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Quantitative Changes of the Alkaloid Complex in a Submerged Culture of *Claviceps paspali*

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ABSTRACT. *Claviceps paspali* FA produced high concentrations of alkaloid under submerged conditions. Their production was found to depend on the developmental stage and treatment of the filamentous culture inoculum. A medium containing Bacto-peptone with a constant composition of amino acids was selected for the preparation of the inoculum. A two-week fermentation in a synthetic medium with mannitol at 24 ± 1 °C resulted in an increased production of total alkaloids from the original value of 100–200 μg/mL to more than 2 000 μg/mL. Addition of tryptophan did not further increase the production of alkaloids but resulted in changes of the spectrum of some metabolites. 2,3-Dihydroxybenzoic acid accompanied the alkaloids in the fermentation medium. α-Hydroxyethyllysergamide was the predominant component of extracellular alkaloids (80 % in the first days of fermentation). During fermentation the level of this alkaloid continuously decreased while the concentration of the accompanying alkaloids, *i.e.* lysergamide and the corresponding minor isomers, increased.

It follows from a literature review of fermentation processes utilizing *C. paspali* strains for the preparation of lysergic acid derivatives that individual cultures are highly variable and that the procedures used are nonuniform. It was the aim of the present work to choose a suitable saprophytic strain of *Claviceps paspali*, increase the production of alkaloids and follow the changes of their spectrum during the fermentation process.

MATERIALS AND METHODS

Microorganisms. Saprophytic *Claviceps paspali* cultures maintained in the Collection of the Institute of Microbiology, Czechoslovak Academy of Sciences, were used. The stock culture of *C. paspali* FA (CCM F-731) maintained on a solid Sabouraud medium at 4 °C was transferred every six months.

Media and cultivation. Vegetative inoculum (1st fermentation stage) was prepared on Sabouraud's medium (Burton 1949) containing (g/L): peptone 10, glucose 40, malt extract Difco 26, agar 20, tap water; pH 5.8 after sterilization (25 min, 100 kPa). Unless otherwise stated, the medium was inoculated with an aqueous suspension of the culture grown for two or three weeks at 24 °C on Sabouraud's agar slants. Flasks (500 mL containing 80 mL of medium) were incubated on a rotary shaker (frequency 3.7 Hz, amplitude 70 mm) at 24 °C in the dark.

Fermentation medium (2nd fermentation stage) contained 3 % mannitol (MP) or glucose (GP) and a basic solution of the following composition: succinic acid 30, propylene glycol 30, KH_2PO_4 1, $MgSO_4.7H_2O$ 0.3, tap water. Prior to sterilization

(35 min, 100 kPa) the pH of the medium was adjusted to 5.2—5.3 with ammonia. Salt solution (P) containing (mg/L) $Ca(NO_3)_2.4H_2O$ 30, $ZnSO_4.7H_2O$ 5, $CuSO_4.5H_2O$ 5, $MnSO_4.4H_2O$ 10, $NaNO_3$ 100, was added after sterilization by filtration. $FeSO_4.7H_2O$ (50 mg/L) was added separately after sterilization (35 min, 100 kPa).

The dry mass of the mycelium was determined gravimetrically in 3- or 5-mL samples, washed with distilled water and dried to constant mass (105 °C, 18 h).

Analytical methods. Alkaloids were analyzed after chromatographic separation of samples in butanol—acetic acid—water (4 : 1 : 5) (Hais and Macek 1954), their fluorescence was followed at 254 and 366 nm (Hanovia lamp) and their presence was further verified by detection with ninhydrin, 4-dimethylaminocinnamaldehyde and Draggendorf's reagent (Hais and Macek 1954; Stahl 1969). Total alkaloids were determined colorimetrically with 4-dimethylaminobenzaldehyde using the method of Allport and Cocking (1932). Ergometrin maleate served as standard. Compounds were separated by means of HPLC (Varian LC 8500) equipped with a device for gradient elution and UV detector Variscan LC (Varian Aerograph, Walnut Creek, CA, USA) at 310 nm (Wurst *et al.* 1978). During gradient elution the initial concentration of ethanol (3 % in diethyl ether) was increased by 1 % in 1 min. The analysis was terminated in 18 min at a solvent ratio of 79 : 21. The individual components of the mixture of alkaloids were analyzed qualitatively by comparing their elution volumes with those of standard compounds obtained by preparative liquid chromatography of a given mixture. The structure of the compounds was verified by mass and ^{13}C-NMR spectroscopy. A Varian CDS 111C integrator was used to determine individual alkaloids in the mixture.

Total phosphorus was determined colorimetrically using the method of Berenblum and Chain (1938) as modified by Liebl and Bass (1958). Succinic acid was followed by descending chromatography on Whatman paper no. 1. Samples (about 500 μg in 20 μL water) were developed for 14 h at room temperature in 1-pentanol—5 M formic acid (1 : 1) and detected with bromocresol green. Standards (mannitol, glucose, fructose) were applied as a 1 % solution in 10 μL aliquots on Whatman paper no. 1, developed three times in 1-butanol—acetic acid—water (4 : 1 : 5) (Hais and Macek 1954) and detected according to Green and Stone (1952). The values of the reducing compounds were related to glucose (R_{Glc} of mannitol was 1.2).

Microscopy. Cultures were examined in a light microscope (Zeiss, type Nf) and in a scanning electron microscope (Jeol, type JSM S 1). Preparations stained with Sudan III were obtained by adding the dye (0.1 g in glycerol—ethanol) to mycelial suspension on a slide and inspected after 5 min.

RESULTS AND DISCUSSION

Evaluation of strains

It followed from the comparison of 10 strains of *C. paspali* cultivated under relatively identical submerged conditions in a medium with propylene glycol (Gröger and Tyler 1963; Tyler 1965) that individual strains differ in their ability to produce compounds reacting with 4-dimethylaminobenzaldehyde. This reaction was used to compare the production of total extracellular alkaloids. The intensity of the reaction was proportional to the intensity, occasionally to the number of UV-fluorescent spots detected after chromatographic separation of samples. Of the several strains that differed (Řičicová and Cudlín 1969) morphologically, the strain *C. paspali* FA was chosen. In submerged conditions, this strain produced compounds reacting intensively with 4-dimethylaminobenzaldehyde.

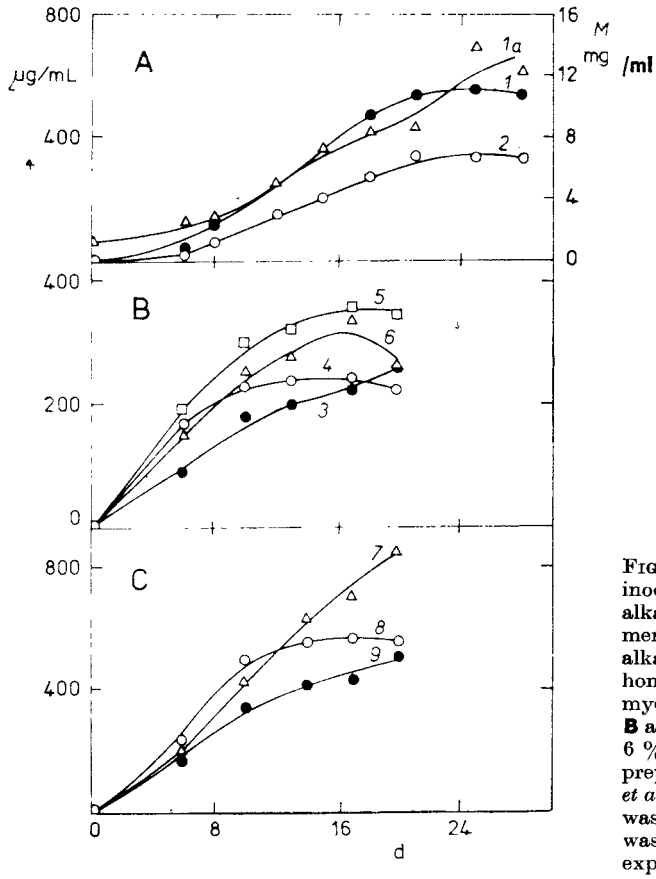

FIG. 1. Effect of treatment of the inoculum on total concentration of alkaloids in *C. paspali* FA (2nd fermentation stage, medium MP). Total alkaloids (µg/mL) after inoculation: **A** homogenized (*1*) and non-homogenized (*2*) mycelium; *M* dry mass (mg/mL, *curve 1a*); **B** amount of inoculum 1 % (*3*), 3.5 % (*4*), 6 % (*5*) and 12 % (*6*); stock culture prepared in the medium of Kobel (Kobel *et al.* 1964); **C** inoculation with mycelium washed once (*7*), twice (*8*) and non-washed (*9*); results of three independent experiments.

Increase of total alkaloid production by variation of the fermentation process

Effect of initial pH of the medium on growth of *C. paspali* FA under submerged conditions. The temperature of 24 °C was found to be suitable for the growth. The optimal pH for cultivation of strains of the genus *Claviceps* is usually described to lie within a wide range: 3.0—5.0 and 5.7—7.0 (Windisch and Bronn 1960), 5.2—5.4 (Taber and Vining 1957*b*), 5.0—6.0 (Abe 1946 cited from Vining and Taber 1963). The optimum growth was observed here between pH 5.4 and 6.8. With respect to the preconditioning effect of the stock culture obtained from the solid medium on further development of the strain it followed that the initial pH of 5.6—6.2 was most favourable for growth of the culture on Sabouraud's medium.

Morphological character and treatment of the submerged inoculum. Macroscopically visible multiplication of the culture, *i.e.* formation of colonies with fine filaments, occurred after cultivating for 2—4 d. The mycelium consisted of regularly thick filaments without septa, with round ends (Plate 1A, B). The picture from the scanning electron microscope revealed filaments of a 5-d-old submerged mycelium with only sparsely occurring intercalar cells (Plate 2A, B). Distinct disperse lipophilic elements could be observed in mycelium older than 5 d. After about 9 d these elements were stained red, orange or yellow with Sudan and formed structures of uneven size (Plate 1C, D).

Filaments were terminated by short, often thin protrusions (Plate 1E). The number and volume of vacuoles in the mycelium gradually increased. Compounds reacting with 4-dimethylaminobenzaldehyde were not released into the cultivation medium. No diffusing pigment was produced.

The mycelium of the submerged inoculum was not homogenous (Plate 2A, B), it was formed by colonies of uneven diameter and by released particles of filaments. Due to this, the distribution of the inoculum in the subsequent cultivation stage was uneven and the culture developed differently in individual flasks. Uniform growth of the culture in the subsequent fermentation stage in the MP medium was reached by homogenization of the mycelium for ± 5 s in an MSE homogenizer (Measuring and Scientific Equipment Ltd., England) adapted for sterile work (Workshops of the Institute of Microbiology, Czechoslovak Academy of Sciences, Plate 2C). The release of filaments from the clusters of colonies extended the area of contact of the mycelium with substrate. The curve of dry mass of the mycelium (Fig. 1A) was of continuous character with a maximum after a 25-d cultivation, similarly to that in flasks inoculated with a nonhomogeneous inoculum. This treatment also increased the production of extracellular compounds reacting with 4-dimethylbenzaldehyde (Fig. 1A).

Literature data showed that views concerning the effect of homogenization of mycelium on the production of alkaloids differ considerably. Taber and Vining (1959) accelerated the production of alkaloids during stationary cultivation of *C. purpurea* PRL 1578 by homogenization, Arcamone *et al.* (1961) obtained reproducible yields with *C. paspali* F; likewise, Pacifici *et al.* (1962) eliminated the nonuniformity of the inoculum from agar slants in the isolate *C. paspali* C-60 by homogenization. However, Gröger and Tyler (1963) described a decreased concentration of total alkaloids after homogenization of the inoculum of *C. paspali* Li 189. Even when using the homogenized mycelium it is necessary to work out an inoculation procedure for every individual culture (Dorrell and Page 1947). The favorable effect of homogenization on the production of alkaloids is probably associated with the character of the used strain: *e.g.*, homogenization of a 4-d inoculum of C. species 47 did not exhibit any favorable effect on the alkaloid yields. As compared with *C. paspali* FA, whose growth curve, after inoculation with homogenized inoculum, had an increasing trend in parallel with the production of alkaloids up to 25 d, in the strain 47 A maximum growth occurred after 14 d when using homogenized inoculum and after 10 d with the nonhomogenized inoculum. The production of total alkaloids was of two-phase character with a decrease after a 14-d fermentation. *C. paspali* 47 A differed from *C. paspali* FA in the formation of conidia.

When inoculating flasks with the submerged mycelium of *C. paspali* FA we introduced various amounts of the inoculum into the subsequent fermentation stage, depending on growth intensity of the inoculum (Fig. 1B). When using inoculum of the same age at a concentration of 1 % (29.2 mg dry mass), 3.5 % (87.6 mg), 6 % (146 mg) and 12 % (292 mg) per flask optimal results were obtained with 6 % inoculum. With increasing concentration of the inoculum (to 12—20 % = 290 to 380 mg) the maximum production of alkaloids was lower. Even on inoculation with a very small amount of inoculum (0.5 mg per flask), with respect to data presented in Fig. 1A, (flasks inoculated with 95.8 mg) the maximum concentration of alkaloids was decreased to only 470 μg/mL and the maximum dry mass of the mycelium to 12.8 mg/mL. In spite of the fact that the amount of homogenized mycelium could be reduced to a very low level under the experimental conditions described, we used a 6 % inoculum with reproducible results. When using this

TABLE I. Effect of washing of the mycelium on growth of *C. paspali* FA in the 2nd fermentation stage (MP)

Cultivation	Dry mass of the mycelium (mg/mL)		
	inoculum washed		control
	1×	2×	
0	0.49	0.91	0.67
10	7.0	9.28	11.01
14	10.26	12.5	15.4
17	7.35	12.31	12.34

inoculum, maximum growth of the culture was reached and the variability of the results caused by a small volume of the inoculum was simultaneously eliminated (Taber and Vining 1957*a*).

The maximum production of alkaloids (480—430 μg/mL after a 20-d cultivation) did not substantially differ in cultures inoculated with a 3-d inoculum as compared with an older inoculum (7-d). The older the inoculum used, the shorter the lag phase in the subsequent 2nd fermentation stage and, thus, the sooner (although even lower) the maximum concentration of total alkaloids. Inoculum older than 7 d was not suitable for the fermentation — the maximum yields decreased to 350 μg/mL. With respect to the amount of the introduced mycelium, yields of total alkaloids were higher in flasks inoculated with a younger mycelium (3—5 d), which is also of practical importance.

In the submerged conditions of the 2nd fermentation stage the alkaloid production was associated with pigmentation. The older the vegetation mycelium used for inoculation, the more intense production of a diffuse yellow-brown pigment. The pigmentation gradually increased both in the mycelium and the fermentation liquid. Washing of the mycelium with distilled water resulted in a visible decrease of the pigmentation intensity in the subsequent stage and in a simultaneous increase of the production of total alkaloids (Fig. 1C). Washing of the mycelium before or after its homogenization did not exhibit a pronounced effect on the production of alkaloids. A single-step washing was more suitable than a repeated one. The growth of washed cultures was less intense than that of non-washed ones (Table I). The relationship between growth intensity of the submerged culture and alkaloid and pigment production is inverse.

Washing of a 7-d mycelium of *C. paspali* FA stimulated and prolonged the phase of alkaloid production (with a maximum of 1500 μg/mL MPP fermentation liquid) at a lower level of 2,3-dihydroxybenzoic acid (with a maximum of 700 μg/mL) measured colorimetrically according to Arnow (1937). Its presence in the medium was demonstrated by chromatographic analysis (compound of R_F 0.8—0.9; Bate-Smith and Westall 1950). On inoculation with the non-washed mycelium the same maximum values of the investigated metabolites were obtained (about 1200 μg/mL). The demonstration of 2,3-dihydroxybenzoic acid in the fermentation liquid confirms the identity of the strain *C. paspali* FA.

On the basis of the above results the fermentation medium was inoculated with 6 % of a 4—5 day inoculum washed with distilled water and homogenized for 5 s.

Stability of the production activity of C. paspali *FA*

The production activity of the culture did not substantially change on storage for 8 weeks on Sabouraud's solid medium at 4 °C. However, it decreased by 50 %

TABLE II. Color of ninhydrin-positive compounds in *C. paspali* FA filtrate

Compound[a]	Medium	
R_F	MP[b]	MPP[c]
0.13[d]	violet	0
0.18	violet	0
0.23	yellow	yellow
0.29	grey-pink	grey-pink
0.42	violet	violet
0.51	violet	violet
0.63	violet	violet

[a] Separation in the system butanol—acetic acid—water (4 : 1 : 5).
[b] Total alkaloids: 635 μg/mL.
[c] Total alkaloids: 910 μg/mL.
[d] It corresponds to glutamine.

after 15 weeks. In a long-maintained culture (4 °C) recovery of growth and metabolic activity was reached after a two- to three-fold transfer of the culture at short three-week intervals.

Fermentation in the medium with mannitol

Glucose, fructose, sucrose, maltose, mannitol and glucitol were suitable carbon sources for growth of *C. paspali* FA under submerged conditions in Tyler's medium containing propylene glycol. The medium with mannitol (MP) was used to evaluate the properties of the vegetative inoculum with respect to the highest possible concentration of total alkaloids. Tyler (1965) described a temperature range of 22—28 °C for the submerged fermentation of *C. paspali* ATCC 14988, Windisch *et al.* (1960) demonstrated values of 25—30 °C for *Claviceps* sp. Under the conditions used in the present work cultivation temperature above 25 °C influenced unfavorably the production of alkaloids: at 24 °C the maximum concentration was 640 μg/mL, at

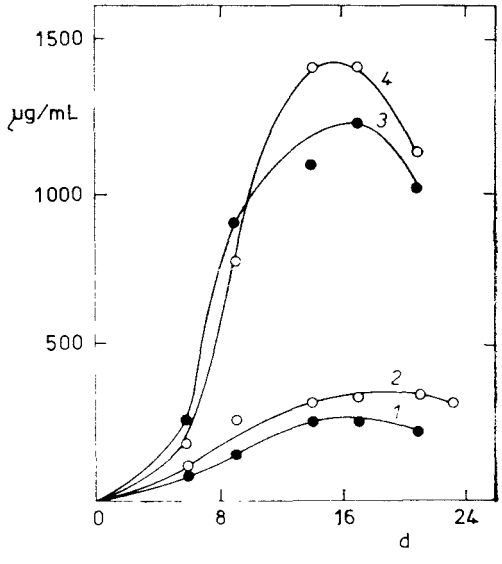

FIG. 2. Effect of inorganic salts on the concentration of total alkaloids in the fermentation liquid of *C. paspali* FA; total alkaloids (μg/mL) in MP (*1*, *2*), MPPFe (*3*, *4*), distilled water (*1*, *3*), and tap water (*2*, *4*).

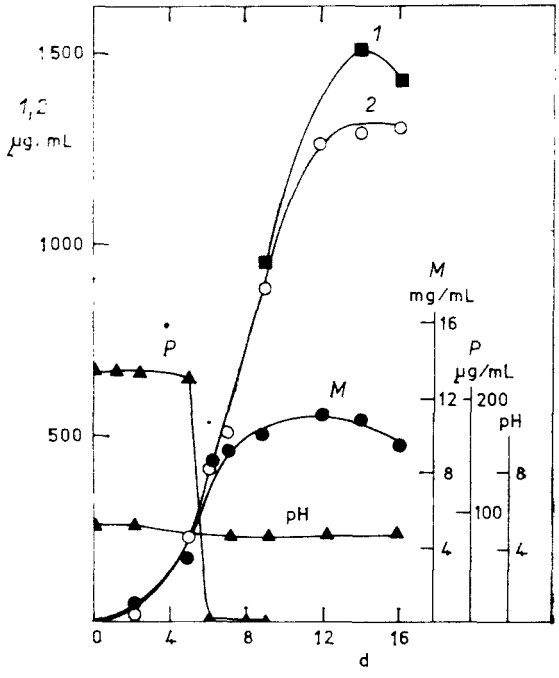

FIG. 3. Changes in the medium containing mannito (MPPFe) during fermentation of *C. paspali* FA total alkaloids (µg/mL) in MPP (*1*), MPPFe (*2*) inorganic phosphate (*P*, µg/mL), dry mass (*M* mg/mL) and pH.

28 °C only 100 µg/mL. The three-week developmental phase of the strain FA in the MP medium was followed and concentration of alkaloids of about 300—400 µg/mL was reached (Fig. 1A, curve 2), dry mass of the mycelium increasing continuously up to 20-d cultivation. According to chromatographic analysis mannitol was present in the medium during the whole period. After 12 d further reducing compounds of R_{Glc} 1.48, 1.72, 3.25 and 4.57, demonstrated by means of the method of Green and Stone (1952) occurred in the medium. In the reaction with silver nitrate the spot of R_{Glc} 4.57 reacted more rapidly than other compounds. Exhaustion of mannitol from the medium after 20 d was associated with a sharp increase of total alkaloid content. Succinic acid was present in the medium during the whole fermentation and influenced the pH (Taber and Siepmann 1966) which lay between 5.0 and 5.5 (Fig. 3, 4). Taber and Vining (1960) demonstrated the participation of succinic acid in the metabolism of producing and non-producing *Claviceps* strains, Taber (1968) verified it by radioisotope experiments.

During fermentation in the MP medium glutamine was demonstrated as one of the ninhydrin-positive nonfluorescing compounds (Table II). This metabolite, known to occur widely not only in microorganisms (Elliott 1953; Mothes 1965), was present from the 7th day of cultivation at a relatively low concentration of total alkaloids (330 µg/mL). It was not detected after increase of the alkaloid production up to 770 µg/mL (21 d) and, thus, its favorable effect on biosynthesis of these compounds can be assumed.

By adding a mixture of inorganic salts in the modification of Rosazza *et al.* (1967) to the basic medium containing mannitol (MPP) it was possible to increase significantly the yield of alkaloids without a proportional increase of growth intensity of the culture (Fig. 1A, 2, 3, 4). The favorable effect of tap water on the production of alkaloids cannot be attributed to the influence of iron as the presence of ferrous

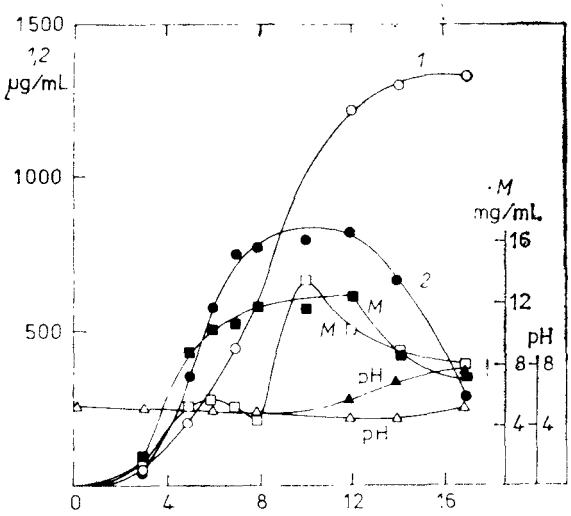

FIG. 4. Submerged cultivation of *C. paspali* FA in media containing glucose (GPP, *closed symbols*) and mannitol (MPP, *open symbols*); total alkaloids (µg/mL, *1* and 2), dry mass (*M*, mg/mL) and pH.

sulphate (50 mg/L in MPPFe) decreased the production of alkaloids (Figs. 2, 3). Mannitol was present only up to 9 d and disappeared when growth of the culture reached its maximum. Beginning with the 7th day compounds reducing ammoniacal silver solution, of R_{Glc} 1.46, 1.72, 3.78 and 4.66, corresponding to compounds demonstrated in the medium MP according to chromatographic analysis, occurred in the medium. The concentration of these compounds gradually increased from the 12th day, (*i.e.* after the period when the maximum dry mass was reached and the level of alkaloids increased.

Development in the medium with mannitol was associated with the production of a diffuse pigment, which was yellowish at the beginning and clearly violet from the 8th day of cultivation. The pigment occurred in the medium after exhaustion of inorganic phosphate (*i.e.* after 6—8 d), before the maximum production of alkaloids (Fig. 3). Inorganic salts did not influence the rate of disappearance of the pigment. Conditions of the pigment production can be compared with those of Rosazza *et al.* (1967), who described a violet pigment in other *C. paspali* strains cultivated in media containing less than 900 mg inorganic phosphate in 1 L. The production of both the intra- and extracellular pigment correlated with the presence of iron. The pigmentation was weaker when ferrous sulphate was replaced with ferrous chloride or an equivalent quantity of sulphuric acid. The growth curve of the culture was continuous in the presence of ferrous sulphate. The favourable effect on the production of alkaloids occurred already before the 10th day of fermentation. During subsequent days the concentration of total alkaloids in the presence of the pigment decreased (Fig. 3). Alkaloids were not bound to the pigment. They could be separated from the pigment by extraction with dichloromethane or with another solvent at alkaline pH (Voigt and Wichmann 1961). After a three-fold extraction the pigment remained in the aqueous phase. It was insoluble in methanol, ethanol, propanol, butanol, chloroform, acetone, benzene, ethyl acetate, diethyl ether, petroleum ether and dichloromethane.

TABLE III. Effect of peptone in the inoculation medium on total alkaloid production in *C. paspali* FA[a]

Peptone type	Content, %		Introduced inoculum[b]	Total alkaloids μg/mL in medium[c]		
	Trp	Tyr		MP	MPPFe	MPP
Bacto-peptone Difco	0.29	0.98	24.5	—	2240 (12)	2280 (12)
			33.6	1275 (20)	1970 (12)	2440 (12)
Bacto-peptone Spofa	—	—	33.5	360 (12)	1235 (20)	1000 (15)
			27.3	460 (15)	1320 (20)	1080 (20)
Proteose-peptone	0.51	2.51	14.8	—	1899 (15)	2000 (12)
			36.15	—	2050 (12)	2000 (12)
Bacto-peptone	1.03	2.99	19.65	1190 (20)	1965 (12)	2050 (12)

[a] Flasks were inoculated with a homogenized suspension from agar slant.
[b] Dry mass, mg per flask.
[c] Day of maximum production is shown in parentheses.

Effect of glucose on the production of total alkaloids

It follows from a comparison of the production of alkaloids in *C. paspali* FA cultivated in a medium containing mannitol (MPP) with that in a medium containing glucose (GPP) that the production of alkaloids on mannitol reached 1300 μg/mL, whereas on glucose it was only 850 μg/mL, both after a 12-d cultivation (Fig. 4). Development in the presence of glucose was faster; after 12 d a rapid decrease of alkaloids occurred. The maximum dry mass of mycelium was lower. The initial growth of the culture on mannitol was considerably less intense than on glucose but it became more intense after 10 d and was associated with an increased production of alkaloids. The phase of alkaloid production was longer in the medium containing mannitol. The maximum production of alkaloids was reached after 14 d and the production did not decrease but its intensity remained stable up to 17 d. Glucose also influenced culture pigmentation: after 6 d a yellow-brown pigment occurred, after 8 d the mycelium formed black-brown pellets.

Utilization of mannitol and glucose under submerged conditions was followed in identical nutritional conditions, in contrast to the paper of Brar *et al.* (1968) who cultivated *C. paspali* TA-1 in an ammonium-succinate medium containing glucitol (initial pH 5.4) and in a complex organic medium containing glucose (initial pH 6.5). Composition of both media differed qualitatively from the medium used in the present work. On the basis of growth ability of *C. paspali* FA (carbon utilization test) on solid medium (Oxoid Manual 1961) mannitol was used for the submerged fermentation. It was found that this strain utilizes both glucose and mannitol for growth better than glucitol does. Brar *et al.* (1968) obtained identical results with the TA-1 strain cultivated in a semisynthetic medium under submerged conditions. However, after a 16-d cultivation, they observed substantially smaller differences between the production of alkaloids on mannitol (above 600 μg/mL) and on glucose (500 μg/mL).

Brar *et al.* (1968) characterized the production of alkaloids under various nutritional conditions regulating the growth of *C. paspali* TA-1. Alkaloids were produced both after termination of total biomass production (pattern 1) and during slow biomass production (pattern 2). Under cultivation conditions used here the value of total alkaloids in the MP medium increased simultaneously with the increasing amount of biomass (Fig. IA) corresponding to characteristics of "pattern 2". In the

TABLE IV. Concentration of alkaloids (%) during submerged cultivation of *C. paspali* FA as analyzed by HPLC

Band	Cultivation, d				Alkaloids
	6	10	14	17	
—	—	—	0.66	0.97	—
1	6.12	7.9	11.38	15.44	α-Hydroxyethylisolysergamide
2	11.47	15.62	19.27	24.14	C_{α}-epimer of α-hydroxyethylisolysergamide
3	7.12	7.44	7.05	10.0	isolysergamide
—	—	—	0.02—0.05	—	—
4	42.44	33.56	28.0	23.53	α-Hydroxyethyllysergamide
5	22.31	27.14	22.49	19.0	lysergamide
6	10.52	7.57	8.54	0.25	C_{α}-epimer of α-hydroxyethyllysergamide
—	490	990	1210	970	total alkaloids, μg/mL

MPP medium the maximum amount of total alkaloids followed the maximum accumulation of biomass (Fig. 3, 4) corresponding to characteristics of "pattern 1". On cultivation in the medium containing glucose (GPP), during which the level of alkaloids was substantially lower, the increase of biomass and total alkaloid production, their maxima and their decrease were simultaneous, corresponding to "pattern 2" (Fig. 4). It follows from the above relationships that the biosynthetic ability of alkaloid production in *C. paspali* FA can be regulated by the carbon source and that the production of alkaloids can be directed either according to "pattern 1" or "pattern 2".

Effect of tryptophan

Peptone was the basic nitrogen source in the Sabouraud's medium used for the preparation of inoculum. Peptone may contain various tryptophan concentrations (Difco Manual 1953) and thus influence production of alkaloids, both as a precursor and as inducer of their biosynthesis. Therefore, different available peptones were tested, and, irrespective of minor differences in the yield of alkaloids, the maximal production of 2400 μg/mL was reached on Bacto-peptone Difco (Table III). The difference in the content of tryptophan in the peptones tested (0.29—1.03 %) was not significantly reflected in the production of total alkaloids. At the beginning of fermentation in the production medium MPP free tryptophan and 5-hydroxytryptophan could not be detected after 3—4 d. It can be assumed that tryptophan was converted to 2,3-dihydroxybenzoic acid (Gröger and Erge 1964) corresponding, according to chromatographic analysis, to a fluorescing spot of R_F 0.8—0.9 in the experiments referred to here.

After the addition of tryptophan (500 mg/L) to the medium MP this compound was not utilized up to 16 d, the production of alkaloids in the control culture did not exceeded 500 μg/mL. In the MPP medium tryptophan was present up to 9 d and did not occur in the medium after 12 d. The curve of total alkaloids was considerably lower after 10 d. Chromatographic analysis of filtrates of fermentation liquid after a 12-d cultivation of the strain with added tryptophan revealed a pronounced fluorescence of spots R_F 0.48 and 0.58, the spot of R_F 0.8—0.9 could not be demonstrated. Tryptophan suppressed growth of the culture and stimulated the production of the yellow-brown pigment.

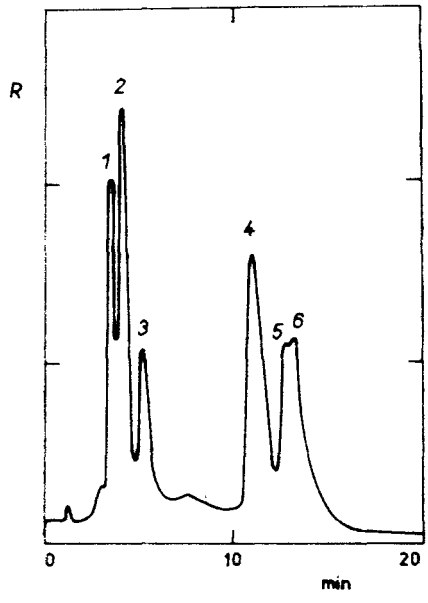

FIG. 5. Spectrum of alkaloids produced by *C. paspali* FA in MPP medium analyzed by HPLC; *1* α-hydroxyethylisolysergamide, *2* C_α-epimer of α-hydroxyethylisolysergamide, *3* isolysergamide, *4* α-hydroxyethyllysergamide, *5* lysergamide, *6* C_α-epimer of α-hydroxyethyllysergamide; *R* detector response.

Changes in the composition of alkaloids released into the MPP medium during fermentation

Adjustment of fermentation conditions led to the maximum concentration of total alkaloids of above 2 000 μg/mL after a 14—16-d cultivation. In the filtrate of a 3-d culture, analyzed chromatographically in butanol-acetic acid-water, two blue-fluorescing compounds of R_F 0.48—0.51 and 0.8—0.9, that could be discriminated according to fluorescence hue at 254 nm, were demonstrated. After 5—6 d the number of fluorescing spots increased to four: R_F 0.39, 0.48, 0.58 and 0.86. The spot with R_F 0.48 exhibited the most striking fluorescence. It was identified as α-hydroxyethyllysergamide according to a comparison with the standard. The spot of isolysergamide also migrated within the above range (R_F 0.47—0.48). The presence of lysergamide could not be demonstrated (except for a single rare case).

Therefore, HPLC was used for the accurate analysis of alkaloids. Six compounds produced at measurable concentrations during fermentation could be demonstrated in the filtrate (Fig. 5). Changes in concentration of these compounds up to a 14-d cultivation are shown in the record of liquid chromatograph (Table IV). The analyzed compounds represented at concentrations of 1—80 % of total alkaloids form two groups of compounds with close values of retention time: lysergic acid derivatives (Fig. 5, *bands 4, 5, 6*) and isolysergic acid derivatives (*bands 1, 2, 3*). By means of mass spectrometry and ^{13}C-NMR spectroscopy isolated of these compounds were identified as α-hydroxyethyllysergamide (*band 4*) and its isomers (*bands 1, 2, 6*) and further as lysergamide (*band 5*) and its isomer (*band 3*). Stereoisomers (*bands 2, 6*) differed in the absolute configuration (*R* or *S*) on C of the side chain (Arcamone *et al.* 1967). α-Hydroxyethyllysergamide, the concentration of which decreased from 80 to 20 % of total alkaloids during 3—22 d (Table IV), was the main component of the compounds produced. In a 15-d culture with a concentration of 1250 μg/mL the main product represented 45 % of the total content of alkaloids. Even under nonsuitable

cultivation conditions at 28 °C, at a low level of alkaloids (250 μg/mL), α-hydroxyethyllysergamide (22 %) and its isomer (20 %) represented the main components of the alkaloid mixture produced.

It follows from the above analyses that this amide was the basic product of lysergic acid at the beginning of fermentation. Due to its instability in aqueous solution it was degraded under submerged conditions (*see also* Kleinerová and Kybal 1972), as reflected by the increasing proportion of its isomer (*band 1*) which did not exceed 15 % of total alkaloids at the end of fermentation. The maximum amount of lysergamide in the fermentation liquid was lower than 30 %, the concentration of its isomer was always lower. Concentration of lysergamide at the end of the fermentation process was always lower than one-half of that of α-hydroxyethyllysergamide. It is likely that during paper chromatographic analysis lysergamide was degraded or converted to isolysergamide.

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The plates will be found at the end of the issue.

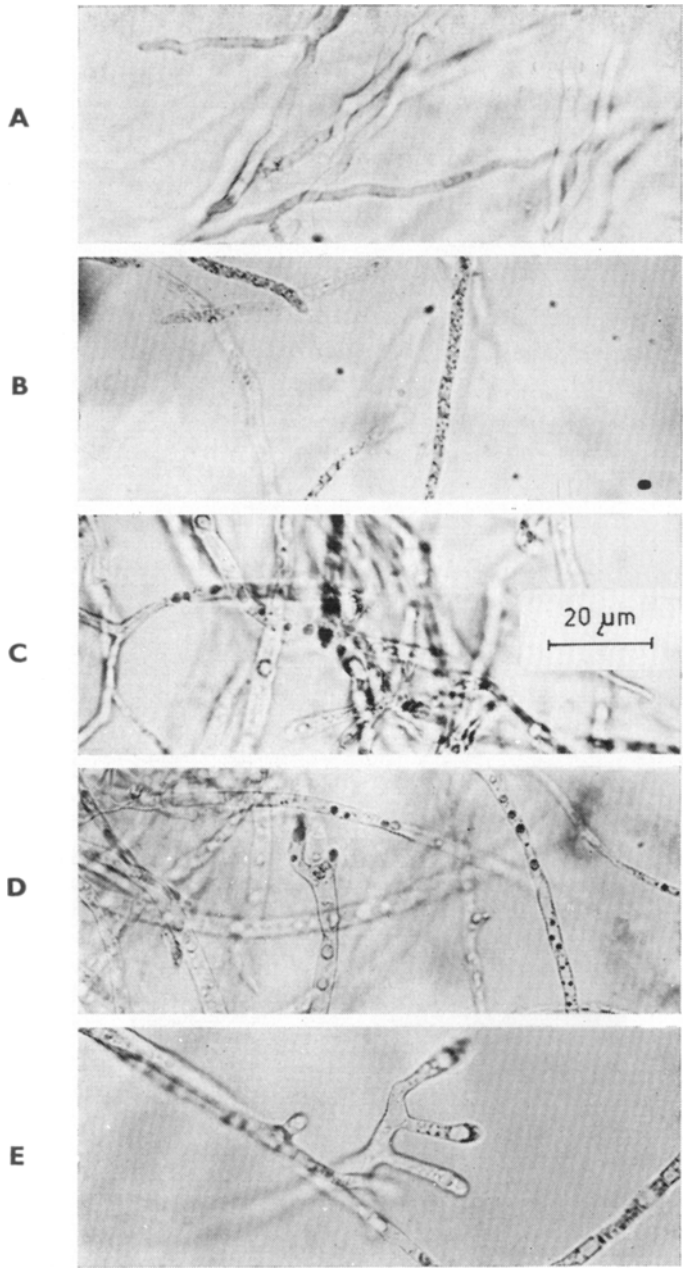

PLATE 1. Submerged mycelium of *C. paspali* FA on Sabouraud's medium (24 °C); A 2 d, B 4 d, C 7 d, D, E 10 d; Sudan B, C, D.

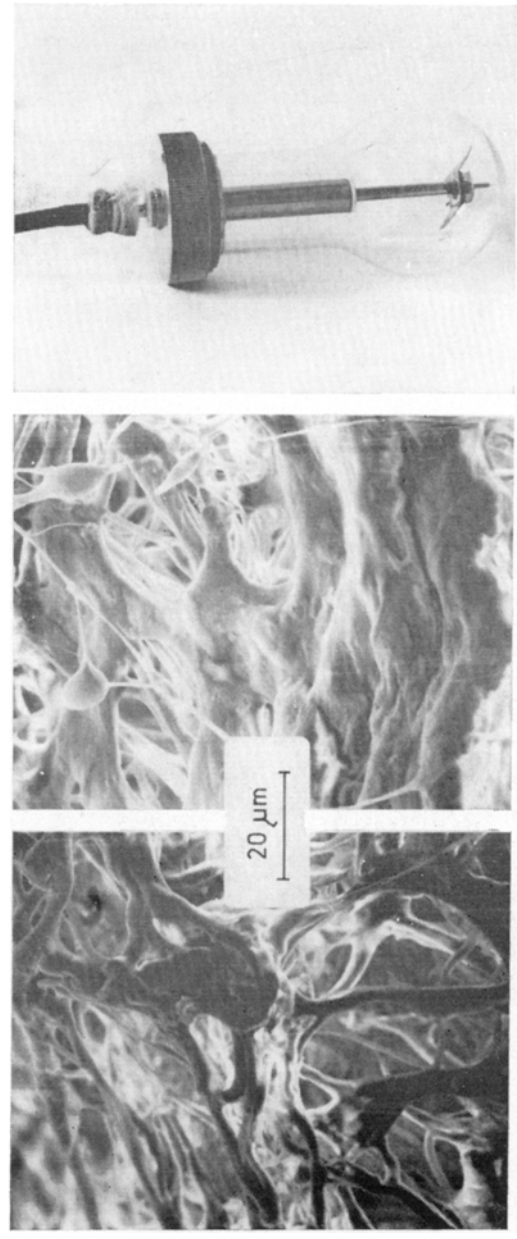

PLATE 2. A, B: submerged mycelium of *C. paspali* FA in scanning electron microscopy (24 °C, 5 d); C: MSE-homogenizer adapted for sterile work.