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Full Research Paper

Combination of On-line pH and Oxygen Transfer Rate Measurement in Shake Flasks by Fiber Optical Technique and Respiration Activity MOnitoring System (RAMOS)

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Abstract: Shake flasks are commonly used for process development in biotechnology industry. For this purpose a lot of information is required from the growth conditions during the fermentation experiments. Therefore, Anderlei et al. developed the RAMOS technology [1, 2], which proviedes on-line oxygen and carbondioxide transfer rates in shake flasks. Besides oxygen consumption, the pH in the medium also plays an important role for the successful cultivation of micro-organisms and for process development. For online pH measurement fiber optical methods based on fluorophores are available. Here a combination of the on-line Oxygen Transfer Rate (OTR) measurements in the RAMOS device with an on-line, fiber optical pH measurement is presented. To demonstrate the application of the combined measurement techniques, *Escherichia coli* cultivations were performed and on-line pH measurements were compared with off-line samples. The combination of on-line OTR and pH measurements gives a lot of information about the cultivation and, therefore, it is a powerful technique for monitoring shake flask experiments as well as for process development.

Keywords: fiber optical pH measurement, shaking flask, oxygen transfer rate, RAMOS, *E. coli*, process development

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1. Introduction

Up to now shaking bioreactors are the most commonly used reaction vessels in microbiology and biotechnology [3]. Several thousand shake flask experiments are carried out annually for strain development, screening processes and media optimization in large companies [4, 8]. For such applications, especially for media optimization, monitoring of cultivation parameters is essential. The information obtained from these experiments with on-line monitoring gives a better insight into limitations, inhibitions and the physiological state of the organisms during the cultivation, thus, allowing the development of optimized production processes in the biotechnology industry.

Anderlei et al. presented the Respiration Activity MOnitoring System (RAMOS) for on-line measurements of the respiration activity parameters (OTR, carbon dioxide transfer rate (CTR) and the respiratory quotient (RQ)) in shaking flasks [1, 2]. Measuring OTR online during cultivation is the most suitable way to quantify the physiological state of aerobic micro-organisms. For example, oxygen limitations, product inhibition and diauxic growth could be identified. This device was successfully employed in different projects [6, 9, 11, 15-17, 21, 22, 25]. Losen et al. used the RAMOS for the optimization of culture conditions and nutrient composition of the medium for *Escherichia coli* fermentations in shake flasks [15]. The RAMOS technology is especially suitable for the optimization of screening cultures. Stoeckmann et al. demonstrated the impact of oxgen limitations during screening processes with *Hansenula polymorpha* [25, 26] and Zimmermann et al. for *Corynebcterium glutamicum* [28].

Other critical parameters during fermentation processes are pH values and pH changes. There are different factors that affect the pH during the growth of micro-organisms. For example, in aerobic culture with high glucose concentrations *E. coli* produces acetate due to overflow metabolism, which causes a decreasing pH of the medium. After the glucose is depleted *E. coli* may consume the acetate as a second carbon source in a diauxic manner so that the pH increases again. Furthermore, the pH value in *E. coli* fermentations in mineral medium with glucose or glycerol as sole carbon source is highly dependent on ammonium consumption. Uptake of one ammonium molecule generates one proton [5, 23] and, therefore, the pH value in the medium declines.

For the pH monitoring of dairy starter cultures in 96-well micro titer plates John et al. presented an optical method based on two different fluorophores [12]. One fluorophore is pH sensitive (indicator) and the other is pH insensitive (reference). To determine the actual pH in the solution the fluorescence intensities of both fluorophores are measured and with the ratio of both values the pH is calculated [12]. Another method for the fiber optical pH measurement in small scale fermentation processes is the dual lifetime referencing (DLR). This method was published by Huber et al. for optical measurement of seawater salinity [10]. DLR is based on the measurement fluorescence decay times of an indicator. The intensity of the excitation light is modulated at a specific frequency and the over-all phase shift of the light emitted by a pH-indicator and a reference fluorophore is evaluated. The company Presens (Precision Sensing GmbH, Regensburg, Germany) commercialized this technology in form of sensor spots, e.g. for the pH measurement in micro titer plates (Hydroplates). This technique was used by Puskeiler et al. for at-line pH-determination in micro titer plates [18]. Kensy et al. demonstrated the application of the technology for on-line monitoring of dissolved oxygen and pH in continuous shaken *E. coli* cultivations performed in 24-well micro titer plates [13].

In this work a fiber optical online pH-measurement was combined with the OTR-measurement in the RAMOS device. The successful combined application of both measurement techniques were demonstrated in *E. coli* cultivations.

2. Results and Discussion

2.1. On-line measurement of OTR and pH in the Respiration Activity MOnitoring System (RAMOS)

E. coli BL 21 pLys pRset eYFP-IL6 was cultivated in the RAMOS device both with and without a sensor spot for pH on-line monitoring. Figure 1 shows the development of the OTR and the pH in the RAMOS-flask with sensor spot.

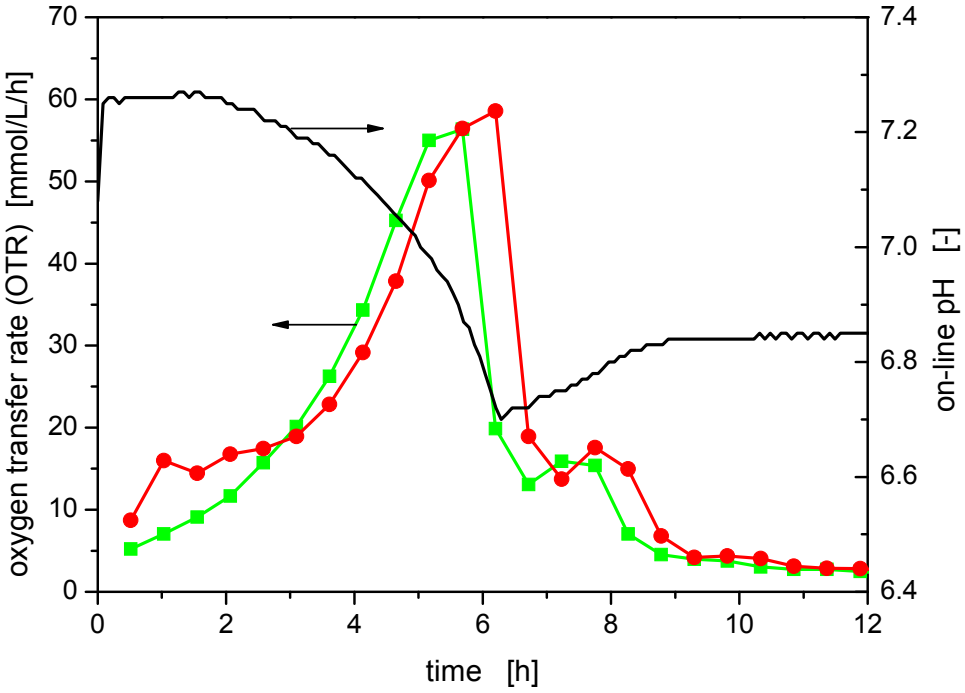

Figure 1: On-line measurement of OTR and pH in *E. coli* BL 21 pLys pRset eYFP-IL6 cultivations; modified Wilms & Reuss medium; cultivation conditions: T = 37°C; d_0 = 50 mm; n = 350 rpm; V_L = 10 mL; OD_α = 0.5; pH_α = 7.3; legend: OTR (flask without pH measurement); OTR (flask with on-line pH measurement); on-line pH

The OTR of the cultivations with and without sensor spot proceeded more or less in parallel. In the flask with sensor spot the OTR curve is only slightly delayed. This might be due to slightly different inocula.

Without any lag-phase the bacteria started to grow so that the OTR increases directly. At the beginning of the cultivation the pH stays constant, because the acidification of the medium caused by the metabolic activity of the organisms is completely compensated by the buffer capacity. During the exponential growth of the micro-organisms after 2 hours fermentation time, the pH declines due to the increasing consumption of ammonium and the production of acetate in the overflow metabolism as

discussed by Christensen et al. [5]. After 6 h the first carbon source glucose is exhausted and therefore the OTR declines rapidly. Thereafter, the OTR shows a second peak, while simultaneously the pH rises. From this point the organisms consume acetate as second carbon source. Due to the removal of acidic acetate, the pH of the medium increases until the acetate is depleted and the micro-organisms enter the stationary phase after 9 h. In the stationary phase the pH stays constant and the OTR declines to a low level.

2.2. Comparison of off-line and on-line pH measurement

To compare on-line and off-line pH measurements *E. coli* BL 21 pLys pRset eYFP-IL6 was cultivated in the RAMOS device and in parallel in normal shake flasks for sampling. Figure 2 shows the courses of both measured pH values.

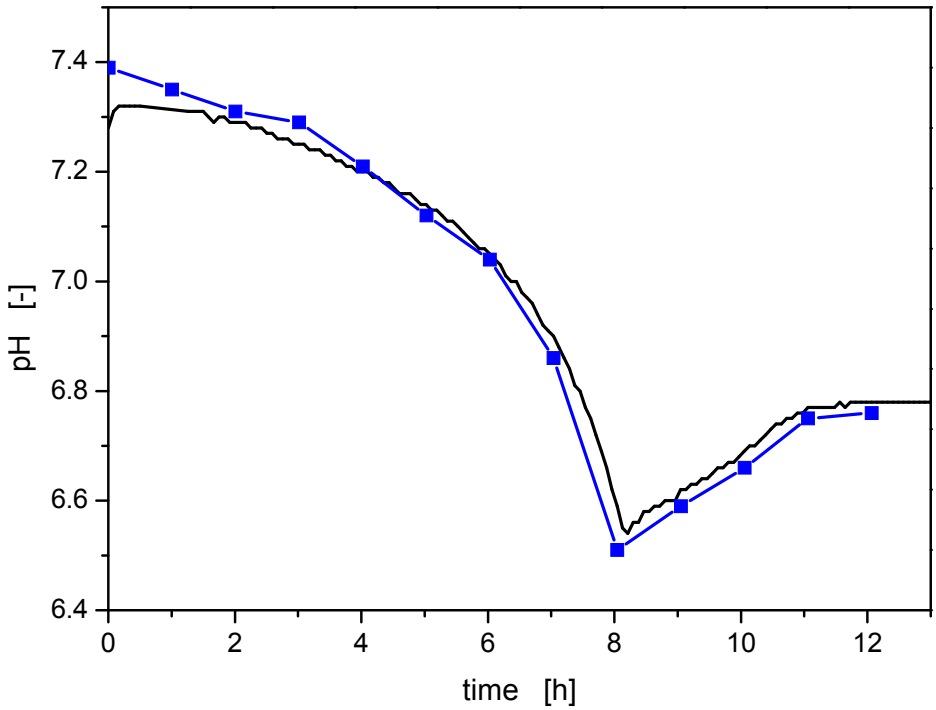

Figure 2: Comparison of off-line and on-line pH measurements in an *E. coli* BL 21 pLys pRset eYFP-IL6 cultivation; modified Wilms & Reuss medium ; cultivation conditions: T = 37°C; d_0 = 50 mm; n = 350 rpm; V_L = 10 mL; OD_α = 0.5 pH_α = 7.3; legend: ——— on-line pH; ——■—— off-line pH

The pH courses show typical shapes for cultivations of *E. coli* BL 21 pLys pRset eYFP-IL6 under the given conditions, which was already discussed in Figure 1. Both curves proceed in parallel and the maximum difference between on-line and off-line pH measurements was ± 0.05 pH. The highest difference occurs at the beginning of the cultivation.

The error in the different measurements averages to ± 0.02 pH, which lies in sum of the accuracies of the Eutech pH meter (Eutech Instruments Europe B.V., Nijkerk, Netherlands) and the pH-mini (Precision Sensing GmbH, Regensburg, Germany) with 0.01 pH, respectively (according to manufacturers). Therefore, the on-line pH measurement in the RAMOS device gives reliable results compared to the off-line measurement.

3. Conclusion

The combination of the fiber optical, on-line pH and OTR measurements in the RAMOS device was successfully applied. This presented technique enables pH measurements in RAMOS flasks without sampling and stopping the shaking machine. Therefore, mass transfer and mixing are not interrupted during the cultivation. Seletztky et al. showed that interruptions could lead to anaerobic periods during cultivation and changes in the metabolic activity of the organisms [21]. Furthermore, the filling volume in the flasks does not change due to sampling, which allows an undisturbed growth of microorganisms. Moreover, a better comparability of the pH values during cultivations in RAMOS flasks and normal shake flasks is achieved.

The pH effects on the OTR during growth of micro-organisms, e.g. inhibited growth due to too low pH values, can easily be identified with this measuring setup. Furthermore, the on-line pH measurement gives a higher resolution than the off-line pH measurement. For instance, pH changes could be resolved more precisely in the on-line pH compared to the off-line measurement, providing better information about the process.

The here presented measurement technique is especially useful for media and cultivation optimization. Oxygen supply and buffer concentrations of media can be checked and adapted to the requirements of the microbial growth. Adaptation and regulation of growth conditions is of utmost importance for organisms with complex growth behavior, which produce and/or consume different pH affecting substances like acetate, lactate or glutamate. For instance, *Gluconobacter oxydans* produces 5-keto-D-gluconate, 2-ketogluconate and 2,5-diketogluconate on glucose as sole carbon source, whereas the product formation is highly dependent on the pH-value and the oxygen supply during the fermentation [7, 14, 19, 24]. Thereby the pH profile of the *G. oxydans* cultivation must be adjusted to the desired product [15] and the acidification caused by the products must be considered.

The combination of the on-line OTR and pH measurement gives a lot of information about the cultivation and, therefore, is a powerful technique for the monitoring of shake flask experiments as well as for process development.

4. Experimental Section

Organism and Cultivation Conditions

E. coli BL 21 pLys pRset eYFP-IL6 [20] was maintained at -80°C in LB medium with 100µg/mL ampicilin. Stock solutions contained 200 g/L glycerol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

All cultivations were performed at 37°C in 250 mL normal shaking flasks or RAMOS flasks, respectively, with 10 mL filling volume (V_L). Shaking machines (LS-W in case of RAMOS device; ISF-4-W in case of normal shaking flasks) with a shaking diameter (d_0) of 50 mm from Adolf Kühner AG, Birsfelden, Switzerland were used. The shaking frequency (n) was 350 rpm.

Media and Solutions

A modified Wilms & Reuss [27] medium was used for the cultivations. The basic solution consists of 20 g/L glucose; 5 g/L $(NH_4)_2SO_4$; 0.5 g/L NH_4Cl; 3 g/L K_2HPO_4; 2 g/L Na_2SO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 41,85 g/L 3-(N-Morpholino)-propanesulfonic acid (MOPS); 0.1 g/L ampicillin; 0.01 g/L thiamine hydrochloride; 1 mL/L trace element solution [0.54 g/L $ZnSO_4 \cdot 7H_2O$; 0.48 g/L $CuSO_4 \cdot 5H_2O$; 0.3 g/L $MnSO_4 \cdot H_2O$; 0.54 g/L $CoCl_2 \cdot 6H_2O$; 41.76 g/L $FeCl_3 \cdot 6H_2O$; 1.98 g/L $CaCl_2 \cdot 2H_2O$; 33.39 g/L Na_2EDTA (Titriplex III)]. The pH was adjusted to 7.3 with NaOH. All reagents were of analytical grade and purchased from Carl Roth GmbH & Co. KG, Karlsruhe, Germany.

Online-Measurement of Oxygen Transfer Rates with the RAMOS Device

The RAMOS device for the on-line measurement of the OTR in shake flasks was introduced by Anderlei *et al.* [1, 2]. The cultivations in the RAMOS device were performed in modified 250 mL Erlenmeyer flasks. In the RAMOS flasks the hydrodynamic conditions and the concentrations in the gas-phase of the head space are the same as in regular Erlenmeyer flasks with cotton plugs [2].

Samples and off-line pH-Measurement

Büchs 2001 [3] described the problem of oxygen limitation while taking samples from the RAMOS flasks during the cultivation. Therefore, additional Erlenmeyer flasks were used for sampling. The cultivations in these flasks were performed in parallel and under the same conditions as the cultivations in the RAMOS device. For each sample the shaking machine was stopped and two flasks were taken from the machine. After taking a 1 mL sample the filling volume was refilled with sterile, purified water. Each flask was used for two samples.

The off-line pH was measured with a CyberScan pH 510 (Eutech, Nijkerk, The Netherlands) pH meter at 37°C.

On-line pH Measurement by Fiber Optical Technique

Commercially available sterile pH sensitive sensor spots (Presens, Regensburg, Germany) were applied for the fiber optical on-line pH measurement in the RAMOS flask. For gluing senor spots into the RAMOS flasks silicone rubber compound (RS, Mörfelden-Walldorf, Germany) was used. The spot was glued with a wet stick under the clean bench at the inside glass wall of autoclaved flasks at the point with the highest flask diameter (Fig. 3). A pH-1 mini (Presens, Regensburg, Germany) with an optical fiber to illuminate and collect the emitted fluorescence from the sensor spot, was used as pH meter.

The calibration was performed unsterile prior to a cultivation experiment with one sensor spot. For calibration six different buffers adjusted to different pH values between pH 4 and 9 were used to cover the measuring range. It was proven that the calibration was stable for the following on-line measurements with further sterile sensor spots from the same batch (data not shown). After 5 experiments a new calibration was performed.

For the analysis of the optical pH measurement a Visual Basic application in Mircosoft Excel was kindly provided by Frank Kensy (m2p-laps, Aachen, Germany).

Combination of On-line pH Measurement and the RAMOS device

To fix the optical fiber on the RAMOS plate, a holder was mounted next to one of the RAMOS flasks (Fig. 3), so that the fluorescence intensities of the fluorophores in the sensor spot could be measured.

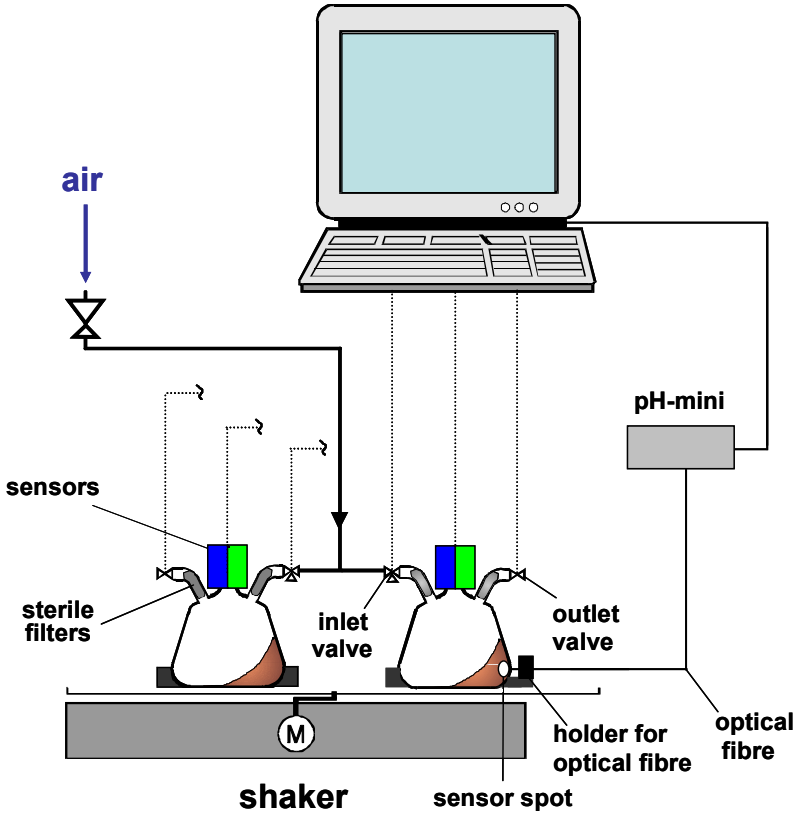

Figure 3: Principal set-up of RAMOS in combination with fiber optical, on-line pH measurement.

Nomenclature

CTR	carbon dioxid transfer rate [mol/Lh]
d_0	orbital shaking diameter [mm]
n	shaking frequency [rpm]
OTR	oxygen transfer rate [mol/Lh]
RAMOS	Respiration Activity Monitoring System
RQ	respiratory quotient [-]
T	temprature [°C]
V_L	filling volume [mL]

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References and Notes

1. Anderlei, T.; Büchs, J. Device for sterile online measurement of the oxygen transfer rate in shaking flasks. *Biochemical Engineering Journal* **2001**, *7*(2), 157-162.
2. Anderlei, T.; Zang, W.; Papaspyrou, M.; Büchs, J. Online respiration activity measurement (OTR, CTR, RQ) in shake flasks. *Biochemical Engineering Journal* **2004**, *17*(3), 187-194.
3. Büchs, J. Introduction to advantages and problems of shaken cultures. *Biochemical Engineering Journal* **2001**, *7*(2), 91-98.
4. Büchs, J. Biochemical Engineering Journal - Preface. *Biochemical Engineering Journal* **2004**, *17*(3), 153-153.
5. Christensen, M.L.; Eriksen, N.T. Growth and proton exchange in recombinant Escherichia coli BL21. *Enzyme and Microbial Technology* **2002**, *31*, 566–574.
6. Danielson, P.B.; Büchs, J.; Stockmann, C.; Fogleman, J.C. Maximizing cell densities in miniprep-scale cultures with H15 medium and improved oxygen transfer. *Biochemical Engineering Journal* **2004**, *17*(3), 175-180.
7. De Ley, J.; Gillis, M.; Swings, J. The genus Gluconobacter. In *Bergey's manual of systematic bacteriology*, 1st Ed.; Krieg N.R., Holt J.G., Eds.; Williams and Wilkins: Baltimore, 1984; *1*, 267-278.
8. Freyer, S.A.; König, M.; Künkel, A. Validating shaking flasks as representativ screening systems. *Biochemical Engineering Journal* **2004**, *17*, 169-173.
9. Hermann, R.; Walther, N.; Maier, U.; Büchs, J. Optical method for the determination of the oxygen-transfer capacity of small bioreactors based on sulfite oxidation. *Biotechnology and Bioengineering* **2001**, *74*(5), 355-363.
10. Huber, C.; Klimant, I.; Krause, C.; Werner, T.; Mayr, T.; Wolfbeis, O.S. Optical sensor for seawater salinity. *Fresenius' Journal of Analytical Chemistry* **2000**, *368*(2), 196-202.
11. Jeude, M.; Dittrich, B.; Niederschulte, H.; Anderlei, T.; Knocke, C.; Klee, D.; Büchs, J. Fed-batch mode in shake flasks by slow-release technique. *Biotechnol Bioeng* **2006**, *95*(3), 433-45.
12. John, G.T.; Goelling, D.; Klimant, I.; Schneider, H.; Heinzle, E. pH-sensing 96-well microtitre plates for the characterization of acid production by dairy starter cultures. *J Dairy Res* **2003**, *70*(3), 327-33.
13. Kensy, F.; John, G.T.; Hofmann, B.; Buchs, J. Characterisation of operation conditions and online monitoring of physiological culture parameters in shaken 24-well microtiter plates. *Bioprocess and Biosystems Engineering* **2005**, *28*(2), 75-81.
14. Levering, P.R.; Weenk, G.; Olijve, W.; Dijkhuizen, L.; Harder, W. Regulation of gluconate and ketogluconate production in Gluconobacter oxydans ATCC 621-H. *Archives of Microbiology* **1988**, *149*(6), 534-539.
15. Losen, M.; Frolich, B.; Pohl, M.; Büchs, J. Effect of oxygen limitation and medium composition on Escherichia coli fermentation in shake-flask cultures. *Biotechnology Progress* **2004**, *20*(4), 1062-1068.

16. Maier, U.; Losen, M.; Büchs, J. Advances in understanding and modeling the gas-liquid mass transfer in shake flasks. *Biochemical Engineering Journal* **2004**, *17*(3), 155-167.
17. Peter, C.P.; Lotter, S.; Maier, U.; Büchs, J. Impact of out-of-phase conditions on screening results in shaking flask experiments. *Biochemical Engineering Journal* **2004**, *17*(3), 205-215.
18. Puskeiler, R.; Kusterer, A.; John, G.T.; Weuster-Botz, D. Miniature bioreactors for automated high-throughput bioprocess design (HTBD): Reproducibility of parallel fed-batch cultivations with Escherichia coli. *Biotechnol Appl Biochem* **2005**.
19. Qazi, G.N.; Parshad, R.; Verma, V.; Chopra, C.L.; Buse, R.; Trager, M.; Onken, U. Diketo-gluconate fermentation by Gluconobacter oxydans. *Enzyme and Microbial Technology* **1991**, *13*(6), 504-507.
20. Samorski, M.; Müller-Newen, G.; Büchs, J. Quasi-continuous combined scattered light and fluorescence measurements: a novel measurement technique for shaken microtiter plates. *Biotechnol Bioeng* **2005**, *92*(1), 61-8.
21. Seletzky, J.; Noack, U.; Fricke, J.; Hahn, S.; Büchs, J. Metabolic activity of Corynebacterium glutamicum grown on l -lactic acid under stress. *Applied Microbiology and Biotechnology* **2006**, *72*(6), 1297-1307.
22. Seletzky, J.M.; Noack, U.; Hahn, S.; Knoll, A.; Amoabediny, G.; Büchs, J. An experimental comparison of respiration measuring techniques in fermenters and shake flasks: exhaust gas analyzer vs. RAMOS device vs. respirometer. *J Ind Microbiol Biotechnol* **2007**, *34*(2), 123-30.
23. Siano, S.A. On the use of the pH control reagent addition rate for fermentation monitoring. *Biotechnology and Bioengineering* **1995**, *47*(6), 651-665.
24. Silberbach, M.; Maier, B.; Zimmermann, M.; Buchs, J. Glucose oxidation by Gluconobacter oxydans: characterization in shaking-flasks, scale-up and optimization of the pH profile. *Applied Microbiology and Biotechnology* **2003**, *62*(1), 92-98.
25. Stöckmann, C.; Losen, M.; Dahlems, U.; Knocke, C.; Gellissen, G.; Büchs, J. Effect of oxygen supply on passaging, stabilising and screening of recombinant Hansenula polymorpha production strains in test tube cultures. *FEMS Yeast Res* **2003**, *4*(2), 195-205.
26. Stöckmann, C.; Maier, U.; Anderlei, T.; Knocke, C.; Gellissen, G.; Büchs, J. The oxygen transfer rate as key parameter for the characterization of Hansenula polymorpha screening cultures. *Journal of Industrial Microbiology & Biotechnology* **2003**, *30*(10), 613-622.
27. Wilms, B.; Hauck, A.; Reuss, M.; Syldatk, C.; Mattes, R.; Siemann, M.; Altenbuchner, J. High-cell-density fermentation for production of L-N-carbamoylase using an expression system based on the Escherichia coli rhaBAD promoter. *Biotechnology and Bioengineering* **2001**, *73*(2), 95-103.
28. Zimmermann, H.F.; Anderlei, T.; Buchs, J.; Binder, M. Oxygen limitation is a pitfall during screening for industrial strains. *Appl Microbiol Biotechnol* **2006**, *72*(6), 1157-60.