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Mini Review

PHB Production in Biofermentors Assisted Through Biosensor Applications

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Abstract

PHAs are biodegradable and biocompatible polymers synthesized and accumulated in intracellular compartments in several bacterial species. Recombinant *E. coli* systems were studied to produce PHB using metabolic engineering. In biofermentors, the critical points are the excess of fermentable sugars and the ratio of nutrients versus cell optical density. In order to allow production in biofermentors in automated system, sensors are envisaged to evaluate critical parameters such as sugar consumption, bacteria concentration and level of synthesis of PHA. In this review these parameters are discussed, as well as possible solutions.

Keywords: *Ralstonia eutropha*; *E. Coli*; Biofermentor; Biosensing; Exponential growth; Biosynthesis

Abbreviations

PHA: Poylhydroxyalanoate; PHB: Polyhydroxybutyrate; PHB-V; mixed type PHA: Containing Hydroxyvalerate; EIS: Electrochemical Impedance Spectroscopy; FTIR: Fourier Transformation-IR Spectrometry; SERS: Surface Enhanced Rama Spectroscopy

Introduction

Polyhydroxyalcanoates (PHAs) are biodegradable and biocompatible polymers synthesized by bacteria and accumulated in intracellular compartments. Poly(3-Hydroxybutyrate) (PHB), have been considered to be good candidates for completely biodegradable polymers due to their similar mechanical properties to petroleum-derived polymers and complete biodegradability. PHB, the most common PHA, is synthesized by numerous prokaryotes as *Cupriavidus necator* (*Ralstonia eutropha*) [1], in response to limitation of nitrogen [2] in presence of high carbon sources. Several strategies are used to produce P(3HB), one-stage batch [3], two-stage batch [4,5] (Chen and Page 1997, Singhaboot and Kaewkannetra 2015) or high-cell-density fed-batch cultures [6,7].

PHA can be synthesised and produced using recombinant microorganisms [8,9]. The use of recombinant bacteria enables to escape the need to limit nitrogen sources [10], while sugars (glucose, lactose, fructose) are continuously added to the medium to sustain exponential growth and PHB synthesis [11,12], as well as for shunt of PHB substrates by modification of glycolysis and other metabolic pathways [13,14].

In the composition of medium used to feed the bacteria, attempts have been made to reduce the costs by extracting the nutrients from wastes and byproducts. Significant research was performed on agro–industrial even agro–waste streams as feedstock for fermentation. Researchers realized a high-productivity fermentation of P(3HB) [15], and implemented successfully a P(3HB) fermentation process using chicory roots (*Cichorium intybus*) [16] after hydroponic cultivation as a carbon source.

Complex organic by-products are a good opportunity, both from an environmental and an economic perspective, to produce Polyhydroxybutyrate (PHB) and organic acids; this was also the topic of a recently concluded EU project [17].

The advantages of using recombinant *E. coli* for the production of PHB include rapid growth, accumulation of PHB greater than 50% of cell weight [18-22], and the ability to utilize inexpensive carbon sources [23,24]. Key process operating variables (i.e., nutritional and aeration conditions) affect the biomass production rate and the PHB accumulation in the cells and its associated molecular weight distribution. Previous studies have demonstrated that PHB production using recombinant systems such as *E. coli* have been hindered upon scaling up in part due to the use of large amounts of oxygen required for high bacterial growth and PHB generation [25,26]. Recently, bubble gas microaeration and sparging has been shown useful to increase the oxygenation of the medium and the synthesis of PHB [27]. The process optimization may lead to high intracellular PHB accumulation (up to 95% of PHB/g of dry cell weight). Applications of PHA are coatings, packaging films and in bottling, medicine, drug delivery and bioplastic components [28,29]. The principal bottleneck is the cost of production, being higher than 2 dollars/kg, due to costs of running the fermentors, and for extraction and purification. Therefore, it is highly desirable to optimise bioreactor conditions to improve the yield, and to scale up the processing capacity of fermentors.

Various companies produce PHB and PHB-V heteropolymers. TephaElast (by Tepha), Biopol (by Metabolix), Mirel (by Telles), Biogreen (by Mitsubishi), Enmat (by Tinan), Nodex (by Kaneka), Biocycle (by PHB Ind.). Of these, Telles has the highest production capacity, projected to reach 50.000 tonn/year in 2020, and SIRIM in Malaysia has a fully automated fermentor system [30]. In 2016, Metabolix announced the intention to sell the patents and assets for PHA to Cheil Jidang, making this company one of the strongest in this field for the next years.

Bioreactors are provided with sensors to monitor physico-

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chemical parameters, such as temperature probe and pH sensor (linked to pumps to add NaOH or HCl), stirring speed, air flux regulation or micro-bubble dispersion by sparging (BIOSTAT Q Multi-Fermentor Bioreactor System with dissolved oxygen probe), to provide dissolved oxygen, needed for aerobic growth. Turbidity (OD600) and glucose consumption need to be measured, at 0,4,8,12,24,48,72 and 96 h. PHA production need to be evaluated too, since after PHA accumulation bacterial cells are collected for PHA extraction. In order to make the process sustainable and economically convenient, two factors need to be optimised: high Optical Density (OD) of cell suspension, and continuous presence of 5-10% sugars. The bacteria need to reach an OD close to 50, to obtain an optimum ratio of cells/volume, exploiting the maximum volume capacity of bioreactors, without diluting the sugars.

Recently researchers have described a combined metabolic/polymerization/macroscopic modelling approach, relating the process performance with the process variables, controlling the key process operating variables (i.e., nutritional and aeration conditions) affecting biomass production rate and PHB accumulation in the cells and PHB molecular weight distribution [31].

The potential of application of sensors and biosensors in the bioreactors applied to monitoring the exponential phase growth, the level of nutrients, and the synthesis of PHA is envisaged for a feasible and sustainable increase in production of bioplastics at industrial level. There is a need to control the availability of sugar substrate, to monitor the synthesis of PHB, an to check the viability of bacteria after exponential growth and at growth curve saturation.

Sensors based on an enzymatic reactions can measure sugars concentration. This is made at laboratory scale using microwell plates and spectrofluorometer reads, based on enzymatic reactions. A sensor must be provided with an autosampler, a microfluidic pump, and a reaction chamber where enzyme produced NADH is quantified by its absorbance.

Microfluidic systems allow the controlled flow of operations like solvent and solute transport, valving, mixing, separation, concentration and detection with a dedicated biosensor (chemical, physicochemical, or biological method). All the components, from micro-reaction chambers, delivering small volumes through servo drives, high-precision mechanical components, and pumping systems with pulsation free fluid streams, syringe pumps, pump modules may be assembled in a Lab-on-Chip (LoC) system, under automated control, reducing operation times and operator errors.

Other types of sensors can be used to determine bacterial concentration, alternative to optical density measures, unsuitable when working with such high density of bacteria. Biosensors for whole-cell bacterial detection have been recently reviewed [32-35]. Various detection systems have been applied in bacteria quantification, from spectrophotometric detection, as Fourier Transformation-IR spectrometry (FTIR) and Reflectometric Interference Spectroscopy (RIfS), to Surface Enhanced Raman Spectroscopy (SERS) [36], to electrochemical biosensors, such as Alternate Current (AC) susceptometry measuring the magnetic field, suitable for bacteria concentration evaluation, to impedance-based systems, as Electrochemical Impedance Spectroscopy (EIS) [34,36].

Finally, a sensor needs to be dedicated to the detection and evaluation of PHB production. This is a critical point in industrial fermentation, since keeping the process for the shortest time possible is economically convenient, and bacteria that do not synthesise still consume sugars and keep the fermentors busy. Traditional PHB screening methods for PHB quantification in whole cells have exploited the Nile Blue staining and fluorescence of PHB containing bacteria [37]. Nile Blue dye stains PHB and other neutral lipids in bacteria. Quantitative assessments of PHB based on Nile Blue fluorescence involve various fixing steps, executed with an alcohol or acetone treatment, that facilitate the permeation of the dye through the membrane. The time required is between one and two hours and some manual passages. New methods have been proven less time consuming than standard Nile Blue colorimetric staining of PHB, and may be run in automatic, providing measures even over-night. Recently a more sensitive measurement has been obtained detecting bacteria fluorescence on a laser scanner (unpublished results) [17]. In this way, a colour scale was obtained, from blue, green, red, to white as the highest saturation signal. The chip slides loaded with serial dilutions of bacteria, are slightly dried for fixing the pellets to the glass, and incubated with the solutions for staining, centrifuged and loaded onto the scanner.

To this aim of automated detection of PHB in cells still in the fermentors, it is envisaged that SERS methods [36] could be efficiently applied not only to quantification of PHB, but also to discrimination of the types of polyhydroxyalkanoic acids produced. This may support the technologists and substitute the standard HPLC analyses for quality and quantity of PHA products.

The combination of these three sensors could make possible the exploitation of the full potential of bioreactors in optimization of the time of use (bacterial growth cycles) and maximization of bacterial synthesis of PHB in the shortest time possible.

Among the measuring methods or biosensors that can be applied to determine bacterial concentration, since the achievement is the determination of the maximum density, the methods most favoured for industrial applications are those cost effective and with few equipment maintenance problems. Therefore EIS, SPR and other applications requiring calibration curves are less favoured, while optical measurements requiring minimal liquid handling, such as few diluting steps and sample reproduction are most probable candidates.

Concerning the measurement of sugars to be quantified, there are already several methods on stick or test strip, exploiting viscosimetric [38] or amperometric detection of glucose oxidase activity. It has to be kept in mind that fermentors may need to work with higher concentration of sugars, proximal to 10% of the volume used. Nanoencapsulation of enzymes to read glucose concentration has been achieved [39].

Lab-on-a-chip (LOC) devices have a strong potential to be used in the field since they can be miniaturized and automated; being also potentially fast and very sensitive. There are still several issues to be solved before application in field applications, including the pre-treatment of a sample, such as dilution of bacteria within the linear range of calibration curve, proper storage of reagents, full integration into a battery-powered or energy-failure proof system, range of

linearity measurements and comparability of each method, and easiness of operations, to allow the operator to overcome troubleshoots of the system. Each biosensor technique has its own advantages and disadvantages in terms of equipment required, sensitivity, simplicity and cost effectiveness, being optical sensors affordable and with maintenance procedures easy to be performed, and enzyme based sensors highly reliable and based on well established protocols in medical applications.

In industrial fermentation for production of PHB at cost-effective scale, in addition to sensors controlling the standard parameters temperature, pH, oxygen, three additional sensors are proposed, a sensor to evaluate bacteria concentration, and a sensor for sugar concentration, and a sensor to monitor PHB synthesis over the time and the amount of PHB produced. The perspectives and future vision for using biosensors for industrial synthesis of PHA and related processes based on industrial fermentors (bio based biochemicals industry) are sound, but not disclosed and discussed due to protection of intellectual property rights and economic interests. Therefore, it is requested from academia to disclose the applications of sensor components with a broader view, such as the production in fermentors of recombinant proteins or organic acids as intermediate products for the green chemistry.

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