Bioresource Technology 142 (2013) 603–610

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

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

Constraint-based genome-scale metabolic modeling of *Clostridium acetobutylicum* behavior in an immobilized column

Janne Wallenius *, Matti Viikilä, Shrikant Survase, Heikki Ojamo, Tero Eerikäinen

Aalto University, School of Chemical Technology, Department of Biotechnology and Chemical Technology, P.O. Box 6100, FIN-02015, Finland

HIGHLIGHTS

- A novel modeling approach for *Clostridium acetobutylicum* solvenotogenic phase is proposed.
- Continuous fermentation with immobilized cells is studied.
- The solvent fluxes are predicted quantitatively.
- A novel optimization procedure is introduced for constraint-based modeling.

ARTICLE INFO

Article history:
Received 19 March 2013
Received in revised form 21 May 2013
Accepted 23 May 2013
Available online 30 May 2013

Keywords:
Genome-scale metabolic modeling
Flux balance analysis
Clostridia
Continuous fermentation
Cell immobilization

ABSTRACT

In this study a step-wise optimization procedure was developed to predict solvent production using continuous ABE fermentation with immobilized cells. The modeling approach presented here utilizes previously published constraint-based metabolic model for *Clostridium acetobutylicum* without direct flux constraints. A recently developed flux ratio constraint method was adopted for the model. An experimental data set consisting of 25 experiments using different sugar mixtures as substrates and differing dilution rates was simulated successfully with the modeling approach. Converted to end product concentrations the mean absolute error for acetone was 0.31 g/l, for butanol 0.49 g/l, and for ethanol 0.17 g/l. The modeling approach was validated with another data set from similar experimental setup. The model errors for the validation data set was 0.24 g/l, 0.60 g/l, and 0.17 g/l for acetone, butanol, and ethanol, respectively.

1. Introduction

Genome-scale models offer a possibility to study quantitatively the cellular behavior of an organism *in silico*. These models can be used as tools to predict for example growth rates, uptake and secretion rates, and internal metabolic fluxes. They provide a framework to predict cellular behavior before laborious experimental work and thus help the design of experiments. Constraint-based modeling has been applied for various purposes, such as (i) a support for genome annotation (Reed et al., 2006), (ii) a support for metabolic engineering (Xu et al., 2013), (iii) study of multi-species interactions (Hanly et al., 2012), (iv) identification of essential drug targets (Kim et al., 2011), and (v) predicting optimal cultivation conditions (Teusink et al., 2006). Genome-scale constraint-based models have a vast solution space. Thus, the more detailed knowledge of the phenotypic state of an organism is desired, the more constraints are needed. Different constraining strategies have been developed, they have been reviewed by Reed (2012). Recently a novel flux ratio based constraining methodology by McAnulty et al. (2012) has been applied in addition to the more commonly used constraints for fluxes (Becker et al., 2007; Reed, 2012). Flux distributions can be studied by minimizing or maximizing a certain optimization objective function using linear or non-linear programming. Balancing fluxes according to a stoichiometric matrix maximizing specific growth rate as an objective function has become a commonly acknowledged method (Schuetz et al., 2007). Also other objective functions have been applied (Schuetz et al., 2007).

Bacterial solvent production has been studied with clostridial species since 1920s (Lutke-Eversloh and Bahl, 2011). Butanol and ethanol produced by *Clostridium acetobutylicum* through acetone, *n*-butanol and ethanol (ABE) fermentation can be used for biofuel purposes. ABE fermentation has been widely studied, and has been recently reviewed in the literature (Lutke-Eversloh and Bahl, 2011; Jurgens et al., 2012; Jang et al., 2012). Traditional batch fermentation has limitations regarding low cell density, low volumetric productivity, high down times, nutritional limitations, and product inhibition (Survase et al., 2012). Thus, continuous process approach could be more attractive for industrial purposes. Different

* Corresponding author. Tel.: +358 504160139.
E-mail address: janne.wallenius@aalto.fi (J. Wallenius).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.05.085

continuous process approaches with immobilized cells have been widely studied (Survase et al., 2012; Qureshi and Maddox, 1995; Qureshi et al., 2000; Zhang et al., 2009; Napoli et al., 2010).

In the literature, there exist multiple constraint-based genome-scale metabolic models for clostridial species, e.g., for *C. acetobutylicum* presented by Senger and Papoutsakis (2008a,b), Lee et al. (2008), and McAnulty et al. (2012), for *Clostridium beijerinckii* by Milne et al. (2011), for *Clostridium thermocellum* by Roberts et al. (2010), and for *C. cellulolyticum* by Salimi et al. (2010). *C. acetobutylicum* has two distinct product formation phases. In the acidogenic phase acetate and butyrate are mainly produced while in the solventogenic phase acetone, butanol, and ethanol are produced. These phases have been modeled separately by Lee et al. (2008) and by using specific proton flux by Senger and Papoutsakis (2008b).

In this paper, we report a constraint-based genome-scale metabolic model for *C. acetobutylicum* adapted to predict quantitatively solvent and acid output from continuous ABE fermentation using immobilized cells. There are no previous reports on modeling a continuous process with a genome-scale constraint-based approach to authors' knowledge. In the presented model no information about the cell dry weight is needed. This is a major advantage of the method as it is difficult to obtain reliable data on the cell dry weight from cultivation with immobilized cells. Also a novel step-wise optimization procedure is presented for flux balance analysis (FBA).

2. Methods

2.1. Outline of the model

A genome-scale model for *C. acetobutylicum* DSM 792 was expanded from the previously published model by Senger and Papoutsakis (2008a,b). The model contains 444 metabolites involved in 592 reactions, including 87 transport reactions, and 7 flux ratio based constraints (McAnulty et al., 2012). The new reactions added to the original Senger and Papoutsakis model included transport and utilization reactions for the substrates used in the experimental case. These reactions were obtained from the KEGG database (Kanehisa and Goto, 2000). Reactions for bifurcated TCA cycle were added according to the study by Amador-Noguez et al. (2010) but the fluxes through reductive TCA cycle, i.e., via malate and fumarate were closed for the solventogenesis modeling (Amador-Noguez et al., 2011). Also reactions were added to enable glycine formation from threonine instead of serine and isoleucine formation through citralamate pathway (Amador-Noguez et al., 2011). 30 reactions were added to the Senger and Papoutsakis model excluding the transport reactions. The extreme pathways (Price et al., 2002) which resulted to unnatural ATP generation were manually curated setting fluxes to zero according to Senger and Papoutsakis model (Senger and Papoutsakis, 2008b) when necessary. The equation for biomass formation was adopted from Senger and Papoutsakis model (2008b). The metabolic network of the applied model is included as appendix A in SBML format.

2.2. Experimental data

The experimental data was gathered from continuous ABE fermentations with immobilized cells as described by Survase et al. (2012). The whole data set of 25 experiments used for the model simulations consists of the results from four continuous fermentation runs (unpublished results).

2.2.1. Preparations and operation

C. acetobutylicum DSM 792 was activated from sporulated cells and further inoculated as described by Survase et al. (2012) with the exception of having two consecutive pre-cultivations instead of one. The first pre-cultivation step was carried out for 20 h at 37 °C in a 100 mL sterile reinforced clostridial medium (RCM) in 125 mL air-tight flasks. For the second pre-cultivation step 40 mL of the first pre-cultivation was transferred into 400 ml of fresh RCM in 500 mL flask and incubated for another 20 h at 37 °C. Both pre-cultivation media were treated similarly. Glucose concentration of 30 g L^{-1} was used for the pre-cultivations instead of 5 g L^{-1} used by Survase et al. (2012). The production medium was prepared and treated as described by Survase et al. (2012) with the exception that different mixtures of mannose, xylose, and glucose were used instead of 60 g L^{-1} glucose. A few drops of Struktol J647 (Schill+Seilacher, Germany) were added to prevent the foaming of the media. The immobilization column reactor was prepared as reported by Survase et al. (2012). The wood pulp was evenly rolled in polyethylene mesh. The column was sterilized for 48 h with 67% ethanol and washed with sterile water before inoculation. Cell adsorption and column operation was carried out as reported by Survase et al. (2012).

2.2.2. Sampling and analyses

Samples were taken from the top of the column as triplicates. Between the samplings the total flow through the system was at least three times the column void volume to ensure the steady state. Samples were centrifuged with Eppendorf 5424 centrifuge (Germany) at 14,850 rpm for 5 min. The supernatant was diluted 1:10 with Milli-Q water. These diluted samples were used for the analysis of the substrates and products.

The solvent concentrations were measured by using gas chromatography with the same device specifications as described by Survase et al. (2012). Glucose and xylose concentrations were quantified by high performance liquid chromatography (WATERS 2695 Alliance, USA) with an Aminex HPX-87P column (Bio-Rad, USA) packed with Pb^{2+}, 300·7.8 mm particles. The column was heated at 70 °C, and the eluent (water) flow rate was 0.60 mL min^{-1}. A refractive index detector (WATERS 2414, USA) was used for the quantification. Residual mannose in the production samples was quantified by using high performance anion exchange chromatography (Dionex ICS-3000, USA) with a Dionex Carbopac PA20 column. The column was at 20 °C, with eluent (water) flow rate of 0.40 mL min^{-1}. Injection volume of 20 μl was used and a pulsed electrochemical detector (Dionex, USA) was used for the quantification. The relevant bioprocess parameters were calculated as described by Survase et al. (2012).

2.3. Model constraints

The model was used to simulate continuous ABE fermentation with immobilized cells. Considering the difficulty to obtain data on the amount of cell mass in the column the unit used for the fluxes in the model was mmol/h instead of the more commonly used mmol/g CDW/h. This was based on the chemostat steady state assumption, i.e., with respect to growth and product formation the amount of active cells remains the same at each dilution rate within an experiment. At each dilution rate, the substrate consumption and product formation was allowed to reach steady state. However, because of the missing data on the amount of cell mass the flux constraints in the model for a minimal medium by Senger and Papoutsakis (2008b) could not be directly utilized. Instead the fluxes were constrained to be open or closed. The open fluxes were set to 1000 or −1000 depending on the direction of the reaction. The closed fluxes were set to 0. Flux constraints from 137 reactions were altered in this manner compared to the model by Senger and Papoutsakis (2008b) excluding the transport reactions. The constraints for the measured substrates were set the same as the actual flux of a substrate in an experiment. The actual