

Cite this: *Green Chem.*, 2019, **21**, 6491

# Microbial production of sebacic acid from a renewable source: production, purification, and polymerization†

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Sebacic acid (SA) is an aliphatic ten-carbon dicarboxylic acid (1,10-decanedioic acid) with a variety of industrial applications, including the production of plasticizers, lubricants, cosmetics, and plastics. Currently, SA is produced exclusively from alkaline pyrolysis of castor oil. Herein, we present an environmentally friendly green route of SA production from plant oil-derived sources by microbial  $\omega$ -oxidation. We genetically engineered  $\beta$ -oxidation-blocked diploid yeast *Candida tropicalis*, and created an effective microbial cell factory with an increase of 46% in SA production by overexpression of genes involved in  $\omega$ -oxidation of hydrocarbons compared to the original strain. A biotransformation process of SA production from decanoic acid methyl ester was developed to overcome the challenges of high-density cell culture, substrate feed, substrate/intermediate toxicity, and foam generation. Fed-batch production of engineered *C. tropicalis* resulted in a molar yield of above 98%, a productivity of 0.57 g L<sup>-1</sup> h<sup>-1</sup>, and a final titre of 98.3 g L<sup>-1</sup> in a 5-litre fermenter and the results were successfully reproduced using a larger scale 50-litre fermenter. The produced SAs were successfully purified to >99.8% using acid precipitation and recrystallization. Finally, bio-nylon 610 was successfully synthesized by polymerization of the purified SA with hexamethylenediamine and showed thermal properties very similar to those of commercially available nylon 610. The processes developed and described in this study can be employed to produce and isolate SA for the synthesis of bio-nylons, using environmentally friendly procedures based on microbial biotransformation with potential industrial applications.

Received 4th July 2019,  
Accepted 31st October 2019  
DOI: 10.1039/c9gc02274k  
rsc.li/greenchem

## Introduction

Sebacic acid (SA) is an aliphatic saturated 10-carbon dicarboxylic acid (DCA) with multiple applications.<sup>1</sup> Its most common application in the plastics industry is the production of polyamide polymers (e.g. PA 6,10, PA 4,10, and PA 10,10) where it confers important properties such as flexibility, hydrophobicity, durability, and low melting temperatures. SA is also

applied to dibasic polyesters to yield good flexibility, stability, chemical resistance, and solvent resistance.<sup>2</sup> Moreover, SA is used in the production of lubricants, such as dioctyl sebacate (DOS) and dihexyl sebacate (DHS).<sup>3</sup> Another interesting application of SA is the development of chitosan- and collagen-based 3D scaffold materials, which are biocompatible and mechanically resistant.<sup>4</sup>

Most of the worldwide SA production occurs in China (over 20 000 metric tons are exported each year, more than 90% of the global trade).<sup>5</sup> It has been traditionally produced by chemical cleavage of ricinoleic acid or castor oil to SA and capryl alcohol (2-octanol) under high temperature (about 280 °C) and alkaline conditions (caustic pyrolysis).<sup>6</sup> This process consumes considerable amounts of H<sub>2</sub>SO<sub>4</sub> and releases Na<sub>2</sub>SO<sub>4</sub>-containing solutions, which represent serious environmental risks.<sup>7</sup> Additionally, Casda Biomaterials Co., Ltd, the world leader in SA manufacture, announced increases in the price of SA due to the strong decline in the price of 2-octanol, a by-product of SA production.<sup>8</sup> Nowadays, China's new environmental policy has

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†Electronic supplementary information (ESI) available: Data on impurity characteristics. See DOI: 10.1039/c9gc02274k

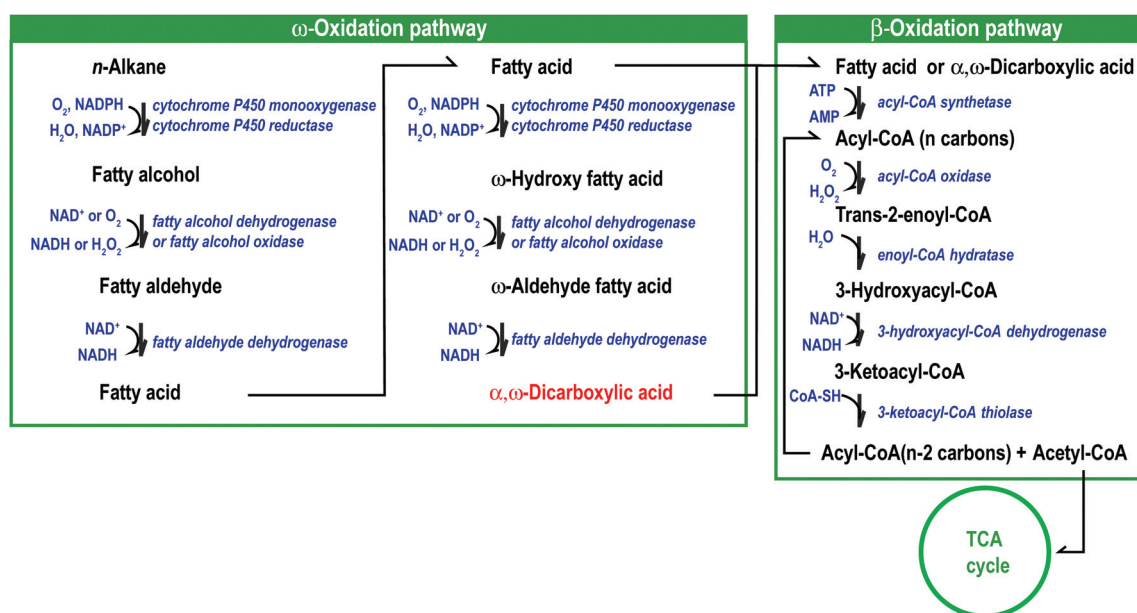
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been associated with factory shutdowns and arrested chemical production of several compounds, including SA, owing to environmental regulations.<sup>9</sup>

Microbial biotransformation and other biotechnological approaches are gaining importance and have been increasingly used to overcome the limitations and environmental issues associated with chemical processes. Several natural metabolic pathways have the potential to be explored for production of a wide variety of molecules with several biotechnological applications. Yeast, for example, can convert long-chain fatty acids and *n*-alkanes into the corresponding DCAs *via* the  $\omega$ -oxidation pathway, whereby terminal ( $\omega$ -position) carbons are oxidized (Fig. 1).<sup>10,11</sup> However, DCAs are naturally degraded by  $\beta$ -oxidation in the peroxisome, where their aliphatic backbone is continuously shortened *via* the cyclic cleavage of two carbon acetyl-CoA moieties. The resulting acetyl-CoA molecules are then metabolized through the tricarboxylic acid cycle, which sustains growth or energy production.<sup>12</sup> Using genetic engineering techniques, one can block or stimulate a given pathway, resulting in the accumulation of one or more desired metabolites. Microbial biotransformation of long-chain DCAs (C12–C18) from *n*-alkane or fatty acids has been performed in various alkane-assimilating yeasts like *Candida tropicalis*, *Yarrowia lipolytica*, *Torulopsis bombicola*, *Candida cloacae*, and *Wickerhamiella sorbophila* by blocking the  $\beta$ -oxidation pathway.<sup>13–17</sup> Moreover, non-alkane-assimilating *Saccharomyces cerevisiae* was engineered for the production of DCAs from fatty acids by a synthetic  $\omega$ -oxidation pathway.<sup>18</sup> Among bacteria, alkane-assimilating *Pseudomonas aeruginosa* converted *n*-pentadecane to the corresponding DCA (C15).<sup>19</sup>

*Escherichia coli* was engineered for the production of DCAs (C12 and C14) by heterologous introduction of the  $\omega$ -oxidation pathway.<sup>20</sup> However, their DCA titres were much lower ( $<1 \text{ g L}^{-1}$ ) than those of the engineered alkane-assimilating yeast strains. Engineering of  $\beta$ -oxidation reversal in *E. coli* was exploited to produce SA from glucose, but the titre was only  $0.061 \text{ g L}^{-1}$ .<sup>21</sup> A strain of *S. cerevisiae*, engineered by introducing a heterogeneous cytochrome P450, produced SA with a very low titre of  $0.012 \text{ g L}^{-1}$ .<sup>22</sup>

*Candida tropicalis* has been used as a sole strain for industrial production of long-chain DCA because of its prominent ability to oxidize alkanes and fatty acids due to strong  $\omega$ -oxidation activity, as reviewed by Huf *et al.* (2011) and Lee *et al.* (2019).<sup>23,24</sup> *C. tropicalis* have 10 cytochrome P450 genes, (CYP52A12-20, CYP52D2) which have various substrate specificities and activities depending on the carbon length of the substrates.<sup>1,25</sup> This yeast species have been metabolically engineered to transform long-chain fatty acids into DCAs by blocking the  $\beta$ -oxidation pathway.<sup>13</sup> The amplification of genes encoding  $\omega$ -hydroxylase complex components, which catalyse the rate-limiting reaction in  $\omega$ -oxidation pathway, enhances the titre and productivity of DCA by *C. tropicalis*.<sup>26</sup> In addition, the production of DCA by microorganisms can be improved by the optimization of parameters, such as medium composition, culture conditions, and substrate feeding strategies.<sup>27–30</sup> Recently, we investigated the toxicity of decanoic acid and 10-hydroxydecanoic acid on *C. tropicalis*. To improve the production of SA, we used the continuous substrate feeding method and the titre was  $34.5 \text{ g L}^{-1}$ .<sup>31</sup> These studies have improved the production of long-chain DCAs with high titres



**Fig. 1** Oxidation pathways for *n*-alkanes and fatty acids in yeast. The oxidation of *n*-alkane and fatty acids can occur by the  $\omega$ -oxidation pathway of the terminal methyl group to yield the  $\alpha, \omega$ -dicarboxylic acid. Fatty acids and  $\alpha, \omega$ -dicarboxylic acids are subjected to subsequent  $\beta$ -oxidation. Deletion of the genes encoding acyl-CoA oxidase in  $\beta$ -oxidation is intended to solely oxidize *n*-alkane or fatty acids by  $\omega$ -oxidation, thereby allowing long-chain  $\alpha, \omega$ -dicarboxylic acid to accumulate.

and productivity. Recently, Cathay Biotechnology Inc. (Shandong, China), leading microbial production of DCAs, is currently producing C11-C18 long-chain DCAs from paraffin as raw material, with an annual production of 20 000 metric tons.<sup>32,33</sup> Unlike other long-chain DCAs, relatively little research has been carried out on the biological production of SA.

Herein, we report an industrially applicable biotransformation protocol using a genetically engineered *C. tropicalis* strain to produce SA from vegetable oil-derived substrates. Its separation and purification process was also optimized, and the purified SA molecules were polymerized for the synthesis of bio-based nylon.

## Materials and methods

### Microorganisms

*Escherichia coli* DH5 $\alpha$  [F<sup>−</sup> *endA1 hsdR17*(r<sub>K</sub><sup>−</sup>m<sub>K</sub><sup>−</sup>) *supE44 thi-1*  $\lambda$ <sup>−</sup> *recA1 gyrA96*  $\phi$ 80*dlacZ* $\Delta$ M15] was used as the host strain for cloning and manipulation of plasmids. For the production of SA, *C. tropicalis* strains used in this study are shown in Table 1. These strains were genetically engineered from the  $\beta$ -oxidation-blocked ATCC® 20962<sup>TM</sup> strain obtained from the American Type Culture Collection (Manassas, VA, USA).

### Amplification of genes involved in $\omega$ -oxidation pathway

The gene encoding orotidine 5-phosphate decarboxylase (*URA3*) was amplified by PCR using as template the genomic DNA of ATCC® 20336<sup>TM</sup> and primers URA-1 and URA-2, which were designed based on the *URA3* sequence (GenBank accession number AB006207). *URA3* was inserted between the *ApaI* and *MluI* sites of pGEM-T Easy Vector (Promega, Madison, WI, USA) using infusion cloning kit (Takara, Kusatsu, Japan). The resulting plasmid was termed as pGEM-*URA3*. For pop-out of the *URA3* selection marker by DNA recombination, two identi-

cal *hisG* DNA sequences from *Bacillus subtilis* were amplified by PCR using two sets of primers SA-1/SA-2 and SA-3/SA-4. *HisG* fragments were inserted between the *BglII* and *EcoRI* sites on left side of *URA3* and *NotI* and *BamHI* sites on right side of *URA3* in pGEM-*URA3* respectively. The resulting plasmid was termed as pGEM-HUH.

The promoter-CYP52A19-terminator was amplified by PCR using genomic DNA of ATCC® 20336<sup>TM</sup> as template and primers CYP-1 and CYP-2. The promoter-CYP52A19-terminator fragment was inserted between the *BglII* site of pGEM-HUH using infusion cloning kit (Takara, Kusatsu, Japan) and the resulting plasmid was termed as pC19HUH. The promoter-CYP52A18-terminator and promoter-CPRB-terminator were amplified by PCR using the same genomic DNA as template using primers CYP-3/CYP-4 and CPR-1/CPR-2 respectively. Each fragment was inserted between the *BglII* site of pGEM-HUH in the same manner, the resulting plasmids were termed as pC18HUH and pCRHUH respectively. The promoter-FAO-terminator was constructed by fusion PCR with TEF promoter and FAO-terminator. TEF promoter and FAO-terminator were amplified by PCR using primers TEF-1/TEF-2 and FAO-1/FAO-2 respectively. The promoter-FAO-terminator was inserted between the *BglII* site on pGEM-HUH, and the resulting plasmid was termed as pFOHUH. The constructed plasmid maps for each expression cassette are shown in the ESI Fig. S1.†

For the transformation of *C. tropicalis* an expression cassette containing the target gene was inserted into the genome site-specifically. The uracil auxotroph strain was constructed by disrupting the *URA3* selection marker gene of *C. tropicalis* ATCC® 20962<sup>TM</sup> for the insertion of the cassette. Each expression cassette containing the selectable marker gene was inserted into the *Candida* genome through homologous recombination with a sequence homologous to a portion of *POX2*, *POX4*, and *POX5* genes encoding acyl-CoA oxidases. The URA<sup>+</sup> transformants selected from the YNB plate were cultured on the YNB-5FOA plate to pop-out the selectable marker gene. The resulting *URA3* auxotroph was transformed again by insertion of an additional expression cassette. The primers used in this study are shown in Table 2.

**Table 1** *Candida tropicalis* strains used in this study

Strain	Genotype
Ct0 (ATCC 20962)	<i>URA3/URA3 POX2/POX2 pox4/pox4 pox5/pox5</i>
Ct1	<i>ura3/ura3 POX2/POX2 pox4<math>\Delta</math>::CYP52A18-URA3/pox4 pox5/pox5</i>
Ct2	<i>ura3/ura3 pox2<math>\Delta</math>::CYP52A19-URA3/POX2 pox4/pox4 pox5/pox5</i>
Ct3	<i>ura3/ura3 POX2/POX2 pox4/pox4 pox5<math>\Delta</math>::CPRB-URA3/pox5</i>
Ct4	<i>ura3/ura3 pox2<math>\Delta</math>::CYP52A19/POX2 pox4/pox4 pox5<math>\Delta</math>::CPRB-URA3/pox5</i>
Ct5	<i>ura3/ura3 pox2<math>\Delta</math>::CYP52A19/POX2 pox4<math>\Delta</math>::CYP52A18/pox4 pox5<math>\Delta</math>::CPRB-URA3/pox5</i>
Ct6	<i>ura3/ura3 pox2<math>\Delta</math>::CYP52A19/POX2 pox4/pox4<math>\Delta</math>::FAO1-URA3 pox5<math>\Delta</math>::CPRB/pox5</i>
Ct7	<i>ura3/ura3 pox2<math>\Delta</math>::CYP52A19/POX2 pox4<math>\Delta</math>::CYP52A18/pox4<math>\Delta</math>::FAO1-URA3 pox5<math>\Delta</math>::CPRB/pox5</i>

### Preparation of C10 fatty acid methyl ester (FAME)

Decanoic acid derived from plant oil was purchased in bulk from Emery Oleochemicals (Teluk Panglima Garang, Malaysia). Decanoic acid (100 kg), 55.8 kg of methanol (55.8 kg) and *p*-toluene sulfonic acid (1 kg) were injected into a reactor and then refluxed for 7.5 h. After removal of residual methanol in the reactor by evaporation, 55.8 kg of methanol was injected into the reactor and reflux was performed for 6 h. The same procedure was repeated with 28 kg of methanol and 1 hour of reflux. Then, 157 g of NaOH and 20 kg of distilled water were added to the reactor, treated at 60 °C for 30 min, and the lower NaOH solution was removed after layer separation. Distilled water (20 kg) was added to the reactor and the lower layer of distilled water was removed through heat treatment and layer separation in the same manner as before. Next, the remaining crude C10 fatty acid methyl ester (FAME) was

Table 2 Primers used in this study

Primers	Sequences (5' → 3')	Description
Ura-1	ccgcacctccgaattcgatctggttgattgttgag	URA3 cloning
Ura-2	accggtctggcgccgcgatcttgaagctctgttg	
SA-1	aattgggcccagatctcagaccggttcagacaggat	
SA-2	aaaccagatcgaattcggaggtgcggatatgaggta	
SA-3	ttcaagatcgcggcgccagaccggttcagacaggat	pop-out vector cloning
SA-4	tccaacgcgtggtatccggaggtgcggatatgaggta	
CYP-1	aattgggcccagatctggcgacacactttcaacgg	
CYP-2	aaccggtctgagatctcgttcacgttgtaggcacgc	
CYP-3	aattgggcccagatcttcaatgctggcgcccg	CYP52A18 cassette
CYP-4	aaccggtctgagatctgggggattttgcatgcacc	
CPR-1	aattgggcccagatctccaccctcgcaggat	CPRb cassette
CPR-2	aaccggtctgagatctccaatgccaatgccaagaagt	
TEF-1	aattgggcccagatctcgcagaagaataacattagtaataga	TEF promoter
TEF-2	gccatattggtgattatttttttagatgttagag	
FAO-1	ggtatgtagctcgcagctacaacttggtctgttccaag	FAO1 cassette
FAO-2	gaaataatcaatccatctggtccatttttcccgcac	
SA-5	gtcacgaccaccaacaaaag	Cassettes insertion
SA-6	ccagaccgttgaagtgtgtcgcccccgaacaaccataaagctgc	
SA-7	atcgctacctcatatccgcacctcccaagttctccaaggacttg	
SA-8	cgagtgcagaacgggtcaac	
SA-9	cctgacctagatcatccatc	
SA-10	aacaagtcggggcccagcattgaagtgttgatgacaaactcgtcag	
SA-11	atcgctacctcatatccgcacctccgcaagaccgtcagaggttcc	
SA-12	acaatcatgtcggattgttg	
SA-13	cgtacgagcctctgatattcttg	
SA-14	gctctattactaatgttatttttgcatacaactagcgagctacag	
SA-15	atcgctacctcatatccgcacctcccaactaatgttcaaatcttcacg	
SA-16	ctcgtcagattcctgtcaaatgtg	
SA-17	cccaagaaagatccgagatcatc	
SA-18	tcaatgattcctgcgaggggtgggcaatctggcgtagacggagc	
SA-19	atcgctacctcatatccgcacctccctccaacgtcagaatccaag	
SA-20	ccacaagcttgacgagcttc	

collected and injected into a distillation apparatus for purification. The distillation was carried out using a 30-stage distillation column at a pressure of 10 to 20 torr, a reactor bottom temperature of 149 to 164 °C, an upper reactor temperature of 110 to 138 °C, and a reflux ratio of 1. The purity of C10 FAME made in this study was  $99.45 \pm 0.15\%$  by GC analysis.

### Cell culture

A single colony of each *C. tropicalis* strain grown on yeast extract-peptone-dextrose (YPD) (2% glucose, 1% yeast extract, 2% Bacto-peptone, 2% agar) agar plate was inoculated into 20 mL of YPD liquid medium and incubated overnight at 30 °C on a rotary shaker (200 rpm). The culture was transferred into an Erlenmeyer flask (1 L) containing 200 mL of YPD medium, and incubated overnight at 30 °C on the rotary shaker (200 rpm). After initial glucose was exhausted, 100 mL of the culture was transferred into another Erlenmeyer flask (1 L) with 100 mL of biotransformation medium (100 g L<sup>-1</sup> glycerol, 13 g L<sup>-1</sup> yeast nitrogen base without amino acids, 6 g L<sup>-1</sup> yeast extract, 3.64 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 3 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, and 1 g L<sup>-1</sup> decane). After the total consumption of decane, 5 g L<sup>-1</sup> of C10 FAME was added twice at 24-hour intervals.

### Fermentation

A 5-litre fermenter system (CNS Inc., Daejeon, Korea) was used for lab-scale biotransformation of C10 FAME to SA.

Inoculation was carried out by adding 10% (v/v) of the pre-culture, which was performed in 2-litre Erlenmeyer flask with 200 mL of YPD media. The fermenter culture contained (per litre) 90 g of glycerol, 1 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 20 g of yeast extract, 8 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g of KH<sub>2</sub>PO<sub>4</sub>, 0.1 g of NaCl, 0.1 g of CaCl<sub>2</sub>·2H<sub>2</sub>O, and 1 mL of trace elements. When initial glycerol was exhausted, a feeding medium containing (per litre) 700 g of glucose and 20 g of yeast extract was continuously fed. For activation of  $\omega$ -oxidation, decane (Junsei Chemical, Kyoto, Japan) was added for 4 h after the start of feeding media, and subsequently C10 FAME was added. The temperature was controlled at 30 °C. The pH was kept constant at 5.5 for cell growth phase and then it was increased up to 7.8 for biotransformation phase with 6 N NaOH. Dissolved oxygen was maintained above 30% by increasing the agitation speed, aeration and use of pure oxygen.

This protocol was scaled up using a 50-litre fermenter system (CNS Inc., Daejeon, Korea) for biotransformation of C10 FAME to SA. Inoculation was carried out by adding 10% (v/v) of the pre-culture, which was performed in four 5-litre Erlenmeyer flasks with 500 mL of YPD media, in a final volume of 2 L. Fermentation conditions in the 50-litre fermenter, including the medium, temperature, pH, dissolved oxygen, and procedures, were the same as in the 5-litre system experiments.

## Analytical methods

The growth of yeast cells was monitored by measuring the optical density at 600 nm using a UVICON930 spectrophotometer (Kontron Analytical, Lucerne, Switzerland). Cell dry weight was inferred from a predetermined conversion factor of 0.32 g per L per OD<sub>600</sub>. To determine residual glycerol or glucose concentrations, 1 mL of culture broth was centrifuged, and the concentration in the supernatant was measured using a YSI 2900 model biochemistry analyser (YSI, Yellow Springs, OH, USA).

Decane, C10 FAME, decanoic acid, and SA were analysed with a gas chromatograph (Master GC; Dani Instruments, Cologno Monzese, Italy) equipped with an RTX-5 column (Restek, Bellefonte, PA, USA). The Flame Ionization Detector (FID) was used for GC analysis (model OPT 100 M – FID 86/C Detection System). Oven temperature ranged from 70 to 237 °C, and the injector and detector temperatures were 280 and 300 °C, respectively. A 100 µL aliquot of 6 N H<sub>2</sub>SO<sub>4</sub> was added to 100 µL of culture broth. The sample was then mixed with 300 µL of diethyl ether with tetradecanedioic acid, used as an internal standard. Then, 100 µL of the upper organic layer was collected and analysed by gas chromatography. For decanoic acid and SA, this organic layer was silylated with the same volume of *N,O*-bis-trimethylsilyl-trifluoroacetamide before gas chromatography analysis.

## Precipitation and purification of sebacic acid

The culture solution was centrifuged to separate the cells from the medium. The pH of the supernatant was acidified to 4 by the addition of HCl to precipitate SA. The precipitated SA was separated from the supernatant by filtration and washed twice using distilled water to remove soluble impurities. The crude SA was mixed with decane at 150 °C and 500 rpm during 3 h for recrystallization. The mixed solution was cooled at a rate of 1 °C min<sup>-1</sup>, followed by filtration to obtain SA. Recrystallized SA was mixed with acetone for extraction and then filtered for the removal of media-derived impurities (ESI Table S1†). Immediately after extraction, the acetone solution containing SA was decolorized by passing through activated carbon and filtered using a rotary vacuum filter. Drying and granulation were used to recover the purified SA.

## Polymerization of nylon 610

For poly-condensation, the purified SA was mixed with hexamethylenediamine in water solution in a heated reaction vessel under stirring. The reactor was heated to an inner temperature of 230 °C and maintained at this temperature for 1 h with an inner pressure of 17.2 bar. Then, the overpressure was released within 1 h and the temperature was raised to 270 °C during this step. Thereafter, the reaction mixture was maintained under a nitrogen stream for 80 min to support the post-condensation reaction and to increase the molecular weight. The polymer was then released through a die plate into water for palletization. The melting temperature (*T<sub>m</sub>*) and crystallization temperature (*T<sub>c</sub>*) of the produced nylon polymers were

measured by differential scanning calorimetry (DSC, Q200, TA Instruments, New Castle, DE, USA) according to ASTM D3418. Then, the nylon 610 sample was melted gradually at 10 °C min<sup>-1</sup> and then cooled and then reheated at the same rate from 30 to 250 °C. Degradation temperature (*T<sub>d</sub>*) of the produced nylon was measured thermogravimetric analysis (TGA, Q2950, TA Instruments) according to ASTM E1131. Then, the nylon 610 sample was heated from 40 to 900 °C at 20 °C min<sup>-1</sup> under N<sub>2</sub>.

## Statistics

Error bars represent the standard error of the mean from at least three independent replicates. Minitab 17 (Minitab Inc, State College, PA, USA) was used for statistical analyses of the results. Paired *t*-tests were used to compare the production of SA by differently genetically engineered strains; *p* values below 0.05 were considered to indicate significant differences.

## Results and discussion

The overall processes for microbial production of SA from renewable source, its purification and polymerization developed in this study are summarized in Fig. 2.

### Strain engineering

In an effort to develop a microbial cell factory for industrial SA production, we engineered the β-oxidation-blocked *C. tropicalis* ATCC® 20962™ to overexpress genes involved in ω-oxidation of *n*-alkane and fatty acids. Cytochrome P450 monooxygenase (CYP450) and cytochrome P450 reductase complex (CPRb) constitute the ω-hydroxylase enzyme complex, which catalyses the rate-limiting first step of ω-oxidation pathway (Fig. 1).<sup>34</sup> Accordingly, the coordinated amplification of CYP450 and CPRb genes in *C. tropicalis* enhances the production of 1,12-dodecanedioic acid or 1,14-tetradecanedioic acid.<sup>35</sup> In the second step of ω-oxidation, fatty alcohol oxidase (FAO1) plays a major role in the terminal oxidation of the alcohol group to an aldehyde.<sup>36</sup> Based on these observations, we attempted to amplify the endogenous genes encoding the CYP52A18 and CYP52A19 which are known to have effective oxidation activities on C10 substrates,<sup>35</sup> CPRb, and FAO1 in *C. tropicalis*. The target genes were site-specifically inserted into the genome by URA3 split-marker recombination and bipartite cassettes (Fig. 3a). As a result, several strains with various combinations of gene amplifications were engineered (Fig. 3b and Table 1).

To evaluate the effect of the coordinated amplification of genes involved in SA production, flask cultivation was performed with the engineered *C. tropicalis* strains. Compared to the parent strain (*C. tropicalis* 20962), individual overexpression of CYP52A18, CYP52A19, and CPR increased SA production by 15%, 9% and 6%, respectively (Fig. 3c). When two or more of these genes were co-overexpressed, SA production increased by 30% for CYP52A19 and CPR, and 32% for CYP52A19, CYP52A18 and CPR compared to *C. tropicalis* 20962. These observations indicate that the combined over-

## Schematic diagram of this study

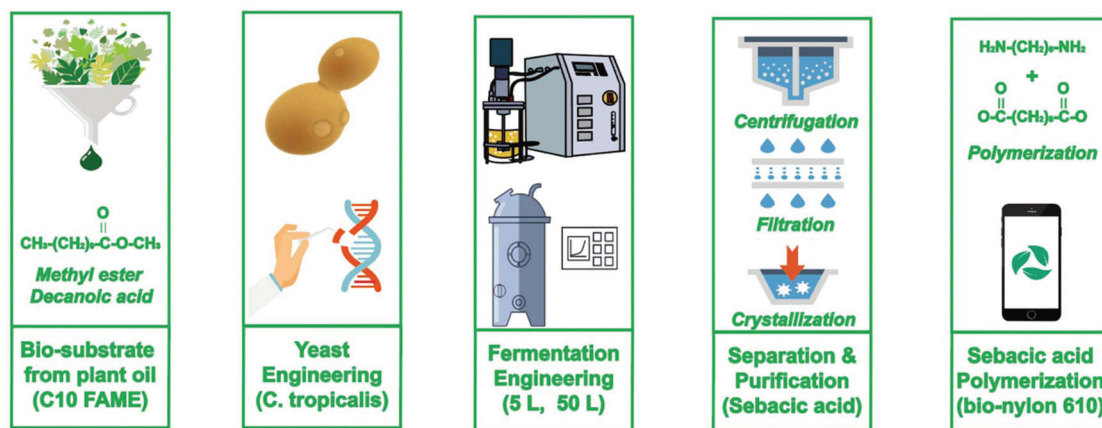


Fig. 2 Schematic representation of microbial sebacic acid (SA) production, purification, and polymerization. Bio-polyamide 610 was produced by polymerization of hexamethylenediamine with the purified SA.

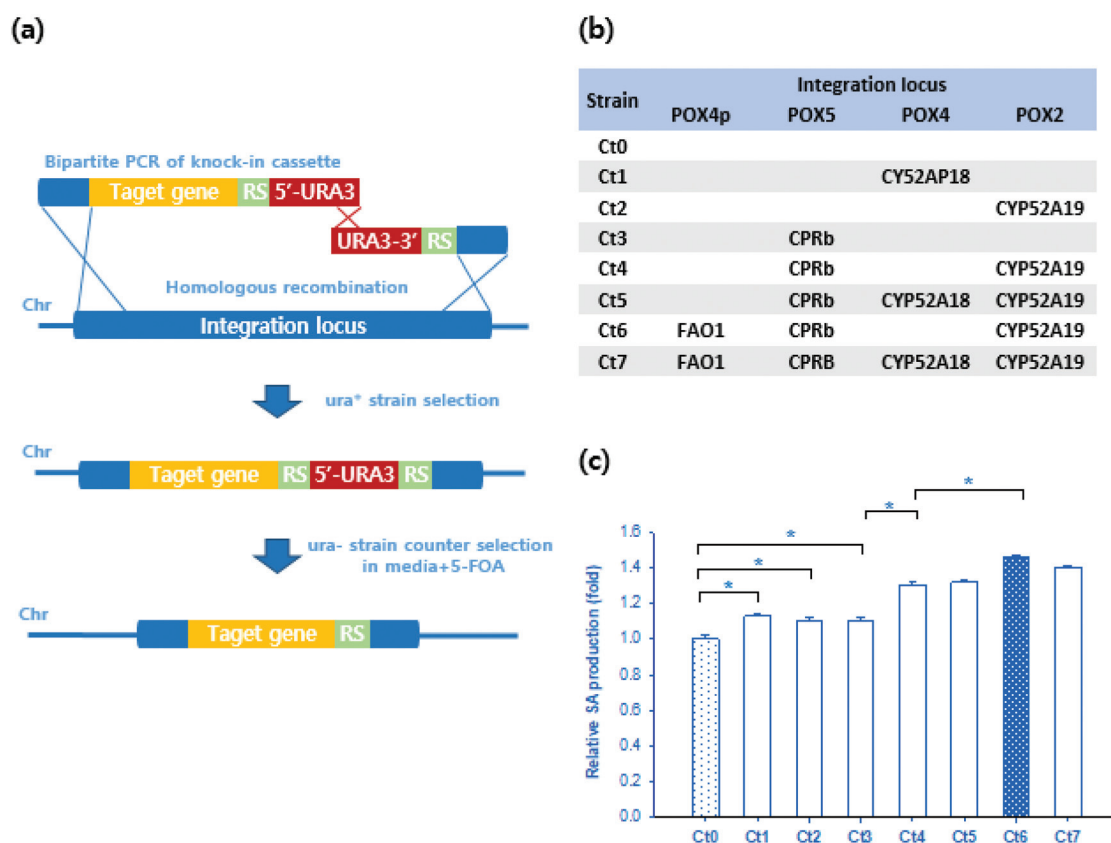


Fig. 3 Engineering the  $\beta$ -oxidation-blocked *Candida tropicalis* ATCC® 20962™ by overexpressing endogenous genes involved in the  $\omega$ -oxidation pathway of *n*-alkanes and fatty acids. (a) Schematic representation of the expression cassettes and chromosome integration by homologous recombination in *C. tropicalis*. (b) The chromosome integrated genes and integration locus in the engineered strains. (c) Comparison of DCA production from C10 FAME in the engineered strains. The asterisks denote significant differences between the indicated groups as determined by paired *t*-tests ( $p < 0.05$ ).

expression of monooxygenase and oxidoreductase was effective in augmenting SA production, whereas expression of both CYP52A18 and CYP52A19 with CPRb or FAO1 did not further increase the SA yield significantly. Co-overexpression of CYP52A19, CPR, and FAO1 (*i.e.* Ct6 strain) elicited an increase of 46% in SA concentration compared to the parent strain (Fig. 3c). Such an effect of FAO1 gene overexpression on the production of SA stems from the accelerated conversion of hydroxyl fatty alcohol to SA. Therefore, the strain Ct6, which had the highest SA productivity in flask culture, was used for the biotransformation process with fermenters in the next experiments.

### Fermentation for sebamic acid production

Whole-cell biotransformation process using fatty acid derivatives as a substrate is very challenging because of product/substrate toxicity and foam generation issues, which limit productivity and process stability. For these reasons, we have developed a biotransformation process that uses high-density

cell culture and substrate feed, and minimizes substrate/intermediate toxicity and foam generation. The biotransformation process was divided into three culture phases: cell growth, activating  $\omega$ -oxidation metabolism with alkane addition, and biotransformation from C10 FAME to SA (Fig. 4b). The conditions at each step were first optimized using a 5-litre fermenter.

The initial phase for the biotransformation process is to obtain cells at high density to act as biocatalysts. To do so, glycerol and glucose were tested as initial carbon sources. At  $60 \text{ g L}^{-1}$  of initial glucose or glycerol, cells reached to the density of  $72.6 \pm 5.4 \text{ OD}_{600}$  for glucose and  $103.4 \pm 8.5 \text{ OD}_{600}$  for glycerol. Furthermore, when glucose was used as a substrate, organic acids and ethanol were produced, while glycerol, a non-fermentable substrate, did not produce any of these by-products (data not shown). Therefore, to achieve high cell densities, around  $150 \text{ OD}_{600}$ ,  $90 \text{ g L}^{-1}$  of glycerol was further used as initial carbon source. However, the obstacle of glycerol utilization was the deficit of NADPH formation because of minimal glycolytic flux from glyceralde-

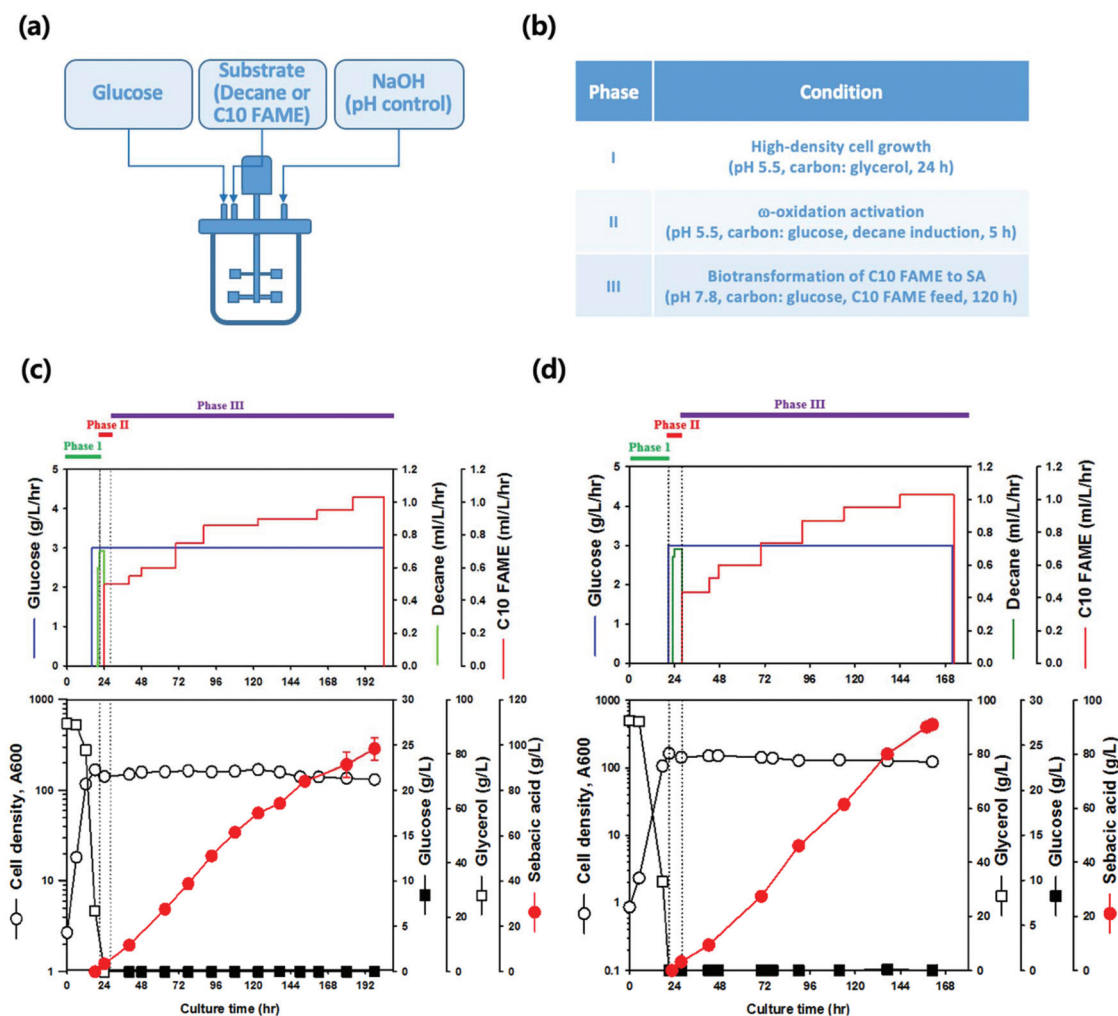


Fig. 4 Microbial sebamic acid (SA) production process from C10 fatty acid methyl ester (FAME). (a) Schematic representation and (b) the three phases of the microbial SA production process. (c, d) Kinetic profiles of microbial SA production processes in the (c) 5-litre fermenter system and (d) 50-litre fermenter system.

hyde-3-phosphate.<sup>37</sup> Therefore, when the initial glycerol was exhausted, glucose was fed at a low feed rate ( $3.0 \text{ g L}^{-1} \text{ h}^{-1}$ ) to promote NADPH regeneration, a key factor for the production of SA. This feeding rate maintained the carbon-limited state in the fermenter.

The second phase was designed to induce the expression of the enzymes involved in  $\omega$ -oxidation. Alkanes are known to act as universal inducers for activating the enzymes involved in  $\omega$ -oxidation. Among the alkanes, decane was chosen since its metabolism leads to the production of SA, other alkanes could be converted to other unwanted DCAs as by-products. To improve the SA production, decane induction was previously compared between a single addition and continuous feeding method.<sup>31</sup> That study demonstrated that continuous feeding was more effective than a single addition because it reduces the accumulation of C10 hydroxy fatty acid (HFA), a toxic intermediate to the cell. Continuous decane feeding was kept for 5 hours at  $0.7 \text{ mL L}^{-1} \text{ h}^{-1}$ , 60% of maximum decane uptake rate ( $q_{\text{decane}}$ ) determined by a single bolus addition. The pH was gradually increased from 5.5 to 7.8, the optimal pH value for DCA production.<sup>27</sup> Glucose was continuously fed at the rate of  $3.0 \text{ g L}^{-1} \text{ h}^{-1}$  for cell maintenance and NADPH regeneration. Decane was converted to the corresponding SA, and cell concentration was about 142 OD<sub>600</sub> at the end of this phase.

The final phase was designed for the biotransformation of C10 FAME into SA. The feed rate of the substrate is a crucial parameter for optimization process due to its toxic effects on the cells at high concentrations<sup>31</sup> and, therefore, various substrate feed rates were tested. The feed rates above  $1.2 \text{ mL L}^{-1} \text{ h}^{-1}$  resulted in the accumulation of toxic intermediates, such as decanoic acid and C10 HFA, which cause foaming and fermenter overflow. Therefore, C10 FAME feeding was started at  $0.5 \text{ mL L}^{-1} \text{ h}^{-1}$ , 70% of decane feeding rate in the second phase ( $\omega$ -oxidation activation). Then, the feed rate was increased step-wise to  $1.03 \text{ mL L}^{-1} \text{ h}^{-1}$  to enhance SA productivity. Because the produced disodium SA generates foam in the fermenter by reducing the surface tension, the air flow rate was reduced to 0.1 vvm. The DO level (above 30%) was maintained by the supply of pure O<sub>2</sub> to 0.025 vvm to compensate for the reduced air flow. The final SA concentration reached  $98.3 \text{ g L}^{-1}$  with a volumetric productivity of  $0.57 \text{ g L}^{-1} \text{ h}^{-1}$  and a molar yield above 98% (Fig. 4c). To our knowledge, the titre, productivity, and process scale for SA production described herein are the highest levels reported so far in the world.

Next, the developed biotransformation process of SA production was tested in a 50-litre system to test its feasibility for industrial production. The biotransformation process in the 50-litre fermenter was performed following the same three phases described above. The initial phase was carried out for 21 h and the maximum cell concentration was 162.1 OD<sub>600</sub>. To minimize the generation of foams, the air flow rate was reduced to 0.1 vvm and the DO level (above 30%) was maintained by the supply of pure O<sub>2</sub> to 0.02 vvm. This 50-litre biotransformation process allowed a molar yield of above 98%, a productivity of  $0.68 \text{ g L}^{-1} \text{ h}^{-1}$ , and a final titre of  $90.9 \text{ g L}^{-1}$

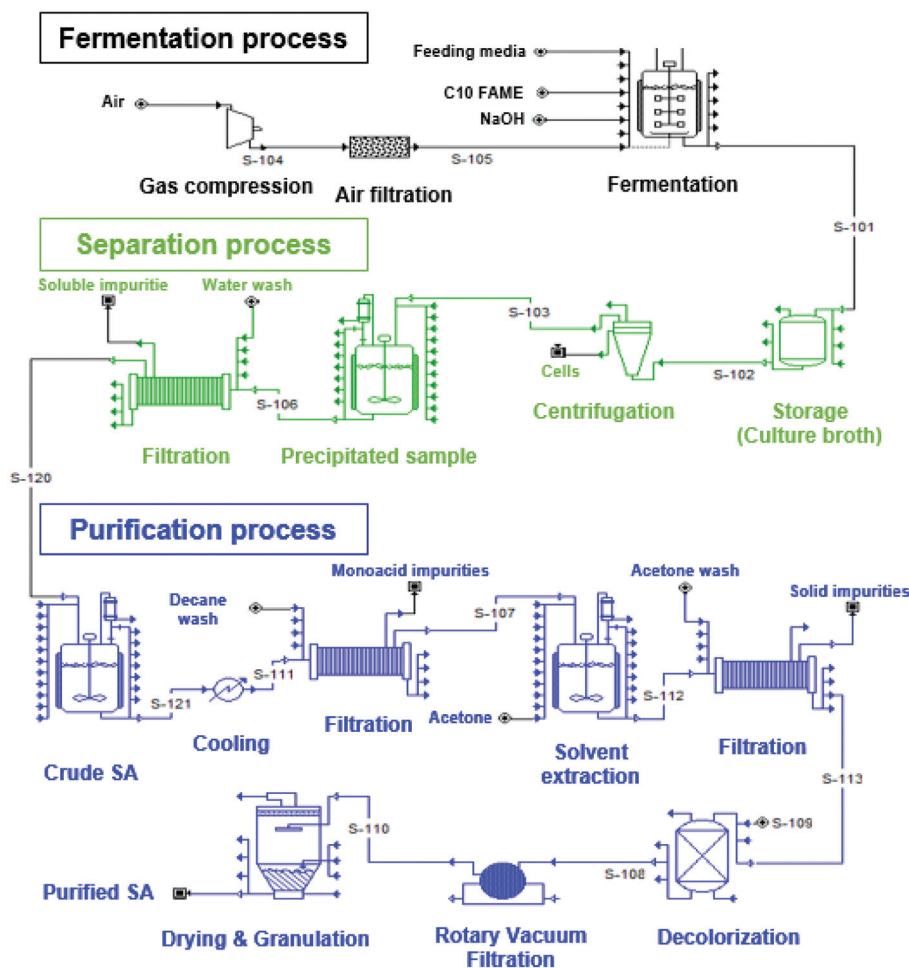
(Fig. 4d). It was confirmed that the scale up of the process for SA production was feasible because the results in 50-litre stainless steel fermenter were similar to those in the 5-litre jar fermenter.

### Precipitation and purification of sebacic acid

To separate SA ( $\text{pK}_{\text{a}} = 4.72$ ) from the other molecules in the culture supernatant, we used acid precipitation by HCl (Fig. 5a). In order to achieve the maximum precipitation yield of SA, the pH was continuously lowered below its  $\text{pK}_{\text{a}}$ , but there was no difference in yield between pH 4 and lower pH values. Therefore, acid precipitation was carried out at pH 4, and the precipitated SA was filtered and washed with distilled water. The precipitate was analysed, and the results indicated that they consisted of 85.4% of SAs, 2.0% of other carbon-chain (C11 and C12) diacids, 1.2% of monoacids (mainly C8 and C10), and 10.1% of impurities. Considering the purity ( $99.45 \pm 0.15\%$ ) of the substrate (C10FAME) used in this study, other carbon-chain diacids may be formed from fatty acids in the cell. Monoacids are formed due to the intermediates in the  $\omega$ -oxidation metabolism as observed in our previous work,<sup>31</sup> but they must be removed because they terminate nylon polymerization. The additional analysis of other impurities indicated that they most likely originated from the media because their components were similar to those found in yeast extract (ESI Table S1†).

We developed a purification process that yields high-purity SA that can be further polymerized. Moreover, this process requires relatively less energy making it an economically feasible process to develop. Noteworthy, it includes steps to remove the monoacids that primarily terminate polymerization, other impurities, and the yellowish colour (Fig. 5a). To purify the crude SA precipitates separated from the culture broth, the recrystallization process was first developed. The proper choice of solvent is crucial in recrystallization and therefore various solvents were tested in this study (data not shown). Non-polar solvents showed higher yield than polar solvents. This result is explained by the loss of residual SA dissolved in the polar solvent during the recrystallization process due to the different solubility of SA in non-polar and polar solvents. Thus, we chose non-polar decane to recrystallize SA, which was able to remove monoacids completely and yielded 99.3% of SA recovery (Table 3). Secondly, solvent extraction process was applied to remove media-derived impurities, and acetone was used as a solvent due to its ability to solubilize SA selectively and its high volatility. The acetone extraction process successfully removed impurities (ESI Table S2†), and SA was obtained at a purity of 96.4%. Finally, the decolorization process was carried out with activated carbon, and the yellowness index of the purified SAs was significantly reduced from  $206 \pm 2$  of the recrystallized SA to  $9 \pm 2$  (Table 3). This value was comparable to that of commercial SAs (10–15), and the final purity and yield of SA were 98.0% (99.6% for diacids) and 95.5%, respectively. It is too difficult to remove other diacids due to their similar chemical properties to SA. Furthermore, they do not terminate the polymerization, and

(a)



(b)

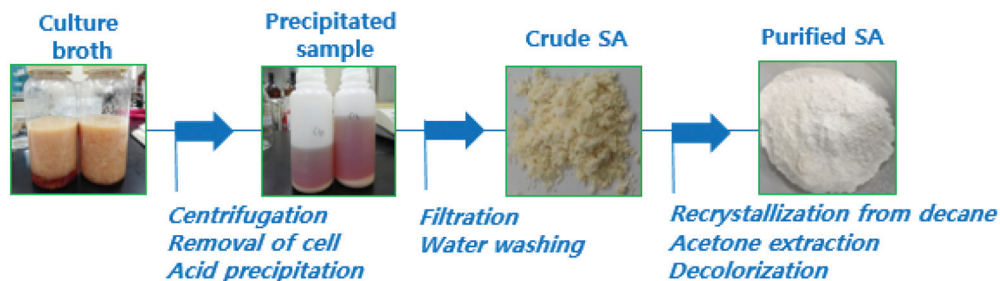


Fig. 5 Separation and purification of sebacic acid (SA) from fermentation broths. (a) Process flow diagram and (b) photographs of the SA products.

Table 3 Summary of purification results of sebacic acid from fermentation broth

Purification step	Sebacic acid (g)	Other diacids (g)	Monoacid (g)	Media-derived impurities (g)	Other impurities (g)	Total weight (g)	Yield (%)	Purity (%)	APHA colour <sup>a</sup>
Crystallized sample	1.025.8	23.6	14.6	121.8	15.0	1200.8	—	85.4	
Recrystallization from decane	1018.3	31.1	0.0	121.8	6.8	1178.0	99.3	86.4	206 ± 2
Acetone extraction	1018.3	31.1	0.0	0.0	6.8	1056.2	99.3	96.4	
Decolorization	979.5	17.7	0.0	0.0	2.7	999.9	95.5	98.0	9 ± 2

<sup>a</sup> APHA colour: a colour standard named for the American Public Health Association and defined by ASTM D1209.

therefore we did not develop additional process to remove them in this study.

There have been few studies about the purification of other long-chain diacids, and C12 diacid (1,12-dodecanedioic acid) has been purified using the falling film crystallization method. However, this process is not easy to apply industrially because it requires great amounts of thermal energy and a sophisticated temperature control system.<sup>38</sup> For short-chain diacids, many studies have reported the purification of succinic acid from microbial fermentation through various processes, such as distillation-crystallization, precipitation, membrane separation, and solvent extraction.<sup>39</sup> Although these purification processes have achieved high purity (>99%), their yields ranged from 60 to 93%. Consequently, this study, for the first time, developed a process for the separation and purification of SAs produced from microbial fermentation, and this process shows the industrial applicability with 98% (99.6% for diacids) of purity and 95.5% of yield at a low cost. Fig. 5b shows the photographs of the SA product during the separation and purification steps of the SA.

### Polymerization with nylon 610

To test whether the purified SA could be polymerized, we attempted to synthesize nylon 610 by its polymerization with hexamethylenediamine ( $\text{NH}_2-(\text{CH}_2)_6-\text{NH}_2$ ). Nylon 610 was successfully synthesized and compared to commercial nylon 610. The chemical properties of the bio-nylon 610 synthesized in this study were analysed using DSC and TGA (Fig. 6).  $T_m$  and  $T_c$  were determined to be 223.5 °C and 188.1 °C, respectively, and  $T_d$ , which indicates heat resistance, was determined to be 470.8 °C. These chemical properties indicated that bio-nylon 610 synthesized in this study is nearly equivalent compared to commercial nylon 610 (Table 4). These thermal properties suggest that the nylon 610 synthesized in this study can be used for coating of bristle like monofilaments of toothbrush and cables since it presented chemical resistance and excellent electric performance.

## Conclusions

In this study, a *C. tropicalis* strain was genetically engineered to improve the SA production. Coordinate amplification of monooxygenase, oxidoreductase, and fatty alcohol oxidase showed the best results for the SA production in flask culture. A biotransformation process was developed for the high-level production of SA to overcome the challenges of high-density cell culture, substrate feed, substrate/intermediate toxicity and foam generation. Subsequently, we used this process in the fed-batch production of engineered *C. tropicalis* produced 98.3 g L<sup>-1</sup> of SA from renewable plant oil-derivatives with a productivity of 0.57 g L<sup>-1</sup> h<sup>-1</sup> at 5-litre system scale. These results were successfully reproduced using a larger 50-litre bench scale system, which resulted in the highest value of SA production so far to our knowledge. However, substrate toxicity limited the SA productivity, and therefore the further develop-

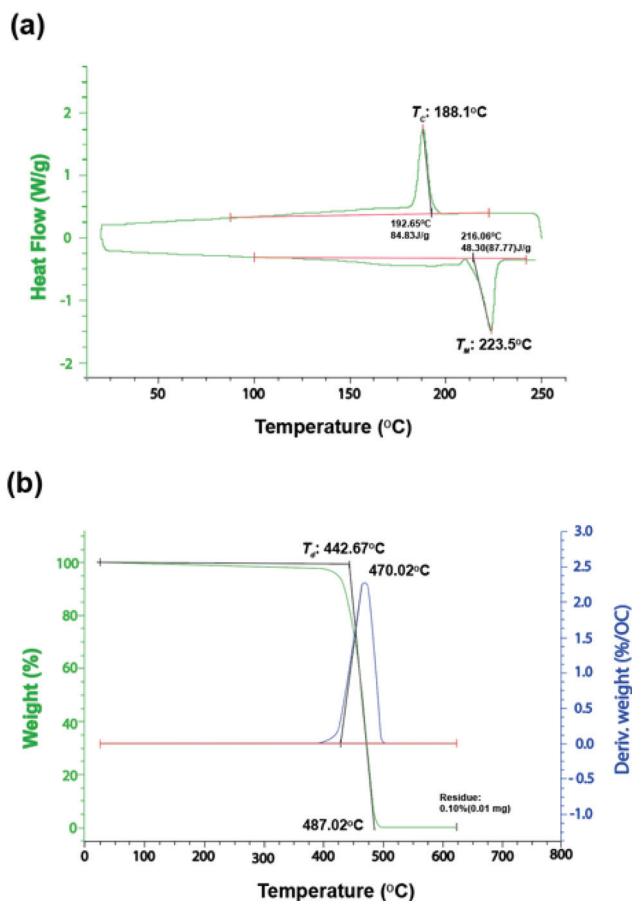


Fig. 6 Analysis of the properties of bio-based nylon 610. (a) Differential scanning calorimetry (DSC) results. (b) Thermogravimetric analysis (TGA) results.

Table 4 Comparison of material properties of bio-based nylon 610 synthesized in this study and commercially available chemical-based nylon 610

Mechanical property	Conventional nylon 610	Bio-nylon 610 in this study
Melting temperature ( $T_m$ , °C)	222.4	223.5
Crystallization temperature ( $T_c$ , °C)	182.1	188.1
Degradation temperature ( $T_d$ , °C)	470.4	470.8

ment of the strain, tolerant to substrate toxicity, will accelerate the industrial application of this achievement. The separation with acid precipitation and purification processes with solvent extraction and recrystallization enhanced the purity of SA to the polymer-grade. Bio-nylon 610 was successfully synthesized by polymerizing hexamethylenediamine and SA. The synthesized bio-nylon 610 showed properties similar to those of commercially available nylon 610. Therefore, the microbial SA biotransformation developed in this study has the potential to replace the traditional caustic pyrolysis process to produce SA, which is leading industries to shut down due to environmental regulations.

## Author contributions

W. J, M. J and G. P designed and conducted the research, and wrote the paper. H. L, S. S., H. L., C. H., H. K., H. L., J. L., Y. H., M. L., and J. L. helped to do the experiments and analyse the samples; H. L. and J. A. conceived and supervised the project. All authors wrote the manuscript together and approved the final version.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

This study was supported by a grant from the KRIBB Research Initiative Program, and industrial Strategic Technology Development Program (10047873 & 20002734) funded by the Ministry of Trade, Industry & Energy (MOTIE, Republic of Korea).

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