De novo Microbial Biosynthesis of a Lactate Ester Platform

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Abstract

Green organic solvents such as lactate esters have broad industrial applications. Manufacturing and use of these biodegradable solvents from renewable feedstocks help benefit the environment. This study presents a microbial conversion platform for direct fermentative biosynthesis of lactate esters from fermentable sugars. To enable the *de novo* microbial biosynthesis of lactate esters, we first designed a sugar-to-lactate ester module, consisting of a lactate dehydrogenase (*ldhA*), a propionate CoA-transferase (*pct*), and an alcohol acyltransferase (*AAT*) to convert sugar to lactate esters. By generating a library of five sugar-to-lactate ester modules with divergent AATs, we screened for the best module(s) capable of producing a wide range of linear, branched, and aromatic lactate esters with an external alcohol supply. By co-introducing a sugar-to-lactate ester module and an alcohol (i.e., ethanol, isobutanol) module into a modular *Escherichia coli* (chassis) cell, we demonstrated for the first time the *de novo* microbial biosynthesis of ethyl and isobutyl lactate esters directly from glucose. In an attempt to enhance ethyl lactate production as a proof-of-study, we re-modularized the pathway into the upstream module to generate the ethanol and lactate precursors and the downstream module to generate lactyl-CoA and condense it with ethanol to produce the target ethyl lactate. By manipulating the metabolic fluxes of the upstream and downstream modules though plasmid copy numbers, promoters, ribosome binding sites, and environmental perturbation, we were able to probe and alleviate the metabolic bottlenecks by improving ethyl lactate production by 4.96-fold.

Keywords: ester; lactate ester; ethyl lactate; isobutyl lactate; acetate ester; alcohol acyltransferase; green solvent; modular cell; *Escherichia coli*.

1. Introduction

Green solvents such as lactate esters are platform chemicals that have a broad range of industrial applications in flavor, fragrance, and pharmaceutical industries [1]. Manufacturing and use of these solvents from renewable feedstocks help benefit the environment. For instance, ethyl lactate is 100% biodegradable, non-carcinogenic, non-corrosive, low volatile, and unhazardous to human health and the environment [2]. Due to the unique beneficial properties of ethyl lactate, it has been approved as a Significant New Alternatives Policy (SNAP) solvent by the U.S. Environmental Protection Agency (EPA) and as food additives by the U.S. Food and Drug Administration (FDA) [2]. Additionally, recent technical and economic analysis conducted by the National Renewable Energy Laboratory (NREL) considers ethyl lactate to be one of the top 12 bioproducts [3].

Lactate esters are generally produced by esterification of lactic acid with alcohols using homogenous catalysts (i.e., sulfuric acid, hydrogen chloride, and/or phosphoric acid) under high temperature reaction conditions [4]. However, the esterification reactions are thermodynamically unfavorable (ΔG = +5 kcal/mol) in aqueous solutions and often encounter significant challenge due to self-polymerization of lactate [5]. Alternatively, microbial catalysts can be used to produce these esters in a thermodynamically favor reaction (ΔG = -7.5 kcal/mol) in an aqueous phase environment at room temperature and atmospheric pressure [6-12]. This reaction uses an alcohol acyl transferase to generate an ester by condensing an alcohol and an acyl-CoA. Currently, the *de novo* microbial biosynthesis of lactate esters directly from fermentable sugars has not yet been demonstrated in microbes.

In this work, we aimed to demonstrate the feasibility of microbial production of lactate esters as green organic solvents, from renewable resources. To establish a *de novo* microbial biosynthesis of a lactate ester platform directly from fermentable sugars, we first started by *in vivo*

screening for an efficient alcohol acyltransferase (AAT) for lactate esters production using a library of five the sugar-to-lactate ester modules with divergent AATs. By co-introducing a sugar-to-lactate ester module and an alcohol module (i.e., ethanol, isobutanol) into an engineered *Escherichia coli* modular cell, we demonstrated direct microbial biosynthesis of ethyl and isobutyl lactate esters from glucose. In an attempt to enhance ethyl lactate production as a proof-of-study, we re-modularized the pathway into the upstream module to generate the ethanol and lactate precursors and the downstream module to generate lactyl-CoA and condense it with ethanol to produce the target ethyl lactate. By manipulating the metabolic fluxes of the upstream and downstream modules though plasmid copy numbers, promoters, ribosome binding sites (RBSs), and environmental perturbation, we were able to probe and alleviate the potential metabolic bottlenecks by improving ethyl lactate production.

2. Materials and methods

2.1. Strain construction

The list of strains used in this study is presented in Table 1. For molecular cloning, *E. coli* TOP10 strain was used. To generate the lactate ester production strains, the modules, including i) the sugar-to-lactate ester modules (pJW002-006), ii) the upstream and/or downstream modules (pJW007-pJW028), and iii) the alcohol modules (pCT24 or pCT13), were transformed into the engineered modular *E. coli* chassis cell, EcDL002 [6], via electroporation [13].

2.2. Plasmid construction

The list of plasmids and primers used in this study is presented in Table 2 and Table 3, respectively. Pathway construction includes i) sugar-to-lactate ester modules, ii) a library of

upstream and downstream modules, and iii) a library of synthetic operons for the upstream and downstream modules.

2.2.1. Construction of sugar-to-lactate ester modules. A library of sugar-to-lactate ester modules with five divergent AATs were constructed to screen for an efficient AAT for production of lactate esters via two rounds of cloning. First, the sugar-to-lactyl-CoA module (pJW001) was constructed by assembling three DNA fragments: i) the *ldhA* gene encoding D-lactate dehydrogenase amplified from *E. coli* MG1655 genomic DNA using the primer pair DL_0032/DL_0033, ii) the *pct* gene encoding propionate CoA-transferase amplified from *Clostridium propionicum* genomic DNA using the primer pair DL_0034/DL_0035, and iii) the backbone amplified from pETite* using the primer pair DL_0001/DL_0002 [14]. Then, the sugar-to-lactate ester modules (pJW002-006) were constructed by assembling three DNA fragments: i) the sugar-to-lactyl-CoA module amplified from pJW001 using the primer pair DL_0032/DL_0014, ii) the *ATF1* gene amplified from pDL004 for pJW002, the *ATF2* gene amplified from pDL005 for pJW003, the *SAAT* gene amplified from pDL001 for pJW004, the *VAAT* gene amplified from pDL006 for pJW005, or the *atfA* gene amplified from pCT16 for pJW006, using the primer pair DL_0015/DL_0016, and iii) the backbone amplified from the pETite* using the primer pair DL_0013/ DL_0002. The genes *ATF1* and *ATF2* are originated from *Saccharomyces cerevisiae* [15] whereas the genes *SAAT*, *VAAT* and *atfA* are derived from *Fragaria ananassa* [16], *F. vesca* [17], and *Acinetobacter* sp. ADP1 [18], respectively.

2.2.2. Construction of a library of upstream and downstream modules with various plasmid copy numbers. A library of upstream and downstream modules were constructed to improve ethyl lactate biosynthesis through a combinatorial pathway optimization strategy using three different plasmids; i) pACYCDuet-1 (P15A origin); ii) pETDuet-1 (ColE1 origin); and iii)

pRSFDuet-1 (RSF1030 origin), having the plasmid copy numbers of 10, 40, and 100, respectively [19].

The upstream modules (pJW007-009) were constructed by assembling three DNA fragments: i) the *ldhA* gene amplified from pJW001 using the primer pair JW_0001/JW_0002, ii) the ethanol module containing *pdc* and *adhB* genes amplified from pCT24 using the primer pair JW_0003/JW_0004, and iii) the backbone amplified from pACYCDuet-1 for pJW007, from pETDuet-1 for pJW008, or from pRSFDuet-1 for pJW009 using the primer pair JW_0005/JW_0006.

The downstream modules (pJW010-012) were constructed by assembling three DNA fragments: i) the *pct* gene amplified from pJW001 using the primer pair JW_0007/JW_0008, ii) the *VAAT* gene amplified from pJW005 using the primer pair JW_0009/JW_0010, and iii) the backbone amplified from pACYCDuet-1 for pJW010, pETDuet-1 for pJW011, or pRSFDuet-1 for pJW012 using the primer pair JW_0011/JW_0012.

The combined upstream and downstream modules (pJW013-015) were constructed by assembling two DNA fragments: i) the upstream module amplified from pJW007 using the primer pair JW_0001/JW_0004 and ii) the backbone containing the downstream module amplified from pJW010 for pJW013, pJW011 for pJW014, or pJW012 for pJW014 using the primer pair JW_0005/JW_0006.

2.2.3. Construction of a library of upstream and downstream modules with various promoters and RBSs. For tighter regulation of biosynthetic pathway of ethyl lactate, we constructed the upstream and downstream modules with tunable promoters and RBSs.

The upstream modules (pJW019-022) were constructed via three rounds of cloning. First, the T7 terminator (T_{T7}) was added between the multiple cloning site 1 (MCS1) and MCS2 of the

pACYCDuet-1 backbone by assembling three DNA fragments: i) the *ldhA* gene amplified from pJW001 using the primer pair JW_0013/JW_0014, ii) the linker containing T_{T7} terminator from pETite* using the primer pair JW_0015/JW_0016, and iii) the backbone from pACYCDuet-1 using the primer pair JW_0017/JW_0018, creating the first intermediate plasmid, pJW016. Next, the original T7 promoter (P_{T7}) in MCS2 of pJW016 was replaced with the AY1 promoter (P_{AY1}; BBa_J23100) or AY3 promoter (P_{AY3}; BBaJ23108) by assembling two DNA fragments: i) the ethanol module amplified from pCT24 under the P_{AY1} promoter for pJW017 or P_{AY3} promoter for pJW018 using the primer pair JW_0019/JW_0020 or JW_0021/JW_0020, respectively, and ii) the backbone amplified from pJW016 for pJW017 or pJW018 using the primer pair JW_0022/JW_0012 or JW_0023/JW_0012, respectively, generating the second intermediate plasmid, pJW017 or pJW018. Lastly, the final four synthetic operons (pJW019-022) were constructed by assembling two DNA fragments: i) the ethanol module amplified from pCT24 with the synthetic RBS sequences with predicted translation initiation rates of 0.33au for pJW019 and pJW021 and 0.03au for pJW020 and pJW022 using the primer pairs JW_0024/JW_0020, JW_0025/JW_0020, JW_0026/JW_0020, and JW_0027/JW_0020, respectively, and ii) the backbone amplified from pJW017 for pJW019 and pJW020 and pJW018 for pJW021 and pJW022 using the primer pairs JW_0028/JW_0012, JW_0029/JW_0012, JW_0030/JW_0012, and JW_0031/JW_0012, respectively. The P_{AY1} and P_{AY3} promoter sequences were obtained from the iGEM Anderson promoter library (http://parts.igem.org/Promoters/Catalog/Anderson) and the strength of promoters were assigned as $P_{AY3} = 0.5 \times P_{AY1}$. The RBS Calculator v2.0 [20, 21] was used to generate four synthetic RBS sequences with predicted translation initiation rates of 0.33 and 0.03 between the P_{AY1} (or P_{AY3}) promoter and *pdc* start codon (Fig. S3).

The downstream modules (pJW027-035) were constructed via three rounds of cloning. First, the T_{T7} terminator was added between the MCS1 and MCS2 of the pRSFDuet-1 backbone by assembling three DNA fragments: i) the *pct* gene amplified from pJW001 using the primer pair JW_0013/JW_0032, ii) the linker containing T_{T7} terminator from pETite* using the primer pair JW_0033/JW_0034, and iii) the backbone from pRSFDuet-1 using the primer pair JW_0017/JW_0018, generating the first intermediate plasmid, pJW023. Then, the original RBS in MCS1 of pJW023 was replaced with synthetic RBSs of various strengths by assembling two DNA fragments: i) the *pct* gene amplified from pJW001 with the synthetic RBS sequences with predicted translation initiation rates at 90, 9000, or 90000au for pJW024, pJW025 or pJW026 using the primer pair JW_0035/JW_0036, JW_0037/JW_0036, or JW_0038/JW_0036, respectively, and ii) the backbone amplified from pJW023 using the primer pair JW_0039/JW_0040 for pJW024, JW_0041/JW_0040 for pJW025, or JW_0042/JW_0040 for pJW026, respectively, generating the second intermediate plasmids, pJW024-026. Lastly, the final nine downstream modules (pJW027-035) were constructed by assembling a combination of two DNA fragments: i) the *VAAT* gene amplified from pDL006 with the synthetic RBS sequences predicted with translation initiation rates of 90, 9000, or 90000au for pJW027/pJW030/pJW033, pJW028/pJW031/pJW034, or pJW029/pJW032/pJW035 using the primer pair JW_0043/JW_0010, JW_0044/JW_0010, or JW_0045/JW_0010, respectively, and ii) the backbone amplified from pJW024, pJW025, or pJW026 for pJW027-029, pJW030-032, or pJW033-035 using the primer pair JW_0046/JW_0012, JW_0047/JW_0012 or JW_0048/JW_0012, respectively. The RBS Calculator v2.0 [20, 21] was used to generate six synthetic RBS sequences with predicted translation initiation rates of 90, 9000, and 90000au between the P_{T7} promoter and *pct* (or *VAAT*) start codon (Fig. S3).

2.3. Culture media and conditions

2.3.1. Culture media. For molecular cloning, seed cultures, and protein expression analysis, Luria-Bertani (LB), comprising of 10 g/L peptone, 5 g/L yeast extract, and 5 g/L NaCl, was used. For high-cell density cultures, pre-cultures of bioreactor batch fermentations, and growth inhibition analysis of lactate esters, the M9 hybrid medium [6] with 20 g/L glucose was used. For bioreactor batch fermentations, the M9 hybrid medium with 50 g/L glucose and 100 µL of antifoam (Antifoam 204, Sigma-Aldrich, MO, USA) was used. 30 µg/mL chloramphenicol (cm), 50 µg/mL kanamycin (kan), and/or 50 µg/mL ampicillin (amp) was added to the media for selection where applicable.

2.3.2. High-cell density cultures. For seed cultures, 2% (v/v) of stock cells were grown overnight in 5 mL of LB with appropriate antibiotics. For pre-cultures, 1% (v/v) of seed cultures were transferred into 100 mL of LB medium in 500 mL baffled flasks. For main cultures, pre-cultures were aerobically grown overnight at 37°C, 200 rpm then centrifuged (4700 rpm, 10 min) and resuspended with an optical density measured at 600nm (OD_{600nm}) of 3 in M9 hybrid medium containing appropriate concentration of isopropyl-beta-D-thiogalatopyranoside (IPTG) and antibiotics. The resuspended cultures were distributed into 15 mL polypropylene centrifuge tubes (Thermo Scientific, IL, USA) with a working volume of 5 mL and grown for 24 hour (h) on a 75° angled platform in a New Brunswick Excella E25 at 37°C, 200 rpm. The tubes were capped to generate anaerobic condition. All high-cell density culture studies were performed in biological triplicates.

2.3.3. Bioreactor batch fermentations. Bioreactor batch fermentations were conducted with a Biostat B+ (Sartorius Stedim, NY, USA) dual 1.5 L fermentation system at a working volume of 1 L M9 hybrid medium. The seed and pre-cultures were prepared as described in high-

cell density cultures in LB and M9 hybrid media, respectively. For main cultures, 10% (v/v) of pre-cultures were inoculated into fermentation cultures. During the fermentation, to achieve high cell density, dual-phase fermentation approach, aerobic cell growth phase followed by anaerobic production phase, was applied. For the first aerobic cell growth phase, the temperature, agitation, and air flow rate were maintained at 37°C, 1000 rpm, and 1 volume/volume/min (vvm) for 4 h, respectively. Then, the oxygen in the medium was purged by sparing nitrogen gas at 2 vvm for 2 h to generate anaerobic condition. For the anaerobic production phase, 0.5 mM of IPTG was added to induce the protein expression and the culture temperature and the nitrogen flow rate were maintained at 30°C and 0.2 vvm, respectively. During the fermentation, the pH was maintained at 7.0 with 5 M KOH and 40% H_3PO_4. Bioreactor batch fermentation studies were performed in biological duplicates.

2.4. Growth inhibition analysis of lactate esters

The seed culture of EcDL002 was prepared as described in high-cell density cultures. 4 % (v/v) of seed cultures were inoculated into 100 µL of the M9 hybrid media, containing various concentration of lactate esters including ethyl- (5~40 g/L), propyl-, butyl-, isobutyl-, isoamyl-, or benzyl lactate (0.5~4 g/L), in a 96-well microplate. Then, the microplate was sealed with a plastic adhesive sealing film, SealPlate® (EXCEL Scientific, Inc., CA, USA) to prevent evaporation of lactate esters and incubated at 37°C with continuous shaking using a BioTek Synergy HT microplate reader (BioTek Instruments, Inc., VT, USA), measuring the OD_{600nm} at 20 min intervals. Growth inhibition studies of lactate esters were performed twice in biological triplicates (n = 6).

2.5. Protein expression and SDS-PAGE analysis

The seed cultures were prepared as described in high-cell density cultures. 1% (v/v) of seed cultures subsequently inoculated in 500 mL baffled flasks containing 100 ml of LB medium. The cells were aerobically grown at 37ºC and 200 rpm and induced at an OD_{600nm} of 0.6~0.8 with 0.5 mM of IPTG. After 4 h of induction, cells were collected by centrifugation and resuspended in 100 mM of sodium phosphate buffer (pH7.0) at the final OD_{600nm} of 10. Cell pellets were disrupted using a probe-type sonicator (Model 120, Fisher Scientific, NH, USA) on ice-water mixture. The resulting crude extracts were mixed with 6x sodium dodecyl sulfate (SDS) sample buffer, heated at 100ºC for 5 min, and then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 14% polyacrylamide gel) with a standard procedure. Protein bands were visualized with Coomassie Brilliant Blue staining.

2.6. Analytical methods

2.6.1. Determination of cell concentrations. The dry cell mass was obtained by multiplication of the optical density of culture broth with a pre-determined conversion factor, 0.48 g/L/OD. The optical density was measured at 600 nm using a spectrophotometer (GENESYS 30, Thermo Scientific, IL, USA).

2.6.2. High performance liquid chromatography (HPLC). Glucose, lactate, acetate, ethanol, isobutanol, isoamyl alcohol, and benzyl alcohol were quantified by using the Shimadzu HPLC system (Shimadzu Inc., MD, USA) equipped with the Aminex HPX-87H cation exchange column (BioRad Inc., CA, USA) heated at 50°C. A mobile phase of 10 mN H_2SO_4 was used at a flow rate of 0.6 mL/min. Detection was made with the reflective index detector (RID) and UV detector (UVD) at 220 nm.

2.6.3. Gas chromatography coupled with mass spectroscopy (GC/MS). All esters were quantified by GC/MS. For GC/MS analysis, analytes in the supernatants were extracted with

dichloromethane (DCM), containing pentanol as an internal standard, in a 1:1 (v/v) ratio for 1 h at 37°C, 200 rpm in 15 mL polypropylene centrifuge tubes. After extraction, supernatant-DCM mixtures were centrifuged and 5 μL of DCM extracts were injected into a gas chromatograph (GC) HP 6890 equipped with the mass selective detector (MS) HP 5973. For the GC system, helium was used as the carrier gas at a flow rate of 0.5 mL/min and the analytes were separated on a Phenomenex ZB-5 capillary column (30 m x 0.25 mm x 0.25 μm). The oven temperature was programmed with an initial temperature of 50°C with a 1°C/min ramp up to 58°C. Next a 25°C/min ramp was deployed to 235°C and then finally held a temperature of 300°C for 2 minutes to elute any residual non-desired analytes. The injection was performed using a splitless mode with an initial injector temperature of 280°C. For the MS system, a selected ion monitoring (SIM) mode was deployed to detect analytes.

The SIM parameters for detecting lactate esters were as follows: i) for pentanol, ions 53.00, 60.00, and 69.00 detected from 5.00 to 7.70 min, ii) for ethyl lactate, ions 46.00, 47.00, and 75.00 detected from 7.70 to 10.10 min, iii) for propyl lactate, ions 59.00, 88.00, and 89.00 detected from 10.10 to 11.00 min, iv) for isobutyl lactate, ions 56.00, 57.00, and 59.00 detected from 11.00 to 11.60 min, v) for butyl lactate, ions 75.00, 91.00, and 101.00 detected from 11.60 to 12.30 min, vi) for isoamyl lactate, ions 46.00, 73.00, 75.00 from 12.30 to 14.50 min, and vii) for benzyl lactate, ions 45.00, 91.00, and 180.00 from 14.50 to 15.08 min. The SIM parameters for detecting acetate esters were as follows: i) for ethyl acetate, ions 45.00, 61.00, and 70.00 detected from 4.22 to 5.35 min, ii) for propyl acetate, ions 57.00, 59.00, and 73.00 detected from 5.35 to 6.40 min, iii) for pentanol, ions 53.00, 60.00, and 69.00 detected from 6.40 to 6.60 min, iv) for isobutyl acetate, ions 56.00, 61.00, and 73.00 detected from 6.60 to 7.70 min, v) for butyl acetate, ions 57.00, 71.00, and 87.00 detected from 7.70 to 9.45 min, vi) for isoamyl acetate, ions 58.00, 70.00, and 88.00 detected

from 9.45 to 13.10 min, and vii) for benzyl acetate, ions 63.00, 107.00, and 150.00 from 13.10 to 15.82 min.

3. Results

3.1. *In vivo* screening of efficient AATs critical for lactate ester biosynthesis

We began the construction of lactate ester biosynthesis pathways by identifying the best AAT candidate because the substrate specificity of AATs is critical to produce target esters [9]. We designed, constructed, and characterized a library of five sugar-to-lactate ester modules (EcJW101-105) carrying five divergent alcohol acyltransferases including *ATF1*, *ATF2*, *SAAT*, *VAAT*, and *AtfA*. For characterization, 2 g/L of ethanol, propanol, butanol, isobutanol, isoamyl alcohol, and benzyl alcohol were added to culture media with 0.5 mM of IPTG for pathway induction to evaluate biosynthesis of six different lactate esters including ethyl lactate, propyl lactate, butyl lactate, isobutyl lactate, isoamyl lactate, and benzyl lactate, respectively, in high-cell density cultures (Fig. 1A).

The results show that most of the strains could produce different types of lactate esters with external supply of alcohols (Fig. 1B, 1C). EcJW104 achieved the highest titer of lactate esters in all cases, producing 1.59 ± 0.04 mg/L of ethyl lactate with a specific productivity of 0.04 ± 0.00 mg/gDCW/h in ethanol doping, 5.46 ± 0.25 mg/L of propyl lactate with a specific productivity of 0.14 ± 0.01 mg/gDCW/h in propanol doping, 11.75 ± 0.43 mg/L of butyl lactate with a specific productivity of 0.29 ± 0.01 mg/gDCW/h in butanol doping, 9.92 ± 0.08 mg/L of isobutyl lactate with a specific productivity of 0.25 ± 0.00 mg/gDCW/h in isobutanol doping, 24.73 ± 0.58 mg/L of isoamyl lactate with a specific productivity of 0.62 ± 0.01 mg/gDCW/h in isoamyl alcohol doping, and 51.59 ± 2.09 mg/L of benzyl lactate with a specific productivity of 1.91 ± 1.10

mg/gDCW/h in benzyl alcohol doping. The lactate ester biosynthesis of EcJW104 exhibited different alcohol substrate preference in the following order: benzyl alcohol > isoamyl alcohol > butanol > isobutanol > propanol > ethanol (Fig. 1B, Supplementary Table S2).

Due to the presence of endogenous acetyl-CoA, we also produced acetate esters in addition to lactate esters (Fig. 1). Among the strains, EcJW101 achieved the highest titers of acetate esters in all cases, producing 115.52 ± 4.83 mg/L of ethyl acetate with a specific productivity of 2.60 ± 0.14 mg/gDCW/h in ethanol doping, 801.62 ± 33.51 mg/L of propyl acetate with a specific productivity of 18.13 ± 0.73 mg/gDCW/h in propanol doping, 1,017.90 ± 20.21 mg/L of butyl acetate with a specific productivity of 25.45 ± 0.38 mg/gDCW/h in butanol doping, 1,210.40 ± 24.83 mg/L of isobutyl acetate with a specific productivity of 29.54 ± 0.71 mg/gDCW/h in isobutanol doping, 692.73 ± 7.65 mg/L of isoamyl acetate with a specific productivity of 20.72 ± 0.31 mg/gDCW/h in isoamyl alcohol doping, and 1,177.98 ± 45.72 mg/L of benzyl acetate with a specific productivity of 34.86 ± 1.30 mg/gDCW/h in benzyl alcohol doping. EcJW101 showed different alcohol substrate preference for the acetate ester biosynthesis in the following order: isobutanol > benzyl alcohol > butanol > propanol > isoamyl alcohol > ethanol (Fig. 1B, Supplementary Table S2). Taken altogether, microbial biosynthesis of lactate esters is highly feasible. Among the library of 12 esters (Fig. 1C), seven of these esters, including ethyl lactate, propyl lactate, butyl lactate, isobutyl lactate, isoamyl lactate, benzyl lactate, and benzyl acetate, were demonstrated for *in vivo* production in microbes for the first time. Since EcJW104, carrying the sugar-to-lactate ester module with *VAAT* (pJW005), achieved the highest titer of lactate esters in all cases, it was selected for establishing the *de novo* biosynthesis pathway of lactate esters from glucose.

3.2. Establishing the *de novo* lactate ester biosynthesis pathways

We next aimed to demonstrate direct conversion of lactate esters from glucose after identifying the efficient AAT for lactate ester biosynthesis. We focused on the biosynthesis of ethyl and isobutyl lactate esters. We designed the *de novo* biosynthesis pathways for ethyl and isobutyl lactate by combining the sugar-to-lactate ester module (pJW005) with the ethanol (pCT24) and isobutanol (pCT13) modules, respectively. By co-transforming pJW005/pCT24 and pJW005/pCT13 into the modular cell EcDL002, we generated the production strains, EcJW201 and EcJW202, for evaluating direct conversion of glucose to ethyl and isobutyl lactate esters.

We characterized EcJW201 and EcJW202 together with the parent strain, EcDL002, as a negative control in high-cell density cultures. The results show EcJW201 and EcJW202 produced ethyl (Fig. 2A) and isobutyl (Fig. 2B) lactate from glucose, respectively, while the negative control strain EcDL002 could not. Consistently, the expressions of metabolic enzymes of the ethyl and isobutyl lactate pathways were confirmed in EcJW201 and EcJW202, respectively, by SDS-PAGE analysis (Supplementary Figure S1). During 24 h fermentation, EcJW201 produced 2.24 ± 0.28 mg/L of ethyl lactate with a specific productivity of 0.04 ± 0.00 mg/gDCW/h while EcJW202 produced 0.26 ± 0.01 mg/L of isobutyl lactate with a specific productivity of 0.01 ± 0.00 mg/gDCW/h. In addition to ethyl or isobutyl lactate biosynthesis, EcJW201 also produced 92.25 ± 9.20 mg/L of ethyl acetate while EcJW202 generated 1.36 ± 0.74 mg/L of ethyl acetate and 0.34 ± 0.07 mg/L of isobutyl acetate (Supplementary Table S3A). Taken altogether, the direct microbial synthesis of lactate esters from fermentable sugar was successfully demonstrated. Since the lactate ester production was low, the next logical step was to identify and alleviate the key pathway bottlenecks for enhanced lactate ester biosynthesis. As proof-of-principle, we focused on optimization of the ethyl lactate production as presented in the subsequent sections.

3.3 Identifying and alleviating key bottlenecks of the ethyl lactate biosynthesis pathway

3.3.1. Evaluating the de novo biosynthesis of ethyl lactate in controlled fermentation as a basis to identify potential pathway bottlenecks. In an attempt to identify the key bottlenecks of the ethyl lactate biosynthesis pathway, we characterized EcJW201 in controlled bioreactors. The results show that EcJW201 produced 9.17 ± 0.12 mg/L of ethyl lactate with a specific productivity of 0.15 ± 0.02 mg/gDCW/h and a yield of 0.19 ± 0.00 mg/g glucose (Fig. 2C, Supplementary Table S3B) in 18 h. Under controlled fermentation, EcJW201 achieved 309%, 275%, and 90% improvement in titer, specific productivity, and yield, respectively, as compared to the strain performance in the high cell density culture. Even though the strain performance in controlled bioreactors was enhanced, the ethyl lactate production was relatively low. We observed a significant accumulation of precursor metabolites (19.35 ± 0.29 g/L of lactate and 10.31 ± 0.41 g/L of ethanol, Supplementary Table S3B) in the medium at 18 h while the titer of ethyl lactate did not increase during the fermentation. This result suggests that (i) rate-limiting conversion of lactate into lactyl-CoA by Pct and/or condensation of lactyl-CoA with an ethanol by VAAT and/or (ii) toxicity of ethyl lactate on *E. coli* health might have limited lactate ester biosynthesis. Therefore, to enhance ethyl lactate production, it is important to elucidate and alleviate these identified potential bottlenecks.

3.3.2. Ethyl lactate exhibited minimal cytotoxicity on cell growth among lactate esters. To determine whether lactate esters inhibited cell growth and hence contributed to low lactate ester production, we cultured the parent strain, EcDL002, in a microplate reader with or without supply of various concentrations of lactate esters including ethyl, propyl, butyl, isobutyl, isoamyl, or benzyl lactate. The results show that ethyl lactate was the least toxic among the six lactate esters characterized where the growth rate (0.47 ± 0.04 1/h) and cell titer (OD = 0.42 ± 0.03) were slightly reduced to 94% and 90%, respectively, upon cell exposure to 5 g/L ethyl lactate. On the other

hand, isobutyl lactate was the most toxic among the lactate esters, where cell exposure to only 0.5 g/L ester caused 82% and 85% decrease in the growth rate (0.41 ± 0.02 1/h) and OD (0.40 ± 0.03), respectively (Supplementary Figure S2A). The toxicity of lactate esters can be ranked in the following order: isoamyl lactate > benzyl lactate > butyl lactate > isobutyl lactate > propyl lactate > ethyl lactate. This trend was consistent with literature, illustrating that increasing toxicity of esters is highly correlated with increasing chain length of alcohol moieties [22]. Taken altogether, ethyl lactate is the least toxic and was not likely the main reason for the low production of ethyl lactate observed. It was likely the downstream pathway, responsible for conversion of lactate into lactyl-CoA by Pct and/or condensation of lactyl-CoA with ethanol by VAAT, might have been contributed to the inefficient ethyl lactate biosynthesis.

3.3.3. Downstream pathway of the lactate ester biosynthesis is the key bottleneck. To identify and alleviate the *de novo* ethyl lactate biosynthesis pathway, we re-modularized it with two new parts: i) the upstream module carrying *ldhA*, *pdc*, and *adhB* for production of lactate and ethanol from sugar and ii) the downstream module carrying *pct* and *VAAT* for converting lactate into lactyl-CoA and condensing lactyl-CoA and ethanol (Fig. 3A). We controlled metabolic fluxes of these modules by controlling their plasmid copy numbers and levels of promoter induction with IPTG. By introducing the plasmids pJW007-015 into EcDL002, we generated the strains EcJW106-108 and EcJW203-208, respectively (Fig. 3B). To evaluate the performance of these constructed strains for ethyl lactate production, we characterized them in high cell density cultures induced with various concentrations of IPTG (0.01, 0.1, and 1.0 mM).

The results show that EcJW204, carrying the upstream module with a low copy number plasmid (P15A origin) and the downstream module with a high copy number plasmid (RSF1030 origin) induced by 0.01 mM of IPTG, achieved the highest titer of ethyl lactate at 11.10 ± 0.58

mg/L with a specific productivity of 0.22 ± 0.02 mg/gDCW/h and a yield of 0.54 ± 0.04 mg/g glucose. As compared to EcJW201, EcJW204 achieved 396%, 450%, and 440% improvement in titer, specific productivity, and yield of ethyl lactate, respectively (Fig. 3B, Supplementary Table S5). Interestingly, the best ethyl lactate producer EcJW204 produced the highest titer of lactate and the lowest ethanol production among the characterized nine strains (Fig. 3F and 3G, Supplementary Table S4), suggesting redistribution of the carbon flux from ethanol to lactate likely helped improve ethyl lactate production. Although EcJW204 showed better performance in ethyl lactate production than EcJW201, the accumulation of lactate and ethanol was still observed (Fig. 3F and 3G, Supplementary Table S4), indicating the pathway bottleneck remained. In particular, the downstream module flux was outcompeted by the upstream module flux and hence failed to turn over the precursor metabolites quickly enough. To truly identify the key metabolic bottlenecks of the downstream module and understand its interaction with the upstream module, we further manipulated components of these modules by modulating promoters, RBSs, and precursor supplementation.

***High ethanol synthesis of the upstream module was critical for ethyl lactate biosynthesis due to low specificity and activity of alcohol acyl transferase*.** In attempt to balance the metabolic fluxes of the upstream and downstream fluxes, we next analyzed the effect of lowering the ethanol flux of the upstream module and redistributing carbon flow from ethanol to lactate. Starting from the module upstream module pJW007 of the best performer EcJW204, we reconfigured and constructed four new upstream modules (pJW019-022) with a library of two weaker promoters and the four weaker synthetic RBSs (Fig. 4A, Supplementary Figure S3A). By introducing each constructed upstream module into EcDL002 together with the downstream module pJW012 used

in EcJW204, we generated the strains EcJW209-212 and then characterized them in high cell density cultures induced with 0.01 mM IPTG.

The results show the carbon flux was successfully redistributed from ethanol to lactate, with 83~86% decrease in ethanol production and 67~159% increase in lactate production (Supplementary Table S6A). However, the production of ethyl lactate and ethyl acetate was reduced 87~92% and 92~95%, respectively in all characterized strains as compared to that of EcJW204 (Fig. 4B, Supplementary Table S6B). The low ethyl ester production suggests that a high level of ethanol is critical for VAAT to produce esters. To support this conclusion, we evaluated the effect of external ethanol supply on production of ethyl esters in high cell density cultures of EcJW209-212 induced with 0.01 mM IPTG. Indeed, with external ethanol supply, we observed enhanced production of both ethyl lactate and ethyl acetate in EcJW209-212. Specifically, with the addition of 2 g/L of ethanol, the ethyl lactate and ethyl acetate production increased by 127~233% and 27~107%, respectively (Supplementary Table S6). Further addition of ethanol up to 10 g/L improved the ethyl lactate and ethyl acetate production by 278~426% and 309~592%, respectively (Supplementary Table S6). Interestingly, while the total titer of ethyl esters increased with the increasing addition of ethanol (Fig. 5A), the proportion of ethyl lactate in the total ester slightly increased in the range of 3.2~7.0% (Fig. 5B), suggesting that VAAT prefers acetyl-CoA over lactyl-CoA with ethanol as a co-substrate. Notably, we observed a strong linear correlation between ethyl esters production and the amount of added ethanol (i.e., for ethyl lactate, R^2 = 0.85~0.94; for ethyl acetate, R^2 = 0.99~1.00) (Supplementary Figure S4A). The results suggest abundant availability of ethanol is essential to achieve high production of ethyl esters. A combination of low affinity of VAAT for ethanol and low specificity of VAAT for lactyl-CoA contributed to low ethyl lactate biosynthesis.

AAT was the most rate limiting step of the downstream module. To determine whether Pct for conversion of lactate to lactyl-CoA or VAAT for condensation of lactyl-CoA and an alcohol was the most rate limiting step of the downstream module, we redesigned and constructed nine downstream modules (pJW027-035) derived from pJW012 of the best performer EcJW204 using a combination of three synthetic RBSs for Pct expression ($synRBS_{pct\#1\text{-}3}$) and three synthetic RBSs for VAAT expression ($synRBS_{VAAT\#1\text{-}3}$) (Fig. 4A, Supplementary Figure S3B). We introduced each downstream module into EcDL002 together with the upstream module (pJW007) used in EcJW204 to generate EcJW213-221. We characterized the constructed strains in high cell density cultures induced with 0.01 mM IPTG.

The results show that the strains harboring the stronger RBSs for VAAT expression achieved the higher titers of ethyl lactate and ethyl acetate regardless of the RBS strengths for Pct expression (Fig. 4C, Supplementary Table S7). There is a strong linear correlation between ethyl ester production and the strength of RBS for VAAT expression (Supplementary Figure S4B). To further validate these results without the influence of the upstream module, we additionally constructed the strains EcJW109-117 by introducing nine individual downstream modules (pJW027-035) into EcDL002 and then characterized these strains in high cell density cultures with addition of 2 g/L of lactate, 2 g/L of ethanol, and 0.01 mM of IPTG. We could observe the same strong linear correlation between ethyl ester production and high VAAT expression without the upstream module (Fig. 5C). Taken altogether, these results suggest that VAAT not Pct was the most rate limiting step of the downstream module of the ethyl lactate biosynthesis pathway.

4. Discussion

Recent interest in lactate esters is increasing due to environmental regulation and compelling demand for the eco-friendly solvents derived from renewable sources. In this study, we have successfully developed a lactate ester production platform and demonstrated for the first time the *de novo* microbial biosynthesis of lactate esters directly from fermentable sugars in an *E. coli* modular cell.

To produce lactate esters, the precursor metabolites, lactate or lactyl-CoA, an alcohol, and enzyme catalyzing condensation of lactate or lactyl-CoA and an alcohol are needed. While metabolic engineering of lactate [23-27] or lactyl-CoA [28-30] and alcohols including ethanol [31, 32], propanol [33], isopropanol [34], butanol [35], isobutanol [36], amyl alcohol [37], isoamyl alcohol [38], and benzyl alcohol [39] has been established in *E. coli*, the enzyme(s) required to catalyze the condensation of lactate or lactyl-CoA and an alcohol have not been well studied. To date, only lipases have been reported for condensation of lactate (not lactyl-CoA) and alcohol. However, use of organic solvents required for the lipase-based esterification reaction under hydrophobic environment [40-43] is neither environmentally benign nor economical. To tackle this problem, we identified AATs capable of synthesizing lactate esters in aqueous solution at ambient temperature [7]. *In vivo* screening of a library of five AATs including ATF1, ATF2, SAAT, VAAT, and AtfA [9] identified VAAT to be the most suitable for lactate ester biosynthesis. EcJW104, harboring the sugar-to-lactate module with *VAAT*, produced 6 out of 6 target lactate esters including ethyl, propyl, butyl, isobutyl, isoamyl, and benzyl lactate with the highest titers. Using VAAT, we demonstrated for the first time the direct biosynthesis of ethyl and isobutyl lactate from fermentable sugars.

Despite successful demonstration of the *de novo* biosynthesis of ethyl lactate from glucose, the current production of target esters was relatively low. Through detailed pathway

characterization, we found that the inefficient lactate ester pathway flux, not the ester cytotoxicity, hindered ethyl lactate production in the *E. coli* modular cell. By modularizing the ethyl lactate pathway into upstream and downstream modules and manipulating their metabolic fluxes with various plasmid copy numbers, promoters, and RBSs, we could identify the inefficient downstream module hindered efficient lactate ester biosynthesis. Specifically, VAAT was the most rate limiting step of the downstream module where it has low affinity to ethanol and low specificity to lactyl-CoA. While overexpression of the downstream module increased lactate ester biosynthesis by ~5 folds, the production level was still low. Future study exploring diversity of AATs and rational protein engineering of these enzymes is warranted for improving product synthesis and expanding a large library of lactate esters to be synthesized.

Since the endogenous *E. coli* D-lactate dehydrogenase (LdhA) was overexpressed in the *ldhA*-deficient modular cell of our study, it is anticipated that D-(-)-lactate and the associated D-(-)-lactate esters were mainly produced. In principle, the lactate ester platform can be controlled to produce enantiomers with broad industrial applications. To date, production of optically pure D-(-)- [23] and L-(+)-form [26] of lactate from glucose in *E. coli* [25] has been well established. In addition, *pct* from *C. propionicum* [28] and *Megasphaera elsdenii* [29, 30] have been used for converting D-(-)-lactate into D-(-)-lactyl-CoA in polylactic acid (PLA) production in *E. coli* and their catalytic activity towards L-(+)-lactate has also been demonstrated [44, 45]. Thus, by combining stereospecific Ldh and Pct enzymes together with AATs, it is highly feasible to extend our lactate ester platform for microbial production of stereospecific lactate esters from renewable resources.

In summary, we have developed the *de novo* microbial biosynthesis of a lactate ester platform from glucose in a modular *E. coli* cell. This study defines a stepping stone for the

microbial production of lactate esters as green solvents from renewable resources with novel industrial applications.

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References

1. Weissermel, K. and H.-J. Arpe, *Industrial Organic Chemistry*. 3rd ed. 1997: VCH Publishers Inc.
2. Pereira, C.S.M., V.M.T.M. Silva, and A.E. Rodrigues, *Ethyl lactate as a solvent: Properties, applications and production processes - a review.* Green Chemistry, 2011. **13**(10): p. 2658-2671.
3. Biddy., M.J., C. Scarlata., and C. Kinchin., *Chemicals from Biomass: A Market Assessment of Bioproducts with Near-Term Potential*. 2016: National Renewable Energy Laboratory.
4. Gao, C., C.Q. Ma, and P. Xu, *Biotechnological routes based on lactic acid production from biomass.* Biotechnology Advances, 2011. **29**(6): p. 930-939.
5. Hasegawa, S., M. Azuma, and K. Takahashi, *Stabilization of enzyme activity during the esterification of lactic acid in hydrophobic ethers and ketones as reaction media that are miscible with lactic acid despite their high hydrophobicity.* Enzyme and Microbial Technology, 2008. **43**(3): p. 309-316.
6. Layton, D.S. and C.T. Trinh, *Engineering modular ester fermentative pathways in Escherichia coli.* Metabolic Engineering, 2014. **26**: p. 77-88.
7. Rodriguez, G.M., Y. Tashiro, and S. Atsumi, *Expanding ester biosynthesis in Escherichia coli.* Nature Chemical Biology, 2014. **10**(4): p. 259-+.
8. Tai, Y.S., M.Y. Xiong, and K.C. Zhang, *Engineered biosynthesis of medium-chain esters in Escherichia coli.* Metabolic Engineering, 2015. **27**: p. 20-28.
9. Layton, D.S. and C.T. Trinh, *Expanding the Modular Ester Fermentative Pathways for Combinatorial Biosynthesis of Esters From Volatile Organic Acids.* Biotechnology and Bioengineering, 2016. **113**(8): p. 1764-1776.

10. Wang, J., et al., *Engineering a bacterial platform for total biosynthesis of caffeic acid derived phenethyl esters and amides.* Metabolic Engineering, 2017. **44**: p. 89-99.

11. Kruis, A.J., et al., *Ethyl acetate production by the elusive alcohol acetyltransferase from yeast.* Metabolic Engineering, 2017. **41**: p. 92-101.

12. Layton, D.S. and C.T. Trinh, *Microbial synthesis of a branched-chain ester platform from organic waste carboxylates.* Metabolic Engineering Communications, 2016. **3**: p. 245-251.

13. Green., M.R. and J. Sambrook, *Molecular Cloning*, in *A Laboratory Manual*. 2001, Cold Spring Harbor Laboratory Press, United States.

14. Gibson, D., et al., *Enzymatic assembly of DNA molecules up to several hundred kilobases.* Nat Methods, 2009. **6**: p. 343 - 345.

15. Verstrepen, K.J., et al., *Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters.* Applied and Environmental Microbiology, 2003. **69**(9): p. 5228-5237.

16. Aharoni, A., et al., *Identification of the SAAT Gene Involved in Strawberry Flavor Biogenesis by Use of DNA Microarrays.* Plant Cell, 2000. **12**(5): p. 647-662.

17. Beekwilder, J., et al., *Functional characterization of enzymes forming volatile esters from strawberry and banana.* Plant Physiol, 2004. **135**(4): p. 1865-78.

18. Shi, S., et al., *Functional expression and characterization of five wax ester synthases in Saccharomyces cerevisiae and their utility for biodiesel production.* Biotechnol Biofuels, 2012. **5**: p. 7.

19. Wu, J.J., et al., *Metabolic engineering of Escherichia coli for (2S)-pinocembrin production from glucose by a modular metabolic strategy.* Metabolic Engineering, 2013. **16**: p. 48-55.

20. Borujeni, A.E., A.S. Channarasappa, and H.M. Salis, *Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites.* Nucleic Acids Research, 2014. **42**(4): p. 2646-2659.

21. Salis, H.M., E.A. Mirsky, and C.A. Voigt, *Automated design of synthetic ribosome binding sites to control protein expression.* Nature Biotechnology, 2009. **27**(10): p. 946-U112.

22. Wilbanks, B. and C.T. Trinh, *Comprehensive characterization of toxicity of fermentative metabolites on microbial growth.* Biotechnology for Biofuels, 2017. **10**(1): p. 262.

23. Zhou, S., et al., *Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered Escherichia coli W3110.* Appl Environ Microbiol, 2003. **69**(1): p. 399-407.

24. Liu, H., et al., *Production of lactate in Escherichia coli by redox regulation genetically and physiologically.* Appl Biochem Biotechnol, 2011. **164**(2): p. 162-9.

25. Chang, D.E., et al., *Homofermentative production of D- or L-lactate in metabolically engineered Escherichia coli RR1.* Appl Environ Microbiol, 1999. **65**(4): p. 1384-9.

26. Niu, D.D., et al., *Highly efficient L-lactate production using engineered Escherichia coli with dissimilar temperature optima for L-lactate formation and cell growth.* Microbial Cell Factories, 2014. **13**.

27. Chen, X.Z., et al., *Efficient bioconversion of crude glycerol from biodiesel to optically pure D-lactate by metabolically engineered Escherichia coli.* Green Chemistry, 2014. **16**(1): p. 342-350.

28. Cho, J.H., et al., *Cells or plants that can producepolylactate or its copolymers and to a method for preparing polylactateor its copolymers using the same*. 2006.

29. Taguchi, S., et al., *A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(45): p. 17323-17327.

30. Matsumoto, K., et al., *Improved production of poly(lactic acid)-like polyester based on metabolite analysis to address the rate-limiting step.* Amb Express, 2014. **4**.

31. Ingram, L.O., et al., *Genetic-Engineering of Ethanol-Production in Escherichia-Coli.* Applied and Environmental Microbiology, 1987. **53**(10): p. 2420-2425.

32. Trinh, C.T., P. Unrean, and F. Srienc, *Minimal Escherichia coli cell for the most efficient production of ethanol from hexoses and pentoses.* Applied and Environmental Microbiology, 2008. **74**(12): p. 3634-3643.

33. Choi, Y.J., et al., *Metabolic engineering of Escherichia coli for the production of 1-propanol.* Metabolic Engineering, 2012. **14**(5): p. 477-486.

34. Hanai, T., S. Atsumi, and J.C. Liao, *Engineered synthetic pathway for isopropanol production in Escherichia coli.* Applied and Environmental Microbiology, 2007. **73**(24): p. 7814-7818.

35. Atsumi, S., et al., *Metabolic engineering of Escherichia coli for 1-butanol production.* Metabolic Engineering, 2008. **10**(6): p. 305-311.

36. Atsumi, S., et al., *Engineering the isobutanol biosynthetic pathway in Escherichia coli by comparison of three aldehyde reductase/alcohol dehydrogenase genes.* Applied Microbiology and Biotechnology, 2010. **85**(3): p. 651-657.

37. Tseng, H.C. and K.L.J. Prather, *Controlled biosynthesis of odd-chain fuels and chemicals via engineered modular metabolic pathways.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(44): p. 17925-17930.

38. Connor, M.R. and J.C. Liao, *Engineering of an Escherichia coli strain for the production of 3-methyl-1-butanol.* Applied and Environmental Microbiology, 2008. **74**(18): p. 5769-5775.

39. Pugh, S., et al., *Engineering Escherichia coli for renewable benzyl alcohol production.* Metabolic Engineering Communications, 2015. **2**: p. 39-45.

40. Roenne, T.H., X.B. Xu, and T.W. Tan, *Lipase-catalyzed esterification of lactic acid with straight-chain alcohols.* Journal of the American Oil Chemists Society, 2005. **82**(12): p. 881-885.

41. Koutinas, M., et al., *Application of commercial and non-commercial immobilized lipases for biocatalytic production of ethyl lactate in organic solvents.* Bioresource Technology, 2018. **247**: p. 496-503.

42. From, M., P. Adlercreutz, and B. Mattiasson, *Lipase catalyzed esterification of lactic acid.* Biotechnology Letters, 1997. **19**(4): p. 315-317.

43. Inaba, C., et al., *Efficient synthesis of enantiomeric ethyl lactate by Candida antarctica lipase B (CALB)-displaying yeasts.* Applied Microbiology and Biotechnology, 2009. **83**(5): p. 859-864.

44. Schweiger, G. and W. Buckel, *On the Dehydration of (R)-Lactate in the Fermentation of Alanine to Propionate by Clostridium-Propionicum.* Febs Letters, 1984. **171**(1): p. 79-84.

45. Niu, W. and J. Guo, *Stereospecific microbial conversion of lactic acid into 1,2-propanediol.* ACS Synth Biol, 2015. **4**(4): p. 378-82.

46. Trinh, C.T., et al., *Redesigning Escherichia coli metabolism for anaerobic production of isobutanol.* Appl Environ Microbiol, 2011. **77**(14): p. 4894-904.

47. Wierzbicki, M., et al., *Engineering an Escherichia coli platform to synthesize designer biodiesels.* Journal of Biotechnology, 2016. **224**: p. 27-34.

Table 1. A list of strains used in this study

Strains	Genotypes	Sources
E. coli TOP10	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
E. coli MG1655	F^- λ^-	ATCC 47076
Clostridium propionicum	Wildtype	ATCC 25522
EcDL002	TCS083 (λDE3) Δ*fadE*	[6]
EcJW101	EcDL002/pJW002; amp^R	This study
EcJW102	EcDL002/pJW003; amp^R	This study
EcJW103	EcDL002/pJW004; amp^R	This study
EcJW104	EcDL002/pJW005; amp^R	This study
EcJW105	EcDL002/pJW006; amp^R	This study
EcJW201	EcDL002/pJW005 pCT24; amp^R kan^R	This study
EcJW202	EcDL002/pJW005 pCT13; amp^R kan^R	This study
EcJW106	EcDL002/pJW013; cm^R	This study
EcJW203	EcDL002/pJW007 pJW011; cm^R amp^R	This study
EcJW204	EcDL002/pJW007 pJW012; cm^R kan^R	This study
EcJW205	EcDL002/pJW008 pJW010; cm^R amp^R	This study
EcJW107	EcDL002/pJW014; amp^R	This study
EcJW206	EcDL002/pJW008 pJW012; amp^R kan^R	This study
EcJW207	EcDL002/pJW009 pJW010; cm^R kan^R	This study
EcJW208	EcDL002/pJW009 pJW011; amp^R kan^R	This study
EcJW108	EcDL002/pJW015; kan^R	This study
EcJW209	EcDL002/pJW019 pJW012; cm^R kan^R	This study
EcJW210	EcDL002/pJW020 pJW012; cm^R kan^R	This study
EcJW211	EcDL002/pJW021 pJW012; cm^R kan^R	This study
EcJW212	EcDL002/pJW022 pJW012; cm^R kan^R	This study
EcJW213	EcDL002/pJW007 pJW027; cm^R kan^R	This study
EcJW214	EcDL002/pJW007 pJW028; cm^R kan^R	This study
EcJW215	EcDL002/pJW007 pJW029; cm^R kan^R	This study
EcJW216	EcDL002/pJW007 pJW030; cm^R kan^R	This study
EcJW217	EcDL002/pJW007 pJW031; cm^R kan^R	This study
EcJW218	EcDL002/pJW007 pJW032; cm^R kan^R	This study
EcJW219	EcDL002/pJW007 pJW033; cm^R kan^R	This study
EcJW220	EcDL002/pJW007 pJW034; cm^R kan^R	This study
EcJW221	EcDL002/pJW007 pJW035; cm^R kan^R	This study
EcJW109	EcDL002/pJW027; kan^R	This study
EcJW110	EcDL002/pJW028; kan^R	This study
EcJW111	EcDL002/pJW029; kan^R	This study
EcJW112	EcDL002/pJW030; kan^R	This study
EcJW113	EcDL002/pJW031; kan^R	This study
EcJW114	EcDL002/pJW032; kan^R	This study
EcJW115	EcDL002/pJW033; kan^R	This study
EcJW116	EcDL002/pJW034; kan^R	This study
EcJW117	EcDL002/pJW035; kan^R	This study

Table 2. A list of plasmids used in this study

Plasmids	Genotypes	Sources
pACYCDuet-1	Two sets of MCS, T_7 promoter, P15A ori; cm^R	Novagen
pETDuet-1	Two sets of MCS, T_7 promoter, ColE1 ori; amp^R	Novagen
pRSFDuet-1	Two sets of MCS, T_7 promoter, RSF1030 ori; kan^R	Novagen
pETite*	T_7 promoter, pBR322 ori; kan^R	[6]
pCT24	pETite* P_{T7}::*pdc*::*adhB*::T_{T7}; kan^R	[6]
pCT13	pCOLA P_{T7}::*alsS*::*ilvC*::*ilvD*–P_{T7}::*kivd*::*adhE*::T_{T7}; kan^R	[46]
pDL004	pETite* *ATF1*; kan^R	[9]
pDL005	pETite* *ATF2*; kan^R	[9]
pDL001	pETite* *SAAT*; kan^R	[9]
pDL006	pETite* *VAAT*; kan^R	[9]
pCT16	pETite* *atfA*; kan^R	[47]
pJW001	pETite* P_{T7}::*ldhA*::*pct*::T_{T7}; amp^R	This study
pJW002	pJW001 P_{T7}::*ldhA*::*pct*-P_{T7}::*ATF1*::T_{T7}; amp^R	This study
pJW003	pJW001 P_{T7}::*ldhA*::*pct*-P_{T7}::*ATF2*::T_{T7}; amp^R	This study
pJW004	pJW001 P_{T7}::*ldhA*::*pct*-P_{T7}::*SAAT*::T_{T7}; amp^R	This study
pJW005	pJW001 P_{T7}::*ldhA*::*pct*-P_{T7}::*VAAT*::T_{T7}; amp^R	This study
pJW006	pJW001 P_{T7}::*ldhA*::*pct*-P_{T7}::*ATFA*::T_{T7}; amp^R	This study
pJW007	pACYCDuet-1 P_{T7}::*ldhA*::*pdc*::*adhB*::T_{T7}; cm^R	This study
pJW008	pETDuet-1 P_{T7}::*ldhA*::*pdc*::*adhB*::T_{T7}; amp^R	This study
pJW009	pRSFDuet-1 P_{T7}::*ldhA*::*pdc*::*adhB*::T_{T7}; kan^R	This study
pJW010	pACYCDuet-1 P_{T7}::*pct*::*VAAT*::T_{T7}; cm^R	This study
pJW011	pETDuet-1 P_{T7}::*pct*::*VAAT*::T_{T7}; amp^R	This study
pJW012	pRSFDuet-1 P_{T7}::*pct*::*VAAT*::T_{T7}; kan^R	This study
pJW013	pACYCDuet-1 P_{T7}::*ldhA*::*pdc*::*adhB*-P_{T7}::*pct*::*VAAT*::T_{T7}; cm^R	This study
pJW014	pETDuet-1 P_{T7}::*ldhA*::*pdc*::*adhB*-P_{T7}::*pct*::*VAAT*::T_{T7}; amp^R	This study
pJW015	pRSFDuet-1 P_{T7}::*ldhA*::*pdc*::*adhB*-P_{T7}::*pct*::*VAAT*::T_{T7}; kan^R	This study
pJW016	pACYCDuet-1 P_{T7}::*ldhA*::T_{T7}-P_{T7}::T_{T7}; cm^R	This study
pJW017	pACYCDuet-1 P_{T7}::*ldhA*::T_{T7}-P_{AY1}::T_{T7}; cm^R	This study
pJW018	pACYCDuet-1 P_{T7}::*ldhA*::T_{T7}-P_{AY3}::T_{T7}; cm^R	This study
pJW019	pACYCDuet-1 P_{T7}::*ldhA*::T_{T7}-P_{AY1}::$synRBS_{pdc\#1}$::*pdc*::*adhB*::T_{T7}; cm^R	This study
pJW020	pACYCDuet-1 P_{T7}::*ldhA*::T_{T7}-P_{AY1}::$synRBS_{pdc\#2}$::*pdc*::*adhB*::T_{T7}; cm^R	This study
pJW021	pACYCDuet-1 P_{T7}::*ldhA*::T_{T7}-P_{AY3}::$synRBS_{pdc\#3}$::*pdc*::*adhB*::T_{T7}; cm^R	This study
pJW022	pACYCDuet-1 P_{T7}::*ldhA*::T_{T7}-P_{AY3}::$synRBS_{pdc\#4}$::*pdc*::*adhB*::T_{T7}; cm^R	This study
pJW023	pRSFDuet-1 P_{T7}::*pct*::T_{T7}-P_{T7}::T_{T7}; kan^R	This study
pJW024	pRSFDuet-1 P_{T7}::$synRBS_{pct\#1}$::*pct*::T_{T7}-P_{T7}::T_{T7}; kan^R	This study
pJW025	pRSFDuet-1 P_{T7}::$synRBS_{pct\#2}$::*pct*::T_{T7}-P_{T7}::T_{T7}; kan^R	This study
pJW026	pRSFDuet-1 P_{T7}::$synRBS_{pct\#3}$::*pct*::T_{T7}-P_{T7}::T_{T7}; kan^R	This study
pJW027	pRSFDuet-1 P_{T7}::$synRBS_{pct\#1}$::*pct*::T_{T7}-P_{T7}::$synRBS_{VAAT\#1}$::*VAAT*::T_{T7}; kan^R	This study
pJW028	pRSFDuet-1 P_{T7}::$synRBS_{pct\#1}$::*pct*::T_{T7}-P_{T7}::$synRBS_{VAAT\#2}$::*VAAT*::T_{T7}; kan^R	This study
pJW029	pRSFDuet-1 P_{T7}::$synRBS_{pct\#1}$::*pct*::T_{T7}-P_{T7}::$synRBS_{VAAT\#3}$::*VAAT*::T_{T7}; kan^R	This study
pJW030	pRSFDuet-1 P_{T7}::$synRBS_{pct\#2}$::*pct*::T_{T7}-P_{T7}::$synRBS_{VAAT\#1}$::*VAAT*::T_{T7}; kan^R	This study
pJW031	pRSFDuet-1 P_{T7}::$synRBS_{pct\#2}$::*pct*::T_{T7}-P_{T7}::$synRBS_{VAAT\#2}$::*VAAT*::T_{T7}; kan^R	This study
pJW032	pRSFDuet-1 P_{T7}::$synRBS_{pct\#2}$::*pct*::T_{T7}-P_{T7}::$synRBS_{VAAT\#3}$::*VAAT*::T_{T7}; kan^R	This study
pJW033	pRSFDuet-1 P_{T7}::$synRBS_{pct\#3}$::*pct*::T_{T7}-P_{T7}::$synRBS_{VAAT\#1}$::*VAAT*::T_{T7}; kan^R	This study
pJW034	pRSFDuet-1 P_{T7}::$synRBS_{pct\#3}$::*pct*::T_{T7}-P_{T7}::$synRBS_{VAAT\#2}$::*VAAT*::T_{T7}; kan^R	This study
pJW035	pRSFDuet-1 P_{T7}::$synRBS_{pct\#3}$::*pct*::T_{T7}-P_{T7}::$synRBS_{VAAT\#3}$::*VAAT*::T_{T7}; kan^R	This study

Table 3. A list of primers used in this study

Primers	Sequences (5'→3')
Sugar-to-lactyl-CoA module	
DL_0001	CATCATCACCACCATCACTAA
DL_0002	ATGTATATCTCCTTCTTATAGTTAAAC
DL_0032	TAGAAATAATTTTGTTTAACTATAAGAAGGAGATATACATATGAAACTCGCCGTTTATAG
DL_0033	GGGAACCTTTCTCATTATATCTCCTTTTAAACCAGTTCGTTCGGGC
DL_0034	ACGAACTGGTTTAAAAGGAGATATAATGAGAAAGGTTCCCATTAT
DL_0035	GCCGCTCTATTAGTGATGGTGGTGATGATGTCAGGACTTCATTTCCTTCAG
Sugar-to-lactate ester module	
DL_0013	GAGCCTCAGACTCCAGCGTA
DL_0014	ATATCAAGCTTGAATTCGTTACCCGG
DL_0015	GGAGGAACTATATCCGGGTAACGAATTCAAGCTTGATATTAATACGACTCACTATAGGG
DL_0016	GTCCAGTTACGCTGGAGTCTGAGGCTC
Upstream module	
JW_0001	GGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCATGAAACTCGCCGTTTATAGC
JW_0002	CTAAATAGGTACCGACAGTATAACTCATTATATCTCCTTTTAAACCAGTTCGTTCGGGC
JW_0003	CGAAACCTGCCCGAACGAACTGGTTTAAAAGGAGATATAATGAGTTATACTGTCGGTACC
JW_0004	CGCAAGCTTGTCGACCTGCAGGCGCGCCGAGCTCGAATTCTTAGAAAGCGCTCAGGAAG
JW_0005	GGATCCTGGCTGTGGTGATGA
JW_0006	GAATTCGAGCTCGGCGCG
Downstream module	
JW_0007	GTATATTAGTTAAGTATAAGAAGGAGATATACATATGATGAGAAAGGTTCCCATTATTAC
JW_0008	GAAATTATACTGACCTCAATTTTCTCCATTATATCTCCTTTCAGGACTTCATTTCCTTC
JW_0009	AATGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATAATGGAGAAAATTGAGGTCAG
JW_0010	CAAATTTCGCAGCAGCGGTTTCTTTACCAGACTCGAGTCAATATCTTGAAATTAGCGTCT
JW_0011	CATATGTATATCTCCTTCTTATACTTAACT
JW_0012	CTCGAGTCTGGTAAAGAAAC
Synthetic operons for upstream module	
JW_0013	GGGAATTGTGAGCGGATAACAATTCCCCAAGGAGATATAATGAAACTCGCCGTTTATAGC
JW_0014	TTATGCTAGTTATTGCTCAGCGGTGGCGGCCGCTCTATTATTAAACCAGTTCGTTCGG
JW_0015	TCTGGAAAAAGGCGAAACCTGCCCGAACGAACTGGTTTAATAATAGAGCGGCCGC
JW_0016	GATTATGCGGCCGTGTACAATACGATTACTTTCTGTTCGATTTCTACCGAAGAAAGGC
JW_0017	CATTATATCTCCTTGGGGAATTGTTATCCGC
JW_0018	TCGAACAGAAAGTAATCGTATTG
JW_0019	AAATTTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCATGAGTTATACTGTCGGTACC
JW_0020	GCGTTCAAATTTCGCAGCAGCGGTTTCTTTACCAGACTCGAGTTAGAAAGCGCTCAGGAA
JW_0021	AAATCTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCATGAGTTATACTGTCGGTACC

JW_0022	CATGCTAGCACTGTACCTAGGACTGAGCTAGCCGTCAAATTTCGATTATGCGGCC
JW_0023	CATGCTAGCATTATACCTAGGACTGAGCTAGCTGTCAGATTTCGATTATGCGGCC
JW_0024	TACAGTGCTAGCAGCTTAGCGACAACCCTAGGCGCTCGCATGAGTTATACTGTCGGTACC
JW_0025	GTATAATGCTAGCTTAGCAGTACCAGGACGTACCGGAGTATGAGTTATACTGTCGGTACC
JW_0026	TAGGTACAGTGCTAGCACTAGGCCTAGCGATTCCGCTAAATGAGTTATACTGTCGGTACC
JW_0027	TATAATGCTAGCAGTTTACCTAGGGCAATAGCGTACCGAATGAGTTATACTGTCGGTACC
JW_0028	CATGCGAGCGCCTAGGGTTGTCGCTAAGCTGCTAGCACTGTACCTAGG
JW_0029	CATTTAGCGGAATCGCTAGGCCTAGTGCTAGCACTGTACCTAGG
JW_0030	CATACTCCGGTACGTCCTGGTACTGCTAAGCTAGCATTATACCTAGG
JW_0031	CATTCGGTACGCTATTGCCCTAGGTAAACTGCTAGCATTATACCTAGG
Synthetic operons for downstream module	
JW_0032	TTATGCTAGTTATTGCTCAGCGGTGGCGGCCGCTCTATTATCAGGACTTCATTTCCTTCA
JW_0033	TGCAGAAGGCTTAATGGGTCTGAAGGAAATGAAGTCCTGATAATAGAGCGGCCGC
JW_0034	GATTATGCGGCCGTGTACAATACGATTACTTTCTGTTCGATTTCTACCGAAGAAAGGC
JW_0035	GATATAGCTCGAACGCGGAAAGAGATGAGAAAGGTTCCCATTATTAC
JW_0036	TCAGGACTTCATTTCCTTCA
JW_0037	GCAACCTATTTTAATCCAAGGAAGATCTAATGAGAAAGGTTCCCATTATTAC
JW_0038	GCAATAACAACTAGGAGAGACGACATGAGAAAGGTTCCCATTATTAC
JW_0039	TAATGGGAACCTTTCTCATCTCTTTCCGCGTTCGAGCTATATCGGGGAATTGTTATCCGC
JW_0040	TGCAGAAGGCTTAATGG
JW_0041	GGAACCTTTCTCATTAGATCTTCCTTGGATTAAAATAGGTTGCGGGGAATTGTTATCCGC
JW_0042	TAATGGGAACCTTTCTCATGTCGTCTCTCCTAGTTGTTATTGCGGGGAATTGTTATCCGC
JW_0043	TAACCAAAACACTAACGCAAGATGGAGAAAATTGAGGTCAGT
JW_0044	AGGGCACGAGGAGGAACCAGTAGAATGGAGAAAATTGAGGTCAGT
JW_0045	GCAACCAACACAACGAGGAGGCATTTAATGGAGAAAATTGAGGTCAGT
JW_0046	TACTGACCTCAATTTTCTCCATCTTGCGTTAGTGTTTTGGTTAGGGGAATTGTTATCCGC
JW_0047	CTCAATTTTCTCCATTCTACTGGTTCCTCCTCGTGCCCTGGGGAATTGTTATCCGC
JW_0048	CTCAATTTTCTCCATTAAATGCCTCCTCGTTGTGTTGGTTGCGGGGAATTGTTATCCGC

Figure Legends

Figure 1. *In vivo* characterization of various alcohol acyltransferases for biosynthesis of lactate esters. **(A)** Biosynthesis pathways of lactate and acetate esters with external supply of alcohols. **(B)** Ester production of EcJW101, EcJW102, EcJW103, EcJW104, and EcJW105 harboring *ATF1*, *ATF2*, *SAAT*, *VAAT*, and *AtfA*, respectively in high cell density cultures. Red arrow indicates the selected strain for further metabolic engineering. Each error bar represents 1 standard deviation (s.d., *n=3*). **(C)** The library of esters produced. Green check marks indicate the esters produced in this study while red star marks indicate the esters produced for first time in engineered strains.

Figure 2. Design, construction, and validation of the *de novo* lactate ester biosynthesis pathways in *E. coli*. **(A)** Engineered *de novo* biosynthesis pathway of ethyl lactate from glucose and its production in high cell density culture of EcJW201. **(B)** Engineered *de novo* biosynthesis pathway of isobutyl lactate from glucose and its production in high cell density culture of EcJW202. In Fig. 2A and 2B, all of the strains were induced at 0 h with 0.5 mM IPTG. Each error bar represents 1 s.d. (*n=3*). **(C)** Production of ethyl lactate from glucose in controlled batch fermentation of EcJW201. The strain was induced at 6 h with 0.5 mM IPTG. Each error bar represents 1 s.d. (*n=2*).

Figure 3. Combinatorial modular pathway optimization of enhanced ethyl lactate biosynthesis by varying plasmid copy number. **(A)** Re-modularization of the ethyl lactate biosynthesis pathway. Sugar-to-lactate ester and ethanol modules were re-modulated into upstream and downstream modules using plasmids with different copy numbers. **(B)** Ethyl lactate production, **(C)** OD_{600}, **(D)** consumed glucose, **(E)** acetate, **(F)** lactate, **(G)** ethanol, and **(H)** ethyl acetate of EcJW106-108

and EcJW203-208 in high cell density cultures induced with various concentrations of IPTG. Green rectangle: low copy number plasmid (10); P15A: origin of pACYCDuet-1; Blue rectangle: medium copy number plasmid (40); ColE1: origin of pETDuet-1; Red rectangle: high copy number plasmid (100); RSF1030: origin of pRSFDuet-1; P_{T7}: T7 promoter; T_{T7}: T7 terminator. All of the strains were induced at 0 h with 0.01, 0.1, or 1.0 mM IPTG, respectively. Each error bar represents 1 s.d. (*n=3*). Red arrows indicate the selected strain with an optimum concentration of IPTG for the further studies.

Figure 4. Probing and alleviating the potential metabolic bottlenecks of the upstream or downstream modules of EcJW204 by varying the strength of promoters and/or ribosome binding sites. **(A)** Design of synthetic operons for the upstream and downstream modules. For the upstream module, the T7 promoter in MCS2 and the RBS between T7 promoter in MCS2 and the start codon of *pdc* were replaced with the combination of AY1 or AY3 promoter and 0.3 or 0.03au RBS. For the downstream module, the RBS between T7 promoter in MCS1 and the start codon of *pct* gene and the RBS between T7 promoter in MCS2 and the start codon of *VAAT* gene were replaced with the combination of 90, 9000, or 90000au RBS and 90, 9000, or 90000au RBS, respectively. Production of ethyl lactate in high cell density cultures of **(B)** EcJW209-212 and **(C)** EcJW213-221. Green rectangle: low copy number plasmid (10); P15A: origin of pACYCDuet-1; Red rectangle: high copy number plasmid (100); RSF1030: origin of pRSFDuet-1; P_{T7}: T7 promoter; T_{T7}: T7 terminator. All of the strains were induced at 0 h with 0.01 mM IPTG. Each error bar represents 1 s.d. (*n=3*).

Figure 5. (A) Total esters and **(B)** composition of total esters produced in high cell density cultures of EcJW209-212 with or without addition of ethanol. **(C)** Ethyl lactate production of EcJW109-117 with addition of 2 g/L of lactate and ethanol. Red rectangle: high copy number plasmid (100); RSF1030: origin of pRSFDuet-1; P_{T7}: T7 promoter; T_{T7}: T7 terminator. All of the strains were induced at 0 h with 0.01 mM IPTG. Each error bar represents 1 s.d. (*n=3*).

Figure 1

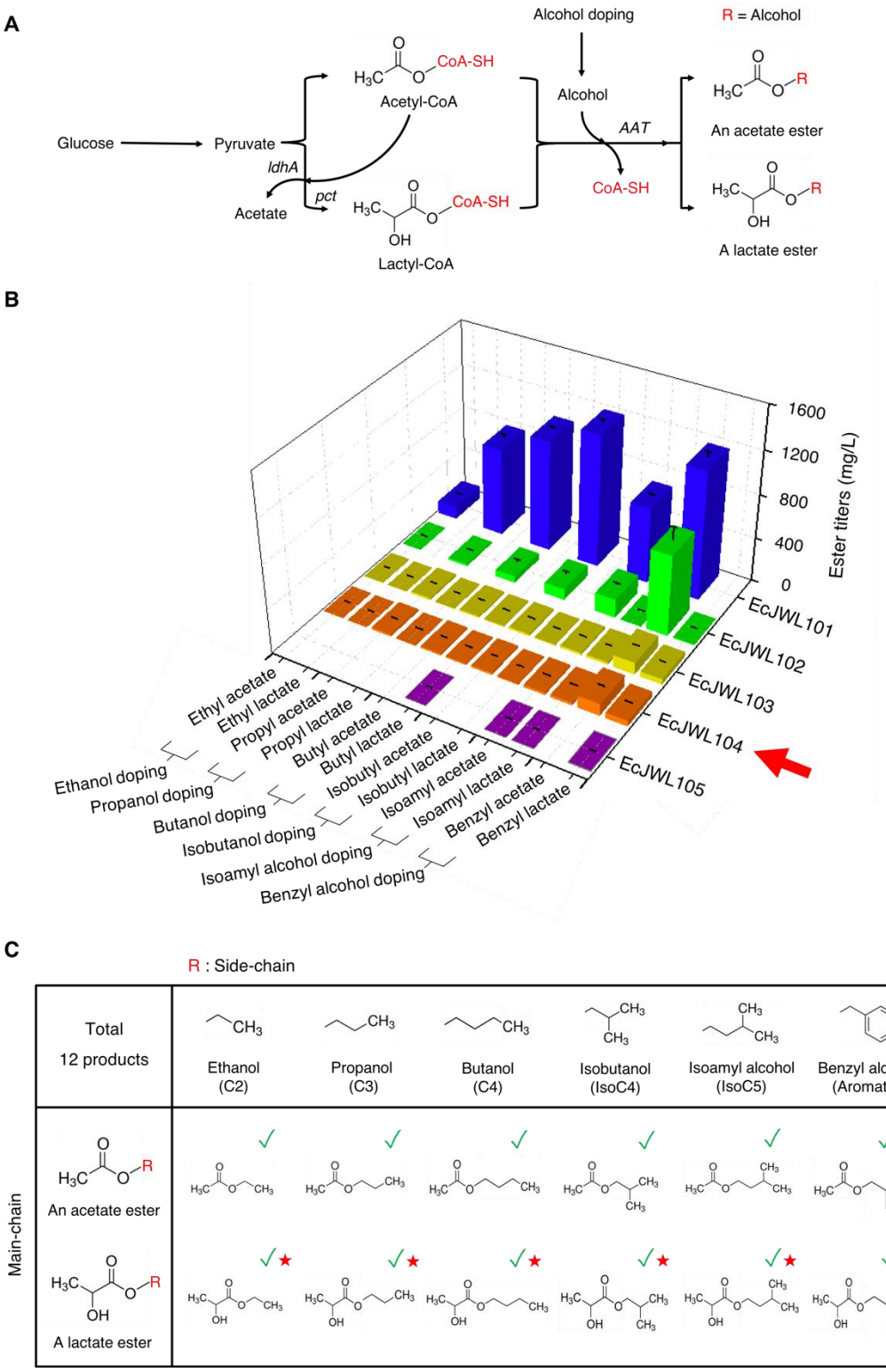

A
Alcohol doping
R = Alcohol
Glucose
Pyruvate
Acetyl-CoA
CoA-SH
Alcohol
AAT
An acetate ester
ldhA
pct
Acetate
CoA-SH
Lactyl-CoA
A lactate ester
B
Ester titers (mg/L)
1600
1200
800
400
0
EcJWL101
EcJWL102
EcJWL103
EcJWL104
EcJWL105
Ethyl acetate
Ethyl lactate
Propyl acetate
Propyl lactate
Butyl acetate
Butyl lactate
Isobutyl acetate
Isobutyl lactate
Isoamyl acetate
Isoamyl lactate
Benzyl acetate
Benzyl lactate
Ethanol doping
Propanol doping
Butanol doping
Isobutanol doping
Isoamyl alcohol doping
Benzyl alcohol doping
C
R : Side-chain
Total
12 products
Ethanol (C2)
Propanol (C3)
Butanol (C4)
Isobutanol (IsoC4)
Isoamyl alcohol (IsoC5)
Benzyl alcohol (Aromatic)
Main-chain
An acetate ester
A lactate ester

Figure 2

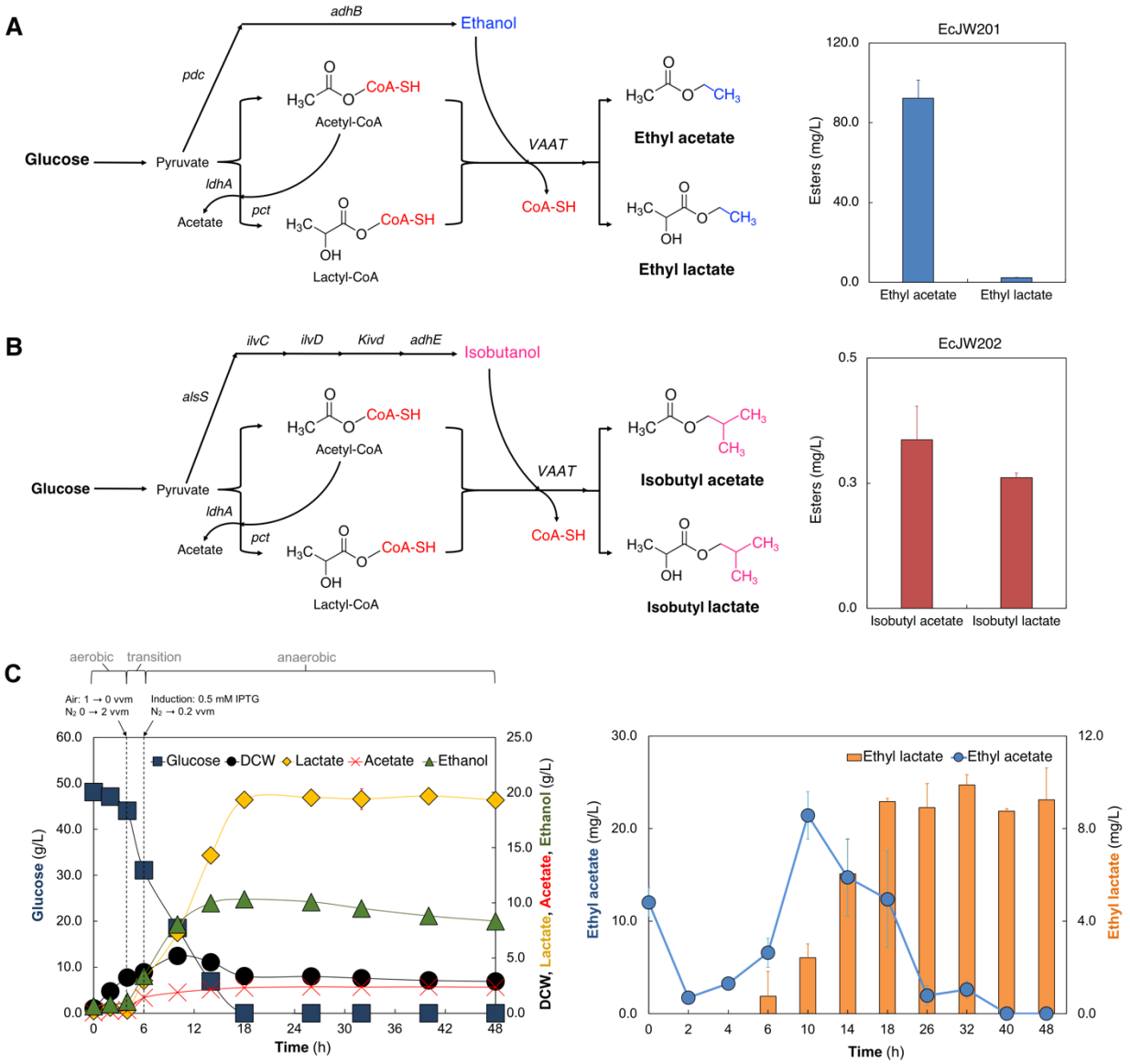

A
adhB
Ethanol
pdc
Glucose
Pyruvate
ldhA
Acetate
pct
Acetyl-CoA
Lactyl-CoA
CoA-SH
VAAT
Ethyl acetate
Ethyl lactate
EcJW201
Esters (mg/L)
Ethyl acetate
Ethyl lactate
B
ilvC
ilvD
Kivd
adhE
Isobutanol
alsS
Isobutyl acetate
Isobutyl lactate
EcJW202
C
aerobic
transition
anaerobic
Air: 1 → 0 vvm
N₂ 0 → 2 vvm
Induction: 0.5 mM IPTG
N₂ → 0.2 vvm
Glucose
DCW
Lactate
Acetate
Ethanol
Glucose (g/L)
DCW, Lactate, Acetate, Ethanol (g/L)
Time (h)
Ethyl lactate
Ethyl acetate
Ethyl acetate (mg/L)
Ethyl lactate (mg/L)
Time (h)

Figure 3

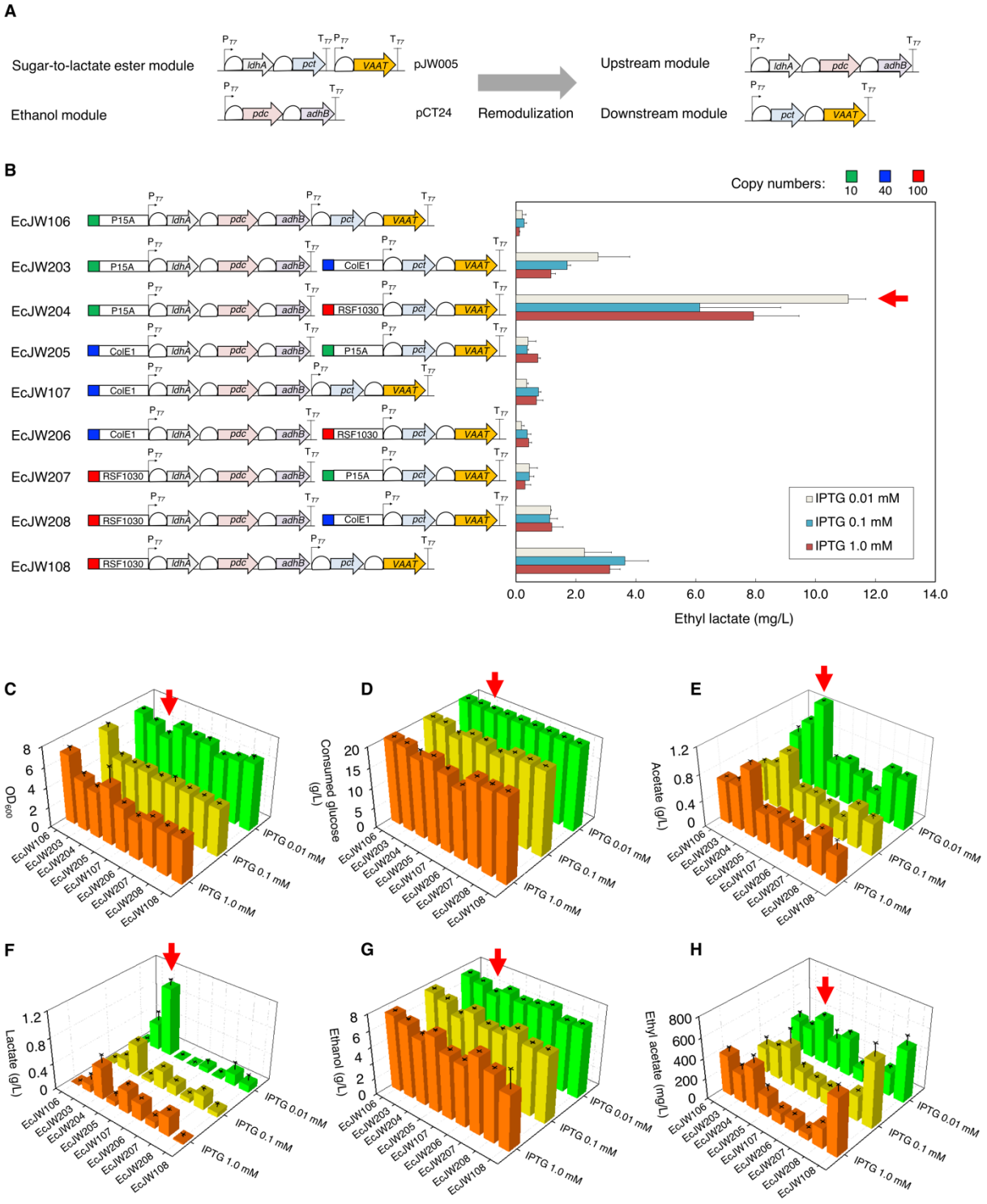

A
Sugar-to-lactate ester module
Ethanol module
pJW005
pCT24
Remodulization
Upstream module
Downstream module
B
Copy numbers: 10 40 100
EcJW106
EcJW203
EcJW204
EcJW205
EcJW107
EcJW206
EcJW207
EcJW208
EcJW108
IPTG 0.01 mM
IPTG 0.1 mM
IPTG 1.0 mM
Ethyl lactate (mg/L)
C
D
E
F
G
H
OD600
Consumed glucose (g/L)
Acetate (g/L)
Lactate (g/L)
Ethanol (g/L)
Ethyl acetate (mg/L)

Figure 4

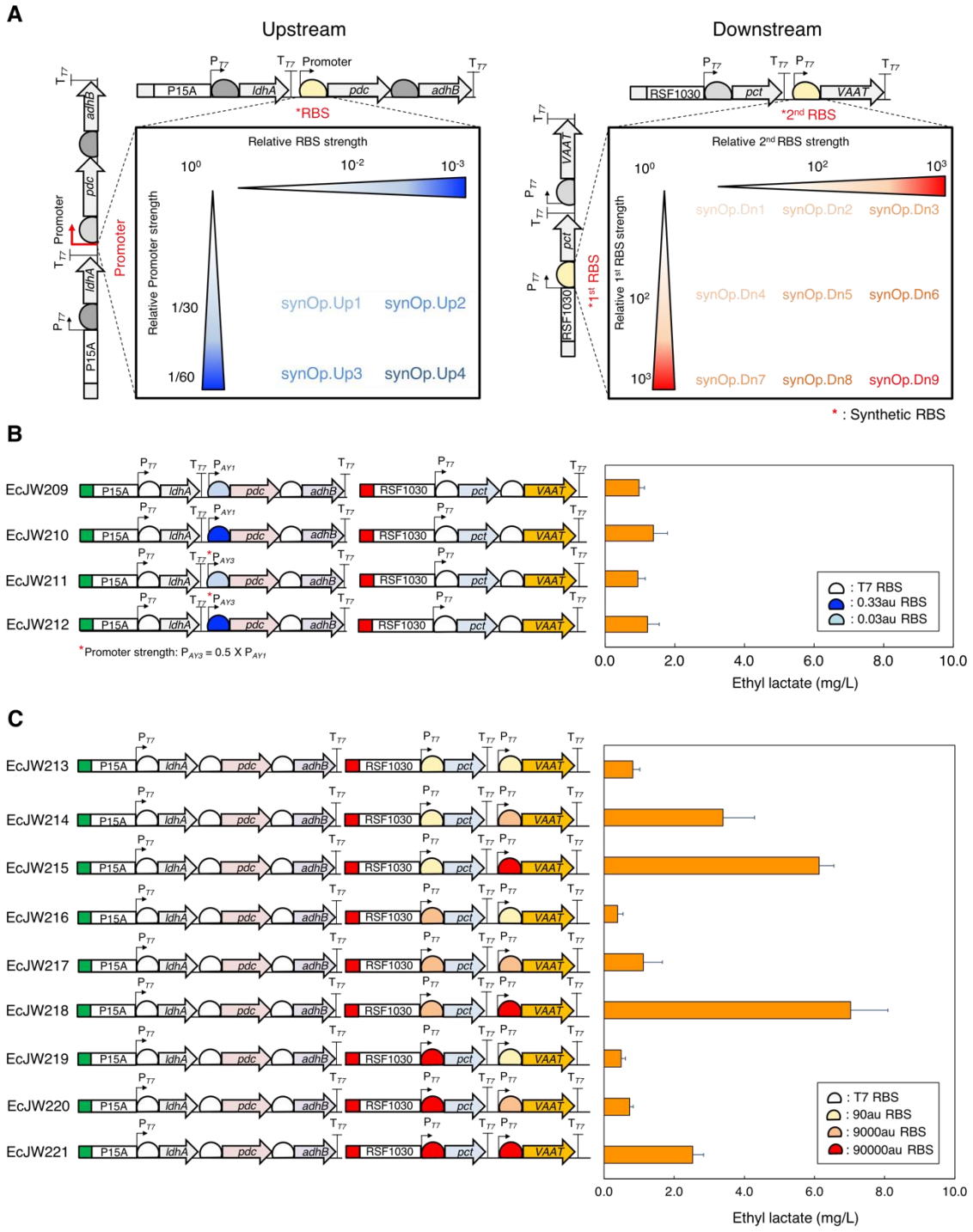
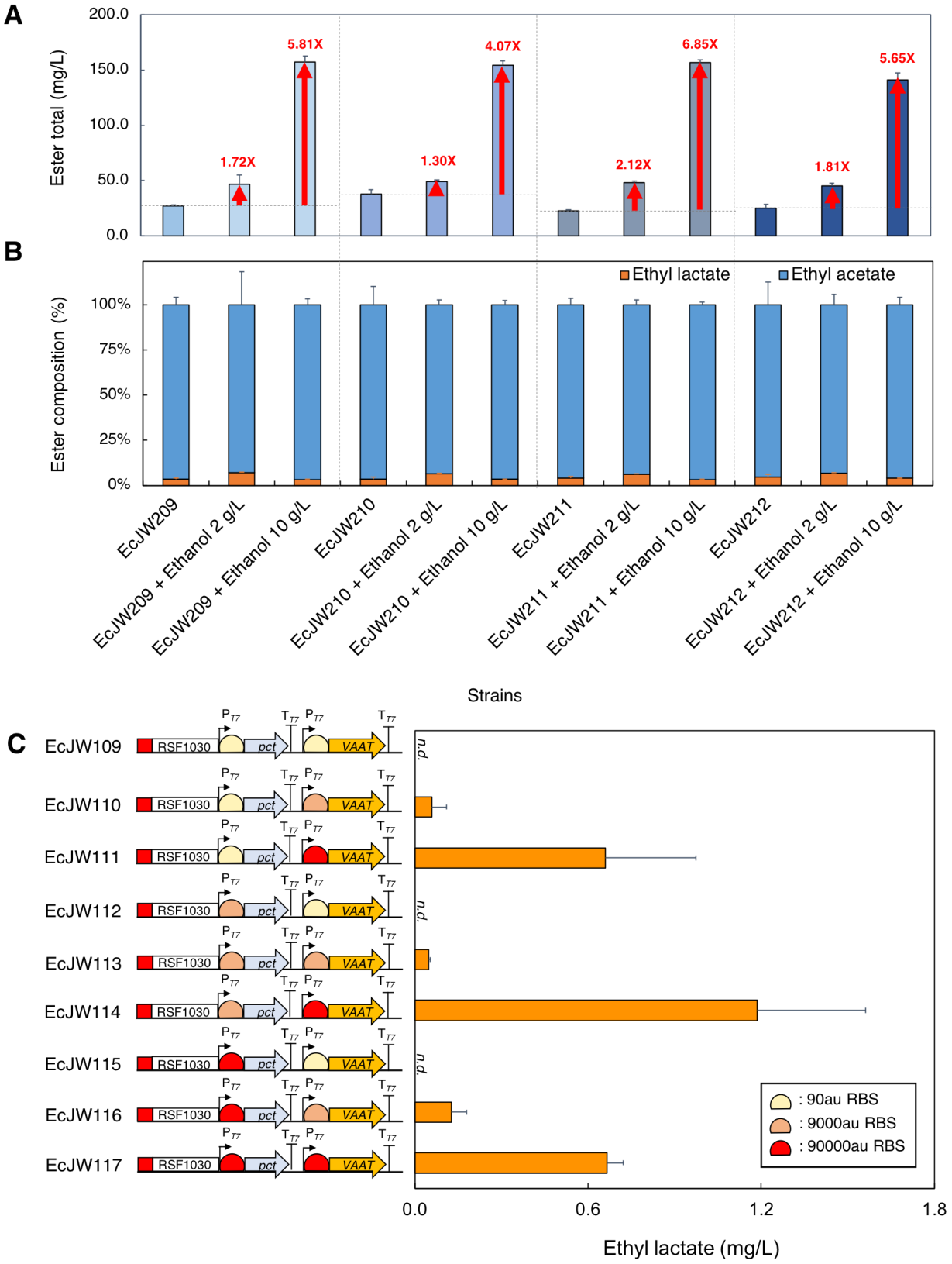

A
Upstream
Downstream
P15A
ldhA
pdc
adhB
*RBS
RSF1030
pct
VAAT
*2nd RBS
Promoter
Relative RBS strength
Relative Promoter strength
Relative 2nd RBS strength
Relative 1st RBS strength
*1st RBS
synOp.Up1
synOp.Up2
synOp.Up3
synOp.Up4
synOp.Dn1
synOp.Dn2
synOp.Dn3
synOp.Dn4
synOp.Dn5
synOp.Dn6
synOp.Dn7
synOp.Dn8
synOp.Dn9
* : Synthetic RBS
B
EcJW209
EcJW210
EcJW211
EcJW212
*Promoter strength: P_AY3 = 0.5 X P_AY1
: T7 RBS
: 0.33au RBS
: 0.03au RBS
Ethyl lactate (mg/L)
C
EcJW213
EcJW214
EcJW215
EcJW216
EcJW217
EcJW218
EcJW219
EcJW220
EcJW221
: T7 RBS
: 90au RBS
: 9000au RBS
: 90000au RBS
Ethyl lactate (mg/L)

Figure 5


A
Ester total (mg/L)
200.0
150.0
100.0
50.0
0.0
1.72X
5.81X
1.30X
4.07X
2.12X
6.85X
1.81X
5.65X
B
Ester composition (%)
100%
75%
50%
25%
0%
Ethyl lactate
Ethyl acetate
EcJW209
EcJW209 + Ethanol 2 g/L
EcJW209 + Ethanol 10 g/L
EcJW210
EcJW210 + Ethanol 2 g/L
EcJW210 + Ethanol 10 g/L
EcJW211
EcJW211 + Ethanol 2 g/L
EcJW211 + Ethanol 10 g/L
EcJW212
EcJW212 + Ethanol 2 g/L
EcJW212 + Ethanol 10 g/L
Strains
C
EcJW109
EcJW110
EcJW111
EcJW112
EcJW113
EcJW114
EcJW115
EcJW116
EcJW117
RSF1030
pct
VAAT
n.d.
: 90au RBS
: 9000au RBS
: 90000au RBS
0.0
0.6
1.2
1.8
Ethyl lactate (mg/L)