Applications of Microbial Engineering

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APPLICATIONS OF MICROBIAL ENGINEERING

Foreword

Humans have used microbes for centuries in the preparation of food and drink. Over these centuries, strain improvements have come randomly, through spontaneous, chemical or radiological mutation. In many cases, we do not know the nature of the changes to an organism that increase (or decrease) its productivity. In a sense, the cell factory is largely an enigma—the input is controlled and the output is predictable—what happens between input and output is not well understood. Taking an engineering approach to controlling the metabolic output of microbes is a recent advance relative to the amount of time that humans have made use of domesticated microbes.

Today we use microbes for the production of enzymes, fuels, commodity chemicals, food, alcohol, crops and pharmaceuticals. Therefore, the field of microbial engineering covers a huge breadth of research whose applications touch our lives on a daily basis. Moreover, the wide variety of approaches used for the engineering of microbes is also massive. Functional genomics, metabolic engineering, systems biology and synthetic biology are all terms used to describe various ingredients needed to successfully engineer microbes. Thus, despite the importance of microbes, it is a daunting challenge for researchers, students and educators to grasp the breadth of endpoints for engineered microbes.

The chapters of this book survey topics across the landscape of microbial engineering. The applications of microbial engineering that are covered by chapters range from food and drink to plant-microbe interactions to biofuel and organic acid production. The phylogenetic space covered by the variety of microbes is equally broad. It is therefore, the broad group of researchers, students and educators whose interest converge on the topic of microbial engineering that will benefit from the information contained in this book.

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Preface

Microbial engineering technologies have been identified as an essential and important subject area of bio-engineering and applied biological sciences. Currently, the world economy needs innovative research for sustainable and environmentally safe industrial processes. Microbial engineering is significant, not only due to the fact that microbes are able to perform chemical conversions in a more environment friendly manner, but also because of the advantages in the production of compounds, over classical chemistry. Scientists and engineers, alike, are motivated to develop sustainable green technology to underpin the predicted industrial revolution that will increasingly depend on microorganisms. However it should be emphasized, that expertise in the use of microbes for industrial applications has to be further developed, either through genetic manipulations or conventional mutagenesis.

Microbial engineering encompasses diverse areas including biotechnology, chemical engineering, and alternative fuel development. The discipline seeks to exploit fungi, bacteria and algae as workhorses for industry. A microbial engineer works on the biological, chemical and engineering aspects of biotechnology, manipulating microbes and developing new uses for bacteria and fungi.

With both introductory and specialized chapters on recent research work, this book will contribute to a better understanding of industrially important microbial processes and can highlight the potential of diverse organisms for production of enzymes or chemicals, which in many cases have become indispensable in daily life. The contributions by specialists on the respective topics provide a profound scientific basis for further research.

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Editors

Contents

1

Aspergillus: A Cell Factory with Unlimited Prospects

Markus R.M. Fiedler, Benjamin M. Nitsche, Franziska Wanka and *Vera Meyer**

ABSTRACT

The genus *Aspergillus* covers a diverse group of filamentous fungi including industrially important species like *A. niger, A. oryzae, A. awamori, A. sojae* and *A. terreus*. Species of this genus have been exploited in large scale industrial production processes for almost 100 years. As microbial cell factories, filamentous fungi are outstanding with respect to their tolerance of extreme cultivation conditions, their ability to grow on plant biomass, their high secretion capacities and versatile secondary metabolism. The array of *Aspergillus* products includes bulk chemicals, enzymes for food and feed processing, homologous and recombinant proteins as well as bioactive compounds. This chapter aims at providing a comprehensive overview of the advances made during the last decade to further establish and improve Aspergilli as industrial production platforms. It starts with a description of the molecular genetics toolbox that has been developed for rational strain improvement, followed by various genetic strategies that have been applied to improve production of heterologous proteins including optimization of transcription, translation, secretory fluxes, product

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degradation and morphology. The second part of this chapter provides an overview of omics tools established for Aspergilli and highlights recent omics studies on *Aspergillus* as producer of organic acids, plant polysaccharide degrading enzymes and secondary metabolites. Finally, the future prospects of *Aspergillus* as a cell factory are discussed.

Introduction

The kingdom of fungi covers a large and diverse group of lower eukaryotes which includes about 100,000 known species and presumably a million yet to be described and characterized (Hawksworth 1991). Fungi range from unicellular (yeasts) to multicellular organisms (filamentous fungi) and are diverse in morphology, physiology and ecology. Among the group of filamentous fungi, the genus *Aspergillus* is of considerable importance for industrial biotechnology. Their ability to grow on rather simple and inexpensive substrates as well as their natural capacity to secrete high amounts of hydrolytic proteins into the environment combined with its ability to synthesize and secrete various organic acids have attracted considerable interest to exploit them as production organisms in biotechnology and food industry. Important industrial production hosts include *A. niger, A. awamori* (a subspecies of the *Aspergillus* section Nigri (Perrone et al. 2011)), *A. oryzae, A. sojae* and *A. terreus*. In general, most members of the genus *Aspergillus* are saprophytic and are of vital importance for nutrient cycling and the function of ecosystems. However, few *Aspergilli* are pathogenic causing detrimental effects on plants and humans such as *A. flavus, A. parasiticus* and *A. fumigatus*.

Aspergilli exploited at an industrial scale, have a long history of safe use and many of their products have acquired the GRAS status meaning that they are generally regarded as safe by the American Food and Drug Administration (Table 1). The groundwork for *Aspergillus* as microbial cell factory was laid at the dawn of the twentieth century that was accompanied by advances in microbiology, biochemistry and fermentation technology. The pioneering works of Jokichi Takamine (production of amylase from Japanese koji mold, *Aspergillus oryzae*, 1894), James Currie (development of fungal fermentation for citric acid production 1917) and Alexander Fleming (discovery of penicillin production by *Penicillium notatum*, 1928) stimulated scientists to further explore fungal metabolic capacities and, moreover, triggered engineers to develop large-scale production processes for filamentous fungi. The findings of James Curie, for example, led to the establishment of the first industrial scale production process with a filamentous fungus by Pfizer already in 1919. Improvements of fungal capacities to produce metabolites of interest were, however, mainly restricted to classical mutagenesis techniques followed by tedious selection strategies.

Table 1. Selected examples of industrially important compounds produced by *Aspergilli.*

Product	Host	Company
Organic acids		
Citric acid	*A. niger*	Adcuram, ADM, Anhui BBCA Biochemical, Cargill, Jungbunzlauer, Gadot Biochemical Industries, Iwata Chemical Co. Ltd., Tate & Lyle
Itaconic acid	*A. terreus*	Itaconix, Shandong Kaison Biochemical, Qingdao Langyatai Group
Kojic acid	*A. oryzae*	Chengdu Jinkai Biology Engineering Industry, MHC INDUSTRIAL CO., LTD., Sansyo Pharmaceutical Co. Ltd., Wuxi syder Bio-products Co. Ltd.
Enzymes		
α-Amylase	*A. oryzae*	Amano Enzyme Co. Ltd., Biocon, DSM, Novozymes, Dupont IB, Novo Nordisk, Hunan Hong-Ying-Xiang Bio-Chemistry, Shin Nihon Chemical Co. Ltd.
Arabinase	*A. niger*	DSM, Shin Nihon Chemical Co. Ltd.
Asparaginase	*A. niger*, *A. oryzae*	DSM, Novozymes
Catalase	*A. niger*	DSM, Dupont IB, Novozymes, Shin Nihon Chemical Co. Ltd.
Cellulase	*A. niger*	Biocon, DSM, Dyadic, Genencor INT, Haihang Industry, Shin Nihon Chemical Co. Ltd., TNO
Chymosin	*A. niger*	Christian Hansen
β-Galactosidase	*A. niger*, *A. oryzae*	Amano Enzyme Co. Ltd., DSM, Dupont IB, Genencor INT, Novozymes, Shin Nihon Chemical Co. Ltd.
Glucoamylase	*A. niger*	Amano Enzyme Co. Ltd., Cangzhou Kangzhuang Chemical, DSM, Dyadic, Novozymes, Dupont IB, Shandong Longda Bio-Products
Glucose oxidase	*A. niger*, *A. oryzae*	Amano Enzyme Co. Ltd., DSM, Dupont IB, Dyadic, Novozymes
Hemicellulase	*A. niger*	Amano Enzyme Co. Ltd., BASF, Biocon, DSM, Dupont IB, Genencor INT, Novozymes, Shin Nihon Chemical Co. Ltd.
Lactoferrin	*A. niger*	DSM, Agennix
Lipase	*A. niger*, *A. oryzae*	DSM, Dupont IB, Novozymes, Novo Nordisk
Pectinase	*A. niger*	Biocon, DSM, Dupont IB, Novozymes, Shandong Longda Bio-Products
Phytase	*A. niger*, *A. oryzae*	BASF, DSM, Novozymes, TNO
Proteases (Acid, Neutral, Alkaline)	*A. niger*, *A. oryzae*, *A. saitoi*	Amano Enzyme Co. Ltd., DSM, Novozymes, Mitsubishi Foods Co. Ltd., Shin Nihon Chemical Co. Ltd.
Tannase	*A. oryzae*, *A. ficuum*	ASA Spezialenzyme GmbH, Biocon, Kikkoman Corp.
Secondary metabolites		
Fumagillin	*A. fumigatus*	Merck
Lovastatin	*A. terreus*	Biocon, Merck

After (Ward 2011, Meyer 2008)

New classical genetic techniques such as (para) sexual processes and protoplast fusion became available around the 1950's and further advanced the productivity of industrial processes. The birth of molecular biology in 1941 with the demonstration of the 'one gene-one enzyme' relationship in the filamentous fungus *Neurospora crassa* (Beadle and Tatum 1941) and the development of recombinant DNA technologies for filamentous fungi, shown for the first time in 1979 for *N. crassa* (Case et al. 1979), has finally revolutionised *Aspergillus* biotechnology. Since then, it became possible to obtain insights into the molecular basis of product formation and to improve traditional fungal fermentations by rational genetic engineering approaches, not only allowing production of homologous proteins but also production of proteins from non-fungal origin. For example, Novozymes has been the first company in the world which commercialised a recombinant lipase using *A. niger* as production host in 1984. Nowadays, the growing demand for industrial enzymes and organic aicds is met by *Aspergilli* (Table 1), which can be cultivated in large-scale stirred tank reactors reaching volumes up to 300,000 litres (Elander 2003). The advantage of *Aspergilli* over other microbial cell factories of bacterial or yeast origin is that they can tolerate extreme cultivation conditions covering a broad spectrum of pH (2–10), temperature (10–50°C), salinity (0–34%) and water activity (0.6–1) (Meyer et al. 2011b). As they are also able to efficiently degrade plant-derived polysaccharides such as starch, cellulose, hemicellulose, pectin and inulin, the importance of *Aspergilli* and its hydrolytic enzymes might even rise in the near future. For example, the efficiency of the saccharification process of second-generation feedstocks used for bioethanol production might become improved by *A. niger* derived (hemi)cellulases (Rumbold et al. 2009, 2010, Pel et al. 2007, de Souza et al. 2011).

The challenge for current and future strain development programs aiming at full exploitation of *Aspergilli* as multi-purpose expression platform is the full understanding of molecular cell biology of these hosts, the identification of pathway limitations and the substantiate prediction of beneficial metabolic engineering strategies. The aim of this chapter is to explore the possibilities and limitations of *Aspergillus* as a cell factory for the production of platform chemicals, proteins and pharmaceuticals. We review the progress made gestrichen in recent years to implement new molecular genetic engineering tools for rational strain improvements and discuss current technologies for the determination and evaluation of transcriptomic, proteomic and metabolomics data from different industrial *Aspergilli*. We highlight representative systems biology approaches which have uncovered some key players and regulatory mechanisms involved in protein secretion and the formation of primary and secondary metabolites. We also summarise the current knowledge of compartmentalized product biosynthesis as well as transport and traffic phenomena in *Aspergillus* as

this is key to fully understand the link between product formation, secretion and morphology in these versatile expression hosts.

The Molecular Genetic Toolbox for *Aspergillus*

The basis for every rational experimental approach that applies genetic modification in any organism is a well-equipped molecular toolbox including suitable vectors, selection markers and transformation protocols. Although several plasmids have been found in bacteria, yeast and filamentous fungi such as *N. crassa*, no naturally occurring plasmids are present in *Aspergillus* (Griffiths 1995). Nevertheless, it has been shown that artificial plasmids with replication sites targeting the *Aspergillus* replication machinery are able to autonomously replicate in *Aspergilli* and distribute during mitosis. The introduction of the autonomous maintenance in *Aspergillus* (AMA1) sequence from a genomic library of *A. nidulans* resulted in plasmids which displayed autonomous replication properties similar to plasmids with homologous sequences used in *S. cerevisiae* (Verdoes et al. 1994b, Khalaj et al. 2007, Gems et al. 1991, Carvalho et al. 2010). However, the mycelium is heterogenic even under selection pressure, tolerating hyphae devoid of plasmid without showing any phenotype or growth defects (Aleksenko and Clutterbuck 1997). In addition, AMA1-based plasmids can get lost during long-term cultivation, especially under non-selective pressure. Due to these reasons, protein overexpression approaches mainly target the genome of *Aspergilli*. However, AMA1-based vectors carrying auxotrophic (*pyrG*) or dominat (*hygB*) markers are very helpful for complementation approaches of gene deletion mutants (Carvalho et al. 2010).

Several selection markers are available for genetic modification of *Aspergillus*. Well established are nutritional and auxotrophic markers like *argB*, *pyrG*, *pyrE*, *trpC*, *amdS* and *niaD* as well as antibiotic resistance markers based on hygromycin B (*hygB*) and phleomycin (*phle*) resistance (Meyer et al. 2010b). The advantage of using *pyrG* or *pyrE* as selection marker is that *pyrG*$^-$ or *pyrE*$^-$ strains can easily be obtained by direct selection on 5′-fluoroorotic acid medium (FOA) without any mutagenic treatment. Another advantage of these markers is that they can be used repeatedly, i.e. after integration of a *pyrG*- or *pyrE*-containing plasmid into the genome, transformants can be cured from *pyrG* or *pyrE* by cultivating them on FOA plates. The *amdS* gene can be used, much like *pyrG* and *pyrE*, as a bidirectional marker by counterselecting for the loss of *amdS* with media containing the antimetabolite fluoroacetamide (FAA). Detailed protocols for obtaining *pyrG*$^-$ *pyrE*$^-$ or *amdS*$^-$ recipient strains of *A. niger* have recently been published (Meyer et al. 2010b).

Other versatile selection markers have recently been added to this collection. For example, three mutant alleles encoding subunits of a

succinate dehydrogenase (*sdhB, sdhC, sdhD*) have been isolated from a carboxin resistant strain of *A. oryzae*. One of them (*sdhB*) has been shown to provide resistance to carboxin in *A. parasiticus* (Shima et al. 2009). Another auxotrophic marker for *A. niger* is based on the *sC* gene encoding an ATP-sulfurylase homologous to the *Saccharomyces cerevisisae MET3* gene. Complementation with a fully functional copy of the *sC* gene from a wild type *A. niger* strain confers cysteine prototrophy (Varadarajalu and Punekar 2005). Finally, an efficient selection system targeting arginine catabolism of *Aspergillus* has most recently been established. An *A. niger* strain deficient in arginase (*agaA*) and hence unable to grow on arginine as the sole nitrogen source can efficiently be complemented with an arginase expression plasmid (Dave et al. 2012).

Counterselection of bi-directional markers is an effective approach to re-use selection markers in *Aspergilli*. As discussed above, counterselection with antimetabolites such as FOA and FAA allow the re-use of markers such as *pyrG* or *amdS*. The rationale is that the antimetabolite used is intracellularly converted into a toxic substance when an intact copy of the marker gene *pyrG* or *amdS* is present in the genome and actively expressed. Such a selection pressure allows the isolation of natural mutants which accumulate loss-of-function mutations within *pyrG* or *amdS* and/or the isolation of induced mutants which have lost the selection marker due to homologous recombination between direct repeats flanking *pyrG* or *amdS*, respectively (Meyer et al. 2010b, Carvalho et al. 2010). An alternative system for marker recycling has been developed for filamentous fungi by adaptation of the *cre/loxP* system from the bacteriophage P1. Here, the marker gene is flanked by 34 bp long DNA sequences (*loxP*), which become specifically recognized by the recombinase Cre. This enzyme efficiently catalyses the excision of any DNA sequence located between both *loxP* sites, which was successfully demonstrated in *A. nidulans*, *A. fumigatus* and *A. oryzae* (Krappmann et al. 2005, Forment et al. 2006, Mizutani et al. 2012).

For gene targeting approaches in *Aspergillus*, two challenges have to be met. Firstly, any recombinant DNA has to pass the cell wall and cell membrane of the recipient strain. Secondly, the DNA introduced has to become targeted to the desired locus efficiently. Several transformation techniques have been established to transform *Aspergilli*, among which polyethylene glycol (PEG)-mediated transformation of protoplasts is the most frequently used method. Other transformation methods such as *Agrobacterium tumefaciens*—mediated transformation (Michielse et al. 2008, de Groot et al. 1998), electroporation or biolistic transformation have been established as well, although their transformation efficiencies are not as high compared to PEG-mediated transformation of protoplasts (Meyer et al. 2003, Meyer 2008). Detailed protocols for PEG-mediated transformation of *A. niger* providing step-by-step instructions and helpful

advices on how to avoid potential pitfalls have recently been published (Arentshorst et al. 2012, Meyer et al. 2010b). Basically, young mycelium is incubated with cell wall degrading enzymes or enzyme mixtures including Lysing Enzyme® from *Trichoderma harzianum*, chitinase from *Streptomyces griseus* or β-glucuronidase from *Helix pomatia* to degrade the cell wall, thus releasing protoplasts (de Bekker et al. 2009). Protoplasts are suspended in buffers with high salt or sugar concentrations (0.7–1.2 M sucrose, sorbitol or NaCl), in order to protect them from burst due to osmotic imbalance. DNA uptake is mediated by calcium chloride (10–50 mM) in combination with high concentrations of PEG. Although PEG-mediated transformation of cells is a well-established method for bacteria, yeast and filamentous fungi since the 1980s, the actual mechanism on how PEG enables DNA to enter the cell membrane has remained cryptic for almost three decades. Recently, it was uncovered that high concentrations of PEG mediate the attachment of dissolved DNA to cells and protoplasts of *S. cerevisiae* and facilitate the uptake of DNA via endocytosis (Kawai et al. 2010, Zheng et al. 2005). However, compared to transformation efficiencies obtained with *Escherichia coli* or *S. cerevisiae*, the transformation efficiencies of *Aspergilli*, and in general of filamentous fungi, are considerably lower, reaching only about 10–100 transformants per µg DNA (Fincham 1989).

A second challenge, when working with *Aspergilli* is their low frequency of homologous recombination, which hampered efficient functional gene analyses for a long time. Only after observing that disruption of the non-homologous end-joining (NHEJ) pathway in *N. crassa* resulted in homologous recombination frequencies up to 100% (Ninomiya et al. 2004), respective mutants were established in various filamentous fungi, thereby allowing genome-wide functional genomics studies to become feasible (see Kück and Hoff 2010, Meyer 2008). In brief, the NHEJ pathway is a eukaryotic mechanism which bridges broken DNA ends by the joint activities of the Ku heterodimer (Ku70/Ku80-protein complex) and the DNA ligase IV-Xrcc4 complex (Dudásová et al. 2004, Krogh and Symington 2004). In eukaryotes, the NHEJ pathway competes with another repair mechanism, the homologous recombination (HR) pathway, which mediates interaction between homologous DNA sequences, whereas the NHEJ pathway ligates double-strand breaks without the requirement of any homology (Shrivastav et al. 2008). By deleting either *ku70*, *ku80* or *lig4* genes, the HR frequency is dramatically increased in *Aspergilli* (Table 2). Another advantage of this high efficiency of gene targeting is that essential genes can easily be identified by the so called heterokaryon rescue technique as shown for *A. nidulans* and *A. niger* (Nayak et al. 2006, Carvalho et al. 2010). However, several studies have shown that inactivation of the NHEJ pathway makes fungal strains vulnerable to DNA damaging conditions thus increasing their sensitivity towards UV, X-ray or chemical mutagens (Meyer et al. 2007a,

Table 2. Homologous recombination frequencies in NHEJ defective mutants of different *Aspergilli*.

Species	Length of homologous sequence (bp)	Homologous recombination frequency (%)	Reference
A. fumigatus	100	(75)[a]	(Krappmann et al. 2006)
	500	(84)[a]	
	1000	96	
	1500	96	
	2000	95	
A. nidulans	500	89	(Nayak et al. 2006)
	1000	92	
	2000	90	
A. niger	100	18	(Meyer et al. 2007a)
	200	33	
	500	88	
	1000	95	
	1500	98	
A. sojae	500	14.3	(Takahashi et al. 2006)
	1000	71	
	1400	75	
	2000	87	
Neurospora crassa	100	10	(Ninomiya et al. 2004)
	500	91	
	1000	100	

[a]Frequencies given in brackets might not be significant due to low numbers of obtained transformants

Malik et al. 2006, Kito et al. 2008, Snoek et al. 2009). To eliminate the risk that NHEJ deficiency influences or obscures phenotypic analyses, *A. nidulans* and *A. niger* strains were established being transiently silenced in NHEJ, and respective strains have proven to perform as efficient as constitutive silenced NHEJ strains with respect to gene targeting (Carvalho et al. 2010, Nielsen et al. 2008).

In summary, the molecular genetic toolbox for *Aspergillus* has considerably been extended in recent years and provides the research community with versatile tools to genetically modify this genus in a rational and user-specified way.

Genetic Strategies to Improve *Aspergillus* as Protein Producer

Aspergilli are extraordinary in their ability to secrete high amounts of proteins into the environment. Concentrations up to 20 g/l culture medium are no peculiarities for a wide range of host specific proteins (Finkelstein 1987). Secreted fungal proteins like amylases, lipases or proteases are