J. Basic Microbiol. 26 (1986) 9, 533—539

("Boris Kidrič" Institute of Chemistry and LEK, Pharmaceutial and Chemical Works[1], Ljubljana, Yugoslavia)

Developmental studies of *Claviceps paspali* seed cultures for the submerged production of lysergic acid derivatives

H. Sočič, V. Gaberc-Porekar, E. Pertot, A. Puc[1] and S. Miličić[1]

(Received June 23, 1986)

Metabolic pattern of mycelial *Claviceps paspali* seed cultures during the submerged cultivation was established. By comparing it with conidial and mycelial *Claviceps purpurea* strains it was found that the biosynthesis of RNA, DNA, and proteins followed a similar course in all *Claviceps* strains, so the fall of RNA content in mycelium may be considered a general biochemical indicator for optimally developed inoculum. But, two different patterns of carbohydrate and lipid metabolism were observed one for conidial and one for mycelial strains.

During the growth and development of fungi in submerged fermentation differentiating cells pass through a series of structural and functional alterations. Each stage of development is preceeded by some disturbance of the metabolic pattern which may arise from various factors not yet entirely understood. The fungi *Claviceps* seem to have been less intensively studied in this respect than other microorganisms.

The development of saprophytic *Claviceps* strains including morphological and biochemical changes was mainly investigated in connection with alkaloid biosynthesis, in the production phase of the fermentation process (Mary *et al.* 1965, Amici *et al.* 1967, Arcamone *et al.* 1970, Řeháček *et al.* 1971, Banks *et al.* 1974). Very little information, however, is available on the development of *Claviceps* seed cultures, particularly on their biochemical characterization. Since inoculum represents a complex biochemical and regulatory system maintaining its effect throughout the fermentation, it is of the outmost importance to get more data on the factors influencing its development.

In our previous work (Sočič *et al.* 1985) we described some biochemical and morphological characteristics of the inoculum of a conidial *C. purpurea* strain producing ergotoxine alkaloids. To gain additional information, in this paper, we tried to establish metabolic changes of the seed cultures of a mycelial *C. paspali* strain which differs in morphology and alkaloid spectrum from the mentioned *C. purpurea* strain.

Materials and methods

Microorganism and culture conditions: Studies were carried out with a *Claviceps paspali* (Stevens and Hall) L-52, selected strain ATCC 13892, which produces simple lysergic acid derivatives with α-hydroxyethylamide of lysergic acid as the main component. The microorganism was kept on potato-glucose agar plates and 21-day-old colonies were used for inoculating the seed flasks. After 6 days of incubation, the shake cultures were homogenized and 10—14 vol. % (8—10 g/l of mycelium dry weight) were used to inoculate the laboratory fermenter for inoculum development studies.

The seed medium (Arcamone *et al.* 1961) was modified and composed of (g/l): mannitol 40, glucose 10, succinic acid 10, KH_2PO_4 3, $MgSO_4 \cdot 7H_2O$ 0.3 and chick pea meal 2 in tap water. The pH 5.2 was adjusted with ammonia before sterilization. The course of fermentation was followed in a laboratory fermenter (Chemap) with total capacity of 7 l, air flow 1/1/min and 50—200 rpm, at 24 °C.

Synthesizing activities of seed cultures were followed in shake flasks containing the medium composed of (g/l): mannitol 150 and peptone 50 in distilled water (Sočič *et al.* 1982). The initial pH 6.8 was unregulated.

Claviceps purpurea ATCC — 20103, L-4 selected mycelial strain capable of producing ergotamine was maintained as described previously (Puc and Sočič 1972). The seed medium was prepared according to Wack *et al.* (1967), containing (g/l): sucrose 100, succinic acid 10, proflo 10, $Ca(NO_3)_2$ 1, KH_2PO_4 0.25, $MgSO_4 \cdot 7\ H_2O$ 0.25, KCl 0.12 in distilled water. pH 5.2 was adjusted with ammonia.

Analytical methods: Biomass determination: Culture broth (50 ml) was filtered through weighed filter paper on a Büchner funnel and the mycelium washed twice with distilled water and dried at 85 °C to constant weight.

Mycelium components were determined in lyophilized samples of washed mycelium. Lipids were determined by extracting the mycelium with chloroform:ethanol (2:1) according to Banks *et al.* (1974). The quantity of nucleic acids was determined according to modified Schneider procedure (Herbert *et al.* 1971) as follows: after lipids removal, acid soluble material was extracted with cold perchloric acid and nucleic acids were thereafter extracted with the hot perchloric acid. DNA was determined in the extract colorimetrically according to Burton (1956) and RNA by measuring the absorbances at 260 nm and 290 nm with reference to the yeast RNA standard (Gottlieb 1964), and the percentage of DNA was substracted.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Total carbohydrates were estimated by the anthrone method (Vahouny 1960).

Total nitrogen was determined by Kjeldahl procedure.

Nitrogen (NH_4^+) was estimated by the method described by Banks *et al.* (1974).

Inorganic phosphate was measured by the method of Berenblum and Chain (1938).

Mannitol in the culture filtrate was estimated by the polarimetric method of Richtmyer and Hudson (1951), as modified by Arcamone *et al.* (1961).

Succinic acid in the filtrate was determined according to the method of Sočič and Gaberc-Porekar (1980).

Alkaloids were determined in suitably diluted filtrate (2 ml) by adding van Urk reagent (4 ml) prepared as described by Agurell (1966). The blue colour was determined spectrophotometrically with reference to a standard solution of ergometrine base.

Microscopy: Cultures were examined in a light microscope (Zeiss — Jenaval) and a scanning electron microscope (Leitz AMP 1600 T).

Results and discussion

During the growth and development of *Claviceps paspali* cultures in the seed tank no significant changes in morphology could be observed. As shown in the picture from the scanning electron microscope (Fig. 1) the mycelium of the developed inoculum was formed by aggregation of elongated hyphae which became only thicker with the development. So developed hyphae examined by light microscopy varried in their length, having 3—5 μm in diameter with septa 30—50 μm long. The hyphae were vacuolized and contained fat droplets distributed throughout the cytoplasm.

Changes in chemical composition of mycelium as well as of cultivation medium during the development of *C. paspali* seed cultures were more expressed. The initial growth phase was characterized by rapid consumption of glucose whereas other medium components such as mannitol, succinic acid, phosphate and ammonium nitrogen were taken up slowlier and were not depleted till the end of the process (Fig. 2). Exponential growth was observed which influenced not only the cell mass but also its composition.

As shown in Fig. 3 the RNA synthesis in mycelium increased with the increasing specific growth rate. The percentages of RNA, DNA, proteins and total nitrogen (Figs. 3 and 4) reached their maxima at about the third day of cultivation, simultaneously with the maximal specific growth rate. Intensive macromolecular synthesis at this stage of inoculum development is very important, promoting not only high fungal biomass but also a high level of enzymes responsible for primary metabolic pathways

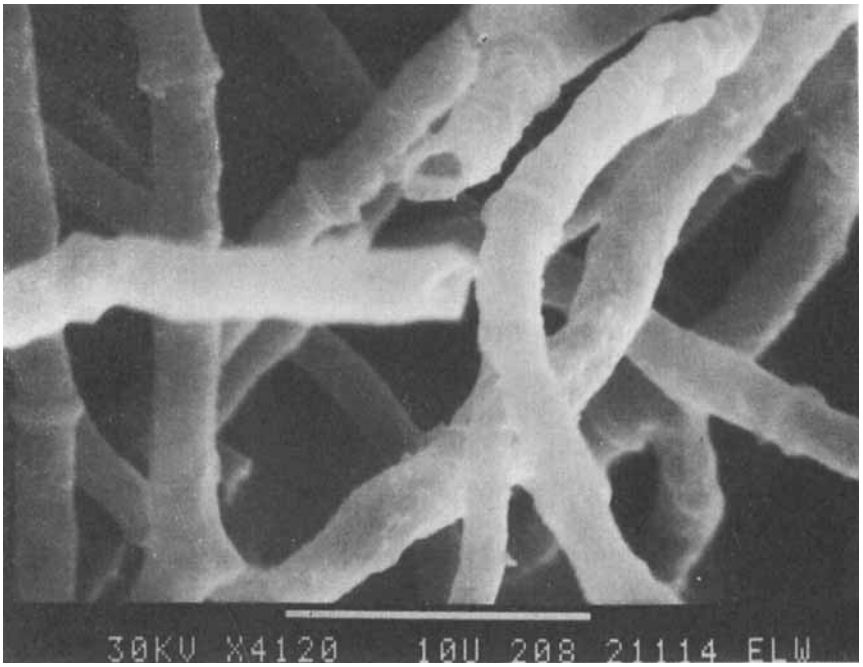

Fig. 1
Scanning electron micrograph of mycelium of 4-day old *C. paspali* seed cultures

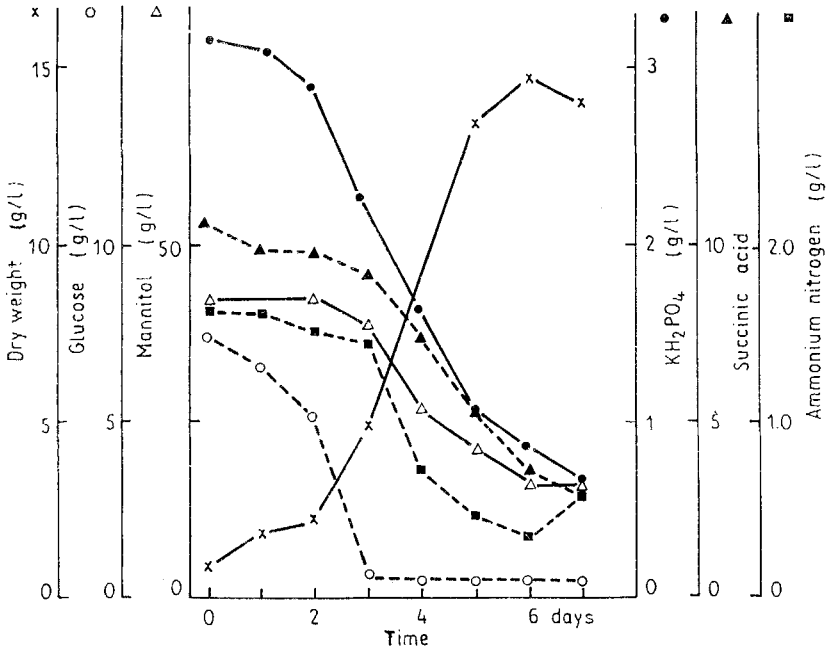

Fig. 2
Metabolic changes during the development of *C. paspali* seed cultures in laboratory fermentor

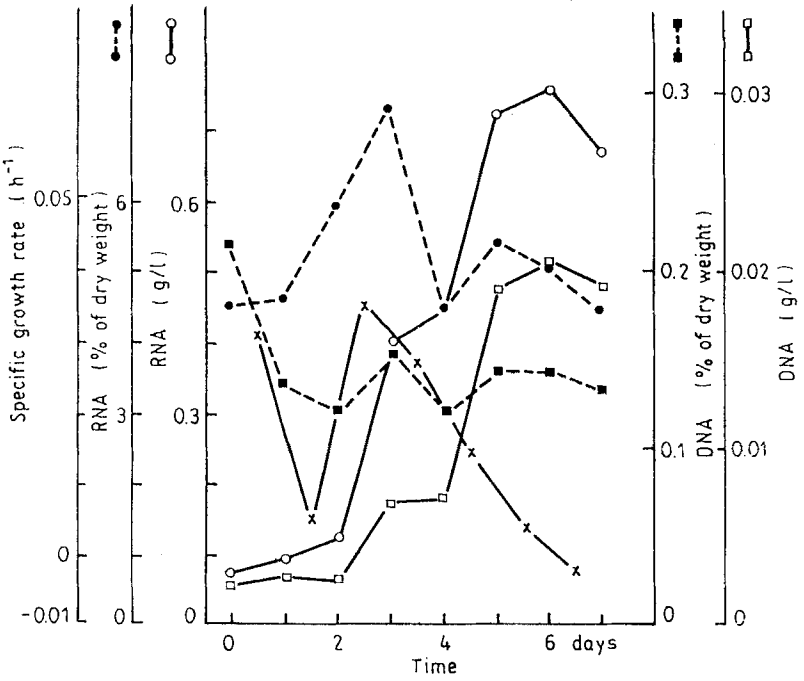

Fig. 3
Synthesis of nucleic acids in the submerged *C. paspali* seed cultures in correlation with specific growth rate (×—×)

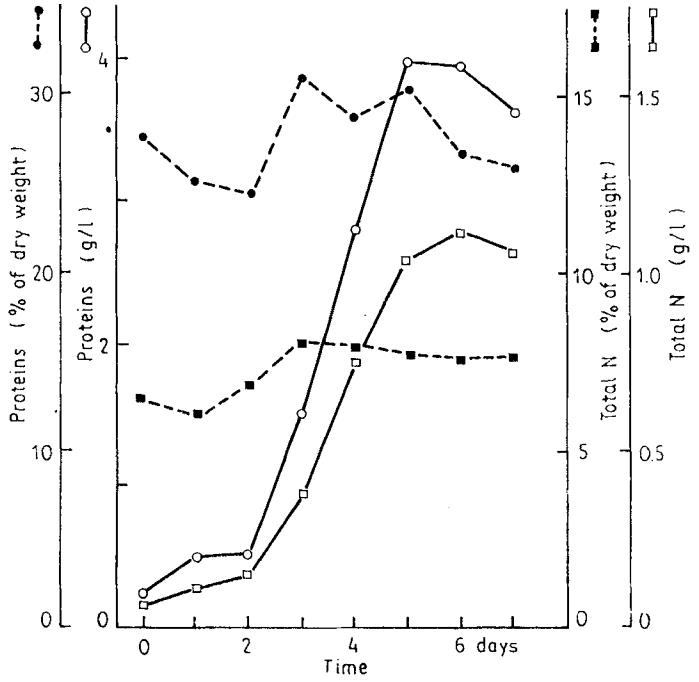

Fig. 4
Synthesis of nitrogeneous compounds during the submerged development of *C. paspali* seed cultures

which affect also secondary metabolites formation. Further development of seed cultures connected with the fall of specific growth rate, was accompanied by a rapid fall of RNA synthesis on the fourth day of cultivation. Similar changes but not as significant were observed in relative protein and DNA syntheses.

By checking the alkaloid synthesizing activity of *C. paspali* seed cultures at different developing stages, the highest alkaloid yields were obtained with 4-day-old cultures (Table 1). The RNA minimum which obviously coincides with the optimal activity of inoculum may be considered, therefore, as a biochemical indicator for the optimally developed inoculum for starting the production phase. According to PROKOFIEVA *et al.* (1959) a decrease of the mycelial RNA is a feature characterizing also the transient phase of growth and might be associated with the changes in the metabolism of fungus. This statement is supported also by our investigations where the RNA decrease reflects, probably, the transition from the primary to the secondary metabolism.

Table 1
Synthesizing activity of *Claviceps paspali* seed cultures at different developing stages

Age of seed cultures (day)	Dry weight (g/l)	Alkaloids (mg/l)
2	30.77	861
3	24.17	2698
4	26.26	2715
5	24.81	2372
6	24.56	2188

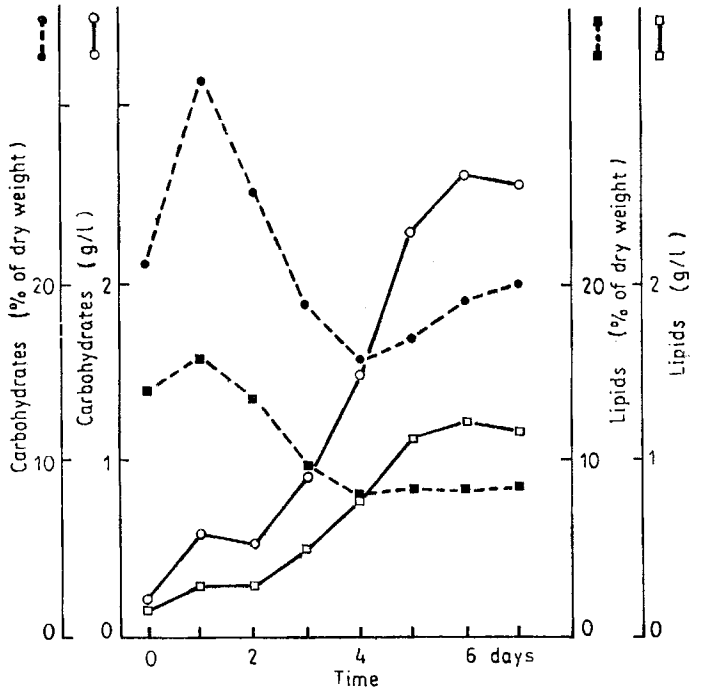

Fig. 5
Carbohydrate and lipid synthesis during development of the submerged *C. paspali* seed cultures

The results obtained on the biochemical characterization of *C. paspali* seed cultures show similarities with our previous investigations of *Claviceps purpurea* seed cultures (Sočič *et al.* 1985) although they produced different type of alkaloids and showed a considerable morphological differentiation. By comparing both, *C. paspali* and *C. purpurea* strains, we have found that biosynthesis patterns of macromolecular substances get along fairly well, but significant differences in the rates of carbohydrate and lipid synthesis were observed. The percentage of these "storage compounds" gradually increased throughout the development of *C. purpurea* seed cultures which underwent the whole developing process from conidia through hyphae into sclerotia-like cells. On the contrary, relative amounts of lipids and carbohydrates of *C. paspali* seed cultures, starting from mycelial fragments, reached their maximum early in the growth phase and declined to the minimal values on the fourth day of cultivation (Fig. 5). We assume that such different metabolic patterns of carbohydrate and lipid synthesis as we found in *C. paspali* strain, might be connected with biologically more differentiated mycelium fragments we used for starting the inoculum production.

To confirm this assumption we checked the synthesis of mycelial components during the vegetative phase of another mycelial strain *C. purpurea* producing ergotamine under submerged conditions. Similar biosynthesis patterns of carbohydrate and lipids as in *C. paspali* seed cultures were established while the synthesis of RNA, DNA and proteins proved to be very much a-like in all *Claviceps* strains. As shown in Fig. 6 the relative amounts of carbohydrates and lipids of mycelial *C. purpurea* were the highest at the beginning of inoculum development and a rapid fall between the second and third day of cultivation followed. The percentage of these compounds started to increase again with the transition from the primary to secondary metabolism which

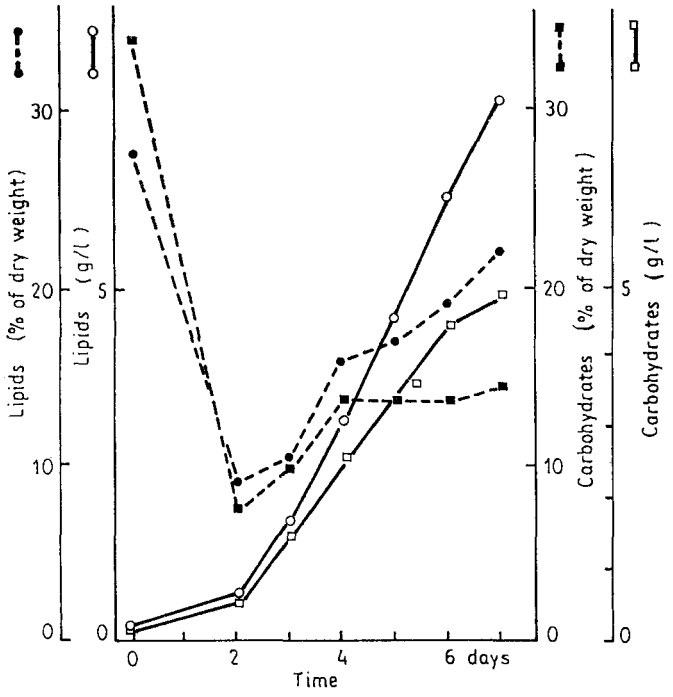

Fig. 6
Carbohydrate and lipid synthesis in seed cultures of mycelial *C. purpurea* strain

was associated also with differentiation of hyphae into sclerotia-like cells. Since the same metabolic patterns of carbohydrates and lipids were established in both mycelial strains, we suppose that such metabolic pattern might be a characteristic of mycelial *Claviceps*.

It is possible to say in conclusion that biochemical patterns can serve as a useful tool for the inoculum production studies, especially in those filamentous fungi where no significant morphological differentiation could be observed.

References

Amici, A. M., Minghetti, A., Scotti, T., Spalla, C. and Tognoli, J., 1967. Ergotamine production in submerged culture and physiology of *Claviceps purpurea*. Appl. Microbiol., **15**, 597–602.

Arcamone, F., Chain., E. B., Ferreti, A., Minghetti, A., Pennella, P., Tonolo A. and Vero, L., 1961. Production of a new lysergic acid derivative in submerged culture by a strain of *Claviceps paspali*. Proc. R. Sco. Lond./Biol./, **155**, 26–54.

Arcamone, F., Casinelli, G., Ferni, G., Penco B., Pennella, P. and Pol, C., 1970. Ergotamine production and metabolism of *Claviceps purpurea* strain 275 FI in stirred fermentors. Can. J. Microbiol., **16**, 923–931.

Agurell, S., 1966. Biosynthesis of ergot alkaloids in *Claviceps paspali*. Acta Pharm. Suecica, **3**, 11–22.

Banks, G. T., Mantle, P. G. and Szcyrbak Ch. A., 1974. Large-scale production of clavine alkaloids by *Claviceps fusiformis*. J. Gen. Microbiol., **82**, 345–361.

Berenblum, I. and Chain, E., 1938. An improved method for the colorimetric determination of phosphate. Biochem. J., **32**, 295–298.

Burton, K., 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. Biochem. J., **62**, 315–323.

Gottlieb, D. and van Etten, J. L., 1964. Biochemical changes during the growth of fungi. J. Bacteriol., 88, 114–121.

Herbert, D., Phipps, P. J. and Strange, R. E., 1971. Chemical analysis of microbial cells. In: Methods in Microbiology (Eds.) Norris, J. R., Ribbons, D. W. Academic Press, London-New York, vol. 5B pp. 340–326.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**, 265–277.

Mary, N. Y., Kelleher, J. and Schwarting, A. E., 1965. Production of lysergic acid derivatives in submerged cultures. Lloydia, **28**, 218–229.

Puc, A. and Sočič, H., 1977. Carbohydrate nutrition of *Claviceps purpurea* for alkaloid production related to the osmolality of media. European J. Appl. Microbiol., **4**, 283–287.

Prokofieva-Belgorskaya, A. A. and Popova, L. A., 1959. The influence of phosphorous on the development of *Streptomyces aureofaciens* and on its ability to produce chlortetracycline. J. Gen. Microbiol., **20**, 462–472.

Řeháček, Z., Sajdl, P., Kozová, J., Malik, K. A., and Ričicová, A., 1971. Correlation of certain alterations in metabolic activity with alkaloid production by submerged *Claviceps*. Appl. Microbiol., **22**, 949–956.

Richtmyer, N. K. and Hudson, C. S., 1951. The rotation of polyols in ammonium molybdate solution. J. Am. Chem. Soc., 7, 2249–2250.

Sočič, H. and Gaberc-Porekar, V., 1980. Micromethod for the quantitative determination of succinic acid in the fermentation media. Europ. J. Appl., Microbiol. Biotechnol., **9**, 53–58.

Sočič, H., Gaberc-Porekar, V. and Didek-Brumec, M., 1985. Biochemical characterization of the inoculum of *Claviceps purpurea* for submerged production of ergot alkaloids. Appl. Microbiol. Biotechnol., **21**, 91–95.

Sočič, H., Pertot, E. and Didek-Brumec, M., 1982. The effect of media composition on the fermentative production of clavine ergot alkaloids. Vestn. Slov. Kem. Drus., **29**, 323–329.

Vahouny, G. V., Mayer, R. M., Roe, J. H., Treadwell, C. R., 1960. Determination of 3α-hydroxy sterols with anthrone reagent. Arch. Biochem. Biophys., **86**, 210–214.

Wack, G., Perényi, T., Udvardy-Nagy, E. and Zsóka, I., 1967. Hung. patent, 153738.

Mailing address: Prof. Dr. Helena Sočič, "Boris Kidrič" Institute of Chemistry, Hajdrihova 19, 61000 Ljubljana, Yugoslavia