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REVIEW

Aspergillus as a multi-purpose cell factory: current status and perspectives

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Abstract Aspergilli have a long history in biotechnology as expression platforms for the production of food ingredients, pharmaceuticals and enzymes. The achievements made during the last years, however, have the potential to revolutionize *Aspergillus* biotechnology and to assure *Aspergillus* a dominant place among microbial cell factories. This mini-review will highlight most recent breakthroughs in fundamental and applied *Aspergillus* research with a focus on new molecular tools, techniques and products. New trends and concepts related to *Aspergillus* genomics and systems biology will be discussed as well as the challenges that have to be met to integrate omics data with metabolic engineering attempts.

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Introduction

There are about 250 named species in the genus *Aspergillus* (Geiser et al. 2007), including important industrially exploited species (*A. niger*, *A. oryzae*, *A. awamori, A. sojae, A. terreus*) and harmful species being pathogenic to animals and/or plants (e.g. *A. fumigatus, A. parasiticus, A. flavus*). Due to their extraordinary metabolic versatility, aspergilli are used in biotechnology for the production of a variety of products such as organic acids, pharmaceuticals, proteins and enzymes (for reviews see (Meyer 2008; Lubertozzi and Keasling 2009)). *Aspergillus* stands out from other microbial cell factories of bacterial or yeast origin due to its ability to tolerate extreme cultivation conditions (Raper and Fennel 1965; Kis-Papo et al. 2003; Machida and Gomi 2010). For example, *Aspergillus* can be cultivated over a wide range of temperatures (10–50°C), pH (2–11), salinity (0–34%), water activity (0.6–1) and under oligotrophic or nutrient-rich conditions. Hence, *Aspergillus* can be used for solid-state or submerged fermentations and respective fermentation protocols have been established for large-scale industrial processes.

Most importantly, *Aspergillus* can degrade and utilize diverse biopolymers such as starch, (hemi-)cellulose, pectin, xylan and proteins, allowing it to be cultivated on renewable resources such as plant biomass.

As of 2010, ten genome sequences of the most important industrial and medical Aspergilli are publically accessible, making this genus one of the best to be studied by comparative genome analysis. This resource has spurred multiple research activities, including functional genomics and systems biology attempts aiming at the identification of new leads for strain development and at the understanding of the onset and progression of *Aspergillus* diseases. The aim of this mini-review is to highlight recent breakthroughs in fundamental and applied *Aspergillus* research, focusing on new tools, techniques and products and to discuss new trends, concepts and challenges important for future strain development programs in the post-genomic era.

Genetic tools

Although *Aspergillus* shares with bacterial and yeast cell factories much of their ease of cultivation, the knowledge on how to genetically dissect and engineer industrial *Aspergillus* strains lagged behind for a long time. However, the past decade of *Aspergillus* research has been a fascinating period of new discoveries and breakthroughs, which resulted in many new genetic tools and techniques. These include the establishment of (i) efficient genetic transformation systems, (ii) high-throughput gene targeting tools, (iii) expression systems for high level and controlled protein production and (iv) live-imaging techniques for cell biological studies. In the following, some of these development will be touched, however, for more in-depth information, the reader is directed to the following reviews (Meyer 2008; Hickey and Read 2009; Lubertozzi and Keasling 2009; Fleissner and Dersch 2010; Kück and Hoff 2010; Meyer et al. 2010a).

Genetic manipulation

For a long time, genetic manipulation of industrially used *Aspergillus* strains was hampered due to the lack of efficient genetic transformation systems, limited availability of selection markers and low gene targeting efficiencies (usually around 1–5%). All three problems have been solved during the last decade. Protoplast-mediated transformation became the method of choice to efficiently transform *A. niger*, *A. oryzae*, *A. sojae* and *A. terreus*, whereas *Agrobacterium*-mediated transformation is most efficient for *A. awamori* (reviewed by (Michielse et al. 2005; Meyer 2008)). In addition, versatile selection systems including antibiotic resistance markers (*hph*, *ble, oliC3*), auxotrophic markers (*pyrG*, *pyrE*, *argB*, *adeA*, *adeB*, *niaD*, *trpC, sC*) and nutritional markers (*amdS*, *ptrA*) in combination with integrative and autonomously replicating vectors have been developed, offering nowadays highest flexibility for genetic manipulation of industrial *Aspergillus* strains (Jin et al. 2004; Carvalho et al. 2010a; Fleissner and Dersch 2010; Meyer et al. 2010a). In this context, it is worth emphasizing that the usage of the counter-selectable markers *pyrE*, *pyrG* and *amdS* does fulfill the requirements of the European Union for self-cloning approaches. An interesting alternative option for efficient marker recycling and removal of heterologous DNA sequences makes use of the yeast FLP/*FRT* recombination system. This system was recently adapted for the penicillin producer *Penicillium chrysogenum* (Kopke et al. 2010) and is likely to be applicable to *Aspergillus* as well.

A quantum leap for molecular *Aspergillus* research was based on a finding made in the fungal model organism *Neurospora crassa*. Here it was observed that homologous recombination frequencies can increase up to 100%, when the recipient strain is deficient for the non-homologous end joining (NHEJ) pathway (Ninomiya et al. 2004). This finding was confirmed in many aspergilli including *A. oryzae, A. sojae* and *A. niger* (Takahashi et al. 2006; Meyer et al. 2007; Mizutani et al. 2008; Carvalho et al. 2010a) and many recipient strains are now available in which the NHEJ pathway is permanently or transiently inactivated (reviewed in (Meyer 2008; Kück and Hoff 2010)). This discovery made genome-wide deletion projects feasible, the first of which was launched in 2007 for *N. crassa* (www.dartmouth.edu/~neurosporagenome/) followed by the *A. nidulans* project in 2010 (www.fgsc.net/Aspergillus/KO_Cassettes.htm).

Expression systems

A comprehensive review on different expression system for recombinant protein production in

Aspergillus was most recently given (Fleissner and Dersch 2010). About two dozen *Aspergillus* promoters are available for high-yield production of homologous and heterologous proteins. The promoters used are either constitutively active (e.g. the glyceraldehyde-3-phosphate dehydrogenase promoter P*gpdA*) or are inducible based on the carbon source present in the fermentation medium (e.g. the glucoamylase promoter P*glaA*, the alcohol dehydrogenase promoter P*alcA*, the α-amylase promoter P*amyA*). However, none of the available promoters allows tight, tunable and carbon source-independent expression control, posing limitations in their use. To overcome this problem, an artificial gene expression system based on the *Escherichia coli* tetracycline-resistance operon (Tet-On/Tet-Off system) was adopted for use in *A. fumigatus* (Vogt et al. 2005). The Tet-On system was further refined for use in *A. niger* and systematically evaluated (Meyer et al. 2010b). The data obtained showed that the Tet-On system is tight under non-induced conditions, is able to respond within minutes after inducer addition and allows tunable gene control in a gene dosage and/or inducer concentration dependent manner. Most importantly, the strength of the *A. niger* Tet-On system can compete with the *gpdA* promoter, making it as a very promising tool for future protein overproductions in *Aspergillus*.

Omics tools

The sequencing of fungal genomes led *Aspergillus* research into a new era referred to as systems biology. Instead of studying one gene or protein at a time, the complete set of genes, proteins and/or metabolites are studied simultaneously, aiming at a comprehensive understanding of the robustness, viability and productivity of industrial *Aspergillus* strains and at the identification of factors important for pathogenicity of medical Aspergilli. For each of the omics subdisciplines (transcriptomics, proteomics, metabolomics) a dedicated set of equipment, techniques and software tools has to be developed, a process which is still on-going for many *Aspergillus* species. For the whole genus, only about 30 omics studies appear yearly in Pubmed (Fig. 1), illustrating that *Aspergillus* systems biology is still in its infancy. Among these, the following publications on industrially used Aspergilli are worth highlighting: (i) a

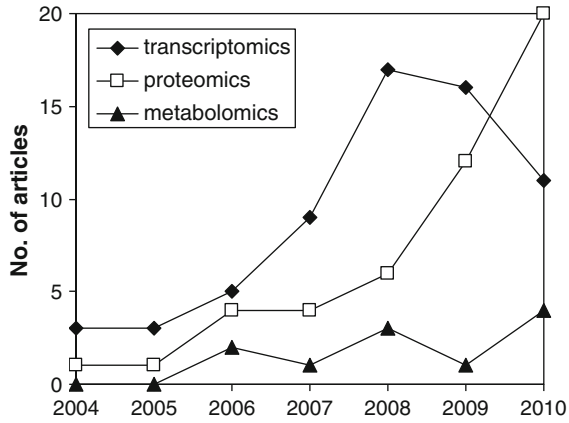

Fig. 1 Number of *Aspergillus* omics articles published over the last years in Pubmed (by October 2010)

metabolic network was established for *A. niger* and *A. oryzae* based on genomic databases and extensive literature surveys (Andersen et al. 2008; Vongsangnak et al. 2008). (Note that a web-based pathway viewer has recently been launched for *A. fumigatus* (Tuckwell et al. 2010.) (ii) The starch-, pectin- and inulin-degrading enzyme network of *A. niger* was identified using a combined database mining and transcriptomic approach (Martens-Uzunova et al. 2006; Yuan et al. 2006, 2008; Martens-Uzunova and Schaap 2009). (iii) An integrated transcriptomics and proteomics approach identified new leads for improved protein secretion of *A. niger* (Guillemette et al. 2007; Levin et al. 2007; Jacobs et al. 2009). (iv) A transcriptomics and genetic study predicted regulatory networks important for the morphological control of *A. niger* growth (Meyer et al. 2009). (v) A transcriptomics and metabolomics study disclosed regulatory genes involved in lovastatin biosynthesis in *A. terreus* (Askenazi et al. 2003). (vi) A transcriptome analysis identified genes important for high-yield solid-phase fermentations in *A. oryzae* (Tamano et al. 2008).

Key for systems biology attempts is the availability of well-annotated genome sequences, standardized analysis tools (e.g. microarray platforms, RNA sequencing technologies, high-throughput chromatographic separations and mass spectrometry tools) and suitably configured databases for data storage and network reconstruction. Most recently, the open access online tool 'BioMet toolbox' has been launched (www.sysbio.se/BioMet/) which allows integration

and systematic analysis of omics data from *A. niger* and *A. oryzae* (Cvijovic et al. 2010). A major challenge in systems biology approaches is the fact that the data gained has varying levels of reliability. Data-sets can be error prone due to technical irreproducibility, e.g. cultivations of *Aspergillus* performed in shake flasks are not reproducible due to continuous changes in pH, temperature, oxygen availability and eventually in morphology. In contrast, cultivations in lab-scale bioreactors allow on-line control on these parameters and thus ensure highest reproducibility (Jorgensen et al. 2010; Meyer et al. 2010a). Besides technical problems, biological variations caused by heterogeneous gene expression in *Aspergillus* populations (Vinck et al. 2005; Levin et al. 2007) will introduce errors as well. Hence, the application of statistical procedures and bioinformatics pipelines for handling and modeling errors as well as internal controls are crucial to gain legitimate interpretations out from high-throughput omics data.

Strain developments

A. niger, A. oryzae and *A. awamori* are well-established expression platforms for the production of secreted proteins and enzymes (Punt et al. 2002). However, their productivities are limited due to several reasons. First, the protein of interest can become degraded by secreted host proteases, the number of which was estimated to be 200 in *A. niger* (Pel et al. 2007). To tailor the host protease profile, several approaches have been followed, including deletion or silencing of selected protease genes in *A. oryzae* or *A. awamori* (Moralejo et al. 2002; Yoon et al. 2009) and deletion of the protease-specific transcription factor PrtT in *A. niger* and *A. oryzae* (Punt et al. 2008). Second, low yields are often observed when proteins of non-fungal origin have to be expressed. It is thought that this results from intracellular misfolding of the target proteins, an event, which initiates compensatory responses in *Aspergillus* such as ERAD (ER-associated degradation; sensing and removal of misfolded proteins via the proteasome) and UPR (unfolded protein response; degradation or refolding of unfolded proteins). To get insights into the genetic repertoire securing protein secretion, ERAD and UPR, transcriptomics and functional genomics approaches have recently been undertaken in *A. niger* (Guillemette et al. 2007; Jorgensen et al. 2009; Carvalho et al. 2010b) and *A. oryzae* (Wang et al. 2010), to name but a few. In order to tackle intracellular degradation of the target protein, the 'carrier approach' is one option, where the protein of interest is genetically fused to an abundantly secreted host protein (Punt et al. 2002). An additional promising approach is the optimisation of the codon usage of the gene of interest according to the preferred codon-usage of the expression host (Roubos and van Peij 2008). Finally, protein engineering approaches aiming at increased protein stabilities hold promise as well (Kumita et al. 2006).

A third, long-lasting and still unsolved problem is related to the morphology of *Aspergillus*. As with all filamentous fungi, either freely dispersed filaments or highly compact pellets can be formed during submerged fermentations, both of which do limit process productivity (Papagianni 2004; Grimm et al. 2005). So far, the formation of these macroscopic morphologies can only descriptively be monitored but not controlled. To overcome this constraint and to optimize the morphology by rational genetic engineering, transcriptomics and functional genomics studies have recently been initiated aiming at the reconstruction of the morphogenetic machinery of *A. niger* (Meyer et al. 2008, 2009).

New products in the post-genomic era

The availability of *Aspergillus* genome sequences has triggered an area of research called 'genome mining' which assumes that *Aspergillus* can be a source of new products such as therapeutics or drugs. Indeed, genome analyses disclosed more genes involved in secondary metabolite (SM) synthesis than anticipated. The respective pathway genes are conveniently arranged in gene clusters (30–40 clusters are typically found in Aspergilli), and have been predicted to be involved in the synthesis of various polyketides or non-ribosomal peptides (Brakhage and Schroeckh 2010). To facilitate the systematic mining and mapping of these clusters, the web-based tool SMURF (Secondary Metabolite Unknown Regions Finder; www.jcvi.org/smurf/) has recently been launched as open access bioinformatics tool (Khaldi et al. 2010). However, most SM clusters are silent or cryptic, i.e. are not expressed under laboratory or

industrial conditions (Pel et al. 2007). To activate expression of silent clusters and to identify the metabolite(s) produced, different strategies based on molecular, epigenetics and cultivation methods have been undertaken in *Aspergillus* and recently reviewed (Chiang et al. 2009; Brakhage and Schroeckh 2010). Among these, four approaches stand out. First, it was reported that controlled activation of a pathway-specific transcription factor in the model *A. nidulans* activated a silent metabolic pathway, as a result of which two novel SM were identified (Bergmann et al. 2007). Second, targeted manipulation of the chromatin state, i.e. removal of a chromatin silencing gene, activated expression of at least two other gene clusters (Bok et al. 2009). Thirdly, it was shown that an intimate physical interaction during fungal-bacterial co-cultivations is the prerequisite for selective activation of another SM cluster (Schroeckh et al. 2009). And finally, cultivation at very low specific growth rates, which almost reached zero-growth conditions, induced expression of SM clusters in *A. niger* (Jorgensen et al. 2010). Hence, multiple approaches can be followed or combined to fully harness the SM potential of Aspergilli.

Lubertozzi and Keasling (2009) list in their comprehensive review many more potential natural *Aspergillus* products with commercial relevance, an impressive list that emphasises the great potential of *Aspergillus* to become a multi-purpose expression platform. Besides polyketides and non-ribosomal peptides as potential therapeutic agents, isoprenoids are interesting as new neutraceuticals or aroma compounds, lipopeptides and AFP-derived proteins as new antifungals and poly-unsaturated fatty acids or lipids as food additives or fuel feedstocks. Moreover, the future product spectrum of industrial *Aspergillus* strains is not restricted to its natural genetic repertoire. The new genetic engineering toolkit for *Aspergillus* and the advent of synthetic biology open new avenues to many more compounds of (non)fungal origin to be expressed and produced in *Aspergillus*.

New concepts and perspectives

To fully explore and exploit *Aspergillus* as a multi-purpose expression platform, conceptually new approaches will be followed, some of which have the potential to revolutionize *Aspergillus* biotechnology. It is likely that newly engineered production strains will be completely devoid of unwanted SM gene clusters and used as chassis for the production of food and feed ingredients, enzymes, pharmaceuticals or even fuel feedstocks. The gene(s) or gene cluster(s) of interest will be of *Aspergillus* or (non)fungal origin and polished according to the *Aspergillus* codon-bias. Expression constructs will be plugged into selected genomic loci allowing highest control over gene expression. The gene(s) of interest will be expressed under the control of constitutive or inducible promoters, depending on the toxicity of the final product. The product repertoire, the timing of product formation and productivity will be optimised using different cultivation strategies. For example, fermentations under zero-growth conditions can uncouple growth from product formation and have the potential to improve SM production (Jorgensen et al. 2010).

In addition, vesicle and organelle engineering approaches offer exciting opportunities. For example, intracellular proteins can become secreted via 'peroxicretion', an artificial route where proteins become targeted to peroxisomes which themselves have genetically been decorated with v-SNARES. They ensure transport of the peroxisomes to the plasma membrane, where the protein cargo becomes released into the medium (Sagt et al. 2009). Moreover, SM production can be improved by optimizing the volume fraction of organelles in which (part of) SM biosynthesis takes place. A proof-of-concept was recently shown for penicillin synthesis in *Penicillium chrysogenum*, where artificially induced proliferation of peroxisomes resulted in increased penicillin titres (Kiel et al. 2005; Meijer et al. 2010). Most interestingly, natural product formation in *Aspergillus* can also be dependent on the circadian rhythm—not only in the case for SM as known for a long time (Brakhage and Schroeckh 2010), but likely also in the case of secreted enzymes as shown for cellulase production in the industrial fungus *Trichoderma reesei* (Castellanos et al. 2010). Hence, understanding circadian regulatory mechanisms might also give new leads for genetic engineering strategies.

What is pivotal to successfully merge *Aspergillus* biotechnology, systems biology tools and metabolic engineering attempts? Controlled cultivation and harvesting conditions for *Aspergillus* as well as standardised methodological and bioinformatics tools are crucial to deduce accurate and meaningful

conclusions out from transcriptome, proteome and metabolome data. Genome-wide metabolic models and metabolic flux analyses are key for the identification of pathway limitations and predicting beneficial metabolic engineering strategies. However, to fully describe and engineer cellular phenomena in *Aspergillus*, the implementation of cell biological studies such as in vivo live imaging are required. Only the understanding of compartmentalized product biosynthesis as well as transport and traffic phenomena will uncover the link between morphology, secretion and product formation in *Aspergillus* and will eventually give significant leads for redesigning this versatile expression host.

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