This is an Open Access-journals’ PDF published in Driouch, H., Roth, A., Dersch, P., Wittmann, C. Filamentous fungi in good shape: Microparticles for tailor-made fungal morphology and enhanced enzyme production (2011) Bioengineered Bugs, 2 (2), .
Filamentous fungi in good shape

Microparticles for tailor-made fungal morphology and enhanced enzyme production

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Filamentous fungi such as Aspergillus niger are important biocatalysts for industrial production of various enzymes as well as organic acids or antibiotics. In suspended culture these microorganisms exhibit a complex morphology which typically has a strong influence on their production properties. In this regard, we have recently shown that the addition of inorganic micro particles to the culture medium is a straightforward and elegant approach to precisely tame fungal morphology. For A. niger a full range of morphological forms from pellets with different diameters to free mycelium could be adjusted by supplementation with talc powder. Aluminium oxide particles similarly affected morphology, showing that this effect is largely independent of the chemical particle composition.

Exemplified for different recombinant A. niger strains enzyme production could be strongly enhanced by the addition of microparticles. This was demonstrated for the production of fructofuranosidase, an important high-value biocatalyst for prebiotic fructooligosaccharides of the inulin type, such as 1-kestose and 1-nystose, prebiotics with substantial commercial interest. In particular, these compounds are highly attractive for human consumption, since they have been shown to reduce the risk of colon cancer.

In summary, the use of microparticles opens a new avenue of engineering fungal morphology into the desired form for specific production processes.

Targeted Morphology Engineering for Improved Enzyme Production

The close link between fungal morphology and productivity has been identified early on in important industrial processes such as the production of several enzymes, citric acid or antibiotics. The formed type of morphology depends on the cultivation conditions and the genotype of the applied strain. So far this only allowed a partial control of morphology by the initial pH-value, the impeller speed or the initial spore concentration. In a pioneering study, the addition of micro particles to the culture was recently shown to alter fungal morphology which was recently utilized to precisely adjust different distinct morphological forms in A. niger.

The strong impact of microparticles on A. niger is exemplified for the addition of talc or aluminium oxide to the culture medium prior to inoculation (Fig. 1). In a normal suspended culture, A. niger forms large pellets with an average diameter of 1.7 ± 0.1 mm. With increasing concentration of microparticles added the pellet size...
**Microparticle-Enhanced Bioprocess for Production of Fructofuranosidase**

This interesting influence of microparticles on important metabolic characteristics of *A. niger* opens a new strategy for superior enzyme production. This was recently demonstrated for fructofuranosidase production by recombinant *A. niger* SKAn1015. Fructofuranosidase is an important industrial biocatalyst for the synthesis of fructooligosaccharides as functional food ingredients (Fig. 4A).\(^5\) Combined with model based medium design, identifying elevated levels of glucose, NaNO\(_3\), MnCl\(_2\).4H\(_2\)O and FeSO\(_4\).7H\(_2\)O as beneficial, and the development of a suitable bioprocess strategy the application of talc micro particles allowed highly efficient enzyme production.\(^1\) When transferred into a fed-batch environment with intermittent feeding of glucose, a
potential of the use of microparticles. To unravel the link between the production behaviour and the obviously affected morphology, enzyme production was spatially resolved during the fed-batch process within the different fungal aggregates, mycelium and pellet, employing a GFP-expressing variant of *A. niger.*

Production was hereby localized in 70 μm thin cross sections through biomass aggregates obtained from cultures with and without micro particles. Protein production in the pellet-based process, however, occurred only within a thin layer at the pellet surface. The inner pellet did not contribute to production, probably due to diffusion limitation of oxygen or of other nutrients. For the micro particle-enhanced process, intensive fluorescence was present across the entire mycelium. This indicates that the interaction with micro particles created a productive biocatalyst remaining highly active during the entire process.
Figure 4. Biosynthesis of fructooligosaccharides of the inulin type by recombinant fructofuranosidase. Enzymatic reaction including transfructosylation towards GF₆, Sucrose; GF₂, 1-kestose, GF₃, 1-nystose and GF₄, 1F-fructofuranosylnystose (A). Batch production of fructooligosaccharides by fructofuranosidase produced by recombinant *A. niger* SKAn1015 in a microparticle enhanced batch process (B). The reaction conditions in the enzymatic conversion were: fructofuranosidase activity 860 U/mL; 500 g/L sucrose; 50°C; pH 5.4. Sugars and fructooligosaccharides were quantified by HPLC using external calibration with pure standards.⁶

A key criterion for the developed process is the selectivity at which the target enzyme fructofuranosidase is produced. Previous separation of the supernatant at the end of the micro particle enhanced cultivation process by 1-D SDS-PAGE revealed the presence of only a few proteins. From enzyme activity measurements and the estimated molecular weights, we concluded that the culture supernatant mainly contains fructofuranosidase as a dominant product and small amounts of glucoamylase. The estimation via the volume of the observed bands in 1D gels revealed that fructofuranosidase accounted for about 85% of the totally excreted protein in the culture supernatant, indicating a highly specific production process. These findings are confirmed by MALDI-TOF MS analysis of the two proteins excised from the gel which allowed clear identification of the two proteins as fructofuranosidase as dominating product (120 kDa) and glucoamylase (80 kDa), respectively (Table 1). Future rational based approaches such as metabolic engineering of *A. niger* SKAn1015 could aim at disruption of the encoding glucoamylase gene to completely prevent its formation, if needed.
The two bands represent the two major proteins produced and previously attributed to fructofuranosidase (band 1) and glucoamylase (band 2) by enzyme activity measurement and molecular weight. For identification, bands were manually excised from the gel, cut into small pieces and prepared for peptide digestion by trypsin addition. Peptide mass fingerprints and peptide fragmentation data were analysed using an UltrafleXtermeTOF/TOF mass spectrometer (Bruker Daltonic GmbH, Bremen, Germany) and subsequently processed using FlexAnalysis™ 3.0 and the Biotools™ 3.2 program of the software-package Masslynx™. The Mascot 2.2 search program was used for protein identification with the annotated A. niger genome (EMBL: www.ebi.ac.uk/genomes/eukaryota.html). Proteins with a score of more than 52 were regarded as significant.

<table>
<thead>
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<th>Band</th>
<th>Accession coverage (%)</th>
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<th>Searched/matched peptides</th>
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</table>

The high level of about 450 g/L fructooligosaccharides is indicated by the formed products comprised the high value compounds 1-kestose (55%), 1-nystose (38%) and 1F-β-fructofuranosynystose (7%), respectively. By extension of the incubation time, the product spectrum could be successfully shifted towards the higher weight fructooligosaccharides, i.e., 1F-β-fructofuranosynystose. During this time a slight decrease of the total amount of fructooligosaccharides was observed which might be due to hydrolysis. The transfructosylating and hydrolytic activity ratio was 25, matching nicely with previous observations for this recombinant fungal fructofuranosidase. Variation of the substrate to sucrose analogues could be an interesting option to extend the product range of the produced recombinant fructofuranosidase. Overall, the results demonstrate that the novel approach of targeted morphological engineering is an effective strategy for biotechnological enzyme production by filamentous fungi. We did not observe any negative interference with subsequent application of the enzyme produced for biocatalysis. In fact, with minimal pretreatment the enzyme obtained allowed highly efficient bioconversion towards prebiotics of commercial interest. Due to the wide use of filamentous fungi the application potential of microparticles in fungal fermentations appears large.

### Acknowledgements

The authors gratefully acknowledge financial support provided by the German Research Foundation (DFG) through the Collaborative Research Center SFB 578 “From Gene to Product” at the Technische Universität Braunschweig, Germany. The authors further acknowledge the contribution by Manfred Nimtz on protein identification by MALDI-TOF MS.

### References