INTERNATIONAL JOURNAL OF ENGINEERING
AND APPLIED SCIENCE
ISSN 2709-8117
DEC. 2020 VOL. 1, NO. 1

Challenges Ahead Before Biobased Ester Production can Move to the Industrial Level.

Aleksander E. Mars
Laboratory of Microbiology, Wageningen University & Research, the Netherlands

ABSTRACT

There are some major challenges ahead before biobased ester production can move to the industrial level. The key ester producing enzyme AAT is not understood well, and studies that focus on fundamental aspects of its function are scarce. For example, it is still not clear why many AATs display thioesterase and esterase activities, and how to control these activities. In case of Eat1, ethanol is the determining factor that shifts the enzyme from a hydrolase to an AAT (Kruis et al., 2017). The controlling factors in other AATs have not been established yet.

KEYWORDS

Metabolic engineering; Alcohol; Chemical; Microbial synthesis.

1. Introduction

Carboxylate esters are versatile compounds that find various applications in the food and chemical industry. They are naturally produced by yeasts such as Saccharomyces cerevisiae and define the taste and odour of fermented beverages (Saerens et al., 2010). As natural food additives they are used to enhance the flavour and odour profile of various food products (Berger, 2009). Ethyl acetate, isoamyl acetate or propyl acetate are common fragrance and aroma compounds, that also find application as bulk chemicals (Fig 1) (Carroll et al., 2016; Rodriguez et al., 2014). Various esters are used as industrial solvents due to their biodegradability and low toxicity, or as plasticizers and polymer additives (Białecka-Florjańczyk and Florjańczyk, 2007; Durrans and Davies, 1971; Löser et al., 2014). Further, they find applications as lubricants, coatings and are explored for their potential as drop-in fuels or biodiesels (Fig 1) (Chuck and Donnelly, 2014; Kalscheuer et al., 2006; Lange et al., 2010).

Traditional ester production processes make use of the FischerSpeier esterification (Fischer and Speier, 1895). Alcohols and carboxylic acids, produced from fossil resources, are condensed in the presence of an acid catalyst at elevated temperatures (Jyoti et al., 2018). Water is released in the process, which leads to the formation of the desired ester. The process however, is dictated by an equilibrium that reduces the reaction rate with time and prevents a complete conversion of all acid and alcohol substrates. In addition, water has been found to inhibit the catalytic activity of the acid catalyst (Liu et al., 2006). This review article focuses on

how and why microorganisms synthesize esters, particularly those that can be applied as commodity chemicals. We provide a detailed overview of the enzymatic reactions that produce esters in microorganisms and how these enzymes have been applied to improve ester formation. An overview of the metabolic engineering strategies aimed at increasing ester production is provided and notable examples are highlighted. We also consider the physical and chemical properties of esters and how they might benefit the production of other valuable bulk chemicals, such as alcohols, carboxylic acids, and α,ω-diols. Lastly, the major challenges that lie ahead of biobased ester production are summarised.

2. Microbial Ester Production

Natural ester production by microorganisms, such as yeasts and lactic acid bacteria is well established and has historically been applied in food production. Volatile esters are among the most important aroma compounds in fermented foods, such as beer, wine and dairy products. In low concentrations, esters impart a sweet, fruity aroma, but are also considered as off-flavours when present in high amounts (Liu et al., 2004). Ethyl acetate is the most abundant volatile ester in food. Its concentration ranges from ~50 to 100 mg/L in dairy products and from ~8 to 64 mg/L in beer and wine (Liu et al., 2004; Saerens et al., 2010). Other volatile esters, such as isoamyl acetate, phenylethyl acetate, ethyl hexanoate and many others do not exceed concentrations of 1 mg/L. These concentrations lie around or just above the human detection threshold (Dzialo et al., 2017; Saerens et al., 2010) and therefore greatly affect the aroma of food products. The amounts of esters naturally produced by microorganisms are typically low, although some exceptions exist, such as bulk ethyl acetate-producing yeast or waxester accumulation by Euglena gracilis. This microalga can accumulate wax esters to as much as 65% of the cell dry weight under anaerobic conditions (Tucci et al., 2010).

The ability of certain yeasts to produce high amounts of ethyl acetate was observed more than 120 years ago (Beijerinck, 1892). Yeasts such as Kluyveromyces marxianus, Wickerhamomyces anomalus and Cyberlindnera jadinii are able to synthesise ethyl acetate from sugars or ethanol (Armstrong et al., 1984; Löser et al., 2014; Meersman et al., 2016; Tabachnick and Joslyn, 1953a,b; van Rijswijck et al., 2017). Growth under iron-limited conditions is the main trigger for bulk ethyl acetate production in yeasts (Armstrong and Yamazaki, 1984; Kruis et al., 2018b; Urit et al., 2012). In some yeasts, ethyl acetate formation has also been induced by oxygen limitation (Fredlund et al., 2006; Kruis et al., 2017). Ethyl acetate production in K. marxianus has been investigated in most detail. Several strains have been identified that form ethyl acetate from whey sugars (Kallel-Mhiri et al., 1993; Löser et al., 2011), glucose (Willetts, 1989), and cassava bagasse supplemented with glucose (Medeiros et al., 2001). K. marxianus is able to catabolise lactose and can utilise whey, a side stream of the cheese industry, to produce ethyl acetate. Moreover, this yeast is able to grow at elevated temperatures, which facilitates ethyl acetate removal from the fermentation broth. These traits make K. marxianus an attractive cell factory for the production of biobased ethyl acetate (Fonseca et al., 2008; Löser et al., 2011, 2014; Urit et al., 2013b). In one study, lactose was converted to ethyl acetate by K. marxianus at 42 °C. The yield reached 56.2% of the pathway maximum, which is the highest reported yield for a natural ethyl acetate-producer reported to date (Urit et al., 2013a).

Because of the structural and chemical diversity of esters, no singular physiological role can be defined for ester synthesis and some are even still debated. Ethyl acetate is a major fermentation product of certain yeast species and contributes to balancing their central carbon metabolism under sub-optimal growth conditions (Fredlund et al., 2004a; Kruis et al., 2018b; Löser et al., 2014). High concentrations of ethyl acetate also repress growth of competitive organisms (Fredlund et al., 2002, 2004b). Volatile esters serve as metabolic intermediates during growth on alkanes or cyclic alcohols in some bacterial species (Iwaki et al., 2002; Kotani et al., 2007). Some esters, such as isoamyl acetate, may help yeast to disperse in the environment by attracting insects (Christiaens et al., 2014). Some specialised esters can even act as bacterial virulence factors (Onwueme et al., 2005). Wax esters are produced as intracellular storage compounds in Acinetobacter baylyi and Marinobacter

hydrocarbonoclasticus (Ishige et al., 2003; Wältermann and Steinbüchel, 2005), or as an anaerobic fermentation product in Euglena gracilis (Müller et al., 2012).

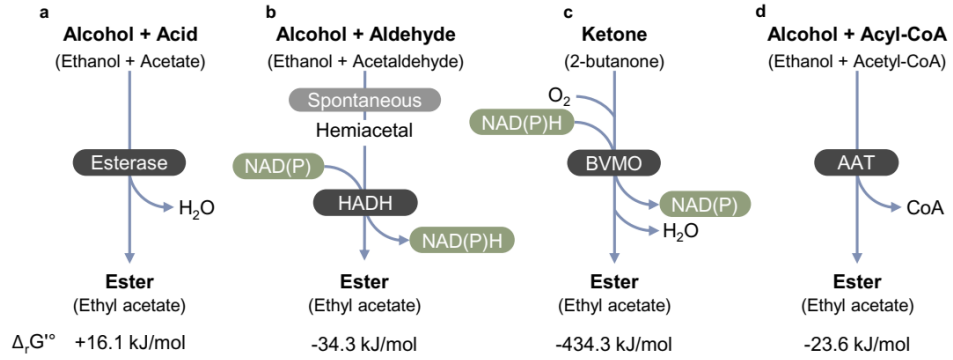

Figure 1. Enzymatic reactions that result in ester production. The ΔrG'° of the reactions were estimated using Equilibrator

3. Metabolic Engineering for Denovo Ester Synthesis

Competing with the petrochemical industry is challenging due to the low market prices of commodity chemicals. Bioconversion of substrates into products must therefore be as efficient as possible (Van Dien, 2013). The amounts of esters naturally produced by microorganisms are generally too low to support cost-competitive biobased processes and considerable metabolic engineering efforts have been invested to enhance this production. The crucial factors in any metabolic engineering strategy are the selection of a suitable catalyst and a sufficient supply of metabolic precursors. Most studies on ester production with microorganisms use sugars, particularly glucose as substrate and are the focus of this review.

Four main enzymatic reactions are available for engineering ester production in vivo (Fig. 2). Esterases and lipases have been applied extensively for ester synthesis via transesterification or reverse esterase activity in nearly non-aqueous environments (Hari Krishna and Karanth, 2002; Kumar et al., 2016; Levisson et al., 2009; Stergiou et al., 2013). However, the reverse esterase reaction is thermodynamically unfavourable in aqueous conditions under which microbial fermentations occur. Metabolic engineering of ester production using HADH has not been reported yet. To produce esters via this route, accumulation of aldehydes would be necessary. This may be challenging due to their toxicity (Kunjapur and Prather, 2015). Furthermore, hemiacetal formation is spontaneous and requires an acid catalyst, which is not present under physiological pH-neutral conditions.

Biotransformation of cyclic ketones into lactones (cyclic esters) by BVMOs have been studied extensively (Leisch et al., 2011). Several BVMOs exist that are also active towards linear ketones, but they are rarely applied in vivo (Ceccoli et al., 2017; Pereira et al., 2018; Rehdorf et al., 2009). Such in vitro processes rely on external supply of expensive cofactors, such as NAD(P)H. Direct synthesis of esters from cheaper substrates like sugars may therefore be more economical. BVMO-catalysed ester production also depends on the supply of ketones, which are not common microbial metabolites.

4. Beyond Esters as Final Products

The various metabolic engineering strategies and increased understanding of microbial ester synthesis should boost developments towards production of biobased esters as high value compounds and bulk chemicals. As esters can be easily hydrolysed to organic acids and alcohols, which are valuable bulk chemicals in their own

right (Chen and Nielsen, 2016), the biobased production of these compounds may profit from enhanced ester production as well, e.g. by decreasing product toxicity or facilitating product removal. In addition, esterification of intermediates improves microbial production of α,ω-diols as they act as a protective group during conversions.

By adding a hydrolysis step, the organic acid and alcohol portion of the produced esters can be readily recovered in a potential bioprocess. The following section highlights a few cases in which alcohol or acid production via an ester intermediate might be beneficial based on their physical properties.

A minimum product titre of 50 g/L is considered acceptable when implementing a biobased process or the downstream processing (DSP) steps would become too cost intense (Van Dien, 2013). Product toxicity often prevents reaching sufficiently high titres during microbial production of chemicals, especially regarding alcohols and acids. The presence of 4 g/L (50 mM) 2-butanol already negatively affected the growth rate of S. cerevisiae, E. coli and B. subtilis, while concentrations of 16 g/L butanol even inhibit growth and continuation of the fermentation in solventogenic Clostridia (Ezeji et al., 2005; Pereira et al., 2016). A similar effect is observed for organic acids such as acetic acid, propionic acid, or butyric acid where growth is completely inhibited at concentrations exceeding 5 g/L, 11 g/L or 6 g/L, respectively (Vázquez et al., 2011). Only for ethanol, fairly tolerant hosts were found, like S. cerevisiae, where ethanol tolerances may exceed 100 g/L ethanol (Casey and Ingledew, 1986; Ghareib et al., 1988).

Most of the industrially relevant alcohols, acids, as well as esters (Fig. 1) are in the toxic range based on their corresponding logPo/w values (Table 4). Nevertheless, microbial production of these esters may be more promising than microbial production of their alcohol or acid precursors. In line with the logPo/w values, C2 to C8 esters were generally less toxic to E. coli than alcohol or acid equivalents (Wilbanks and Trinh, 2017). Moreover, the C-mol-based Heat of Vaporization at standard conditions (Hvap°), the energy needed to evaporate a compound of interest, shows that short and medium chain lengths esters consistently require less energy input than their acid or alcohol counterpart (Table 4). In situ product removal (ISPR) by, for instance gas stripping or phase extraction has proven an efficient way to increase final yields and titres and is better applicable to esters than to alcohols and acids. Introducing a biphasic system using hexadecane, enabled a yield of 80%, reaching a final titre of 17 g/L isobutyl acetate (Rodriguez et al., 2014). With gas stripping, isobutyl acetate yields could be increased from 28.8% without stripping to 42% of the theoretical maximum, and reached 50% of the theoretical maximum yield in an ethyl acetate stripping experiment (Tai et al., 2015; Urit et al., 2011). For ethyl acetate this would correspond to liquid titres twice as high as the predicted critical concentration, for isobutyl acetate the increase is even more than 10-fold (Table 4). Therefore, this approach offers a way to keep up high productivities by avoiding accumulation of inhibitory product concentrations.

5. Challenges and Perspectives

This review provides an overview of microbial ester production on a fundamental level, with special attention towards the potential applications in the bulk chemical arena. There are some major challenges ahead before biobased ester production can move to the industrial level. The key ester producing enzyme AAT is not understood well, and studies that focus on fundamental aspects of its function are scarce. For example, it is still not clear why many AATs display thioesterase and esterase activities, and how to control these activities. In case of Eat1, ethanol is the determining factor that shifts the enzyme from a hydrolase to an AAT (Kruis et al., 2017). The controlling factors in other AATs have not been established yet. It is furthermore unclear how efficiently AATs are expressed and translated in heterologous hosts, which may hinder the development of efficient bioprocesses.

Another major challenge is how to fully employ the vast array of AATs that are available in nature. Metabolic engineering studies have hitherto utilised a relatively limited subset of AATs. However, it has been shown that even homologs of the same AAT can show remarkable differences in their substrate specificities. Mining the unknown AATs may provide us with enzymes that are able to perform conversions that are currently either not possible or inefficient. AATs with narrower substrate specificities than the ones employed currently should also be identified. Catalytic promiscuity can be useful as the same enzyme can form different products. This can simplify the development of ester producing-strains, particularly in laboratory research where many metabolic engineering strategies are typically tested at the same time. However, promiscuity can also be a disadvantage, especially on large scale, where a single fermentation product is desired. Special consideration should also be given to discovering catalytically efficient AATs. In many studies aimed at ester production, the titres achieved were relatively low. The reasons for this are not well understood, but it is likely that some AATs may be catalytically inefficient to allow metabolic flux towards ester synthesis. To enable development of esterproducing processes, efficient AATs should be discovered, or alternatively evolved from known AATs.

The advances in metabolic engineering of ester production are closely linked to the developments in engineering towards production of acyl-CoA (acids) and alcohols. The metabolic diversity of the alcohol and acyl-CoA pathways indicate that a practically limitless number of esters can be designed, assuming an AAT with the appropriate specificity is available. The real challenge of engineering ester production will be to balance the supply of the alcohol and acyl-CoA substrates. Ideally, both substrates will be produced in a 1:1 ratio to ensure efficient conversion of sugars into esters. This is challenging as both substrates are produced in complex and intertwined metabolic pathways.

The commercialisation of ester production will require efficient DSP development. Based on the physical properties of esters, they can be removed by gas stripping, extracted into an organic phase, or may even form their own phase. In comparison to their alcohol or acid precursor ISPR is benefitting from the absence of polar groups, making esters generally easier to remove from aqueous systems. Small, volatile esters can freely diffuse from the cell. However, this is not the case for large, insoluble esters, such as wax esters of FAEE. These esters mostly accumulate in the cell and need to be extracted, adding costs to the DSP.

Finally, the true potential of esters as a platform chemical for alcohol and carboxylic acids should be investigated. From a bioprocess engineering perspective, ester formation could be favourable to alcohol and acid formation due to lower toxicity and easier extractability. Esterification could also facilitate the production of high value compounds such as α,ω-diols and α,ω-dicarboxylic acids and unleash the full potential of biobased ester production.

References

Abeijón Mukdsi, M.C., Medina, R.B., Alvarez, M.D.F., González, S.N. “ster synthesis by lactic acid bacteria isolated from goat’s and ewe’s milk and cheeses.” Food Chem, 7, pp. 241-247, 2009. https://doi.org/10.1016/j.foodchem.2009.03.105.

Abeijón Mukdsi, M.C., Maillard, M.B., Medina, R.B., Thierry, A. “Ethyl butanoate is synthesised both by alcoholysis and esterification by dairy lactobacilli and propionibacteria.” LWT-Food Sci. Technol, 89, pp. 38-43, 2018. https://doi.org/10.1016/j.lwt.2017.10.012.

Aharoni, A., Keizer, L.C., Bouwmeester, H.J., Sun, Z., Alvarez-Huerta, M., Verhoeven, H.A., Blaas, J., van Houwelingen, A.M., De Vos, R.C., van der Voet, H., Jansen, R.C., Guis, M., Mol, J., Davis, R.W., Schena, M., van Tunen, A.J., O’Connell, A.P. “Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays.” Plant Cell, 12, pp. 647-662, 2000.

Akhtar, M.K., Turner, N.J., Jones, P.R. “Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities.” Proc. Natl. Acad. Sci, 110, pp. 87-92, 2013. https://doi.org/10.1073/pnas.1216516110.

Armstrong, D.W., Yamazaki, H. "Effect of iron and EDTA on ethyl acetate accumulation in Candida utilis." Biotechnol, Lett, 6, pp. 819-824, 1984. https://doi.org/10.1007/ Bf00134726.

Armstrong, D.W., Martin, S.M., Yamazaki, H. "Production of ethyl acetate from dilute ethanol solutions by Candida utilis." Biotechnol Bioeng, 26, pp. 1038-1041, 1984. https:// doi.org/10.1002/bit.260260905.

Atsumi, S., Hanai, T., Liao, J.C. "Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels." Nature, 451, pp. 86-89, 2008. https://doi.org/10. 1038/nature06450.

Banella, M.B., Gioia, C., Vannini, M., Colonna, M., Celli, A., Gandini, A. "A sustainable route to a terephthalic acid precursor." ChemSusChem, 9, pp. 942-945, 2016. https:// doi.org/10.1002/cssc.201600166.

Bar, R., Gainer, J.L. "Acid fermentation in water-organic solvent two-liquid phase systems." Biotechnol. Prog, 3, pp. 109-114, 1987. https://doi.org/10.1002/btpr.5420030208.

Barney, B.M. "Metabolic engineering: The sweet smell of biosynthesis." Nat Chem Biol, 10, pp. 246-247, 2014. https://doi.org/10.1038/nchembio.1480.

Becerra, M., Cerdán, M.E., González-Siso, M.I. "Biobutanol from cheese whey.Microb." Cell Fact, 14, 2015. https://doi.org/10.1186/s12934-015-0200-1.

Beekwilder, J., Alvarez-Huerta, M., Neef, E., Verstappen, F.W.A., Bouwmeester, H.J., Aharoni, A. "Functional characterization of enzymes forming volatile esters from strawberry and banana." Plant Physiol, 135, pp. 1865-1878, 2004. https://doi.org/10.1104/pp.104.042580.

Beijerinck, M.W. "Zur Ernährungsphysiologie des Kahmpilzes." Cent. für Bakteriol. und Parasitenkd, 11, pp. 68-76, 1892.

Beld, J., Lee, D.J., Burkart, M.D. "Fatty acid biosynthesis revisited: Structure elucidation and metabolic engineering." Mol. Biosyst, 11, pp. 38-59, 2015. https://doi.org/10.1039/c4mb00443d.

Copyrights