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Optimization of polyhydroxyalkanoates fermentations with on-line capacitance measurement

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HIGHLIGHTS

- On-line capacitance values reflect the change of microbial morphology and viability.
- Real-time specific oxygen uptake rate and specific growth rate can be calculated.
- The viable cell number can be controlled by regulating the phosphate concentration.
- The fed-batch control strategy directed by capacitance improves the PHAs production.

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ABSTRACT

The aim of this work was to provide an effective methodology for optimization of the polyhydroxyalkanoates (PHAs) fermentation with *Ralstonia eutropha* by the on-line capacitance measurement. The present study found the capacitance values could reflect variations of microbial morphology and viability. Furthermore, oxygen uptake rate, specific oxygen uptake rate and specific growth rate were measured in real-time and compared with the capacitance value. In addition, a fed-batch control strategy based on the on-line capacitance measurement was proposed to improve the PHAs production by 22%.

1. Introduction

In a bioprocess, the amount of viable biomass is a crucial physiological parameter, which is highly correlated to the cell growth, metabolism and productivity (Carvell and Dowd, 2006). Dry cell weight (DCW), optical density (OD) and packed mycelial volume (PMV) are the conventional off-line indicators of biomass concentration. Nevertheless, these measurements are time-consuming and cannot reflect the microbial viability. Although the number of colony forming units (CFU) measures merely viable cells, the measurement is also time-consuming and difficult to reproduce (Xiong et al., 2008).

Currently, the employment of advanced instruments to monitor and control the microbial physiological state has become popular in the industrial fermentations. On-line measurements of physiological parameters are beneficial to comprehend the variation of cell status and culture condition in time. The constant physiological parameters such as oxygen uptake rate (OUR) have been a scale-up criterion to promote the erythromycin production (Zou et al., 2009). Redox electrode can measure redox potential to optimize the ethanol fermentation (Yu and Lin, 2012). Respiratory quotient (RQ) is controlled in micro-aerobic fermentation of ethanol production to improve the yield (Franzen, 2003).

In the latest studies, a few of instruments for biomass determinations have been developed, such as in situ near-infrared measurement (Arnold et al., 2002), impedance spectroscopy measurement (Sarro et al., 2012), fluorescence measurement (Zhao et al., 2011) and capacitance measurement (Markx et al., 1991). Among them, Biomass Monitor (Aber Instruments Ltd., Aberystwyth, UK) has become an effective tool to evaluate the concentration of viable biomass. Cells with intact plasma membranes are considered as tiny capacitors after polarized under the influence of a radio frequency electric field (Kiviharju et al., 2008). After choosing an optimal frequency with β-dispersion, the capacitance values are proportional to concentrations of viable cells. Dead cells and other particles without intact cell membrane do not contribute to the capacitance signals (Carvell and Dowd, 2006). This

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technology satisfies Process Analytical Technology (PAT) requirements initiated by FDA (2004). It has been applied successfully to monitor concentrations of various cells, such as bacteria (Kedia et al., 2013), yeasts (Xiong et al., 2008), plant cells (Markx et al., 1991), insect cells (Zeiser et al., 1999) and mammalian cells (Neves et al., 2000).

In this study, Biomass Monitor is adopted during the polyhydroxyalkanoates (PHAs) fermentation by *Ralstonia eutropha* (reclassified from *Alcaligenes eutrophus*). PHAs are a class of biological polyesters, which can be biosynthesized by microbe utilizing starch as carbon resource (Pan et al., 2012). In previous study, it has been found that the *R. eutropha* is capable of producing the PHAs containing approximately 10 mol% 3-hydroxybutyrate (3HB) and 90 mol% 3-hydrocyvalerate (3HV). Because PHAs are nontoxic and biodegradable, they turn out to be the potential substitutes of fossil-fuel plastics. Typically, in this fermentation process when the bacteria encounter an excess supply of carbon but limitation of nitrogen, phosphorus or oxygen, bacterial sizes begin to expand with the PHAs accumulation (Madison and Huisman, 1999). As the morphological changes are closely related to the PHAs production, an automated method is developed for the quantification of microbial morphology. Meanwhile, the capacitance measurement can also display the dynamic changes regarding the visibility of biomass and the morphology, and is utilized as a key physiological parameter for the fed-batch control strategy in this work.

So far, few investigations focus on analyzing capacitance combined with the microscope image of bacteria and other parameters. In addition, a fed-batch control strategy judging by capacitance is first proposed, which is a convenient and feasible method for improving the industrial PHAs production.

2. Methods

2.1. Strain, media and cultivation

The strain used in this study is *R. eutropha* TA-032, which is kindly provided by Tianan Pharmacy Corporation (Zhejiang, China).

1 L pre-cultured *R. eutropha* are inoculated in 29 L of fermentation medium in a 50 L bioreactor (NCBIO) using Biostar database at 34 °C. The medium consists of, in (g/L): yeast extract (8.0), glucose (20.0), Na_2HPO_4 (6.78), KH_2PO_4 (3.0), NH_4Cl (1.0), NaCl (0.5), $CaCl_2$ (4.0) and $MgSO_4$ (0.24). During the fed-batch culture, the concentration of residual sugar maintains at 15.0 ± 2.0 g/L by continuously feeding glucose (600 g/L). The feed rate of NaH_2PO_4 solution (10 g/L) is controlled based on designed capacitance values. After the fed-batch culture, the final working volume is about 45 L. The pH is kept at 6.8 by adding $NH_3{\cdot}H_2O$. The agitation and aeration are adjusted to obtain adequate dissolved oxygen (DO $\geqslant$ 15%). Meanwhile, OUR is detected by a process mass spectrometer (MAX300-LG, Extrel).

2.2. Capacitance measurement

Biomass Monitor 220 measures on-line capacitance of the broth in the bioreactor. In this study, the selected frequency is 580 kHz and the low pass is 10, so the correct and flat capacitance curve will be observed.

2.3. Analytical methods

Optical density (OD) is obtained after multiplying the absorbance value by the dilution multiple. The measurement wavelength is 540 nm and the cell samples are diluted properly with H_2O to make sure the absorbance values are in the range of 0.2–0.8.

Dry cell weight (DCW) is harvested using plastic tubes. 10 mL of cell sample is centrifuged at 3000*g* for 10 min. Then the cell cluster is washed twice with H_2O and dried for 24 h at 80 °C.

The number of colony forming units (CFU) is counted after the cell samples are properly diluted and cultivated on LB agar plates for 48 h at 34 °C.

Concentration of phosphate ions in the supernatant is measured using the cuvette test kit (Merck, No. 1.00616.0001) (Knabben et al., 2010).

Concentration of glucose is measured with glucose-oxidase kit (Shanghai Kexin biotechnology Co., Ltd).

The intracellular PHAs is converted to crotonic acid and pentenoic acid by treatment with 4 M H_2SO_4, and then analyzed by a high-performance liquid chromatography (HPLC) method (Karr et al., 1983).

2.4. Automated quantification of microbial morphology

In order to automatically determine the bacterial sizes during the fermentation processes, Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MA) is used to export the information of image analysis. Cell samples are stained with safranine. First of all, light microscope images of bacteria acquired by a CCD camera (cells are magnified 10 × 100 times) are handled with best-fit equalization and contrast enhancement. Then, the effects of edge enhancement, noise cancellation and smooth are realized by binarization processing. Finally, the counted dark areas are projected cell areas. In parallel, the objects that are not interested in must be hidden, and the adhesive objects must be split. In this investigation, three repetitions are conducted to accomplish 50 microscope images taken from a cell sample at every time point.

3. Results

3.1. Comparison of on-line and off-line biomass measurements

A typical time course of the PHAs fermentation by *R. eutropha* is shown in Fig. 1. On-line capacitance measurement is compared with three conventional off-line biomass measurements: the number of colony forming units (CFU), optical density at 540 nm (OD_{540}) and dry cell weight (DCW). The number of CFU represents viable biomass, while OD_{540} and DCW are used to estimate total biomass concentration. After the inoculation, the four biomass measurements increase synchronously during 8–32 h. Subsequently, both capacitance and the number of CFU approximate steady states with slight increase. However, there are great changes of OD_{540} and DCW in comparison to capacitance after 32 h in the PHAs fermentation.

The relationship between DCW and on-line capacitance is analyzed. Two stages are divided for calculation, including cell growth phase and PHAs biosynthesis phase. The cell growth phase characterized by linearly rising capacitance (0.105 pF/cm) is easily distinguishable from the PHAs synthesis phase with capacitance (0.026 pF/cm) in spite of increasing DCW. In contrast, an excellent correlation is achieved between capacitance and number of CFU during the entire fermentation process. Therefore, it is confirmed that the capacitance measurement has a good linear relationship with the number of living cells, rather than DCW. Many previous reports also show non-linear correlations between capacitance and DCW, which are attributed to growth phase shift and cell expansion with increasing biosynthesis of the PHAs in vivo (Junker et al., 1994; Maskow et al., 2008).

Many previous reviews show the linear correlations between capacitance and off-line biomass in yeast and mammalian fermentations (Davey et al., 1993; Ducommun et al., 2001; Fehrenbach