Sector: Transforming Biomanufacturing with Synthetic Biology

Use case for Biomanufacturing: Scaling Cell-Free Systems

End Product: Variety of biotherapeutics and molecular and biomolecular products

Organism(s) if applicable: Microbial Cultures

Cell-free systems offer the opportunity to harness the manufacturing potential of cells, without the problems associated with keeping cells alive and the complications of the manufacturing process happening inside a cell membrane or cell wall. Cell-free systems can be used to make therapeutics, sensors, and even educational kits, but scaling these for practical and economical use more broadly poses several technical challenges.

Cell-free systems can greatly expand the reach of biotechnologies to address real-world challenges and enable new biomanufacturing workflows. By leveraging only as much of a cell as is needed for a purpose at hand, cell-free systems are more accessible, more tractable, and arguably safer than cell-based workflows and products. Expanding our capabilities to include scaling up cell-free workflows to volumes that are relevant industrially will go a long way towards realizing the potential for cell-free systems in the future bio-economy.

Desired outcome(s) that stretch current capabilities

- Scale-up practical cell-free workflows to span volumes from approximately 10 µL to 10^3 L
- Products produced at competitive costs relative to existing manufacturing methods, where applicable
- Predictive modeling of cell-free reactions
- Measurement tools and methods for testing models and process control
- Expand cell-free workflows to an increasing number of non-model organisms

Sector: Transforming Biomanufacturing with Synthetic Biology

Use Case for Biomanufacturing: Minimizing Supply Chain Gaps

End Product: Value added products or critical precursors such as butanetriol trinitrate (BTTN)

Organism(s) if applicable: *Escherichia coli* with genes from *Caulobacter crescentus, Pseudomonas putida*

Synthetic Biology can help to mitigate supply chain gaps for critical DoD precursors. For example, synthetic biology can be used to optimize bioproduction of 1,2,4-butanetriol (BT) and butanetriol trinitrate (BTTN). 1,2,4-Butanetriol (BT) is a precursor to butanetriol trinitrate (BTTN) which is a liquid ester that is used in US weapon systems. Research and development to synthesize D, L-1, 2 ,4 – butanetriol (D-BT) using synthetic biology was funded via Office of Naval Research starting in 2004. A successful demonstration o*f Escherichia coli* W3110 produced 10g/L by conversion of D-xylose and then strain was modified to WN13 in order to scale up from 1L to 100 L fermenter working volumes at MBI International. The D-BT was purified using filtration, ion exchange, evaporation, and distillation. Naval Surface Warfare Center then nitrated the "green" D-GT and showed comparable performance metrics. In 2007, "production costs of generating synthetic D, L-1,2,4-butanetriol make scale-up from current capacity of 15, 000 pounds per years to a desired capacity of 180, 000 pounds per year economically infeasible." MBI did achieved 10g/ L BT titers after 60 hours of operation and pilot scale cost was $89.90/lb (2018 dollars), however this was determined to be not economical nor commercially viable. The services have identified a potential performer (Duke University) who is able to produce BT at 62 g/L more than 6-fold improvement as low a cost $11.14 /kg. There is a need to optimize the production as well as the subsequent nitration steps for this critical weapons material. Currently Naval Air Warfare Center has a 6.4 Advance Development program (Basic Science – Demonstration and Validation in realistic operating environment to assess performance and/or cost reduction potential) to replace existing fielded system propellant from current sources.

Desired outcome(s) that stretch current capabilities

- Sustainable low-cost process for the conversion of biomass sugars to BTTN
- Increase in on-demand production of critical precursors, such as BTTN for use in both RDT&E and weapon systems
- Elimination or significant decrease in hazardous waste streams
- Improvements in Separation Technologies which can be applied to wide range of chemicals

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RESEARCH ARTICLE

Engineering in Life Sciences

Coupled biosynthesis and esterification of 1,2,4-butanetriol to simplify its separation from fermentation broth

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1,2,4-Butanetriol (BT) is a valuable chemical with versatile applications in many fields and can be produced through biosynthetic pathways. As a trihydric alcohol, BT possesses good water solubility and is very difficult to separate from fermentation broth, which does complicate the production process and increase the cost. To develop a novel method for BT separation, a biosynthetic pathway for 1,2,4-butanetriol esters with poor water solubility was constructed. Wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase (Atf) from *Acinetobacter baylyi*, *Mycobacterium smegmatis*, and *Escherichia coli* were screened, and the acyltransferase from *A. baylyi* (AtfA) was found to have higher capability. The BT producing strain with AtfA overexpression produced 49.5 mg/L BT oleate in flask cultivation. Through enhancement of acyl-CoA production by overexpression of the acyl-CoA synthetase gene *fadD* and deleting the acyl coenzyme A dehydrogenase gene *fadE*, the production was improved to 64.4 mg/L. Under fed-batch fermentation, the resulting strain produced up to 1.1 g/L BT oleate. This is the first time showed that engineered *E. coli* strains can successfully produce BT esters from xylose and free fatty acids.

KEYWORDS
1,2,4-butanetriol esters, acyltransferase, biosynthesis, separation, trihydric alcohol

1 | INTRODUCTION

1,2,4-Butanetriol (BT) is a valuable fine chemical that has versatile applications in many fields. For instance, BT can be used as raw material for making polyurethane foams with better elastic properties [1], and potential precursor for the synthesis of various pharmaceuticals [2]. BT is most widely applied to the production of 1,2,4-butanetriol trinitrate, an energetic plasticizer to replace nitroglycerin [3]. BT synthesis has aroused much interest as its potential applications.

Traditionally, BT is mainly manufactured through chemical routes using malate as the starting material [4,5]. However, the chemical synthesis is not competitive because of the harsh reaction conditions and poor selectivity. And $NaBH_4$ is required as the reducing agent resulting with plenty of borate salts generated [6]. In recent years, BT was successfully produced from cheap sugars through biological pathways [3,7,8], which possesses mild conditions and can reduce the environmental pollution compared to the traditional petrochemical routes. A patent claimed that 18 g/L BT could be achieved from xylose in a single strain with a yield of 0.55 mol/mol [9]. However, no reports were focused on its separation so far.

As a polyol, BT has three hydrophilic hydroxyl groups, which means good water solubility and very difficult to separate from water. Many methods were studied on the separation of polyol including evaporation, distillation, membrane

Abbreviations: Atf, wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase; BT, 1,2,4-butanetriol; FFA, free fatty acid; IPTG, isopropyl-β-D-thiogalactopyranoside; TAG, triglyceride

filtration, ion exchange chromatography, and extraction. However, no single method proved simple, efficient, and low cost.

Among these methods, liquid–liquid extraction can be easy to scale-up and has low energy consumption, but it is difficult to find an effective solvent for polyols [10]. The distillation method operates easily, but it consumes too much energy, especially the azeotropic phenomenon exists between water and polyols, which increases production cost. It is estimated that the separation of the polyols from fermentation broth makes about 50–70% of the total costs in their whole production process [11]. Salting-out is another alternative separation method, which was investigated on the recovery of 1,3-propanediol from fermentation broth [10]. Although the yield achieved more than 90%, much salt needed to add as salting-out agent.

Esters, usually derived from carboxylic acid and alcohol, confer poor water-solubility and can be easily separated from fermentation broth. Some previous studies have focused on the extraction and purification of lactic acid by coupling esterification and hydrolysis [12–14]. In these researches, lactate or ammonium lactate was esterified to methanol, ethanol, or butanol, following hydrolysis of the purified ester into lactate again to extract and purify. Both the yield and the purity reached about 90%, and no calcium salts generated. It seemed that the esterification and hydrolysis method was much more desirable compared with the conventional process, and actually inspired us to develop a similar method to recovery BT. The purpose of this work is to develop a biosynthetic pathway for 1,2,4-butanetriol esters production. We firstly introduced different wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase to a BT producing strain to esterify BT. To improve the acyl-CoA as the ester precursor, the *fadE* gene encoding acyl coenzyme A dehydrogenase was deleted to block fatty acid β-oxidation, and *fadD* gene encoding acyl-CoA synthetase was overexpressed. The key enzymes *glpK*, *plsB*, and *pgpB* of triglyceride (TAG) biosynthesis were finally over-expressed to check whether it can enhance the BT esters production. The entire metabolic pathway used in this study is proposed in Fig. 1. The engineered strain cultivation was performed in fed-batch fermentation to evaluate the potential for large-scale production. This research will provide a new idea for the separation of polyol production from fermentation broth.

PRACTICAL APPLICATION

Due to good water solubility, polyhydric alcohols are very difficult to separate from fermentation broths. As a trihydric alcohol with versatile applications, 1,2,4-butanetriol can be produced by biological fermentation with good yield. However, no research was focused on its separation so far which hamper its industrialization promotion. In this study, BT esters with poor water-solubility was successfully produced by recombinant strains from xylose and oleate for the first time, and the fed-batch fermentation showed a promising prospect for the large-scale production. With further research, BT can be recovered more easily and cost-effective by centrifugation coupling with hydrolysis. The present study not only proved that BT esters can be produced through biosynthetic pathway but also provided an alternative method for trihydric alcohol separation from fermentation broth.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and plasmids construction

The strains and plasmids used in this study were presented in Table 1, and the primers used for plasmids construction for gene cloning plasmid maintenance, and *E. coli* BL21 star (DE3) was used as the host strain for gene expression. The genes *xylAB* deleted strain *E. coli* BL21 star (DE3) Δ*xylAB* was constructed in our previous study [15]. The chromosomal *fadE* gene encoding acyl coenzyme A dehydrogenase was knocked out using the λ-Red recombination method resulting strain *E. coli* BL21 star (DE3) Δ *xylAB* Δ *fadE*.

The plasmid pE-*mdlC-xylBC* and pA-*adhP-yjhG* were constructed in our previous study [8]. The wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase from *Acinetobacter baylyi* (*atfA*), and *Mycobacterium smegmatis* (*atfM*) were codon optimized and chemically synthesized. The *atfE* gene amplified from *Escherichia coli*, *atfA*, and *atfM* were cloned into pA-*adhP-yjhG* between *EcoRI* and *SalI* sites to construct plasmids pA-*adhP-atfE-yjhG*, pA-*adhP-atfA-yjhG*, and pA-*adhP-atfM-yjhG*, respectively. All the *glpK*, *plsB*, *pgpB*, and *fadD* genes were PCR amplified from *E. coli* BL21. The *fadD* was cloned into pCOLADuet-1 vector between *AflII* and *KpnI* sites to generate pC-*fadD*, and the *glpK* was cloned into pC-*fadD* between *EcoRI* and *SacI* sites to create pC-*glpK-fadD*. The *plsB* was cloned into pC-*glpK-fadD* between *SacI* and *SalI* sites to obtain pC-*glpK-plsB-fadD*. Finally, the *pgpB* was amplified along with T7 promoter and then cloned into pC-*glpK-plsB-fadD* between *SalI* and *AflII* sites to get pC-*glpK-plsB-pgpB-fadD*. The resulting plasmid was verified by colony PCR, and nucleotide sequenc-

and allele verification were listed in Supporting Information Table 1. *E. coli* DH5α purchased from Invitrogen was used ing. And all the genes used in this study was listed in Supporting Information Table 2.

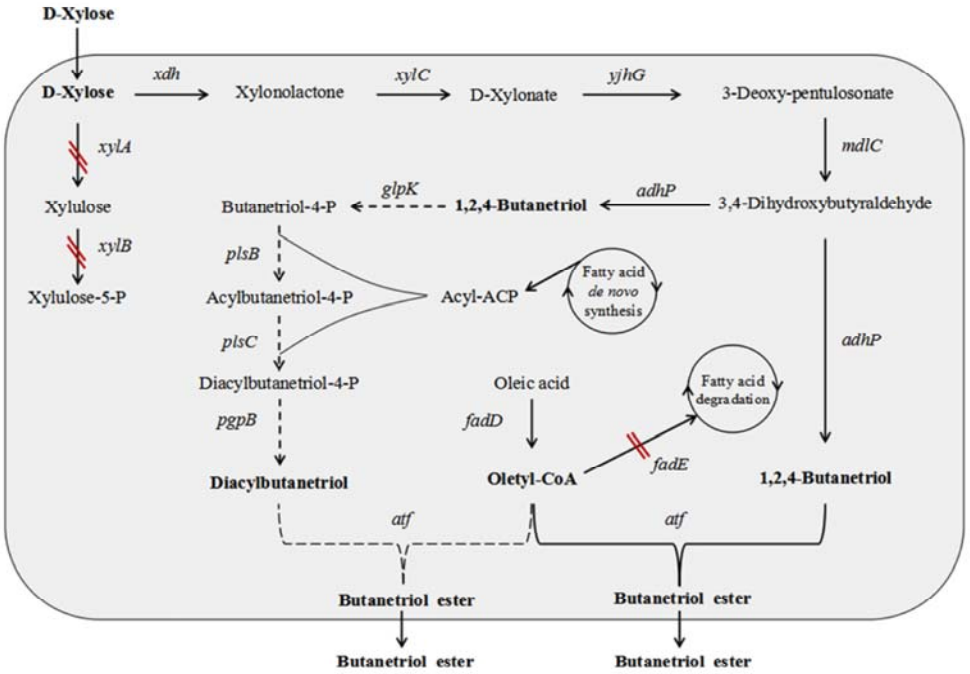

FIGURE 1 The metabolic pathway from xylose and oleate in engineered *E. coli* to BT oleate. The solid lines are the pathway used in this study for BT esters production, and the dashed lines are the presumed pathway. Genes: *xdh*, xylose dehydrogenase; *xylC*, xylonolactonase; *yjhG*, xylonate dehydratase; *mdlc*, 2-keto acid decarboxylase; *adhp*, aldehyde reductase; *xylA*, xylose isomerase; *xylB*, xylulose kinase; *glpK*, glycerol kinase; *plsB*, glycerol-3-phosphate acyltransferase; *plsC*, 1-acyl glycerol-3-phosphateacyltransferase; *pgpB*, diacylglycerol-3- phosphate phosphatase; *fadD*, acyl-CoA synthetase; *fadE*, coenzyme A dehydrogenase; atf, wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase.

2.2 | Protein expression and gel electrophoresis analysis

To check the expression of the recombinant proteins, single colonies of *E. coli* BL21 (DE3) star harboring different recombinant plasmids were cultured in LB medium containing appropriate antibiotics at 37°C overnight and then diluted 1:100 into fresh LB medium and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD_{600} of 0.6~0.8. The cells were collected from 10 mL bacteria cultures 4h after induction and washed with phosphate buffer (pH 6.8). The washed cells were suspended in 1 mL buffer and subjected to ultrasonication. The cell lysates were centrifuged and the supernatant was analyzed by SDS-PAGE [16].

2.3 | Shake flask cultivation

To evaluate the ester production using different engineered strains, shake flask cultivation was carried out with 100 mL of liquid LB medium in 250 mL nonbaffled flasks with appropriate antibiotics. The strains were inoculated to the medium and incubated in an orbital incubation shaker at 37°C with 180 rpm. A 0.5 mM IPTG was added into the medium to induce the enzymes expression when the cells reached about 0.6 OD_{600}. 20 g/L xylose was added for BT production and 2 g/L sodium oleate was added as precursor for esters. After induction, the temperature was set at 30°C for further 24 h cultivation.

2.4 | Fed-batch fermentation

For large-scale production, fed-batch fermentation was carried out in a Biostat B plus MO5L bioreactor (Sartorius Stedim Biotech GmbH, Germany) containing 2 L growth medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 5 g/L $K_2HPO_4 \cdot 3H_2O$, 0.12 g/L $MgSO_4$, 0.1 g/L of thiamine hydrochloride and 1 mL trace elements per liter [8]). Two hundred milliliters of overnight seed culture was inoculated into the fermentor to start the fermentation at 37°C. During the fermentation, sterilized air was supplied at 1 vvm and ammonia was added automatically to control the pH 7.0. The agitation speed was set at 400 rpm and then associated with the dissolved oxygen to maintain the concentration at 20% saturation. Fed-batch mode was commenced by feeding 5 × LB when the dissolved oxygen increased. When the cell density reached to an OD_{600} of 10, the recombinant proteins were induced by 0.5 mM IPTG, 20 g/L xylose and 10 g/L sodium oleate were added for BT esters production. 10 ml of the fermentation broth was withdrawn at intervals to determine cell density and products.

LE 1 Strains and plasmids used in this study

ins or plasmids	Genotype/description	Source
ns		
li DH5α	F⁻ *recA endA1 Φ80dlacZΔM15 hsdR17*(r_K^- m_K^+) λ^-	Invitrogen
li BL21 star (DE3)	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm rne131* (DE3)	Invitrogen
li BL21 star (DE3) Δ *xylAB*	Knockout of *xylA* and *EcxylB* encoding xylose isomerase and xylulose kinase	Ref. 15
li BL21 star (DE3) Δ *xylAB* Δ *fadE*	Knockout of *xylA*, *EcxylB* and *fadE* encoding xylose isomerase, xylulose kinase and acyl-CoA dehydrogenase	This study
45	*E. coli* BL21 star (DE3)Δ *xylAB* / pACYCDuet-*adhP-atfA-yjhG* & pETDuet-*mdlC-xylBC*	This study
46	*E. coli* BL21 star (DE3)Δ *xylAB* / pACYCDuet-*adhP-atfE-yjhG* & pETDuet -*mdlC-xylBC*	This study
47	*E. coli* BL21 star (DE3)Δ *xylAB* / pACYCDuet-*adhP-atfM-yjhG* & pETDuet -*mdlC-xylBC*	This study
14	*E. coli* BL21 star (DE3) Δ *xylAB* Δ *fadE* / pET-*mdlC-xylBC* & pACYCDuet-*adhP-atfA-yjhG* & pCOLA- *fadD*	This study
16	*E. coli* BL21 star (DE3) Δ *xylAB* Δ *fadE* / pET-*mdlC-xylBC* & pACYCDuet-*adhP-atfA-yjhG* & pCOLA-*glpK-plsB -pgpB-fadD*	This study
nids		
LADuet-1	*Kan*^{r} *oriColA lacI*^{q} *T7*$_p$	Novagen
57-*atfM*	puC57 harboring *M. smegmatis atf*	Genewiz
57-*atfA*	puC57 harboring *A. baylyi atf*	Genewiz
dlC-xylBC	pETDuet-1 harboring *P. putida mdlC* and *C. crescentus CCxylB* and *xylC*	Ref. 8
dhP-yjhG	pACYCDuet-1 harboring *E. coli yjhG* and *adhP*	Ref. 8
dhP-atfA-yjhG	pACYCDuet-1 harboring *E. coli yjhG*, *adhP* and *A. baylyi atf*	This study
dhP-atfE-yjhG	pACYCDuet-1 harboring *E. coli yjhG*, *adhP* and *atf*	This study
dhP-atfM-yjhG	pACYCDuet-1 harboring *E. coli yjhG*, *adhP* and *M. smegmatis atf*	This study
dD	pCOLADuet-1 harboring *E. coli fabD*	This study
lpK-plsB-pgpB-fadD	pCOLADuet-1 harboring *E. coli glpK, plsB, pgpB, and fabD*	This study

TAB	Strai
	Strai
	E. co
	E. co
	E. co
	E. co
	Q25
	Q25
	Q25
	Q27
	Q30
	Plas
	pCO
	puC
	puC
	pE-*n*
	pA-d
	pA-d
	pA-d
	pA-d
	pC-*fl*
	pC-*g*

2.5 | Analytic methods

BT esters were extracted by adding 50 mL of organic solvent containing chloroform and methanol (the ratio is 2:1 by v/v) to an equal volume of whole culture with 0.1 mg nonadecanoic acid methyl ester added as an internal standard and vortexing for 30 seconds. After phase separation, the organic phase was evaporated by a rotary evaporator and redissolved in 2 mL of organic solvent containing chloroform and methanol.

Samples were analyzed by LC-MS. UHPLC analysis was carried out using an Ultimate 3000 UHPLC (Thermo, USA) with a Thermo Acclaim RSLC C18 column (2.1 mm × 100 mm, 2.2 μm) with Thermo online UHPLC filter (2.1 mm, 0.2 μm) used for the chromatographic separation. The mobile phase A consisted of 0.1% of formic acid and 2 mM ammonium formate in 2-propanol/water (98: 2), and the mobile phase B was composed of methanol/water with 0.1% formic acid and 2 mM ammonium formate (98: 2). The elution gradient was started at 100% B for the first 3 min with flow rate of 0.2 mL/min, stepping to 75% B at 3.1 min, linearly ramped to 50% B at 20 min, linearly ramped to 17% B at 36 min, stepping to 0% B and 0.25 mL/min at 36.2 min, holding at 0% B until 43 min, and returning to 100% B with the increased flow rate of 0.2 mL/min at 43.1 min, holding these conditions at 50 min and stopping the controller. The injection volume was 2 μL and the column temperature was 30°C.

A Compact Q-TOF mass spectrometry (Bruker Daltonics, Billerica, USA) with an ESI source in positive ion mode with HyStar 3.2 software was used to link the LC and the MS, using the following operation parameters: capillary voltage 4500 V, dry temperature 200°C, nebulizing gas of 1.5 bar, drying gas (N_2, purity 99.999%) flowing of 5.5 L/min. High resolution MS and MS/MS spectra were acquired in the range 50–1300 *m/z*. The collision gas was high purity nitrogen (purity 99.99%). The data were collected by auto MS/MS acquisition with a MS scan rate of 1 spectra/s and MS/MS scan rate of three precursor was acquired per cycle, active exclusion after 3 spectra and 1.0 min. OTOF Control software was used to carry out mass spectrometer control and data acquisition and Compass Data Analysis soft was applied for data analysis.

3 | RESULTS AND DISCUSSION

3.1 | Product verification and Atf screening

A novel bifunctional wax ester synthase/acyl-CoA: diacylglycerol acyltransferase was found in *Acinetobacter baylyi* ADP1 exhibiting acyl-CoA: fatty alcohol acyltransferase as well as acyl-CoA: diacylglycerol acyltransferase activity, oleate production in metabolically engineered *Escherichia coli*, which means short chain alcohols can also be used as substrate for AtfA [18]. Based on its broad substrate range, we speculated that BT can be esterified with fatty acids by AtfA.

In order to obtain BT esters, the AtfA was introduced into a BT producing strain constructed in our previous study [8], the resulting strain Q2545 was cultured in LB medium with xylose and sodium oleate. After fermentation, the extracted production was analyzed by LC-MS. As the skeletal structure, the BT molecule can be esterified with 1, 2, or 3 oleate molecules. The proposed formulas and ions of the mass spectra for the identified BT esters are shown in Table 2. As an example, when one BT molecule was esterified with two oleate molecule, the ions at 635.6254 *m/z* ($[M + H]^+$), 652.6258 *m/z* ($[M + NH_4]^+$), and 657.6095 *m/z* ($[M + Na]^+$) can be observed in the mass spectrum. As Supporting Information Figure 1 demonstrated, except that the BT esterification products with three oleate molecules only obtained the ion of 916.8315 *m/z* ($[M + NH_4]^+$), all the other specific ions appeared. The reason for this phenomenon might be the fairly low amount of the product. For the MS^2 spectrum, the characteristic spectra of 265 *m/z* ($C_{18}H_{34}O_2$-OH) were observed in all the three different scenarios. The results described above clearly proved that BT oleate biosynthesis is feasible in recombinant *E. coli* with heterologous expression of AtfA.

Until now, enzymes similar to AtfA have been identified in several different types of bacteria [19]. To enhance the production, the key enzyme was screened. Two more genes encoding Atf from *Mycobacterium smegmatis* (*atfM*, GeneID: 4537099) and *E. coli* (*atfE*, GeneID: 947324) were cloned and used for BT esters production. After 24 h cultivation postincubation, the strain Q2545 carrying *atfA* produced 49.5 mg/L BT oleate (Figure 2). Atf in *M. smegmatis* mc^2 *155* was up to 41% amino acid identity to AtfA, and the activity of AtfM was

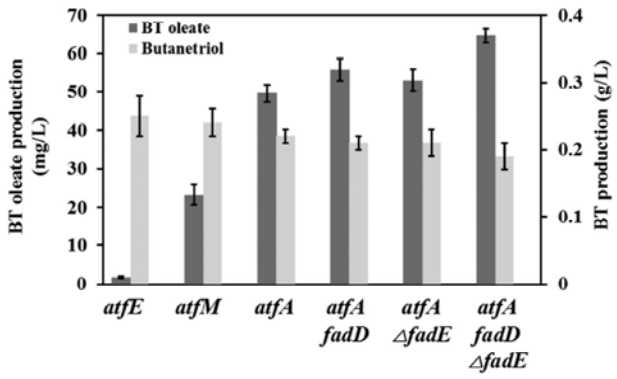

FIGURE 2 Comparison of BT esters production in flask cultivation by different engineered strains. Data were obtained after

which can utilize C_{12}-C_{20} carbon length fatty acids and fatty alcohols [17]. In another report, the AtfA was used for ethyl

each strain was induced for 24 h in liquid LB medium. All the experiments were performed in triplicate.

TABLE 2 Proposed fragmentations of the tandem mass spectra for the identified BT esters

Skeletal structure	Esterification with oleate	MS^1 formula, and ion (*m/z*)
1,2,4-butanetriol, $C_4H_{10}O_3$	1 oleic acid, $C_{22}H_{42}O_4$	$[M + H]^+$, 371.35
		$[M + NH_4]^+$, 388.38
		$[M + Na]^+$, 393.34
	2 oleic acid, $C_{40}H_{74}O_5$	$[M + H]^+$, 635.63
		$[M + NH_4]^+$, 652.65
		$[M + Na]^+$, 657.61
	3 oleic acid, $C_{58}H_{106}O_6$	$[M + H]^+$, 899.81
		$[M + NH_4]^+$, 916.83
		$[M + Na]^+$, 921.78

106.54 pmol/(mg min) that is higher than AtfA with 90.37 pmol/(mg min) [17]. However, the strain carrying *atfM* just produced 23.3 mg/L BT oleate that is less than half of that of strain Q2545. It was speculated that AtfM has higher substrate specificity than AtfA and the underlying reason need revealed in future study. A predicted acyltransferase from *E. coli* (*atfE*) was also cloned for BT oleate. Due to AtfE prefer short-chain acyl-CoA [20], the production in the present study reached just 1.8 mg/L.

3.2 | Expression and identification of recombinant enzymes

To make sure all the genes used in this study were successfully expressed in the host strain, the recombinant plasmids were transformed into *E. coli* BL21 star (DE3) and the expression level of the recombinant proteins were verified by SDS-PAGE. All the strains were cultured in liquid LB medium and 0.5 mM IPTG were added to induce the expression of the recombinant proteins. The bands with the expected size of the recombinant proteins from crude extracts of the recombinant strains appeared obviously in comparison with the control strain containing empty vector. As shown in Supporting Information Figure 2, the various recombinant strains carrying the MdlC, XylB, XylC, AdhP, and YjhG enzymes that constitute the entire BT metabolic pathway from xylose revealed the corresponding bands with molecular weights of 56.4 kDa, 26.6 kDa, 31.6 kDa, 35.4 kDa, and 70 kDa. And the Atf from *A. baylyi* (50.5 kDa for AtfA) was also successfully expressed, presenting the expected protein band. Finally, all the enzymes for BT ester production were properly expressed in the resulting strain.

3.3 | Effect of *fadD* overexpression and *fadE* deletion

A sufficient amount of precursors is necessary to the efficient synthesis of the final products. The amount of acyl-CoA can free fatty acids (FFA) can be esterified to acyl-CoAs by the acyl-CoA synthetase FadD [21]. There are few studies describing significant triacylglycerols (TAG) synthesis in *E. coli* by overexpressing *fadD* and *atfA* [22]. As an alternative strategy for enhanced yields of FFA, *fadE* has been deleted in several studies to interrupt fatty acid degradation [23,24]. To improve the BT oleate production, overexpression of *fadD* or deleting of *fadE* was performed, *fadD* was cloned into vector pCOLADuet-1 between sites *BglII* and *KpnI*, and the *fadE* gene was deleted using the λ-Red recombination method. Under the same cultivation conditions, the production of BT oleate was a little higher than the strain of Q2545, with *fadD* overexpressed or *fadE* deleted independently (Figure 2). And the resulting strain Q2714 obtained 64.4 mg/L BT oleate that is 1.3-fold higher than the strain Q2545 (Figure 2).

The acyltransferase played an important role in TAG biosynthesis pathway. There are at least five steps for converting glycerol into TAG. Glycerol was firstly converted to glycerol-3-phosphate by glycerol kinase GlpK, then converted into diacylglycerol step by step by glycerol-3-phosphate acyltransferase PlsB, 1-acyl glycerol-3-phosphateacyltransferase PlsC, and diacylglycerol-3-phosphate phosphatase PgpB, and finally esterified by acyltransferase with acyl-CoA [25]. BT (1,2,4-butanetriol) has the similar structure as that of glycerol (propanetriol), which enlightened us to enhance the BT oleate production using the TAG synthesis pathway. So, the genes *glpK*, *plsB*, and *pgpB* were cloned and overexpressed to verify the influence. However, the yield of the product was not further improved by the resulting strain Q3016 (63.2 mg/L). Moreover, more complex mass spectrum was observed which means some other products produced along with the TAG biosynthesis pathway, and further study was needed in the future to confirm this speculation.

3.4 | Fed-batch fermentation

be increased by activating the fatty acid de novo synthesis or by blocking their degradation via β-oxidation. In *E. coli*,

In order to test the potentiality of the recombinant strain for larger scale production of BT oleate, fed-batch fermentation

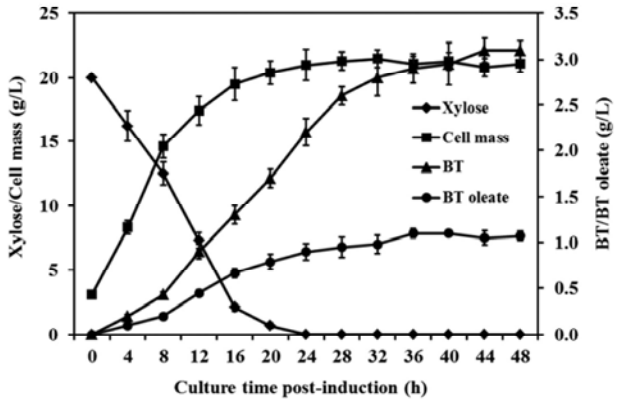

FIGURE 3 The time profiles of cell growth, residual xylose, BT, and BT oleate concentrations in culture broth during the aerobic fed-batch fermentation of the engineered strain Q2714. Cultures were performed in a 5-L laboratory bioreactor.

was established in a 5 L-scale laboratory fermenter using Q2714. The concentrated LB was used for cell growth, 0.5 mM IPTG was added to induce the recombinant proteins when the cell density reached to about 10 OD_{600}, and 20 g/L xylose and 10 g/L sodium oleate were used for BT oleate biosynthesis. Cell growth, residual xylose and product accumulation were monitored over the course of the fermentation. As shown in Figure 3, the bacteria grew rapidly and the cell mass reached to about 21.5 g/L at 32 h postinduction. A 20 g/L xylose for BT biosynthesis was almost exhausted within 24 h. BT oleate accumulated after induction in the culture broth and obtained 1.1 g/L at 36 h postinduction and then gradually to a stable value, corresponding to a productivity of 30.6 mg/L/h. An amount of BT was still detected in the broth during the fermentation, which means that it can't be converted into esters effectively. It is necessary to screen high-efficiency acyltransferase or improve the catalytic activity with enzyme engineering. Also, incorporate the *atf* gene in the genome, optimize the gene expression through promoter selection, and other methods should be considered in future study. As the cultivation conditions might affect the product synthesis and the conversion efficiency, it is worthy to optimize the fermentation mode, aeration condition, and substrate addition strategy in further research. The BT production can be recovered by centrifugation coupling with hydrolysis reaction. Compared to the traditional distillation, evaporation and other methods, the procedure is simplified and consumes lower energy, which can reduce the process cost. It is believed that this research will provide a new idea for simplifying the separation and purification process and reduce the cost of BT production. Considering potential problems, maybe it still need to combine the present method with other technologies

4 | CONCLUDING REMARKS

1,2,4-Butanetriol was successfully esterified with oleate in recombinant *E.coli* strains with wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase in this study. When *atf* from *Acinetobacter baylyi* and *fadD* from *E. coli* were overexpressed in the BT producing strain with *fadE* gene deletion, 64.4 mg/L BT oleate was accumulated in flask cultures, and the yield of the product reached to 1.1 g/L in fed-batch fermentation. This is the first time to achieve BT esters through biosynthetic pathway.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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to establish a more energy saving and efficient method for BT separation.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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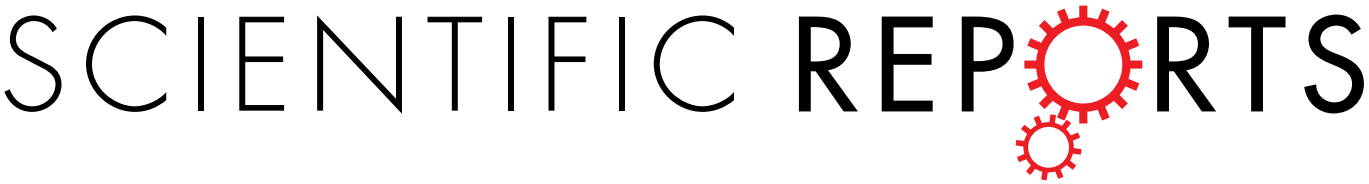

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Biotechnological production of 1,2,4-butanetriol: An efficient process to synthesize energetic material precursor from renewable biomass

Yujin Cao[1], Wei Niu[2], Jiantao Guo[2], Mo Xian[1] & Huizhou Liu[1]

1,2,4-Butanetriol (BT) is a valuable chemical with extensive applications in many different fields. The traditional chemical routes to synthesize BT suffer from many drawbacks, e.g., harsh reaction conditions, multiple steps and poor selectivity, limiting its industrial production. In this study, an engineered *Escherichia coli* strain was constructed to produce BT from xylose, which is a major component of the lignocellulosic biomass. Through the coexpression of a xylose dehydrogenase (*CCxylB*) and a xylonolactonase (*xylC*) from *Caulobacter crescentus*, native *E. coli* xylonate dehydratase (*yjhG*), a 2-keto acid decarboxylase from *Pseudomonas putida* (*mdlC*) and native *E. coli* aldehyde reductase (*adhP*) in *E. coli* BL21 star(DE3), the recombinant strain could efficiently convert xylose to BT. Furthermore, the competitive pathway responsible for xylose metabolism in *E. coli* was blocked by disrupting two genes (*xylA* and *EcxylB*) encoding xylose isomerase and xyloluse kinase. Under fed-batch conditions, the engineered strain BL21ΔxylAB/pE-mdlCxylBC&pA-adhPyjhG produced up to 3.92 g/L of BT from 20 g/L of xylose, corresponding to a molar yield of 27.7%. These results suggest that the engineered *E. coli* has a promising prospect for the large-scale production of BT.

1,2,4-Butanetriol (BT) is a four-carbon polyol with three hydrophilic hydroxyl groups. As an important fine chemical, BT has versatile applications in many different fields and attracted considerable interest in the past few years. For instance, BT can be used for making polyurethane foams. These foams have the same compression-bending characteristics as natural rubber[1]. BT severs as the recording agent of high-quality inks and renders the inks having well balanced anti-feathering and penetrability effects[2]. Optically active BT is also a potential building block for the synthesis of various pharmaceuticals, such as Crestor and Zetia[3]. Finally, BT is the direct precursor for the manufacture of butanetriol trinitrate, an excellent energetic plasticizer to replace nitroglycerin, which is less volatile, thermally more stable and offers better low-temperature properties[4,5].

BT is traditionally manufactured through chemical routes using glycidol[6], 2-butene-1,4-diol[7], 3,4-dihydroxybutanoate[8] or malate[9,10] as the starting materials. Among them, the high pressure catalytic hydrogenation of esterified malate is currently commercially available. However, this process requires $NaBH_4$ as the reducing agent. For each ton of BT to be synthesized, multiple tons of borate salts are generated as the by-products, thus resulting in high production costs and severe environmental pollution[11]. In addition, all of the chemical synthetic routes suffer from similar drawbacks, e.g., harsh reaction conditions, multiple steps and poor selectivity. These difficulties create hurdles that hamper further applications of the petrochemical-based BT.

In recent years, biomass utilization for the production of value-added chemicals is attracting considerable interest[12,13]. Xylose is the second most abundant sugar in nature and a major constituent of hemicellulose in lignocellulosic biomass[14]. A number of wild-type microbial strains or metabolically engineered strains have been isolated or constructed to ferment xylose for the production of ethanol[15], xylitol[16], succinate[17], lactate[18] and other important

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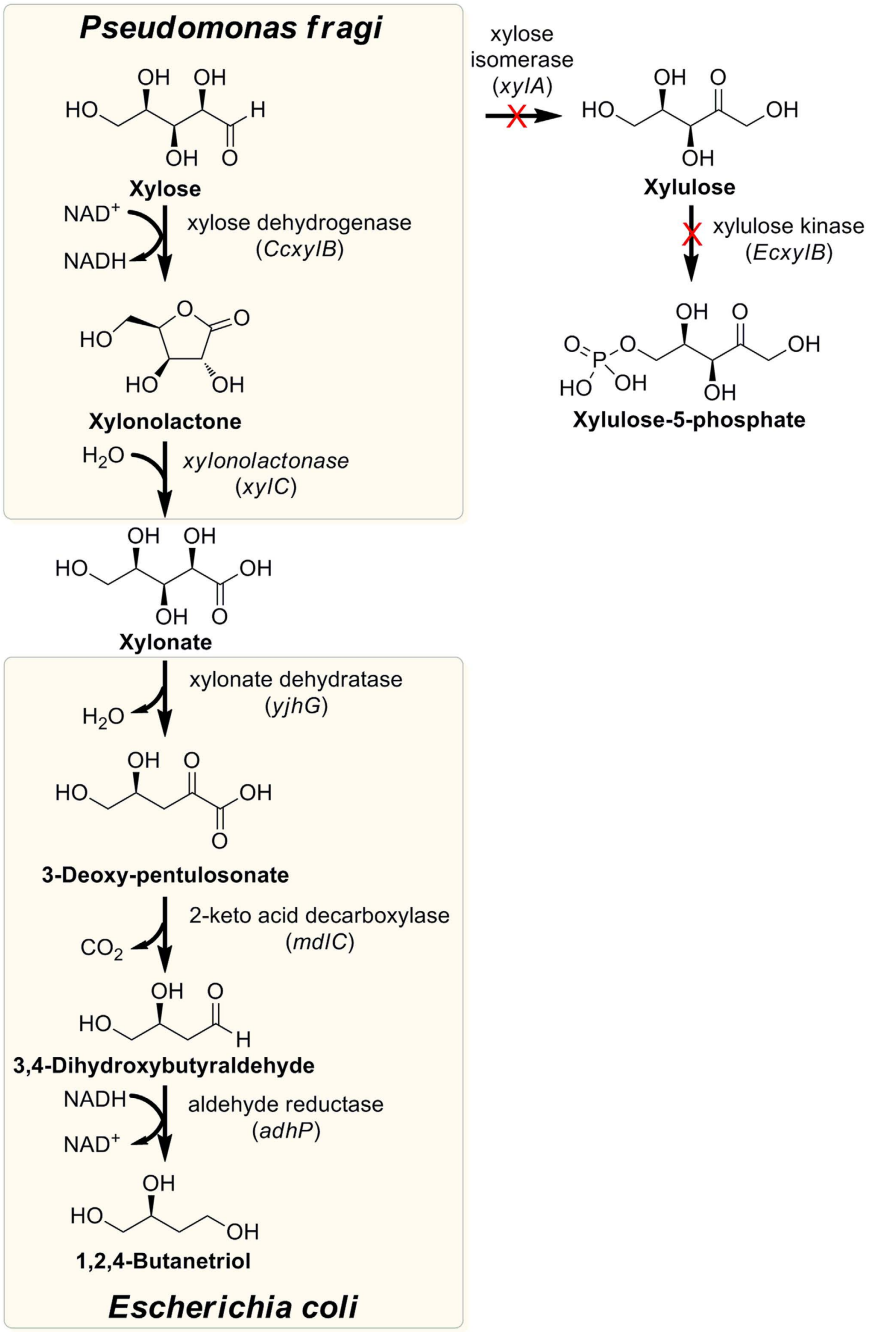

Figure 1. The metabolic pathway from xylose to BT. Xylose is oxidized to xylonolactone by the xylose dehydrogenase (encoded by *CcxylB*). Xylonolactone is hydrolyzed to xylonate by the xylonolactonase (encoded by *xylC*). Xylonate dehydratase (encoded by *yjhG*) catalyzes the dehydration of xylonate to produce 3-deoxy-pentulosonate. Then 3-deoxy-pentulosonate is decarboxylated by 2-keto acid decarboxylase (encoded by *mdlC*) to form 3,4-dihydroxybutyraldehyde. At last, 3,4-dihydroxybutyraldehyde is reduced by the aldehyde reductase (encoded by *adhP*) to generate BT. Xylose isomerase (encoded by *xylA*) and xylulose kinase (encoded by *EcxylB*) responsible for xylose metabolism are knocked out to block the branched pathways.

products. The utilization of biomass-derived xylose to produce bio-based chemicals offers many advantages over the traditional petrochemical routes in that it is made from renewable resources, possesses mild transformation conditions and reduces the environmental pollution. Researchers also explored new routes for BT production and finally they established a new process to produce BT from xylose by microbial conversion[19]. Through the introduction of a 2-keto acid decarboxylase (*mdlC*) from *Pseudomonas putita*[20] to *Escherichia coli*, the recombinant strain could efficiently covert xylonate to BT. Meanwhile, the feedstock xylonate could be obtained by microbial oxidation of xylose by *Pseudomonas fragi*[21]. The entire metabolic pathway from xylose to BT is proposed in Fig. 1. Although many wild-type bacterial species can produce xylonate in high yields[21–23], these natural producers always

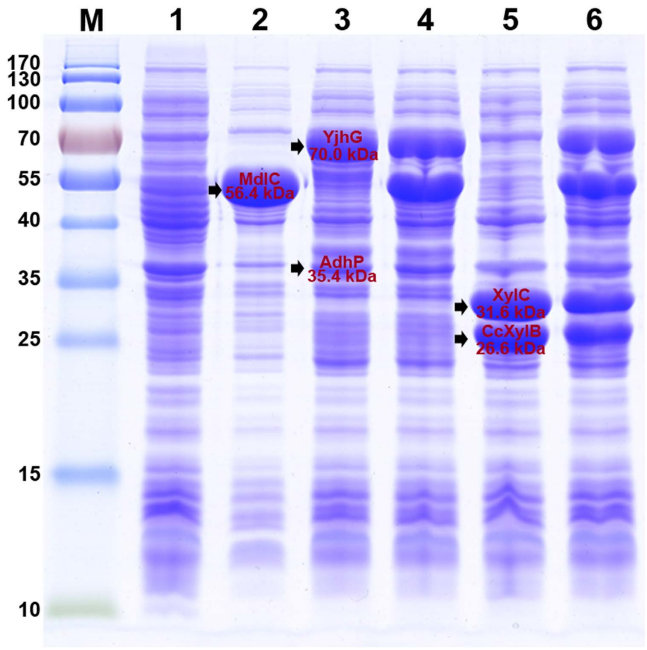

Figure 2. Validation of the expression of different recombinant enzymes in *E. coli* through SDS-PAGE analysis. Recombinant protein expression was induced using 0.5 mM IPTG for a cultivation time of 4 h at 37 °C. Lane M, prestained protein molecular weight marker; lane 1, crude cells extracts from *E. coli* BL21 star(DE3) harboring pET28a; lane 2, crude cells extracts from strain BL21/pET-mdlC; lane 3, crude cells extracts from strain BL21/pA-adhPyjhG; lane 4, crude cells extracts from strain BL21/pET-mdlC&pA-adhPyjhG; lane 5, crude cells extracts from strain BL21/pA-xylBC; lane 6, crude cells extracts from strain BL21/pE-mdlCxylBC&pA-adhPyjhG. The bands corresponding to the individual proteins were indicated by an arrow.

require expensive nutrient media and take several days to reach the maximum titer. Meanwhile, separation and purification of xylonate from the fermentation broth is also a difficult task. As is known to all, the downstream processing of organic acids is estimated to make up the major cost in its microbial production[24]. Therefore, the two-step fermentation process would be economically unfeasible for large-scale production. A one-pot conversion from xylose to BT would be more reliable for industrial applications.

The construction of a direct BT-producing *E. coli* strain from xylose was presented in this study. The *Caulobacter crescentus* xylose dehydrogenase (*CCxylB*), xylonolactonase (*xylC*) and *P. putita mdlC* were coexpressed with native *yjhG* (encoding xylonate dehydratase) and *adhP* (encoding aldehyde reductase/alcohol dehydrogenase) in *E. coli* BL21 star(DE3). To avoid the consumption of xylose for cell growth, the endogenous xylose catabolic pathway was blocked by disrupting two genes, *xylA* (encoding xylose isomerase) and *EcxylB* (encoding xylulose kinase) responsible for xylose utilization. The engineered strain was cultured under fed-batch conditions to evaluate the potential for large-scale BT production.

Results

Identification of recombinant enzymes expressed in *E. coli*. *E. coli* BL21 star(DE3) was chosen as the host for recombinant proteins expression and BT production in this work. This strain has a genotype that promotes mRNA stability and protein yield and thus is ideal for use with low copy number, T7 promoter-based plasmids. In order to express the CCXylB, XylC, YjhG, MdlC and AdhP enzymes which constitute the entire metabolic pathway from xylose to BT, we cloned the coding regions of these genes into the expression vectors pET28a, pTrcHis2B, pETDuet-1 and pACYCDuet-1, respectively. The expression constructs were checked by restriction enzyme digestion and DNA sequencing. To verify the expression levels of these recombinant proteins, *E. coli* BL21 star(DE3) was transformed with the expression vectors and grown in liquid LB medium followed by induction with 0.5 mM IPTG. Figure 2 showed the gel electrophoresis patterns of protein samples visualized by Coomassie Brilliant Blue staining. We noted distinct bands of the expected sizes from crude extracts of the recombinant strains when compared with the control strain BL21/pET28a. SDS-PAGE analysis of the recombinant strain carrying pET-mdlC showed a band corresponding to the molecular weight of 56.4 kDa while strain BL21/pA-yjhGadhP revealed two protein bands (35.4 kDa and 70.0 kDa). The recombinant strain harboring both of the two plasmids gave all three bands. Strain BL21/pA-xylBC revealed the recombinant proteins corresponding to the molecular weights of 26.6 kDa and 31.6 kDa. And the final strain harboring both pE-mdlCxylBC and pA-yjhGadhP overexpressed all of the five enzymes.

Enhancement of BT production from xylonate by coexpression of the entire pathway. It has been demonstrated that heterologous expression of *P. putida mdlC* gene in *E. coli* DH5α led to the synthesis of BT from xylonate[19]. However, this biotransformation process could not be achieved using *E. coli* BL21(DE3) as

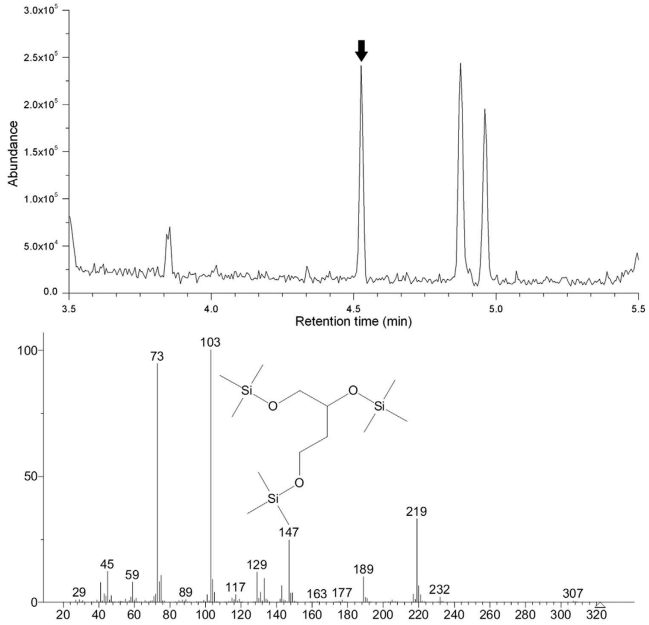

Figure 3. Identification of BT accumulated in the culture broth by GC-MS. (**A**) total ion current (TIC) chromatogram of the silylation products of the fermentation supernatant from *E. coli* BL21 star(DE3) harboring pET-mdlC and pA-adhPyjhG after being induced for 48 h. (**B**) mass spectrum of silylation product of BT (corresponding to the retention time of 4.53 min).

the host strain. Frost and co-workers have postulated a potential metabolic pathway from xylonate to BT (Fig. 1). Two enzymes, xylonate dehydratase (encoded by *yjhG*) and aldehyde reductase (encoded by *adhP*) are required for the necessary steps of the bioconversion. *E. coli* BL21(DE3) genome does not carry any xylonate dehydratase[25] and thus cannot metabolize xylonate. To render strain BL21 the ability of producing BT from xylonate, we cloned the *yjhG* and *adhP* genes from strain DH5α into the expression vector pACYCDuet-1 sequentially, resulting pA-yjhG and pA-adhPyjhG. To evaluate the effects of coexpression of the entire pathway on BT production, *E. coli* DH5α, W3110, JM109(DE3) and BL21 star(DE3) was transformed with pTrc-mdlC, pET-mdlC, pA-yjhG, pA-adhPyjhG or the combination of these plasmids. The recombinant strains were cultured using liquid LB medium supplemented with 5 g/L of potassium xylonate under shake-flask conditions. After being induced for 48 h, the bacterial cells were separated by centrifugation and BT produced in the supernatant was derivatized and analyzed by gas chromatography—mass spectrometry (GC-MS). Figure 3 showed the total ion current (TIC) chromatogram and mass spectrum of the silylation products of the fermentation supernatant from strain BL21/pET-mdlC&pA-adhPyjhG. 1,2,4-Tri(trimethylsilyl)butanetriol (corresponding to the retention time of 4.53 min) was identified by matching a standard NIST library. BT production of different engineered strains in the culture supernatant was then directly determined by high-performance liquid chromatography (HPLC). As shown in Fig. 4, cell growth was comparable between these recombinant strains. The titer of BT for strain DH5α/pTrc-mdlC reached 0.11 g/L after 48 h of induction. Similar results were obtained by strain W3110/pTrc-mdlC and JM109/pTrc-mdlC. When the *yjhG* gene was coexpressed with *mdlC*, BT production was enhanced to 0.17 g/L. In the strain JM109/pET-mdlC&pA-adhPyjhG and BL21/pET-mdlC&pA-adhPyjhG coexpressing the entire BT biosynthesis pathway, the final titer of BT was further elevated to 0.31 g/L, which is 1.8-fold higher than the original strain. It has been demonstrated that the aldehyde reductase AdhP could convert aldehydes to alcohols[26] and reduce the toxicity of the intermediate 3,4-dihydroxybutanal, thus leading to an enhanced production of BT.

Direct bioconversion of xylose to BT. Previous studies employed a two-step process to synthesize BT from xylose because *E. coli* was incapable of producing xylonate from xylose. The conversion of xylose to xylonate requires two enzymes, xylose dehydrogenase and xylonolactonase. Recently, the xylose dehydrogenase (*CCxylB*) and xylonolactonase (*xylC*) were identified from the freshwater bacterium *C. crescentus*[27]. Here, the two genes were coexpressed in *E. coli* BL21 star(DE3) using the recombinant vector pA-xylBC. Accumulations of xylonolactone and xylonate were detected in the cultures of the recombinant strain. As shown in Fig. 5, 0.38 g/L of xylonolactone and 3.19 g/L of xylonate were produced from 5 g/L of xylose after being induced for 24 h. *E. coli* could utilize xylose via the pentose phosphate pathway for cell growth. The first two genes responsible for xylose catabolism have been recognized as xylose isomerase (encoded by *xylA*) and xylulose kinase (encoded by *EcxylB*)[28]. In order to block the native xylose catabolic pathway, we disrupted the *xylA* and *EcxylB* genes in strain BL21 star(DE3) to create BL21ΔxylAB. The final titers of xylonolactone and xylonate in strain BL21ΔxylAB/pA-xylBC were enhanced to 0.49 g/L and 4.07 g/L, respectively. The molar yield of xylonolactone and xylonate on xylose also reached 83.5%, which is 18.1% higher than strain BL21/pA-xylBC.

In order to integrate the xylonate and BT producing pathway, the *CCxylB* and xylC genes were coexpressed with *mdlC* using the recombinant vector pE-mdlCxylBC. Both pE-mdlCxylBC and pA-adhPyjhG were co-transformed

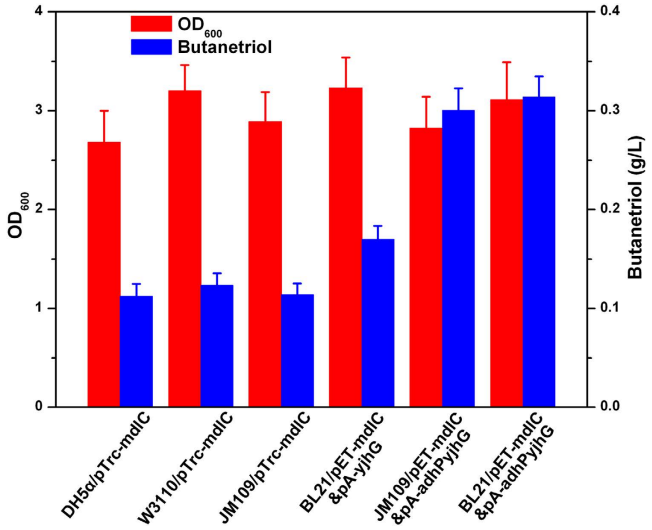

Figure 4. Comparison of BT productions of several different strains. DH5α/pTrc-mdlC, strain DH5α *P. putida* 2-keto acid decarboxylase; BL21/pET-mdlC&pA-yjhG, strain BL21 star(DE3) expressing *P. putida* 2-keto acid decarboxylase and native *E. coli* dehydratase; BL21/pET-mdlC&pA-adhPyjhG, strain BL21 star(DE3) expressing *P. putida* 2-keto acid decarboxylase and native *E. coli* dehydratase and aldehyde reductase. Data were obtained after each strain was induced for 48 h in liquid LB medium supplemented with 5 g/L of potassium xylonate as the substrates for BT production.

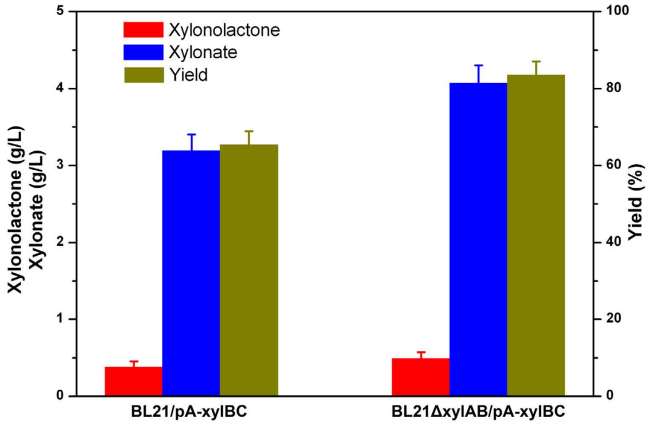

Figure 5. Production of xylonate and xylonolactone through the upstream pathway. BL21/pA-xylBC, strain BL21 star(DE3) expressing *C. crescentus* xylose dehydrogenase and xylonolactonase; BL21ΔxylAB/pA-xylBC, knockout of native *xylA* and *EcxylB* while coexpressing *C. crescentus* xylose dehydrogenase and xylonolactonase. Data were obtained after each strain was induced for 24 h in liquid LB medium supplemented with 5 g/L of xylose.

to BL21ΔxylAB competent cells. The resulting strain BL21ΔxylAB/pE-mdlCxylBC&pA-adhPyjhG was grown in LB medium containing 5 g/L of xylose. As expected, accumulation of BT was found in the culture of this engineered strain. BT production after being induced for 48 h reached 0.30 g/L, which is comparable with the strain converting xylonate to BT. The molar yield of BT to xylose reached 8.5%. As xylose, xylonate and many other organic acids shared a similar retention time in the HPLC chromatograph, we use the ion chromatography (IC) to separate these metabolites. IC analysis showed that the initial xylose was completely exhausted at 48 h post-induction, but intermediate metabolites of the BT biosynthesis pathway were detected in the culture broth. This fact indicated that the downstream pathway (xylonate to BT) instead of the upstream pathway (xylose to xylonate) was the rate-limiting step for BT biosynthesis.

BT production in fed-batch cultivation. In order to test the suitability of the recombinant *E. coli* strains for larger-scale production of BT, we established fed-batch fermentation based on the results obtained with shake-flask cultures. Strain BL21ΔxylAB/pE-mdlCxylBC&pA-adhPyjhG was cultured in a 5 L-scale laboratory fermenter. Glycerol was selected as the carbon source instead of glucose to avoid catabolite repression of xylose uptake. Cell density, residual xylose and BT accumulation were monitored over the course of the experiment. The residual

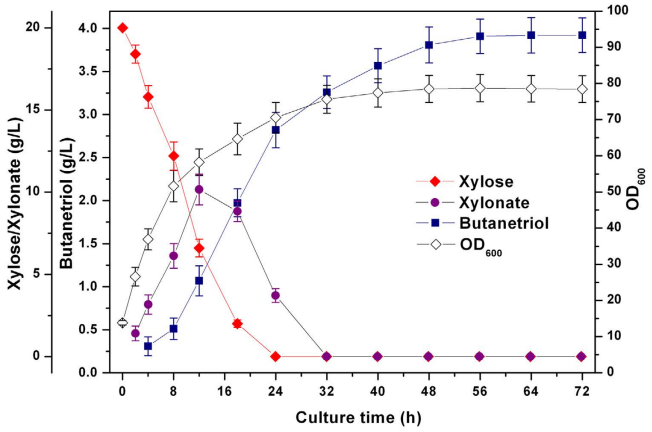

Figure 6. Time profiles for cell density (OD_{600}), residual xylose and BT concentrations in the culture broth during fed-batch culture of the engineered strain BL21ΔxylAB/pE-mdlCxylBC&pA-adhPyjhG. Cultures were performed in a 5L laboratory fermentor. 20 g/L of xylose was used as the substrates for BT production. Error bars represent the range of three independent fermentations.

glycerol concentration was controlled at a low level to avoid acetate formation, which might hamper cell growth and BT detection. Figure 6 showed the time profiles of cell density and concentrations of different metabolites during the whole fermentation period. For approximately 32 h post-induction, the bacteria grew rapidly to an OD_{600} of 75 or so. Most of the xylose was utilized in the first 18 h of fermentation. The intermediate metabolite xylonate reached a maximum titer of 10.2 g/L at 10 h post-induction and was then gradually consumed by the downstream pathway. BT also accumulated rapidly in the culture broth during the first 40 h post-induction and then gradually to a stable value. The highest BT production was obtained after being induced for 56 h, that is, 3.92 g/L, corresponding to a volumetric productivity of 0.89 mg/(L·h·OD_{600}). The final molar yield of BT on xylose reached 27.7%. The above results achieved at the fermenter level greatly enhanced the titer of BT in the fermentation broth and demonstrated that this engineered *E. coli* strain had the potential to produce BT in a large scale.

Discussion

In this work, we integrated the two steps for biotechnological BT production in a single *E. coli* host and the metabolic pathway was further engineered to enhance the carbon flux to BT. Both the titer and yield of BT were enhanced to some extent compared with the original study[19]. These results demonstrated the power of rational design to improve the ability of microorganisms to produce bio-based chemicals. The one-step conversion process also makes the cells maintain redox balance. Reducing equivalent supply is crucial for the biosynthesis of value-added chemicals[29]. The metabolic pathway from xylonate to BT consists of three steps: dehydration, decarboxylation and reduction. One mole of NADH is required for each mole of BT formed. Therefore, the cells should provide additional NADH to facilitate the reduction of 3,4-dihydroxybutanal. The NADH balance forces more carbon flux to be channeled into the TCA cycle, leading to excessive consumption of the carbon sources. The upstream metabolic pathway from xylose to xylonate could produce one mole of NADH for each mole of xylose to be oxidized[30]. NADH consumed by the production of BT could be regenerated through the oxidation of xylose. Therefore, the integration of the upstream pathway and the downstream pathway in a single host could provide appropriate reducing power, which might contribute to BT biosynthesis.

The engineered strain in current study has greatly improved BT production. However, the yield of BT on xylose is still far from the theoretical limits. This result might be due to the following issues. First of all, the rate-determining step for BT biosynthesis is the decarboxylation of 3-deoxy-pentulosonate catalyzed by MdlC. The original activity of this enzyme is a benzoylformate decarboxylase participating in mandelate degradation[20]. 3-Deoxy-pentulosonate is not the natural substrate and thus it shows a much lower substrate specificity and catalytic activity. Therefore, protein engineering of the MdlC decarboxylase to improve its catalytic activity towards 3-deoxy-pentulosonate would be helpful to BT production[31]. On the other hand, there are many competing pathways associated with the BT biosynthesis pathway. For instance, 3-deoxy-pentulosonate can be split to pyruvate and glycolaldehyde[32] as well as forming 2-amino-4,5-dihydroxypentanoate catalyzed by aminotransferase[33]. The oxidation of 3,4-dihydroxybutanal to 3,4-dihydroxybutyrate might also compete with its reduction to BT. Inactivation of these branched metabolic pathways would be expected to further improve BT production. However, these reactions are always catalyzed by multiple enzymes or key enzymes essential for the cell's normal metabolism. It is difficult to completely block these branched pathways without affecting cell viability. Therefore, regulating the metabolic flux to BT biosynthesis would be critical to enhance its production.

Materials and Methods

Bacterial strains and plasmids construction. A list of bacterial strains and recombinant plasmids was presented in Table 1. Primers used for plasmids construction was provided in Table 2. *E. coli* DH5α was used for gene cloning and *E. coli* BL21 star(DE3) was used as the host for the expression of the recombinant proteins. The chromosomal *xylA* and *EcxylB* genes of strain BL21 star(DE3) responsible for xylose utilization were knocked out using the λ-Red recombination strategy in a previous study, resulting strain BL21ΔxylAB[34].

Strains or plasmids	Genotype/Description	Source
Strains		
E. coli DH5α	*F⁻ recA endA1 Φ80dlacZΔM15 hsdR17(r_K^- m_K^+) λ⁻*	Invitrogen
E. coli W3110	*F⁻ λ⁻ rph-1 INV(rrnD rrnE)*	Coli Genetic Stock Center
E. coli JM109 (DE3)	*recA endA1 lacZΔM15 (lac-proAB) hsdR17(r_K^- m_K^+)* IDE3	Promega
E. coli BL21 star (DE3)	*F⁻ ompT hsdS_B* (r_B^- m_B^-) *gal dcm rne131* (DE3)	Invitrogen
E. coli BL21 star (DE3) ΔxylAB	Knockout of *xylA* and *EcxylB* encoding xylose isomerase and xylulose kinase	34
Plasmids		
pET28a	*Kan^r oripBR322 lacI^q T7p*	Novagen
pTrcHis2B	*Amp^r oripBR322 lacI^q Trcp*	Invitrogen
pETDuet-1	*Amp^r oripBR322 lacI^q T7p*	Novagen
pACYCDuet-1	*Cm^r oriP15A lacI^q T7p*	Novagen
pET-mdlC	pET28a harboring *P. putida mdlC*	This study
pTrc-mdlC	pTrcHis2B harboring *P. putida mdlC*	This study
pA-xylBC	pACYCDuet-1 harboring *C. crescentus CCxylB* and *xylC*	34
pE-xylB	pETDuet-1 harboring *C. crescentus* CC*xylB*	This study
pE-mdlCxylB	pETDuet-1 harboring *P. putida mdlC* and *C. crescentus CCxylB*	This study
pE-mdlCxylBC	pETDuet-1 harboring *P. putida mdlC* and *C. crescentus CCxylB* and *xylC*	This study
pA-yjhG	pACYCDuet-1 harboring *E. coli yjhG*	This study
pA-adhPyjhG	pACYCDuet-1 harboring *E. coli yjhG* and *adhP*	This study

Table 1. Strains and plasmids used in this study.

Oligonucleotide primers	Description
xylB_F_NdeI	GGGAATTCCATATGTCCTCAGCCATCTATCCC
xylB_R_KpnI	CGGGGTACCTCAACGCCAGCCGGCGTCGAT
mdlC_F_NcoI	CATGCCATGGCTTCTGTTCACGGTACCACC
mdlC_R_EcoRI	CCGGAATTCTTATTTAACCGGAGAAACGGTAG
T7xylC_F_EcoRI	CCGGAATTCTAATACGACTCACTATAGGGGAATTG
T7xylC_R_NotI	AAGGAAAAAAGCGGCCGCTTAAACCAGACGAACTTCGTGCTG
yjhG_F_NdeI	GGAATTCCATATGTCTGTTCGCAATATTTTTGC
yjhG_R_XhoI	CCGCTCGAGTCAGTTTTTATTCATAAAATCGCG
adhP_F_NcoI	CATGCCATGGGCATGAAGGCTGCAGTTGTTACG
adhP_R_EcoRI	CCGGAATTCTTAGTGACGGAAATCAATCACC

Table 2. Primers used in this study for plasmids construction or allele verification.

The 2-keto acid decarboxylase from *P. putita* (*mdlC*, GenBank accession no.: AY143338) was codon optimized, chemically synthesized and cloned into pET28a or pTrcHis2B vector between *Nco*I and *Eco*RI sites to create pET-mdlC or pTrc-mdlC. The *CCxylB* (GenBank Accession No.: NACL94329) and *xylC* (GenBank Accession No.: NACL94328) genes from *C. crescentus* were also cloned into pACYCDuet-1 vector simultaneously to generate plasmid pA-xylBC in our previous study[34]. Then *CCxylB* was PCR amplified from vector pA-xylBC and cloned into pETDuet-1 vector between *Nde*I and *Kpn*I sites to create pE-xylB. The *mdlC* gene was digested from pET-mdlC using *Nco*I and *Eco*RI, and then cloned into the same restrict sites of pE-xylB, resulting pE-mdlCxylB. The *xylC* gene was amplified from pA-xylBC along with T7 promoter and the PCR product T7xylC was then cloned into pE-mdlCxylB between *Eco*RI and *Not*I sites, to create pE-mdlCxylBC. The coding sequences of native *E. coli yjhG* (Gene ID: 6060334) and *adhP* (Gene ID: 6059208) were generated by PCR using the primers based on the selected sequences around the start or stop codons of the genes and with restriction enzyme sites at 5′ ends. The resulting PCR product of *yjhG* was cloned into pACYCDuet-1 between *Nde*I and *Xho*I sites leading to plasmid pA-yjhG. The *adhP* gene was then cloned into pA-yjhG between *Nco*I and *Eco*RI sites to create pA-adhPyjhG. Successful gene cloning was verified by colony PCR, restriction mapping and direct nucleotide sequencing.

Protein expression and gel electrophoresis analysis. For the expression of different recombinant proteins, single colonies of *E. coli* BL21 star(DE3) harboring different recombinant plasmids were used to inoculate Luria-Bertani (LB) medium containing appropriate antibiotics and grown at 37 °C overnight. The saturated culture was diluted 1:100 into fresh LB medium and incubated under the same conditions. When the optical density at 600 nm (OD_{600}) of the culture reached about 0.6, recombinant protein expression was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and growth was continued for 4 h. Cells were collected from 5 ml of bacteria cultures by centrifugation and washed with sterile distilled water. The washed pellets were suspended in 500 μl Tris-HCl buffer (pH 8.0) and subject to ultrasonication. The cell lysates were centrifuged and the supernatant

obtained was mixed with 2 × sodium dodecyl sulfate (SDS) sample buffer, heated at 100 °C for 10 min and then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) according to standard protocols[35].

Shake-flask cultivation. To evaluate the BT producing ability of different engineered strains, shake-flask experiments were carried out in triplicate series of 100 ml Erlenmeyer flasks containing 20 ml of liquid LB medium supplemented with appropriate antibiotics. *E. coli* strains were inoculated to the culture medium and incubated in a gyratory shaker incubator at 37 °C and 180 rpm. When the OD_{600} of the culture reached about 0.6, IPTG was added to a final concentration of 0.5 mM to induce the expression of different enzymes. 5 g/L of potassium xylonate or xylose was added to the culture at the same time as the substrates for BT production. Samples were taken at different intervals to determine cell density, residual xylose and BT accumulated in the culture broth during the whole fermentation courses.

Fed-batch fermentation. For large-scale production of BT, fed-batch cultures were performed in a Biostat B plus MO5L fermentor (Sartorius, Germany) containing 3 L of growth medium (20 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl and 5 g/L $K_2HPO_4{\cdot}3H_2O$) that was sterilized at 121 °C for 20 min. Glycerol (10 g/L), $MgSO_4$ (0.12 g/L) and trace elements (1 ml per liter, 3.7 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 2.9 g/L $ZnSO_4{\cdot}7H_2O$, 24.7 g/L H_3BO_3, 2.5 g/L $CuSO_4{\cdot}5H_2O$, 15.8 g/L $MnCl_2{\cdot}4H_2O$) were autoclaved or filter-sterilized separately and added prior to initiation of the fermentation. Fermentation was started by inoculating 100 ml of overnight seed culture prepared in LB medium. During the fermentation process, continuous sterile air was supplied at a flow rate of 3 L/min. The temperature was controlled at 37 °C and the pH was controlled at 7.0 via automatic addition of ammonia. Antifoam was added to prevent frothing if necessary. The agitation speed was set at 400 rpm and then associated with the dissolved oxygen (DO) to maintain the DO level above 20% air saturation. Fermentation was first operated in a batch mode until the initial glycerol was nearly exhausted. Then fed-batch mode was commenced by feeding 70% of glycerol at appropriate rates. When the cells were grown to an OD_{600} of about 15, 0.5 mM of IPTG, 20 g/L of xylose and 0.1 g/L of thiamine hydrochloride were added to the culture broth to induce recombinant protein expression and BT production. Samples of the fermentation broth were determined the same as shake-flask cultivation.

Analytic methods. BT produced in the culture broth was identified using gas chromatography-mass spectrometry (GC-MS). The culture supernatant was mixed with 9 volume of ethanol to deposit proteins and concentrated by reduced pressure distillation. The concentrated sample was then derivatized by bis(trimethylsilyl) trifluoroacetamide (BSTFA): appropriate amounts of the samples were added 1 ml of BSTFA and 0.2 ml of pyridine, and then reacted at 70 °C for 30 min[36]. The reaction mixture was diluted by hexane and then subjected to GC-MS. GC-MS analysis was performed with an Agilent 7890 GC system coupled to a quadrupole rods detector. The GC-MS conditions were as follows: a 30 m HP-5 ms column (internal diameter 0.25 mm, film thickness 0.25 μm); an oven temperature program composed of an initial hold at 100 °C for 2 min, ramping at 10 °C per min to 250 °C, and a final hold at 250 °C for 3 min; an ion source temperature of 220 °C and EI ionization at 70 eV.

BT concentrations in the culture supernatant was determined by an Agilent 1200 series HPLC system equipped with an Aminex HPX-87H (Bio-Rad, Hercules, CA) column (300 × 7.8 mm). All samples were filtered through 0.22 μm syringe filter. Ultrapure water with 5 mM H_2SO_4 was used as the eluent at a flow rate of 0.5 ml/min. The oven temperature was maintained at 55 °C. Peaks were detected by a refractive index detector (RID). Quantitation of BT was performed by using the external standard method.

The determination of xylose in the culture broth was performed on an ICS-3000 (Dionex, Sunnyvale, CA) ion chromatography (IC) system. The IC was equipped with an IonPacAS11 anion chromatography column (4.0 mm × 250 mm) and an AG-11 guard column (4.0 mm × 50 mm). Suppression was achieved with anion suppressor (ASRS 300 4 mm). Peaks were detected using electrochemical detector. A mixture of 250 mM NaOH (2%) and H_2O (98%) was used for elution at a flow rate of 1 ml/min. For sample analysis, another elution step with 80% of 250 mM NaOH was employed to remove the residual components. Data collection and handling were carried out by Dionex Chromeleon software.

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Author Contributions

M.X. and H.L. conceived the project; Y.C., M.X. and H.L. designed the experiments; Y.C. performed the experiments; Y.C., W.N., J.G. and M.X. analyzed the primary data; Y.C. and M.X. drafted the manuscript; W.N., J.G. and H.L. revised the manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

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CELL-FREE (Comparable Engineered Living Lysates For Research Education and Entrepreneurship) **Workshop Report**

Workshop held February 7, 2019
J. Craig Venter Institute, La Jolla, CA

Report completed May 9, 2019

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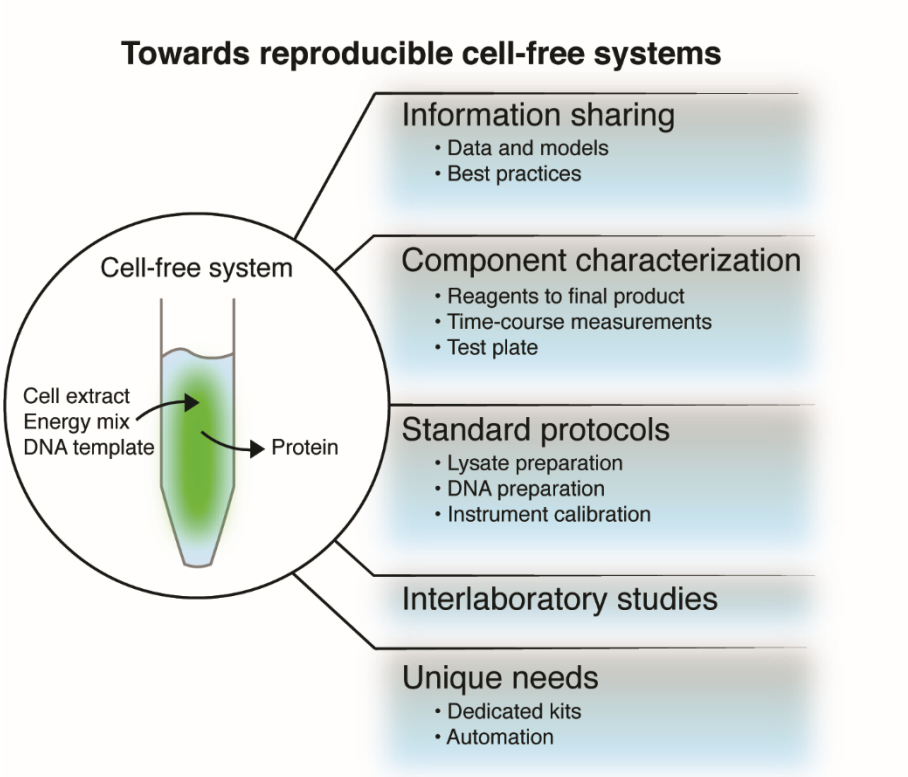
> ***"In principle, cell-free systems are simpler [than cell-based systems], but in practice, that hasn't been true, at least not yet."*** – Matthew Lux, workshop participant and researcher using cell-free systems [1]

Scope
The CELL-FREE Workshop sought to identify and prioritize actionable steps towards more reproducible and comparable cell-free systems for practical applications in bioengineering and biomanufacturing.

Findings
- Cell-free systems generate broad excitement for their potential as an enabling technology platform, but their full capabilities and suitable applications remain unclear.
- A common repertoire of protocols and methods for typical cell-free systems will aid adoption and reproducibility.
- Improved access to data and sharing of information and expertise across laboratories will also aid adoption and reproducibility.
- Improved methods and tools for measuring the components and performance of cell-free systems at all stages in a typical workflow from reagent preparation to final product are needed to advance reproducibility and applications of cell-free systems.
- Cell-free systems may differ from cell-based systems in ways that significantly impact the performance of cell-free systems, when protocols, materials, and measurements for cell-based systems are applied naively to cell-free systems.

Recommendations
- Focus the research community and spur investment by identifying specific foundational studies and application areas well-served by cell-free systems.
- Develop and disseminate standard protocols for DNA template and lysate preparation for common use cases.
- Encourage engagement with existing online resources for community and information sharing, such as the BuildACell/CellFree page on OpenWetWare [2].

- Perform interlaboratory studies to identify and test best practices for reproducible lysate and DNA template preparation.
- Develop a standard test plate and protocol to assess reagent quality and facilitate characterization, performance, and reproducibility of cell-free reactions.
- Rigorously assess all assumptions associated with the use of protocols, materials, and measurements for cell-based systems applied to cell-free systems.



Motivation

Living systems are not only complex, they are relentless in their demands to be kept alive, with all the attendant limitations and challenges for experimentation and measurement. So-called "cell-free systems" inhabit the space between chemistry and biology proper, obviating some of the requirements of living, cell-based systems and enabling biochemical processes and experimental interventions toxic, contrary, or simply inconvenient to the enterprise of being fully alive. Cell-free systems are typically composed from the minimally-prepared extracts of lysed cells or reconstituted from purified biochemical components, supplemented with the desired DNA template and a chemical mixture to supply energy, amino acids, and small molecules to the system. A recent review of current organisms, methods, and applications is offered by [3].

Despite a relaxation of the stringent demands of living, users of cell-free systems must still contend with the inherent complexity of life that is both the compelling strength and confounding liability of cell-free systems. Consequently, a lack of reproducibility persists within and across laboratories engaged in research using cell-free systems. Complexity in cell-free systems can

manifest as variability in function, such as protein production, which generally cannot be designed out or optimized away at the state of the art. Although biological variability undoubtedly contributes to generally poor reproducibility in uses of cell-free systems, this Workshop focused instead on modeling, laboratory practices, and measurement technology to improve reproducibility. Biological variability may then be approached as an enabling feature of engineered biological systems for emerging applications. This view deliberately tempers a pervasive myopic affinity for deterministic systems, which would miss the opportunity to learn control approaches unique to biological systems and harness these for safe and robust applications of engineering and synthetic biology broadly.

Stakeholders of the National Institute of Standards and Technology (NIST) in industry, government, and academia have requested measurement tools and methods to improve reproducibility, primarily for protein expression using cell-free systems, to support intra- and interlaboratory collaboration, assist nascent industry, and improve fundamental understanding of biology. As part of a broader portfolio of investments in synthetic biology and engineering biology, NIST is building technical competence and measurement capabilities in cell-free systems. This Workshop and report constitute early outputs of these efforts.

Approach and organization

The need for this Workshop became clear in conversation with numerous stakeholders across the community of cell-free researchers. Following the example of existing online discussion forums initiated by, for example, the Murray laboratory [2, 4] and Build-a-Cell [5], NIST hosted documents online to gather relevant information [6] ahead of the in-person meeting. This online interaction enabled the open exchange of ideas, concerns, and so on, regarding the formation of Working Groups and the Workshop agenda, as well as facilitated the exchange of notes and other materials after the Workshop. Working Group topics were selected for their immediate relevance to the central task of identifying actionable, near-term steps to improve reproducibility for cell-free systems. Working Group leaders were chosen for their knowledge and respected standing in each of the Working Group topics. A summary follows, collated from notes taken during each Working Group discussion. This Report offers the opinions presented by participants, as captured through this imperfect process. The authors of this Report make no claims to a comprehensive or balanced survey of the field of cell-free systems broadly.

Modeling Working Group, led by William Poole (California Institute of Technology) and Richard Murray (California Institute of Technology)

Several general goals and guidelines surfaced during discussion in the Modeling Working Group. First, modeling efforts should aim towards predictive, rather than simply descriptive, models of cell-free systems. Second, models should build understanding and inform capabilities that bridge *in vitro*, cell-free systems and *in vivo,* cell-based systems. Third, models should be compatible with practical experimental testing according to existing capabilities, to ensure relevance and speed model validation. Fourth, specific, near-term applications of modeling include aiding in the design of biological circuits, optimizing resource sharing and substrate blocking, improving protein folding, and realistic models of metabolism. Last, with regard to reproducibility, modeling should inform both fundamental understanding of biology and practical approaches to tune variability in the performance of cell-free systems for protein production.

A variety of biological systems were discussed as potential focal points for modeling efforts for cell-free systems, along with the advantages and challenges of each system. Today, efforts produce largely generic models applicable to extracts from common laboratory organisms, such as *E. coli* and the reconstituted PURExpress system [7-12]. While cell extract includes biological complexity ignored and absent from models, the PURExpress system itself arguably avoids much of that complexity altogether. The PURExpress system may therefore serve as an attractive and tractable intermediate step towards modeling more complex lysates, composed of cell extract supplemented with an energy buffer, or chemical mixture to supply energy, amino acids, and small molecules to the system. The behavior of DNA, RNA, and metabolites could be modeled in the PURExpress system, but not all relevant mechanisms and parameters are currently well-defined.

To move from an understanding of the PURExpress system to extract-based and *in vivo* systems, participants recommended incorporating explicitly the effects of molecular crowding. Because molecular crowding changes biomolecular dynamics within a biological system, this potentially offers a practical experimental approach for testing and optimization. A well-mixed model may not adequately account for affects due to molecular crowding. Rather, spatial structure should be modeled at spatial scales associated with relevant interactions within the cell-free system. However, the spatial structure of cell-free systems at different length scales and over time remains unclear, and control over this structure, for example through molecule scaffolds or microfabricated environments, could provide another experimental tool for optimization.

Participants recommended that models of cell-free systems also include time dependence. Models could predict the performance of a cell-free system throughout the life cycle of the reaction, and experimental data at regular time points could be obtained in a straightforward manner. Today, most cell-free systems are measured only after the reaction has run to completion, but more frequent measurements, especially for early time points, could provide important data to inform time-dependent, predictive models. Such models could address resource utilization, inform methods to remove detrimental byproducts, troubleshoot how reactions "die," and offer paths towards increasing or otherwise tuning reaction longevity.

This Working Group called for the generation of new experimental data and sharing data from existing studies to inform more accurate, useful, and predictive models. Standardization of experimental samples and protocols could assist in comparability across datasets and model development. Integrated data management tools and artificial intelligence could help to collect, store, and analyze the simulated and experimental data. Ideally, experimental data and models would be accessible broadly in a repository. However, using, integrating, and modifying existing models remains challenging, with no standardized language, code, or methodology in use. Existing options include, for example, subsbml to combine models [13], SED-ML for sharing simulations [14], the COMBINE archive for documenting models [15], and SBML for sharing models [16].

Lysate Preparation Working Group, led by Zachary Sun (Tierra Biosciences [17])

This Working Group gave a bleak characterization of the state of lysate preparation for cell-free systems, which is largely non-standardized, expensive, and irreproducible. Laboratories widely choose to produce their own lysate, even for common organisms, because existing commercial kits are prohibitively expensive, have limited applications, and are not customizable. Commercial kits do not seem to be any more reproducible or characterized than their laboratory-prepared analogs. Consequently, each laboratory typically has one person with so-called "magic hands" who, for undetermined and perhaps indeterminable reasons, is best able to produce suitable lysate for all users and collaborators for that laboratory. Protocols tend to follow historical precedent without optimization, suggesting the possibility that the full range of performance attainable by cell-free systems through optimization of the lysate remains unexplored and potentially considerable.

It is unclear the extent to which reproducibility in lysate preparation may contribute significantly – if at all – to reproducibility in the ultimate performance of a cell-free reaction. Complicating the matter is that no accepted criteria exist to determine how reproducible is reproducible enough generally or for a given application or purpose. Lysates are inherently heterogeneous, variation between nominally identical batches is common, and even nominally identical lysate from a common batch stored in different aliquots may perform differently. Equipment for preparing lysate varies across laboratories, such as sonication, bead beating, French press, and others, and operation of the same type of equipment varies between laboratories. In one study, sonication was more reproducible than other methods [18, 19]. Beyond hardware, human operators are reportedly a large source of variability [CCDC Chemical and Biological Center manuscript in preparation], the length of typical protocols offer many opportunities for operator error, and the level of detail in typical protocols leaves room for interpretation.

Consider, for example, the numerous factors that may introduce variability in uses of *E. coli* lysates for cell-free systems. The choice of *E. coli* strain and whether a runoff reaction [20] was performed generally affect the final cell-free reaction. Cell growth is affected by media composition, and the use of defined media could help standardize the preparation of cell lysates. Researchers may find, for example, that B strains of *E. coli* tend to perform better than K strains for no discernible reason. Similarly, researchers may prefer lysate derived from cells grown at nominally different incubation temperatures. Lysates may be further affected by the specific starter culture, presence of phage, use of a glycerol stock, use of an ill-defined "overnight culture," technique for colony picking, pellet lyophilization, amount and composition of gas headspace in culture vessels, type of flasks, shaking speed, optical density at harvest, lysis method, and use of clarification steps. Generally, cells harvested from exponential growth through stationary phase have been shown to work adequately for lysates as part of cell-free reactions [21]. Measurements of optical density should be calibrated properly to ensure reproducibility for cell growth prior to lysis [22].

Participants in this Working Group called for interlaboratory studies to assess lysate composition, performance, and reproducibility to improve the state of the art. Similar work with reference yeast strains [23], oligonucleotide microarrays [24], and a triservice interlaboratory study [CCDC Chemical and Biological Center manuscript in preparation] could serve as guiding

examples. The study would perhaps compare the performance of an *E. coli* cell lysate prepared using a common protocol across interested laboratories. Several detailed protocols are available, such as the Murray protocol in JOVE [25], which was the first known attempt to standardize a protocol with an associated video that demonstrates each step, the Hasty protocol [26], and protocols from the Jewett laboratory [18, 27]. Potential parameters to standardize in a protocol for lysate preparation could include, for example, protein abundance and activity, concentration and purity of supplemented energy, amino acids, small molecules, amount of residual native genomic DNA, and the use of crowding agents. This is envisioned as a collaborative endeavor partnering academic researchers, government agencies, and private industry.

In addition to improving lysate preparation within individual laboratories, this Working Group advocated for a shared production facility for cell lysate to advance accessibility, affordability, and reproducibility of cell-free systems. For example, a non-profit cost center may produce standardized and characterized lysate to meet the needs of the majority of users working with cell-free systems. The center could encourage publications in optimization approaches that improve reproducibility and performance. Lysate produced from *E. coli* is an obvious initial focus, along with promoting good practices and protocols to modify standardized lysates for specific application areas of common interest. In this way, the cost to produce cell lysate of sufficient quantity, quality, and characterization suitable for most cell-free applications may be reduced. Currently, the main cost to produce cell lysate is labor, which presumably inflates the cost of commercial kits from an ideal cost of approximately $0.03/μL [25] to, for example, prohibitive values currently of $0.88/μL for myTXTL [28] and for $1.044/μL for the PURExpress system [29].

DNA Template Preparation Working Group, led by Vincent Noireaux (University of Minnesota)

Active discussion surrounds the topic of DNA template preparation, despite the wide availability of commercial kits and extensive knowledge and experience accumulated in the biosciences for this purpose. Perhaps commercial kits for DNA preparation for cell-based systems would benefit from modification and optimization, such as improved quality control, additional clean-up steps, and product pamphlets with instructions specifically aimed at researchers using the prepared DNA in cell-free reactions, to yield DNA templates better suited for use with cell-free systems. Participants in this Working Group asserted that accessible, standardized DNA preparation protocols would facilitate the wider adoption of cell-free systems. Developing such protocols would require guidance and methods for relevant quantitative measurements. Variability in the performance of cell-free systems could arise, in principle, not only from the amount of DNA template introduced into a cell-free reaction but also from the quality of the DNA itself and contaminants in the solution containing the DNA.

Improvements to quantifying DNA templates are straightforward with existing measurement technologies and good laboratory practices. For example, rather than assume that prepared DNA does not change during storage, the authors of this Report observed an approximate 10 % mean change in the concentration of extracted and purified DNA using high copy number, 3.2 kbp plasmids when stored for 30 days at 4 °C in nuclease-free water in LoBind tubes, as measured

using both the Nanodrop and the Qubit Broad Range Assay (unpublished). Consultation with experts in DNA storage at NIST [30] revealed that this loss of DNA should have been expected, due to adsorption of DNA to the tube surfaces and/or hydrolysis of the DNA in water. Teflon tubes and storage in a low concentration salt buffer could mitigate this loss [30] but may not be realistic, due to the prohibitive cost of the custom tubes and potential effects of the buffer salt in the subsequent cell-free reaction. Mitigation may not even be necessary once the effects of DNA loss are acknowledged, quantified, and compensated for when assembling cell-free reactions. In this case, the prepared DNA was likely still adequate for most cell-based purposes, but the change in DNA concentration may have impacted the measured amount of protein produced when added to cell-free reactions (unpublished). Measurements of DNA quantity during this process were obtained using ultraviolet spectrophotometry (Nanodrop [31]) and fluorometry (Qubit Assay [32]) [33] calibrated using the NIST Human DNA Quantitation Standard [34].

Beyond the quantity of the prepared DNA template, the quality of each DNA molecule may impact whether that molecule is functional in a cell-free reaction. Typical measurement methods for the amount of DNA may not accurately represent the functional amount of DNA, whether impacted by physical damage, chemical modification, or other changes to a DNA molecule. Both plasmids and linear DNA templates, especially for DNA molecules longer than approximately (10^3 to 10^4) bp, are susceptible to shearing and breakage from pipetting, vortexing, passage through separation columns included in some common kits for DNA template preparation, and other manipulation. Some researchers with experience with cell-based systems may disagree, perhaps due to typical cell-culture and colony selection steps that select out broken or otherwise nonfunctional DNA. Still, most researchers agree on the difficulty in handling very long, genomic DNA, which is possible but imposes practical limits at the state of the art on delivery and use of that DNA for applications in cell-free systems. Genomic DNA may be delivered, for example, encased in agar or another gel matrix, but the additional processing steps add unwelcome complexity to experimental protocols [35].

The aqueous solution in which the DNA template is suspended during preparation and assembly in a cell-free reaction may also influence the functional amount of DNA. Consider that a significant volume of a typical cell-free system is the DNA solution. For example, a 12 μL myTXTL reaction may require 5 nM DNA, which, for the authors, was approximately 1 μL DNA solution comprising a considerable 8.3% of the overall volume of the cell-free reaction (unpublished). Contaminants are known to pass through steps of the DNA purification and preparation protocols and remain in the final solution containing the DNA template as added to the cell-free reaction. At nearly 10% of the final reaction volume, this may be a significant concern in cell-free workflows that accumulate contaminants and consequences from all preparatory steps for each ingredient in the final reaction. Because plasmid DNA templates are purified from cells, DNA plasmids are arguably more susceptible than linear DNA templates to contaminants that may not be adequately removed during extraction, such as salts and Ribonuclease A (RNase A).

Anecdotally, plasmid DNA extracted from cells using various commercial kits give different and inconsistent results when used in cell-free systems. Workshop participants report that midi and

maxi prep kits [36-38], although more time consuming than mini prep kits [38, 39], generally yield DNA templates that perform better for protein production using cell-free systems than mini prep kits, which require additional purification steps for DNA templates of suitable quality. The Murray laboratory [4] improves the quality of DNA templates after extraction through three consecutive ethanol washes, while the Noireaux laboratory [40] purifies DNA templates after extraction with a cleanup kit developed for use with polymerase chain reaction (PCR) products to obtain a higher protein yield relative to that same DNA template prepared without the additional purification step [41]. Unfortunately, these approaches increase the length of protocols for DNA template preparation, allowing correspondingly more opportunities for DNA breakage, DNA loss, and operator error.

Currently, DNA templates for cell-free systems [42]are typically plasmid DNA, despite the increased labor and time required for preparation as compared to linear DNA. Working Group participants noted that protocols that enable the routine use of linear DNA templates in cell-free systems are highly desirable. Linear DNA is susceptible to degradation in most lysates due to the RecBCD complex, an exonuclease in *E. coli* extracts essential for double-strand break repair [43]. Two successful approaches to protect linear DNA in cell-free systems from degradation by endogenous recBCD use the bacteriophage protein GamS [44] and modified linear dsDNA with six χ-sites (Chi6) [45]. Unproven, impractical, and/or unoptimized alternatives to these include using a modified *E. coli* that removes RecBCD but leads to decreased lysate performance, the addition of small molecules to inhibit RecBCD, and unpublished work suggesting linear DNA modified with unnatural bases may prevent degradation.

Measurement Needs Working Group, led by Eugenia "Jane" Romantseva (National Institute of Standards and Technology)
The Measurement Needs Working Group focused on determining the specific measurement tools, methods, and needs for more reproducible outcomes in cell-free systems. The topics pertained to many aspects of cell-free systems and overlapped substantially with discussions in the other Working Groups. The summary provided here would benefit from a more comprehensive treatment to arrive at prioritized recommendations to best guide the field. The Measurement Needs Working Group identified four areas of the typical workflow for cell-free systems in which better measurement tools and methods could contribute to improved outcomes and reproducibility: instrument calibration and characterization, lysate preparation, DNA template preparation, and protein production.

Regarding the first area, instruments should be calibrated and characterized to understand bias and sources of uncertainty in measured quantities. Instruments common to cell-free workflows that require calibration and characterization include, for example, scales, pipettors, incubators, shakers, plate readers, sonicators, homogenizers, spectrophotometers, and fluorometers. Measurands for this process may include temperature, shaking speed, illumination intensity, energy transferred to the sample, humidity, oxygen concentration, carbon dioxide concentration, and the variation of these across the area or volume in the tool relevant to the sample. In one example, temperature differences across a plate may affect measurements of a standard solution of green fluorescent protein (GFP), and temperature gradients across an individual well may also

be significant. To mitigate this, wells containing temperature sensitive indicators, such as cobalt chloride, could be used to measure temperature and temperature uniformity across a plate. In a second example, and especially for the small reaction volumes typical of cell-free systems, evaporation across a plate could be characterized over the course of the reaction. Unfortunately, not every measurement need has an accessible commercial off-the-shelf solution. While many options exist for calibration plates for temperature, humidity, absorbance, and fluorescence for plate readers, the authors are unaware of a test plate with more than one accelerometer to measure shaking at more than one location across a plate. In a third example, measurements of optical density of cell cultures are a routine measurement included in protocols for lysate preparation, but measurements of optical density are neither quantitative [22] nor comparable across instruments. In a final example, including multiple fluorescent calibration curves on the plate could aid in comparability of fluorescence data across time, operators, sites, and equipment. The authors found sampling the calibration curve in many locations beneficial for accounting for variability across the plate of fluorescent measurements during cell-free protein production.

Measurement needs for the second area of lysate preparation are driven by a desire to know the amount and activity of every component of a cell-free reaction. This desire is motivated partly by the belief and hope that such comprehensive characterization will result in better predictive models, more reproducibility and control, and fully rational design for applications of cell-free systems, as well as significantly broaden our fundamental understanding of biology. This reductionist bias overlooks the biological complexity still present in cell-free systems. The mismatch between a fully realized reductionist understanding of cell-free systems and the measured, experimental reality may serve as a means to study the "aliveness" of these and, ultimately, cell-based systems, perhaps even enabling synthetic and minimal cells [5].

Measurements to adequately characterize the components of cell extracts, the energy buffer, and the fully assembled lysates prior to the addition of the DNA template could inform sources of variability. Measurement needs for the cell extract include, for example, a starting strain that has been validated with proteomics and metabolomics, determining the quantity and quality of residual native genomic DNA after lysate preparation, the abundance and activity of endogenous proteins, and the concentration of components in the extract compared to *in vivo.* On the topic of cell lysis using sonicators and homogenizers, Working Group participants offered anecdotal evidence suggesting that sonicators yield lysates with more reproducible cell-free reactions than other approaches, despite the challenge of calibrating the energy deposited into a sample during sonication. Because vendors of sonication equipment typically report energy output as energy at the piezoelectric element rather than at the probe tip, the sample is exposed to an unknown amount and distribution of energy across the sample. Working Group participants suggested characterizing commercial sonicators using a suspension of beads, dispersed at various sonication settings, and evaluated for sample opacity using well-calibrated plate readers or spectrophotometers. Alternative methods for cell lysis using French press and homogenizers lead to visible oily residue in lysates. It is unclear if and how this residue affects the lysate performance of the assembled cell-free reaction. Regarding residual genomic DNA and endogenous proteins, the presence and abundance of native genomic DNA in the cell extract can be confirmed by using gel electrophoresis to separate the genomic DNA from the sample, followed by commercially available kits to extract the DNA from the gel and fluorometry or

PCR analysis for quantitation. Similarly, to identify abundance and activity of endogenous proteins, Working Group participants suggested fluorescent thermal shift assays, such as ThermoFluor, which incorporate a hydrophobic dye that fluoresces upon binding to molten globules and thermal denaturization intermediates, for high-throughput analyses of protein stability [46].

The measurement needs for the energy buffer supplementing the cell extract include, for example, concentration and purity of component ions (typically magnesium and potassium), amino acids, crowding agents (such as polyethylene glycol (PEG)), cofactors (such as NADH and NAD+), and nucleoside triphosphates (such as ATP, GTP, CTP, and UTP). Although well-established methods exist to characterize the components of the energy buffer, few such analyses are routine in cell-free applications. For example, high performance liquid chromatography (HPLC) or capillary electrophoresis [47] can identify and quantitate ions and amino acids. Commercial assay kits are available for high-throughput measurements of the amount of PEG in biological samples [48, 49], as well as common cofactors, such as NADH and NAD+ [50-52], with colorimetric, fluorometric, or bioluminescent readouts. Nucleoside triphosphates can be analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), hydrophilic interaction liquid chromatography (HILIC), and liquid chromatography–triple quadrupole tandem mass spectrometry with hydrophilic liquid interaction chromatography (LC-TQ-MS-HILIC), which was developed recently for use with cells [53].

Working group participants expressed the strong desire for similar knowledge and characterization of their laboratory prepared lysates as for the PURExpress system. This includes for example, quantifying transcription rates, translation rates, DNA replication rates, and lysate concentration as compared to *in vivo*. Many Working Group participants suggested performing time course proteomics, metabolomics, and LC-MS/MS of a cell-free reaction, while simultaneously admitting to the prohibitive cost, time, and difficulty in interpreting the results. Fluorescent aptamers, such as malachite green, are used routinely to measure RNA production and decay rates, but anecdotal evidence points to poor performance and degradation in cell-free systems. Alternatively, whole transcriptome shotgun sequencing (RNA-Seq) can be used to determine RNA quantity, screen for novel transcripts, and analyze transcript structure. Molecular beacons [54] currently used to image RNA molecules in real-time in living systems could also be adapted for cell-free systems. Ribosome profiling (Ribo-Seq) techniques [55], such as RiboLace [56], offer positional information about ribosomes flowing along the transcript and can characterize translation. These measurements are rarely performed in cell-free applications and remain unpublished. To characterize and troubleshoot overall cell-free performance, Working Group participants suggested standardized plasmids to comparably monitor and track lysate performance. These plasmids would produce only a single specific known output, such as a transcript, a transcription factor, an orthogonal promoter, or an enzyme, such as kinases and phosphatases, and would include the promotor(s) appropriate for use with the intended organism(s).

Discussion regarding measurement needs for the third area of DNA template preparation focused on the desire for high quality, high quantity, reproducible, and robust DNA templates. Ideally,

this would also be achievable for both circular and linear DNA templates and with high-throughput. Existing protocols could, in principle, be expanded to include automation methods, improve reproducibility and throughput, while lowering the cost of DNA template preparation in some cases [57]. DNA templates, which often contain an origin of replication, and any residual native genomic DNA may also replicate in a cell-free reaction, and DNA replication or interactions between DNA and other components of a cell-free reaction may introduce unwanted effects, such as unanticipated resource consumption, that could decrease protein yield or confound reproducibility. These effects could be detected using fluorescent labels or quantitative polymerase chain reaction (qPCR) and may be compatible with high-throughput screening [58]. The Working Group missed the opportunity to define metrics or other criteria related to DNA preparation; measurement methods and needs would have been implicit in such metrics.

For the fourth area of protein production, Working Group participants focused on several ways to potentially improve reproducibility. Accurate and reproducible methods to quantify low concentrations of protein are needed, because plate readers have prohibitively poor sensitivity to low fluorescence signals. This necessitates the use of DNA templates with so-called "strong" promoters to produce enough product to obtain a detectable signal in the cell-free reaction. Additionally, measurements of common fluorescent reporters, such as various green fluorescent proteins, are not reproducible at low concentrations, require accurate instrument calibration, and may not be reproducible across instruments, sites, and operators. Alternative reporters are desirable, including brighter fluorescent reporters with faster folding, alternative colorimetric readouts, and nonfluorescent techniques with high specificity, accuracy, and resolution. Ideally, these would each also be optimized for use in cell-free applications. More direct measurements are needed than those relying on fluorescent reporters. Existing alternatives could include acoustic mass spectrometry [59], cryo-electron microscopy, and chip-based systems coupled to single molecule fluorescence, which remain under development and are not readily scalable. While these methods are prohibitively expensive and time consuming, they may prove useful for visualizing biomolecular conformation.

Working Group participants agreed that a tangible path forward towards more complete and quantitative characterization of a cell-free reaction would be the development of a standard 96-well test plate. The test plate could characterize the cell extract, energy buffer, and cell-free lysate, including initial and final composition. The test plate could also be used to measure the abundance of native genomic DNA and endogenous proteins in the raw cell extract, as well as rates of transcription, translation, and decay. Different chemical and physical conditions could be examined, along with time-course information throughout the duration of a cell-free reaction. In aggregate, this comprehensive information could aid in identifying sources of variability and optimizing reproducibility and performance. A protocol to execute such a test plate could be developed in partnership between NIST and the community of cell-free researchers and include integrated automation. The results of the test plate could provide an internal check on the quality and performance of a cell-free reaction, offer a means to quantitatively compare results across experiments and laboratories, and perhaps even be reported routinely in publications relying on data from cell-free systems. In this way, laboratory-made cell-free systems could also be compared to commercial alternatives. The proof of concept design of the test and associated

protocol could then be passed to commercial manufacturers for additional optimization, production, and distribution. Ideally, the test plate would be designed to characterize and understand cell-free reactions made using cell lysate with the same or more mechanistic detail currently possible with the PURExpress system.

Automation may improve reproducibility in the preparation of cell lysates and DNA templates and the assembly of cell-free reactions. In general, automation systems can reduce user error and uncertainty associated with pipetting, which can be measured. For example, the Labcyte Echo liquid handlers are most commonly reported in automated protocols for cell-free systems, for their ability to accurately dispense small volumes of liquid [60]. The existing anecdotal evidence for the advantages of automation warrants further investigation.

Working Group participants also considered how industry vendors could better serve the community of researchers using cell-free systems. Suggestions included more complete and quantitative manuals for distribution with commercial kits used for preparing components of the cell-free reaction. For example, kits for preparing DNA templates should specify what exactly is eluted at each step of the protocol, as well as the tendency for that step to shear, degrade, or otherwise result in nonfunctional DNA. Quality control for plasmids supplied by commercial vendors could be improved, to spare researchers the cost and time of verifying the sequence themselves. To improve reproducibility for the preparation of cell lysate, purchased or shared strains could ship with specification sheets or other information relevant to easily and rapidly validating the strain. Commercial cell-free kits could provide detailed information on quality control, lot size, and statistics for expected protein yield. For example, the manual for myTXTL requires updating to include the use of an optimized test plasmid that gives approximately double the protein yield than reported in the supplied manual (unpublished results). Vendors could also provide automation protocols with kits and products, as appropriate. Finally, participants called for an overall 10× reduction in the costs for commercial kits used with cell-free systems, both for DNA template preparation and commercial lysates, including the recombinant PURExpress system.

Interlaboratory studies could help identify opportunities to increase reproducibility, develop standardized and reproducible protocols, and clarify the full potential of cell-free systems. To the best of our knowledge, the triservice interlaboratory study is the largest such study performed to date [CCDC Chemical and Biological Center manuscript in preparation]. A much smaller effort was reported as part of iGEM 2018 [61], with an expanded study to include more teams planned for iGEM 2019 [62, 63]. Both of these studies focused primarily on reproducibility in the endpoint measurement of protein yield after completion of the cell-free reaction. Separate studies could optimize the conditions for DNA template extraction, purification, handling, and storage. These results could inform interlaboratory collaborations focused on developing and testing standardized protocols for both circular and linear DNA. NIST is assessing the feasibility and impact of a potential study in this area.

As uses of cell-free systems mature, it is appropriate to engage in rigorous, systematic, and quantitative studies to explore issues around measurement assurance and measurement science

for these systems. For example, it remains unclear what degree reproducibility, as well as the number of biological and technical replicates, is required or desirable for cell-free systems. The consensus urges for more reproducibility and more measurements than we have now. Each experiment and measurement must ultimately be fit for purpose and settling these issues will depend upon the intended use of the information gained from each measurement of a cell-free reaction for each application. While cell-free systems are inherently different from cell-based systems, many of the related challenges in reproducibility and measurement assurance are also relevant to biological systems beyond cell-free systems. An open question remains whether and to what extent absolute quantitation, as opposed to the relative quantitation typical of measurements of biological systems, is important to advance reproducibility and applications of cell-free systems.

Sharing and Reporting Information Working Group, led by Kate Adamala (University of Minnesota)
This Working Group was disbanded, because the topics of sharing and reporting information were drawn extensively through the discussions of the other Working Groups. Instead, William Poole (California Institute of Technology) introduced the BuildACell/CellFree page on OpenWetWare [2], which he administers as a graduate student in the Murray laboratory [4] and is available to researchers in cell-free systems as an initial location to share experimental advice and data.

Outlook

This Report offers practical, actionable recommendations to advance reproducibility in cell-free systems. Applications well-served by cell-free systems will emerge, as the full capabilities of these systems become clearer, in part through more complete understanding of variability and reproducibility. NIST investment and outputs, in close partnership with researchers using cell-free systems, will advance progress in this technology space to support economically-viable applications for bioengineering and biomanufacturing. Cell-free systems may also deepen our fundamental understanding of life itself, for example, by offering an attractive platform towards building artificial and minimal cells [5].

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Disclaimer

Certain commercial equipment, instruments, or materials are identified to adequately specify experimental procedures. Such identification implies neither recommendation nor endorsement by the National Institute of Standards and Technology nor that the materials or equipment identified are necessarily the best available for the purpose.

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Agenda

Time	Activity	Room
12:00 pm	Registration (and lunch/coffee/snacks)	Lobby/patio
12:30 pm	Welcome, scope, and deliverables (Elizabeth Strychalski and Jane Romantseva)	Auditorium
1:00 pm	Cell-free wiki (William Poole)	Auditorium
1:15 pm	Breakout sessions: Modeling Lysate preparation DNA template preparation Measurement needs Sharing and reporting information (disbanded)	TBD
3:45 pm 4:00 pm 4:15 pm 4:30 pm 4:45 pm	Reports from breakout sessions: Modeling Lysate preparation DNA template preparation Measurement needs Sharing and reporting information	Auditorium
5:00 pm	Group discussion	Auditorium
5:30 pm	Wrap-up, homework, and concluding remarks (Elizabeth Strychalski and Jane Romantseva)	Auditorium
6:00 pm	Adjourn for joint dinner	Lobby/patio

Workshop participants

Last name	First name	Affiliation	Working Group
Adamala	Kate	University of Minnesota	Lysate preparation
Bashirzadeh	Yashar	University of Michigan	DNA preparation
Benjamin	David	Synlife	Lysate preparation
Buschnyj	Justin	University of Minnesota	Measurement needs
Cole	Stephanie	US Army ECBC	Lysate preparation
Deich	Chris	University of Minnesota	DNA preparation
Ehrenreich	Ian	University of South Carolina	DNA preparation
Garza	Erin	JCVI	Lysate preparation
Gaut	Nathan	University of Minnesota	Lysate preparation
Glass	John	JCVI	Lysate preparation
Heus	Hans	Radboud University	Lysate preparation
Huck	Wilhelm	Radboud University	Modeling
Hutchison	Clyde	JCVI	Modeling
Jackson-Smith	Anton	Stanford University	Measurement needs
Jurado	Zoila	Caltech	DNA preparation
Karas	Bogumil	Western University	DNA preparation
Kempes	Chris	Santa Fe Institute	Modeling
Larsson	Elin	Caltech	Modeling
Lux	Matthew	US Army ECBC	Measurement needs
Maheshwari	Akshay	Stanford University	Measurement needs
Martinez	Heather	Qiagen	DNA preparation
McGovern	Sammi	BioBlaze	DNA preparation
McManus	John	Caltech	Modeling
Merryman	Chuck	JCVI	Modeling
Meyerowitz	Joseph	Caltech	Measurement needs
Moser	Felix	Synlife	Modeling
Murray	Richard	Caltech	Modeling
Nguyen	Michael Truong-Giang	Aarhus University	DNA preparation

Niederholtmeyer	Henrike	UCSD	Lysate preparation
Noireaux	Vincent	University of Minnesota	DNA preparation
Pandey	Ayush	Caltech	Modeling
Panganiban	Jeremy	BioBlaze	Lysate preparation
Poole	William	Caltech	Modeling
Romantseva	Jane	NIST	Measurement needs
Rothschild	Lynn	NASA	Lysate preparation
Shaffer	Jonathan	Qiagen	Measurement needs
Smith	Hamilton	JCVI	DNA preparation
Strychalski	Elizabeth	NIST	Measurement needs
Styczynski	Mark	Georgia Tech	Lysate preparation
Sun	Zachary	Synvitrobio	Lysate preparation
Suzuki	Yo	JCVI	Unknown
Swanson	Haley	San Diego State University	Modeling
Valipour	Michael	Bioblaze	DNA preparation
Walper	Scott	Naval Research Laboratory	Lysate preparation
Wei	Eric	Stanford University	Modeling
Wise	Kim	JCVI	Lysate preparation