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Microbial synthesis of a branched-chain ester platform from organic waste carboxylates

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ABSTRACT

Processing of lignocellulosic biomass or organic wastes produces a plethora of chemicals such as short, linear carboxylic acids, known as carboxylates, derived from anaerobic digestion. While these carboxylates have low values and are inhibitory to microbes during fermentation, they can be biologically upgraded to high-value products. In this study, we expanded our general framework for biological upgrading of carboxylates to branched-chain esters by using three highly active alcohol acyltransferases (AATs) for alcohol and acyl CoA condensation and modulating the alcohol moiety from ethanol to isobutanol in the modular chassis cell. With this framework, we demonstrated the production of an ester library comprised of 16 out of all 18 potential esters, including acetate, propionate, butanoate, pentanoate, and hexanoate esters, from the 5 linear, saturated C_2-C_6 carboxylic acids. Among these esters, 5 new branched-chain esters, including isobutyl acetate, isobutyl propionate, isobutyl butyrate, isobutyl pentanoate, and isobutyl hexanoate were synthesized *in vivo*. During 24 h *in situ* fermentation and extraction, one of the engineered strains, EcDL208 harnessing the SAAT of *Fragaria ananassa* produced ~63 mg/L of a mixture of butyl and isobutyl butyrates from glucose and butyrate co-fermentation and ~127 mg/L of a mixture of isobutyl and pentyl pentanoates from glucose and pentanoate co-fermentation, with high specificity. These butyrate and pentanoate esters are potential drop-in liquid fuels. This study provides better understanding of functional roles of AATs for microbial biosynthesis of branched-chain esters and expands the potential use of these esters as drop-in biofuels beyond their conventional flavor, fragrance, and solvent applications.

1. Introduction

The natural, efficient consolidated bioprocessing of lignocellulosic biomass or organic wastes is anaerobic digestion (Agler et al., 2011; Jonsson and Martin, 2016). In this process, a consortium of mixed microbes (*e.g.* anaerobic digesters) can degrade organic wastes directly into carboxylates (*e.g.*, linear and saturated C_2-C_6 organic acids) without the stipulation of any pretreatment (Batstone and Virdis, 2014; Thanakoses et al., 2003). While these carboxylates have low values and are inhibitory to microbes, they can be biologically upgraded to a large space of high-value chemicals such as esters that are widely used in flavor, fragrance, and solvent industries. Certain carboxylate-derived esters have high hydrophobicity for easy separation from fermentation and encompass high combustion properties that can be used as biodiesels or jet fuels (Chuck and Donnelly, 2014; Contino et al., 2011; Kallio et al., 2014).

Biologically upgrading the carboxylate to ester platforms has recently been demonstrated (Layton and Trinh, 2016). This conversion was achieved by a modular cell (Trinh et al., 2015) tightly integrated with an engineered acid-to-ester production module – a modular heterologous pathway comprised of an alcohol production submodule, an acid to acyl CoA synthesis submodule, and alcohol and an acyl CoA condensation submodule. The flexible design of these modules served several purposes: (i) expanding the biosynthesis of the ester platform in a plug-and-play fashion using a pure culture or a consortium of mixed cultures and (ii) screening alcohol acyl transferases (AATs) for their novel *in vivo* activities. Understanding the catalysis of the AAT condensation reaction is critical for efficient ester biosynthesis but is currently limited. Some recent studies have aimed at understanding AAT specificities using various techniques, from whole-cell *in vivo* approaches using the carboxylates as substrates (Layton and Trinh, 2016) or acid additions from the 2-keto acid synthesis pathway (Rodriguez et al., 2014) to *in vitro* enzymatic assays (Lin et al.,

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

Plasmids/Strains	Genotypes	Sources
Plasmids		
pETite*	kan^R	Layton and Trinh (2014)
pDL001	pETite* SAAT; kan+	Layton and Trinh (2016)
pDL004	pETite* *atf1*; kan+	Layton and Trinh (2016)
pDL006	pETite* VAAT; kan+	Layton and Trinh (2016)
pCT13	pCOLA-P_{T7}:RBS:*alsS*:RBS:*ilvC*:RBS:*ilvD*−P_{T7}:RBS:*kivd*:RBS:*adhE*:T_{T7}; kan^R	Trinh et al. (2011)
pDL014	pETite* P_{T7}:RBS:*pct*:RBS:*atf1*:T_{T7}; amp^R	This study
pDL015	pETite* P_{T7}:RBS:*pct*:RBS:SAAT:T_{T7}; amp^R	This study
pDL016	pETite* P_{T7}:RBS:*pct*:RBS:VAAT:T_{T7}; amp^R	This study
Strains		
C. propionicum	Wildtype	ATCC 25522
EcDL002	TCS083 (λDE3) Δ*fadE*:kan^- (cured)	Layton and Trinh (2014)
EcDL207	EcDL002 pCT13+pDL014; kan^R amp^R	This study
EcDL208	EcDL002 pCT13+pDL015; kan^R amp^R	This study
EcDL209	EcDL002 pCT13+pDL016; kan^R amp^R	This study

2016) and *in silico* protein modeling (Morales-Quintana et al., 2011, 2012, 2013, 2015). To date, the biological upgrading of the carboxylate to ester platforms has only been demonstrated using the ethanol production module, and understanding of whether the targeted AATs have activity towards other alcohols has not yet been investigated.

In this study, we biologically upgraded the carboxylate to branched-chain ester platforms by modulating the alcohol submodule from ethanol to isobutanol. Using the engineered *Escherichia coli* modular cell, we explored the functional roles of three AATs of the acid-to-ester module for the potential synthesis of 18 unique esters from the 5 linear, saturated C_2-C_6 carboxylic acids commonly found in the carboxylate platform. Microbial biosynthesis of the ester platform with longer- and branched-chain alcohols beyond ethanol modulates the ester flavor and fragrance properties as well as improves the energy density of these esters that can potentially be used as pure or blended biodiesels and jet fuels.

2. Materials and methods

2.1. Plasmids and strains

The list of plasmids and strains used in this study is presented in Table 1. The fermentative branched-chain ester pathway was designed as an exchangeable production module comprised of an alcohol submodule and an acyl-CoA transferase (ACT) plus AAT submodule (Layton and Trinh, 2016). Each submodule carried genes organized in operons of a plasmid under T7 promoters. The isobutanol submodule pCT13 was previously constructed (Trinh et al., 2011). Each ACT plus AAT submodule (*e.g.*, pDL014, pDL015, or pDL016) was created by assembling 3 DNA fragments including (i) the propionyl-CoA transferase (PCT, belonging to the general class of ACT) gene amplified from the genomic DNA of *Clostridium propionicum* using the primers DL_0023/DL0024, (ii) the ATF1 gene (amplified from the plasmid pDL004 using primers DL_0025/DL_0020), the SAAT gene (pDL001, DL_0012/DL_0027), or the VAAT gene (pDL006, DL_0018/DL_0028), and (iii) the pETite* backbone amplified using the primers DL_0001/DL_0002. Primers used for this study are presented in Table 2.

The engineered *E. coli* modular chassis cell, EcDL002, was deployed as the ester production host (Layton and Trinh, 2014). By transforming the submodules pCT13 and pDL014-pDL016 into EcDL002 via electroporation (Sambrook, 2001), we created the ester production strains EcDL207-209, respectively.

2.2. Media and cell culturing conditions

The medium (pH∼7) used for the acid-to-ester production experiments contained 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 solution (1 g/L), 1 mL/L of stock trace metals solution (Trinh et al., 2008), 5 g/L yeast extract, 2 g/L organic acid (*e.g.*, acetic, propionic, butyric, pentanoic, or hexanoic acid), 20 g/L glucose, 25 μg/mL kanamycin, and 50 μg/mL ampicillin. 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. The organic acids used for the acid-to-ester production experiments are the dominant chemicals present in the carboxylate platform (Holtzapple, 2015).

Ester production was carried out via *in situ*, high-cell density fermentation and extraction using the hexadecane organic overlay as previously described (Layton and Trinh, 2016). Briefly, the fermentation was conducted in a 75° angled platform in a New Brunswick Excella E25 at 37 °C and 175 rpm for 24 h under anaerobic conditions. Whole-cells and cell supernatants were collected and stored at −20 °C for subsequent metabolite analysis while hexadecane overlay was stored at room temperature for ester analysis. All experiments were performed with at least three biological replicates.

2.3. Analytical methods

Sugars, organic acids, and alcohols from culture supernatants were analyzed by the high pressure liquid chromatography (HPLC) technique. Produced esters were captured by hexadecane organic

Table 2
A list of primers for plasmid construction.

Primers	Sequences
DL_0001	5′-CATCATCACCACCATCACTAA-3′
DL_0002	5′-ATGTATATCTCCTTCTTATAGTTAAAC-3′
DL_0012	5′-GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGTTAAATTAAGGTCTTTGGAG-3′
DL_0018	5′-GGCGGCCGCTCTATTAGTGATGGTGGTGATGATGCGGATAACATACGTAGACCG-3′
DL_0020	5′-GCCGCTCTATTAGTGATGGTGGTGATGATGCTAAGGGCCTAAAAGGAGAG-3′
DL_0023	5′-AAATAATTTTGTTTAACTATAAGAAGGAGATATACATATG AGAAAGGTTCCCATTATTAC-3′
DL_0024	5′-TCAGGACTTCATTTCCTTCAG-3′
DL_0025	5′-CTGAAGGAAATGAAGTCCTGAAAGGAGATATACATATGAATGAAATCGATGAGAAAAATC-3′
DL_0027	5′-TGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACATATGGAGAAAATTGAGGTCAG-3′
DL_0028	5′-TGGGTCTGAAGGAAATGAAGTCCTGAAAGGAGATATACATATGGAGAAAATTGAGGTCAG-3′

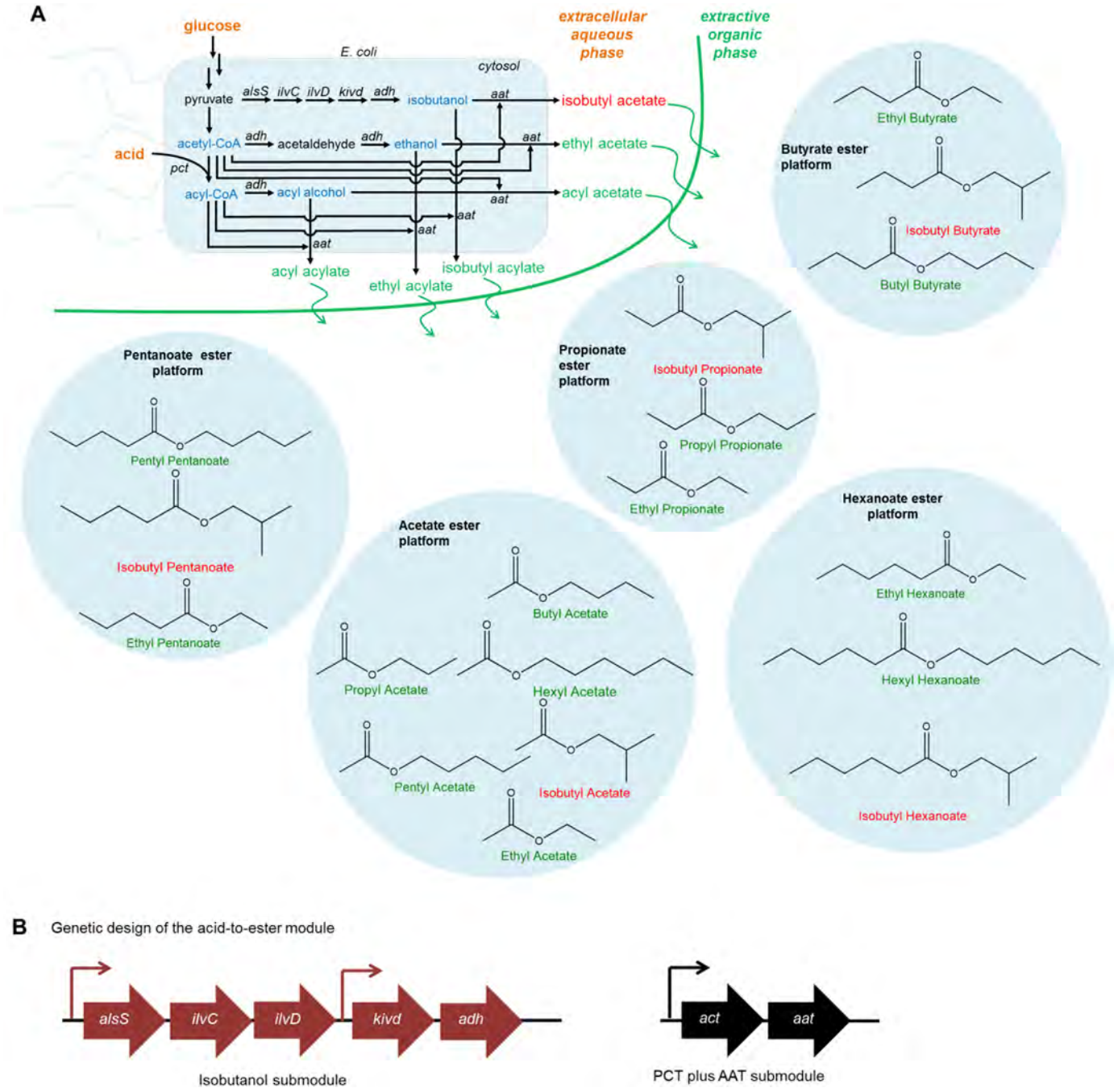

Fig. 1. (A) Microbial biosynthesis of branched-chain esters from carboxylates. (B) Genetic design of the acid-to-ester module.

overlay and were quantified by gas chromatography coupled with mass spectroscopy (GC/MS) technique. The sample preparation for HPLC and GC/MS, data analyses, and instruments were described in detail previously (Layton and Trinh, 2016) while the running methods were slightly modified to capture the new branched chain esters (see Supplementary Texts S1 for details).

2.4. Calculation of octane normalized mass energy density (ONMED)

The standard heat of combustion of each chemical (kJ/kg) was estimated based on average bond energies (Zumdahl, 2000). The ONMED of a chemical is defined as the ratio of its standard heat of combustion to that of octane.

3. Results and discussion

3.1. Design of microbial biosynthesis of branched-chain ester platform

The general framework for biological upgrading of the carboxylate to branched-chain ester platforms utilizes the acid-to-ester pathway (Fig. 1A). This framework was built upon our previously established foundation (Layton and Trinh, 2016) by modulating the alcohol submodule from ethanol to isobutanol. In brief, the designed acid-to-ester pathway contained the isobutanol submodule for conversion of pyruvate to isobutanol and the PCT plus AAT submodule that converts carboxylates to acyl CoAs and

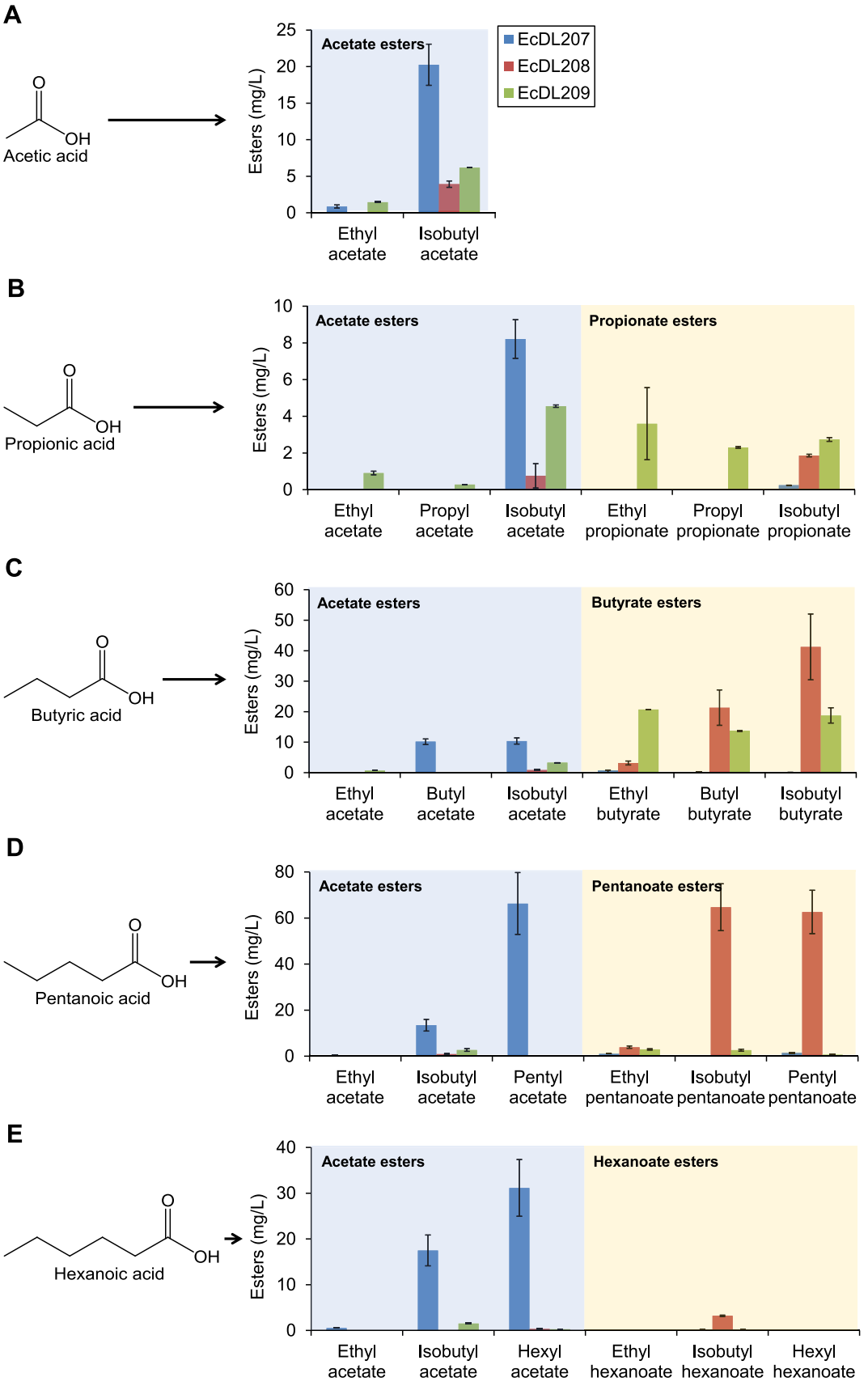

Fig. 2. Ester production of EcDL207, 208, and 209 after 24 h from co-fermentation of (A) glucose and acetic acid, (B) glucose and propionic acid, (C) glucose and butyric acid, (D) glucose and pentanoic acid, and (E) glucose and hexanoic acid.

condenses them with alcohols to produce esters (Fig. 1B). As the isobutanol submodule contains the overexpression of an *E. coli* alcohol/aldehyde dehydrogenase (AdhE), it can reduce acyl CoAs from carboxylates to alcohols that can be used for ester biosynthesis by the PCT plus AAT submodule. In our design, we used the PCT of *C. propionicum* because it exhibits broad substrate specificity towards C_2-C_6 carboxylates to produce their respective CoA counterparts (Layton and Trinh, 2016; Schweiger and Buckel 1984). We also used three highly active AATs, including ATF1 of *Saccharomyces cerevisiae*, SAAT of *Fragaria ananassa,* and VAAT of *F. vesca,* that encompass various substrate preferences to test for the branched-chain ester biosynthesis (Layton and Trinh, 2016). Specifically, ATF1 exhibits substrate preference towards longer-chain acetate esters, while SAAT and VAAT have substrate preferences towards C_4-C_6 ethyl acylates and C_2-C_4 ethyl acylates, respectively. In addition, SAAT has specificity towards acyl acylates.

By modulating the alcohol submodule from ethanol to isobutanol, the designed framework can expand the ester production library from 13 to 18 potential esters by co-fermentation of glucose and five linear, saturated C_2-C_6 carboxylates (Fig. 1A). The five new branched-chain esters that can be synthesized microbially from the carboxylate platform include isobutyl acetate, propionate, butyrate, pentanoate, and hexanoate (Fig. 1B). Ester synthesis depends on the availability of precursor metabolites, acyl CoAs and alcohols, and the broad substrate activities of AATs. In this study, we characterized three strains harnessing the acid-to-ester pathways with various AATs while other heterologous genes and their constructs were identical. These engineered strains are EcDL207, 208, and 209 and carry ATF1, SAAT and VAAT, respectively.

3.2. Expanding combinatorial biosynthesis of ester platforms

3.2.1. Microbial biosynthesis of an acetate ester platform

A total of 5 targeted acetate esters including ethyl, propyl, butyl, pentyl, and hexyl acetates could be potentially synthesized from 5 carboxylates. Our results show that (i) a mixture of ethyl and isobutyl acetates could be produced from co-fermentation of glucose and acetate (Fig. 2A); (ii) a mixture of ethyl, propyl, and isobutyl acetates from co-fermentation of glucose and propionate (Fig. 2B); (iii) a mixture of ethyl, butyl, and isobutyl acetates from co-fermentation of glucose and butyrate (Fig. 2C); (iv) a mixture of ethyl, isobutyl, and pentyl acetates from co-fermentation of glucose and pentanoate (Fig. 2D); and (v) a mixture of ethyl, isobutyl, and hexyl acetates from co-fermentation of glucose and hexanoate (Fig. 2E). Due to the various AAT specificities and precursor availability, it is anticipated that each characterized strain might not be able to produce all expected acetate esters for each co-fermentation of glucose and a targeted carboxylate as previously observed (Layton and Trinh, 2016).

Among the characterized strains and acetate esters, EcDL207 produced pentyl acetate at the highest level of 66.30 ± 13.44 mg/L after 24 h from the co-fermentation of glucose and pentanoate (Fig. 2D). EcDL207 also synthesized 31.17 ± 0.32 mg/L hexyl acetate (Fig. 2E), 20.26 ± 2.82 mg/L isobutyl acetate (Fig. 2A) and 10.22 ± 0.85 mg/L butyl acetate (Fig. 2C) at much higher titers than EcDL208 and EcDL209. The observed phenotypes of EcDL207 were consistent with our previous study (Layton and Trinh, 2016) where ATF1 exhibited high specificity towards acetate esters. If the application were tailored for production of an acetate ester platform, ATF1 would be a strong candidate to use for the acid-to-ester pathway.

Both carboxylates and acetate esters have distinct physical properties. The fruity smell of esters makes them unique presenting broad applications in flavor, fragrance, and solvent industries. While acetate and most of the carboxylates are very soluble in water, causing toxicity to microbes during fermentation, the acetate esters have significant reduction in water solubility (Fig. 3A) and can be easily extracted during fermentation as implemented in our study. In addition, biological upgrading of acetate to acetate esters resulted in the improved ONMED values making them suitable for biofuel applications (Fig. 3B). For instance, isobutyl acetate (0.639) has a higher ONMED value than ethanol (0.615) and acetic acid (0.312), and has been tested as a biofuel blend (Olson et al., 2003). Lower solubility of isobutyl acetate in water (~7 g/L) in comparison to acetic acid (complete solubility) and isobutanol (~88 g/L) is very advantageous for *in situ* fermentation and extraction.

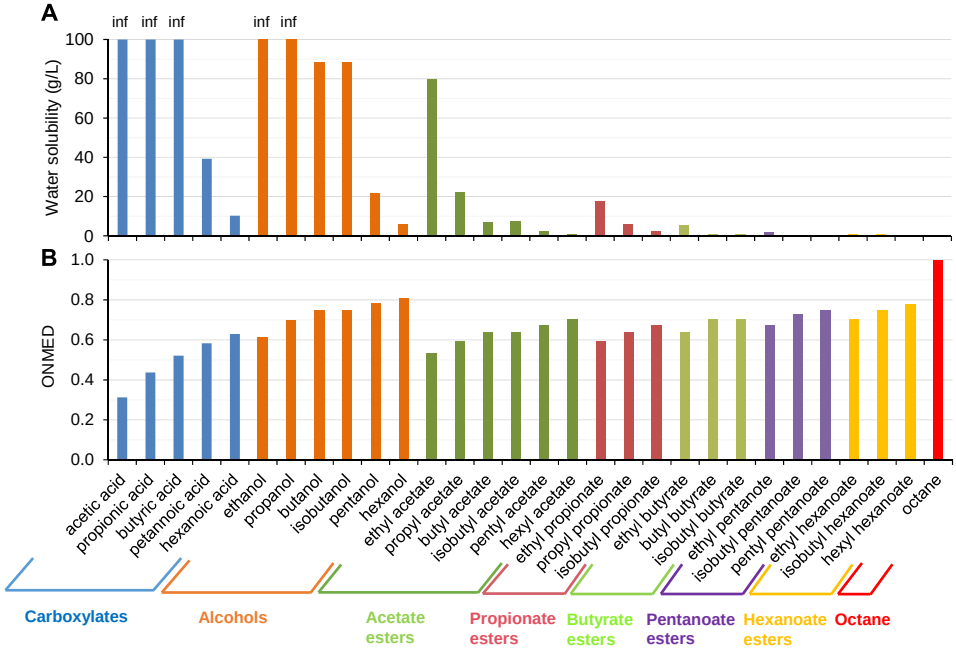

Fig. 3. Physical properties of carboxylates, alcohols, acetate esters, propionate esters, butyrate esters, pentanoate esters, and hexanoate esters. (A) Water solubility (g/L). inf: complete solubility. (B) Octane normalized mass energy density (ONMED).

3.2.2. Microbial biosynthesis of a propionate ester platform

Co-fermentation of glucose and propionate could generate a propionate ester library comprised of ethyl, propyl, and isobutyl propionates (Fig. 2B). Among the characterized strains, only EcDL209 could produce all three propionate esters while EcDL207 and EcDL208 could synthesize only isobutyl propionate. Isobutyl propionate was produced at the highest titer of 3.60 ± 1.96 (mg/L) by EcDL209 among propionate esters and characterized strains.

It is interesting to notice that EcDL209 harnessing VAAT produced little ethyl propionate. In our previous study, however, we observed that the VAAT exhibited a relatively high activity towards ethyl propionate production with a titer up to 67.24 ± 10.41 mg/L when the ethanol module was used instead of the isobutanol module (Layton and Trinh, 2016). Altogether, these results suggest that the insufficient generation of ethanol in EcDL207, 208 and 209 might have resulted in low ethyl propionate production. Among acylate esters, the production of propionate esters was the lowest (Fig. 2).

Propyl acetate, propyl propionate, and isobutyl propionate, each have unique intrinsic physical properties including ONMED values 0.594, 0.639 and 0.675, respectively as well as fruity odors and relatively low water solubility (Fig. 3). The ONMED values of these propionate esters are all slightly lower than propanol (0.697) but higher than ethanol (0.615), which allow them to be blended in biofuels for increasing their octane.

3.2.3. Microbial biosynthesis of a butyrate ester platform

From co-fermentation of glucose and butyrate, all characterized strains EcDL207-209 produced the targeted butyrate ester library, consisting of ethyl, butyl, and isobutyl butyrates. EcDL208 produced 21.34 ± 13.76 mg/L butyl butyrate and 41.25 ± 18.76 mg/L isobutyl butyrate at the highest levels among the characterized strains while EcDL209 produced ethyl butyrate at the highest titer of 20.76 ± 0.00 (mg/L). Consistent with the previous study (Layton and Trinh, 2016), SAAT of EcDL208 shows substrate preferences towards C_4-C_6 acyl CoAs and short-chain alcohols while VAAT of EcDL209 exhibits substrate preferences toward C_2-C_4 acyl CoAs and ethanol. As expected, ATF1 of EcDL207 produced some amount of butyl and isobutyl acetates because it has substrate preferences towards acetyl CoA and short-chain alcohols.

Butyric acid is completely soluble in water and is very toxic to microbes (Fig. 3). However, biological upgrading of butyrate can generate a butyrate ester library with unique properties. The most distinct feature is the odor difference between rancid butyrate and its derived pleasant butyrate esters. Both butyl and isobutyl butyrate have very low water solubility (< 0.7 g/L), which is advantageous for simultaneous fermentation, separation, and extraction process development (Fig. 3). These butyrate esters also have higher ONMED value than ethanol and propanol, which make them suitable for biofuel application. For instance, butyl butyrate has been recently tested as a potential jet fuel alternative (Chuck and Donnelly, 2014).

3.2.4. Microbial biosynthesis of a pentanoate ester platform

Biological upgrading of pentanoate could expand the pentanoate ester platform to include ethyl, pentyl, and isobutyl pentanoates. Among the characterized strains, EcDL208 could produce all three targeted pentanoate esters at the highest titers, including 3.90 ± 0.50 mg/L ethyl pentanoate, 64.71 ± 10.19 mg/L isobutyl pentanoate, and 62.63 ± 0.75 mg/L pentyl pentanoate. The high production of the targeted pentanoate ester library conferred the substrate preference of SAAT used in EcDL208 (Layton and Trinh, 2016).

Like butyric acid, pentanoic acid exhibits a rancid odor. However, biologically-upgraded pentanoate esters have pleasant smells and tastes, and hence are known for their wide use in flavor and fragrance industries. Unlike pentanoic acid, its derived pentanoate esters are mostly insoluble and are advantageous for *in situ* fermentation and extraction. Since ethyl, isobutyl, and pentyl pentanoates have ONMED values of 0.675, 0.727, and 0.747, respectively, which are close to the isobutanol ONMED (0.749), these esters can be potentially used as drop-in biofuels beyond their conventional flavor, fragrance, and solvent applications. For instance, ethyl pentanoate has undergone road trials and demonstrated stable performance when blended (10%) with gasoline (Lange et al., 2010).

3.2.5. Microbial biosynthesis of a hexanoate ester platform

Co-fermentation of hexanoate and glucose could potentially yield a hexanoate ester library including ethyl, isobutyl, and hexyl hexanoates. The characterized strains, however, could only synthesize isobutyl hexanoate, neither ethyl hexanoate nor hexyl hexanoate. EcDL208 produced isobutyl hexanoate with the highest titer of 3.21 ± 0.15 mg/L. SAAT of EcDL208 was also first shown to have the substrate specificity for isobutanol and hexanoyl CoA to produce isobutyl hexanoate. Different from the previous study where the ethanol submodule was used instead of the isobutanol submodule (Layton and Trinh, 2016), both SAAT and VAAT exhibited activities towards ethyl hexanoate production despite low titers. These results suggest that inefficient supply of precursor metabolites in EcDL208 and EcDL209 might have limited ethyl hexanoate biosynthesis investigated in this study. Currently, we have not been able to synthesize hexyl hexanoate likely due to low activity of the characterized AATs towards this ester.

Not only does isobutyl hexanoate have one of the highest ONMED values at 0.747 (comparable with pentyl pentanoate) among the biologically upgraded esters, but it also exhibits little to no solubility in aqueous solutions (Fig. 3). Interestingly, ethyl octanoate that also has the same ONMED as isobutyl hexanoate has recently been tested and demonstrated for its use in A-1 jet fuel (Chuck and Donnelly, 2014). The physical property of isobutyl hexanoate makes it a potential candidate for jet fuel application.

4. Conclusion

Biological upgrading low-value carboxylates, derived from lignocellulosic biomass or organic wastes, to high-value esters has significant potential. In this study, we expanded our general, flexible framework for this biological upgrading. By deploying the ester production strains harnessing the acid-to-ester modules with various AATs, we demonstrated the microbial biosynthesis of 16 out of the total 18 potential esters including 5 new branched-chained esters – isobutyl acetate, isobutyl propionate, isobutyl butyrate, isobutyl pentanoate, and isobutyl hexanoate from the carboxylates. Not only did we confirm the substrate preferences of ATF1 (EcDL207) towards long-chain acetate esters, SAAT (EcDL208) towards acyl acylates, and VAAT (EcDL209) towards ethyl C_2-C_4 acylates, but also demonstrated their activities towards branched-chain esters. Since our study aimed to expand the carboxylate to ester platforms, there is much room to enhance ester production that is currently low in future studies (Supplementary Tables 1–3). Many promising strategies can be employed to improve the ester production such as pathway optimization (*e.g.*, modulating promoter, ribosome binding site, gene orthologs to balance and optimize pathway fluxes) and process conditions (*e.g.* temperature, medium, pH, substrate feeding, *in situ* extraction and fermentation).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.meteno.2016.08.001.

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