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Journal of Biotechnology 114 (2004) 199–208
JOURNAL OF Biotechnology
www.elsevier.com/locate/jbiotec

On-line cell mass monitoring of *Saccharomyces cerevisiae* cultivations by multi-wavelength fluorescence

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Received 28 January 2004; received in revised form 27 May 2004; accepted 28 May 2004

Abstract

The catalyst in bioprocesses, i.e. the cell mass, is one of the most challenging and important variables to monitor in bioprocesses. In the present study, cell mass in cultivations with *Saccharomyces cerevisiae* was monitored on-line with a non-invasive in situ placed sensor measuring multi-wavelength culture fluorescence. The excitation wavelength ranged from 270 to 550 nm with 20 nm steps and the emission wavelength range was from 310 to 590 nm also with 20 nm steps. The obtained spectra were analysed chemometrically with the multi-way technique, parallel factor analysis (PARAFAC), resulting in a decomposition of the multivariate fluorescent landscape, whereby underlying spectra of the individual intrinsic fluorophors present in the cell mass were estimated. Furthermore, gravimetrically determined cell mass concentration was used together with the fluorescence spectra for calibration and validation of multivariate partial least squares (PLS) regression models. Both two- and three-way models were calculated, the models behaved similarly giving root mean square error of prediction (RMSEPs) of 0.20 and 0.19 $g\,l^{-1}$, respectively, when test set validation was used. Based on this work, it is evident that on-line monitoring of culture fluorescence can be used for estimation of the cell mass concentration during cultivations.

Keywords: On-line monitoring; Two-dimensional fluorescence; Chemometrics; Cell mass

1. Introduction

For optimisation of bioprocesses as well as for ensuring high, consistent product quality, accurate real-time monitoring of different physical, chemical and biological parameters is necessary. In the years to come, increasing focus will be given to on-line/in-line techniques for process monitoring, primarily driven by FDAs initiative regarding process analytical technology (PAT) (www.fda.gov/cder/OPS/PAT.htm) and the industry's never ending need for process optimisation.

Traditionally, physical parameters, e.g. temperature, pressure, agitation and power input, and chemical parameters, e.g. pH, dissolved O_2, and CO_2 have been monitored. However, to gain better process knowledge, monitoring of biological and biochemical parameters like viable cell concentration, metabolic activity and product concentration is needed. It is the viable cell population that is the biocatalyst in bioreactors and hence, monitoring of the cell mass, indirectly or directly, can indicate the physiological

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doi:10.1016/j.jbiotec.2004.05.009

state of the process (Olsson et al., 1998; Sonnleitner, 1999; Harms et al., 2002).

Equipment used for monitoring of biological parameters often has a sampling unit, e.g. a membrane unit in front of the actual sensor. This might cause problems, as the medium used in industrial bioprocesses often is complex in nature, and therefore often contains smaller particles, which puts extra stress on the sampling equipment and may cause fouling. Optical sensor technologies circumvent these problems by being non-invasive, thus having no direct contact with the medium. Several reviews, discussing optical sensor techniques, have been published in recent years (Wolfbeis, 2002; Ulber et al., 2003).

Among non-invasive optical techniques, fluorescence spectroscopy has been used for monitoring of cultivation processes for many years. In early publications, single excitation/emission wavelength combinations were used for monitoring of NAD(P)H with excitation around 360 nm and emission monitoring around 460 nm (Duysens and Amesz, 1957; Zabriskie and Humphrey, 1978). Later, systems for monitoring of multiple excitation spectra have been employed (Li et al., 1991; Horvath et al., 1993). For monitoring of the cellular fluorophores, tryptophan, pyridoxine, NAD(P)H and riboflavin, five different excitation wavelengths were used. The intrinsic fluorescence of tryptophan was suggested as a possible indicator of cell concentration (Li et al., 1991; Horvath et al., 1993).

In many industrial bioprocesses, the medium consists of a complex matrix of substrates and particles. For these applications, the use of only a few excitation–emission wavelengths can be severely limited due to overlap in spectra and inner filter effects caused by the medium. An example of this is penicillin cultivations, where the fluorescence spectra of penicillin V overlap with that of corn steep liquor (which is often used as a substrate during production of penicillin). This has been shown to cause a drop in sensitivity, especially at high cell mass concentrations (Nielsen et al., 1994).

Recently, monitoring of bioprocesses by two-dimensional fluorescence has been applied (Marose et al., 1998). The usefulness of two-dimensional fluorescence spectroscopy combined with chemometric modelling has been demonstrated for monitoring of CO_2 and O_2 in the exhaust gas and for monitoring of the substrate, succinate in *Pseudomonas fluorescens* cultivations (Skibsted et al., 2001). Furthermore, two-dimensional fluorescence has also been used for on-line quantitative monitoring of cell mass concentration and alkaloid formation in *Claviceps purpurea* cultivations with root mean square error of prediction (RMSEP) values of 2.5 and 8.4 $mg\,l^{-1}$, respectively (Boehl et al., 2003). As on-line measurements of two-dimensional fluorescence create large amounts of multivariate data, the interpretation of these data is of utmost importance, and chemometric modelling becomes a very useful tool. Chemometrics can be used for explorative analysis of processes, and, furthermore, calibration models can be computed if the purpose is to use the sensor for on-line quantification of biological parameters.

In early studies on two-dimensional spectroscopy, two-way (data in matrix form) partial least squares (PLS) calibration has been used for prediction of various variables in bioprocesses (Skibsted et al., 2001; Boehl et al., 2003). However, series of excitation/emission fluorescence spectra recorded on-line will be of a three-dimensional structure (data in cube structure) and a more direct approach would be to model the data with three-way chemometrics. In a recent study by Solle et al. (2003), a yeast cultivation has been monitored with multi-wavelength fluorescence. In this work, three-way PLS calibration models were constructed based on on-line measured fluorescence and simulated cell mass concentration. This way it was possible to circumvent the use of off-line measured data in the calibration step, and using two components a RMSEP value of 0.5 $g\,l^{-1}$, when predicting cell mass concentration, was reported.

In the present study, two-dimensional fluorescence was measured on-line during *Saccharomyces cerevisiae* cultivations with the BioView® system (Delta Light & Optics, Denmark) and data was used for two different purposes. First, the fluorescence spectra were analysed with the parallel factor analysis (PARAFAC) method. PARAFAC is a generalisation of principal component analysis (PCA) to higher order arrays, e.g. three-dimensional data. Applying PARAFAC on multi-wavelength fluorescence data gives the opportunity to elucidate the underlying chemical structure of the multivariate spectra as PARAFAC in contrast to PCA only has one true solution (Bro, 1997). The same spectra were also used for evaluating the use of

multi-wavelength fluorescence for on-line prediction of cell mass concentration. For comparison both two- and three-way PLS calibration models were computed. In order to obtain a realistic value of the predictive performance of the models, test set validation was employed.

2. Materials and methods

2.1. Growth conditions for the cultivations

The strain used in this study was the laboratory strain *Saccharomyces cerevisiae* CEN.PK.113-7D. Using this strain, 10 batch cultivations were conducted in a 4 l MBR bioreactor (new MBR AG, Switzerland) with a working volume of 3 l. In all cultivations, a defined laboratory medium with glucose as carbon and energy source was used according to Verduyn et al. (1992), the media also contained antifoam 289 (A5551, Sigma) (50 $\mu l\,l^{-1}$). The initial concentration of glucose was varied from 10 to 40 $g\,l^{-1}$ in the different cultivations, one started with 10 $g\,l^{-1}$, three with 20 $g\,l^{-1}$, three with 30 $g\,l^{-1}$ and three with 40 $g\,l^{-1}$ glucose. Concentrations of trace metal (1000×) and vitamins (1000×) were 1 $ml\,l^{-1}$ for glucose concentrations of 20 $g\,l^{-1}$. For other glucose concentrations than 20 $g\,l^{-1}$, the concentration of the trace metal and vitamin solutions were varied proportionally to the glucose concentration. The temperature was in all cultivations controlled at 30 °C and the pH was controlled at 5.0 by addition of 2 M KOH. The airflow was kept constant at 3 $l\,min^{-1}$ and the agitation rate was kept at 750 rpm for all cultivations. The off gas was analysed for carbon dioxide and oxygen by a Brüel and Kjær 1308 acoustic gas analyzer (Brüel & Kjær, Denmark). The bioreactor was inoculated with an exponentially growing preculture from a 500 ml Erlenmeyer shake flask containing 100 ml defined medium with $(NH_4)SO_4$ (7.5 $g\,l^{-1}$), KH_2PO_4 (14.4 $g\,l^{-1}$), $MgSO_4{\cdot}7H_2O$ (0.5 $g\,l^{-1}$), vitamins (1000×) (1 $ml\,l^{-1}$), trace metals (1000×) (2 $ml\,l^{-1}$), antifoam 289 (A5551, Sigma–Aldrich) (50 $\mu l\,l^{-1}$) and 10 $g\,l^{-1}$ glucose at pH 6.5. All batches were inoculated to an initial OD_{600} between 0.0001 and 0.001, sampling for dry weight measurements were started the following day once the OD_{600} of the culture reached 0.5.

2.2. Off-line analysis

S. cerevisiae dry cell mass was determined gravimetrically off-line during all cultivations. Samples were withdrawn with syringes during the cultivations and injected into 0 °C cold sample glasses for fast cooling. Volumes between 5 and 30 ml, depending on the concentration of cell mass, were, within 2–5 min after sampling, filtered through nitrocellulose filters with pore size 0.45 μm (Pall Corporation, Ann Arbor, MI). The filters had been pre-dried for 10 min at 150 W in a microwave oven and weighed after cooling in a desiccator. The filter cakes were washed with distilled water, twice the sample volume, and dried in a microwave oven for 15 min at 150 W. All measurements of dry cell mass were done in duplicates and the average values were used for modelling.

Samples for measurement of extracellular metabolites were also collected. These samples were withdrawn from the bioreactor with syringes and immediately filtered through a 0.45 μm pore size sterile filter, to avoid that cell metabolism would influence the composition of the samples during handling, and then stored at −20 °C. Analysis of glucose and the products ethanol, glycerol and acetate were done by HPLC using an Aminex HPX-87H column (Bio-rad, Hercules, CA) kept at 65 °C, eluted by 5 mM H_2SO_4 at a flow of 0.6 $ml\,min^{-1}$. The compounds were determined refractometrically using either a Waters 410 differential refractometer of a Shodex RI-71.

2.3. On-line multi-wavelength fluorescence measurements

The BioView® instrument (Delta Light & Optics, Denmark) was used for measuring multi-wavelength fluorescence spectra. The BioView® instrument has already been described in detail in the literature by Lindemann et al. (1998) and Marose et al. (1998) and only a short description will be given here. Optical fibres were used to connect the internal parts of the BioView® instrument with the probe. After autoclavation of the reactor, the fluorescence probe was inserted into a standard 25 mm port in the side of the reactor mounted with an optical well containing a bottom surface of sapphire glass. Consequently, there was no contact between the cultivation broth and the probe. A standard O-ring gasket was used when inserting the

optical well in the side of the reactor. No sterilisation problem aroused as a consequence of using the optical well. After cultivation the probe was removed from the optical well and wiped with a cloth. The BioView® system was set up to excite with wavelengths in the range from 270 to 550 nm in steps of 20 nm, and subsequently measured fluorescence light emitted from the cultivation broth with wavelengths in the range from 310 to 590 nm after each step. This gives rise to a three-dimensional spectrum where the excitation and emission wavelength are the *x*- and the *y*-axis and the fluorescence intensity is the *z*-axis. The instrument was set to collect a full spectrum every 5 min with a gain of 1450 units and normal sensitivity.

2.4. Multi-wavelength fluorescence measurements of reference standards

Multi-wavelength fluorescence spectra of tryptophan, NADH and riboflavin were collected with the BioView® instrument (Delta Light & Optics, Denmark). The measurements were conducted at 30 °C in the same bioreactor as described previously. pH was kept at 6.8, which has been reported to be the intracellular pH level of exponentially growing *S. cerevisiae* cells when the external pH was kept at 5.0 (Imai and Ohno, 1995). The medium used for the off-line measurements had the same composition as the medium used during cultivations. Tryptophan concentration was 60 mg l^{-1}, NADH 40 mg l^{-1} and riboflavin 60 mg l^{-1}.

2.5. Chemometric model building

The chemometric models were built in MATLAB for Windows (Version 6.5, The MathWorks, Inc., Natrick, MA, USA) using the *N*-way toolbox available from the net at www.models.kvl.dk (Andersson and Bro, 2000). With this toolbox, it is possible to analyse the three-dimensional fluorescent spectra without unfolding it into a two-dimensional structure. The toolbox contains algorithms both for explorative data structure analysis (PARAFAC) and for regression (*n*-PLS). The rationale behind all multivariate data analysis is to decompose the data in order to detect hidden phenomena and to be able to model these. A basic assumption is that the directions with maximum variance are related to the hidden structure in data. Accordingly, the PARAFAC method decomposes the data structure into components that are optimised to explain the variance in the data array. The components will, in three-way fluorescence arrays, consist of one score and two loading vectors (one for excitation and one for emission). A special feature for the PARAFAC method is that it returns a unique solution. Hence, the excitation and emission loadings will represent the underlying pure spectra of the complex fluorescence array, given that the right number of components has been estimated and that the data has a suitable signal-to-noise ratio (Bro, 1997).

When using *n*-PLS, the result is also a breakdown of the data into a few components with one score and two loading vectors. The difference compared to the data structure analysis method is, that when computing a regression model the covariance between the independent (*X* data) and the dependent (*Y* data) is maximised rather than just the variance in *X* (Bro, 1996).

In the present study, models describing the exponential growth on glucose were estimated. In total, 106 samples from the exponential growth phase of 10 cultivations were used. The samples (corresponding off-line dry weight samples and on-line multi-wavelength spectra) were divided into two sets, a calibration set and a validation set, composed of samples from five cultivations each. In the calibration set, data from one 10 g l^{-1} glucose, one 20 g l^{-1} glucose, one 30 g l^{-1} glucose and two 40 g l^{-1} glucose cultivations were placed. The validation set contained data from two 20 g l^{-1} glucose, two 30 g l^{-1} glucose and one 40 g l^{-1} glucose cultivations. Two types of models were calculated, namely, PARAFAC models used for identifying underlying chemical structures and PLS models able to predict the dry cell weight concentration. When calibrating the PLS models, only the calibration data set was used, the validation data set was afterwards used to estimate the model error and thus the validity of the model. The PARAFAC model was built using all 106 samples.

3. Results

3.1. Cultivation characteristics

In order to thoroughly examine the possibility of using the BioView® (Delta Light & Optics, Denmark)

Table 1
Growth characteristics of the calibration and validation set

Data set	μ_{max} (h^{-1})	$Y_{cell\ mass}$ (C-mol C-mol^{-1})	$Y_{ethanol}$ (C-mol C-mol^{-1})	$Y_{glycerol}$ (C-mol C-mol^{-1})	Y_{CO_2} (C-mol C-mol^{-1})
Calibration set					
Average	0.38	0.14	0.51[a]	0.06	0.29
Standard deviation	0.03	0.01	0.00	0.01	0.05
Validation set					
Average	0.38	0.15	0.51	0.06	0.31
Standard deviation	0.02	0.01	0.03	0.01	0.02

All cultivations were conducted with *Saccharomyces cerevisiae* CEN.PK.113-7D. Yields were calculated based on consumed glucose.
[a] Ethanol yield from fermentation number five was not included as this yield was unreasonably low, probably due to evaporation of ethanol.

instrument as an on-line sensor for cell mass, 10 yeast cultivations were conducted. The cultivations differed in the initial substrate concentration, but otherwise the cultivations were conducted under similar conditions. A low batch-to-batch variation was found in the different cultivations (Table 1) as the maximal specific growth rate of the individual cultivations was 0.38 h^{-1} (±0.03). The average yield of dry cell mass was 0.15 C-mol C-mol^{-1} (±0.01), the ethanol yield was 0.51 C-mol C-mol^{-1} (±0.02), the glycerol yield was 0.06 C-mol C-mol^{-1} (±0.01) and the CO_2 yield was 0.30 C-mol C-mol^{-1} (±0.04). Carbon balances for all the cultivations, except one (Table 1), closed with a maximum error of 6%.

3.2. Elucidation of intrinsic culture fluorescence with PARAFAC models

Multi-wavelength fluorescence spectra were collected during the exponential growth on glucose for the 10 cultivations. A three-dimensional model (PARAFAC) was estimated from the 106 spectra in order to elucidate the underlying individual spectra of the intrinsic fluorophors composing the real-time multi-component spectra. When estimating chemometric models describing the underlying chemical structure, it is essential to use the right number of components, otherwise the model will not be representative of the process investigated. In order to estimate the right number of components in spectral data several options are available (Bro, 1996; Bro and Kiers, 2003). Three different methods were applied on the current data set. During the model building, the residual sum of squares was plotted against the number of components (Fig. 1). A flattening of the curve going from a three-components to a four-components model (Fig. 1) indicated that no more systematic variation was left in the data, as adding an additional component did not decrease the error further.

Split-half analysis is another method for assessing the correct number of components. Results from such an analysis performed on the calibration and validation set, separately, also indicated that three components should be used as the scores and loadings from the resulting two models were very alike (data not shown).

The final method for assessing the right number of components in the present work was the use of process knowledge. When investigating a fluorescent spectrum from one time point in the exponential growth phase, three clear peaks could be seen

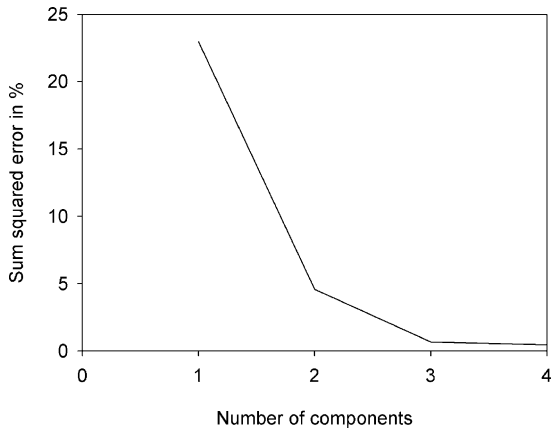

Fig. 1. Sum squared error in percent for PARAFAC models with up to four components. A flattening of the curve indicates that the correct number of components has been reached.

in the fluorescent landscape. This finding corresponds well with earlier investigations where three major intrinsic fluorescent compounds in *S. cerevisiae* have been identified (Horvath et al., 1993). Therefore, also this method pointed towards three components as the right number for the PARAFAC model. Consequently, a three-component PARAFAC model explaining 99% of the variation in the spectral data was estimated. Due to the uniqueness of the PARAFAC solution and the three-dimensional data structure, the emission and excitation loadings corresponded to pure emission and excitation spectra of the fluorescent compounds present in the cultivations (Fig. 2).

As the emission and the excitation loadings are estimates of the underlying fluorescent structure in the data, the loadings should be comparable with pure spectra of intrinsic fluorescence compounds of the yeast cells. To establish causality of the estimated PARAFAC components, off-line spectra of compounds reported to be involved in the intrinsic fluorescence of yeast cells were collected. Pure emission and excitation spectra of tryptophan, NADH and riboflavin were obtained and merged into an emission and an excitation plot (Fig. 2B and D). The emission and excitation loadings of the three estimated PARAFAC components were similar to the pure emission spectra of tryptophan, NADH and riboflavin (Fig. 2A and C), thus supporting the idea of tryptophan, NADH and riboflavin as the intrinsic fluorescent compounds in exponentially growing yeast. The largest dissimilarities between the estimated spectra and the spectra obtained with pure spectra were found in the emission plot in the area between 390 and 430 nm.

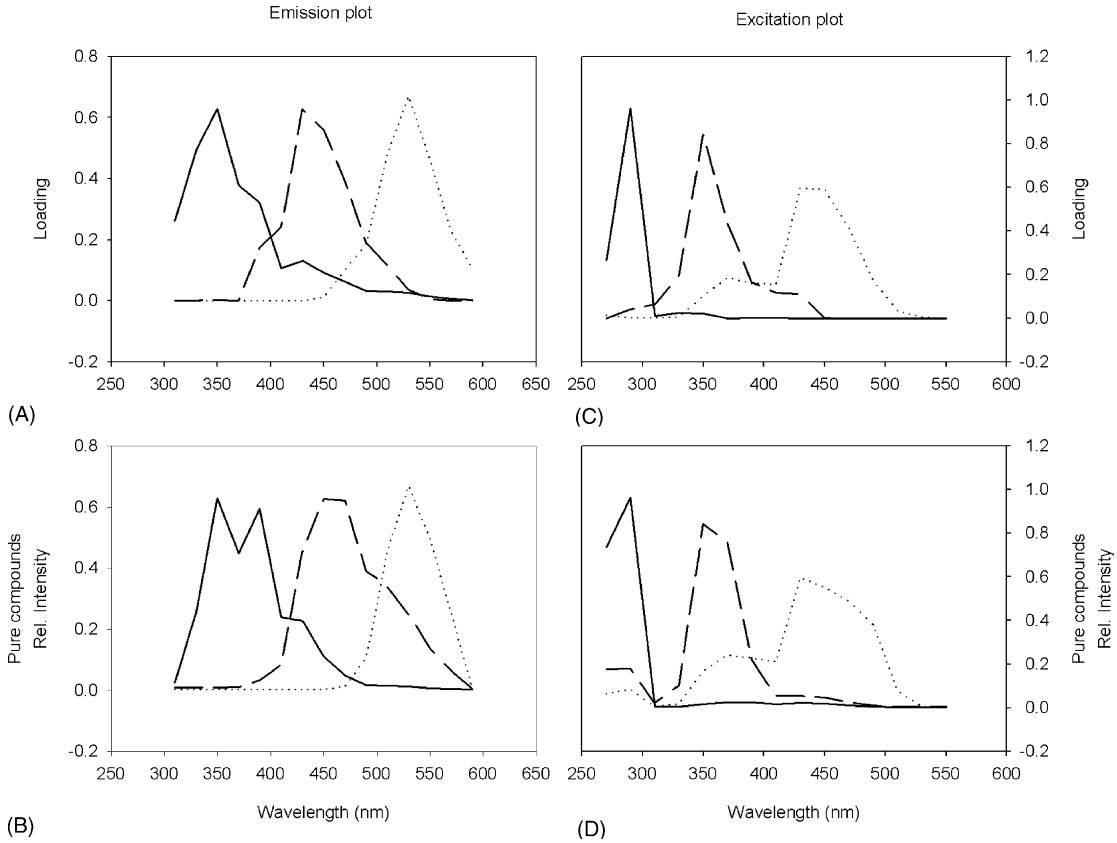

Fig. 2. Emission and excitation plot of the three estimated PARAFAC components (A and C) and emission and excitation spectra of pure tryptophan, NADH and riboflavin (B and D). In A and C, the three lines represent the three estimated PARAFAC components. In B and D, the lines represent tryptophan (–), NADH (––) and riboflavin (· · ·), respectively.

3.3. Prediction of dry weight concentration with PLS models

One way of extracting information from the multi-wavelength fluorescence spectra is to split them up into the individual spectra of the intrinsic yeast fluorescence as described in the previous section. But, fluorescence data can also, together with reference data, be used to train a chemometric model. In the present work, fluorescence data provided by the on-line sensor, BioView®, and the off-line determined cell mass concentration, were used to build calibration models able to estimate the dry cell mass content during exponential growth on glucose. A chemometric three-way PLS model was constructed from the five cultivations making up the calibration set. The data were centred over the sample dimension and no scaling was applied. A model with two components explained 75% of the variance in the *X* space and 96% in the *Y* space (Fig. 3). This two-component model gave the lowest root mean square error of prediction (RMSEP) value of 0.19 g l^{-1} (Fig. 3).

The RMSEP calculations were based on predicted values of the cell mass in the cultivations composing the validation set. When the number of components included in the model was increased, an increasing amount of the variance in the *X* and *Y* space could be explained (Fig. 3). However, a concurrent increase in the RMSEP value was observed, which will give an inferior prediction performance of the model. The increase in explained *X* and *Y* space variance in response to increasing component number was, therefore, most likely due to modelling of noise and a two-component model was chosen as the most appropriate model to determine the dry cell mass from the fluorescence spectra.

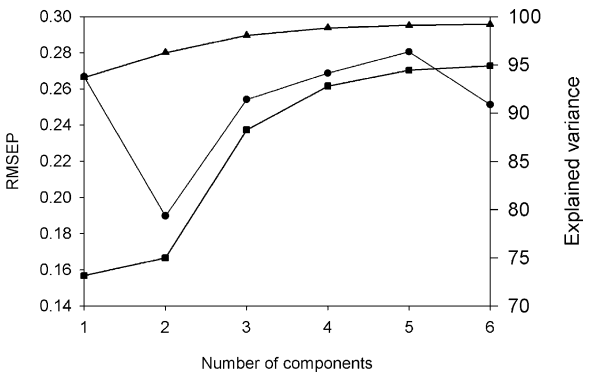

Fig. 3. RMSEP vs. number of components in the model. The minimum RMSEP (●) value was found after two components where it was 0.19. Explained variance in the *X* (■) and *Y* (▲) space were plotted against the number of components included in the model.

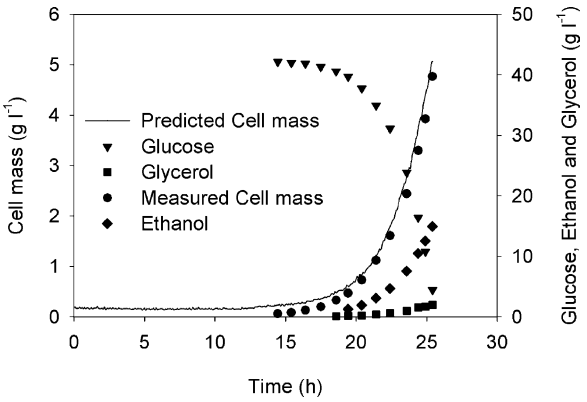

Fig. 4. Predicted dry cell mass during the time course of one of the cultivations from the validation set. Measured values together with major fermentation products are also depicted. As the data from this fermentation was placed in the validation set it was not used for calibrating the model.

One of the cultivations included in the validation set was used to illustrate the accuracy of the chemometric model. A good correspondence of the estimated dry cell mass to the off-line determined values was observed (Fig. 4). The model had difficulties in determining cell mass concentrations below 0.5 g l^{-1} precisely. For cell mass values above 0.5 g l^{-1}, the error with respect to the off-line determined cell mass was on an average basis 12%. Compared to the error on the reference method, which had an average absolute error of 2% over all samples in the present study, the prediction error was higher.

Not only cell mass was determined off-line, also the concentration of the major fermentation products were determined. Glycerol, ethanol and cell mass were the main products during the exponential growth on glucose (Fig. 4). All product concentrations increased exponentially while the substrate was consumed. PLS models estimating the concentration of these products could be constructed with similar precision as the cell mass model due to the constant yields of these compounds during the period investigated. However, as none of these compounds show intrinsic fluorescence,

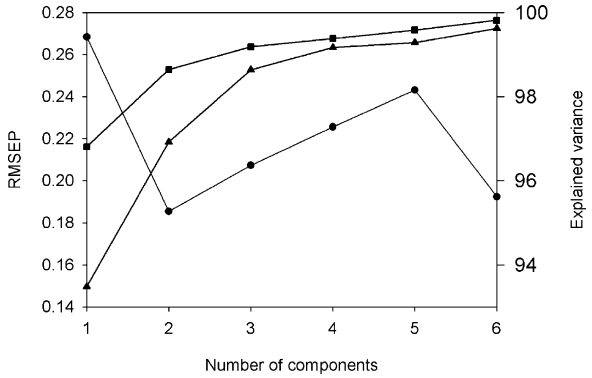

Fig. 5. RMSEP (●) vs. number of components in the model based on the unfolded data structure. The minimum RMSEP value was found after two components where it was 0.20. Explained variance in the X (■) and Y (▲) space were plotted against the number of components included in the model.

correlation models to other products than cell mass were not found relevant as these would only reflect their correlation to the cell mass.

The same calibration and test sets were used to evaluate the performance of two-way PLS (two-dimensional data structure) compared to three-way PLS calibration (three-dimensional data structure). The three-way data structure was unfolded, that is, transferred into a two-dimensional matrix structure with samples in the rows and the different ex–em combinations in the column. The data sets were mean centred after unfolding. A two-way PLS model with two components gave the best performance, with a RMSEP of $0.20\,g\,l^{-1}$ (Fig. 5).

4. Discussion

This work demonstrates that on-line monitoring using in situ multi-wavelength fluorescence together with chemometric modelling can give important qualitative and quantitative information concerning the cell mass during submerged cultivations. Both three-way explorative data analysis, i.e. PARAFAC, and two- and three-way PLS regression models showed good results and can in a complementary way be used for monitoring of the cell mass in bioreactors.

The PARAFAC algorithm was used to estimate the spectra of the individual intrinsic fluorophores present in the cell mass. The estimated spectra fitted very well with individual spectra of tryptophan, NADH and riboflavin, obtained off-line. Especially the estimated excitation spectra fitted well, with the exception of a small shoulder on the riboflavin spectrum that was not included in the estimated spectrum (Fig. 2). The emission spectra were more difficult to estimate. The estimated spectra of tryptophan and NADH seemed to be mixed up in the region where they overlap in the multivariate spectra (390–430 nm). This may be due to an overestimation of the emission loading of the second component (resembling NADH) at 390 nm and a underestimation of the emission loading of the first component (resembling tryptophan). The emission loadings of the component resembling riboflavin (the third one) fitted very well with individual spectra of riboflavin.

Two-dimensional fluorescence in combination with chemometric modelling (PARAFAC) has been applied for simultaneously determination of three pesticides in low concentrations (Rodriguez-Cuesta et al., 2003). The estimated spectra showed very good resemblance to spectra of the individual pesticides. PARAFAC and two-dimensional fluorescence have also been used for determination of doxorubicin, an antibiotic used against human neoplasm, in plasma (Trevisan and Poppi, 2003). Also in this case, the estimated spectra closely resembled the individual spectra of the antibiotic. In both of these studies, equipment with only a few nanometres (0.5–2 nm) between each excitation or emission wavelength have been used, resulting in very detailed spectra over a narrow range. Thereby, the estimated spectra had a very high standard and could be used for direct calibration to the concentration of the compound, in question (Rodriguez-Cuesta et al., 2003; Trevisan and Poppi, 2003). In contrast, the instrument used in the present study has steps of 20 nm between each excitation and emission wavelength, and therefore, a wide range of compounds, with very different fluorescence characteristics could be measured in a short time span. However, it evidently, also caused the estimated spectra to be of a lesser quality, than they would have been if the steps had been shorter. The estimated loadings resembled the underlying fluorescent structure, and most of them fitted well with individual spectra measured off-line, but they were not identical. The method used in this study, with the current instrument setting, is thus not

suited for very solid identification of the fluorophores, but certainly as an indication of which intrinsic fluorophores that are present during the bioprocess, and in which area of the multivariate spectrum they are present. To our knowledge, this is the first reported use of on-line collected two-dimensional fluorescence and decomposition with PARAFAC.

The loadings of the estimated spectra discussed in the previous paragraph were not of such quality, that they could be used for direct calibration to the amount of cell mass in the cultivations and instead *n*-way calibration was used for this purpose. Two PLS models were calibrated, with almost similar results. The RMSEP values of the three-way model and the two-way model, both with two components, were 0.19 and $0.20\,g\,l^{-1}$, respectively, when using test set validation, which should give a more conservative prediction performance compared to, e.g. cross validation, as the test data are independent of the calibration data. This also shows that the BioView® instrument gives reproducible data as the data from the 10 cultivations were collected over a period of 6 months and still the RMSEPs were low. In the study by Solle et al. (2003), the three-way model they report on also consisted of two components and the RMSEP value of $0.5\,g\,l^{-1}$ was only slightly higher than the RMSEP of $0.19\,g\,l^{-1}$ we report here. The prediction performance of the two-way PLS model was not significantly inferior to the three-way model, but the two-way model contained 120 loadings for each component, whereas the three-way model only contained 30 loadings for every component. This makes the three-way PLS model simpler and also more robust compared to the two-way model and would, thus, be the preferred choice of model when the data are of a three-dimensional structure (Bro, 1996).

Monitoring of cell mass is a difficult discipline due to the complex chemical nature of cell mass. Cell mass is composed of a large number of different chemical compounds, displaying different characteristics under different physiological and morphological conditions. This is important to take into account when using fluorescence measurements for calibration purpose, as a given calibration will not necessarily hold under other physiological conditions. In case a global model is needed, it will be necessary to make use of a method applying a separate calibration model for each physiological state as was done by Solle et al. (2003).

Different methods for on-line determination of the cell mass in cultivations have been applied during the last few decades. These sensors can generally be grouped into optical- (optical density or fluorescence), electrical property- (capacitance or conductivity) or software sensors (based on linear or non-linear models), according to the measuring principle they employ (Sonnleitner et al., 1992). None of these have been applied widely as cell mass sensor due to limitations in dynamic range, interference from aeration or agitation, inner filter effects and interference from media components. The BioView® system overcomes some of these limitations and it has its real potential in industrial applications where the medium is complex or the producing cells create a harsh environment for in situ sensors, e.g. filamentous growth. A limitation in the use of the BioView® system would be if the medium used in a given process had a high background fluorescence thus overshadowing the fluorescence from the biological parameters of interest. The main advantages of the BioView® system in an industrial perspective are two-fold. Firstly, the sensor is non-invasive and thus, has no contact with the medium, giving fewer problems with fouling of the sensor or maintaining a non-contaminated environment. In addition, the BioView® probe is easily cleaned and the measurements show a high degree of reproducibility. Secondly, the BioView® can together with chemometric methods be used for monitoring of several compounds simultaneously as illustrated in this study where the BioView® was used for monitoring the cell mass and also for qualitative explorative analysis of the fluorophores present in cell mass. Thus, using three-way analysis (PARAFAC, three-way PLS) together with on-line fluorescence gives more information than using a two-way chemometric method.

Acknowledgements

Delta Lights & Optics, Denmark has provided us with the BioView®, for which we are grateful. This research was performed within the framework of Center for Combination of Sensors, which was financed by the Danish Agency for Trade and Industry, Contract No.1999-603/4020-3.

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