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PHYSIOLOGICAL DIFERENTIATION OF ALKALOID PRODUCING STRAINS OF *CLAVICEPS PURPUREA* (FR.) TUL.

SREĆKO MATOŠIĆ, MILENA MEHAK, JAGODA ŠUŠKOVIĆ

(Faculty of Food Technology and Biotechnology, University of Zagreb)

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Four different strains of *Claviceps purpurea* IC/39/20–B, G, R, and W have been selected and isolated using common selection method. The relationship between intensity of pigmentation of the culture and the accumulation of ergot alkaloids has been noticed. Maximal alkaloid yields have been obtained with more pigmented strains R and W in the sucrose-asparagine medium (1.30 and 1.50 g/L respectively) and with less pigmented strains B and G in the sucrose-peptone medium (0.30 and 0.80 g/L).

Introduction

Successful production of ergot alkaloids depends on the strain used and the conditions of its cultivation. Both the regulation of the process and production of alkaloids could be substantially improved by elucidating the macroscopic kinetic mechanism of biosynthesis.

Published data of peptide alkaloid fermentation carried out with industrial strains developed in the laboratory refer to levels not higher than 1.0–1.8 g/L (Udvardy 1980, Puc 1987). There are some possibilities of developing strains for alkaloid fermentation.

Either a mutation program (Kobel 1973. Rehaček 1983, Kren 1986, Didek-Brumec 1987) or interspecific fusion of protoplasts (Spalla 1982) are used as in the case of antibiotics, or it is possible to follow a certain directed selection in which it is intended to save sclerotial feature and sclerotial functions of the fungus putting it into and keeping it under saprophytic-submerged conditions (Amici 1969, Puc 1977, Udvardi 1980). The problem of saprophytic alkaloid production is not only a problem

of genetics, but also of nutrition and of the phase of development of culture (Bekesy 1973).

This paper reports the results of a physiological comparison between an ergotamine producing strain (Galeffi 1974, Matošić 1984) and four strains spontaneously obtained i. e. selected from it.

Materials and Methods

Microorganism. Claviceps purpurea IC/39/20, originally from the Collection of Instituto Superiore di Sanita, Rome, was used in this study.

Culture media. Media for maintenance and selection of cultures and seed cultivation had the following composition (g/L): sucrose 100, L-asparagine 10, $Ca(NO_3)_2$ 1, KH_2PO_4 0.25, $MgSO_4 \cdot 7H_2O$ 0.25, KCI 0.125, $FeSO_4 \cdot 7H_2O$ 0.033, $ZnSo_4 7H_2O$ 0.027, cisteine 0,01, yeast extract 0.1, agar 20 (not for the seed medium), distilled water to 1 L. The pH 5.2 of media was adjusted with NH_4OH before sterilization.

The production media of different composition contained (g/L):

SA – was of the same composition as the seed medium but contained 300 g/L of sucrose. In the medium experiments were carried out with quantity of sucrose from 100 to 500 g/L and L-asparagine from 5 to 30 g/L.

MS – mannitol 100, succinic acid 40, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.0033, $ZnSO_4 \cdot 7H_2O$ 0.0015, distilled water to 1 L. The pH was adjusted with NH_4OH to 5.2 before sterilization.

SC – sucrose 300, citric acid 10, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.0033. $ZnSO_4 \cdot 7H_2O$ 0.0015, distilled water to 1 L. The pH was adjusted with NH_4OH to 5.2 before sterilization.

SP – sucrose 200, peptone 3, tap water to 1 L. The pH 6.2 was adjusted before sterilization. In the medium experiments were carried out with quantity of sucrose from 100 to 400, and peptone from 10 to 40.

MP – mannitol 150 or 200, peptone 30, tap water to 1 L. The pH 6.2 was adjusted before sterilization.

Sterilization for all media 120°C/20 min.

Culture conditions. Selection of the strains were made on agar plates. Dillutions of cultures were prepared from a single colony, or from the fermentation broth of seed-stage fermentation (6 days old). Cultures were maintained on agar plates, and 2-weeks old single colony was used for inoculating the medium in each seed flask. All fermentation experiments were carried out in two stages: a seed-stage fermentation for 6 days and a production-stage fermentation for 10 days inoculated with 10% of the seed culture. Cotton-wool-plugged, 500 mL Erlenmeyer flasks containing 100 mL of culture media were incubated at 23 to 25°C on a rotary shaker with a 6 cm stroke.

Alkaloids. These were determined in fermentation broth filtrate and a mycelium by van Urk reagent (Banks 1974) spectrophotometrically with reference to a standard solution of ergotamine base. Intracellular alkaloids were previously extracted from mycelium with 4% tartaric acid.

Mycelial dry weight. A sample of culture broth was filtered, washed twice with distilled water and dried at 95 °C

Sucrose. Concentrations of reducing sugars were determined by means of method of Schoorl Luff.

Results and Discussion

Four new strains were obtained from the original strain *C. purpurea* IC/39/20, using common selected method on agar-plate. The first *C. purpurea* IC/39/20–B, or original strain segregated spontaneously during the selection and strains IC/39/20–G, IC/39/20–R and IC/39/20–W were obtained. All the strains exhibited sclerotial feature of mycelium, and grew on agar-plate forming bulky colonies, compact and rather sharp at the edge. Morphologically, colonies of different strains differed from each other in colours and intensity of pigmentation (*C. purpurea* produced ergochromes and anthrachinone carbon acids pigments, Franck 1965). Strain B produced white colonies on agar-plate and practically colourless pigmentation of other strains (extraction of pigments from mycelium with acetone) vary from orange-yellow (strain G), to intensive red (strain R) and dark purplish-violet (strain W) (Matošić 1976). In the preliminary experiments positive correlation was established between intensity of pigment biosynthesis and accumulation of alkaloids in the culture of different strains. The lowest intensity of alkaloid biosynthesis was obtained in the colourless culture of the strain B and maximal yields of alkaloids were reached in intensively pigmented culture of the strain W.

All isolated strains also exhibited different physiological characteristics i. e. alkaloid synthesis and growth in the different cultivation media (Fig. 1.)

Maximal alkaloid yields were obtained in culture of more pigmented strains R and W grown in the medium with sucrose and L-asparagine (1.3 and 1.5 g/L respectively) and with less pigmented strains B and G in sucrose-peptone medium (0.3 and 0.8 g/L) (Figs. 1, 2 and 3).

The course of typical fermentations in the appropriate medium, carried out with these four strains is presented in Figs. 4, 5, 6 and 7. There is a possible explanation of the relatively high frequency of the strain segregation obtained in the present work. It could be interpreted by taking into consideration that the cells of hyphae of *Claviceps* are plurinucleate i. e. that the mycelium of producing strains is heterokaryotic (Spalla 1980, 1982). This caryologic condition is strongly advantageous for production of alkaloids, and it can be lost by separation into constituent nonproductive homokaryons.

A more probable explanation (Řeháček 1991) classified the differences in the intensity of alkaloids formations in the category of cytoplasmic variations. Changes in the strain ability to produce alkaloids obviously occur not only due to genetic but also due to exceptional sensitivity of the strain to changes in cultivation medium.

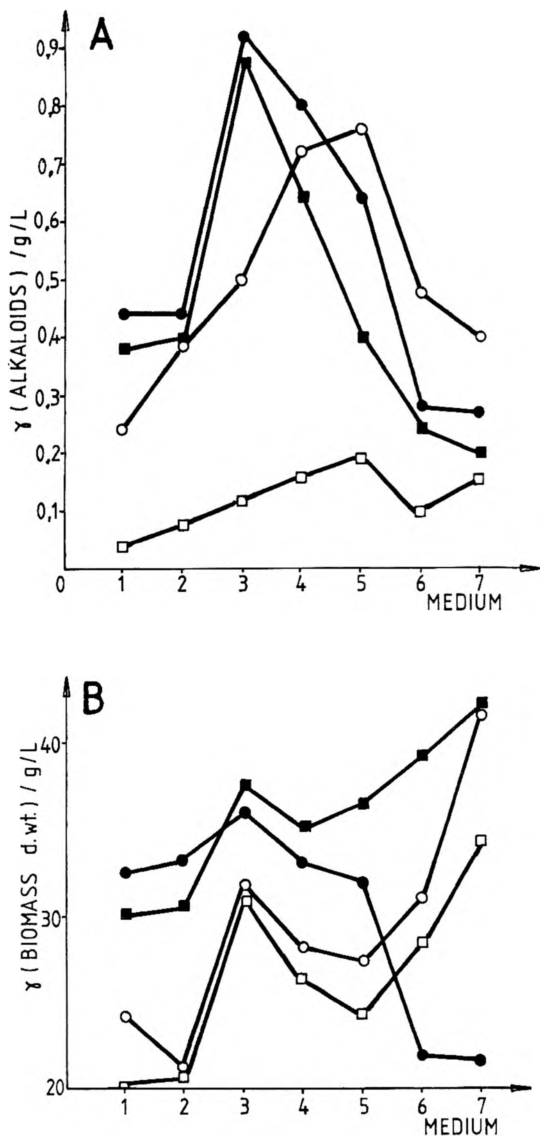


Fig. 1. Effect of the medium composition on (A) the intensity of alkaloid biosynthesis and (B) the biomass yield in the culture of different strains of *C. purpurea:*
□ – IC/39/20–B; ○ – IC/39/20–G; ■ – IC/39/20–R; ● – IC/39/20–W. Media composition: 1 – SC; 2 – MS; 3 – SA; 4 – SP with 30% sucrose; 5 – SP with 20% sucrose; 6 – MP with 5% mannitol; 7 – MP with 20% mannitol.

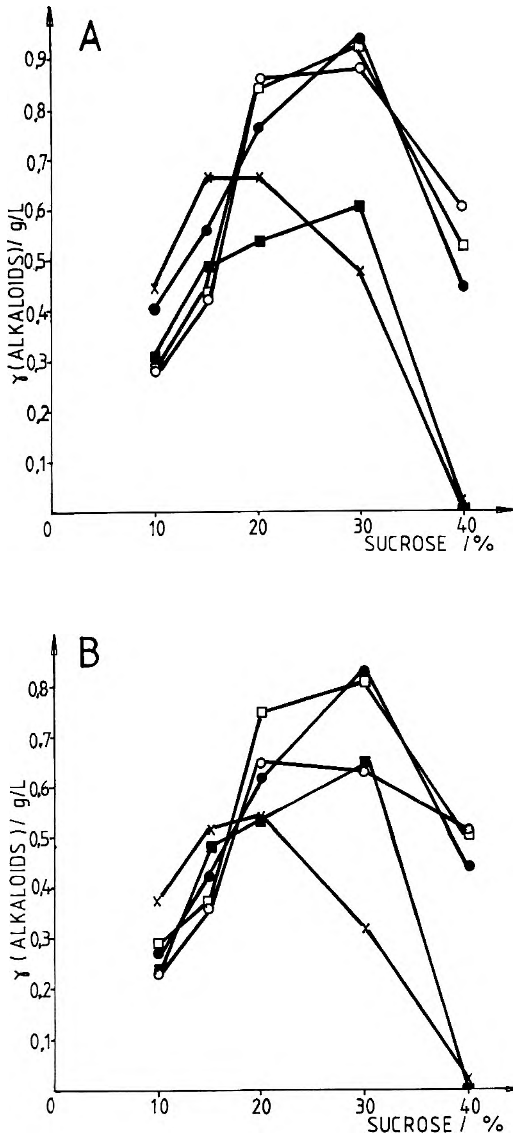
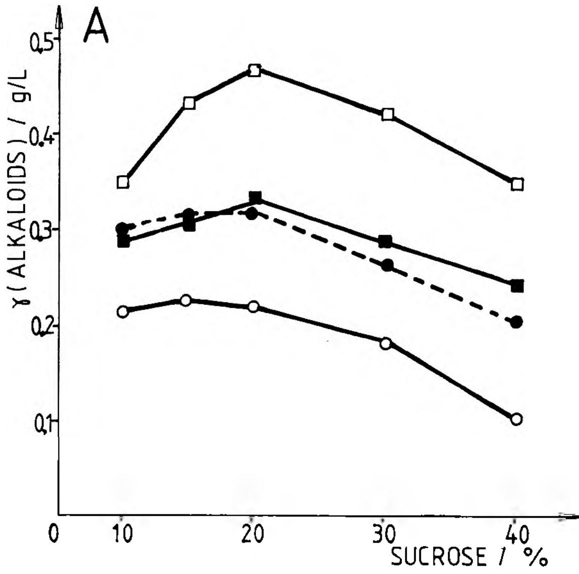


Fig. 2. Biosynthesis of alkaloids in relation to the concentrations of sucrose and L-asparagine in the medium (SA) for cultivation of *C. purpurea* (A) strain IC/39/20–W and (B) strain IC/39/20–R. L-asparagine concentrations (%): o – 0.5; • – 1.0; □ – 1.5; ■ – 2.0; x – 3.0.



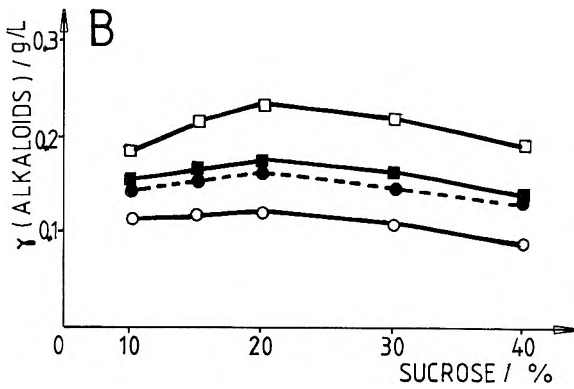


Fig. 3. Biosynthesis of alkaloids in relation to the concentrations of sucrose and peptone in the medium (SP) for cultivation of *C. purpurea* (A) strain IC/39/20-G and (B) strain IC/39/20-B. Peptone concentrations (%): ○ – 1; ● – 2; □ – 3; ■ – 4.

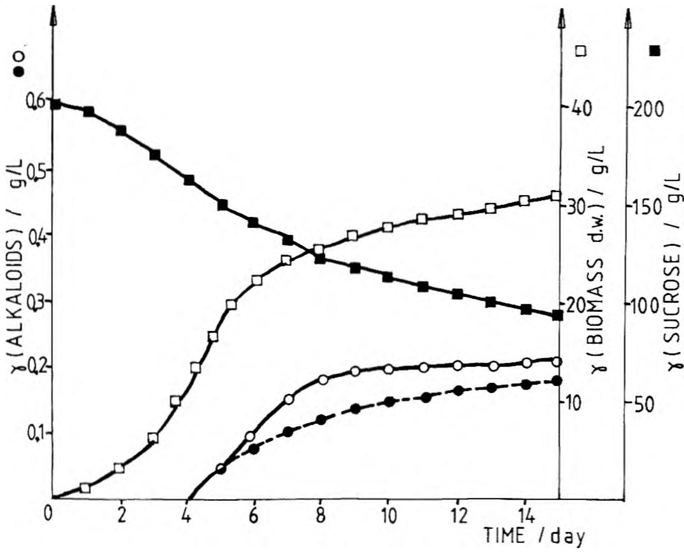


Fig. 4. Course of the process of alkaloid biosynthesis in the culture of *C. purpurea* strain IC/39/20–B. Medium: SP with 20% sucrose. Alkaloids: ○ – total and ● – intracellular.

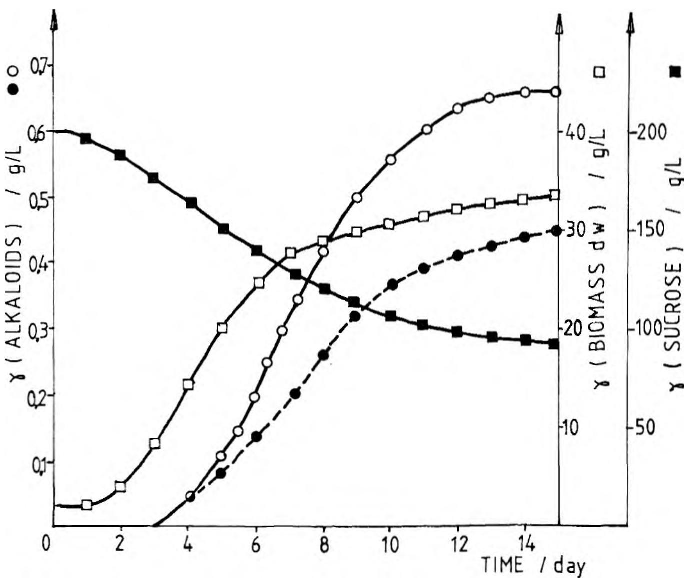


Fig. 5. Course of the process of alkaloid biosynthesis in the culture of *C. purpurea* strain IC/39/20 – G. Medium: SP with 20% sucrose. Alkaloids: ○ – total and ● – intracelullar.

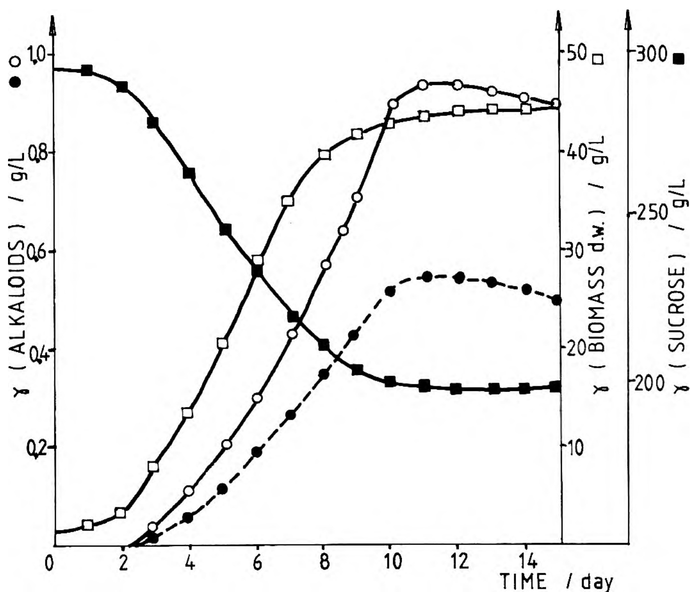


Fig. 6. Course of the process of alkaloid biosynthesis in the culture of *C. purpurea* strain IC/39/20–R. Medium: SP with 30% sucrose. Alkaloids: ○ – total and ● – intracellular.

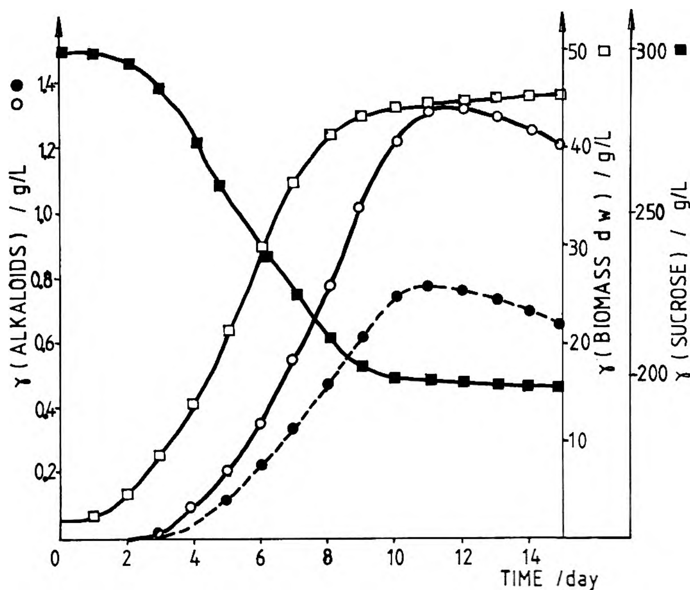


Fig. 7. Course of the process of alkaloid biosynthesis in the culture of *C. purpurea* strain IC/39/20–W. Medium: SP with 30% sucrose. Alkaloids: ○ – total and ● – intracellular.

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SAŽETAK

FIZIOLOŠKA DIFERENCIJACIJA SOJEVA GLJIVE *CLAVICEPS PURPUREA* (FR.) TUL. PRI PROIZVODNJI ERGOT-ALKALOIDA

Srećko Matošić, Milena Mehak, Jagoda Šušković

(Prehrambeno-biotehnološki fakultet Sveučilišta u Zagrebu)

Izolirana su četiri soja gljive *Claviceps purpurea* IC/39/20-B, G, R i W primjenom uobičajenih selekcijskih metoda. Ustanovljena je povezanost intenziteta pigmentacije kulture i intenziteta biosinteze ergot-alkaloida. Intenzivnije pigmentirani sojevi (R i W) ostvaruju maksimalne prinose alkaloida u podlozi

s L-asparaginom i saharozom (1,30 odnosno 1,50 g/L). Slabije pigmentirani sojevi (B i G) ostvaruju maksimalnu sintezu u hranjivoj podlozi s peptonom i saharozom (0,30 i 0,80 g/L).

Prof. dr. Srećko Matošić
Mr. Milena Mehak
Mr. Jagoda Šušković

Zavod za biokemijsko inženjerstvo
Prehrambeno biotehnološki fakultet
Sveučilišta u Zagrebu
Pierottijeva 6,
41 000 Zagreb, Hrvatska (Croatia)