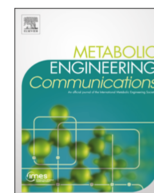
Metabolic Engineering Communications 3 (2016) 245–251

Contents lists available at ScienceDirect

Metabolic Engineering Communications

journal homepage: www.elsevier.com/locate/mec



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

Donovan S. Layton [a,c], Cong T. Trinh [a,b,c,*]

[a] Department of Chemical and Biomolecular Engineering, The University of Tennessee, Knoxville, The United States of America
[b] Bredesen Center for Interdisciplinary Research and Graduate Education, The University of Tennessee, Knoxville, The United States of America
[c] Bioenergy Science Center (BESC), Oak Ridge National Laboratory, Oak Ridge, The United States of America

ARTICLE INFO

Article history:
Received 6 June 2016
Received in revised form
15 July 2016
Accepted 5 August 2016
Available online 6 August 2016

Keywords:
Carboxylate platform
Ester platform
Branched-chain ester
Modular cell
Biological upgrading
Organic waste
Lignocellulosic biomass
Isobutyl esters

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.
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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.,

* Corresponding author at: Department of Chemical and Biomolecular Engineering, The University of Tennessee, Knoxville, The United States of America.
E-mail address: ctrinh@utk.edu (C.T. Trinh).

http://dx.doi.org/10.1016/j.meteno.2016.08.001
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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′

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