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Improvement of glucaric acid production in *E. coli* via dynamic control of metabolic fluxes

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ABSTRACT

D-glucaric acid can be used as a building block for biopolymers as well as in the formulation of detergents and corrosion inhibitors. A biosynthetic route for production in *Escherichia coli* has been developed (Moon et al., 2009), but previous work with the glucaric acid pathway has indicated that competition with endogenous metabolism may limit carbon flux into the pathway. Our group has recently developed an *E. coli* strain where phosphofructokinase (Pfk) activity can be dynamically controlled and demonstrated its use for improving yields and titers of the glucaric acid precursor *myo*-inositol on glucose minimal medium. In this work, we have explored the further applicability of this strain for glucaric acid production in a supplemented medium more relevant for scale-up studies, both under batch conditions and with glucose feeding via *in situ* enzymatic starch hydrolysis. It was found that glucaric acid titers could be improved by up to 42% with appropriately timed knockdown of Pfk activity during glucose feeding. The glucose feeding protocol could also be used for reduction of acetate production in the wild type and modified *E. coli* strains.

1. Introduction

D-glucaric acid was identified by the United State Department of Energy as a top value-added chemical for production from biomass (Werpy and Petersen, 2004). It has a number of potential applications including use in biopolymers (Kiely and Chen, 1994) and as a detergent builder and corrosion inhibitor (Smith et al., 2012). Glucaric acid can be produced through nitric acid oxidation of glucose (Mehltretter and Rist, 1953) but a biological route to glucaric acid production could potentially provide several advantages, including mild processing conditions and high selectivity for the product of interest.

Production of D-glucaric acid in *Escherichia coli* was previously demonstrated by our group via expression of heterologous enzymes from three different organisms (Moon et al., 2009). Titers of 1.13 g/L glucaric acid were achieved in strain BL21(DE3) in LB medium supplemented with 10 g/L glucose. Following demonstration of the initial pathway, some increases in glucaric acid titers were achieved through improved strategies for expression of the *myo*-inositol oxygenase (MIOX) enzyme, one of the limiting factors in glucaric acid production in LB supplemented with glucose or *myo*-inositol (Moon et al., 2010; Shiue and Prather, 2014). However, competition for glucose-6-phosphate (G6P) between native *E. coli* enzymes (phosphoglucosisomerase and glucose-6-phosphate dehydrogenase) and the first enzyme in the glucaric acid pathway, *myo*-inositol-1-phosphate synthase (INO1), is also a concern. High level expression of INO1 is required for detectable *myo*-inositol and glucaric acid production, indicating it competes poorly with endogenous metabolism for substrate (Moon et al., 2009). Additionally, the second pathway enzyme, MIOX, appears to be stabilized by its substrate, *myo*-inositol, so more rapid accumulation of *myo*-inositol may help reduce limitations in MIOX

Abbreviations: aTc, anhydrotetracycline; G6P, glucose-6-phosphate; INO1, *myo*-inositol-1-phosphate synthase; IPTG, β-D-1-thiogalactopyranoside; MIOX, *myo*-inositol oxygenase; Pfk, phosphofructokinase

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activity as well (Moon et al., 2010).

With this in mind, our group has explored strategies for development of strains capable of accumulating G6P and directing greater fluxes of this metabolite into production of glucaric acid and *myo*-inositol. By eliminating the pathways for glucose catabolism in the production strain, and feeding alternative carbon sources, higher yields of glucaric acid from glucose could be achieved (Shiue et al., 2015). However, the rate of glucose uptake in this K-12 host strain was quite slow, especially in minimal medium, and its use was limited to mixed sugar substrates.

While gene knockouts provide a static solution for redirecting fluxes in the cell (Kogure et al., 2007; Shiue et al., 2015), under many conditions, it may be advantageous to develop cells where dynamic changes in enzyme levels can be used to switch between substrate consumption for biomass formation and substrate conversion into product. Dynamic control of key enzymes can be used to facilitate more rapid initial accumulation of biomass, overcoming potential reductions in growth rate, and can eliminate the need for supplementation of the medium or addition of secondary carbon sources required with some gene knockouts (Anesiadis et al., 2008; Gadkar et al., 2005). At the desired time, activity of the target enzyme(s) can be reduced through decreasing transcription (Scalcinati et al., 2012; Solomon et al., 2012; Soma et al., 2014) or translation (Williams et al., 2015) of the enzyme, or initiating rapid degradation (Brockman and Prather, 2015; Torella et al., 2013). Coupling such controls with sensors capable of reporting on intracellular metabolite levels allows for the development of more complex systems capable of continuously adjusting enzyme levels to balance metabolite pools or maintain cellular state (Dahl et al., 2013; Farmer and Liao, 2000; Xu et al., 2014; Zhang et al., 2012).

It was recently shown that by inducing degradation of phosphofructokinase I (Pfk-I) activity in the cell, the pools of G6P could be increased during growth on glucose minimal medium, along with the yields and titers of the glucaric acid precursor *myo*-inositol (Brockman and Prather, 2015). In this work, we explore the expanded utility of this system for production of glucaric acid from glucose in a semi-defined medium under batch conditions and a fed-batch condition simulated by glucose release from *in situ* enzymatic starch hydrolysis. To explore the interplay of production conditions with metabolic intervention through Pfk-I degradation, initial screening runs were carried out in 48-well plates in a BioLector benchtop bioreactor. Follow-up experiments were then carried out at altered conditions or altered scale (shake flask) to understand the robustness of the results. Improvements in glucaric acid titer of up to 42% were achieved through appropriately timed induction of Pfk activity knockdown during the fermentation.

2. Materials and methods

2.1. Strains and plasmids

E. coli strains and plasmids used in this study are listed in Table 1. Strains IB1863 and IB1379 were constructed by our group previously (Brockman and Prather, 2015). To eliminate catabolism of glucaric acid and the pathway intermediate glucuronic acid in strain IB1863, knockouts of *gudD* and *uxaC* were carried out via sequential P1 transduction from Keio collection donor strains (Baba et al., 2006). The kanamycin resistance cassette was removed after each transduction via expression of FLP recombinase from pCP20 (Datsenko and Wanner, 2000). The λDE3 lysogen was integrated into this strain using a λDE3 Lysogenization Kit (Novagen, Darmstadt, Germany), generating strain IB1486. To generate the Δ*pfkA* control strain IB2255, serial P1 transductions were also carried out in IB1379 to knock out *pfkA, gudD,* and *uxaC*, and the λDE3 lysogen was integrated as described above. An additional control strain without any degradation tag on *pfkA*, IB2472, was generated using lambda red recombination combined with Cas9-based counterselection (Reisch and Prather, in press). Using this method, the tag sequence was removed from the *pfkA* locus without any addition of antibiotic resistance cassettes or FRT scars. Construction of plasmids for production of glucaric acid, pRSFD-IN-MI and pTrc-udh, was described previously (Moon et al., 2009; Yoon et al., 2009).

2.2. Culture medium and conditions

For plasmid preparation and genetic manipulations, strains were cultured in Luria–Bertani (LB) medium at either 30° or 37 °C. Temperature sensitive plasmids were cured at 42 °C.

Glucaric acid production experiments were carried out in T12 medium containing 7.5 g/L yeast extract, 7.5 g/L soy peptone, 7 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 3 g/L $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 100 μg/ml carbenicillin, 50 μg/ ml kanamycin, and the indicated amount of glucose and/or soluble starch (Sigma-Aldrich S9765) plus amyloglucosidase (Sigma-Aldrich A7095). For experiments in the BioLector (m2p-labs, Baesweiler, Germany), starter cultures were incubated in culture tubes at 30 °C and 250 rpm

Table 1
Strains and plasmids used in this study.

Strain/plasmid	Genotype	Reference/source
Strains		
LG1458	MG1655(DE3) *ΔgudD ΔuxaC*	Prather Lab
IB1863	MG1655 *ΔendA Δzwf ΔpfkB ΔsspB pfkA::114-pfkA(DAS+4) HK022::tetR-Ptet-sspB*	(Brockman and Prather, 2015)
IB1379	MG1655 *ΔendA Δzwf ΔpfkB*	(Brockman and Prather, 2015)
IB1486	MG1655(DE3) *ΔendA Δzwf ΔpfkB ΔsspB pfkA::114-pfkA(DAS+4) HK022::tetR-Ptet-sspB ΔgudD ΔuxaC*	This study
IB2255	MG1655(DE3) *ΔendA Δzwf ΔpfkB ΔpfkA ΔgudD ΔuxaC*	This study
IB2472	MG1655(DE3) *ΔendA Δzwf ΔpfkB ΔsspB pfkA::114-pfkA HK022::tetR-Ptet-sspB ΔgudD ΔuxaC*	This study
JW2758-5	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *ΔgudD785::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	(Baba et al., 2006)
JW3887-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *ΔpfkA775::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	(Baba et al., 2006)
JW3603-2	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *ΔuxaC782::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	(Baba et al., 2006)
IB1486-GA	IB1486 / pRSFD-IN-MI / pTrc-udh	This study
LG1458-GA	LG1458 / pRSFD-IN-MI / pTrc-udh	This study
IB2255-GA	IB2255 / pRSFD-IN-MI / pTrc-udh	This study
IB2472-GA	IB2472 / pRSFD-IN-MI / pTrc-udh	This study
Plasmids		
pCP20	Rep[a], Amp^R, Cm^R, FLP recombinase expressed by λ p_r under control of λ cI857	CGSC #7629
pRSFD-IN-MI	pRSR1030 *ori*, lacI, Kan^R, INO1 (*S. cerevisiae*) and MIOX (*M. musculus*) expressed under control of T7 promoter	(Moon et al., 2009)
pTrc-udh	pBR322 *ori*, lacI, Amp^R, Udh (*P. syringae*) expressed under control of Trc promoter	(Yoon et al., 2009)