ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Improved Enzyme Production by Bio-Pellets of *Aspergillus niger*: Targeted Morphology Engineering Using Titanate Microparticles

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Received 12 July 2011; accepted 17 August 2011

Published online 24 August 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23313

ABSTRACT: The present study describes the design of bio-pellet morphologies of the industrial working horse *Aspergillus niger* strains in submerged culture. The novel approach recruits the intended addition of titanate microparticles ($TiSiO_4$, 8 μm) to the growth medium. As tested for two recombinant strains producing fructofuranosidase and glucoamylase, the enzyme titer by the titanate-enhanced cultures in shake flasks was increased 3.7-fold to 150 U/mL (for fructofuranosidase) and 9.5-fold to 190 U/mL (for glucoamylase) as compared to the control. This could be successfully utilized for improved enzyme production in stirred tank reactors. Stimulated by the particles, the achieved final glucoamylase activity of 1,080 U/mL (fed-batch) and 320 U/mL (batch) was sevenfold higher as compared to the conventional processes. The major reason for the enhanced production was the close association between the titanate particles and the fungal cells. Already below 2.5 g/L the micromaterial was found inside the pellets, including single particles embedded as 50–150 μm particle aggregates in the center resulting in core shell pellets. With increasing titanate levels the pellet size decreased from 1,700 μm (control) to 300 μm. Fluorescence based resolution of GFP expression revealed that the large pellets of the control were only active in a 200 μm surface layer. This matches with the critical penetration depth for nutrients and oxygen typically observed for fungal pellets. The biomass within the titanate derived fungal pellets, however, was completely active. This was due a reduced thickness of the biomass layer via smaller pellets as well as the core shell structure. Moreover, also the created loose inner pellet structure enabled a higher mass transfer and penetration depths for up to 500 μm. The creation of core-shell pellets has not been achieved previously by the addition of microparticles, for example, made of talc or alumina. Due to this, the present work opens further possibilities to use microparticles for tailor-made morphology design of filamentous fungi, especially for pellet based processes which have a long and strong industrial relevance for industrial production.

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Biotechnol. Bioeng. 2012;109: 462–471.

KEYWORDS: morphology engineering; core shell pellet; filamentous fungi; critical pellet diameter; fructofuranosidase; glucoamylase

Introduction

Filamentous microorganisms cover the production of commercially important products. As one of the major industrial working horses, *Aspergillus* species produce technical, food and animal feed enzymes, organic acids, antibiotics, or eukaryotic gene products such as tissue plasminogen activators or lactoferrins (Wucherpfennig et al., 2010). The morphology of filamentous microorganisms in submerged cultivation has a strong influence on productivity and has been therefore a subject of considerable interest since many years (McIntyre et al., 2001b). As observed from different studies optimal production requires a distinct morphology which is likely to be process specific. Interesting attempts have employed targeted metabolic engineering (McIntyre et al., 2001a; Müller et al., 2002; Thykaer et al., 2009), evolutive adaptation in chemostat (van de Vondervoort et al., 2004) or variation of operational parameters (Moreira et al., 1996; Spohr et al., 1998; Xu et al., 2000) to influence the morphology in filamentous microorganisms. However, the whole picture is quite complex. Morphology appears difficult to be controlled especially with regard to superior production performance. Obviously many factors including specific strain properties, process variables, rheology, or mass transfer are involved in the underlying macro and micromorphogenesis (McIntyre

et al., 2001b). A breakthrough in the targeted control of fungal morphology was the recent discovery that the addition of talc or alumina microparticles into the culture medium strongly influences the morphological development of *A. niger* and other filamentous microorganisms (Kaup et al., 2007). This could be further elaborated into a targeted engineering of fungal morphology towards elevated enzyme production in different recombinant strains involving the creation of highly productive mycelia by the talc or alumina based micromaterial (Driouch et al., 2010a,b,c). Although obviously beneficial for production, the created mycelium, in contrast to pelleted growth, poses potential problems on the process performance such as increased viscosity of the culture broth with reduced mass transfer as well as a more elaborative separation of the biomass during product purification (Cui et al., 1998). Moreover, pellets have been reported as desired morphology for production of itaconic acid (Metz, 1976), citric acid (Gomez et al., 1988), or penicillin (Nielsen et al., 1995). What limits the application of pellets is the low mass transfer inside the fungal aggregate (Wittler et al., 1986; Nielsen, 1996; Hille et al., 2005). Pellets are fully supplied with nutrients or oxygen only up to a critical diameter. For *A. niger* pellets this diameter is about 400 μm (Hille et al., 2005). Recent fluorescence analysis of GFP reporter strains of *A. niger* confirmed that only a thin layer at the pellet surface contributes to protein production, whereas the large inner part of the pellet was found inactive (Driouch et al., 2010a). At this stage, it appeared promising to see weather microparticles can be also recruited to generate pellets with improved performance and thus extend the available morphology space of filamentous fungi.

In this regard, the present work demonstrates the design of bio-pellets of *A. niger* adding 8 μm titanate particles to the culture, a micromaterial previously not considered. Exemplified for different recombinant *A. niger* strains, expressing the enzymes fructofuranosidase, glucoamylase, and the model protein GFP the effect of this material on growth, production, and morphology was studied in detail. The results could be used to create high-producing core-shell pellets. Applying titanate addition in batch and fed-batch processes, the glucoamylase production of *A. niger* could be enhanced up to sevenfold.

Materials and Methods

Strains and Maintenance

The fructofuranosidase overproducing strain *A. niger* SKAn1015 carrying the *suc1* gene under the control of the constitutive *pki*A promoter was previously obtained from *A. niger* AB1.13 by transformation (Zuccaro et al., 2008; Driouch et al., 2010b,c). Similarly, *A. niger* ANip7-MCS-gfp2 which co-expresses the genes for glucoamylase (*gla*A) and green fluorescent protein (GFP) under the control of the *gla*A promoter was derived (Driouch et al., 2010b). The *gla*A promoter is repressed by xylose and inducible by carbon sources such as glucose, maltose, or starch. Both strains were maintained as frozen spore suspension in 50% glycerol at −80°C.

Seed Culture

A spore inoculum of *A. niger* was prepared by growing thawn spores from the maintenance culture on 30 g/L potato dextrose agar (Sigma–Aldrich, Seelze, Germany) at 30°C for 3 days. Spores were then harvested as suspension from the plate into 20 mL 0.9% NaCI solution, which was spread onto the plate. After filtration (Miracloth, 25 μm pore size, CalBioChem, Darmstadt, Germany) the spore concentration was determined photometrically at 600 nm. All cultivations were inoculated from this suspension to an initial concentration of 10^6 spores/mL.

Cultivation Media

The cultivation medium of *A. niger* SKAn1015 contained per litre: 20 g D-glucose, 6 g $NaNO_3$, 0.5 g KCI, 1.5 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 10 mg EDTA, 4.4 mg $ZnSO_4{\cdot}7H_2O$, 1.01 mg $MnCl_2{\cdot}4H_2O$, 0.32 mg $CuSO_4{\cdot}5H_2O$, 1 mg $FeSO_4{\cdot}7H_2O$, 0.32 mg $CoCl_2{\cdot}6H_2O$, 1.47 mg $CaCl_2{\cdot}2H_2O$, and 0.22 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. The cultivation medium of *A. niger* ANip7-MCS-gfp2 contained per litre: 10 g D-xylose, 6.6 g $(NH_4)_2SO_4$, 2.5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}H_2O$, 5 mg citric acid$\cdot H_2O$, 5 mg $ZnSO_4{\cdot}7H_2O$, 1 mg $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 0.16 mg $CuSO_4$, 0.05 mg H_3BO_3, 0.05 mg $Na_2MoO_4{\cdot}H_2O$, and 0.037 mg $MnSO_4{\cdot}H_2O$). Maltose was added as indicated to induce the glucoamylase production. In fed-batch cultivation of *A. niger* ANip7-MCS-gfp2 a 200 g/L maltose solution was applied as feed. In all cases the solutions containing the carbon source, the inorganic salts, and the trace element were sterilized separately. The maltose solution was sterilized by filtration. All other solutions were sterilized by autoclaving at 121°C for 20 min and cooled down to room temperature prior to mixing. Titanium silicate oxide ($TiSiO_4$) was obtained as dry powder from Sigma–Aldrich. The mean particle diameter of this micromaterial ($X_{50} = 8$ μm) was estimated experimentally by particle size analysis. Talc applied for reference measurement of viscosity was used as described before (Driouch et al., 2010a). In all cultivations with microparticle addition, the material was added at the beginning of process. Prior to use, the microparticles were re-suspended in 50 mM Na-acetate buffer (pH 6.5), autoclaved at 121°C for 20 min and added to the sterile growth medium prior to inoculation.

Cultivation Conditions

Cultures of both *A. niger* strains were grown in 250 mL baffled shake flasks with 50 mL medium at 120 min^{-1} on a rotary shaker (Certomat BS-1, 50 mm, Sartorius, Göttingen,

Germany). The growth temperature was 37°C (*A. niger* SKAn1015) and 30°C (*A. niger* ANip7-MCS-gfp2), respectively. All shake flask studies were carried out in triplicate. The glucoamylase producing strain *A. niger* ANip7-MCS-gfp2 was additionally cultivated in a 3.0 L stirred tank bioreactor (Applikon, Schiedam, The Netherlands). All bioreactor cultivations were carried out in duplicate. Hereby the aeration rate was maintained constant at 1.0 L/min using a mass flow controller (red-y compact, Vögtlin Instruments, Hamburg, Germany). The agitation speed was kept at 200 min^{-1}. The pH was automatically kept constant at pH 5.0 by the addition of 2 M NaOH and 2 M HCI. The temperature was maintained at 30°C. Antifoam (Ucolub N115, Mühlheim, Germany) was added manually when required. For batch cultivation a working volume of 2.2 L was employed. Fed-batch processes involved an initial batch phase with 1.5 L starting volume. Intermittent feeding of the maltose solution was controlled such that the substrate level remained above 5 g/L, which was monitored by measurement in the broth. The total feed volume added was 1 L. The agitation speed was maintained at 200 min^{-1} during the first batch phase. Subsequently, the agitation speed was increased stepwise every 30 min to 300 and 400 min^{-1}, meaning that the latter value was then maintained for the rest of the process.

Quantification of Biomass

The cell dry mass was determined gravimetrically. For this purpose, 5 mL culture broth was filtered through a pre-weighted cellulose acetate filter (pore size 20 μm, Sartorius). Subsequently, the filter was rinsed twice with deionized water and then dried at 100°C until weight constancy. After cooling in a desiccator the cell dry mass was determined by re-weighing. All measurements were corrected for the micromaterial added and were performed in triplicate.

Enzymatic Assays

The specific activity of fructofuranosidase in the culture supernatant was determined as described previously (Driouch et al., 2010a). Shortly, the reaction mixture (220 μL) consisted of 200 μL of 1.65 M sucrose dissolved in 0.05 M phosphate buffer (pH 5.4) and 20 μL of sample. The reaction was started by addition of the substrate solution to the sample. After incubation at 40°C for 20 min the reaction was stopped by heating at 95°C for 10 min. After cooling, the reaction mixture was centrifuged at 13,000*g* for 10 min at 4°C. Glucose formed from cleavage of sucrose by the enzyme was then quantified. To account for residual glucose in the culture broth, negative controls were carried out by using samples in which fructofuranosidase was inactivated by heating at 95°C for 10 min prior to incubation. Glucoamylase activity was determined in crude cell extract by the method of (Withers et al., 1978) using *p*-nitrophenyl-α-glucopyranoside (pNPG) as substrate. For this purpose cells were harvested from 10 mL culture broth by filtration (pore size 20 mm, Sartorius) including two washing steps with 10 mM sodium-acetate buffer (pH 4.5). Cells were then suspended in fresh buffer, disrupted by a mill mortar for 6 min (RMO, Retsch, Haan, Germany). The cell extract was then obtained by filtration (0.2 mm pore size, Sartorius). Subsequently, 500 μL of pNPG solution (0.1% w/v, in sodium-acetate buffer, pH 4.8) was added to 250 μL cell extract. After incubation for 20 min at 60°C, the enzymatic reaction was stopped by addition of 750 μL of 0.1 M sodium borate. After filtration (0.2 mm pore size, Sartorius) the absorption at 400 nm was measured. All enzymatic assays were done in triplicate.

Microscopy

The culture morphology was analyzed by 3D photographs using a stereo-microscope (Stemi 2000-C, ZEISS, Jena, Germany) with an AxioCamMRc5 camera (Stemi 2000-C, ZEISS). Via light microscopy and image analysis of 100 aggregates from culture samples, the average pellet diameter (d_{pellet}) was measured and used to determine the volume-related specific surface (S_v) assuming all the pellets to have the same spherical shape (Eq. 1).

$$S_v = \frac{6}{d_{pellet}} \quad (1)$$

In addition, confocal laser scanning microscopy (CLSM) was applied to analyze the inner structure and the spatial distribution of GFP within thin sections through cellular aggregates as described previously (Driouch et al., 2010b,c).

Rheological Analysis

Rheological measurements of broth samples were performed in compliance with ISO-2431 using a rotational viscosimeter (Type Bohlin CS10, Malvern Instruments, Worcestershire, UK), equipped with a double gap system (DG 40/50, Malvern Instruments). The given viscosity values are as collected for 30 mL sample at a shear rate of 100 s^{-1}. All measurements were performed in triplicate, values given represent total mean. The data were corrected for the different biomass concentrations to account for the impact of morphology, but exclude concentration dependent interference.

Results

Improvement of Enzyme Production in *Aspergillus niger* by Titanate Microparticles

In initial screening experiments in shaken flasks, the impact of titanate microparticles (titanium silicate oxide, $TiSiO_4$, 8 μm) on growth and enzyme formation in recombinant *A. niger* was investigated. The study comprised comparative

cultivations of the fructofuranosidase producer *A. niger* strain SKAn1015 and the glucoamylase producer ANip7-MCS-gfp2 with different titanate levels in the range of 0–50 g/L over 72 h (Fig. 1A–D). Obviously, production of both enzymes was enhanced by the added particles (Fig. 1B). The highest extracellular enzyme activity was achieved when a titanate concentration of 25 g/L was used. Under these conditions, the resulting activities for fructofuranosidase (150 U/mL) and glucoamylase (185 U/mL) were 4-fold and 10-fold higher as compared to the control, respectively. Similarly, growth seemed to be enhanced for both strains, although the effect was not so pronounced (Fig. 1A). The pellet size could be designed rather precisely over almost one order of magnitude (Fig. 1D). Without titanate in the control experiment, the average pellet diameter at the end of the cultivation was 1.7 mm. Upon addition of titanate particles, the pellets formed were much smaller. The average diameter decreased to about 0.3 mm. *A. niger* maintained its pelleted growth even at rather high levels of titanate. The formation of mycelia or hyphal fragments was not observed. The formed pellets only slightly increased the viscosity. In comparison to the control, broth viscosity after 72 h (end of cultivation) was below 10 mPa s for all titanate levels, reflecting the presence of pellets (Table I). Additional experiments with talc microparticles (6 μm) resulted in the previously described formation of free mycelium (Driouch et al., 2010a). The corresponding viscosity was fourfold higher, reaching a maximum of approximately 40 mPa s. The pH value of the cultures was unaffected by the addition of titanate (Fig. 1C).

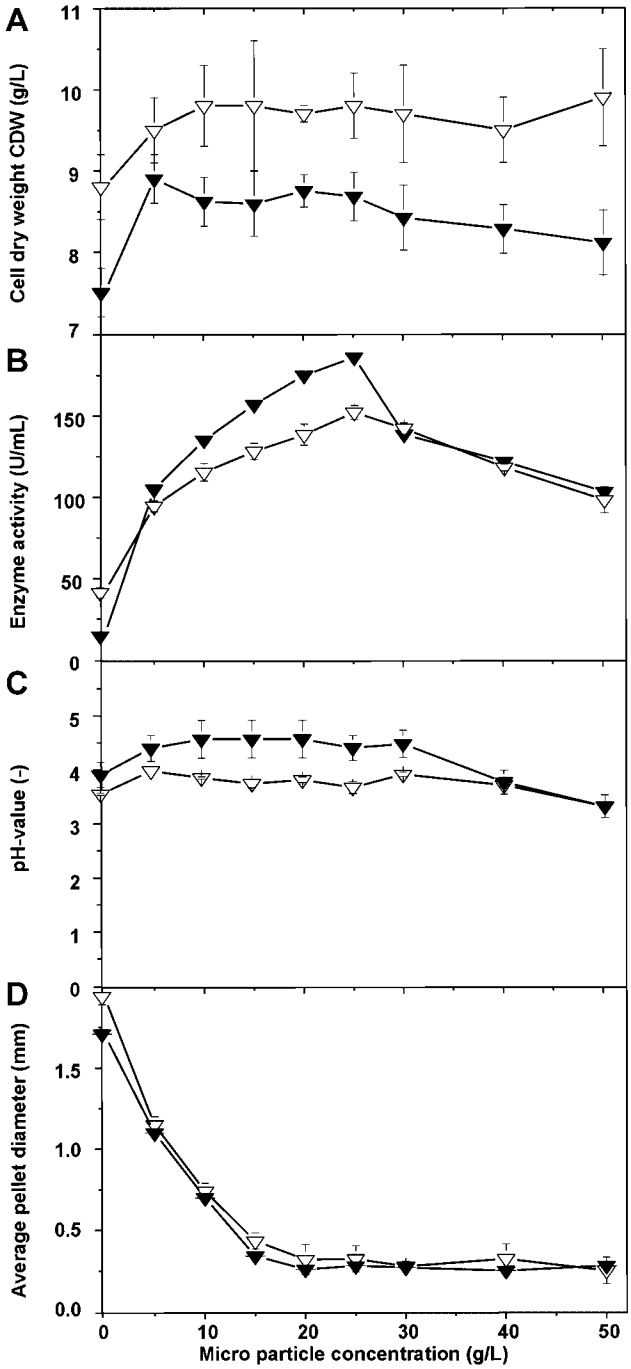

Figure 1. Production of fructofuranosidase and glucoamylase by *Aspergillus niger* SKAn1015 and *Aspergillus niger* ANip7-MCS-gfp2 in batch culture without (control) and with addition titanate (8 μm) microparticles. The data comprise cell dry mass (**A**), enzyme activity (**B**), pH of the culture (**C**), and average pellet size diameter (**D**) after 72 h cultivation time as mean value from triplicate cultivations with corresponding deviation.

Morphology of *Aspergillus niger* in the Presence of Titanate Microparticles

Microscopic analysis revealed that depending on the amount of titanate present, different types of pellets were formed. The control pellet of about 2 mm size consisted of a rather dense outer layer of biomass and exhibited an unfilled centre (Fig. 2A). Elevated levels of the micromaterial resulted in a remarkable change of the morphology. Most strikingly, the titanate particles were to a large extent associated with the biomass and occurred inside the pellets. Up to 5 g/L titanate (Fig. 2B,C) the overall pellet size was almost unaffected. Interestingly, the micromaterial was partly present inside the pellet. This obviously created a loose interior structure with a better biomass filling in the pellet core. Hereby, the particles were randomly distributed within the aggregate. At higher levels, core shell aggregates were formed (Fig. 2D–I). Hereby, the titanate obviously aggregated in the initial phase of the cultivation. The formed particle aggregates provided a solid support for growth of *A. niger* which subsequently covered the titanate cores with a thin layer of mycelium during the culture. Partly, hyphae could be also observed in the inner zone between the titanate particles. Interestingly, the titanate cores varied in size between 20 and 150 μm in diameter depending on the amount of particles initially added. Together with the outer cell layer this resulted in pellets of differed sizes, amounts, and composition. The smaller pellets mainly consisted of cells, whereas the larger pellets were mainly composed of

Table I. Broth viscosity for different morphological forms of *Aspergillus niger* SKAn1015 obtained by the addition of microparticles.

	Viscosity (mPa s)	Morphology	Average pellet size diameter (mm)
Without particle addition	5.5 ± 0.3	Pellet (control)	1.71 ± 0.11
With titanate[a] microparticle			
2.5	7.2 ± 0.3	Pellet	1.4 ± 0.1
5	6.8 ± 0.1	Pellet	1.1 ± 0.1
10	7.6 ± 0.5	Pellet	0.7 ± 0.1
15	7.4 ± 0.1	Pellet	0.3 ± 0.1
20	8.6 ± 0.2	Pellet	0.3 ± 0.1
25	7.5 ± 0.4	Pellet	0.3 ± 0.1
30	10 ± 0.3	Pellet	0.3 ± 0.1
40	9.4 ± 0.1	Pellet	0.3 ± 0.1
50	12 ± 0.3	Pellet	0.3 ± 0.1
With talc[b] microparticle			
2.5	18.5 ± 0.3	Pellet	0.9 ± 0.1
5	29.9 ± 0.3	Mycelium	—
10	30.2 ± 0.5	Mycelium	—
15	29.0 ± 0.1	Mycelium	—
20	32.7 ± 0.3	Mycelium	—
25	27.7 ± 0.4	Mycelium	—
30	39.0 ± 0.3	Mycelium	—
40	34.9 ± 0.3	Mycelium	—
50	36.1 ± 0.6	Mycelium	—

The measurements were performed for culture broth after 72 h of cultivation.
[a]Titanium silicate oxide (titanate, $TiSiO_4$, 8 μm particle size).
[b]Hydrous magnesium silicate (Talc, 6 μm particle size).

titanate. Overall, a full range of macro-morphological pellet forms from dense pellets to core shell forms could be generated.

Spatial Resolution of Protein Production in *Aspergillus niger* Pellets

It was now interesting to see, how the obviously increased production was distributed across the designed bio-pellets. The biomass of filamentous fungi is typically rather heterogeneous. Therefore, the extent of enzyme synthesis was spatially resolved across the different zones of the mycelial aggregates formed in the presence or absence of microparticles. This was investigated for *A. niger* ANip7-MCS-gfp2 which co-expresses glucoamylase together with green fluorescent protein (GFP2) under control of the same promoter. GFP2 expression was localized via fluorescence intensity in 70 μm thin cross sections through the biomass aggregates formed. For different titanate levels, the overall structure and the corresponding fluorescence intensity across a pellet are displayed in Figure 3A–J. In the control, protein production was highest within a thin layer of about 50 μm thickness at the pellet surface. Inside the pellet, fluorescence was strongly reduced. Obviously, only a small fraction of the entire biomass was contributing to product formation. The interaction with the microparticles created smaller and highly active bio-pellets of basically three different types. At low titanate concentration, a gradient of GFP expression within the pellet was still visible, although the thickness of the high-producing surface layer as well the contribution of the inner core were substantially higher, probably related to the looser inner pellet structure created (Fig. 3C,D). In the medium concentration range, the pellets were smaller and exhibited a highly active biomass layer surrounding the inner titanate core (Fig. 3F–H). With higher concentration of titanate, the GPF expression was equally high across the whole 400 μm pellets. Above this optimum concentration, the overall production was reduced (Fig. 3J).

Bioprocess Development Using Titanate-Designed Bio-Pellets

The potential of titanate microparticles on enzyme production was now tested in batch and fed-batch processes operated in an industrially relevant stirred tank bioreactor with the glucoamylase producer *A. niger* ANip7-MCS-gfp2. Hereby, a concentration of 25 g/L titanate, identified as optimum value (Fig. 1), was chosen. In batch mode, the microparticles resulted in faster growth of the strain and a higher biomass level (Fig. 4A,B). Most strikingly, glucoamylase production was almost sevenfold higher in the presence of titanate. This is reflected by the final titre of 320 U/mL after 100 h of cultivation as compared to that of the normal cultivation (50 U/mL). Operated in fed-batch with intermittent maltose feeding glucoamylase production could be even further enhanced (Fig. 4C,D). After an initial batch phase of 34 h, the feed was started, which contained a concentrated form of maltose to induce the *gla*A promoter. The intermittent feed with a total volume of 1 L was then adjusted such that the maltose level in the culture was maintained above 5 g/L ensuring sufficient supply of the culture with the carbon source. In the microparticle enhanced process, the extracellular glucoamylase activity reached a final value of 1,080 U/mL after 200 h. The corresponding value in the control fed-batch was only 140 U/mL, meaning that the addition of the microparticles increased production dramatically. The total biomass concentration of both processes was similar, although the biomass in the control fermentation probably contained a certain fraction of inactive cells inside the large pellets.

Discussion

Here we demonstrate the targeted engineering of the morphology of *A. niger* into core shell pellets by the addition of titanate microparticles. Through appropriate addition of the micromaterial, pellet size, and inner pellet structure could be rather precisely adjusted. Obviously, this was rather

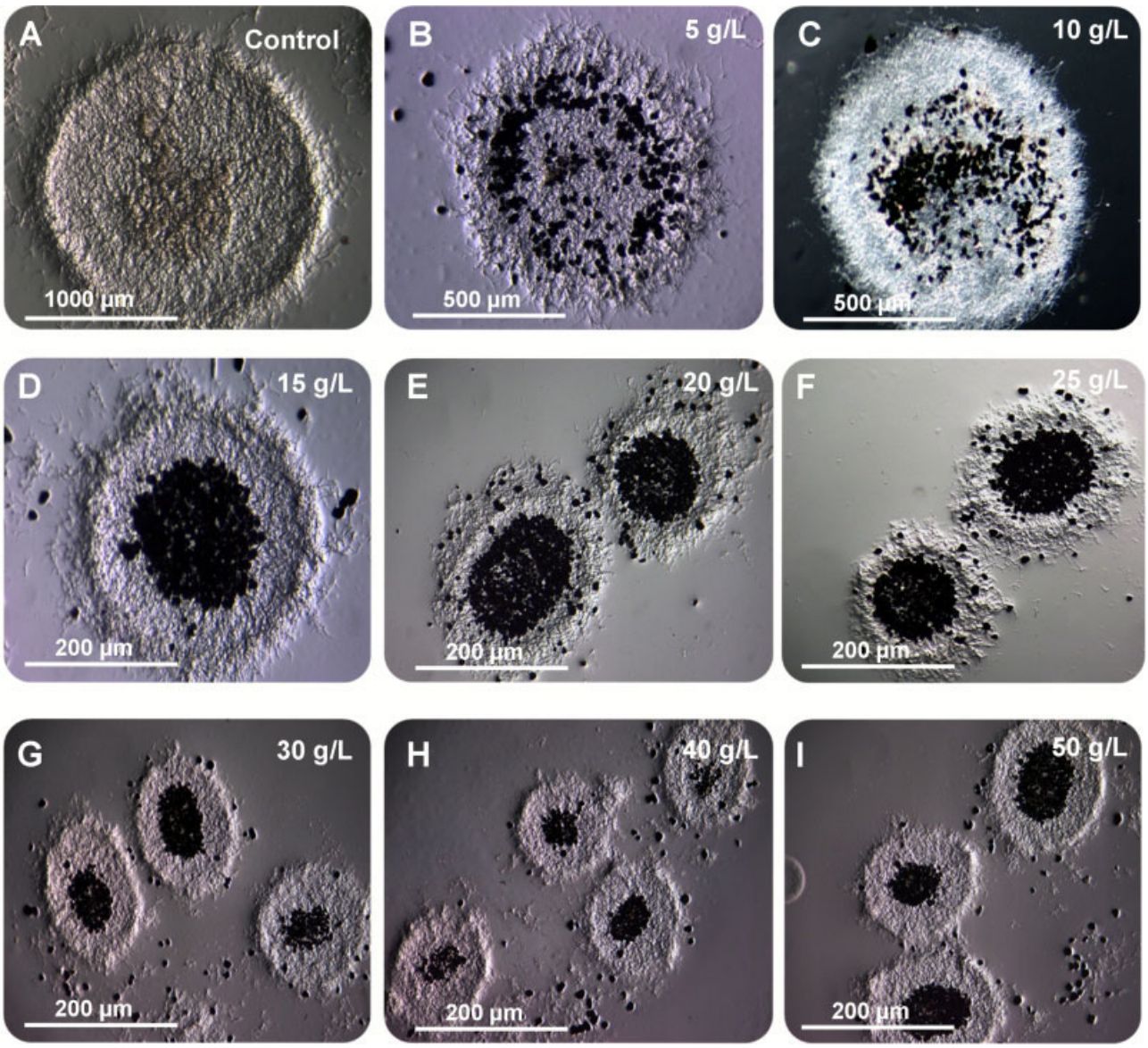

Figure 2. Influence of titanate microparticles on the morphology of *Aspergillus niger* SKAn1015 aggregates in submerged culture in shaken flasks. Titanium silicate oxide (titanate, $TiSiO_4$, 8 µm particle size) was added at varied concentration: control without addition (**A**), 5 g/L (**B**), 10 g/L (**C**), 15 g/L (**D**), 20 g/L (**E**), 25 g/L (**F**), 30 g/L (**G**), 40 g/L (**H**), and 50 g/L (**I**). Image analysis was performed by light microscopy after 72 h of cultivation.

beneficial for the production of recombinant enzymes in *A. niger*. Utilizing the optimized titanate concentration (25 g/L) (Fig. 1B), the process was successful then transferred from shake flask (250 mL) into a batch and a fed-batch-operated bioreactor (3 L). In batch and fed-batch processes, the intended addition of titanate microparticles allowed the engineering of the morphology of *A. niger* into a highly active bio-pellet form which strongly boosted enzyme production (Table II). Exemplified for the industrial enzyme glucoamylase, the finally obtained activity in the supernatant of 1,080 U/mL (fed-batch) and 320 U/mL (batch) was about sevenfold higher as compared to the corresponding conventional operation without titanate. Additionally, also the space time yield was markedly higher.

Major reasons for this optimized production behavior are related to the characteristics of pelleted growth by filamentous fungi (Wittler et al., 1986; Nielsen, 1996; Hille et al., 2005). It is often observed and generally regarded as a severe disadvantage that the mass transfer within the typically dense pellet is rather low. Accordingly, pellets are fully supplied with nutrients or oxygen only up to a critical diameter. Although partly depending on the cultivation

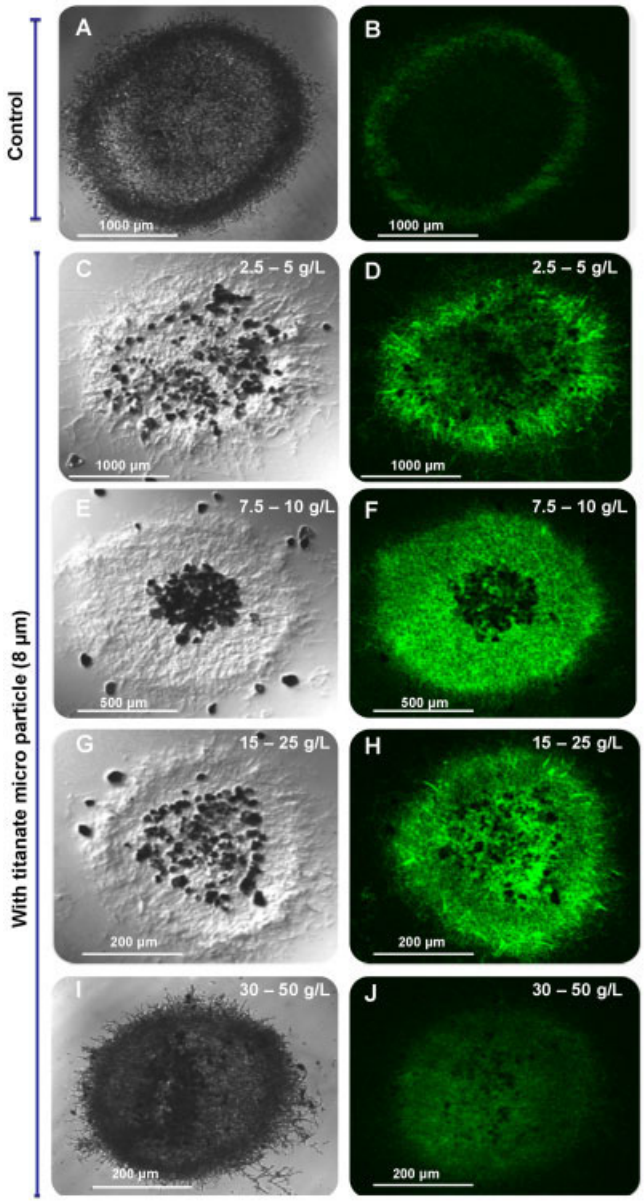

Figure 3. Spatial resolution of fluorescent protein production (GFP2) in recombinant *Aspergillus niger* ANip7-MCS-gfp2 pellets. The GFP2 expression was induced by start of the maltose feed after 40 h of cultivation. Samples were taken after induction 72 h of cultivation time. The pictures were obtained from 70 µm cross-sections through fungal aggregates by confocal laser scanning microscopy.

conditions and the pellet structure, previously measured values for the critical pellet diameter are rather similar for different *Aspergillus* species. As example, *A. niger* pellets exhibit a value of 410 µm (Hille et al., 2005), whereas the critical pellet diameter was estimated as 370 µm for the related species *A. awamori*. Obviously, the supply of oxygen and other nutrients to cells in the interior of larger aggregates is thus limited beyond the critical penetration depth of half of the critical diameter, that is, 200 µm. These phenomena can be visualized via fluorescence related detection of the expression of GFP. Biosynthesis of this model protein from sugar, salts, and oxygen requires the entire carbon core metabolism of *A. niger* including the supply of the amino acid building blocks from various precursor compounds as well as high amounts of ATP and reduction equivalents. The detection of fluorescence is thus a good indicator to which extent the cells are sufficiently supplied with their key nutrients. In this regard, the large pellets of the control culture exhibit a 200 µm active layer at the pellet surface nicely matching with the above derived critical penetration depth (Fig. 5A). With respect to production, however, the active layer represented only a small fraction of the entire pellet. The dominant fraction of cells, occurring inside the pellet was probably limited. The inspection of the pellets formed via addition of titanate unravels the underlying reasons for their improved performance. Hereby different positive effects are combined. First, the overall inner structure was improved. With titanate supplementation, core shell pellets with a 500 µm layer of fungal biomass resulted (Fig. 5B). Supported by the titanate core the entire biomass layer was fully active. Obviously, the created inner pellet structure was rather loose. This enabled a higher mass transfer and a more than doubled penetration depth as compared to the control. At higher titanate level, the derived smaller pellets were also fully supplied (Fig. 5C). The major reason for the optimized pellet structure is the surprising influence of the micro-material. It was mainly found within the pellets. At low titanate concentration a statistical distribution of single particles inside the pellet could be observed, which changed into a rather dense titanate core in the pellet centre at higher levels. In our opinion this is a major driver to overcome limiting biomass growth, regarded as one of the main problems for achieving performance with filamentous fungi (Nielsen, 1996). It was interesting to note, that protein production was obviously not restricted to hyphal tips as previously reported (Gordon et al., 2000; Vinck et al., 2005). This might display a further advantage of the novel cultivation approach.

Second, titanate addition of titanate created smaller pellets (Fig. 1D). The diameter of the fungal pellets was reduced from 1,700 µm in the control cultivation to 300 µm, meaning that a substantially larger pellet surface for transport and nutrient uptake resulted (Fig. 6A). This was directly correlated to the production performance (Fig. 6B). The close correlation further confirms that mass transfer limitations play a major role in this system. The large surface area allowing higher supply of the fungal cells with nutrients and oxygen was thus beneficial for production. This was rather independent from the enzyme produced suggesting a general advantage of titanate supplementation.

The response of cells to the titanate microparticles greatly differed from the effects to talc or alumina materials recently investigated (Kaup et al., 2007). In contrast to titanate, the addition of talc and alumina to cultures of *A. niger* and other

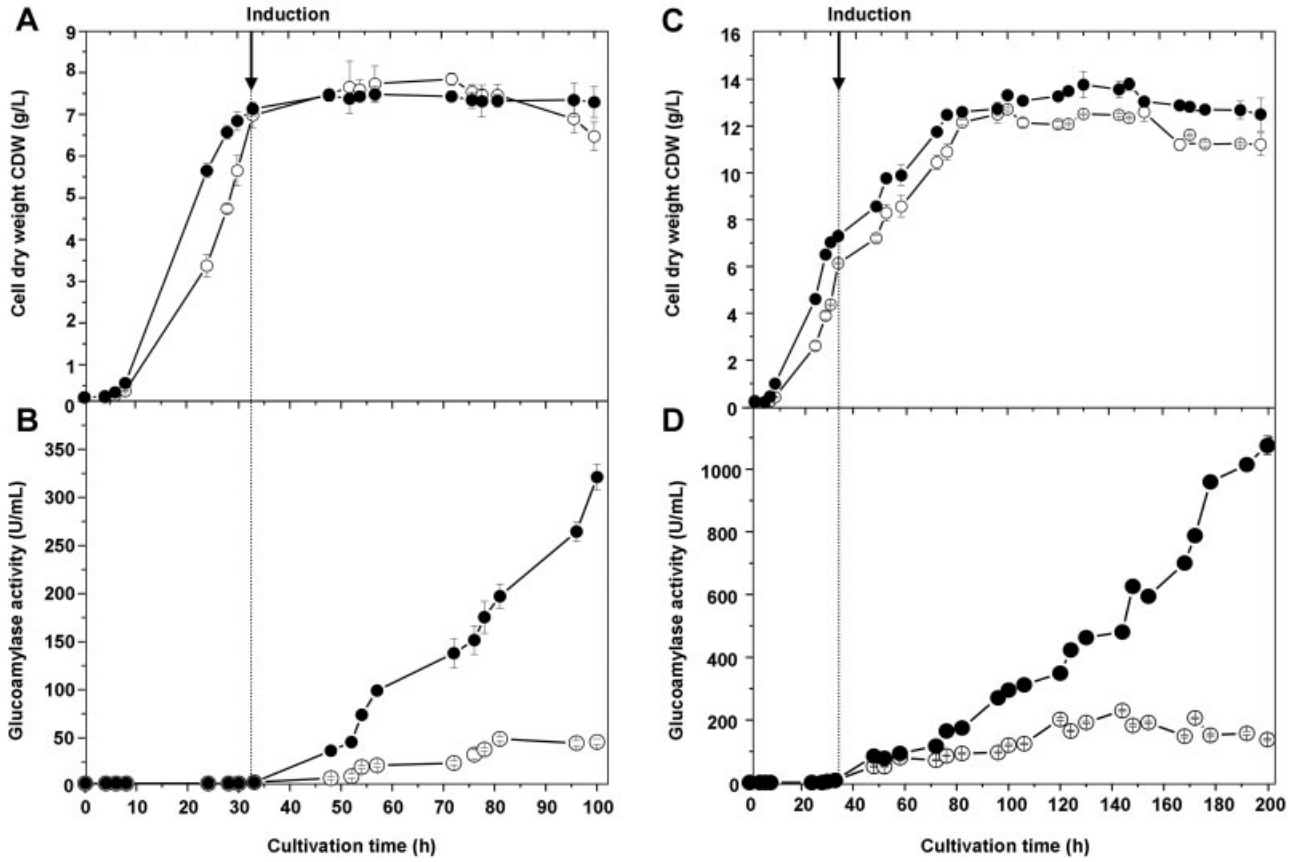

Figure 4. Production performance of *Aspergillus niger* ANip7-MCS-gfp2 in a stirred tank bioreactor operated in batch (**A**,**B**) and in fed-batch (**C**,**D**) mode. This involved conventional cultivations (open symbols) and cultivations supplemented with titanate microparticles (25 g/L, 8 μm, closed symbols). The data given represent the biomass concentration and the glucoamylase activity in the culture supernatant as mean values from two replicate cultivations with corresponding deviations.

filamentous fungi leads to free mycelium and even hyphal fragments. Hereby, these materials are found typically outside the fungal aggregates and occur freely in the cultivation medium. Titanate particles, however, are able to form aggregates from early on and are then obviously trapped within the fungal aggregates so that pelleted growth is achieved even at high levels of the micromaterial. What remains open at this point is a clear understanding of probably complex underlying interactions between particles and fungal cells. In future work, this important issue might be investigated via thorough measurement of forces between microparticles, spores and hyphae under different environmental conditions, with different particle materials or filamentous fungal strains. A promising method for the determination of the underlying adhesion forces seems atomic force microscopy (Wargenau and Kwade, 2010). An improved understanding of the corresponding physical and chemical interactions seems valuable for an even more targeted design of particle-based processes.

Overall, the resulting high-producing bio-pellets display a valuable complementation of recent approaches suggesting microparticles based on talc and alumina to engineer fungal morphology into free mycelium (Kaup et al., 2007). Beyond the present work, this seems especially useful for processes with filamentous fungi where pellets are preferred, such as the production of itaconic acid (Metz, 1976), citric acid (Gomez et al., 1988) or penicillin (Nielsen et al., 1995). Beyond direct production characteristics the pelleted growth allowed a reduced viscosity of the culture fluid. In this regard, the enzyme production in fungal processes is detrimentally affected by high broth viscosity, which leads to reduced oxygen mass transfer (Bhargava et al., 2003; Gupta and Srivastava, 2007; Wucherpfennig et al., 2010). Although the measurements here were performed at low biomass levels so that the resulting viscosity values are rather low, it becomes obvious that titanate treated cultures exhibit a substantially lower viscosity. Extrapolated to higher concentrations, one might expect improved mixing and mass transfer within the broth (Wucherpfennig et al., 2010) as well as facilitated downstream processing by a simplified separation of biomass and culture fluid. Overall, the present work widens the avenue for future work, especially towards

Table II. Production of glucoamylase by *Aspergillus niger* ANip7-MCS-gfp2 in shaken flasks (250 mL) and in stirred tank bioreactor operated in batch and in fed-batch mode (3 L).

	Process (cultivation time)					
	Batch				Fed-batch	
	Shake flasks (72 h)		Bioreactor (100 h)		Bioreactor (200 h)	
Titanate particle addition[a]	−	+	−	+	−	+
Cell dry weight, CDW (g/L)	8.8 ± 0.4	9.8 ± 0.4	6.5 ± 0.3	7.3 ± 0.4	11.2 ± 0.5	12.5 ± 0.7
Volumetric activity (U/mL)	15 ± 1	190 ± 2	50 ± 3	320 ± 8	140 ± 3	1,080 ± 7
Specific activity (U/gCDW)	1.7	19	8	44	13	90
Productivity (U/L h)	110	1,370	460	3,200	2,600	21,500

[a]−/+ Without/with microparticles of titanium silicate oxide (titanate, $TiSiO_4$, 25 g/L, 8 μm).

understanding of the molecular mechanisms that link morphology with the underlying metabolic and regulatory networks. The use of different microparticle materials allows a targeted and rather precise engineering of cellular morphology into free mycelium (Kaup et al., 2007) and now also into bio-pellets (this work) which creates novel possibilities for future design and optimization of bioprocesses with *A. niger* and also other fungi.

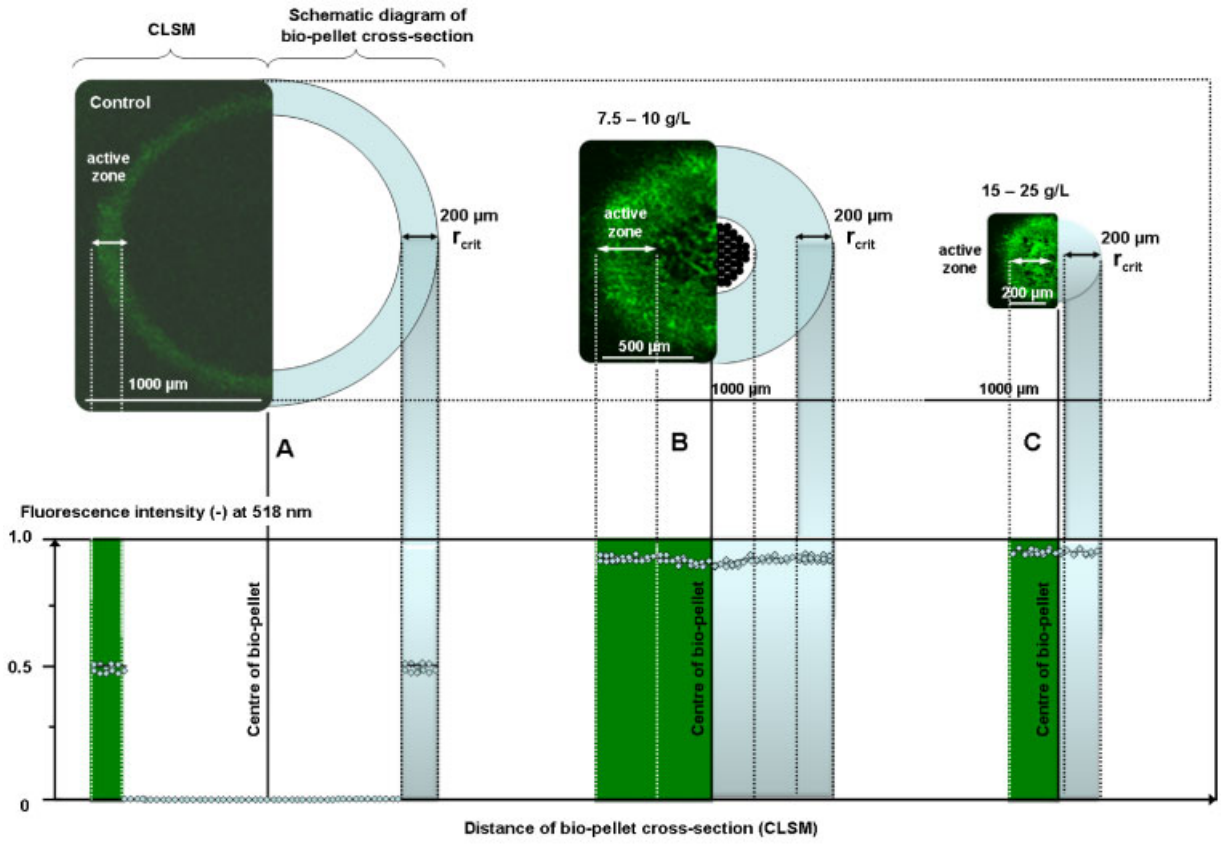

Figure 5. Visualization of metabolic activity and nutrient supply in aggregates of *Aspergillus niger* ANip7-MCS-gfp2 formed at different levels of titanate microparticles (8 μm) present in the medium via the active zone of GFP production obtained by confocal laser scanning microscopy (left) and the indication of the critical penetration depth of 200 μm (right) taken from previous measurements of different *Aspergillus* species.

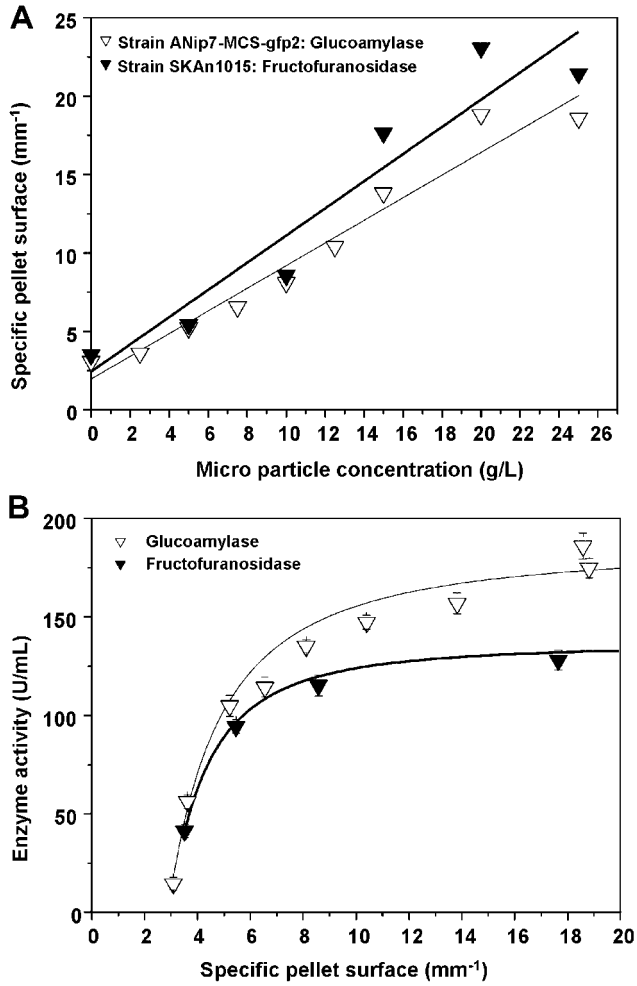

Figure 6. Impact of titanate microparticles on enzyme production in *Aspergillus niger* involving the resulting specific pellet surface (S_v) (**A**) and the production of glucoamylase by strain ANip7-MCS-gfp2 and of fructofuranosidase by strain SKAn1015 (**B**).

This work was financially supported as sub-project B11 within the framework of the Collaborative Research Center SFB 578 "From Gene to Product" by the German Research Foundation (DFG).

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