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OPINION

Pseudomonas putida as a platform for medium-chain length α,ω-diol production: Opportunities and challenges

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Abstract

Medium-chain-length α,ω-diols (mcl-diols) play an important role in polymer production, traditionally depending on energy-intensive chemical processes. Microbial cell factories offer an alternative, but conventional strains like *Escherichia coli* and *Saccharomyces cerevisiae* face challenges in mcl-diol production due to the toxicity of intermediates such as alcohols and acids. Metabolic engineering and synthetic biology enable the engineering of non-model strains for such purposes with *P. putida* emerging as a promising microbial platform. This study reviews the advancement in diol production using *P. putida* and proposes a four-module approach for the sustainable production of diols. Despite progress, challenges persist, and this study discusses current obstacles and future opportunities for leveraging *P. putida* as a microbial cell factory for mcl-diol production. Furthermore, this study highlights the potential of using *P. putida* as an efficient chassis for diol synthesis.

INTRODUCTION

Biomanufacturing contributes to the transition from a fossil fuel-based economy to a sustainable and circular one. This innovative approach harnesses the metabolic capabilities of microorganisms, cells, and enzymes to successfully synthesize many valuable products, such as biofuels, bioplastics, pharmaceuticals, food and beverages, and other specialty chemicals (Garces Daza et al., 2023; Lu et al., 2017; Orsi, Beekwilder, et al., 2021; Werner et al., 2021; Zhang et al., 2017, 2019; Zhao et al., 2019). Biomanufacturing has shown its potential to address the crises humankind faces including climate change, population growth, food security, and plastic accumulation. However, the current product portfolio derived from renewable sources remains narrow. For example, medium-chain-length aliphatic α,ω-diols (mcl-diols, C_5-C_{12}) are important building blocks for polymer production and used as surfactants, lubricants, and cosmetics ingredients (Figure 1) (Bramson et al., 2020; Erle et al., 2012; Gross et al., 2010; Kluge et al., 2020; Llevot et al., 2015; Youngquist et al., 2013). Their production heavily relies on chemical processes that require intensive energy and expensive catalysts, while leading to greenhouse gas emissions. Meanwhile, the need for mcl-diols is growing at a rate of 8% per year. The market volume of 1,6-hexanediol, the most commonly used monomer, is expected to reach $1401 million by 2025 (Kim et al., 2021). While microbial synthesis of short-chain diols (scl-diols) like 1,4-butanediol has seen significant success in engineered *E. coli* strains with well-established and concise biosynthetic pathways (Burgard et al., 2016), progress in mcl-diols production has been relatively constrained. The synthetic pathway of scl-diols is rooted in intermediates of the tricarboxylic acid cycle, a well-studied process. On the other hand, for mcl-diols, the core synthetic module involves either the fatty acid synthetic pathway or

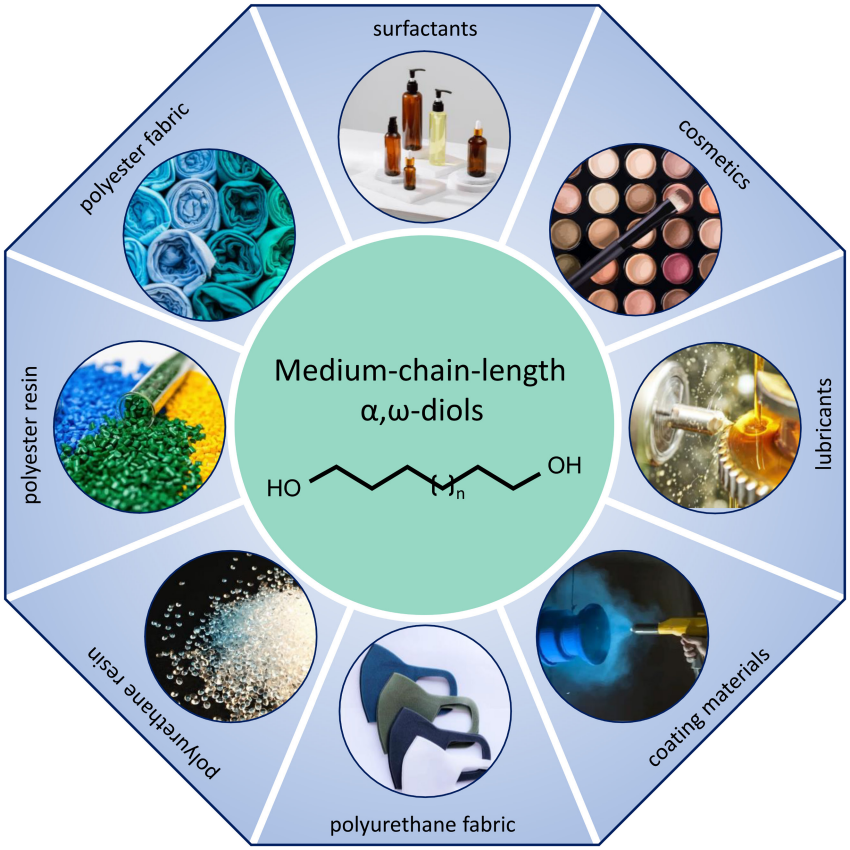

FIGURE 1 Applications of medium-chain-length aliphatic α,ω-diols.

the reversed beta-oxidation pathway. Both pathways for mcl-diol production encounter challenges including insufficient availability of medium-chain precursors and inefficiencies in microbial cell factories.

The model microorganisms such as *E. coli* and *Saccharomyces cerevisiae* are commonly used chassis for industrial bioproduction. However popular, they are not always the ideal chassis. Medium-chain fatty acids and alcohols that are the important intermediates of mcl-diols synthesis were produced from diverse feedstocks via either a fatty acid synthesis pathway or reversed beta-oxidation pathway. Due to their toxicity, low product titres were usually reported in model microorganisms. Non-model microorganisms, therefore, need to be explored and investigated for such applications. Furthermore, the use of cheap feedstocks would make the process more economically feasible, given the low price of diols and their inefficient biosynthesis. Considering the properties of mcl-diols and their precursors, and the features of the synthetic pathway, an ideal chassis should possess (1) tolerance to high concentrations of organic solvents, alcohols, and acids; (2) robust flux of fatty acid synthesis; (3) available genome sequence and genetic toolboxes; (4) flexible metabolism, utilizing cheap raw materials; (5) metabolic models. Metabolic engineering and synthetic biology have emerged as enabling technologies for the microbial synthesis of numerous desired compounds from sustainable feedstocks. Using these techniques, various non-model strains are constructed and subsequently optimized through the iterative 'Design-Build-Test-Learn' (DBTL) cycle to improve titres (g/L), rates (g/L/h), and yields ($g_{product}/g_{substrate}$) (Nielsen & Keasling, 2016; Orsi, Claassens, et al., 2021).

Pseudomonas putida KT2440 as a non-model strain has attracted more and more interest in academia and industry. This gram-negative soil bacterium is viewed as a promising microbial cell factory owing to its high metabolic versatility, low maintenance demands, tolerance to harsh conditions (including high concentrations of organic solvents and oxidative stress), efficient genetic toolbox, available genome-scale metabolic model, and excellent capacity to produce fatty acid-based storage materials, such as polyhydroxyalkanoate (PHA) and rhamnolipids (Batianis et al., 2020; Belda et al., 2016; Volke et al., 2020; Wirth et al., 2019). The commercial production of PHA using engineered *P. putida* has proven its robust flux channelling substrates via fatty acid synthesis or reversed beta-oxidations pathway (Bluepha® and PhaBuilder®, China). In the past decade, an increasing number of tools have been developed for genome modification and gene regulation in *P. putida* (Martin-Pascual et al., 2021). Overall, the biosynthesis of mcl-diols in *P. putida* is seen as a viable alternative.

In this study, we summarize the characteristics of *P. putida* as a non-model microbial cell factory

and the progress of mcl-diol production in *P. putida*. Furthermore, we point out the opportunities and challenges of using *P. putida* as a platform for diol production. Finally, we proposed future prospects that can further improve its performance.

PSEUDOMONAS PUTIDA AS A RISING MICROBIAL CELL FACTORY

PHA and rhamnolipid synthesis

The polyhydroxyalkanoate (PHA) synthesis in *P. putida* exemplifies its capability for industrial-scale production (Van Der Walle et al., 1999). Two metabolic pathways provide 3-hydroxy acyl precursors for PHA synthesis. One is for PHA-unrelated substrates (e.g. glucose, gluconate, and ethanol) via the *de novo* fatty acid pathways (Madison & Huisman, 1999). The resulting acyl-ACP intermediates are converted into (*R*)-3-hydroxy acyl-CoA by the specific transacylase PhaG (Hoffmann et al., 2000). The other is for aliphatic substrates such as fatty acids through the beta-oxidation pathway. The 3-hydroxy acyl precursors are then converted into PHA (Mozejko-Ciesielska et al., 2019). So far, PHAs have been successfully produced from glucose, glycerol, acetate, fatty acids, plant oils, benzoate, p-coumaric acid, vanillic acid, and ferulic acid. Up to 90% of the cell dry weight of PHA was produced in *P. putida* (Yadav et al., 2021), suggesting its robust flux and potential for the production of other products with the common precursors.

Rhamnolipids, known as versatile biosurfactants, have been used in a wide range of industrial applications in past decades (Jiang et al., 2022; Varjani et al., 2021; Zhao et al., 2021). Naturally, they are synthesized by the opportunistic pathogen *Pseudomonas aeruginosa* and certain *Burkholderia* species (Soberón-Chávez et al., 2021). *P. putida* was reported as a natural producer of rhamnolipids too. Due to its robust fatty acid synthesis flux, the introduction of a plasmid encoding the *rhlAB* operon from *P. aeruginosa* PAO1 significantly improved its ability to produce rhamnolipids. Rhamnosyltransferase 1, composed of RhlA and RhlB, is responsible for the synthesis of mono-rhamnolipid using dTDP-L-rhamnose and the fatty acid dimers–3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) as substrates. RhlC is another rhamnosyltransferase involved in di-rhamnolipid biosynthesis. Engineered *P. putida* strains now serve as efficient heterologous hosts and exhibit relatively high yields in rhamnolipids production (Beuker et al., 2016). Evonik is successfully producing rhamnolipids with a genetically modified *P. putida* on an industrial scale (Bettenhausen, 2022; Kumar et al., 2023).

Versatile metabolism

P. putida KT2440 is known for its versatile metabolism. The metabolic versatility of *P. putida* is reflected by its ability to utilize a wide range of substrates (Nikel & de Lorenzo, 2018) and thrive under various environmental conditions (Martins Dos Santos et al., 2004). It can metabolize substances including glucose, acetate, succinate, fatty acids, fatty alcohols, polyols, and aromatic compounds such as p-coumaric acid. Its central metabolism is mainly composed of a robust Entner-Doudoroff pathway combined with a gluconeogenic partial Embden-Meyerhof-Parnass pathway (Elmore et al., 2020). This enables a rapid regeneration of NADPH, thereby endowing *P. putida* with an advantage for NADPH-dependent conversions. Notably, *P. putida* also holds the potential to be a suitable strain for the efficient production of chemicals that require NADH (Ebert et al., 2011). When equipped with the appropriate plasmids, toluene (Nakazawa & Yokota, 1973), naphthalene (Dunn & Gunsalus, 1973), 4-chloronitrobenzene (Zhen et al., 2006), 2,4-xylenol (Dean et al., 1989), and phenol (Kurbatov et al., 2006) can be assimilated. *Pseudomonas* spp. has been found to degrade plastics such as polyethylene (Balasubramanian et al., 2010; Gyung Yoon et al., 2012), polypropylene (Arkatkar et al., 2010), and polyvinyl chloride (Giacomucci et al., 2019). Moreover, *P. putida* is recently engineered to use formate and methanol for cell growth (Bruinsma et al., 2023; Turlin et al., 2022). The aforementioned traits and pathways enable the wider application of *P. putida* in bioremediation and industrial biotechnology, such as plant biomass valorization and plastic upcycling (Mohamed et al., 2020; Sullivan et al., 2022).

Carbon catabolite repression

Carbon catabolite repression (CCR) enables *P. putida* to prioritize the use of energetically efficient carbon sources. This strategy helps the bacterium outcompete other species in its niche, providing a competitive advantage in environments with mixed and fluctuating nutrient reserves. *P. putida* exhibits a distinct preference for carbon assimilation in the following order: organic acids and specific amino acids (Pro, Ala, Glu, Gln, His); sugars such as glucose and fructose; other amino acids (Arg, Lys, Asp, Asn); benzoate; and hydrocarbons (Rojo, 2010). To execute this preference in *Pseudomonas*, three principal mechanisms involved in CCR are (i) Crc global regulator (integrating Crc and Hfq, the two-component system CbrA/B, and the sRNAs CrcZ and CrcY), (ii) cytochrome o ubiquinol oxidase (Cyo), and (iii) phosphotransferase system (PTS). The latter two mechanisms are not well-understood yet (Rojo, 2010). Cyo is a terminal oxidase that is present during growth in the presence of sufficient oxygen (Nikel et al., 2014). The PTS^{Ntr} is not involved in sugar uptake but is involved in signal transduction. The Crc/Hfq complex is the main mediator of CCR. It is a post-transcriptional

regulator of many (>130) target genes (Bharwad & Rajkumar, 2019; Moreno et al., 2009; Rojo, 2010). The function of Crc requires the accessory protein Hfq that recognizes an A rich-motif (AANAANAA, also known as attachment site) near the start codon of the target mRNAs. This interaction then determines the initiation of translation (Moreno et al., 2009). The binding site for the Hfq protein is variable in length and position relative to the start codon. Depending on the target mRNAs, it can be upstream of the Shine-Dalgarno (SD) sequence, overlapping with the ribosomal binding site (RBS), or even after the start codon (such as in AlkS) (Hernández-Arranz et al., 2013; Lu, Ramalho, et al., 2023; Moreno et al., 2012). The Crc/Hfq activity in *P. putida* is controlled by two sRNAs, CrcZ, and CrcY. In the presence of a preferred carbon source, CrcZ and CrcY levels remain low, allowing a high concentration of Crc/Hfq. The complex can bind to the mRNAs of non-preferred substrate-assimilating genes, thereby inhibiting their translation. Upon the preferred substrate being depleted, sRNA levels increase, releasing mRNAs bound to the Crc/Hfq complex. This, in turn, enables the utilization of the non-preferred carbon source. Although the CCR conferred an advantage to *P. putida* in the environment, it prevents it from being an efficient microbial cell factory for biological applications. For instance, Crc/Hfq limits the alkane monooxygenase system (AlkBGT) from being efficiently expressed, downregulates the conversion of aromatic lignin monomers to cis,cis-muconic acid, and hinders major components of lignocellulosic biomass from being co-utilized (Bentley et al., 2020; Elmore et al., 2020; Lu et al., 2021). Fortunately, these hurdles have been eliminated by different approaches such as deletion of *crc* and manipulation of attachment sites.

MODULAR SYNTHESIS OF MCL-DIOL IN *PSEUDOMONAS PUTIDA*

The mcl-diol synthesis pathway has not been found in nature. To achieve sustainable diol production in *P. putida*, its synthetic pathway needs to be constructed. A modular approach would facilitate such a long synthetic pathway. According to the reported medium-chain fatty acid and esterified diol production (Deng et al., 2020; Feng et al., 2018; Hernández Lozada et al., 2020; van Nuland et al., 2017), we propose a four-module diol synthesis pathway, consisting of (a) mcl-fatty acid supply module from glucose or other substrates, (b) fatty acid reduction module, (c) mono-(di-)ester synthesis module, and (d) diester hydrolysis module (Figure 2). Module a is responsible for the production of fatty acids. This can also be achieved through the reverse β-oxidation pathway. However, it was usually less efficient for the medium-chain molecules. The formed fatty acids are reduced by Module b into the corresponding alcohols, which are esterified and ω-oxidized into diesters by Module c, otherwise, alcohols are over-oxidized into acids. Module d releases diol and the by-product acetate from the diester. Each module has been separately reported and tested in engineered *P. putida* KT2440 strains (Lu, Akwafo, et al., 2023; Lu et al., 2021, 2022; McNaught et al., 2023; Valencia et al., 2022).

Fatty acid supply module

Fatty acids are the appropriate intermediates to achieve sustainable diol production using renewable feedstocks. Bioproduction of fatty acids and their derivates such as biodiesel has been intensively investigated in many microbial cell factories in the last decades (Hernández Lozada et al., 2018; Hu et al., 2020; Rutter et al., 2015). Two main pathways are commonly used, fatty acid synthesis (FAS) and reversed beta-oxidation (rBOX). Hitherto, the FAS is used in *P. putida* for chain-tailored fatty acid production. Blocking the beta-oxidation pathway and deleting CoA ligase genes (*PP_0763*, *PP_4549*, and *PP_4550*) completely impaired its ability to catabolize fatty acids. Overexpression of the optimized acyl-ACP thioesterase I ('TesA) in *P. putida* produced 670.9 mg/L total free fatty acids and 253.6 mg/L octanoic acid (Valencia et al., 2022). Tailor-designed thioesterases are the critical chain control step in medium-chain fatty acids synthesis. Other engineered thioesterases such as CpFatB1 have been reported in other microbial cell factories (Hernández Lozada et al., 2020; Krishnan et al., 2020; Südfeld et al., 2022).

Fatty acid reduction module

Fatty alcohols can be either produced from the reduction of fatty aldehydes and acids or directly reduced from acyl-CoA and acyl-ACP. However, the latter option is rather inefficient because of the low activity and narrow substrate acceptance range of acyl-CoA reductase and acyl-ACP reductase (Krishnan et al., 2020). Advancements in protein engineering have led to the development of the new variant MmCAR (RF1 + 303) by narrowing the substrate binding pocket of carboxylic acid reductase (CAR). This variant can efficiently reduce medium-chain fatty acids into the corresponding alcohols. Overexpression of MmCAR in engineered *S. cerevisiae* produced 252 mg/L of medium-chain fatty alcohols from glucose (Hu et al., 2020). Nevertheless, a similar test in *P. putida* KT2440 was incapable of accumulating fatty alcohols

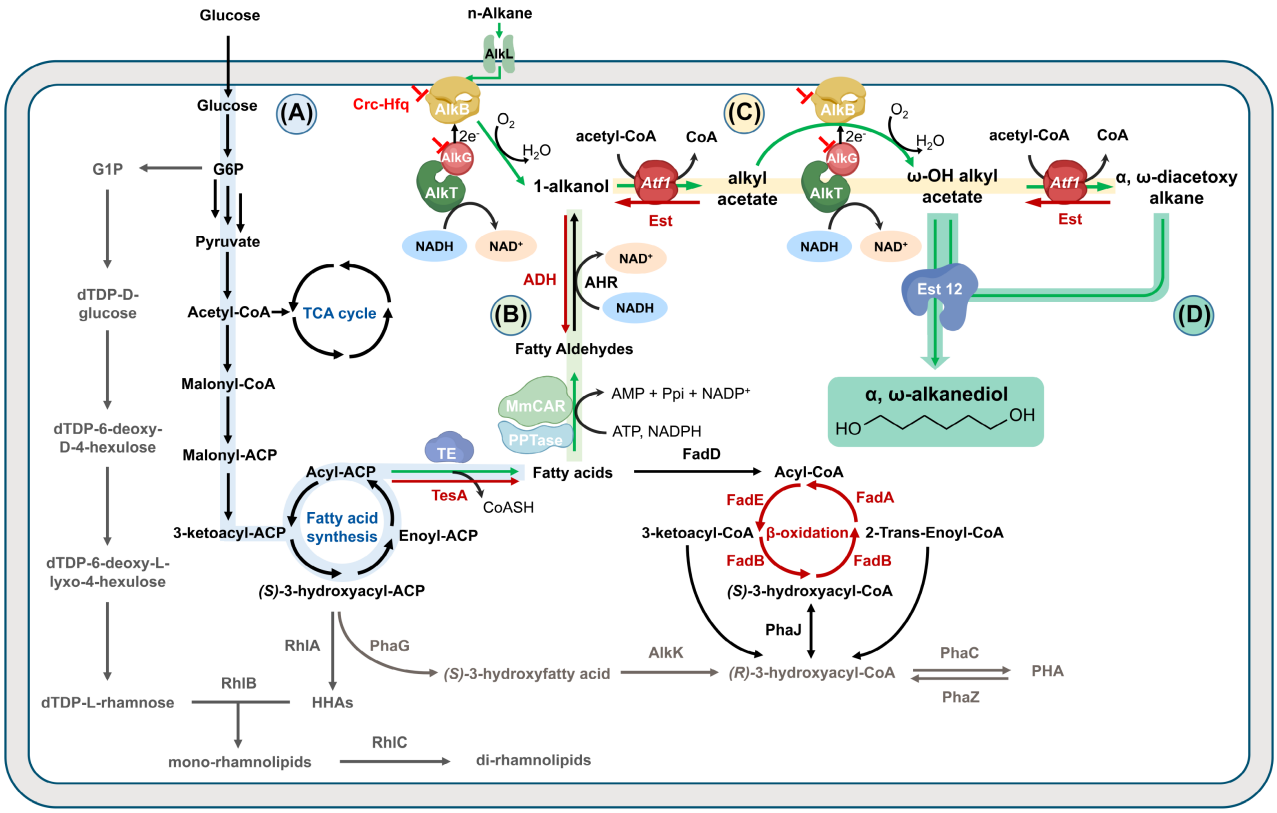

FIGURE 2 The proposed four-module mcl-diol synthesis pathway in *P. putida* KT2440. Genes deleted are represented in red arrow while genes overexpressed are in green arrow. Black arrows present the native genes of *P putida*. Grey arrows showcase the robust PHA and rhamnolipids synthesis pathways. (A) Module a: mcl-fatty acid supply module from glucose; (B) Module b: fatty acid reduction module; (C) Module c: monoester and diester synthesis module, and (D) Module d: diester hydrolysis module. ADH, alcohol dehydrogenase; AHR, aldehyde reductase; AlkB, alkane monooxygenase; AlkG, rubredoxin; AlkK, acyl-CoA-synthase; AlkL, outer membrane protein; AlkT, rubredoxin reductase; Atf1, alcohol acetyltransferase; Crc, catabolite repression control protein; Est, esterase; MmCAR, carboxylic acid reductase from *Mycobacterium marinum*; FadA, 3-ketoacyl-CoA thiolase; FadB, enoyl-CoA hydratase/3-hy-droxyacyl-CoA dehydrogenase; FadD, long-chain fatty acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; G1P, D-glucose-1-phosphate; G6P, D-glucose-6-phosphate; HAAs, 3-(3-hydroxyalkanoyloxy)alkanoic acids; Hfq, RNA-binding protein; PHA, polyhydroxyalkanoate; PhaC, PHA polymerase; PhaG, 3-hydroxy acyl-ACP acyl-transferase; PhaJ, (*R*)-specific enoyl-CoA hydratase; PhaZ, PHA depolymerase; PPTase, phosphopantetheinyl transferase; RhlABC, rhamnosyltransferases; TE, thioesterase; TesA, Acyl-CoA thioesterase I.

(Lu, Akwafo, et al., 2023). This is partly because of the presence of various alcohol dehydrogenases. By deleting *pedF* (*PP_2675*) and *adhP* (*PP_3839*) and coexpressing of Atf1, alkyl acetate was successfully detected. Coexpression of the fatty acid transporter ScTP further increased hexanol and hexyl acetate production in *P. putida*.

Mono- and di-ester synthesis module

Without the esterification step, alcohols will be over-oxidized to aldehydes and acids. This has been reported in different microbial cell factories (Lu et al., 2021; van Nuland et al., 2016). The alcohol acetyltransferase Atf1 is commonly used to esterify medium-chain fatty alcohols because acetyl-CoA acts as co-substrates and is abundant in cells. Coexpression of alkane monooxygenase system AlkBGTL and Atf1 converted monoesters (alkyl acetate) to diesters (α,ω-diacetoxyalkane) (van Nuland et al., 2017). Total production of 6.9 mM diacetoxyhexane was produced from 1% v/v hexyl acetate in *P. putida* (Lu et al., 2021). This is lower than that in *E. coli*. CCR negatively regulates AlkBGT expression in *P. putida* because glucose, a preferred substrate, was fed to supply acetyl-CoA. Within the AlkBGT monooxygenase system, the mRNAs of AlkB and AlkG contain the recognition sequence of the Crc/Hfq mechanism. The removal of attachment sites of AlkB and AlkG increased their performance (Lu et al., 2021). Thanks to the broad substrate specificity of AlkBGT and Atf1, this method is applicable to all medium-chain substrates. In addition, the formation of esters also promotes the ω-oxidation of alcohols and acids, because AlkBGT exhibited higher activity on medium-chain fatty acid alkyl esters compared with fatty acids (van Nuland et al., 2016).

Diester hydrolysis module

Although the esterification step has multiple advantages, this costs extra substrates to supply acetyl-CoA. It significantly reduces the yield of diols, especially shorter chain diols. Hence, recovery or reuse of acetate would be beneficial. For this, the produced diester needs to be hydrolysed to release diol and acetate. Various esterases have been reported to hydrolyse alkyl esters in different microbes (Hong et al., 2012; Millar et al., 2017). In particular, *P. putida* possesses multiple potent esterases, such as Est12, EstC, and EstZ (Lu et al., 2021). They can efficiently hydrolyse alkyl acetate and diacetoxyhexane. Furthermore, *P. putida* has been shown to preferentially utilize acetate over glucose, fatty alcohols, and diols for cell growth and maintenance (Lu et al., 2021, 2022; Rojo, 2010), demonstrating its capability and potential to reuse the by-product acetate. In the case of the conversion from glucose ($C_6H_{12}O_6$) to diacetoxyhexane ($C_{10}H_{18}O_4$), the maximum theoretical yield is 0.48 mol/mol, calculated based on the degree of reduction. Similarly, the value of the conversion from glucose ($C_6H_{12}O_6$) to hexanediol ($C_6H_{14}O_2$) is 0.71 mol/mol. By reusing acetate, the maximum theoretical yield would be increased by 47%.

MCL-DIOLS PRODUCTION IN *PSEUDOMONAS PUTIDA*: OPPORTUNITIES AND CHALLENGES

Theoretically, assembling the aforementioned four mcl-diol synthesis modules will generate diol from substrates such as glucose. However, this has not been reported yet. It is challenging to build up such a long pathway including eight heterologous enzymes, especially considering the low conversion efficiency of each module. On the other hand, developing non-model chassis is expected to face issues such as unrevealed genes and regulations, and inefficient genome modifications (Lammens et al., 2020).

Opportunities

Despite the difficulties of using non-model strain *P. putida* as a microbial cell factory and the poor activity of diol synthetic enzymes, most of them have been solved or mitigated with the rapid advances in genome editing tools, protein engineering, and gene annotation. For instance, I-SceI-mediated recombination and self-curing plasmids enable rapid gene deletion (Volke et al., 2020; Wirth et al., 2019). In addition, the tailor-optimized carboxylic acid reductase (MmCAR) and thioesterases ('$TesA^{RD-2}$ and CpFatB1*) specifically for medium-chain fatty acids facilitate the biosynthesis of mcl-diols (Deng et al., 2020; Hernández Lozada et al., 2020; Hu et al., 2020). Furthermore, the annotation of alcohol dehydrogenases (AdhP), cytochrome c oxidase (PedF), and short-chain fatty acid transporter (ScTP) removed the hurdles of diol production in *P. putida* (Lu, Akwafo, et al., 2023).

The versatile metabolism also brings new potential opportunities and applications. The potent esterase Est12 has been successfully used to hydrolyse a range of acetate esters (Lu et al., 2021). It is speculated that its signal peptide is what makes it stand out and could be used to further enhance the activity of other enzymes or extracellularly expressed proteins. A similar strategy has been used in the optimization of other enzymes (Cho et al., 2018; Tozakidis et al., 2020; Yoo et al., 2019). Other identified six esterases such as EstC and EstZ can be applied in ester hydrolysis. The activity of PPTase and ScTP was confirmed for the first time (Lu, Akwafo, et al., 2023). The activity of MmCAR with the assistance of EcPPTase and PpPPTase is comparable, showcasing its ability as an alternative enzyme used for acid reduction. ScTP is a putative short-chain fatty acid transporter. Its function was verified by promoting fatty acid uptake. Theoretically, ScTP can be further applied for the transport of alcohols and fatty acid methyl esters, like AlkL and FadL (He et al., 2019; Tan et al., 2017; van Nuland et al., 2016).

It has been widely reported that the major carbon catabolite repression mechanism, Crc-Hfq, enables *P. putida* to utilize substrates in the default hierarchy (Rojo, 2010). The regulated mRNAs are recognized and bound via attachment sites by Crc-Hfq. The structure and location of an attachment site have a significant influence on the translation of the mRNAs (Moreno et al., 2009). Direct modification of attachment sites is expected to deliver different expression levels. Besides, a scientifically intriguing question is whether this Crc-Hfq mechanism can serve as a general toolbox controlling gene expression. The successful repression of AlkBGT in *E. coli* by transplanting Crc revealed the practicability of this method (Lu, Ramalho, et al., 2023). Although the off-target effect and Hfq sequestration are needed to be handled carefully, the Crc protein together with Hfq provides us with a novel multiplex regulation of post-transcriptional gene expression. Due to its faster response compared with the other known gene regulation tools that act at the transcriptional level, it holds promise for further tool development.

Computational approaches, including genome-scale metabolic models, have become integral in guiding metabolic engineering and strain improvements (Chung et al., 2010). These models are widely used to characterize cellular metabolism and improve the production of various chemicals and biopharmaceuticals. For *P. putida*, multiple genome-scale metabolic models such as iJN746, PpuQY1140, and iJN1411 have been

established (Nogales et al., 2008; Tokic et al., 2020). Notably, iJN1411 contains 2057 metabolites, 2581 reactions, and 1411 genes. It has been further developed into a kinetic metabolic model that accounts for dynamic metabolic responses to changes in cellular and process parameters and incorporates regulation at the enzymatic and post-translational level. Utilizing such models makes it possible to evaluate the efficiency and capability of each module in mcl-diol production, predict potential targets for genetic manipulation, and refine the model based on experimental data. Moreover, this iterative might help to achieve the optimal combination of the four modules. Once the engineered *P. putida* strain can produce mcl-diol, approaches such as growth-coupled overproduction and adaptive laboratory evolution (ALE) could help to further enhance mcl-diol production. The concept of growth-coupled product synthesis is to make the desired product a mandatory by-product, thereby using the growth of organism as a driving force for production. It has been used for a variety of compounds, including 1,4-butanediol, 2,3-butanediol, and fatty acids (Ranganathan et al., 2012; Von Kamp & Klamt, 2017). ALE helps to rapidly improve the fitness, growth, and tolerance of production strains to certain harsh conditions (Sandberg et al., 2019).

Challenges

The versatile metabolism and regulation mechanism of *P. putida* is not always beneficial to mcl-diol production. The metabolic versatility, largely mediated by the Crc-Hfq regulatory mechanism and demonstrated through the presence of esterases, alcohol dehydrogenases (ADH), and thioesterases (TE), can be troublesome. The repression imposed by Crc-Hfq on AlkBGT prevented the efficient hydroxylation of n-hexane and ω-oxidation of hexyl acetate in the presence of both n-hexane and glucose (Lu et al., 2021). Another example is the presence of alcohol dehydrogenases. *P. putida* can utilize butanol or octanol as the sole carbon source for cell growth due to its many ADHs (Cuenca et al., 2016; Rühl et al., 2009; Simon et al., 2015; Wehrmann et al., 2020), thereby leading to very low alcohol yields.

There may be unrevealed competitive genes or regulations of diol production in *P. putida*. The alcohol acetyltransferase (Atf1) was used to protect the terminal hydroxyl group of alcohol. 30 mM hexyl acetate was produced when 1-hexanol (80 mM) and glucose (55.4 mM) were fed as substrates, indicating esterification is not the limiting step, nor is the supply of acetyl-CoA. However, only 3.7 mM hexyl acetate was produced when n-hexane was fed as substrate, suggesting that the terminal oxidation step by AlkBGT is the bottleneck. Moreover, although hexyl acetate was successfully produced, 1,6-diacetoxyhexane was not found. It is still unclear what hampers it. In *P. putida*, when multiple substrates (in this case, glucose, n-hexane, hexanol, and hexyl acetate) are available, unrevealed regulations or genes might be activated to prevent further oxidation. Besides, the introduction of carboxylic acid reductase (MmCAR) did not lead to the excretion of 1-hexanol. However, the coexpression of alcohol acetyltransferase (Atf1) resulted in the production of hexyl acetate, suggesting that 1-hexanol was produced but not excreted. Similarly, the strain *P. putida* G38 is incapable of growing on C_5-C_{10} alcohols. Nonetheless, the strain G23 derived from G38 by knocking out *pedF* (*PP_2675)* restores its ability to utilize C_9 and C_{10} alcohols (Lu, Akwafo, et al., 2023). It remains unclear how this was triggered. It has been reported that *P. putida* usually has multiple backup genes (such as esterases, alcohol dehydrogenases, thioesterases, etc.) for a specific function (Lu, Akwafo, et al., 2023; Lu et al., 2021; Ma et al., 2021). While this confers a versatile metabolism, it simultaneously complicates the process of strain engineering.

Low activity and a broad substrate specificity pose additional challenges to diol production, particularly for enzymes like TE and CAR. Although 'TesARD-2 and CpFatB1* are tailor-designed thioesterases for the formation of the C_8 molecule (Deng et al., 2020; Hernández Lozada et al., 2020; Krishnan et al., 2020; Liu et al., 2014), there are no thioesterases specifically available for other carbon-chain-length compounds. Given the comparable properties of medium-chain molecules, the development and optimization of these enzymes will streamline the production of specific fatty acids, simplifying downstream processing. Concerning CARs, MmCAR, one of the most studied CARs, has been optimized for C_8 fatty acid production through protein engineering approaches, including directed evolution, structure-guided semi-rational design, and rational design (Hu et al., 2020). However, the optimal MmCAR still suffers from low activity. Additionally, the toxicity of medium-chain fatty acids and alcohols is another threat, as reported in various microbial cell factories (Wu et al., 2019).

Finally, optimizing the combination of ester formation and diester hydrolysis is crucial. While *P. putida* utilizes several esterases for mcl-diols release from the corresponding esters, direct coexpression of AlkBGTL-Atf1 and Est12 has proven inefficient (Lu et al., 2022). Efficient 1,6-hexanediol production was achieved by separating diester synthesis and hydrolysis steps, albeit with increased operational costs. The ideal one-step approach is challenging due to the conflicting reactions by Atf1 and Est. Considering the entire mcl-diol synthetic pathway, proper expression of 10 enzymes (TE, MmCAR, PPTase, Atf1, AlkB, AlkG, AlkT, AlkL, ScTP, and Est12) is necessary. Depending on substrates, transporters AlkL and ScTP might be disregarded.

However, such protein expression poses a substantial proteome burden, potentially limiting availability for other processes (Nielsen, 2019). Harmonizing expression levels of these enzymes in a single host could alleviate the proteome burden.

FUTURE PERSPECTIVE

To further render *P. putida* into a more efficient and effective chassis for mcl-diols production, multiple DBTL cycles are required to refine its performance. The primary focus of future investigations should be on the synthetic modules, which serve as the main workhorses for mcl-diol synthesis.

Increasing the supply of medium-chain molecules

The production of mcl-diols from starting materials like glucose depends on the availability of medium-chain molecules. This is by far the rate-limiting step. Only 'TesA was evaluated in *P. putida*. Other tailor-optimized thioesterases such as 'TesA^{RD-2} and CpFatB1* have been used to produce medium-chain fatty acids in other microbes such as *E. coli* and microalgae (Cho et al., 2020; Hernández Lozada et al., 2020; Lennen & Pfleger, 2013; Südfeld et al., 2022). Similarly, these thioesterases can be used to further improve the fatty acid supply in *P. putida*. In addition, taking advantage of the robust PHA synthesis pathway should be considered. First, triggering and maximizing the robust flux of PHA should improve the availability of precursors (mcl acyl-ACP) for mcl-diol biosynthesis. Previous studies have been focused on nutrient limitation (e.g. N, P, and S), reinforcing reducing equivalent (NADH or NADPH) supply, morphological engineering to enlarge the cell size, removing competing pathways, and using different cheap substrates such as glycerol and lignin-derived aromatic compounds (Xu et al., 2021). The aforementioned strategies should be investigated to increase the production of mcl-fatty acids in *P. putida*. Second, PHA synthesis needs to be prevented to keep its precursors to be channelled into medium-chain fatty acids. The knockout of *phaG* can reduce 60% of PHA formation from glucose (Hoffmann et al., 2000). To fully block PHA production, the deletion of two medium-chain PHA synthases-PhaC1 (PP_5003) and PhaC2 (PP_5005) would be sufficient. Besides, to avoid the complex regulation network, the deletion of the whole *pha* cluster may be considered. (Mezzina et al., 2021; Poblete-Castro et al., 2012). Concerning trans-enoyl-CoA hydratases (PhaJs), deleting PhaJ4 (PP_4817) is probably sufficient because it was reported to be the primary one in *P. putida* KT2440 (Liu et al., 2023).

Accelerating the conversion from fatty acids to diesters

Increasing the conversion rate of acids to alcohols and monoesters is therefore desirable. Overexpression of rate-limited enzymes is often used to increase the flux from accumulated intermediates to the desired products (Huang et al., 2021; Michener et al., 2012). When MmCAR, PPTase, Atf1, and ScTP were overexpressed, 1-hexanol however accumulated and was not fully esterified (Lu, Akwafo, et al., 2023). In this scenario, these enzymes were equally overexpressed. Fine-tuning the expression ratio of Atf1 and other enzymes using different RBS, copy numbers, and promotors is a viable solution. Besides, ensuring a sufficient supply of acetyl-CoA is another factor in minimizing the accumulation of alcohols. This has been improved by tuning the expression level of *aceEF* coding for encoding the E1 and E2 PDH (pyruvate dehydrogenase) subunits (Batianis et al., 2023). Given that *P. putida* predominantly relies on the Entner-Doudoroff pathway (ED pathway), known for its higher NADPH regeneration rate (Ng et al., 2015), this becomes advantageous in conversion processes requiring ATP and NADPH. For example, the production of shikimate pathway-dependent chemicals has been reported to benefit from the higher NADPH flux in *P. putida* because the conversion of 3-dehydroshikimate to shikimate requires NADPH as a cofactor (Yu et al., 2016). Converting fatty acids to fatty aldehydes costs ATP and NADH too. The conversion rate of hexanoic acid to hexanol and hexyl acetate is 63% (Lu, Akwafo, et al., 2023), indicating that this step is rate-limiting. Therefore, increasing or saving ATP and NADPH should be further explored. A practical and easy way is to use the engineered strain *P. putida* EM42, derived from *P. putida* KT2440 by deleting 300 genes (~4.3% of the total genome) and demonstrating increased net ATP and NADPH availability (Martínez-García et al., 2014; Martin-Pascual et al., 2021).

Once the conversion of fatty acids to monoesters is boosted, enhancing ω-oxidation needs to be taken into consideration. For this step, the AlkBGT system is the main workhorse at the cost of NADH. Increasing the *Kcat* of AlkB is encouraged, especially towards medium-chain compounds. AlkB has been evolved for higher activity on shorter chain ($<C_6$) n-alkanes (Koch et al., 2009). The rapid advances in protein engineering, protein structure prediction tools, and high-throughput screening will offer new opportunities and shorten time costs for the evolution of AlkB. Furthermore, improvement of electron transfer efficiency among AlkB, AlkG, and AlkT holds promise for efficient conversion. Inefficient electron transfer is detrimental to the oxidative activity of cytochrome P450 and its reductase (Nazor et al., 2008). Constructing a self-sufficient P450 reduction enzyme by fusing cytochrome P450 to the reductase domain

was successfully used to overcome this bottleneck (Sadeghi & Gilardi, 2013; Zhao et al., 2016). Similarly, fusing the monooxygenase AlkB to reductases AlkG and AlkT could be a potential way to increase the catalytic activity of the AlkBGT system. Apart from that, a balanced allocation of reducing equivalents between NADH and NADPH needs to be considered, because CAR requires NADPH while AlkBGT needs NADH. Otherwise, turning the cofactor dependence of AlkBGT from NADH to NADPH may be helpful. This strategy has been successfully used for various oxidoreductases (Brinkmann-Chen et al., 2013; Li et al., 2019). Furthermore, to avoid the possible regulations involved in cofactor regeneration in *P. putida*, applying non-canonical redox cofactors (NCDH and NMNH) is another feasible orthogonal circuit for electron transfer (Weusthuis et al., 2020).

Preventing the degradation of intermediates and products

P. putida was shown to degrade most of the intermediate of mcl-diols production to some extent (Lu et al., 2021, 2022). Deleting *est12*, *adhP*, and *pedF* hampers *P. putida* from degrading esters and alcohols (Lu, Akwafo, et al., 2023). However, this might be not sufficient under growth conditions. Concerning fatty acids, although *P. putida* prefers to consume glucose over hexanoic acid and octanoic acid (Lu et al., 2022), it is necessary to completely abolish the degradation of fatty acids, thereby allowing them to be accumulated when the preferred substrates deplete. Disruption of key genes (*fadB*, *fadA*, and *fadE*) of beta-oxidation rendered *P. putida* incapable of growth on hexanoic acid and octanoic acid (unpublished results). Therefore, knocking out the above-mentioned genes is recommended to thoroughly halt the consumption of alcohols, acids, and esters during diol production.

CONCLUSION

P. putida KT2440 has demonstrated its potential as a non-model microbial cell factory for mcl-diol production. The construction and evaluation of a four-module diol synthesis pathway have been successful, removing some barriers to mcl-diol synthesis. However, the full exploitation of its robust flux and capacity remains untapped, with a need to address remaining hurdles in future research. Furthermore, the ability to utilize diverse and economical resources (e.g. lignocellulosic biomass, wasted plastics) could position *P. putida* to produce cost-effective diols, giving it a competitive edge over other microbial cell factories. A deeper understanding of pseudomonal metabolism and regulations will be instrumental in transforming *P. putida* into a more efficient microbial cell factory for the sustainable production of mcl-diols.

AUTHOR CONTRIBUTIONS

Chunzhe Lu: Conceptualization (equal); visualization (equal); writing – original draft (equal). **Rene Wijffels:** Writing – review and editing (equal). **Vitor A. P. Martins dos Santos:** Writing – review and editing (equal). **Ruud A. Weusthuis:** Supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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