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Fluorescence spectroscopy as a novel method for on-line analysis of biocatalytic C–C bond formations

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ABSTRACT

On-line analysis of bioprocesses is of increasing interest avoiding the time delay for off-line sample preparation and the following analyses via chromatographic methods. Moreover, continuous monitoring of the reaction components during chemo- or biocatalytic transformations provides a direct control of the process. Since productivity of the processes can be controlled simultaneously, on-line monitoring of the processes is attractive for industrial applications. The reliable in situ monitoring of biocatalyzed reactions has been a challenge where reactions run in aqueous solutions. Limited work has been published on the use of spectroscopic methods for on-line analysis of biocatalytic reactions up to now. However, in this communication two dimensional (2D)-fluorescence spectroscopy has been proved to be an effective tool for on-line monitoring of the carboligation reactions catalyzed by wild type benzoylformate decarboxylase (BFD) from *Pseudomonas putida*. BFD is a thiamine diphosphate (ThDP)-dependent enzyme that catalyzes the asymmetric C–C bond formation to (*S*)-2-hydroxypropiophenone ((*S*)-2-HPP) starting from benzaldehyde and acetaldehyde. The analysis of the fluorescence spectra was achieved by chemometric modeling performing principle component analysis (PCA) and partial least square (PLS) regression. The derived chemometric models were used for the validation of concentrations of yielded 2-HPP and the substrate benzaldehyde with low root mean square error of calibration (RMSEC).

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1. Introduction

In recent years many procedures for on-line monitoring of biotransformations have been reported, e.g., ultraviolet (UV) spectroscopy [1], fluorescence spectroscopy [2], bioluminescence [3], flow calorimetry [4], dielectric spectroscopy [5], or Fourier transformed infrared (FT-IR) spectroscopy [6]. With on-line analysis methods reaction systems can be monitored faster and more efficiently than with conventional methods. On-line analysis techniques enable production control in order to enhance procedures and processes, as well as for quality control. Thus, on-line monitoring results in improved ecologic and economic process management. Moreover, rapid enzyme screening is achieved by colorimetric assays where the color formation can be continuously monitored via UV measurements. The reliable in situ monitoring of biocatalyzed reactions has been a challenge where reactions run in aqueous solutions. Since water is the dominant component and the reacting species are at low concentrations, obtained spectra are usually of poor quality in infrared (IR) spectroscopy. Among the developed methods, two dimensional (2D)-fluorescence analysis has been successfully applied for the on-line monitoring of cultivations where the NAD(P)H-pool inside living organisms is followed. Via fluorescence measurements bioprocess parameters can be derived and be controlled in order to achieve high biomass yield [7]. In addition to cultivation applications, 2D-fluorescence spectroscopy has also been applied for the analysis of enzymatic catalyses. Applications are found to examine pseudoenantiomeric reactions catalyzed by proteases and esterases [2(a,b)] and to analyze the NADH-dependent biotransformations catalyzed by an oxidoreductase [2(c)].

The BioView® sensor (DELTA, Denmark) was developed as a robust instrument for 2D-fluorescence measurement of bioprocesses especially in an industrial environment [7]. The main advantage is the absence of a direct contact between sensor and medium which eliminates the risk of contamination. No sampling is necessary to get a direct insight into the process itself. Therefore, 2D-fluorescence spectroscopy is a promising analytic method

Abbreviations: BFD, benzoylformate decarboxylase; BSA, bovine serum albumin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HPP, hydroxypropiophenone; PCA, principal component analysis; PLS, partial least square; RFI, relative fluorescence intensity; RMSEC, root mean square error of calibration; ThDP, thiamine diphosphate; Wt, wild type.

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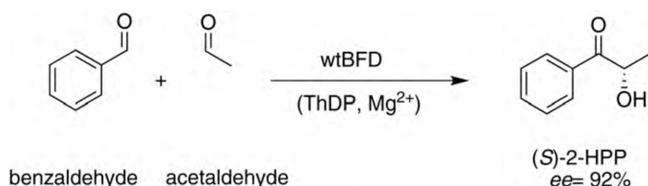

Fig. 1. The synthesis of (*S*)-2-HPP from benzaldehyde and acetaldehyde catalyzed by wtBFD.

for the on-line monitoring of bioprocesses including the enzymatic reactions which are performed under harsh conditions, especially under high pressure [2(a,b)]. Our future goal is to transfer the applicability of 2D-fluorescence spectroscopy into the biocatalyses which are performed under high pressure conditions.

The bi-functional nature (keto- and hydroxy groups) and the presence of a stereocentre make the chiral 2-hydroxyketones important as building blocks for several pharmaceuticals. Thus, synthesis of these key intermediates is of high interest. Most importantly, these crucial compounds can be synthesized via biocatalyzed asymmetric C–C bond formations with high enantiomeric excesses (*ee*) [8]. One of the enzymes applied in the synthesis of these chiral intermediates is benzoylformate decarboxylase from *Pseudomonas putida* (BFD, EC 4.1.1.7). BFD is a homotetrameric thiamine diphosphate (ThDP)-dependent enzyme which catalyzes the synthesis of chiral 2-hydroxyketones accepting a broad range of aldehydes as substrates. Besides ThDP, divalent metal cations, preferable Mg^{2+} is required as a second cofactor in biotransformations. Wild type (wt) BFD is catalyzing the carboligation of benzaldehyde and acetaldehyde yielding 2-hydroxypropiophenone (HPP) with *ee* of 92% (*S*) (Fig. 1) [9]. Synthesis of 2-HPP and its derivatives has been intensively studied [9(a,b)].

Limited work has been published on the use of spectroscopic methods in biocatalysis up to now. Due to disturbance of water IR-spectroscopy gives often suboptimal results. However, this communication demonstrates the successful monitoring of the 2-HPP synthesis catalyzed by wtBFD via 2D-fluorescence spectroscopy where reactions run in aqueous medium.

2. Experimental

2.1. Material

All chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany) and Carl Roth GmbH (Karlsruhe, Germany).

2.2. General methods

2.2.1. Overexpression, fermentation and purification

Plasmid hosting wild type BFD was kindly supplied by PD. Dr. Martina Pohl, Institute of Molecular Enzyme Technology/Heinrich-Heine University of Düsseldorf, Germany. Precultivation of the variants was done in shaking cultures (200 mL, pH 7.0 in 1 L flasks) using a complex batch medium containing glucose (2.0 g/L), $MgSO_4 \cdot 7H_2O$ (1.0 g/L), NH_4Cl (0.2 g/L), $(NH_4)_2SO_4$ (2.0 g/L), KH_2PO_4 (13 g/L), K_2HPO_4 (10 g/L), $NaH_2PO_4 \cdot H_2O$ (6 g/L), yeast extract (3 g/L), ampicillin (0.1 g/L), and kanamycin (0.05 g/L). Fed-batch fermentation was performed in a 2 L foil-fermenter (Bioengineering AG, Wald, Switzerland) using the complex medium defined before with 0.2% (v/v) Desmophen as an antifoam agent and 25% ammonia solution for pH control at 7.1. The fermenter was inoculated by the addition of 1% (v/v) active preculture having an $OD_{600\,nm}$ = 1.0. After 12 h the feed solution was supplied containing glucose (600 g/L), yeast extract (15 g/L), $MgSO_4 \cdot 7H_2O$ (400 g/L), ampicillin (0.1 g/L), and kanamycin (0.05 g/L). The oxygen saturation was adjusted to at least 40% by varying the stirrer speed and by the aeration with compressed air and pure oxygen.

After 24 h the overexpression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1.5 mM at an $OD_{600\,nm}$ = 50. Temperature was decreased from 37 °C to 28 °C and 18 h after induction cells were harvested by centrifugation (10,000 rpm for 30 min at 4 °C) and stored at −20 °C. Purification of the wtBFD was performed by immobilized metal affinity chromatography Ni-NTA column as previously describes [10]. BFD was applied in biotransformations as freeze-dried preparations. Protein amounts in the solutions were determined by the standard Bradford method [11] using bovine serum albumin (BSA) as a standard.

2.2.2. Biotransformations catalyzed by wtBFD

Reactions were carried out in a steel reactor with a total volume of 14 mL and thermostated at 20 °C for approximately 2 h. Two independent process runs were carried out where 15 and 14 off-line samples were taken during the first and during the second run, respectively. 40 mM/20 mM benzaldehyde and 400 mM acetaldehyde were prepared in 50 mM phosphate buffer pH 7.5 containing 2 mM $MgSO_4$ and 0.5 mM ThDP. The reaction was started by the addition of wtBFD (8 U) dissolved in the same buffer; 50 mM phosphate buffer pH 7.5 containing 0.5 mM ThDP and 2 mM $MgSO_4$. Here, one unit of activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of 2-hydroxypropiophenone (HPP) in 1 min at 30 °C. Activity analysis for 2-HPP synthesis was performed with 40 mM benzaldehyde and 400 mM acetaldehyde in 50 mM phosphate buffer containing 0.5 mM ThDP and 2 mM $MgSO_4$ at pH 7.5.

2.2.3. Off-line analysis

Reactions were analyzed by HPLC (Agilent 1100, Hewlett Packard) equipped with a LiChrosphere RP-8 column (Hypersil, 250 mm × 4 mm, Merck) using triethanolamine (0.2%, pH 3.5): methanol (50:50, v/v) as an eluent at a flow rate of 1.0 mL min^{-1} at 40 °C. Typical retention times were: 2-HPP 4.9 ± 0.1 min, benzaldehyde 5.7 ± 0.1 min. The reactions were quenched by the addition of a stop-buffer (90% acetonitrile, 5% H_2O, and 5% H_3PO_4) with the ratio of 1:1 (sample: stop-buffer, v/v) followed by intense mixing and centrifugation of the precipitate. During the process runs 15 (first process) or 14 (second process) samples were taken simultaneously to one of the 63 fluorescence spectra or 48 fluorescence spectra, respectively.

2.2.4. On-line monitoring via fluorescence spectroscopy and chemometry

On-line monitoring of the carboligations catalyzed by wtBFD was achieved using a 2D-fluorescence sensor (BioView® sensor). The fluorescence sensor is equipped with a xenon flash lamp for the excitation light. The sensor is connected directly by a bifurcated liquid light conductor (Lumatec, Germany) to a quartz window in a 17-mm electrode standard port of a steel reactor. The sensor uses two independently rotating filter wheels with 16 different filters for excitation and emission and a photomultiplier for detection of emission light. Each spectrum has 150 combinations of excitation (Ex) emission (Em) wavelength pairs in a region of 270–550 nm for excitation and in the region of 310–590 nm for emission with a bandwidth of 20 nm. Intensities obtained from full excitation and emission wavelength range are also included. Full fluorescence spectra were recorded every 1.5 min during biotransformations. Thus, 63 spectra of the first process and 48 spectra of the second process were collected. To eliminate the differences in fluorescence intensities occurred by varying the initial conditions, difference spectra to the first spectra of each process are calculated.

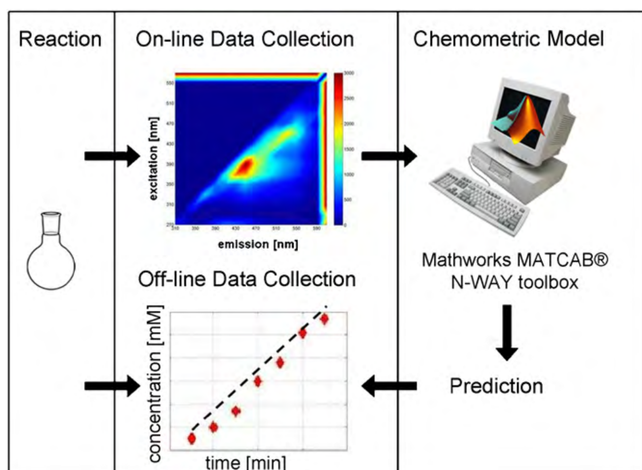

Fig. 2. General procedure for the development of the chemometric model in order to predict the concentrations (dashed line).

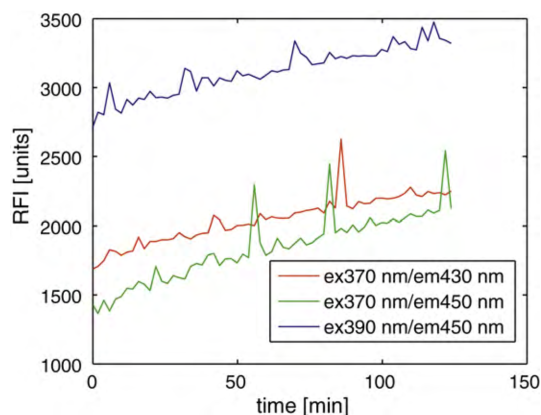

Fig. 3. Course of fluorescence intensities in the regions of Ex390 nm/Em450 nm, Ex370 nm/Em450 nm and Ex370 nm/Em430 nm during the carboligation of benzaldehyde and acetaldehyde (first process) catalyzed by wtBFD.

Principle component analysis (PCA) and partial least square (PLS) regression are widely used techniques in multivariate data analysis in order to reduce the huge amount of data to a small number of components [12]. The analysis of the fluorescence spectra was achieved by PCA and PLS regression. To evaluate the number of latent variables (principal components) and to compare both processes a principle component analysis is performed. Chemometric models, like PLS are developed to correlate the information from the fluorescence spectra with the off-line data set of the respective process variable. In the present work two process runs were analyzed where off-line samples were used to build a well performance model. For each process variable (substrate and product) an own PLS regression model was performed using the data of both process runs together. The model was developed using multilinear partial least square regression in the *MathWorks*™ MATLAB® utilizing the N-way toolbox [13] (Fig. 2) with unfolded spectra.

3. Results and discussion

Fluorescence spectroscopy has been applied so far to monitor fluorophores such as amino acids (e.g., tryptophan, tyrosine, and phenylalanine), vitamins (e.g., pyridoxine (vitamin B6), riboflavin (vitamin B2)) and coenzymes (e.g., NAD(P)H, FAD, and FMN). Main applications of fluorescence spectroscopy are found for monitoring of the cultivations where cell dry mass concentration, product concentrations, and the actual metabolic state of the cell are followed. In this contribution the applicability of 2D-fluorescence spectroscopy to monitor the synthesis of 2-hydroxyketone was demonstrated without addition of a fluorescence marker to follow the reaction components.

3.1. *Monitoring of carboligation reactions catalyzed by wtBFD*

The best of our knowledge, the monitoring of the carboligation reactions via 2D-fluorescence spectroscopy yielding asymmetric hydroxyketones has been achieved for the first time. The synthesis of 2-HPP which is the main carboligation product starting from benzaldehyde and acetaldehyde was successfully followed by on-line analysis.

During the wtBFD catalyzed asymmetric C–C bond formations 2D-fluorescence measurements were performed. A steady increase in fluorescence intensities in the region of Ex390 nm/Em450 nm was detected during the course of both biotransformations and verified by highest PCA-loadings. Besides the region of Ex390 nm/Em450 nm where the increase in relative fluorescence intensity (RFI) units was more significant, remarkable increases in RFI units were also detected in the regions of Ex370 nm/Em430 nm and Ex370 nm/Em450 nm. Fig. 3 displays the increase in RFI units in the described regions from the first process run. In Fig. 3 next to the general increase of the fluorescence intensities, noise in spectra can be seen. In order to avoid loss of information related to other Ex/Em regions all collected wavelength pairs were used for the development of a chemometric model since chemometric modeling can handle noisy spectra very well.

Background spectra of reaction buffer (50 mM phosphate buffer with cofactors ThDP and Mg^{+2}), substrate solutions (benzaldehyde or 3-methoxybenzaldehyde with acetaldehyde), as well as 2-HPP product solution were analyzed. The remarkable intensity value of the buffer system (2730 RFI unit; Ex390 nm/Em450 nm) can be explained due the presence of cofactor ThDP which is the biologically active form of vitamin B1 (given in supporting information).

3.2. *Principal component analysis (PCA) of fluorescence data*

For evaluation of the fluorescence data and for comparison of both processes principle component analysis is performed. In the score plot of the raw data both process runs are separated by the second principal component which indicates that both processes have different initial conditions (Fig. 4, left). Thus, the difference in starting benzaldehyde concentrations is recognized which is the only varying parameter between two process runs. However, small deviations in reaction temperatures (20.3 °C, first process and 19.9 °C, second process) have also been taken into account since fluorescence measurements are sensitive to any temperature deviation. Different from the PCA based on the raw data, when PCA is performed based on the difference spectra both process runs show similarities with some runaway spectra. The score plot of the difference spectra demonstrates that a clear separation is not observed (Fig. 4, right). In order to predict the latent information PCA based on the raw data shows that three components are required whereas PCA based on the difference spectra indicates that two components are adequate.

The difference between two processes can be displayed as a difference spectrum between the respective initial spectra of two reactions runs (Fig. 5). In most Ex/Em regions lower RFI units were observed which can be resulted from the small deviation in reaction temperatures. However, significant differences were observed in the regions of Ex370 nm/Em450 nm and Ex450 nm/Em530 nm where these regions are well known for NADH and flavin, respectively.

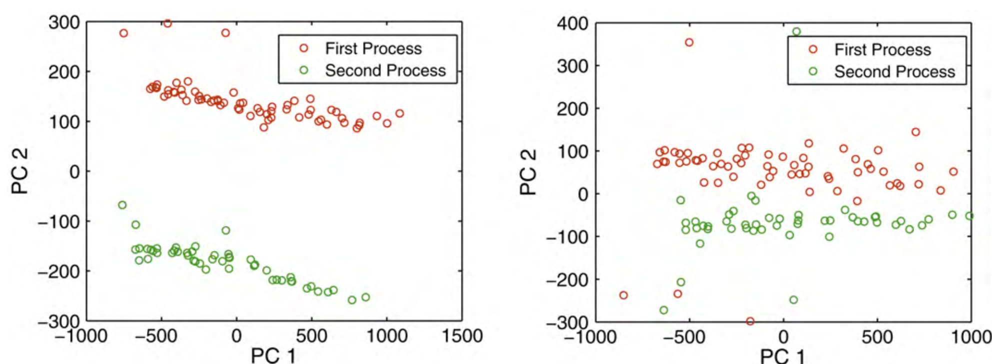

Fig. 4. PCA analysis of the fluorescence spectra. The score plot of the raw data (left) and the score plot of the difference spectra (right).

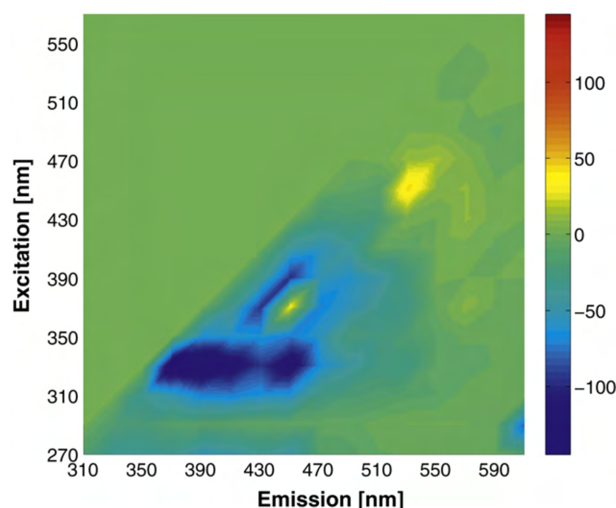

Fig. 5. Difference spectrum of two process runs displayed with respect to their initial spectra.

Since the analyzed reactions are only differ in initial substrate concentrations, observed high intensities in the difference spectrum can only result from benzaldehyde. Therefore, for validation of 2-HPP concentrations a chemometric model is developed based on the difference spectra whereas for validation of benzaldehyde concentrations a model is built based on the raw data.

3.3. *Chemometric modeling for the fluorescence based on-line monitoring*

Fluorescence spectroscopy offers the possibility to perform multi-wavelength monitoring of the bioprocesses. Using suitable mathematical tools such as chemometric regression models concentrations of the reaction components could be predicted on the basis of the collected fluorescence spectra. Subsequently, two PLS regression models were developed to correlate the measured concentrations of the carboligation product 2-HPP and the substrate benzaldehyde with the courses of fluorescence intensities in difference spectra. Two latent variables were adequate to obtain a good correlation. Fig. 6 demonstrates the good calibration of off-line data and on-line spectra for the synthesis of 2-HPP and conversion of benzaldehyde for the first process run.

Table 1
RMSEC of process variables of carboligations starting with benzaldehyde and acetaldehyde catalyzed by wtBFD.

Process variable	RMSEC
2-HPP	1.080 mM (4.73%)
Benzaldehyde	0.765 mM (2.25%)

Similarly, Fig. 7 shows the calibration of 2-HPP and the substrate benzaldehyde for the second process.

The quality of prediction was quantified by analyzing the root mean square error of calibration (RMSEC) determined from the square root of the mean squared deviations between the off-line measurements and the on-line determinations (Table 1).

The internal validation (full-cross validation) of the developed chemometric model exhibits the quality of the predicted concentrations for the time intervals where no off-line information was available. Thus, internal validation reveals the model performance over the whole examined time range. Besides the internal validation, an external validation of the chemometric model was analyzed which allows us to investigate the quality of the developed model to be applied for the next set of experiments. Thus, using the on-line fluorescence data of the next reaction with the previously developed model the concentrations of the reaction components of the next reaction were predicted. Developed models were assured by

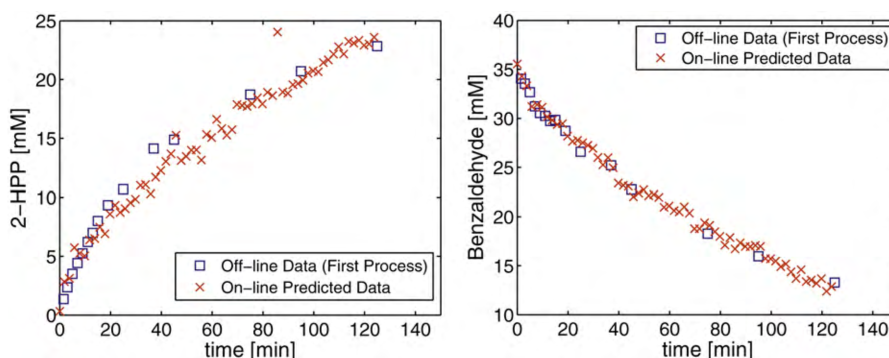

Fig. 6. Analysis of 2-hydroxypropiophenone synthesis (left) and benzaldehyde conversion (right) for the first process. On-line predicted data and off-line HPLC data.

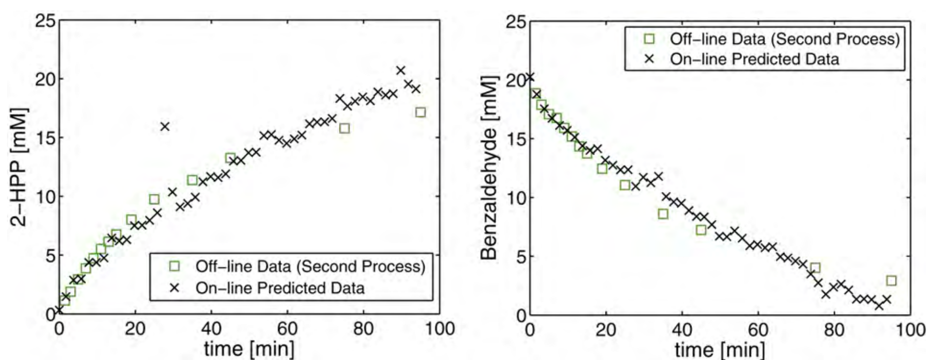

Fig. 7. Analysis of 2-hydroxypropiophenone synthesis (left) and benzaldehyde conversion (right) for the second process. On-line predicted data and off-line HPLC data.

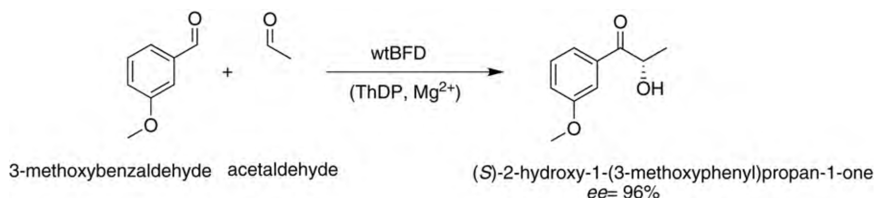

Fig. 8. The carboligation of 3-methoxybenzaldehyde and acetaldehyde catalyzed by wtBFD.

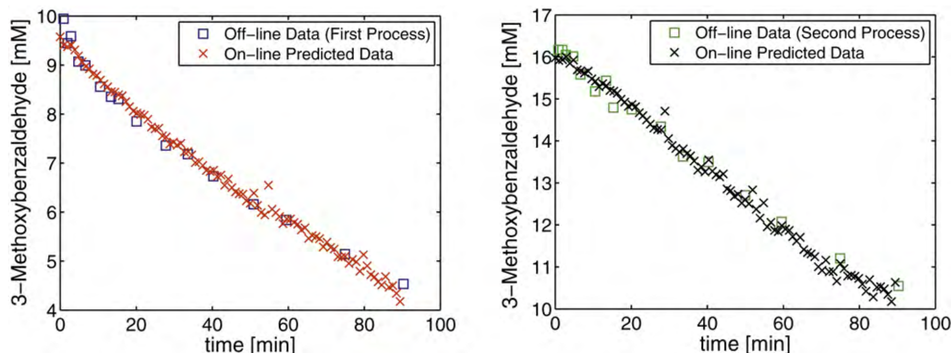

Fig. 9. Analysis of 3-methoxybenzaldehyde conversion for the synthesis of 2-hydroxy-1-(3-methoxyphenyl)propan-1-one, first process (left) and second process (right). On-line predicted data and off-line HPLC data.

performing full-cross validation where observed noises were eliminated reducing the quality of prediction.

A wide variety of benzaldehyde derivatives are accepted by wtBFD [9(a)]. The highest conversions were detected with meta-substituted benzaldehyde derivatives whereas ortho-substituted benzaldehyde derivatives were mainly identified as bad substrates [9(a)]. The influence of electronic effects on the enantiomeric excess was demonstrated to depend on the *Hammett*-correlation as well as on steric effects of substituents on the aromatic substrates [9(a,b)]. Thus, alternative to benzaldehyde, carboligation of 3-methoxybenzaldehdye (*m*-anisaldehyde) and acetaldehyde was analyzed (Fig. 8). Addition of any organic solvent was not required since 3-methoxybenzaldehyde was soluble at analyzed reaction conditions.

Two reactions were carried out containing 10 mM/16 mM 3-methoxybenzaldehyde and 400 mM acetaldehyde prepared in 50 mM phosphate buffer in the presence of the cofactors 0.5 mM ThDP, 2 mM $MgSO_4$ at pH 7.5 and 20 °C. During analyses 16 off-line samples were taken with previously described procedure until 90 min. The biotransformation was started by the addition of wtBFD (15 U) solution dissolved in 50 mM phosphate buffer, 0.5 mM ThDP and 2 mM $MgSO_4$ at pH 7.5. Since the reference 2-hydroxy-1-(3-methoxyphenyl)propan-1-one product was not available for calibration, Fig. 9 demonstrates only the off-line determined concentrations and the on-line predicted data of 3-methoxybenzaldehyde. For 3-methoxybenzaldehyde an own PLS regression model was developed for each process. Self-carboligation of 3-methoxybenzaldehyde yielding 2-hydroxy-1,2-bis(3-methoxyphenyl)-ethanone was not detected in any reaction analyzed (chromatograms are found in provided supporting information).

RMSEC for the prediction of 3-methoxybenzaldehyde conversion was determined as 0.199 mM (2.00%) for the first process and 0.169 mM (1.05%) for the second process which demonstrates that the applicability of fluorescence spectroscopy can be broadened to substituted benzaldehyde derivatives.

4. Conclusions

On-line analysis of bioprocesses is of high interest that enables direct control of process parameters in order to achieve improved process efficiencies. Among the developed on-line analysis techniques fluorescence spectroscopy has been mainly applied in monitoring of cultivations to obtain high biomass yield. The biggest advantage of fluorescence spectroscopy is its applicability to aqueous systems providing high quality spectra of all fluorescent

compounds at low concentrations. In this contribution the applicability of fluorescence spectroscopy for monitoring the synthesis of 2-hydroxypropiophenone (HPP), a chiral 2-hydroxyketone, was successfully demonstrated. Wild type BFD catalyzed carboligations were monitored by the BioView® fluorescence sensor (DELTA, Denmark) with a very good correlation. A reliable chemometric model was developed where the concentrations of reaction components were validated with satisfying RMSEC values. Besides the synthesis of 2-HPPs which is the main carboligation product, the conversion of benzaldehyde was also successfully monitored via 2D-fluorescence spectroscopy. Therefore, this study demonstrates for the first time that 2D-fluorescence spectroscopy can be successfully applied in biocatalyzed asymmetric C–C bond formations, even in water as reaction medium with low starting material concentrations. Via chemometric models concentrations/conversions of reaction components can be predicted in a small range of errors. On-line determined data for substrate conversion and product formation during the course of biotransformations can be used in progressive curve analyses. Thus, the enzyme kinetic parameters can be further identified by developing appropriate kinetic models which is going to be included in our next communication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.04.006.

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