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Bioproduction of mcl-PHAs Biopolymers – Process States and Control

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Abstract: The presented paper deals with the experimental and theoretical work related to mcl-PHAs biopolymer production process using fed-batch cultivations of the bacterium *Pseudomonas putida* KT2442. The focus is on the definition and identification of process states in the form of physiological situations relevant to intracellular biopolymer production as well as the design of appropriate process control strategies.

1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are microbial polyester polymers synthesized by numerous bacteria as carbon and energy storage material. These polymers are deposited intracellularly in the form of inclusion bodies and can amount up to 90% of dry weight of cells. PHAs belong to promising candidates for biodegradable plastics and elastomers of industrial interest and of environmental value. Potential fields of their application include *e.g.* drug delivery, bone replacement applications, etc.

PHAs are in general classified in two major classes: short-chain-length PHAs (scl-PHAs) with C4-C5 monomers and medium-chain-length PHAs (mcl-PHAs) with C6-C14 monomers. At present, only few PHAs, typically from the structurally simpler class of scl-PHAs are industrially produced. Especially in the case of the more complex mcl-PHAs the disadvantage of the high production cost has prevented its wide use despite their promising properties.

Even though fermentation processes were developed employing different kinds of bacteria to improve PHA productivity there is still considerable room for further improvements, including the area of process control. The work done in this field by ICTP Prague has been concentrated on the development of a physiological state control approach for the production of mcl-PHAs biopolymers by cultivation of the *Pseudomonas putida* KT2442 strain.

2. MATERIALS AND METHODS

The inoculum (bacterium *Pseudomonas putida* KT2442) for fed-batch cultivations was prepared at 30 °C in shaking flasks in a rotary incubator (incubation duration: 16-18 h). The fed-batch cultivation conditions were as follows: temperature at 30 °C, pH = 7, stirrer speed 900 min^{-1}, air flow 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 octanoic acid was continually supplied as carbon source using feeding strategies described further in the text.

All cultivations were carried out in a 7-litre laboratory bioreactor (newMBR). The bioreactor was equipped with an IMCS 2000 analogue control unit, a programmable logic controller and the proprietary Biogenes II control system. The dissolved oxygen tension was measured by an oxygen probe (Mettler Toledo); the oxygen and carbon dioxide 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 and base were also recorded.

The capacitance measurement reflecting the amount of live biomass 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 and the capacitance difference (ΔC=C(0.47 MHz)-C(15.65 MHz)). Biomass concentration in the bioreactor was determined off-line gravimetrically as dry cell weight.

The intracellular 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 deionized 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.

In order to characterize quantitatively the process of biopolymer accumulation in the cultivated bacterial cells, several new variables computed from off-line analyses of PHA and biomass concentrations were introduced. Besides standard cumulative productivities and yields of biomass, residual biomass and PHA, there are specifically:

the specific PHA synthesis rate based on the amount of residual biomass - qPHA(Xr), defined as (dPHA/dt)/Xr (g PHA . g Xr^{-1} . h^{-1}),

the specific PHA synthesis rate based on the amount of PHA - qPHA(PHA), defined as (dPHA/dt)/PHA (g PHA . g PHA^{-1} . h^{-1}).

The specific PHA synthesis rate based on the amount of PHA has been found to be particularly useful for the evaluation of the PHA production process and the definition of individual physiological situations (see Figures 1 and 2).

3. RESULTS AND DISCUSSION

3.1 Definition of physiological situations

From the standpoint of industrial operation it is important to note that the type of metabolism used by the cultivated microorganism for the processing of substrates has decisive impact on process performance measured by indicators like productivity and yield. Therefore the design of bioprocess control strategies has to be focused not only on the issue of cell environment control but should ideally also aim at the control of the cell physiology itself. This issue has been addressed by the introduction of a control concept referred to as physiological state control by Konstantinov and Yoshida (1989). In contrast to conventional control strategies operating in closed loop in respect to the cell environment, the physiological state control scheme creates a closed loop in respect to the cell state. Consequently the environment is not a goal but a tool for manipulating cell physiology.

First step in the design of a control scheme based on the concept of the physiological state control is thus the definition and subsequent classification of bioprocess states related to the physiology of the cultivated microorganism – referred to as physiological situations. In the presented case this approach is demonstrated using the experimental data from two cultivations carried out in the Bioprocess Control Laboratory at ICT Prague (named Experiment 100210 and Experiment 100513).

Although the final classification was done for the process of fed-batch cultivations of the strain *Pseudomonas putida* KT2442 grown on octanoic acid for production of mcl-PHAs biopolymers, it is also valid for all fed-batch processes which use toxic substrates and where precise carbon source feeding is necessary.

The physiological situations are defined according to the feeding quality with respect to the state of the cells themselves (see Table 1).

Name	Description	Definition
BATCH	Batch or Return from Overfeeding	Fm = 0, DO ↓, DO spectrum I
OPT	Optimal feeding (optimal growth with optimal substrate utilization)	Fm > 0, DO ↓, DO spectrum II
OF	Overfeeding (growth inhibited owing to excessive substrate concentration)	Fm > 0, DO ↑, DO spectrum I
UF	Underfeeding (growth limited owing to lower substrate concentration)	Fm > 0, DO ↑, DO spectrum II
DOLIM	DO limitation	2 % < DO < 10 %, CO2 ↑
DOZER	DO zero or DO near zero value	DO < 2 %, CO2 ↓

Tab. 1. Physiological situations definitions (Fm – carbon source feedrate, CO2 – carbon dioxide offgas concentration, DO – dissolved oxygen concentration, DO spectrum I, II – different spectra of the dissolved oxygen concentration signal)

Two different DO limitation situations were introduced, because different DO limitations were observed to have different effect on cell growth and PHA production respectively. The situations can be estimated on-line from the carbon source feeding rate Fm and the trends of DO and CO2 variables.

In the two above mentioned experiments 100210 and 100513 the physiological situations were identified as follows from Table 2 and Table 3 and depicted in Fig. 1 and Fig. 2.

Cultivation Time (min)	Physiological Situation
0-250	BATCH
255-405	OPT
405-565	OF
565-750	BATCH
750-790	UF
790-1180	OPT
1180-1380	UF
1380-1440	OPT
1440-1580	UF
1580-1660	OPT
1660-1850	DOLIM
1850-2040	DOZER

Tab. 2. Physiological situations - Experiment 100210

Cultivation Time (min)	Physiological Situation
0-320	BATCH
320-420	OPT
420-520	OF
520-600	BATCH
600-650	UF
650-1560	OPT
1560-2070	DOZER

Tab. 3. Physiological situations - Experiment 100513

From Figures 1 and 2 it can be seen that optimal situation for mcl-PHA production according to the specific PHA synthesis rate based on the amount of PHA - variable qPHA(PHA) – is Optimal feeding, during which this variable is increasing. On the other hand this variable is decreasing in Overfeeding and also Underfeeding situations, not to mention DO limitations.

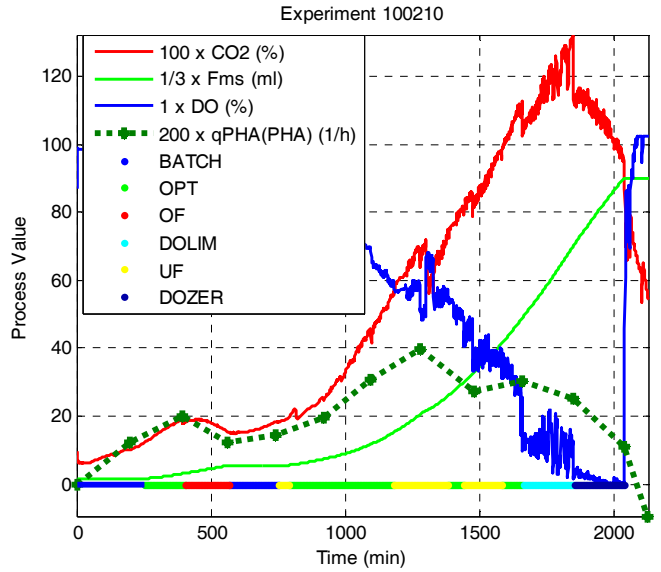

Fig. 1. Physiological situations during Experiment 100210

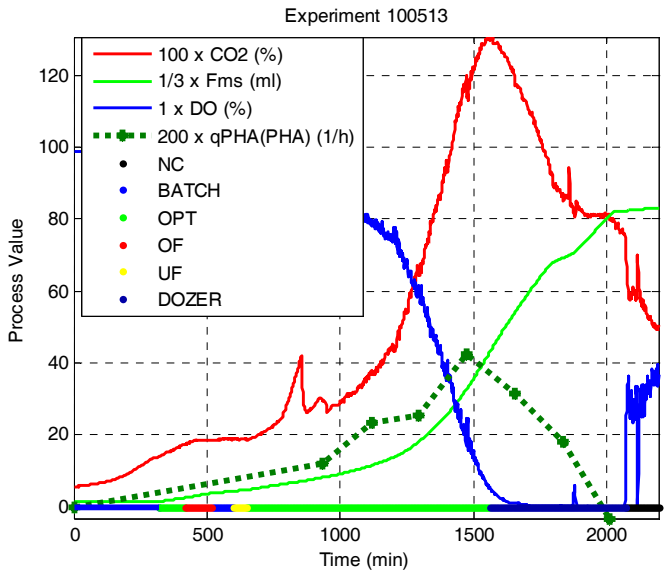

Fig. 2. Physiological situations during Experiment 100513

Both experiments were also visualized and analysed in the 3D space of the first three principal components (see Figures 3 and 4). These components were computed using Principal Component Analysis (PCA) from nine selected physiological variables (specific uptake/production rates and rate ratios), without significant loss of information about the process. The selected variables are all of the ratio type and were chosen from the complete set of computed physiological variables to eliminate the time trend effect on the fermentation trajectory depiction in the PCA space.

It can be observed that during fed-batch cultivations the fermentation goes through different physiological states (points in the space) even if it remains in the same physiological situation, as e.g. Optimal feeding.

Summary of the results of both experiments is presented in Table 4.

Experiment:	100210	100513
Cell concentration ($g \cdot l^{-1}$)	64.53	42.34
PHA concentration ($g \cdot l^{-1}$)	33.26	25.50
PHA content (%)	54.94	65.19
Overall PHA yield ($g \cdot g^{-1}$)	0.48	0.56
Overall PHA productivity ($g \cdot l^{-1} \cdot h^{-1}$)	3.36	3.67

Tab. 4. Experimental Results – Summary of attained maxima

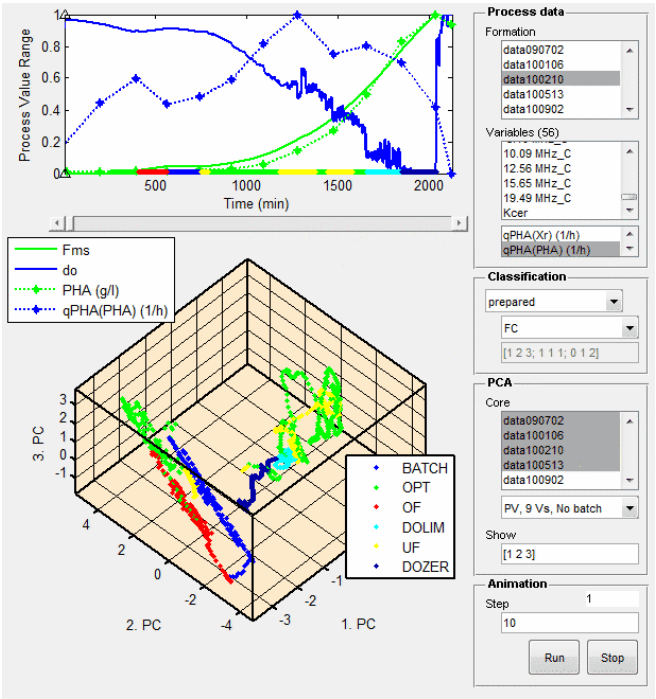

Fig. 3. Physiological situations during Experiment 100210 (PCA)

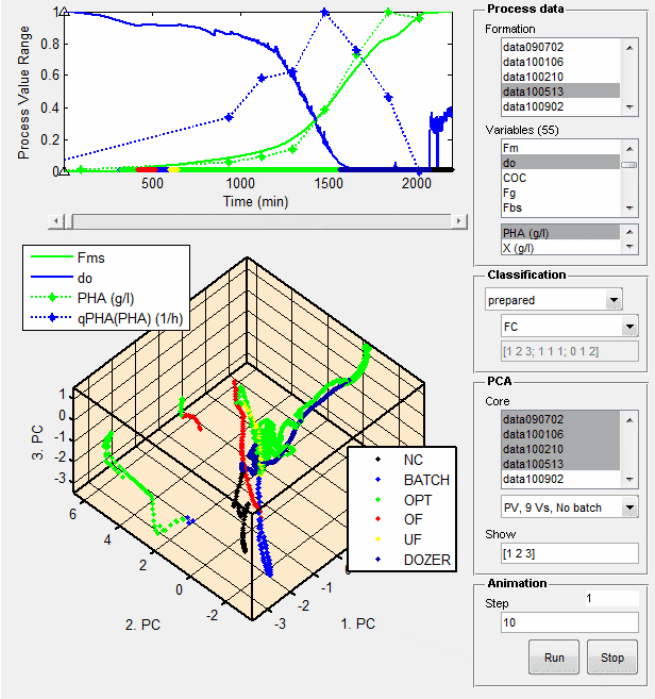

Fig. 4. Physiological situations during Experiment 100513 (PCA)

3.2 Process control strategies

Microbial bioproduction processes are traditionally mostly operated in fed-batch mode capable of achieving high-cell density cultures in an efficient way. The key issue related to the design of such processes is the choice of suitable substrate feeding strategies to control key nutrient concentrations, being in most cases the carbon source. The ideal solution, i.e. control strategies based on a direct measurement of the particular key nutrient concentration, is in many cases not feasible due to the limited availability of the necessary analytical devices. The carbon source used in the mcl-PHAs fed-batch bioproduction - octanoic acid - is a typical example of such a case, where sensor system suitable for routine on-line measurement of its concentration is not readily available. A common solution to this problem is often the use of predetermined exponential feeding profiles, i.e. feeding recipes calculated apriori from historical data with the aim to achieve a prescribed cell growth rate. However, the main drawback of this approach is its lack of flexibility vis-a-vis unexpected process events, since it cannot compensate for process disturbances that may severely affect the cell physiology. More appropriate are therefore control strategies where the key nutrient feeding is based on cell physiology, typically via one or several physiological variables that are calculable or even directly measurable on-line.

Specifically for the mcl-PHAs fed-batch bioproduction process, two such new process control strategies for the phases of optimal cell growth (CER agent) and oxygen limitation (DOPID) respectively have been designed and implemented.

CER Agent - control strategy description

- carbon-source (octanoic acid) feeding strategy based on CO2 evolution (production) rate CER (Eq. 1).
- $$C-source\_feeding\_rate = k_{CER} \cdot CER \quad (1)$$
  where $k_{CER} \approx \frac{1}{Y_{CO_2/C-source}}$
- based on similar strategy applied by Sun et al. (2006) in high-cell-density fed-batch cultivation of *P. putida* KT2440 with glucose as the carbon source
- contrary to common feeding strategies based on predetermined exponential feeding profile this strategy is able to adjust the feeding profile in response to the variability of individual bacterial cultures
- using this strategy both high mcl-PHAs concentration and content have been achieved
- the strategy can be tuned by just one parameter - kCER, which is adjusted by process operator using a process state classification scheme (based on CO2, DO online measurements and carbon-source feeding rate), automation of this adjustment strategy is envisaged. This new classification scheme turned out to be better (more robust and easier to implement on-line) than the classification scheme based on the so called "obesity quotient" studied previously (Hrnčiřík et al. 2010).

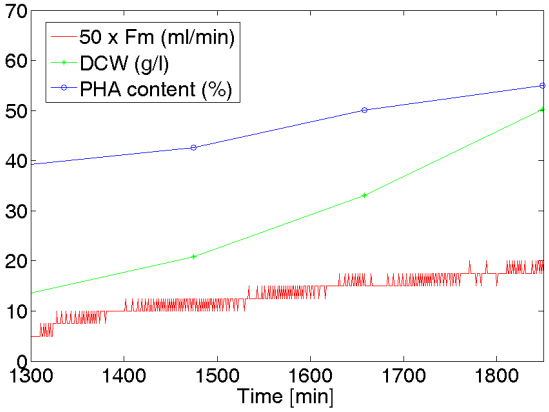

Fig. 5. Cell growth controlled by the CER agent strategy (Experiment 100210, Fm = substrate feeding rate, DCW = dry cell weight, PHA content = intracellular biopolymer content)

DOPID - control strategy description

- carbon-source (octanoic acid) feeding strategy based on dissolved oxygen (DO) concentration
- aiming to stabilize the DO level above critical values to avoid excessive carbon-source overfeeding that might lead to inhibiting accumulation of the carbon source (octanoic acid) in the cultivation medium
- switched to from the CER agent strategy after oxygen limitation sets in (at approx. DO=15%)
- implementation by a standard PID control loop
- oscillatory behaviour of the controlled variable (DO) serves as an immediate indicator of an adequate feeding level

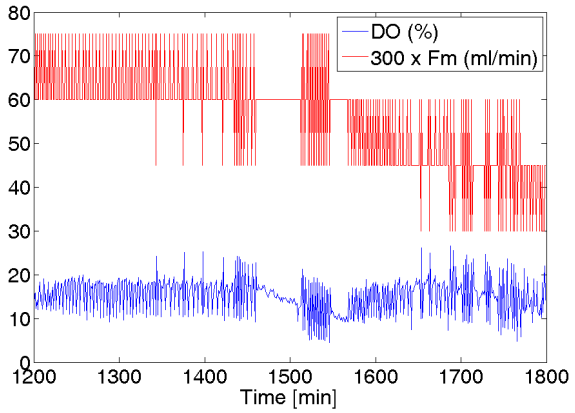

Fig. 6. Process control during the oxygen limitation phase - DOPID strategy (Experiment 110317, DO = dissolved oxygen concentration, Fm = substrate feeding rate)

3.3 Conclusion

Using these strategies in combination with the classification of physiological situations it was possible to maintain the process in the optimal feeding regime just on the border between underfeeding and overfeeding situations, thereby bringing about the optimal exploitation of cell growth potential and PHA production capacity.

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