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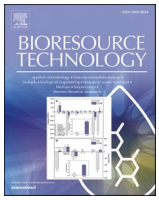

Microbial production of medium-chain-length α, ω-diols via two-stage process under mild conditions

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HIGHLIGHTS

- The highest 1,6-hexanediol microbial production from n-hexane was reported.
- Est12 hydrolyzes C6-C10 diesters with over 98% substrate hydrolysis efficiency.
- Est12 is partially secreted into the medium.
- *Pseudomonas putida* KT2440 is an efficient chassis for ester hydrolysis.
- The two-stage approach addresses the conflict of ester synthesis and hydrolysis.

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ABSTRACT

Medium-chain-length α, ω-diols (mcl-diols) are versatile compounds widely used as building blocks of coating materials and polymers. Mcl-diols are currently synthesized through energy intensive chemical process. Recently, esterified diols have been produced from n-alkanes in *E. coli* by co-expression of the alkane monooxygenase module (AlkBGTL) and the esterification module (Atf1), thereby establishing the technical feasibility of the process. However, esterified diols need to be hydrolyzed for further applications. In this study, we developed bio-catalysts for mcl-diol production from n-alkanes under mild conditions. The engineered *P. putida* KT2440 with overexpression of Est12 can efficiently hydrolyze esterified diols (C_6-C_{10}). Later, the engineered strain was co-cultured with an *E. coli* strain (AlkBGTL-Atf1) to produce mcl-diols. In a two-stage approach, 5 mM 1,6-hexanediol was produced, 61.5 times of one-stage test, from n-hexane by biocatalysts for the first time. In conclusion, the present work indicates that bio-catalysis offers a green biobased alternative for synthesis of mcl-diols.

1. Introduction

Medium-chain-length α, ω-diols (mcl-diols) are essential and versatile chemicals used in fuel, detergent, and polymer applications (Kawaguchi et al., 2017; Kruis et al., 2019; Lligadas et al., 2010; Vivek et al., 2021; Youngquist et al., 2013). Among these diols, 1,6-hexanediol is the most widely used monomer for industrial polymer production. The market price of 1,6-hexanediol is $4400 per ton (Kim et al., 2021a). The market volume was $902 million in 2019 and is expected to reach $1401 million by 2025 at an annual growth rate of 8% (Kim et al., 2021b). Currently, 1,6-hexanediol is mainly produced by multistage chemical processes with cyclohexane as the substrate in industrial production (Noack et al., 2011). This chemical route is energy intensive and requires expensive catalysts, resulting in environmental issues. To tackle these challenges, a biocatalytic cascade for 1,6-hexanediol production from cyclohexane in a one-pot-one-step manner was developed (Zhang et al., 2020). The conversion was successfully achieved by a microbial consortium under mild conditions. However, cyclohexane as the starting material is mostly derived via the hydrogenation of benzene, which still requires hydrogen and massive energy input. Besides, considering that other medium chain cycloalkanes are not abundantly available, it is therefore necessary to develop alternative routes for mcl-diols synthesis.

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Microbial synthesis of mcl-diols from n-alkanes or 1-alkanols is attracting more and more attention given 1) the availability of efficient alkane monooxygenases (Grant et al., 2011; Schaffer and Haas, 2014; Schrewe et al., 2014; Yoo et al., 2019); 2) mild conversion conditions with less by-product formation compared to chemical synthesis (Ahsan et al., 2018; Scheps et al., 2013; van Nuland et al., 2017) and 3) the potential to supply the intermediate alcohols from sustainable substrates such as glucose and lignin through fatty acid synthesis pathway (Deng et al., 2020; Mezzina et al., 2020; Vivek et al., 2021). Microbial production of esterified diols (α,ω-diacetoxyalkanes) from n-alkanes was achieved in *E. coli* through a combination of terminal oxidation and esterification steps (van Nuland et al., 2017). The pathway consists of the alkane monooxygenase system AlkBGTL from *Pseudomonas putida* GPo1 and alcohol acetyltransferase Atf1 from *Saccharomyces cerevisiae* (Fig. 1). AlkB, an integral membrane protein, is an alkane monooxygenase, specifically hydroxylating terminal carbon-hydrogen bonds of methyl group. The soluble rubredoxin AlkG, reduced by the rubredoxin reductase AlkT at the expense of NADH, is in charge of delivering electrons to AlkB. AlkL is a transporter located on the outer membrane that facilitates the uptake of hydrophobic compounds (Julsing et al., 2012; Yoo et al., 2019). Since AlkBGT can also over-oxidize hydroxyl groups into carboxylic acid groups, Atf1 was employed by van Nuland and colleagues to protect the hydroxyl group from overoxidation through esterifying it into acetate ester bonds (van Nuland et al., 2017). This strategy not only protected the hydroxy group from overoxidation but also enabled ω-hydroxylation and subsequent esterification, forming esterified diols (α,ω-diacetoxyalkanes). However, diols and not diacetoxyalkanes are the required compounds for further applications (such as polyester and polyurethane production) and the solution for the release of diols from the esterified diols under mild conditions was not provided yet. This can be implemented by expression of enzymes such as esterases. Thus, biocatalysts which combine diester synthesis and ester hydrolysis for diol production are desired.

Esterases are ubiquitous enzymes in many microorganisms, known for their ability to hydrolyze ester bonds in aqueous environments (Hasona et al., 2002; Kruis et al., 2019; Lee et al., 2013; Truongvan et al., 2016). For diol production, esterified mcl-diols need to be efficiently hydrolyzed. Therefore, potent esterases for mcl-diols production are demanded. It was reported that *Pseudomonas putida* KT2440 possesses multiple effective esterases such as EstB, EstC, EstZ and Est12 (Lu et al., 2021). Among them, Est12 was proven to be dominant for hydrolysis of 1,6-diacetoxyhexane to 1,6-hexanediol. *P. putida* KT2440 is not able to consume most mcl α,ω-diols (except 1,8-octanediol), but can grow on α,ω-diacetoxyalkanes, consuming only acetate as the sole carbon source (Lu et al., 2021). It has a robust tolerance to high concentrations of solvents (Gómez-Álvarez et al., 2022; Li et al., 2022; Mohamed et al., 2020). *P. putida* KT2440 is therefore regarded as a suitable chassis for hydrolysis of diesters, except for its ability to metabolize 1,8-octanediol. This can be abolished by deleting a LysR-type transcriptional regulator (Ltr, PP_2046) of an operon encoding beta-oxidation related proteins and an alcohol dehydrogenase (Li et al., 2020). However, the synthesis of esterified mcl-diols in *P. putida* KT2440 is less efficient than that in *E. coli* due to its versatile metabolism and carbon catabolite repression (Lu et al., 2021; Rojo, 2010). Co-expression of esterase with AlkBGTL-Atf1 in one host probably conflicts with the esterification step of Atf1, leading to poor diol production and overoxidation. So, biosynthesis of mcl-diol from alkanes using a combination of engineered *E. coli* and *P. putida* needs to be established and well comprehended.

In this study, we developed a biocatalytic conversion of n-alkanes to mcl-diols (Fig. 1). To construct a strain suitable for C_6-C_{10} diol production, *est12* was overexpressed in the deletion mutant of LysR-type transcriptional regulator (PP_2046) of *P. putida*. The hydrolysis rate of 1,6-diacetoxyhexane by the engineered *P. putida* strain was evaluated. The secretion of Est12 in *P. putida* during the diester hydrolysis process was investigated. The engineered *E. coli* (AlkBGTL-Atf1) was combined with the engineered *P. putida* strain in a one-stage and two-stage manner for 1,6-hexanediol production from n-hexane.

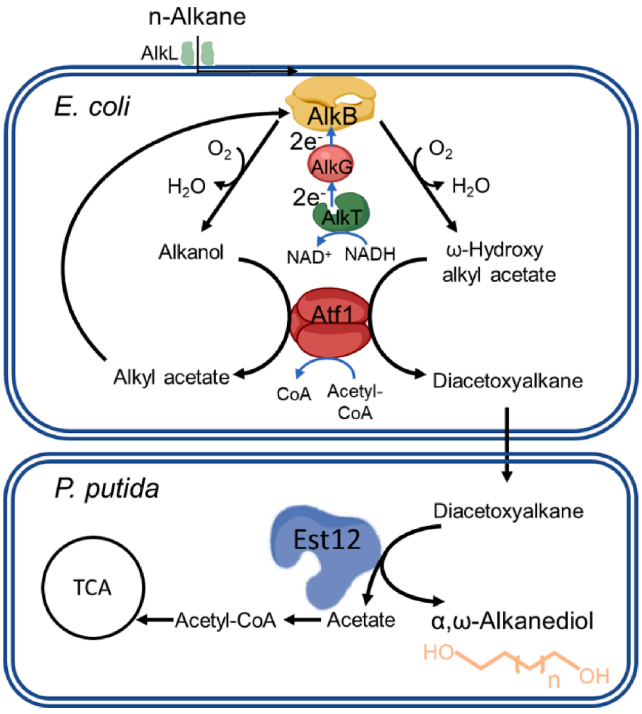

Fig. 1. The proposed α, ω-alkanediol biosynthesis pathway by combining *E. coli* AlkBGTL-Atf1 with *P. putida* Est12 using n-alkanes as starting material. AlkB: alkane monooxygenase; AlkG: rubredoxin; AlkT: rubredoxin reductase; Atf1: alcohol acetyl transferase; Est12: esterase 12, TCA: tricarboxylic acid cycle.

2. Materials and methods

2.1. Plasmids and strains

Plasmids and strains used in this study are listed in Table 1. *E. coli* DH 5α was used for gene cloning. *P. putida* KT2440, and *P. putida* KT2440 ΔLtr were used for the whole cell diester hydrolysis assay. The cellular location of Est12 was studied in *P. putida* KT2440. The plasmid pSEVAb658-est12 were constructed using a 2X HiFi assembly master mix (NEB) based on the supplier's instructions. The primers were designed for amplification of the backbone pSEVAb658 and the insert *est12*. PCR amplifications were performed with Q5 polymerase (NEB) according to the protocol of the supplier. Gene deletion in *P. putida* KT2440 was carried out based on Daniel's method using the suicide plasmid pGNW and the helper plasmid pQURE6•H (Volke et al., 2020). The plasmid pGNW-ΔLtr was constructed using SevaBrick Assembly (Damalas et al., 2020). The suicide plasmid pGNW-ΔLtr harboring GFP is first integrated into the genome of *P. putida* at the target place via homologous recombination. After the selection of GFP colonies, the helper plasmid pQURE6•H harboring the meganuclease I-SceI is introduced. The expression of I-SceI will generate either the knockout genotype or a wild-type genotype. Plasmids were verified by colony PCR and sequencing. Kanamycin (Km) and/or gentamicin (Gm) were added at 50 μg/mL and 10 μg/mL, respectively, as needed.

2.2. Protein expression and whole-cell assay

The engineered *P. putida* and *E. coli* strains were inoculated in LB medium containing the relevant antibiotics and cultured at 30 °C, 250 rpm overnight. The preculture (1% v/v) was transferred to 50 mL of defined M9 minimal medium consisting of 1x M9 minimal salts, 0.2 mM $MgSO_4 \cdot 7H_2O$, 55 mM glucose and 1 mL/L trace elements US^{Fe} and cultured overnight. This overnight culture was used to inoculate 50 mL

Table 1
List of plasmids and strains used in this study.

Name	Description	Reference
Plasmids		
pSEVAb658	Expression vector; *oriV(RSF1010)*; *XylS, Pm*; Gm^R	(Damalas et al., 2020)
pSEVAb658-est12	pSEVA658 vector harbouring *est12* gene from *P. putida* KT2440	This study
pSEVAb658-AlkBA	pSEVA658 harbouring the AlkBGTL-Atf1 module (optimized for expression in *P. putida* KT2440)	(Lu et al., 2021)
pCOM	pMB1 origin for *E. coli* and rep for *P. putida*, KanR	(Julsing et al., 2012)
pCOM-AlkL	pCOM plasmid harbouring a gene for the outer membrane protein AlkL	(van Nuland et al., 2017)
pCOM-AlkBA	pCOM plasmid harbouring the AlkBGTL-Atf1 module	(van Nuland et al., 2017)
pGNW2	Suicide vector used for gene deletion in *P. putida* KT2440; *oriV(R6K)* containing *P14g → msfGFP*; Km^R	(Volke et al., 2020)
pGNW2-ΔLtr	Derivative of vector pGNW2 containing HRs to delete *Ltr* (*PP_2046*)	This study
pQURE6•H	Conditionally-replicating vector; oriV(RK2), XylS/Pm → I-SceI and P14g(BCD2) → mRFP;GmR	(Volke et al., 2020)
Strains		
E. coli NEBT7	*fhuA2 lacZ::T7 gene1* [*lon*] *ompT gal sulA11 R(mcr-73::miniTn10*–TetS*)2* [*dcm*] *R(zgb-210::Tn10*–TetS*) endA*1 Δ*(mcrC-mrr)114::IS10*	New England Biolabs®
E.coli-AlkL	*E. coli* NEBT7 containing pCOM-AlkL	This study
E.coli-AlkBGTL-Atf1	*E. coli* NEBT7 containing pSEVA658-AlkBA	This study
P. putida KT2440	Wild-type strain, derived from *P. putida* mt-2(Worsey and Williams, 1975)	(Bagdasarian et al., 1981)
KT2440-est12	*P. putida* KT2440 containing pSEVAb658-est12	This study
KT2440-ΔLtr	*P. putida* KT2440 with the knocked-out LysR-type transcriptional regulator (PP_2046)	This study
ΔLtr-est12	*P. putida* KT2440 ΔLtr containing pSEVAb658-est12	This study

of defined M9 minimal medium at an initial OD_{600} of 0.25. When the OD_{600} reached 0.6–0.8, 3-methylbenzoate (3-MB) was added at a final concentration of 1 mM to induce *est12* expression. Except for gene expression, culture conditions for engineered *E. coli* strains are the same as those for *P. putida*. The culture was induced by the addition of 0.025 % v/v of dicyclopropylketone (DCPK) for AlkL and AlkBGTL-Atf1 expression. After 4–6 h of induction, the cells were harvested by centrifugation at 4255×*g* for 10 min.

The harvested cells were resuspended in resting-cell buffer (0.5% glucose, 2 mM $MgSO_4$, and 50 mM KPi with pH of 7.4) to a final concentration of $1g_{dcw}$/L. The resting cell suspension (1 mL) was transferred to 15 mL closed Pyrex tubes and the substrates were added at different concentrations according to the corresponding assay. Following that, the tubes were transferred to a rotary shaker at 30 °C, 250 rpm for conversion. The reactions were halted by adding 1% v/v of phosphoric acid and the tube was immediately transferred to ice. The reactions were extracted by diethyl ether containing 0.2 mM dodecane as internal standard. For the two-stage assay, after conversion by the Ec-AlkL or Ec-AlkBGTL-Atf1, reaction suspension was acidified to the pH of 2 for 30 min, then was adjusted to 7.4. Later, resting cells of *P. putida* were added to the reaction suspension and incubated for 2 h on a rotary shaker before stopping the reaction. The organic phase was used for gas chromatography (7890A) analysis. The water phase was analyzed by gas chromatography (7890B) and high-performance liquid chromatography (HPLC).

2.3. Acetate consumption tests

Tests were conducted in pH-controlled bioreactors (Applikon, The Netherlands) with 0.5 L of working volume. The M9 precultures of *P. putida* KT2440-est12 were used to inoculate bioreactors at the initial OD_{600} of 0.25. Bioreactor cultivations were done in batch operation mode with a working volume of 0.5 L. The temperature was kept at 30 °C and pH of 7 was maintained by the addition of 2 M NaOH or H_2SO_4 if needed. The dissolved oxygen was kept above 30% by stirring 500–670 rpm and sparging with air 500–700 mL/min. Foam formation was prevented by the addition of 400 μL of the 10% Antifoam B. For the strain KT2440-est12, the plasmid was maintained by adding kanamycin and gene expression was induced immediately upon inoculation with 1 mM 3-methylbenzoate (3-MB). For each time point, samples were taken for the OD_{600} measurements and quantification of substrates and products by gas chromatography (GC) and high-performance liquid chromatography (HPLC).

2.4. Signal peptide prediction of Est12 and its secretion tests

Signal sequences were predicted using SignalP 5.0 online tool (SignalP). Protein sequences of *est12* in FASTA format were uploaded. Gram-negative was set as the organism group. Long output was set as output format.

For Est12 secretion tests, *P. putida* KT2440 and *P. putida* KT2440-est12 were inoculated in LB medium overnight. The preculture was used to inoculate M9 glucose medium at an initial OD_{600} of 0.25. When the OD_{600} reached 0.8, 2.5 mM 3-methylbenzoate (3-MB) was added to induce *est12* expression for 4 h. After induction, cell pellets were harvested by centrifugation and the corresponding supernatant were collected too. The cell pellets ($1mg_{dcw}$) was washed twice with 50 mM phosphate buffer, pH 7.4 and resuspended in resting-cell buffer for diester hydrolysis test. The corresponding supernatant was filtered through 0.22 μm Nalgene® polyethersulfone (PES) filter (ThermoFisher) twice to exclude cell pellets and afterwards used for diester hydrolysis test. The cell pellets and the supernatant were added with 20 mM 1,6-diacetoxyhexane and cultured at 30 °C, 250 rpm for 20 h conversion. Samples were prepared and analyzed as beforementioned methods.

2.5. Gas chromatography analysis

GC analysis was done on an Agilent 7890A gas chromatograph equipped with a flame ionization detector (FID) using the HP-5 column (30 m × 30 μm × 0.25 μm). 1 μL sample was injected with split-less mode, using the following temperature program: 50 °C for 3 min, then 15 °C/min increase to 180 °C, 7 °C/min increase to 230 °C, and 30 °C/min increase to 350 °C, which was held for 3 min. The quantification was carried out using available standards. If standards were not commercially available, quantification was done based on structurally related compounds with similar numbers of carbon/hydrogen atoms. For example, mono-hexyl adipate was quantified based on mono-ethyl sebacate. For acids, samples were derivatized with a 10% v/v solution of 0.2 M trimethyl sulfonium hydroxide (TMSH).

Diols in liquid samples were measured by an Agilent 7890B gas chromatograph equipped with a flame ionization detector (GC-FID) and an Agilent 7693 autosampler. Samples were analyzed by injecting 1 μL of liquid sample into a NukolTM column (30 m × 0.53 mm, 1.0 μm coating, Supelco) with a split ratio of 10. The column temperature was maintained at 50 °C for 1 min and increased to 200 °C at a rate of 75 °C/min. 5 mM 1-Butanol was applied as an internal standard.

2.6. High-performance liquid chromatography

Acetic acid was analyzed by HPLC on an Agilent 1290 LC II system, equipped with an Agilent 1290 Infinity Binary Pump, an Agilent 1290 Infinity Autosampler, an Agilent 1290 Infinity diode array detector operating at 210 nm, and an Agilent 1260 Infinity RI detector (RID) operated at 45 °C. A Rezex ROA-Organic Acid H + (Phenomenex) column was used with a mobile phase of H_2SO_4 (0.008 mM). The HPLC was run at 0.8 mL/min and 60 °C. 50 mM propionic acid was employed as an internal standard.

3. Results and discussion

3.1. Construction of engineered P. putida for production of mcl-diols

To produce mcl-diols from the corresponding diesters (α, ω-diacetoxyalkanes) by *P. putida*, *est12* needs to be overexpressed. A plasmid containing *est12* under the control of the inducible XylS/P*m* expression system was introduced in wild type strain *P. putida* KT2440, giving rise to KT2440-est12. The wild-type strain was used as a negative control strain. 1,6-Diacetoxyhexane (20 mM) was added to the resting-cell suspension of both strains as substrate. As *P. putida* harbors multiple native esterases (Lu et al., 2021), the wild-type strain can hydrolyze 1,6-diacetoxyhexane to 1,6-hexanediol and 6-hydroxy hexyl acetate, resulting in less than 2 mM 1,6-hexanediol after 20 h (Fig. 2a). With Est12 overexpression, KT2440-est12 produced 19.5 mM 1,6-hexanediol with a 97.5% of substrate conversion yield. This indicates that the overexpression of *est12* in *P. putida* results in an efficient production of diol from its diester.

P. putida KT2440 is reported to be able to utilize 1,8-octanediol (Li et al., 2020). Indeed, when KT2440-est12 was incubated in the presence of 35 mM 1,8-diacetoxyoctane only 25 mM 1,8-octanediol was retrieved after 72 h while ΔLtr-est12 produced 35 mM 1,8-octanediol, indicating that the remainder was consumed by KT2440-est12 (Fig. 2b). To construct a strain suitable for production of C_6-C_{10} diols, genes involved in 1,8-octanediol utilization need to be silenced. The deletion of a LysR-type transcriptional regulator (Ltr, PP_2046) was reported to disable growth of *P. putida* on 1,8-octanediol.(Li et al., 2020) Since Ltr regulates an adjacent operon encoding an alcohol dehydrogenase and beta-oxidation related proteins, it is necessary to evaluate the effect of its deletion on 1,8-octanediol production in the ΔLtr mutant. Ltr was knocked out in strain KT2440-est12 giving rise to strain ΔLtr-est12. This strain was fed with 35 mM 1,8-diacetoxyoctane. As shown in Fig. 2b, the strain completely hydrolyzed 1,8-diacetoxyoctane into 1,8-octanediol, and the complete 35 mM substrate was retrieved as product. Based on these results, ΔLtr-est12 was used for further tests.

After the selection and evaluation of engineered strains, the production of $C_{6\text{-}10}$ diols from the corresponding diesters was tested. When 20 mM diester substrate was added to resting-cell suspensions of tested strains, the ΔLtr mutant was still able to hydrolyze part of them (9–41%), due to the presence of native esterases (Lu et al., 2021). In the same setup, ΔLtr-est12 converted over 98% of substrates to diols (Fig. 2c). This again demonstrates that the engineered *P. putida* strain ΔLtr-est12 is a potent biocatalyst for mcl-diols production from α, ω-diacetoxyalkanes.

3.2. The diol formation rate of engineered P. putida

In the above tests, 20 mM diester substrate was almost completely converted into the respective diol within 20 h. To further evaluate the performance of the biocatalyst ΔLtr-est12, 50 mM 1,6-diacetoxyhexane was added to the resting-cell suspensions. Within 2 h, 97% of 1,6-diacetoxyhexane was hydrolyzed to 1,6-hexanediol and 6-hydroxy hexyl acetate. After 4 h, 97% of the substrate was fully hydrolyzed to 1,6-hexanediol (Fig. 2d). Subsequently, 200 mM 1,6-diacetoxyhexane was fed to the resting-cell suspension of ΔLtr-est12. In the first half hour, 49.9

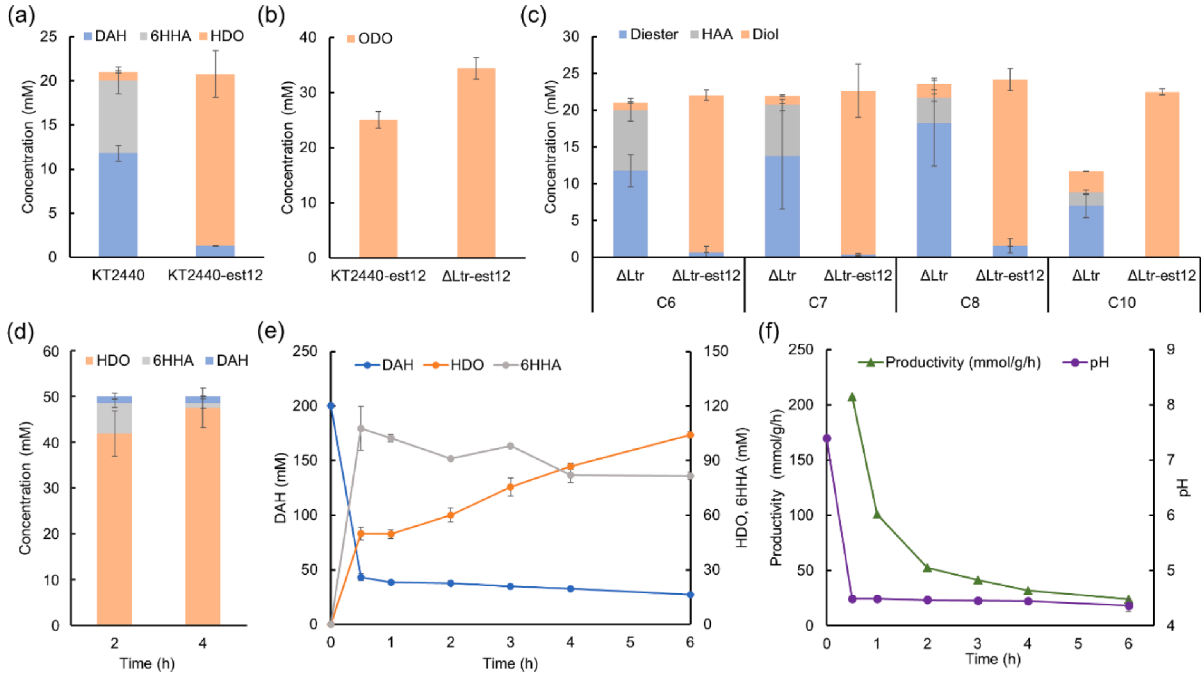

Fig. 2. Engineered *P. putida* expressing est12 for diol production under resting-cell conditions. (a) The effect of est12 expression in the wild type strain on 1,6-diacetoxyhexane (20 mM) hydrolysis in 20 h. DAH: 1,6-diacetoxyhexane; 6HHA: 6-hydroxy hexyl acetate; HDO: 1,6-hexanediol. (b) The effect of ΔLtr on the hydrolysis of 1,8-diacetoxyoctane (35 mM) when est12 was overexpressed in 72 h. Ltr: a LysR-type transcriptional regulator of an operon encoding beta-oxidation related proteins and an alcohol dehydrogenase; ODO: 1,8-octanediol. (c) The engineered strains for C_6-C_{10} diols production by hydrolysis of their corresponding diesters (20 mM) hydrolysis in 20 h. Diester: α, ω-diacetoxyalkane (C_6-C_{10}); HAA: ω-hydroxy alkyl acetate; Diol: α, ω-diacetoxyalkane (C_6-C_{10}). (d) 50 mM 1,6-diacetoxyhexane hydrolysis test by ΔLtr-est12 in 4 h under resting-cell conditions. (e) Hydrolysis test by ΔLtr-est12 in 6 h with 200 mM 1,6-diacetoxyhexane. Each conversion experiment was conducted in triplicate. (f) The change of HDO productivity rate and pH over time.

mM 1,6-hexanediol and 107.6 mM 6-hydroxy hexyl acetate were formed, corresponding to 78.4% of the original 1,6-diacetoxyhexane (Fig. 2e). The concentration of 1,6-hexanediol increased over time, while that of 6-hydroxy hexyl acetate and 1,6-diacetoxyhexane reduced. Within 6 h, 104 mM 1,6-hexanediol was produced, corresponding to 52% of the original substrate. After the first half hour, there is a significant drop in the performance of ΔLtr-est12. To better evaluate the hydrolysis ability of ΔLtr-est12, the formation rate of the products - 6-hydroxy hexyl acetate and 1,6-hexanediol - was calculated. One molecule of 1,6-diacetoxyhexane was first hydrolyzed into one molecule of 6-hydroxy hexyl acetate and one molecule of acetate by Est12. Later, the formed 6-hydroxy hexyl acetate was further hydrolyzed to one molecule of 1,6-hexanediol and another molecule of acetate. This means that formation of one molecule of 1,6-hexanediol requires Est12 function twice, while one molecule of 6-hydroxy hexyl acetate needs Est12 function once (equation I). The formation rate significantly decreased from 207.5 mmol/h/g_{dcw} at 0.5 h to 52.9 mmol/h/g_{dcw} at 2 h, and later gradually dropped to 21.1 mmol/h/g_{dcw} at 6 h. At the same time, the pH of the suspension reduced from 7.4 to 4.5 (Fig. 2f). The decrease of ΔLtr-est12 activity is in line with Millar's observation (Millar et al., 2017) showing the optimal pH for Est12 is between 7.5 and 8.5 with a significant reduction in Est12 activity at lower pH values.

$$RatePF(mmol/h/gcdw) = \frac{(1*CONC_{6-OHHA} + 2*CONC_{1,6-Hexanediol})*Volume}{Time_{conversion}*Biomass_{initial}} \quad (1)$$

3.3. Diol formation in a bioreactor

The decrease in pH was caused by the release of acetate from the esters. To prevent the decrease in pH, conversion tests with ΔLtr-est12 were performed in pH-controlled 0.5-L bioreactors. The inducer and 1,6-diacetoxyhexane (40 mM) were added at 0 h. In the first 4 h, 1,6-diacetoxyhexane was rapidly hydrolyzed, while acetate, 6-hydroxy hexyl acetate and 1,6-hexanediol were generated (Fig. 3). After 3 h, acetate and 6-hydroxy hexyl acetate reached their maximum concentration of 55.5 mM and 24.6 mM, respectively, after which they were consumed until they were depleted after about 10 h. Given both 1,6-hexanediol and acetate were released in culture, the consumption of acetate by *P. putida* could prove beneficial as it cannot interfere with the downstream process for diol extraction. Glucose was added at the start of the experiment, and was consumed simultaneously with acetate. It would be interesting to test whether glucose addition is necessary at all, and whether the released acetate could serve as the only carbon- and energy source.

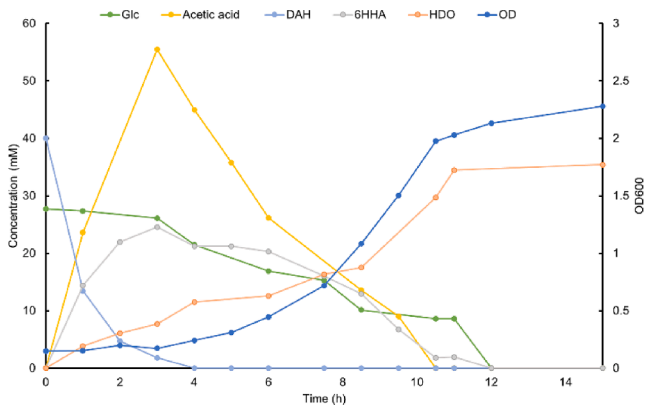

Fig. 3. Hydrolysis of 40 mM 1,6-diacetoxyhexane by ΔLtr-est12 over time in a 0.5-L bioreactor. Glc: glucose; 6HHA: 6-hydroxy hexyl acetate; DAH: 1,6-diacetoxyhexane; HDO: 1,6-hexanediol; OD: optical density at 600 nm. The experiment was performed multiple times, the graph show a typical example.

3.4. Est12 secretion

In our previous study, it was found that *P. putida* KT2440 encodes several esterases and their activities were verified (Lu et al., 2021). Among these esterases, Est12 showed the highest activity on alkyl acetate and 1,6-diacetoxyhexane. Est12 (EstK) was referred to as an extracellular carboxylesterase, but no experimental evidence on its localization was provided (Millar et al., 2017). The SignalP-5.0 (SignalP) prediction tool was used to check the existence of a signal peptide in all esterases. The output showed a high probability (99.5%) for the presence of a signal peptide only in Est12 (Fig. 4a). We presumed that the high activity of Est12 might be because of the presence of this signal peptide, resulting in secretion of Est12 protein into medium, facilitating the hydrolysis of the esters.

To check whether Est12 was excreted from cells, diester hydrolysis tests were conducted with washed and resuspended cell pellets and their corresponding supernatants. 20 mM 1,6-diacetoxyhexane was used as substrate. The supernatant from wild type *P. putida* hydrolyzed 1,6-acetoxyhexane to 6-hydroxyhexyl acetate, whereas the washed cell pellets of wild type *P. putida* hydrolyzed 1,6-acetoxyhexane into 1,6-hexanediol and 6-hydroxyhexyl acetate (Fig. 4b). When Est12 was overexpressed, 1,6-acetoxyhexane was completely hydrolyzed by the supernatant and cell pellets. In the cell pellets case, 1,6-acetoxyhexane was completely hydrolyzed into 1,6-hexanediol. In the supernatant case, 1,6-acetoxyhexane was not fully hydrolyzed to 1,6-hexanediol, accounting for 50% of the total products and the rest was 6-hydroxyhexyl acetate. All in all, this indicates that Est12 remained mainly in the cell pellets and was partially secreted into the medium.

3.5. One-stage synthesis of HDO

To produce 1,6-hexanediol directly from n-hexane, two modules are

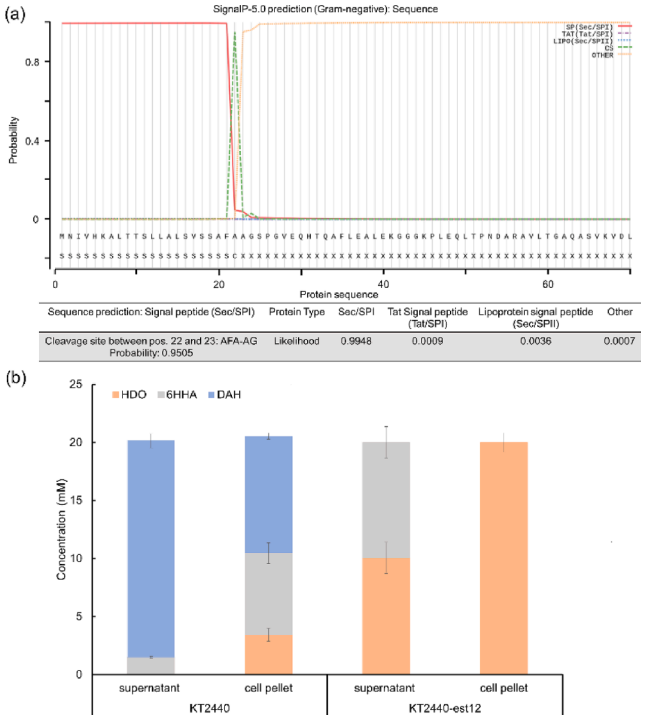

Fig. 4. (a) The likelihood of existence of signal peptide in Est12. The SignalP-5.0 (SignalP) prediction tool was used. (b) DAH hydrolysis tests by cell pellets and the corresponding supernatant. 6HHA: 6-hydroxyhexyl acetate; DAH: 1,6-diacetoxyhexane; HDO: 1,6-hexanediol. 20 mM diacetoxyhexane was fed as substrate. 1 g_{dcw}/L Cell pellets and the corresponding supernatant were used. Cell pellets were washed twice with 50 mM phosphate buffer, pH 7.4. The supernatant was filtered through a 0.22 μm filter.

required: the diester synthesis module (AlkBGTL-Atf1) and diester hydrolysis module (Est12). However, they are in conflict with each other when being expressed in the same host. To counter the conflict of co-expression of two modules in a host, the combination of engineered *E. coli* harboring AlkBGTL-Atf1 and engineered *P. putida* harboring Est12 in a one-stage manner could be a solution as diester synthesis is happening in *E. coli* whilst hydrolysis of ester would be mainly in *P. putida*.

The strains used for one-stage diol synthesis are listed (Fig. 5a). For 1,6-hexanediol production from 1,6-acetoxyhexane, ΔLtr is not required (Fig. 2a,c). Therefore, we chose *P. putida* KT2440-est12 for diester hydrolysis. Three negative controls were used: (1) *E. coli* AlkL only, (2) *E. coli* AlkL cocultured with *P. putida* KT2440 and (3) *E. coli* AlkL cocultured with *P. putida* KT2440-est12. Three positive controls were used: (1) *E. coli* AlkBGTL-Atf1 only, (2) *E. coli* AlkBGTL-Atf1 cocultured with *P. putida* KT2440 and (3) *E. coli* AlkBGTL-Atf1 cocultured with *P. putida* KT2440-est12. The one-stage synthesis of diol was performed according to the procedure (Fig. 5b). The engineered *E. coli* and *P. putida* strains were separately cultured and induced. The cell pellets were harvested and resuspended together in resting-cell buffer for further one-stage conversion. n-Hexane (1% v/v, 76.5 mM) was fed in all one-stage tests as the substrate. All negative controls produced no products (Fig. 5d). When only *E. coli* AlkBGTL-Atf1 was present, 1,6-diacetoxyhexane and 6-hydroxylhexyl acetate were produced, indicating the AlkBGTL-Atf1 is properly activated. The absence of products like hexanoate shows that the esterification effectively prevented overoxidation of hexane.

When *E. coli* AlkBGTL-Atf1 was co-cultivated with wild-type *P. putida* KT2440, the production of all products was decreased compared to that of only *E. coli* AlkBGTL-Atf1 case. It is likely caused by the presence of multiple native esterases in *P. putida* KT2440. Apparently, the esters formed by *E. coli* are excreted in the medium and taken up by *P. putida*, which hydrolyses the esters and excretes the corresponding alcohols. These in turn are taken up by *E. coli* and either used for esterification or overoxidation (Fig. 5c). As a result, only a small amount of 1,6-hexanediol (less than 0.1 mM) was detected. This view is supported by the data obtained from the test where *E. coli* AlkBGTL-Atf1 was co-cultivated with *P. putida* KT2440-est12, in which Est12 was overexpressed. The enhanced esterase activity resulted in a full hydrolysis of hexyl acetate into 1-hexanol (2.3 mM). The excess 1-hexanol was partly overoxidized by AlkBGT to hexanoic acid (Fig. 5d). In addition, the total production when *P. putida* KT2440 or *P. putida* KT2440-est12 is present is comparable and less, respectively, than the test with only *E. coli* AlkBGTL-Atf1. It was concluded that the presence of esterase in a one-stage method reduced the efficiency of the whole conversion.

3.6. Two-stage synthesis of HDO

Considering the low production of 1,6-hexanediol and the occurrence of overoxidation in a one-stage way, a two-stage synthesis approach was investigated. The strains used are listed (Fig. 6a). *E. coli* AlkBGTL-Atf1 was incubated as a negative control. *E. coli* AlkBGTL-Atf1 cocultured with *P. putida* KT2440 and *P. putida* KT2440-est12 were used as positive control. The synthesis of diol proceeded according to the procedure (Fig. 6b). In this approach, after *E. coli* AlkBGTL-Atf1 converts n-hexane to 1,6-diacetoxyhexane, it was inactivated by acidification before the addition of *P. putida* KT2440-est12 resting cells. This way the synthesis of 1,6-diacetoxyhexane and its hydrolysis are separated into two stages (Fig. 6b). This strategy would avoid overoxidation of 1-hexanol and low efficiency of 1,6-diacetoxyhexane synthesis in the one-stage tests (Fig. 6c).

E. coli AlkBGTL-Atf1 produced 5.5 mM precursors of 1,6-hexanediol, 6-hydroxylhexyl acetate and 1,6-diacetoxyhexane (Fig. 6d). In presence of *P. putida* KT2440 and *E. coli* AlkBGTL-Atf1, 1.9 mM 1,6-hexanediol, 0.6 mM 1,6-diacetoxyhexane and 1.6 mM 6-hydroxylhexyl acetate were produced. The accumulation of 1,6-diacetoxyhexane and 6-hydroxylhexyl acetate implies that native esterases of *P. putida* KT2440 are not enough to completely hydrolyze the diol precursors. When Est12 was overexpressed, the combination of *P. putida* KT2440-est12 and *E. coli* AlkBGTL-Atf1 produced 5 mM 1,6-hexanediol as the main product and 0.4 mM 1-hexanol. There were no 1,6-hexanediol precursors left, indicating that all of them were hydrolyzed by the overexpressed Est12 into 1,6-hexanediol. Besides, acetate concentration increased to a maximum of 3 mM, after which it was consumed, whereas glucose consumption was not observed in this test. The released acetate was possibly enough for cell maintenance during the diol formation

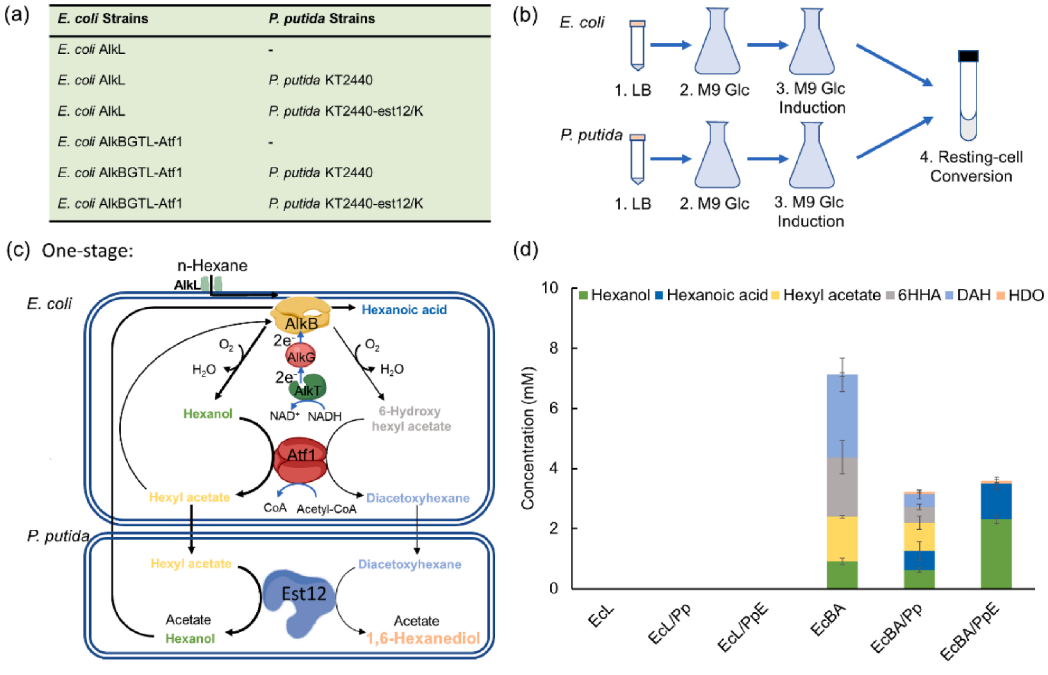

Fig. 5. One-stage synthesis of 1,6-hexanediol from n-hexane. (a) The different combinations of strains were used in this one-stage assay. AlkB: alkane monooxygenase; AlkG: rubredoxin; AlkT: rubredoxin reductase; Atf1: alcohol acetyl transferase; est12: esterase 12 from *P. putida* KT2440. (b) Overview diagram of the one-stage synthesis of 1,6-hexanediol. LB: Luria-Bertani broth medium; M9: Minimal medium; Glc: glucose. (c) The fate of n-hexane through the one-stage assay within *E. coli*-AlkBGTL-Atf1 and *P. putida*-est12. (d) Conversion products by different combinations of strains in presence of 1% v/v n-hexane in 20 h. 1g_{dcw}/L *E. coli* strain and 1g_{dcw}/L *P. putida* strain were used for conversion tests. 6HHA: 6-hydroxy hexyl acetate; DAH: 1,6-diacetoxyhexane; HDO: 1,6-hexanediol. EcL: *E.coli* AlkL; EcL/Pp: *E.coli* AlkL and *P. putida* KT2440; EcL/PpE: *E.coli* AlkL and *P. putida* KT2440-est12/K; EcBA: *E.coli* AlkBGTL-Atf1; EcL/Pp: *E.coli* AlkL and *P. putida* KT2440; EcBA/PpE: *E.coli* AlkBGTL-Atf1 and *P. putida* KT2440-est12.

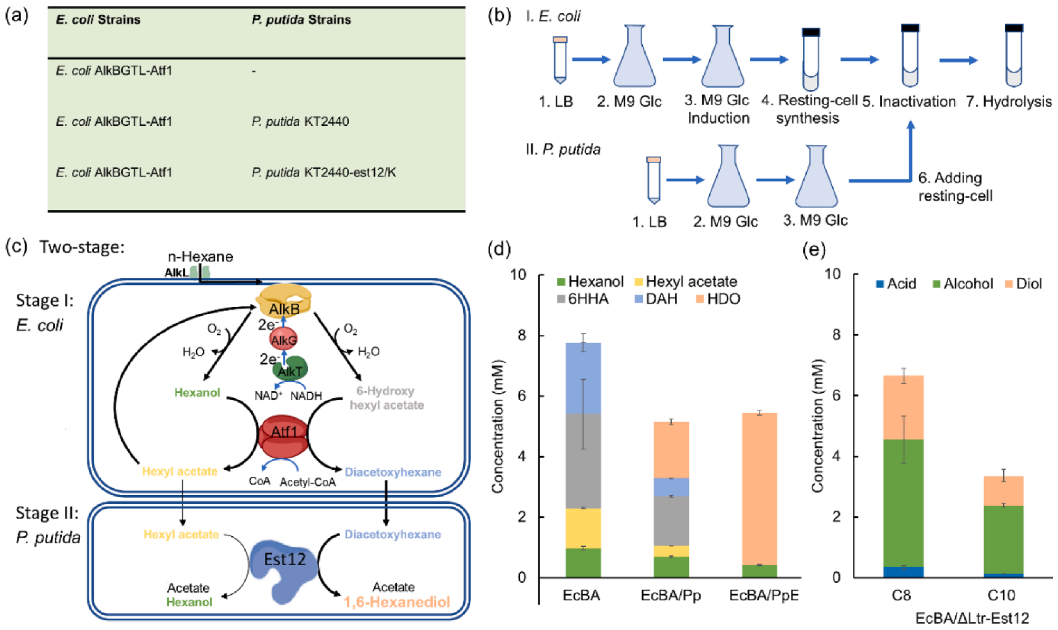

Fig. 6. Two-stage synthesis of 1,6-hexanediol from n-hexane. (a) The different combinations of strains were used in the two-stage assay. AlkB: alkane monooxygenase; AlkG: rubredoxin; AlkT: rubredoxin reductase; Atf1: alcohol acetyl transferase; est12: esterase 12 from *P. putida* KT2440. (b) Overview diagram of the one-pot synthesis of 1,6-hexanediol. LB: Luria-Bertani broth medium; M9: Minimal medium; Glc: glucose. (c) The fate of n-hexane through the two-stage assay within *E. coli*-AlkBGTL-Atf1 and subsequently *P. putida*-est12. (d) Products converted by different two-stage assays in presence of 1% v/v n-hexane in 20 h. $1g_{dcw}$/L *E. coli* strain or $1g_{dcw}$/L *P. putida* strain were used for conversion tests. 6HHA: 6-hydroxy hexyl acetate; DAH: 1,6-diacetoxyhexane; HDO: 1,6-hexanediol. EcBA: *E. coli* AlkBGTL-Atf1; EcL/Pp: *E.coli* AlkL and *P. putida* KT2440; EcBA/PpE: *E. coli* AlkBGTL-Atf1 and *P. putida* KT2440-est12. Each conversion experiment was conducted in triplicate. (e) 1,8-Octanediol and 1,10-decanediol production through two-stage approach in 20 h. $1g_{dcw}$/L *E. coli* strain or $1g_{dcw}$/L *P. putida* strain were used for conversion tests. EcBA/ΔLtr-Est12: *E. coli* AlkBGTL-Atf1 and *P. putida* KT2440 ΔLtr -Est12; Acid: octanoic acid or decanoic acid; Alcohol: octanol or decanol; Diol: 1,8-octanediol or 1,10-decanediol.

process. The production of 1,6-hexanediol with Est12 overexpression is 1.6 times of that with native esterases from wild type *P. putida* KT2440. In addition, compared to that of the one-stage method, the 1,6-hexanediol production in two-stage approach was increased by 61.5 times when *P. putida* KT2440-est12 and *E. coli* AlkBGTL-Atf1 were co-cultivated. Besides, the production of total products in the two-stage assay was increased by 51% compared with the one-pot assay when the engineered *E. coli* and *P. putida* strains were combined. Given the performance of the two-stage conversion for 1,6-hexanediol, 1% v/v of n-octane and n-decane were separately added as substrate. *E. coli* AlkBGTL-Atf1 and *P. putida* ΔLtr-est12 were used in these tests, producing 2.1 mM 1,8-octanediol and 1 mM 1,10-decanediol (Fig. 6e). This proves the feasibility of the two-stage approach for other medium chain diol production. 1-Octanol and 1-decanol were detected at higher concentrations than diols. Minor amounts of acids were observed in both cases, indicating that overoxidation occurred. This is in line with our previous work (van Nuland et al., 2017), where octyl acetate and decyl acetate were main products instead of esterified diols and acids were formed in *E. coli* AlkBGTL-Atf1.

With these tests, we showed that medium-chain-length α, ω-diols are successfully produced from n-alkanes under mild conditions by biocatalysts. In our two-stage approach, the production of 1,6-hexanediol was significantly increased by separating hydrolysis of diacetoxyhexane from its synthesis. This strategy is applicable to the production of C_6-C_{10} α, ω-alkanediols. In addition, considering its performance when Est12 was overexpressed and its preference to consume acetate, *P. putida* was shown to be a suitable biocatalyst for diester hydrolysis. Given the broad substrate scope of AlkBGT (Grant et al., 2011; Julsing et al., 2012; Schrewe et al., 2011; van Nuland et al., 2017), the pathway is supposed to be applicable for C_5-C_{16} diol production. In other studies, CYP153 monooxygenase was used to directly synthesize medium-chain α, ω-alkanediols from n-alkanes by engineered microbes (Ahsan et al., 2018; Fujii et al., 2006; Scheps et al., 2011). However, the diol titers obtained in these studies were quite low. 1,6-Hexanediol (51 μM) and 1,8-octanediol (1.8 mM) were produced in the engineered *E. coli* strain from the corresponding alkane (Fujii et al., 2006). Considering the low activity of CYP153 monooxygenase on medium-chain molecules (Liu et al., 2021; Wang et al., 2011), the approach used in this work could be a promising solution for potential industrial applications. In a recent study, 13 mM 1,6-hexanediol was successfully produced from cyclohexane by an *E. coli* consortium with a total OD_{600} of 20 (Zhang et al., 2020). In our study, 5 mM (591 mg/L) 1,6-hexanediol was produced with a total OD_{600} of 6 (*E. coli* AlkBGTL-Atf1 and *P. putida* est12). The content of 1,6-hexanediol in our method is 0.28 mM/g_{dcw}, 27% higher than the value (0.22 mM/g_{dcw}) reported by Zhang *et al.* (Zhang et al., 2020) The productivity of 1,6-hexanediol is 0.31 μmol/g_{dcw}/h, 80% of the amount of Zhang's work (0.39 μmol/g_{dcw}/h), mainly because of the rate-limited ω-oxidation of hexyl acetate by the monooxygenase system AlkBGT. In addition, only 6.5% of n-hexane was completely converted into 1,6-hexanediol in our study. This indicates that hydrolysis of diacetoxyhexane was not the limiting step for 1,6-hexanediol production with the potent esterase Est12, whilst diester synthesis in *E. coli* with expression of AlkBGTL-Atf1 is the bottleneck of this process. This limitation also occurs in the octane and decane conversion tests. Thus, the monooxygenase system AlkBGT needs to be comprehensively studied in future to increase the conversion efficiency. To further facilitate the diol production process, future work will focus on the availability of oxygen, carbon source and the removal of products and intermediates which result in product inhibition by addition of carrier solvents.

4. Conclusions

To conclude, we developed a microbial synthesis pathway of α,ω-alkanediols from n-alkanes by combining the oxidation/esterification module with the diester hydrolysis module. In comparison to the existing industrial production routes, which require intensive energy

and expensive catalysts, the biosynthesis route in this study paves the way to efficient diol production under mild conditions. The present work offers an appealing approach for not only diol production but also biosynthesis of other alcohol/diol-based compounds. Furthermore, this work lays foundation for future integration of the diol synthesis pathway into fatty acid synthesis to achieve sustainable diol production from plenty of renewable source.

CRediT authorship contribution statement

Chunzhe Lu: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Software, Visualization, Writing – original draft, Writing – review & editing. **Nina Leitner:** Investigation, Methodology, Data curation. **Rene H. Wijffels:** Project administration, Formal analysis, Supervision, Writing – review & editing. **Vitor A.P. Martins dos Santos:** Project administration, Supervision, Writing – review & editing. **Ruud A. Weusthuis:** Project administration, Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127111.

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