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From gene to product - Development of biotechnological processes by integrating genetic and engineering methods

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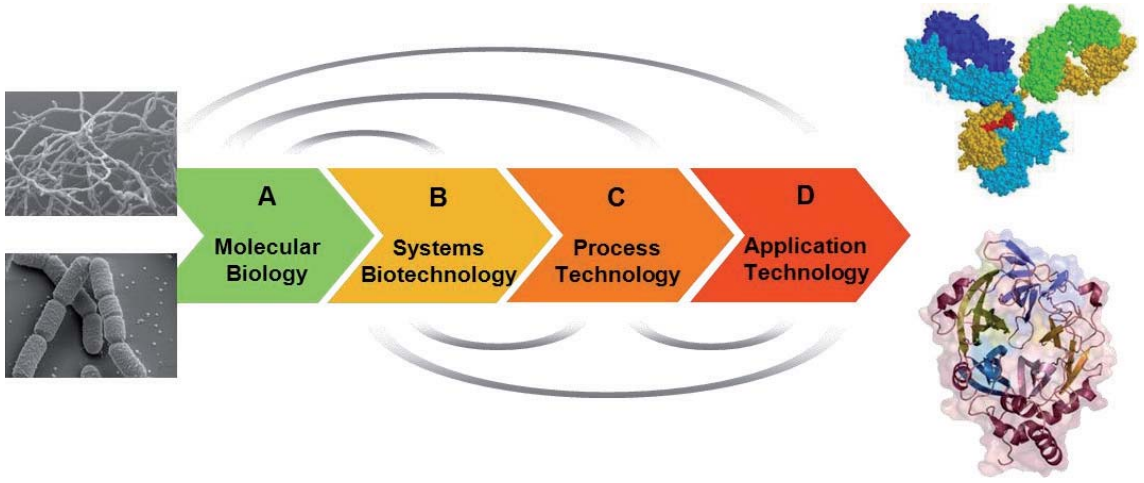
Preface

General goals, strategic approach and used methodology

Since 2001, the Collaborative Research Centre SFB 578, "*Development of biotechnological processes by integrating genetic and engineering methods - From gene to product*", has been dedicated to designing and implementing novel concepts and strategies for integrated strain and process development for industrial bio-production. Right from the beginning, research in SFB 578 employed a truly global and integrated approach for the optimization of production hosts and the corresponding production processes, a strategy which has meanwhile been coined systems biotechnology. For systems-wide strain and process engineering, disciplines from engineering and life sciences were integrated and combined to provide high-value biotechnological products in biologically active form at high titer and yield. This new integrated approach is now changing the way we design and develop industrial processes in biotechnology.

Strategically, we focused our research for the whole funding period on two model organisms and two product classes to achieve close cooperation and a synergistic interaction. The filamentous fungus *Aspergillus niger* and the bacterial host *Bacillus megaterium* were selected as work horses of SFB 578. Both microorganisms have a high industrial relevance. In addition, they share a substantial potential to produce and secrete recombinant proteins, thus promising that the obtained data and strategies will have broad relevance. Across different, closely interlinked research areas, high-value recombinant proteins were selected as relevant model products. These included biocatalysts involved in sugar metabolization and pharma proteins of the antibody and bone morphogenic factors. Among the products to be investigated, glycosyltransferases are receiving increasing interest for supplying pre-biotic sugars, which have been gaining more and more applications in the food and pharma sector. Moreover, recombinant antibodies for therapy and diagnostics represent the largest and most rapidly growing fraction of pharma proteins.



Development of Biotechnological Processes by Integrating Genetic and Engineering Methods - From Gene to Product.

Practically, results from quantitative physiology were complemented by information from an efficient omics technology platform, providing insights into metabolome, fluxome, transcriptome and proteome of the production host of interest. Bioinformatics was used to intelligently store, interpret and combine obtained results with literature data. Thus, a solid basis for regulatory and metabolic network modeling was generated. Model-based understanding of the underlying biological systems was used for prediction concerning the optimization of the overall production process, verified via experimentation and used for interative model optimization. Finally, obtained recombinant products were further developed with the goal of applying them in drug delivery, and for lab-on-the-chip and other diagnostic tools.

Research program and partners

The central overall aim of this SFB 578 was a holistic approach to analyze, quantify and model the biological and biochemical engineering processes for optimal product formation, purification and application of biotechnological cultivations. At the beginning of this last funding period, a solid basis of know-how from the last two funding periods of this SFB already exited. Genetically engineered production strains of both model systems, *A. niger* and *B. megaterium,* have been created for intra- and extracellular formation of antibodies and glycosyltransferases. A broad methodological repertoire for their analyses has been developed. Secreted target proteins can now be localized *in vivo* and characterized *in vitro* through biochemical and structural analysis. Modelling approaches have been established, and elaborated bioinformatics tools are at hand, the latter based on complete genome databases. The basis for quantitative determination of the transcriptome, proteome, metabolome and fluxome to characterize and optimize cultivation processes under different environmental conditions has been successfully established. Further, purification strategies for the target proteins as well as innovative strategies for their applications were developed. Thus, substantially all required methodological tools have been created. The central goal in the last funding period was to bring these pieces together, including data integration for the overall process and its optimization.

To attain the goals of SFB, a close and intensive collaboration between all partners was required. In this SFB, the core competence lay in the disciplines of Biochemical Engineering, Microbiology and Biotechnology of the Technische Universität Braunschweig. Furthermore, Institutes of Microtechnology, Technical Chemistry, Electrical and Pharmaceutical Engineering were involved. They were supported by working groups of the Helmholtz Centre for Infection Research (HZI), Braunschweig, and the Institute for Process Engineering, Otto-von-Guericke-University, Magdeburg. Overall, the SFB was divided into four project areas in 16 subprojects with 23 project managers and 27 PhD-students.

Project area A: Molecular biology of product formation

SFB 578 provided the basis for the establishment of a complete tool kit for recombinant intra- and extracellular protein production using *A. niger* and *B. megaterium*. To date, over 20 different expression vectors encoding various inducible systematically optimized

promoters, including those for phage RNA polymerases, in combination with perfect ribosome binding, are available for *B. megaterium*. Currently, 1.3 g per liter GFP model protein can be produced intracellularly. Various added affinity tags allow for fast affinity chromatographic purification. Furthermore, different high efficient signal sequences for protein export via the Sec system into the growth medium were added to the system, yielding up to 0.2 g per liter single chain antibody fragment and more than 1 g per liter of levansucrase exported into the growth medium. The system is commercialized with MoBiTec GmbH, Göttingen. High yield protein production was the result of a systematic systems biotechnological optimization strategy for both, *A. niger* and *B. megaterium*. This included transcriptome, proteome, metabolome and fluxome measurements and their bioinformatics based integration. Gene regulatory and metabolic modeling yielded predictions for process optimization. In this context, the genome of two *B. megaterium* strains was solved and annotated.

Furthermore, multiple sugar metabolizing enzymes were produced recombinantly by using *A. niger* and *B. megaterium,* and subsequently employed for the synthesis of high value sugars. The crystal structure of one central enzyme of interest was elucidated and used for designing substrate specificity changes.

Project area B: Systems biotechnology of product formation

Results obtained with *A. niger* and *B. megaterium* played a central role towards understanding the investigated cellular and bio-process systems and providing strategies for their tailor-made improvement. In this line, different projects were aimed at obtaining quantitative, highly informative data sets from genomics, transcriptomics, proteomics, metabolomics and fluxomics. In pioneering concepts, these should be integrated into the process environment on the level of shear stress, cellular morphology and fluid dynamics to provide the most comprehensive picture of the process to be optimized. Targets on the genetic as well as on the process level should be identified to be implemented by research groups from all other areas of SFB 578.

Project area C: Process technique

The downstream purification process of isolation, concentration and analytical detection of the target proteins from culture media was a further focus of this SFB 578. For protein produced by the recombinant *A. niger* and *B. megaterium* strains, a purification chromatographic method and functionalized nanoparticles were used and studied in depth. The purification of antibodies was performed by continuous chromatographic separation according to the simulated moving bed technology. The main task was the development of the theoretical basis for a simulation of the entire separation process. In addition, stabilized water-compatible functionalized iron oxide nanoparticles with covalently bound functionalization were developed for protein purification, prepared and applied for the purification of recombinantly produced antibody fragments and glycosyltransferases. The realization of a molecular functionalization was targeted, without large macromolecules such as polymers to shield the magnetic cores and reduce the active surface of the

system. Furthermore, the development of magnetic separation methods with homogenous gradient fields and thus homogenous adsorbance force was investigated to achieve highly specific separation and filtration of the protein-carrying magnetic particles. For a production-related on-line analysis of enzymes and antibodies, magnetorelaxometry was developed on the basis of a differential fluxgate setup. For the targeted bio-products sophisticated processing routines are available that allow generation of important, time-resolved analyses of data of high quality for complex systems biotechnological approaches.

Project area D: Application technique

The focus of this project area within this SFB 578 was the use of antibodies for different applications. The first example was the development of hydrogel drug delivery systems as attractive approaches for the administration of therapeutically relevant biomacromolecules such as antibodies. The main focus here was the engineering and delivery of the drug dosage precisely at the desired target and indicated dosing rate. The investigations were mainly based on biocompatible hydroxyethyl starch, modified with a crosslinkable side group (e.g. hydroxyethyl methacrylate). Single chain antibody fragment variables were incorporated in this hydrogel system. The application demonstrated a detailed realization of a drug delivery system involving synthesis characterization of the hydrogel and mobility of encapsulated substances, but also of *in vivo* studies and correlation of *in vitro* to *in vivo* release rates. Finally, this method allowed individual animals to be monitored over an extended period of time. This led to improved quality of data and, additionally, a much lower number of animals was required than that used in conventional animal studies.

The second application dealt with the development of a medical analysis laboratory in chip size (lab-on-chip) for point-of-care diagnostics as a tool for a much quicker diagnosis and the start of therapy. C-reactive protein (CRP) has been identified as a superior indicator for inflammatory diseases, but its analysis requires sophisticated and time consuming detection, which are not suitable for point-of-care applications. Consequently, CRP was used as a model molecule for the development towards a point-of-care analysis system. The developed method now provides different possibilities for application, since the detector antibody can be changed to measure a large variety of clinically relevant parameters. Furthermore, the microfluidic setup will allow facile parallelization in future setups to determine a larger number of samples or parameters simultaneously.

***Model system* Aspergillus niger**

During the years of funding, SFB 578 provided a range of innovations on systems biotechnology of *A. niger*. These included efficient genetics enabled the creation of production mutants for recombinant enzymes such as glucoamylase and fructofuranosidase, but also of GFP-based reporter strains. For systems-wide analysis of *A. niger*, a rich portfolio of experimental and computational methods was established. This allowed detailed investigations of gene expression and protein production and secretion, intracellular metabolite

levels or pathway fluxes. This was complemented by different methods to analyze morphological forms of *A. niger* under various process conditions. Coupled to automated image analyses, these studies allowed a precise estimation of morphological shape via newly derived dimensionless morphology numbers. All these tools are now available to the academic and industrial research. These could be exploited to fine tune morphology for bio-production. Novel approaches used the addition of inorganic micro particles to the culture to control the morphology of the fungus. Integrated with model-based medium design and process development, production of recombinant enzymes can be highly efficient. Exemplified for fructofuranosidase, SFB 578 created a production process for this valuable enzyme, which was more than tenfold more efficient than any other process reported to data, and perfectly underlining the great potential of systems biotechnology based strain and process engineering.

***Model system* Bacillus megaterium**

Multiple expression plasmids for the extra- and intracellular recombinant production of proteins in *B. megaterium* were constructed and commercialized in cooperation with the local company MoBiTec. These plasmids enable the gram per liter production of desired proteins, as well as their purification via affinity tags. In recent years, this productions system has been frequently employed world-wide by industrial companies. The described system was successfully developed to high productivity during bioreactor growth. For this purpose, novel integrated systems biology approaches were established. A genome database (MegaBac) and various novel bioinformatics tools for microarray and proteome data interpretation (JProGo, JVirGel), signal peptide prediction (PrediSi) and codon adaptation index calculation (JCat) were established and are now frequently utilized. Novel strategies for the inexpensive synthesis of high value sugar using recombinant enzymes and their variants were developed. The catalytic enzyme mechanisms that are involved were elucidated at the molecular level. Furthermore, high yield bioreactor based production processes for recombinant antibodies were implemented and optimized using the outlined systems biotechnological approach.

SFB key numbers

The last funding period of the SFB (2008 - 2012) involved 15 subprojects with 23 projects managers in four project areas. Additionally, in 2009 the SFB started the transfer project "*Recombinant production of new phytases by* Bacillus megaterium" (Jahn) together with the BASF AG, Ludwigshafen, developing methods, know-how and major new aspects of the SFB expertise into industrial application and ongoing fundamental research.

Within the projects of this SFB during the last funding period, approximately 125 publications were published in high-reputation journals (e.g. *Appl. Microbiol. Biotechnol., Appl. Environ. Microbiol., Bioinformatics, Biotechnol,. Biotechnol. Bioeng., ChemBioChem, Chem. Eng. Sci., Eng. Life Sci., J. Appl. Phys., J. Biotechnol., J. Chromatography, Lab on a Chip, Microb. Cell Fact., Nature Biotechnol.*). Additionally, 23 PhD-theses, which had been financed by SFB, were successfully completed, several more will follow soon. A vital

exchange with external research groups was established. Within the eleven-year-term of our SFB approximately 120 seminars with invited speakers from academia and industry were conducted. For the 100th colloquium anniversary in June 2010, two volumes of the renowned series of *Advances in Biochemical Engineering/Biotechnology* were published, nicely integrating the SFB research into the overall developments in systems biotechnology. Different status conferences with the entire SFB 578 community focused on project cooperations and on strengthening interdisciplinary efforts. Additionally, a vital and continuous exchange with direct team work between the PhD students developed that supported work in the different projects and areas. On the occasion of the completion of the SFB 578 the editorial board of the *Journal of Biotechnology* has invited the SFB 578 to provide a special issue dedicated to the "*Development of Biotechnological Processes by Integrating Genetic and Engineering Methods – From Gene to Product*", containing more than 20 scientific contributions of both research papers and review articles.

The SFB 578 initiated successful technology transfer from basic research to industrial application. The constructed vector systems for *Bacillus megaterium* were commercialized by the company MoBiTec GmbH, Göttingen. This expression system is now successfully used in several laboratories worldwide in academia as well as in industry for production and secretion of different kinds of recombinant proteins. A further industrial cooperation was established with Bayer Pharma AG, Bergkamen, on the development of indicators for the quality of filamentous seed cultures. Here, elaborated instrumental and experimental methods to evaluate spore quality are now established for routine use in industrial cultivation.

SFB 578 caused major structural changes at the Technische Universität Braunschweig

During the granting periods of SFB 578, a strong systems biology unit with a broad spectrum of Omics technologies in combination with various bioinformatics groups was established at our university. Due to its success, the university decided to focus its research strategy on systems biology in the Life Sciences. Consequently, the Faculties of Life Science and Mechanical Engineering adapted their hiring strategies to this research focus. Multiple professorships were filled with experts for example in proteomics (W2, Riedel), metabolomics and bioinformatics (W3, Schomburg), and fluxomics and bio-engineering (W3, Wittmann). In the next stage, the Technische Universität Braunschweig, in close cooperation with the local Helmholtz Center for Infection Research, is building a novel "*Braunschweig Integrated Center fo Systems Biology*" (BRICS) on the university campus close to the biocenter for 25 million Euro. The construction process will start in early 2013 and the center will host various groups (Wittmann, Jahn, Schomburg) involved in this SFB 578. The systems biology expertise of BRICS also constitutes a major component of the recently granted SFB-TR 51 ("*Ecology, Physiology and Molecular Biology of the Roseobacter Clade*"), the currently renewed DFG-Forschergruppe FOR 1220 ("*PROTRAIN - Prosthetic Groups: Transport and Insertion*") and the BMBF consortium UroGenOmics ("*Strain Specific Systems Biology of Uropathogenic Bacteria"*). Another

important offspring from SFB 578 is the new Pharma process engineering center (PVC) which will be built for 29 million Euro also on the campus of the university. Both centers, BRICS and PVC, are part of the local Translational Alliance in Niedersachsen (TRAIN), a joint venture of the Technische Universität Braunschweig, the local Helmholtz Center (HZI), the Medical School Hannover, the Leibniz Universität Hannover and the University of Veterinary Medicine Hannover.

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Dieter Jahn (Speaker)
Christoph Wittmann (Deputy speaker)
Rainer Krull (Managing director)

Project area A: Molecular biology of product formation

Project-No.	Topic	Disciplines and field of work	Project manager, institution
A1	Production of recombinant glycosyltransferases using *Bacillus megaterium* and *Aspergillus niger*	Microbiology, Bacterial and fungal genetics, Recombinant protein production and secretion	D. Jahn and P. Dersch, Institute of Microbiology, TU BS
A6	Systems biology of chaperones for the production of antibodies with *Bacillus megaterium*	Biotechnology	S. Dübel and M. Hust, Department of Biotechnology, TU BS
A7	Structural biology of glycosyltransferases for the optimization of biotechnical processes	Organic chemistry, Structural biology, Biocatalysis, Glycobiology	D. Heinz, Department of Structural Biology HZI BS / J. Seibel, Institute of Organic Chemistry, University of Würzburg

3.1 General information on the completed project A1

3.1.1 Topic

***Production of recombinant glycosyltransferases using* Bacillus megaterium *and* Aspergillus niger**

3.1.2 Disciplines and field of work

Microbiology, bacterial and fungal genetics, recombinant protein production and secretion

3.1.3 Project manager

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3.2 Development of the subproject

3.2.1 Report

3.2.1.1 Current knowledge at the last application and initial question

The production, secretion and purification of recombinant proteins in eukaryotic as well as prokaryotic systems are major topics of industrial biotechnology. During the first application period (2001-2004) microbial expression systems for the filamentous nonpathogenic fungi *Aspergillus niger* as well for the Gram positive bacterium *Bacillus megaterium* were established. It has an efficient secretion systems which makes it applied in industrial production processes. The proteins produced by this organism possess posttranslational modifications. *B. megaterium* is nonpathogenic and able to metabolize numerous carbon sources. Further, it shows a great potential for protein secretion and, important for recombinant protein production, has a stable plasmid replication system. The major goal of the first funding period of SFB 578, the establishment of a system for the secretion of the recombinant proteins, which included different homologous and heterologous glycosyltransferases into the growth medium was reached.

During the second funding period (2004-2008) the expression systems for both organisms were further enhanced. This included the integration of different small affinity tags for purification of the intra- as well the extracellular produced recombinant proteins. Proteins could now be easily purified and used for different activity tests. Here, new glycosyltransferases recombinantly overproduced in *A. niger* and *B. megaterium* were characterized, respectively [*Homann et al., 2009, Zuccaro et al., 2008*]. New promoters and leader peptides were identified by secretome analysis for both hosts. These promoters could be induced using simple cheap carbon sources. For *B. megaterium* a further

promoter system based on a two vector system was developed. With this phage dependent system it was possible to produce recombinant proteins intracellularly.

Now, within the third application (2008-2012) the questions and requirements were phrased:

1. Enhancing and optimizing existing promoter systems using different strategies
2. Characterization of new promoter systems
3. Construction of strains coproducing rare tRNA
4. Sequencing and annotation of the genome of *B. megaterium* DSM319
5. Adaptation of the vector systems to the cloning host *E. coli* for cloning toxic genes
6. Establishment of microarray experiments

3.2.1.2 Results and applied methods

3.2.1.2.1 *Aspergillus niger*

Characterization of inducible promoters of *Aspergillus niger*

Suitable inducible promoter systems have fundamental impact for recombinant protein production using microorganisms. These systems are characterized by stringent control, cheap inducers and high inducibility. During the last application period different inducible promoters for recombinant protein production using *A. niger* were identified and further characterized using the green fluorescent protein Gfp as a model. These promoters include the sucrose inducible β-fructofuranosidase promoter P_{suc1} [*Zuccharo et al., 2008*], the promoter of the *phiA* gene which is induced by osmotic stress and the inducible gluco-amylase promoter P_{glaA} [*Ganzlin and Rinas, 2008*].

In this application period the promoter P_{suc1} was further characterized. For this, the strain ARAn1 (P_{suc1}-*gfp*) was constructed while the strain AB1.13 was used as a control. After verifying stringent inducibility of P_{suc1}, the influence of different carbon sources (maltose, xylose, fructose, sucrose, glucose) in minimal medium on growth and Gfp production was tested. Highest cell dry weight (CDW) was achieved if growing with maltose or sucrose (around 15 g L^{-1}). Further, the different carbon sources showed an influence on recombinant Gfp production (**Fig. 1**). While all strains showed fluorescent properties the production of Gfp with specific antibodies was only detected if grown with sucrose or with a mixture of sucrose and glucose. The specific fluorescence detected could be identified as background signal. These results clearly indicated that glucose did not cause catabolite repression.