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A new set up for multi-analyte sensing: *At-line* bio-process monitoring

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ABSTRACT

A multi-analyte sensing device is described, for simultaneous *at-line* monitoring of glucose, ethanol, pO_2-value and cell density. It consists of a dual biosensor, a modified microscope and a fiber optical pO_2-sensor that are integrated into a flow analysis (FA) system.

The biosensor is based on a conventional thin layer flow-through cell equipped with a gold (Au) dual electrode (serial configuration). The biosensors with no cross-talking were produced by modifying the electrochemical transducers. Each Au surface was initially modified by self-assembled monolayer (SAM) of cysteamine. Alcohol oxidase (AOx) and pyranose oxidase (PyOx) were immobilized each onto a gold surface by means of PAMAM (polyamidoamine) dendrimer via glutaraldehyde cross-linking. The responses for glucose and ethanol were linear up to 0.5 mM. The operational stability of the biosensors was very promising, after 11 h continuous operation, only 6.0% of the initial activity was lost. The potential of the described biosensor was demonstrated by parallel determination of ethanol and glucose in yeast fermentation process.

Simultaneously the cell density of the culture was monitored with an *in situ* microscope (ISM), which was integrated into the FA system. Both the used *in situ* microscope and the image processing algorithm used for the analysis of the acquired image data are described. Furthermore the pO_2-value was monitored using a fiber optical sensor, which was embedded in a flow cell. The multi-sensor device allows the *at-line* monitoring of several process values without the need for further sampling or time consuming offline measurements.

1. Introduction

Soon after biosensors appeared in the 1960s (Clark and Lyons, 1962), efforts began to improve their characteristics in order to achieve more selective measurements. Especially enzyme based biosensors have been demanded recently in the biomedical, biotechnological, industrial and environmental fields (Palmisano et al., 2000). Incorporation of different enzymes that are selective to different analytes provides each biological recognition element to give an individual response (O'Connell and Guilbalt, 2001). The most commonly analyzed compounds in fermentation processes are glucose, lactic acid, ethanol and glycerol (Guzman-Vazquez de Prada et al., 2004; Min et al., 1998; Niculescu et al., 2003; Razumiene et al., 2001).

Among the analytes that need to be monitored in yeast fermentation processes, ethanol and glucose are of highest importance. In most fermentation processes glucose is the growth-limiting substrate. Ethanol is a product of yeast processes, besides it has dramatic effects on the cell growth. Ethanol concentrations above 14% destroy metabolic enzymes, inhibiting further growth. Thus, it is essential to monitor ethanol as well as glucose concentrations during yeast fermentation (Niculescu et al., 2003). In the fabrication of the multi-analyte bio-monitoring devices, some important requirements should be fulfilled. For instance, they must be easy to operate, should possess good operational stability, should not consume high amounts of sample and must be interference and cross-talk free (Palmisano et al., 2000). In fact, integration of the small dimensioned sensors causes well-known problems such as interference and cross-talk. Those effects are represented when dealing with H_2O_2 between adjacent electrodes due to inter-electrode diffusion of the generated hydrogen peroxide and also while applying higher potentials. Palmisano et al. describe their system as being cross-talk free due to the whole design and the flow analysis (FA) measurement method, which would sweep away generated H_2O_2. In our design we deal with consumption of O_2, therefore we applied

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negative potentials, which is no media compounds can interfere with the signals.

In situ microscope (ISM) was originally developed for non-invasive monitoring of fermentative processes in bioreactors. Several ISM systems have already been developed, differing mostly in the limitation of the sampling zone and the layout of the illumination system (Bluma et al., 2010; Camisard et al., 2002; Prediger et al., 2011; Suhr et al., 1995; Wei et al., 2007). For the described experiments, a transmitted light/bright-field microscope (TB-ISM) with finite corrected optics was used. The measurement principle of the TB-ISM is shown in Fig. 1.

For this study the sampling zone of the TB-ISM was transformed into a flow cell allowing it to be integrated into the flow analysis system. A sophisticated image processing algorithm is used to analyze the acquired image data. In combination with this algorithm it is possible to determine the cell density of the taken samples. Furthermore it allows the monitoring of the cell morphology.

Fiber optical oxygen sensors contain fluorophores whose fluorescence is quenched by molecular oxygen. Commonly used fluorophores are ruthenium diimine complexes. In principle the oxygen concentration can be calculated directly from an intensity measurement. However, photo bleaching of the fluorophores makes long term intensity measurements unreliable. Therefore the phase shift of the fluorescence is measured while modulating the excitation intensity and subsequently the oxygen concentration can be calculated (Klimant and Wolfbeis, 1995; Klimant et al., 1997).

A sensitive and fast responding flow through based amperometric biosensors for ethanol and glucose detection has been developed in our laboratory. Since the applied immobilization procedure works well, the same protocol was used for the construction of the dual electrochemical flow cell in this study. Our previous transducing schemes for the selective detection of ethanol (Akin et al., 2010) and glucose (Yuksel et al., 2010) were incorporated into the multi-analyte flow electrochemical system. In addition, an ISM and a pO_2 detection device as well were integrated into the system. The designed set-up was used for the quantification of ethanol, glucose, cells density and pO_2. *At-line* monitoring of all described analytes in fermentation process was achieved successfully.

2. Experimental

2.1. Materials

Pyranose oxidase (PyOx, EC 1.1.3.10, 10.8 Units/mg), alcohol oxidase (AOx, EC 1.1.3.13. from *Pichia pastoris* species, 22 Units/mg of protein), poly(amidoamine) (PAMAM) 25% C_{12} Dendrimer Generation 4, glutaraldehyde solution (25%, v/v), and D-glucose were acquired from Sigma–Aldrich (Dorset, UK). Cysteamine hydrochloride and sodium borohydride were obtained from Fluka (Steinheim, Germany) and used without further purification. All of the other compounds were of analytical-reagent grade. Sodium phosphate buffer (50 mM, pH 7.0) was used for making the solutions.

2.2. Apparatus

FA experiments were performed using an electrochemical flow through cell of the cross-flow type with gold dual working (3.0 mm diameter and 1.0 mm electrode gap), Ag/AgCl reference and Pt auxiliary electrodes (CHI130, Austin, US). A peristaltic pump (FIAtron, Oconomovoc, WI, USA) equipped with silicon tubing (0.89 mm inner diameter) pumped the working buffer solution as the carrier into the flow line with a flow rate of 1.3 mL min^{-1}. Four thin-layer flow cell gaskets 0.10 mm thick (CH Instruments, Inc. Austin, USA) were used as cell spacers. The FA system was connected to a PalmSens potentiostat and PalmSens multiplexer with 8-channel configuration (Jumper 2, 5 and 6 settings) for the simultaneous electrochemical measurements. The FA system was controlled by software via RS232 interfaces.

2.3. Electrochemical measurements

All FA based electrochemical experiments were performed with a detection potential of −0.7 V versus Ag/AgCl in sodium phosphate buffer (0.5 M; pH 7.0). The depletion of molecular oxygen in the enzymatic reactions was monitored at given potential. PyOx oxidizes glucose to 2-dehydro-D-glucose and AOx oxidizes ethanol to acetaldehyde. The FA signals were correlated indirectly to the substrate concentrations consumed during enzymatic reactions. Obtained currents were recorded as ΔI (μA) at −0.7 V by the computer program (PSlite 1.8; Informer Technologies, Inc.) and were plotted versus substrate concentrations.

2.4. Biosensor preparation

Our previous biosensor configuration for the selective detection of ethanol (Akin et al., 2010) and glucose (Yuksel et al., 2010) were incorporated into the multi-analyte flow electrochemical system. The surface modification of each gold electrode was achieved by dropping the solutions onto the gold discs to cover the entire surfaces. First, gold electrode surfaces were modified with self-assembled monolayer (SAMs) of cysteamine, second PAMAM dendrimers were attached to the surfaces. Afterwards the surfaces were dried. At the enzyme adding step, given amount of enzyme solutions were dropped to the surfaces very carefully one by one to avoid cross-contamination. A typical PyOx–AOx dual gold electrode was prepared as follows: 3.0 μL of AOx (0.141 mg/mL; 4.167 Unit) from stock enzyme solution was mixed with 6.0 μL of 1.0% glutaraldehyde solution (25% glutaraldehyde solution diluted 1/25 (v/v) in sodium phosphate buffer pH 7.0, 50 mM). PyOx solution was prepared by mixing 5.0 μL of buffer containing 0.9 mg solid enzyme (180 mg/mL; 9.4 Unit) with 50 μL of 1.0% glutaraldehyde solution. The final AOx and PyOx solutions were pipetted carefully onto the surfaces of the Au/cysteamine/PAMAM modified electrodes avoiding any mixing of the two solutions. After air-drying at room temperature for at least 90 min, dual biosensor was washed with buffer and used directly in the measurements.

2.5. pO_2-value determination

For the determination of the pO_2-value a fiber optical sensor (Presens GmbH, Regensburg, Germany) was used. The sensor spot was enclosed in a flow cell that allowed the implementation into the analytical flow system. Prior to measurements the sensor was calibrated according to the respective user manual.

2.6. In situ microscopy

In situ microscopy measurements were carried out using an III XTF-microscope (Sartorius Stedim Biotech GmbH, Goettingen, Germany). This is a transmitted-light bright field microscope with finite corrected optics. All images were obtained using a 4-fold magnifying objective and a XCD-SX910 CCD-camera from Sony. A yellow LED (λ = 588 nm) was used for illumination. The measuring zone of the microscope was modified to act as a flow cell and integrated into the FA system. Prior to measurements the measuring zone was opened 250 μm resulting in a total flow cell volume of 4.5 mm^3. For the monitoring of the fermentation process every hour a recording cycle of 60 images was carried out. The exposure time for each image was 5.33 ms while the time between two images accounted for 1 s. The images were subsequently analyzed using

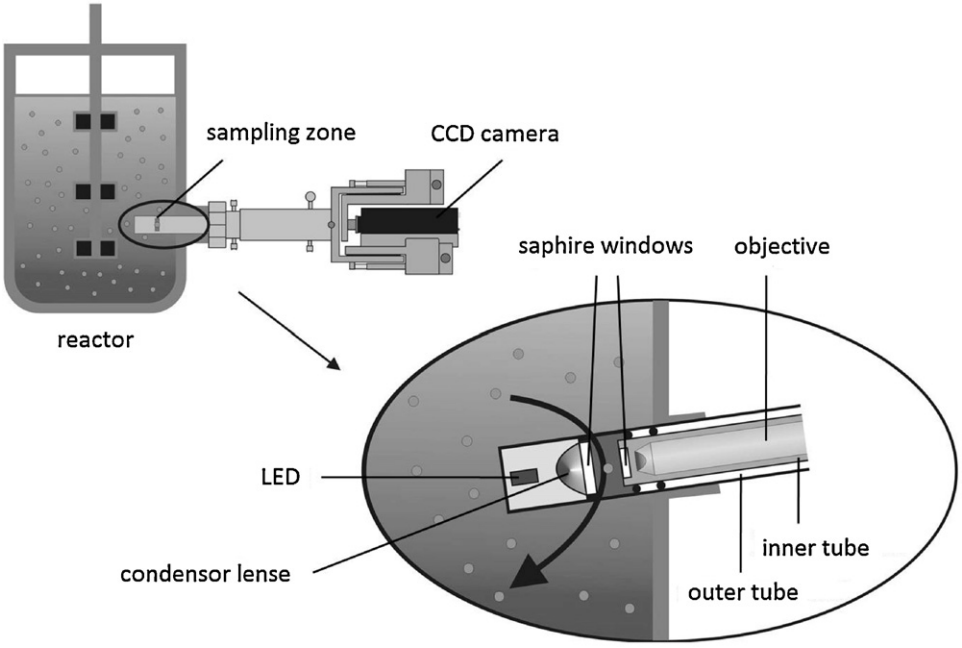

Fig. 1. Measuring principle of *in situ* microscopy.

an image processing algorithm which is described in the following paragraph.

2.7. Image processing software

The utilized image processing algorithm is based on an algorithm that was first described by Canny (1986). In a first step a 2-dimensional Gauss-Filter is applied to eliminate random noise. Consecutively grey value gradients in the image are calculated. If they exceed a defined threshold they are recognized as object edges. The algorithm then connects detected edges to form objects. The size of all recognized objects is calculated. Consecutively objects over and under defined thresholds are eliminated. Every object found in this manner is defined as a cell. The number of recognized cells per recording cycle is calculated and stored in a text file.

Offline cell counting measurements with a Neubauer-chamber for the determination of the cell-density were carried out and compared to the results generated via ISM.

2.8. At-line set up

The proposed FA based multi-analyte biosensor system was applied to monitor a *Saccharomyces cerevisiae* H620 cultivation process. Cultivation experiments were carried out according to a procedure described in our previous study (Akin et al., 2010). The analytical procedure consists of a sequence of pre-defined steps, during which liquid volume elements are displaced according to the chosen pumping direction, the pump speed and the pump time. This sampling cycle is programmed as a software module. An operation cycle consists of: (i) pumping buffer solution through the flow cell to get a stable carrier stream; (ii) loading of a defined volume of sample towards the pump; (iii) pumping of sample through *in situ* microscope; (iv) pumping of sample through pO_2 flow cell; (v) dilution by transporting a pre-defined sample volume to the dilution chamber and adding a defined volume of buffer; (vi) loading a fixed volume of the diluted sample from the dilution chamber and pumping of this liquid through the electrochemical dual cell. The whole set up is shown in Fig. 2.

The consumption of glucose and the production of ethanol by yeast during fermentation was monitored *at-line* for 13 h. Furthermore the cell density and the pO_2-value were monitored. For the biosensor measurements samples from culture were automatically diluted 300-fold by the FA system. Measurements were automatically carried out hourly except for offline sampling for reference methods.

3. Results and discussion

There are many reports dealed with simultaneous determination of ethanol and glucose in various samples. Some of them required a second method such as colorimetry (Valach et al., 2009), a second biosensor system (Reshetilov et al., 1998) or pre-separation step with HPLC (Guzman-Vazquez de Prada et al., 2004). To the best of our knowledge, there are no reported literature reports related to the *at-line* monitoring of glucose and ethanol contents in fermentation medium without any parallel or further analysis method. In addition, we successfully monitored pO_2-value and cell density with the integration of pO_2 sensor and *in situ* microscopy. This study definitely composes a novel multi measurement system to perform simultaneous analysis of several parameters during yeast cultivation.

The above described device possesses good characteristics required for the analysis of culture broth samples in terms of linearity, stability, repeatability and selectivity for multiple analytes. A simple cross-linking via multi-branched dendrimers allows multipoint covalent attachment of enzymes through amid bonds resulting in the prevention of enzyme deactivation and good sensitivity (Akin et al., 2010). The surface did not introduce significant distortion of FA peaks because of the high-level precision of the self-assembled layers of both surfaces.

3.1. Characterization of dual working biosensor

First, linearity of the dual electrode was estimated by applying standard solutions that contain equal glucose and ethanol contents directly to the electrochemical flow cell. Linear response ranges for dual biosensor were found as 0.025–0.5 mM for both

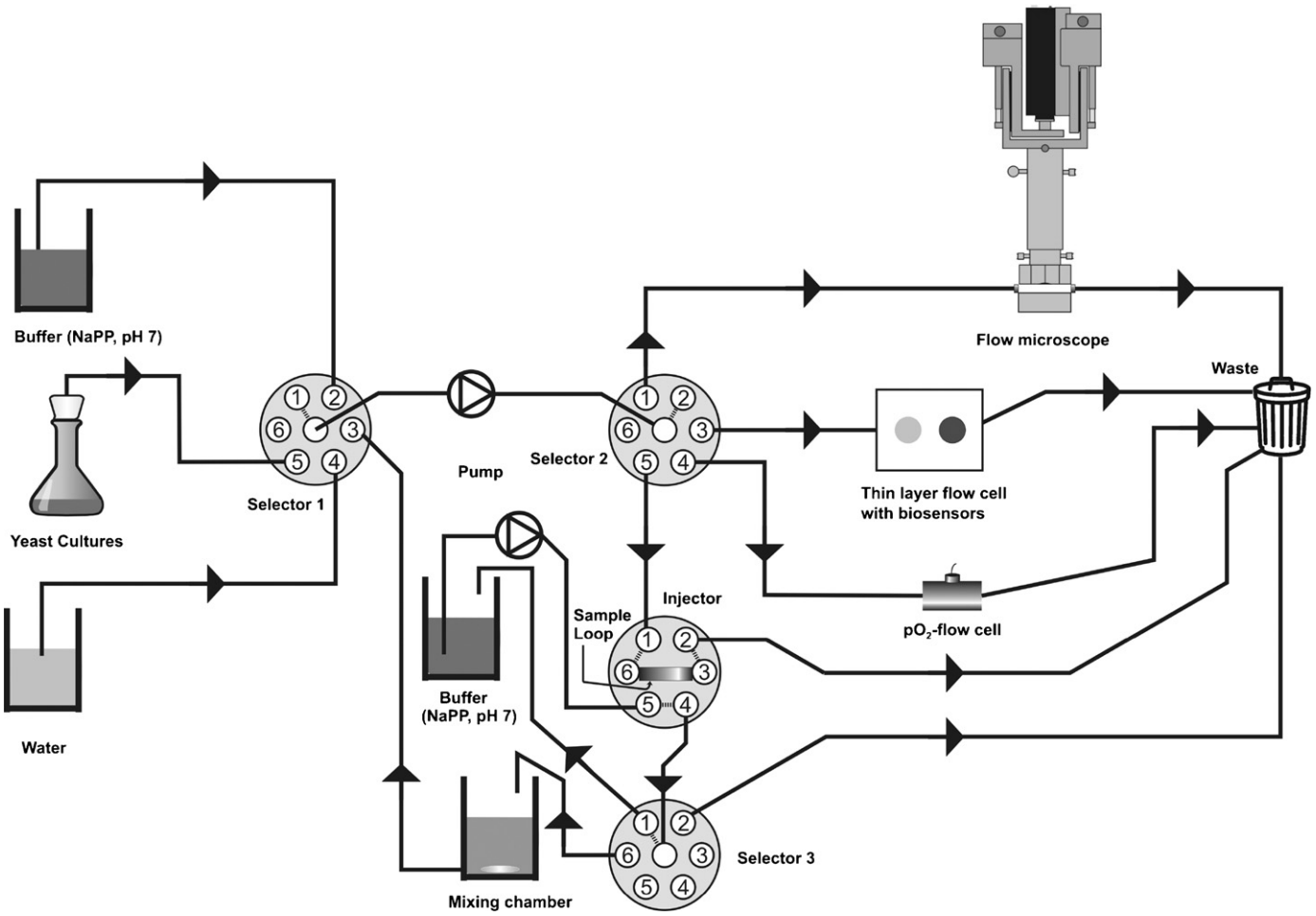

Fig. 2. Configuration of FA system.

ethanol and glucose biosensors (Fig. 3). Repetitive injections of 0.5 mM mixed standard solution gave relative standard deviation values of 0.87% ($n = 5$) and 4.51% ($n = 5$) for glucose and ethanol, respectively. After every biosensor preparation, biosensors were calibrated before sample application. The linear range of glucose and ethanol biosensors remained the same but the electrochemical signals were variable with a coefficient of variation range of 3–10%.

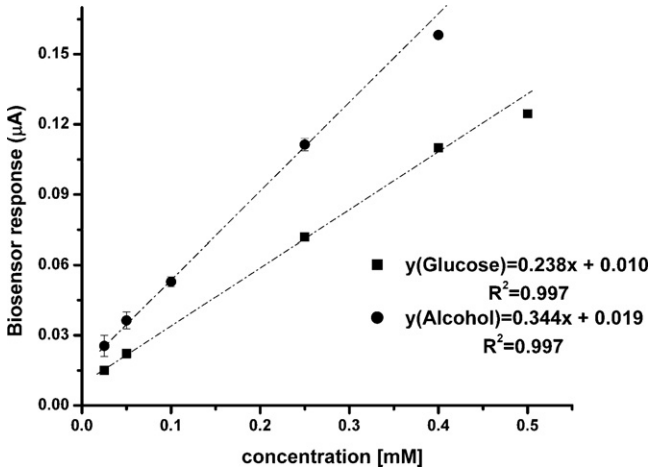

Fig. 3. Calibration curves for glucose/ethanol dual biosensor system (in 50 mM sodium phosphate buffer, pH 7.0; −0.7 V).

Recovery of the signals was determined by first applying a given amount of standard ethanol or glucose solutions separately and applying mixtures afterwards. The relative biosensor responses were calculated and results were summarized in Table 1 (signals were measured two or three times). According to the results, the analytes do not interfere to each other thus the proposed dual biosensor system responses to each analyte correctly in mixed solutions.

As outlined in the introduction section the cross-talk problem is more effective when dealing with hydrogen peroxide monitoring at positive potentials (Min et al., 1998; Yang et al., 1998). Absence of this effect is one of the most important prerequisites for simultaneous determination of glucose and ethanol without a pre-separation step.

However, it could be also possible that the hydrogen peroxide produced through PyOx- or AOx-catalyzed reaction would be

Table 1
Recovery of the dual biosensor signals (in 50 mM sodium phosphate buffer, pH 7.0; −0.7 V).

Solution	Glucose (mM)	Recovery (%)	Ethanol (mM)	Recovery (%)
1	0.05	98.20 ± 1.91	0.05	99.22 ± 1.114
2	0.1	99.16 ± 2.43	0.1	90.46 ± 0.852
3	0.25	99.42 ± 2.06	0.25	102.69 ± 4.718
4	0.5	99.30 ± 2.56	0.5	98.20 ± 3.263
5	0.05	97.73 ± 1.93	0.5	97.42 ± 8.30
6	0.5	98.35 ± 3.56	0.05	97.66 ± 3.31

reduced on the electrode surface under the negative potential of −0.7 V. This increase in the cathodic current corresponding the reduction of hydrogen peroxide may superimpose on the current decrease followed by the oxygen consumption, so as to reduce the electrode response. In our case, we have monitored the response of PyOx electrode in the presence of glucose itself (0.5 mM which is the final concentration of linear range) and glucose (0.5 mM) in the presence of hydrogen peroxide (1.0 mM) at −0.7 V. Similar response signals were observed for each trial. However, higher concentrations than 1.0 mM hydrogen peroxide caused an opposite signal responses and reduced signals were observed for glucose under the same conditions. This means that the sensor responses were free of the interference effect of the liberated hydrogen peroxide under the working conditions such as the substrate concentrations in linear ranges (0.025–0.5 mM) and working potential. On the other hand, despite the detection method with electrochemical measurements at negative potentials, a small cross-talk response was observed at the alcohol sensor while higher amount of glucose (>0.5 mM) were applied. In this case the observed effect cannot be due to diffusion of enzymatically generated hydrogen peroxide from the adjacent electrode but might originate from enzyme cross-contamination (i.e. migration of loosely bound PyOx from the glucose to the ethanol electrode) during sensor preparation (Guerrieri et al., 2006) and also might be due to serial configuration of the dual flow cell. The automatic 300-fold dilution of the samples to adjust the glucose content to the biosensor range was effective in the elimination of the problem; indeed, the resulting biosensor was completely cross-talk free. In addition we have already demonstrated in previous studies that no compounds contained in the used fermentation medium interfere with the biosensor signals at −0.7 V (Akin et al., 2010; Yuksel et al., 2010).

3.2. Stability

A loss of sensitivity under operational conditions limits the practical utility of the biosensors. Besides enzyme leaching, it can be ascribed to enzyme denaturation. The dual working biosensor operational stability was assessed by a continuous long term experiment. A solution containing both analytes at 0.5 mM concentration level was injected during 11 h and totally 85 repetitive measurements were carried out. In the whole explored period of time signals for glucose remained 98% and ethanol signals remained 94.1% of the initial ones. The immobilization strategy that was applied for the construction of the biosensors seems effective in stabilizing PyOx and AOx activities during performance.

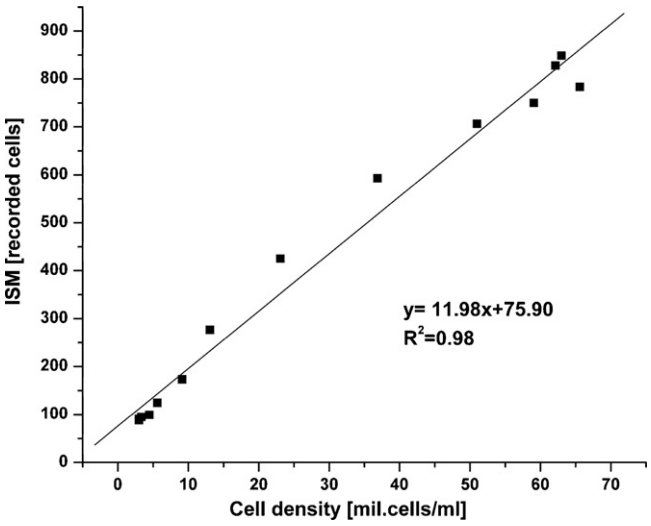

Fig. 4. Comparison of detected cells per image and off-line determined cell density via Neubauer chamber.

3.3. In situ microscopy

The offline cell counting with a Neubauer-chamber was compared to the results of the image processing software analyzing the acquired image data by ISM. The result is shown in Fig. 4.

A strong linear correlation between the number of detected cells per recording cycle and the calculated cell density via Neubauer cell-counting can be observed. Using the shown data as a calibration the cell density can be determined directly by the combination of ISM and image processing software.

The increase of the cell density during the fermentation is reported in Fig. 5. A lag phase can be observed between the start of the cultivation and the measurement after 3 h. It is followed by an exponential growth phase until it reaches a stationary phase between 9 h and 10 h cultivation time.

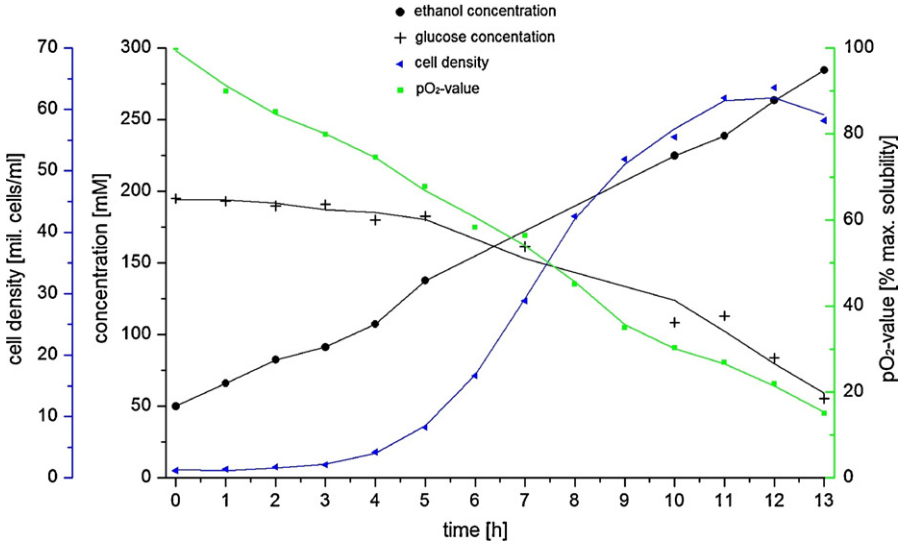

Fig. 5. Monitoring of glucose consumption, ethanol production, cell density and pO_2 by multi-analyte sensing system.

3.4. pO_2-measurements

The concentration of oxygen in the fermentation medium was monitored for 13 h. The result is shown in Fig. 5. At the beginning of the fermentation the pO_2 value is 90%. During the fermentation it decreases linearly until it reaches 15% after 13 h of cultivation. No bleaching was observed over the whole fermentation period.

3.5. At-line fermentation experiments

The designed new multi-analyte sensing device enables sampling from the fermenter, the automatic dilution of the sample, the real-time evaluation of the ethanol/glucose concentrations in the fermentation broth, observation of cell density as well as the monitoring of the pO_2-value. The obtained results are shown in Fig. 5. Ethanol production, glucose consumption, growth of yeast cells, as well as the decrease of oxygen concentration can be observed.

4. Conclusions

A multi-analyte sensing device for monitoring of biotechnological processes was designed by the integration of a modified ISM, a pO_2 flow cell and a dual electrochemical biosensor flow cell into one flow analysis system. The system can be controlled via a computer and acquires the process data automatically.

In combination with an image processing software the determination of the cell density at any given point in the cultivation can be determined without additional sampling or time consuming cell counting. Moreover, the multi-analyte sensor device allows the monitoring of the pO_2-value over the whole fermentation period.

For the dual biosensor flow cell the enzyme immobilizing procedure ensured satisfactory selectivity towards related analytes and good operational stability. A previously described automated flow injection analyzer (Akin et al., 2010) was adapted to the specific requirements of *at-line* ethanol and glucose determination by integration of a dilution chamber and adaptation of the software. Thus an interference and cross-talk free device for the *at-line* monitoring of ethanol and glucose concentrations in laboratory scale bioprocesses was successfully established.

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