Turning the non-pathogenic yeast *Starmerella bombicola* into a powerful long-chain dicarboxylic acid production host

Keywords

Bio-based LCDA, fatty acid, β-oxidation, ω-oxidation, bioprocess optimization

Abstract

Bio-based long-chain dicarboxylic acids (LCDAs) are in high demand in the polymer industry. These compounds have diverse applications as building blocks for polymers with distinct features, which lead to a fast-growing global LCDA market. However, bio-based LCDA production is currently limited in Europe as established processes are using the pathogenic yeast C*andida tropicalis*. Therefore, this study aimed to establish safe and sustainable LCDA production using an industrially relevant non-pathogenic yeast, *Starmerella bombicola*. The metabolic network was successfully controlled to channel fatty acids from rapeseed oil into the ω-oxidation for the high production of LCDAs. Importantly, the engineered yeast strain produced 5.5 g/l of total LCDAs in shake flasks. Furthermore, pH optimization of the bioprocess resulted in a significant improvement of the total LCDA titer up to 117.8 g/l. The outcomes strongly demonstrate that *S. bombicola* can serve as a safe and efficient platform microorganism for industrial LCDA production.

1. Introduction

Long-chain dicarboxylic acids (LCDAs) are defined as harbouring 12 or more carbon atoms and containing two carboxylic groups at both ends of the long-chain compounds. As building block monomers with versatile features based on the different chain lengths and varying degrees of unsaturation, LCDAs are usable to produce diverse high-grade polymers, lubricants, coating, corrosion inhibitors, perfumes, adhesives and macrolide antibiotics (Huf et al., 2011; Werner & Zibek, 2017; Zhu et al., 2024). The global LCDA market value was $227.0 million in 2022. It was also predicted to increase with a compound annual growth rate (CAGR) of 7.0% from 2023 to 2030 (Grand View Research, 2023).

Traditional fossil-based chemical synthesis of short and medium-chain saturated dicarboxylic acids (DAs) requires expensive and harsh chemical processes (Polen et al., 2013; Bart and Cavallaro, 2015; Li et al., 2018). Furthermore, a self-metathesis for unsaturated long-chain dicarboxylic acids from methyl oleate is a non-environmentally friendly reaction using a second-generation Grubbs catalyst, which leads to high byproduct formation (Stempfle et al., 2016). On the other hand, bio-based LCDA production using the pathogenic yeast, *Candida tropicalis* has been established (Picataggio et al., 1992). A recent study demonstrated that CRISPR-based genetic modification and metabolic engineering of *Candida viswanathii* led to 224 g/l of dodecanedioic acid production (Pham et al., 2023). However, implementation of this unsafe bioprocess is hampered in Europe since *C. tropicalis* is classified as a class two risk group organism. Therefore, safety precautions and related legislation are required for industrial production, which lead to elevated production costs. In addition, extensive metabolic engineering of non-natural LCDA producing platform

microorganisms, *Escherichia coli* and *Saccharomyces cerevisiae*, has not shown any promising production due to poor activity of the heterologous cytochrome P450 monooxygenases (CYP) coupled with the cytochrome P450 reductase (CPR), redox partners, catalysing the terminal oxidation of fatty acids (Zimmer et al., 1995; Sathesh-Pradu & Lee, 2015; Qi et al., 2024). As a result of evaluating alternative LCDA producing yeasts with active terminal oxidation capability, *Candida guilliermondii* has been engineered to accumulate LCDAs, but further strain development with its fatty acid metabolism is necessary to improve the titer (Werner et al., 2017). Although the oleaginous yeast *Yarrowia lipolytica* has been engineered for the production of LCDAs with its advantages of being Generally Regarded As Safe (GRAS) and a tremendous amount of knowledge about its lipid metabolism, the LCDA production titer was inferior compared to *C. tropicalis* (23 g/l versus 100 g/l; Nicaud et al., 2006; Werner & Zibek, 2017). Up to now, the only high LCDA production with a non-pathogenic microorganism was achieved with an alkane-assimilating yeast, *Wickerhamiella sorbophila* using methyl laurate as the substrate (92.5 g/l in fed-batch; Lee et al., 2018). Nevertheless, this established bioprocess is limited to C12 dicarboxylic acid (dodecanedioic acid) production.

A non-pathogenic and industrially relevant yeast, *Starmerella bombicola* has high potential to be a good platform organism to produce LCDAs since it is capable of producing high amounts of fatty acid-derived products, such as sophorolipids and hydroxy fatty acids (Pekin et al., 2005; De Graeve et al., 2019). In addition, its genome sequence (NCBI database GCA_001599315.1, GCA_004124885.1, GCA_000950655.1, GCA_003033785.1) and sophisticated molecular engineering tools (e.g., homologous recombination and CRISPR-Cas9 system) for genetic modification are available (Li et al., 2016; De Graeve et al., 2018; Shi et al., 2022).

Importantly, the sophorolipid biosynthetic gene cluster is well-studied and the optimal bioprocess for large-scale production has been established (Kim et al., 2009; Van Bogaert et al., 2013; To et al., 2022).

In *S. bombicola*, the biosynthesis of LCDAs from fatty acids through the ω-oxidation pathway competes with the β-oxidation, sophorolipid biosynthesis and the fatty acid acyl-CoA forming route (Fig. 1). The main route in which the fatty acid input and the biosynthesized LCDAs can be lost is energy generation through the β-oxidation, which is initiated by fatty acyl-CoA oxidase (POX1; Lee et al., 2018). The sophorolipid biosynthesis is initiated by a cytochrome P450 monooxygenase (CYP52M1) coupled with its redox partner CPR, creating a hydroxy fatty acid, which is conjugated with UDP-glucose by glucosyltransferase 1 (UGTA1; Van Bogaert et al., 2009; Saerens et al., 2011a). In the case of LCDA biosynthesis, additional terminal oxidation of hydroxy fatty acids takes place within the ω-oxidation. Fatty acyl-CoA synthase (FAA1) is responsible for converting free fatty acids to fatty acyl-CoAs; in this way shuttling them to various cellular processes, such as triacylglyceride build-up, integration in cell membranes or protein lipidations (Ledesma-Amaro et al., 2016; Fig. 1). All of the three above described routes should be carefully reconsidered when aiming for LCDA synthesis.

This study aims to establish safe and sustainable LCDA production in *S. bombicola* using rapeseed oil as a renewable fatty acid source by inactivating the β-oxidation, sophorolipid biosynthesis and the fatty acyl-CoA forming route. In addition, the optimal pH condition for LCDA production in *S. bombicola* was investigated to set an ideal bioprocess for the best engineered strain to achieve the high LCDA production. The newly established bio-based LCDA production process using the engineered *S. bombicola* strain will serve as a safe and sustainable way for future LCDA production.

2. Materials and methods

2.1. Cloning and strain engineering

Standard molecular cloning procedures were performed using *E. coli* Top10 cells (Invitrogen, Carlsbad, USA), cultivated in lysogeny broth (LB) medium (5 g/l of yeast extract, 10 g/l of tryptone and 10 g/l of NaCl). If necessary, either 100 mg/ml of ampicillin or 50 mg/ml of kanamycin was supplemented for antibiotic selection, and 20 g/l of agar was added. For the gene deletion construct (*pox1*, GenBank: MK947130; *ugta1,* GenBank: HM440973.1; *faa1,* GenBank: MK952999.1), 1 kb long upstream and downstream regions of the target open reading frame were amplified via PCR. The gene fragments and a selection marker gene cassette (e.g., nat^R and *ura3*) were assembled through CPEC (Quan & Tian, 2009). The gene deletion construct was amplified to introduce into *S. bombicola* competent cells via electroporation (2.5 kV; 200 Ω; Saerens et al., 2011b). For nourseothricin resistance selection, the transformants were selected on YPD agar plates (10 g/l of yeast extract, 20 g/l of peptone, 20 g/l of glucose and 20 g/l of agar) containing 600 µg/ml of nourseothricin (Jena Bioscience, Jena, Germany, AB-102L). The successful homologous recombination event was confirmed with colony PCR. Before knocking out the *faa1* gene, the *ura3* marker, which was used for the deletion of *ugta1*, was recycled by homologous recombination with an amplified gene fragment containing the combined 1 kb long upstream and downstream regions of the *ugta1* locus. The absence of the *ura3* gene was verified on SD agar plates (6.7 g/l of yeast nitrogen base without amino acid, 0.77 g/l of complete supplement mixture of amino acids, 20 g/l of glucose and 20 g/l of agar) supplemented with 1 g/l of 5-fluoroorotic acid (5-FOA, Thermo Fisher Scientific, Waltham, USA, R0812). The marker recycling for the desired strain

(Δ*pox1*Δ*ugta1*Δ*ura3*) was additionally confirmed with colony PCR. For the *faa1* gene deletion construct, 1 kb long upstream and downstream regions of the target open reading frame were amplified via PCR. The gene fragments and a selection marker gene cassette (*ura3*) were assembled through CPEC. The gene deletion construct was amplified to introduce into *S. bombicola* competent cells via electroporation. The transformants were selected on SD-ura agar plates (6.7 g/l of yeast nitrogen base without amino acid, 0.77 g/l of complete supplement mixture without uracil, 20 g/l of glucose and 20 g/l of agar). The successful homologous recombination event to obtain the triple deletion strain (Δ*pox1*Δ*ugta1*Δ*faa1*) was confirmed with colony PCR. The engineered strains were maintained in YPD medium. If necessary, 20 g/l of agar was added. Information about the used DNA oligonucleotides is available (see supplementary material).

2.2. Shake flask experiment

The engineered *S. bombicola* strains were grown in 50 ml of YPD medium to compare the cell growth for 12 hours (starting OD_{600} of 1) at 30 °C and 200 rpm. To test LCDA production of the engineered strains, the cells were grown on 3C plates (10 g/l of yeast extract, 1 g/l of urea, 100 g/l of glucose and 20 g/l of agar) to adapt to a high glucose condition before pre-culture in 5 ml Lang medium (120 g/l of glucose, 4 g/l of yeast extract, 5 g/l of $C_6H_5Na_3O_7 \cdot 2H_2O$, 1.5 g/l of NH_4Cl, 1 g/l of KH_2PO_4, 0.16 g/l of K_2HPO_4, 0.7 g/l of $MgSO_4 \cdot 7H_2O$, 0.5 g/l of NaCl, $CaCl_2 \cdot 2H_2O$, pH 5.8; Lang et al., 2000) for 2 days at 30 °C and 200 rpm. For monitoring cell growth and the production of LCDAs, the pre-culture was diluted to a starting OD_{600} of 0.5 in a 250 ml volume non-baffled flask containing a total of 50 ml culture in Lang medium (120 g/l glucose; starting pH 5.8) at 30 °C and 200 rpm. The cell growth was monitored by determining colony

forming units (CFU) after spreading 10^{-6} diluted cell cultures on 3C agar plates and incubation at 30 °C for 3 days. In addition, the pH changes during the cultivation were monitored. After 48 hours of cell growth, the production phase was initiated by adding 37.5 g/l of rapeseed oil (Vandemoortele, Ghent, Belgium). To compare the biomass between the parental strain (Δ*pox1*Δ*ugta1*) and the triple deletion strain (Δ*pox1*Δ*ugta1*Δ*faa1*), the dry cell weight (DCW) after 8 days was measured. Furthermore, the cell growth of the engineered strains in 20 ml Lang medium (OD_{600} of 0.5; starting pH 5.8) at 30 °C and 200 rpm was monitored in Cell Growth Quantifier (CGQ; Scientific Bioprocessing, Baesweiler, Germany).

2.3. Glucose quantification

To monitor the glucose consumption during the shake flask cultivation and fermentation, cells were removed by centrifugation at 16,200 × g, for 10 min to harvest the supernatant. The glucose concentration was quantified using an HPLC-RID (Shimadzu, Kyoto, Japan) equipped with a Rezex™ ROA-Organic Acid H^+ LC Guard column (50 × 7.8 mm; Phenomenex, Torrance, USA, 03B-0138-K0). 5 mM sulfuric acid was used as the eluent at flow rate 0.1 ml/min. Glucose was used for the calibration curve.

2.4. LCDA extraction and thin layer chromatography (TLC)

For extraction and detection of the produced LCDAs, the cell culture after 8 days was vigorously mixed with an equal volume of ethyl acetate. Continuously the mixture was shaken at 200 rpm for 1 h. The ethyl acetate phase was harvested at 7,690 × g for 20 min and dried. The dried LCDAs were dissolved in 100% (v/v) methanol. TLC on silica-coated plates was performed with the running solvent (*n*-heptane/dimethyl ether/acetic

176 acid (50:50:1, by vol). The TLC plates were stained with 0.4 g/l of bromocresol green
177 (Merck, Darmstadt, Germany, 114359) dissolved in 100% (v/v) ethanol to detect
178 LCDAs.
179
180 *2.5. LCDA quantification*
181 For quantification of the produced LCDAs, dried LCDAs were dissolved in 100% (v/v)
182 ethanol. The LCDA profile was analysed using a UPLC-MS (Shimadzu) equipped with
183 a Kinetex® 2.6 µm Polar C18 100 Å, LC column (100 × 2.1 mm; Phenomenex, 00D-
184 4759-AN). Different LCDAs were separated using an acetonitrile/water gradient (0.1%
185 formic acid) 0-1 min, 25%, 1-8 min, 100%, 8.10-10 min 25%, flow rate 0.35 ml/min. To
186 quantify the LCDAs, commercial LCDAs (C12:0, Merck, D1009; C14:0, Merck,
187 D221201; C16:0, Merck, 177504; C18:0, TCI, Tokyo, Japan, O0222; C22:0, Merck,
188 306673) were used for the calibration curves.
189
190 *2.6. Fermentation and optimization*
191 The fermentation processes were performed in a 1 l fermentor with a working volume
192 of 0.7 l (SciVario® twin 1 l vessel; Eppendorf, Hamburg, Germany) in Lang medium
193 (pH 5.8). A late stationary phase cell culture grown in Lang medium was inoculated at
194 10% (v/v). To adjust the cells to an oil condition, 0.5% (v/v) of rapeseed oil was added.
195 After the growth phase of 48 h, rapeseed oil was continuously fed at 0.21 ml/h until the
196 end of the fermentation process. The stirring rate was held at 600 rpm and the airflow
197 rate and temperature were maintained at 42 l/h (1 vvm) and 30 °C, respectively. For
198 testing different pH conditions during the production phase, pH was adjusted to pH 3.5,
199 4.6, 5.2, 5.8, and 8 by the addition of 25% (w/v) of NaOH. The initial of pH 5.8 was
200 allowed to freely drop until the desired pH (3.5, 4.6 and 5.2), and then the pH condition

was constantly controlled until the end of the fermentation. In the case of pH 5.8, pH was maintained during the entire fermentation process. In the case of pH 8, pH was adjusted to pH 5.8 during the growth phase, and then gradually increased to pH 6.5 (48 h), pH 7.0 (72 h), pH 7.5 (96 h), pH 7.7-8.0 (120 h). For fed-batch fermentation, glucose was supplemented to maintain the concentration above 40 g/l. The harvested cell culture was acidified with 5 M HCl before the LCDA extraction except for the fermentation run at pH 3.5, and a secondary extraction was performed.

3. Results and discussion

3.1. Creation and evaluation of the first engineered strain: Δpox1Δugta1

To offer an alternative method of safe and efficient bio-based LCDA production, the industrially relevant yeast, *S. bombicola* was genetically engineered to produce LCDAs from vegetable oil-derived fatty acids. The core metabolic network was conceptually divided into three parts: the β-oxidation, ω-oxidation and sophorolipid biosynthesis (Fig. 1). Initially, to detect biosynthesized LCDAs and reach a considerable production level (g/l scale), simultaneous inactivation of two precursor-losing pathways, the β-oxidation and sophorolipid biosynthesis, was performed so that the first engineered *S. bombicola* strain is capable to actively convert supplemented fatty acids (C16-C18) into LCDAs through the endogenous ω-oxidation pathway in the cytosol instead of incorporating them into sophorolipids or metabolizing them in the β-oxidation. Moreover, fatty acids and produced LCDAs could enter the degradation steps in the peroxisomal β-oxidation pathway for energy generation. Blocking the β-oxidation pathway has been commonly performed by knocking out *pox* genes encoding fatty acyl-CoA oxidases to protect fatty acids and their derivative compounds as precursors or final products in other yeasts

(Picataggio et al., 1992; Li et al., 2014; Lee et al., 2018; Lee et al., 2024). This strategy was also successfully applied in *S. bombicola* for the high production of free fatty acids and hydroxy fatty acids (De Graeve et al., 2019; Salvador Lopez et al., 2022). Therefore, the deletion of *pox1* is essential for the safe channeling of fatty acids into the ω-oxidation pathway and preventing the LCDA degradation via the β-oxidation pathway. In contrast to most other LCDA producing strains, *S. bombicola* only harbors one *pox* gene, making it less challenging to engineer compared to the other producers (De Graeve et al., 2019). *S. bombicola* is a natural producer of sophorolipids, this unlike the best LCDA producer, *C. tropicalis*. The sophorolipid biosynthesis pathway shares the same precursors (e.g., ω-hydroxy fatty acids) with the LCDA biosynthesis. Furthermore, the biosynthesis of sophorolipids is a major glucose-losing route in *S. bombicola* because the sophorolipid is a conjugation result of a glucose disaccharide sophorose and a hydroxy fatty acid. Hence, the first step of sophorolipid synthesis is still required (i.e., hydroxylation of the fatty acid by CYP), but further activation by glucosyltransferase UGTA1 should be avoided (see Fig. 1). Indeed, a previous report compared the glucosyltransferase activity between cell lysates of the *S. bombicola* wild-type strain and the *ugta1* deletion strain and confirmed that the *ugta1* deletion strain is unable to accumulate sophorolipids (Saerens et al., 2011a).

The *pox1* in the genome was successfully deleted by replacing it with the nourseothricin resistance marker gene (*nat*^{R}) cassette by homologous recombination. Colony number 2 showed the expected amplicon sizes for correct *pox1* disruption (see supplementary material) The cell growth of the single *pox1* deletion strain was compared with the cell growth of the parental *ura3*-negative strain derived from *S. bombicola* ATCC 22214. No cell growth change caused by *pox1* deletion was observed (see supplementary material). The strain development was continued to inactivate the

sophorolipid biosynthesis pathway by deletion of *ugta1* encoding glucosyltransferase 1. The *ugta1* gene in the genome was replaced by the *ura3* cassette. Uracil auxotrophic selected colonies were subjected to the colony PCR and colony number 2 showed the correct amplicon sizes (see supplementary material). The knock-outs of *pox1* and u*gta1* were successfully executed in one strain. In addition, the cell growth between the single *ugta1* deletion strain and the double deletion strain (Δ*pox1*Δ*ugta1*) was compared. The growth of the double deletion strain was slightly slower than that of the single *ugta1* deletion strain (see supplementary material). To understand the characteristics of the double deletion strain in Lang medium for LCDA production, three different parameters were analysed: cell growth, glucose consumption and pH change over the cultivation time in shake flasks (Fig. 2). After 2 days, 37.5 g/l of rapeseed oil was added to provide fatty acids for the bioconversion to LCDAs in the production phase. A significant decrease in cell growth after the rapeseed oil feeding was observed (Fig. 2A). As the cells entered the stationary phase, the glucose consumption speed was increased. Approximately half of the initial glucose (120 g/l) remained after 8 days of cultivation (Fig. 2B). This could be explained by the fact that the double deletion strain (Δ*pox1*Δ*ugta1*) is unable to conjugate the hydroxy fatty acids and UDP-glucose for sophorolipid production. A similar observation was reported during the hydroxy fatty acid production in a non-sophorolipid producing *S. bombicola* strain (Δ*ugta1*Δ*pox1*Δ *fao1*; De Graeve et al., 2019). From the beginning of the cultivation, pH was significantly decreased from pH 5.8 to pH 3 (Fig. 2C). This pH profile during the cultivation is similar to the previous reports for the production of sophorolipids and hydroxy fatty acids in *S. bombicola* (Kim et al., 2009; De Graeve et al., 2019).

To evaluate the LCDA production capability of the first engineered strain, LCDAs from the cell culture was extracted in ethyl acetate and subjected to TLC with reference

LCDAs. A strong LCDA signal (lower band) was detected on a TLC plate when the height of the band was compared with the bands of reference LCDAs (Fig. 2D). The LCDA production profile was analysed and the prominent LCDAs, mainly C16 and C18 LCDAs, were quantified. The total amount of LCDAs produced in the *pox1* and *ugta1* double deletion strain in the shake flask after 8 days reached 1 g/l (Fig. 2E). In conclusion, the genetically engineered first strain is capable of efficient bioconversion of fatty acids into LCDAs using the endogenous ω-oxidation pathway.

3.2. pH optimization for high production of LCDAs

The first engineered strain (Δ*pox1*Δ*ugta1*) produced considerable amounts of LCDAs (1 g/l) in shake flask experiments. This occurred at acidic conditions, which is contrast to the optimal pH values (e.g., pH 8) reported for high LCDA production processes in other yeasts. It has been known that a pH above 8 is desired for high production of LCDAs in *C. tropicalis* strains (Funk et al., 2017b; Bauwelinck et al., 2021; Zhang et al., 2021; Pham et al., 2023). In addition, an engineered *W. sorbophila* strain was able to produce 92.5 g/l of dodecanedioic acid using methyl laurate by gradually increasing the pH up to 8 during the production phase (Lee et al., 2018). Therefore, the double deletion strain was subjected to the pH optimizing in a fermentor set-up with convenient and accurate pH adjustments, which might be difficult to perform in shake flasks. It turned out that a different pH strategy compared to the *C. tropicalis* and *W. sorbophila* procedures needs to be followed: a gradual pH increase from pH 5.8 to pH 8 during the production phase caused cell stress as can be seen in a drop of CFUs and lack of glucose consumption (Fig. 3A-B). There was no difference in the cell growth at pH 3.5 (value optimal for endogenous sophorolipid production) and pH 5.8. The cultivation medium contained a high amount of glucose (120 g/l) at the beginning of the

bioprocess, which was adapted from the optimized condition for sophorolipid production in *S. bombicola* (Lang et al., 2000). The glucose consumption rate during the production phase was slightly reduced at pH 5.8 compared to the rate at pH 3.5, and additional glucose feeding at 12 days (pH 3.5) did not increase the total LCDA titer (Fig. 3B). The final total LCDA titer at pH 8 was the lowest among the tested conditions (Fig. 3C). Additional pH optimization processes at other pH values (e.g., pH 4.6 and pH 5.2) did not improve the titer (see supplementary material). During the fermentation optimization process, the highest LCDA production (10 g/l; maximum productivity of 0.076 g/l/h between 192 h and 240 h) was achieved with the double deletion strain at pH 5.8 (Fig. 3C).

The finding that the optimal pH for LCDA production in *S. bombicola* is 5.8 is unexpected. The effect of higher pH (e.g., pH 8) on the LCDA production in *C. tropicalis* has been reported by comparing the cell growth and the production between pH 5.8 and pH 8 during the production phase. Although higher cell biomass was obtained when pH was constantly maintained at pH 5.8, the productivity was significantly reduced to about 3-fold compared to the fermentation at pH 8 (Funk et al., 2017a). The gradual increase of the pH up to 8 during the production phase significantly reduced the biomass and the LCDA production of the engineered *S. bombicola* strain (Δ*pox1*Δ*ugta1*). Furthermore, the industrial production of sophorolipids in *S. bombicola* strains is optimally conducted at pH 3.5 during the production phase (Kim et al., 2009). However, this condition was unfavourable for LCDA production even though both biosynthesis pathways share the same precursors, fatty acids. In essence, optimization of the bioprocess pH is important for successful bioconversion of LCDAs in different microbial hosts.

3.3. Creation and evaluation of the Δpox1Δugta1Δfaa1 strain

Intracellular fatty acids are activated as fatty acyl-CoAs by fatty acyl-CoA synthase (FAA1), and then further processed through the Kenndy pathway to form mainly TAGs in the lipid bodies (Ledesma-Amaro et al., 2016). Controlling the lipid metabolism-related pathway is a requisite to maintain high levels of intracellular fatty acids. For example, for free fatty acid production in *S. bombicola*, additional deletion of *faa1* in the fatty acid production strains significantly improved the titers (Jezierska et al., 2019; Salvador Lopez et al., 2022). This strategy can also be applied to the next strain generation strategy for the active channeling of fatty acids toward LCDA synthesis. To establish this third deletion, the *ura3* marker present at the former *ugta1* locus was recycled. This strain was successfully created, and the *ura3*-negative double deletion strain was used to introduce the *faa1* deletion. The correct triple deletion strain (Δ*pox1*Δ*ugta1*Δ*faa1*) was obtained (see supplementary material).

To understand the characteristics of the triple deletion strain (Δ*pox1*Δ*ugta1*Δ*faa1*), the cell growth and pH profile during shake flask cultivation were determined (Fig. 4). The strain significantly grew slower compared to the parental strain (Fig. 4A). For the triple deletion strain, only a μ_{max} value of 0.032 ± 0.007 h^{-1} was obtained, while this was 0.063 ± 0.002 h^{-1} for the parental strain ($\mu = (\log_{10}\text{Backscatter } t_2 - \log_{10}\text{Backscatter } t_1)/(t_2-t_1)$). Furthermore, there was a significant reduction of the dry cell weight of the triple deletion strain compared to the parental strain after 8 days of cultivation (Fig. 4B). The slow growth phenotype caused by the *faa1* deletion in *S. bombicola* has been observed previously (Jezierska et al., 2019). The culture medium pH of the triple deletion strain was also drastically decreased during the growth phase as it was observed with the parental strain. However, the pH during the production phase (2-8

days) did not significantly change, while the pH value from the culture medium of the parental strain was continuously decreased to pH 3 (Fig. 4C).

The high LCDA concentrations become visible in the culture broth as they turn it into a turbid mixture (Fig. 5A). Despite the affected growth, the triple deletion strain clearly produced more LCDAs, especially if a longer cultivation time to compensate for the slower growth was applied. In addition, C16 and C18 LCDAs were prominent among the total LCDA amounts as a result of the bioconversion using rapeseed oil, which contains high amounts of C16 and C18 fatty acids. Upon similar cultivation time (8 days), a 5-fold increase compared to the parental strain was observed, and even higher titers were obtained upon longer cultivation time (12 days). In this case, the titer was improved from 1.3 g/l for the parental strain to 5.5 g/l; a very significant increase that could have a great contribution to the development of an industrially relevant process (Fig. 5B). This indicates that preventing fatty acid activation is key to shuttle high levels of intracellular fatty acids toward the ω-oxidation pathway. In essence, a powerful *S. bombicola* strain for the high production of LCDA is at hand to explore for industrial applications.

3.4. Fed-batch fermentation with the best LCDA producing strain

Since the additional deletion of *faa1* significantly improved the total LCDA titer in shake flasks, the next step was to evaluate the best LCDA producing *S. bombicola* strain (Δ*pox1*Δ*ugta1*Δ*faa1*) in a fed-batch fermentation with acquired knowledge of the optimal pH 5.8. The fermentation ran for 30 days to test the LCDA production capability of the best strain and monitor any toxic effects and strain stability over time. The dry cell weight (DCW), glucose and LCDA concentrations in the fermentor were measured (Fig. 6). For cell viability and stable metabolic activity during the long fermentation,

additional glucose was supplemented to maintain the glucose concentration above 40 g/l in the fermentation broth (Fig. 6A-B; De Graeve et al., 2019). During the whole fed-batch fermentation process continuous LCDA production was observed. The measured maximum titer of LCDAs was 99.9 g/l (28 days). Note that due to the high LCDA concentration and the continuous rapeseed oil feeding during the production phase, there were some deposits in the fermentor (walls, probes, lid), hampering correct sampling. Hence, a total extraction of the broth was performed at the end (30 days), which yielded 117.8 g/l LCDA. Maximum productivity was reached between days 16 and 18 (0.64 g/l/h; Fig. 6C). During the entire bioprocess, a total of 147.86 ml of rapeseed oil was added and in the final extraction, 38.64 ml was recovered. This means that about 74% of the rapeseed oil was converted to LCDAs. In addition, the total amount of glucose added was 159 g, of which 108.5 g was consumed (68%).

As a first report on the high LCDA production in the triple deletion *S. bombicola* strain (Δ*pox1*Δ*ugta1*Δ*faa1*), information about the long-term production trend will play a significant role in the future optimization processes to meet the requirements of industrialization. The process might be more economically favorable to be stopped after 16-18 days or try to extend or mimic this most productive phase. The glucose consumption stagnates at the end of the cultivation time, again offering room for improvement to better adjust glucose addition and consumption to one another without hampering LCDA production requiring higher glucose concentrations. In addition, the feeding rate of rapeseed oil will be further optimized to increase the bioconversion rate from fed rapeseed oil to LCDAs. Although the continuous rapeseed oil feeding and slow adaptation in the oil condition avoided the cell growth defect during the production phase and resulted in high LCDA production, there was remaining rapeseed oil in the fermentation broth. A dynamic feeding control linked to monitoring the remaining

rapeseed oil amount might be important to improve the bioconversion rate and avoid difficulties of separation of pure LCDAs from the rapeseed oil remaining fermentation broth during the downstream processing. The best LCDA producing strain (Δ*pox1*Δ*ugta1*Δ*faa1*) produced a mixture of LCDAs with diverse chain lengths (mainly C16-C18 LCDAs). This is on the one hand caused by the fatty acid profile of the applied rapeseed oil and on the other hand by the specificities of the hydroxylating enzyme CYP52M1 and others (Van Bogaert et al., 2009). Nevertheless, generation of a mixture of LCDAs is not necessarily a problem for low-cost polymer manufacturing instead of using pure LCDAs. In the future, advancing membrane separation technology to isolate pure forms at low cost will broaden the use of bio-based LCDAs, enabling the application in high grade polymers that require specific type of LCDAs, beyond just simple polymer products. In summary, a safe and efficient bioprocess for C16-C18 LCDA production using rapeseed oil in the best LCDA producing *S. bombicola* strain was achieved.

4. Conclusions

This study reports the first efficient and sustainable bio-based C16-C18 long-chain dicarboxylic acid production using rapeseed oil in a non-pathogenic, oleaginous and industrially well-established yeast, *S. bombicola*. The outstanding production level showed evidence that *S. bombicola* is genetically well-equipped for this process. The achieved bioprocess will be designed to utilize cheap and renewable fatty acids sources, such as used cooking oils and grease trap waste, aligning with bioeconomy principles. This approach could pave the way for sustainable polymer production in the future.

Data availability statement

E-supplementary data for this work can be found in e-version of this paper online.

Figures

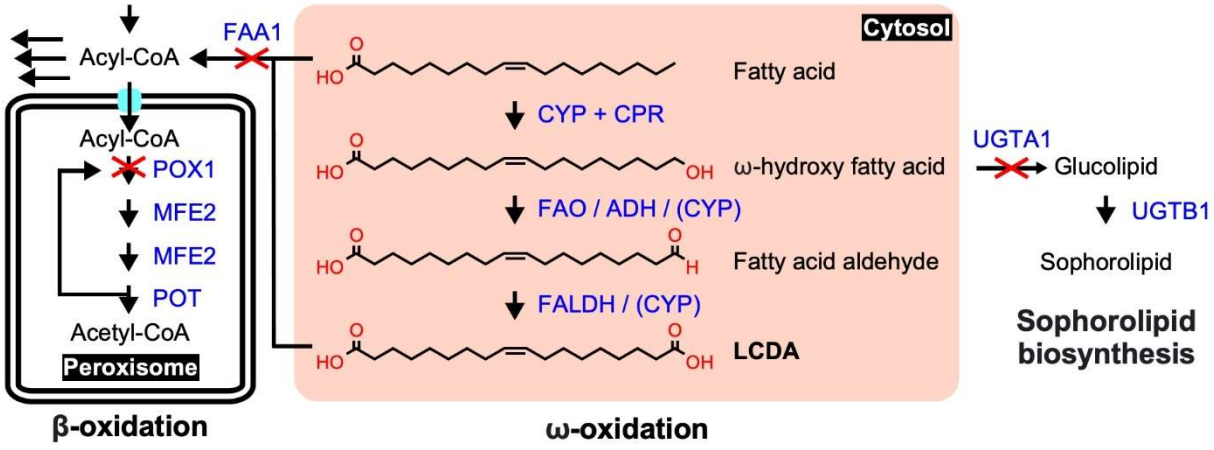

Fig. 1. Graphical representation of the expected LCDA biosynthesis from fatty acids and its competing pathways in *S. bombicola*. Enzymes are indicated in blue (CYP, cytochrome P450 monooxygenase; CPR, cytochrome P450 reductase; FAO1, fatty alcohol oxidase; ADH, alcohol dehydrogenase; FALDH, fatty aldehyde dehydrogenase; FAA1, fatty acyl-CoA synthase; POX1, fatty acyl-CoA oxidase; MFE2, multifunction enzyme type 2; POT, 3-ketoacyl-CoA thiolase; UGTA1, glucosyltransferase 1; UGTB1, glucosyltransferase 2). CYP enzymes could be involved in multiple enzymatic reactions within the ω-oxidation pathway. The three metabolic engineering strategies, blocking the precursor loss at branch points, are indicated with red crosses.

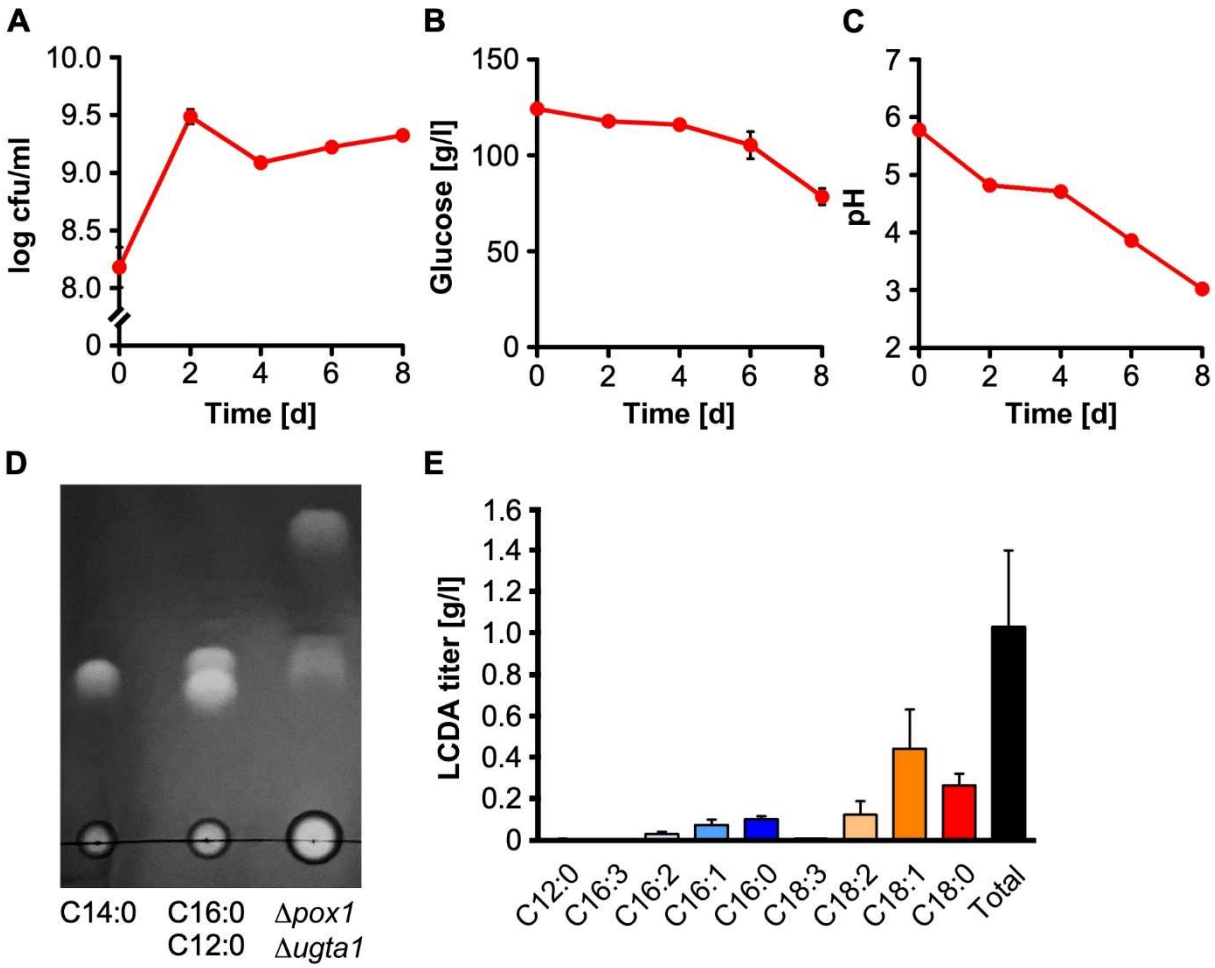

Fig. 2. Characterization of the genetically engineered strain carrying *pox1* and *ugta1* deletions and the LCDA production in shake flasks. A. The cell growth for 8 days. B. The glucose consumption over the cultivation time. C. The pH change during the cultivation. D. LCDA detection on a TLC plate. Reference LCDAs (saturated C12-16) were used to compare the position of the biosynthesized LCDAs. E. LCDA production profile of the strain and the concentrations. Three replicates (n = 3) were carried out. Error bars indicate the standard deviation of the mean.

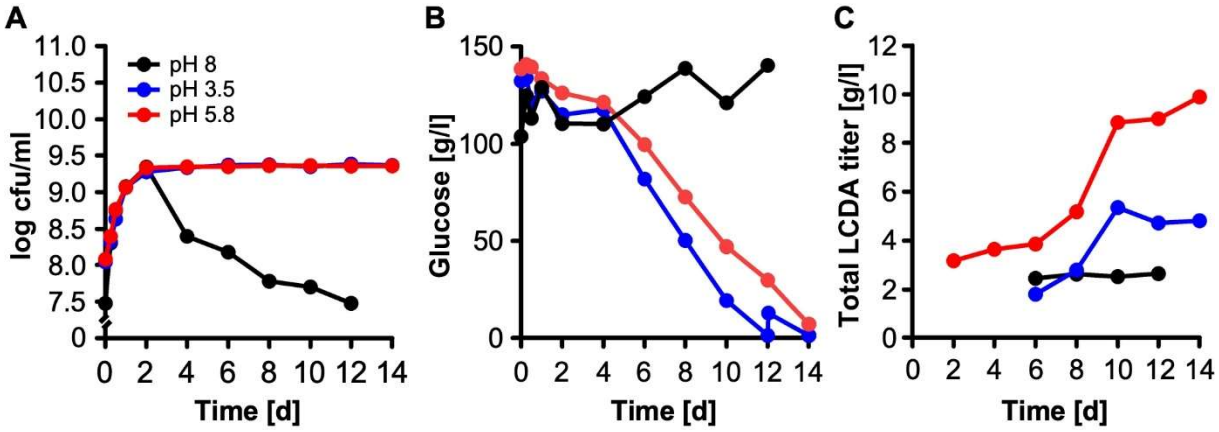

Fig. 3. Optimization of the fermentation for LCDA production in the double deletion *S. bombicola* strain (Δ*pox1*Δ*ugta1*). A. Cell growth comparison (CFU) at different pHs. B. Glucose consumption at different pHs. C. Time course LCDA production at different pHs during the fermentation.

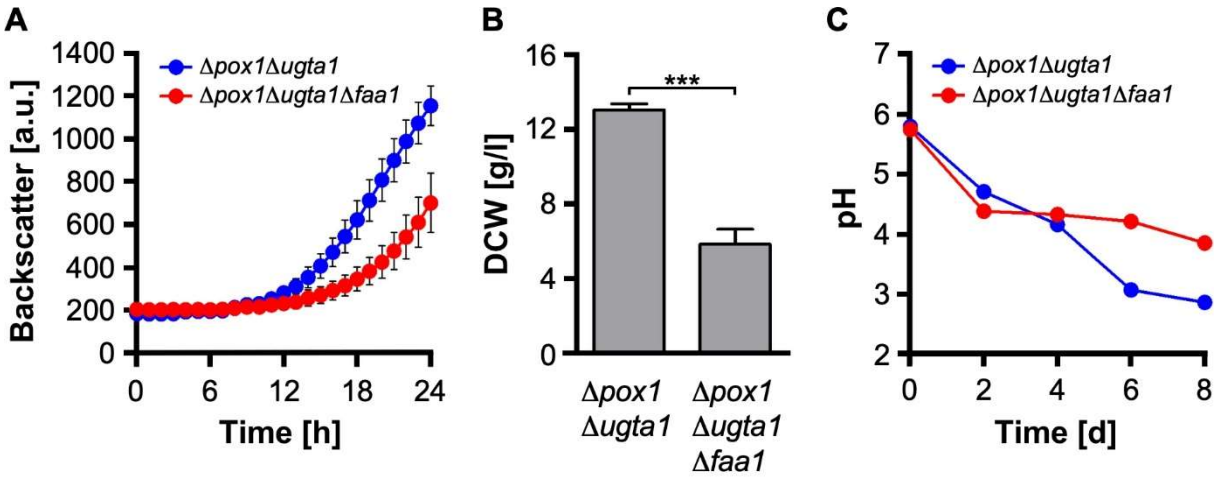

Fig. 4. Characterization of the genetically engineered strain carrying *pox1*, *ugta1* and *faa1* deletions compared to the parental strain with two deletions (Δ*pox1*Δ*ugta1*) in shake flasks for 8 days. A. Cell growth comparison in CGQ for 24 h. B. Final DCW after 8 days. C. pH profile during the cultivation. Three replicates (n = 3) were carried out. Error bars indicate the standard deviation of the mean. Statistical significance was calculated using the unpaired two-tailed *t*-test and the *p*-value was indicated above as **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

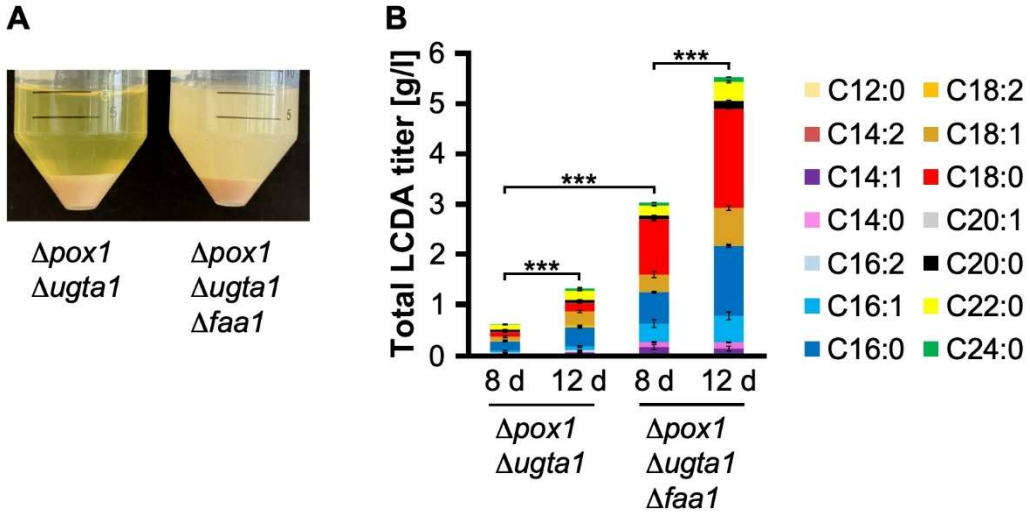

Fig. 5. LCDA production in the triple deletion strain (Δ*pox1*Δ*ugta1*Δ*faa1*) compared to the parental double deletion strain (Δ*pox1*Δ*ugta1*) in shake flasks after 8 days or 12 days. A. Difference in appearance of the culture liquid after sampling and sedimentation of the cells. B. LCDA chain length profile and quantities for the two strains. Three replicates (n = 3) were carried out. Error bars indicate the standard deviation of the mean. Statistical significance was calculated using the unpaired two-tailed *t*-test and the *p*-value was indicated above as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

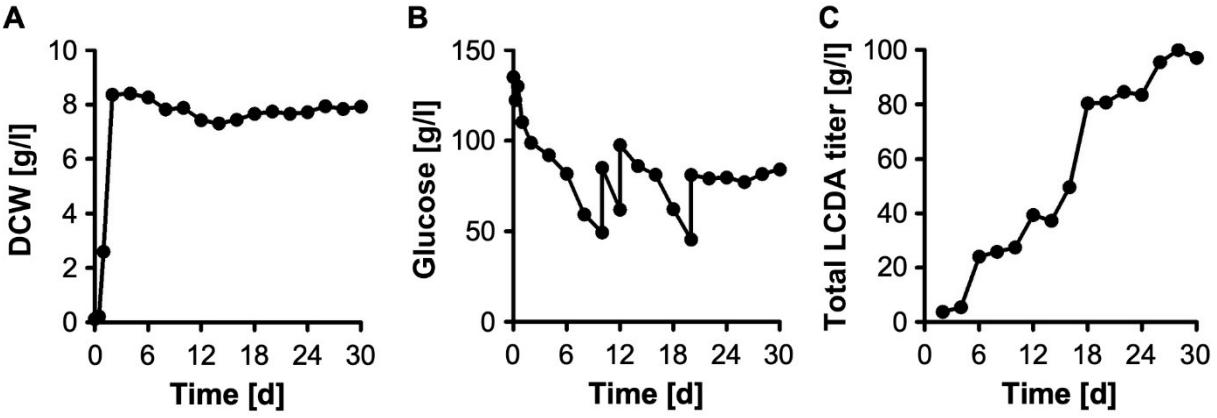

Fig. 6. The fermentation process for LCDA production in the *S. bombicola* triple deletion strain (Δ*pox1*Δ*ugta1*Δ*faa1*) at pH 5.8. A. Monitoring the cell growth (DCW). B.

Glucose concentration in a fed-batch manner. C. Time course of LCDA concentration during the fermentation.

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