Metabolic Engineering 27 (2015) 20–28

Contents lists available at ScienceDirect

Metabolic Engineering

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

Engineered biosynthesis of medium-chain esters in *Escherichia coli*

Yi-Shu Tai, Mingyong Xiong, Kechun Zhang *

Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, MN 55455, USA

ARTICLE INFO

Article history:
Received 26 September 2013
Received in revised form
28 September 2014
Accepted 20 October 2014
Available online 29 October 2014

Keywords:
E. coli
Renewable chemicals
Metabolic engineering
Pathway manipulation
Ester
Isobutyl acetate
Isoamyl acetate

ABSTRACT

Medium-chain esters such as isobutyl acetate (IBAc) and isoamyl acetate (IAAc) are high-volume solvents, flavors and fragrances. In this work, we engineered synthetic metabolic pathways in *Escherichia coli* for the total biosynthesis of IBAc and IAAc directly from glucose. Our pathways harnessed the power of natural amino acid biosynthesis. In particular, the native valine and leucine pathways in *E. coli* were utilized to supply the precursors. Then alcohol acyltransferases from various organisms were investigated on their capability to catalyze esterification reactions. It was discovered that ATF1 from *Saccharomyces cerevisiae* was the best enzyme for the formation of both IBAc and IAAc in *E. coli*. *In vitro* biochemical characterization of ATF1 confirmed the fermentation results and provided rational guidance for future enzyme engineering. We also performed strain improvement by removing byproduct pathways (Δ*ldh*, Δ*poxB*, Δ*pta*) and increased the production of both target chemicals. Then the best IBAc producing strain was used for scale-up fermentation in a 1.3-L benchtop bioreactor. 36 g/L of IBAc was produced after 72 h fermentation. This work demonstrates the feasibility of total biosynthesis of medium-chain esters as renewable chemicals.

1. Introduction

Petroleum is the major material basis of modern society. With increasing demand and dwindling reserves, there is a pressing need to find alternative resources for fuels and chemicals. Metabolic engineering has the potential to be a key player in facilitating the transition from traditional chemical production to a green and sustainable process (Connor and Liao, 2009; Keasling, 2010; Stephanopoulos, 2007). Over past decade, this powerful biotechnology has been successful in converting renewable sugars into various fuels (Atsumi et al., 2008; Lennen and Pfleger, 2012; Liu et al., 2010; Steen et al., 2010; Xu et al., 2013) and chemicals (Achkar et al., 2005; Alper et al., 2005; Atsumi et al., 2008; Dellomonaco et al., 2011; Handke et al., 2011; Tseng and Prather, 2012; Yan et al., 2005; Zhu et al., 2008).

Medium-chain esters (C6–C10) are useful chemicals with various industrial applications. They are flavor components of the majority of fruits (Beekwilder et al., 2004). For example, isobutyl acetate (IBAc), naturally existing in raspberries, pears, and pineapples, has a fruity odor and can be used as a colorless solvent (Panda, 2010). IBAc is also an economical and environmentally friendly replacement for methylisobutyl ketone, or toluene in many formulations. It is widely used in coatings, adhesives, printing inks, fragrances, and cosmetics. The global consumption of IBAc is 187 million pounds in 2005 (Linak, 2006). The other target chemical, isoamyl acetate (IAAc), has one more carbon than IBAc and is used extensively in the food and cosmetic industries. Due to its banana flavor, it is used as flavoring additives in foods and drinks (Vadali et al., 2004a). Similar to IBAc, IAAc can also be used as a green solvent in surface coatings and pharmaceuticals.

Another potential application of these esters is biofuel. For example, several valerate esters (C6–C10) have been proved to be fully compatible with transportation fuels (Lange et al., 2010). These medium-chain esters contain high energy content that is compatible with existing infrastructures (Lange et al., 2010). Moreover, they all have very low solubility in water which is beneficial for a separation process (Perry and Green, 1997). Medium-chain esters are not hygroscopic like ethanol, which are suitable for cold starting and will prevent corrosion. Currently, ethanol (Luli et al., 2008) and biodiesels (Liu et al., 2010; Steen et al., 2010) are the primary targets for biofuel production. However, ethanol has low energy content and is highly hygroscopic. Although biodiesel contains higher energy density, it will gel under cold weather (Alleman et al., 2011) and is not efficient for extracellular secretion due to its large molecular size (Steen et al., 2010). Therefore, medium-chain esters have the potential to address these significant challenges in current biofuel production.

There are several methods that have been developed for the production of medium-chain esters. They are mainly manufactured via Fischer esterification, but this process may raise

* Corresponding author.
E-mail address: kzhang@umn.edu (K. Zhang).

http://dx.doi.org/10.1016/j.ymben.2014.10.004
1096-7176/

environmental concerns since the reactants are often derived from crude oil. Extraction from plant materials has low yield and results in high processing cost (Chulalaksananukul et al., 1992). Direct esterification or trans-esterification reactions can be catalyzed by enzymes such as lipase. However, to shift the reaction equilibrium towards ester synthesis, a significant amount of organic solvent such as *n*-hexane has to be used which makes the process environmentally unfriendly (Chulalaksananukul et al., 1992). Naturally, several sake yeasts are able to produce IBAc and IAAc in low levels (Rojas et al., 2001; Watanabe et al., 1990). Recently, there are several studies on the production of IAAc using externally supplemented 3-methyl-1-butanol (3MB) and intracellular acetyl-CoA (San et al., 2002; Vadali et al., 2004a, 2004b). Both pathway and cofactor manipulations were implemented to enhance IAAc production. Since 3MB was fed to fermentation culture, this process is not truly renewable and is costly. To improve the economics of the ester manufacturing, in this work we designed and engineered metabolic pathways by expanding 2-keto acids synthetic pathways for the direct biosynthesis of IBAc and IAAc in *Escherichia coli*.

The production of higher alcohols (Atsumi et al., 2008; Zhang et al., 2008) and carboxylic acids (Dhande et al., 2012; Zhang et al., 2011) exploiting amino acid precursors has proven to be successful. These amino acid pathways generate 2-keto acids that can be converted into desired products. Since it utilizes hosts' native pathways, a variety of hosts can be explored. In this work, the metabolic platform for IBAc and IAAc production was constructed by expanding native valine and leucine biosynthetic pathways in *E. coli* (Fig. 1). Acetyl-CoA is a key metabolite and is ubiquitous in *E. coli*. For the production of both IBAc and IAAc, a common intermediate is 2-ketoisovalerate (KIV). KIV is the precursor for valine biosynthesis and its production was improved by overexpressing three enzymes, acetolactate synthase (*alsS,* accession number:NC_014479 region: complement (3501924–3504150)) (Renna et al., 1993), 2,3-dihydroxy isovalerate oxidoreductase (*ilvC,* accession number: NC_000913 region: 3957748–3959665), and 2,3-dihydroxy isovalerate dehydratase (*ilvD,* accession number: NC_000913 region: 3953199–3955605) (Daniels et al., 1992). Followed by the last two steps in Ehrlich pathway (Hazelwood et al., 2008), KIV is then converted to isobutyraldehyde by 2-keto-acid decarboxylases (*kivd,* accession number: NC_013656 region: complement (1447696–1449836)) (de la Plaza et al., 2004) and then to isobutanol by alcohol dehydrogenases (*yqhD,* accession number: NC_007779 region: 3153836–3155347) (Sulzenbacher et al., 2004). For IAAc production, to elongate the carbon chain of KIV, the leucine pathway which overexpresses 2-isopropylmalate synthase (*leuA,* accession number: NC_007779 region: complement (81721–83764)), isopropylmalate isomerase complex (*leuC,* accession number: NC_007779 region: complement (79253–81073), *leuD,* accession number: NC_007779 region: complement (78756–79543)) and 3-isopropylmalatedehydrogenase (*leuB,* accession number: NC_007779 region: complement (80702–82121)) was introduced. KIV is converted to 2-keto-4-methyl-pentanoate and then to 3-methyl-1-butanol (Connor and Liao, 2008) by the last two steps in Ehrlich pathway. The final step for the production of our target compounds IBAc and IAAc was achieved by the overexpression of the alcohol acyltransferase (AAT) that catalyzes the reaction: condensing isobutanol or 3-methyl-1-butanolwith acetyl-CoA to form IBAc or IAAc respectively. To further enhance the production of these medium-chain esters, the acetate production pathway (*poxB-pta*) and the lactate production pathway (*ldh*) were inactivated to increase intracellular levels of both acetyl-CoA and the targeted alcohols. In this work, we demonstrated the production of two medium-chain esters, IBAc and IAAc, directly from glucose. Particularly, we successfully scaled up IBAc biosynthesis in a 1.3-L benchtop bioreactor and produced 36 g/L IBAc in 72 h.

2. Materials and methods

2.1. Bacterial strains

All strains and plasmids are listed in Table 1. Oligonucleotides used are listed in Table S-1 in Supplementary materials. *E. coli* strain BW25113 ($rrnB_{T14}\Delta lacZ$WJ16 *hsdR*514 $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) was used as the wild-type host (WT) (Datsenko and Wanner, 2000) for this study. XL1-Blue and XL10-Gold competent cells from

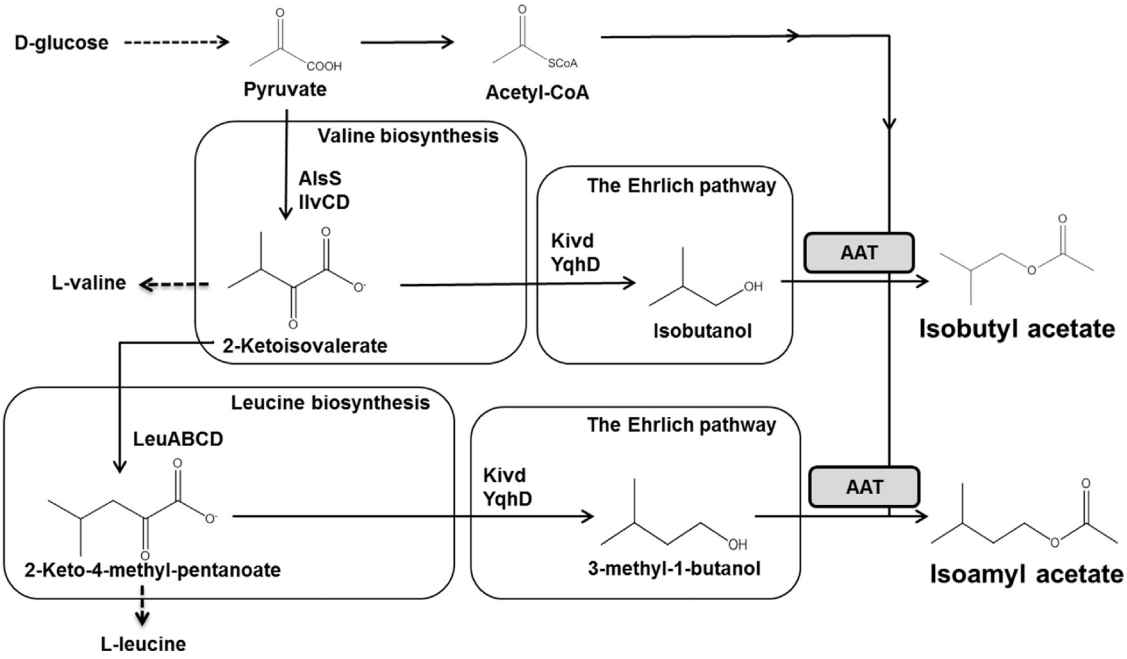

Fig. 1. Metabolic pathway from glucose to isobutyl acetate (IBAc) and isoamyl acetate (IAAc). AATs were screened from five different enzymes. Abbreviations: AAT (Alcohol acyltransferase).