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Chemometric modelling with two-dimensional fluorescence data for *Claviceps purpurea* bioprocess characterization

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Abstract

Modern bioprocess control requires fast data acquisition and in-time evaluation of bioprocess variables. On-line fluorescence spectroscopy and the application of chemometric methods accomplish these goals. In order to demonstrate how time-consuming off-line analysis methods can be replaced for bioprocess monitoring, fluorescence measurements were performed during different cultivations of the fungus *Claviceps purpurea*. To predict process variables like biomass, protein, and alkaloid concentrations, chemometric models were developed on the basis of the acquired fluorescence spectra. The results of these investigations are presented and the applicability of this approach for bioprocess monitoring is discussed.

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1. Introduction

Recently, different spectroscopic techniques have been studied intensely for potential application in bioprocess monitoring (Wolfbeiss, 2002). Of particular interest are optical techniques using fiber technology, which provide the possibility for non-invasive monitoring of bioprocesses. Examples of bioprocess monitoring by fluorescence spectroscopy were described by Ulber et al. (2000) and Stärk et al. (2002). These measurements are not only useful for qualitative assessment of cultivations but can also be incorporated into chemometric models. This has been demonstrated by Christensen et al. (1999) and Solle et al. (2003), who used principal component analysis to determine the correlation of fluorescence data and bioprocess variables.

The fungus *Claviceps purpurea* produces different ergotalkaloids like ergotamine and α-ergocryptin, which have a variety of pharmaceutical applications. The biotechnological production of the ergotalkaloids requires optimal process conditions such as nutrient and oxygen supply (Kobel and Sanglier, 1986). Furthermore, the measurement of the alkaloid concentration and other process variables is very time-consuming. Optimisation of this bioprocess would clearly benefit

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from a good monitoring system. Because ergotalkloids fluoresce (350 nm excitation and 430 nm emission), monitoring of this component by fluorescence measurements during the cultivation is possible. Other components (e.g. amino acids and coenzymes) can also be detected by fluorescence spectroscopy, and the resulting information used to monitor the growth of microorganisms. The advantage of fluorescence spectroscopy is its high sensitivity and the fact that the measurement need not interfere with the medium or microorganisms. This non-invasive technique offers the possibility to directly follow different analyte concentrations and even the metabolic state of the biomass. Thus, changes in the process — controlled or uncontrolled — can be followed without time delays. The goal of this work was to apply this technique in a complex cultivation process for the production of ergotalkaloids.

2. Materials and methods

2.1. Bioprocesses

Cultivations of *C. purpurea* 1029 NS were performed in a 2-L bioreactor with addition of perfluorocarbon (PFC) (Hostinert 216, Hoechst, Germany) according to the protocols of Menge et al. (2001). Strain 1029 N5 produces several ergotalkaloids, and 60% of the produced alkaloids are secreted.

Cell suspension (200 ml) was added to 1800 ml of production medium (Menge et al., 2001) complemented by antifoam agent (Desmophen 3900, Bayer, Germany) and 20% PFC in a 2-L stainless steel tank reactor, which was constructed for on-line fluorescence measurements. A Biostat B unit (B. Braun Biotech International, Germany) was used as the process control system and data acquisition and monitoring were performed with a real time integrating software platform developed in house. The aeration was controlled by the mass-flow meter of the Biostat B unit and the exhaust gas was analysed by an EGAS 2 system (Hartmann & Braun, Germany). The cultivations were performed at a constant temperature of 24 °C and the pH was maintained at 5.2. The stirrer speed and the aeration rate were adjusted if necessary to ensure a minimum pO_2 level of 25%.

2.2. Off-line analysis

The growth of the fungus was determined by measuring the dry weight of biomass and the extracellular protein content. Samples were withdrawn during the cultivation, filtered, and the biomass dried together with the filter at 75 °C. Protein content was determined with the DC protein assay (Bio-Rad Laboratories, Hercules, USA) and the alkaloid content in the filtrate was measured by the modified van Urk test (Michelon and Kelleher, 1963).

2.3. Fluorescence spectroscopy

The fluorescence measurements were performed with the BioView® sensor (DELTA Light & Optics, Denmark). The experimental set-up is shown in Fig. 1. The BioView® sensor is equipped with a xenon flash lamp for the excitation light. The sensor uses two different filter wheels with 16 different filters for excitation and emission and a photomultiplier for detection of the emission light. The liquid light guide is connected to a quartz window of the bioreactor. The measurements were carried out in steps of 20 nm in the excitation wavelength range from 270 to 550 nm and the emission wavelength range from 310 to 590 nm. Other filters with transmittance in the full excitation and emission wavelength range (neutral density (nd) filters) were also used.

2.4. Chemometric evaluation

The chemometric modelling was performed using Unscrambler® software (Camo, Norway). For every process variable, a single partial least square model (PLS-1 model) was calculated. The centered data of the fluorescence spectra were used as independent variables and the values of the process variables determined off-line were the dependent variables. For further preprocessing, subtraction fluorescence spectra were calculated by subtraction of the arithmetic average value of five spectra (recorded within the first hour of the

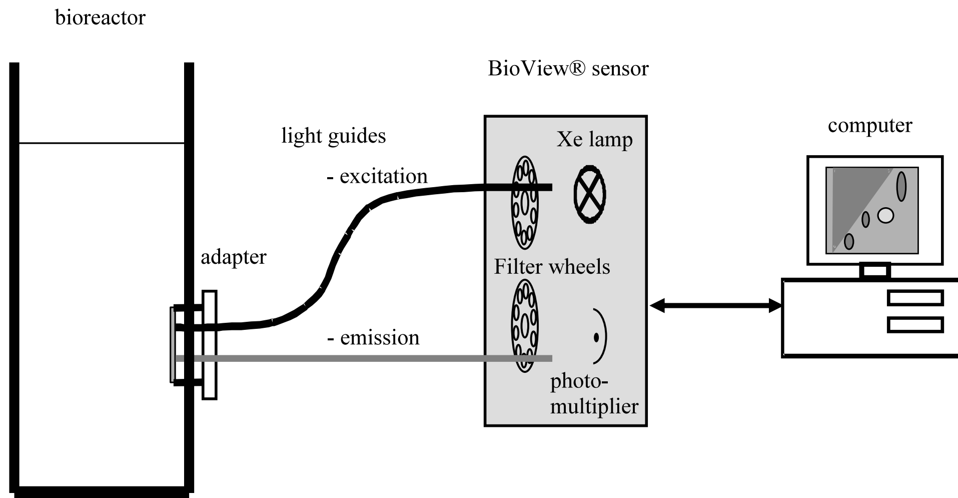

Fig. 1. Experimental set-up of the BioView®-sensor.

culture) from every spectrum recorded during the cultivation. Using this procedure, the variableness in the spectra with respect to different cultivations is compensated. The averaging procedure was performed to reduce noise in the measurement of the intensities, which was averaged 2% (mean percentage error).

For validation of the chemometric models, full cross-validation was used and a comparison of the predicted values with the values determined off-line was performed. Furthermore, the regression coefficients of the calculated models were scaled and plotted in sensitivity spectra.

The models were used for prediction of process variables of another cultivation of *C. purpurea*. The average difference between predicted and measured values is expressed by the root mean square error of prediction (RMSEP).

3. Results and discussion

3.1. Bioprocesses

Throughout the cultivations of *C. purpurea*, fluorescence signals were recorded and three process parameters (biomass, extracellular protein, and extracellular alkaloid concentrations) were analysed. The time courses of these process parameters during one of the bioprocesses are shown in Fig. 2. Growth of the fungus is indicated by the increase of biomass and the protein concentration. After a lag phase of approximately 100 h, the fungus grew continuously over the next 200 h. Production of the secondary metabolites started approximately after 200 h. The alkaloid concentration at the end of the cultivation reached 195 mg l^{-1}.

3.2. Fluorescence data

Two fluorescence spectra recorded during the cultivation of *C. purpurea* are shown in Fig. 3. The spectrum in Fig. 3a, recorded 1 h after inoculation, shows fluorescence peaks at ex/em 390/450 nm and ex/em 450/530 nm. These peaks are due to the biogenic fluorophors NADPH and flavine (Schulmann, 1985). The spectrum in Fig. 3b was recorded after 284 h. The fluorescence intensities of the NADPH and flavine peaks increased because of the growth of the fungus, and a new fluorescence peak developed in the range of ex/em 350/430 nm, which can be related to the production of alkaloids.

The time courses of the fluorescence intensity of several biogenic fluorophors throughout the cultivation are shown in Fig. 4. At the beginning of the cultivation, a slight increase of the fluorescence intensity of the alkaloids (ex/em 350/430 nm) can be observed. After 130 h, the fluorescence intensity

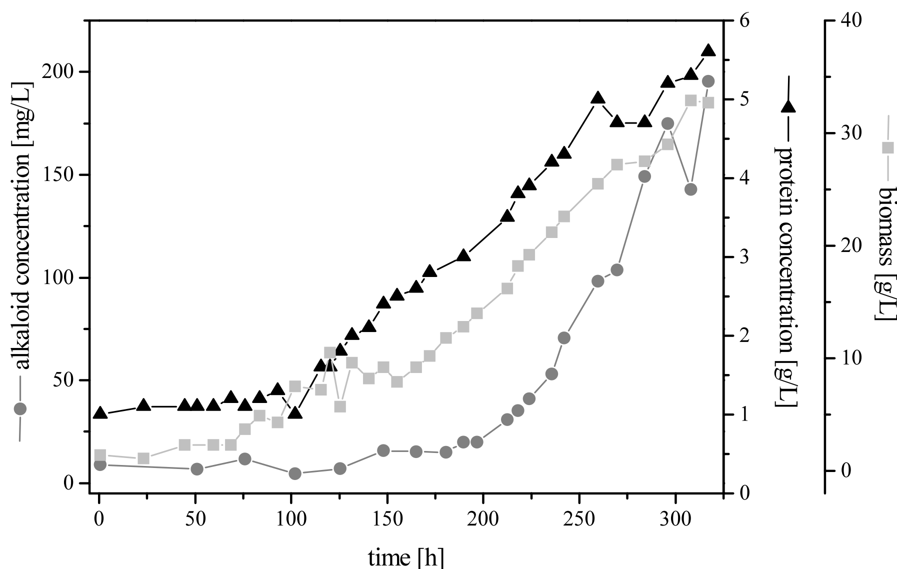

Fig. 2. Courses of the bioprocess variables dry biomass, protein concentration and alkaloid concentration throughout the cultivation of *C. purpurea*.

increased strongly and reached a maximum after 240 h (during the exponential production phase) before declining. In contrast, the concentration of alkaloids rose continuously until the end of the cultivation (Fig. 2). This effect might be due to a fluorescence energy shift, since the fluorescence emitted at 430 nm can be adsorbed by the ergotalkaloids or other components again (see Fig. 3b) followed by a fluorescence emission at 530 nm. Thus the increase of ergotalkaloids is not directly followed by a fluorescence increase at 430 nm and no linear correlation is possible. A chemometric model using all spectral information is necessary.

Another difference between the measured alkaloid concentration and the associated fluorescence (ex/em 350/430 nm) is the different times at which they increased. The fluorescence intensity of the alkaloids increased after 130 h (Fig. 4), whereas the measured concentration of ergotalkaloids rose after 196 h (Fig. 2). The reason for this can be found in the fact that the sensitivity of the off-line analysis method of alkaloids (van Urk test) is not sufficient in this concentration range. With fluorescence spectroscopy, the detection of ergotalkaloids in very low concentrations is possible. This is important for effective bioprocess optimization, since the monitoring is performed not only non-invasively but the process variables can be directly changed and the subsequent influence on the production can be followed in a very sensitive manner.

The fluorescence intensity of other excitation/emission wavelength combinations, such as those associated with proteins and amino acids (ex/em 270/390 nm and ex/em 290/350 nm) increased slightly at the beginning of the cultivation. As the cultivation progressed, the fluorescence intensities in this area also rose significantly. However, a direct correlation between a process variable and one wavelength combination is not possible. All of these effects reveal that the modelling of all recorded fluorescence data is required for bioprocess monitoring. For this purpose, the application of chemometric methods was performed.

Chemometric evaluation of fluorescence data recorded during cultivations of *C. purpurea* PLS-1 models for every process parameter were calculated using the fluorescence spectra as well as the off-line measurements from one cultivation. The PLS models for biomass and protein content were based on two principal components. Three principal components were used for the modelling of the alkaloid concentration.

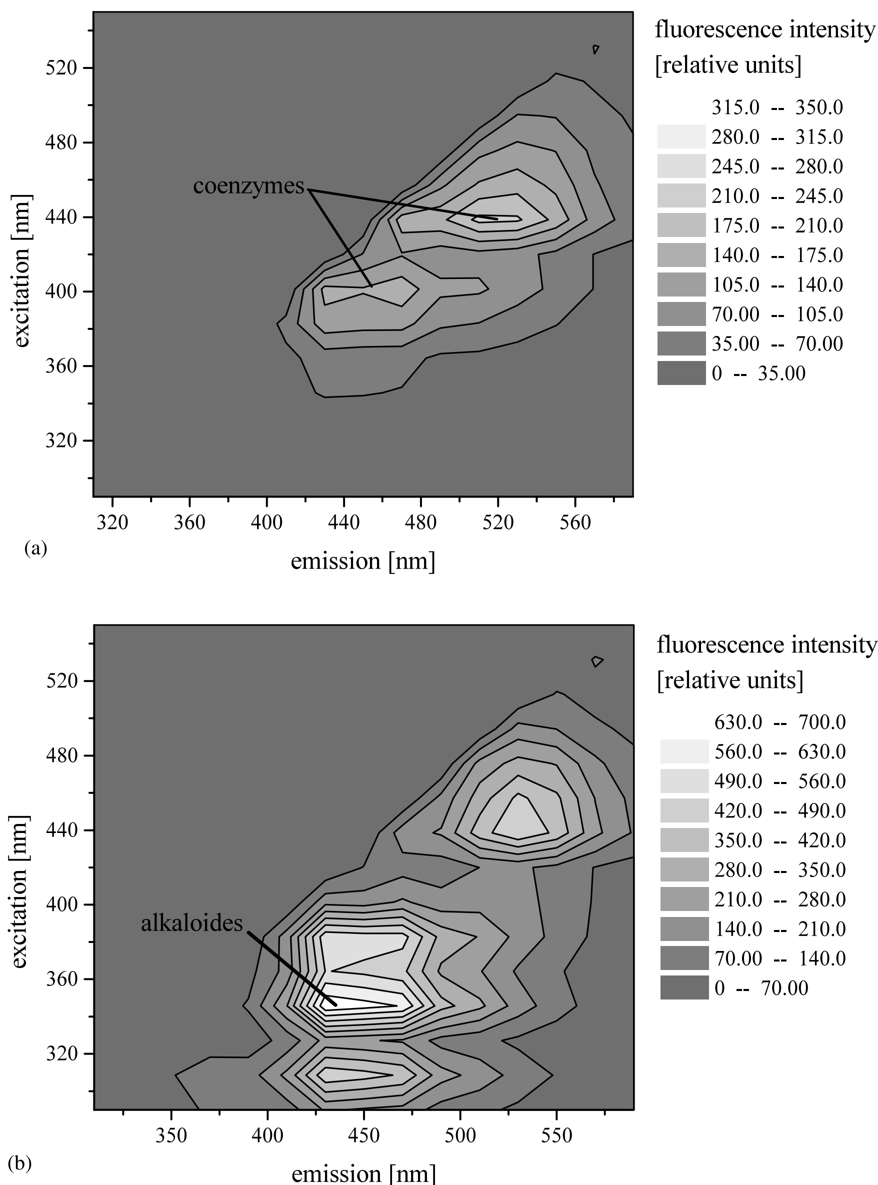

Fig. 3. (a) Fluorescence spectrum recorded 1 h after inoculation of the cultivation of *C. purpurea*. (b) Fluorescence spectrum recorded after 284 h of the cultivation of *C. purpurea*.

A first interpretation of the chemometric models can be made by inspection of the sensitivity of the model prediction to the intensity distribution. The sensitivity spectra in Fig. 5 show the scaled regression coefficients of every wavelength combination calculated with spectra gathered during the process run. The sensitivity values in the spectra can be interpreted as a measure of relevance of the corresponding wavelength combination for the prediction of the process variable. Therefore, the wavelength combinations with importance to the prediction of the process variables can be easily

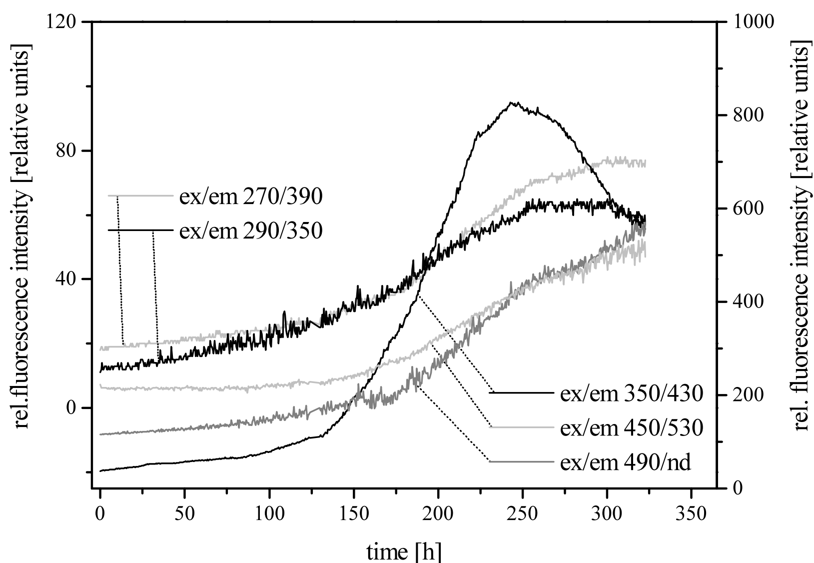

Fig. 4. Courses of fluorescence intensity throughout the cultivation of *C. purpurea*.

identified. In the sensitivity spectrum for the biomass concentration (Fig. 5a), the highest values of the coefficients are found in the area of flavins (ex/em 450/530 nm) and in the area of the neutral density filters (ex/em 490–590 nm/nd and ex/em 510–550 nm/nd). Both areas showed continuous increase during the cultivation and the correlation with the course of biomass is reasonable. Marose et al. (1998) showed that these areas of the fluorescence spectra can be correlated with the growth of microorganisms. The same conditions were found for the prediction model of the protein concentration (sensitivity spectra not shown) because the biomass as well as the protein concentration both describes the growth of the fungus and therefore showed a parallel course during the cultivation (Fig. 2).

The sensitivity spectrum of the alkaloids (Fig. 5b) reveals a high sensitivity in the region of ex/em 350/430 nm. This is the typical area of fluorescence of ergotalkaloids (Wolfbeis, 1985). As discussed earlier, a simple correlation of the product concentration with the fluorescence of the ergotalkaloids is not possible. To compensate for this interference, additional information from the area of flavins (ex/em 450/530 nm) was used for correlation of the alkaloid concentration and fluorescence data when chemometric modelling was applied.

The validation of the models was performed by prediction of the process parameters of another cultivation of *C. purpurea*. Fig. 6 shows the predicted courses of the biomass and ergotalkaloid concentrations in comparison to their off-line measurements. The predicted values of the alkaloid concentration agree very well with measurements during the cultivation. The deviation of the predicted values from the off-line recorded data is very low (3.3%, Table 1). It can be concluded that the calculated model can be used very efficiently for the on-line determination of alkaloid concentration by fluorescence spectroscopy.

The predicted values of the biomass concentration agree with the trend of the corresponding measured values during the cultivation but the predicted values were low. The cause of these errors is the slight differences in the composition and coloring of the medium and the age of the preculture was used for inoculation. To balance these factors, the models can be calculated on the basis of the subtraction spectrum (Fig. 7). With this procedure, only the changes in the fluorescence spectra during the cultivation are considered and the sensitivity and the quality of the models

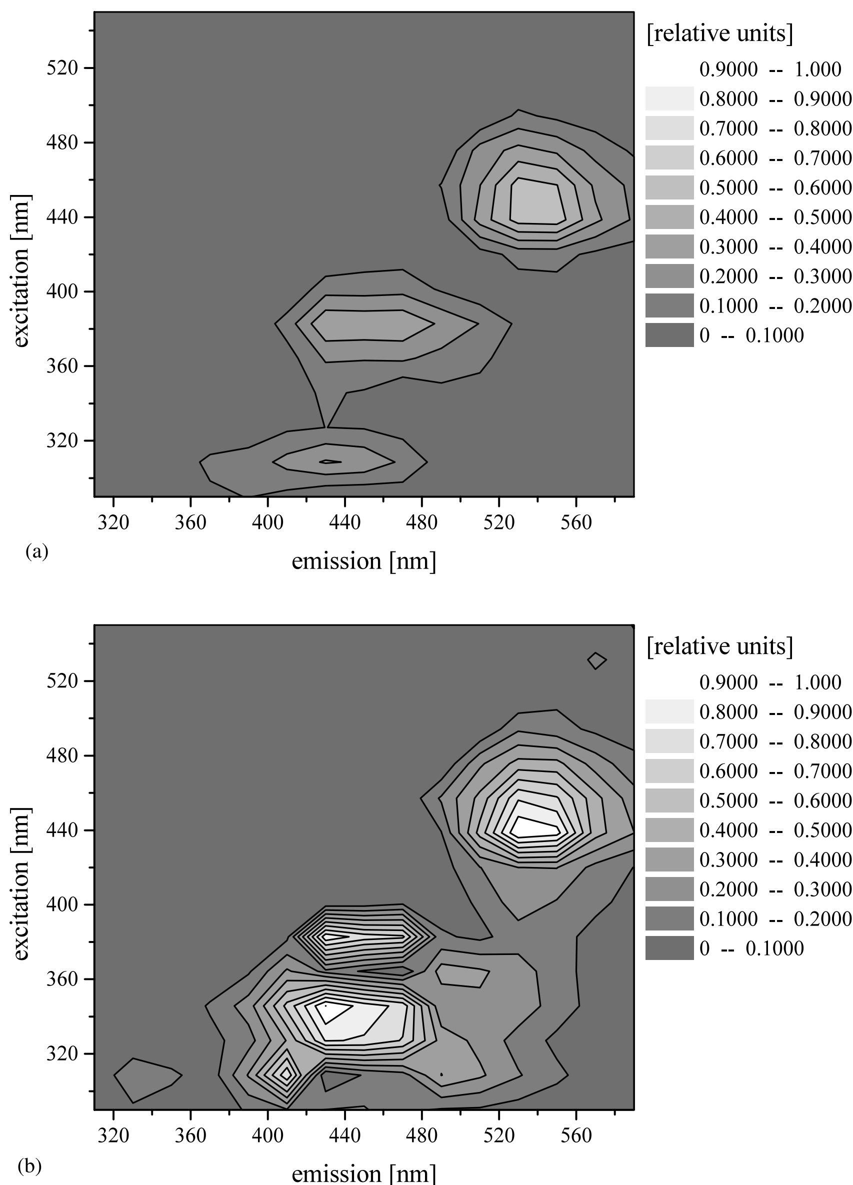

Fig. 5. (a) Sensitivity spectrum of the biomass model. (b) Sensitivity spectrum of the alkaloid model.

are not affected. The predictions based on the subtraction spectra are also plotted in Fig. 6 and the deviations of the prediction to the off-line determined values are given in Table 1.

The use of this calculation method resulted in predicted values of biomass that are significantly closer to the off-line measurements, as reflected in the lower RMSEP (7.26%). The use of subtraction spectra had no influence on the prediction of the alkaloid concentration. The reason for the greater errors in prediction of biomass and protein concentration can be found in the higher variance of the off-line measurements of biomass and protein concentration.

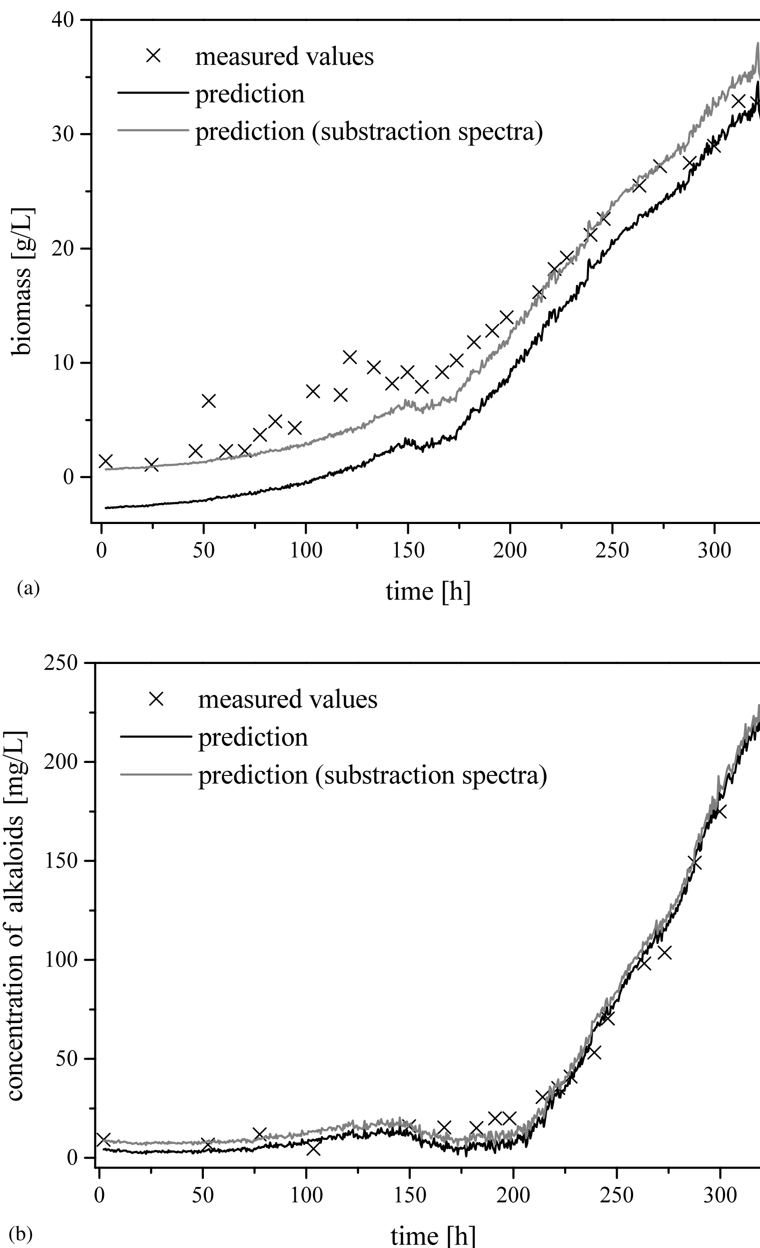

Fig. 6. (a) Comparison of predicted and the measured values of biomass. (b) Comparison of predicted and measured values of alkaloid concentration.

4. Conclusion

These results reveal that on-line monitoring of cultivations of *C. purpurea* by chemometric evaluation of fluorescence spectra is possible. The formation of the product can be monitored very well, owing to the fluorescence characteristics of the alkaloids. Therefore, a prediction with only minor errors can be achieved. Using PLS models, the prediction can be made directly from the

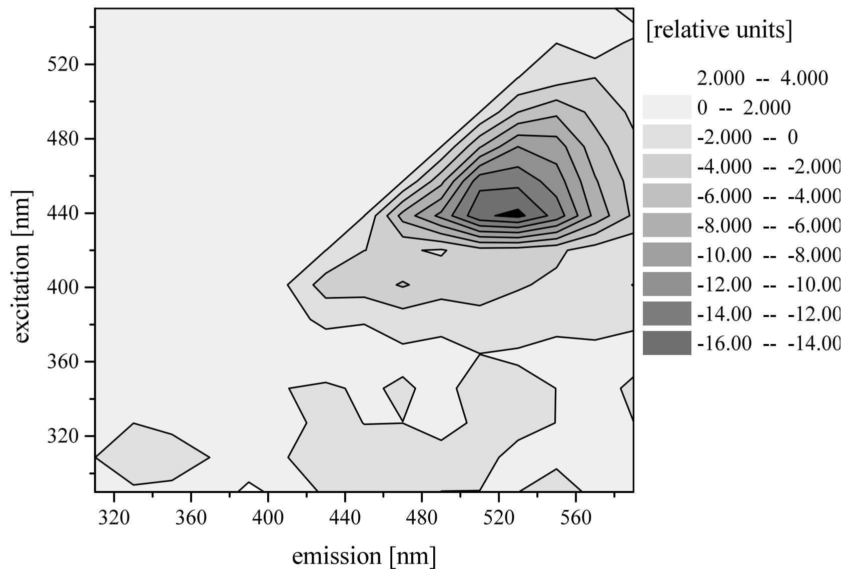

Fig. 7. Subtraction spectrum calculated of the spectrum recorded after 284 h and the average spectrum (five spectra recorded within the first hour of the culture).

Table 1
RMSEP of process variables of cultivation of *C. purpurea*

Process variable	RMSEP prediction 1	RMSEP prediction 2 (use of subtraction spectra)
Biomass (dry weight)	4.99 g l^{-1} (14.25%)	2.54 g l^{-1} (7.26%)
Concentration of proteins	0.65 g l^{-1} (10.82%)	0.34 g l^{-1} (5.74%)
Concentration of alkaloids	8.24 mg l^{-1} (3.30%)	8.443 mg l^{-1} (3.37%)

fluorescence data. For the prediction of the growth of the fungus, differences in the starting conditions must be considered. The combination of fluorescence spectroscopy and chemometric evaluation is suitable for on-line bioprocess monitoring and can replace time-consuming off-line measurement of process variables. With chemometric modelling, the information content of the fluorescence data can be used optimally for prediction of process variables and therefore for efficient bioprocess monitoring.

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