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Metabolic characterization and modeling of fermentation process of an engineered *Geobacillus thermoglucosidasius* strain for bioethanol production with gas stripping

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HIGHLIGHTS

- Metabolic flux distributions are visualized by dynamic metabolic flux analysis.
- Extreme pathway analysis reduces the network into a five-macro-reaction scheme.
- A dynamic model is established according to the scheme.
- The model is validated and global sensitivity analysis is performed.
- Model predicts chemostat offers both higher ethanol productivity and higher yield.

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ABSTRACT

The recently engineered *Geobacillus thermoglucosidasius* strain is an industrially potent thermophilic ethanologen. We employ a systematic approach to improve our understanding of the fermentation process using cellobiose as the substrate. Dynamic metabolic flux analysis clearly shows that the fluxes from pyruvate to lactate and formate are both strictly constrained throughout the process and that both the maximum ethanol yield (0.46C/C) and the maximum specific productivity (19.8 mmol C $(g\ DCW)^{-1}\ h^{-1}$) occur at late-exponential growth phase. Accordingly, extreme pathway analysis reduces the metabolic network into a macro reaction scheme, on which a dynamic metabolic model is built. The model is validated with experimental data, parameters are identified with confidence intervals, and global sensitivity analysis (Sobol' method) is performed. Model-based optimization predicts that ethanol productivity could increase from 34.2 in a typical batch process to 55.3 mmol $L^{-1}\ h^{-1}$ in an optimum fed-batch process with higher ethanol yield. Furthermore, the optimal operating regime was identified to be continuous fermentation process with gas stripping, in which a high ethanol productivity of 113 mmol $L^{-1}\ h^{-1}$, *i.e.*, 26.8 mmol C $(g\ DCW)^{-1}\ h^{-1}$, corresponding to 90.2% of the maximum theoretical ethanol yield could be achieved.

1. Introduction

An efficiently operated biorefinery using cellulosic substrates, the so-called second-generation biorefining, would be able to deliver an 80% reduction in greenhouse gas emissions compared to its fossil fuel equivalent based on ethanol production (Wertz and Bédué, 2013). Cellulosic biorefineries for ethanol production using microbial fermentation have been studied extensively. By comparison, growing at a lower optimum temperature, one commonly used organism *Saccharomyces cerevisiae* (Antoni et al., 2007) cannot naturally ferment C5 sugars, a major component of lignocellulosic biomass; another often used organism, *Clostridium thermosaccharolyticum* (Lin and Tanaka, 2006), is a strict anaerobe

Abbreviations: AcCoA, Acetyl CoA; Ace, acetate; aKG, alpha-ketoglutarate; Cit, citrate; E4P, erythrose 4-phosphate; Eth, ethanol; F6P, fructose 6-phosphate; Fom, formate; Fum, fumarate; Glc, glucose; G6P, glucose 6-phosphate; Lac, lactate; Mal, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglyceric acid; Pyr, pyruvate; R5P, ribose 5-phosphate; Rul5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate; Suc, succinate; T3P, glyceraldehyde 3-phosphate; TCA, tricarboxylic acid cycle.

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with lower growth rate and cell yield, and usually does not tolerate high ethanol concentrations (Fong et al., 2006). A recently engineered *Geobacillus thermoglucosidasius* organism (Cripps et al., 2009) has been demonstrated to be a potent thermophilic ethanologen (and a potential producer of other bio-based chemicals) with significant advantages over widely used microorganisms, such as *S. cerevisiae* and *C. thermosaccharolyticum*. First, the thermophilic organism grows optimally at higher temperatures (50–70 °C), which promotes higher rates of feedstock conversion and reduces risk of contamination and cooling costs in fermentation. Second, many glycolytic thermophiles are potentially able to ferment a broader substrate spectrum of polymeric or short oligomeric carbohydrates (Shaw et al., 2008), which is an innate asset for consolidated bioprocess development (Lynd et al., 2002, 2005; Olson et al., 2012). Finally, the higher fermentation temperature facilitates ethanol removal by applying a mild vacuum or using an inert stripping gas, by which the ethanol is vaporized (Ennis et al., 1986; Gong et al., 1999; Taylor et al., 1998), and consequently ethanol inhibition is partly relieved, resulting in higher productivity. This will also produce a stronger "beer" and therefore reduce downstream separation costs.

Although the molecular tools for gene modification have been developed and some fundamental aspects of the biochemistry have been explored (Cripps et al., 2009), the organism obtained is still relatively poorly understood at the level of the fermentation process, *i.e.*, how it behaves under complex fermentation conditions. Additionally, in order to exploit its potential, metabolic characterization and dynamic modeling are prerequisites for process optimization, especially for a process with continuous ethanol recovery via *in situ* gas stripping (Matthias Hild, 1998).

In this work, besides off-line measurements of metabolites, O_2, CO_2, and gaseous ethanol concentrations in effluent gas have been monitored online, providing dynamic information about the metabolic regulation of the organism during the fermentation process. To analyze these data, we have applied recently developed methods (Niu et al., 2012, 2013) for metabolic model building, model validation and model-based optimization (as shown in Appendix A). These involve dynamic metabolic flux analysis to understand the metabolic regulation under different fermentation/physiological conditions, extreme pathway analysis to systematically reduce the metabolic network and generate a macroscopic reaction scheme (as a result, no intercellular measurements are required and the complexity of dynamic metabolic flux balance model is systematically and significantly reduced) for control design, and process optimization based on the previously obtained model.

2. Materials and methods

2.1. Strain and fermentation

The engineered *G. thermoglucosidasius* strain TM242 (*ldh, pfl, pdh*^{up}) was grown in a 1.6 L Braun Biostat® B plus fermenter (Sartorius-Stedim UK, Surrey) with 1.0 L modified urea sulfate medium (USM) containing 12% cellobiose, 2%(w/v) yeast extract, 25 mmol/L NaH_2PO_4, 50 mmol/L urea, 25 mmol/L K_2SO_4, 5 mmol/L citric acid, 3 mmol/L $MgSO_4$, 50 mmol/L $CaCl_2$, 0.3 mmol/L biotin and 12.5 ml/L trace-element solution (60 mmol/L·H_2SO_4, 1.44 g/L $MnSO_4{\cdot}7H_2O$, 5.56 g/L $FeSO_4{\cdot}7H_2O$, 1.69 g/L $MnSO_4{\cdot}H_2O$, 0.25 g/L $CuSO_4{\cdot}5H_2O$, 0.56 g/L $CoSO_4{\cdot}7H_2O$, 0.06 g/L H_3BO_3, 0.89 g/L $NiSO_4{\cdot}6H_2O$).

The fermenter was inoculated with 100 ml of a mid-log phase seed (OD_{600}=2). Temperature, pH, and aeration flow rate were maintained at 60 °C, 6.65 ± 0.2, and 1.0 l/min during both aerobic and anaerobic processes. Aerobic growth was switched to anaerobic fermentation at 4 h and 100% air flow was replaced with 0.2 l/min air and 0.8 l/min nitrogen. Samples were taken regularly. All experimental work including sample analysis was carried out by TMO Renewables (U.K.).

2.2. Analytical methods

Cell concentration was monitored by measuring the optical density at 600 nm (OD_{600}). Dry cell weight (DCW) was determined and an OD_{600} value of 1 corresponds to 0.39 g DCW/L. The Ash content of biomass was determined to be 7% (w/w). The typical elemental composition of *Bacillus subtilis* was cited (Dauner et al., 2001a, 2001b) to represent that of *G. thermoglucosidasius*, *i.e.*, $CH_{1.608}O_{0.364}N_{0.235}$.

Cellobiose, organic acids (acetate, citrate, formate, fumarate, lactate, pyruvate, and succinate) and ethanol were quantified in clarified culture samples by HPLC using a Hewlett Packard 1100 system. Detection was done by UV 215 nm (organic acids) and refractive index (sugars and ethanol using a Knauer Well ChromK-2301RI detector). Further details were as follows: column, Nucleogel Sugar 810H (Macherey Nagel); flow rate, 0.6 mL/min; temperature, 30 °C; mobile phase, 0.01 N H_2SO_4; 10 mL injection; run time, 27 min.

2.3. Exhaust gas analysis

The fermenter was equipped with a tandem CO_2 and O_2 gas analyzer (Magellan Instruments, Middlesex, UK). The exhaust gas was more extensively analyzed, including ethanol content, using a VG Prima δB mass spectrophotometer (Thermo Electron Corporation) linked to a fermentation off-gas management system (Thyson Technology Ltd., Ellesmere Port, UK), which ensured that all the ethanol in the off-gas remained in the vapor phase. OUR, CER and EthTR were calculated on line (Niu et al., 2012).

2.4. Determination of measured extracellular flux $\boldsymbol{f}_{extra}$

Hereinafter, a variable with a bar denotes the mean value of measurements while a symbol with a circumflex accent represents an estimate. To smooth raw data, cumulative production/consumption was calculated for polynomial regression over time. The cumulative production of biomass at sampling time point t_{k+1} was:

$$Cum_X = \overline{X}_{t_{k+1}} V_{t_{k+1}} - \overline{X}_{t_1} V_{t_1} \tag{1}$$

Similarly, the cumulative consumption of substrates or production of products is defined as:

$$Cum_{C_i} = \sum_{j=1}^{k} [\overline{C}_{i,t_{j+1}} V_{t_{j+1}} - \overline{C}_{i,t_j} V_{t_j} - F(t_{j+1} - t_j) C_{i,\mathrm{in}}] \tag{2}$$

Then, the first time derivative of the polynomial regression function ($fun(Cum_X)$ or $fun(Cum_{S_i})$) was taken, and was further divided by the corresponding biomass to estimate the flux, *i.e.*,

$$\mu = \frac{dfun(Cum_X)/dt}{XV_t}, f_{C_i} = \frac{dfun(Cum_{C_i})/dt}{XV_t} \tag{3}$$

Totally extracellular fluxes of 12 ($\boldsymbol{f}_{\mathrm{extra}} \in \Re^{12\times 1}$) and 11 ($\boldsymbol{f}_{\mathrm{extra}} \in \Re^{11\times 1}$) species were determined based on measurements during aerobic and anaerobic phases, respectively. For details, please refer to the worksheets of Supplementary Material 2. The flux was positive when associated with a component that was produced or negative when it was consumed.