Biochemical Engineering Journal 116 (2016) 113–121

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Building a predictive model for PHB production from glycerol

Cristina Pérez Rivero, Chenhao Sun, Constantinos Theodoropoulos, Colin Webb*

School of Chemical Engineering and Analytical Science, University of Manchester, M13 9PL, UK

ARTICLE INFO

Article history:
Received 12 December 2015
Received in revised form 5 April 2016
Accepted 18 April 2016
Available online 6 May 2016

Keywords:
Biorefinery
Intracellular polymer
Predictive modelling
Growth kinetics
Production kinetics
Dual substrate model

ABSTRACT

Poly-3-hydroxybutyrate (PHB) is a biodegradable biopolyester with plastic like properties, which on its own or as part of a heteropolymer, finds application in everyday products, competing directly with fossil fuel based plastics in terms of physical and mechanical properties. In nature, PHB is produced as an energy reservoir for the host cell, when environmental conditions limit growth. It is this inherent condition for PHB synthesis (*i.e.* an environment unsuitable for growth) that challenges design of conventional batch production systems. Balance between growth (driven by nitrogen availability) and PHB production (enhanced by an excess of carbon) is the critical aspect for consideration in such designs. However, selecting the best operating conditions is not obvious for this system and so a systematic approach has been used in this paper, utilising simulations based on a purpose built model to supplement experimental studies.

The interaction between the carbon and nitrogen sources (glycerol and ammonium sulphate respectively) was carefully evaluated and incorporated into a low-structured model able to describe the dynamics of substrate consumption and product accumulation during *Cupriavidus necator* DSM 545 cultivation at small scale. The kinetic parameters thus determined have been assumed to be constant, fixed accordingly, and the model used to predict the fermentation profiles for different operating conditions. Results showed good agreement with experimental data, supporting the efficacy of this approach. The dual utilization of multiple substrates suggests there is a system capacity to which both growth and PHB production contribute and that sets the maximum total biomass concentration. A logistic type term added to both growth and product rate equations enabled the effective decoupling of cell proliferation and PHB accumulation for a wide range of scenarios. In this way, the combination of predictive modelling and experimental verification potentially reduces, by a significant amount, the number of experiments required to establish operational targets such as specific growth rate and productivity as well as identifying often sought criteria such as optimum C:N ratio.

1. Introduction

Interest in poly-3-hydroxybutyrate rose due to different reasons since its discovery in 1927. For example, in the 1970s, matching the peak in oil prices, substitutes to petrol-derived plastics seemed necessary to cover the polymer demand. More recently, environmental awareness has motivated the research into non-chemical routes for producing bioplastics, particularly from renewable resources to be used as feedstocks for microbial cultivation [1]. Through such products, the disposal problems at the end of life of plastics are set to disappear. PHB is biodegradable and the carbon dioxide released when it degrades is balanced in part by that used for the growth of the biomass, decreasing net greenhouse gas emissions and solids wastes sent to landfills [2]. Besides, the independence from fossil fuel sources remains a key factor for pushing research and development in this area.

The physical characteristics of PHB are similar to those of polypropylene, making it a suitable candidate to replace traditional plastics in applications such as food packaging films, biodegradable carriers for medicines and insecticides, disposable cosmetic products, surgical devices and starting compounds for chiral substances [3]. Nevertheless, technology lags behind that necessary to reach all these markets. Expensive raw materials, insufficient fermentation optimization and poor product recovery are highlighted as the most important reasons for the scarce industrial implementation [4]. To deal with these issues, more efficient microorganisms that utilize low cost substrates together with a better control of the production process need to be established.

Though many bacteria have been isolated and characterised as natural PHB producers, with *Azohydromonas lata* (formerly known

* Corresponding author.
E-mail address: colin.webb@manchester.ac.uk (C. Webb).

http://dx.doi.org/10.1016/j.bej.2016.04.016
1369-703X/

Nomenclature

List of symbols:

Gly	Glycerol concentration (g/L)
k_d	Apparent dissociation constant (g/L)
k_G	Half saturation constant for glycerol (g/L)
k_{IG}	Glycerol inhibition constant (g/L)
k_{IN}	Nitrogen inhibition constant (g/L)
k_{INP}	Inhibition constant for PHB production (g/L)
k_N	Half saturation constant for nitrogen (g/L)
k_P	Half saturation constant for PHB (g/L)
K_x	Carrying capacity (g/L)
n	Hill coefficient (–)
N	Nitrogen concentration (g/L)
S	Substrate concentration (g/L)
$Y_{PHB/Gly}$	Yield of PHB based on glycerol (g/g)
$Y_{x_R/N}$	Yield of non-PHB biomass based on nitrogen (g/g)
$Y_{x_R/Gly}$	Yield of non-PHB biomass based on glycerol (g/g)
β	Non growth associated specific rate constant (1/h)
μ_m	Maximum specific growth rate (1/h)
μ_p	Specific production rate (1/h)
μ_{x_R}	Specific growth rate (1/h)

as *Alcaligenes latus*) and *Azotobacter* and *Pseudomonas* species as some of the most studied, there is no real competitor to *Cupriavidus necator* (formerly *Alcaligenes eutrophus*, *Ralstonia eutropha*) as an industrial strain. *C. necator* can utilize many different organic or inorganic carbon sources for accumulating large amounts of PHB (or its copolymers when feeding more than one carbon substrate). Special efforts have been put into using inexpensive renewable raw materials as fermentation feedstocks [5]. For example, under chemolithoautotrophic growth conditions, recycled mixtures of H_2, CO_2 and O_2 have been used to produce 61.9 g/L PHB in 40 h, matching normal levels in a two stage fermentation of conventional sugars [6].

Mixed cultures have been explored to overcome two main limitations: the inability of *C. necator* to metabolize some common sugars and the additional substrates, besides glucose, required by some other bacteria in order to synthesize significant amounts of PHB [7]. When using microbial consortia, abundant sugars can be converted into organic acids by the latter species and those organic acids later used as substrate by *C. necator* to produce the biopolymer. In order to achieve higher productivities or, as commented, finding microorganisms that can readily utilize low-cost substrates, polyhydroxyalkoanate (PHA) biosynthesis genes from *C. necator* and *Azohydromonas australica* have been cloned into *Escherichia coli* [8]. Although there are several studies on limiting the amount of oxygen as a strategy to promote PHB production during the accumulation stage, the desirable high growth rate of the *E. coli* cells requires a large oxygen supply. The alternative of employing anaerobic *E. coli* has the drawback of slower growth [9].

Glycerol is an example of those sustainable substrates that *C. necator* can readily utilize [10]. The current surplus of this polyalcohol, obtained as an intrinsic by-product from the transesterification reaction in biodiesel manufacture, has lowered the selling price and turned it into a waste rather than a coproduct. Its use as raw material in biological processes would contribute to the sustainable development of biorefineries for biodiesel production [11]. Methanol and sodium chloride have been found as the principal impurities in crude glycerol that affect the fermentation process by inhibiting cell growth [12,13].

PHB production in *C. necator* cells is attributed to unbalanced nutritional conditions in which carbon is in excess over other key elements, such as nitrogen, phosphorus, oxygen or sulphur [14]. Under stress conditions, cells experience a switch from predominant biosynthesis of cellular components to production and accumulation of PHB, used as a carbon and energy storage system [15]. Many studies have explored the desirable conditions for yielding large amounts of PHB, *e.g.* the ratio of carbon to nitrogen concentrations and it is commonly accepted that the initial C:N ratio determines the concentration of PHB that can be obtained through batch cultivation [16,17]. However, such studies are most often empirical and the lack of a suitable bioreactor model limits the value of experimental data, prolonging the optimization process and reducing the general applicability of the results.

With essentially fixed yields (at least until more genetic information is released), and the substrate choice limited by the microorganism or by regional availability, reducing the cost gap between petroleum based plastics and bioplastics requires better fermentation process design, which brings the need for reliable kinetic and design models [18]. These can be of very different types, depending on the sort of information used for their development. Mechanistic models are based on the understanding of the behaviour of the system's components. They combine the kinetics of the bioprocess with mass balances and mass transfer phenomena. Some are developed from the review of earlier models with modifications being made to accommodate new findings. Thus, for example the differentiation between PHB and non-PHB biomass led to some authors referring to a catalytic fraction of the total biomass as the part that needs to be included in the Monod-type expression usually adopted for describing cell growth [19] or the fact that a severe shortage of nitrogen as well as an excess of it can prevent PHB formation [20]. The substrate inhibition model proposed by Luong in 1987 [21] and the logistic equation appear often to achieve a good fit for the experimental data [20,22,23].

Two-stage fermentation is the preferred configuration for the PHB production system, where the aim of the first stage is to achieve good growth; PHB production is then boosted during the second stage following exhaustion of, usually, a key nutrient. Although single stage continuous cultivation could result in a simpler and cheaper process, the low dilution rates necessary would reduce productivity and this mode of operation has only been used with certain microorganisms that exhibit high production rate during the exponential phase.

Good modelling provides a tool for design but only if constants can be transferred across systems and conditions. Lee et al. [24] searched for the optimal profiles for the feeding of carbon and nitrogen through different control strategies in order to maximize PHB production at the end of the fermentation. Macroscopic modelling together with information from metabolic fluxes was used by Katoh et al. [23] to refine the control strategy based on the addition of glucose (substrate for *Lactobacillus delbrueckii*) pulses whenever lactate (substrate for *C.necator*) concentration in the bioreactor fell below a set-point. In contrast with past practices, Tohyama et al. [25] proposed a rate equation in which the specific growth rate was expressed as a function of dissolved oxygen concentration. More recently, Špoljarić et al. [26] developed a mathematical model to describe the conversion of substrates from biodiesel production (glycerol and fatty acid methyl esters) into poly[(*R*)-3-hydroxyalkanoate] by *C. necator*. Optimization of the fed-batch cultivation on glycerol, based on a low structured kinetic model, was then presented in a subsequent publication [27].

To achieve a more realistic representation of the cell machinery, cybernetic models add the action of internal regulatory controls, such as specific enzyme activities [28] while metabolic models consider the whole metabolic network. These provide in-sights into the internal workings of the cell and help identify bottlenecks and constraints, so that genetic engineering can be used to relieve them. In order to study the metabolic network of *C. necator* DSM 545, Lopar