Slovak University of Technology in Bratislava
Institute of Information Engineering, Automation, and Mathematics

PROCEEDINGS

17th International Conference on Process Control 2009

Hotel Baník, Štrbské Pleso, Slovakia, June 9 – 12, 2009

ISBN 978-80-227-3081-5

http://www.kirp.chtf.stuba.sk/pc09

Editors: M. Fikar and M. Kvasnica

Hrnčiřík, P., Vovsík, J., Jurníček, V., Náhlík, J.: On-Line Monitoring of Biopolymer Content in Bacterial Cells Using Radio-Frequency Impedance, Editors: Fikar, M., Kvasnica, M., In *Proceedings of the 17th International Conference on Process Control '09*, Štrbské Pleso, Slovakia, 254–258, 2009.

Full paper online: http://www.kirp.chtf.stuba.sk/pc09/data/abstracts/022.html

ON-LINE MONITORING OF BIOPOLYMER CONTENT IN BACTERIAL CELLS USING RADIO-FREQUENCY IMPEDANCE

P. Hrnčiřík*, J. Vovsík*, V. Jurníček*, and J. Náhlík*

** Department of Computing and Control Engineering,*
Institute of Chemical Technology Prague,Technická 5, 166 28 Praha 6, Czech Republic
fax : +420 220 445 053 and e-mail : Pavel.Hrncirik@vscht.cz

Abstract: This paper describes the implementation of a radio-frequency (RF) impedance-based sensor for on-line monitoring of a biopolymer - medium chain length poly(3-hydroxyalkanoates) (mcl-PHAs, shortly PHA) production process by the *Pseudomonas putida* KT2442 strain with emphasis on on-line in-situ monitoring of PHA content in bacterial cells. First, motivation for the application of the commercially available RF impedance-based sensor technology is presented. Subsequently, experimental results obtained from fed-batch cultivations of the *Pseudomonas putida* KT2442 strain are presented and discussed, and a novel scheme for on-line estimation of the PHA content in bacterial cells under the conditions of aerated cultivations combining the capacitance and offgas composition measurements is proposed.

Keywords: on-line monitoring, biopolymer production, fed-batch cultivation, radio-frequency impedance measurement.

1 INTRODUCTION

Radio-frequency impedance based biomass measurements have recently enjoyed wide acceptance both in academic and industrial applications as a relatively accurate and reliable method for on-line quantification of viable cellular biomass. This method is based on the fact that an aqueous cell suspension can be interpreted, from an electrical point of view, as a suspension of moreless spherical tiny capacitors the capacitance of which can be measured by an appropriate sensor. As a result, the measured signal then reflects the total viable cell biovolume. For a more detailed description of the theoretical fundaments see the paper by Carvell *et al.* (2006).

The Biomass Monitor series (Aber Instruments Ltd., UK) represents in this context one of commercially available RF impedance-based sensors (Carvell *et al.* 2006). Specifically, the Biomass Monitor 210 is not only able to determine living biomass concentration on-line in-situ using either single capacitance reading or capacitance difference between two frequencies to eliminate the influence of aeration and stirring, but it can also measure a capacitance spectrum using the frequency scanning option. For the living biomass concentration measurement, a linear correlation between cell number (biomass concentration) and capacitance is generally assumed. However, as has been shown recently, this assumption is not always valid. It was found that *e.g.* the cell size and morphology can influence the capacitance signal during animal cell cultivations (Cannizzaro *et al.* 2003).

There are also other factors, especially those influencing the intracellular conductance that may potentially violate the assumption of linear correlation. Specifically for yeast cells, it was found that the presence of cellular lipid inclusions has considerable impact on the capacitance signal. Furthermore, it has been shown that the degree of deviation from linearity depended directly on the intracellular lipid content (Maskow *et al.* 2008b).

Therefore, one of the main objectives of this experimental study was to examine the potential of the RF impedance-based sensor (Biomass Monitor 210) for on-line in-situ monitoring of PHA content in bacterial cells (*Pseudomonas putida* KT2442) because currently there are no on-line sensors available for this task. The PHA intracellular content is usually assessed off-line using flow cytometry and/or determined gravimetrically.

2 MATERIALS AND METHODS

2.1 *Microorganism and cultivation conditions*

The inocula (*Pseudomonas putida* KT2442) for fed-batch cultivations were prepared at 30 °C in shaking flasks in a rotary incubator (incubation duration: 16-18 h). Composition of the incubation medium per liter: 4.7 g $(NH_4)_2SO_4$, 0.8 g $MgSO_4.7H_2O$, 12 g $Na_2HPO_4.7H_2O$, 2.7 g KH_2PO_4, 3 g nutrient broth. Productive medium for the fed-batch phase contained per litre: 4.7 g $(NH_4)_2SO_4$, 0.8 g $MgSO_4.7H_2O$, 9 g $Na_2HPO_4.7H_2O$, 2.03 g KH_2PO_4, 1 g octanoic acid and 10 ml trace element solution (composition per litre: 10 g $FeSO_4.7H_2O$, 3 g $CaCl_2$, 2.2 g $ZnSO_4.H_2O$, 0.5 g $MnSO_4.4H_2O$, 0.3 g H_3BO_3, 0.2 g $CoCl_2.6H_2O$, 0.15 g $Na_2MoO_4.2H_2O$, 0.02 g $NiCl_2.6H_2O$, 1 g $CuSO_4.5H_2O$).

2.2 *Experimental setup*

The fed-batch cultivations (*Pseudomonas putida* KT2442) were carried out under the following conditions: temperature at 30 °C, pH = 7, stirrer speed 900 min^{-1}, air flow rate 9.5 $l.min^{-1}$. Base (14 % NH_4OH) and acid (17 % H_3PO_4) solutions were added to the cultivation medium to control pH. Following the initial batch phase carbon source (octanoic acid) was continually supplied with a feeding rate set by the operator. Feeding strategies varied by individual cultivation runs, generally there was a phase of an exponential feeding followed by underfeeding and starvation, respectively.

All cultivations were carried out in a 7-litre laboratory bioreactor (newMBR, Switzerland) at the Bioprocess Control Laboratory at the Department of Computing and Control Engineering of the Institute of Chemical Technology in Prague (ICT Prague). The bioreactor was equipped with an IMCS 2000 analogue control unit (temperature, pH, stirrer speed, antifoam level, and airflow control), a programmable logic controller (Modicon Compact PC-E984-265, Schneider Electric, France) and the proprietary Biogenes II control system (based on Factory Suite 2000 software package, Wonderware, USA). The dissolved oxygen tension (*DO*) was measured by an oxygen probe (Mettler Toledo); the oxygen (O_2) and carbon dioxide (CO_2) concentrations in the off-gas were measured by SERVOMEX 1100 and 1440 analysers, respectively. For the substrate supply to the bioreactor a DP200 peristaltic pump (New Brunswick) was used. Control variables feeding rate, acid, base and antifoam addition were also recorded.

The capacitance measurement in the bioreactor was carried out by an Aber Biomass Monitor 210 (Aber Instruments Ltd., UK) operating in scanning mode and equipped with a highly sensitive four-annular ring probe AberProbe. The measuring device measured both the capacitance spectrum (25 data points corresponding to increasing frequencies on a logarithmic scale from 0.1 MHz to 19.49 MHz) and the capacitance difference ($\Delta C = C$(0.47 MHz)-C(15.65 MHz)).

In order to assess the PHA cell content, flow cytometric measurement was applied to determine relative fluorescence intensity of cells stained with Nile red probe. Evaluation of presence, localization, distribution and frequency of PHA droplets was processed by fluorescence microscopy. Flow cytometer PAS III Partec (Partec, Germany) equipped with 20 mW argon-ion laser (excitation wavelength 488 nm) and data analysis software PartecFlow were used. Forward scatter and side scatter threshold level were applied to separate cell and non-cell particles and to reduce background noise. *FL2* fluorescence (orange – 590 nm) was detected and quantified for single-cell particles. The mean fluorescence value was calculated from approximately 100 000 analyzed particles. All assays and sample preparations were carried out in duplicates and data were averaged.

In selected samples the PHA content was also determined gravimetrically. Biomass for PHA gravimetric determinations was prepared by centrifuging samples (50 ml). The pellet was washed three times with deonized water and lyophilised. PHA was extracted from the lyophilised cells by Soxhlet extraction with hot chloroform (150 ml) for 24 h. Excessive chloroform was then distilled off to obtain cca 5 ml residue. PHA was subsequently precipitated in 10 volumes of cold methanol. The precipitated polymer was separated by decantation, the solvent evaporated to dryness and the purified PHA was then weighed.

3 RESULTS

Several fed-batch cultivations were carried out to generate data for process analysis and to examine the monitoring capabilities of the RF impedance-based sensor (Biomass Monitor 210) for PHA producing microbial cultures. In the following an exemplary cultivation will be briefly described.

Cultivation PP-OA-08-3 (Figs. 1, 2): From the point of view of culture growth the cultivation can be divided into 3 phases. The first phase comprises periods A to D and represents exponential growth of the cells. The second phase – stagnation – comprises periods E and F, where the limitation by oxygen and consequently by the substrate occurred. In the periods G, H and I no substrate was added and this phase can be described as starvation phase.

Relative PHA content in the cells during cultivation was determined off-line using flow cytometer. The course of the obtained values is shown in figure 2 along with dry cell weight and corresponding values of the capacitance difference signal from Biomass Monitor 210. The PHA content in the cells increased initially in the first half of the growth phase under

3.1 *Cultivation description*

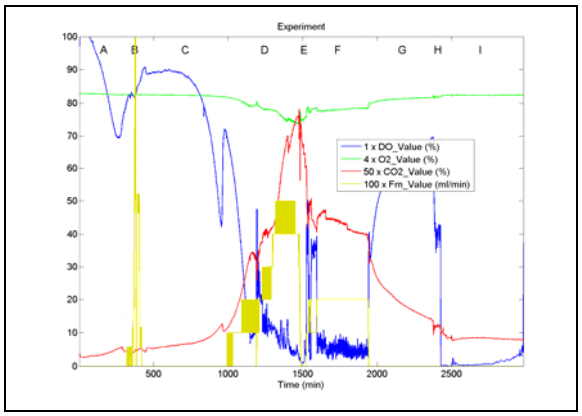

Fig. 1 Course of principal concentrations (*DO*, O_2, CO_2) and carbon source flow rate (*Fm*) during PP-OA-08-3 cultivation with outlined periods

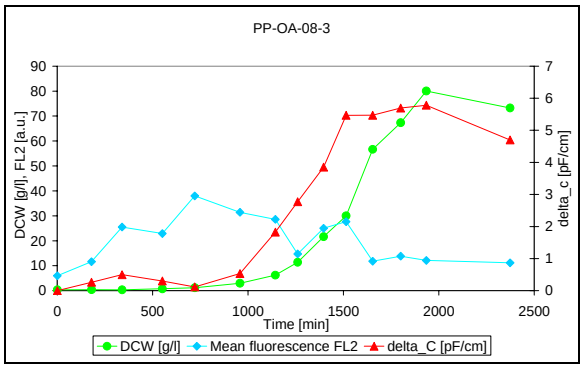

Fig. 2 Off-line measurements of dry cell weight (*DCW*), flow cytometry (Mean fluorescence *FL2*) and corresponding data points of the capacitance difference signal from Biomass Monitor 210 (*delta_C*) from PP-OA-08-3 cultivation

3.2 *PHA content estimation*

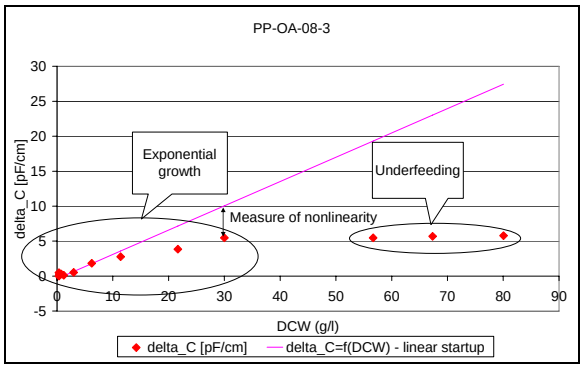

Fig. 3 Deviation from linearity between dry cell weight and capacitance difference

low cell densities. Next, when the cell density increased, the PHA content was slowly decreasing. The decrease can be caused by the limitation by substrates that did not appear under low cell densities. In the stagnation and even in the starvation phases, the PHA content was basically constant. It implies that the cells do not deplete all intracellular reserves of PHA in the case of absence of octanoic acid in the broth but go into "idle state" instead.

As is apparent from experimental data (Figs. 2, 3), the loss of linearity between dry cell weight and the capacitance difference takes place also in the case of bacterial culture of *Pseudomonas putida*.

For further analysis the deviation from linearity can be quantified in terms of capacitance as difference between the actual readings and the corresponding theoretical values of an extrapolated initial linear trend (*MON* – measure of nonlinearity, Eq. 1).

$$MON = \Delta C_{lin} - \Delta C = k \cdot DCW + b - \Delta C \qquad (1)$$

Subsequently it must be examined whether this measure of nonlinearity is correlated to the cellular content of PHA. A plot of the approximative measure of PHA cell content (*DCW* × *FL2*) against the measure of nonlinearity (Fig. 4) indicates linear correlation during the phase of exponential growth (approx. 700–1500 min). In the subsequent phases of underfeeding (stagnation) and starvation, respectively (approx. 1500–2400 min), this linear correlation is however no longer valid, probably due to important changes in cell size and morphology, which can also influence the capacitance signal.

For on-line applicability of this linear correlation for PHA content estimation the dry cell weight (*DCW*), which is determined off-line, has to be replaced by an on-line measurable variable. For the phase of exponential growth, this can be realised by using the cumulative oxygen consumption (*COC* – Eq. 2).

$$COC(T) = \int_0^T OUR(t)dt \qquad (2)$$

PHA content can then be estimated on-line during the exponential growth phase using Eqs. 3, 4, 5:

$$DCW_{est} = k_1 \cdot COC \qquad (3)$$

$$MON = k \cdot DCW_{est} + b - \Delta C \qquad (4)$$

$$PHA_{est} = k_2 \cdot MON \qquad (5)$$

where k, b, k_1, k_2 are calibration constants and DCW_{est} is dry cell weight estimated on-line from cumulative oxygen consumption (*COC*).

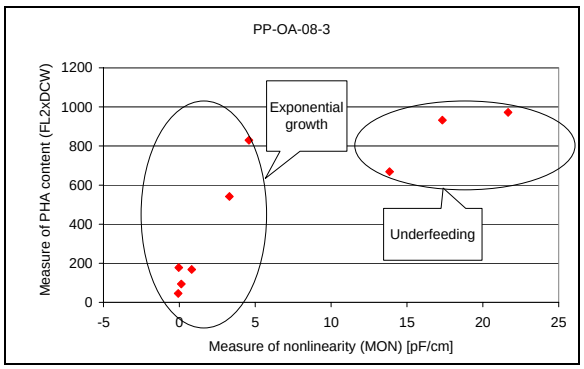

Fig. 4 Relation between the measure of PHA content in cells (*FL2* × *DCW*) and measure of nonlinearity between dry cell weight and capacitance difference (*MON*)

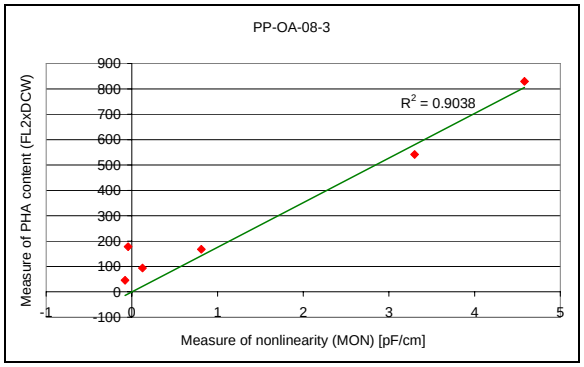

Fig. 5 Linear correlation between the measure of PHA content in cells (*FL2* × *DCW*) and measure of nonlinearity between dry cell weight and capacitance difference (*MON*) in the exponential growth phase

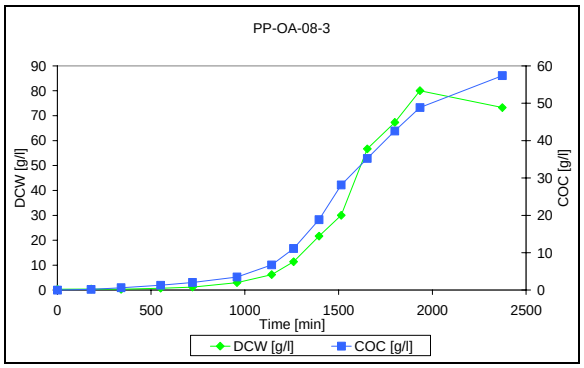

Fig. 6 Course of dry cell weight and cumulative oxygen consumption

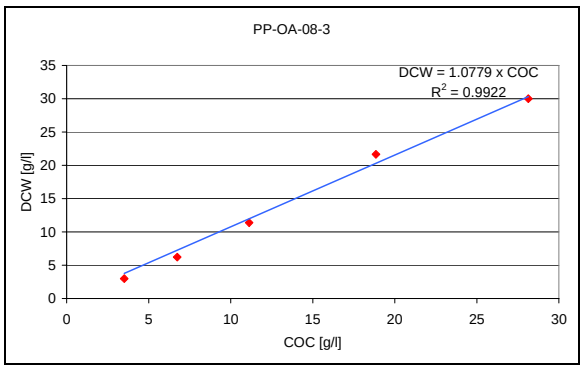

Fig. 7 Linear correlation between dry cell weight and cumulative oxygen consumption

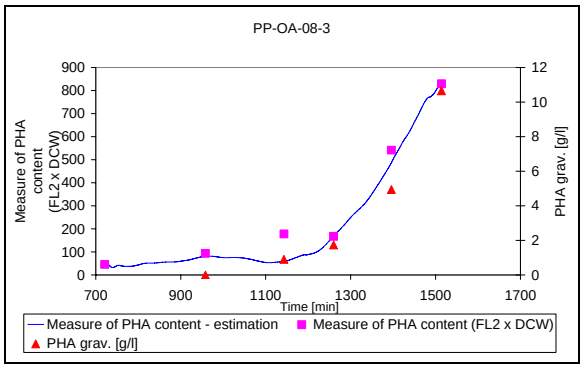

Fig. 8 Estimation of PHA content based on cumulative oxygen consumption and capacitance difference (exponential growth phase) by the derived on-line estimator

4 CONCLUSION

A radio-frequency impedance-based sensor (Biomass Monitor 210, Aber Instruments, UK) has been applied for on-line monitoring of the PHA bioproduction process by the *P. putida* KT2442 strain. The first results reported here show that the RF impedance-based sensor has an important potential for on-line monitoring of the PHA bioproduction process – for PHA content estimation (capacitance difference). Subsequently a novel scheme for on-line estimation of the PHA content in bacterial cells during the exponential growth phase combining the capacitance and offgas composition measurements has been proposed. This research forms part of a larger project comprising a design of process control strategies aimed at maximising efficiency of the PHA biopolymer production.

5 ACKNOWLEDGMENTS

This work was supported by the 6th Framework Programme of the European Community under contract No. NMP2-CT-2007-026515 "Bioproduction project - Sustainble Microbial and Biocatalytic Production of

Advanced Functional Materials" and by the fund No. MSM 6046137306 of the Czech Ministry of Education. This support is very gratefully acknowledged.

6 REFERENCES

Cannizzaro, C., Guegerli, R., Marison, I., von Stockar, U. (2003) On-line biomass monitoring of CHO perfusion culture with scanning dielectric spectroscopy. *Biotechnology and Bioengineering* **84**, 597–610

Carvell, J.P., Dowd, J.E. (2006) On-line measurements and control of viable cell density in cell culture manufacturing processes using radio-frequency impedance. *Cytotechnology* **50**, 35–48

Maskow T., Röllich A., Fetzer I., Ackermann J.U., Harms H. (2008a) On-line monitoring of lipid storage in yeasts using impedance spectroscopy. *Journal of Biotechnology* **135**, 64-70

Maskow T., Röllich A., Fetzer I., Yao J., Harms H. (2008b) Observation of non-linear biomass-capacitance correlations: Reasons and implications for bioprocess control. *Biosensors & Bioelectronics* **24**, 123-128