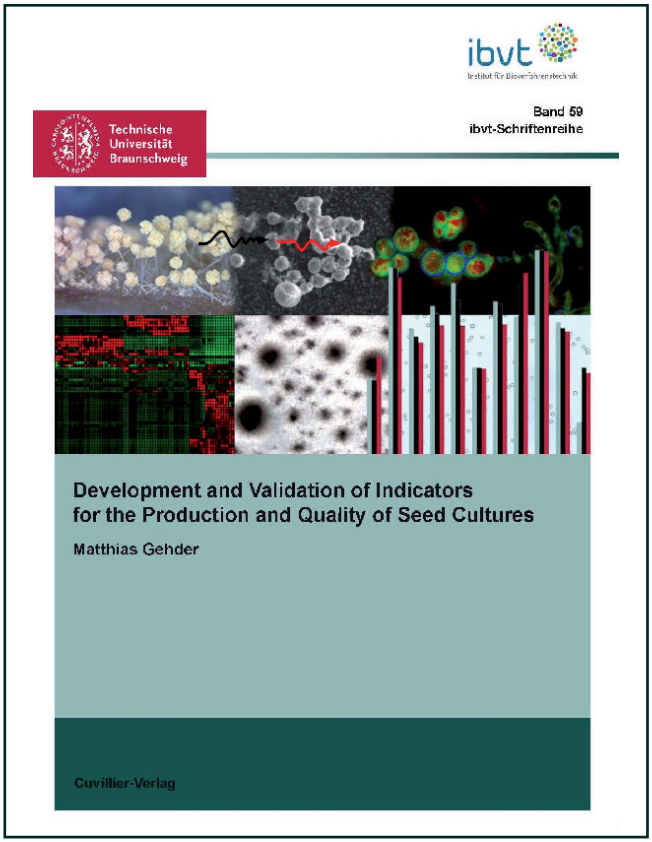
Matthias Gehder (Autor)

Development and Validation of Indicators for the Production and Quality of Seed Cultures



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1 Introduction and theoretical background

1.1 Aim and structure of the thesis

Filamentous fungi are of tremendous interest in research and industry. Their ability to produce a large spectrum of economically relevant substances makes them appealing organisms for the entire biotechnological community. Their use hence ranges from large scale applications like the production of citric acid all the way to therapeutic proteins requiring complex glycosylation patterns (Demain, 2000; Grimm et al., 2005a; Punt et al., 2002). Furthermore, filamentous fungi are widely used in bioremediation and biotransformation (Da Costa et al., 2007). In fact, the 11-alpha hydroxylation of progesterone by the filamentous fungus *Mucorales* spp. shown by Peterson and Murray (1952), the same biotransformation that is in the focus of this work, is today considered the first 'green chemistry' process as this single step cultivation process replaced a 31-step chemical synthesis (Dunn et al., 2010).

The increasing awareness for ecologically clean production alternatives, the rising application of technical enzymes and the growing demand for protein-based therapeutics have hence raised the interest in filamentous fungi dramatically. Tremendous efforts are being invested to understand and optimize the complex cultivation processes. Power input through stirring and aeration, initial pH and pH shift of the cultivation broth, addition of microparticles to the medium, aggregation behavior of spores, hyphae and steel as well as rupture resistance of hyphae walls have been investigated, only to name a few, recently reviewed in Wucherpfennig et al. (2010). Advances in this field have been contributed especially from the collaborative research center SFB 578, based in Braunschweig, Germany.

The inoculum, however, has been neglected throughout most of those studies. If at all, the effect of the concentration of the inoculum was assessed. But disregarding the inoculum in the holistic analysis jeopardizes the entire scrutiny as the seed culture marks the absolute starting point of any cultivation. Without detailed knowledge of the characteristics of the inoculum, the cultivation outcome cannot be interpreted properly. As with any process, the output is an immediate function of the input and hence maximum care has to be taken with the inoculum. Inferior seed cultures will result in suboptimal products and an inoculation with spores of unknown properties will turn the result of any cultivation into a game of 'trial and error' while also strongly hindering the correct interpretation of the same. Overcoming of these two immense stumbling blocks, i.e. usage of mediocre inocula and working with inocula of unknown properties, is the overall goal of this investigation.

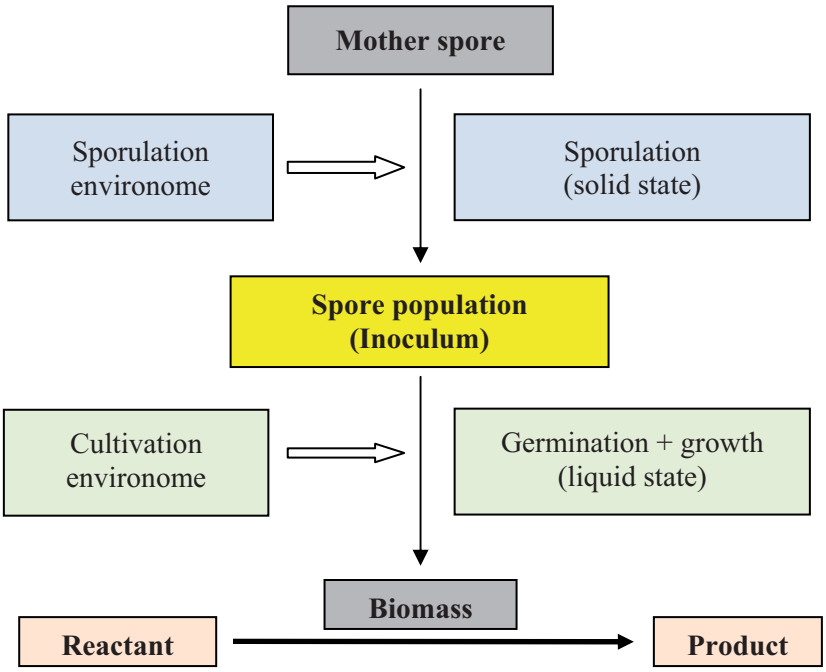

Fig. 1.1. Schematic illustration of the process sequence for cultivations with filamentous fungi including the often neglected inoculum generation.

Figure 1.1 schematically illustrates the process sequence for cultivations including the generation of the inoculum which is strongly affected by the sporulation environome. Previously reported studies, however, often focused exclusively on the cultivation part of the process, i.e. the actual cultivation while the inoculum was regarded as a 'black box'. Hence, the first step has to be the establishment of indicators that allow the assessment of spore quality. Only very little is known about the constitution of spores even though it has been shown very early that spores are capable of conducting biotransformations (Vezina et al., 1963).

Analysis of conidial properties such as cell wall characteristics and the resulting aggregation behavior, determination of intra-conidial carbon reservoirs and stress-protectors as well as metabolic characteristics are possible starting points. However, the methods for the proper analyses of the here suggested criteria need to be established as hardly any work has previously been conducted in this field. Possible means for their analysis are biochemical and spectroscopic techniques as well as extraction of key compounds and consecutive analysis by means of chromatography.

Subsequently, in a second step, the methods have to be validated as indicators are only meaningful if they exhibit biological responses upon changing parameters during the sporulation. Further, at least for industrial applications, they are additionally interesting when showing correlations to properties of the cultivation inoculated with the respective spore population. This validation can be combined with the assessment

of typical sporulation parameters such as solid medium, incubation duration and storage which together resemble a part of the ‘sporulation environome’. Summarized, spore characterization indicators have to be established and consequently validated. This hence represents the structure of the presented work:

In the first results section (chapter three) nine methods are introduced which have been set up and employed throughout this work. As the establishment of these methods represents a significant part of the results of this work, they are reported there rather than in the standard ‘materials and methods’ section. Further, the significance is exemplified for every assessment and, where applicable, possible relevant correlations to other criteria are illustrated.

In the second results section (chapter four), the nine previously presented methods are applied to spore populations generated under alternating conditions (where reasonable). This hence represents a permutation of the nine methods and five parameters of relevance, i.e. sporulation media, sporulation duration, inoculum concentration, storage and initiating germination.

Regardless of the section, the focus throughout the entire study is the introduction of novel meaningful methods to facilitate the assessment of spore characteristics in order to avoid working with mediocre inocula and/or inocula of unknown quality. As it has been increasingly difficult to interpret the escalating details of submerged cultivations, this study may foster the understanding of these processes by elucidating the influence of the inoculum and therefore eliminating an unknown but important parameter in this multivariant system. Further, determination of the requirements for the generation of optimal seed cultures will result in more homogenous and improved cultivations and eventually lower production costs.

1.2 Biology of *Aspergillus ochraceus*

Aspergillus ochraceus is a wide spread filamentous fungus. It is typically found in soil of tropical and subtropical regions but also in cereals, nuts and many more food products. It was first characterized in 1877 and is best known as a feed spoiler due to his production of the mycotoxin Ochratoxin A (Al-Anati and Petzinger, 2006; Kozakiewicz, 1990; O'Callaghan et al., 2006; Van Der Merwe et al., 1965). *A. ochraceus* can be classified as follows (Schwantes, 1996):

- Phylum: Ascomycota
- Class: Ascomycetes, Subclass: Eurotiomycetes
- Order: Eurotiales
- Family: Trichocomaceae
- Genus: *Aspergillus*
- Species: *Aspergillus ochraceus*

A. ochraceus is in many aspects a typical representative of the Ascomycota. This phylum contains numerous very common fungi covering several thousand species, including famous edible fungi like morels and truffles. The Phylum received its name from the typical ascus-cell where the ascospores, the spores for sexual reproduction, are produced. However, not all fungi categorized as Ascomycota actually form an ascus. The categorization can therefore be misleading. In fact, for many fungi, including the majority of Aspergilli, no sexual propagation has been witnessed so far. These species only reproduce asexually by the formation of conidiospores and were therefore formerly united in the informal taxon Deuteromycota and denoted as fungi imperfecti for not having a sexual stage (Geiser, 2009; Hibbett et al., 2007; Schwantes, 1996).

A close investigation of the current literature reveals an equivocal use of the numerous terms for the entire spore developing process even amongst mycologists. Therefore, the notions employed in this work are briefly shown below summarizing the definitions given by Axelrod (1972), Champe et al. (1987), Cahagenier et al. (1993) and Davis and Perkins (2002):

- Ascospore: haploid spore for sexual reproduction
- Conidiophore: specialized fungal hypha that bears and generates the conidiospores
- Conidiospore: synonym for conidium
- Conidium (pl. conidia): spore for asexual reproduction, generally haploid
- Macrospore / Microspore: synonym for conidium, used to differentiate between smaller and larger spores from the same organism
- Mitospore: synonym for conidium

For this study, ‘conidium’ and ‘spore’ are used synonymously.

Fig. 1.2. On this cross section of an A. ochraceus *colony grown on malt extract agar the substrate penetrating hyphae, the aerial hyphae and the conidiophores carrying the spores can be identified.*

Figure 1.2 shows a cross section of *A. ochraceus* growing and sporulating on solid substrate. The spores are of ocher to brown color, giving the species its name. They are spherical with a diameter of approx. 3.5 µm and a slightly rough surface. Their generation is identical to the typical spore formation of Ascomycota and involves the following seven steps (Adams et al., 1998; Broderick and Greenshields, 1981; Christensen, 1982; Schwantes, 1996):

- Formation of thick-walled footcells on the solid medium.
- Development of conidiophores, aerial unbranched stalks that elongate by apical extension on top of each footcell.
- Growth of the conidiophores orthogonally to the medium surface up to 1.5 mm in length.
- Formation of a conidiophore vesicle at the distal end of each conidiophore.
- Development of metulae surrounding the conidiophore vesicles.
- Budding of metulae to produce a layer of uninucleate sterigmata termed phialides.
- Phialides then continuously produce a chain of conidia.

As phialides undergo repeated asymmetric division to produce chains of spores while maintaining their own identity, they have been considered ‘fungal stem cells’. The spore chain growing from each phialide can contain several hundred spores with the youngest conidium being located immediately next to the phialide-cell at the bottom of the spore chain and the oldest spore at the distal end of the tip (Timberlake, 1990).

The spore formation in *A. ochraceus* can be categorized as blastic conidiogenesis as the spores are constricted after conidiation (i.e. the spore is evident before it separates

from the phialide), in contrast to thallic conidiogenesis where constriction occurs before conidiation. In all cases, ripening of the conidia occurs after constriction and like in most Ascomycota the spores as well as the vegetative cells of *A. ochraceus* are haploid (Schwantes, 1996).

Further, international research groups are currently combining efforts to understand the complex role of light for conidiogenesis. However, the results for the different genera and even for different species within some genera seem to differ significantly and are still very equivocal (Friedl et al., 2008). First regulatory mechanisms are just being revealed for *A. nidulans* and *Neurospora crassa* but no publications were found examining the effect in *A. ochraceus*. The role of light is hence not discussed deeply within this work.

Before the availability of rapid rRNA analysis for the establishment of phylogenetic trees, *A. ochraceus* was considered as a group of fungi containing 15 species, the key representative being '*Aspergillus alutaceus* var. *alutaceus* Berkeley et Curtis'. After 1990 some regrouping efforts were undertaken and strains were renamed. Hence, some older references referring to *A. alutaceus* are now ascribed to *A. ochraceus* Wilhelm (Chelack et al., 1991; Christensen, 1982; El-Kady and Youssef, 1993).

1.3 Industrial relevance of *A. ochraceus* and the synthesis of steroids

The first documented utilization of fungi for the production or enhancement of food dates back to the early settlements in the Middle East and Egypt some 5 000 years ago. The production of bread, beer, wine and fermented meat are well known examples how microorganisms were exploited yet in ancient times and their use has been broadened since then. Today, filamentous fungi are well established biocatalysts for various reactions like condensations, oxidations, reductions and isomerizations. (Bennett, 1998). The commercially most important example for the industrial employment of fungi is the production of citric acid with a volume of approx. one million tons by *A. niger* and the synthesis of numerous antibiotics with a market volume of approx. 25 billion US$ by a multitude of species (Crolla and Kennedy, 2001; Janssen, 2006).

Lately, the expression of heterologous proteins has gained significant interest as well. Most fungi are unpretentious with respect to their growth conditions on the one hand, but at the same time capable to introduce complex glycosylation patterns. This fact makes them promising production hosts for technical enzymes and therapeutic proteins

alike filling the gap between bacteria and cell culture (Punt et al., 2002; Sharma et al., 2009). Furthermore, the use of fungi, especially spores of fungi, as a biological pest control has gained broad attention during the last years. Particularly, conidia of *Trichoderma* spp. are widely used to protect crops from other fungi, nematodes, insects and weeds (Verma et al., 2007).

The relevant commercial use of *A. ochraceus* focuses on two applications: the decomposition of the xenobioticum triphenylmethane which accumulates during staining in the textile industry (Parshetti et al., 2007; Saratale et al., 2006) and the enantioselective hydroxylation of various steroids and similar substances which has been exploited and expanded for more than fifty years (Dulaney et al., 1955a; He et al., 2010). The chemical family of steroids comprises a variety of naturally occurring substances in fungi, plants and animals that all share the same structure depicted in **figure 1.3**.

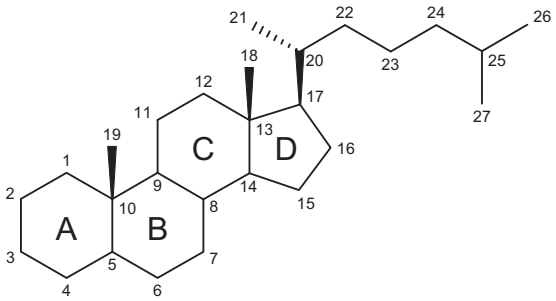

Fig. 1.3. Steroid skeleton including the IUPAC recommended ring lettering and atom numbering.

Steroids can be categorized into the following four major groups (Berg et al., 2003):

- Sterols, e.g. Cholesterol
- Bile acids
- Steroid hormones, i.e. steroids that function as hormones
  These are again divided into five groups according to the type of receptor they typically bind to:
    - Glucocorticoids, e.g. Cortisone*
    - Mineralocorticoids, e.g. Aldosterone*
    - Androgens, e.g. Testosterone*
    - Estrogens, e.g. Estradiol*
    - Progestagens, e.g. Progesterone*
- Other steroid derived molecules
    - Secosteroids, e.g. Calcitriol*
    - Insect hormones, e.g. Ecdyson
    - Heart active steroids, e.g. Digitoxin
    - Steroid alkaloids, e.g. Solanidin

The steroid hormones indicated with an asterisk (*) are found in humans. Glucocorticoids and mineralocorticoids are often summarized as ‘corticosteroids’. This super-group contains the hormones naturally synthesized in the adrenal cortex as well as similar synthetic substances. The biosynthesis of all steroid hormones initiates at cholesterol. With the exception of calcitriol they carry no or only small side chains of two carbon atoms. Characteristic is an oxo-group at C-3 and the conjugated double-bond between C-4/C-5 in ring A. Only estradiol is aromatic in ring A, its hydroxyl-group is hence of phenolic tendency. Characteristic differences between the hormones can be found in the residues of rings C and D. Calcitriol, however, differs from this scheme that is conserved throughout the vertebrates. It contains the entire carbon skeleton from the cholesterol but ring B has been opened through a light dependent reaction transforming it into a secosteroid (Koolman and Röhm, 1998).

It is true for all steroid hormones that they play an important role in the regulation of the human body. They are relevant for the carbohydrate and electrolyte metabolism, the circulatory system and the blood pressure, sexual development, the nervous system, the regulation of inflammations and the immune system. They are hence prescribed for a wide range of indications such as rheumatism, arthritis, allergies, asthma, gastrointestinal diseases, anemia, leukemia, meningitis and many more (Hildebrandt, 1994).

Here it quickly becomes evident that there is a tremendous demand for synthetically produced steroids in medicine. Due to their complex structure, however, their chemical synthesis has been limited for many years and was a tedious, expensive undertaking. Starting from ox bile acid, a 31 step chemical synthesis was necessary to produce cortisone, twelve of those only for the rearrangement of the hydroxyl group from position 12 to position 11 (Kardinahl et al., 2006).

After Peterson and Murray in 1952 discovered the ability of several *Rhizopus* spp. to directly introduce an 11-alpha-hydroxyl group to progesterone, i.e. the one step bio-synthesis of 11-alpha-OH-progesterone, biotransformations have rapidly replaced the unthrifty chemical process (Peterson and Murray, 1952). The reaction is depicted in **figure 1.4**.