6. PHYSIOLOGICAL REGULATION OF ERGOT ALKALOID PRODUCTION AND SPECIAL CULTIVATION TECHNIQUES

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6.1. REGULATION OF ERGOT ALKALOID BIOSYNTHESIS

The studies on the ergot physiology were mostly motivated by industrial process improvements. These studies nevertheless brought many basic findings from physiology of filamentous fungi.

The results of these studies that were performed with the strains not generally available can hardly be transferred to other strains and, therefore, general applicability or even reproducibility is sometimes questionable. Critical assessment of such results is always necessary. Therefore, all results obtained with, e.g. *Claviceps fusiformis* SD-58 that was made generally available by Prof. D. Gröger from Halle an der Saale (Germany) are especially valuable.

Deep knowledge of the regulation of the alkaloid synthesis and the metabolism of the producer is a prerequisite for successful design of biotechnological process of alkaloid production. Some products of primary metabolism serve in secondary metabolism as precursors. They, however, exhibit also regulatory effects (tryptophan) or act as structural elements (lipids) influencing thus the biosynthesis of secondary metabolites in a complex way.

6.1.1. Carbon Sources and Saccharides

Saccharides are the main carbon and energy source for *Claviceps* both under parasitic and saprophytic conditions. Sucrose is an important component of phloem sap of the host plants (Basset *et al*., 1972). The best substrates for alkaloid production are slowly metabolizable Saccharides, e.g., sucrose, maltose and polyols (mannitol, sorbitol) (Křen *et al*., 1984).

Sucrose is metabolised faster at the very beginning of the submerge *C. purpurea* cultivation (*Figure 1*). Monosaccharides released are quickly utilized (Křen *et al*., 1984). From the sucrose molecule, mostly glucose is utilized. Fructose, by the action of transglycosylating \( \beta \)-fructofuranosidase, is oligomerized starting with one sucrose molecule (Perényi *et al*., 1968).
Glucose is not suitable as a sole carbon source because it supports mycelial growth and the alkaloid formation is virtually inhibited by glucose-catabolite repression (Křen et al., 1987a). Glucose in some strains, e.g., *C. fusiformis*, also strongly supports extracellular polysaccharide (β-glucan) formation that increase viscosity and complicate the oxygen transport (Buck et al., 1968). However, glucose, when added in an optimised proportion and at respective time, supports alkaloid production.

Secondary carbon-sources, i.e., intermediates of citrate or glyoxalate cycle are essential for good saccharide utilisation by saprophytically cultivated *Claviceps* strains.

*C. purpurea* growing on sucrose as sole C-source produces lactate and severe acidification of the medium diminishes activity of Krebs cycle enzymes, e.g., malatedehydrogenase and citratesynthase (Pazoutová and Řeháček, 1981). Addition of citrate or succinate to the medium maintains the citrate cycle active, ensures catabolism of pyruvate and by ATP production helps to control the rate of glycolysis.

Key regulatory enzyme of the glycolysis in *Claviceps* is phosphofructokinase that has rather atypical regulatory properties—feedforward regulation and hyperbolic saturation kinetics—(Křen and Řeháček, 1984).

Hexosomonophosphate pathway prevails in glucose breakdown during the vegetative phase of fermentation, the share of the glycolytic pathway becomes

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**Figure 1** Sucrose metabolism in a submerged culture of *C. fusiformis* (former *purpurea*) 129/35. *P*₁, *P*₂ production phases of the culture; *T* transition (growth) phase (Křen et al., 1984).
more pronounced during alkaloid synthesis (Gaberc-Porekar et al., 1990). Supply of NADPH is important for ergot alkaloid biosynthesis, e.g., for cyt P-450-catalysed steps.

6.1.2. Phosphate

Inorganic phosphate has an exquisite role among the inorganic nutrients in C. purpurea cultivation. In most of the microbial secondary metabolites (e.g., antibiotics) a suboptimal level of phosphate has a stimulative role for their production (Martín, 1977). In high alkaloid producing Claviceps strains the optimum level of phosphate in medium ranges from 1 to 4 mM. The phosphate is taken up by the fungus during first 2–4 days of the cultivation and it is accumulated in the mycelium. Higher phosphate concentrations stimulate vegetative growth of the mycelium, glucane production and inhibit alkaloid biosynthesis. Drop of the intracellular phosphate coincides with the stoppage of the mycelial growth and the onset of the alkaloid production (Pazoutová and Řeháček, 1984). High levels of phosphate also induce alkaloid degrading enzymes, lowering thus the yields (Robbers et al., 1978).

Some strains as, e.g., C. paspali, demand for alkaloid production higher phosphate concentration.

6.1.3. Lipids

Ergot alkaloid biosynthesis and lipid metabolism have some common regulatory points. Hydroxymethylglutaryl-coenzyme-A reductase is the key regulatory enzyme of isoprenyl unit production. The activity pattern of this enzyme in Claviceps (Křen et al., 1986) reveals that the mevalonate distribution is shared at the beginning of the fermentation by sterol and alkaloid biosynthesis and later it is used solely for alkaloid building.

Substances known to modulate lipid biosynthesis (and cell lipid composition), e.g., chlorophenoxy acids are also able to influence ergot alkaloid biosynthesis and to amend their yields (Křen et al., 1990a). Chlorophenoxypropionate in minute doses significantly stimulates alkaloid production in C. purpurea strain 59 (Figure 2).

6.1.4. Medium Osmolarity

Claviceps in its parasitic stadium grows under high sucrose concentration of the phloem sap. This fact lead to assumption (Amici et al., 1967) (see Chapter 1) that high sucrose concentration should be favourable for the high alkaloid production. Optimum sucrose concentration was identified to be 30–35%. At present, most media for saprophytic alkaloid production by Claviceps have at least 100 g sucrose per litre. Later it was found that part of sucrose (or manitol) can be replaced by NaCl (or KC1) to maintain high osmolarity (Puc and Sočič, 1977).
6.2. SEASONAL CYCLES IN ERGOT CULTIVATION

Most people working longer with *Claviceps* strains admit that there exist substantial difference in alkaloid production and strain growth during the year. These effects occur mostly in the Autumn season (September till beginning of December—in Europe) when the strains grow worse and the production is lower. Some people ascribe these effects to higher risk of aerial contamination in this period when, due to the fruit ripening, there exist high concentration of airborne germs. However, these effects occur also in noncontaminated cultures. This seasonal effect might have a correlation with time rhythms of the parasite growing in nature. Not all strains exert this effect and it is not always of the same magnitude.

To my knowledge, this observation has never been published as it should have been proved by a large statistical set. However, both people in research and in industry should bear this possibility in their minds when comparing the results from various seasons of the year.

Figure 2 Effect of DL-2(4-chlorophenoxy)-propionate on ergot alkaloid production in *Claviceps purpurea* 59 on 14th (Δ) and 21st (○) day of cultivation (Křen et al., 1990a).
6.3. BIOTECHNOLOGY OF SUBMERGED CULTIVATION OF ERGOT

The prerequisite for successful, high yielding cultivation is a high-quality strain. There exist many *Claviceps* strains (Schmauder, 1982), some of them of unclear origin. Primarily, all the strains were isolated from the parasitic sclerotia and later were mutagenized and selected. Some of the strains are deposited in public collections, however, most of high-producing strains are in the possession of pharmaceutical companies (see also Chapter 12).

The *Claviceps* strains display high variability and they often tend to degeneration. Therefore continuous selection and standard storage techniques are integral part of the bioprocess. The best method for strain storage is deep freezing in liquid nitrogen or freeze drying in a preserving solution consisting of sucrose and skimmed milk.

Cultivation conditions for *Claviceps* strains do not basically differ from other cultivations of the filamentous fungi, e.g., *Penicillia*. A crucial step is the inoculum preparation. If the inoculum is of bad quality, the production stage cannot be usually recovered. Suitability of the inoculum can be judged also by morphological and biochemical parameters, e.g., RNA content (Sočič *et al.*, 1985). Production stage must be inoculated by at least 10% of inoculum. During the fermentation all the key parameters must be controlled, pH being 4.5–6.2 (optimum 5.4), aeration 0.5–1.51 of air/1 of medium, mixing rate 75–200rpm and temperature 24 °C. Some synthetic antifoam reagents diminish yields. Good results are obtained by use of plant oils (or ergot oil from sclerotia) that is partly utilised. The inoculum stage takes place for 6–10 days (or longer in multistage inoculum), the production stage takes place for 12–18 days. Long cultivation puts high demand on strict sterility of the process. Sometimes, low amount of broad spectrum antibiotics (chloramphenicol) could be added (Křen *et al.*, 1986c) to protect against prokaryotic contamination.

For most *Claviceps* strains synthetic media can be used. In industry sucrose or sorbitol is used (seldom other C-sources) in concentrations of 70–250 g/l. As a secondary C-source usually citrate in concentration of 5–10 g/l is used. NH₄⁺ salts, asparagine or NO₃⁻ salts serve as N-source and phosphate—*vide supra*—and other inorganic salts are supplemented. Inoculation medium differs from the production medium by higher phosphate concentration—up to 10 mM, different N-source, some additional vitamins (biotin, aneurine) and by complex nutrients added—yeast extract, corn steep, malt extract etc.

More than 120 various media with approx. 50 components were described for *Claviceps* strains cultivation. Choice of the suitable inoculation and production medium is rather important because each strain considerably differs in nutrient demands and the respective media optimization should be always performed.
6.4. CULTIVATION OF ERGOT ON THE PLANT TISSUE CULTURES

Plant tissue culture technique provides completely controlled conditions to elucidate the growth and physiology of the cells and to study host-parasite interactions at the cellular level. There exists probably single paper on this interesting technique in ergot research on cultivation of \textit{C. fusiformis} on a \textit{Pennisetum typhoides} cell culture. The authors (Roy and Kumar, 1985) established the methodology and studied nutritional demands of the mixed culture with respect to the growth and alkaloid production.

6.5. ALKALOID PRODUCTION BY IMMOBILISED CELLS OF \textit{CLAVICEPS}

The use of immobilised cells of producing strain is a promising method for extending the production period of the ergot cultures.

\textit{Claviceps purpurea} was immobilised in Ca-alginate by Kopp and Rehm (1983, 1984). The immobilised cells maintained the alkaloid production, for approximately 150 days under semicontinuous regime. Higher matrix (alginate) concentration promoted the overall production but mostly alkaloids in lower oxidation state were produced. The immobilised cells formed sklerotia-like structures suggesting the immobilisation (matrix) can simulate parasitic conditions.

Physiology of immobilised \textit{C. fusiformis} during long-term semicontinuous cultivation was studied by Krén \textit{et al.} (1987b). The immobilised cells maintained the alkaloid production for 770 days. The cells underwent profound morphological changes—vacuolisation, mitochondrial degeneration. The beads remained mechanically stable for the whole time.

Also \textit{C. paspali} has been immobilized and semicontinuous production of simple lysergic acid derivatives has been achieved (Rozman \textit{et al.}, 1989).

Immobilisation of \textit{Claviceps} cells in other matrices (carrageenan, pectate) and their coimmobilisation with oxygen supplying systems was studied by Krén (1990). The immobilised \textit{Claviceps} cells can be used not only for alkaloid production but also for biotransformations for e.g., stabilization of \textit{C. purpurea} protoplasts (Komel \textit{et al.}, 1985).

6.6. PROTOCOL

Following procedure as a practical example describes submerged cultivation of \textit{Claviceps fusiformis} SD-58 for clavine alkaloid production. The strain can be obtained, e.g., from American Type Culture Collection (ATCC).

The culture from a slant agar medium (Sabouraud) is inoculated into inoculation medium TI (60 ml/300 ml conical flasks) and aseptically cultivated on rotary shaker for 10 days. 5 ml of the mycelial suspension is transferred into same amount of production medium CS2 and shaken for 20 days (temperature...
24°C). Ergot alkaloid production (agroclavine, elymoclavine, chanoclavine) is 0.8–2.5 g/l. After biomass separation the liquid is alkalised by ammonia and extracted by ether. Alkaloid can be separated by TLC (SiO2, CHC13: MeOH: NH4OH=8:2:0.02) and the spots visualised by Ehrlich reagent. Media: TI, (g/1): sucrose, 100; L-asparagin, 10; (NH4)2SO4, 10; KH2PO4, 0.25; Ca(NO3)2, 1; yeast extract, 0.1; L-cystein, 0.1; inorganic salts and trace elements, pH 5.5. CS2, (g/1): sucrose, 100; citric acid, 16.8; (NH4)2SO4, 10; KH2PO4, 0.25; CaCl2, 1; inorganic salts and trace elements, pH 5.5.

REFERENCES


