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Autonomous production of 1,4-butanediol via a *de novo* biosynthesis pathway in engineered *Escherichia coli*

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ABSTRACT

1,4-Butanediol (BD) is an important chemical that is widely used in industry with an annual demand of one million metric tons. Here we report a modular development of engineered bacteria for successful BD bio-production. Using a systems engineering concept, we partitioned our development into two parts: namely BD biosynthesis and production control. The former was implemented through a *de novo* pathway that functions as an enzymatic reactor, while the latter was accomplished via synthetic circuits serving as genetic controllers. To facilitate development, the carbon utilizations were also partitioned into two routes. D-xylose was exclusively designated for BD production with other carbon sources utilized for cellular growth. Additionally, a quorum-sensing mechanism was exploited for production control, and the resulting strain was capable of autonomous production of BD. This study represents an example of the synergy between synthetic biology and metabolic engineering, affirming the need for deeper integration of the two fields.

1. Introduction

The development of metabolically engineered microorganisms represents an attractive solution to the growing demand for consumables, the declining availability of fossil fuels, and the increasing concerns for the environment (Lee et al., 2008; Zhang et al., 2011; Lee, 2012; Butterbach-Bahl and Kiese, 2013). Over the last two decades, an extraordinarily wide spectrum of products, including biofuels, bioplastics, and biopharmaceuticals, have been manufactured from renewable biomass by engineered microbes (Niu et al., 2003; Atsumi et al., 2008; Zhang et al., 2008; Celinska, 2010; Brat et al., 2012; Felnagle et al., 2012). With the emergence of synthetic biology, systems biology, and evolutionary engineering, metabolic engineering has lately undergone a paradigm shift from the construction and optimization of individual pathways to tuning entire metabolic networks at the systems level (Nielsen and Keasling, 2011; Keasling, 2012; Nielsen and Pronk, 2012; Nielsen et al., 2014; Xu et al., 2014b). This transformation has enabled more efficient and cheaper manufacturing of existing bio-products, and the successful production of new biomass based-compounds. One such example is the bacterial synthesis of 1,4-butanediol (Yim et al., 2011).

1,4-Butanediol (BD) is an important commodity chemical that is widely used in industry for production of plastics, elastic fibers, polyurethanes and pharmaceuticals. Its annual demand is about one million metric tons. However, there is no known microorganism on the planet that naturally produces BD. It could only be manufactured from petroleum-based feedstocks such as acetylene, propylene, and butadiene until the recent work by Yim et al. (2011)—computer aided pathway design was paired with genome-scale metabolic modeling to confer the successful development of BD-producing *Escherichia coli* strains.

One key consensus that emerged through the synergy of metabolic engineering and related disciplines, particularly synthetic biology, is the need for a system-level view of metabolic engineering (Ajikumar et al., 2010; Lee et al., 2011; Rabinovitch-Deere et al., 2013; Paddon and Keasling, 2014). That is, the design, construction, and optimization of biosynthesis pathways can be made more feasible with the adoption of engineering principles, including systems abstraction, part standardization and characterization, and modularity, particularly as system complexity increases (Henry et al., 2010; Lee et al., 2012; Chou and Keasling, 2013; Chaudhary et al., 2013; Lim et al., 2013; Srivastava et al., 2013). In engineering sciences, it is well acknowledged that decomposition of a complex system into multiple parts (modules) can reduce its complexity,

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enable parallel construction and characterization, and accommodate future uncertainty. In addition to modularity, dynamic and fine control of metabolic and genetic networks has also been adopted as a critical system-level methodology for optimal bio-production (Holtz and Keasling, 2010; Mukherjee et al., 2013; Way et al., 2014). Pertinent examples include dynamic response of inhibitory intermediate metabolites (Zhang et al., 2012; Xu et al., 2014a), stress-regulated expression of recombinant proteins (Dragosits et al., 2012; Dahl et al., 2013; Nakashima et al., 2014), and cell density-dependent control of heterologous gene expression (Tsao et al., 2010).

Here we report an alternative solution to microbial BD production inspired by the above systems engineering paradigm. As shown in Fig. 1a, we argue that any metabolically engineered system, including those for BD biosynthesis, can be partitioned into two parts according to desired functionality: An enzymatic reactor, responsible for the conversion of substrates and intermediates to the desired product (BD in this case), and genetic controllers, responsible for the control of the enzymatic reactor in a dynamic and programmable fashion. Meanwhile, in terms of resource consumption, we can designate cellular chassis and engineered pathways, the two key parts of a cell factory, into two independent carbon-utilization routes, which will minimize the crosstalk between the two parts for simplification of

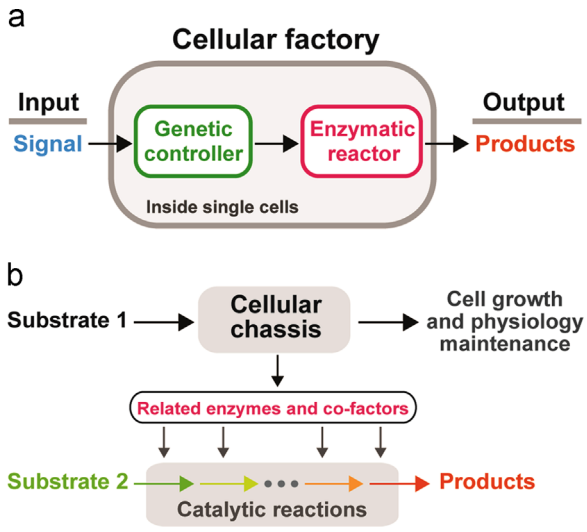

Fig. 1. A conceptual, system-level design of a cellular factory: (a) partition of a cell factory into two functional parts: the enzymatic reactor module and the genetic controller module. (b) Partition of carbon source utilization into two parallel routes with one for designed pathway and one for cellular chassis maintenance.

Table 1
Strains used in this study.

Strain	Function/characteristic	Resource/reference
W3110	*E. coli W3110* F$^-$, λ^- IN (rrnD-rrnE)1	ATCC no. 27325
EWH	*E. coli W3110* Δ*xylA*Δ*yjhH*Δ*yagE*	This work
XH	EWH/*pTrc-xdh-hvo*	This work
XX	EWH/*pTrc-xdh-xylX*	This work
K	EWH/*pTrc-kivD*	This work
M	EWH/*pTrc-mdlC*	This work
XHK	EWH/*pTrc-xdh-hvo, pTrc-kivD*	This work
XHM	EWH/*pTrc-xdh-hvo, pTrc-mdlC*	This work
XXK	EWH/*pTrc-xdh-xylX, pTrc-kivD*	This work
XXM	EWH/*pTrc-xdh-xylX, pTrc-mdlC*	This work
EWC1	EWH/*pTrc-GFP*	This work
EWC2	EWH/*pLux-GFP*	This work
EWC3	EWH/*LuxI-pLux-GFP*	This work
EWCB1	EWH/*pTrc-xdh-xylX-mdlC*	This work
EWCB2	EWH/*pLux-xdh-xylX-mdlC*	This work
EWCB3	EWH/*LuxI-pLux-xdh-xylX-mdlC*	This work

construction and optimization of each (Fig. 1b). Such a division of tasks and recourses may expedite the process of pathway engineering, confer dynamic (even autonomous) control of BD biosynthesis, and also offer a higher theoretical yield.

To realize our conceptual cell factory, we first designed a *de novo* pathway that is capable of BD production from D-xylose. We then assembled enzyme genes through different combinations to build an optimal version of the pathway that serves as the core of the enzymatic reactor module. A cellular chassis was concurrently built to orthogonalize carbon fluxes between the *de novo* pathway and cellular central metabolism. Subsequently, we constructed three genetic circuits to enable inducible and self-regulated control of gene expression by using green fluorescence protein (GFP) as a reporter. We finally integrated the genetic controllers with the identified enzymatic reactor as a complete, metabolically engineered system for autonomous BD bio-production.

2. Materials and methods

2.1. Strains

E. coli W3110 was purchased from ATCC (No. 27325). *E. coli W3110*Δ*xylA*Δ*yagE*Δ*yjhH* was constructed from *E. coli* W3110 by knocking out the genes *xylA*, *yagE* and *yjhH*. For gene deletion, the plasmid pKD3 was used as the template for amplification of disruption cassettes, the plasmid pKD46 was used as the Red recombinase expression vector, and pCP20 was used for eliminating the antibiotic resistance gene. All strains used in this study are listed in Table 1.

2.2. Plasmid construction for the enzymatic reactor module

The codon-optimized sequences of *xdh*, *xylX*, *hvo*, *mdlC* and *kivD* were synthesized. To construct the plasmids *pTrc-xdh-hvo* and *pTrc-xdh-xylX*, *xdh* was first inserted into *pKM212* at the *EcoRI* and *BamHI* restriction sites to generate *pTrc-xdh*, followed by the insertion of *hvo* and *xylX* individually at the *sbfI* and *HindIII* sites. *pTrc-xdh-hvo* and *pTrc-xdh-xylX* were subsequently transformed into *E. coli W3110*Δ*xylA*Δ*yagE*Δ*yjhH* via electroporation to generate the XH and XX (Table 1) strains respectively. To construct the plasmids *pTrc-mdlC* and *pTrc-kivD*, *mdlC* and *kivD* were individually ligated into *pTrcHis2A* by using *BamHI and EcoRI*. The four strains XHK, XXK, XHM, and XXM were obtained by transforming the plasmids *pTrc-mdlC* and *pTrc-kivD* into *E. coli W3110*Δ*xylA*Δ*yagE*Δ*yjhH* containing *pTrc-xdh-hvo* and *pTrc-xdh-xylX* (Table 1).

2.3. Plasmid construction for the genetic controller module

To construct controller 1, the *GFP-ColE1-Kan* fragment from plasmid *pTD103LuxI-GFP* (Danino et al., 2010) and the *LacI-pTrc* fragment from plasmid *pTrcHis2A* were amplified by PCR and further assembled together using Gibson assembly method (Gibson et al., 2009). The resulted plasmid was denoted as *pTrc-GFP*. Controller 2 was constructed by digesting *pTD103LuxI-GFP* using the restriction enzyme *AvaI* to remove the AHL producing gene *luxI* and then ligating the remaining fragment, generating the plasmid *pLuxI-GFP*. For controller 3, we directly utilized the intact *pTD103LuxI-GFP* plasmid.

2.4. Plasmid construction for the integration of the two modules

The *xdh-xylX* fragment of *pTrc-xdh-xylX* was amplified and ligated into the plasmid *pTrc-mdlC* via the *NcoI* site to generate the plasmid *pTrcHis2A-xdh-xylX-mdlC*. Here, all of the three genes share an identical RBS-spacer sequence. Meanwhile, we replaced the *GFP* gene in controller 1–3 with the *xdh-xylX-mdlC* fragment