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Scalable production and simplified purification of bio-based dodecanedioic acid from renewable plant oil

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Research Article

Keywords: dodecanedioic acid, Candida tropicalis, pilot-scale fermentation, downstream processing, crystallization

Posted Date: October 14th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11090787/v1

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Abstract

Background

Dodecanedioic acid (DDDA) is an important bio-based monomer for engineering plastics such as nylon 6.12; however, its commercial production relies predominantly on petrochemical processes. In this study, we developed a pilot-scale bioprocess for sustainable DDDA production from renewable methyl laurate derived from coconut oil, with particular emphasis on scalable production and simplified downstream purification.

Methods

An engineered *Candida tropicalis* strain efficiently converted methyl laurate into DDDA, achieving a titer of 102 g L^{-1} with a productivity of 0.85 g L^{-1} h^{-1} in a 5-L fermenter. The process was successfully translated to a 300-L pilot-scale fermenter without loss of performance, reaching 105 g L^{-1} and 1.07 g L^{-1} h^{-1}, demonstrating robust scalability for industrial application. To reduce downstream processing complexity and cost, a direct crystallization process based on acetic acid was developed, replacing conventional sulfuric acid precipitation and eliminating additional decolorization and washing steps.

Results

The simplified purification process consistently produced DDDA with 99.4% purity and commercial-grade color quality at pilot scale while minimizing inorganic salt generation and wastewater production.

Conclusions

These results demonstrate an integrated and scalable production–purification platform for high-purity bio-based DDDA and provide a practical foundation for the industrial manufacture of renewable nylon monomers.

Introduction

Dodecanedioic acid (DDDA) is a saturated aliphatic 12-carbon diacid with diverse industrial applications, including the production of high-quality polyester (PE), polyamide (PA), polyurethane, surfactants, perfumes, and antiseptics (Craft et al., 2003). Notably, DDDA serves as a crucial monomer in the synthesis of premium engineering plastics, particularly in the production of PE and PA materials (Kyulavska et al., 2017). Of particular significance is polyamide 6.12 (PA 6.12), a thermoplastic with a distinctive chemical structure that enables lower water absorption compared with the widely used polyamide 6 (PA 6) and PA 6.6 (Akro-Plastic, 1988).

At present, DDDA is primarily produced from petroleum resources using a multi-step chemical process involving the trimetallization of butadiene to form cyclododecatriene, which is subsequently hydrogenated to yield cyclododecane. This cyclododecane is then oxidized using nitric acid to synthesize DDDA (Mills et al., 2018). However, the hydrogenation of cyclododecatriene to cyclododecane requires a Raney nickel catalyst and harsh conditions like high temperatures (170–180°C) and high pressures (26–28 bar absolute). These harsh conditions and the use of heavy metal catalysts pose significant environmental risks. Notably, Evonik Industries, a leading cyclododecatriene manufacturer, faced a tragic incident resulting in fatalities and production disruptions owing to an explosion and fire (HazardEx, 2021; Reuters, 1851). Consequently, the production of essential components, such as plastics for fuel and brake lines, was severely affected, impacting automotive component companies in the United States and Europe. Furthermore, since butadiene is derived from fossil fuels, the petrochemical synthesis of DDDA is highly dependent on raw materials, making it susceptible to extreme price volatility due to supply limitations and increased demand across various markets.

To address the challenges associated with conventional chemical processes, there has been a growing interest in utilizing microbial transformation for DDDA production. Microorganisms such as yeasts are well-suited for biomass-based biotransformation because they produce enzymes of the family that helps convert various substrates, including alkanes and fatty acids (Irina et al., 2017). Hydrocarbon-assimilating yeast strains can efficiently produce α,ω-dicarboxylic acid (DCA). This process, known as ω-oxidation, involves the oxidization of the terminal methyl group of alkanes and fatty acids (Sumita et al., 2022; Scheller et al., 1998). However, DCAs are naturally degraded through β-oxidation in the peroxisome and used for growth or energy production (Hettema and Tabak, 2000). *Candida tropicalis*, known for its excellent ability to metabolize alkanes and fatty acids via ω-oxidation and β-oxidation pathways, has been metabolically engineered to accumulate DCAs by blocking the degradation pathway (Picataggio et al., 1991). Additionally, overexpression of genes related to the ω-hydroxylase complex has been studied to address the rate-limiting issue in the first step of ω-oxidation, which is involved in the conversion to DCA. Recombinant *Saccharomyces cerevisiae* strains expressing cytochrome P450 (CYP) monooxygenases and the corresponding reductases from *Candida maltosa* or *C. tropicalis* could synthesize DDDA from lauric acid (Zimmer et al., 1995; Sanglard et al., 1990). Co-expression of CYP monooxygenase and reductase genes encoding ω-hydroxylase complex components enhanced the productivity of tetradecanedioic acid (C14 DCA) via *C. tropicalis* by 30% (Picataggio et al., 1992). Optimization studies of parameters such as medium composition, culture conditions, and substrate supply strategies have improved DCA production by microorganisms (Liu et al., 2004; Zibek et al., 2009). However, despite these advancements, all DDDAs are produced commercially from fossil fuels, not bio-derived sources.

Recently, we reported the microbial production process for sebacic acid (decanedioic acid) from vegetable oil-derived feedstocks using an engineered *Candida tropicalis* strain, demonstrating the feasibility of producing long-chain dicarboxylic acids from renewable resources (Jeon et al., 2019). Building upon this foundation, herein, we describe a process for the production of DDDA from plant oil-derived methyl laurate. Emphasis was placed on establishing a robust pilot-scale fermentation process

and a simplified downstream purification strategy suitable for large-scale manufacturing. We demonstrate reproducible DDDA production at the 300-L pilot scale together with a direct crystallization process that enables the recovery of high-purity DDDA without conventional decolorization or extensive washing steps. These results provide a practical and scalable platform for the sustainable industrial production of bio-based DDDA from renewable feedstocks (Fig. 1).

Materials and Methods

Microorganisms

The strains, plasmids, and oligonucleotide primers used in this study are comprehensively listed in Table 1. Detailed methodologies for strain construction are described below.

Table 1
Strains used in this study

Strain	Genotype	Reference
C. tropicalis ATCC 20962	ura3A/uraB pox4A/pox4B pox5/pox5 ::URA3A	Picataggio et al., 1991
C. tropicalis ATCC 20962△ura3	ura3A/uraB pox4A/pox4B pox5/pox5 ::ura3A	Jeon et al., 2019
C. sorbophila DS02	wild type	Lee et al., 2017
E. coli BW25113 ΔfadD (DE3)	F- DE(araD-araB)567 lacZ4787(del)::rrnB-3 LAM- rph-1 DE(rhaD-rhaB)568 hsdR514, ΔfadD	Bae et al, 2014
C. tropicalis GK5	ura3A/uraB pox4A/pox4B pox5/pox5 ::ura3AhisG::WsFAO-URA3A	This study

Comparison of various fatty alcohol oxidase 1 (FAO1) types

Three FAO1 genes, with Genbank accession numbers CAG83115, AY538780, and XM_024807606, originating from *C. tropicalis*, *Yarrowia lipolytica*, and *Wichermyces sorbophila*, respectively, were amplified by polymerase chain reaction (PCR) using the designated oligonucleotide primers, namely, YlF1_F and YlF1_R, CtF1-F and CtF1-R, and WsF-F and WsF-R, respectively.

For constructing the FAO1 expression vectors, the amplified YlFAO1, CtFAO1, and WsFAO genes were inserted into the *NdeI/NotI* sites of the pET24ma vector (Novagen, Pretoria, South Africa). Subsequently, the resulting plasmids were introduced into *Escherichia coli* BW25113 (DE3) using the heat shock method (Hanahan, 1983) with some modification. Each transformant was cultivated in Luria–Bertani media supplemented with 50 μg/L kanamycin (Duchefa Biochemie, Netherlands) and stored at 37°C overnight. For enzyme overexpression, *E. coli* strains from overnight cultures were inoculated into 50-mL Terrific broth (Thermo Fisher Scientific, Waltham, MA, USA) contained in 250-mL baffled flasks. Cultures

were allowed to grow for approximately 2 h until the optical density at 600 nm (OD_{600}) reached 0.5–0.8. Subsequently, fatty acid oxidase (FAO) expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (LPS Solution, Daejeon, Korea). Following induction, the cultures were transferred to a shake incubator set at 20°C and cultured further for 16 h. Cells were then harvested and washed twice with 100 mM HEPES buffer (pH 7.6) for subsequent whole-cell biotransformation. Whole-cell biotransformation was conducted using 100 mM HEPES buffer (pH 7.6) supplemented with 30 OD_{600} of each transformant, 10 mM 12-hydroxydodecanoic acid, and 20 g/L glucose at 30°C.

Codon optimization of the WsFAO1 gene

To enable functional expression in *C. tropicalis* ATCC 20962 (American Type Culture Collection, VA, USA), codon optimization of the WsFAO gene was performed. The amino acid sequence of WsFAO was reverse-translated to the corresponding nucleotide sequence, considering the preferred codons of *C. tropicalis*. Subsequently, the codon-optimized WsFAO gene was synthesized by GenScript (Piscataway, NJ, USA) (Supplementary Table 2).

Construction of the C. tropicalis strain expressing WsFAO1

The codon-optimized WsFAO1 gene was PCR-amplified using the primers WsFAO1-F and WsFAO1-R. For the promoter, 1481-bp sequences upstream of the glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* gene (GenBank accession no. NW003020061.1; Bethesda, MD, USA) were amplified from the genomic DNA of *C. tropicalis* ATCC 20962 using the primers GAPp-F and GAPp-R. Further, 600-bp 5' and 3' homologous arms of the URA3A genes (GenBank accession no. QLNQ00000000.1) were amplified for target gene integration from the genomic DNA of *C. tropicalis* ATCC 20962 using primers URA3 HR-F1, URA3 HR-R2, URA3 HR-F3, and URA3 HR-R4. A 300-bp fragment downstream of the *GAPDH* gene sequence (GenBank accession no. HQ171163.1) was amplified for use as a terminator from the genomic DNA of the ATCC 20962 strain using primers GAPt-F and GAPt-R. A 438-bp fragment of the *hisG* gene (GenBank accession no. CP020101.1) was amplified for use as a flanking sequence for pop-out from the genomic DNA of *Salmonella enterica* using primers hisG-F and hisG-R. A *URA3* marker cassette was constructed according to a previously published protocol (Bae et al., 2014). The *C. tropicalis URA3A* fragment was inserted into the *Eco*RI/*Not*I sites of the pGEM-T easy vector, and the *hisG* gene was inserted into the *Eco*RI/*Not*I sites at both ends of the *URA3* as a repeating sequence in the vector. The resulting plasmid contained the *hisG-URA3-hisG* cassette and was termed pGEM-*huh*. To assemble the *FAO1* gene expression cassette, the *GAPDH* promoter, codon-optimized *WsFAO1* gene, and *GAPDH* terminator were inserted into the *Apa*I/*Bgl*II sites of the pGEM-*huh* vector. The resulting plasmids were named pGEM-GAPDHp-WsFAO. *C. tropicalis* ATCC 20962△*ura3* was transformed using the lithium acetate method with some modification (Waltz, 2012). After transformation of cells with 1 μg of the linearized pGEM-GAPDHp-WsFAO1, the cell pellet was suspended in 1 mL of yeast extract peptone dextrose (YPD) and incubated for 2 h at 30°C with continuous shaking at 180 rpm. Subsequently, the cells were spread on the yeast synthetic drop-out medium supplements without uracil (Y1501; Sigma-

Aldrich Co., St Louis, MO, USA) and incubated for 3 days at 30°C. Candidates were confirmed by colony PCR. Yeast colony PCR was performed to select transformants. Single colonies grown on the selection medium were suspended in 30 µL of distilled water, treated with 20 U of lyticase (SIGMA-ALDRICH Co., MO, USA), and incubated for 1 h at 37°C. The resultant solution was directly used as a template for PCR. The insertion of the expression cassettes into the chromosome was confirmed by colony PCR using primers URA3 out-F and CsFco mid-R (Supplementary Table 1).

Biotransformation of methyl laurate into DDDA

A 5-L fermenter system (CNS Inc., Daejeon, Korea) was utilized for lab-scale production of DDDA. Inoculation was performed by adding 10% (v/v) of the pre-culture of *C. tropicalis* strains, which was performed in a 2-L Erlenmeyer flask containing 200 mL of YPD media. The fermenter culture consisted of the following components per liter: 50 g of glucose, 5 g of sucrose, 10 g of corn steep powder, 10 g of yeast extract, 5 g of KH_2PO_4, 3 g of KNO_3, 0.5 g of NaCl, and 1 mL of trace elements (13.2 g/L of $CaCl_2 \cdot 2H_2O$, 8.4 g/L of $FeSO_4 \cdot 7H_2O$, 2.4 g/L of $MnSO_4 \cdot 4H_2O$, 2.4 g/L of $ZnSO_4 \cdot 7H_2O$, 0.48 g/L of $CuSO_4 \cdot 5H_2O$, 0.48 g/L of $CoCl_2 \cdot 6H_2O$, 0.24 g/L of $NaMnO_4 \cdot 2H_2O$, and 0.06 g/L of $K_2B_4O_7 \cdot 4H_2O$ in 1 M HCl). Upon depletion of the initial glucose, a feeding medium containing (per L) 800 g of glucose and 10 g of yeast extract was continuously supplied at the rate of 2–3 g/L/h. To trigger ω-oxidation, dodecane (Junsei Chemical, Kyoto, Japan) was introduced 4 h after the initiation of feeding, when the cell density reached an OD of approximately 70–80. After the consumption of dodecane, methyl dodecanoate was subsequently added as the substrate. The temperature was maintained at 30°C. The pH was maintained at 5.5 during the cell growth phase and subsequently increased to 7.8 for the biotransformation phase using 6 N NaOH to enhance the solubility of DDDA. Dissolved oxygen levels were maintained above 30% by increasing the agitation speed to enhance aeration.

This protocol was scaled up using a 300-L fermenter system (CNS Inc., Daejeon, Korea). Inoculation was performed by adding 10% (v/v) of the pre-culture, which was cultured in three 5-L Erlenmeyer flasks, each containing 500 mL of YPD media to reach a final volume of 1.5 L. The culture was then transferred to a 30-L fermenter containing 13.5 L YPD media, and subcultured cells were transferred to a 300-L fermenter containing 135 L production media, as described previously. Fermentation conditions in the 300-L fermenter, including the medium, temperature, pH, dissolved oxygen, and procedures, were consistent with those of the 5-L systems.

Separation and purification of DDDA

The culture solution was centrifuged to separate the cells from the medium. The supernatant was poured into 5- or 30-L scale reactors for precipitation and crystallization. The DDDA crystallization method employed the commonly used pH adjustment approach; 6 N H_2SO_4 was used to precipitate the DDDA from its alkaline dissolved state. Sulfuric acid (20% of the culture medium volume) was added, and the precipitated crude DDDA was washed with water (10 times the volume of the culture medium),

followed by drying in an oven at 80°C for 1 day. The direct crystallization purification process using acetic acid was performed as follows. The pH of the supernatant was adjusted to 4 by adding 10% acetic acid solution to precipitate DDDA. The precipitated DDDA was completely dissolved in acetic acid by heating the reactor to 90°C. Then, the dissolved DDDA solution was filtered using a filter paper with a pore size ≤ 1.0 μm. The filtered DDDA solution was immediately crystallized for purification. The DDDA solution was cooled at a rate of 30°C /min during the crystallization process.

Polymerization of PA 6.12

DDDA (Sigma-Aldrich Co., 99% or Verdezyne, 99.6% and Bio-derived DDDA purified in this study), hexamethylene diamine (HMDA) (99.5%; Acros Organics, Geel, Belgium), sodium hypophosphite (99%; Sigma-Aldrich Co.), and deionized water were used for the synthesis of poly(hexamethylene dodecanamide) (PA 6.12). For relative viscosity (RV) and gel chromatography (GPC) analysis, *m*-cresol was employed as the dissolution solvent (99% for RV [Alfa Aesar, Ward Hill, MA, USA]; 99% for GPC [Sigma-Aldrich Co.]). The polymerization of PA 6.12 was performed using a melting process in a 1-L-scale reactor. DDDA (0.65 mol), HMDA (0.66 mol), and sodium hypophosphite (0.13 mmol) were prepared in distilled water (47 wt% of the entire amount of reacting materials) and placed in the reactor. After the reactor was closed tightly, the oxygen inside the reactor was removed by nitrogen gas at room temperature. To obtain the nylon 612 salt, the reaction temperature was increased to 130°C and maintained for 30 min. After salt formation, prepolymerization was performed. The reactor was heated to 215°C, following which the pressure was gradually increased. Once the pressure reached 17.2 bar, water vapor was removed slowly to maintain this pressure. Consequently, the pressure did not increase further, and the water vapor was slowly eliminated to obtain an ambient pressure at 215°C. Prepolymerization was terminated when the ambient pressure was reached. Furthermore, the polymerization reaction was continuously performed at 270°C for 70–80 min using a temperature-raising time under 1 atm, and then for 10–15 min under full vacuum conditions. The reaction was stopped by monitoring the torque gauge. The synthesized PA 6.12 was extruded under nitrogen pressure.

Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm using a UVICON930 spectrophotometer (Kontron Analytical, Lucerne, Switzerland). To determine residual glucose concentrations, the supernatant of the culture broth was measured using a YSI 2900 model biochemistry analyzer (YSI, Yellow Springs, OH, USA). Analysis of dodecane, methyl laurate, dodecanoic acid, and DDDA was performed using a gas chromatograph (Master GC; Dani Instruments, Cologno Monzese, Italy) equipped with an RTX-5 column (Restek, Bellefonte, PA, USA). The oven temperature ranged from 70 to 237°C, while the injector and detector temperatures were maintained at 280 and 300°C, respectively. For sample preparation, a 100-mL aliquot of 6 N H_2SO_4 was added to 100 μL of the culture broth. The sample was mixed with 300 μL diethyl ether, with tetradecanedioic acid used as an internal

standard. Then, 100 µL of the upper organic layer was collected and analyzed using gas chromatography. For dodecanoic acid and DDDA, this organic layer was silylated with the same volume of *N,O*-bis-trimethylsilyl-trifluoroacetamide before gas chromatography analysis. The purity of purified DDDA was determined by gas chromatography based on the relative peak area of DDDA after derivatization. The successful synthesis of the polymer was confirmed by proton nuclear magnetic resonance spectroscopy (500 MHz; Agilent DD2, Agilent, Santa Clara, CA, USA) using 1,1,1,3,3,3-hexafluoro-2-propanol (99%; TCI Chemicals, Tokyo, Japan) and chloroform-d (99.8%; Cambridge, Tewksbury, MA, USA) solvents. Chemical shifts were expressed as parts per million. Melting temperature (Tm) and crystallization temperature (Tc) were measured by differential scanning calorimetry (DSC) (Q200; TA Instruments, New Castle, DE, USA). The DSC profiles were recorded by melting the sample gradually at a rate of 10°C/min, followed by cooling, and finally reheating at the same rate from 20 to 300°C under a nitrogen environment. RV was measured using an Ubbelohde viscometer (Witeg, Wertheim, Germany) at 25°C according to the method specified in ISO307. The concentration of the sample was 1 g/dL in *m*-cresol. Molecular weight was determined by performing gel permeation chromatography (GPC; PL220C; Agilent) at 100°C using *m*-cresol solvent and a polystyrene standard.

Results and discussion

Development of a production strain toward efficient DDDA production

We utilized methyl laurate (methyl ester dodecanoic acid), a bio-based substrate obtained through the transesterification of coconut oil with methanol and subsequent purification via vacuum distillation, facilitated by Lotte company (Korea). To convert methyl laurate into DDDA, we employed a β-oxidation-deficient strain of *C. tropicalis* as the production host, as it is an established industrial strain used in the DCA bioprocess using *n*-paraffin (Waltz, 2012). Within the ω-oxidation pathway of hydrocarbons, the ω-hydroxylase complex, comprising CYP monooxygenase and cytochrome P450 reductase (CPR), catalyzes the conversion of the terminal methyl group of hydrocarbon into a hydroxyl group (Fig. 1a). This step is widely recognized as the rate-limiting step in this process (Craft et al., 2003; Sumita et al., 2002). In a previous study, we enhanced C10 DCA (sebacic acid) production in *C. tropicalis* by overexpressing the CYP and CPR genes (Jeon et al., 2019). However, the FAO activity in *C. tropicalis*, representing the second step in the ω-oxidation pathway, showed higher levels of C12 fatty alcohol but significantly lower levels of 12-hydroxy fatty acid (HFA) (Cheng et al., 2005; Eirich et al., 2004). Notably, when fatty acids or methyl ester fatty acids are used as substrates, HFAs corresponding to the carbon number of the substrates were produced as an intermediate in the ω-oxidation pathway, instead of fatty alcohol (Fig. 2a). Furthermore, our recent research has revealed that in the presence of HFA, the consumption of fatty acids in the ω-oxidation reaction is 24–32% lower compared to when HFA is absent. This leads to the accumulation of toxic fatty acids, negatively impacting DCA production (Lee et al., 2019; Sugiharto et al., 2018). Recent studies have shown that overexpression of the FAO gene in the *C. tropicalis* strain increases the production of DDDA from dodecane without the formation of HFA

intermediates (Pham et al., 2023). Moreover, we recently confirmed that our isolated *W. sorbophila* strain exhibits remarkable ω-oxidation capability when exposed to medium-chain alkanes and fatty acid methyl ester (FAME) substrates (Liu et al., 2004). Consequently, we conducted a comparative analysis of the FAO1 activity derived from *C. tropicalis*, *W. sorbophila*, and *Y. lipolytica*, known for their specialization in fatty acid metabolism. This analysis focused on their performance regarding 12-HFA in *E. coli*. The results revealed that the FAO1 from *W. sorbophila* achieved a remarkable conversion rate of 61% over a 12-h period, starting from 10 mM of 12-HFA and ultimately leading to the production of DDDA. In contrast, FAO1s from *Y. lipolytica* and *C. tropicalis* exhibited conversion rates of 6.9% and 8.5%, respectively. The FAO1 from *W. sorbophila* (*Ws*FAO) exhibited a DDDA production capability that was more than seven times higher than that of other FAOs (Fig. 2b). To assess the potential positive impact of the *Ws*FAO on DDDA production during the fermentation process using the *C. tropicalis* strain, we codon-optimized the *Ws*FAO gene for *C. tropicalis* and site-specifically integrated the gene into the genome of *C. tropicalis* through URA3 split-marker recombination (Fig. 2c).

Pilot-scale biotransformation process for DDDA production

For large-scale DDDA production, we developed a comprehensive biotransformation process using methyl laurates as a substrate. The process was structured into three distinct phases: cell growth, activation of ω-oxidation metabolism with alkane addition, and the actual biotransformation from methyl laurate to DDDA (Fig. 3a). We compared the DDDA production capabilities of the strains with and without the insertion of WsFAO during the biotransformation process. The development and testing of this process were performed in both 5- and 300-L fermenters to assess its industrial feasibility.

In the first phase, glucose served as the initial carbon source, and the optical density of the cells reached approximately 60–70 at 600 nm (OD_{600}) when initial glucose was depleted, achieving a 12-h advancement compared to that in our previous study using glycerol as the initial carbon source. The second phase aimed to induce the expression of the enzymes involved in ω-oxidation. After initial glucose consumption, dodecane was added as an inducer, constituting 1.5% of the total culture volume. The pH was gradually increased from 5.5 to 7.8, the optimal pH for DDDA production (Jeon et al., 2016). Continuous glucose feeding at the rate of 2–3 g/L/h sustained cell maintenance and NADPH regeneration of the coenzyme of the P450 enzymes (Straathof, 2011). The final phase was designed for converting bio-based methyl laurate to DDDA. Methyl laurate was chosen owing to its low melting point (5°C) and reduced toxicity compared to lauric acid (melting point: 43°C). Since the temperature within the fermenter, where biotransformation occurs, was maintained at 30°C, the substrate was supplied in methyl ester form to ensure it remained in the liquid state. Additionally, it was observed that while dodecanoic acid exhibited toxicity even at a concentration of 0.2 g/L, the methyl ester form did not inhibit the growth of the strain at concentrations as high as 10 g/L (data not shown). To manage the toxic effects of the high substrate concentration on the cells, the substrate feed rate was controlled from 0.5 to 1.5 g/L/h (Fig. 3c). Through the above fermentation process, cultivating the parent strain and the WsFAO1-expressing strain (GK5 strain) resulted in similar levels of maximum cell density during the first stage of cell growth, with respective OD values of 68.1 and 63.8. Conversely, during the DDDA production

phase, for the parent strain, the substrate was fed at a rate of 0.5–1.4 g/L/h to prevent substrate accumulation. In the case of the GK5 strain, however, a higher substrate feeding rate of 0.65–1.5 g/L/h was achievable. As a result, the GK5 strain, expressing the WsFAO1 gene, demonstrated a DDDA concentration of 102 g/L with a volumetric productivity of 0.85 g/L/h, calculated based on the duration from substrate addition. Compared with the parent strain, the GK5 strain increased the DDDA production and productivity by 45% and 31%, respectively (Fig. 3c).

The developed biotransformation process for DDDA production was subsequently tested in a 300-L system to assess its feasibility for industrial production. The process in the 300-L fermenter replicated the same three phases described earlier. In the case of a 300-L culture, the scale-up from the 5-L culture necessitated a gradual increase in the shaking speed from 100 to 330 rpm to maintain the dissolved oxygen, whereas for the 5-L culture, the shaking speed was maintained between 200 and 900 rpm. In the DDDA production phase, similar to the 5-L setup, the substrate feeding rate was maintained at 0.8–1.5 g/L/h to prevent substrate accumulation. Consequently, both the substrate and 12-HFA did not accumulate for 72 h after substrate feeding. This resulted in a productivity of 1.07 g/L/h and a final titer of 105 g/L in the 300-L biotransformation process (Fig. 3d). In Table 2, we present a comparative overview of various studies on DDDA production using different substrates, strains, and production methods. Our study achieved the highest titer when using 12-FAME as the substrate and showcased reproducible results at the pilot scale (300 L). By introducing FAO from *W. sorbophila* into *C. tropicalis*, we successfully developed a robust production process, mitigating the accumulation of intermediates detrimental to DDDA production

Table 2
Summary of the results of some studies on DDDA production

Strain	Production condition	Substrate	Titer (g/L)	Scale (L)	Reference
C. tropicalis	Fed-batch culture	Methyl laurate	102	5	This study
C. tropicalis	Fed-batch culture	Methyl laurate	105	300	This study
C. tropicalis	Fed-batch culture	Methyl laurate	66	2	Funk et al. (2017)
C. tropicalis	Fed-batch culture	Dodecane	140	5	Picataggio et al. (1992)
C. tropicalis	Fed-batch culture	Dodecane	181.6	7.5	Cao et al. (2017)
C. tropicalis	Fed-batch culture	Dodecane	224	3	Pham et al. (2023)
C. cloacae	Fed-batch culture	Lauric acid	5	2	Green et al. (2000)
C. cloacae	Resting cells	Dodecane	8.2	0.5	Uchio et al. (1972)
Y. lipolytica	Fed-batch culture	Dodecane	11	0.6	Gatter et al. (2014)
Y. lipolytica	Batch culture	Dodecane	8	0.5	Smit et al. (2005)
E. coli	Resting cells	Lauric acid	0.16	0.25	Sathesh-Prabu et al. (2015)

Purification of polymer-grade DDDA through simplified process

To develop a practical biotechnological process for DDDA production, process simplification and operational efficiency are key considerations. In the case of fermentation products, the cost contribution of the downstream processes involved in their production approaches 50–55% at high-purity levels (Hang et al., 2013). Moreover, for practical industrial applications, downstream processing must be straightforward and designed to minimize waste. Based on these considerations, we developed a downstream process for DDDA extraction and purification from the fermentation broth.

To polymerize DDDA into nylon 6.12, it is imperative to establish effective separation and purification processes to achieve a high level of purity. DDDA possesses amphiphilic properties owing to hydrophobic carbon chains and hydrophilic carboxyl groups at its terminals. Consequently, the solubility of DDDA varies depending on the pH of the solution, causing it to precipitate at pH values below its pKa (4.5). Given that the pH reaches 7.8 at the end of the fermentation process, the produced DDDA exists in a soluble salt form (Fig. 4). Our initial step involved removing cells and solid particles from the

fermentation broth through centrifugation. Subsequently, to isolate the DDDA product from the fermentation broth, we used a crystallization process through acid precipitation with sulfuric acid. An experiment to determine the required amount of sulfuric acid revealed that the molar amount of sulfuric acid should be 1.25 times its theoretical molar amount to completely precipitate the DCA within the DDDA present in the culture medium. At this point, the DDDA in the precipitates had a purity of 83%. The sodium sulfate (Na_2SO_4) generated during the DDDA precipitation process with sulfuric acid exhibited reduced solubility in water at or below 32°C. To remove Na_2SO_4, the precipitates containing DDDA were washed with water at 32°C, following which the DDDA purity increased by 4.7%. The b-value (the degree of yellowness) and L-value (the degree of whiteness among color values) decreased by 21% and increased by 16%, respectively. Additionally, the content of inorganic elements, namely Na and S, decreased by over 90%. However, it is imperative to acknowledge that the utilization of strong acids such as sulfuric acid in this process entails several drawbacks, encompassing safety concerns, fixed facility costs, and operating expenses. Furthermore, the challenge of managing wastewater containing Na_2SO_4 necessitates attention. Approximately 30 L of wastewater, containing 50 g/L of Na_2SO_4, has been reported to be discharged per ton of sebacic acid produced (Cho et al., 1996).

To mitigate these challenges, we adopted a direct crystallization process involving continuous precipitation and crystallization, using acetic acid as the solvent for precipitation and crystallization. The direct crystallization and purification process minimizes the cost incurred during purification by obviating the need for culture solution discoloration and water-washing process. Furthermore, this approach prevents the formation of insoluble inorganic salts such as Na_2SO_4, which commonly occurs when using sulfuric acid. This contributes to increasing the purity of DDDA. Thus, implementing this enhanced direct crystallization and purification process yielded a DDDA purity of 99.2%, which is comparable to that of the commercial product (Verdezyne). Additionally, the L^* value measured at 97.83 was equal to or superior to that of the commercial product within the margin of error (Table 3). To perform separation and purification of high-purity DDDA from a DDDA culture medium prepared by pilot-scale (300 L) fermentation, we employed a direct crystallization process. In the scale-up process, cells were initially removed from 200 L of the culture medium in a 300-L fermenter, resulting in 170 L of the culture medium. The concentration of DDDA in the separated culture medium was 105 g/L, and, ultimately, 18.7 kg of crude DDDA was obtained from the culture medium by acetic acid precipitation. Then, 3.7 kg of the crude DDDA was dissolved in 20 L of acetic acid, recovering 2.5 kg of DDDA through the crystallization process. Considering that the purity of crude DDDA is 97.4%, the recovery yield is relatively low at 69.3%. However, this can be improved by implementing a recycling process. Throughout the scale-up process, the purity of DDDA remained consistently high at 99.4%, which was equal to or greater than that of the commercial product (Verdezyne). An excellent L^* value of 97.99, within the margin of error of that of the commercial products (Verdezyne), was obtained. The b^* value was 0.60 lower, which was also within the margin of error, compared to that of the commercial product (Verdezyne). Therefore, the purity and color of the obtained DDDA were equivalent or superior to those of the existing commercial products.

Table 3
Comparison between the direct crystallization and purification method and the conventional purification method

	Precipitation process	Discoloration process	Washing process	Purity (%)	Color		
					L*	a*	b*
Verdezyne DDDA	Sulfuric acid	0	0	99.4	96.8	0.10	0.74
DDDA (in this study)	Sulfuric acid	X	X	99.7	97.5	0.03	1.33
DDDA (in this study)	Acetic acid	X	X	99.2	97.8	0.06	0.87

Polymerization of PA 6.12 using bio-DDDA

The condensation polymerization of nylon typically involves hydrolytic polymerization using water as a solvent, with phosphate catalysts, monoacids, and monoamines serving as terminators (Hang et al., 2013). In this study, we utilized sodium hypophosphite (NaH_2PO_2) and phosphoric acid (H_3PO_4) as catalysts, while acetic acid and *N*-butylbenzenesulfonamide were employed as terminators. After synthesizing the salt with purified bio-DDDA, we opted for the salt method for polymerization because it facilitates an increase in the degree of polymerization, which is easier than direct polymerization from monomers. We established the optimal polymerization conditions using 0.65 moles of salt and 0.02 moles of catalyst. Under these ideal conditions, bio-PA 6.12 was synthesized, exhibiting a Tm and Tc of 216 and 186°C, respectively. The number-average and weight-average molecular weights were determined to be 111 kDa and 195 kDa, respectively (Table 4) (Fig. 5c, d). These results indicate that the chemical properties of the bio-PA 6.12 synthesized in this study are almost similar to those of the commercially available bio-PA 6.12, with only a slightly lower molecular weight. The bio-PA 6.12 synthesized in this study exhibits excellent durability and heat resistance, making it suitable for various industrial applications. It can be utilized in automotive components, including engine parts, fuel systems, and brake systems, as well as in the textile industry for products such as sports apparel and socks, highlighting its versatility.

Table 4
Comparison of PA 6.12 from bio-derived DDDA and commercial DDDA

DDDA	T_m (°C)	T_c (°C)	RV	M_n (g/mol)	M_w (g/mol)	PDI
Bio-DDDA Purified (99.4%)	216	186	3.2	111K	195K	1.8
Commercial (Verdezyne, >99.4%)	215	187	3.7	146K	217K	1.5

M_n, number-average molecular weight; M_w, weight-average molecular weight; RV, relative viscosity; T_c, crystallization temperature; T_m, melting temperature

Conclusions

We successfully established a robust production process for DDDA by integrating FAO from *W. sorbophila* into *C. tropicalis*. This strategic bioengineering approach effectively mitigated the accumulation of intermediates that hinder DDDA production. Utilizing 12-FAME as the substrate, our process achieved one of the highest reported DDDA titers and successfully maintained its performance upon scale-up from a 5-L to a 300-L fermenter. In the fed-batch production using engineered *C. tropicalis*, we attained a significant DDDA yield of 102 g/L from renewable plant oil, with a productivity rate of 0.85 g/L/h in a 5-L system. This efficiency was further validated in a 300-L bench-scale system, where we observed even higher production values.

Our advancements in the separation and purification processes, particularly through acid precipitation and crystallization, have streamlined the procedure to achieve DDDA with commercial-level purity. This research paves a promising path for the sustainable polymerization of PA 6.12 using bio-derived DDDA. Our findings signify a substantial step toward environmentally friendly nylon production and highlight the potential of bio-based materials for diverse industrial applications. The bio-PA 6.12 synthesized in this study, notable for its durability and heat resistance, shows great promise for use in automotive components and the textile industry. This research contributes significantly to the field of green and sustainable chemistry, and we believe it will inspire further advancement in this field.

Abbreviations

CPR, cytochrome P450 reductase

CYP, cytochrome P450

DSC, scanning calorimetry

DDDA, dodecanedioic acid

DCA, a,w-dicarboxylic acid

FAME, fatty acid methyl ester

FAO, fatty acid oxidase

GPC, gel chromatography

HMDA, hexamethylene diamine

PA, polyamide

PE, polyester

RV, relative viscosity

Tc, crystallization temperature

Tm, melting temperature

YPD, yeast extract peptone dextrose

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The data that support the findings of this study are included within the article and the supplementary data.

Competing interests: The authors declare that they have no competing interests.

Funding: This study was supported by a grant from the KRIBB Research Initiative Program, Bioindustry Core Technology Development Project (RS-2024-00467186), Material and Component Technology Development Project (RS-2024-00432188), Industrial Technology Alchemist Project (20025698) funded by the Ministry of Trade, Industry, Energy (Republic of Korea) and 2026 Hongik University Innovation Support program Fund, and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: RS-2025-02263556).

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Ji-Yoon Hyun: Investigation, Validation

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Hong-Weon Lee: Supervision, Project administration, Funding acquisition, Writing – review & editing

Jung-Oh Ahn: Supervision, Project administration, Funding acquisition, Writing – review & editing

Acknowledgements: Not applicable.

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Figures

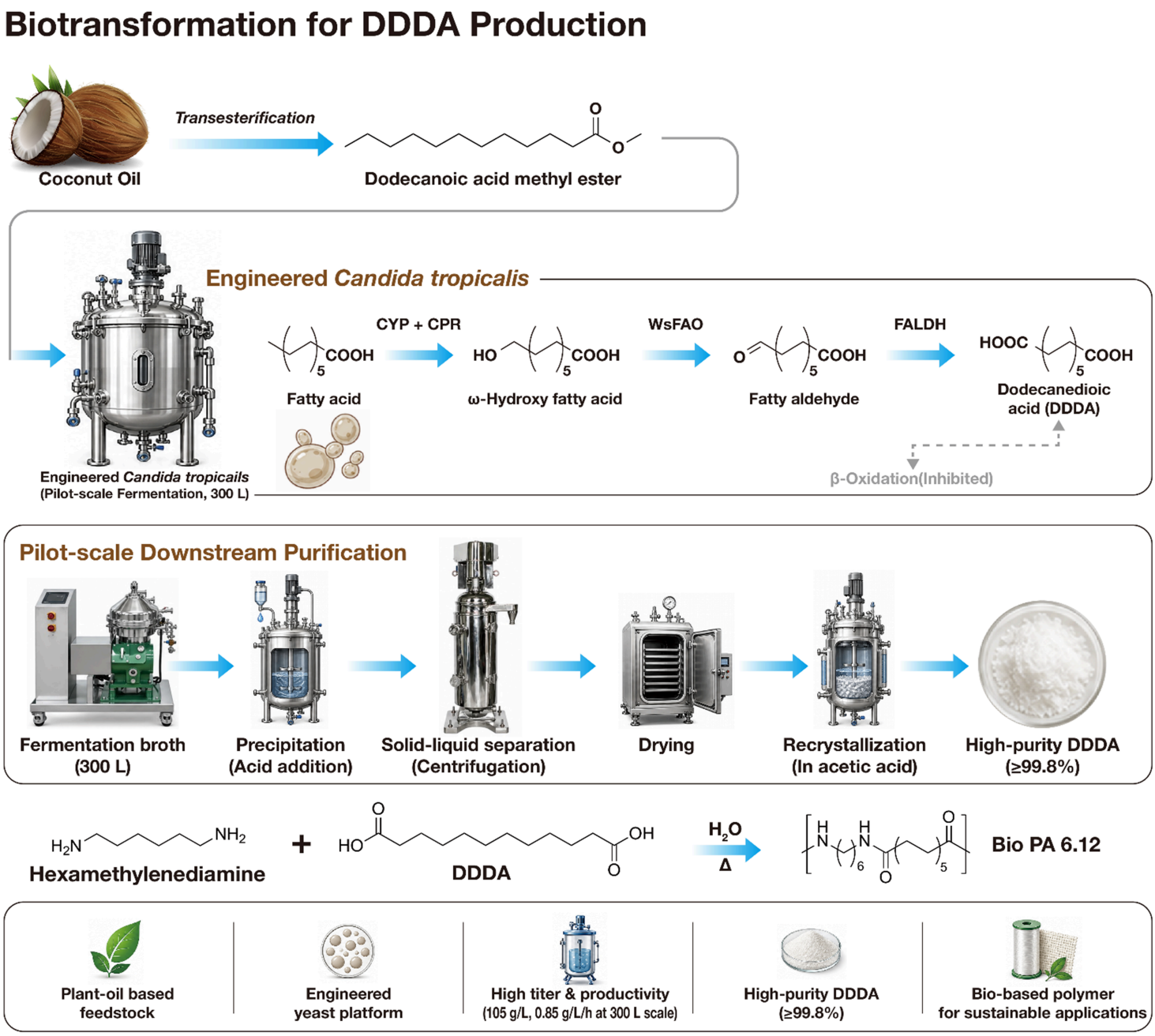


Figure 1

Overview of the integrated bioprocess for the production of bio-based dodecanedioic acid (DDDA) and bio-nylon 6.12 from coconut oil-derived methyl laurate. The process comprises substrate preparation, microbial biotransformation using engineered *Candida tropicalis*, pilot-scale fermentation (300 L), downstream purification to obtain high-purity DDDA, and subsequent polymerization into bio-based nylon 6.12.

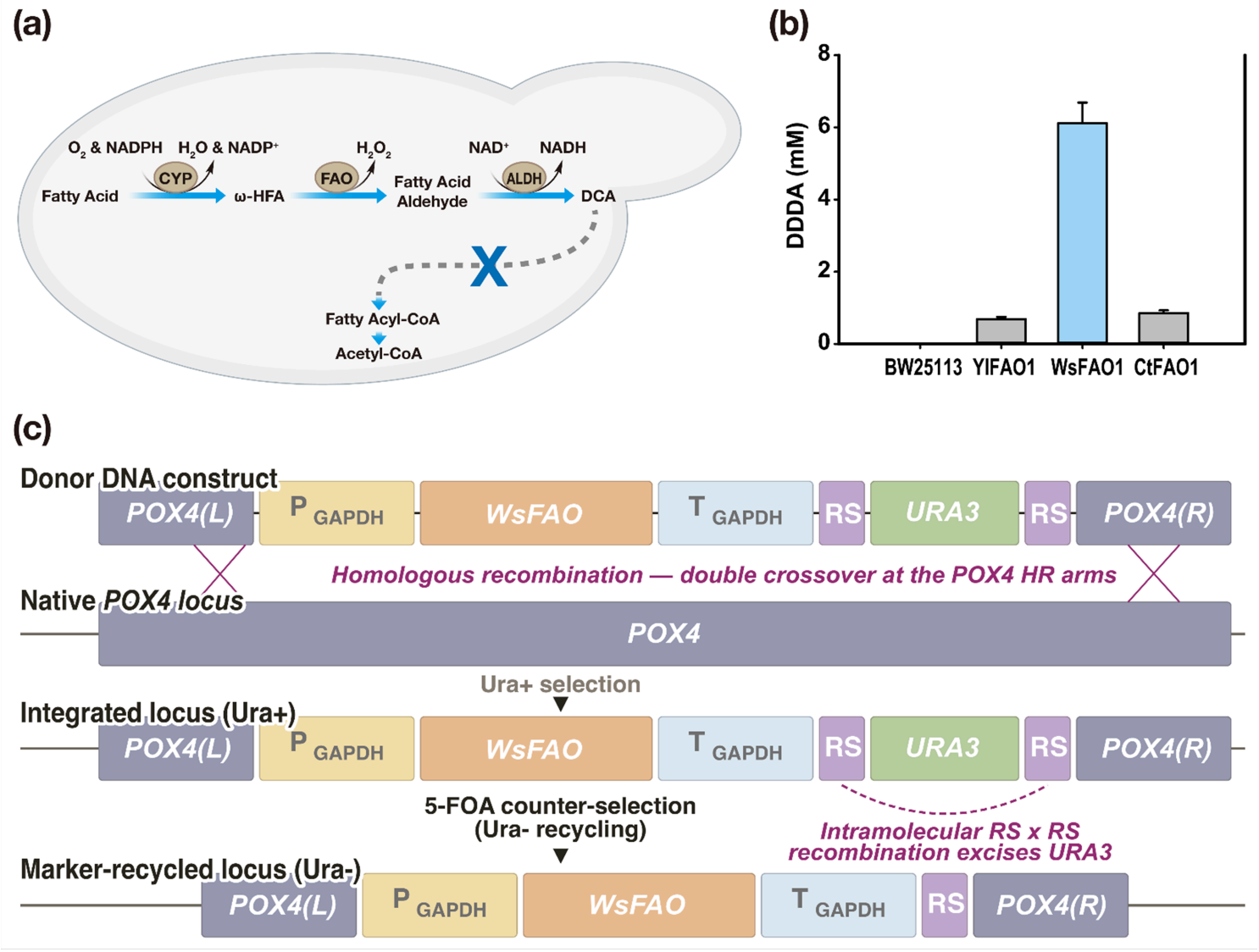


Figure 2

Engineering β-oxidation-blocked *C. tropicalis* 20962 through selected heterologous fatty acid oxidase (FAO) expression in whole-cell biotransformation. (a) Pathway of a,w-terminal and β-oxidation of fatty acids in *C. tropicalis*. (b) Formation of DDDA from 10 mM 12-HFA using resting *E. coli* cells expressing FAO1s from *Yarrowia lipolytica*, *Wichermyces sorbophila*, and *C. tropicalis*. (c) Schematic representation of the expression cassettes and chromosome integration by homologous recombination in *C. tropicalis*.

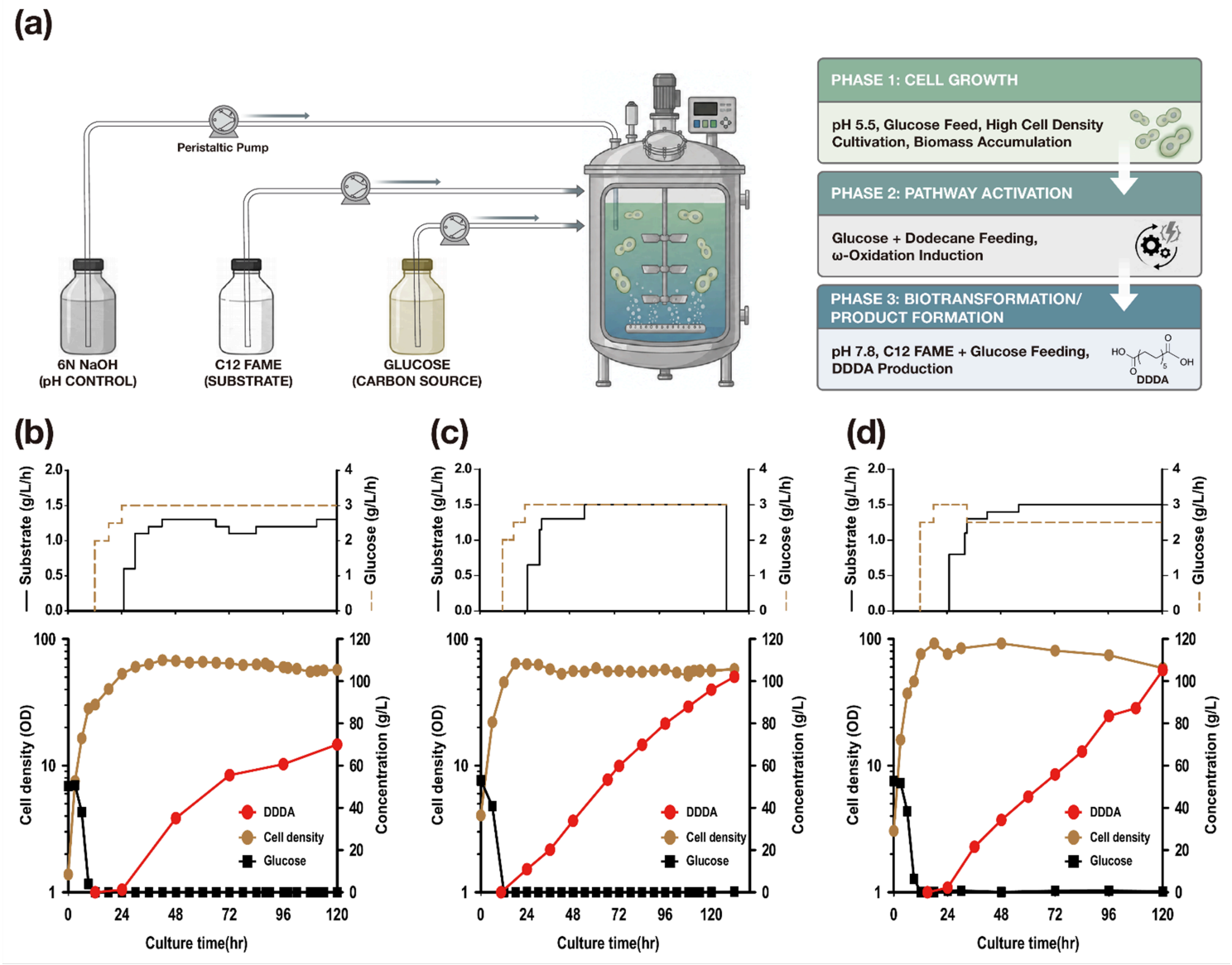


Figure 3

Microbial DDDA production from C12 FAME. (a) Schematic representation and the three phases of microbial DDDA production process. (b) DDDA production in the 5-L fermenter system with *C. tropicalis* 20962. (c) DDDA production in the 5-L fermenter system with heterologous FAO-expressed strain. (d) DDDA production in the 300-L fermenter system with heterologous FAO-expressed strain.

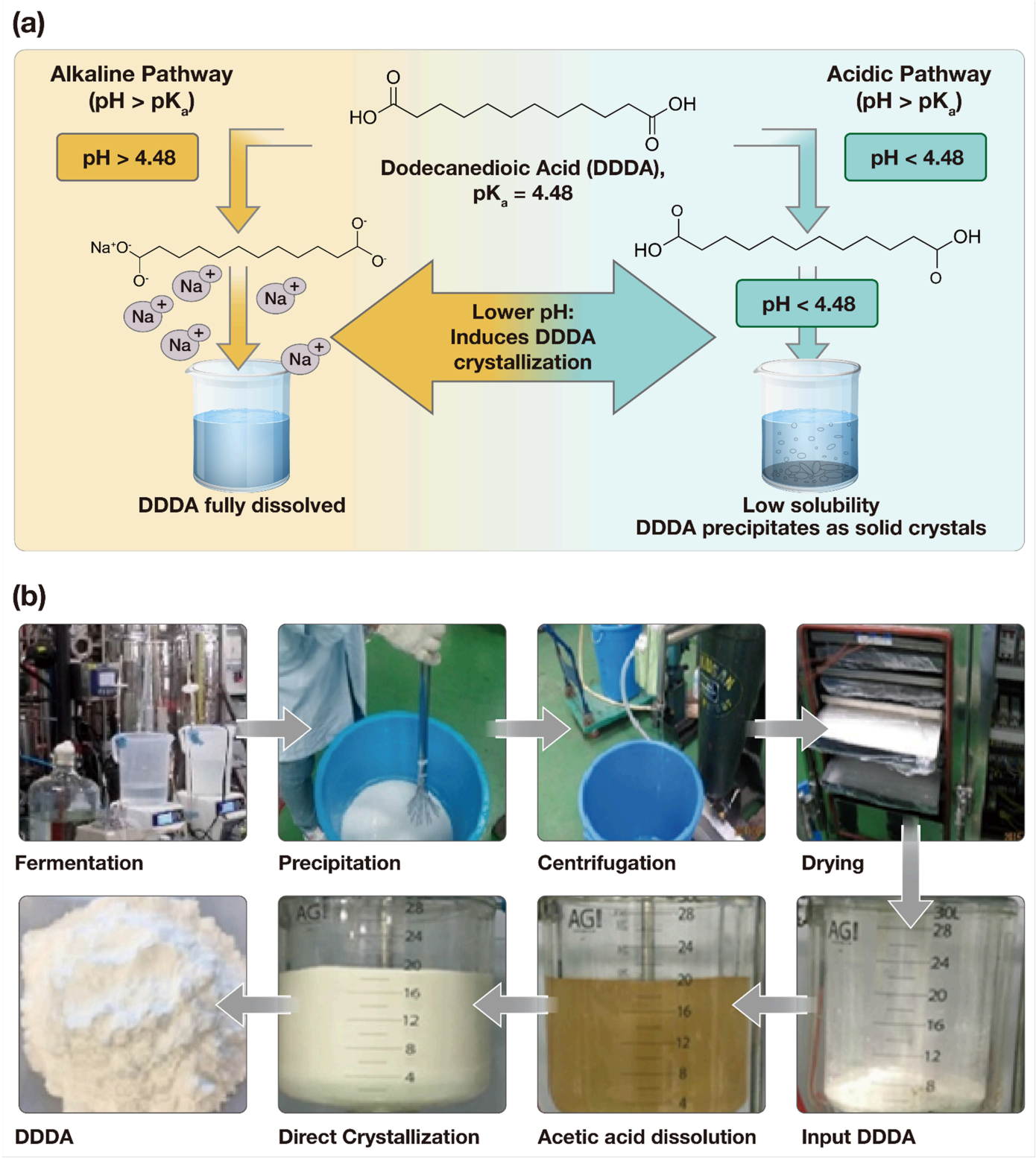


Figure 4

Separation and purification of DDDA from fermentation broths. (a) The precipitation mechanism of DDDA in the fermentation broth. (b) Process flow diagram of separation and purification. The simplified purification process employs direct crystallization via acetic acid precipitation, eliminating the need for decolorization, precipitation, and washing steps.

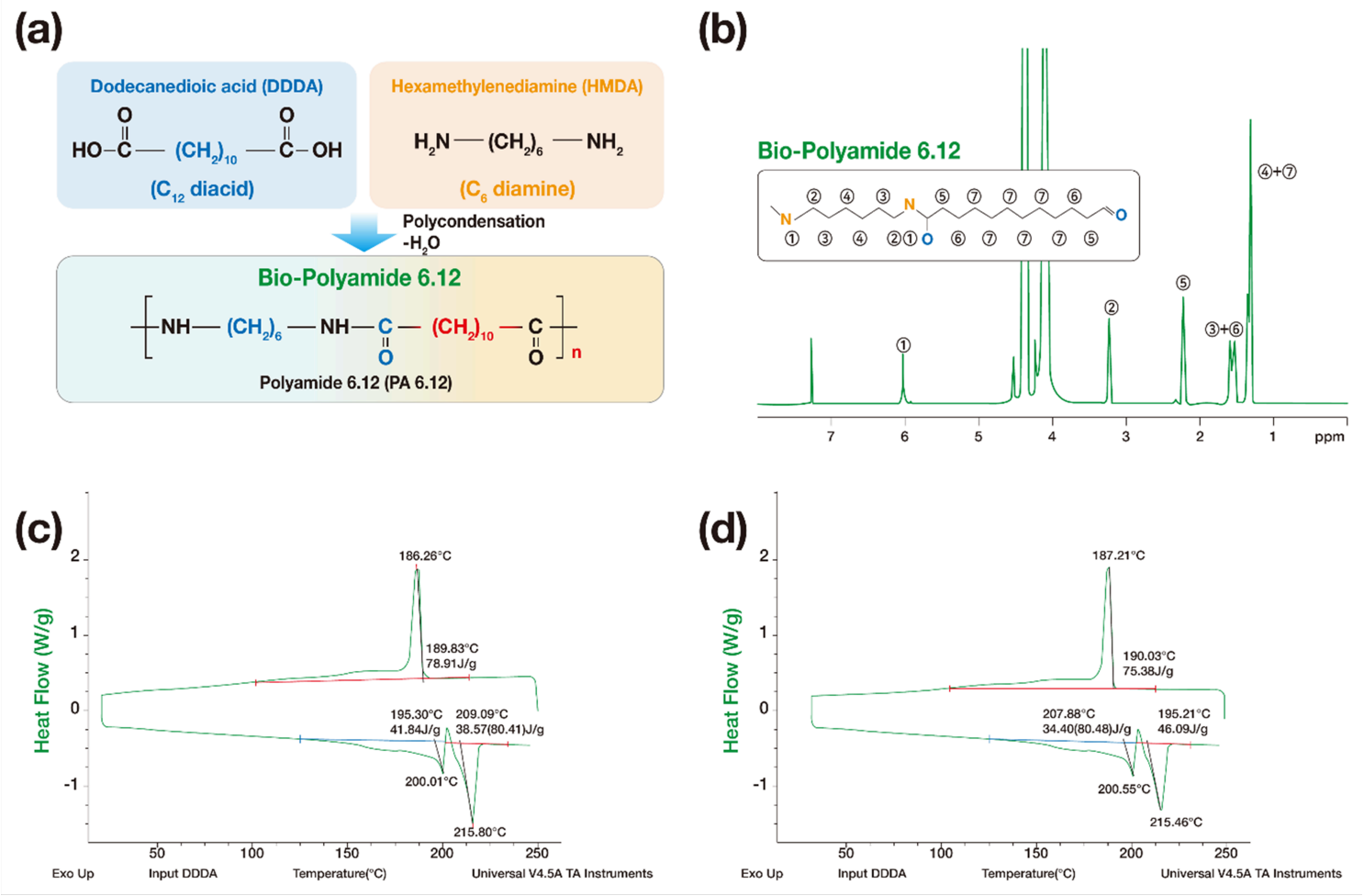


Figure 5

Polymerization of bio-based nylon 6.12. (a) Schematic representation of polymerization. (b) 1H-NMR spectra. (c) DSC profiles of PA 6.12 with purified DDDA. (d) DSC profiles of PA 6.12 with commercial DDDA.

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