12. SAPROPHYTIC CULTIVATION OF *CLAVICEPS*

ZDENĚK MALINKA

Galena a.s., 747 70 Opava 9, Czech Republic

12.1. INTRODUCTION

12.1.1. History

Biological, non-parasitic production of ergot alkaloids is carried out by saprophytic cultivation of production strains of different species of the genus *Claviceps*. The saprophytic cultivations of *Claviceps* spp. were experimentally performed as early as in the last century (Bové, 1970). Mycelial saprophytic cultures in nutrient media were reported since the 1920s (Bonns, 1922; McCrea, 1931; Schweizer, 1941; De Tempe, 1945). These experiments provided the basis of cultivation of the fungi *Claviceps* under artificial nutritional conditions but did not yet serve for alkaloid production or were not reproducible (McCrea, 1933).

Successful work oriented at the directed use of the saprophytic cultivation for alkaloid manufacture depended on the isolation of clavine alkaloids from saprophytic cultures (Abe, 1951; Abe *et al.*, 1951, 1952, 1953). The processes developed in a number of laboratories aimed at the industrial production of therapeutically applicable alkaloids or their precursors (Stoll *et al.*, 1953; Stoll *et al.*, 1954a; Rochelmeyer, 1959; Rutschman and Kobel, 1963a, b; Rutschman *et al.*, 1963). (For the history of *Claviceps* fermentation see Chapter 1 of this book.)

Fermentation makes possible to produce ergopeptines, paspalic acid, simple derivatives of lysergic acid and clavine alkaloids. Ergopeptines can be used for therapeutical purposes directly or after semisynthetic modification. Simple derivatives of lysergic acid, paspalic acid as well as clavines serve as a basal structure for the subsequent semisynthetic production of pharmaceutically utilizable alkaloids. From the simple derivatives of lysergic acid only ergometrine is used in therapy. (For details see Chapter 13.)

12.2. PRODUCTION MICROORGANISMS

12.2.1. Sources

Fermentation production of ergot alkaloids based on saprophytic cultivation of production strains selected from different species of the genus *Claviceps* represents the most important way of biological production of the alkaloids. However, also other filamentous fungi, able to produce ergot alkaloids, can serve as a source of production strains. (For more details see Chapter 18 of this

book.) The patent literature mentiones, besides *Claviceps,* only fungi of genera *Aspergillus* (Siegle and Brunner, 1963), *Hypomyces* (Yamatoya and Yamamoto, 1983) and *Penicillium* (Kozlovsky *et al.,* 1979). In the *Claviceps* fungi, selection of strains has been described for the species *C. purpurea, C. paspali* and *C. fusiformis*. The use of tissue cultures of plants of the *Convolvulaceae* family represents so far only theoretical possibility.

Saprophytic cultures can easily be obtained from sclerotia of the appropriate species of *Claviceps*. After pre-soaking with ethanol or propanol, the sclerotium surface is sterilized by a suitable agent—resorcinol, mercury dichloride or Lugol solution (Desai *et al.,* 1982b; Mantle, 1969; Strnadová *et al.,* 1986). After washing under sterile conditions, the plectenchymatic tissue of the sclerotium is cut and the slices are transferred on the surface of an agar growth medium. Another method of inoculum preparation by mechanical decomposition of a sclerotium was described by Řičičová and Řeháček (1968). Preparation of a saprophytic culture from the honeydew of a host plant invaded by *Claviceps* sp. was also described (Janardhan and Husain, 1984). A surface saprophytic mycelium starts growing on the medium and different asexual spores are generated on the hyphal tips. They are mostly classified as conidia. These spores are used for the further transfer and culture propagation, monosporic isolation (Kybal *et al.,* 1956; Necásek, 1954) and the following stabilization of the culture.

Culture isolation from ascospores is another method. A fungal sclerotium, after a cold storage period, forms under suitable conditions fruiting bodies. After ripening they release sexual ascospores, which germinate on the surface of an agar medium and form the saprophytic mycelium (Vásárhelyi *et al.,* 1980b). Monosporic isolation can be performed both directly with the ascospores or with asexual spores formed during the further saprophytic cultivation of a mycelium grown up from an ascospore.

12.2.2. Breeding and Selection of Production Strains

Classical methods of selection pressure, mutagenesis and recombination or their mutual combinations, can be applied to breeding production strains for fermentative alkaloid production. With the *Claviceps* fungi these methods are to a certain extent complicated by an incomplete information about the cell nucleus for a number of potential sources. The production strains are often highly heterogeneous and include both heterokaryotic and homokaryotic ones (Amici *et al.,* 1967c; Didek-Brumec *et al.,* 1991a; Mantle and Nisbet, 1976; Olasz *et al.,* 1982; Spalla *et al.,* 1969; Strnadová and Kybal, 1974). (See also Chapter 5 on the genetics of *Claviceps*.)

Nutrient components are mostly used for selection pressure. Such principle of selection in growth media, simulating the composition of phloem juice of a host plant, is described by Strnadová *et al.* (1986). Another example is, *e.g.,* acquisition of new strains with modified production qualities through regeneration of protoplasts (Schumann *et al.,* 1982, 1987).

The principles of mutagenesis of ergot alkaloid producers are the same as those used in bacteria and fungi. According to the literature, physical mutagenes are often used as mutational agents—UV light (Nordmann and Bärwald, 1981; Strnadová, 1964a, b), X-rays and gamma irradiation (Zalai *et al.,* 1990). Chemical agents include derivatives of N-nitrosoguanidine and nitrosourea, ethyl methane sulfonate (Keller, 1983) or their combinations (Řeháček *et al.,* 1978a), and nitrous acid (Strnadová and Kybal, 1976).

The simplest way is to expose to the chemical or physical mutagens a suspension of fungal spores. This technique facilitates the subsequent simple monosporic isolation, cultivation and selection of isolates originating from a single cell. Problems arise when asporogenic fungal strains are to be selected. In this case a suspension of hyphae or hyphal fragments can be directly exposed to a mutagenic agent, but it brings difficulties with culture heterogeneity in the subsequent transfer and selection. In this case it is advantageous to perform the mutagenesis on protoplasts (Křen *et al.,* 1988c). Protoplasts can be also prepared from spores of sporulating strains. When protoplasts are used, the mutation frequency is much higher (Baumert *et al.,* 1979b; Keller, 1983; Olasz *et al.,* 1982; Zalai *et al.,* 1990).

Mutants were also prepared with the ergot alkaloid biosynthetic pathway blocked on different levels (Maier *et al.,* 1980a; Pertot *et al.,* 1990). When supplemented by a modified precursor these strains can be employed for effective mutational biosynthesis (Erge *et al.,* 1981; Maier *et al.,* 1980b).

With *Claviceps* spp., breeding using DNA recombination can be done in two basic ways—meiotic recombination and fusion of protoplasts. In former method a corresponding strain is cultivated parasitically to form a sclerotium which, after its germination, then serves as the source of sexual ascospores (Tudzynski *et al.,* 1982; Vásárhelyi *et al.,* 1980b). Protoplast fusion methods have been therefore elaborated for the common species *C. purpurea, C. paspali* and *C. fusiformis*. A problem of genetic markers had to be solved since the markers of auxotrophy or resistance against fungicides in most cases negatively influence the alkaloid production level of progeny strains (DidekBrumec *et al.,* 1991b). To eliminate these disadvantages, methods were developed resulting in nearly 30% increase of production compared to parent strains (Didek-Brumec *et al.,* 1992, 1993). Interspecies hybrids that have been prepared by fusion of protoplasts from *C. purpurea*+*C. paspali* (Spalla and Marnati, 1981) and *C. purpurea*+*C. fusiformis* (Nagy *et al.,* 1994) represent further possibilities of selection of production strains. A question remains to what extent these hybrids will be stable during manifold transfers.

Application of the above methods is followed by selection of isolates having higher production capability or altered in some other way. The testing of all isolates in submerged cultivation on a shaker or by stationary surface cultivation imposes a high material requirements. Correlations were therefore studied between morfological and physiological characteristics and alkaloid production (Srikrai and Robbers, 1979). Selection methods based on pigmentation

(Borowski *et al.,* 1976; Kobel and Sanglier, 1976; Molnár *et al.,* 1964; Udvardy Nagy, E. *et al.,* 1964; Wack *et al.,* 1973), specific colour reaction (Zalai *et al.,* 1990), fluorescence (Gaberc-Porekar *et al.,* 1981, 1983), enzyme activity profiles (Schmauder and Gröger, 1983) or antimicrobial effects of clavine alkaloids (Homolka *et al.,* 1985) were worked out.

In the following steps an intimate selection can be performed on shakers or in laboratory fermentors for submerged or stationary cultivation. For the most promising isolates these works are organically interconnected with optimization of a medium and production conditions.

Developments of molecular biology and genetics of the genus *Claviceps* open new perspectives in obtaining of suitable production organisms (for more details see Chapter 4 in this book).

12.2.3. Maintenance Improvement

Selected high-yielding strains of *Claviceps* spp., similarly as those of other microorganisms, degenerate (Kobel, 1969). Also, problems of transfer of original cultures in a fermentation technological process are connected with this fact. Different producers of clavine alkaloids born to be transferred 6–9 times without decrease of producing capability (Malinka *et al.,* 1988).

It is necessary to maintain the optimal qualities of a selected production strain by two parallel ways—conservation and dynamic ones. The conservation way consists in keeping of stock cultures of the production strain under conditions of maximal possible elimination of biological effects given by transfer of cultures, ageing and other external influences. The dynamic way comprises systematically performed selection in the frame of the maintenance improvement, which consists in continual testing of monosporic isolates (in sporogenic lines) or at least hyphal isolates (in asporogenic lines) made from stock cultures of the production strain, and positive choice of a culture with optimal producing qualities for the subsequent work. This activity can be a part of optimization of other factors having an influence on a level and parameters of production of the final product. Here, there is also possible to apply the before-cited procedures of rational selection and apply selection pressure methods.

12.2.4. Long-Term Preservation of Production Strains

For long-term preservation of the production strains of *Claviceps* common methods used for other fungi can be applied as reviewed *e.g.* by Kirsop and Snell (1984) and by Hunter-Cervera and Belt (1996). Besides preservation of sporulated cultures on rye grains placed in a refrigerator or a deep-freezer it is also possible to keep frozen dried suspensions or gelatine disks. First of all non-sporulating strains, being more sensitive to different conservation procedures, can be preserved as cultures on agar plates under a mineral oil or, for a single use, as a suspension of mycelium at—18°C (Křen *et al.,* 1988c). Keeping of

lyofilized cultures and cultures frozen in liquid nitrogen are probably the most universal methods, though technically more complicated. Břemek (1981) compared these methods with different *Claviceps* strains and found that the both are suitable. During lyofilization diverse protective media are applied, *e.g.* serum, milk, peptone, sugars, sodium glutamate or combinations of previous (Chomátová *et al.,* 1985; Ustyuzhanina *et al.,* 1991). Procedures of lyofilization of non-sporulating strains are described by Křen *et al.* (1988c) and Pertot *et al.* (1977). A modification of the lyofilization process for preservation of cultures from regenerated protoplast was worked out by Baumert *et al.* (1979b).

As a theoretical alternative seems a method according that strains, which produce ergot alkaloids saprophytically, are preserved as sclerotia formed on an infected, proper host plant, *e.g.* rye for strains of the species *Claviceps purpurea*. Viability of the sclerotia when stored in refrigerator is several years. Questions of contingent changes of strain production characteristics due to alternation of saprophytic and parasitic phases were treated by Breuel and Braun (1981), and Breuel *et al.* (1982). During surface stationary production of peptide alkaloids it was possible to keep production strain in the form of dried mycelium at 4°C for 3 years without any influence to production capability (Kybal, Malinka, unpublished results).

As a source of production strains serve internationally established culture collections (*e.g.* ATCC, CBS, CCM, NRRL) or collections in certain institutes (*e.g.* MZKIBK—Cimerman *et al.,* 1992). However, industrially usable strains are mostly patented; in the collections they are stored according to the Budapest Convention and not commonly accessible.

12.3. FERMENTATION TECHNOLOGY

All species of ergot alkaloid producers from the *Pyrenomycetes* class as well as an overwhelming majority of other fungal producers are parasites of different plants and fungi. Principle of saprophytic cultivation is growth of a production fungi on a synthetic medium. The saprophytic cultivation makes possible better optimization of a production level, elimination of biosynthesis of accompanying undesirable matters and regulation of ergot alkaloids production through rational outside interventions. On the other side it is much more exacting on the technological equipment. All industrially adopted processes have the same basic aim—the maximal production of a matter with the minimum of undesirable compounds, got in the shortest time with minimized costs of medium, equipment and labour.

For ergot alkaloid manufacture different fermentation technologies can be employed. In principle, they can be produced by (i) stationary cultivation, when microorganisms are growing on the surface of a cultivation medium, both liquid (Abe, 1951; Kybal and Vlček, 1976; Malinka, 1988) and solid (Trejo-Hernández *et al.,* 1992; Trejo-Hernández and Lonsane, 1993), or (ii) submerged cultivation

with agitation of a suspension of microorganisms (Abe *et al.,* 1951; Amici *et al.,* 1966; Arcamone *et al.,* 1960; Bianchi *et al.,* 1976; Kobel and Sanglier, 1986). Semicontinuous and continuous cultivations (Kopp and Rehm, 1984; Křen *et al.,* 1986b) as well as those using immobilized microorganisms (Komel *et al.,* 1985; Kopp and Rehm, 1983; Křen *et al.,* 1989a) represent specific modifications of the submerged cultivation. General reviews of biosynthesis and production of ergot alkaloids were published by a number of authors (Esser and Düvell, 1984; Kobel and Sanglier, 1986; Křen *et al.,* 1994; Mantle, 1975; Řeháček, 1983a, b; 1984, 1991; Řeháček and Sajdl, 1990; Robbers, 1984; Sočič and Gaberc-Porekar, 1992; Udvardy Nagy, 1980). (For special cultivation procedures see the Chapter 7.)

In all types of fermentation it is necessary to use optimized media. Generally, the cultivation media should fulfill the same requirements as those for saprophytic cultivation of other fungi, *i.e.* they have to contain sources of energy, carbon, nitrogen, phosphorus and with advantage also certain trace elements and some complex matters. Price of the medium should always be taken into account and optimal variants be chosen from the point of view of costs per an unit operation. (For media components and physiology of production see Chapter 6.)

During the production phase, according to a kind of fermentation and elaboration of a given fermentation process, it is desirable to follow utilization of individual nutrients, activities of particular enzymes and a course of the proper synthesis of the alkaloids. It is also necessary to control and regulate basic physical and physico-chemical parameters of the culture—pH, dissolved oxygen concentration, dissolved carbon dioxide concentration, concentration of carbon dioxide in outlet, temperature, pressure, agitator speed or other physical characteristics typical for a given kind of fermentation and a type of fermentor used. During the pre-inoculation and inoculation phases, depending on requirements of their optimal course, only some fermentation parameters should be controlled and measured. Values of these parameters and their course during cultivation cannot be generalized for they are very often specific for the each production strain and the sort of the end product. To industrial production of the ergot alkaloids there are related pertinent regulations and requirements of the state and international institutions and offices (Priesmeyer, 1997). Basic pharmacopoieal demands on fermentation processes are presented by Anonymous (1997).

Specific problems of fermentation production of the ergot alkaloids consist in microbial contamination. Compared to an overwhelming majority of other secondary metabolites production processes, fermentation of the ergot alkaloids is marked by two negative factors—slow growth of mycelium on rich media and none or weak antibiotic activity of the produced alkaloids. In spite of reported antibacterial effect of clavine alkaloids (Eich and Eichberg, 1982; Eich *et al.,* 1995) probability of their pronounced exercise in autoprotection against contamination is only small. For these reasons an effect of broad spectrum antibiotics on the production strain of *C. fusiformis* W1 was investigated (Břemek *et al.,* 1986b; Křen *et al.,* 1986a); chloramphenicol was found as the most suitable antibiotic. Its use can be advantageous also in semicontinuous and continuous

processes (Křen *et al.,* 1985; Křen *et al.,* 1986b). Kopp (1987) having been working with immobilized cells of *Claviceps* used streptomycin. The positive effect of streptomycin and the negative one of oxytetracycline and nystatin on production of alkaloids by not closer specified strain of *Claviceps* described Slokoska *et al.* (1992).

12.3.1. Stationary Surface Cultivation

The stationary cultivation is commonly used for stock and starting cultures (growth on the surface of an agar medium) without respect what a kind of cultivation process will be used in the production phase. On the production scale there are in particular described processes where fungal mycelium capable to produce ergot alkaloids was growing on the surface of a liquid medium (Adams, 1962; Kobel *et al.,* 1962; Kybal *et al.,* 1960; Molnár *et al.,* 1964; Rochelmeyer, 1965; Stoll *et al.,* 1953; Strnadová *et al.,* 1981, 1986). Necessity of gaining the surface as large as possible under aseptic condition and difficulties of automation represent the main problems of that kind of cultivation. An equipment for the stationary cultivation representing a simple stationary fermentors (Figure 1) was developed (Vlček and Kybal, 1974; Kybal and Vlček, 1976) with plastic bags filled by the inoculated medium. The bags are manufactured by cross welding of a sterile polyethylene hose of a proper width. After that the bags are filled up by the inoculated medium and equipped by manifolds for controlled aeration (Malinka, 1982). During the following stationary fermentation, which is performed in a tempered room, the mycelium is growing on the surface of the medium. The water soluble alkaloids are excreted into the medium whilst the hydrophobic ones remain in the mycelium as intracellular metabolites. Manipulation with the filled cultivation bags can be performed by a high lift truck, commonly used in stores (Figure 2). Large cultivation area is an advantage of the plastic bag cultivation while the fact that each bag during long-term cultivation behaves as a separated fermentor made problems with product standardization. Even if manipulation with the bags is mechanized the load of workers during bag filling and harvesting of a mycelium produced is increased. More exacting cleaning of the used polyethylene foil prior to recycling represents a non-negligible aspect, as well. Into the industrial scale this method was introduced for production of ergocornine, and α- and *ß*-ergokryptine.

The stationary surface cultivation in the plastic bags can also be adopted for production of physiologically active asexual spores of production strains of *Claviceps purpurea* (Fr.) Tul., which are used as an infection agent at field parasitic cultivation of ergot (Harazim *et al.,* 1984; Valík and Malinka, 1992). This methods is employed for cyclosporin A production (Mat'ha, 1993; Mat'ha *et al.,* 1993), for cultivation of entomopathogenic fungi with the aim of production of spores used in manufacturing of bioinsecticides, and for cultivation of the mould *Trichoderma harzianum* producing mycofungicide (Kybal and Nesrsta, 1994; Nesrsta, 1989).

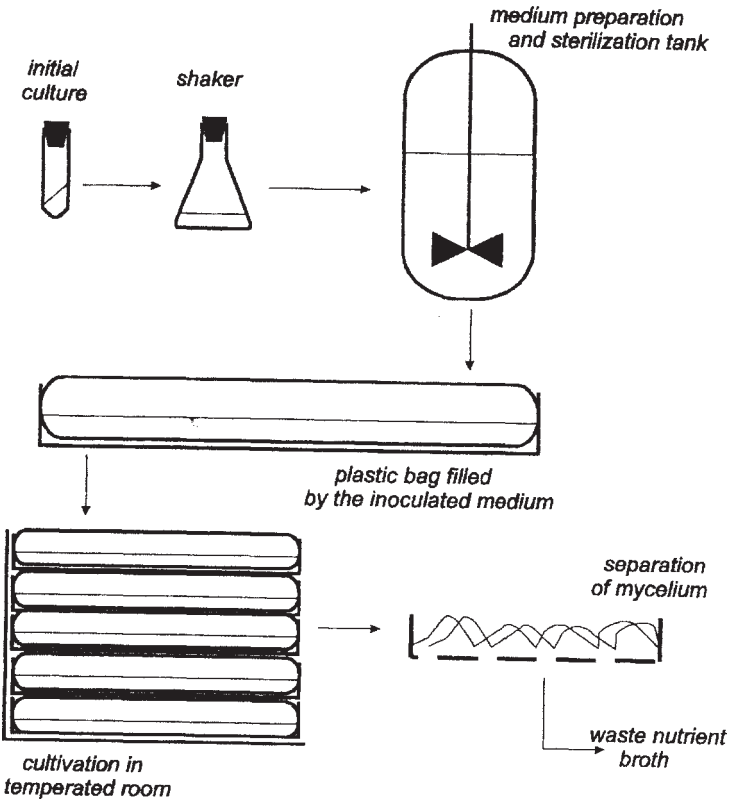

Figure 1 Flowsheet of stationary surface cultivation in plastic bags

Kybal and Strnadová (1982) described also other, technically more complicated equipments for stationary surface cultivation to prepare inoculum for field parasitic cultivation of ergot.

12.3.2. Submerged Cultivation

The submerged cultivation is used for manufacturing of different microbial products and of ergot alkaloids as well. By the means of laboratory scale submerged cultivation most of knowledge of biogenesis of the ergot alkaloids and physiology of their producers was gained. Contemporary expertise makes possible to control effectively individual production stages, influence biosynthesis of alkaloids and to a considerable extent eliminate unfavorable factors typical for selected high producing strains, such as *e.g.* loss of sporulating capability, production of glucans and minimal adaptation to variable cultivation conditions.

Figure 2 Stationary cultivation equipment

A basis of the submerged fermentation on laboratory scale as well as the primary step in an overwhelming majority of industrial scale processes is a shaker culture (Figure 3). In industrial scale production the aim of this cultivation is to obtain a sufficient amount of inoculum for the next cultivation step. Medium composition is subordinated to the aim of reaching fast germination of spores of production microorganism and fast growth of mycelial hyphae, or, as the case may be in specific processes, fast sporulation, and obtaining a mixture of hyphae and asexual spores.

For inoculation there is usually used a suspension of spores and/or hyphae of the aerial mycelium from the surface of primal cultures growing on agar solid media, or hyphal fragments when non-sporulating strains are worked with. Lyofilized cultures or microorganisms kept in liquid nitrogen can be also used as an inoculating material.

Preparation of the shaker cultures is usually made on rotary shakers. If for some production strains less mechanical stress of hyphae is more suitable, reciprocal shakers can be used. The shaker culture can be replaced by a culture from a laboratory fermentor.

The following steps are always run in fermentors. Volume of the end production step is decisive for the number of previous cultivation steps for propagation of necessary amount of inoculum. During the inoculum preparation it can be also advantageously manipulated to evoke an optimal state of the

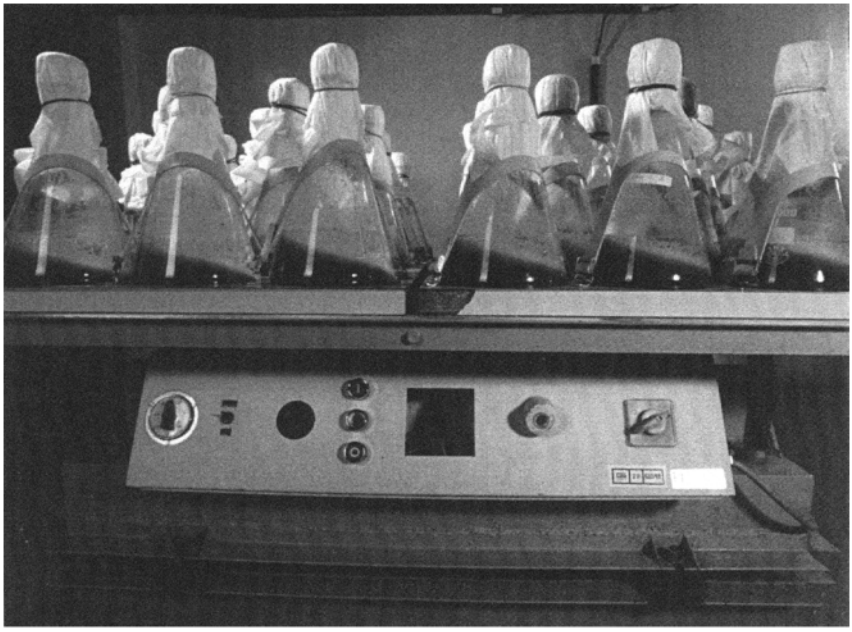

Figure 3 Cultivation of shaker culture

culture for biosynthesis of ergot alkaloids in the production step (Sočič *et al.*, 1985, 1986). Most often there are three cultivation steps, *viz.* cultivation in pre-inoculating tanks, seed tanks and production fermentors (Figure 4). Increasing the number of the cultivation steps is usually undesirable for with increasing transfers production capability of the culture is diminished. This fact limits also the possibility of recirculation of a part of the cultivation medium from the production step for inoculation of the following cultivation.

Similarly as at other filamentous fungi, during *Claviceps* cultivation fermentors have to be used enabling to work with viscous media. Non-newtonian character of liquid flow becomes obvious only during the course of cultivation, on the one hand due to growth of hyphal filaments, on the other hand because of production of glucans.

12.3.3. Alternative Fermentation Processes

Ergot alkaloids can be manufactured also by alternative fermentation processes, *e.g.* by those using nontraditional substrates or immobilized cells of *Claviceps* spp. or their subunits. Semicontinuous or continuous cultivations represent other alternatives of the saprophytic cultivation.

Stationary solid state cultivation on the surface of solid substrates soaked by a liquid medium has been reported by Trejo Hernández *et al.* (1992) and Trejo Hernández and Lonsane (1993). In these studies, growth and production of

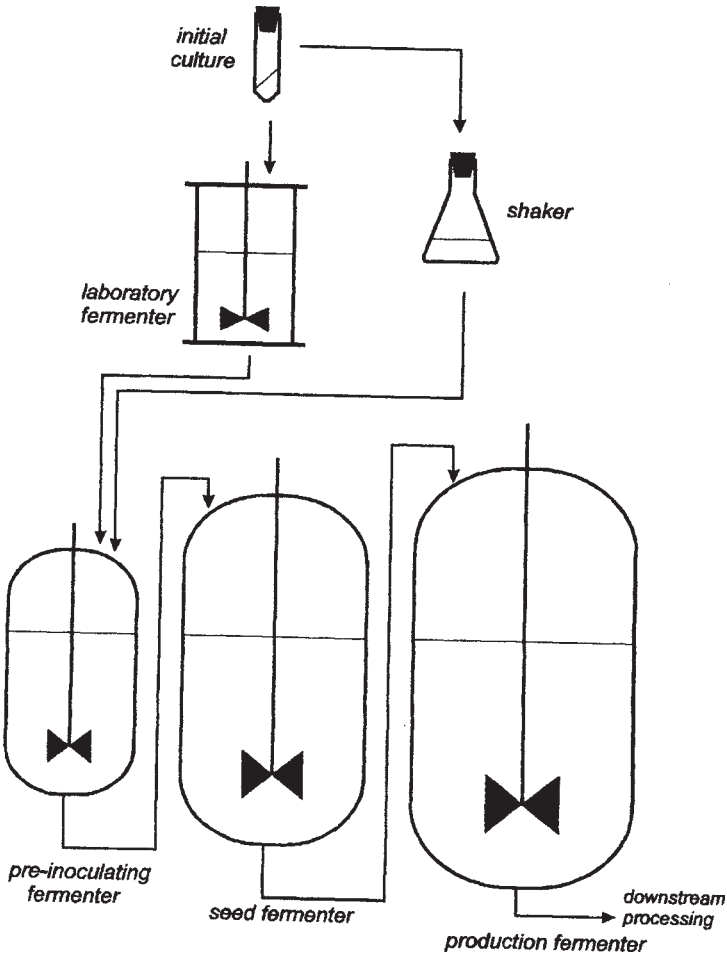

Figure 4 Flowsheet of submerged cultivation

alkaloids were investigated with different species of the genus *Claviceps* on sugarcane pith bagasse and significant dependence of the production level and the spectrum of synthesized alkaloids on composition of the medium used for solid substrate impregnation was found. However, there is discussible a possibility of application of this process on the industrial scale.

Other alternative types of cultivation are described in Chapter 6.

12.4. MANUFACTURE OF CLAVINE ALKALOIDS

After transfer to a saprophytic culture a number of parasitic strains of ergot, both wild or improved, is able to synthesize only clavine alkaloids and lose the

ability to perform subsequent biosynthetic steps. For this reason a vast number of production strains exists, most frequently of the species *Claviceps purpurea, e.g.* CP 7/274 CCM F-632 (Řeháček *et al.,* 1978b), 88-EP/1988 (Křen *et al.,* 1988c), IBP 182 ZIMET 43673 (Schumann *et al.,* 1984), IBP 180 ZIMET PA 138 (Baumert *et al.,* 1979a), Pepty 695/e (Erge *et al.,* 1984), Pepty 695/ch-I (Gröger *et al.,* 1991; Maier *et al.,* 1988a,b), 59 CC 5/86 (Řeháček *et al.,* 1986a), SL 096 CCM F-733 (Flieger *et al.,* 1989b), EK 10 (Pazoutová *et al.,* 1990), AA218 (Harris and Horwell, 1992), CBS 164.59 (Kopp, 1987) and *C. fusiformis, e.g.* W1 (Křen *et al.,* 1985), F 27 (Křen *et al.,* 1985), MNG 00211 (Trinn *et al.,* 1983), NCAIM 001107 (Trinn *et al.,* 1990), CF 13 (Rozman *et al.,* 1985, 1987).

The species *Claviceps paspali* has been reported only sporadically, *e.g.* strain DSM 2838, a producer of festuclavine (Wilke and Weber, 1985a) and strains Li 342 (ATCC 34500) (Erge *et al.,* 1972) and Li 342/SE 60 (Gröger, 1965), producers of chanoclavine-I; the same holds for not closely identified strains of the genus *Claviceps, e.g.* DSM 2837, which produces chanoclavine (Wilke and Weber, 1985b), IBFM-F-401, a producer of elymoclavine (Kozlovsky *et al.,* 1978), and the strains 47A and 231, from whose cultures norsetoclavine was isolated for the first time (Ramstad *et al.,* 1967).

The patent literature reports, besides the genus *Claviceps,* also strains *Hypomyces aurantus* IFO 773, which produce ergocornine, agroclavine, elymoclavine and chanoclavine (Yamatoya and Yamamoto, 1983), and *Penicillium corylophillum* IBFM-F-152 (Kozlovsky *et al.,* 1979), which produces epoxyagroclavine I.

A number of clavine alkaloids, in addition to the mentioned fungal species, was isolated also from *Claviceps gigantea, Claviceps* spp. originating from different host plants, fungi of the *Penicillium, Aspergillus, Rhizopus* and other genera, and from seeds of plants of the *Convolvulaceae* family. A review was compiled by Flieger *et al.* (1997). Apart from the genus *Claviceps,* nothing is known about other fungal species employed in the selection of industrially applicable production strains, except for the two above cases. It is, however, possible that some isolates of *Claviceps* sp., mentioned in connection with the isolation of certain clavine alkaloids, served as a starting material in the selection of production strains (Stoll *et al.,* 1954b).

12.4.1. Production of $\Delta^{8,9}$-Ergolenes

Industrial processes for manufacture of agroclavine and elymoclavine are best elaborated among the methods for acquiring of clavine alkaloids. Due to the direct biosynthetic succession of these alkaloids they are usually produced in mixtures (Adams, 1962; Baumert *et al.,* 1979b; Břemek *et al.,* 1986c, 1989; Erge *et al.,* 1984; Řeháček *et al.,* 1978a, b, c; 1984a; 1986b; Řeháček and Rylko, 1985; Takeda Pharm. Ind. 1956; Trinn *et al.,* 1983; Wack *et al.,* 1966; Windisch and Bronn, 1960; Yamatoya and Yamamoto, 1983).

As a substrate for subsequent chemical operations elymoclavine is superior to agroclavine and efforts were therefore made to develop processes leading to elymoclavine production with maximal possible elimination of agroclavine. Generally, this goal can be achieved by selecting proper production strains and by optimizing cultivation conditions. The direct biosynthetic succession of the two alkaloids makes it possible to use also bioconversion processes.

Production strains for elymoclavine manufacture and general cultivation conditions have been described in a number of patents (Kozlovsky *et al.,* 1978; Křen *et al.,* 1985, 1988c; Řeháček *et al.,* 1984b; Schumann *et al.,* 1984; Trinn *et al.,* 1983, 1990). In addition to the patent literature many works refer to results of investigation of individual aspects of physiology and biochemistry of clavine alkaloid synthesis. These works were done either directly with production strains, *e.g. C. purpurea* 129 (later classified as *C. fusiformis*) producing 4500–7000 mg L^{-1} and isolates selected from it (Desai and Řeháček, 1982; Křen and Řeháček, 1984; Křen *et al.,* 1984, 1987; Pazoutová *et al.,* 1977, 1980, 1981; Pazoutová and Řeháček, 1978, 1981a, b, 1984; Řeháček *et al.,* 1977; Sajdl *et al.,* 1978; Voříšek *et al.,* 1981) or with strains of different provenance, *e.g. Clavices* sp. SD-58 (ATCC 26019) (later classified as *C. fusiformis;* Desai *et al.,* 1982a, 1983, 1986; Eich and Sieben., 1985; Kozikowski *et al.,* 1993; Křen *et al.,* 1987; Otsuka *et al.,* 1980; Patel and Desai, 1985; Robbers *et al.,* 1972, 1978, 1982; Robertson *et al.,* 1973; *Rylkoetal.,* 1986, 1988a; Schmauder *et al.,* 1981a, b, 1986; Vaidya and Desai, 1981a, b, 1982, 1983a, b), *C. purpurea* 59 *(C. fusiformis)* (Pazoutová *et al.* 1986, 1987a, b, 1988, 1989, 1990; Pazoutová and Sajdl, 1988; Sajdl *et al.,* 1988b), *Claviceps* sp. CP II (Krustev *et al.,* 1984; Slokoska *et al.,* 1981, 1985, 1988) and *Claviceps* sp. PRL 1980, ATCC 26245 (Kim *et al.,* 1981; Taber, 1964).

Bioconversion of agroclavine to elymoclavine can be done by both free and immobilized cells of suitable production strains (Břemek *et al.,* 1986a; Křen *et al.,* 1989a) with efficiency of up to 97% (Malinka and Břemek, 1989). In addition to strains producing clavine alkaloids, also those synthesizing simple derivatives of lysergic acid can be used for the conversion. In this case a preferential bioconversion of agroclavine to elymoclavine can be brought about by a simple modification of cultivation conditions (Flieger *et al.,* 1989a; Harazim *et al.,* 1989). Flieger *et al.,* 1989b described also a process of purification of clavine alkaloids combined with a conversion to elymoclavine and lysergic acid α-hydroxyethylamide; these products can be easily separated and used for semisynthesis. Other strains able to convert agroclavine to elymoclavine are *C. fusiformis* SD-58, and *Claviceps* sp. KK-2, Se-134 and 47A (Sieben *et al.,* 1984). An exhaustive review on the bioconversion of ergot alkaloids was worked out by Křen (1991) and a review can be also found in Chapter 10.

During the cultivation of commonly used strains, growth of a culture and production of clavine alkaloids are accompanied by the concurrent biosynthesis of glucans. These compounds unfavourably influence medium rheology,

complicate proper mixing and aeration, slow down oxygen transfer and make the cultivation medium foam. Processes elaborated to eliminate glucan production employ a special composition of an inoculation medium, two-stage preparation of inoculum and a special composition of a production medium (Břemek *et al.,* 1986c). In this way, physiological conditions are reached which decrease or eliminate the synthesis of glucans and, at the same time, have a positive effect on alkaloid biosynthesis. Production of up to 4600 mg L^{-1} of total alkaloids (out of which 2300 mg L^{-1} is due to elymoclavine) in shaker cultures has been reported; in fermentors the production reaches 2836 mg L^{-1} (2322 mg L^{-1} of elymoclavine). The concentration of undesirable of glucans can be decreased by the addition of 0.4–0.5 g L^{-1} of sodium phenobarbitale from the original 38.4–42.2 g L^{-1} to 0–11 g L^{-1} (Řeháček and Rylko 1985). An addition of barbiturates into a medium, influencing cytochrome P-450, was also described by Trinn *et al.* (1983) but without relationship to glucan suppression. Processes with feedback inhibition of glucan-synthesizing enzymes induced by addition of glucans into cultivation media during inoculation were proposed (Kybal, personal communication). The use of specific production strains with lowered or eliminated glucan production, such as *Claviceps purpurea* CP 7/5/35 CC-2/1985 (Řeháček *et al.,* 1984a), seems to be economically optimal. This strain produces a mixture of 10–30% of elymoclavine, 65–90% of agroclavine and 1–5% of chanoclavine—I. However, the employment of the strain *Claviceps purpurea* 88-EP/1988 (Křen *et al.,* 1988c) is more advantageous since the strain produces nearly 2500 mg L^{-1} of elymoclavine and this alkaloid represents almost 90% of total alkaloids. The use of two inoculation stages for clavine producers is suitable not only for elimination of glucan production but also for reaching an optimum physiological state for maximal biosynthesis of the alkaloids. The production of alkaloids by the strain *C. fusiformis* W1 is decreased by 11.4–57.8% (Malinka *et al.,* 1986) when a single-stage inoculum is used.

An ihibition effect of phosphate ions upon biosynthesis of alkaloids plays an important role and a positive effect of phosphate deficiency was described already by Windisch and Bronn (1960). The problem consists in the fact that phosphate is necessary for biomass growth; it is therefore necessary to find an optimal ratio between biomass growth (and proliferation of cells able to produce alkaloids) and the alkaloid synthesis rate. Most of the processes described here employ a low content of phosphate in production media combined with the use of a dense inoculum. Some production strains are marked by a higher resistance of alkaloid biosynthesis to phosphates (Řeháček *et al.,* 1984a). This problem was solved in a particular way in the patent of Břemek *et al.* (1989) by using gradually utilized hexaamidotriphosphazene as a phosphate source and at the same time as a supplementary source of nitrogen.

Křen *et al.* (1989b) described a process of production of fructosides of elymoclavine, namely elymoclavine-*O*-*ß*-D-fructofuranoside (Floss *et al.,* 1967) and elymoclavine-*O*-*ß*-D-fructofuranosyl-(2→1)-*O*-*ß*-D-fructofuranoside (Flieger *et al.,* 1989d). The efficiency of glycosylation fluctuated between 10

and 62% . The strain *C. purpurea* 88-EP-47 was selected for the preparation of fructosides of elymoclavine (Křen *et al.,* 1989c); during fermentation this strain produces fructosides in a concentration of 920 mg L^{-1} while the concentration of the total alkaloids reached 2800 mg L^{-1}. Due to the high glycosylation activity the strain could be used for production of fructosides of alkaloids added to cultures (Křen *et al.,* 1989b). Glycosides of ergot alkaloids exhibit interesting physiological effects and can be also used as substrates for the preparation of semisynthetic derivatives.

12.4.2. Production of 6, 7-secoergolenes

Besides agroclavine and elymoclavine, also other clavine alkaloids can be used for the preparation of certain semisynthetic derivatives. Although no derivatives prepared by modification of the clavine molecule are used in therapy, some preparation procedures yielding such clavine alkaloids are protected by patents. Among 6, 7-secoergolenes, *i.e.,* alkaloids with an open ring D of the ergoline structure, chanoclavine-I and chanoclavine-I aldehyde have been patented.

Production processes employing specific production strains are also protected by patents. Thus the production of chanoclavine-I or a mixture of chanoclavine-I and chanoclavine-I aldehyde has been described because all chanoclavine-I aldehyde represents a suitable substrate for subsequent semisynthesis. Wilke and Weber (1985b) described a method of chanoclavine manufacture with the strain *C. purpurea* DSM 2837 giving 390 mg L^{-1} of the alkaloid. Baumert *et al.* (1979a) reported on the use of the strain *C. purpurea* IBP 180, ZIMET PA 138, in which the total production of alkaloids was 500–600 mg L^{-1} and this amount comprised 80% of chanoclavine-I and 20% of chanoclavine-I aldehyde. Maier *et al.* (1980a, b) and Baumert and Gröger (1982) described another strain, denoted Pepty 695/ch, which produced chanoclavine-I and chanoclavine-I aldehyde; these secoergolenes were produced in a concentration of 300–350 mg L^{-1} in a ratio of 3:1 (Erge *et al.,* 1984). A substantially higher production was mentioned by Řeháček *et al.* (1986a) for the strain *C. purpurea* 59 CC5/86 selected from the parent strain *C. purpurea* 129 (Pazoutová *et al.,* 1987a) which produced as much as 3000–6000 g L^{-1} of total alkaloids, composed of 40–60% chanoclavine-I, 20–30% chanoclavine-I aldehyde, 10–15% elymoclavine and 5–10% agroclavine. Besides the patent literature, Gröger (1965) described the strain *C. paspali* Li 342/SE 60 producing 400 mg L^{-1} of alkaloids, 40% of which was chanoclavine. Chanoclavine-I was isolated not only from fungi of the genus *Claviceps* (Abe *et al.,* 1959; Agurell and Ramstad, 1965; Hofmann *et al.,* 1957; Stauffacher and Tscherter, 1964), but also from other fungi—*Penicillium concavo-rugulosum* (Abe *et al.,* 1969), *Aspergillus fumigatus* (Yamano *et al.,* 1962) and *Hypornyces aurantius* (Yamatoya and Yamamoto, 1983).

12.4.3. Production of Ergolines

Processes for the production of festuclavine and epoxyagroclavine I from the group of ergolines are described in patents. Festuclavine was isolated from cultures of *Aspergillus fumigatus* and from sclerotia of *Claviceps gigantea* (Agurell and Ramstad, 1965). In the patent of Wilke and Weber (1985a) a method of production of festuclavine is described using the production strain *C. paspali* 2338. During a 7–9-day cultivation the concentration of festuclavine reached 2280 mg L^{-1}. Epoxyagroclavine I has so far been found only as a metabolite of *Penicillium corylophilum* (Kozlovsky *et al.,* 1982) and process of its production is patented (Kozlovsky *et al.,* 1979).

12.4.4. Production of $\Delta^{9,10}$-Ergolenes

Lysergol as well as isolysergol from the group of $\Delta^{9,10}$-ergolenes can be used as suitable substrates for the production of semisynthetic derivatives. Lysergol, together with lysergene and lysergine were isolated from the saprophytic fungi *Claviceps* spp. originating from ergot parasitizing on *Elymus mollis* (Abe *et al.,* 1961). Isolysergol was isolated from the saprophytic cultures of *Claviceps* sp. 47 A derived from ergot parasitizing on *Pennisetum typhoideum* (Agurell, 1966).

12.5. LYSERGIC ACID, ITS SIMPLE DERIVATIVES AND PASPALIC ACID

This group of ergot alkaloids encompasses both compounds directly applicable in therapy (ergometrine) and compounds, which can be employed for the production of semisynthetic alkaloids (lysergic acid, ergine, lysergic acid α-hydroxyethylamide and their isomers, paspalic acid).

Lysergic acid and paspalic acid were isolated in 1964 from cultures of *C. paspali* (Kobel *et al.,* 1964) and in 1966 from cultures of *C. purpurea* (Castagnoli and Mantle, 1966). Ergine (Arcamone *et al.,* 1961; Kobel *et al.,* 1964) and lysergic acid α-hydroxyethylamide (Arcamone *et al.,* 1960; Flieger *et al.,* 1982) were also isolated from the cultures of *C. paspali*. Ergometrine (ergonovine) was isolated from both *C. purpurea* (Stoll, 1952) and *C. paspali* (Kobel *et al.,* 1964).

Analogously to clavine alkaloids, a number of ergolene-production strains has been isolated. Strains of the genus *Claviceps* for direct biosynthesis of lysergic acid, paspalic acid and lysergic acid α-hydroxyethylamide were obviously selected only from the species *Claviceps paspali* that grows on grasses of the genus *Paspalum* in diverse parts of the world, *e.g.* strains *C. paspali* F-140 (ATCC 13895), F-S 13/1 (ATCC 13892), F-237 (ATCC 13893), F-240 (ATCC 13894) (Chain *et al.,* 1960), NRRL 3027, NRRL 3166 (Rutschmann and Kobel, 1963b), NRRL 3080, NRRL 3167 (Kobel and Schreier, 1966; Rutschmann *et al.,* 1963), ATCC 14988 (Tyler, 1963), C-60 and its derivatives (Mary *et al.,* 1965), FA

CCM F-731 (Řičicová *et al.,* 1982b), CP 2505, YU 6 (Harazim *et al.,* 1986), CCM 8061 (Flieger *et al.,* 1989a), CCM 8063 (Harazim *et al.,* 1989), CCM 8176 (Satke *et al.,* 1994).

The sporogenic strain *C. paspali* MG-6 played for ergolenes a similar role as the strain *Claviceps fusiformis* SD-58 for clavine alkaloids—the basic knowledge of physiology and biochemistry of formation of simple derivatives of lysergic acid was gained using this organism (Bumbová-Linhartová *et al.,* 1991; Linhartová *et al.,* 1988; Řeháček and Malik, 1971; Řeháček *et al.,* 1971; Rylko *et al.,* 1988d). The same holds for the strains *C. paspali* 31 (Rosazza *et al.,* 1967) and L-52, identical with the strain ATCC 13892 (Sočič *et al.,* 1986). Mantle (1969) described the production of a mixture of lysergic and paspalic acids by saprophytic strains, not selected by mutagenesis, isolated from ergotoxine containing sclerotia of *C. purpurea*. Philippi and Eich (1984) demonstrated the bioconversion of elymoclavine to lysergic acid by the strain *C. paspali* SO 70/5/2, Maier *et al.* (1988b) reported on an analogous bioconversion using a microsomal fraction of the ergopeptine producer *C. purpurea* Pepty 695/S. Besides the genus *Claviceps* formation of lysergic acid and its derivatives has been reported in a number of strains of different species of the genus *Aspergillus (A. clavatus, A. repens, A. umbrosus, A. fumigatus, A. caespitosus, A. nidulans, A. ustus, A. flavipes, A. versicolor, A. sydowi, A. humicola, A. terreus, A. niveus, A. carneus, A. niger, A. phoenicus)* (Siegle and Brunner, 1963).

12.5.1. Production of Simple Derivatives of Lysergic Acid

Production of amides of lysergic acid is described more often than the production of the acid itself. These amides are isomers of lysergic acid α-hydroxyethylamide; lysergic acid can be prepared from them by bioconversion. Amici *et al.* (1963) described bioconversion with 95% efficiency in cultures of *Claviceps purpurea* without further specification. Some production strains are very sensitive to surplus iron ions (Chain *et al.,* 1960; Řičicová *et al.,* 1982b) or they require ions of iron and zinc, and sometimes also other inorganic ions, in defined proportions (Mary *et al.,* 1965; Rutschmann and Kobel, 1963b). The process reported by Chain *et al.* (1960) needs so-called virulentation of the strain in a rye embryo to get sufficient production. Concentrations between 450 and 1600 mg L^{-1} are reached during the submerged cultivation. Iron ions did not interfere with the process described by Tyler (1963). The procedure according to Řičicová *et al.* (1982a) employed the asporogenic production strain *C. paspali* FA CCM F-731; that brought problems with the preparation of a standard inoculum for the production phase. The strain produced over 2000 mg L^{-1} of alkaloids from which 80% was lysergic acid α-hydroxyethylamide. Řičicová *et al.* (1981, 1986) also reported on the strain *C. paspali* F 2056 that produced nearly 2000 mg L^{-1} of alkaloids with the same proportion of lysergic acid α-hydroxyethylamide. Production of max. 2000 mg L^{-1} of simple derivatives of lysergic acid was described using the strain *C. paspali* ATCC 13892 and optimized cultivation

conditions (Pertot *et al.*, 1984). Rutschmann and Kobel (1963b) reported that the strain *C. paspali* NRRL 3027 had formed over 1000 mg L^{-1} of alkaloids. The concentration of total alkaloids in a shaker culture of the strain NRRL 3166 reached as much as 2210 mg L^{-1} from which 80% was formed by amides of lysergic and isolysergic acids; in a fermentor the concentration was 1820 mg $L^{-1,}$ with 87% of amides. Harazim *et al.* (1986) dealt with the optimization of the inoculation phase of lysergic acid α-hydroxyethylamide production. The asporogenic strain *C. paspali* CP 2505 and the sporogenic one YU 6, selected from natural material of a different geographic origin, were found to have the same requirements for optimal media composition. Pertot *et al.* (1990) reported the strain *C. paspali* L-52 which produced as much as 2647 mg L^{-1} of a mixture of ergometrine, lysergic acid amide and lysergic acid α-hydroxyethylamide, and from it selected a mutant CP 2 with a totally blocked synthesis of ergometrine and with the production of as much as 1552 mg L^{-1} of lysergic acid derivatives. Flieger *et al.* (1989a) and Harazim *et al.* (1989) described the production strains of *C. paspali* mentioned earlier in connection with biotransformation of agroclavine to elymoclavine. The strain CCM 8061 (Flieger *et al.*, 1989a) produced 1220 mg L^{-1} of simple derivatives of lysergic acid; at the same time it showed a high activity of bioconversion (almost 95%) of clavine alkaloids to simple derivatives. Also, by adding clavines the actual biosynthesis of lysergic acid derivatives was increased by 33.5%. The strain could also be used in the immobilized form for semicontinuous production of lysergic acid derivatives by *de novo* biosynthesis and/or by clavine conversion. The strain CCM 8063 (Harazim *et al.*, 1989) is characteristic by the production of lysergic acid α-hydroxyethylamide in concentrations of up to 2200 mg L^{-1}. When clavine alkaloids were added to the medium nearly, 5070 mg L^{-1} of lysergic acid α-hydroxyethylamide was produced as a consequence of their concurrent conversion. A semicontinuous process was also described using cells of this strain entrapped in alginate. Procedures reported for these two strains were later worked out to produce simple derivatives of lysergic acid, first of all its α-hydroxyethylamide, ergometrine and partially also ergine, by means of aggressive bioconversion of clavine alkaloids (Flieger *et al.*, 1989b); induction of lysergic acid derivatives took place at the same time. In a batch cultivation, the concentration of lysergic acid derivatives reached almost 5400 mg L^{-1} while in a large scale industrial fermentor the concentration was 2920 mg L^{-1}. The concentration of total alkaloids in cultures of the strain *C. paspali* CCM 8062 after clavine conversion reached 2130 mg L^{-1}; out of this amount 78% was ergometrine, 11% ergine and 11% lysergic acid α-hydroxyethylamide. A mixed cultivation of the strain *C. purpurea* CCM F-733 (producer of clavine alkaloids) and *C. paspali* CCM 8061 yielded 4890 mg L^{-1} of alkaloids during a fortnight cultivation; alkaloids suitable for semisynthesis made up 97.3% (lysergic acid α-hydroxyethylamide 73%, elymoclavine 24.3%). A mixture of lysergic acid α-hydroxyethylamide and ergine was also produced by the strain *C. paspali* MG-6. Derivatives of lysergic and paspalic acids—8-hydroxyergine and 8-hydroxyerginine (Flieger *et al.*, 1989c), and

10-hydroxy-*cis*- and 10-hydroxy-*trans*-paspalic acid amide (Flieger *et al.,* 1993)—were isolated from the culture medium of this strain in the post-production phase. The bioconversion of elymoclavine to ergine by the strain *C. paspali* LI 189 + was described by Mothes *et al.* (1962). Matošić *et al.* (1988a, b) used an immobilized strain of *C. paspali* which produced a mixture of lysergic acid α-hydroxyethylamide and ergometrine. He also tried to increase the production by means of surfactants. During a 60-day cultivation with six medium replacements the total production of alkaloids reached 8290 mg L^{-1}.

12.5.2. Production of Ergometrine

Ergometrine was isolated from both *Claviceps purpurea* (Stoll, 1952) and *C. paspali* (Kobel *et al.,* 1964) cultures. There is a number of described strains of the both species that produce ergometrine: *C. paspali* CCM 8062 (Flieger *et al.,* 1989b), NRRL 3081, NRRL 3082 (Rutschmann and Kobel, 1963a), ATCC 13892 (Gaberc-Porekar *et al.,* 1987), *C. paspali* without additional marking, isolated from *Paspalum commersonii* (Janardhan and Husain, 1984), *C. purpurea* IMET PA 130 (ZIMET 43769), IMET PA 135 (ZIMET 43695) (Borowski *et al.,* 1976; Volzke *et al.,* 1985), NCAIM 001106 (Zalai *et al.,* 1990), OKI 22/1963 (Molnár *et al.,* 1964; Udvardy-Nagy, I. *et al.,* 1964), OKI 620 125 (Molnár and Tétényi, 1962), Pepty 695 (Baumert and Gröger 1982, Erge *et al.,* 1972), PRL 1578 (ATCC 14934) (Taber and Vining, 1958). Ergometrine is formed biosynthetically via the intermediate lysergylalanine, the common precursor of ergoptinyle (Řeháček and Sajdl, 1990). *Claviceps purpurea* which, unlike *C. paspali,* is able to synthesize ergopeptine alkaloids, normally produces ergometrine together with a certain amount of ergopeptines. Both components are easily separable and most of ergopeptines find application in therapy.

Stoll *et al.* (1953) described a surface cultivation of *Claviceps purpurea* during which low amounts of ergometrine and ergotamine are formed in strict dependence on the concentration of iron and zinc ions, similarly as in lysergic acid amide production (Rutschmann and Kobel, 1963b). Windisch and Bronn (1960) reported on cultivations in which production of clavines, ergometrine and ergopeptines was induced by anaerobic conditions elicited by respiration inhibition. The process could hardly be implemented on the industrial scale, because of a very low production and other factors. Later, more efficient processes were developed having with the aid of better production strains. Molnár *et al.* (1964) described the production of a mixture of ergometrine and ergotoxine, rich in ergocristine, by submerged as well as surface cultivation yielding a minimum concentration of alkaloids 300 mg L^{-1}. A patent of Molnár and Tétényi (1962) described a production of a mixture of ergometrine, ergokryptine and ergocornine during both stationary and submerged cultivations. In the surface cultivation, the concentration of alkaloids in the mycelium was 0.6%, 30% of which was ergometrine, in the submerged one the total alkaloid production was 480 mg L^{-1}. The process according to Rutschmann

and Kobel (1963a) made use of the production strains *C. paspali* NRRL 3081 and 3082; they formed higher concentrations of a product without ergopeptines. Gaberc-Porekar *et al.* (1987) published data on the asporogenic strain *C. paspali* ATCC 13892 that produced 1200 mg L^{-1} of alkaloids consisting from 50–60% by ergometrine and from 25–30% by lysergic acid α-hydroxyethylamide. Using a mutagenic effect of gamma irradiation, they selected from this strain a daughter one able to form conidia. In the process of Zalai *et al.* (1990) the production strain *C. purpurea* NCAIM 001106 was selected by mutagenesis of protoplasts. The produced mixture of alkaloids contained 1100 mg L^{-1} of ergometrine, 450 mg L^{-1} of ergocornine and 600 mg L^{-1} of ergokryptine. Borowski *et al.* (1976) described submerged cultivations of the strain *C. purpurea* IMET PA 130 where the total alkaloid concentrations reached 2430–2460 mg L^{-1}, out of which the ergotoxine group ergopeptines comprised 1150–1300 mg L^{-1} and ergometrine 340–550 mg L^{-1}. The procedures were further elaborated by Volzke *et al.* (1985) who used the strains IMET PA 130 (ZIMET 43769) and IMET PA 135 (ZIMET 43695); by changing the limitation and/or nutrient sources they were able to change the proportions of ergometrine and individual alkaloids of the ergotoxine group. The total concentration of alkaloids was as high as 4000 mg L^{-1}; under different cultivation regimes ergometrine was produced in concentrations between 420 and 800 mg L^{-1}. The maximal proportion of ergometrine was reached when urea was used together with partial limitation by the phosphorus source, oxygen saturation was kept at 48–86% and pH under 7. The process described by Flieger *et al.* (1989b), in which the strain *C. paspali* CCM 8062 produced 1660 mg L^{-1} of ergometrine and small amounts of ergine and lysergic acid amide when clavine alkaloids were added as precursors, has been mentioned earlier. The paragraph concerning clavine alkaloids also report on the production of ergometrine mixed with clavine alkaloids by immobilized cells of the strain *C. purpurea* CBS 164.59 (Kopp, 1987).

12.5.3. Production of Paspalic Acid

Paspalic acid is another suitable substrate for preparation of semisynthetic derivatives. The patent literature contains description of its production by strains *C. paspali* NRRL 3080, and NRRL 3167 (Kobel and Schreier, 1966, Rutschmann *et al.*, 1963) and *C. paspali* CCM 8176 (Satke *et al.*, 1994). The strain NRRL 3167 formed 3330 mg L^{-1} of total alkaloids, out of which paspalic acid represented 89%. The strain CCM 8176, in dependence on sugar and organic acid components used, produced as much as 7927 mg L^{-1} of total alkaloids. Paspalic acid formed 54.4% (4257 mg L^{-1}) and the rest was formed by isopaspalic, lysergic and isolysergic acids. A cell-free extract of the strain *C. purpurea* PCCE1 was able to convert elymoclavine to paspalic acid with a 95% efficiency (Kim and Anderson, 1982; Kim *et al.*, 1983).

12.6. PRODUCTION OF ERGOPEPTINES

12.6.1. Production of Ergotamine Group Alkaloids

So far, ergotamine is the only natural alkaloid from the ergotamine group of ergopeptines which has found a therapeutical use. It was detected only in the strain *Claviceps purpurea* (Flieger *et al.,* 1997; Stoll, 1952). The first isolation from saprophytic mycelia was shown by Kybal and Starý (1958). The fermentative production of ergotamine was performed with many strains—*C. purpurea* IBP 74, IMET PA 135 (Baumert *et al.,* 1979b, c), JAP 471 (Erge *et al.,* 1984; Schmauder and Gröger, 1986), JAP 471/1 (Maier *et al.,* 1983), I.M.I. 104437 (ATCC 15383) (Amici *et al.,* 1964), 275 F.I. (Amici *et al.,* 1966, 1967a; Crespi-Perellino *et al.,* 1981; Floss *et al.,* 1971b), F.I. 32/17 (ATCC 20102) (Amici *et al.,* 1968; Keller *et al.,* 1980) and its derived strain 1029 (Keller *et al.,* 1988; Lohmeyr and Sander, 1993); L-4 (ATCC 20103) (Komel *et al.,* 1985), CP II (Sarkisova and Smirnova, 1984), 312-A (Sarkisova, 1990; Ustyuzhanina *et al.,* 1991).

In addition to the process mentioned earlier, which produces small amounts of ergotamines (Windisch and Bronn, 1960), other processes were successively developed with higher industrial utility. Kybal *et al.* (1960) described both surface and submerged cultivation of non-specified strains *Claviceps purpurea*. In the surface cultivation the yield of ergotamine was 0.14% in dry biomass, while in the submerged one 0.07% . Amici *et al.* (1964) working with the strain I.M.I. 104437, obtained as much as 1300 mg L^{-1} of ergotamine. In their experiments with the strain 275 F.I., which produced 1–150 mg L^{-1} of alkaloids, Amici *et al.,* found correlation between the production capability of alkaloids and lipids (Amici *et al.,* 1967a). Procedures described by Amici *et al.* (1968) with the strain F.I. 32/17 served to increase the production of ergotamine and α-ergokryptine in shaker cultures up to 2000 mg L^{-1} with an approximately equal proportion of the two components; in a fermentor the production reached 1200 mg L^{-1}. Baumert *et al.* (1979b) described, *e.g.,* a procedure of selection of the production strain IBP 47, IMET PA 135; Baumert *et al.* (1979c) developed cultivation processes for this strain. The total alkaloid production reached 900–1500 mg L^{-1}; the total alkaloid mass was composed of 75–80% ergotamine, 10–15% chanoclavine, 5–6% ergometrine, 5% ergokryptine, a maximum of 4% ergosine and traces of other clavines. The strain JAP 471 gave about 800 mg L^{-1} of alkaloids out of which 70% was ergotamine and 30% was clavine alkaloids (Erge *et al.,* 1984). In the submersion mycelium of the strain *C. purpurea* II, the content of alkaloids reached 0.4% of dry mass (Sarkisova and Smirnova, 1984). The strain L-4 (ATCC 20103) produced about 1500 mg L^{-1} of ergotamine (Komel *et al.,* 1985).

Long-term production of a mixture of ergotamine and ergokryptine by immobilized cells was studied by Dierkes *et al.* (1993) in semicontinuous and continuous systems. When cells of the strain *C. purpurea* 1029/N5 entrapped

in alginate were cultivated in a 500 mL bubble column reactor for 30 days, alkaloid productivity was 17–40 mg L^{-1} per day.

Ergotamine producing strains were also used in different studies as model organisms for research on various aspects of *Claviceps* biology and alkaloid biosyntesis, *e.g.* the original parasitic ergotamine strain *C. purpurea* Pla-4 (Majer *et al.,* 1967; Řeháček and Kozová, 1975), or the strain PCCE1 (Quigley and Floss, 1981).

Ergosine, another representative of the ergotamine group, exerts very similar pharmacological effects as ergotamine. In spite of the fact that it has not yet been used in therapy, processes of its production are described in the patent literature. Amici *et al.* (1969) described a concurrent production of ergocornine and ergosine by the strain *C. purpurea* F.I. 43/14, ATCC 20106, when the production of total alkaloids was 950–1100 mg L^{-1} and ergosine content 40–45% . Gröger *et al.* (1977) and Maier *et al.* (1981) employed the strains *C. purpurea* MUT 168 and MUT 168/2 for both surface and submerged cultivation with a production of 300–350 mg L^{-1} of alkaloids, containing 90% of ergosine and ergosinine together with 10% of clavine alkaloids, or 80% of ergosine and 20% of chanoclavine-I, respectively. Baumert *et al.* (1979b, 1980) described the selection of the production strain IBP 179, IMET PA 136 and its submerged cultivation. The concentration of total alkaloids was in this case 900–1300 mg L^{-1}, with 80–90% of ergosine and ergosinine. The ergosine strain *C. purpurea* MUT 170 (Baumert and Gröger, 1982; Schmauder and Gröger, 1986) produced a mixture of ergosine and clavine alkaloids in amounts of about 700 mg L^{-1} (Erge *et al.,* 1984).

Dihydroergopeptines (dihydroergotamine, dihydroergocristine *etc.*) which are produced from common ergopeptines by chemical methods have significant therapeutic use. The only dihydroergopeptine found in nature is dihydroergosine (Mantle and Waight, 1968) isolated from *Claviceps africana* (formerly *Sphacelia sorghi*); its biosynthetic precursors are dihydroelymoclavine and dihydrolysergic acid (Barrow *et al.,* 1974). These findings opened the possibility of fermentative production of dihydroergopeptines by common strains when these precursors were used.

12.6.2. Production of Alkaloids of the Ergotoxine Series

Ergocristine, ergocornine, α-ergokryptine and *ß*-ergokryptine from alkaloids of this group are used in therapy. As drugs they are used both separately (*e.g.* ergocristine) and in mixtures (ergocornine, α- and *ß*-ergokryptine), with the native molecule or hydrogenated. All these alkaloids were isolated from the species *C. purpurea* (Schlientz *et al.,* 1968; Stoll, 1952). Besides the above mentioned production strain *Hypomyces aurantus* (Yamatoya and Yamamoto, 1983) and the only one described production strain *Claviceps paspali* (Wilke and Weber, 1984), all the strains mentioned in the literature originated from the species *C. purpurea:* CCM F-508 (Strnadová and Kybal, 1976), CCM

F-725 (Strnadová *et al.,* 1981), CCM 8043 (Strnadová *et al.,* 1986), IBP 84, ZIMET 43768 (Schumann *et al.,* 1986), IMET PA 130, ZIMET 43769 (Ludwigs *et al.,* 1985; Volzke *et al.,* 1985), DH 82, ZIMET 43695 (Erge *et al.,* 1982), F.I. 101a (Amici *et al.,* 1967b), F.I. 43/14, ATCC 20106 (Amici *et al.,* 1969), F.I. S40, ATCC 20103 (Minghetti *et al.,* 1967), F.I. 7374 (Bianchi *et al.,* 1974), Exy 20, Ech K 420 (Kobel and Sanglier, 1976) Ecc 93 (Keller *et al.,* 1988), MNG 022, MNG 0083, MNG 00186 (Udvardy-Nagy *et al.,* 1981; Wack *et al.,* 1981), OKI 88/1972 (Richter Gedeon V.G., 1973), 231 F.I., ATCC 20106 (Bianchi *et al.,* 1976, Crespi-Perellino *et al.,* 1987, 1992, 1993), 563 E (Miličić *et al.,* 1984), L–16 (Puc *et al.,* 1987), L–17 (Didek-Brumec *et al.,* 1991a, b; Gaberc-Porekar *et al.,* 1990; Miličić *et al.,* 1987, 1989; Sočič *et al.,* 1985), L–18 (Didek-Brumec *et al.,* 1988), Pepty 695 (Maier *et al.,* 1971; Schmauder and Gröger, 1986), Pepty 695/S (Erge *et al.,* 1984; Maier *et al.,* 1980b, 1988b), 1029 (Lohmeyer and Sander, 1993; Lohmeyer *et al.,* 1990).

The original procedures elaborated for fermentation production of ergotoxine alkaloids were not introduced into practice both for practical reasons, as, *e.g.,* in the patent of Windisch and Bronn (1960), and for economic ones, given by the very low productivity. For example, surface cultivation was developed producing 0.18 g of total ergotoxine alkaloids per 100 g of dry mass with the ergocristine/ergocornine/ergokryptine ratio of 3:1:2 (Kybal *et al.,* 1960). At that time no technological process was available for the industrial application of this cultivation but later the process of cultivation in plastic bags was developed (Kybal and Vlček, 1976; Vlček and Kybal, 1974) and the high-producing strains *C. purpurea* CCM F-725 (Strnadová *et al.,* 1981) and CCM 8043 (Strnadová *et al.,* 1986) were selected. On a rich medium the strain *C. purpurea* CCM F-725 formed mycelia containing 1.5% of alkaloids per dry mass. This product contained ergocornine, α-ergokryptine and *ß*-ergokryptine in a 6:5:1 ratio, small amounts of ergometrine and traces of ergosine, ergocristine, ergotamine and ergoxine. Later the strain *C. purpurea* CCM 8043 was selected which produced as much as 3.5% alkaloids per mycelia dry mass; the mixture of alkaloids contained α-ergokryptine, ergocornine, *ß*-ergokryptine, ergometrine and traces of ergosine. From the end of the 1970s pharmacopoeias requirements became more strict as regards the mutual proportion of α- and *ß*-ergokryptine in ergotoxine substances and drugs. The mutual ratio of biologically synthesized alkaloids of the ergotine group can be influenced by the addition of amino acids that form the peptidic moiety of the ergopeptine structure (Kobel and Sanglier 1978). Kybal *et al.* (1979) described a surface cultivation giving a controlled proportion of ergocornine, α-ergokryptine and *ß*-ergokryptine. Threonine, the biosynthetic precursor of isoleucine, was also used besides the amino acids forming the peptidic part of ergopeptines. Experiments with additions of threonine, leucine and isoleucine into media provided 0.51–0.86% alkaloids per dry mass, with the ratio of ergokryptines to ergocornine 1.5–3:1 and α-ergokryptine to *ß*-ergokryptine 1:5–100:1. When precursors were employed, three new alkaloids were isolated—*5´*-epi-*ß*-ergokryptine from the ergopeptine group, and *ß*-ergokryptame and *ß,*

ß-ergoanname (Flieger *et al.*, 1984). The procedure was further optimized by using the economically more favourable threonine; as a result, production strains D3–18 and D2–B1 were able to produce mycelia containing the precise proportion of ergocornine and ergokryptine components in ergotoxine preparations required by the Pharmacopoeia (Malinka *et al.*, 1987). A process for the controlled biosynthesis of ergocornine, a-ergokryptine and ß-ergokryptine was also elaborated for submerged cultivation (Udvardy-Nagy *et al.*, 1981). Production strains MNG 0022, MNG 0083 a MNG 00186 provided 80–200 mg L^{-1} of ergocornine, 15–150 mg L^{-1} of a-ergokryptine, nearly 80–100 mg L^{-1} of ß-ergokryptine and 150–180 mg L^{-1} of a mixture of ergocorninine and ergokryptinines. A broader spectrum of compounds was used as precursors—besides threonine and isoleucine also homoserine, homocysteine, methionine and α-ketobutyric acid. Wack *et al.* (1981) reported on the use of valine and isoleucine as precursors in the cultivation of strain MNG 00186 also. The precursor addition enhanced the original production of 150 mg L^{-1} of ergocornine, 40 mg L^{-1} of α-ergokryptine and 90 mg L^{-1} of ß-ergokryptine to 320 mg L^{-1} of ergocornine, 60 mg L^{-1} of α-ergokryptine and 160 mg L^{-1} of ß-ergokryptine. Increased production of a-ergokryptine using leucine as a precursor in cultures of *C. purpurea* strains IMET PA 130 or ZIMET PA 43769 was described by Ludwigs *et al.* (1985). When 2–5 g L^{-1} of L-leucine was added to the medium, the concentration of ergotoxine alkaloids reached 900–1200 mg L^{-1} or 1400–2500 mg $L^{-1,}$ with 65–85% of α-ergokryptine. Puc *et al.* (1987) described the use of valine as a precursor with strain L–16. Depending on the amount of valine added into a submerged culture of the strain producing 1800 mg L^{-1} of total alkaloids, with a proportion of ergocornine to ergokryptines 1:2, this proportion was changed up to 4.5:1. Another method of production control, in addition to leucine precursoring, is described in the patent of Volzke *et al.* (1985). The production of ergocornine by *C. purpurea* strains IMET PA 130 and ZIMET 43769 can be supported by partial phosphate limitation and by continuous addition of ammonium ions; production of α-ergokryptine can be increased by simultaneous addition of urea or ammonium salts and phosphate. In addition to these procedures with directed precursoring, a number of patents describes the production of ergocornine or ergokryptines without precursors. The production strain *C. purpurea* IBP 84, ZIMET 43768 used for production of a mixture of a-ergokryptine and ergosine formed 700–1400 mg L^{-1} of total alkaloids with 80% of a-ergokryptine and 20% of ergosine (Schumann *et al.*, 1986). According to the patent of Amici *et al.* (1967b), the production of ergokryptine in cultures of the strain *C. purpurea* F.I. 101a reached 1100–1500 mg $L^{-1.}$ Production of a mixture of ergokryptine and ergotamine was mentioned earlier (Amici *et al.*, 1968), and so was the production of ergokryptinine, ergokryptine and other alkaloids by a fungi of the genus *Hypomyces* (Yamatoya and Yamamoto, 1983). Wilke and Weber (1984) reported the production of 525 mg L^{-1} of α-ergokryptine with the asporogenic strain *C. paspali* DSM 2836. Patent of Richter Gedeon V.G. (1973) described the production of a mixture of ergocornine and α-ergokryptine; when the strain *C. purpurea* OKI 88/1972 was

employed, 1046–1246 mg L^{-1} of total alkaloids were produced from which a mixture of ergocornine and ergokryptine represented 646 mg L^{-1} and the concentration of ergometrine was 202–310 mg L^{-1}. A mixture of ergocornine and ergokryptine was also the main component of the 2000 mg L^{-1} alkaloids which were produced by the sporogenic strain L-17 bred by combined mutagenesis and selection from an originally parasitic strain (Didek-Brumec *et al.,* 1991a, b). The production of a mixture of ergocornine and ergosine was reported by Amici *et al.* (1969). The strain *C. purpurea* F.I. 43/14 ATCC 20106 formed in different cultivation media 950–110 mg L^{-1} of a mixture containing 40–45% of ergosine and 55–60% of ergocornine. Special strains for *ß*-ergokryptine production are referred to by Bianchi *et al.* (1974, 1976). The strains *C. purpurea* 231 F.I. and F.I. 7374 produced the total amount 1200 mg L^{-1} of ergopeptines with 30% of ergokryptine. The patent of Kobel and Sanglier (1976) described the production of ergocornine and ergokryptine by the strain *C. purpurea* Exy 20, and the production of ergocristine by the strain *C. purpurea* Ech K 420; a so called pre-culture was used in the process. The cultivation production of total ergopeptines was 770 mg L^{-1} from which ergokryptine and ergokryptinine comprised 203 mg L^{-1}, ergocornine and ergocorninine 200 mg L^{-1}, ergocristine and ergocristinine 206 mg L^{-1} and other alkaloids 170 mg L^{-1}. The fermentative production of ergocristine was described in patent of Minghetti *et al.* (1967). The process employed the production strain *C. purpurea* F.I. S40 (ATCC 20103) which, when cultivated in a fermentor, gave 920 mg L^{-1} of ergocristine. Another process described by Erge *et al.* (1982) employed the strain *C. purpurea* DH 82, ZIMET 43695; in a fermentor the production of alkaloids reached 600–1000 mg L^{-1} of which ergocristine represented 400–550 mg L^{-1}. Didek-Brumec *et al.* (1988) referred to the asporogenic strain L-18 that formed 2000 mg L^{-1} of ergocristine. The production about l000–1200 mg L^{-1} of total alkaloids by the strain Pepty 695/S, which contained 50–60% of ergotoxines composed of a mixture of ergocornine, ergokryptine and a 20% of ergometrine, was reported by Maier *et al.* (1980b, 1988b) and Erge *et al.* (1984). The original parent strain Pepty 695 showed the total alkaloids production of about 400–450 mg L^{-1}, with 50% of ergotoxines (ergocornine to ergokryptine ratio 3:1) and 15–20% of ergometrine (Floss *et al.,* 1971 a). Gaberc-Porekar *et al.* (1990) used the strain *C. purpurea* L–17 to produce 2400 mg L^{-1} of total ergotoxine alkaloids, mostly ergocornine and ergokryptine. In the same study, devoted to carbohydrate metabolism, the hexose monophosphate shunt metabolizing glucose during the vegetative phase of fermentation was shown to be replaced by glycolysis during the period of increasing production of alkaloids. This strain served for further research on biochemistry and physiology of high-producing strains, *e.g.* the correlation between the intermediary metabolism and secondary metabolite synthesis (Gaberc-Porekar *et al.,* 1992a). In the case of ergopeptines, the further direction of production processes development—immobilization of producers and possible continualization—is only at its beginning (Lohmeyer and Sander, 1993).

12.6.3. Derivatives and Analoga of Ergopeptines

The fungus *Claviceps purpurea* is able to incorporate the amino acids, present in the medium, into the peptidic moiety of ergopeptines and to perform similar reaction also with their precursors. This fact was used in a controlled fermentation with the directed application of precursors, as mentioned in previous paragraphs. *C. purpurea* is also able to incorporate a number of other different amino acids and their analoga into the peptidic moiety (Beacco *et al.,* 1978). Thus ergobutine (from the group of ergoxines) and ergobutyrine (from the group of ergotoxines) were isolated from the saprophytic cultures of the strain *C. purpurea* 231 F.I. (Bianchi *et al.,* 1982). 5′-Epi-ß-ergokryptine from the ergotoxine group, ß-ergokryptame from the ergotaxame group and ß, ß-ergoanname from the ergoanname group, isolated from saprophytic surface cultures of the strain *C. purpurea* D-3–18 on addition of different stereomers of isoleucine and threonine as precursors (Flieger *et al.,* 1984), were mentioned earlier. Addition of L-norvaline as a precursor into cultures of *C. purpurea* 231 F.I. yielded unnatural ergopeptines—ergorine, ergonorine and ergonornorine (Crespi-Perellino *et al.,* 1992). These capabilities of *C. purpurea* were used in the development of processes for preparation of ergopeptines analoga. The procedure according to Beacco *et al.* (1977) employed the specially selected mutant strains *C. purpurea* ATCC 15383, ATCC 20103 and ATCC 20019, dependent upon different nonhydroxylated amino acids—leucine, phenylalanine, halogenated phenylalanine, thienylalanine, pyrazolylalanine, furylalanine, pyridylalanine, *etc.* A number of derivatives of ergopeptine with the adrenolytic effect (blockade of α-receptors), *e.g.* 5′-debenzyl-5′-*p*-chlorobenzyl-dihydroergocristine or 5′-debenzyl-5′-*p*-fluorobenzyl-ergotamine, was obtained. The process proposed by Baumert *et al.* (1981) employed the strains *C. purpurea* IBP 179 and MUT 168, which produced ergosine. Addition of 3–6 g L^{-1} of the proline analogue—the anticancer substance 1, 3-thiazolidine-4-carboxylic acid-resulted in the synthesis of 1′ ß-methyl-5'α-isobutyl-9′-thiaergopeptine (Thiaergosine). The biosynthesis of similar compounds has been described in the patent of Kobel *et al.* (1982). Addition of appropriate precursors to cultures of the strain *C. purpurea* NRRL 12043, which produces ergotamine and ergotaminine, and to those of the strain NRRL 12044, that produces ergocristine and ergocristinine, yielded a number of substances. These derivatives of peptidic alkaloids exert a spectrum of physiological and therapeutical effects (dopaminergic stimulation, prolactin inhibition, vasoconstriction activity, *etc.*). 9′-Thia-ergocristine and 9′-thia-ergotamine can be mentioned as representatives of such substances.

12.7. CONTROL AND MODELLING OF ERGOT ALKALOID FERMENTATION

Processes in which final yields of products were influenced by precursor addition or by limitation and dosing of individual nutrients were mentioned earlier.

Tryptophan, the building unit of the ergoline nucleus (Floss, 1976), can also be used to increase production of many alkaloids. Detailed research into the topic was done by, *e.g.,* Gaberc-Porekar *et al.* (1992b). A comparative study with a number of strains producing different ergot alkaloids was performed by Erge *et al.* (1984). More details are given in Chapter 7.

Bianchi *et al.* (1981) and Crespi-Perellino *et al.* (1994) carried out certain generalization of results with controlled precursor addition promoting ergopeptine production. An amino acid at position 3 of the ergopeptine molecule is specific, amino acids at positions 1 and 2 can be changed. Amino acids with a lipophilic side chain can be introduced into the ergopeptine molecule depending on the number of C atoms in the side chain.

Some model procedures for a more complex control of fermentative production of ergot alkaloids were elaborated based on, *e.g.* mathematical models of clavine and ergopeptine alkaloid production in batch cultivation. A model based on the concentration of extracellular and intraceliular phosphate was published for clavine alkaloids (Votruba and Pazoutová, 1981). A mathematical simulation of different technological alternatives of clavine production was done on this basis (Pazoutová *et al.,* 1981b). Apart from this model, also a hypothesis was published on gene expression in *Claviceps* biosynthetic pathways (Pazoutová and Sajdl, 1988). A regulation model of the gene expression for alkaloid biosynthesis was proposed according to which the tryptophan-induced synthesis is mediated by an activator binding tryptophan and stimulating the transcription of pertinent genes. The kinetics of clavine alkaloids production was also investigated (Flieger *et al.,* 1988). Based on these results, processes were developed in which elimination of feed-back inhibition by fermentation products lead to a higher production of clavine alkaloids and ergometrine (Flieger *et al.,* 1987).

In the case of ergopeptine alkaloids, batch submerged cultivation was modelled on the basis of the predicted concentrations of biomass, alkaloids and sucrose. Good agreement was achieved between the calculated and found values of the former two parameters (Grm *et al.,* 1980). Using a previously found correlation between growth and alkaloid biosynthesis on the one hand (Miličić *et al.,* 1987) and the effects of cultivation conditions on morphology and alkaloid synthesis on the other (Miličić *et al.,* 1989), Miličić *et al.* (1993) elaborated a more general model. Models of microorganism "life span", "microbial growth" and "alkaloid synthesis" were elaborated on the basis of the specific growth rates and morphological analysis of proliferation.

Preliminary studies, whose results could be used for model building were performed with producers of simple derivatives of lysergic acid. Bumbová-Linhartová *et al.* (1991) divided the production process of these derivatives into three phases—production, post-production and degradation ones—and set up their characteristics.

Other procedures leading to increased effectivity of the production processes are also described in the patent literature. Rochelmayer (1965) stimulated

alkaloid biosynthesis by adding parts of the Thallophyta, especially fungi and bacteria, into the medium. A similar principle was adopted in the patent of Fiedler *et al.* (1989) where elicitors were used to enhance the activity of biosynthetic enzymes in microorganisms and higher plants. Another way increasing of alkaloid biosynthesis by more than 100%, described by Rylko *et al.* (1988a, b, c), made use of suitable inducers of cytochrome P-450. A positive effect of substances modifying cell lipids of production strains has also been demonstrated (Křen *et al.,* 1988a,b). These substances increased alkaloid production by almost 74%; the same effect has been shown with high-producing strains (Sajdl *et al.,* 1988a). The relationship between morphology of saprophytic cells and production capability has not been explicitly elucidated yet (Esser and Tudzynski, 1978; Didek-Brumec *et al.,* 1991 a). The production of alkaloids is supported by such cultivation conditions that cause mycelial differentiation to sclerotium cells (Kybal, 1981; Wichmann and Voigt, 1962) and are connected with specific manifestations of the primary metabolism (Kleinerová, 1975; Kybal *et al.,* 1978, 1981; Zalai and Jaksa, 1981). These findings have been complemented by Lösecke *et al.* (1980, 1981, 1982) by the data on the relationship between the ultrastructure of cells from submerged culture and alkaloid production.

12.8. PRODUCTION OF INOCULATION MATERIAL FOR PARASITIC ERGOT PRODUCTION

The infection material for inoculation has been mentioned by Németh in Chapter 11 "Parasitic production of ergot alkaloids". Asexual sporesconidia—are exclusively used as a source of the primary infection in the parasitic production of ergot. When the infection inoculation material is to be prepared, the saprophytic cultivation aims at obtaining the maximum amount of vital infectious spores. Nutritional sources and the cultivation process itself are adapted to support growth and differentiation of hyphae to obtain massive conidiation.

Cultivation processes are generally identical with those for the production of alkaloids. It is possible to employ cultivation on solid substrates as well as stationary or submerged cultivation in liquid media. Grains, which were reported as the solid-state medium of choice since the 1940s, has been mentioned by Chapter 11 (see also Kybal, 1955; Sastry *et al.,* 1970b). The grains supplemented by nutrients (Kybal, 1963) was still used in the 1980s as an optimum substrate for production of high-quality inoculation material and a reference standard for comparison with other inoculation materials. This material, or conidia from surface agar cultures, were used also in experimental parasitic cultivations (Corbett *et al.,* 1974; Kybal and Strnadová., 1968; Singh *et al.,* 1992) while inoculation material from submerged cultivations has been used less frequently (Košir *et al.,* 1981). Cultivations on the surface of liquid media are performed

with the plastic bags (Harazim *et al.,* 1984; Kybal and Vlček, 1976; Strnadová *et al.,* 1986) or other suitable equipment (Kybal and Strnadová, 1982). Submerged cultivations, depending on the properties of the parasitic production strains, are multi-stage. The produced infection material can be conserved in a sucrose solution (Kubec *et al.,* 1974), mixed with an inert filler, granulated and dried (Kiniczky *et al.,* 1982; Kybal *et al.,* 1990) or frozen in an osmotically stabilized medium (Yásárhelyi *et al.,* 1980a). In experiments done by Czech authors, optimum results were achieved with an inoculation material dried together with SiO_2 (Valík and Malinka, 1992).

Quality evaluation of the inoculation material can be done by vital staining of conidia, but methods based on germination ability (Švecová, 1985) and determination of the unit infection dose appears more optimal. An optimal number of conidia and a procedure of infection of a host spike should be experimentally determined for each kind of inoculation material and a type of host (Sastry *et al.,* 1970a, c).

12.9. PRODUCTION OF OTHER SUBSTANCES BY *CLAVICEPS*

Fungi of the genus *Claviceps* have been shown to produce not only ergot alkaloids but also other substances. Tryptophan is used as a starting material for biosynthesis of ergot alkaloids. The use of the *Claviceps* fungi is mentioned in the patent of Enatsu and Terui (1967) describing L-tryptophan production. In the process reported by Dinelli *et al.* (1972), enzyme complexes isolated among others from *Claviceps* are employed for the production of L-tryptophan from indole and serine. The patent of Lapis *et al.* (1978) describes the manufacture of antitumor basic proteins with molecular weight of 1, 8–3, 5 kDa from the mycelium of *C. purpurea* and *C. fusiformis*.

The rice leaf binding component, produced in aerobic cultures of the strain *Claviceps purpurea* ATCC 9605 or of a number of other microorganisms (Oishi *et al.,* 1984), can be used to increase the rice crop. The active component increases the yield and shortens the production period. Also other metabolites of *Claviceps* can find application in agriculture practice. Patent of Dowd *et al.* (1988) describes the use of tremorgenic mycotoxins as insecticides against corn earworm and fall armyworm. Gubański and Lowkis (1964) have demonstrated the inhibition of the tobacco mosaic virus by a substance isolated from *C. purpurea*.

Detoxification of methyl-*N*-methylanthranilate to methyl-anthranilate using a number of microorganisms, among others *Claviceps* spp., is described in the patent of Page *et al.* (1989). The detoxification was carried out in a 4–8-day fermentation process.

There are two patents describing production of lipids. The patent of Fukuda (1986) referred to a method of isolation of lipids from lipid-producing algae and fungi, including *Claviceps*. In the patent of Sarkisova *et al.* (1987) the strain *C. purpurea* 312A produced lipids with composition similar to that of

cotton-seed oil. After a 10-day cultivation in a liquid medium, the biomass contained 27–46% of lipids.

Production of carbohydrates is also mentioned in two patents. Glucans, undesirable in ergot alkaloid fermentation but applicable in a number of other industrial branches including the pharmaceutical industry, are produced according to the patent of Johal and Cash (1989) by different filamentous fungi, including *Claviceps*. Glucans are remarkable for their pharmacological, especially immunomodulatory, effects and their future therapeutical use can be envisaged. The patent of Senda *et al.* (1989) describes the production of another type of carbohydrates—oligoinulosaccharides—using *ß*-fructofuranosidase from different microorganisms (also from *Claviceps purpurea*). These oligosaccharides can be employed, *e.g.,* in the food industry.

12.10. DOWN-STREAM PROCESSES

Procedures for the subsequent processing of fermentation products depend on their properties; they are different for products of stationary cultivations and of submerged ones, and also for water soluble (clavines, lysergic acid and its derivatives, ergometrine) or insoluble (ergopeptines) substances.

The concentration of ergopeptines in a medium during their surface production is negligible; the mycelium is processed in this case. Isolation procedures are very similar to those used for the isolation of alkaloids from ergot sclerotia grown during field parasitic cultivation. When clavines, lysergic acid or its derivatives are produced by surface fermentation it is advantageous to process both the mycelia and the medium.

In the case of submerged cultivation the whole volume of a medium with the mycelium is processed. Due to the mechanical stress and the resulting injury to the hyphae a non-negligible amount of hydrophobic alkaloids can be contained in the medium.

To decide what isolation process should be used it is necessary to take into account also other substances produced by the fungi. Besides the already mentioned glucans, which complicate manipulation with the end product of fermentation and make the isolation more expensive, these effects can be exerted also by lipids and pigments.

Isolation processes used in industry are detailed in Chapter 13.

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