Anaerobic growth of *Saccharomyces cerevisiae* CEN.PK113-7D does not depend on synthesis or supplementation of unsaturated fatty acids

Wijb J.C. Dekker†, Sanne J. Wiersma†, Jonna Bouwknegt, Christiaan Mooiman and Jack T. Pronk*

Delft University of Technology, Department of Biotechnology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands

*Corresponding author: Delft University of Technology, Department of Biotechnology, Van der Maasweg 9, 2629 HZ, Delft, The Netherlands. Tel: +31 15 2783214; E-mail: j.t.pronk@tudelft.nl

One sentence summary: *Saccharomyces cerevisiae* CEN.PK113-7D and a congenic ole1 null mutant grow anaerobically without supplementation of unsaturated fatty acids, which have long been considered as essential anaerobic growth factors for this yeast.

†These authors have contributed equally to this publication and should be considered co-first authors.

Editor: Prof. Hyun Ah Kang

ABSTRACT

In *Saccharomyces cerevisiae*, acyl-coenzyme A desaturation by Ole1 requires molecular oxygen. Tween 80, a poly-ethoxylated sorbitan-oleate ester, is therefore routinely included in anaerobic growth media as a source of unsaturated fatty acids (UFAs). During optimization of protocols for anaerobic bioreactor cultivation of this yeast, we consistently observed growth of the laboratory strain *S. cerevisiae* CEN.PK113-7D in media that contained the anaerobic growth factor ergosterol, but lacked UFAs. To minimize oxygen contamination, additional experiments were performed in an anaerobic chamber. After anaerobic precultivation without ergosterol and Tween 80, strain CEN.PK113-7D and a congenic ole1Δ strain both grew during three consecutive batch-cultivation cycles on medium that contained ergosterol, but not Tween 80. During these three cycles, no UFAs were detected in biomass of cultures grown without Tween 80, while contents of C10 to C14 saturated fatty acids were higher than in biomass from Tween 80-supplemented cultures. In contrast to its UFA-independent anaerobic growth, aerobic growth of the ole1Δ strain strictly depended on Tween 80 supplementation. This study shows that the requirement of anaerobic cultures of *S. cerevisiae* for UFA supplementation is not absolute and provides a basis for further research on the effects of lipid composition on yeast viability and robustness.

Keywords: *S. cerevisiae*; OLE1; unsaturated fatty acids; anaerobic; oxygen requirement; membrane composition

INTRODUCTION

The large majority of known yeast species ferment glucose to ethanol when grown under oxygen limitation (Barnett, Payne and Yarrow 1979; van Dijken et al. 1986). This observation implies that most yeasts do not exclusively depend on mitochondrial respiration for energy metabolism. However, only few yeasts, including *Saccharomyces cerevisiae*, are able to grow on glucose in the complete absence of oxygen (Visser et al. 1990; Merico et al. 2007). The molecular basis for the non-dissimilatory oxygen requirements of most facultatively fermentative non-*Saccharomyces* yeasts is still not completely understood (Snoek and Steensma 2006; Merico et al. 2009).
Anaerobic growth of *S. cerevisiae* imposes special nutritional requirements. Already in the 1950s, Andersen and Steir (1953a, 1953b) reported that strictly anaerobic growth of *S. cerevisiae* required supplementation of media with a sterol and an unsaturated fatty acid (UFA). Ever since these original observations, synthetic laboratory media for anaerobic growth of *S. cerevisiae* are routinely supplemented with a sterol (usually ergosterol) and a UFA source. The latter is generally provided as Tween 80, a poly-ethoxylated sorbitan ester of oleic acid (Wheeler and Rose 1973; Bulder and Reinink 1974; Fekete, Ganzler and Fekete 2010). While synthesis of nicotinic acid by *S. cerevisiae* is also oxygen dependent (Panzuzzo et al. 2002), this vitamin is not generally considered an anaerobic growth factor, as it is also routinely included in synthetic media for aerobic cultivation of this yeast.

The growth-factor-dependent ability of *Saccharomyces* yeasts to grow anaerobi-cally plays a key role in several of their large-scale industrial applications. In beer fermentation, wort is intensively aerated before inoculation to enable brewing yeast to build up stores of sterols and UFAs for the subsequent anaerobic fermentation process (Casey, Magnus and Ingledew 1983; Depaeretere et al. 2008). In artisanal wine fermentation, *S. cerevisiae* starts to dominate other ‘wild’ yeast species once oxygen has been depleted during the initial phases of fermentation (Mauricio, Milla and Ortega 1998; Holm Hansen et al. 2001).

Sterols and fatty acids are important constituents of cellular membranes. Sterols play a key role in maintenance of membrane integrity and fluidity (Rodriguez et al. 1985; Lintwood and Simons 2010), and have also been implicated in specific cellular processes such as endocytosis and nutrient uptake (Umebayashi and Nakano 2003; Pichler and Riezman 2004). The degree of (uns)aturation of the fatty-acyl moieties in phospholipids is an important determinant of membrane fluidity (de Kroon, Rijken and de Smet 2013). In addition, fatty acids are involved in energy storage and post-translational modification of proteins (Klug and Daum 2014).

De novo biosynthesis of ergosterol, the major sterol in aerobi-cally grown *S. cerevisiae*, involves a monoxygenase (*Erg1*), demethylase (*Erg3*), oxidase (*Erg25*) and desaturases (*Erg3* and *Erg5*) and requires 12 moles of O2 per mol of sterol (Summons et al. 2006). The oxygen requirement of *S. cerevisiae* for synthesis of UFAs (mainly palmityoleic acid, C16:1, and oleic acid, C18:1, (Martin, Oh and Jiang 2007)) originates from the essential role of the Δ9-fatty acid desaturase Ole1. In the presence of ferrocenochromeb5, Ole1 catalyzes the oxygen-dependent introduction of a cis double bond in palmityl-CoA and stearyl-CoA, yielding palmitoleyl-CoA (C16:1-CoA) and oleoyl-CoA (C18:1-CoA), respectively (Martin, Oh and Jiang 2007; Tehlivets, Scheuringer and Kohliwein 2007). The importance of this reaction is illustrated by the strict UFA auxotrophy of ole1 null mutants in aerobic cultures (Resnick and Mortimer 1966; Wisnieski and Kiyamoto 1972; Stukey, Mondonough and Martin 1989).

While no indications for oxygen-independent sterol biosynthesis have been found in nature, neither in living organisms nor in the fossil record (Summons et al. 2006), microbial UFA biosynthesis does not universally require oxygen. For example, during acyl-CoA synthesis by bacterial multicompartment type-II fatty-acid synthase (FAS) systems, unsaturated fatty-acyl-CoA intermediates are formed during chain elongation. Following dehydration of the acyl-chain, the double bond of this intermediate of the elongation cycle can be isomerized. This isomerization precludes saturation in subsequent steps and thereby conserves the double bond (White et al. 2005). Furthermore, in contrast to the cytosolic *S. cerevisiae* type-I FAS complex that only produces saturated fatty acids (Tehlivets, Scheuringer and Kohliwein 2007), some bacterial type-I FAS proteins are capable of oxygen-independent UFA synthesis (Stuible, Meurer and Schweizer 1997; Radmacher et al. 2005).

Based on reported biomass contents, oxygen requirements for UFAs and sterols in *S. cerevisiae* each amount to ~0.1 mmol O2 (g biomass)−1 (Bulder and Reinink 1974; Otero et al. 2010; Caspeta et al. 2014). However, it should be noted that UFA and sterol contents strongly depend on strain background and culture conditions (Deytieux et al. 2005). In laboratory-scale cultures, which have a high surface-to-volume ratio, extensive precautions have to be taken to prevent such small amounts of oxygen from entering cultures. For example, cultivation in serum flasks requires removal of oxygen by autoclaving and use of septa that are highly resistant to oxygen diffusion (Miller and Wolin 1974). Minimizing entry of small amounts of oxygen into bench-top laboratory bioreactors is even more challenging and requires use of ultrapure nitrogen gas, applying overpressure and using special materials for tubing and septa (Visser et al. 1990; da Costa et al. 2018). Furthermore, as indicated by the practice of aerobically ‘loading’ brewing yeasts (Casey et al. 1983; Depaeretere et al. 2008), intracellular stores of ergosterol and UFAs of aerobically pregrown yeast cells may support several generations of growth upon transfer to anaerobic media that lack these anaerobic growth factors.

This paper describes how, during experiments aimed at optimizing bioreactor cultivation protocols for anaerobic growth of the laboratory strain *S. cerevisiae* CEN.PK113-7D (Entian and Köter 2007; Nijkamp et al. 2012), growth was consistently observed in synthetic media that were not supplemented with UFAs, while elimination of both sterols and UFAs almost completely blocked growth. These observations led to the hypothesis that, in contrast to the common assumption in the literature on anaerobic yeast physiology, *S. cerevisiae* does not absolutely require UFAs for anaerobic growth. To test this hypothesis, we analyzed growth of *S. cerevisiae* CEN.PK113-7D and a congenic ole1 null mutant in cultures grown in an anaerobic chamber and analyzed the lipid composition of anaerobically grown biomass.

**MATERIALS AND METHODS**

**Strains, media and maintenance**

*S. cerevisiae* strains used and constructed in this study (Table 1) were derived from the CEN.PK lineage (Entian and Köter 2007; Nijkamp et al. 2012). Yeast extract peptone dextrose medium (YPD; 10 g L−1 Bacto yeast extract, 20 g L−1 Bacto peptone, 20 g L−1 glucose) was used for making frozen stock cultures. Synthetic medium with 20 g L−1 glucose (SMD) was prepared as described previously (Verduyn et al. 1992). Synthetic urea medium (SMD-urea), in which ammonium sulfate was replaced by 2.3 g L−1 urea and 6.6 g L−1 K2SO4 was prepared as described earlier (Luttik et al. 2000). Similarly, for selection of transformants carrying the amdS marker cassette, ammonium sulfate in SMD was replaced by 10 mM acetamide and 6.6 g L−1 glucose and 6.6 g L−1 K2SO4 was prepared as described earlier (Luttik et al. 2000). SIM media and YP media were autoclaved at 121 and 110 °C, respectively, for 20 min. Where indicated, unsaturated fatty acids and/or sterols were added to autoclaved medium as Tween 80 (polyethylene glycol sorbitol monooleate, Merck, Darmstadt, Germany) and ergosterol (≥95% pure, Sigma-Aldrich, St. Louis, MO), respectively. Concentrated stock solutions of these anaerobic growth factors were prepared by dissolving 8.4 g Tween 80
(equivalent to 7.8 mL) and 0.2 g ergosterol in 17 mL of absolute ethanol, or by dissolving 0.2 g ergosterol in 25 mL absolute ethanol. These stock solutions were incubated at 80°C for 20 min before diluting them 800-fold in growth medium, yielding final concentrations of 420 mg L\(^{-1}\) Tween 80 and/or 10 mg L\(^{-1}\) ergosterol. Escherichia coli XL1-Blue was grown in Lysogeny Broth (LB; 10 g L\(^{-1}\) Bacto tryptone, 5 g L\(^{-1}\) Bacto yeast extract and 5 g L\(^{-1}\) NaCl). For selection of transformants, LB was supplemented with 100 mg L\(^{-1}\) ampicillin. After addition of sterile glycerol (30% v/v), culture samples were frozen and stored at −80°C. ’Super optimal broth’ (SOB) medium contained 0.5 g L\(^{-1}\) yeast extract, 2 g L\(^{-1}\) Bacto tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl\(_2\), 6H\(_2\)O, 10 mM MgSO\(_4\), 7H\(_2\)O, and was autoclaved at 121°C for 20 min. To prepare ‘super optimal broth medium with catabolite repression’ (SOC), a concentrated solution of glucose, separately autoclaved at 110°C for 30 min, was added to SOB to a final concentration of 20 mM.

**Molecular biology techniques**

To amplify DNA fragments for plasmid construction, Phusion\textsuperscript{®} High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA) was applied as specified in the manufacturer’s protocol, using PAGE-purified oligonucleotide primers (Sigma-Aldrich). Diagnostic polymerase chain reaction (PCR) was performed with DreamTaq PCR Master Mix (Thermo Scientific), according to the manufacturer’s protocol and with desalted oligonucleotide primers (Sigma-Aldrich). PCR-amplified linear integration cas- settes were purified from 1% (w/v) agarose gels (TopVision Agarose, Thermo Fisher) with TAE buffer (50x, Thermo Fisher) using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). E. coli XL1-Blue competent cells were transferred by heat shock for 40 s at 42°C and, after 1 h recovery at 37°C in SOC medium, plated on selective LB ampicillin media. The GenElute Plasmid Miniprep Kit (Thermo Fisher Scientific) was used to isolate plasmids from overnight cultures in 15 mL Greiner tubes on selective medium. S. cerevisiae was transformed with the lithium-acetate method (Gietz and Woods 2002). Transformants were selected on solid agar with acetamide as sole nitrogen source. Single-cell lines of transformants were obtained by three consecutive re-streaks on solid selective medium.

**Plasmid and strain construction**

Markerless CRISPR/Cas9-based genome editing of S. cerevisiae was performed as described previously (Mans et al. 2015). Oligonucleotides and plasmids used in this study are listed in Tables 2 and 3, respectively. A unique guide-RNA (gRNA) sequence targeting OLE1 was designed using YeastRictron (Mans et al. 2015) and synthesized as a 103 bp oligonucleotide (Sigma). To construct the OLE1-targeting CRISPR plasmid pUDR319, the plasmid backbone of pROS11 was first PCR-amplified with the double-binding primer 6005. The gRNA-targeting sequence was then introduced as 5’ primer overhang with the double-binding primer 11986, using pROS11 as template. Subsequently, both PCR products were gel purified, digested with DpnI (Thermo Scientific) and mixed in equimolar ratio. Gibson assembly was performed in a final volume of 5 μL with the NEBuilder HiFi DNA assembly master mix (NEB, Ipswich, MA), according to the manufacturer’s instructions. Assembled plasmids were transformed into E. coli and selected on solid LB-ampicillin medium. To delete OLE1 in S. cerevisiae, 500 ng of the gRNA plasmid (pUDR319) was transformed to strain IMX585, together with 400 ng of the annealed 120 bp double-strand DNA repair fragment (oligonucleotides 11239 & 11240). This repair fragment consisted of homologous 60 bp sequences immediately up- and downstream of the OLE1 coding sequence. Cells were selected on solid SM with acetamide as nitrogen source for plasmid selection and Tween 80 to supplement UFA auxotrophic transformants. Deletion of OLE1 was verified by diagnostic PCR amplification with primers 11249 & 11250. The CRISPR gRNA plasmid was removed by cultivation in YPD with Tween 80 and subsequent single-cell selection on SMD agar plates with Tween 80. Plasmid loss was checked by streaking the resulting single-colony isolates on SMD with 5-fluoroacetamide (Solis-Escalante et al. 2013).

**Aerobic growth studies in shake flasks**

Aerobic growth studies of S. cerevisiae strains were performed in 500-mL round-bottom shake flasks filled with 100 mL SMD containing 20 g L\(^{-1}\) glucose as carbon source, with or without supplementation of Tween 80. Precultures were inoculated from frozen glycerol stocks and grown overnight on the same medium and used to inoculate fresh flasks, at an initial optical density at 660 nm (OD\(_{660}\)) of 0.2. OD\(_{660}\) was monitored at regular time intervals using a 7200 visible spectrophotometer (Jenway, Staffordshire, UK). All aerobic shake-flask experiments were carried out in duplicate, in an Innova shaker incubator (New Brunswick Scientific, Edison, NJ) set at 30°C and 200 rpm.

**Anaerobic bioreactor cultivation**

Anaerobic bioreactor batch cultivation was performed in 2-L laboratory bioreactors (Applikon, Schiedam, the Netherlands) with a working volume of 1.2 L. Before autoclaving, bioreactors were tested for gas leakage by applying 0.3 bar overpressure while completely submerging them in water. Anaerobic conditions were maintained by continuous flushing of the headspace of bioreactor cultures with 500 mL N\(_2\) min\(^{-1}\) (≤0.5 ppm O\(_2\), H\(_2\)Q Nitrogen 6.0, Linde Gas Benelux, Schiedam, the Netherlands) and, after inoculation, by maintaining an overpressure of 0.2 bar in the headspace. Oxygen diffusion was minimized by using Fluran tubing (14 Barrer O\(_2\), F-5500-A, Saint-Gobain, Courbevoie, France) and Viton O-rings (Eriks, Alkmaar, the Netherlands). Furthermore, bioreactor cultures were grown on SMD-urea (Luttik et al. 2000) to eliminate the need for pH control and, thereby, to prevent oxygen entry via alkali titration or diffusion through pH probes. The autoclaved mineral salts solution
was supplemented with 0.2 g L⁻¹ sterile antifoam emulsion C (Sigma-Aldrich, St. Louis, MA). Bioreactors were continuously stirred at 800 rpm and temperature was controlled at 30 °C. The outlet gas of bioreactors was cooled to 4 °C and overpressure (0.2 bar) was applied throughout empty-refill cycles. To further minimize oxygen contamination, gassing was temporarily switched from headspace to sparging during refilling. To remove any oxygen contamination in stagnant medium, the 5-L glass medium reservoir vessel was sparged with N5.5 grade nitrogen gas (Linde Gas Benelux) for at least one h before refilling. Immediately before refilling, ∼20 mL medium was purged from the medium inlet line to remove any oxygen contamination in stagnant medium. To further minimize oxygen contamination, gassing was temporarily switched from headspace to sparging during refilling and overpressure (0.2 bar) was applied throughout empty-refill cycles.

**Anaerobic growth studies in shake flasks**

Anaerobic shake-flask experiments were performed in a Shel Lab Bactron 300 anaerobic workstation (Sheldon Manufacturing Inc., Cornelius, OR) at 30 °C. The anaerobic gas mixture used for flushing the work space and air lock consisted of 85% N₂, 10% CO₂ and 5% H₂. An IKA® KS 260 Basic orbital shaker platform (Dijkgraaf Verenigde BV, Lelystad, The Netherlands) placed in the anaerobic chamber was set at 200 rpm. During anaerobic experiments, the air lock was used fewer than three times per week. To minimize oxygen entry during this procedure, a regenerated Pd catalyst for H₂-dependent oxygen removal was introduced into the chamber whenever the air lock was used. Cultures were grown in 50-mL round-bottom shake flasks containing 40 mL SMD-urea supplemented with either 20 or 50 g L⁻¹ glucose. Concentrated solutions of ergosterol and/or Tween 80 were added as indicated. Sterile growth media were preincubated in the anaerobic chamber for at least 48 h prior to inoculation to allow for complete removal of oxygen. Growth experiments in the anaerobic chamber were started by inoculating anaerobic shake flasks, containing SMD-urea without both ergosterol and Tween 80 and containing 50 g L⁻¹ glucose, at an initial OD₆₀₀ of 0.2, from an exponentially growing aerobic preculture on SMD. Growth was measured by high-performance liquid chromatography (HPLC) as described previously (Verhoeven et al. 2017). Biomass dry weight measurements in SBR cultures were performed at the end of each cultivation cycle, using preweighed nitrocellulose filters.
(0.45 μm, Gelman Laboratory, Ann Arbor, MI). After filtration of 10 or 20 mL culture samples, filters were washed with demineralized water prior to drying in a microwave oven (20 min at 360 W).

Fatty acids in biomass were analyzed as methyl-ester derivatives by gas chromatography with flame-ionization detection (GC-FID). Biomass samples were harvested by centrifuging at least 30 mL of culture broth at 3000 g for 5 min. Pellets were washed once with demineralized water and stored at −80 °C. Frozen samples were lyophilized overnight in a freeze-dryer (Alpha 1–4 LD Plus, Christ, Osterode am Harz, Germany) and 20 to 30 mg of lyophilized material was weighed into glass methylation tubes (Article no. 10044604, PYREX™ Borosilicate glass, Thermo Fisher Scientific). After adding 2 mL methanol (Honeywell, Mexico City, Mexico), samples were vortexed thoroughly every 15 min, and then rapidly chilled on ice to room temperature. After addition of 2 mL of Milli-Q water (Merck), samples were again vortexed, and centrifuged at 3000 g for 5 min to ensure phase separation. The upper heptane phase was transferred to a 2 mL Eppendorf tube (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) containing 10–20 mg dried Na2SO4 (Merck) to remove remaining traces of water and shaken vigorously. After centrifugation (5 min at 5000 g), the liquid phase was transferred to a GC vial (11 mm crimp-neck vial (10326042) and cap (11821653) with butyl rubber septum (Thermo Fisher Scientific)). The sample was concentrated by evaporating the solvent under a stream of N2. Fatty acid methyl esters were analyzed on an Agilent Technologies 7890A GC-FID system equipped with an FID-1000–220 Gas Station (Parker Balston, Haverhill, MA, USA) and an Agilent Technologies 7693 Autosampler. A VF-5 ms column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness, Agilent part no. CP9013) was used for separation, and nitrogen was used as a carrier gas at a constant flow of 0.4 mL min⁻¹. The oven temperature, which was initially 50 °C, was increased to 220 °C at 60 °C min⁻¹, then kept constant for 3 min, increased to 250 °C at 8 °C min⁻¹, again kept constant for 3 min, and finally increased to 320 °C at 60 °C min⁻¹ and kept constant for another 6 min. Inlet temperature was set at 150 °C, and FID temperature at 280 °C. The Supelco FAME mix C8-C24 (Sigma-Aldrich, MO, USA) was used to calibrate the GC-FID system for quantification of individual fatty acid methyl esters. A separate 10-point calibration curve was made with methyl oleate (∼99%, Sigma-Aldrich). Data were adjusted for internal standard concentrations and expressed per g of lyophilized biomass.

RESULTS
Minimal growth in anaerobic batch bioreactors of S. cerevisiae in the absence of sterols and unsaturated fatty acids

In view of reported technical challenges in achieving strictly anaerobic growth conditions in laboratory bioreactor cultures of yeasts (Visser et al. 1990; da Costa et al. 2018), we attempted to eliminate sources of oxygen contamination in bioreactor batch cultures of S. cerevisiae. The bioreactor headspace was continuously flushed with ultrapure nitrogen gas and kept under pressure; special tubing and septa were used to minimize oxygen diffusion and no pH or oxygen sensors were used. To assess whether these measures were successful, bioreactor batch cultures were grown on synthetic medium without the anaerobic growth factors ergosterol and Tween 80. After inoculation with an aerobically grown preculture, CO2 production rapidly increased until, after 17 h, it reached a maximum and subsequently decreased (Fig. 1A). At this stage, the biomass concentration in the cultures had increased from 0.21 ± 0.00 to 0.60 ± 0.02 g L⁻¹ while the glucose concentration was still above 14 g L⁻¹ (Figure S1, Supporting Information). Reactors were then emptied, leaving 25 mL culture broth in the reactor, and refilled with fresh medium without ergosterol and Tween 80. In the subsequent batch culture, CO2 production was much lower than in the first culture and remained stable for 24 h (Fig. 1A). Optical density measurements showed that fewer than two biomass doublings had occurred over this period, leading to a biomass concentration of ∼0.2 g L⁻¹. These results strongly suggested that growth in the first anaerobic batch culture was supported by ‘carry-over’ of anaerobic growth factors from aerobically pregrown inoculum. An initial anaerobic cultivation cycle on medium without ergosterol and Tween 80 (‘carry-over culture’) was therefore implemented in all subsequent experiments in anaerobic bioreactors, as well as in growth experiments in an anaerobic chamber.

Although the slow increase of the biomass concentration during the second cultivation cycle suggested that oxygen entry had not been completely eliminated, the experimental set-up was considered suitable for further studies on anaerobic growth-factor requirements. As a pilot experiment, ergosterol (0.55 mg L⁻¹) was administered 24 h into the second anaerobic cultivation cycle. After ergosterol addition, CO2 production rapidly accelerated and the biomass concentration increased to 0.86 ± 0.03 g L⁻¹ (Fig. 1A), indicating that, during the second cultivation cycle, growth in the anaerobic bioreactors was restricted by sterol availability. Since no Tween 80 was added, this observation raised questions about the requirement of these anaerobic cultures for UFAs.

Omission of UFAs does not prevent growth in anaerobic SBR cultures

To further investigate the observed anaerobic growth of S. cerevisiae CEN.PK113-7D in synthetic medium supplemented with ergosterol, but not with Tween 80, experiments were performed in SBRs. After an initial carry-over cycle, four consecutive SBR cycles on medium without Tween 80 showed a pronounced CO2 production and corresponding increase of the biomass concentration (Fig. 1B). Specific growth rates estimated from the exponential phases of CO2 production, as well as estimated biomass yields on glucose, were similar throughout these four cycles (Table 4). To investigate whether growth without Tween 80 supplementation reflected de novo UFA biosynthesis, enabled by inadvertent entry of oxygen into the bioreactors, fatty acids were extracted from biomass harvested at the end of each SBR cycle and analyzed by gas chromatography. At the end of the ‘carry-over’ cycle, but also at the end of the subsequent four cycles on medium from which Tween 80 was omitted, small quantities of palmitoleate (C16:1) and oleate (C18:1) were detected (Fig. 2A; Table S2, Supporting Information). Since the four SBR cycles led to an ~5·10⁶-fold dilution of any UFAs remaining in yeast biomass after the initial carry-over cycle, presence of these UFAs most probably indicated de novo UFA synthesis due to leakage of oxygen into the reactors.
UFA-independent growth of a reference strain and an ole1 null mutant in an anaerobic chamber

To further reduce oxygen contamination, UFA-independent anaerobic growth of the reference strain S. cerevisiae IMX585 (CEN.PK113-7D with a chromosomally integrated Cas9 expression cassette, Mans et al. 2015) was studied in an anaerobic chamber equipped with a H2/Pd catalyst system to scavenge traces of oxygen. Since ole1Δ strains of S. cerevisiae are unable to synthesize UFAs (Resnick and Mortimer 1966; Stukey, Mcdonough and Martin 1989), growth of the congenic ole1Δ mutant was studied in the same system to exclude the possibility of de novo UFA synthesis. As observed in SBR cultures, both strains grew during an initial anaerobic ‘carry-over’ culture on medium without sterols or UFAs. However, upon transfer to a second anaerobic shake-flask culture without these supplements, virtually no growth was observed for the two strains over a period of 180 h (Fig. 3).

After the initial carry-over culture, both the reference strain and the ole1Δ mutant grew to similar optical densities in medium supplemented with both Tween 80 and ergosterol, indicating that deletion of ole1 did not negatively affect growth in UFA-supplemented anaerobic cultures. In addition, both strains grew in three consecutive transfers in anaerobic shake flasks containing synthetic medium supplemented with only ergosterol. During these serial transfers, similar maximum optical densities were again reached for both strains (Fig. 3). These observations suggested that, at least in the CEN.PK genetic background, synthesis or supplementation of UFAs is not required for anaerobic growth of S. cerevisiae. This hypothesis was further tested by analyzing the lipid content and composition of yeast biomass in the serial transfer experiments.

At the end of the carry-over cultures, UFAs were detected in both strains (Fig. 2B and C). Since growth in the carry-over cultures ceased before glucose was depleted (Table S1, Supporting Information), this observation suggested that depletion of sterols rather than depletion of UFAs caused growth to stop. No UFAs were detected during three subsequent transfers in medium without Tween 80, neither in the reference strain nor in the ole1Δ mutant. Instead, contents of palmitic acid (C16:0) and short-chain saturated fatty acids (C10-C14) were higher in cultures supplemented with Tween 80 (Fig. 2B and C; Table S2, Supporting Information).

When cells from a stationary-phase carry-over culture were instead transferred to medium containing both ergosterol and Tween 80, oleic acid (C18:1), which is the main UFA side-chain of Tween 80 (Buider and Reinink 1974), was the dominant fatty acid in yeast biomass (Fig. 2B and C; Table S2, Supporting Information).

UFA synthesis or supplementation is essential for aerobic growth

Several previous studies reported that ole1Δ strains constructed in other S. cerevisiae genetic backgrounds are unable to grow aerobically without UFA supplementation (Resnick and Mortimer 1966; Stukey, Mcdonough and Martin 1989; Giaever et al. 2002).
Figure 2. Fatty acid composition of anaerobic S. cerevisiae cultures. Fatty acid composition, analyzed by GC-FID, of anaerobically grown cultures in SBRs (A) and anaerobic-chamber shake-flask cultures (B, C). (A) Fatty acid composition of the reference strain CEN.PK113–7D during anaerobic SBR cultivation; 'CO': carry-over cycle; 1–4: subsequent SBR cycles 1–4 on synthetic medium supplemented with ergosterol but not Tween 80. (B) and (C) Fatty acid composition of strains IMX585 (CEN.PK113–7D with Cas9 integrated in genome) and its congenic ole1/Delta1 mutant IMK861, respectively, during serial-transfer shake-flask cultivation in an anaerobic chamber; 'CO': carry-over culture; 'TE': cultures grown on synthetic medium supplemented with both anaerobic growth factors; 1–3: transfers 1–3, respectively, of cultures grown on synthetic medium supplemented with ergosterol but not Tween 80. Each panel shows data from independent duplicate bioreactor or shake-flask cultures grown on synthetic medium.

Figure 3. UFA-independent anaerobic growth of the reference strain S. cerevisiae IMX585 and the ole1/Delta1 strain IMK861. Strains IMX585 (A) and IMK861 (ole1/Delta1) (B) were grown in shake-flask cultures placed in an anaerobic chamber. Aerobic precultures were used to inoculate an anaerobic preculture ('carry-over culture', open squares, gray box) on SMD containing 50 g L$^{-1}$ glucose but no anaerobic growth factors. When the OD$_{600}$ no longer increased, cultures were transferred to fresh SMD, supplemented either with Tween 80 and ergosterol (closed squares), only ergosterol (closed circles) or neither (open circles). The initial culture to which only ergosterol was added (closed circles, first line) was sequentially transferred to the same medium (closed circles, second and third line). The data are from a single representative experiment of biological duplicate cultures. Data of the duplicate experiment are shown in Figure S2 (Supporting Information).
These results do not entirely exclude a minute UFA requirement in SMD without Tween 80 (closed symbols) and in SMD with Tween 80 (open symbols). Data represent mean and standard error of the mean of independent biological duplicate cultures. The carry-over culture had been diluted by 500-fold. Although our results indicate that ole1 null mutants to aerobic conditions should provide a relevant experimental system to further explore this interesting problem. Although other environmental stresses (Okuyama et al. 1979; Klose et al. 2012). A similar adaptation was previously observed in promitochondria of cells after anaerobic incubation without a source of UFAs (Paaltaufl and Schatz 1969) and in a recent chemostat study on severe oxygen limitation in chemostat cultures without UFA or sterol supplementation (da Costa et al. 2019).

**DISCUSSION**

For over six decades, yeast researchers have based the design of anaerobic growth media on the assumption that anaerobic growth of *S. cerevisiae* strictly requires UFA supplementation. While this study confirms previous reports that synthesis or supplementation of UFAs is required for aerobic growth of *S. cerevisiae* (Stukey, Mcdonough and Martin 1989; Giaever et al. 2002), it indicates that, surprisingly, the UFA requirement for anaerobic growth of *S. cerevisiae* is not absolute.

Since nonrespiratory oxygen requirements of *S. cerevisiae* are small (Rosenfeld and Beauvoit 2003), interpretation of results can easily be obscured by oxygen contamination and by ‘carry-over’ of anaerobic growth factors from aerobic or growth-factor-supplemented precultures. Indeed, oxygen contamination of bioreactor experiments was evident from synthesis of small amounts of palmitoleic and oleic acid (Fig. 2A; Table S1, Supporting Information). This UFA synthesis occurred despite extensive precautions to prevent oxygen entry, which sufficed to severely restrict growth in the absence of both ergosterol and Tween 80. Residual production of unsaturated fatty acids, despite extensive measures to exclude oxygen, was also observed in a recent chemostat study in which both ergosterol and Tween 80 were omitted from growth media (da Costa et al. 2019). No *Km* values for oxygen of *S. cerevisiae* Ole1 or related Δ9 desaturases have been reported in the literature. However, these results suggest that Ole1 has a very high affinity for oxygen which, even under extreme oxygen limitation, enables yeast cells to efficiently scavenge oxygen for UFA synthesis.

Serial-transfer experiments in an anaerobic chamber, equipped with a Pd/H2 system to remove traces of oxygen, did not show detectable UFA levels in biomass grown on synthetic medium without Tween 80. Nevertheless, after an initial ‘carry-over’ culture, growth of a reference strain and of an ole1Δ mutant continued during three consecutive transfers in UFA-free medium. UFA contents were already below detection limit after the first cycle and, after the second cycle, biomass of the carry-over culture had been diluted by ∼500-fold. Although these results do not entirely exclude a minute UFA requirement for anaerobic growth, any remaining UFA levels in the serial batch cultures were too low to account for maintenance of membrane fluidity (Degreif et al. 2017). Anaerobic cultures in UFA-free medium showed increased contents of medium-chain (C10 to C14) fatty acids. This adaptation is in line with the demonstrated flexibility of the yeast lipidome in response to other environmental stresses (Okuyama et al. 1979; Klose et al. 2012). A similar adaptation was previously observed in promitochondria of cells after anaerobic incubation without a source of UFAs (Paaltaufl and Schatz 1969) and in a recent chemostat study on severe oxygen limitation in chemostat cultures without UFA or sterol supplementation (da Costa et al. 2019).

Anaerobic growth of *S. cerevisiae* is only rarely studied in media that contain sterols, but not UFAs. In the original work of Andreasen and Stier, cell counts that reached in cultures that were supplemented with only ergosterol were slightly higher than in controls with only UFA supplementation or in the absence of both growth factors (Andreasen and Stier 1953b). One reason for the routine inclusion of Tween 80 is that this surfactant aids distribution of highly hydrophobic sterols in aqueous media (Bikhazi and Higuchi 1971). A requirement of anaerobic *S. cerevisiae* cultures for UFA supplementation is often inferred from the well-documented UFA auxotrophy of ole1 null mutants in aerobic cultures. Stukey, Mcdonough and Martin (1989) showed that aerobic growth of an ole1 null mutant ceased when the contribution of UFAs decreased below 7.3% mol of the total fatty acid content. The sustained anaerobic growth of an ole1Δ mutant in UFA-free media, with undetectable intracellular UFA contents (Figs 2 and 3), reveals that UFA requirements of *S. cerevisiae* strongly depend on oxygen status.

While experimentally addressing the question why UFA requirements of aerobic and anaerobic *S. cerevisiae* cultures differ is beyond the scope of this study, at least two hypotheses can be formulated based on the literature. Esterification of sterols with fatty acids, predominantly with oleate (C18:1-Δ9), plays a key role in the complex regulation of sterol homeostasis (Ferreira et al. 2004) and steryl-ester synthesis has been demonstrated to decrease during anaerobiosis (Valachovič, Hronská and Hapala 2001). The lower sterol content of anaerobic *S. cerevisiae* cultures may well render them less sensitive to UFA depletion. In mammalian cells, oleate prevents mitochondrial generation of reactive oxygen species (ROS) under palmitate stress (Yuzefovich, Wilson and Rachek 2010). If the same mechanism occurs in yeast mitochondria, absence of respiratory ROS generation in anaerobic cultures could offer an explanation for their tolerance to UFA depletion. Shifting anaerobically grown, UFA-free cultures of an ole1Δ mutant to aerobic conditions should provide a relevant experimental system to further explore this interesting problem.
elimination of Tween 80 from growth media negatively affected growth rate and biomass yield. SBR cultures supplemented with ergosterol but not Tween 80, in which the biomass still contained small amounts of palmitoleic and oleic acid (Table S2, Supporting Information), showed an estimated specific growth rate of 0.14 and 0.20 h\(^{-1}\) (Table 4). This value is significantly lower than reported for anaerobic batch cultures of S. cerevisiae CEN.PK113-7D supplemented with both Tween 80 and ergosterol (Bisschops et al. 2015). Biomass yields on glucose (0.04 g biomass (g glucose)\(^{-1}\), Table 4) were ∼2-fold lower than in anaerobic chemostat cultures grown with Tween 80 supplementation (Boender et al. 2009). This low biomass yield might reflect increased leakage of protons and/or other solutes across UFA-depleted membranes, for example caused by mislocalization of proteins in membranes with a high proportion of saturated lipids (Budin et al. 2018). Increased membrane permeability may also contribute to the lag phases observed in anaerobic chamber experiments upon transfer of stationary-phase cultures, grown without Tween 80, to fresh UFA-free medium (Fig. 3).

We hope that our results, which were generated with yeast strains belonging to a single genetic background and under a limited set of experimental conditions, will inspire further research into the physiology and ecological relevance of UFA-independent growth of yeasts. As illustrated by the strain and context dependency of lipid composition in aerobic S. cerevisiae cultures (Daum et al. 1999; Otero et al. 2010; Madsen et al. 2011), it is relevant to explore whether UFA-independent growth also occurs in other S. cerevisiae genetic backgrounds and in related species and genera. Further research is also needed to investigate the impact of UFA depletion on robustness of S. cerevisiae cultures, for example, at low pH and at different temperatures. Furthermore, availability of a S. cerevisiae strain that can grow without UFA supplementation provides an interesting starting point for laboratory evolution experiments and studies on membrane engineering for improved cellular performance (Degreif et al. 2017; Ma et al. 2019).

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSYR online.

**FUNDING**

This work was funded by an Advanced Grant of the European Research Council to JTP (grant #694633).

**ACKNOWLEDGEMENTS**

We gratefully acknowledge the technical advice and support of Erik de Hulster for bioreactor research, and Marijke Luttik and Patricia van Dam for GC analysis. We thank Taufik Abdullah, MSc, for preliminary observations on UFA-independent growth during his MSc research project and our colleagues in the Industrial Microbiology group of TU Delft for stimulating discussions.

**Conflicts of Interests.** None declared.

**REFERENCES**

Andreasen AA, Stier TJB. Anaerobic nutrition of Saccharomyces cerevisiae I ergosterol requirement for growth in a defined medium. J Cell Comp Physiol 1953a;43:271–81.

Andreasen AA, Stier TJB. Anaerobic nutrition of Saccharomyces cerevisiae II unsaturated fatty acid requirement for growth in a defined medium. J Cell Comp Physiol 1953b;41:23–6.

Barnett JA, Payne RW, Yarrow D. A Guide to Identifying and Classifying Yeasts. Cambridge: Cambridge University Press, 1979.

Bikhazi AB, Higuchi WI. Interfacial barriers to the transport of sterols and other organic compounds at the aqueous polysorbate 80-hexadecane interface. Biochim Biophys Acta 1971;233:676–87.

Bisschops MM, Vos T, Martínez-Moreno R et al. Oxygen availabilty strongly affects chronological lifespan and thermostolerence in batch cultures of Saccharomyces cerevisiae. Microb Cell 2015;2:429–44.

Boender LGM, de Hulster EA, van Maris AJ et al. Quantitative physiology of Saccharomyces cerevisiae at near-zero specific growth rates. Appl Environ Microbiol 2009;75:5607–14.

Budin I, de Rond T, Chen Y et al. Viscous control of cellular respiration by membrane lipid composition. Science 2018;7925:1–10.

Bulder CJEA, Reinkink M. Unsaturated fatty acid composition of wild type and respiratory deficient yeasts after aerobic and anaerobic growth. Antonie Van Leeuwenhoek 1974;40:445–55.

Casey GP, Magnus CA, Ingledew WM. High gravity brewing: nutrient enhanced production of high concentrations of ethanol by brewing yeast. Biotechnol Lett 1983;5:429–34.

Caspeta L, Chen Y, Ghiaic P et al. Altered sterol composition renders yeast thermostolerant. Science 2014;346:75–8.

da Costa BLV, Basso TO, Raghavendran V et al. Anaerobiosis revisited: growth of Saccharomyces cerevisiae under extremely low oxygen availability. Appl Microbiol Biotechnol 2018;102:2101–16.

da Costa BLV, Raghavendran V, Franco LFM et al. Forever panting and forever growing: physiology of Saccharomyces cerevisiae at extremely low oxygen availability in the absence of ergosterol and unsaturated fatty acids. FEMS Yeast Res 2019;19:foz054.

Daum G, Tüller G, Nemec T et al. Systematic analysis of yeast strains with possible defects in lipid metabolism. Yeast 1999;15:601–14.

Degreif D, de Rond T, Bértl A et al. Lipid engineering reveals regulatory roles for membrane fluidity in yeast flocculation and oxygen-limited growth. Metab Eng 2017;41:46–56.

de Kroon AIPM, Rijken PJ, de Smet CH. Checks and balances in membrane phospholipid class and acyl chain methylation, the yeast perspective. Prog Lipid Res 2013;52:374–94.

Depraetere SA, Delvaux F, De Schutter D et al. The influence of wort aeration and yeast preoxygenation on beer staling processes. Food Chem 2008;107:242–9.

Deytieuex C, Mussard L, Biron MJ et al. Fine measurement of ergosterol requirements for growth of Saccharomyces cerevisiae during alcoholic fermentation. Appl Microbiol Biotechnol 2005;68:266–71.

Entian K-D, Kötter P. 25 Yeast genetic strain and plasmid collections. Methods Microbiol 2007;36:629–66.

Fekete S, Ganzler K, Fekete J. Fast and sensitive determination of polysorbate 80 in solutions containing proteins. J Pharm Biomed Anal 2010;52:672–9.

Ferreira T, Régnaucq M, Alamardani P et al. Lipid dynamics in yeast under haem-induced unsaturated fatty acid and/or sterol depletion. Biochem J 2004;378:899–908.

Giaever G, Chu AM, Ni L et al. Functional profiling of the Saccharomyces cerevisiae genome. Nature 2002;418:387–91.
Gietz RD, Woods RA. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polycytidylyglycol method. Methods Enzymol 2002;350:87–96.

Holm Hansen E, Nissen P, Sommer P et al. The effect of oxygen on the survival of non-Saccharomyces yeasts during mixed culture fermentations of grape juice with Saccharomyces cerevisiae. J Appl Microbiol 2001;91:541–7.

Klose C, Surma MA, Gerl MJ et al. Flexibility of a eukaryotic lipidome-Insights from yeast lipidomics. PLoS One 2012;7:e35063.

Klug L, Daum G. Yeast lipid metabolism at a glance. FEMS Yeast Res 2014;14:369–88.

Lingwood D, Simons K. Lipid rafts as a membrane organizing principle. Science 2010;327:46–50.

Luttik MA, Köttner P, Salomons FA et al. Metabolic fluxes in yeasts: growth requirements and electron spin resonance method. Am J Physiol Endocrinol Metab 1973;247:E361–9.

Ma T, Shi B, Ye Z et al. Aerobic and anaerobic NAD+ metabolism in Saccharomyces cerevisiae. FEBS Lett 2002;517:97–102.

Pichler H, Riezman H. Where sterols are required for endocytosis. Biochim Biophys Acta 2004;1666:51–61.

Radmacher E, Alderwick LJ, Besra GS et al. Two functional FAS-I type fatty acid synthases in Corynebacterium glutamicum. Microbiology 2005;151:2421–7.

Resnick MA, Mortimer RK. Unsaturated fatty acid mutants of Saccharomyces cerevisiae. J Bacteriol 1966;92:597–600.

Rodriguez RJ, Low C, Bottema CD et al. Multiple functions for sterols in Saccharomyces cerevisiae. Biochim Biophys Acta 1985;837:336–43.

Rosenfeld E, Beauvoit B. Role of the non-respiratory pathways in the utilization of molecular oxygen by Saccharomyces cerevisiae. Yeast 2003;20:1115–44.

Snoek ISI, Steensma YH. Why does Kluyveromyces lactis not grow under anaerobic conditions? Comparison of essential anaerobic genes of Saccharomyces cerevisiae with the Kluyveromyces lactis genome. FEMS Yeast Res 2006;6:1–10.

Solis-Escalante D, Kuipers NG, Bongaerts N et al. amdSYM, a new dominant recyclable marker cassette for Saccharomyces cerevisiae. FEMS Yeast Res 2013;13:126–39.

Stuible HP, Meurer G, Schweizer E. Heterologous expression and biochemical characterization of two functionally different type I fatty acid synthases from Brevibacterium ammoniagenes. Eur J Biochem 1997;247:268–73.

Tsuchiya K, Mcdonough VM, Martin CE. Isolation and characterization of OLE1, a gene affecting fatty acid desaturation from Saccharomyces cerevisiae. J Biol Chem 1989;264:16537–44.

Summons RE, Bradley AS, Jahnke LH et al. Steroids, triterpenoids and molecular oxygen. Phil Trans R Soc 2006;361:951–68.

Tehlivets O, Scheueringer K, Kohlwein SD. Fatty acid synthesis and elongation in yeast. Biochim Biophys Acta 2007;1771:255–70.

Umebayashi K, Nakano A. Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane. J Cell Biol 2003;161:1117–31.

Valachovič M, Hronska L, Hapala I. Anaerobiosis induces complex changes in sterol esterification pattern in the yeast Saccharomyces cerevisiae. FEMS Microbiol Lett 2001;197:41–5.

van Dijken JP, van den Bosch E, Hermans JH et al. Alcoholic fermentation by ‘non-fermentative’ yeasts. Yeast 1986;2:123–7.

Verduyn C, Postma E, Scheffers WA et al. Effect of benzoic acid on metabolic fluxes in yeast: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 1992;8:501–17.

Verhoeven MD, Lee M, Kamoen L et al. Mutations in FMR1 stimulate xylolose isomerase activity and anaerobic growth on xylose of engineered Saccharomyces cerevisiae by influencing manganese homeostasis. Sci Rep 2017;7:46155.

Visser W, Scheffers WA, Batenburg-van der Vege WH et al. Oxygen requirements of yeasts. Appl Environ Microbiol 1990;56:3785–92.

Wheelier GE, Rose DAH. Location and properties of an esterase activity in Saccharomyces cerevisiae. J Gen Microbiol 1973;74:189–92.

White SW, Zheng J, Zhang YM et al. The structural biology of type II fatty acid biosynthesis. Annu Rev Biochem 2005;74:791–831.

Wissiack BJ, Kyiymoto RK. Fatty acid desaturation mutants of yeast: growth requirements and electron spin resonance spin-label distribution. J Bacteriol 1972;109:186–95.

Yuzefovich L, Wilson G, Rachek L. Different effects of oleate vs. palmitate on mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells: role of oxidative stress. Am J Physiol Endocrinol Metab 2010;299:E1096–1105.