or high viscosity formed identical spherical particles but a 4% concentration resulted in the formation of cylindrical particles with the same diameter. Immobilized mycelium was washed with 0.9% NaCl and put into the PM.

In semicontinuous fermentation, the beads were separated from the production medium every 10, 12 or 14 days, washed with 0.9% NaCl and transferred to fresh PM. Fermentations took place in 500-ml erlenmeyer flasks with 100 ml PM on a rotary shaker (220 rpm) at 24° C.

Analytical methods. The total alkaloid concentration was determined spectrophotometrically by means of the van Urk procedure (Agurell 1966a) directly from the filtered PM.

Individual alkaloids were determined from 2 ml filtered PM adjusted to pH 8 with ammonia; an extract was made with 4 ml chloroform. Two millilitres of this extract was dried under nitrogen and stored in the dark at −20° C. Thin layer chromatography took place on Merck G 60 (Merck, Darmstadt, FRG) silica gel plates with 85% chloroform and 15% methanol as the mobile phase.

For identification of particular alkaloids standard solutions of LA (Lek, Pharmaceutical and Chemical Works, Ljubljana, Yugoslavia), ALA (Lek), MCLA ("Boris Kidrič" Institute of Chemistry, Ljubljana, Yugoslavia) and EMT-maleate (WHO Centre for Chemical Reference Substances, Genève, Switzerland) were used. Since lysergic acid derivatives isomerize very readily under a variety of conditions (Floss 1976), standard solutions of EMT, MCLA and ALA were allowed to stand at pH 8 in a bright room for a couple of days to identify their iso-forms.

The chromatographic plates were scanned densitometrically by measurement of fluorescence at excitation 313 and cut-off filter by Camag TLC scanner II (Muttenz, Switzerland). The amount of a particular alkaloid was represented as the sum of its normal and iso-form.

Results

Claviceps paspali L-52 produced MCLA, EMT and ALA in mannitol-succinate production medium (Fig. 1). Sometimes traces of unidentified alkaloids were observed. Ergot alkaloid production began with MCLA, followed by EMT. ALA is not the real biosynthetic product of *C. paspali* (Agurell 1966b). It arises from chemical decomposition of MCLA and its quantity depends upon the fermentation conditions. In a 10-day fermentation, free *C. paspali* cells synthesized 49% MCLA, 48% EMT and 1% ALA (Fig. 2).

Immobilization in different types and concentrations of alginates

In preliminary experiments Na-alginates of high, medium and low viscosity were tested as immobil-

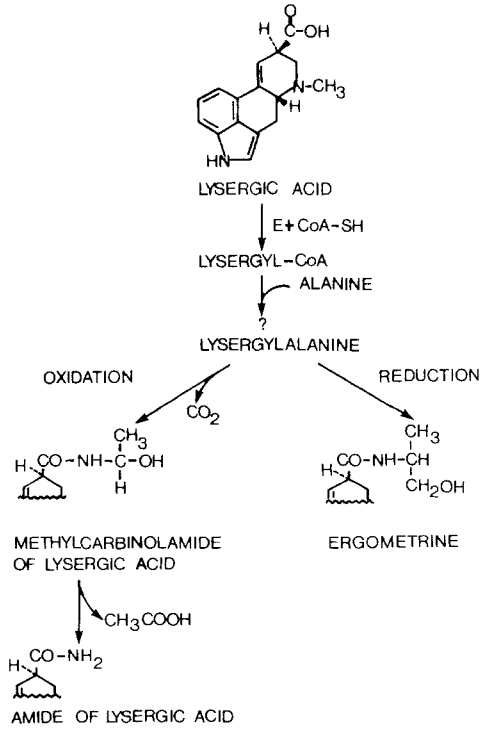

Fig. 1. The biosynthetic pathway from LA to its simple derivatives produced by *Claviceps paspali* (Agurell 1966b)

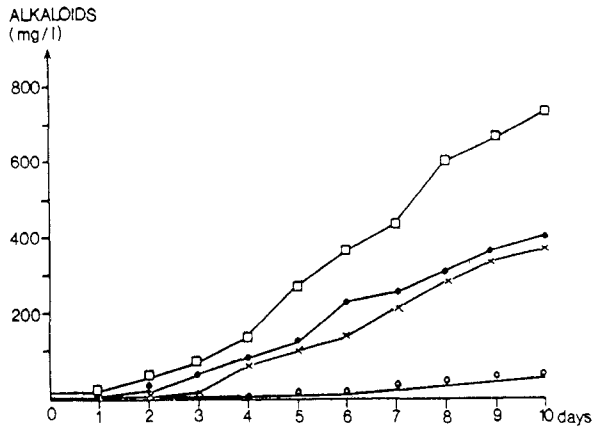

Fig. 2. Kinetics of alkaloid production in a 10-day fermentation with free *C. paspali* cells: □, total alkaloid amount; ●, amount of MCLA; ×, amount of EMT; ○, amount of ALA

ization agents in 2% and 4% concentrations with 6-day-old mycelium of *C. paspali* in the vegetative phase as an inoculum. Since growth of *C. paspali* hyphae inside alginate beads is limited (Pertot et al. 1988), a five times higher concentration of mycelium in comparison to fermentation with free cells was taken for immobilization (500 mg DMW/20 ml alginate per 100 ml PM). In all gel types and in both concentrations used, EMT synthesis represented more than 50% of the total alkaloid production (Fig. 3). The alginates of medium and low viscosity had better mechanical properties than that of high viscosity. They were used in further investigations since by using them

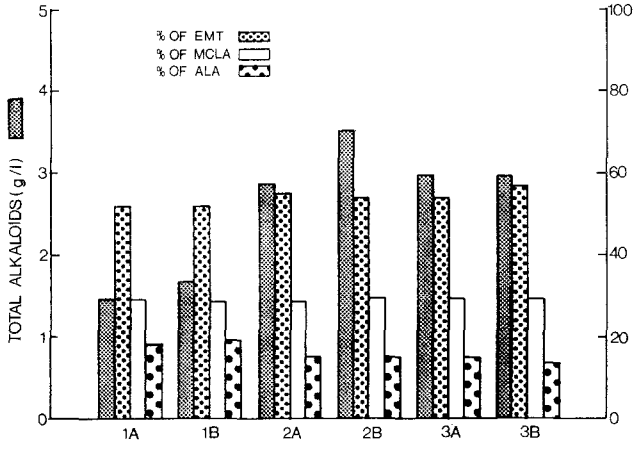

Fig. 3. Total alkaloid production and relative content of simple LA derivatives in a semicontinuous fermentation with *C. paspali* cells, immobilized in different types and concentrations of Na-alginate gel. *1A*, 2% alginate of high viscosity (Type IV); *1B*, 4% alginate of high viscosity (Tye IV); *2A*, 2% alginate of medium viscosity (Type VI); *2B*, 4% alginate of medium viscosity (Type VI); *3A*, 2% alginate of low viscosity (Type VII); *3B*, 4% alginate of low viscosity (Type VII). (*1A*, *1B*, after 36 days; *2A*, *2B*, *3A*, *3B*, after 60 days)

it was possible to make more reincubations and to obtain a greater cumulative amount of alkaloids.

Effect of the age of inoculum

According to our experience in fermentations with free *C. paspali* cells, three different ages of mycelium were used for immobilization: (1) 6-day-old mycelium of the vegetative phase, i.e. the mycelium which is commonly used as an inoculum in free-cell fermentations; (2) 6-day-old production mycelium (the mycelium of the middle production phase); (3) 11-day-old production mycelium (the mycelium of the late production phase). The concentrations of mycelia were chosen according to the growth curve and are described in Materials and methods.

In four independent experiments with four 10-day reincubations, the highest cumulative yield of alkaloids was, in all cases, observed with mycelium of the vegetative phase (Table 1). The relative content of ergometrine did not vary significantly with age of the inoculum.

Effect of the quantity of inoculum

The measurements of mannitol concentration after each cycle of semicontinuous fermentation, when 500 mg of 6-day-old vegetative *C. paspali* mycelium was used for immobilization, showed that nutrients in the production medium were not consumed completely (Pertot et al. 1988). Accordingly, higher amounts of inoculum were also tested: 750 mg, 1000 mg and 1500 mg (all DMW/10 ml PM) of *C. paspali* vegetative mycelium were

Table 1. Total alkaloid production and relative content of EMT in semicontinuous fermentation with *Claviceps paspali* mycelium at different growth phases immobilized in 4% alginate of low viscosity

	VEG		6P		11P	
	Alk (mg/l)	EMT (%)	Alk (mg/l)	EMT (%)	Alk (mg/l)	EMT (%)
	2670	56	2000	57	2500	59
	3130	55	2600	57	2840	56
	2330	57	1730	57	2140	59
	1800	58	1350	59	1670	57
Average	2480	56.5	1920	57	2290	57.7
(%)	100		77		92	

VEG, mycelium of the vegetative phase; 6P and 11P, 6- and 11-days-old mycelium in the production phase; Alk, total alkaloids

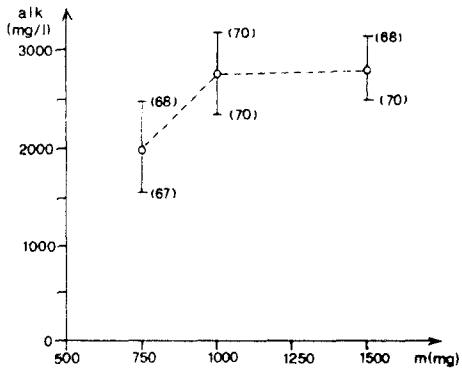

Fig. 4. The effect of the concentration of vegetative *C. paspali* mycelium (immobilized in low viscosity 4% alginate) on total alkaloid production. The *numbers* in the *brackets* indicate the percentage of EMT at the particular alkaloid yield

immobilized in different amounts of 4% alginate of medium viscosity so that the concentration of the fungus inside the gel remained constant (25 mg DWM/1 ml of alginate). In two experiments with four consecutive incubations, each taking 10 days, the cumulative yield of alkaloids showed a saturation curve (Fig. 4).

Effect of the length of a particular cycle on alkaloid production

Fermentations were carried out with 1000 mg of 6-day-old mycelium in the vegetative phase immobilized in 4% alginate of medium viscosity in 100 ml PM. By recycling every 10, 12 or 14 days it was possible to continue the fermentation up to 90, 96 or 98 days. The highest cumulative yield of alkaloids was observed in fermentation with 10-day cycles and the lowest with 12-day cycles (Fig. 5).

Figure 6 shows the average total alkaloid production and the relative content of particular alkaloids from five different fermentations with 10- and 14-day cycles. Two fermentations of 70 days (seven 10-day, or five 14-day cycles), two of 40–42 days (four 10-day or three 14-day cycles) and one of 28–30 days (three 10-day or two 14-day cycles) were carried out. It is evident that fermentations with 10-day cycles gave about 10% greater cumulative yields of alkaloids in the same time. The percentage of EMT was slightly higher in fermentations with 14-day cycles.

Fermentation when no fungus was found outside the gel beads

To prevent outgrowth of mycelium as far as possible, the lowest amount of vegetative mycelium

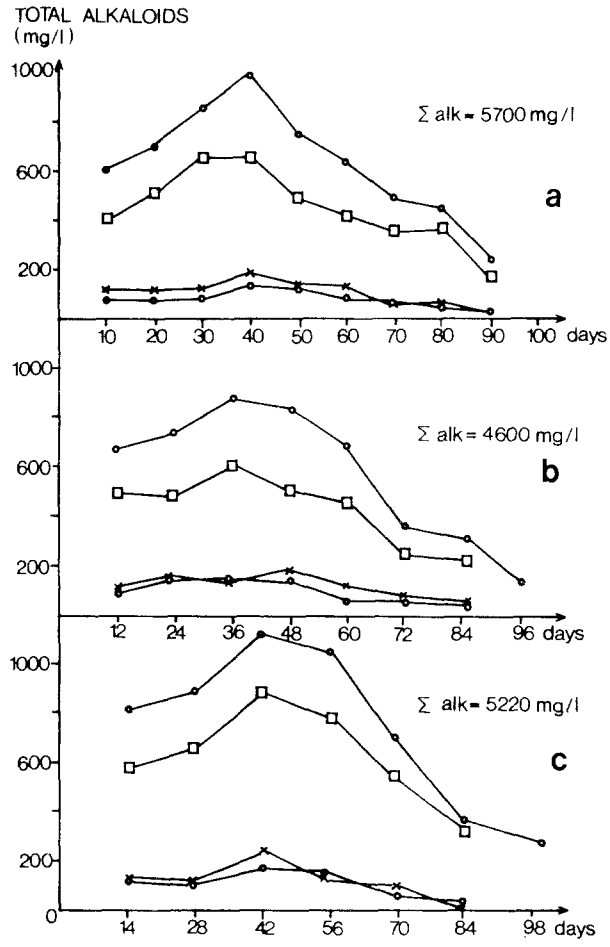

Fig. 5. Semicontinuous production of alkaloids with immobilized *C. paspali* cells by recycling every 10 **(a)**, 12 **(b)**, or 14 **(c)** days: ○, total alkaloids; □, EMT; ×, MCLA; ○, ALA; ∑alk = the sum of alkaloid production at the end of fermentation

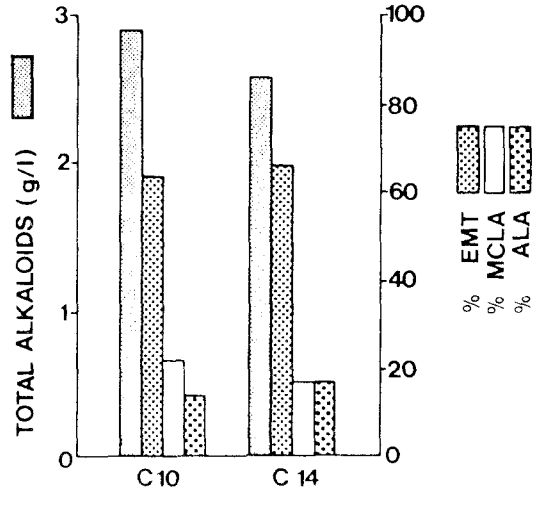

Fig. 6. The average result of five different fermentations with 10- and 14-day cycles: *C10*, recycling every 10 days; *C14*, recycling every 14 days

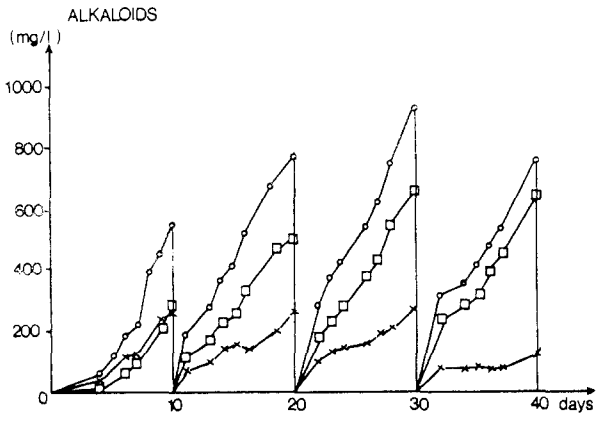

Fig. 7. Kinetics of alkaloid production with immobilized *C. paspali* cells under conditions with no fungus outside the gel beads: O, total alkaloids; □, EMT; ×, MCLA+ALA

(500 mg DWM/100 ml PM) was immobilized in 4% alginate of medium viscosity. Before the beads were embedded in production medium, they were washed several times with 0.85% NaCl solution. Only in the first four cycles was it possible to obtain an immobilized system without outgrowing hyphae. Results presented in Fig. 7 show that under these circumstances the amount of EMT increased in each cycle, from 52% of the total alkaloid yield in the first cycle, to 85% at the end of the fourth cycle.

Discussion

Data obtained in semicontinuous fermentation with immobilized *C. paspali* cells showed that the age and quantity of *C. paspali* mycelium, as well as the length of a particular cycle affected total alkaloid production, but dit not strongly influence the relative content of the lysergic acid derivatives EMT, MCLA (and ALA), which were between 1%–3%. Immobilization in alginates of medium and low viscosity enabled a long semicontinuous fermentation. The 2%–4% concentrations of alginate did not seem to affect the changes in the alkaloid spectrum, as found also for *C. fusiformis* cells (Kren et al. 1987), but not for *C. purpurea* cells (Kopp and Rehm 1983).

The results indicated that it was better to immobilize *C. paspali* mycelium during the growth phase. The alkaloid yield was on average 8% lower when 11-day-old mycelium in the production phase was used, and 23% lower when 6-day-old mycelium in the production phase was taken. The optimum quantity of inoculum should be between 750 mg and 1000 mg (DWM/100 ml PM) of *C. paspali* mycelium, since with higher amounts of mycelium (1500 mg DWM/100 ml PM) the amount of alkaloids no longer increased, probably because of nutrient limitations. Since the daily increase in alkaloids diminished during the last days of the production period, it was proposed that optimization of the semicontinuous fermentations with immobilized *C. fusiformis* cells should involve shortening the production cycle (Rozman et al. 1987). The normal production period in *C. paspali* fermentation lasts for 14 days. Recycling every 12 days gave worse results but recycling every 10 days resulted in a 10% greater cumulative alkaloid yield in comparison to fermentations with 14-day cycles.

While in free-cell fermentation MCLA synthesis prevailed and the content of EMT after 10 days never exceeded 50%, immobilized cells in the same period synthesized more than 50% EMT irrespective of the conditions. The impaired diffusion of oxygen through the alginate beads, by which biosynthetic reactions that need less molecular oxygen are preferred (Gosmann and Rehm 1986), could probably be the reason for improved EMT synthesis with immobilized *C. paspali* cells, since reduction is the key biosynthetic reaction from LA to EMT (Agurell 1966b).

The reason for the lower sensitivity of the alkaloid spectrum to the immobilization and cultivation conditions could lie in the fact that the alkaloid spectra of some fungi are relatively stable, as proposed by Kren et al. (1987). Our results offer another explanation. In each period some branches of outgrowing mycelia fragmented and were released into the medium, forming separate hyphae (Pertot et al. 1988). This small amount of free mycelium synthesized alkaloids in different proportions from immobilized cells and the alkaloid composition of the medium remained apparently unchanged. By establishing a system with only immobilized *C. paspali* cells (choosing a low mycelium/alginate ratio and with laborious washing of gel beads before embedding them into the new PM) it was possible to obtain a great increase in EMT production from 52% of the total alkaloid amount initially, to 85% at the end of the fourth cycle. Although the different fermentation conditions did not strongly affect the relative content of EMT, the increased total alkaloid amounts also represents an improvement in the absolute EMT yield.

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