Ganzlin, M., Marose, S., Lu, X., Hitzmann, B., Scheper, T., Rinas, U.
In situ multi-wavelength fluorescence spectroscopy as effective tool to simultaneously monitor spore germination, metabolic activity and quantitative protein production in recombinant Aspergillus niger fed-batch cultures
(2007) Journal of Biotechnology, 132 (4), pp. 461-468.

In-situ multi-wavelength fluorescence spectroscopy as effective tool to simultaneously monitor spore germination, metabolic activity and quantitative protein production in recombinant *Aspergillus niger* fed-batch cultures

Markus Ganzlin [1, #], Stefan Marose [2], Xin Lu [1], Bernd Hitzmann [2], Thomas Scheper [2] and Ursula Rinas [1, *]

[1] Helmholtz Centre for Infection Research
(former German Research Centre for Biotechnology – GBF)
Inhoffenstr. 7, 38124 Braunschweig, Germany

[2] Institut für Technische Chemie, Universität Hannover, Callinstr. 3,
D-30167 Hannover, Germany

* Corresponding author: Tel: 0049-531-6181-7014, Email: uri@helmholtz-hzi.de
# Current address: AstraZeneca, DECS/GPS&S, 13S28 Mereside, Alderley Park, Macclesfield SK10 4TG.

Running title: On-line monitoring of fungal cultures
Keywords: multi-wavelength fluorescence spectroscopy, *Aspergillus niger,* GFP, *on-line* monitoring

Abstract

The production of a mutant green fluorescent protein (S65TGFP), controlled by the maltose inducible glucoamylase promoter, was followed *in-situ* in fed-batch cultures of recombinant *Aspergillus niger* using multi-wavelength fluorescence spectroscopy. Disturbance of quantitative product analysis by interfering fluorescence signals was resolved by using a set of defined combinations of excitation and emission wavelengths (λ_{ex} / λ_{em}). This technique resulted in excellent linearity between *on-line* signal and *off-line* determined S65TGFP concentrations. Spore germination was detectable *in-situ* by monitoring the back scattered light intensity. Moreover, flavin-like fluorophores were identified as the dominating fungal host fluorophores. The time-dependent intensity of this fluorophore, potentially fungal flavin-containing oxidoreductase(s), did not correlate with the biomass concentration but correlated well with the fungal metabolic activity (e.g. respiratory activity). Other fluorophores commonly found in microbial cultures such NADH, pyridoxine and the aromatic amino acids, tryptophan, phenylalanine and tyrosine did not contribute significantly to the culture fluorescence of *A. niger*. Thus, multi-wavelength fluorescence spectroscopy has proven to be an effective tool for simultaneous *on-line* monitoring of the most relevant process variables in fungal cultures, e.g. spore germination, metabolic activity, and quantitative product formation.

1. Introduction

Optical sensors are attractive tools for *on-line* bioprocess monitoring (e.g. Sonnleitner et al., 1991; Locher et al., 1992; Mulchandani and Bassi, 1995; Marose et al., 1998; Marose et al., 1999; Scheper et al., 1999; Stark et al., 2002). These sensor systems are usually very sensitive, do not interfere with the monitored bioprocess, and, the data, obtained from these measurements, are available in *real-time*, and thus, provide decision making information already during the cultivation.

In particular, multi-wavelength or two-dimensional (2-D) fluorescence spectroscopy has proven its usefulness in monitoring a variety of different bioprocesses (Tartakovsky et al., 1996; Marose et al., 1998; Mukherjee et al., 1999; Surribas et al., 2006; Hantelmann et al., 2006, Haack et al., 2007). The fluorescence intensity of the culture is scanned over a wide range of excitation and emission wavelengths, delivering a multitude of data sets for various wavelengths combinations. In contrast to one-dimensional (1-D) fluorescence spectroscopy, an increased quantity as well as an improved quality of information can be obtained. By comparing time course data of fluorescence intensities of the culture, using different combinations of excitation and emission wavelengths, the quality and reliability of information can be improved and directly related to the most relevant process variables. Nowadays, 2-D fluorescence spectroscopy combined with chemometric methods are most often applied to estimate substrate consumption as well as the formation of low molecular weight products and cell biomass. In addition, 2-D fluorescence data have been also used to estimate enzyme activities in microbial cultures (Mortensen and Bro, 2006; Haack et al., 2007).

Fluorescent proteins such as the green fluorescent protein (GFP) offer interesting options for process development due to their easy detection via optical sensors (Tsien, 1998; March et al., 2003; Su, 2005). In general, monitoring of fluorescent proteins like GFP in bioreactor cultivations occurs through 1-D fluorescence spectroscopy (Randers-Eichhorn et al., 1997; Poppenborg et al., 1997; Albano et al., 1998; DeLisa et al., 1999; Chae et al., 2000; Li et al., 2000; Gilbert et al., 2000; Reischer et al., 2004; Su et al., 2005). However, it has been demonstrated that the riboflavin fluorescent signals overlap with that of GFP (Hisiger and Jolicoeur, 2005; Surribas et al., 2007), thereby impeding accurate quantitative GFP measurements based on single wavelength fluorescence spectroscopy. To date, GFP-fluorescence intensity monitoring in bioreactor processes has been restricted to unicellular production organisms and not yet been applied to multicellular organism displaying a complex growth morphology such as filamentous fungi.

In this study, we report a straightforward method for quantitative *on-line* monitoring of induced production of a mutant green fluorescent protein (S65TGFP) in fed-batch cultures of a recombinant strain of the filamentous fungus *Aspergillus niger* based on 2-D fluorescence spectroscopy. Moreover, we also demonstrate the applicability of this technique to detect the onset of spore germination, a critical time point in fungal bioprocesses and to monitor the metabolic activity.

2. Materials and Methods

2.1. Recombinant strain

The construction of the recombinant *A. niger* (AB6.4Δ*pepE*[pAN52-10S65TGFPn/s]Ac5, producing S65TGFP under the control of the glucoamylase promoter (*glaA* promoter) has been described previously (Siedenberg et al., 1999). Transformants expressing the mutant GFP showed bright green fluorescence on maltose agar plates while wtGFP transformants were not fluorescent probably because of proteolytic degradation of the wtGFP gene product (Siedenberg et al., 1999).

2.2. Medium and culture conditions

Spores were obtained from 3.8% ($w \cdot v^{-1}$) potato-dextrose-agar plates, suspended in 0.9% ($w\ v^{-1}$) NaCl solution and used for inoculation. The final spore concentration after inoculation was 10^6 spores$\cdot mL^{-1}$. A defined growth medium (Vogel's medium without addition of vitamins) was used (Vogel, 1956). The composition of the basic defined batch medium was as follows: $Na_3 \cdot Citrate \cdot 2H_2O$, 2.85 $g \cdot L^{-1}$; KH_2PO_4, 5 $g \cdot L^{-1}$; $(NH_4)_2SO_4$, 7 $g \cdot L^{-1}$; $CaCl_2 \cdot 2H_2O$, 0.1 $g \cdot L^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.2 $g \cdot L^{-1}$, and trace elements (citric acid$\cdot H_2O$, 5 $mg \cdot L^{-1}$; $ZnSO_4 \cdot 7H_2O$, 5 $mg \cdot L^{-1}$; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1 $mg \cdot L^{-1}$; $CuSO_4 \cdot 5H_2O$, 0.16 $mg \cdot L^{-1}$; $MnSO_4 \cdot H_2O$, 0.037 $mg \cdot L^{-1}$; H_3BO_3, 0.05 $mg \cdot L^{-1}$, and $Na_2MoO_4 \cdot 2H_2O$, 0.05 $mg \cdot L^{-1}$). The trace element solution was stored as 10,000 times concentrated sterile-filtered stock solution. The salt solution ($Na_3 \cdot Citrate \cdot 2H_2O$, KH_2PO_4, NH_4NO_3, and $CaCl_2 \cdot 2H_2O$) was stored as 50 times concentrated sterile-filtered stock solution. The sugar carbon source xylose (initial concentration 10 $g \cdot L^{-1}$), uridine (initial concentration 2.44 $g \cdot L^{-1}$), and antifoam reagent Ucolub N115 (0.1 $ml \cdot L^{-1}$) were sterilized for 30 min at 121°C prior to inoculation.

A 50-L bioreactor (Type U50K; B. Braun Diessel Biotech GmbH / Sartorius AG, Melsungen / Göttingen, Germany), equipped with extensive analytical devices and a process control system for control and data-sampling (Type μXL; Yokogawa Deutschland GmbH, Ratingen, Germany), was used. The initial culture volume was 25 L, pH was kept constant at pH 5.5 using NaOH (5.0 $mol \cdot L^{-1}$). Stirrer speed (400 rpm) and aeration rate (13 $L \cdot min^{-1}$) were also kept constant. At the end of the batch phase, feeding started immediately after an initial pulse of maltose (5.0 $g \cdot L^{-1}$) with a feeding rate of 0.513 $g \cdot min^{-1}$. The feeding rate increased linear (0.091 $g \cdot min^{-1} \cdot h^{-1}$). The composition of the feeding solution was as follows: maltose, 170

$g \cdot kg^{-1}$ and uridine, 6 $g \cdot L^{-1}$, $Na_3 \cdot Citrate \cdot 2H_2O$, 5.7 $g \cdot L^{-1}$; KH_2PO_4, 10 $g \cdot L^{-1}$; $(NH_4)_2SO_4$, 14 $g \cdot L^{-1}$; $CaCl_2 \cdot 2H_2O$, 0.2 $g \cdot L^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.4 $g \cdot L^{-1}$, and trace elements (citric acid$\cdot H_2O$, 10 $mg \cdot L^{-1}$; $ZnSO_4 \cdot 7H_2O$, 10 $mg \cdot L^{-1}$; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2 $mg \cdot L^{-1}$; $CuSO_4 \cdot 5H_2O$, 0.32 $mg \cdot L^{-1}$; $MnSO_4 \cdot H_2O$, 0.074 $mg \cdot L^{-1}$; H_3BO_3, 0.1 $mg \cdot L^{-1}$, and $Na_2MoO_4 \cdot 2H_2O$, 0.1 $mg \cdot L^{-1}$).

2.3. On-line *multi-wavelength fluorescence spectroscopy*

On-line 2-D fluorescence spectroscopy was performed using a fluorescence spectrophotometer (Type F-4500 Hitachi, Japan), which was connected by means of a light conductor (Type 300, Lumatec, Munich, Germany) to a planar quartz window in an electrode port of the bioreactor. The double-track light conductor used separate tracks for excitation and emission with an active diameter of 3 mm per track and an opening angle of $2\alpha=72°$. The detection of the emission light was performed by using an open-end 180° measurement technique. The light transmission of this liquid light conductor was above 50% in the wavelength range 250 - 600 nm. The conditions for 2-D fluorescence spectroscopy were as follows: excitation band-width: 10 nm, emission band-width: 20 nm, photomultiplier voltage 700 Volt, scan speed 30000 $nm \cdot s^{-1}$, wavelength range of excitation 250 - 550 nm, wavelength range of emission 260 - 600 nm. Within these ranges, the measurements occurred at various consecutive combinations of one excitation and one emission wavelength. In this fashion 31 different excitation and 35 emission wavelengths were scanned. These wavelengths were obtained by increasing the lowest value (250 nm and 260 nm, respectively) in steps of 10 nm. Emission was scanned in 35 steps for each of the 31 excitation wavelengths. This resulted in a total of 1085 wavelength combinations, which determined the excitation and emission range of one 2-D spectrum. The measuring time for each 2-D spectrum was about 1 min. The chosen interval between two subsequent 2-D spectrum scans was 10 min. The relative fluorescence intensities (RI) corresponding to these different wavelength combinations (λ_{ex} / λ_{em}) are depicted as 3-dimensional contour plots as described previously (Marose et al., 1999). In addition to fluorescence intensities, these three dimensional graphs also show the intensities of back scattered light, situated in the diagonal area ($\lambda_{em} \cong \lambda_{ex}$) of the contour plot. Scattered light originating from higher order scattering in the grating monochromators appeared in the lower right corner of the contour plot with emission wavelengths two times higher than the corresponding excitation wavelengths. Areas within the *in-situ* 2-D fluorescence spectrum of the culture, which showed a significant variation of fluorescence

intensity during the course of the cultivation, were determined. Four such areas were identified and the course of their fluorescence intensity was compared with other cultivation data. Their respective excitation and emission wavelengths are listed in Table I. The relative intensity of an area (RI"area name") was determined as the average value of the relative intensities corresponding to all wavelength combinations of this area.

Purified S65TGFP (Clontech, Palo Alto, USA) was analyzed in a thermostated (temperature, 25°C) 10 mL cell via 2-D fluorescence spectroscopy. It was diluted in a buffer (pH 5.65; KH_2PO_4, 63.3 mmol·L^{-1} and Na_2HPO_4, 3.3 mmol·L^{-1}). Thus, conditions of this measurement were almost identical with those chosen for bioprocess monitoring. The only difference was a reduced scan speed (200 nm·s^{-1}) to improve the signal-to-noise ratio.

2.4. In-situ *calibration of optical* on-line *signals*

An *in-situ* calibration analysis was carried out to determine the correlation of the concentration of fluorescent biomass and the optical *on-line* signals. The biomass was removed at the end of cultivation using sieves (0.1 - 1.6 mm, Retsch, Haan, Germany) and kept on ice while the bioreactor was cleaned and refilled with buffer (NaCl, 15.4 mmol·L^{-1}; KH_2PO_4, 1 mmol·L^{-1}; KH_2PO_4, 64.1 mmol·L^{-1}; Na_2HPO_4, 2.6 mmol·L^{-1}; Na_2HPO_4, 2.6 mmol·L^{-1}; pH 5.5) to a final volume of 25 L. Subsequently, the biomass was added in seven consecutive steps into the bioreactor fully equipped with all optical sensors. Before a fraction of biomass was added, it was washed with buffer (NaCl, 15.4 mmol·L^{-1}; KH_2PO_4, 1 mmol·L^{-1}; KH_2PO_4, 64.1 mmol·L^{-1}; Na_2HPO_4, 2.6 mmol·L^{-1}; pH 5.5) and its wet weight determined. The intensity of the optical *on-line* signal was correlated with the total amount of biomass returned into the bioreactor. The ratio of cell wet weight to cell dry weight was determined afterwards for analysis of the specific S65TGFP concentrations (S65TGFP per cell dry weight [mg g^{-1}]). The settings for temperature (25°C), pH (pH 5.5), agitation (400 rpm) and aeration (13 L·min^{-1}) were kept constant as during the cultivation. The fluorescence spectrometer settings were as during analysis of purified S65TGFP.

2.5. Other on-line *analytical methods*

The intensity of back scattered light was also measured *on-line* in the near-infrared range using a turbidity probe (type AF 44 S; Aquasant-Messtechnik AG., Bubendorf, Switzerland) which was installed in an electrode port of the bioreactor. This probe detects only the

reflected light at an angle of 180° relative to the light source (900 nm). The concentrations of oxygen and carbon dioxide in the exhaust gas were determined by paramagnetic and infrared gas analysis systems, respectively (Maihak, Hamburg, Germany). Data acquisition was accomplished by means of the process control system (Modell: μXL; Yokogawa Germany GmbH, Ratingen, Germany).

2.6. Off-line *analytical methods*

The *off-line* measurement of S65TGFP in the fungal biomass was carried out by a fluorometric assay with pure S65TGFP for calibration (Clontech Product Protocol: Living Colors^{TM}, GFP Application Notes (PT2040-1), Clontech, USA). These measurements were performed with a PERKIN ELMER Luminescence Spectrometer LS 50 B, using the software package FL WinLab Vers. No. 2.00 (PERKIN ELMER) under following conditions: excitation at 488 nm (band-width, 3 nm), emission at 511 nm (band-width, 7 nm), temperature: 25°C ± 0.1°C. The buffer composition was: NaCl, 200 mmol·L^{-1}; NaH_2PO_4, 50 mmol·L^{-1} and Tris/HCl, 10 mmol·L^{-1} (pH 8.0). A calibration curve [concentration of pure S65TGFP (mg·L^{-1}) versus relative fluorescence units (RFU), linear regression coefficient 0.99] was always obtained directly prior to sample measurements. Extracellular S65TGFP concentrations in the culture medium were measured by means of a fluorescence multi-well plate reader (Type CytoFluor^{TM} 4000, PerSeptive Biosystems, Farmingham, USA). A calibration curve was obtained by dilutions of pure S65TGFP in filtered culture medium, which was sampled and frozen immediately after inoculation. The conditions for extracellular fluorescence measurements were as follows: range of excitation wavelengths, 485-505 nm; range of emission wavelengths, 530-560 nm; temperature, 25°C. In addition, detection of S65TGFP in the fungal biomass was carried out by fluorescence microscopy (Axiovert 120, Zeiss, Jena, excitation 488 nm, emission at 515 nm).

The fluorescence properties of S65TGFP in the fungal biomass and the medium was preserved by immediate freezing in liquid nitrogen and subsequent storage at -70°C. Cell disruption for determination of cytoplasmic S65TGFP was carried out using a vibration mill (Type MM2000, Retsch, Haan, Germany) and metal beads with a diameter of 4.0 mm (10 min, setting for amplitude, 100). The cell dry mass was determined from 10 mL aliquots of the culture. The fungal biomass was collected on a filter paper for quantitative analysis (red band, Schleicher & Schüll), washed with distilled water, and dried at 40°C under vacuum (Vacutherm, Heraeus / oil vacuum pump: << 50 mbar) to constant weight.

3. Results and Discussion

3.1. On-line-*detection of* in vivo *S65TGFP fluorescence using* in-situ *2-D fluorescence spectroscopy*

The recombinant S65TGFP is produced as a non-fused protein under the control of the glucoamylase promoter without a signal sequence and thus, accumulates as a cytoplasmic protein in the fungal biomass (Fig. 1). Synthesis of the recombinant fluorescent protein is repressed during growth on the non-inducing carbon source xylose and strongly induced during growth on maltose.

The change of the culture fluorescence in fed-batch cultures was followed *on-line* using *in-situ* 2-D fluorescence spectroscopy. The culture was started as batch culture with xylose as carbon source, and after depletion of xylose, feeding of the inducing carbon source maltose occurred. A comparison of the 2-D fluorescence spectrum obtained in the beginning and at the end of the cultivation revealed a significant increase of relative fluorescence intensities in different areas of the contour plot (Fig. 2A and C). An increase of light emission was not only detected in the diagonal area ($\lambda_{em} \approx \lambda_{ex}$) representing back scattered light, but also in the fluorescent region ($\lambda_{em} > \lambda_{ex}$) (Fig. 2C). The increase in back scattered light intensity was caused by the increasing biomass and the increase in light emission in the fluorescent region by the production of S65TGFP. The similarity between the 2-D fluorescence spectrum of the culture and the 2-D fluorescence spectrum of purified S65TGFP confirmed that S65TGFP was the dominant fluorophore at the end of the cultivation (Fig. 2C and D, respectively). Both contour plots revealed a fluorescence spectrum with a maximum characteristic for S65TGFP (λ_{ex} 488 nm and λ_{em} 511 nm, Heim et al., 1995). However, S65TGFP was not the only fluorophore in the culture at the end of the fed-batch cultivation; another prominent fluorophore, designated "UF1", unknown fluorophore (Fig. 2B and C), also contributed to the culture fluorescence. This fluorophore was already observed during the batch phase of the cultivation prior to the induction of S65TGFP synthesis with a maximum of λ_{ex} 415 nm and λ_{em} 505 nm (Fig. 2B). At the end of the fed-batch cultivation, the fluorescence intensity of UF1 was low compared to the intensive S65TGFP fluorescence, thus, no distinct maximum was anymore detectable (Fig. 2C).

3.2. Process monitoring of A. niger *fed-batch cultures using multi-wavelength fluorescence spectroscopy.*

The time-dependent changes in culture fluorescence were followed during fed-batch cultivations of *A. niger* and correlated with other process variables (Fig. 3).

3.2.1. On-line *detection of spore germination in* real time

Spore germination was detected by microscopy 53 h after inoculation. This event caused an instantaneous increase in the scattered light intensity [the entire diagonal area ($\lambda_{em} \approx \lambda_{ex}$) of the contour plot], shown here as the rapid increase of the RI"scattered light 500 nm" (Fig. 3C, designated area 4 in Fig. 2C; Table I). The correlation of spore germination with increased scattered light intensity was additionally verified using a turbidity probe from Aquasant which measures the back scattered light in the near infrared range (Fig. 3B).

3.2.2. Quantitative on-line *monitoring of S65TGFP production*

Multi-wavelength analysis of *A. niger* culture fluorescence clearly showed that accurate quantitative GFP measurements are difficult to obtain solely through single wavelength fluorescence spectroscopy in fungal cultures (Fig. 2). On the one hand, the maximum of S65TGFP fluorescence is close to the diagonal line of the contour plot, thus interference with scattering light from biomass is expected. On the other hand, monitoring S65TGFP fluorescence away from its maximum increases the risk of interference with the fluorescence of UF1.

The fluorescence intensities of two different areas were evaluated for quantitative prediction of product formation ("S65TGFP-maximum" and "S65TGFP-adjacency", designated areas 2 and 3, respectively, in Fig. 2C; Table I). The area "S65TGFP-maximum" comprised wavelength combinations (λ_{ex} and λ_{em}) close to the maximum of S65TGFP fluorescence, whereas the area "S65TGFP-adjacency" formed a semicircle adjacent to the "S65TGFP-maximum" (Fig. 2C, Table I).

Quantitative *off-line* analysis of S65TGFP in the fungal biomass revealed that the fluorescent protein already accumulated during batch growth on the non-inducing carbon source xylose to 7 mg L^{-1} of culture and rapidly increased to 110 mg L^{-1} in the fed-batch phase after the onset of maltose feeding (Figs. 3B and C). *On-line* monitoring of S65TGFP production based on the RI"S65TGFP-maximum" clearly showed an interference with the scattering light, detectable,

for example, through a sharp increase in the RI"S65TGFP-maximum" signal caused by the appearance of germinating spores (Fig. 3C). Analysis of S65TGFP production based on the RI"S65TGFP-adjacency", however, remained unaffected by back scattering light during spore germination (Fig. 3B). In this case, signals were subjected to interference with UF1 fluorescence, noticeable, for example, through a small local maximum of the RI"S65TGFP-adjacency" signal at the onset of the fed-batch phase caused by overlapping UF1 fluorescence (Fig. 3B). The perturbation of the RI"S65TGFP-adjacency" signal by UF1 fluorescence became neglectable at S65TGFP concentrations above 15 mg L^{-1} from 90 h cultivation time onwards. From this time on, the RI"S65TGFP-adjacency" *on-line* signal was in excellent agreement with the *off-line* determined S65TGFP concentration (Fig. 3B). The RI"S65TGFP-maximum" signals were less reliable for prediction of low concentrations of S65TGFP, here the back scattering light obscured the proportionality between *on-line* and *off-line* signal to a greater extent leading to accurate predictions of S65TGFP only above concentrations of 60 mg L^{-1} (Fig. 3C).

The correlation of the RI"S65TGFP-maximum" signal with the *off-line* determined intracellular S65TGFP concentration revealed a linear correlation with considerable axis intercept ($C_M \approx 137$ RI) resulting from the interference of RI"S65TGFP-maximum" signals with scattering light (Fig. 4). In contrast, the correlation of the RI"S65TGFP-adjacency" signals with the *off-line* determined S65TGFP concentration resulted in an tenfold smaller intercept ($C_A \approx 11$ RI) (Fig. 4), thus rendering the RI"S65TGFP-adjacency" signal superior for quantitative *on-line* S65TGFP monitoring.

The reliability of this approach was additionally verified by *in-situ* calibration. The fungal biomass recovered at the end of the fed-batch cultivation was added, after washing, back to the bioreactor in a stepwise fashion and *off-line* determined dry mass concentrations were correlated with the turbidity probe (Fig. 5A) and the RI"S65TGFP-adjacency" signals (Fig. 5B). Both correlations revealed excellent linearity (linear regression coefficient: 0.996) demonstrating no significant interference with the so called inner filter effect (Li and Humphrey, 1992) for the chosen range of cell dry mass (2-11 $g \cdot L^{-1}$).

The amount of S65TGFP released by cell lysis into the medium remained low (maximal extracellular S65TGFP concentration ≈ 5.6 $mg \cdot L^{-1}$ at the end of the cultivation), thus *on-line* determined S65TGFP concentrations by 2-D fluorescence spectroscopy reflect the cytoplasmic concentration of the recombinant protein. All optical sensors are, however, interference-prone to film-forming reagents; addition of antifoam reagent, for example, can

cause a sudden temporary decrease in the signal of all optical sensors including the 2-D fluorescence signals (Fig. 3, indicated by the arrow).

3.3. Flavin-like fluorophores are the dominating fungal host fluorophores

Except for S65TGFP fluorescence, only one other major fluorophore, designated UF1, contributed to the culture fluorescence of *A. n*iger (Figs. 2B and C). The 2-D spectrum of this fluorophore (Fig. 2B) shows similar characteristics as the biogenic flavin fluorophores, two well resolved absorption maxima and a defined excitation maximum above 500 nm (520 nm for free riboflavin, FMN and FAD; Marose et al., 1998). Flavins when bound to proteins, however, can change their spectroscopic properties, e.g. their emission maximum can vary in the range of 500 to 530 nm depending on the redox state and the local environment of the bound flavin (Ghisla et al., 1974; Ghisla, 1980). Other fluorophores commonly found in microbial cultures such NADH, pyridoxine and the aromatic amino acids, tryptophan, phenylalanine and tyrosine did not contribute significantly to the culture fluorescence of *A. niger* (Figs. 2B and C; Marose et al., 1998).

The time-dependent light emission of UF1 was monitored below its maximum by measuring the fluorescence intensity of "area 1" (Fig. 2B and C), thereby reducing the risk of interference with the light emission of S65TGFP. The fluorescence intensity of this area reached a maximum during rapid growth in the batch phase of the culture, started to decrease concomitantly with the exhaustion of xylose and the decline of the carbon dioxide evolution rate, and finally dropped to about 50% of its maximum during the fed-batch phase of the cultivation (Fig. 3B). Thus, the UF1 fluorescence intensity can be used as a marker of fungal metabolic activity.

4. Conclusions

Multi-wavelength fluorescence spectroscopy has proven to be an effective tool for simultaneous *on-line* monitoring of the most relevant process variables in fungal cultures, e.g. spore germination, metabolic activity, and quantitative product formation. The RI"S65TGFP-adjacency" signal was recognized as the most reliable signal to quantitatively forecast cytoplasmic S65TGFP formation. A sudden strong increase in the scattering light intensity could be used to detect the time-point of sporulation in the early batch culture of the fungus. Moreover, a flavin-like fluorescence was identified as indicator of fungal metabolic activity. Although this fluorophore could not be identified, potential candidates are fungal flavoproteins such as flavin-containing oxidoreductases which are major constituents of the fungal proteome (unpublished). Other biogenic fluorophores commonly detected in microbial cultures such as such NADH, pyridoxine and the aromatic amino acids, tryptophan, phenylalanine and tyrosine did not contribute significantly to the culture fluorescence of *A. niger*.

Acknowledgements

This work was supported mainly by the EC grant BIO4CT96-0535 and in part by the Sonderforschungsbereich 578 (Project B1/B4).

References

Albano, C.R., Randers-Eichhorn, L., Bentley, W.E., Rao, G., 1998. Green fluorescent protein as a real time quantitative reporter of heterologous protein production. Biotechnol. Prog. 14, 351-354.

Chae, H.J., DeLisa, M.P., Cha, H.J., Weigand, W.A., Rao, G., Bentley, W.E., 2000. Framework for *online* optimization of recombinant protein expression in high-cell-density *Escherichia coli* cultures using GFP-fusion monitoring. Biotechnol. Bioeng. 69, 275-285.

DeLisa, M.P., Li, J.K., Rao, G., Weigand, W.A., Bentley, W.E., 1999. Monitoring GFP-operon fusion protein expression during high cell density cultivation of *Escherichia coli* using an *on-line* optical sensor. Biotechnol. Bioeng. 65, 54-64.

Ghisla, S., 1980. Fluorescence and optical characteristics of reduced flavins and flavoproteins. Methods Enzymol, 66, 360-373.

Ghisla, S., Massey, V., Lhoste, J.M., Mayhew, S.G., 1974. Fluorescence and optical characteristics of reduced flavines and flavoproteins. Biochemistry 13, 589-597.

Gilbert, P.A., Garnier, A., Jacob, D., Kamen ,A., 2000. *On-line* measurement of green fluorescent protein (GFP) fluorescence for the monitoring of recombinant adeno virus production. Biotechnol. Lett. 22, 561-567.

Haack, M.B., Lantz, A.E., Mortensen, P.P., Olsson, L., 2007. Chemometric analysis of in-line multi-wavelength fluorescence measurements obtained during cultivations with a lipase producing *Aspergillus oryzae* strain. Biotechnol. Bioeng. 96, 904-913.

Hantelmann, K., Kollecker, M., Hull, D., Hitzmann, B., Scheper, T., 2006. Two-dimensional fluorescence spectroscopy: a novel approach for controlling fed-batch cultivations. J. Biotechnol. 121, 410-417.

Heim, R., Cubitt, A.B., Tsien, R.Y., 1995. Improved green fluorescence. Nature 373, 663-664.

Hisiger, S., Jolicoeur, M., 2005. A multiwavelength fluorescence probe: is one probe capable for on-line monitoring of recombinant protein production and biomass activity? J. Biotechnol. 117, 325-336.

Li, J., Wang, S., VanDusen, W.J., Schultz, L.D., George, H.A., Herber, W.K., Chae, H.J., Bentley, WE, Rao, G., 2000. Green fluorescent protein in *Saccharomyces cerevisiae*: real-time studies of the *GAL1* promoter. Biotechnol. Bioeng. 70, 187-196.

Li, J., Humphrey, A.E., 1992. Factors affecting culture fluorescence when monitoring bioreactors. J. Ferment. Bioeng. 74, 104-111.

Locher, G., Sonnleitner, B., Fiechter, A., 1992. On-line measurement in biotechnology; techniques. J. Biotechnol. 25, 23-53.

March, J.C., Rao, G., Bentley, W.E., 2003. Biotechnological applications of green fluorescent protein. Appl. Microbiol. Biotechnol. 62, 303-315.

Marose, S., Lindemann, C., Scheper, T., 1998. Two-dimensional fluorescence spectroscopy: a new tool for *on-line* bioprocess monitoring. Biotechnol Prog. 14, 63-74.

Marose, S., Lindemann, C., Ulber, R., Scheper, T., 1999. Optical sensor systems for bioprocess monitoring. Trends Biotechnol. 17, 30-34.

Mortensen, P.P., Bro, R., 2006. Real-time monitoring and chemical profiling of a cultivation process. Chemom. Intell. Lab. Syst. 84, 106-113.

Mukherjee, J., Lindemann, C., Scheper, T., 1999. Fluorescence monitoring during cultivation of *Enterobacter aerogenes* at different oxygen levels. Appl. Microbiol. Biotechnol. 52, 489-494.

Mulchandani, A., Bassi, A.S., 1995. Principles and applications of biosensors for bioprocess monitoring and control. Crit. Rev. Biotechnol. 15, 105-124.

Poppenborg, L., Friehs, K., Flaschel, E., 1997. The green fluorescent protein is a versatile reporter for bioprocess monitoring. J. Biotechnol. 58, 79-88.

Randers-Eichhorn, L., Albano, C.R., Sipior, J., Bentley, W.E., Rao, G., 1997. *On-line* green fluorescent protein sensor with LED excitation. Biotechnol. Bioeng. 55, 921-926.

Reischer, H., Schotola, I., Striedner, G., Potschacher, F., Bayer, K,. 2004. Evaluation of the GFP signal and its aptitude for novel on-line monitoring strategies of recombinant fermentation processes. J. Biotechnol. 108, 115-125.

Scheper, T., Hitzmann, B., Staerk, E., Ulber, R., Faurie, R., Sosnitza, P., Reardon, K.F., 1999. Bioanalytics: detailed insight into bioprocesses. Anal. Chim. Acta 400, 121-134.

Siedenberg, D., Mestric, S., Ganzlin, M., Schmidt, M., Punt, P.J., van den Hondel, C.A.M.J.J., Rinas, U., 1999. *GlaA* promoter controlled production of a mutant green fluorescent protein (S65T) by recombinant *Aspergillus niger* during growth on defined medium in batch and fed-batch cultures. Biotechnol. Prog. 15, 43-50.

Sonnleitner, B., Locher, G., Fiechter, A., 1991. Automatic bioprocess control. 1. A general concept. J. Biotechnol. 19, 1-18.

Stark, E., Hitzmann, B., Schügerl, K., Scheper, T., Fuchs, C., Koster, D., Märkl, H., 2002. In-situ-fluorescence-probes: a useful tool for non-invasive bioprocess monitoring. Adv. Biochem. Eng. Biotechnol. 74, 21-38.

Su, W.W., 2005. Fluorescent proteins as tools to aid protein production. Microb. Cell Fact. 4, 12.

Su, W.W., Liu, B., Lu, W.B., Xu, N.S., Du, G.C., Tan, J.L., 2005. Observer-based online compensation of inner filter effect in monitoring fluorescence of GFP-expressing plant cell cultures. Biotechnol. Bioeng. 91, 213-226.

Surribas, A., Geissler, D., Gierse, A., Scheper, T., Hitzmann, B., Montesinos, J.L., Valero, F., 2006. State variables monitoring by in situ multi-wavelength fluorescence spectroscopy in heterologous protein production by *Pichia pastoris*. J. Biotechnol. 124, 412-419.

Surribas, A., Resina, D., Ferrer, P., Valero, F., 2007. Rivoflavin may interfere with on-line monitoring of secreted green fluorescence protein fusion proteins in *Pichia pastoris*. Microb. Cell Fact. 6, 15.

Tartakovsky, B., Sheintuch, M., Hilmer, J.M., Scheper, T., 1996. Application of scanning fluorometry for monitoring of a fermentation process. Biotechnol. Prog. 12, 126-131.

Tsien, R.Y., 1998. The green fluorescent protein. Annu. Rev. Biochem. 67, 509-544.

Vogel, H.J., 1956. A convenient growth medium for *Neurospora* (Medium N). Microbial. Genet. Bull. 243, 112-119.

Tables

Table I. Analyzed fluorophores defined by the following excitation/emission wavelengths combinations from 2-D fluorescence spectra selected for *on-line* analysis (Areas also indicated in Figure 2)

Unknown fluorophore (UF1) Area 1		**S65TGFP-Maximum** Area 2		**S65TGFP-Adjacency** Area 3		**scattered light 500 nm** Area 4	
λ_{ex} [nm]	λ_{em} [nm]	λ_{ex} [nm]	λ_{em} [nm]	λ_{ex} [nm]	λ_{em} [nm]	λ_{ex} [nm]	λ_{em} [nm]
370	490-540	480	500	450	500-520	490	480-500
380	490-540	490	510	460	490-530	500	490-510
390	490-540	500	520	470	500-550	510	500-520
400	490-540	510	530	480	510-560		
410	490-540			490	520-560		
				500	530-560		
				510	540-550		

A set of distinct emission wavelengths was defined by indicating the lowest and highest value with a stepwise increase of 10 nm in between (emission band-width 20 nm); e.g. 480-500 corresponds to emission at 480 nm, 490 nm, and 500 nm.

Figure captions

Figure 1. Analysis of *A. niger* (AB6.4Δ*pepE*[pAN52-10S65TGFPn/s]Ac5) by fluorescence microscopy. Lower part: Longitudinal cryoscopic section of hyphae; arrows indicate septum positions. Bar represents 10 μm.

Figure 2. Analysis of culture fluorescence of *A. niger* producing S65TGFP in fed-batch culture using multi-wavelength fluorescence spectroscopy. 2-D fluorescence spectra were measured *in situ* A) directly after inoculation, B) 65 h after inoculation during the batch phase of the cultivation before the induction of S65TGFP production, and C) at the end of the fed-batch cultivation. D) 2-D fluorescence spectrum of pure S65TGFP. The areas indicated in B) and C) correspond to the analyzed fluorophores defined in Table I: Area 1: unknown fluorophore (UF1); area 2: S65TGFP-maximum; area 3: S65TGFP-adjacency; area 4: scattered light 500 nm. Time-course data of these fluorophores are shown in Figure 3.

Figure 3. Time course analysis of culture fluorescence of *A. niger* producing S65TGFP in fed-batch culture. Cells were first grown in batch culture on xylose as carbon source. The vertical line indicates the onset of maltose feeding to induce the production of S65TGFP. A) Cell dry mass and xylose concentration and concentration of carbon dioxide in the offgas. B) Volumentric concentration of S65TGFP and the fluorescence of “S65TGFP adjacency” and of the unknown fluorophore (“UF1”) in relative intensities. Time course data of the turbidity probe are also shown in relative intensities. C) Volumentric concentration of S65TGFP and the fluorescence of “S65TGFP maximum” and the “scattered light 500nm” in relative intensities. For definition of fluorophores see Table I. The arrow indicates interference through the addition of 20 g antifoam reagent.

Figure 4. Correlation of *on-line* fluorescence intensity with the volumetric *off-line* determined S65TGFP concentration. Data were obtained from the fed-batch cultivation (62-106h cultivation time) and the *on-line*-fluorescence signals (RI"S65TGFP-maximum" and "S65TGFP-adjacency") were plotted against the corresponding volumetric *off-line* determined S65TGFP concentrations.

Figure 5. *In-situ* calibration of optical *on-line* measurements. The *on-line*-signals A) RI"S65TGFP-adjacency" and B) the turbidity probe signal are plotted against the cell dry weight of the fluorescent mycelium which was added to the bioreactor as indicated in the Materials and Methods section.

Figures

Figure 1

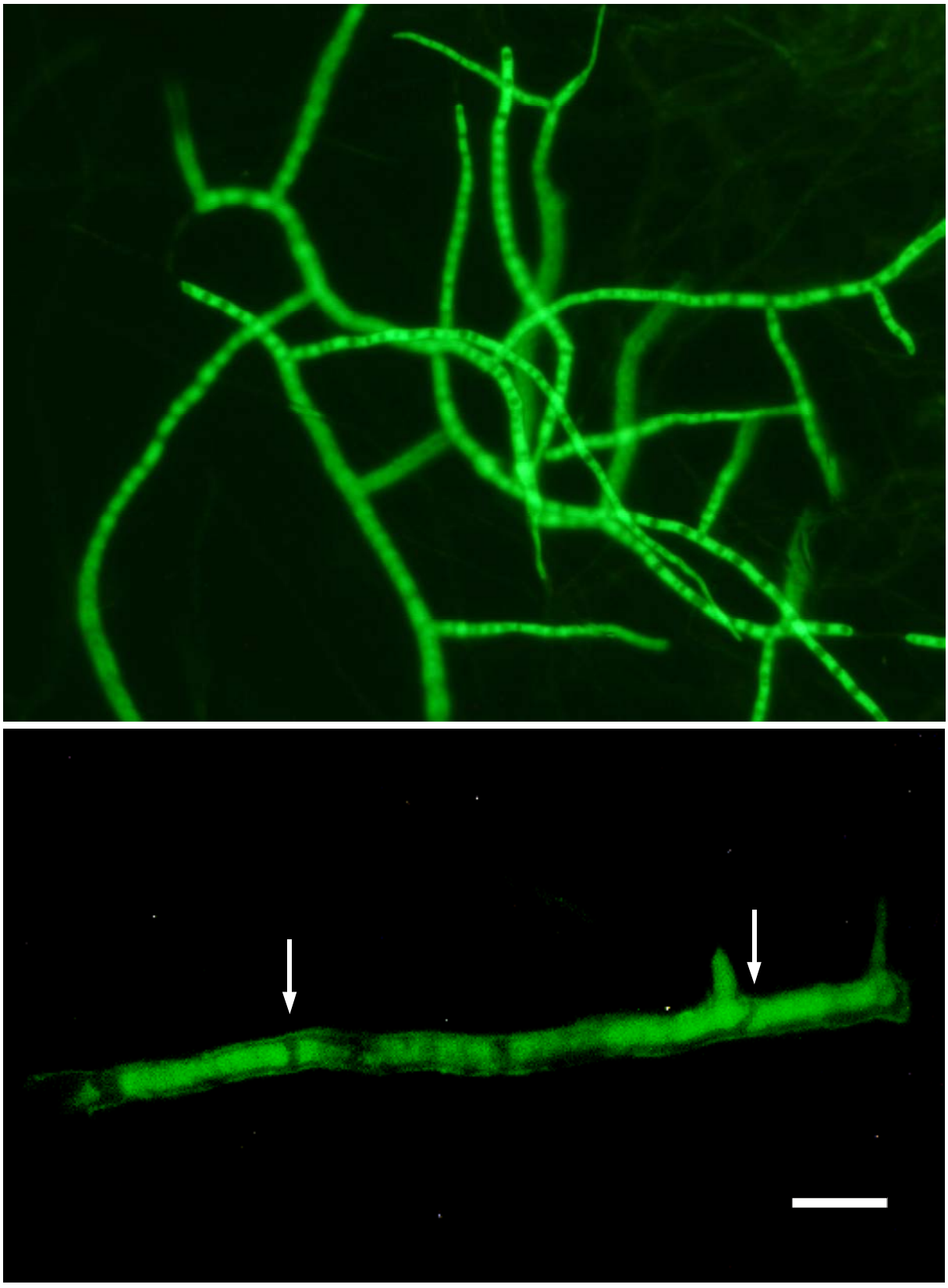


Figure 2

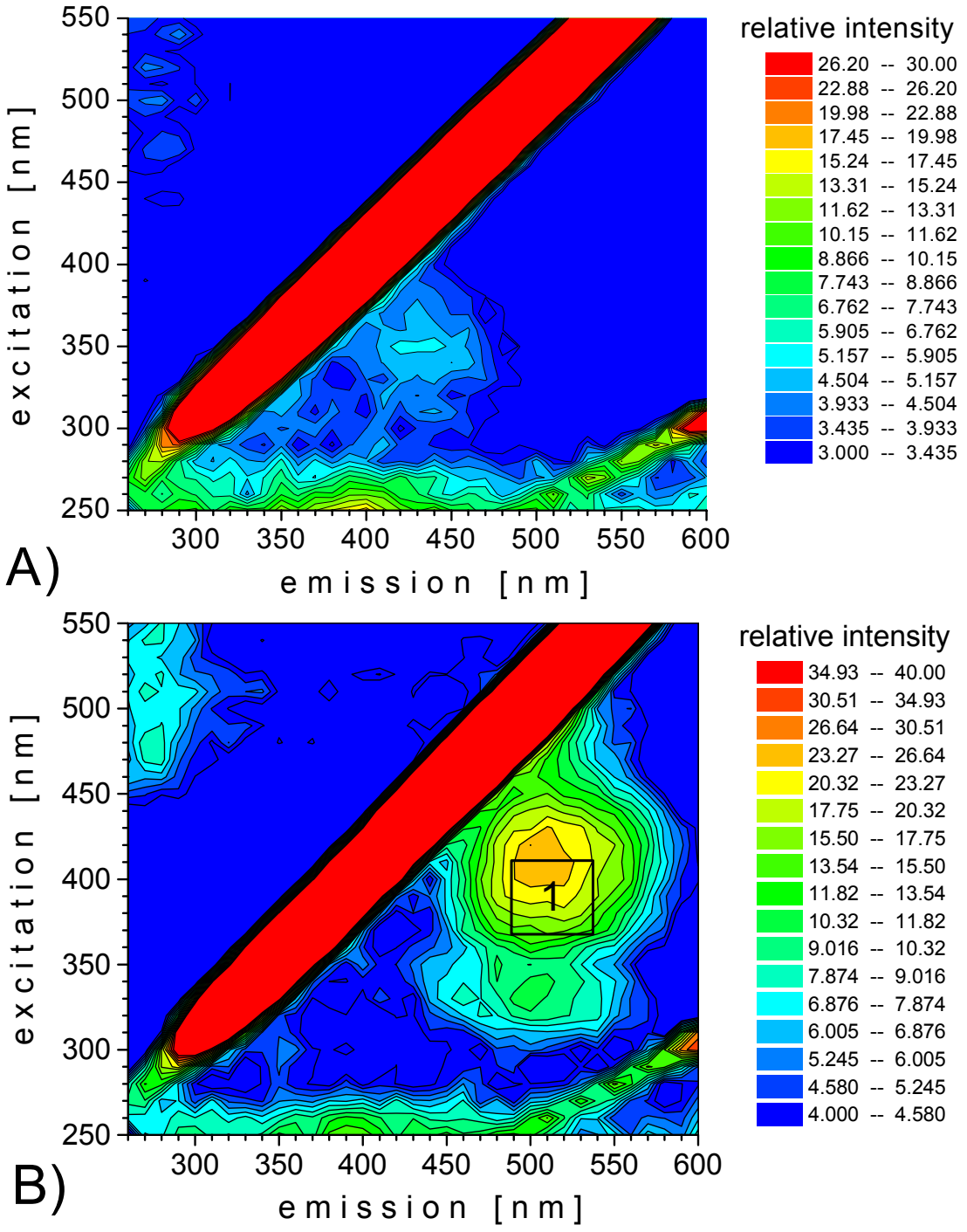

A)
excitation [nm]
emission [nm]
relative intensity
26.20 -- 30.00
22.88 -- 26.20
19.98 -- 22.88
17.45 -- 19.98
15.24 -- 17.45
13.31 -- 15.24
11.62 -- 13.31
10.15 -- 11.62
8.866 -- 10.15
7.743 -- 8.866
6.762 -- 7.743
5.905 -- 6.762
5.157 -- 5.905
4.504 -- 5.157
3.933 -- 4.504
3.435 -- 3.933
3.000 -- 3.435
B)
excitation [nm]
emission [nm]
relative intensity
34.93 -- 40.00
30.51 -- 34.93
26.64 -- 30.51
23.27 -- 26.64
20.32 -- 23.27
17.75 -- 20.32
15.50 -- 17.75
13.54 -- 15.50
11.82 -- 13.54
10.32 -- 11.82
9.016 -- 10.32
7.874 -- 9.016
6.876 -- 7.874
6.005 -- 6.876
5.245 -- 6.005
4.580 -- 5.245
4.000 -- 4.580
1

Figure 2

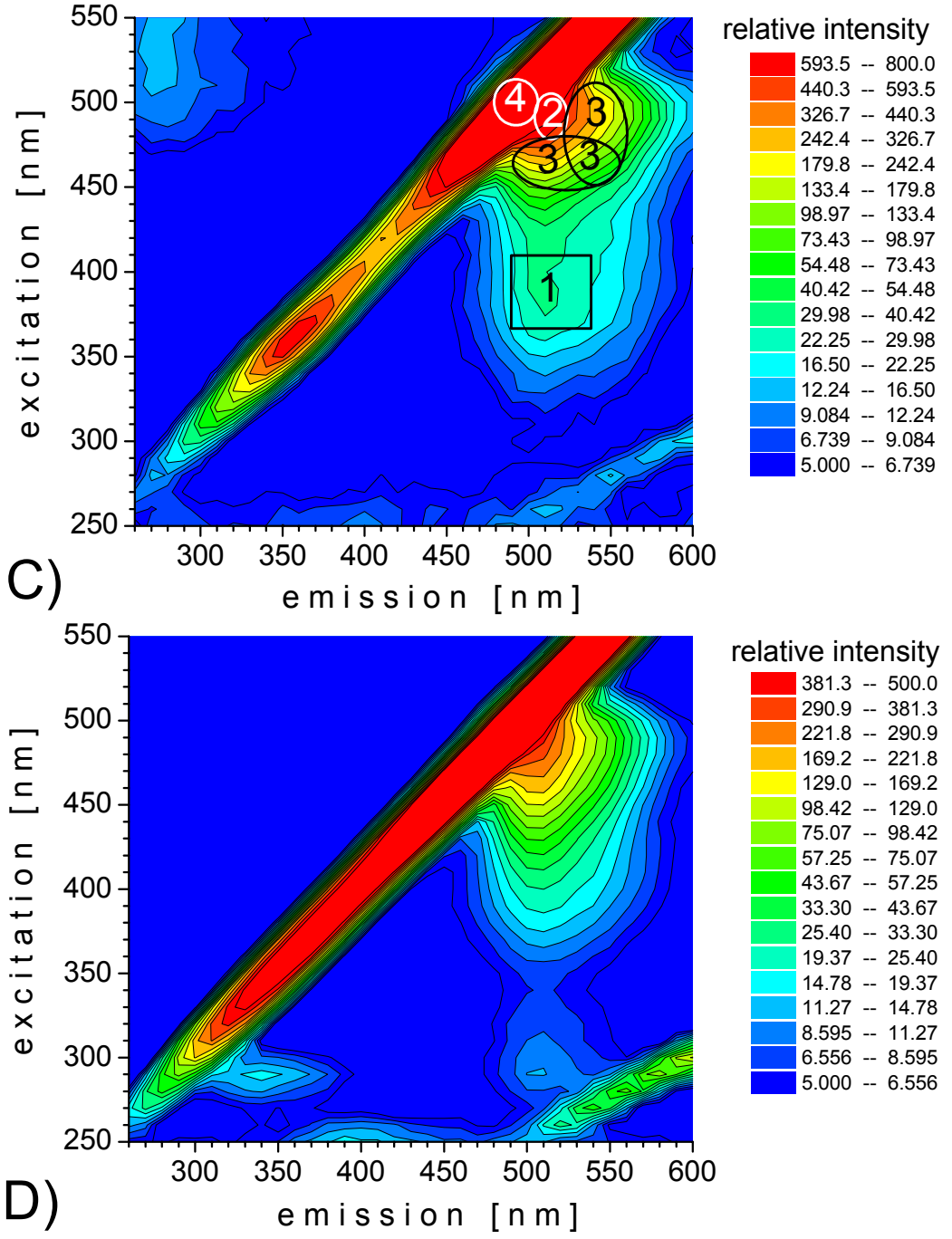

C)
excitation [nm]
emission [nm]
relative intensity
593.5 -- 800.0
440.3 -- 593.5
326.7 -- 440.3
242.4 -- 326.7
179.8 -- 242.4
133.4 -- 179.8
98.97 -- 133.4
73.43 -- 98.97
54.48 -- 73.43
40.42 -- 54.48
29.98 -- 40.42
22.25 -- 29.98
16.50 -- 22.25
12.24 -- 16.50
9.084 -- 12.24
6.739 -- 9.084
5.000 -- 6.739
D)
excitation [nm]
emission [nm]
relative intensity
381.3 -- 500.0
290.9 -- 381.3
221.8 -- 290.9
169.2 -- 221.8
129.0 -- 169.2
98.42 -- 129.0
75.07 -- 98.42
57.25 -- 75.07
43.67 -- 57.25
33.30 -- 43.67
25.40 -- 33.30
19.37 -- 25.40
14.78 -- 19.37
11.27 -- 14.78
8.595 -- 11.27
6.556 -- 8.595
5.000 -- 6.556

Figure 3

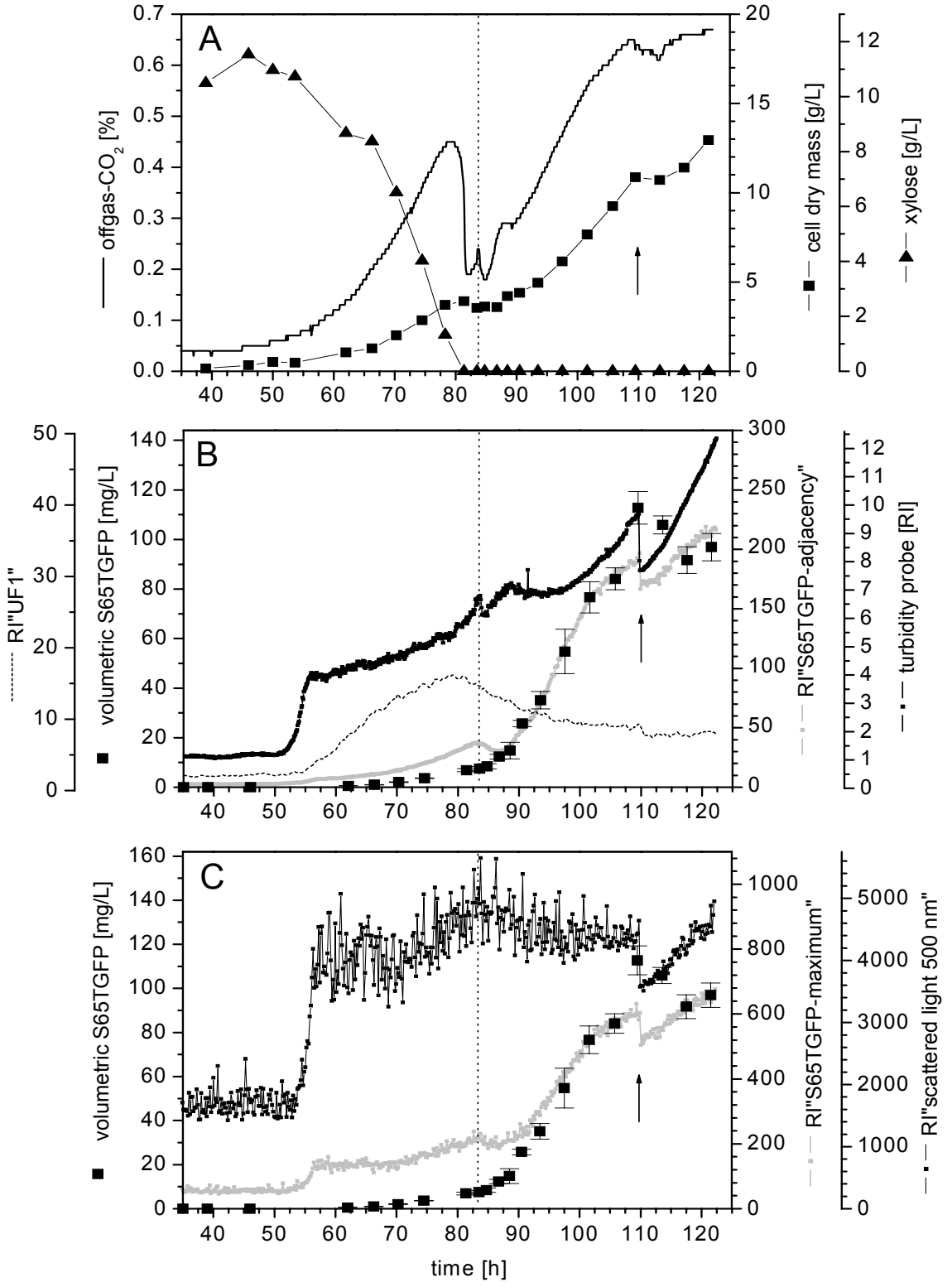

A
offgas-CO2 [%]
cell dry mass [g/L]
xylose [g/L]
B
RI"UF1"
volumetric S65TGFP [mg/L]
RI"S65TGFP-adjacency"
turbidity probe [RI]
C
volumetric S65TGFP [mg/L]
RI"S65TGFP-maximum"
RI"scattered light 500 nm"
time [h]

Figure 4

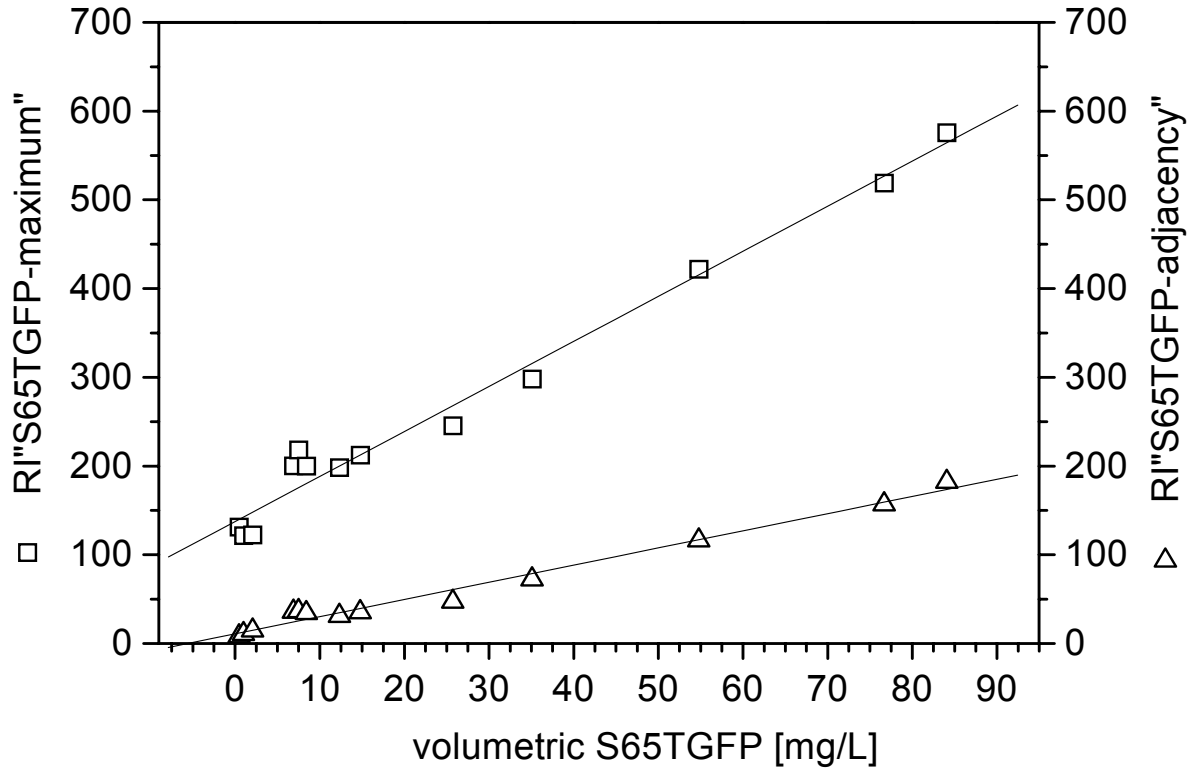
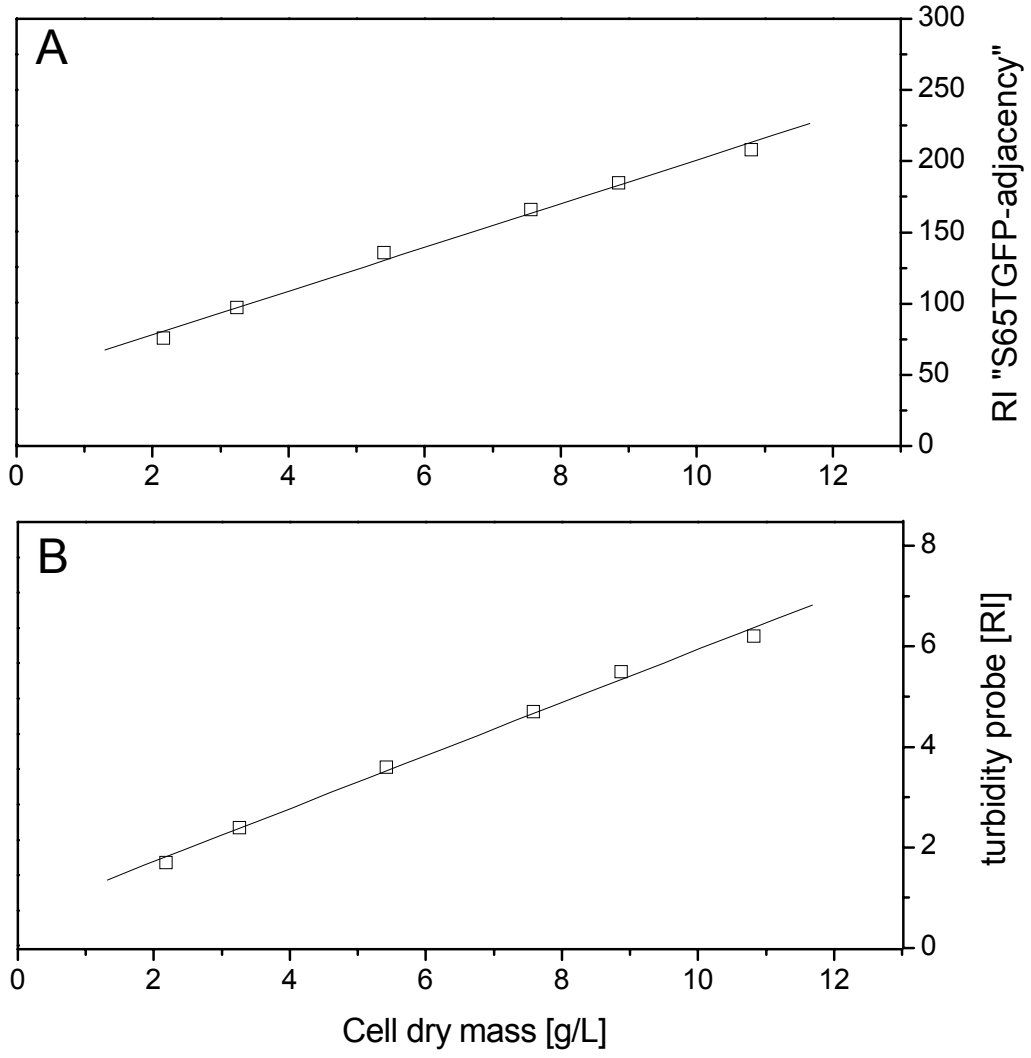

RI"S65TGFP-maximum"
RI"S65TGFP-adjacency"
volumetric S65TGFP [mg/L]
0
100
200
300
400
500
600
700
0
10
20
30
40
50
60
70
80
90

Figure 5


A
B
RI "S65TGFP-adjacency"
turbidity probe [RI]
Cell dry mass [g/L]
0
50
100
150
200
250
300
2
4
6
8
10
12