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# Engineering modular ester fermentative pathways in *Escherichia coli*

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ABSTRACT

Sensation profiles are observed all around us and are made up of many different molecules, such as esters. These profiles can be mimicked in everyday items for their uses in foods, beverages, cosmetics, perfumes, solvents, and biofuels. Here, we developed a systematic 'natural' way to derive these products via fermentative biosynthesis. Each ester fermentative pathway was designed as an exchangeable ester production module for generating two precursors – alcohols and acyl-CoAs that were condensed by an alcohol acyltransferase to produce a combinatorial library of unique esters. As a proof-of-principle, we coupled these ester modules with an engineered, modular, *Escherichia coli* chassis in a plug-and-play fashion to create microbial cell factories for enhanced anaerobic production of a butyrate ester library. We demonstrated tight coupling between the modular chassis and ester modules for enhanced product biosynthesis, an engineered phenotype useful for directed metabolic pathway evolution. Compared to the wildtype, the engineered cell factories yielded up to 48 fold increase in butyrate ester production from glucose.



## 1. Introduction

Chemicals and transportation fuels are mainly derived from petroleum-based feedstocks, which are not renewable or sustainable. Their increasing demand has adversely affected market prices, the environment, and national energy security (Hill et al., 2006). To tackle this problem, recent research has focused on exploiting microbial conversion routes to produce these chemicals and fuels from renewable and sustainable biomass feedstocks (Lynd et al., 2008; Somerville et al., 2010; Stephanopoulos, 2008). Some of the challenges are to overcome biomass recalcitrance, and engineer novel microbial biocatalysts that can efficiently convert lignocellulosic biomass into target biochemicals and biofuels and replace those synthesized from petroleum-based feedstocks in a competitive manner (Himmel et al., 2007; Stephanopoulos, 2007).

Esters are valuable chemicals that have broad applications in foods, beverages, cosmetics, perfumes, solvents, and biofuels. Most esters $R_1COOR_2$ are currently produced by the Fisher esterification of an organic acid $R_1COOH$ and an alcohol $R_2OH$ derived from the petroleum-based feedstocks under hazardous conditions using corrosive acid/base and high temperature (Riemenschneider and Bolt, 2000). The diversity of $R_1$ and $R_2$ moieties consisting of linear, branched, even, odd, saturated, unsaturated, and/or aromatic carbon structures can cover a large combinatorial space of esters with unique properties. Since esters are commonly found in living species, such as plants and microbes, there is great potential to produce these esters via microbial conversion routes from renewable and sustainable feedstocks.

In living cells, esters can be esterified from an ac(et)yl-CoA (or aryl-CoA) and an alcohol via an alcohol acyltransferase (AAT). Alcohols such as ethanol (Ohta et al., 1991; Trinh et al., 2008), propanol (Jain and Yan, 2011; Jun Choi et al., 2012; Shen and Liao, 2008; Srirangan et al., 2013), isopropanol (Hanai et al., 2007; Lee et al., 2012), butanol (Bond-Watts et al., 2011; Inui et al., 2008; Lee et al., 2008; Nielsen et al., 2009), and isobutanol (Atsumi et al., 2008; Trinh et al., 2011) can be synthesized from either fermentative or Ehrlich pathways, and ac(et)yl-CoAs from the *de novo* fatty acid biosynthesis (Handke et al., 2011; Magnuson et al., 1993) or reverse β-oxidation cycle (Dellomonaco et al., 2011) (Fig. 1). With the diversity of alcohol and ac(et)yl-CoA synthesis, a combinatorial library of esters can be synthesized, and many of these esters can be found in nature such as fruits – bananas (Beekwilder et al., 2004; Harada et al., 1985), melons (El-Sharkawy et al., 2005; Lucchetta et al., 2007; Shalit et al., 2001; Yahyaoui et al., 2002), strawberries (Aharoni et al., 2000; Olías et al., 2002; Perez et al., 1993, 1996), and plant waxes (Kunst and Samuels, 2003, 2009; Samuels et al., 2008) or brewery yeast (Suomalainen and Lehtonen, 1979). For instance, esterification of isobutanol and butyryl-CoA yields isobutyl butyrate, which has a fruity aroma and can be used as food supplement or solvent for lacquer and nitrocellulose. Some of these "natural" esters can also be synthesized by recombinant hosts such as *E. coli*, *Lactococcus lactis*, and *Clostridium acetobutylicum* expressing different AATs (Aharoni et al., 2000; Beekwilder et al., 2004; El-Sharkawy et al., 2005;

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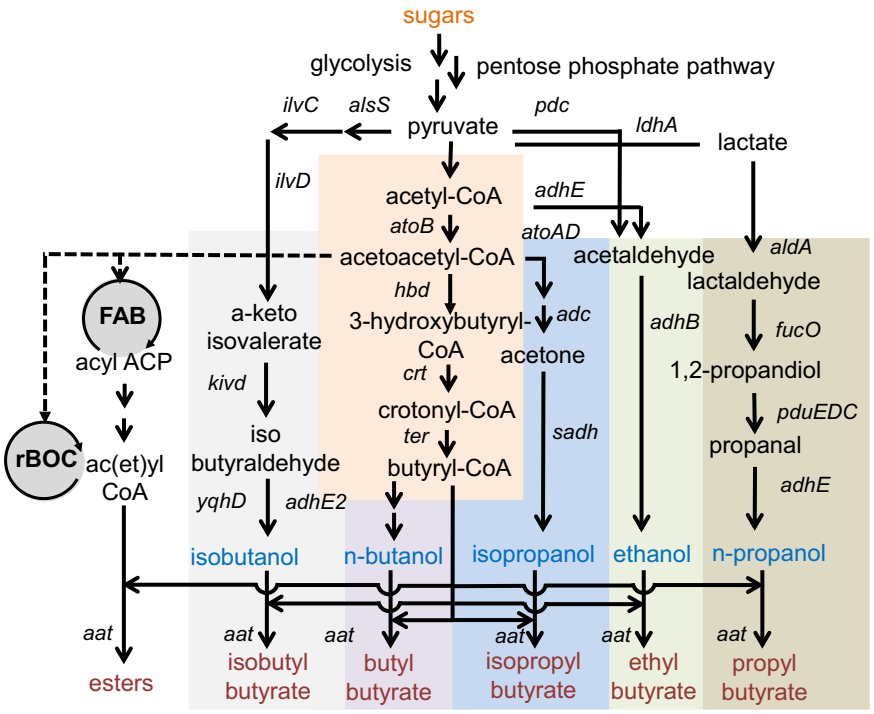


**Fig. 1.** A simplified metabolic network demonstrating the production of esters by esterification of alcohols (e.g., ethanol, propanol, isopropanol, butanol, and isobutanol) and acyl-CoAs from biomass-derived fermentable sugars by microbial conversion routes. Acyl-CoAs can be derived from the fermentative pathways, the fatty acid biosynthesis pathway (FAB), or the reverse β-oxidation cycle (rBOC).

Harada et al., 1985; Hernandez et al., 2007; Lucchetta et al., 2007; Olías et al., 2002; Park et al., 2009; Perez et al., 1993, 1996; Rodriguez et al., 2014; Shalit et al., 2001; Vadali et al., 2004; Yahyaoui et al., 2002). Recently, recombinant *S. cerevisiae* and *E. coli* were also engineered to produce fatty acid methyl (Menendez-Bravo et al., 2014; Nawabi et al., 2011), ethyl (Kalscheuer et al., 2006; Runguphan and Keasling, 2014; Shi et al., 2014; Steen et al., 2010; Yu et al., 2011) and short-chain esters as biodiesels (Guo et al., 2014), which expands the ester solution space beyond what has been previously mentioned.

In this study, we designed and constructed modular ester producing pathways that tightly couple with a modular *E. coli* chassis designed for efficient combinatorial biosynthesis of novel esters under anaerobic conditions. Anaerobic fermentation is the most efficient and economical route to produce biochemicals and biofuels such as esters due to the following reasons: (1) the reducing equivalent NADH generated from sugar degradation can be effectively recycled by modular ester fermentative pathways to enhance ester production and (2) scale-up fermentation processes are much simpler and less expensive since the supply and precise control of air are not required and contamination issues can be minimized (Blanch, 2012; Huang and Zhang, 2011; Trinh, 2012; Trinh et al., 2011). We designed each ester fermentative pathway as an exchangeable ester production module for producing two precursors, alcohols and acyl-CoAs, which were then condensed by an alcohol acyltransferase to produce a combinatorial library of unique esters. As a proof-of-principle, we coupled each of the fermentative ester modules with the modular chassis in a plug-and-play fashion to create modular microbial cell factories for enhanced production of a butyrate ester library. We demonstrated these cell factories could secrete esters extracellularly and enhance ester production via *in situ* fermentation and extraction. By performing kinetic characterization of the optimized ethyl butyrate production strain, we demonstrated tight coupling between the modular chassis and the ester production modules for enhanced butyrate ester production, an engineered novel phenotype useful for the directed metabolic pathway evolution.

## 2. Materials and methods

### 2.1. Strain construction

Table 1 lists strains used in this study. *E. coli* TOP10 was primarily used for molecular cloning. *E. coli* MG1655 and its derivatives were used for ester production studies. Mutants with chromosomal gene deletion were constructed by using the P1 transduction (Trinh et al., 2006). To construct mutants for expressing T7 promoter, the prophage λDE3 was used to insert a T7 polymerase gene into the specific site of the mutant chromosome by using a commercial kit (cat#69734-3, Novagen Inc.) Mutants with gene deletion or/and addition were confirmed by PCR amplification with the primers listed in Table 2.

### 2.2. Plasmid/pathway construction

Modular metabolic pathway engineering strategy (Tseng and Prather, 2012; Xu et al., 2013, 2012) was applied to design ester fermentative pathways as exchangeable ester production modules that are derived from multiple sub-modules (Figs. S1–S3). In general, each ester production module consists of an acyl-CoA production submodule, an AAT production submodule, and an alcohol production submodule. Each submodule is designed as one operon that contains at least one gene under a consecutive T7 promoter. The acyl-CoA and AAT production submodules are organized in a pETite∗ vector backbone using the ampicillin selection marker. The alcohol production submodule is organized in a different pETite∗ vector using the kanamycin selection marker. This modular metabolic pathway engineering enables quick replacement of ribosome binding sites, promoter strengths, and origin of replications for manipulating plasmid copy number to control and optimize metabolic fluxes through the ester fermentative pathways.

Genes encoding the ester fermentative pathways are organized in plasmids. Table 1 shows a list of plasmids used and generated in this study, and Table 2 presents a list of primers used for the plasmid

**Table 1**
A list of strains and plasmids used in this study.

| | Genotypes | Sources |
|---|---|---|
| **Plasmids** | | |
| pCP20 | *flp*, *bla*, *cat*, *cI*857λts | Yale collection |
| pCOLA | kan$^+$ | Novagen |
| pETite C-His | pBR322 *ori*; kan$^+$ | Lucigen |
| pETite∗ | kan$^R$ | this study |
| pCT13 | pCOLA $P_{T7}$::RBS::*alsS*::RBS::*ilvC*::RBS:: *ilvD* – $P_{T7}$::RBS::*kivd*::RBS::*adhE*::$T_{T7}$; kan$^+$ | Trinh 2011 |
| pCT24 | pETite∗ $P_{T7}$::RBS::*pdc*::RBS::*adhB*::$T_{T7}$; kan$^+$ | This study |
| pCT79 | pETite∗ $P_{T7}$:: RBS::*atoD*:: RBS::*atoA*::RBS::*thl*:: RBS::*adc*::RBS::*sadh*::$T_{T7}$; kan$^+$ | This study |
| pAY7 | pETite∗ $p_{T7}$::*atoD*::*atoA*; kan$^+$ | This study |
| pDL1 | pETite∗ SAAT; kan$^+$ | This study |
| pDL2 | pETite∗ $P_{T7}$::RBS::*atoB*::RBS::*hbd*::RBS:: *crt*::RBS::*ter*::$T_{T7}$; kan$^+$ | This study |
| pDL3 | pETite∗ $P_{T7}$:: RBS::*atoB*:: RBS::*hbd*::RBS:: *crt*:: RBS::*ter*:: RBS::$T_{T7}$::*saat*::$T_{T7}$; amp$^+$ | This study |
| **Strains** | | |
| *T. denticola* | Wildtype | ATCC 35405 |
| *C. acetobutylicum* | Wildtype | ATCC 824 |
| TOP10 | F-*mcrA* Δ(*mrr-hsd*RMS-*mcr*BC)Φ80*lacZ* ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G | Invitrogen |
| JW5020-1 | BW25113 Δ*fadE::kan*$^+$ | CGSC#11134 |
| MG1655 | F$^-$ λ$^-$ | ATCC 700926 |
| TCS083 | MG1655, Δ*zwf*::Δ*ndh*::Δ*sfcA*::Δ*maeB*::Δ*ldhA*::Δ*frdA*:: Δ*poxB*::Δ*pta*::kan$^-$ (cured) | Trinh 2008 |
| EcDL001 | MG1655 (λDE3) Δ*fadE*::kan$^-$ (cured) | This study |
| EcDL002 | TCS083 (λDE3) Δ*fadE*::kan$^-$ (cured) | This study |
| EcDL101 | EcDL001 carrying pDL3; amp$^+$ | This study |
| EcDL201 | EcDL001 carrying pDL3 and pCT24; kan$^+$ and amp$^+$ | This study |
| EcDL202 | EcDL001 carrying pDL3 and pCT13; kan$^+$ and amp$^+$ | This study |
| EcDL203 | EcDL001 carrying pDL3 and pCT79; kan$^+$ and amp$^+$ | This study |
| EcDL204 | EcDL002 carrying pDL3 and pCT24; kan$^+$ and amp$^+$ | This study |
| EcDL205 | EcDL002 carrying pDL3 and pCT13; kan$^+$ and amp$^+$ | This study |
| EcDL206 | EcDL002 carrying pDL3 and pCT79; kan$^+$ and amp$^+$ | This study |

construction and validation. All plasmids were constructed using the pETite∗ backbone that was modified from pETite to be compatible with the BglBrick Gene Assembly method (Anderson et al., 2010). The XhoI restriction site in the kanamycin gene of pETite∗ and EcoRI restriction site downstream of the T7 promoter was removed via site-directed mutagenesis (cat#210519-5, Agilent Inc.) by using the primers P004_f/P004_r and P013_f/P013_r, respectively. A new BglII restriction site that is 50 nucleotides upstream of the T7 promoter was created by using the primers P012_f/P012_r.

### 2.2.1. Construction of the butyryl-CoA submodule

The design of the butyryl-CoA production submodule is pDL2, pETite∗ $P_{T7}$::RBS::*atoB*::RBS::*hbd*::RBS::*crt*::RBS::*ter*::$T_{T7}$, where $P_{T7}$ and $T_{T7}$ are T7 promoter and T7 terminator, respectively. This submodule converts acetyl-CoA to butyryl-CoA that is based on the fermentative butanol pathway of *C. acetobutylicum* and was previously demonstrated to be functional in *E. coli* (Inui et al., 2008). The plasmid pDL2 was assembled by the Gibson Gene Assembly method (Gibson et al., 2009) using 5 DNA fragments: the backbone fragment amplified from pETite∗ using the primers DL_0001/DL_0002, the gene *atoB* amplified from the genomic DNA of *E. coli* MG1655 using the primers DL_0003/DL_0004, the genes *hbd* and *crt* amplified from *C. acetobutylicum* using the primers DL_0005/DL_0006 and DL_0007/DL_0008, respectively, and the gene *ter* amplified from *Treponema denticola* using the primers DL_0009/DL_0010.

### 2.2.2. Construction of the butyryl-CoA plus AAT submodule

The SAAT gene was used to construct the butyryl-CoA plus AAT submodule – pDL3 (pEtite∗ $P_{T7}$::RBS::*atoB*::RBS::*hbd*::RBS::*crt*::RBS::*ter*::RBS::$T_{T7}$::*saat*::$T_{T7}$). SAAT was derived from the strawberry alcohol acyltransferase and provided by Dr. Jules Beekwilder (Plant Research International) as a kind gift. This gene was amplified from the plasmid pRSET-SAAT (Aharoni et al., 2000) by using the primers DL_0011/DL_0012 and inserted into pETite∗ to create pDL1. The assembly of the butyryl-CoA plus SAAT submodule to form pDL3 was performed by using the Gibson Gene Assembly method with 3 DNA pieces: the butyryl-CoA operon amplified from pDL2 using the primers DL_0003/DL_0014, the AAT operon amplified from pDL1 using the primers DL_0015/DL_0016, and the backbone pETite∗ amplified using the primers DL_0013/DL_0002.

### 2.2.3. Construction of the ethanol production submodule

The ethanol production submodule (pCT24, pETite∗ $P_{T7}$::RBS::*pdc*::RBS::*adhB*::$T_{T7}$) was designed to convert pyruvate to ethanol. The plasmid pCT24 was assembled by the BglBrick Gene Assembly method using 3 DNA pieces: the gene *pdc* amplified from pLOI297 using the primers P006_f/P006_r and digested with NdeI/BamHI, the gene *adhB* amplified from pLOI297 using the primers P007_f/P007_r and digested with BglII/XhoI, and the vector backbone pETite∗ doubly digested with NdeI/XhoI.

### 2.2.4. Construction of the isopropanol production submodule

The isopropanol production submodule – pCT79 (pETite∗ $P_{T7}$::RBS::*atoD*::RBS::*atoA*::RBS::*thiL*::RBS::*adc*::RBS::*sadH*::$T_{T7}$) was designed to convert acetyl-CoA to isopropanol based on the previous study (Hanai et al., 2007). We first constructed pAY7 (pETite∗ $P_{T7}$::RBS::*atoD*::RBS::*atoA*::$T_{T7}$) by amplifying the genes *atoD* and *atoA* from the genomic DNA of *E. coli* MG1655 with the primers AY.22f/AY.22r and AY.23f/AY.23r, respectively and ligating them into the pETite∗ by the BglBrick Gene Assembly method. We then constructed pCT79 by the Gibson Gene Assembly method with 4 DNA pieces: the backbone fragment amplified from pETite∗ with the primers NN35/DL_0002, the genes *thiL*, *adc* and *sadH* amplified from the genomic DNA of *C. acetobutylicum* using the primers NN36/NN37, NN38/NN39, and NN40/MW.sadH.r, respectively.

### 2.2.5. Construction of the isobutanol production submodule

The isobutanol production submodule – pCT13 pCOLA $P_{T7}$::RBS::*alsS*::RBS::*ilvC*::RBS::*ilvD* – $P_{T7}$::RBS::*kivd*::RBS::*adhE*::$T_{T7}$ was previously constructed (Trinh et al., 2011).

**Table 2**
A list of primers for plasmid and strain construction and validation.

| Primer name | Sequences |
|---|---|
| **Primers used to build the butyryl-CoA submodule (pDL2)** | |
| DL_0001 | 5′–CATCATCACCACCATCACTAA–3′ |
| DL_0002 | 5′-ATGTATATCTCCTTCTTATAGTTAAAC-3′ |
| DL_0003 | 5′-TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACAT ATGAAAAATTGTGTCATCGT-3′ |
| DL_0004 | 5′-TTAATTCAACCGTTCAATCAC-3′ |
| DL_0005 | 5′-CGGTCAGGGAATTGCGATGGTGATTGAACGGTTGAATTAAA AAGAGGAGAAAATGAAAAAGGTATGTGTTATAG-3′ |
| DL_0006 | 5′-TTATTTTGAATAATCGTAGAAAC-3′ |
| DL_0007 | 5′-AAGAAAATCAGGAAAAGGTTTCTACGATTATTCAAAATAAA AAGAGGAGAAAATGGA ACTAAACAATGTCATC-3′ |
| DL_0008 | 5′-CTATCTATTTTTGAAGCCTTC-3′ |
| DL_0009 | 5′-CATAGAGAAAAGAAAAATTGAAGGCTTCAAAAATAGATAG AAAGAGGAGAAAAT GATTGTAAAACCAATGGTTAG-3′ |
| DL_0010 | 5′-GCCGCTCTATTAGTGATGGTGGTGATGATGTTAAATCCTGTC GAACCTT T-3′ |
| **Primers used to build the ethanol submodule (pCT24)** | |
| P006_f | 5′-GAAGGAGATATACATATGAGTTATACTGTCGGTACCTATTT AGCGGAG-3′ |
| P006_r | 5′-GTGATGGTGGTGATGATGCTCGAGTTAGGATCCCTAGAGGA GCTTGTTAACAGG-3′ |
| P007_f | 5′-AAAAAACTCGAGTTAGGATCCTTAGAAAGCGCTCAGGAAGAG -3′ |
| P007_r | 5′-AAAAAAGAATTCATGAGATCTAAGGAGATATAATGGCTTCT TCAACTTTTTAT-3′ |
| **Primers used to build the isopropanol submodule (pCT79)** | |
| AY.22f | 5′-AAAAAACATATGAAAACAAAATTGATGACATTA-3′ |
| AY.22r | 5′-AAAAAAAGATCTTTATTTGCTCTCCTGTGAAAC-3′ |
| AY.23f | 5′-AAAAAAGGATCCAAGGAGATATAATGGATGCGAAACAAC GTATT-3′ |
| AY.23r | 5′-AAAAAACTCGAGTCATAAATCACCCCGTTGCGT-3′ |
| NN35 | 5′-ACTTACTATTACTACATCTCTcatTATATCTCCTTtcaTAAATCACCCCGTTGCGTATT-3′ |
| DL_0002 | 5′-ATGTATATCTCCTTCTTATAGTTAAAC-3′ |
| NN36 | 5′-AATACGCAACGGGGTGATTTATGAAAGGAGATATAATGAGAGAT GT AGTAATAGT AAGT-3′ |
| NN37 | 5′-TTTAATTACTTCATCCTTTAACATTATATCTCCTTTTAGTCTCTTTC AA CTACGAGAG C-3′ |
| NN38 | 5′-GCTCTCGTAGTTGAAAGAGACTAAAAGGAGATATAATGTTAAAG GAT GAAGTAATT AAA-3′ |
| NN39 | 5′-ACCTAGCATTGCAAAACCTTTCATTATATCTCCTTTTACTTA AGATAATCATATATAAC-3′ |
| NN40 | 5′-GTTATATATGATTATCTTAAGTAAAAGGAGATATAATGAAA GGTTTTGCAATGCTAGGT-3′ |
| MW.sadh.r | 5′-GTGGCGGCCGCTCTATTAGTGATGGTGGTGATGATGTTATA ATATAACTACTGCTTTAAT-3′ |
| **Primers used to build the AAT submodule (pDL1)** | |
| DL_0001 | 5′-CATCATCACCACCATCACTAA-3′ |
| DL_0002 | 5′-ATGTATATCTCCTTCTTATAGTTAAAC-3′ |
| pETiteDL_0011 | 5′-GAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATG GAGAAAATTGAGGTCAG-3′ |
| pETiteDL_0012 | 5′-GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGTTAAATT AAGGTCTTTGGAG-3′ |
| **Primers used to build the butyryl-CoA plus AAT submodule (pDL3)** | |
| DL_0003 | 5′-TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATA TGAAAAATTGTGTCATCGT-3′ |
| DL_0014 | 5′-ATATCAAGCTTGAATTCGTTACCCGG-3′ |
| pETiteDL_0015 | 5′-GGAGGAACTATATCCGGGTAACGAATTCAAGCTTGATATTA ATACGACTCACTATAGGG-3′ |
| pETiteDL_0016 | 5′-GTCCAGTTACGCTGGAGTCTGAGGCTC-3′ |
| DL_0013 | 5′-GAGCCTCAGACTCCAGCGTA-3′ |
| DL_0002 | 5′-ATGTATATCTCCTTCTTATAGTTAAAC-3′ |
| **Primers used for knockout verification** | |
| P043_KO_ldhA_f | 5′-TTTCTGGCGGATTTTTATCG-3′ |
| P043_KO_ldhA_r | 5′-CGTCAACGGCACAAGAATAA-3′ |
| P051_KO_frdA_f | 5′-AGTTGATGCAACCGGAGAAC-3′ |
| P051_KO_frdA_r | 5′-ACGGCGAGACAAATTTTACG-3′ |
| maeA_fwd_KO | 5′-CAGCGTAGTAAATAACCCAACC-3′ |
| maeA_rev_KO | 5′-GACAGCTTAACGGCTTTGTAG-3′ |
| P044_KO_zwf_f | 5′-CGATGAACGGTCGAAGTTTT-3′ |
| P044_KO_zwf_r | 5′-TGCCATAGCAGCAATACTCG-3′ |
| P049_KO_ndh_f | 5′-GCAGACGCACAAATTCAAGA-3′ |
| P049_KO_ndh_r | 5′-ACGGGAACACCTCCTTCTTT-3′ |
| maeB_fwd_KO | 5′-GATGATAATGGCGAATGGAC-3′ |
| maeB_rev_KO | 5′-CGTTCTTTATCCATGAGTCG-3′ |
| P045_KO_pta_f | 5′-TCACTGGTGGTATCGGTGAA-3′ |
| P045_KO_pta_r | 5′-GAATGCGAAATGAGTGTGGA-3′ |
| P046_KO_poxB_f | 5′-ATGGATATCGTCGGGTTTGA-3′ |
| P046_KO_poxB_r | 5′-AAGCAATAACGTTCCGGTTG-3′ |
| fadE_fwd_KO | 5′-CGCATTATTCGGCCTACGGTTC-3′ |
| fadE_rev_KO | 5′-CCAGACTCCGTTTGTAATGCAACAC-3′ |
| DE3_fwd | 5′-ATGAACACGATTAACATCGC-3′ |
| DE3_rev | 5′-TTACGCGAACGCGAAGTC-3′ |

All plasmid constructs were checked by enzyme digestion, PCR amplification, and sequencing before characterization.

### 2.3. Medium and cell culturing techniques

#### 2.3.1. Culture media

For molecular cloning, Luria-Bertani (LB) was used with supplementation of antibiotic where applicable. Antibiotics at working concentrations of 50 μg/mL kanamycin (kan), 100 μg/mL ampicillin (amp) were used to maintain the selection of single plasmids. For dual plasmids, two antibiotics were used for selection and concentrations of each antibiotic were used at half of their working concentrations for single plasmid selection.

For ester production experiments, the M9 hybrid medium (pH∼7) was used, consisting of 100 ml/L of 10X M9 salts, 1 ml/L of 1 M $MgSO_4$, 100 μL/L of 1 M $CaCl_2$, 1 ml/L of stock thiamine HCl solution (1 g/L), 1 ml/L of stock trace metals solution (Trinh et al., 2008), 5 g/L yeast extract, and appropriate antibiotics. Unless

specified, 20 g/L glucose was used in the M9 hybrid medium. The stock 10X M9 salt solution contained 67.8 g/L $Na_2HPO_4$, 30 g/L $KH_2PO_4$, 5 g/L NaCl, and 10 g/L $NH_4Cl$. For fermentation experiments using glucose greater than 20 g/L, the M9 hybrid medium was also supplemented with 50 mM MOPS for buffering capacity.

#### 2.3.2. Strain characterization for ester production

For high-cell density fermentation experiments, cells were grown overnight in culture tubes containing the M9 hybrid medium, and subcultured the next morning until the exponential phase ($OD_{600\ nm}$ ~ 2.0) was reached. Next cells were transferred in a fresh M9 hybrid medium with an initial $OD_{600\ nm}$ ~ 0.05, grown to $OD_{600\ nm}$ ~ 5.0, and then induced with IPTG at a working concentration of 0.5 mM for 30 min to activate production modules. To set consistent characterization conditions among strains, cells were then spun down, resuspended in a fresh M9 hybrid medium containing 0.5 mM IPTG, and transferred into 15 mL polypropylene centrifuge tubes with a working volume of 10 mL. For exogenous alcohol addition experiments, the stock alcohols were added to reach a concentration of ~2 g/L. The tubes were wrapped in PTFE tape to seal tube threading and capped to ensure complete anaerobic conditions. The residue oxygen in the medium and head space should be exhausted less than 1 h. For endogenous ester production experiments, characterization was carried out in the same fashion as the exogenous alcohol additions for ethyl butyrate, isopropyl butyrate, and isobutyl butyrate production. For isopropyl butyrate production, ~10 g/L of acetate was also added into the medium due to the necessary conversion of acetate to produce isopropanol.

Cells were grown on a 75° angled platform in a New Brunswick Excella E25 at 37 °C and 175 rpm. After 24 h, the culture tubes were submerged in an ice water bath for at least 1 h to condense any volatiles. Whole-cells and cell supernatants were stored at −20 °C for subsequent metabolite analysis.

For the *in situ* high-glucose fermentation and extraction experiments, cells were first grown overnight in culture tubes with the M9 hybrid medium, subcultured into capped flasks the next morning to an $OD_{600\ nm}$ ~ 0.05, and grown to $OD_{600\ nm}$ ~ 1.0. Cells were then subcultured a second time into anaerobic bottles with fresh medium with dodecane overlay (a 1:10 volume ratio of dodecane to medium), an initial $OD_{600\ nm}$ ~ 0.1, and a final working volume of 120 mL. Cells were grown to $OD_{600\ nm}$ ~ 2.0 and induced with IPTG at a working concentration of 0.2 mM. Sampling and pH (~7) adjustment with 5 M KOH were performed every 24 h. The anaerobic bottles were set up in a way to reduce the $CO_2$ pressure build up by rerouting the pressure to an external tube with dodecane to create a closed, anaerobic, system to produce ethyl butyrate. All characterization experiments were performed at least in biological triplicates.

### 2.4. Analytical methods

#### 2.4.1. High performance liquid chromatography (HPLC)

Metabolites from cell supernatants were quantified by using a Shimadzu HPLC system equipped with RID and UV–Vis detectors, and an Aminex HPX-87H cation exchange column (BioRad Inc.) A sample was first filtered through a 0.2-μm filter unit, then loaded into the column operated at 50 °C, and eluded with the 10 mN $H_2SO_4$ mobile phase running at a flow rate of 0.6 mL/min (Trinh et al., 2011).

#### 2.4.2. Gas chromatography coupled with mass spectroscopy (GC/MS)

For the aqueous phase analysis, 500 μL of samples (cells plus supernatant) were transferred to a 2 mL polypropelyene microcentrifuge tube with a screw cap containing 100–200 mg of glass beads (0.25–0.30 mm in diameter), 60 μL of 6 N HCl, and 500 μL of hexane solution containing ~3 mg/L amyl acetate as an internal standard. The cells were lysed by bead bashing for 5 min using a Biospec Mini BeadBeater 16 and then centrifuged at 13,300*g* for 1 min. The extractants of the organic layer were diluted 10 fold in hexane with internal standard and used for the GC/MS analysis. For the organic phase analysis conducted in the *in situ* fermentation and extraction experiments, samples in the dodecane layer were used directly for the GC/MS analysis.

All esters were analyzed by using the HP6890 GC/MS system equipped with a 30 m × 0.25 mm i.d., 0.25 μm film thickness column plus an attached 10 m guard column (Zebron ZB-5, Phenomenex Inc.) and a HP 5973 mass selective detector. A selected ion mode (SIM) method was deployed to analyze 1 μL of samples. The GC method was programmed with an initial temperature of 50 °C with a 1 °C/min ramp up to 58 °C then a 25 °C/min ramp was deployed to 180 °C. The final ramp was then issued to a final temperature of 300 °C at a rate of 50 °C/min. The injection was performed using a splitless mode with an initial MS source temperature of 160 °C. The carrier gas used was helium flowing at a rate of 0.5 mL/min. The detection was delayed 6.5 min due to solvent delay and then esters were detected using the following SIM parameters: ions 56.10 and 73.10 detected from 6.50 to 7.20 min for isobutyl acetate, ions 71.1, 88.10, and 116.0 detected from 7.20 to 7.85 min for ethyl butyrate, ions 56.10 and 73.00 detected from 7.85 to 8.50 min for butyl acetate, ions 71.00 and 89.10 detected from 8.50 to 10.15 min for isopropyl butyrate and propyl butyrate, ions 70.10 and 101.00 detected from 10.15 to 10.70 min for amyl acetate, ions 71.10 and 114.10 detected from 10.70 to 11.25 min for isobutyl butyrate, and ions 71.10, 89.10, and 116.00 detected from 11.25 to $t_{final}$ min for butyl butyrate.

## 3. Results

### 3.1. Establishing the butyrate ester fermentative pathways in E. coli

Butyrate esters can be produced directly from sugars by esterification of butyryl-CoA and alcohols via alcohol acyl transferases (AATs). High production of butyrate esters depends on not only the high fluxes of butyryl-CoA- and alcohol-producing pathways, but also enzyme specificity and activity of AATs. To establish the butyrate ester fermentative pathway in *E. coli*, first we tested the function of the butyryl-CoA plus SAAT production submodule under anaerobic conditions. SAAT was chosen because it had broad substrate specificity (Aharoni et al., 2000).

#### 3.1.1. Investigating targeted butyrate ester production by alcohol addition

We first constructed the *E. coli* base strain, EcDL001 derived from *E. coli* MG1655, containing the disrupted acyl-CoA dehydrogenase FadE which blocks the degradation of acyl-CoA pool required for the ester biosynthesis (Clark, 1981). We then introduced the Butyryl-CoA plus SAAT production module, pDL3, into EcDL001 to yield EcDL101. We characterized EcDL101 in high-cell density fermentation experiments to test the function of pDL3 and its alcohol specificity. This was done by externally adding various short-chain alcohols including ethanol, propanol, isopropanol, butanol, and isobutanol at working concentrations of 2 g/L. The results show that EcDL101 successfully produced the targeted butyrate esters with exogenous addition of alcohols under anaerobic conditions (Fig. 2).

EcDL101 produced ethyl butyrate with a titer of 6.00 ± 0.13 mg/L and a specific ester production rate of 80.02 ± 3.26 μg/gDCW/h after 24 h by exogenous ethanol addition to the medium (Fig. 2B). Similarly, addition of propanol yielded 5.21 ± 0.10 mg/L and 69.16 ± 1.65 μg/gDCW/h of propyl butyrate; addition of isopropanol produced 4.31 ± 0.28 mg/L and 57.13 ± 2.97 μg/gDCW/h of isopropyl butyrate; addition of isobutanol achieved 2.08 ± 0.07 mg/L and 27.00 ± 1.02 μg/gDCW/h of isobutyl butyrate; and addition of butanol produced 2.37 ± 0.04 mg/L and

30.55 ± 1.24 μg/gDCW/h (Fig. 2B). Of note, EcDL101 also produced ethyl butyrate as byproduct in some alcohol addition experiments but at lower concentrations and rates than the controlled experiments. This was likely due to the availability of endogenous acetyl-CoA and the function of native aldehyde/alcohol dehydrogenases such as *adhE* for converting acetyl-CoA to ethanol. Taken together, EcDL101 achieved the highest ethyl butyrate production and exhibited different specificity towards different added alcohols with the following order: ethanol > propanol > isopropanol > butanol > isobutanol.

### 3.1.2. Demonstrating extracellular secretion of butyrate esters

We hypothesized that *E. coli* produced and secreted butyrate esters extracellularly due to the aroma produced. To test this hypothesis, we analyzed the butyrate ester production by using two different extraction methods with hexane as an extraction solvent. The first method extracted esters from the whole-cell culture which were mechanically lysed. The second method extracted esters by using only the cell supernatant. The results show that at least 76% of butyrate esters were secreted extracellularly by EcDL101 (Fig. 3A). It is interesting to observe that 99% of ethyl butyrate was excreted extracellularly while only 76% to ~89% was excreted for the longer, bulkier esters such as isobutyl butyrate. This difference could be due to the limitations placed on molecular diffusion through the cell membrane or due to active pumping mechanisms. This discovery leads to future investigation of the limits placed on the ester secretion mechanisms found in *E. coli* and perhaps allow for engineering of targeted pumps for further excretion for the enhancement of ester production.

### 3.1.3. Engineering E. coli base strain for endogenous butyrate ester production from glucose

The issue with exogenous alcohol addition for ester production is the inhibitory effect of alcohols on cellular function and product

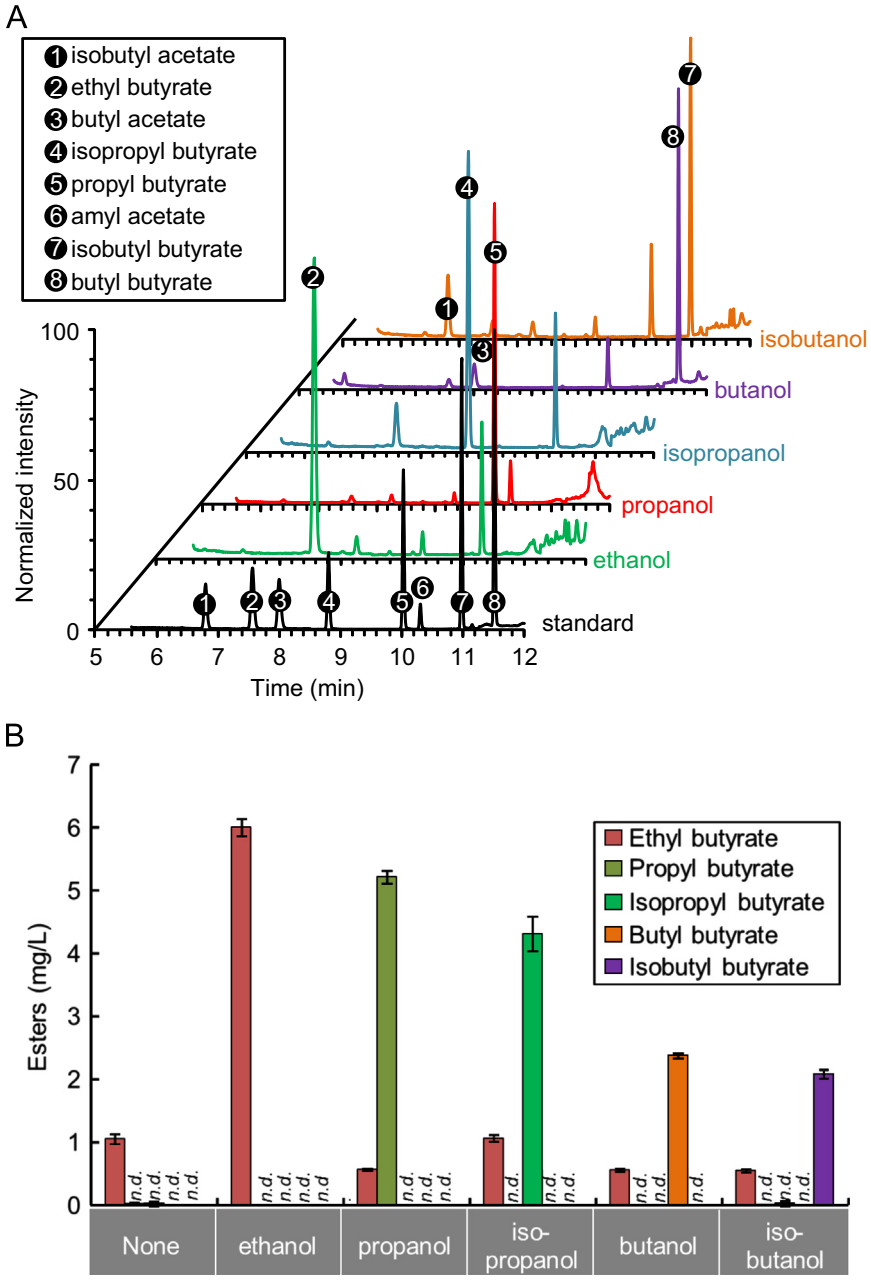


**Fig. 2.** *In vivo* production of butyrate esters by exogenously adding into the medium short chain alcohols—ethanol, propanol, isopropanol, butanol, and isobutanol at a working concentration of 2 g/L: (A) GC/MS chromatograms of butyrate esters produced by EcDL101 and (B) *in vivo* butyrate ester production by EcDL101. *n.d.*: not detected.

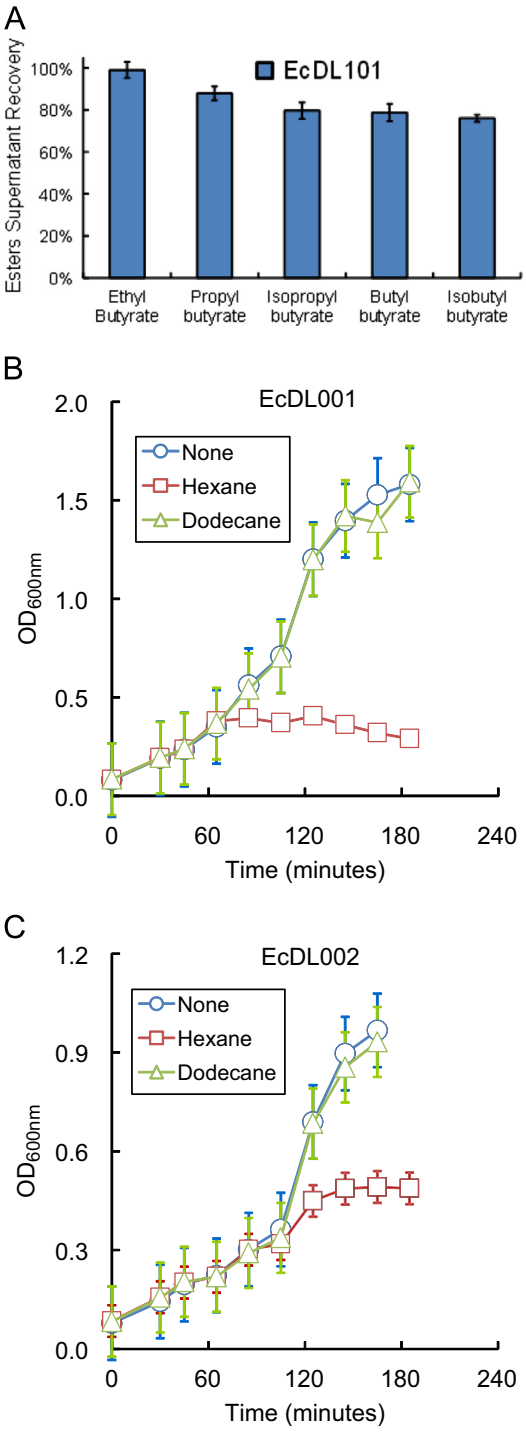


**Fig. 3.** (A) Demonstration of extracellular secretion of butyrate esters by EcDL101. (B) Effect of overlaying organic solvents, hexane and dodecane, on cell growth of EcDL001 and EcDL002 (C).

formation, and the cost of alcohols themselves. Thus, it is advantageous to endogenously produce the alcohols, along with butyryl-CoA, to produce butyrate esters directly from sugars. We constructed the butyrate ester fermentative pathways by simply combining the butyryl CoA plus SAAT production submodule (pDL3) with any alcohol submodules. In this study, we focused on characterizing the ethyl butyrate production module (pDL3/pCT24), isopropyl butyrate module (pDL3/pCT77), and isobutyl butyrate module (pDL3/pCT13). The base strain carrying these modules are named as EcDL201, EcDL202, and EcDL203 and are designed to produce ethyl butyrate, isopropyl butyrate, and isobutyl butyrate, respectively, from glucose under anaerobic conditions.

We characterized EcDL201, EcDL202, and EcDL203 in high-cell density fermentation experiments. The results show all characterized base strains successfully produced the targeted butyrate esters directly from glucose under anaerobic conditions (Fig. 4 and Table 3). After 24 h, EcDL201 produced ethyl butyrate at a titer of $3.21 \pm 0.14$ mg/L and a specific ester production rate of $39.17 \pm 2.00$ μg/gDCW/h; EcDL202 produced isopropyl butyrate at $0.18 \pm 0.02$ mg/L and $2.64 \pm 0.26$ μg/gDCW/h; and EcDL203 produced isobutyl butyrate at $0.26 \pm 0.00$ mg/L and $3.71 \pm 0.06$ μg/gDCW/h. Besides the targeted butyrate esters, EcDL202 and EcDL203 also produced high level of ethyl butyrate as a byproduct. Taken all together, ethyl butyrate was produced with the highest specificity (99%) and rate by EcDL201 but the trend appeared to be reciprocal for production of isopropyl butyrate (29%) by EcDL202 and isobutyl butyrate (19%) by EcDL203. This could be due to flux imbalance for synthesizing the ester precursors, butyryl CoA and alcohols, as well as the specificity of SAAT. Residue alcohols were produced in a range of 1.63–5.97 g/L in total for all characterized strains (Fig. S4).

### 3.2. Optimizing endogenous production of butyrate esters

#### 3.2.1. Endogenous butyrate ester production by the modular strain

Low level of ester production in the base strain is expected because it has multiple competitive fermentative pathways. To enhance butyrate ester production, we next designed and constructed the modular chassis EcDL002 (having 9 gene knockouts and 1 gene knockin, see Table 1). This chassis had the disrupted FadE and was derived from TCS083 that was previously designed by elementary mode analysis (EMA), constructed, and validated for enhanced ethanol production under anaerobic conditions (Trinh et al., 2008). In brief, TCS083 was designed to block all major fermentative pathways, oxidative pentose phosphate pathway, and inefficient electron transport system (see Trinh et al., 2006, 2008 for details). Based on EMA, the modular chassis could tightly couple growth with enhanced production of not only ethanol but also other alcohols (butanol and isobutanol) (Trinh, 2012) and their associated esters under anaerobic conditions (Liu and Trinh, under review) (this novel phenotype experimentally validated and presented later in the text).

We constructed EcDL204, EcDL205, and EcDL206 by transforming the ethyl butyrate, isopropyl butyrate, and isobutyl butyrate production modules into EcDL002, respectively. We then characterized these coupled modular strains in high-cell density fermentation experiments for enhanced butyrate ester production directly from glucose. The result shows that the coupled modular strains successfully produced the targeted butyrate esters endogenously from glucose under anaerobic conditions and outperformed the base strains characterized above (Fig. 4 and Table 3).

EcDL204 produced ethyl butyrate at a titer of $37.16 \pm 0.43$ mg/L (98% specificity) and a specific ester production rate of $536.62 \pm 1.74$ μg/gDCW/h after 24 h, yielding ~14 fold higher ethyl butyrate production than the base strain EcDL201. Likewise, EcDL205 produced isopropyl butyrate at $1.34 \pm 0.05$ mg/L (26% specificity) and $21.37 \pm 0.58$ μg/gDCW/h, reaching ~8 fold higher isopropyl butyrate production than the base strain EcDL202. EcDL206 produced isobutyl butyrate at $1.54 \pm 0.09$ mg/L (29% specificity) and $23.15 \pm 1.44$ μg/gDCW/h, achieving ~6 fold higher isobutyl butyrate production than the base strain EcDL203. Among the engineered modular strains, EcDL204 produced ethyl butyrate with the highest titer, rate, and specificity. Like EcDL202-3, EcDL205-6 produced a large fraction of ethyl butyrate as byproduct with the same reason as previously described.

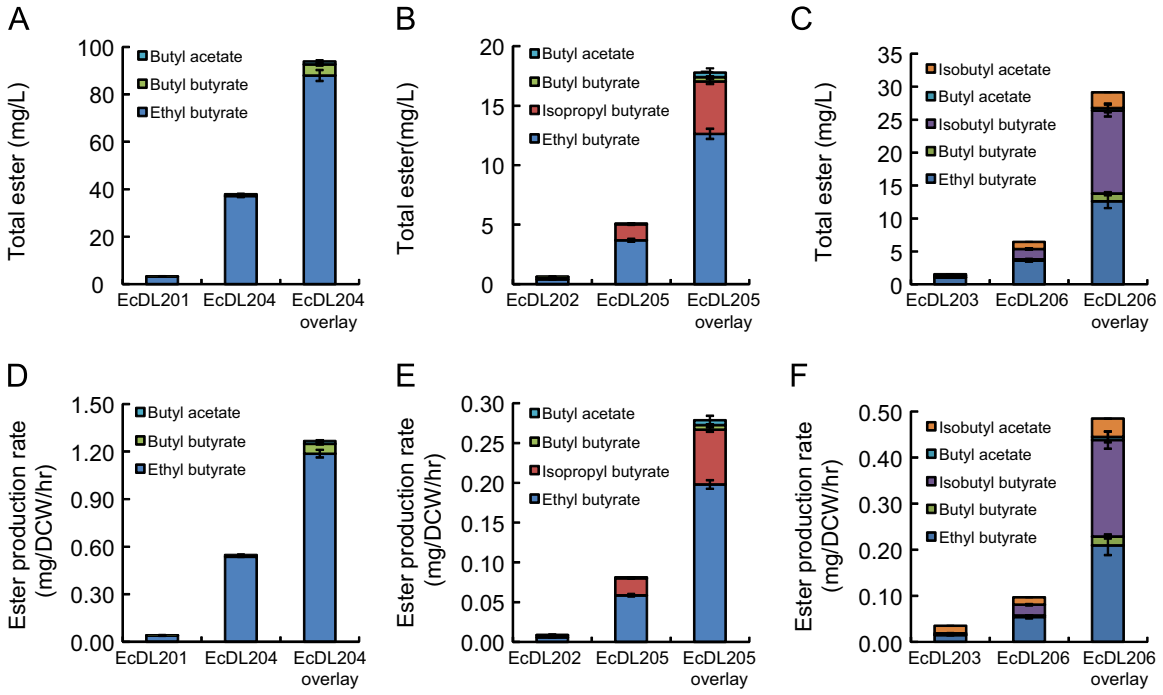


**Fig. 4.** Endogenous production of ethyl butyrate, isopropyl butyrate, and butyl butyrate from glucose by the base strains (EcDL201, EcDL202, and EcDL203) and modular strains (EcDL204, EcDL205 and EcDL206) after 24 h. Panels A–C: ester titers produced, Panels D–F: specific ester production rates. Dodecane overlay was used for solvent extraction during the *in situ* fermentation and extraction.

**Table 3**
Production of butyrate esters by EcDL201-6 in high-cell density fermentation experiments after 24 h. The shaded cells of the table correspond to the expected butyrate esters produced by the engineered strains. Values shown in parenthesis are product specificity. *n.d.*: not detected by GC/MS.

| Strains | Ethyl butyrate | Isopropyl butyrate | Isobutyl butyrate | Butyl butyrate | Isobutyl acetate | Butyl acetate | Total | Total ester yield (mg/g) |
|---|---|---|---|---|---|---|---|---|
| | mg/L (%) | | | | | | | |
| EcDL201 | 3.21 ± 0.14 (99%) | *n.d.* | *n.d.* | 0.02 ± 0.00 (1%) | *n.d.* | *n.d.* | 3.24 ± 0.14 | 0.16 ± 0.01 |
| EcDL202 | 0.41 ± 0.01 (66%) | 0.18 ± 0.02 (30%) | *n.d.* | 0.03 ± 0.00 (4%) | *n.d.* | *n.d.* | 0.62 ± 0.01 | 0.1 ± 0.01 |
| EcDL203 | 1.07 ± 0.01 (69%) | *n.d.* | 0.26 ± 0.00 (2%) | 0.03 ± 0.00 (17%) | 0.19 ± 0.01 (12%) | *n.d.* | 1.37 ± 0.01 | 0.16 ± 0 |
| EcDL204 | 37.16 ± 0.43 (98%) | *n.d.* | *n.d.* | 0.75 ± 0.16 (2%) | *n.d.* | *n.d.* | 37.91 ± 0.41 | 2.69 ± 0.05 |
| EcDL205 | 3.68 ± 0.12 (72%) | 1.34 ± 0.05 (26%) | *n.d.* | 0.07 ± 0.00 (2%) | *n.d.* | *n.d.* | 5.09 ± 0.17 | 0.36 ± 0.01 |
| EcDL206 | 3.61 ± 0.16 (56%) | *n.d.* | 1.54 ± 0.09 (24%) | 0.23 ± 0.02 (3%) | 1.08 ± 0.02 (17%) | *n.d.* | 5.38 ± 0.28 | 1.04 ± 0.02 |
| EcDL204 overlay | 87.93 ± 2.26 (94%) | *n.d.* | *n.d.* | 4.62 ± 0.40 (5%) | *n.d.* | 1.29 ± 0.42(1%) | 93.85 ± 3.05 | 15.3 ± 0.43 |
| EcDL205 overlay | 12.63 ± 0.42 (71%) | 4.40 ± 0.22 (25%) | *n.d.* | 0.37 ± 0.04 (2%) | *n.d.* | 0.38 ± 0.34(2%) | 17.79 ± 0.71 | 1.8 ± 0.07 |
| EcDL206 overlay | 12.64 ± 1.07 (43%) | *n.d.* | 12.60 ± 0.93 (43%) | 1.14 ± 0.24 (4%) | 2.39 ± 0.25 (8%) | 0.40 ± 0.69(2%) | 28.77 ± 2.48 | 2.87 ± 0.23 |

### 3.2.2. *Enhancing ester production by in situ fermentation and extraction*

Since butyrate esters have low solubility in the aqueous phase and are secreted extracellularly, they can be isolated from the fermentation broth via solvent extraction. This simultaneous *in situ* fermentation and extraction strategy can potentially increase ester production, minimize product cytotoxicity on cell growth, and reduce downstream separation costs. Therefore, we first examined some common candidate solvents – hexane and dodecane to determine whether they can be used for the *in situ* fermentation and extraction. We characterized the growth of the base strain (EcDL001) and modular strain (EcDL002) on these two extraction solvents. The result shows that hexane was toxic to cell growth while dodecane was not (Fig. 3B and C). Therefore, we decided to use dodecane for *in situ* fermentation and extraction to enhance ester production in subsequent studies.

We characterized the coupled modular strains EcDL204, EcDL205, and EcDL206 in high cell density experiments with *in situ* extraction for enhanced production of ethyl butyrate, isopropyl butyrate, and isobutyl butyrate, respectively. The results show that all characterized strains significantly improved targeted ester production with *in situ* extraction. After 24 h, EcDL204 increased the ethyl butyrate production about 2.2 fold from 37.16 ± 0.43 mg/L (or 536.62 μg/gDCW/h) to 87.93 ± 2.26 mg/L (1187.35 ± 24.32 μg/gDCW/h). Likewise, EcDL205 improved the isopropyl butyrate production about 3.2 fold from 1.34 ± 0.05 mg/L (or 21.37 ± 0.58 μg/gDCW/h) to 4.40 ± 0.22 mg/L (or 68.96 ± 2.91 μg/gDCW/h). EcDL206 increased the isobutyl butyrate production about 9.0 fold from 1.54 ± 0.09 mg/L (or 23.15 ± 1.44 μg/gDCW/h) to 12.60 ± 0.93 mg/L (or 209.10 ± 18.58 μg/gDCW/h). The yields of ethyl butyrate, isopropyl butyrate, and isobutyl butyrate on glucose were 15.3 ± 0.43, 1.8 ± 0.07, and 2.87 ± 0.23 mg ester/g glucose for EcDL204, 205, and 206, respectively (Table 3).

Overall, deploying the coupled modular strains together with *in situ* fermentation and extraction resulted in ~27 fold increase in ethyl butyrate production, ~24 fold in isopropyl butyrate production, and ~48 fold in isobutyl butyrate production over the base strain.

### 3.3. Demonstrating obligate ester fermentative pathways in the modular strain

We performed the anaerobic growth kinetics of the coupled modular strains to demonstrate that modular ester producing pathways as obligate fermentative pathways. We chose EcDL204 as a model candidate because it can produce ethyl butyrate with high specificity, titer, and rate. First, we performed the ester-module dependent growth experiments to demonstrate tight coupling between the ester module and modular chassis under anaerobic conditions. We characterized EcDL001, EcDL002, and EcDL204 in the anaerobic rubber capped tubes sparged with nitrogen as shown in Fig. 5. The results show that EcDL001, a positive control, could grow without carrying any ester production modules as expected because its genotype is quite similar to the wildtype *E. coli* MG1655. On the contrary, the modular chassis EcDL002, a negative control, could not grow anaerobically because it did not carry any ester production module. However, the coupled modular strain EcDL204 (EcDL002 pDL3/pCT24) could recover growth anaerobically by carrying the ethyl butyrate production module. This result clearly demonstrates the tight coupling between the modular chassis and ester production modules. This engineered phenotype is very useful for the directed metabolic pathway evolution to enhanced ester production because ester-overproducing mutants can be isolated based on a simple and effective growth selection (Fong et al., 2005; Jantama et al., 2008; Shen et al., 2011; Trinh and Srienc, 2009; Unrean and Srienc, 2011).

Next, we investigated the fermentation kinetics of EcDL204 by characterizing it in an *in situ* high-glucose fermentation and extraction experiments with low cell inoculation. The results show that EcDL204 could grow and couple with the ethyl butyrate production module under anaerobic conditions. The coupled modular strain produced ethyl butyrate during both the (0–24 h) growth and (24–96 h) stationary phases (Fig. 6A). After 96 h, EcDL204 produced ethyl butyrate up to 134.00 ± 4.83 mg/L (78% specificity), about 1.52 fold increase from the previous 24 h experiment (Fig. 6B), and 36.83 ± 2.13 mg/L of butyl butyrate. The yield of total ester production was 4.36 ± 0.35 mg/g glucose. It is of interest to note that residue alcohols, 12.39 g/L of ethanol and 0.37 g/L butanol, were also observed at the end of fermentation. Production levels of residue alcohols, suggest (1) low turnover of the alcohol acyltransferase, (2) high ethanol flux, and (3) a relatively high production (22% of total produced esters) of butyl butyrate as a byproduct when compared to the 99% product specificity observed in the high-cell density fermentation experiments. This result provides insight to the efficiency and future optimization of the butyrate ester modules.

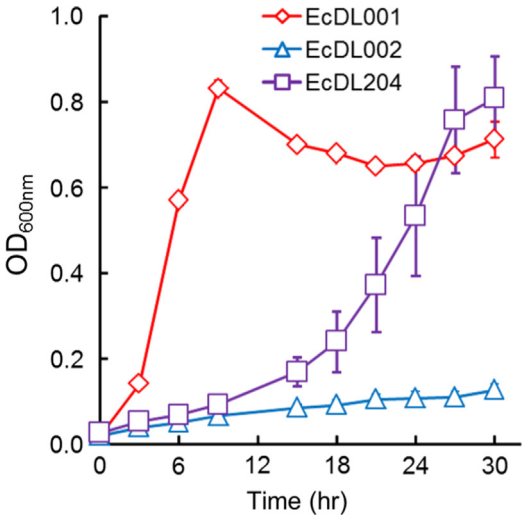


**Fig. 5.** Growth kinetics study demonstrating the tight coupling between the modular chassis and ethyl butyrate production module as an obligate ester fermentative pathway. Positive control strain: EcDL001, negative control strain: EcDL002, and test strain: EcDL204.

## 4. Discussion

Esters represent a diverse class of unique molecules with a broad range of applications that can be synthesized combinatorially by condensation of acyl-CoA and alcohol moieties. By using the modular pathway design, we presented a systematic approach to construct and characterize ester production with the focus on exploring the modular butyrate ester fermentative pathways. The base strain EcDL101 could overproduce a variety of butyrate esters such as ethyl butyrate, propyl butyrate, isopropyl butyrate, butyl butyrate, and isobutyl butyrate with exogenous addition of short-chain alcohols, where each one of these bioesters can be used as an additive for flavor and fragrances. This strain can potentially produce a variety of butyrate esters with exogenous supply of other alcohols, which are currently being explored in our laboratory. Of note, EcDL101 (or EcDL002/pDL3) can be deployed as a platform to screen other novel AATs besides SAAT to make unique butyrate esters. Likewise, this modular design strategy can be exploited to screen for other acyl-CoA plus AAT submodules to produce other esters. This whole-cell screening method is expected to be more sensitive, quicker, and cheaper than the traditional screening method because extracellularly secreted esters can be collected in an organic layer, and this method does not require AATs to be expressed, purified, and characterized with exogenous addition of both acyl-CoAs and alcohols.

Energetic concerns are of high priority when developing modular ester fermentative pathways to ensure cofactor balancing. By utilizing anaerobic conditions we can use the butyrate ester production module as an effective electron sink and remove the competitive effect of an oxygen electron sink to direct reducing equivalents toward enhanced ester production. Based on elementary mode analysis, there exist efficient pathways that could support anaerobic growth and butyrate ester production during growth and no-growth phases. During the no-growth phase, the maximum theoretical yields of ethyl butyrate, isopropyl butyrate, and isobutyl butyrate are 0.67, 0.47, and 0.67 Cmol ester/Cmol glucose (or 0.43, 0.29, and 0.40 g ester/g glucose), respectively (Liu and Trinh, under review). In this study, we successfully demonstrated the design, construction, and characterization of modular butyrate ester pathways as obligate fermentative pathways for combinatorial biosynthesis of butyrate esters directly from fermentable sugars under anaerobic conditions.

Compared to the base strain, the coupled modular strains EcDL204-6 achieved 27, 24, and 48 fold increase in production of ethyl butyrate, isopropyl butyrate, and isobutyl butyrate, respectively, from glucose via fermentation and extraction with dodecane overlay. Kinetic characterization of EcDL204 achieved a titer of 134 mg/L in the high glucose fermentation and extraction, the highest titer reported to date. Despite achieving significant increase in butyrate ester production by the engineered strains, the product yields were still low. It is expected that the target ester

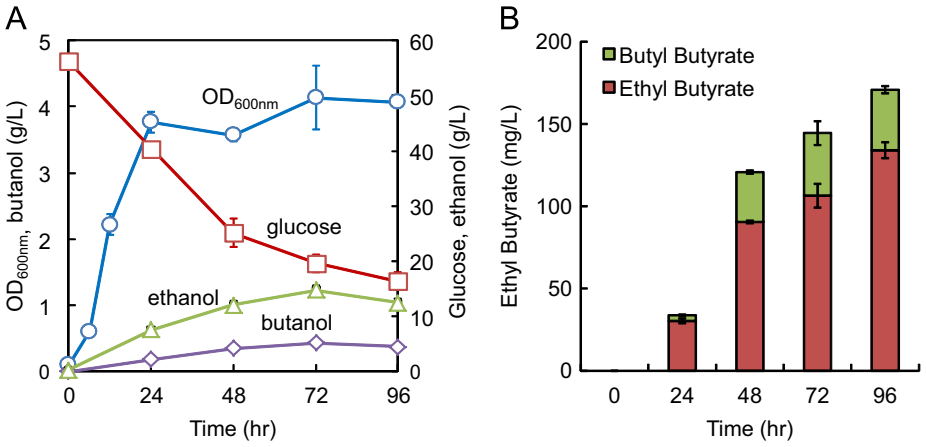


**Fig. 6.** Fermentation kinetics of endogenous ethyl butyrate production by the coupled modular strain EcDL204 in the *in situ* high-glucose fermentation and extraction: (A) metabolic profiles of cell growth ($OD_{600\ nm}$), glucose consumption, and alcohol production. (B) Ethyl butyrate production.

production will significantly be improved with medium optimization and characterization in controlled bioreactors with either *in situ* solvent extraction or *in situ* gas stripping. In addition, it is important to further enhance the butyrate ester production by balancing and enhancing fluxes through the butyrate ester production modules by manipulating promoters (Cox et al., 2007), ribosome binding sites (Anderson et al., 2010; Salis et al., 2009), and plasmid copy numbers (Kittleson et al., 2011) and harnessing efficient AATs.

As shown in Fig. 1, esters constitute a large space of unique molecules seen in nature such as fruits and plants. Our research is fundamentally different from a recent work by Rodriguez et al. (2014). Here, we laid out the foundation to engineer modular ester fermentative pathways as exchangeable ester production modules. We investigated the biosynthesis of butyrate esters (such as ethyl butyrate, isopropyl butyrate, and isobutyl butyrate) directly from glucose under anaerobic conditions while Rodriguez et al. focused on the biosynthesis of acetate esters. It is interesting to observe that ATF1 from *S. cerevisiae* had high specificity towards acetate esters such as isobutyl acetate in Rodriguez et al.'s report but not others such as butyrate esters of this study. In addition, we engineered our modular chassis that coupled with ester production modules for anaerobic production of targeted esters. However, strains employed in Rodriguez et al.'s work were derived from JCL260 and likely cannot support anaerobic production of reduced metabolites such as butanol, isobutanol, and derived esters directly from sugars under anaerobic conditions due to redox imbalance leading to growth inhibition (Trinh, 2012; Trinh et al., 2011).

Since coupled modular strains in this study could produce esters under anaerobic conditions and secrete them for easy extraction, they have great advantages for scale-up fermentation processes. Furthermore, esters produced via microbial conversion routes are considered as "natural" with valuable and broad applications as flavor and fragrance products. The other advantage to produce esters in a recombinant host, such as *E. coli*, is the flexibility to achieve high product selectivity by manipulating orthogonal acyl-CoA, alcohol pathways, and AATs. As demonstrated in this study, we introduced the heterologous butyryl-CoA, ethanol, isopropanol, and isobutanol pathways and SAAT for making butyrate esters with relatively high selectivity with some unavoidable byproducts due to endogenous production of acetyl-CoA and ethanol in *E. coli* and broad substrate specificity of SAAT. We believe this issue can be addressed by screening for novel, highly-specific AATs that are abundant in nature.

Indeed the diversity of alcohol ac(et)yltransferases span over every higher order eukaryote that produces a flavor and fragrance, and are capable of condensing a plethora of alcohols and CoA moieties to form esters. We see this in every fruit we taste, and every flower we smell. Using this enzyme class enables us to engineer alcohol and CoA pathways to produce vibrant, desirable molecules. Nature has already perfected these enzymes, but the issue at hand lies in the screening process for higher specificity, faster turnover. Unfortunately, screening every enzyme found in nature for desired product formation is nearly impossible, but, utilizing techniques such as random mutagenesis, high throughput screening, and molecular dynamic simulations, will allow further understanding and development of specificity across this diverse enzyme class. By coupling plug-and-play designer alcohol and CoA modules with the designed modular chassis, demonstration of a combinatorial product library points toward a library of esters limited only by user's imagination.

## Acknowledgments

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at: http://dx.doi.org/10.1016/j.ymben.2014.09.006.

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