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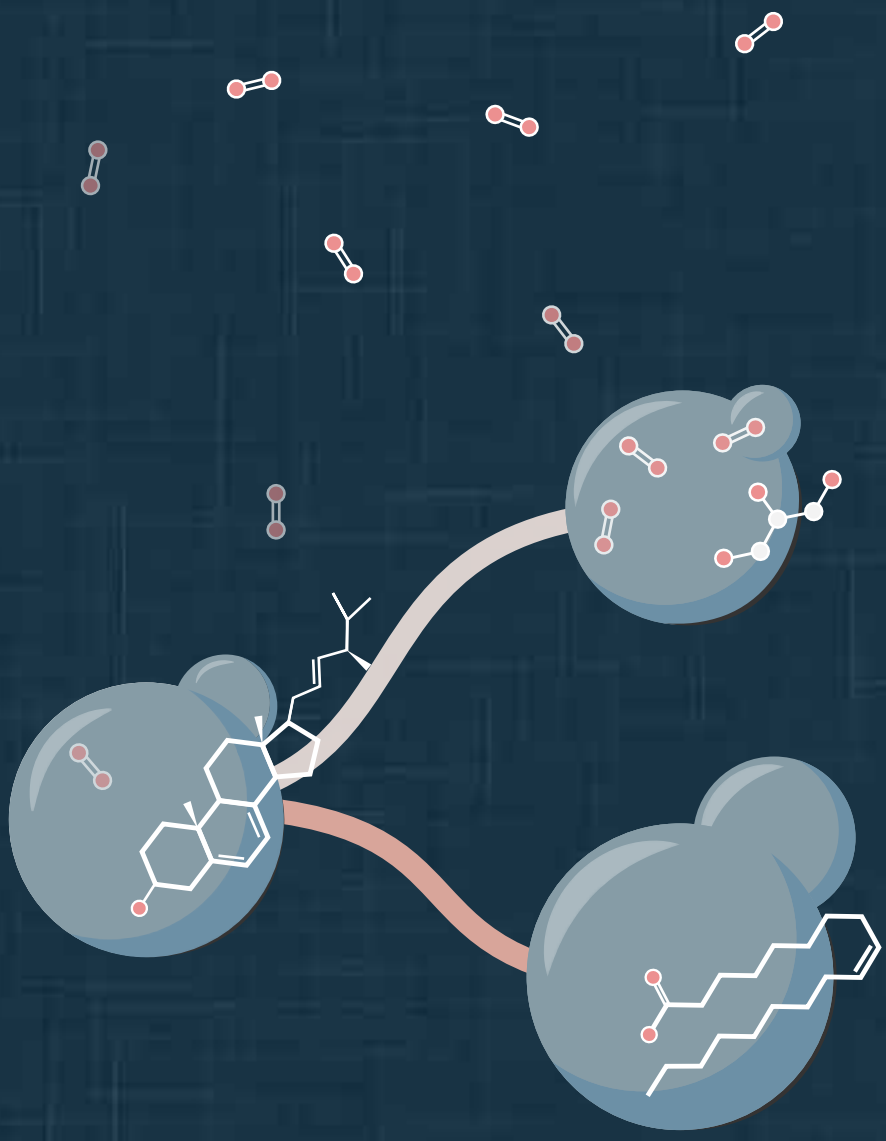

Wijbrand J.C. Dekker

Identification and elimination of biosynthetic oxygen requirements in yeasts

Dissertation

for the purpose of obtaining the degree of doctor

at Delft University of Technology

by the authority of the Rector Magnificus, Prof.dr.ir. T.H.J.J. van der Hagen,

chair of the Board for Doctorates

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Friday 10 September 2021 at 12:30 o'clock

by

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Dedicated to dr. J.M.J. Coremans & B.D. Dekker-Jansen

Samenvatting

De gist *Saccharomyces cerevisiae* is een van oorsprong natuurlijke producent van ethanol. Industriële stammen van deze gist kunnen op een hoog tempo ethanol produceren, met een nagenoeg theoretische opbrengst. *S. cerevisiae* is niet alleen een favoriet micro-organisme voor industriële ethanolfermentatie vanwege deze snelle, efficiënte ethanolfermentatie, maar ook vanwege een microbieel-veilige status (GRAS), toegankelijkheid voor genetische modificatie en een hoge tolerantie voor lage pH-waardes en hoge ethanolconcentraties. Ethanolfermentatie is echter geen unieke eigenschap van *S. cerevisiae* en er zijn meerdere facultatief fermentatieve gistsoorten die talrijke proceseigenschappen delen met *S. cerevisiae*. Sommige van deze gisten hebben zelfs aanvullende positieve eigenschappen voor industriële toepassingen. Echter, de meeste van deze gistsoorten missen een karakteristieke eigenschap van *S. cerevisiae*, namelijk het vermogen om snel te groei in afwezigheid van zuurstof (anaeroob) in simpele voedingsoplossingen (media) met minimale toevoegingen van vitaminen en anaerobe groeifactoren. Deze eigenschap is noodzakelijk voor toepassing van gisten voor productie van ethanol en verwante productie, vanwege de hoge kosten en complexiteit van beluchting die anders noodzakelijk is. Ook kan de nagenoeg theoretische productopbrengst van gistingsproducten alleen behaald worden in de afwezigheid van aerobe verademing (respiratie) van suikers.

Productie van ethanol als biobrandstof op grote schaal is economisch uitdagend vanwege de lage volumetrische prijs van fossiele brandstoffen en de productie- en infrastructuurkosten van het fermentatieprocessen. Voor productieprocessen van bioethanol gebaseerd op lignocellulose-houdende grondstoffen, zoals reststromen uit de landbouw, is een voorbehandeling van deze grondstoffen vereist. Tijdens deze voorbehandeling wordt de aanwezige cellulose en hemi-cellulose eerst afgebroken tot fermenteerbare suikers met enzymen. Idealiter zouden de enzymatische afbraak van cellulose en hemi-cellulose samen met de fermentatie plaatsvinden in een enkele processtap. Deze afzonderlijke processen kunnen nu echter niet efficiënt gecombineerd worden door de grote verschillen in temperatuuroptima van de enzymen en *S. cerevisiae*. Ondanks de recentelijke ontwikkelingen en de vooruitgang in technieken voor het aanpassen van het genomisch DNA en de microbiële stofwisseling blijft het een enorme uitdaging om de temperatuuroptima van deze gist te veranderen. Sommige andere gistsoorten die in de natuur voorkomen zijn uit zichzelf al bestand tegen hoge temperaturen en het toepassen van deze gisten in de industrie zou het combineren van de enzymatische hydrolyse en de fermentatie in een proces dan ook mogelijk kunnen maken. Het gebruik van deze gisten is daarmee erg aantrekkelijk, maar hun onvermogen om te groeien in afwezigheid van zuurstof vormt een groot obstakel voor hun exploitatie. De moleculaire oorsprong van de zuurstofbehoefte van deze "thermotolerante" gisten, die in tegenstelling tot *S. cerevisiae* in hun evolutiegeschiedenis geen volledig genoomduplicatie (WGD) hebben ondergaan, is tot heden onbekend. Het doel van het in dit proefschrift beschreven onderzoek is daarom om de zuurstofbehoefte van thermotolerante, industrieel relevante gisten te identificeren en vervol-

gens te elimineren, als een eerste stap naar hun toepassing in anaerobe industriële processen.

Het systematisch karakteriseren van de groei-afhankelijke zuurstofbehoeften vereist zorgvuldige controle en nauwkeurige metingen van de zuurstoftoevoer aan gistculturen. Cellulaire opname van zuurstof door gist is bijzonder efficiënt en gistcellen kunnen hierdoor al in hun groei-afhankelijke zuurstofbehoeften voorzien bij extreem lage concentraties van opgeloste zuurstof. Hoewel *S. cerevisiae* snel kan groeien zonder zuurstof is deze anaerobe groei afhankelijk van de toevoeging van vitaminen, onverzadigde vetzuren en sterolen aan het medium. Deze vereiste toevoegingen ("anaerobe groeifactoren") zijn direct gerelateerd aan zuurstof-afhankelijke biochemische reacties in de stofwisselingsroutes voor biosynthese van deze verbindingen door *S. cerevisiae*.

Voor het bestuderen van de groeiafhankelijke zuurstofbehoeften van gisten is het cruciaal dat zuurstoflekkage in de gebruikte anaerobe experimentele opstellingen wordt geminimaliseerd. Afwezigheid van groei van *S. cerevisiae* in een medium zonder anaerobe groeifactoren vormt hierbij een cruciale controle en een criterium voor anaerobe experimentele kweekopstellingen. Het doel in **hoofdstuk 2** was daarom om de impact van het weglaten van deze anaerobe groei-elementen op de fysiologie van *S. cerevisiae* te onderzoeken. Het achterwege laten van een bron van sterolen in anaerobe culturen van *S. cerevisiae* resulteerde in complete afwezigheid van groei. Het weglaten van een bron van onverzadigde vetzuren leidde in dezelfde omstandigheden echter niet tot afwezigheid van groei, maar wel tot een zeer sterke afname van de groeisnelheid. Door deze gistcultuur herhaaldelijk door te zetten naar steeds nieuwe anaerobe culturen werd aangetoond dat dit fenomeen persistent aanwezig was en dat eventuele voorraden van onverzadigde vetzuren in de gistcelen compleet waren uitgedund of verbruikt. Analyse van de vetzuursamenstelling van deze gistculturen bevestigde de afwezigheid van onverzadigde vetzuren. De onafhankelijkheid van de anaerobe groei van gist van onverzadigde vetzuren werd verder bevestigd door een deletie-mutant te maken in het gen dat codeert voor het enige vetzuurdesaturase in *S. cerevisiae*. In de aanwezigheid van zuurstof vereiste deze deletie-mutant de toevoeging van onverzadigde vetzuren voor groei. Deze toevoeging van onverzadigde vetzuren bleek echter niet noodzakelijk onder zuurstofloze (anaerobe) omstandigheden. Deze studie toonde daarmee aan dat de nutriëntvereisten van gisten complex zijn en sterk afhangen van de omstandigheden. Bovendien leidden deze resultaten tot een heroverweging van de gevestigde aanname dat onverzadigde vetzuren noodzakelijk zijn voor de anaerobe groei van *S. cerevisiae*.

Het heronderzoeken van de anaerobe groeibehoeften van *S. cerevisiae* vereiste de optimalisatie van de experimentele procedures voor gistcultivatie met als hoofddoel om onbedoelde zuurstofcontaminatie in kweeksytemen zoals schudkolven en bioreactoren te minimaliseren. Deze inspanningen zijn tijdrovend maar noodzakelijk om tegenstrijdige of onduidelijke resultaten te voorkomen. Om relevante experimentele benadering en valkuilen met de wetenschappelijke gemeenschap te delen, zijn in **hoofdstuk 3** de ervaringen van onze projectgroep van de afgelopen vier jaar gedocumenteerd. Belangrijke aandachtspunten die in deze studie

naar voren kwamen, waren onder meer de noodzaak om cellulaire-reserves van anaerobe groeifactoren uit te putten voordat anaerobe groei wordt geanalyseerd, het belang van het minimaliseren van zuurstoflekkage door slangen en het gebruik van controle- of mutantstammen voor correcte interpretatie van resultaten.

Het doel van **hoofdstuk 4** is het karakteriseren en het kwantificeren van de zuurstofbehoeften van de thermotolerante gist *Kluyveromyces marxianus*. Voor het identificeren van deze zuurstofbehoeften werden de fysiologie van *S. cerevisiae* en de zuurstof-afhankelijke gist *K. marxianus* kwantitatief geanalyseerd en vergeleken op basis van chemostaatexperimenten met verschillende beluchtingsregimes. Zuurstof-gelimiteerde chemostaatculturen van *K. marxianus* vertoonden hoge biomassa-specifieke fermentatiesnelheden en een zeer kleine maar significante behoefte van zuurstof voor biosynthese. Op basis van de bekende rol van sterolen en onverzadigde vetzuren voor de anaerobe groei van *S. cerevisiae* werden deze groeifactoren zowel individueel als tegelijk weggelaten uit het medium van de chemostaatculturen. De resultaten van deze experimenten en een transcriptoom-analyse van de impact van zuurstof en groeifactorlimitatie in chemostaatculturen van *S. cerevisiae* en *K. marxianus* suggereerde dat deze gisten sterk verschillen in het vermogen om sterolen vanuit het medium op te nemen. De opname van fluorescent gelabelde sterolen door *S. cerevisiae* en het gebrek daarvan in *K. marxianus* waren consistent met deze hypothese. Beide experimenten toonden daarmee aan dat *K. marxianus* hoogstwaarschijnlijk geen mechanisme heeft om sterolen op te nemen om daarmee de in anaerobe condities geblokkeerde zuurstofafhankelijke sterol synthese te complementeren. De expressie van een heterologe squaleen-tetrahymanol cyclase in *K. marxianus* werd onderzocht om een sterolsurrogaat te synthetiseren en daardoor de zuurstofafhankelijke sterol biosynthese te omzeilen. Na een korte adaptatiefase onder stringente zuurstof-gelimiteerde omstandigheden was de gemodificeerde *K. marxianus* stam in staat om te groeien zonder zuurstof. Deze zuurstof- en sterol-onafhankelijke groei werd ook aangetoond bij kweektemperaturen die hoger waren dan de maximale groeitemperatuur van wildtype *S. cerevisiae* stammen. Alles bij elkaar toonden deze resultaten aan wat de moleculaire basis van de zuurstofbehoeften voor biosynthese van *K. marxianus* is en lieten ze zien het mogelijk is om de zuurstofbehoeften te omzeilen door het metabolisme aan te passen en zo een obligaat aerobe gist te veranderen in een facultatieve anaerobe gist.

Net als *K. marxianus* heeft de methylotrofe gist *Ogataea parapolymorpha* een hoge thermotolerantie. *O. parapolymorpha* is een belangrijke modelgist voor het bestuderen van methanolmetabolisme en wordt gebruikt voor de industriële productie van heterologe eiwitten. In vergelijking met *K. marxianus* is *O. parapolymorpha* genetisch gezien veel minder verwant aan *S. cerevisiae* en is er veel minder informatie beschikbaar over fermentatief metabolisme en zuurstofbehoeften. Het doel van het onderzoek in **hoofdstuk 5** was om de zuurstofbehoeftes, en de biochemische basis hiervoor, van *O. parapolymorpha* te bestuderen. Hiertoe werd de fysiologie van *O. parapolymorpha* gekarakteriseerd onder verschillende beluchtingsregimes en vergeleken met data die in hoofdstuk 4 waren verkregen met *S. cerevisiae* en *K. marxianus*. Hoewel duidelijk ethanolfermentatie optrad in zuurstof-gelimiteerde culturen, was de hoev-

eelheid zuurstof die in deze cultures werd geconsumeerde, uitgedrukt per gram biomassa van *O. parapolymorpha*, aanzienlijk groter dan voor *K. marxianus*. In tegenstelling tot de zuurstof-gelimiteerde culturen van *S. cerevisiae* en *K. marxianus*, die allebei overtollig NADH uit biosynthetische reacties kunnen reoxideren door de productie van glycerol uit glucose, had *O. parapolymorpha* zuurstof-ademhaling nodig voor dit doel. De expressie van de glycerolroute enzymen van *S. cerevisiae* in *O. parapolymorpha* verhoogde de biomassa-opbrengst op zuurstof in zuurstof-gelimiteerde culturen, maar was onvoldoende om de redox-cofactor balans te kunnen herstellen zonder gebruik van zuurstof.

Summary

Saccharomyces cerevisiae is a natural producer of ethanol and industrial strains can produce ethanol at high volumetric rates and near-theoretical yields. In addition to its fast fermentative metabolism, its GRAS (generally recognized as safe) status, ease of genetic engineering, tolerance to low pH and high ethanol concentrations contribute to the popularity of *S. cerevisiae* as an industrial platform organism. Ethanolic fermentation is, however, not unique to *S. cerevisiae*. Other facultatively fermentative yeast species share many performance characteristics with *S. cerevisiae* and may even hold additional advantages for industrial application. However, they typically lack one key distinctive phenotype of *S. cerevisiae*: its capability to grow fast in the absence of oxygen on simple media with minimal addition of vitamins and anaerobic growth factors. This characteristic is essential for industrial application, as aeration of large bioreactors is expensive and near-theoretical yields of fermentation products can only be achieved in the absence of respiratory dissimilation of sugars.

Production of ethanol as biofuel is economically challenging due to the low volumetric price of fossil fuels and the high process and equipment costs of the production process. For bioethanol processes based on lignocellulosic feedstocks, such as agricultural residues, pretreatment of the feedstock requires the enzymatic hydrolysis of the (hemi-)cellulose prior to fermentation. Ideally, enzymatic hydrolysis and ethanolic fermentation would be combined in a single process unit. However, enzymatic hydrolysis and ethanolic fermentation using *S. cerevisiae* cannot be efficiently combined, due to large difference in temperature optima of hydrolytic enzymes and *S. cerevisiae*. Despite the great progress in engineering metabolic traits of *S. cerevisiae*, drastically changing its temperature optima remains a challenge. In contrast, wild-type strains of other yeast species with high thermotolerance have been characterized and their application would be much better compatible with combined saccharification and fermentation. However, in contrast to *Saccharomyces* yeast, these yeasts, whose evolutionary past did not include the whole-genome duplication (WGD) event that helped shape the genomes of *S. cerevisiae* and closely related species, require oxygen for growth. The molecular origin of the requirement for oxygen of these non-WGD yeasts is unknown. The goal of the research described in this thesis was therefore to identify and eliminate the oxygen requirements of industrially relevant, thermotolerant, non-WGD yeasts as a first step towards their application in anaerobic industrial fermentation processes.

Characterization of oxygen requirements for biosynthesis requires the careful control and measurement of oxygen entry into yeast cultures. Cellular uptake of oxygen by yeast cells is particularly efficient and yeasts can meet their biosynthetic oxygen requirements even at extremely low dissolved oxygen concentrations. While *S. cerevisiae* can grow quickly under anaerobic conditions, its anaerobic growth is dependent on the supplementation of vitamins, unsaturated fatty acids and sterols. These requirements can be directly linked to oxygen-requiring reactions in the yeast biosynthetic pathways involved in *de novo* synthesis of these anaerobic growth factors.

Lack of growth of *S. cerevisiae* in the absence of exogenously supplied, known anaerobic growth factors is a key design criterion for anaerobic cultivation set-ups used in studying biosynthetic oxygen requirements of yeasts. The objective of **Chapter 2** of this thesis was therefore to investigate the impact of the omission of anaerobic growth factors on the physiology of *S. cerevisiae*. The omission of a source of sterols led to a virtually complete absence of growth in anaerobic cultures. However, under the same conditions, omission of a source of unsaturated fatty acids reduced the specific growth rate but did not completely abolish growth. Serial transfers of yeast cultures revealed that this phenomenon was persistent and lipid analyses on these cultures showed a complete absence of unsaturated fatty acids. The independence of anaerobic growth of *S. cerevisiae* on supplementation of unsaturated fatty acids was further validated by the construction and testing of a knockout mutant in which Ole1, the only fatty acyl-CoA desaturase in *S. cerevisiae*, was removed. In the presence of oxygen, this mutant still required the complementation of unsaturated fatty acids but, surprisingly, such a nutritional requirement was not necessary under anaerobic conditions. This study highlighted the complexity and context-dependency of the nutritional requirements of yeasts and forced a reconsideration of the widespread assumption that supplementation of unsaturated fatty acids is an absolute requirement for anaerobic growth of *S. cerevisiae*.

Validation of the anaerobic requirements of *S. cerevisiae* required optimization of experimental procedures for yeast cultivation, with the main goal to minimize inadvertent oxygen entry into cultivation systems such as shake-flasks and bioreactors. Such efforts are time-consuming but essential to prevent ambiguous or conflicting results. To share relevant measures and pitfalls with the scientific community, **Chapter 3** outlines the experiences of our project team over a four-year period. Key points of attention highlighted in this study included the need to deplete cellular reserves of anaerobic growth factors before analysis of anaerobic growth, the importance of oxygen leaking through tubing and the relevance of control strains and gene knockouts for interpretation of results.

The aim of **Chapter 4** of this thesis was to characterize and quantify the oxygen requirement for biosynthesis of the thermotolerant yeast *Kluyveromyces marxianus*. For identification of this oxygen requirement, the physiology of both the facultative anaerobic *S. cerevisiae* and the obligate aerobic *K. marxianus* were quantitatively analysed and compared based on chemostat experiments performed under various defined oxygenation regimes. Oxygen-limited chemostat cultures of *K. marxianus* showed a high specific rate of ethanolic fermentation and a minute but significant oxygen requirement for biosynthesis. In view of the known roles of ergosterol and unsaturated fatty acids for the anaerobic growth of *S. cerevisiae* we omitted either or both of these 'anaerobic growth factors' from the medium supply of chemostat cultures. Results from these experiments and chemostat-based transcriptome analysis of the responses of *S. cerevisiae* and *K. marxianus* to oxygen and anaerobic growth-factor limitation suggested that the (in)ability to take up sterols might be a key difference between *S. cerevisiae* and *K. marxianus*. Consistent with this hypothesis, uptake of fluorescent labelled sterol by *S. cerevisiae* was readily apparent but no such activity was found in *K. marxianus*, indicating

that it lacked the ability to transport sterols and thereby complement the oxygen-requirement for sterol biosynthesis. Expression of heterologous squalene-tetrahymanol cyclase in *K. marxianus* was explored to synthesize the sterol surrogate tetrahymanol and thereby allow for a bypass of its oxygen requirements for sterol biosynthesis. After a short adaptation under severely oxygen-limited conditions, the resulting *K. marxianus* strain was able to grow without oxygen. This anaerobic, sterol-independent growth was also demonstrated at cultivation temperatures higher than the maximum growth temperature of wild-type *S. cerevisiae* strains. Together, these observations resolved the molecular basis for the oxygen requirement for biosynthesis of *K. marxianus* and demonstrated that metabolic engineering strategies are possible to turn an obligate aerobic yeast into a facultative anaerobe yeast.

Just like *K. marxianus*, the methylotrophic yeast *Ogataea parapolymorpha* is well characterized for its high thermotolerance. *O. parapolymorpha* is an important model yeast for studying methanol metabolism and is used in industry for heterologous protein production. In contrast to *K. marxianus* however, *O. parapolymorpha* is only distantly related to *S. cerevisiae* and only limited information is available on the regulation of its fermentative metabolism and virtually no data are available on its biosynthetic oxygen requirements. The aim of the research presented in **Chapter 5** was therefore to investigate the quantity of the oxygen requirements and their biochemical basis in *O. parapolymorpha*. To this end, the physiology of *O. parapolymorpha* was characterized in chemostats under various oxygenation regimes and compared to data obtained in Chapter 4 for *S. cerevisiae* and *K. marxianus*. While ethanolic fermentation was readily apparent in oxygen-limited cultures, the amount of oxygen needed for biosynthetic reactions, expressed per gram biomass, was much higher in *O. parapolymorpha* than in *K. marxianus*. In contrast to oxygen-limited cultures of *S. cerevisiae* and *K. marxianus*, which both reoxidized 'surplus' biosynthetic NADH by the production of glycerol from glucose, *O. parapolymorpha* was found to require aerobic respiration for this purpose. Expression of *S. cerevisiae* glycerol pathway enzymes in *O. parapolymorpha* increased the biomass yield on oxygen in oxygen-limited cultures but did not completely solve this redox-cofactor-balancing problem.

Chapter 1

General introduction

Relevance of research

Already for thousands of years, humans use living micro-organisms for their benefit. Although the existence and activities of micro-organisms long remained unknown, their role in the production of bread and fermented drinks such as beer, mead and wine date back to prehistory (1–3). The use of micro-organisms to generate or process foods allowed for the diversification of the human diet and provided human civilization with a powerful tool to harness chemistry. In more recent times, the rapidly increasing body of knowledge on (industrial) microbiology has enabled application of micro-organisms in a wide variety of processes in the pharmaceutical, chemical, and fragrance industry (4–6).

Millions of years of evolution under strong natural selection pressure, including competition for scarce chemical resources, have led to an enormous diversity of microbial life and optimized the thousands of enzyme-catalyzed chemical reactions happening at the same time inside microbial cells. In industrial applications, microorganisms have several advantages over chemical catalysts, including their self-replicating nature and the high enantio- and region-selectivity of the enzyme-catalyzed reactions in their metabolic networks.

The diversity of micro-organisms and their networks of chemical reactions provide many opportunities to develop new industrial bioprocesses and microbially driven chemical conversions for new and existing products. In industrial microbiology, production of chemicals using microbial strains as 'cell factories' is actively explored, both for novel applications and the generation of renewable alternatives for petrochemically derived products (6). Development of efficient cell factories for a diverse array of new products requires the rewiring of metabolic networks and introduction of genes for chemical pathways that in nature only occur in organisms that are not suitable for industrial production processes (7–9).

Except for a number of anaerobic fermentation processes (e.g. alcoholic fermentation, lactic acid fermentation and butanol/acetone fermentation), industrial processes in microbial biotechnology involve aeration. Due to limitations of natural metabolic networks, aerobic processes even need to be applied for some products whose synthesis, on purely thermodynamic grounds, might be feasible in the absence of oxygen. Synthesis of products under anaerobic conditions has received much attention due to substantial cost benefits relative to aerobic processes. However, many eukaryotic cell factories require oxygen, either for energy metabolism or because of its involvement in biosynthesis (10, 11).

The yeast species *Saccharomyces cerevisiae* is among the most extensively used micro-organisms in biotechnology, and its industrial-scale applications for production fuel ethanol from agricultural carbohydrates and for the production of alcoholic beverages rank among the largest-volume biotechnology processes. Facilitated by its amenability to genetic engineering techniques, *S. cerevisiae* is a popular eukaryotic model organism in fundamental research and a platform organism for a wide range of other processes in microbial biotechnology (12, 13).

Although yeast species other than *S. cerevisiae* are often referred to as 'non-conventional yeasts', the ability of *S. cerevisiae* to grow in the complete absence of oxygen is, in fact, unconventional among yeasts, with only a few other yeast species show anaerobic growth (10). Since the ability to grow anaerobically is an important prerequisite for application in anaerobic processes such as ethanol production, it is highly relevant to elucidate its genetic and/or metabolic basis.

Sugar metabolism

While yeasts can use a range of organic substrates for energy metabolism and biomass synthesis, most yeasts show a preference for sugars as carbon and energy source. Sugar dissimilation enables cells to conserve energy in the form of adenosine triphosphate (ATP) or its energetic equivalent guanosine triphosphate (GTP). ATP and GTP are biochemical energy carriers, which shuttle free energy between energy demanding and producing reactions (Figure 1). The pools of ATP and GTP in cells are limited, and their cellular production and consumption (phosphorylation of ADP/GDP and dephosphorylation of ATP/GTP, respectively) rates therefore need to be continuously balanced. ATP can be formed either by substrate-level phosphorylation in glycolysis or, when oxygen is available, by mitochondrial respiration and oxidative phosphorylation. In *S. cerevisiae*, complete respiratory dissimilation of one molecule glucose via glycolysis, TCA cycle and mitochondrial respiration results in the net generation of 4 moles of ATP and 16 moles of ATP, respectively (Figure 2)(14). During respiratory dissimilation, pyruvate derived from glucose by glycolysis is used in the mitochondrial TCA cycle to generate the reduced redox cofactors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) and cellular building blocks. In addition, it involves phosphorylation of GDP to GTP in the succinate thiokinase reaction. NADH and $FADH_2$ transfer their electrons to the electron transport chain embedded in the mitochondrial inner membrane. This process is coupled to proton translocation, resulting in an electrochemical proton gradient across the mitochondrial inner membrane (15). This mitochondrial membrane proton gradient (proton motive force) is then used to generate ATP from ADP by the mitochondrial F_0-F_1 ATP-synthetase complex (16). Depending on the composition of their respiratory chains and, in particular, on the presence of a proton-translocation 'Complex I'-type NADH dehydrogenase, higher ATP yields can be achieved during respiratory sugar dissimilation by yeast species other than *S. cerevisiae* (15, 17).

In contrast to mitochondrial respiration, sugar dissimilation by alcoholic fermentation only conserves ATP by substrate-level phosphorylation in glycolysis, with a net yield of 2 moles of ATP per mole of glucose (18). Obviously, alcoholic fermentation is the preferred mode of sugar dissimilation for production of ethanol and alcoholic beverages, which are therefore performed under anaerobic conditions. In contrast, for production of yeast biomass (e.g. bakers' yeast and yeast extracts) or compounds whose synthesis from sugar requires a net input of ATP, respiratory dissimilation is strongly preferred due to its much higher ATP yield (19). The energy conserved by dissimilatory processes can be used for biosynthesis of macromolecules

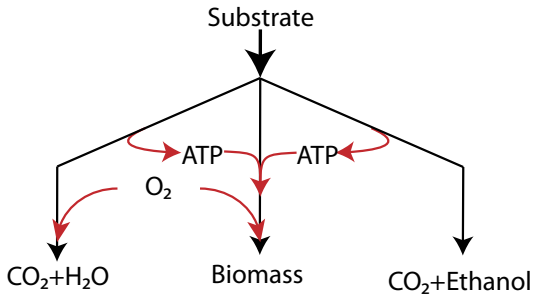

Figure 1. Both under aerobic and anaerobic conditions, sugar substrates are dissimilated by yeasts to conserve free energy ('ATP') and to synthesize biomass via reactions that require a net input of free energy.

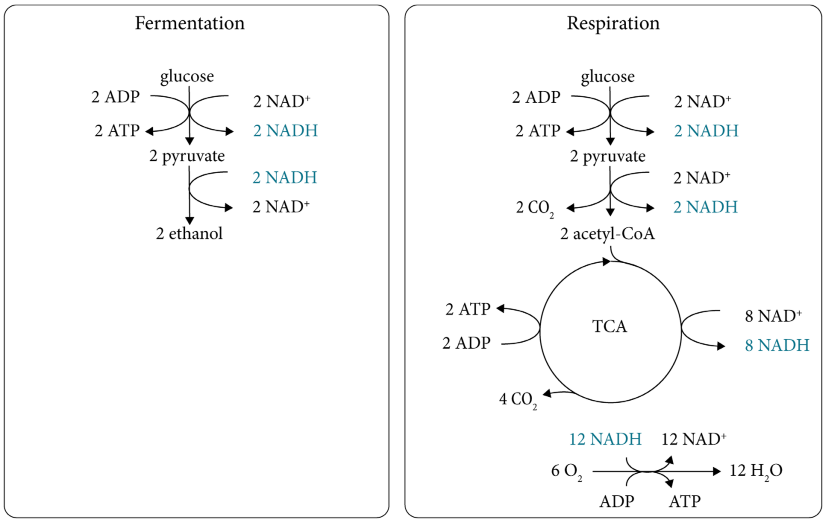

Figure 2. Dissimilation of glucose by yeast can occur either by oxidative respiration or by alcoholic fermentation. Glucose dissimilation via the Embden-Meyerhof glycolysis results in the net formation of 2 NADH and 2 ATP. In the absence of oxygen as external electron acceptor, the 2 molecules of NADH formed in glycolysis can be re-oxidized by reduction of pyruvate to ethanol. Alternatively, in the presence of oxygen, pyruvate can be fully respired in the tricarboxylic acid cycle (TCA) to CO_2 and H_2O with the production of 2 GTP (ATP equivalent) and 8 NADH (considering 2 $FADH_2$ as NADH equivalents). The 12 NADH produced from glucose dissimilation are then reoxidized in the respiratory chain resulting in the generation of a proton motive force which in turn is used to generate 12 ATP (assuming a P/O ratio of 1).

such as DNA, RNA, proteins and lipids, as well as for the translocation of molecules across membranes, product formation, and cellular maintenance (20). In addition, intermediates of glycolysis and TCA cycle serve as building blocks for synthesis of macromolecules, other cellular constituents and, in industrial contexts, for product formation. Use of cellular energy for cellular maintenance encompasses investment of energy to maintain physicochemical conditions inside cells that are permissive for survival and growth, and to recycle damaged macromolecules. These maintenance processes do not contribute to the formation of new biomass. Therefore, in many micro-organisms including *S. cerevisiae*, maintenance-energy requirements are essentially independent of their specific growth rate (20–22).

Most yeast species do not exclusively depend on mitochondrial respiration for sugar dissimilation, but can also conserve energy by fermenting glucose to ethanol (23, 24). Even when ethanolic fermentation is used for sugar dissimilation, TCA-cycle intermediates are required as precursors for biomass formation. However, the quinone-dependent succinate dehydrogenase (complex II) does not function *in vivo* under anaerobic conditions since its activity requires oxygen as terminal electron acceptor for mitochondrial respiration (25). To still meet the requirements for the metabolic precursors and α-ketoglutarate and succinyl-CoA, the TCA cycle in anaerobic yeast cultures operates as a 'horse shoe' with a reductive branch leading to succinyl-CoA and oxidative branch leading to α-ketoglutarate (25, 26). In the reductive branch, the quinone-dependent succinate dehydrogenase is replaced by the soluble fumarate reductases Osm1 and Frd1, which catalyzes fumarate reduction, contributing to succinate excretion by anaerobic cultures (27, 28). In addition, the fumarate reductase Osm1 enables oxygen-independent formation of protein-disulfide bonds by transferring electrons from the FAD cofactor of thiol oxidase Ero1 to fumarate reduction (29).

Based on the possible contribution of respiration and alcoholic fermentation to sugar dissimilation, yeasts can be classified in three categories: non-fermentative, facultatively fermentative and obligately fermentative. Facultatively fermentative yeasts produce ethanol from glucose under oxygen-limited conditions. However, this statement does not imply that, under fully aerobic conditions, all yeasts exclusively use the energetically more efficient option to respire glucose. *S. cerevisiae* belongs to a group of yeasts that exhibit a so-called Crabtree effect, which is defined as the occurrence of a mixed respiro-fermentative mode of sugar dissimilation under fully aerobic conditions (30, 31). The Crabtree effect has been proposed to originate from a trade-off between the higher ATP stoichiometry of respiratory pathways and a lower ATP demand for synthesizing the catalytic machinery (higher protein-efficiency of the fermentative pathway (32). In *S. cerevisiae*, aerobic ethanolic fermentation occurs in the presence of excess glucose and at high specific growth rates, whereas a completely respiratory mode of sugar dissimilation is only observed in aerobic, sugar-limited cultures grown at low and intermediate specific growth rates. The exact tipping point of the specific growth rate at which sugar dissimilation switches from respiratory to respirofermentative in such cultures is strain and condition dependent (33–35).

Nutritional requirements of yeasts

For optimal growth of yeasts, medium compositions need to be carefully adapted to their nutritional requirements. Complex media, for example based on yeast extract and or peptone, allow for easy preparation and support fast growth of yeasts, but are chemically non-defined and can exhibit considerable variations between suppliers and batches. Chemically defined synthetic media formulations are therefore crucial for reproducible experiments and for identification of nutrient limitations and analysis of their impacts on yeast physiology. Moreover, the ability to independently vary the concentrations of individual components in synthetic media offers substantial advantages in the rational design and optimization of media for high-biomass-density cultivation in industrial settings.

The development of synthetic media to support physiological studies enabled systematic investigations into the growth requirements of micro-organisms. Already in the early 20th century, evidence accumulated that yeasts required more than sugar and salts for growth. These additional nutritional requirements could be complemented by an unknown component(s), termed 'bios', in peptone, meat extract, beer wort or boiled yeasts (36, 37). The term 'bios' was later exchanged for 'vitamins'. In human nutrition, vitamins are defined as essential organic compounds that cannot be *de novo* synthesized in sufficient amounts and are therefore an essential part of the diet (38, 39). In current synthetic media for cultivation of yeasts, biotin, pantothenate, inositol, nicotinic acid, para-aminobenzoic acid, pyridoxine and thiamine are common ingredients. Although, also in the context of yeast cultivation, compounds such as biotin, pantothenate and nicotinic acid are commonly referred to as vitamins, their requirement for growth varies among yeast species and strains and, moreover, can be strongly influenced by cultivation conditions (40).

Several synthetic, chemically defined media for cultivation of yeasts are used in laboratories around the world. For example, Delft researchers developed a medium that supports aerobic, glucose-limited growth at biomass concentrations of up to 10 $g \cdot L^{-1}$ biomass of *S. cerevisiae* and several other yeasts (41, 42). Kluyver (1940) was among the first researchers to demonstrate that oxygen availability not only influences the mode of sugar dissimilation in yeast, but can also strongly influence nutritional requirements. In studies on the yeast *Torulopsis utilis* (syn. to *Cyberlindnera jadinii*), he demonstrated that vitamins were only required for cultivation at low oxygen concentrations and could be omitted from growth media under aerobic conditions (43). Andreasen and Stier (1953, 1954) demonstrated that the fast anaerobic growth of the yeast *Saccharomyces cerevisiae* in synthetic media strictly depended on nutritional supplementation with sterols and unsaturated fatty acids (44, 45). In aerobically grown biomass of *S. cerevisiae*, the predominant sterol lipid is ergosterol, while the unsaturated fatty acids oleic acid (C18:1-Δ9) and palmitoleic acid (C16:1-Δ7) are abundantly present (46). Both unsaturated fatty acids and sterols are key building blocks of eukaryotic cell membranes and, in yeasts, their respective contents and composition is strain and growth condition-dependent (46, 47).

Life depends on transport of chemicals over cellular membranes and on the ability to maintain and modulate concentration gradients of a wide range of compounds across these membranes by the activities of a wide diversity of integral membrane proteins. Stability and activity of these membrane transporters and, thereby, their ability to modulate chemical gradients depends on the physicochemical properties of the lipid membrane, including the membrane viscosity, permeability, thickness, curvature, and bilayer asymmetry (48–50). These physiochemical properties are predominantly determined by the lipid membrane composition and lipid spatial distribution. In yeast, the lipid membrane mainly consists of phospholipids, sterols, sphingolipids and isoprenoids such as squalene (46). To maintain cellular homeostasis under different and sometimes dynamic environmental conditions, composition of the lipid membrane is adjusted by *de novo* lipid synthesis, intracellular transport, lipid degradation and remodeling of lipids, including for example the remodeling of the acyl-chain composition (51).

Sterols such as ergosterol are UV-sensitive, lipophilic, and poorly soluble in water, which presents a problem for complementation of the sterol auxotrophy of anaerobic yeast cultures. Bioaccessibility of sterols in synthetic media for anaerobic cultivation of yeasts is usually increased by micellar solubilization with the surfactant Tween 80, while in complex media sterols can be bound and complexed by proteins (52). Tween 80 is a poly-ethoxylated sorbitan esterified to the unsaturated fatty acid oleic acid. Tween 80 is also used as a source of unsaturated fatty acids, although it remains unknown which enzymes in *S. cerevisiae* are responsible for hydrolysis of the esterified oleate. Tween 80 in combination with calcium chloride is often used to test the lipase activity of micro-organisms, since calcium-complexed fatty acids precipitate from solution, but in this test *S. cerevisiae* does not demonstrate lipase activity (53, 54). Tween 80 can chemically degrade slowly over time depending on the environmental conditions and Tween 80 solutions can therefore contain a minor fraction of free unsaturated fatty acids (55, 56).

Although ergosterol is lipophilic, its incorporation into the yeast plasma membrane requires ATP-dependent membrane transport. In *S. cerevisiae* this process can be catalyzed by the ATP-binding cassette (ABC) transporters Pdr11 or Aus1 (57, 58). While most ABC transporters export small organic molecules from the cell, Aus1 and Pdr11 probably only transport sterols in the inward direction (59). Remarkably, cholesterol and other non-native sterols are preferred substrates of Aus1 and Pdr11 relative to the native yeast ergosterol (59). Aus1 and Pdr11 are members of a group of nine *S. cerevisiae* Pleiotropic Drug Resistant transporters (PDR) belonging to the ABC subfamily. In contrast to representatives of other ABC subfamilies present in *S. cerevisiae*, which have remained as single copies through evolutionary history, multiple gene duplications in the PDR family led to expansion and neo-functionalization (60, 61). Interestingly, the yeasts *Brettanomyces bruxellensis* and *Candida albicans* are capable of sterol-dependent anaerobic growth but their genomes do not harbor clear *AUS1/PDR11* orthologs. It therefore remains unclear how sterols are incorporated into these yeasts (62–64).

Despite the demonstrated involvement of PDR transporters in sterol uptake by *S. cerevisiae*, understanding of the exact mechanism by which this transport process occurs is far from complete. Since sterols are hydrophobic, release of free sterol into the cytoplasm is thermodynamically unfavorable and it appears logical that, instead, the sterol is either directly transferred to intracellular proteins or incorporated into the plasma membrane and from there transferred to intracellular membranes and/or lipid droplets. It has been proposed that yeast cell-wall mannoproteins contribute to the uptake of sterols by Aus1 and Pdr11 but their mechanism of action remains unknown (65–68). Extracellular proteins can also bind sterols and it has been demonstrated that serum albumin promotes sterol transport (52, 62, 69, 70).

Sterol metabolism

Yeasts are able to *de novo* synthesize sterols from the precursor acetyl-CoA through the mevalonate and ergosterol pathways. Synthesis of sterols is strictly oxygen dependent and requires 12 molecules of O_2 for a single molecule of ergosterol (Figure 3)(71), which explains its essentiality as growth factor in anaerobic *S. cerevisiae* cultures. Sterol biosynthesis is also energetically expensive, as synthesis of a single molecule of ergosterol requires at least 15 molecules of NADPH and 18 molecules of ATP (excluding additional cofactors). Sterol biosynthesis is initiated by oxygen-independent synthesis of squalene from acetyl-CoA via the mevalonate pathway. In this pathway, cytosolic acetyl-CoA is first extended by acetoacetyl-CoA thiolase, HMG-CoA synthase and reductase to the branched C5-compound isopentenyl-pyrophosphate (IPP). IPP molecules then act as precursors for formation of the branched long-chain C30 molecule squalene. The subsequent conversion of squalene to sterols involves a series of oxygen-dependent reactions. The first of these is the epoxidation of squalene to oxidosqualene which forms the basis for triterpene cyclisation to lanosterol in animals and fungi and cycloartenol in plants (72, 73) (Figure 3).

In contrast to the large NADPH and ATP requirement for sterol synthesis, import of ergosterol via Aus1 or Pdr11 probably only requires 1 mol of ATP per mol of ergosterol (74). Although sterol import would appear to be energetically favorable relative to *de novo* synthesis, the *S. cerevisiae* sterol transporters are only expressed in the absence of oxygen (45, 75). As a consequence, mutants with gene disruptions that eliminate the first steps of the essential sterol biosynthesis pathway are only viable under anaerobic conditions (58).

Sterols are near-ubiquitous in eukaryotic membranes and their synthesis is assumed to already have taken place in the last eukaryotic common ancestor (76). Lanosterol, the first cyclic sterol precursor, is in yeast further modified by oxygen-dependent demethylation and desaturation reactions leading to ergosterol and, via a similar set of reactions in animals, to cholesterol. Cholesterol and ergosterol have comparable functions and similar chemical structures (Figure 3). In contrast to animals and fungi, plants can synthesize a wider variety of sterols, whose functions not only involve structural roles but also signaling and hormonal regulation (77, 78). Sterols are only rarely found in prokaryotes (79). Multiple prokaryotes do, however, convert squalene with squalene-hopene cyclases, resulting in the formation of

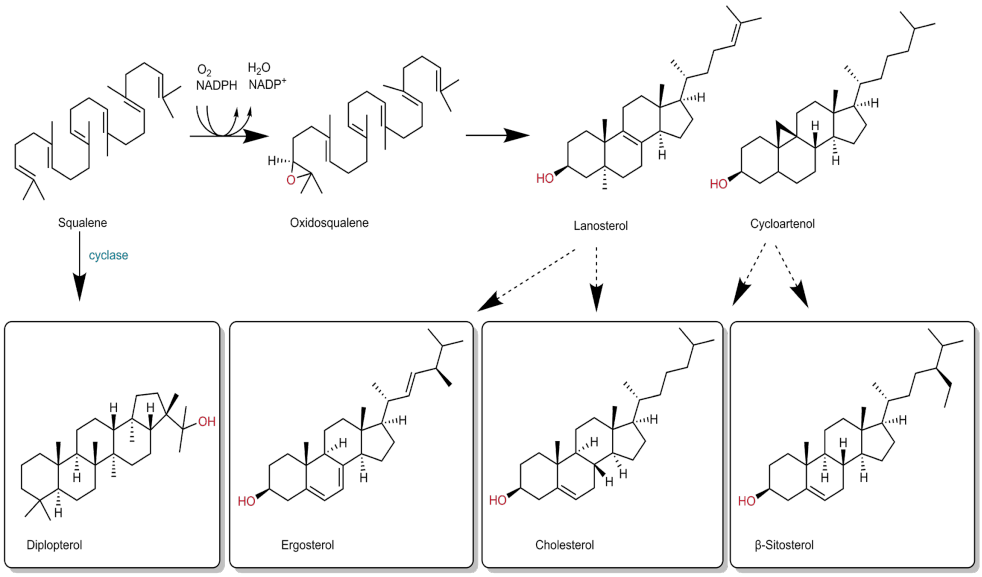

Figure 3. Synthesis of cyclic isoprenoids and sterols. Squalene can be cyclized directly to diplopterol or tetrahymanol. Cyclisation of oxidosqualene, after epoxidation, results in the sterols lanosterol or cycloartenol. In yeast, lanosterol is converted to ergosterol via several oxygen-dependent steps, while in mammals cholesterol is the end product of the sterol pathway. In plants cycloartenol can be converted to β-sitosterol or structurally similar sterols.

hopanoids or, with the additional involvement of an additional synthase, tetrahymanol (80). In Neocallimastigomycota, a group of deep-branching strictly anaerobic fungi, tetrahymanol is synthesized by a tetrahymanol squalene cyclase (Stc) acquired by lateral gene transfer and acts as a sterol surrogate (81). These fungi are therefore rare examples of sterol-free eukaryotes.

The different molecular structures of the sterol intermediates and products of sterol biosynthesis among and within organisms confer different physicochemical properties to their membranes. The sterol structure of cholesterol is planar and confers better membrane properties than to lanosterol, which is more bulky due to the presence of methyl groups. Mutations in the final steps of the ergosterol biosynthesis pathway in *S. cerevisiae*, which prevent the formation of ergosterol, are not lethal. However, such mutants generally display various sub-optimal phenotypes (reviewed in (82)). Interestingly, a mutation in the C-5 sterol desaturase (Erg5) that led to incorporation of the intermediate fecosterol incorporation into the plasma membrane led to improved growth at high temperatures than in wild-type *S. cerevisiae* (83, 84).

Intracellular sterol transport

Many of the reactions of sterol synthesis occur at the endoplasmic reticulum (ER) membrane, and transport of ergosterol from the ER to the plasma membrane (PM) involves the non-secretory pathway (85, 86). Sterols are transferred between membranes by lipid-transfer proteins and through membrane contact sites between ER and PM (87, 88). Sterols taken up from the environment are incorporated into the PM and transported back to the ER for esterification and storage in lipid droplets (89, 90). The acyl-CoA sterol transferases Are1 and Are2 catalyze sterol esterification with fatty acids resulting in steryl esters for storage in lipid particles and thereby protect cells against accumulation of toxic intracellular levels of sterols (91). Steryl ester synthesis is not essential (92, 93). Are2 physically binds to the sterol transporter Pdr11, which suggests that transported sterols are directly transferred from Pdr11 to Are2, thus potentially facilitating direct esterification of sterols at the site of sterol import (89). Sterol esterification, however, is not required for sterol uptake as mutants deficient in sterol esterification are viable under sterol-auxotrophic conditions (94). The sterol O-acetylating enzyme Are1 is located solely in the ER in close proximity to the proteins involved in sterol biosynthesis (89). Steryl ester hydrolysis is catalyzed by membrane-bound lipases Yeh1, Yeh2, Tgl1 and also not essential (95). Steryl esters act as a cellular reserve that can be easily interconverted to free sterols in case sterol synthesis becomes limited. Yeast cells can discriminate between different sterol molecules and remove suboptimal sterol species from membranes by a general acetylation and sterol-structure specific deacetylation cycle (96). In *S. cerevisiae*, acetylation of sterols by acyl-CoA sterol acyltransferases Are1 and Are2 and reduces their membrane incorporation. The membrane-anchored lipases Yeh1, Yeh2, and Tgl1 efficiently deacetylate ergosterol, thereby retaining ergosterol in the membrane whereas other sterols are not deacetylated and exported from the cell (96).

Regulation of sterol synthesis

In *S. cerevisiae*, expression of the genes encoding the enzymes of the ergosterol pathway is strictly regulated, and sterol biosynthesis is influenced by both the cellular sterol content and the oxygen tension. The action of transcription factors Upc2 and the homologous Ecm22 zinc-finger proteins are responsible for feedback regulation of ergosterol on its biosynthetic pathway genes (97). These transcription factors bind ergosterol molecules directly, thereby sensing the intracellular sterol level (98). Upon ergosterol binding, Upc2 is retained in the cytosol. When absence of oxygen prevents sterol biosynthesis, Upc2 relocates to the nucleus. Although sterol biosynthesis requires molecular oxygen, the pathway is up-regulated in the absence of oxygen by Upc2, while Upc2 also activates expression of *AUS1* and *PDR11*, which encode the plasma-membrane sterol transporters. Genes involved in sterol biosynthesis are independently regulated by oxygen by a coupling to the activity of heme biosynthesis through the heme-dependent transcription factor Hap1 and transcriptional repressor Rox1 (99, 100). Using these mechanisms of transcriptional repression and activation. *S. cerevisiae* is able to sense O_2 by the activity of sterol and heme biosynthesis (101). Several mutations in genes

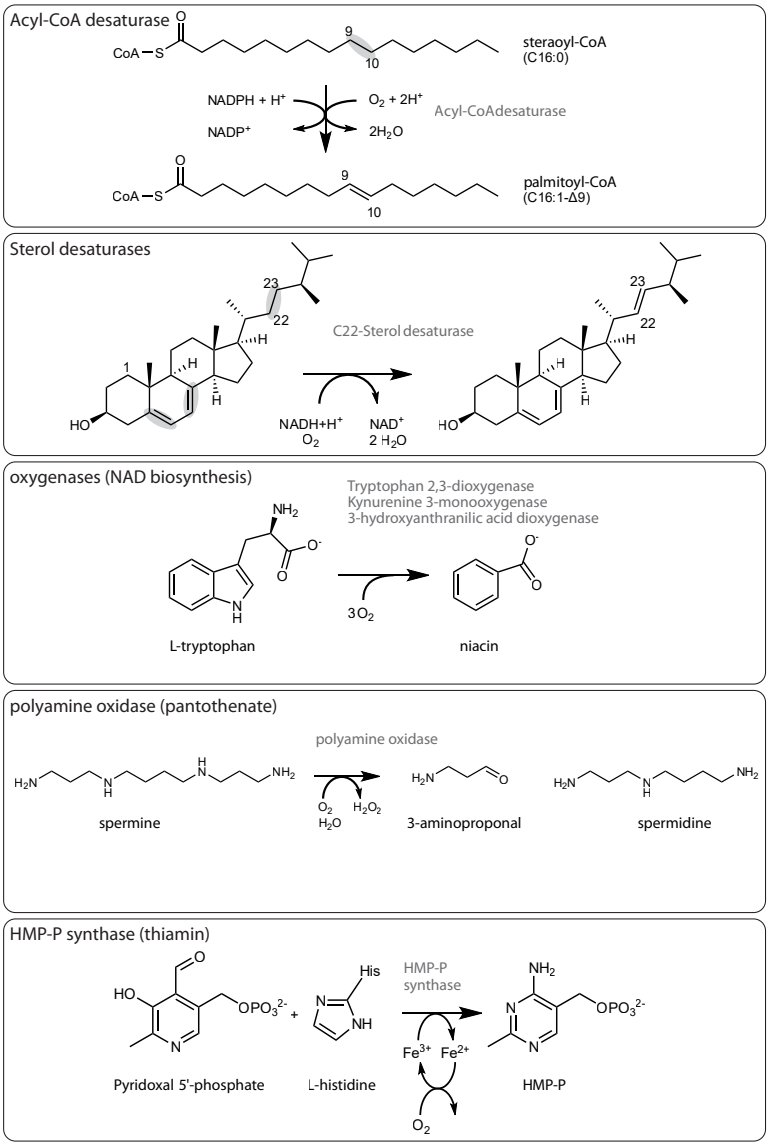

Figure 4. Documented oxygen-requiring biosynthetic reactions in yeasts. Desaturation of fatty acyl-CoA by Ole1 in yeast introduces a double bond at the Δ9th position (grey marking). In the ergosterol pathway, demethylation and desaturation reactions involve molecular oxygen and sites of desaturation are indicated (orange yellow). In the biosynthesis of NAD^+, three mono-oxygenases require oxygen to convert tryptophan to niacin. Polyamine oxidase in pantothenate biosynthesis requires molecular oxygen to convert spermine to 3-aminoproponal and spermidine. The oxygen dependence of HMP-P synthase activity in the thiamin biosynthesis pathway is hypothesized to originate from the oxygen-dependent regeneration of Fe^{3+}.

involved in heme biosynthesis or in *UPC2* have been shown to deregulate expression of sterol transporters and enable sterol uptake under aerobic conditions (75, 102, 103)

In the mevalonate pathway, two isoenzymes of HMG-CoA reductase (Hmg1 and Hmg2 are differentially regulated at the transcriptional level, with Hmg1 being mainly expressed under aerobic conditions and Hmg2 being the predominant isoenzyme under anaerobic conditions (104). HMG-CoA reductase activity is additionally regulated by proteasomal degradation, which is stimulated by farnesyl pyrophosphate (105, 106). Similarly, targeting of squalene epoxidase (Erg1) for proteasomal degradation by the ubiquitin ligase Dao10 is regulated by sterol levels (107).

Vitamins

Although most strains of *S. cerevisiae* contain a complete set of biosynthetic genes for biotin, nicotinic acid, para-aminobenzoic acid, synthetic media for cultivation of this yeast are usually supplemented with these vitamins (40). Oxygen plays an often overlooked role in the requirement of *S. cerevisiae* for these vitamins (Figure 4). To characterize small oxygen requirements for biosynthesis of obligately aerobic, facultatively fermentative yeasts, the role of oxygen in vitamin synthesis therefore needs to be elucidated. The biotin biosynthesis pathway by *S. cerevisiae* has still not been fully resolved and although laboratory evolution can be used to select for fast biotin-prototrophic growth under aerobic conditions (108), the resulting strains cannot grow anaerobically without biotin supplementation, which indicates involvement of oxygen in the initial steps of biotin synthesis (109). Recently, the oxygen requirement for biotin biosynthesis was bypassed by introduction of the biotin pathway from *E. coli* (110).

Pantothenate (vitamin B5) is a key precursor for synthesis of CoA and acyl carrier protein. Pantothenate can be imported by a proton-symporter Fen2 or *de novo* synthesized from 2-keto-isovalerate and spermine (111). Hydroxymethyltransferase Ecm31 catalyzes transfer of a hydroxymethyl moiety to 2-keto-isovalerate resulting in formation 2-dehydropantoate, which is subsequently reduced by NADPH to pantoate by gluconate 5-dehydrogenase Pan5 (112, 113). Spermine is oxidized by the polyamine oxidase Fms1 in an oxygen-dependent reaction that yields 3-aminopropanal, spermidine and hydrogen peroxide. 3-aminopropanal is then oxidized by the NAD-dependent aldehyde dehydrogenases Ald2 and Ald3 to β-alanine. Pantoate and β-alanine are then combined to pantothenate in an ATP-dependent amidation reaction catalyzed by the pantothenate synthase Pan6.

Nicotinic acid (vitamin B3) is a key precursor for the pyridine-nucleotide redox cofactors NAD^+ and $NADP^+$. *S. cerevisiae* can import nicotinic acid or nicotinamide riboside from the environment by the plasma membrane transporters Tna1 and Nrt1, respectively. Nicotinic acid is an intermediate of the NAD^+ salvage pathway, in which it is converted to nicotinamide nucleotide by nicotinate phosphoribosyltransferase (Npt1). Alternatively, in the absence of extracellular sources of nicotinic acid, nicotinamide nucleotide can be *de novo* synthesized

from the aromatic amino acid L-tryptophan, via a pathway that involves three oxygen-requiring oxygenases (114).

Thiamine (vitamin B1) is a precursor for thiamine diphosphate (TDP), a crucial cofactor of transketolases, pyruvate decarboxylases, pyruvate- and 2-oxoglutarate-dehydrogenase complexes and α-acetolacetate synthase. In *S. cerevisiae*, thiamine can be synthesized *de novo* from histidine, pyridoxal 5-phosphate (PLP), NAD^+ and glycine. A source of PLP can be imported either in the form of pyridoxine, pyridoxal or pyridoxamine 5-phosphate. Synthesis of thiamin is initiated by phosphorylation of PLP by the kinase Bud16. The resulting pyridoxiamine-5-phosphate is oxidized in an oxygen-dependent step to PLP by the oxidase Pdx3. The phosphorylation of pyridoxal by Bud16 directly results in the formation of PLP. Alternatively, PLP can be *de novo* synthesized from dihydroxyacetone phosphate (DHAP), glutamine, and keto-D-ribose-5-phosphate by a PLP synthase, which is a protein complex consisting of Sno1-3 and Snz1-3. PLP and L-histidine are substrates for a biochemical Diels-Alder reaction catalyzed by 4-amino-2-methyl-5-hydroxy-pyrimidine (HMP) synthase (115). Based on research on *Candida albicans*, the L-histidine precursor is proposed to originate from the Thi5 protein rather than from free histidine (115). The requirement of this reaction for Fe^{3+}, which in *S. cerevisiae* is only available under aerobic conditions, may explain the oxygen-dependency of thiamin biosynthesis (115). Consistent with this conclusion, anaerobic auxotrophy for thiamin can be rescued by HMP addition (116). HMP can be phosphorylated to HMP-diphosphate by the kinases Thi20 and Thi21. HMP-diphosphate is then condensed with 5-(2-hydroxylethyl)-4-methyl thiazole phosphate to thiamine mono-phosphate. The thiazole is derived from nicotinamide, glycine and from the thiazole synthase Thi4 protein itself. In this reaction, Thi4 acts as a suicide enzyme, which acts as co-substrate in a single-turnover reaction with NAD^+ and glycine to donate sulfur from a cysteine residue to form 5-(2-hydroxylethyl)-4-methyl thiazole phosphate. In yeast, thiamin mono-phosphate is first dephosphorylated to thiamine before being phosphorylated to thiamine diphosphate.

Oxygen and Iron metabolism

Iron is an essential element for pro- and eukaryotes and plays a key role in many cellular redox reactions. Iron is a transition metal that can exist in the -2 to + 6 oxidation states. In living cells, iron participates as a reactive cofactor in the form of mononuclear iron, oxo-diiron, heme, siroheme and iron-sulfur clusters. Under aerobic conditions and at pH values above 5, Fe^{2+} is spontaneously oxidized by molecular oxygen, yielding Fe^{3+}. Fe^{3+} precipitates easily as oxides, or with phosphates, and sulfates and, under these conditions, is essentially insoluble. This solubility challenge makes acquisition of iron a crucial process for microbial growth (117). *S. cerevisiae* extracellularly reduces Fe^{3+} to Fe^{2+}, which can be transported by the high-affinity iron transporters Fet3 and Frt1 (118, 119) or by the low-affinity transporters Fet4 and Smf1 (120). Some processes inside the yeast cell require Fe^{3+} or the derived oxo-diiron, including sterol, dNTP and thiamin biosynthesis (115, 121). Under aerobic conditions, intracellular Fe^{2+} can be oxidized to Fe^{3+} with oxygen by ferroxidase (122). It is generally

assumed that these Fe^{3+}-dependent processes are blocked in anaerobic cultures due to the likely absence of oxygen and Fe^{3+} uptake (123, 124).

Iron can react with oxygen in Fenton reactions, which produce oxygen radicals that are toxic to the cell. Therefore, free iron concentrations inside cells are kept low by complexation with proteins and organic ligands (123, 125). Superoxide radicals can be detoxified by superoxide dismutase into hydrogen peroxide, which in turn can be eliminated by catalases to water and oxygen. The overall stoichiometry of reactions of iron and oxygen is therefore dependent on the oxygen recovery by superoxide dismutase and catalase (126).

Heme is a particular organic coordination complex of iron, which acts as a prosthetic group in many enzymes that mediate electron transfer reactions and oxygen binding. In *S. cerevisiae*, heme biosynthesis is regulated by the availability of oxygen (127, 128). Heme proteins are crucial for respiratory metabolism and in *S. cerevisiae*, transcriptional regulation pathways for cellular heme status and respiration-associated genes are transcriptionally intertwined (129, 130). Iron limitation results in metabolic remodeling of *S. cerevisiae* to preserve iron for crucial metabolic processes while, for example, down-regulating mitochondrial respiration (reviewed in (129)). Facultatively fermentative yeasts such as *S. cerevisiae* are not dependent on respiratory metabolism. Therefore, heme biosynthesis is dispensable as long as essential products of heme-containing enzymes (vitamins, unsaturated fatty acids, sterols, cysteine or methionine) are included in growth media. Molecular oxygen is required in the last steps of heme biosynthesis and alternatively siroheme can be synthesized from the heme biosynthesis intermediate uroporphyrinogen III (131). Siroheme has a similar chemical structure as heme and can replace the function of heme in sulfite and nitrite reductases (132, 133). Anaerobic cultures of *S. cerevisiae* are therefore not auxotrophic for methionine, cysteine or other reduced sulfur compounds.

Ribonucleotide reductases (RNR) are essential enzymes for the *de novo* DNA biosynthesis, for which they provide the deoxyribonucleoside triphosphate (dNTP) building blocks. RNRs catalyze the reduction of ribonucleotides to deoxyribonucleotides (Figure 5) and all known variants of RNR share a common radical reaction mechanism involving the diferric tyrosyl radical (Fe^{3+}_2-Y·) cofactor. However, the mechanism by which this radical is generated differs between RNR's (134). The iron atom in the Fe^{3+}_2-Y· cofactor is derived from Fe-S cluster biosynthesis, and the cofactor is reduced and assembled by collaboration of the electron-transfer protein Dre2, and glutathione-dependent oxidoreductases Grx3 and Grx4 (135). Similar to most other eukaryotes, *S. cerevisiae* contains a class 1α RNR. Representatives of this class are generally assumed to be dependent on oxygen for radical formation. Eukaryotic RNR complexes typically consist of two small and two large subunits (136). Instead, the RNR of *S. cerevisiae* is a heterodimer of Rnr2-Rnr4, in which Rnr4 contributes to correct protein folding and assembly of the cofactor in Rnr2 (137–140). In contrast to the situation in other eukaryotes, the yeast small subunit is heterodimeric, with only a single catalytic site (137, 140). Once the oxygen-dependent radical generation has occurred, the tyrosyl radical can

catalyze multiple turnovers but needs to be regenerated. The timespan over which the radical remains active depends on the presence of oxygen and on the temperature, e.g. in the absence of oxygen the stability of the radical is increased (141). The strict oxygen requirement for radical synthesis and regeneration of class 1 RNR would support the hypothesis that dNTP formation is an oxygen-dependent process. However, this hypothesis is contradicted by the observation that anaerobic *S. cerevisiae* cultures do not require supplementation of dNTPs. It remains to be elucidated how the radical cofactor in the RNR of *S. cerevisiae* is generated in the absence of oxygen. In mammals, a subunit exchange upon hypoxia leads to longer stability of the radical and potentially higher affinity for molecular oxygen (142). Similarly, crucian carps, which can survive and maintain cell division during prolonged periods of hypoxia, have two paralogs of the genes encoding RNR subunits, thus providing a further indication that DNA synthesis in eukaryotes is more flexible with respect to its oxygen requirement than generally assumed (141).

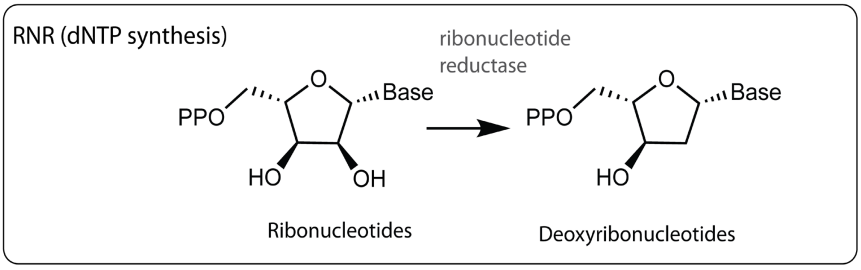

Figure 5. Oxygen-dependent generation of the ribonucleotide reductase (RNR) radical is required for the conversion of ribonucleotides to deoxyribonucleotides.

Bioethanol production

The natural ability of *S. cerevisiae* to produce ethanol from simple mono- and disaccharides has long been exploited for industrial-scale production of alcoholic beverages. Another application of yeast alcoholic fermentation makes use of the fact that alcoholic fermentation concentrates almost the entire heat of combustion of sugars into a smaller number of carbon atoms and yields a liquid product that can be used as a renewable car fuel. As part of a global drive to replace fossil fuels with renewable biofuels, yeast-based production of ethanol has increased and is now estimated to have a global production volume of 87 Mton y^{-1}(143). Techno-economic analyses of low-carbon scenarios include liquid biofuels as a significant contributor towards sustainable economy (144–146). 'First generation' bioethanol production processes rely on the alcoholic fermentation of sugars derived from cane sugar or hydrolyzed corn starch with the yeast *S. cerevisiae*. These large-scale processes are not aerated and, except for a brief initial phase, are completely anaerobic due to vigorous oxygen consumption, vigorous carbon-dioxide production and limited mass transfer. Since ethanolic fermentation of sugars is redox neutral, these bioprocesses are characterized by a high yield of ethanol on substrate. Moreover, improvements in processes and yeast strains have enabled high ethanol titers and high volumetric productivities (147–151). These first-generation processes are,

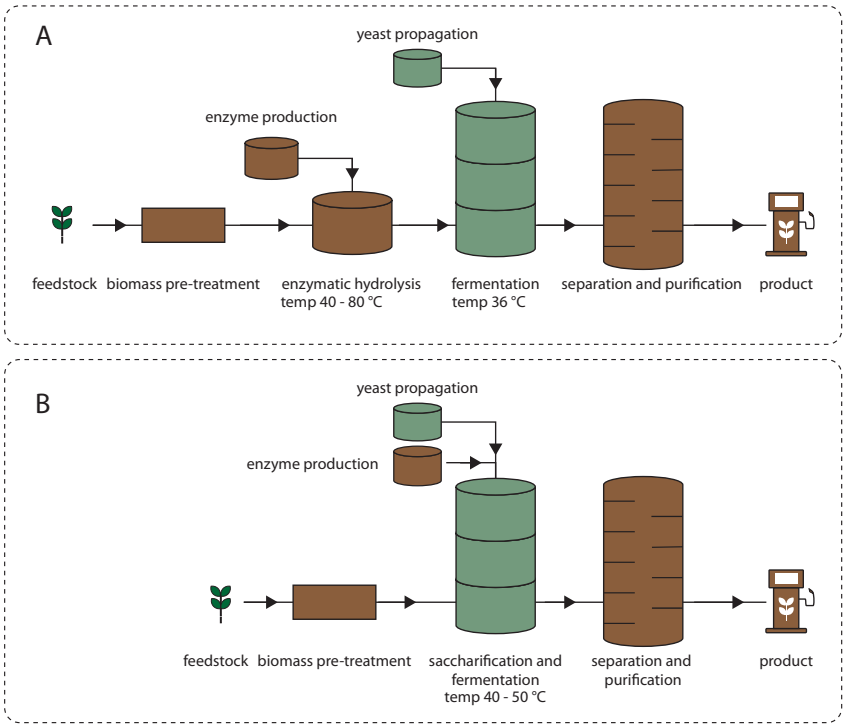

Figure 6. Schematic outline of yeast-based bioethanol production process. (A) separate enzymatic hydrolysis and fermentation or (B) simultaneous saccharification and fermentation (SSF).

however, not without controversy due to an ongoing public debate about competition for land with food production and other human needs (144, 152).

Research into reduction of the carbon footprint and land use of ethanol production have focused on accessing alternative feedstocks which can be sustainably sourced. These could, for example, include fast-growing 'energy crops' harvested from soils that are not compatible with food production. In addition, large amounts of readily available agricultural and forestry waste streams and residues contain potentially fermentable sugars (153). In contrast to the sugar feedstocks used in 'first-generation' bioethanol processes, plant biomass considered for second-generation processes mainly consists of lignocellulosic material, which cannot be directly fermented by *S. cerevisiae*. The potential bioethanol yield from lignocellulosic feedstocks depends on their content of fermentable sugar (154). Lignocellulosic feedstocks vary in chemical composition and the variability is not only plant specific but arises from a multitude of other causes including seasonal effects, harvesting methods, and storage time (154–156).

In second-generation processes for yeast-based bioethanol production, plant biomass is first pretreated using mechanical, thermal and/or chemical methods to deconstruct the compact structure of lignocellulose and open up the carbohydrate polymers for enzymatic hydrolysis (Figure 6). The carbohydrate polymers in lignocellulosic plant biomass are then hydrolyzed (saccharified) by a variety of fungal hydrolytic enzymes to release the fermentable sugars

(157, 158). This method of enzymatic degradation of lignocellulose is favored since it requires low energy and has a minimal environmental footprint, but the process is time consuming and suffers from product inhibition by released sugars (159). After enzymatic hydrolysis, the lignocellulosic hydrolysates are typically fermented to ethanol with *S. cerevisiae*. To this end, *S. cerevisiae* strains have been engineered for efficient fermentation of two pentose sugars, D-xylose and L-arabinose, that occur in copious amount in most second-generation feedstocks (160). Successful development of second-generation bioethanol production processes can serve as an example for similar processes to produce other biological-derived bulk or commodity chemicals.

To successfully replace fossil transport fuels, the price of bioethanol has to be cost-competitive. This requires near-theoretical product yields. Extensive engineering of yeast central metabolism, for example by expression of non-native pathways for reduction of acetyl-CoA to ethanol and for carbon dioxide fixation, have enabled improvements of ethanol yield under industrially relevant conditions (161–164).

Stress tolerance under industrially relevant conditions: beyond *S. cerevisiae*?

The economics of large-scale anaerobic yeast fermentation processes for production of ethanol and other fermentation products does not only depend on the product yield on fermentable sugar. Process intensification (i.e. increasing volumetric productivity of biomass pretreatment and fermentation) is another major factor for process economics, which can however impose significant stress conditions on yeast cells. In particular in second-generation bioethanol processes, yeast cells are exposed to a range of stress factors that may affect their fermentative performance (165–168). Harsh pretreatment of lignocellulosic feedstocks can result in an unfavorable chemical compositions of biomass hydrolysates due to the presence of a range of inhibitors of yeast performance, including acetic acid, furfural and hydroxymethylfurfural. The chemical stress on yeast cells in ethanol product processes is usually dynamic, as the composition of biomass hydrolysates changes during batch fermentation due to consumption of carbon sources and production of growth inhibitors (in particular the product ethanol). Cellular toxicity of inhibitors can originate from inhibition of enzyme reactions, disruption of cellular structures or, especially in the case of weak organic acids such as acetic acid and formic acid, from dissipation of the pH gradient across the yeast plasma membrane (84, 169, 170). Tolerance to inhibitors is often determined by multiple genes and cellular processes and, even in an extensively studied organism such as *S. cerevisiae*, remains incompletely understood (171–174). Laboratory evolution of strains for improved tolerance, combined with whole-genome sequencing and reverse engineering of selected mutations by efficient genome-editing techniques, has proven to be powerful tool to improve tolerance to inhibitors present in stress (175–177).

Increased tolerance to high temperatures would be highly relevant for yeast-based bioethanol production, and in particular for second-generation processes. *S. cerevisiae* strains typically show decreased performance at temperatures above approximately 36 °C (178, 179), while

the enzymes used for plant biomass hydrolysis have considerable higher temperature optima. A process in which enzymatic hydrolysis and fermentation occur in the same reactor (SSF, simultaneous saccharification and fermentation) would reduce feedback inhibition of hydrolytic enzymes by monosaccharides due to the continuous sugar consumption by the yeast. However, temperature optima of fungal hydrolases are higher than those of *S. cerevisiae*. A simultaneous process at high temperature (for example 50 °C) could also reduce costs of cooling, equipment and product recovery and, moreover, decrease microbial contamination issues (180, 181). Since improving the performance of cellulases at sub-optimal temperatures by protein engineering is in practice difficult, application of thermotolerant yeasts would be a key breakthrough (182). So far, research on engineering *S. cerevisiae* for increased thermotolerance has only met limited success, with temperature optima remaining below 40 °C and trade-offs at lower growth temperatures (83, 183).

Outside the genus *Saccharomyces*, multiple 'non-conventional' yeast species exhibit better tolerance to industrially relevant stress factors than *S. cerevisiae* (182, 184–186). This includes several facultatively fermentative yeasts, including *Kluyveromyces marxianus* (187) and *Ogataea parapolymorpha* (188, 189) which can grow at temperatures of up to 50 °C. However, as yet unidentified oxygen requirements restrict the implementation of these and other non-conventional yeasts in large-scale anaerobic processes (10, 23, 190). This thesis therefore focuses on understanding, identifying and eliminating biosynthetic requirements for molecular oxygen in yeasts.

Scope of the thesis

The objectives of the research described in this thesis were to elucidate as yet unknown oxygen-dependent biosynthetic reactions in yeasts, with the ultimate goal to design and implement engineering strategies for their elimination (i.e. to turn an obligately aerobic yeast into a facultative anaerobe). This fundamental research goal addresses a question that has been studied by yeast researchers for at least three decades (10, 190) and is inspired by an ambition to make new yeast species accessible for application in anaerobic industrial processes and to thus exploit of the wide diverse phenotypic traits of yeasts.

Interpretation of anaerobic growth experiments with yeasts and evaluation of engineering strategies to eliminate biosynthetic oxygen requirements critically depend on careful experimental design to minimize oxygen contamination. Even for the model yeast *S. cerevisiae*, which is the corner stone for yeast physiology research, questions still remain about its minimal nutritional requirements for strictly anaerobic growth. Therefore, in **Chapter 2**, the requirement of a popular *S. cerevisiae* laboratory strain for the commonly used 'anaerobic growth factors' ergosterol and Tween 80 were critically reinvestigated, using a combination of sequencing-batch bioreactor cultivation, anaerobic-chamber experiments and use of a specific gene-knockout mutant. In addition to providing a new insight in the non-essentiality of unsaturated fatty acid supplementation for anaerobic growth of *S. cerevisiae*, the research

described in this Chapter helped to provide a baseline for research on non-conventional yeasts.

Design and operation of cultivation systems for studying anaerobic growth requirements of yeasts is technically challenging. In particular in bioreactor cultures, data interpretation is easily complicated by levels of oxygen contamination that mask biosynthetic oxygen-requirements and drastic changes to conventional bioreactor set-ups are required to minimize oxygen entry (10, 191). Throughout this PhD project, which was part of a larger ERC-funded project, possible origins of oxygen contamination in cultivation systems and ways to mitigate them were investigated. In **Chapter 3**, the experience gathered in this project is discussed and protocols are described for anaerobic cultivation in anaerobic chambers and bioreactors.

Chapter 4 is initiated by a thorough continuous-culture characterization of the biosynthetic oxygen requirements of the obligately aerobic, thermotolerant yeast *K. marxianus*. Analysis of transcriptome and physiological data under different oxygen-availability regimes, in which responses of *K. marxianus* were compared to those of the reference yeast *S. cerevisiae*, allowed for the generation of an hypothesis on the molecular origin of the oxygen requirement of *K. marxianus*. This hypothesis was tested by sterol-uptake experiments and validated by construction of a strain engineered for oxygen-independent production of a sterol surrogate. This engineered strain was evaluated for its capability of anaerobic growth and subjected to laboratory evolution for the identification of beneficial mutations and assessment of its thermotolerance.

Just like *K. marxianus*, *O. parapolymorpha* is a fast growing, facultatively fermentative yeast applied in aerobic industrial processes. The goal of the research described in **Chapter 5** was to identify and quantify the oxygen-dependency of this yeast species. As in Chapter 4, application of oxygen-limited continuous cultivation regimes enabled a thorough investigation of the physiology and oxygen requirements of this yeast, which were tested by construction and physiological analysis of engineered *O. parapolymorpha* strains.

This thesis is concluded with a brief outlook section, which discusses the scientific and applied significance and potential of the presented results.

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Chapter 2

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

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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 pre-cultivation without ergosterol and Tween 80, strain CEN.PK113-7D and a congenic *ole1*Δ 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 *ole1*Δ 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.

Introduction

The large majority of known yeast species ferment glucose to ethanol when grown under oxygen limitation (1, 2). 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 (3, 4). The molecular basis for the non-dissimilatory oxygen requirements of most facultatively fermentative non-*Saccharomyces* yeasts is still not completely understood (5, 6).

Anaerobic growth of *S. cerevisiae* imposes special nutritional requirements. Already in the 1950s, Andreasen and Stier reported that strictly anaerobic growth of *S. cerevisiae* required supplementation of media with a sterol and an unsaturated fatty acid (UFA)(7, 8). 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 (9–11). While synthesis of nicotinic acid by *S. cerevisiae* is also oxygen dependent (12), 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 anaerobically 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 (13, 14). In artisanal wine fermentation, *S. cerevisiae* starts to dominate other 'wild' yeast species once oxygen has been depleted during the initial phases of fermentation (15, 16).

Sterols and fatty acids are important constituents of cellular membranes. Sterols play a key role in maintenance of membrane integrity and fluidity (17, 18), and have also been implicated in specific cellular processes such as endocytosis and nutrient uptake (19, 20). The degree of (un)saturation of the fatty-acyl moieties in phospholipids is an important determinant of membrane fluidity (21). In addition, fatty acids are involved in energy storage and post-translational modification of proteins (22).

De novo biosynthesis of ergosterol, the major sterol in aerobically grown *S. cerevisiae*, involves a monooxygenase (Erg1), demethylase (Erg3), oxidase (Erg25) and desaturases (Erg3 and Erg5) and requires 12 moles of O_2 per mol of sterol (23). The oxygen requirement of *S. cerevisiae* for synthesis of UFAs (mainly palmitoleic acid, C16:1, and oleic acid, C18:1, (24)) originates from the essential role of the Δ9-fatty acid desaturase Ole1. In the presence of ferrocytochrome b5, Ole1 catalyses the oxygen-dependent introduction of a cis double bond in palmitoyl-CoA and stearoyl-CoA, yielding palmitoleoyl-CoA (C16:1-CoA) and oleoyl-CoA (C18:1-CoA), respectively (24, 25). The importance of this reaction is illustrated by the strict UFA auxotrophy of *ole1* null mutants in aerobic cultures (26–28).

While no indications for oxygen-independent sterol biosynthesis have been found in nature,

neither in living organisms nor in the fossil record (23), microbial UFA biosynthesis does not universally require oxygen. For example, during acyl-CoA synthesis by bacterial multi-component 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 (29). Furthermore, in contrast to the cytosolic *S. cerevisiae* type-I FAS complex that only produces saturated fatty acids (25), some bacterial type-I FAS proteins are capable of oxygen-independent UFA synthesis (30, 31).

Based on reported biomass contents, oxygen requirements for UFAs and sterols in *S. cerevisiae* each amount to approximately 0.1 mmol O_2 (g biomass)$^{-1}$ (10, 32, 33). However, it should be noted that UFA and sterol contents strongly depend on strain background and culture conditions (34). 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 (35). Minimizing entry of small amounts of oxygen into bench-top laboratory bioreactors is even more challenging and requires use of ultra-pure nitrogen gas, applying overpressure and using special materials for tubing and septa (3, 36). Furthermore, as indicated by the practice of aerobically 'loading' brewing yeasts (13, 14), 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 (37, 38), 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 analysed growth of *S. cerevisiae* CEN.PK113-7D and a congenic *ole1* null mutant in cultures grown in an anaerobic chamber and analysed the lipid composition of anaerobically grown biomass.

Materials and methods

Strains, media and maintenance. *Saccharomyces cerevisiae* strains used and constructed in this study (Table 1) were derived from the CEN.PK lineage (37, 38). YPD medium (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 (39). Synthetic urea medium (SMD-urea), in which ammonium sulfate was replaced by 2.3 g L^{-1} urea and 6.6 g L^{-1} K_2SO_4 was prepared as described earlier (40). 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} K_2SO_4 (41). SM 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 media as Tween 80 (polyethylene glycol sorbate 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. E. 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. 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 $MgCl_2 \cdot 6H_2O$, 10 mM $MgSO_4 \cdot 7H_2O$, and was autoclaved at 121°C for 20 min. To prepare SOC medium, a concentrated solution of glucose, separately autoclaved at 110° 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® 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 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 cassettes were purified from 1% (w/v) agarose gels (TopVision Agarose, Thermo Fisher) with TAE buffer (50x, Thermo Fisher) using a ZymocleanTM Gel DNA Recovery Kit (Zymo Research, Irvine, CA). E. coli XL1-Blue competent cells were transformed 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 GenEluteTM 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 (42). Transformants were selected on SMD 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 (43). Oligonucleotides and plasmids used in this study are listed in Table 2 and 3, respectively. A unique guide-RNA (gRNA) sequence targeting *OLE1* was designed using Yeastriction (43) and synthesised 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

Table 1: Strains used in this study

Name	Relevant genotype	Parental strain	Origin
CEN.PK113-7D	*MATa MAL2-8c SUC2 CAN1*	-	(37)
IMX585	*MATa MAL2-8c SUC2 can1Δ::cas9-natNT2 URA3 TRP1 LEU2 HIS3*	CEN.PK113-7D	(43)
IMK861	*MATa MAL2-8c SUC2 can1Δ::cas9-natNT2 URA3 TRP1 LEU2 HIS3 ole1Δ*	IMX585	This study

Table 2: Primers used in this study

Purpose	Primer nr.	Sequence 5'-> 3'
gRNA primer targeting *OLE1*	11231	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCCTTTTGTTCTTGTTGAATCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
Repair fragment, *OLE1* upper strand	11239	CATAGTAATAGATAGTTGTGGTGATCATATTATAAACAGCACTAAAACATTACAACAAAGGTATCACATTACAATAACAAAACTGCAACTACCATAAAAAAAAATTGAAAAATCATAAAA
Repair fragment, *OLE1* lower strand	11240	TTTTATGATTTTTCAATTTTTTTTTATGGTAGTTGCAGTTTTGTTATTGTAATGTGATACCTTTGTTGTAATGTTTTAGTGCTGTTTATAATATGATCACCACAACTATCTATTACTATG
Diagnostic primer *OLE1* fw	11249	GGTATCCCAGCCTTCTCTGC
Diagnostic primer *OLE1* rv	11250	CTATTGCTCCAGGGCCCAG

Table 3: Plasmids used in this study. gRNA target sequences are indicated in subscript.

Name	Relevant characteristics	Origin
pROS11	2µm ampR *amdSYM* p*SNR52*-gRNA$_{CAN1.Y}$ p*SNR52*-gRNA$_{ADE.Y}$	(43)
pUDR319	2µm ampR *amdSYM* p*SNR52*-gRNA$_{OLE1}$ p*SNR52*-gRNA$_{OLE1}$	This study

a final volume of 5 µL with the NEBuilder HiFi DNA assembly master mix (NEB, Ipswich, MA), according to 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 (41).

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. Pre-cultures 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, Delft, 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 O2, HiQ 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 (40) 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 in a condenser to minimize evaporation of water and volatile metabolites and dried with a PermaPure PD-50T-12MPP dryer (Permapure, Lakewood, NJ) prior to analysis. CO_2 concentrations in the outlet gas were measured with a NGA 2000 Rosemount gas analyser (Emerson, St. Louis, MO). The gas analyser was calibrated with reference gas containing 3.03% CO2 and N6 grade N_2 (Linde Gas Benelux).

Frozen glycerol stock cultures were used to inoculate aerobic 100-mL shake-flask cultures on SMD. After overnight cultivation at 30 °C, a second 100-mL aerobic shake-flask pre-culture on SMD was inoculated at an OD_{660} of 1.0. During the exponential growth phase of this second pre-culture, biomass was harvested by centrifugation at 4000 g for 5 min and washed with sterile demineralized water. The resulting cell suspension was used to inoculate anaerobic bioreactors at an initial OD_{660} of 0.2. No ergosterol or Tween 80 were included in the medium for the first bioreactor batch cultivation cycle ('carry-over cycle'), in order to deplete endogenous stores of sterols and UFAs.

After the carry-over cycle, cultures were continued in sequential batch reactor (SBR) mode. When the percentage of CO2 in the outlet gas dropped sharply to zero, indicating nutrient depletion, a next SBR cycle was manually initiated by removing culture broth with a Masterflex peristaltic pump, until only 25 mL of culture was left in the reactor. The bioreactor was then refilled to 1.2 L with fresh medium with a peristaltic pump and electric level sensor, which corresponded to a 48-fold dilution of the remaining culture sample. The 5-L glass medium reservoir vessel was sparged with N5.5 grade nitrogen gas (Linde Gas Benelux) for at least 1 h before refilling. Immediately before refilling, approximately 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% N_2, 10% CO_2 and 5% H_2. An IKA® KS 260 Basic orbital shaker platform (Dijkstra 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 H_2-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 g L^{-1} or 50 g L^{-1} glucose. Concentrated solutions of ergosterol and/or Tween 80 were added as indicated. Sterile growth media were pre-incubated 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 OD_{600} of 0.2, from an exponentially growing aerobic pre-culture on SMD. Growth was measured by periodic measurements of the optical density at 600 nm with an Ultrospec® 10 cell density meter (Biochrom, Cambridge, UK) placed inside the anaerobic chamber. To prevent frequent use of the air lock, supplies of cuvettes, pipet tips and demineralized water were all placed inside the anaerobic workspace before the start of growth experiments. When the OD_{600} of the pre-culture no longer increased, it was used to inoculate anaerobic cultures on SMD-urea with 20 g L^{-1} glucose at an initial OD_{600} of 0.2.

Analytical methods. Metabolite concentrations in culture supernatants were analysed by high-performance liquid chromatography (HPLC) as described previously (44). Biomass dry weight measurements in SBR cultures were performed at the end of each cultivation cycle, using pre-weighed 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 analysed 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, PYREXTM Borosilicate glass, Thermo Fisher Scientific). After adding 2 mL methanol, samples were vortexed thoroughly. After addition of 30-100 µL of a 2 mg mL^{-1} internal standard solution of heptadecanoic acid (≥ 98% pure, Sigma) 2 mL of 3 M methanolic HCl and 2 mL of n-heptane (Sigma) were added. The resulting mixtures were incubated at 80 °C for 2 h, while vortexing 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 containing 10-20 mg dried Na_2SO_4 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 N_2. Fatty-acid methyl esters were analysed on an Agilent Technologies 7890A GC-FID system equipped with a FID-1000-220 Gas Station (Parker Balston, Haverhill, MA, USA) and an Agilent Technologies 7693 Autosampler. A VF-5ms 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 (3, 36), we attempted to eliminate sources of oxygen contamination in bioreactor batch cultures of *S. cerevisiae*. The bioreactor headspace was continuously flushed with ultra-pure nitrogen gas and kept under overpressure, 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 pre-culture, CO_2 production rapidly increased until, after 17 h, it reached a maximum and subsequently decreased (Figure 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} (Supplementary figure S1). 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, CO_2 production was much lower than in the first culture and remained stable for 24 h (Figure 1a). Optical density measurements showed that fewer than two biomass doublings had occurred over this period, leading to a biomass concentration of approximately 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 pre-grown 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, CO_2 production rapidly accelerated and the biomass concentration increased to 0.86 ± 0.03 g L^{-1} (Figure 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 unsaturated fatty acids (UFAs).

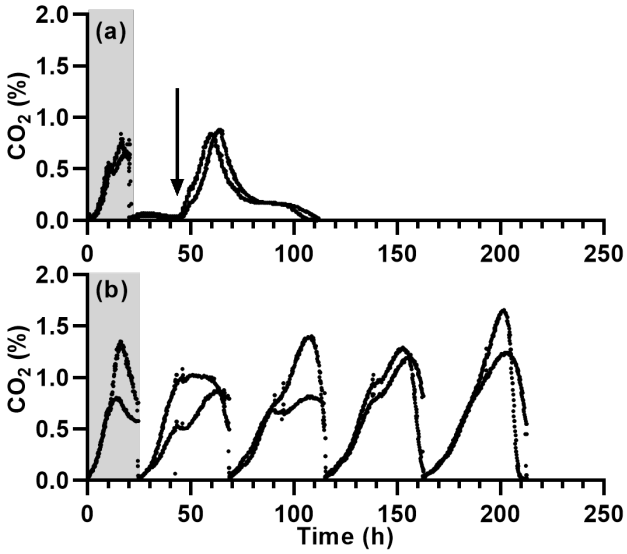

Figure 1. CO_2 production profiles of anaerobic bioreactor batch cultures of *S. cerevisiae* CEN.PK113-7D. Each panel shows data from independent duplicate bioreactor cultures grown on synthetic medium. Experiments were started with a 'carry-over' bioreactor batch culture on synthetic medium without ergosterol and Tween 80 (grey boxes, glucose concentrations in panels A and B were 25 and 40 g L^{-1}, respectively). After emptying and refilling with fresh medium, cultures were continued as follows: (**a**) second cycle of growth on synthetic medium with glucose (25 g L^{-1}) without ergosterol and Tween 80. An ergosterol pulse (0.55 mg L^{-1}) was administered at the time point indicated by the arrow. (**b**) Four sequential batch reactor (SBR) cycles on synthetic medium with glucose (40 g L^{-1}), supplemented with 10 mg L^{-1} ergosterol but not with Tween-80.

Table 4. Anaerobic growth of *S. cerevisiae* CEN.PK113-7D on glucose in anaerobic sequencing batch reactor (SBR) cultures on synthetic medium with glucose, supplemented with ergosterol (10 mg·L^{-1}), but without UFA supplementation. In the initial carry-over cycle (CO, see Figure 1B), also ergosterol was omitted. Specific growth rates were estimated from CO_2 production profiles. Yields of ethanol and biomass were estimated from measurements of biomass, glucose and ethanol at the start and end of each SBR cycle. Data are represented as mean and standard error of the mean of data from independent duplicate cultures.

Cycle	Biomass (g L^{-1})	μ (h^{-1})	$Y_{Ethanol/glucose}$ (g g^{-1})	$Y_{Biomass/glucose}$ (g g^{-1})
CO	0.84 ± 0.09	0.42 ± 0.00	0.37 ± 0.01	0.05 ± 0.00
1	1.30 ± 0.06	0.20 ± 0.02	0.38 ± 0.00	0.04 ± 0.00
2	1.48 ± 0.16	0.17 ± 0.01	0.38 ± 0.02	0.04 ± 0.00
3	1.55 ± 0.02	0.17 ± 0.00	0.37 ± 0.00	0.04 ± 0.00
4	1.57 ± 0.04	0.14 ± 0.02	0.37 ± 0.01	0.04 ± 0.00

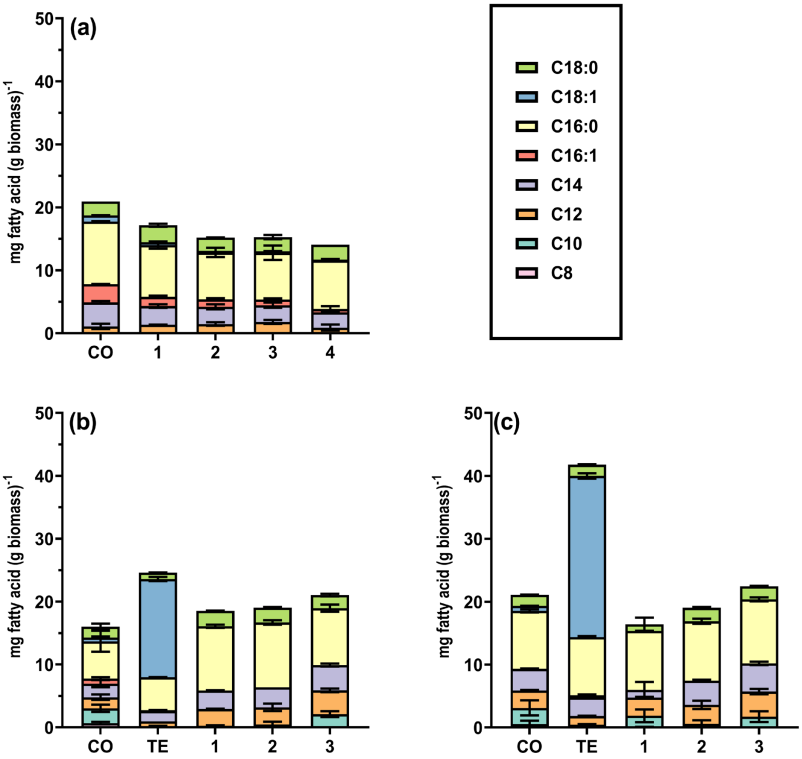

Figure 2. Fatty-acid composition of anaerobic *S. cerevisiae* cultures. Fatty acid composition, analysed by GC-FID, of anaerobically grown cultures in sequential batch bioreactors (SBR, A) and anaerobic-chamber shake-flask cultures (B, C). (**a**) Fatty-acid composition of the reference strain CEN.PK113-7D 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*Δ mutant IMK861 (congenic), 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.

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 sequential batch reactors (SBRs). After an initial carry-over cycle, four consecutive SBR cycles on medium without Tween 80 showed a pronounced CO_2 production and corresponding increase of the biomass concentration (Figure 1b). Specific growth rates estimated from the exponential phases of CO_2 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 analysed 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 (Figure 2a, supplementary Table S2). Since the four SBR cycles led to an approximately $5{\cdot}10^6$-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, (43)) was studied in an anaerobic chamber equipped with a H_2/Pd catalyst system to scavenge traces of oxygen. Since *ole1Δ* strains of *S. cerevisiae* are unable to synthesize UFAs (27, 28), growth of the congenic *ole1Δ* strain IMK861 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 (Figure 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 (Figure 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 analysing 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 (Figure 2b and c). Since growth in the carry-over cultures ceased before glucose was depleted (supplementary data Table S1), 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 than in cultures supplemented with Tween 80 (Figure 2b and c, supplementary Table S2).

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 (10) was the dominant fatty acid in yeast biomass (Figure 2b and c, supplementary Table S2).

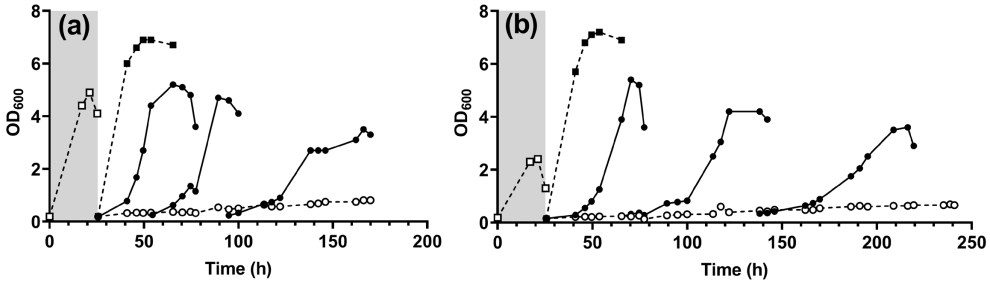

Figure 3. UFA-independent anaerobic growth of the reference strain *S. cerevisiae* IMX585 and the *ole1*Δ strain IMK861. Strains IMX585 (**a**) and IMK861 (*ole1*Δ, **b**) were grown in shake-flask cultures placed in an anaerobic chamber. Aerobic pre-cultures were used to inoculate an anaerobic pre-culture ('carry-over culture', open squares, grey 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 of from a single representative experiment of biological duplicate cultures. Data of the duplicate experiment are shown in supplementary Figure S2.

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 (27, 28, 45). To check if an *ole1* null mutation in the CEN.PK genetic background might have a different phenotype, we investigated aerobic growth of the *ole1Δ* strain IMK861 in shake-flask cultures. These experiments confirmed that, also in the CEN.PK genetic background, aerobic growth on a glucose synthetic medium strictly depended on UFA supplementation after transfer from an aerobic Tween 80-supplemented pre-culture (Figure 4).

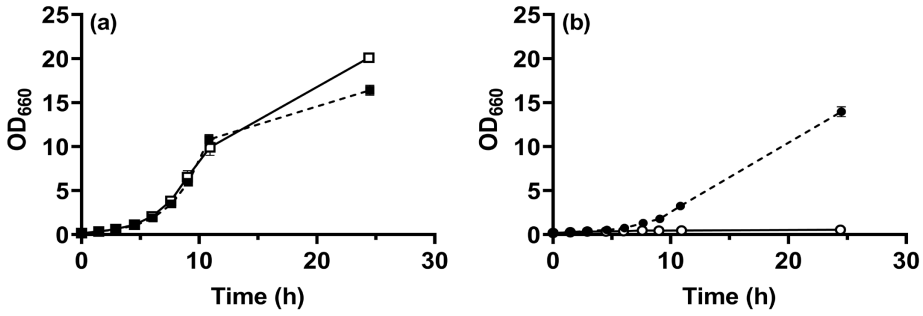

Figure 4. Aerobic growth of *S. cerevisiae* strains IMX585 and IMK861 (*ole1*Δ) in the presence and absence of a source of unsaturated fatty acids. Strains IMX585 (**a**) and IMK861 (*ole1*Δ, **b**) were grown in 100 mL SMD in 500 mL round-bottom shake-flasks at 30 °C and at 200 rpm. Growth was monitored in SMD supplemented with Tween 80 (closed symbols) and in SMD without Tween 80 (open symbols). Data represent mean and standard error of the mean of independent biological duplicate cultures.

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* (27, 45), it indicates that, surprisingly, the UFA requirement for anaerobic growth of *S. cerevisiae* is not absolute.

Since non-respiratory oxygen requirements of *S. cerevisiae* are small (46), interpretation of results can easily be obscured by oxygen contamination and by 'carry-over' of anaerobic growth factors from aerobic or growth-factor-supplemented pre-cultures. Indeed, oxygen contamination of bioreactor experiments was evident from synthesis of small amounts of palmitoleic and oleic acid (Figure 2a, Supplementary Table 1). 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 (47). No K_m 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/H_2 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 approximately 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 (48). 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 (49, 50). A similar adaptation was previously observed in pro-mitochondria of cells after anaerobic incubation without a source of UFAs (51) and in a recent chemostat study on severe oxygen limitation in chemostat cultures without UFA or sterol supplementation (47).

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 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 (8). One reason for the routine inclusion of Tween 80 is that this surfactant aids distribution of highly hydrophobic sterols in aqueous media (52). A requirement of anaerobic *S. cerevisiae* cultures for UFA supplemen-

tation is often inferred from the well-documented UFA auxotrophy of *ole1* null mutants in aerobic cultures. In an earlier study aerobic growth of an *ole1* null mutant ceased when the contribution of UFAs decreased below 7.3% mol of the total fatty acid content (27). The sustained anaerobic growth of an *ole1Δ* mutant in UFA-free media, with undetectable intracellular UFA contents (Figures 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 (53) and steryl-ester synthesis has been demonstrated to decrease during anaerobiosis (54). 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 (55). 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.

Although our results indicate that *S. cerevisiae* CEN.PK113-7D does not absolutely require UFAs during anaerobic growth, 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 2), showed an estimated specific growth rate 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 (56). Biomass yields on glucose (0.04 g biomass (g glucose)$^{-1}$, Table 4) were approximately 2-fold lower than in anaerobic chemostat cultures grown with Tween 80 supplementation (57). 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 (58). 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 (Figure 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 (32, 59, 60), 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 (48, 61).

Acknowledgements

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Supplementary data

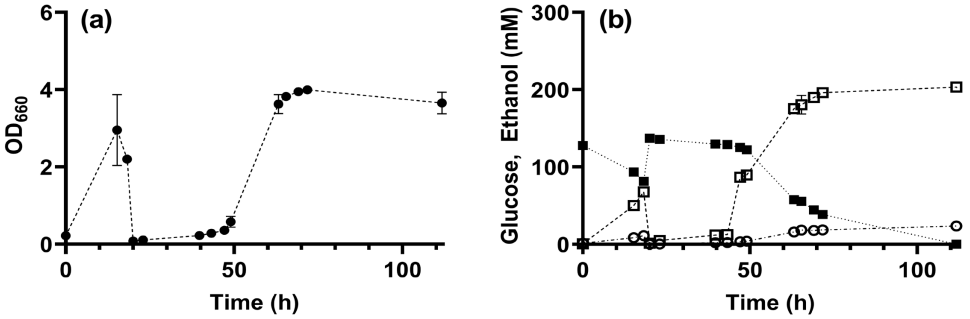

Figure S1. Anaerobic growth profiles of CEN.PK113-7D in bioreactor cultures on SMD-urea. Carry-over bioreactor cultures on synthetic medium with 25 g L^{-1} glucose and, initially, no Tween 80 or ergosterol, were inoculated from aerobic shake-flask cultures. Upon observation of a decrease in optical density a new batch cycle was initiated by emptying and refilling with new media. Subsequently, at approximately 48 h, a pulse of ergosterol dissolved in ethanol (0.55 mg L^{-1}) was added. (**a**) optical density measurements, (**b**) metabolite profiles of glucose (closed squares), ethanol (open squares) and glycerol (open circles) over time. Data represent mean and standard error of the mean from two independent biological cultures.

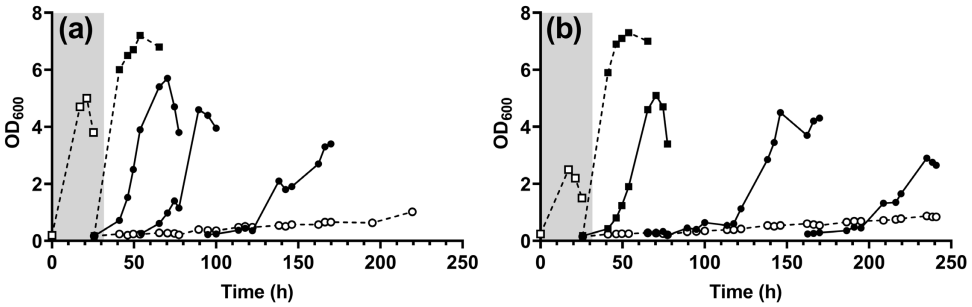

Figure S2. Biological duplicates of anaerobic growth experiment in anaerobic chamber of the reference strain *S. cerevisiae* IMX585 and the *ole1*Δ strain IMK861. Strains IMX585 (**a**) and IMK861 (*ole1*Δ, **b**) were grown on glucose synthetic medium in shake-flask cultures placed in an anaerobic chamber. Aerobic pre-cultures were used to inoculate an anaerobic pre-culture ('carry-over culture', open squares, grey box) on synthetic medium containing 50 g L^{-1} glucose but no anaerobic growth factors. When the OD_{600} no longer increased, cultures were transferred to fresh synthetic medium with 20 g L^{-1} glucose and supplemented with either both anaerobic growth factors (Tween 80 and ergosterol, closed squares), only ergosterol (closed circles), or neither of the growth factors (open circles). The initial culture to which only ergosterol was added (closed circles, first line) was sequentially transferred to medium of the same composition (closed circles, second and third lines). The data are of one representative experiment of biological duplicate cultures.

Table S1. Residual glucose of shake-flask cultures in anaerobic chamber upon termination of the experiment. Residual glucose as measured by HPLC upon termination of the anaerobic shake-flask experiment. 'CO' = carry-over batch, 'TE' = culture supplemented with Tween 80 and ergosterol, 1-3 = subsequent cycles of growth on synthetic medium supplemented with only ergosterol. Data represent mean and standard error of the mean of independent biological duplicate cultures.

Cycle	Residual glucose (mM)	
	IMX585	IMK861 (*ole1*Δ)
CO	91,6 ± 0,8	200,0 ± 0,6
TE	0	0
1	0	0
2	0	0
3	0	0

Table S2. Fatty acid composition of biomass of anaerobic yeast cultures. Data represent mean and standard error of the mean of two biological duplicate cultures. Carry-over batch (CO'), culture supplemented with Tween 80 and ergosterol (TE), subsequent cycles of growth on medium supplemented with only ergosterol (1-4). Data corresponds to Figure 2.

Fatty acid composition in mg fatty acid (g biomass)$^{-1}$									
	C8	C10	C12	C14	C16:1	C16:0	C18:1	C18:0	Total
Anaerobic SBR, CEN.PK113-7D									
CO	-	-	1,10 ±0,42	3,81 ±0,21	2,90 ±0,02	9,94 ±0,05	0,98 ±0,03	2,22 ±0,01	20,93 ±0,73
1	-	-	1,34 ±0,02	2,98 ±0,28	1,48 ±0,20	8,21 ±0,58	0,44 ±0,11	2,71 ±0,25	17,15 ±0,33
2	-	-	1,48 ±0,28	2,73 ±0,40	1,18 ±0,19	7,45 ±0,74	0,25 ±0,01	2,12 ±0,02	15,20 ±1,59
3	-	-	1,80 ±0,31	2,67 ±0,42	0,89 ±0,17	7,45 ±1,15	0,22 ±0,03	2,26 ±0,34	15,28 ±2,01
4	-	-	0,90 ±0,48	2,43 ±0,15	0,53 ±0,45	7,70 ±0,00	0,16 ±0,10	2,37 ±0,00	14,07 ±0,08
Anaerobic shake-flasks, IMX585									
CO	0,68±0,20	2,36 ±0,59	1,78 ±0,44	2,15 ±0,58	0,81 ±0,24	5,94 ±1,66	0,61 ±0,17	1,70 ±0,49	16,01 ±4,35
TE	-	0,20 ±0,08	0,77 ±0,01	1,65 ±0,02	0,15 ±0,02	5,24 ±0,02	15,61 ±0,34	1,01 ±0,02	24,61 ±0,32
1	-	0,27 ±0,05	2,67 ±0,04	2,91 ±0,07	-	10,23 ±0,25	-	2,45 ±0,03	18,52 ±0,45
2	-	0,45 ±0,45	2,74 ±0,60	3,20 ±0,01	-	10,31 ±0,36	-	2,36 ±0,11	19,05 ±1,51
3	-	2,11 ±0,47	3,78 ±0,29	4,04 ±0,23	-	9,03 ±0,57	-	2,08 ±0,21	21,03 ±1,77
Anaerobic shake-flasks, IMK861 (*ole1*Δ)									
CO	0,55±0,55	2,58 ±1,19	2,74 ±0,12	3,49 ±0,04	-	9,19 ±0,23	0,81 ±0,02	1,75 ±0,04	21,10 ±1,53
TE	-	0,45 ±0,10	1,39 ±0,03	2,93 ±0,02	0,36 ±0,13	9,24 ±0,16	25,63 ±0,43	1,78 ±0,08	41,77 ±0,69
1	0,07±0,05	1,80 ±1,02	2,89 ±0,12	1,25 ±1,25	-	9,33 ±0,04	-	1,09 ±1,06	16,42 ±3,45
2	-	0,58 ±0,58	3,03 ±0,66	3,86 ±0,13	-	9,43 ±0,42	-	2,15 ±0,12	19,04 ±0,57
3	-	1,74 ±0,86	3,99 ±0,44	4,48 ±0,26	-	10,18 ±0,32	-	2,08 ±0,06	22,46 ±1,93

Chapter 3
Critical parameters and procedures for anaerobic cultivation of yeasts in bioreactors and anaerobic chambers

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Abstract

All known facultatively fermentative yeasts require molecular oxygen for growth. Only in a small number of yeast species, these requirements can be circumvented by supplementation of known anaerobic growth factors such as nicotinate, sterols and unsaturated fatty acids. Biosynthetic oxygen requirements of yeasts are typically small and, unless extensive precautions are taken to minimize inadvertent entry of trace amounts of oxygen, easily go unnoticed in small-scale laboratory cultivation systems. This paper discusses critical points in the design of anaerobic yeast cultivation experiments in anaerobic chambers and laboratory bioreactors. Serial transfer or continuous cultivation to dilute growth factors present in anaerobically pregrown inocula, systematic inclusion of control strains and minimizing the impact of oxygen diffusion through tubing are identified as key elements in experimental design. Basic protocols are presented for anaerobic-chamber and bioreactor experiments.

Introduction

When grown under oxygen-limited conditions, the large majority of currently known yeast species at least partially ferment glucose to ethanol and carbon dioxide (1–4). The ability of these facultatively fermentative yeasts to generate ATP by substrate-level phosphorylation does not, however, imply they are all able to grow in the complete absence of oxygen. Instead, with few exceptions, even yeast species that vigorously ferment glucose under oxygen-limited conditions cannot sustain growth under strictly anaerobic conditions. This inability reflects small and often undefined oxygen requirements, which are generally attributed to a direct requirement of biosynthetic reactions for molecular oxygen and/or coupling of reactions in biosynthesis to the mitochondrial respiratory chain (3, 5–9).

The model yeast *Saccharomyces cerevisiae* is one of few yeasts capable of fast anaerobic growth on synthetic mineral media supplemented with a fermentable sugar, a defined set of B-type vitamins, a source of sterols and unsaturated fatty acids (UFAs) (10–15). The requirement of anaerobic yeast cultures for sterols and UFAs, which are frequently referred to as 'anaerobic growth factors', is due to the use of molecular oxygen in sterol biosynthesis and fatty acyl-CoA desaturation, respectively (5, 7, 16–18). Although the requirement of anaerobic *S. cerevisiae* cultures for UFAs is not absolute, anaerobic growth in the absence of UFA supplementation is slow (19). The maximum specific growth rate of *S. cerevisiae* in sterol- and UFA-supplemented, glucose-grown anaerobic batch cultures is typically only about 25% lower than in corresponding aerobic cultures (15). This fast anaerobic growth of *Saccharomyces* yeasts is exceptional among yeasts (1, 3) and essential for their large-scale industrial application in brewing, wine fermentation and bioethanol production (20–24). In industrial settings, large-scale fermentation processes are often preceded by an aerobic pre-cultivation phase or, alternatively, by a brief phase of intensive aeration, which enables cells to build intracellular storage of sterols and UFAs. In such set-ups, oxygen requirements for sterol and unsaturated-fatty-acid (UFA) synthesis can still negatively affect strain performance during prolonged anaerobic cultivation. For example, premature depletion of sterols and/or UFAs can cause 'stuck' brewing and wine fermentations (25, 26). In addition, UFAs as well as ergosterol contribute to tolerance of *S. cerevisiae* to ethanol stress (28, 29), which is an important factor for intensification of yeast-based processes for ethanol production.

The magnitude and molecular basis of the oxygen requirements of most yeasts other than *S. cerevisiae*, remain to be fully elucidated (8, 30). Analyzing and understanding oxygen requirements of facultatively fermentative 'non-conventional' yeasts can contribute to our comprehension of the roles of molecular oxygen in eukaryotic metabolism. In addition, such knowledge is essential for designing metabolic engineering strategies to enable application of non-conventional yeasts with industrially relevant traits, such as thermotolerance and inhibitor tolerance, in large-scale anaerobic processes (31–33).

Oxygen requirements for synthesis of key cellular components in *S. cerevisiae* can be estimated from the stoichiometry of biosynthetic reactions and reported data on biomass composition

Table 1 (right page): Estimated oxygen requirements for biosynthesis of ergosterol, unsaturated fatty acids and other components of yeast biomass. Unless otherwise indicated, data are based on reported biomass compositions of *S. cerevisiae* strains in anaerobic experiments with supplementation of Tween 80 (polyoxyethylene sorbitan monooleate), ergosterol and a selection of B-type vitamins. [a] Values are given as: Total UFA's (palmitoleate, $C_{16:1}$ /oleate, $C_{18:1}$). [b] Nicotinate is a precursor for oxygen-independent synthesis of nicotinamide adenine dinucleotides (NAD^+/$NADP^+$). [c] Oxygen-dependent reactions involved in thiamin and biotin biosynthesis by yeasts have not been fully resolved, and the indicated stoichiometries are estimates. For thiamine, the requirement of 3 moles of oxygen for the synthesis of the required NAD^+ moiety is incorporated. [d] Pantothenic acid is a precursor for oxygen-independent synthesis of coenzyme A. [e] *S. cerevisiae* strains do not require oxygen for pyrimidine biosynthesis. Estimated oxygen requirements refer to yeasts in which pyrimidine biosynthesis depends on a respiratory-chain-coupled dihydroorotate dehydrogenase, assuming a DNA and RNA content equal to that of *S. cerevisiae*.

(Table 1). Although biomass composition may vary among strains and depend on cultivation conditions, such an analysis readily identifies synthesis of sterols and UFAs as the major oxygen-requiring biosynthetic processes. However, their combined oxygen requirement of approximately 0.1 mmol O_2 (g biomass)$^{-1}$ (Table 1) requires an oxygen consumption rate of only 0.16 μmol (g biomass)$^{-1}$ min^{-1} to sustain a specific growth rate of 0.10 h^{-1} (doubling time of 6.9 h) that is used as a reference in many yeast chemostat studies (34–36).

In *S. cerevisiae*, biosynthesis of several key cofactors and their precursors requires oxygen (Table 1). In the *de novo* synthesis of the pyridine-nucleotide cofactors NAD^+ and $NADP^+$, the reactions catalysed by Bna2, Bna4 and Bna1 each require one mole of oxygen (6). Similarly, Fms1 catalyses an oxygen-dependent reaction in pantothenate biosynthesis (White 2001), while synthesis of thiamine requires at least three moles of oxygen because NAD^+ acts as a precursor. In addition, *S. cerevisiae* has an incompletely understood oxygen requirement for synthesis of the thiamine precursor hydroxymethylpyrimidine (38). Recent research indicates that also the first, unresolved step of biotin biosynthesis in yeasts, catalysed by Bio1 orthologs, is oxygen dependent (39). Although heme biosynthesis is oxygen dependent, heme-containing proteins in *S. cerevisiae* are strongly associated with aerobic metabolism. Since, moreover, sulfite reductase contains siroheme rather than heme (40), anaerobic cultivation of *S. cerevisiae* does not require heme supplementation.

With the exception of pyridine-nucleotide synthesis, oxygen requirements for cofactor biosynthesis are at least two orders of magnitude lower than those for sterol and UFA biosynthesis (Table 1)and, in laboratory studies with synthetic media, they are usually masked by the routine inclusion of a mix of B-type vitamins (41).

In most non-*Saccharomyces* yeasts, pyrimidine metabolism depends on a mitochondrial, respiratory-chain-coupled dihydroorotate dehydrogenase (42, 43) and therefore contributes a biosynthetic oxygen requirement similar in magnitude to that for sterol and UFA synthesis (Table 1). Pyrimidine biosynthesis in *S. cerevisiae* does not require oxygen, because its soluble cytosolic dihydroorotate dehydrogenase (Ura1) uses fumarate as electron acceptor (44, 45).

Studies on the quantification and elucidation of oxygen requirements of yeasts, as well as physiological studies on the effects of severe oxygen limitation, require the option to reduce

Biomass component *Medium component*	O_2 stoichiometry of biosynthesis (mol O_2 mol^{-1})	Content in *S. cerevisiae* biomass (µmol g $biomass^{-1}$)	*S. cerevisiae* strain and growth conditions	Reference	O_2 requirement for biosynthesis (µmol g $biomass^{-1}$)
Ergosterol *Ergosterol*	12	4.3	CBS2806, glucose-limited chemostat	(59)	30-52
		2.6	CEN.PK113-7D, glucose-limited chemostat	(56)	
		3.9	CEN.PK113-7D, batch culture	(47)	
UFA[a] *Tween 80*	1	21 (1.6 $C_{16:1}$, 19.4 $C_{18:1}$)	CBS2806, glucose-limited chemostat	(59)	21-103
		103 (44 $C_{16:1}$, 59 $C_{18:1}$)	CEN.PK113-7D, glucose-limited chemostat	(56)	
		58 (1.2 $C_{16:1}$, 57 $C_{18:1}$)	CEN.PK113-7D, batch culture	(47)	
Pyridine nucleotides *Nicotinate*[b]	3	3.8	CEN.PK113-7D, glucose limited chemostat	(60)	6.3-14
		4.5	Strain 210NG, aerobic ethanol-stat vitamin fed-batch	(61)	
		2.1-3.9	CEN.PK113-7D, glucose-limited accelerostat (NAD^+/NADH only)	(62)	
Biotin Biotin	~1[c]	0.002-0.009	Industrially produced yeast	(63)	~0.002 - 0.009
		0.002-0.008	Strain 1403-7A, aerobic uptake assay	(64)	
Coenzyme A *Pantothenate*[d]	1	0.43	Glucose limited chemostat (sum of CoA and acetyl-CoA)	(60)	~0.4
		~0.38	CEN.PK2-1C, aerobic shake flask (only acetyl-CoA)	(65)	
Thiamine *Thiamine*	~4[c]	0.025-0.22	Strain 210NG, aerobic ethanol-stat vitamin fed-batch	(61)	~0.0022-0.22
		0.0022-0.0029	Brewing strain #1007, static and shaken wort cultures	(66)	
Pyrimidines[e] *Uracil*	0.5	179	Strain 306, oxygen-limited continuous cultures	(67)	56-90
		111	Biomass equation in genome-scale model	(68)	

oxygen entry into yeast cultivation systems to extremely low levels (3, 19, 46, 47). Here, we focus on two cultivation systems that are commonly used in such anaerobic growth studies with yeasts. Anaerobic chambers, filled with a hydrogen-containing atmosphere and equipped with a Pd catalyst to remove traces of oxygen, are often used for anaerobic batch cultivation of yeasts in shake flasks or on plates (48–50). Laboratory bioreactors are popular systems to perform controlled batch, fed-batch or continuous cultivation of yeasts under anaerobic conditions. Closed systems such as anaerobic jars for cultivation on agar plates, serum flasks and Hungate tubes will not be discussed in view of their limited applicability for quantitative analysis of growth, physiology and gene expression.

Vessels and lids of bioreactors are typically made of oxygen-impermeable materials such as glass and/or stainless steel. Their area-to-volume ratio (A/V), which is sometimes mentioned as a key factor in oxygen diffusion (46, 51), is therefore not in itself a key factor in oxygen leakage. Instead, synthetic tubing, rings and seals, as well as sensors and sampling ports, are among the key potential entry points for oxygen. Since the surface area of these sensitive points (A_S) does not scale with reactor volume, A_S/V is orders of magnitude lower in large-scale industrial bioreactors than in bench-top laboratory set-ups (52). When bioreactors are operated as fed-batch or chemostat cultures, additional precautions are needed to prevent oxygen entry via the medium feed (3).

Experimental challenges involved in preventing oxygen leakage into laboratory cultures have contributed to conflicting reports on the ability of yeast species and strains to grow anaerobically (8, 48, 53–57). Although these challenges are frequently mentioned in the literature (3, 46, 58), we are not aware of publications that combine a discussion of critical points in design of anaerobic yeast cultivation experiments with laboratory protocols. Based on experience in our laboratory spanning three decades (3, 15, 19, 47), this paper aims to discuss pitfalls and challenges and share our current protocols for anaerobic cultivation of yeasts in anaerobic chambers and bioreactors.

Intracellular reserves and carry-over of anaerobic growth factors

Many yeast species accumulate lipids, including UFAs and sterols, in lipid droplets during aerobic growth on glucose (69–71). Toxic effects of intracellular free fatty acids and sterols are prevented by storage as nonpolar steryl esters (SE) and triacylglycerol (TAG) lipids (72). Lipid droplet synthesis from extracellular sources of sterols and UFAs has also been observed in heme-deficient cells (73, 74) and under anaerobic conditions (75, 76). Such intracellular stores of lipids can be mobilized to supply sources for membrane synthesis (77). Redistribution of the released UFAs and sterols over dividing yeast cells may therefore enable multiple generations upon transfer to strictly anaerobic conditions, even if sterols and UFAs are not included in growth media. This phenomenon is applied in industrial brewing, in which a brief aeration phase enables the generation of endogenous sterol and UFA reserves, which then support growth and fermentative capacity during the subsequent anaerobic fermentation process (21).

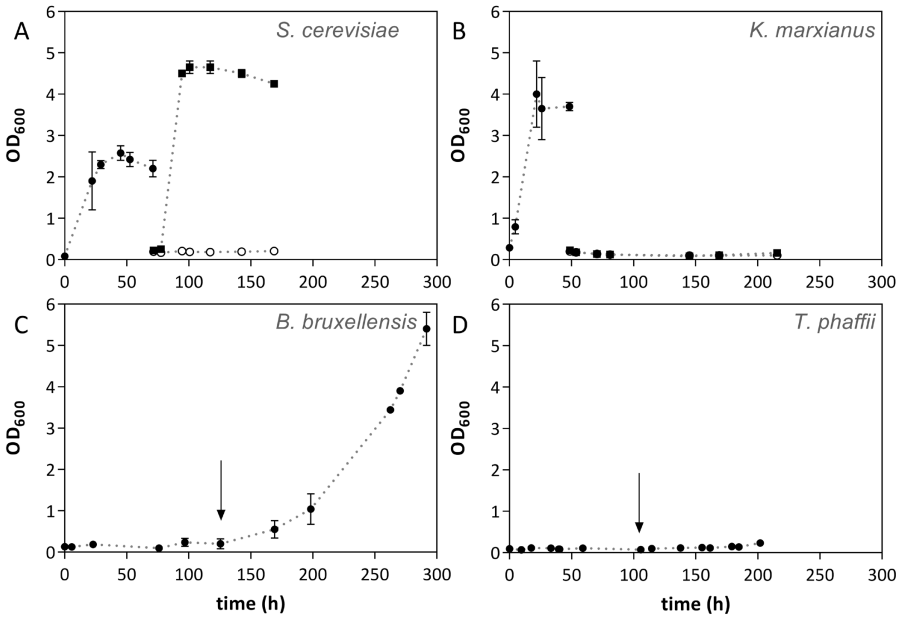

Figure 1. Representative growth profiles of four facultatively fermentative yeast species in standardized anaerobic chamber experiments. Anaerobic-chamber experiments were performed as described in Protocol 1. The yeasts *S. cerevisiae* (**A**), *K. marxianus* (**B**), B. *bruxellensis* (**C**) and *Tetrapisispora phaffii* (**D**) were grown in 50-mL shake-flasks containing 40 mL synthetic medium with urea as nitrogen source (SMU)(80), with supplements as indicated below. An anaerobic pre-culture (closed circles) without ergosterol or Tween 80, supplemented with 50 g L^{-1} glucose, was inoculated within the anaerobic chamber with an inoculum that had been grown aerobically on SMU with 20 g L^{-1} glucose until late exponential phase. When growth had occurred in this anaerobic pre-culture (**A** and **B**), and no further increase of the optical density was observed, aliquots were transferred to flasks with fresh SMU with 20 g L^{-1} glucose, either containing no anaerobic growth factors (open circles), or both Tween 80 and ergosterol (closed squares). When no growth was observed in the anaerobic pre-cultures for at least 100 h (**C** and **D**), a Tween 80 and ergosterol pulse was administered (indicated by arrows) and growth was further monitored. Data are represented as averages and mean deviation of two independent biological replicate cultures for each strain.

'Carry-over' of extracellular and/or intracellular reserves of anaerobic growth factors or their precursors may obscure biosynthetic oxygen requirements of yeasts in laboratory studies (19, 48, 57). For example, significant growth is observed upon inoculation of anaerobic shake-flask cultures of *S. cerevisiae* on glucose synthetic media without sterols or UFAs with aerobically pregrown cells (Figure 1). Increasing the glucose concentration in the anaerobic cultures can help to deplete intracellular reserves before glucose is completely consumed. In such experiments depletion of reserves is reflected by the absence of growth upon transfer of cells to a subsequent anaerobic culture on sterol- and UFA-free medium (Figure 1). The extent to which yeast strains or species grow during such a first cycle of anaerobic growth designed to deplete stores of anaerobic growth factors has no predictive value for their ability to subsequently grow in Tween 80 and sterol-supplemented medium (Figure 1). For example, *Brettanomyces* (*Dekkera) bruxellensis* cannot sustain anaerobic growth on intracellular reserves, but initiates growth upon addition of Tween 80 and ergosterol. Conversely, *Kluyveromyces marxianus*

grows anaerobically in the growth-factor depletion culture, but not upon transfer to fresh medium with or without these growth factors. This pattern may reflect additional nutritional requirements for anaerobic growth and, in *K. marxianus*, has recently been attributed to absence of a functional sterol uptake system (78). These observations underline the necessity, irrespective of the cultivation system, to include a dedicated anaerobic pre-cultivation step to deplete intracellular growth factors in batch-cultivation studies on anaerobic nutritional requirements of yeasts.

Monitoring anaerobicity of yeast cultures

Facultatively fermentative yeasts that cannot grow under strictly anaerobic conditions, typically show fast growth at dissolved-oxygen concentrations that are below the detection level of the polarographic oxygen probes that are commonly used in microbial cultures (2, 30) Measurement of dissolved-oxygen concentrations is, therefore, not a reliable way to assess culture anaerobicity. In anaerobic chambers, indicator cultures that require small amounts of oxygen for growth can be used as a control for oxygen contamination (8). For example, *Kluyveromyces lactis* cannot grow on synthetic glucose medium with Tween 80 and ergosterol under strictly anaerobic conditions, but shows fast fermentative growth in oxygen-limited cultures (30). In our experience, cultures of a wild-type *S. cerevisiae* strain on synthetic glucose medium lacking Tween 80 and ergosterol provides an even more sensitive detection of oxygen leakage (Figure 2).

In bioreactor cultures, indicator strains cannot be in the same anaerobic compartment as the strain of interest. As outlined in several studies on anaerobic yeast cultivation, it is virtually impossible to eliminate oxygen leakage in bench-top bioreactors (3, 19, 46, 47). In studies on oxygen-independent synthesis of (presumed) anaerobic growth factors by wild-type and engineered yeast strains, mutants in which relevant oxygen-dependent reactions have been eliminated therefore provide essential negative controls. For example, sterol-independent anaerobic growth of an *S. cerevisiae* strain expressing a eukaryotic squalene-tetrahymanol cyclase was confirmed by deleting *ERG1* (which encodes an essential enzyme in sterol synthesis (47)).

Anaerobic chambers

Anaerobic chambers are designed to provide gas-tight, near oxygen-free interior workspaces, in which experiments can be performed by using arm-length gloves made of materials, that are resistant to oxygen diffusion, such as butyl rubber. Materials, including flasks, chemicals and inocula can be transferred to and from the anaerobic workspace via an air lock ('pass box'). A Pd catalyst, combined with a hydrogen-containing atmosphere, is generally used to remove traces of oxygen from the workspace (79).

Air locks are designed to remove oxygen before transfer of materials to the anaerobic workspace. However, growth experiments with *S. cerevisiae* in the presence and absence of sterol- and UFA-supplementation indicate that, even when manufacturers' protocols are strictly

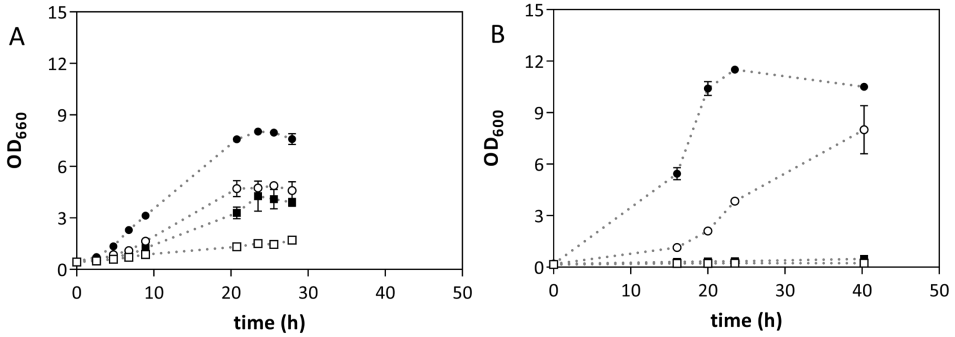

Figure 2. Use of the air lock of an anaerobic chamber as main source of oxygen contamination. Anaerobic-chamber experiments were performed as described in Protocol 1. *S. cerevisiae* CEN.PK113-7D was grown in 50-mL shake-flasks containing 40 mL synthetic medium with urea as nitrogen source (SMU)(80), with supplements as indicated below. An anaerobic pre-culture without ergosterol or Tween 80, supplemented with 50 g L^{-1} glucose, was inoculated within the anaerobic chamber with an inoculum from an exponentially growing aerobic culture on SMU with 20 g L^{-1} glucose. After the optical density increase in the pre-culture levelled off, aliquots were transferred to flasks with fresh SMU with 20 g L^{-1} glucose, supplemented with Tween 80 and ergosterol (closed circles), ergosterol only (open circles), Tween 80 only (closed squares) or SMU without these anaerobic growth factors (open squares). (**A**) Optical density measurements at 660 nm were performed outside the anaerobic chamber, requiring frequent use of the air lock. (**B**) Optical density was measured within the anaerobic chamber at a wavelength of 600 nm (different wavelength due to use of dedicated fixed-wavelength spectrophotometer in anaerobic chamber). This decreased the need to open the doors of the air lock. Data are represented as averages and mean deviation of two independent biological replicate cultures for each condition.

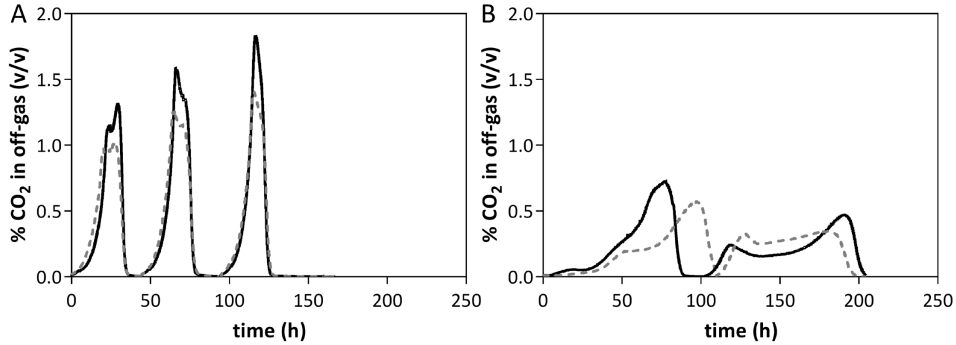

Figure 3. Nitrogen sparging versus headspace supply: impact on growth of *S. cerevisiae* in sequential batch reactors (SBR). Bioreactors were assembled according to Protocol 2. Anaerobic bioreactors were operated in SBR mode, and the CO_2 content of the off-gas was used to monitor growth of *S. cerevisiae* strain IMX585 (83). Cultures were grown on synthetic medium with urea as nitrogen source (SMU)(80). Nitrogen 6.0 HiQ gas (Linde AG, Schiedam, the Netherlands) was supplied to the reactor at 0.5 L min^{-1} either by sparging (**A**) or through the reactor headspace (**B**). When N_2 was supplied by sparging, an initial anaerobic batch culture on SMU with 25 g L^{-1} glucose, lacking ergosterol and Tween 80 was followed by three consecutive SBR cycles on SMU with 20 g L^{-1} glucose supplemented with Tween 80 but not with ergosterol. In the cultures to which N_2 was supplied to the headspace, only two consecutive batch cultures were monitored. Data shown in the figures are from two individual biological replicates for each mode of nitrogen supply, indicated by black and grey lines.

followed, use of the air lock can be a significant cause of oxygen entry (Figure 2). Reducing the void volume of the air lock by inserting inert solid objects helps to reduce oxygen entry (see Protocols). Media should be pre-incubated in the anaerobic air lock and/or in the workspace before inoculation to remove traces of oxygen. Furthermore, experiments should be designed to minimize use of the air lock and, where possible, to synchronize it with catalyst replacement. This minimization implies that routine analyses such as optical density measurements should be performed inside the anaerobic workspace rather than by regular use of the air lock for analyses on external equipment. In view of their restricted options for sampling, sample handling and long-term aseptic operation of cultures, anaerobic chambers are particularly useful for simple, parallel batch-cultivation studies in shake flasks or deep-well plates, for example to compare multiple yeast strains or cultivation conditions.

Bioreactors

Bench-top bioreactors, with working volumes ranging from 0.5 to 5 L, are widely used in quantitative microbial physiology. In contrast to the simple cultivation systems that are generally used in anaerobic chambers, they allow for simultaneous measurement and tight control of multiple process conditions, including pH, temperature, dissolved-oxygen and biomass concentration. For these reasons, laboratory-scale bioreactor cultures are also popular models for design and optimization of large-scale industrial fermentation processes. Bioreactors can be operated in batch, fed-batch or continuous mode which are defined by the medium supply- and broth withdrawal regimes (15, 47, 70, 71).

For anaerobic yeast cultivation in bioreactors, gas with a near zero oxygen content is continuously flushed through the cultures, in most cases in the form of high-purity nitrogen (N6) gas. However, the complexity of laboratory bioreactors makes them prone to permeation of oxygen through seals and tubing, oxygen contamination in gas and liquid flows and/or oxygen leakages through sampling ports, sensors, mass flow controllers and valves. Setting up (near-) anaerobic bioreactor cultures therefore requires great attention for experimental design.

Gas can be supplied to bioreactors either by sparging the stirred liquid phase or by leading gas through the reactor headspace. As bubble formation greatly increases the gas-liquid interface, sparging enables more efficient gas transfer than headspace aeration. In an ideal system, i.e., without any oxygen entry into the reactor, both modes of gas supply should yield the same results. When, instead, inadvertent oxygen entry occurs primarily via the inlet gas, e.g. due to oxygen contamination of high-purity nitrogen (N6) gas, supply through the headspace leads to a lower oxygen transfer to the broth, in which yeast cells maintain a vanishingly low oxygen concentration (2, 19, 30). Conversely, when oxygen predominantly enters via liquid flows or submerged sampling ports, sparging is preferable. Applying overpressure in the reactor may reduce entry of oxygen through small leaks connected to the headspace. However, at the same time, overpressure will facilitate transfer of any oxygen that enters the reactor as contaminations of the inlet gas flow, as it increases the partial pressure gradient for oxygen transfer to the broth. For our 2-L bioreactor set-ups, we have found that headspace aeration, combined

with a small 0.2 bar overpressure to prevent oxygen entry during sampling, results in a lower oxygen availability than sparging (Figure 3).

Polarographic oxygen electrodes do not detect minor oxygen leaks in growing cultures and can therefore be omitted from anaerobic cultivation set-ups. Probes for pH measurement are often made of porous glass and are not sealed in a gas tight manner. In our anaerobic bioreactor set-ups, we therefore generally accept the absence of active pH control (19, 47). To prevent the decrease of culture pH caused by ammonium consumption, use of urea as alternative nitrogen source is a straightforward way to avoid excessive acidification (80, 84). Alternatively, a buffering compound can be included in the medium.

Bioreactors are typically connected to a significant length of synthetic tubing to enable addition of liquids and gasses. Tubes connected to the reactor are opened and closed with clamps or valves or, alternatively, inserted in peristaltic pumps. Since permeation through tubing can be a major source of oxygen entry into reactors (3, 46), choosing the right material is crucial. In selecting tubing materials, not only oxygen permeability but also factors such as tolerance to autoclaving, resistance to tearing at steel-tubing connections, and ruggedness of tubing used in peristaltic pumps need to be considered. For a long time, our group relied on Norprene A-60-G tubing for anaerobic bioreactor set-ups (15). For research on biosynthetic oxygen requirements, we recently changed to Fluran F-5500-A for all gas and liquid tubing, as it has a much lower oxygen permeability than Norprene A-60-G (Table 2). Masterflex C-Flex Ultra has an even lower oxygen permeability to Fluran F-5500-A but in our hands was considerably less resistant to autoclaving, which caused loss of flexibility. In addition, Fluran F-5500-A could also be used in peristaltic pumps, although this requires regular recalibration of pump rates during prolonged operation.

In bioreactors, depletion of intracellular reserves of anaerobic growth factors can be achieved by automated sequential batch-reactor (SBR) cultivation. In SBR set-ups, the reactor is manually or automatically emptied upon reaching a predefined biomass density or CO_2 output, leaving a small volume of culture broth to act as inoculum after automatic refilling with fresh sterile medium. Alternatively, reactors can be operated as fully continuous (chemostat) cultures. These (semi-) continuous modes of operation require that not only the bioreactors themselves, but also the medium reservoirs are gassed with nitrogen (N5.5).

In SBR cultures, medium in the tubing between the medium reservoir and the reactor is stagnant in between empty-refill cycles. We observed that slow permeation through tubing caused entry of oxygen into this stagnant medium. To prevent entry of this oxygenated medium into the bioreactor, the 'fill' phase was preceded by pumping the first 10 mL of sterile medium into a dedicated sample bottle placed between the bioreactor and the medium pump., Subsequently, the medium pump was stopped and the mild overpressure in the reactor was used to also evacuate the tubing between the bioreactor and the sample bottle (Figure 4) (Supplementary file S1) before refilling the reactor.

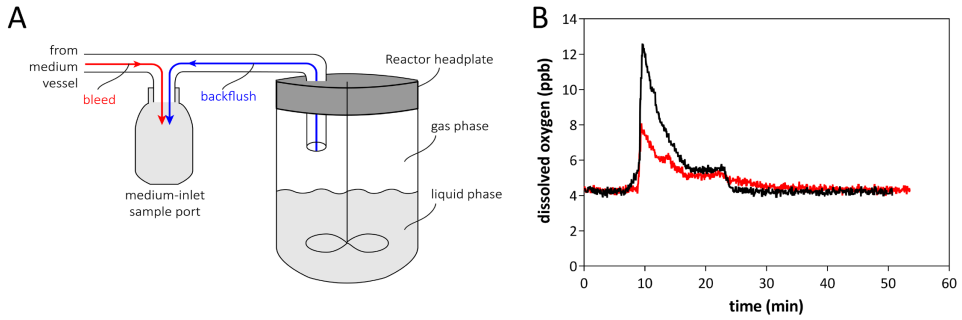

Figure 4. Effect of bleed and backflush of the medium inflow on dissolved oxygen concentration in anaerobic bioreactors. (A) Schematic representation of 'bleed' and 'backflush' to eliminate stagnant medium in the inlet tubing that had acquired oxygen by permeation through tubing during sequencing-batch reactor (SBR) experiments. The bleed operation disposes medium from tubing between medium reservoir and the sampling port. A separate 'backflush' operation uses overpressure in the reactor to push stagnant medium between reactor and sample point into the sample bottle. (B) A bioreactor assembled according to Protocol 2 was filled with tap water. Dissolved oxygen in the liquid phase was measured with a sensitive Hamilton VisiTrace Optical Trace DO 225 (Hamilton, Bonaduz GR, Switzerland) sensor equipped with an optical dissolved oxygen cap (L0-80) during an empty-refill sequence of the bioreactor with bleed, without (black line) and with the backflush operation (red line). Dissolved oxygen data were recorded with Android application ArcAir (Hamilton).

Table 2. Characteristics of tubing material for anaerobic bioreactor cultivation. Silicone Peroxide and Norprene A-60-G tubing are commonly used for liquid and gas flows in aerobic and anaerobic laboratory bioreactor cultivation experiments, respectively. Oxygen permeability is expressed in Barrer (10^{-10} cm^3_{STP}·cm·cm^{-2}·s^{-1}·$(cm\ Hg)^{-1}$); rate of diffusion, at a given pressure, through an area of material with a specified thickness).

Tubing	O_2 Permeability (Barrer)	Autoclavability
Silicone Peroxide	4715	++
Norprene A-60-G	200	+++
Fluran F-5500-F	14	+
Nylon	5.4	+
C-Flex Ultra	1.1	---

Even when a glass medium reservoir is continuously flushed with high-purity (N6) N_2, small amounts of oxygen were found to enter bioreactors with the ingoing medium flow. This problem was most pronounced in chemostat cultures, into which medium is slowly pumped from the reservoir to the reactor and, even when using tubing with a low oxygen permeability, may become contaminated with oxygen due to permeation. Visser *et al.* (1990) identified this mechanism as a major source of oxygen entry and placed a separate sterile, nitrogen-sparged, stirred bioreactor just in front of the actual chemostat bioreactor. We recently found that small, autoclavable membrane-contactor modules commonly used for gas exchange (85-87) are extremely efficient, affordable and practical devices for deoxygenating the medium feed of continuous-cultivation systems (Figure 5). When a membrane-contactor module was placed near the medium entry point of bioreactors and connected to a flow of nitrogen (N5.5), *S.*

cerevisiae chemostat cultures grown on glucose synthetic medium without the anaerobic growth factors ergosterol and Tween 80 completely washed out. This result marks a strong improvement on previous systems in which, under the same conditions, oxygen entry invariably led to reduced but significant steady-state biomass concentrations (46, 56, 78).

Conclusions and protocols

Many aspects of the anaerobic physiology of yeasts can be studied in bioreactors or shake-flasks without requiring the extreme measures discussed above. For example, energy coupling and product yields on glucose can be reliably measured in bioreactors sparged with nitrogen (N5.5) gas and equipped with Norprene tubing (88, 89). Experimental design for investigations into the small oxygen requirements of yeasts for biosynthetic reactions starts with the sober realization that complete elimination of oxygen from bench-top set-ups, and even from anaerobic chambers, is virtually impossible. Unless elaborate measures are taken, such as pure-nitrogen sparging of cultures inside an anaerobic chamber (90), the key challenge is to consistently and verifiably reduce oxygen entry to levels that allow for meaningful experiments. Whenever possible, inclusion of oxygen-dependent control strains and/or verification of conclusions by genetic modification, is therefore essential.

In our research on anaerobic cultivation of yeasts, we identified carry-over of anaerobic growth factors from aerobically grown pre-cultures, frequent use of air locks in anaerobic chambers and oxygen permeation through bioreactor tubing as key points of attention. Serial transfer was found to be an essential and reliable approach to prevent misinterpretation of results caused by intracellular reserves of anaerobic growth factors. Permeation of oxygen diffusion through tubing was found to be particularly relevant for stagnant medium in tubing during SBR cultivation and during slow supply of medium during continuous operation. Use of membrane-contactor modules is a simple and promising approach to address the latter problem.

Below, two basic protocols for anaerobic cultivation of yeasts in anaerobic chambers and bioreactors are presented, along with comments that describe or explain specific points of attention. Clearly, differences in equipment, lab infrastructure and research goals may require other or additional measures. The main goal of these protocols is therefore not to provide a generally applicable manual, but to alert colleagues to potential pitfalls and possible solutions, and thereby aid them in interpreting published studies and in setting up anaerobic yeast cultivation experiments in their laboratories.

Protocol 1 Anaerobic chamber

The following step-by-step description of growth experiments in an anaerobic chamber complements the Materials and Methods section of a publication in which we used this protocol to study UFA-independent anaerobic growth of *S. cerevisiae* (19).

1. Place an orbital shaker platform and a small spectrophotometer, both cleaned with suitable disinfectant, in the previously cleaned workspace of the anaerobic chamber.
2. Generate an anaerobic environment in the workspace of the chamber according to manufacturer's protocol, and check its anaerobicity with a test culture (see Note 1).
3. Thoroughly clean all equipment and materials that will be introduced into the workspace with a suitable disinfectant (see Note 2).
4. Place cleaned containers/flasks containing sterile pipette tips, spectrophotometer cuvettes, demineralized water, concentrated solutions of anaerobic growth factors of interest, along with shake flasks filled with relevant sterile media, calibrated pipettes, a closable waste bin and any other required materials in the air lock of the anaerobic chamber, together with a freshly activated catalyst cartridge (see Note 3). Fill up the void volume of the air lock with oxygen-impermeable materials.
5. Perform at least four vacuum/purge cycles of the air lock, including two with hydrogen-containing gas, to aid removal of oxygen by the Pd catalyst cartridge before opening the inner door of the air lock.
6. Move all required materials into the workspace of the anaerobic chamber (see Note 4).
7. Repeat steps 3-6 until all required materials are in the workspace. Make sure all materials are in the workspace 2 days before starting an experiment, to allow oxygen to be removed, especially from liquid media.
8. Grow the strains of interest and control strains (see Note 1) in an aerobic incubator to mid-exponential phase.
9. Prepare inocula for anaerobic experiments in small volumes (typically up to 2% of final culture volume, to minimize introduction of dissolved oxygen into anaerobic pre-cultures via aerobically pre-grown inocula). Concentrate samples if necessary.
10. Transfer inocula into the workspace as described in steps 3-6.
11. Inoculate (see Note 5) the anaerobic pre-cultures at the desired optical densities.
12. Monitor optical density of the anaerobic pre-cultures over time with a spectrophotometer placed in the workspace (see Note 6). Next steps depend on the growth profile (see Figure 2):
    a. Growth is observed (Figure 2A and B; the culture uses intracellular reserves of anaerobic growth factors or can grow anaerobically in the absence of the growth factor of interest). Continue with step 13.
    b. No growth is observed (Figure 2C and D; the yeast cannot use intracellular reserves,

has additional oxygen requirements, is unable to take up the growth factor of interest or the culture is no longer viable). Continue with step 14.

13. When the optical density no longer increases, transfer a small aliquot of the culture to separate flasks with anaerobic media, supplemented with various combinations of the anaerobic growth factor(s) of interest. Monitor growth as described above until the end of the experiment. Continue with step 15.

14. Add an appropriate volume of the concentrated solution of the anaerobic growth factor(s) of interest to the culture:
    a. Growth is observed (Figure 2C; the yeast grows anaerobically when provided with this anaerobic growth factor).
    b. No growth is observed (Figure 2D; the yeast has additional oxygen requirements, is unable to take up the growth factor of interest or the culture is no longer viable).

15. After terminating the anaerobic growth chamber experiment, move cultures that did not grow out of the anaerobic chamber and incubate them aerobically for a provisional indication of culture viability.

Protocol 2 Anaerobic cultivation in bioreactors

This protocol outlines key steps for anaerobic batch, sequential-batch and chemostat cultivation in bioreactors. Information on equipment and materials used in our laboratory can be found in recent publications (19, 78, 91) (and see supplementary file S2).

Bioreactor batch cultivation

Steps 1–9 describe anaerobic bioreactor batch experiments. Since sequencing-batch and chemostat experiments are usually started as batch cultures, these steps also apply for those modes of cultivation.

1. Before assembling a bioreactor set-up, thoroughly check all tubing, seals, septa and O-rings for wear or damage and replace them when necessary. Minimize and standardize length of tubing for replicate bioreactors (see Note 7).

2. Clamp all tubing, apply an 0.4 bar overpressure and monitor pressure for at least 15 min. If a pressure drop is observed, submerge the bioreactor in water to identify the leak. Prior to autoclaving the bioreactor, remove clamps to ensure gas exchange is possible.

3. Aseptically fill the autoclaved bioreactor with sterile medium to the intended working volume minus the volume of the inoculum.

4. Activate gas analysis equipment, mass flow controllers, pressure valves and equipment used for control of process parameters (e.g. temperature, stirrer speed).

5. Sparge medium in the bioreactor and set overpressure at 0.2 bar (see Note 8). Continue sparging for at least 1 h with high-purity nitrogen (N6 or above) at 0.5 L N_2 (L working volume)$^{-1}$ min^{-1} (see Note 9).

6. Release overpressure, then stop gas flow and inoculate the bioreactor.

7. Redirect inlet nitrogen stream through bioreactor headspace, stop sparging of N_2 through culture broth, and reapply 0.2 bar overpressure
8. Clamp all tubing that is not actively used during the growth experiment as close as possible to the bioreactor (see Note 10).
9. Use the 0.2 bar overpressure for aseptic sampling and take a pre-sample with each sample to discard any stagnant culture from tubing.

For further operation as a sequential batch reactor experiment, continue at step 10. For further operation as a chemostat, continue until step 11 and then proceed to step 21.

10. Assemble glass medium reservoir using oxygen impermeable tubing, O-rings and include sparging equipment (e.g. air stone).
11. Aseptically connect sterile medium reservoir and effluent to bioreactor influent and effluent, respectively, via peristaltic pumps.
12. Under the chosen process conditions, aseptically adjust level sensor to the desired working volume of the subsequent batch-cultivation cycles (see Note 11). Connect the level sensor to influent pump, to stop pumping upon contact.
13. Leave 0.2 – 1.0% of the working volume set in step 12 after the emptying phase. The ratio of residual over the working volume determines the number of generations in each batch-cultivation cycle (see Note 12).
14. Vigorously sparge medium reservoir with nitrogen (N5.5) gas for at least one hour before use and continue sparging until refilling of bioreactor is complete (see Note 9).
15. Empty the bioreactor by manually or automatically switching on the effluent pump (see Note 13).
16. Prior to refilling the bioreactor, take a sample of 10 mL from the medium inlet to discard stagnant medium from the tubing, using a sampling port close to the bioreactor lid. Use the 0.2 bar overpressure to backflush the medium in between the sampling port and the bioreactor (Figure 4A)
17. Activate the influent pump to start filling the bioreactor and switch the gas inflow from headspace to sparging to minimize oxygen entry via medium inflow.
18. Inflow of medium will automatically stop when the medium reaches the electrical level sensor. Gas inflow can be reverted to headspace (see Note 14).
19. Wait until the culture has depleted the limiting medium component, usually indicated by a decrease of the CO_2 concentration in the exhaust gas (see Note 15).
20. To initiate a subsequent empty refill cycle, repeat step 14 to 19 for another empty refill cycle.

After the anaerobic batch reactor experiment (step 1-9), connect the medium vessel (step 10-11) and continue with the next steps for an anaerobic chemostat experiment.

21. Connect the level sensor to the effluent pump, or 'pump on contact' mode (see Note 16).
22. Set the influent pump to a rate corresponding to the desired dilution rate of the continuous culture. Adjust level sensor if required. A culture is considered to be in a steady state when during at least five volume changes the culture parameters and physiology did not differ more than a predefined margin over three subsequent samples taken at least one volume change apart (see Note 17)
23. When steady-state conditions have been reached, start sampling for steady state characterization. The experiment can be stopped, the overpressure released, and the broth weighed to determine the actual working volume.

Notes Anaerobic Chamber

Note 1. As controls in anaerobic growth chamber experiments, we routinely include cultures of *S. cerevisiae* CEN.PK113-7D on glucose synthetic medium with and without Tween 80 and ergosterol. If, after a first anaerobic growth cycle to deplete intracellular reserves, sustained growth is observed on glucose synthetic medium without Tween 80 and ergosterol, this is a strong indication for the presence of oxygen in the workspace. See Figure 2B for representative results of negative control cultures in which only a very slow increase of optical density is observed.

Note 2. Because no fire/Bunsen burner can be used, strict measures are needed to reduce the risk of contamination. Surfaces and equipment must be regularly disinfected with 70% ethanol or other suitable disinfectants. Oxygen permeability of butyl rubber increases upon repeated or prolonged exposure to ethanol. Therefore, avoid spilling of ethanol on gloves while cleaning. We recommend using sterile pipette tips equipped with filters, and to clean pipettes with ethanol in between sampling of different cultures. Even when taking extensive precautions, be alert to the possibility of (cross) contamination of cultures.

Note 3. The Pd catalyst cartridge aids the removal of traces of oxygen by catalysing oxidation of hydrogen in the anaerobic gas mixture (up to 5% H_2, 5-10% CO_2 and N_2). Because this process generates water, the catalyst needs to be regularly reactivated by dry heating. Consult manufacturer's instructions for frequency of recycling, but be aware of trade-offs related to frequent use of the air lock. We limit use of the air lock to twice a week, despite the manufacturer's advice to re-activate the catalyst daily. This limited use of the air lock requires careful scheduling of entry and removal of materials.

Note 4. Frequent use of the air lock is a main cause of oxygen contamination in anaerobic chamber experiments (Figure 2). Preferably, materials introduced via the air lock should be accompanied by an activated catalyst cartridge and incubated in the air lock for at least 30 min to reduce oxygen entry.

Note 5. In the anaerobic pre-culture, intracellular reserves of anaerobic growth factors should be depleted. To prevent premature depletion of glucose we recommend using a high initial concentration (5% w/v) for this pre-culture.

Note 6. Working with gloves complicates taking notes of measurements made within the anaerobic workspace. A small voice recorder attached to the anaerobic chamber facilitates recording of culture number, time and optical density.

Notes Bioreactor

Note 7. To minimize oxygen entry, several modifications were made to our standard bioreactor setups. Silicone sealing rings in the headplate were replaced with less oxygen-permeable Viton rings (Eriks, Rotterdam, NL). Nylon tubing was used for non-aseptic parts of the gas supply and the length of the gas line from cylinder to bioreactor was minimized. When near-empty gas cylinders were replaced for full ones, the gas supply line was purged before reconnection. Where possible, plastic parts (e.g. tubing connectors, sterile cotton-wool filter canisters in gas lines) were replaced with stainless steel parts.

Note 8. Throughout cultivation, experimenters should be aware that the bioreactor is operated under overpressure, which pushes broth out of the bioreactor when the effluent line is opened. In addition, if the gas flow through the bioreactor is interrupted while sparging, broth can be pushed into the gas inlet. Install liquid traps to protect expensive mass flow controllers and be mindful to always release the overpressure from bioreactor before changing the gas flow.

Note 9. During sparging with nitrogen (N5.5 or above), the dissolved oxygen (DO) concentration asymptotically approaches zero. Depending on the volumetric mass-transfer coefficient (k_La), 90% of the oxygen is usually already removed within 1 h. Sparging time prior to the experiment can be increased, but near-complete removal of oxygen may take several hours.

Note 10. Even use of highly oxygen-impermeable tubing (e.g. Fluran F-5500-A) does not completely eliminate oxygen permeation through tubing. Clamping tubes close to the bioreactor head plate helps to minimize this mode of oxygen entry.

Note 11. Adjusting the level sensor towards the top of the turbulent liquid level, while the bioreactor is operating at its mixing, gassing and temperature setpoints, ensures that a correct and constant working volume is maintained. To prevent adjustments of the level sensor from compromising aseptic conditions, 70% ethanol can be applied to the level sensor seal.

Note 12. When biomass concentrations at the end of each batch cultivation cycle are the same, the number of generations per cycle roughly corresponds to the number of doublings of the culture volume (e.g., leaving 25 mL of broth after the emptying phase in an SBR that is subsequently refilled to a working volume of 1600 mL will result in six generations per SBR cycle).

Note 13. It is important to empty the bioreactor as fast as possible. During the majority of the emptying process the broth can still be mixed but as the liquid-gas interface drops below the impellers, the broth becomes stagnant which may lead to sedimentation of yeast to the bottom of the bioreactor. Cells at the bottom of the bioreactor are not removed via the effluent pipe as it is not located at the absolute bottom of the bioreactor, thus selecting for fast sedimenting yeast and reducing the number of generations per cycle (92).

Note 14. After re-filling of the bioreactor is initiated, we recommend to sparge the broth with high-purity nitrogen (N6) to rapidly 'strip' any remaining oxygen in the medium. After filling is completed, we redirect nitrogen supply through the headspace to minimize transfer of traces of oxygen in the nitrogen gas into the liquid phase (see Figure 3).

Note 15. When sugar is the first nutrient to be depleted, this usually coincides with a sharp decline of the CO_2 concentration in the exhaust gas. Continuous monitoring of the CO_2 concentration in the exhaust gas is then a useful trigger mechanism for initiating a new batch. If another nutrient becomes limiting first, this may not lead to an immediate decrease of the CO_2 output, and other trigger mechanisms must be employed.

Note 16. The control loop connected to the level sensor and pump operates in opposite modes in chemostat or sequential batch reactor experiment. In chemostats, contact of the broth with the level sensor is used as a signal to start the effluent pump and thereby keep the volume of the broth constant over time. In contrast, during sequential batch reactor experiments, pumping of fresh medium is terminated upon contact when the desired working volume is reached.

Note 17. Achieving a steady state is an asymptotic process, during which adjustments to the culture introduce undesired dynamics. Adjustments of the cultivation conditions should therefore be performed directly after the batch phase or early on in the chemostat experiment. Steady state is assumed when at least 5 volume changes have occurred after the last change in growth conditions and, moreover, the biomass concentration, the concentration of the growth limiting nutrient and important biomass-specific production and consumption rates differ by less than a predefined margin (e.g. 1, 2 or 5%, depending on the experimental goals) for a further two consecutive volume changes.

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Chapter 4

Engineering the thermotolerant industrial yeast *Kluyveromyces marxianus* for anaerobic growth

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Abstract

Current large-scale, anaerobic industrial processes for ethanol production from renewable carbohydrates predominantly rely on the mesophilic yeast *Saccharomyces cerevisiae*. Use of thermotolerant, facultatively fermentative yeasts such as *Kluyveromyces marxianus* could confer significant economic benefits. However, in contrast to *S. cerevisiae*, these yeasts cannot grow in the absence of oxygen. Responses of *K. marxianus* and *S. cerevisiae* to different oxygen-limitation regimes were analyzed in chemostats. Genome and transcriptome analysis, physiological responses to sterol supplementation and sterol-uptake measurements identified absence of a functional sterol-uptake mechanism as a key factor underlying the oxygen requirement of *K. marxianus*. Heterologous expression of a squalene-tetrahymanol cyclase enabled oxygen-independent synthesis of the sterol surrogate tetrahymanol in *K. marxianus*. After a brief adaptation under oxygen-limited conditions, tetrahymanol-expressing *K. marxianus* strains grew anaerobically on glucose at temperatures of up to 45 °C. These results open up new directions in the development of thermotolerant yeast strains for anaerobic industrial applications.

Introduction

In terms of product volume (87 Mton y^{-1})(1, 2), anaerobic conversion of carbohydrates into ethanol by the yeast *Saccharomyces cerevisiae* is the single largest process in industrial biotechnology. For fermentation products such as ethanol, anaerobic process conditions are required to maximize product yields and to minimize both cooling costs and complexity of bioreactors (3). While *S. cerevisiae* is applied in many large-scale processes and is readily accessible to modern genome-editing techniques (4, 5), several non-*Saccharomyces* yeasts have traits that are attractive for industrial application. In particular, the high maximum growth temperature of thermotolerant yeasts, such as *Kluyveromyces marxianus* (up to 50 °C as opposed to 39 °C for *S. cerevisiae*), could enable lower cooling costs (6–8). Moreover, it could reduce the required dosage of fungal polysaccharide hydrolases during simultaneous saccharification and fermentation (SSF) processes (9, 10). However, as yet unidentified oxygen requirements hamper implementation of *Kluyveromyces* species in large-scale anaerobic processes (11–14).

In *S. cerevisiae*, fast anaerobic growth on synthetic media requires supplementation with a source of unsaturated fatty acids (UFA), sterols, as well as several vitamins (15–18). These nutritional requirements reflect well-characterized, oxygen-dependent biosynthetic reactions. UFA synthesis involves the oxygen-dependent acyl-CoA desaturase Ole1, NAD^+ synthesis depends on the oxygenases Bna2, Bna4, and Bna1, while *de novo* synthesis of ergosterol, the main yeast sterol, even requires 12 moles of oxygen per mole.

Oxygen-dependent reactions in NAD^+ synthesis can be bypassed by nutritional supplementation of nicotinic acid, which is a standard ingredient of synthetic media for cultivation of *S. cerevisiae* (18, 19). Ergosterol and the UFA source Tween 80 (polyethoxylated sorbitan oleate) are routinely included in media for anaerobic cultivation as 'anaerobic growth factors' (AGF) (16, 18, 20). Under anaerobic conditions, *S. cerevisiae* imports exogenous sterols via the ABC transporters Aus1 and Pdr11 (21). Mechanisms for uptake and hydrolysis of Tween 80 by *S. cerevisiae* are unknown but, after its release, oleate is activated by the acyl-CoA synthetases Faa1 and Faa4 (22, 23).

Outside the whole-genome duplicated (WGD) clade of Saccharomycotina yeasts, only few yeasts (including *Candida albicans* and *Brettanomyces bruxellensis*) are capable of anaerobic growth in synthetic media supplemented with vitamins, ergosterol and Tween 80 (12, 13, 24, 25). However, most currently known yeast species readily ferment glucose to ethanol and carbon dioxide when exposed to oxygen-limited growth conditions (12, 13, 26), indicating that they do not depend on respiration for energy conservation. The inability of the large majority of facultatively fermentative yeast species to grow under strictly anaerobic conditions is therefore commonly attributed to incompletely understood oxygen requirements for biosynthetic processes (11). Several oxygen-requiring processes have been proposed, including involvement of a respiration-coupled dihydroorotate dehydrogenase in pyrimidine biosynthesis, limitations in uptake and/or metabolism of anaerobic growth factors, and redox-cofactor balancing constraints (11, 13, 27).

Quantitation, identification and elimination of oxygen requirements in non-*Saccharomyces* yeasts is hampered by the very small amounts of oxygen required for non-dissimilatory purposes. For example, preventing entry of the small amounts of oxygen required for sterol and UFA synthesis in laboratory-scale bioreactor cultures of *S. cerevisiae* requires extreme measures, such as used for anaerobic workstations, leading ultra-pure nitrogen gas over cultures, use of bioreactor tubing, seals and sensors that are resistant to oxygen diffusion and prevention of 'carry-over' of anaerobic growth factors from aerobically grown inocula (12, 28, 29). These technical challenges are likely to have contributed to reports on anaerobic growth of several non-*Saccharomyces* yeasts. In addition, the term 'anaerobic' is often applied to cultivation systems in which the contribution of respiration to energy metabolism is quantitatively insignificant relative to that of fermentation. For example, several publications that report anaerobic growth of *K. marxianus* do not detail key measures required to minimize entry of the small amounts of oxygen required for biosynthetic processes (30–32). Paradoxically, the same small oxygen requirements that, without special measures, go unnoticed in laboratory cultures can represent a real challenge in large-scale bioreactors, in which oxygen availability is limited by low surface-to-volume ratios and vigorous carbon-dioxide production.

Identification of the non-dissimilatory oxygen requirements of non-conventional yeast species is required to eliminate a key bottleneck for their application in industrial anaerobic processes and, on a fundamental level, can shed light on the roles of oxygen in eukaryotic metabolism. The goal of this study was to identify and eliminate the non-dissimilatory oxygen requirements of the facultatively fermentative, thermotolerant yeast *K. marxianus*. To this end, we analyzed and compared physiological and transcriptional responses of *K. marxianus* and *S. cerevisiae* to different oxygen- and anaerobic-growth factor limitation regimes in chemostat cultures. Based on the outcome of this comparative analysis, subsequent experiments focused on characterization and engineering of sterol metabolism and yielded *K. marxianus* strains that grew anaerobically at 45 °C.

Results

K. marxianus and *S. cerevisiae* show different physiological responses to oxygen limitation

In ideally mixed, steady-state chemostat cultures, the applied dilution rate (D, h^{-1}) equals the specific growth rate. Chemostat cultivation therefore enables analysis of the impact of cultivation conditions on microbial physiology and genome expression at a fixed specific growth rate (33). To investigate oxygen requirements of *K. marxianus*, physiological responses of strain CBS6556 to different oxygenation regimes and anaerobic growth-factor (AGF) limitation regimes (Fig. 1a) were studied in glucose-grown chemostat cultures. The chemostats were operated at a D of 0.10 h^{-1}, which is used as a reference value in many yeast physiology studies (34, 35). Physiological parameters of *K. marxianus* in these cultures were compared to those of *S. cerevisiae* CEN.PK113-7D subjected to the same cultivation regimes.

When glucose-limited, aerobic chemostat cultures with a working volume of 1.2 L were supplied with 0.5 L air·min^{-1} (21·10^4 ppm O_2 in inlet gas flow, corresponding to 258 mmol O_2 h^{-1}, Condition 1), the Crabtree-negative yeast *K. marxianus* (36) and the Crabtree-positive yeast *S. cerevisiae* (37) both exhibited a fully respiratory dissimilation of glucose, as evident from absence of ethanol production and a respiratory quotient (RQ) close to 1 (Table 1). Apparent biomass yields on glucose of both yeasts exceeded 0.5 g biomass (g glucose)$^{-1}$ and were approximately 10% higher than previously reported due to co-consumption of ethanol, which was used as solvent for the anaerobic growth factor ergosterol (36, 38).

When the oxygen content in the inlet gas was reduced to 840 ppm (2.1 mmol O_2 h^{-1}, Condition 2), both yeasts showed a much lower biomass-specific respiration rate (q_{O2}) than in the aerobic cultures (Fig. 1b), resulting in a mixed respiro-fermentative glucose metabolism. RQ values close to 50 and biomass-specific ethanol-production rates of 11.5 ± 0.6 mmol·g^{-1}·h^{-1} for *K. marxianus* and 7.5 ± 0.1 mmol·g^{-1}·h^{-1} for *S. cerevisiae* (Table 1), indicated that glucose dissimilation in Condition 2 was predominantly fermentative. In these oxygen-limited cultures, a higher concentration of glucose (20 g·L^{-1}) was included in the medium feed than in the aerobic cultures (7.5 g·L^{-1}) to compensative for the lower biomass yield of oxygen-limited, predominantly fermentative yeast cultures (14, 39). Biomass-specific rates of glycerol production which, under oxygen-limited conditions is responsible for re-oxidation of NADH generated in biosynthetic reactions (40), were approximately 2.5-fold higher ($p = 2.3 \cdot 10^{-4}$) in *K. marxianus* than in *S. cerevisiae*. Glycerol production further confirmed that, for both yeasts, the reduced oxygen-supply rate in Condition 2 constrained mitochondrial respiration (39). However, low residual glucose concentrations (Fig. 1, Table 1) indicated that sufficient oxygen was provided to meet most or all of the biosynthetic oxygen requirements of *K. marxianus*.

To explore growth under an even more stringent oxygen-limitation regime, we exploited previously documented challenges in achieving complete anaerobiosis in laboratory bioreactors (20, 28, 29). Even in chemostats sparged with pure nitrogen, which contained < 0.5 ppm oxygen (< 0.13 µmol min^{-1}, Conditions 3, 4 and 5), *S. cerevisiae* grew on synthetic medium lacking Tween 80 and ergosterol, albeit at an increased residual glucose concentration (Condition 5, Fig. 1, Table 1). In contrast, *K. marxianus* cultures sparged with pure N_2 and supplemented with both AGFs consumed only 20% of the glucose fed to the cultures (Condition 3). These severely oxygen-limited cultures showed a high residual glucose concentration of 15.9 ± 0.3 g·L^{-1} and a low but constant biomass concentration of 0.4 ± 0.0 g·L^{-1} (Fig. 1b, Table 1). This pronounced response of *K. marxianus* to extreme oxygen limitation provided an experimental context for further analyzing its unknown oxygen requirements.

S. cerevisiae can import exogenous sterols under severely oxygen-limited or anaerobic conditions (21). If the latter were also true for *K. marxianus*, omission of ergosterol from the growth medium of severely oxygen-limited cultures would increase biomass-specific oxygen requirements and lead to an even lower biomass concentration. In practice however, omission of ergosterol from the medium feed of severely oxygen-limited chemostat cultures (Condition

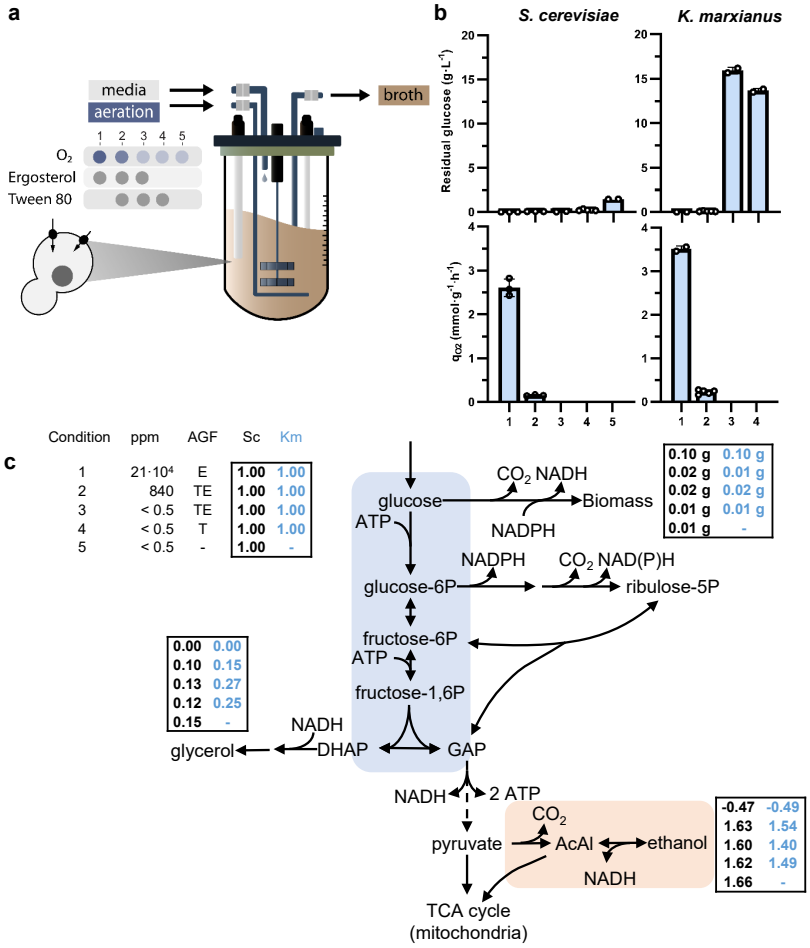

Fig. 1. Chemostat cultivation of *S. cerevisiae* CEN.PK113-7D and *K. marxianus* CBS6556 under different oxygen-supply and anaerobic-growth-factor (AGF) supplementation regimes. Chemostat cultures were grown on SMG-urea, at pH 6.0, at 30 °C and at a dilution rate of 0.10 h^{-1}. The ingoing gas flow of 500 mL·min^{-1} contained either 21·10^4 ppm O_2 (condition 1), 840 ppm O_2 (condition 2), or < 0.5 ppm O_2 (conditions 3, 4 and 5). Cultures were grown with or without supplementation ergosterol (E) and/or Tween 80 (T). **a**, Schematic representation of experimental set-up and growth conditions. Grey dots indicate cultures supplemented with ergosterol and/or Tween 80 and intensity of the blue dots represents oxygen supply to the cultures. Data for each condition were obtained from independent replicate chemostat cultures. **b**, Residual glucose concentrations and biomass-specific oxygen-consumption rates (q_{O2}) in chemostat cultures grown under the different oxygen-supply and AGF-supplementation regimes. Data represent mean and standard deviation of independent replicate chemostat cultures. **c**, Distribution of consumed glucose over biomass and products in chemostat cultures of *S. cerevisiae* (left column) and *K. marxianus* (right column), normalized to a glucose uptake rate of 1.00 mol·h^{-1}. Numbers in boxes indicate averages of measured metabolite formation rates (mol·h^{-1}) and biomass production rates (g dry weight·h^{-1}) for each condition.

Table 1. Physiology of *S. cerevisiae* CEN.PK113-7D and *K. marxianus* CBS6556 in glucose-grown chemostat cultures with different aeration and anaerobic-growth-factor (AGF) supplementation regimes. Cultures were grown at pH 6.0 on SMG-urea containing either 7.5 g·L^{-1} glucose (aerobic cultures, condition 1) or 20 g·L^{-1} glucose (oxygen-limited cultures, conditions 2-5). Data are represented as mean ± standard error of the mean (SEM) of data from independent chemostat cultures for each condition. AGFs ergosterol (E) and Tween 80 (T) were added as indicated. Cultures were aerated at 500 mL·min^{-1} with gas mixtures containing 21·10^4 ppm O_2 (condition 1), 840 ppm O_2 (condition 2) or < 0.5 ppm O_2 (conditions 3-5). Tween 80 was omitted from media used for aerobic cultivation to prevent excessive foaming. Ethanol measurements were corrected for evaporation (Supplementary Fig. 1). Positive and negative biomass-specific conversion rates (q) represent consumption and production rates, respectively. Respiratory quotient (RQ) is equal to qCO_2/qO_2.

Yeast strain	*S. cerevisiae* CEN.PK113-7D					*K. marxianus* CBS6556			
Condition	1	2	3	4	5	1	2	3	4
O_2 in inlet gas (ppm)	21·10^4	840	0.5	0.5	0.5	21·10^4	840	0.5	0.5
AGF	E	TE	TE	T	-	E	TE	TE	T
Replicates	3	3	2	5	2	2	5	2	2
Biomass (g·L^{-1})	4.22 ± 0.06	2.29 ± 0.04	1.98 ± 0.01	1.56 ± 0.03	1.12 ± 0.02	3.79 ± 0.02	1.57 ± 0.10	0.35 ± 0.02	0.50 ± 0.04
Residual glucose (g·L^{-1})	0.00 ± 0.00	0.07 ± 0.00	0.06 ± 0.02	0.23 ± 0.04	1.47 ± 0.01	0.00 ± 0.00	0.10 ± 0.02	16.0 ± 0.3	13.7 ± 0.3
Biomass-specific rates									
Specific growth rate (h^{-1})	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
$q_{glucose}$ (mmol·g^{-1}·h^{-1})	-0.95 ± 0.03	-4.59 ± 0.10	-5.25 ± 0.04	-6.77 ± 0.27	-9.06 ± 0.15	-1.05 ± 0.00	-7.46 ± 0.30	-7.30 ± 0.81	-8.53 ± 0.00
$q_{ethanol}$ (mmol·g^{-1}·h^{-1})	-0.44 ± 0.03	7.48 ± 0.10	8.40 ± 0.02	11.0 ± 0.6	15.0 ± 0.5	-0.52 ± 0.00	11.5 ± 0.44	10.3 ± 0.7	12.7 ± 0.1
$q_{succinate}$ (mmol·g^{-1}·h^{-1})	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.06 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.01 ± 0.01	0.04 ± 0.00
Stoichiometries									
RQ	1.08 ± 0.02	52.2 ± 2.4	-	-	-	1.06 ± 0.01	49.3 ± 7.5	-	-
Y glycerol/biomass (mmol·g^{-1})	0.00 ± 0.00	3.67 ± 0.05	5.58 ± 0.02	6.73 ± 0.25	11.3 ± 0.4	0.00 ± 0.00	9.51 ± 0.46	16.9 ± 0.8	18.5 ± 2.1
Y biomass/glucose (g·g^{-1})	0.57 ± 0.01	0.12 ± 0.00	0.10 ± 0.00	0.08 ± 0.00	0.06 ± 0.00	0.53 ± 0.00	0.08 ± 0.00	0.09 ± 0.00	0.09 ± 0.01
Y ethanol/glucose (g·g^{-1})	-	1.67 ± 0.06	1.63 ± 0.02	1.65 ± 0.02	1.68 ± 0.02	-	1.53 ± 0.03	1.31 ± 0.05	1.40 ± 0.02
Recoveries (out/in)									
Carbon (%)	99.9 ± 0.7	101.2 ± 3.3	100.4 ± 0.1	100.1 ± 1.3	104.0 ± 0.2	100.5 ± 0.1	91.1 ± 2.0	101.6 ± 6.5	99.7 ± 3.9
Degree of reduction (%)	98.4 ± 0.7	100.9 ± 0.8	100.1 ± 0.9	98.1 ± 0.6	100.1 ± 1.8	98.8 ± 0.1	94.5 ± 0.4	97.8 ± 6.2	99.1 ± 3.5

4, Fig. 1b, Table 1) led to a small increase of the biomass concentration and a corresponding decrease of the residual glucose concentration relative to severely oxygen-limited cultures that were supplemented with ergosterol (Condition 3). This observation suggested that, in contrast to *S. cerevisiae*, *K. marxianus* cannot replace *de novo* oxygen-dependent synthesis of sterols by uptake of exogenous ergosterol.

Transcriptional responses of *K. marxianus* to oxygen limitation involve ergosterol metabolism

To further investigate the inability of *K. marxianus* to grow anaerobically, transcriptome analyses were performed on chemostat cultures of *S. cerevisiae* and *K. marxianus*. A genome sequence of *K. marxianus* CBS6556 was only available as draft, non-annotated assembly (41). Therefore, long-read genome sequencing, assembly and *de novo* genome annotation were performed and the annotation was refined by using transcriptome assemblies (see Data availability). Complete transcriptome datasets for the tested combinations of growth conditions and yeast strains shown in Fig. 1 are available (see Data availability).

Analysis of the transcriptome data was focused on orthologous genes in *S. cerevisiae* and *K. marxianus* under a subset of the tested conditions (Fig. 2 and 3). Contrast 31, which captured a comparison between conditions 3 (< 0.5 ppm O_2 in inlet gas, severely oxygen-limited, supplemented with Tween 80 and ergosterol) and 1 (aerobic, glucose-limited), revealed large differences between *S. cerevisiae* and *K. marxianus* (Fig. 2). Of the 3 GO categories (biological processes) that were enriched among *S. cerevisiae* genes upregulated under severe oxygen limitation (Condition 3), only one (rRNA processing) was also found in *K. marxianus* with the same directionality change. Likewise, 12 GO categories that were overrepresented among genes that were down-regulated in this contrast in *S. cerevisiae* cultures, only 7 were similarly overrepresented among down-regulated genes in *K. marxianus*. A comparison of contrast 31 for all orthologous genes in *S. cerevisiae* and *K. marxianus* also revealed large differences (Fig. 2b).

Although low, residual glucose concentrations in the severely oxygen-limited (Condition 3) *S. cerevisiae* cultures were significantly higher than in aerobic, glucose-limited cultures (Condition 1, Table 1). Closer inspection of the enriched GO categories (Fig. 2b) for contrast 31 revealed GO categories that, in *S. cerevisiae*, harbor many genes whose transcription is regulated by glucose. For example, many *S. cerevisiae* genes involved in carnitine metabolism, TCA cycle and glyoxylate cycle are all transcriptionally repressed by glucose (42). The much higher glucose concentration in the severely oxygen-limited *K. marxianus* cultures (Fig. 1, Table 1) may therefore have exerted an even stronger impact on contrast 31 for this yeast. In an attempt to exclude genes for which, in *S. cerevisiae*, glucose regulation masks a transcriptional response to oxygen availability, contrast 31 was studied for a subset of *S. cerevisiae* genes that show consistent transcriptional up- or down-regulation in anaerobic chemostat cultures grown under different nutrient limitations (34). Also for this subset, transcriptional responses of the corresponding *K. marxianus* orthologs to severe oxygen limitation were

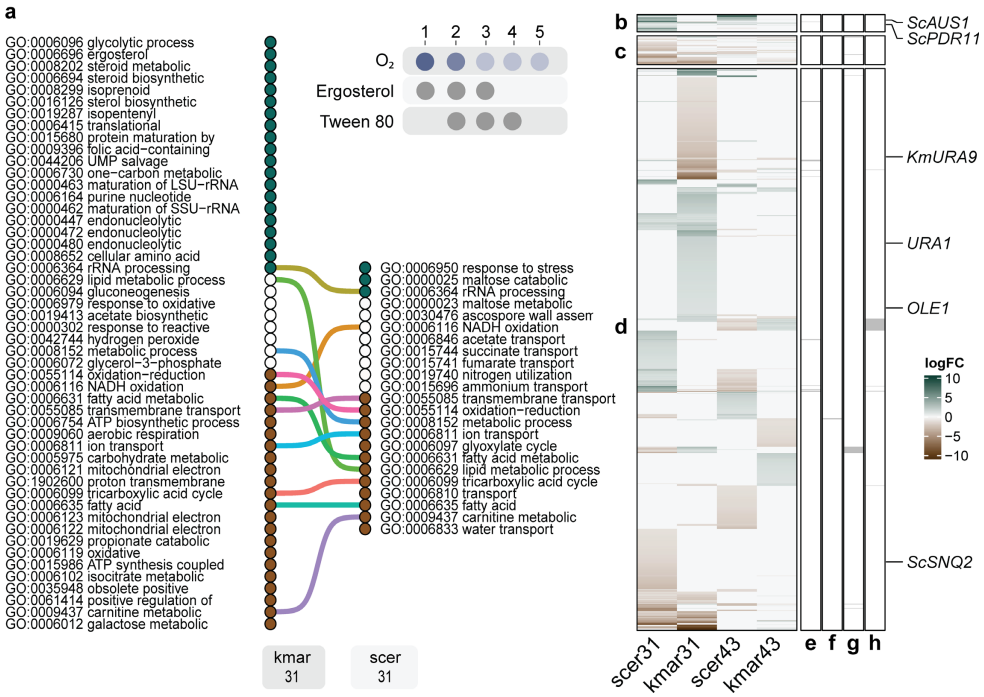

Fig. 2. Global transcriptional responses of *K. marxianus* CBS6556 and *S. cerevisiae* CEN.PK113-7D to oxygen availability in ergosterol-supplemented chemostat cultures and to ergosterol supplementation in severely oxygen-limited chemostat cultures. Chemostat cultures were grown on SMG-urea, at pH 6.0, at 30 °C and at a dilution rate of 0.10 h^{-1}. Transcriptome analysis was performed on independent replicate chemostat cultures for each condition. **a,** Comparison of enriched GO terms in contrast 31 (comparison of cultures with < 0.5 ppm O_2 in the inlet gas, supplemented with Tween 80 and ergosterol (Condition 3) with fully aerobic cultures (Condition 1)) of *S. cerevisiae* and *K. marxianus*. GO-terms, with a truncated description of the associated biological processes, are vertically ordered based on their distinct directionality calculated with Piano (46). GO-terms that showed enrichment of genes that are up-regulated (blue green) under Condition 3, GO-terms with mixed- or no-directionality in the middle (white) and GO-terms enriched for genes that were down-regulated under Condition 3 at the bottom (brown). **b**, **c**, **d:** Overview of differential transcriptional responses of orthologous *S. cerevisiae* and *K. marxianus* genes to severe oxygen limitation (contrast 31) and to omission of ergosterol from the medium of severely oxygen-limited cultures (contrast 43). Relative expression level changes are indicated as logFC; genes for which no orthologs were identified were matched with a placeholder with logFC value of 0 for visualization. **b,** *S. cerevisiae* gene set showing consistent transcriptional upregulation in anaerobic cultures grown under four different nutrient-limitation regimes relative to corresponding aerobic cultures (34) and corresponding *K. marxianus* orthologs. **c**, Gene set showing consistent transcriptional down regulation in anaerobic *S. cerevisiae* cultures grown under four different nutrient-limitation regimes (34) and their *K. marxianus* orthologs. **d**, Transcriptional responses of other *S. cerevisiae* genes and their *K. marxianus* orthologs. **e, f, g, h,** Highlighted gene-sets showing divergent expression patterns across the two yeasts. **e,** *S. cerevisiae* genes upregulated in contrast 31 but downregulated in *K. marxianus*. **f,** *S. cerevisiae* genes downregulated in contrast 31 but upregulated in *K. marxianus*. **g, h,** Similar to e and f but for contrast 43 (Supplementary Fig. 2-5).

mostly different from those in *S. cerevisiae* (Fig. 2bc). This observation suggested that not all differences observed for contrast 31 can be attributed to different glucose concentrations and that, instead, transcriptional responses to oxygen availability of *S. cerevisiae* and *K. marxianus* are substantially different.

K. marxianus harbors two dihydroorotate dehydrogenases, a cytosolic fumarate-dependent enzyme (KmUra1) and a mitochondrial quinone-dependent enzyme (KmUra9). *In vivo* activity of the latter requires oxygen because the reduced quinone is reoxidized by the mitochondrial respiratory chain (43). Consistent with the different oxygen requirements of pyrimidine synthesis via KmUra9 and ScUra1, Km*URA9* showed lower transcript levels under severely oxygen-limited conditions than in aerobic, glucose-limited cultures, while Km*URA1* was upregulated under severe oxygen limitation (Fig. 2b, contrast 31). Upregulation of Km*URA1* coincided with increased production of succinate (Table 1).

To test our hypothesis that absence of a functional sterol uptake system plays a key role in the inability of *K. marxianus* to grow anaerobically, further analysis of the transcriptome data was focused on expression of genes involved in sterol metabolism. In *S. cerevisiae,* import of exogenous sterols by Aus1 and Pdr11 can alleviate the impact of oxygen limitation on sterol biosynthesis (21). Consistent with this role of sterol uptake, sterol biosynthetic genes in *S. cerevisiae* were highly upregulated in severely oxygen-limited cultures when ergosterol in contrast 43, which captured the transcriptional response of oxygen-limited cultures upon omission of ergosterol from the growth medium (Fig. 3b, Supplementary Fig. 6). Also the mevalonate pathway for synthesis of the sterol precursor squalene, which does not require oxygen, was upregulated in this comparison, consistent with a relief of feedback regulation by ergosterol (44). In contrast to *S. cerevisiae*, *K. marxianus* showed a higher transcript levels of genes involved in sterol, isoprenoid and fatty-acid metabolism in severely oxygen-limited cultures supplemented with ergosterol and Tween 80 than in aerobic, glucose-limited cultures (Fig. 2ab, Fig. 3, contrast 31). No further increase of the expression levels of sterol-biosynthesis genes was observed in the latter yeast upon omission of ergosterol from the medium of severely oxygen-limited cultures (Supplementary Fig. 6, contrast 43). These results were consistent with our hypothesis that, in contrast to *S. cerevisiae*, *K. marxianus* is unable to import ergosterol when sterol synthesis is compromised. Moreover, co-orthology prediction with Proteinortho (45) revealed no orthologs of the *S. cerevisiae* sterol transporters Aus1 and Pdr11 in *K. marxianus*.

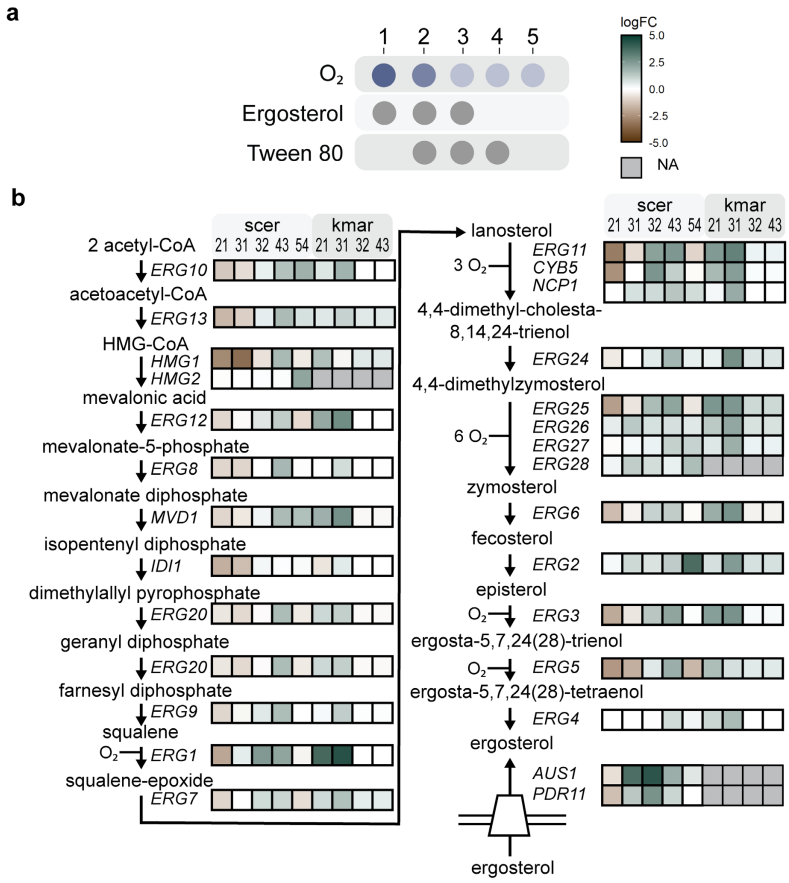

Fig. 3. Different transcriptional regulation of ergosterol-biosynthesis genes in *K. marxianus* and *S. cerevisiae*. **a**, Schematic representation of cultivation conditions (1-5, see Fig. 1) in chemostat cultures of *S. cerevisiae* CEN. PK113-7D and *K. marxianus* CBS6556. **b**, Transcriptional differences in the mevalonate- and ergosterol-pathway genes of *S. cerevisiae* and *K. marxianus*. Contrasts indicate the following comparisons: 21: oxygen-limited (840 ppm O_2 in inlet gas) and supplementation with Tween 80 and ergosterol versus aerobic, glucose limited; 31: severely oxygen limited (< 0.5 ppm O_2 in inlet gas) versus aerobic and glucose limited; 32: severely oxygen limited (< 0.5 ppm O_2 in inlet gas) versus oxygen limited (840 ppm O_2 in inlet gas), both supplemented with Tween 80 and ergosterol; 43 (severely oxygen limited (< 0.5 ppm O_2 in inlet gas), supplemented with only Tween 80 versus severely oxygen limited, supplemented with Tween 80 and ergosterol); 54: severely oxygen limited (< 0.5 ppm O_2 in inlet gas) without supplementation of Tween 80 or ergosterol versus severely oxygen limited with supplementation of only Tween 80. Lumped biochemical reactions are represented by arrows. Colors indicate up- (blue) or down-regulation (brown) with color intensity indicating log 2 fold differences with color range capped to a maximum of 4. Reactions are labeled with the corresponding gene name, *K. marxianus* genes are indicated with the name of the *S. cerevisiae* orthologs. Ergosterol uptake by *S. cerevisiae* requires additional factors beyond the membrane transporters Aus1 and Pdr11 (47). No orthologs of the sterol-transporters or Hmg2 were identified for *K. marxianus* and low read counts for Erg3, Erg9 and Erg20 precluded differential gene expression analysis across all conditions (dark grey). Enzyme abbreviations: Erg10 acetyl-CoA acetyltransferase, Erg13 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, Hmg1/Hmg2 HMG-CoA reductase, Erg12 mevalonate kinase, Erg8 phosphomevalonate kinase, Mvd1 mevalonate pyrophosphate decarboxylase, Idi1 isopentenyl diphosphate:dimethylallyl diphosphate (IPP) isomerase, Erg20 farnesyl pyrophosphate synthetase, Erg9 farnesyl-diphosphate transferase (squalene synthase), Erg7 lanosterol synthase, Erg11 lanosterol 14α-de-

(fig. 3 continued)
methylase, Cyb5 cytochrome b5 (electron donor for sterol C5-6 desaturation), Ncp1 NADP-cytochrome P450 reductase, Erg24 C-14 sterol reductase, Erg25 C-4 methyl sterol oxidase, Erg26 C-3 sterol dehydrogenase, Erg27 3-keto-sterol reductase, Erg28 endoplasmic reticulum membrane protein (may facilitate protein-protein interactions between Erg26 and Erg27, or tether these to the ER), Erg6 Δ24-sterol C-methyltransferase, Erg2 Δ24-sterol C-methyltransferase, Erg3 C-5 sterol desaturase, Erg5 C-22 sterol desaturase, Erg4 C24/28 sterol reductase, Aus1/Pdr11 plasma-membrane sterol transporter.

Absence of sterol import in *K. marxianus*

To test the hypothesis that *K. marxianus* lacks a functional sterol-uptake mechanism, uptake of fluorescent sterol derivative 25-NBD-cholesterol (NBDC) was measured by flow cytometry (48). Since *S. cerevisiae* sterol transporters are not expressed in aerobic conditions (21) and to avoid interference of sterol synthesis, NBDC uptake was analysed in anaerobic cell suspensions (Fig. 4a). Four hours after NBDC addition to cell suspensions of the reference strain *S. cerevisiae* IMX585, median single-cell fluorescence increased by 66-fold (Fig. 4bc). In contrast, the congenic sterol-transporter-deficient strain IMK809 (*aus1*Δ *pdr11*Δ) only showed a 6-fold increase of fluorescence, probably reflected detergent-resistant binding of NBDC to *S. cerevisiae* cell-wall proteins (48, 49). *K. marxianus* strains CBS6556 and NBRC1777 did not show increased fluorescence, neither after 4 h nor after 23 h of incubation with NBDC (< 2-fold, Fig. 4bc, Supplementary Fig. 7).

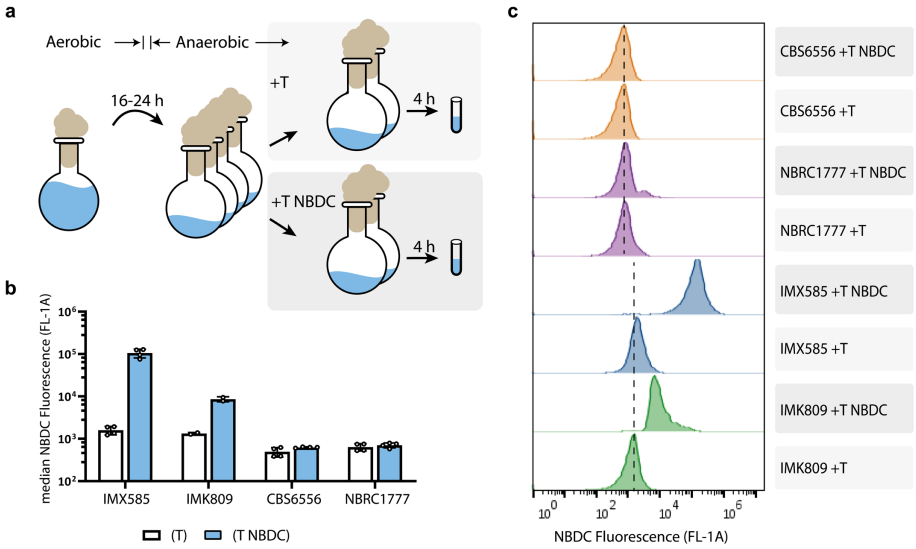

Fig. 4. Uptake of the fluorescent sterol derivative NBDC by *S. cerevisiae* and *K. marxianus* strains. **a**, Experimental approach. *S. cerevisiae* strains IMX585 (reference) and IMK809 (*aus1*Δ *pdr11*Δ), and *K. marxianus* strains NBRC1777 and CBS6556 were each anaerobically incubated in four replicate shake-flask cultures. NBDC and Tween 80 (NBDC T) were added to two cultures, while only Tween 80 (T) was added to the other two. After 4 h incubation, cells were stained with propidium iodide (PI) and analysed by flow cytometry. PI staining was used to eliminate cells with compromised membrane integrity from analysis of NBDC fluorescence. Cultivation conditions and flow cytometry gating are described in Methods and in Supplementary Fig. 8, Supplementary Data set 1 and 2. **b**, Median and pooled standard deviation of fluorescence intensity (λ_{ex} 488 nm | λ_{em} 533/30 nm, FL1-A) of PI-negative cells with variance of biological replicates after 4 h exposure to Tween 80 (white bars) or Tween 80 and NBDC (blue bars). Variance was pooled for the strains IMX585, CBS6556 and NBRC1777 by repeating the experiment. **c**, NBDC fluorescence-intensity distribution of cells in a sample from a single culture for each strain, shown as modal-scaled density function. Dashed lines represent background fluorescence of unstained cells of *S. cerevisiae* and *K. marxianus*. Fluorescence data for 23-h incubations with NBDC are shown in Supplementary Fig. 7.

Engineering *K. marxianus* for oxygen-independent growth

The mechanism of sterol uptake by *S. cerevisiae*, which in addition to a functional plasma-membrane transporter also requires cell wall proteins, has not yet been fully resolved (47, 48). Instead of expressing a heterologous sterol-import system in *K. marxianus*, we therefore explored production of tetrahymanol, which acts as a sterol surrogate in strictly anaerobic fungi (50). Expression of a squalene-tetrahymanol cyclase from *Tetrahymena thermophila* (*TtSTC1*), which catalyzes the single-step oxygen-independent conversion of squalene into tetrahymanol (Fig. 5a), was recently shown to enable sterol-independent growth of *S. cerevisiae* (51).

TtSTC1 was expressed in *K. marxianus* NBRC1777, which is more genetically amenable than strain CBS6556 (52). After 40 h of anaerobic incubation, the resulting strain contained 2.4 ± 0.4 mg·(g biomass)$^{-1}$ tetrahymanol, 0.4 ± 0.1 mg·g^{-1} ergosterol and no detectable squalene, while strain NBRC1777 contained 3.5 ± 0.1 mg·g^{-1} squalene and 3.4 ± 0.2 mg·g^{-1} ergosterol (Fig. 5b). In strictly anaerobic cultures on sterol-free medium, strain NBRC1777 grew immediately after inoculation but not after transfer to a second anaerobic culture (Fig. 5c), consistent with 'carry-over' of ergosterol from the aerobic preculture (20). The tetrahymanol-producing strain did not grow under these conditions (Fig. 5c) but showed sustained growth under severely oxygen-limited conditions that did not support growth of strain NBRC1777 (Fig. 5de). Single-cell isolates derived from these oxygen-limited cultures (IMS1111, IMS1131, IMS1132, IMS1133) showed instantaneous as well as sustained growth under strictly anaerobic conditions (Figure 5f and 5g) from an aerobic pre-culture. Tetrahymanol contents in the first, second and third cycle of anaerobic cultivation of isolate IMS1111 were 7.6 ± 0.0 mg·g^{-1}, 28.0 ± 13.0 mg·g^{-1} and 11.5 ± 0.1 mg·g^{-1}, respectively (Fig. 5b), while no ergosterol was detected.

To identify whether adaptation of the tetrahymanol-producing strain IMX2323 to anaerobic growth involved genetic changes, its genome and those of the four adapted isolates were sequenced (Supplementary Table 1). No copy number variations were detected in any of the four adapted isolates. Only strain IMS1111 showed two non-conservative mutations in coding regions: a single-nucleotide insertion in a transposon-borne gene and a stop codon at position 350 (of 496 bp) in *KmCLN3*, which encodes for a G1 cyclin (53). The apparent absence of mutations in the three other, independently adapted strains indicated that their ability to grow anaerobically reflected a non-genetic adaptation.

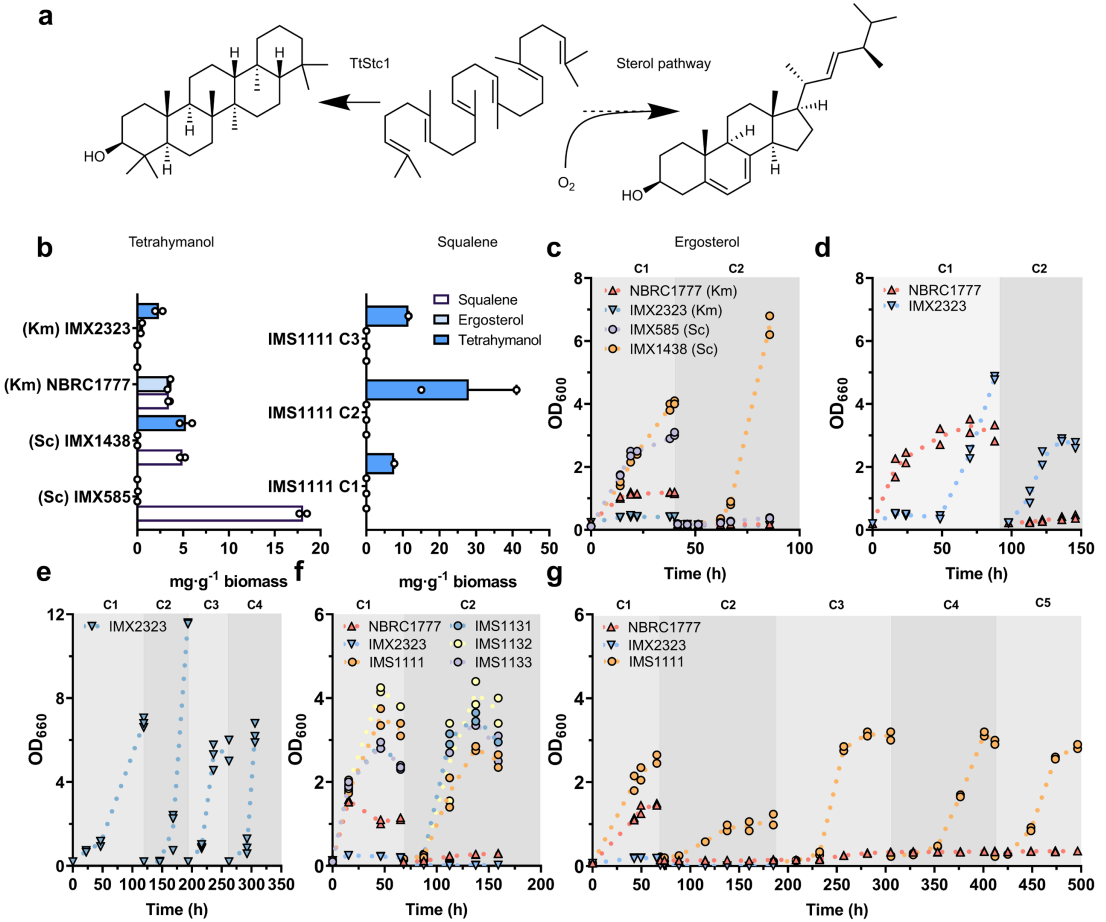

Fig. 5. (see description on the next page)

Fig. 5. (previous page) Sterol-independent anaerobic growth of *K. marxianus* and *S. cerevisiae* strains expressing *TtSTC1*. **a**, Oxygen-dependent sterol synthesis and cyclisation of squalene to tetrahymanol by TtStc1. **b**, Squalene, ergosterol, and tetrahymanol contents of yeast biomass with mean and standard error of the mean. Biomass samples were derived from the stationary phase of anaerobic cultures (**g** and Supplementary Fig. 10) inoculated from aerobically grown cultures and grown on SMG-urea and Tween 80. Left panel: *S. cerevisiae* IMX585 (reference), *S. cerevisiae* IMX1438 (*sga1*Δ::*TtSTC1*), *K. marxianus* NBRC1777 (reference) and *K. marxianus* IMX2323 (*TtSTC1*). Right panel: *K. marxianus* single-cell isolate IMS1111 (*TtSTC1*) (right panel) over 3 serial transfers (C1-C3). Growth of replicate cultures grown in strictly anaerobic (**c**, **f**, **g**) or severely oxygen-limited (**d, e**) shake-flask cultures. Aerobically grown pre-cultures were used to inoculate the first anaerobic culture on SMG-urea and Tween 80. When the optical density started to stabilize, samples were used to inoculate fresh medium. Data depicted are of each replicate culture (points) and the mean (dotted line) from independent biological duplicate cultures, serial transfers cultures are represented with C1-C5. *K. marxianus* strains NBRC1777 (wild-type, upward red triangles), IMX2323 (*TtSTC1*, cyan downward triangle), and the single-cell isolates IMS1111 (*TtSTC1*, orange circles), IMS1131 (*TtSTC1*, blue circles), IMS1132 (*TtSTC1*, yellow circles), IMS1133 (*TtSTC1*, purple circles). *S. cerevisiae* IMX585 (reference, purple circle) and IMX1438 (*TtSTC1*, orange circles). **c**, Extended data with double inoculum size is available in Supplementary Fig. 10. **d**, Extended data are available in Supplementary Fig. 9a.

Selection for faster anaerobic growth and thermotolerance

One of the attractive phenotypes of *K. marxianus* for industrial application is its high thermotolerance, with reported maximum growth temperatures of 46-52 °C (54, 55). To test if anaerobically growing tetrahymanol-producing strains retained thermotolerance, strain IMS1111 was grown in anaerobic sequential-batch-reactor (SBR) cultures with a working volume of 100 mL (Fig. 6). After an initial growth cycle at 30 °C, the growth temperature was shifted to 42 °C. Specific growth at 42 °C progressively accelerated from 0.06 h^{-1} to 0.13 h^{-1} over 17 SBR cycles (corresponding to ca. 65 generations; Fig. 6b). A subsequent temperature increase to 45 °C led to a decrease of the maximum growth rate which, after approximately 70 cycles of selective growth, stabilized at 0.08 h^{-1}. Whole-population genome sequencing of the evolved populations revealed no common mutations or chromosomal copy number variations (Supplementary Table 1). These data show that *TtSTC1*-expressing *K. marxianus* can grow anaerobically at temperatures up to at least 45 °C.

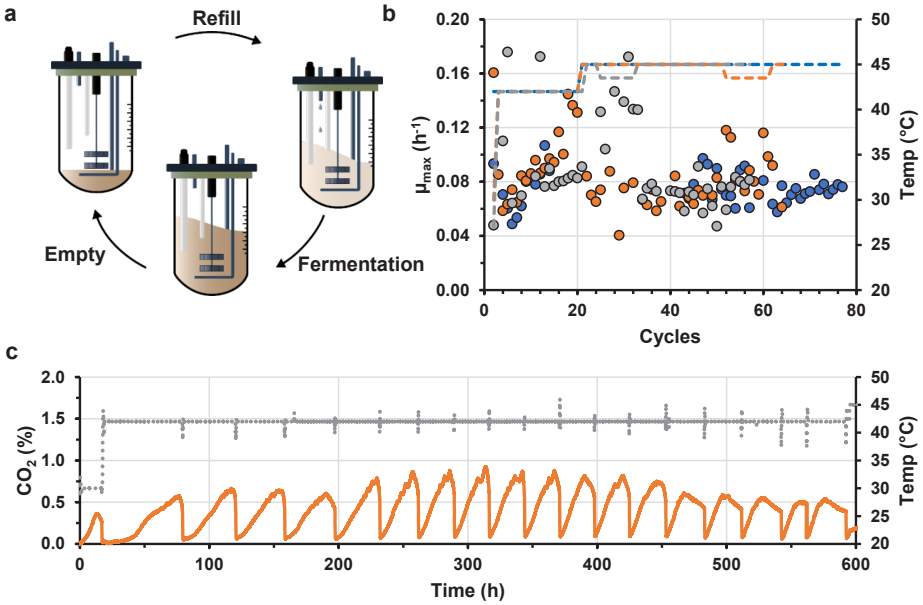

Fig. 6. Thermotolerance and anaerobic growth of tetrahymanol-producing *K. marxianus* strain. The strain IMS1111 was grown in triplicate sequential batch reactor (SBR) cultures with a working volume of 100 mL in synthetic media supplemented with 20 g·L^{-1} glucose and 420 mg·L^{-1} Tween 80 at pH 5.0. **a**, Experimental design of sequential batch fermentation with cycles at stepwise increasing temperatures to select for faster growing mutants, each cycle consisted of three phases; (i) (re)filling of the bioreactor with fresh media up to 100 mL and adjustment of temperature to a new set-point, (ii) anaerobic batch fermentation at a fixed culture temperature with continuous N_2 sparging for monitoring of CO_2 in the culture off-gas, and (iii) fast broth withdrawal leaving 7 mL (14.3 fold dilution ≈ 3.8 generations) to inoculate the next batch. **b**, Maximum specific estimated growth rate (circles) of each batch cycle for the three independent bioreactor cultivations (M3R blue, M5R orange, M6L grey). The growth rate was calculated from the CO_2 production as measured in the off-gas and should be interpreted as an estimate and in some cases could not be calculated. The culture temperature profile (dotted line) for each independent bioreactor cultivation (blue, grey, orange) consisted of a step-wise increment of the temperature at the onset of the fermentation phase in each batch cycle. **c**, Representative section of CO_2 off-gas profiles of the individual bioreactor (M5R) cultivation over time with CO_2 fraction (orange line) and culture temperature (grey dotted line), data of the entire experiment is available in Supplementary Fig. 11 (see Data availability).

Discussion

Industrial production of ethanol from carbohydrates relies on *S. cerevisiae*, due to its capacity for efficient, fast alcoholic fermentation and growth under strictly anaerobic process conditions. Many facultatively fermentative yeast species outside the Saccharomycotina WGD-clade also rapidly ferment sugars to ethanol under oxygen-limited conditions (26), but cannot grow and ferment in the complete absence of oxygen (11–13). Identifying and eliminating oxygen requirements of these yeasts is essential to unlock their industrially relevant traits for application. Here, this challenge was addressed for the thermotolerant yeast *K. marxianus*, using a systematic approach based on chemostat-based quantitative physiology, genome and transcriptome analysis, sterol-uptake assays and genetic modification. *S. cerevisiae*, which was used as a reference in this study, shows strongly different genome-wide expression profiles under aerobic and anaerobic or oxygen-limited conditions (34). Although only a small fraction of these differences were conserved in *K. marxianus* (Fig. 2), we were able to identify absence of a functional sterol import system as the critical cause for its inability to grow anaerobically. Enabling synthesis of the sterol surrogate tetrahymanol yielded strains that grew anaerobically at temperatures above the permissive temperature range of *S. cerevisiae*.

A short adaptation phase of tetrahymanol-producing *K. marxianus* strains under oxygen-limited conditions reproducibly enabled strictly anaerobic growth, which was retained after aerobic isolation of single-cell lines. However, resequencing of the genomes of thus adapted strains did not identify potential causal mutations. In this context, it may be relevant to note that, in contrast to wild-type *K. marxianus*, a non-adapted tetrahymanol-producing strain did not show 'carry-over growth' after transfer from aerobic to strictly anaerobic conditions. In addition, anaerobic cultures of adapted *TtSTC1*-expressing *K. marxianus* strains showed reduced squalene contents (Fig. 5). A similar reduction of squalene content was observed during anaerobic SBR cultivation of an *TtSTC1*-expressing *S. cerevisiae* strain (51). Based on these observations, we speculate that relative contents of tetrahymanol and squalene may be critical for the onset of anaerobic growth. Once microaerobic adaptation has established a balance between these lipids that is permissive for anaerobic growth, this might then be stabilized by native regulation networks. Genome-wide expression studies on adapted and non-adapted cultures and modification of intracellular squalene pools, may elucidate how tetrahymanol interacts with other membrane components and whether such interactions are involved in adaptation to anaerobic growth of tetrahymanol-producing *K. marxianus*.

Comparative genomic studies in Saccharomycotina yeasts have previously led to the hypothesis that sterol transporters are absent from pre-WGD yeast species (11, 56). While our observations on *K. marxianus* reinforce this hypothesis, which was hitherto not experimentally tested, they do not exclude involvement of additional oxygen-requiring reactions in other non-*Saccharomyces* yeasts. For example, pyrimidine biosynthesis is often cited as a key oxygen-requiring process in non-*Saccharomyces* yeasts, due to involvement of a respiratory-chain-linked dihydroorotate dehydrogenase (DHOD)(57, 58). *K. marxianus*, is among a small number of

yeast species that, in addition to this respiration dependent enzyme (KmUra9), also harbors a fumarate-dependent DHOD (KmUra1)(59). In *K. marxianus* the activation of this oxygen-independent KmUra1 is a crucial adaptation for anaerobic pyrimidine biosynthesis. The experimental approach followed in the present study should be applicable to resolve the role of pyrimidine biosynthesis and other oxygen-requiring reactions in additional yeast species.

Enabling *K. marxianus* to grow anaerobically represents an important step towards application of this thermotolerant yeast in large-scale anaerobic bioprocesses. However, specific growth rates of anaerobic SBR cultures of *TtSTC1*-expressing *K. marxianus* strains growing at 45 °C were only approximately 0.08 h^{-1}, which is ca. 4-fold lower than the anaerobic growth rate of *S. cerevisiae* in anaerobic, glucose-grown batch cultures grown at 30 °C (20, 60). In addition, final optical densities of anaerobic batch cultures of *TtStc1*-expressing *K. marxianus* cultures at 30 °C (OD_{600} of approximately 3, Fig. 5g) were over two-fold lower than those of Tween 80 and ergosterol-supplemented *S. cerevisiae* cultures grown under the same conditions (OD_{600} of approximately 7, (20)). A similar phenotype of tetrahymanol-producing *S. cerevisiae* was proposed to reflect an increased membrane permeability (51). Additional membrane engineering or expression of a functional sterol transport system is therefore required for further development of robust, anaerobically growing industrial strains of *K. marxianus* (61).

Methods

Yeast strains, maintenance and shake-flask cultivation . Yeast strains used and constructed in this study (Table 2). *Saccharomyces cerevisiae* CEN.PK113-7D (62, 63) (*MATa MAL2-8c SUC2*) was obtained from Dr. Peter Kötter, J.W. Goethe University, Frankfurt. *Kluyveromyces marxianus* strains CBS 6556 (ATCC 26548; NCYC 2597; NRRL Y-7571) and NBRC 1777 (IFO 1777) were obtained from the Westerdijk Fungal Biodiversity Institute (CBS-KNAW, Utrecht, The Netherlands) and the Biological Resource Center, NITE (NBRC) (Chiba, Japan), respectively. Stock cultures of *S. cerevisiae* were grown at 30 °C in an orbital shaker set at 200 rpm, in 500 mL shake flasks containing 100 mL YPD (10 $g{\cdot}L^{-1}$ Bacto yeast extract, 20 $g{\cdot}L^{-1}$ Bacto peptone, 20 $g{\cdot}L^{-1}$ glucose). For cultures of *K. marxianus*, the glucose concentration was reduced to 7.5 $g{\cdot}L^{-1}$. After addition of glycerol to early stationary-phase cultures, to a concentration of 30% (v/v), 2 mL aliquots were stored at -80 °C. Shake-flask precultures for bioreactor experiments were grown in 100 mL synthetic medium (SM) with glucose as carbon source and urea as nitrogen source (SMG-urea)(18, 64). For anaerobic cultivation, synthetic medium was supplemented with ergosterol (10 $mg{\cdot}L^{-1}$) and Tween 80 (420 $mg{\cdot}L^{-1}$) as described previously (15, 18, 20).

Expression-cassette and plasmid construction. Plasmids used in this study are described in (Table 3). To delete the sterol transporters *AUS1* and *PDR1* in *S. cerevisiae* with CRISPR-Cas9, the gRNA-expression plasmids pUDE659 ($gRNA_{AUS1}$) and pUDE663 ($gRNA_{PDR11}$) were constructed with target sequences described in (Table 4). To this end, the pROS11 plasmid-backbone was PCR amplified using Phusion HF polymerase (Thermo Scientific, Waltham, MA) with the double-binding primer 6005. PCR amplifications were performed with desalted or

PAGE-purified oligonucleotide primers (Sigma-Aldrich, St Louis, MO) according to manufacturer's instructions (see Table 5). To introduce the gRNA-encoding nucleotide sequences into gRNA-expression plasmids, a 2µm fragment was first amplified with primers 11228 and 11232 containing the specific sequence as primer overhang using pROS11 as template. PCR products were purified with genElute PCR Clean-Up Kit (Sigma-Aldrich) or Gel DNA Recovery Kit (Zymo Research, Irvine, CA). The two DNA fragments were then assembled by Gibson Assembly (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Gibson assembly reaction volumes were downscaled to 10 µL and 0.01 pmol·µL^{-1} DNA fragments at 1:1 molar ratio for 1 h at 50 °C. Chemically competent *E. coli* XL1-Blue was transformed with the Gibson assembly mix via a 5 min incubation on ice followed by a 40 s heat shock at 42 °C and 1 h recovery in non-selective LB medium. Transformants were selected on LB agar containing the appropriate antibiotic. Golden Gate assembly with the yeast tool kit (65) was performed in 20 µL reaction mixtures containing 0.75 µL BsaI HF V2 (NEB, #R3733), 2 µL DNA ligase buffer with ATP (New England Biolabs), 0.5 µL T7-ligase (NEB) with 20 fmol DNA donor fragments and MilliQ water. Before ligation at 16 °C was initiated by addition of T7 DNA ligase, an initial BsaI digestion (30 min at 37 °C) was performed. Then 30 cycles of digestion and ligation at 37 °C and 16 °C, respectively, were performed, with 5 min incubation times for each reaction. Thermocycling was terminated with a 5 min final digestion step at 60 °C.

To construct a *TtSTC1* expression vector for integration into the *K. marxianus* genome, the coding sequence of *TtSTC1* (pUD696) was PCR amplified with primer pair 16096/16097 and Golden gate assembled with the donor plasmids pGGkd015 (ori ampR), pP2 (Km*PDC1*p), pYTK053 (Sc*ADH1*t) resulting in pUDE909 (ori ampR Km*PDC1*p-*TtSTC1*-Sc*ADH1*t). For integration of *TtSTC1* cassette into the *K. marxianus* genome upstream and downstream flanking sequences (877/878 bps) of the *lac4* locus were PCR amplified with the primer pairs 14197/14198 and 14199/14200, respectively. An empty integration vector, pGGKd068, was constructed by *BsaI* golden gate cloning of pYTK047 (GFP-dropout), pYTK079 (hygB), pYTK090 (kanR), pYTK073 (ConRE'), pYTK008 (ConLS') together with the two *lac4* homologous nucleotide sequences. Plasmid assembly was verified by PCR amplification with primers 15210, 9335, 16274 and 16275 and by digestion with *BsmBI* (New England Biolabs, #R0580). The integration vector pUDI246 with the *TtSTC1* expression cassette was constructed by Gibson assembly of the PCR amplified pGGKd068 and pUDE909 with primer pairs 16274/16275 and 16272/16273, thereby adding 20 bp overlaps for assembly. For this step, the incubation time of the Gibson assembly was increased to 90 min. Plasmid assembly was verified by diagnostic PCR amplification using DreamTaq polymerase (Thermo Scientific) with primers 5941, 8442, 15216 and subsequent Illumina short-read sequencing.

Table 2. Strains used in this study. Abbreviations: *Saccharomyces cerevisiae* (Sc), *Kluyveromyces marxianus* (Km), *Tetrahymena thermophila* (Tt).

Genus	Strain	Relevant genotype	Reference
S. cerevisiae	CEN.PK113-7D	*MATa URA3 HIS3 LEU2 TRP1 MAL2-8c SUC2*	(62)
S. cerevisiae	IMX585	CEN.PK113-7D *can1*Δ::cas9-natNT2	(66)
S. cerevisiae	IMX1438	IMX585 *sga1*Δ::*TtSTC1*	(51)
S. cerevisiae	IMK802	IMX585 *aus1*Δ	This study
S. cerevisiae	IMK806	IMX585 *pdr11*Δ	This study
S. cerevisiae	IMK809	IMX585 *aus1*Δ *pdr11*Δ	This study
K. marxianus	CBS6556	*URA3 HIS3 LEU2 TRP1*	CBS-KNAW
K. marxianus	NBRC1777	*URA3 HIS3 LEU2 TRP1*	NBRC
K. marxianus	IMX2323	Km*PDC1*p-*TtSTC1*-Sc*ADH1*t-hygB	This study
K. marxianus	IMS1111	Km*PDC1*p-*TtSTC1*-Sc*ADH1*t-hygB	This study
K. marxianus	IMS1112	Km*PDC1*p-*TtSTC1*-Sc*ADH1*t-hygB	This study
K. marxianus	IMS1113	Km*PDC1*p-*TtSTC1*-Sc*ADH1*t-hygB	This study
K. marxianus	IMS1131	Km*PDC1*p-*TtSTC1*-Sc*ADH1*t-hygB	This study
K. marxianus	IMS1132	Km*PDC1*p-*TtSTC1*-Sc*ADH1*t-hygB	This study
K. marxianus	IMS1133	Km*PDC1*p-*TtSTC1*-Sc*ADH1*t-hygB	This study

Table 3. Plasmids used in this study. Restriction enzyme recognition sites are indicated in superscript. US/DS represent upstream and downstream homologous recombination sequences used for genomic integration into the *K. marxianus lac4* locus. Abbreviations: *Saccharomyces cerevisiae* (Sc), *Kluyveromyces marxianus* (Km), *Tetrahymena thermophila* (Tt).

Plasmid	Characteristics	Source
pGGkd015	ori ampR ConLS GFP ConR1	(67)
pGGKd068	ori kanR ^{NotI}*Kmlac4*$_{US}$ ^{BsmBI}ConRE′^{BsaI}sfGFP^{BsaI} ConLS′^{BsmBI} hygB *Kmlac4*$_{DS}$ ^{NotI}	This study
pP2	ori cam^{R} *KmPDC1*p	(52)
pROS11	ori amp^{R} 2µm amdSYM pSNR52-gRNA$_{CAN1}$ pRSNR52-gRNA$_{ADE2}$	(66)
pUD696	ori kanR *TtSTC1*	(51)
pUDE659	ori amp^{R} 2µm amdSYM pSNR52-gRNA$_{AUS1}$ pRSNR52-gRNA$_{AUS1}$	This study
pUDE663	ori amp^{R} 2µm amdSYM pSNR52-gRNA$_{PDR11}$ pRSNR52-gRNA$_{PDR11}$	This study
pUDE909	ori ampR Km*PDC1*p-*TtSTC1-ScADH1*t	This study
pUDI246	ori kanR ^{NotI}*Kmlac4*$_{US}$ *KmPDC1p-TtSTC1*-Sc*ADH1*t hygB *Kmlac4*$_{DS}$ ^{NotI}	This study
pYTK008	ori camR ConLS′	(65)
pYTK047	ori camR GFP dropout	(65)
pYTK053	ori camR *ScADH1*t	(65)
pYTK073	ori camR ConRE′	(65)
pYTK079	ori camR hygB	(65)

Table 4. CRISPR gRNA target sequences used in this study. gRNA target sequences are shown with PAM sequences underlined. Position in ORF indicates the base pair after which the Cas9-mediated double-strand break is introduced. AT score indicates the AT content of the 20-bp target sequence and RNA score indicates the fraction of unpaired nucleotides of the 20-bp target sequence, predicted with the complete gRNA sequence using a minimum free energy prediction by the RNAfold algorithm (68).

Locus	Target sequence (5'-3')	Position in ORF (bp)	AT score	RNA score
AUS1	CATTATTGTAAATGATTTGGTGG	320/4184	0.75	1
PDR11	ATCTTTCATATAAATAACATAGG	1627/4235	0.85	1

Table 5. Oligonucleotide primers used in this study.

Primer	Sequence (5'->3')
11228	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCCATTATTGTAAATGATTTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
11232	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCATCTTTCATATAAATAACATGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
11233	TAGTAAAGACTGCTGTAATTCATCTCTCAGTCCTTGCAGTCTGCTTTTTCTGGAATTAATTACCATTTTTAAATATATTTCTACTTTCTACTTAATAGCAATTTTAATTAATCTAATTAT
11234	ATAATTAGATTAATTAAAATTGCTATTAAGTAGAAAGTAGAAATATATTTAAAAATGGTAATTAATTCCAGAAAAAGCAGACTGCAAGGACTGAGAGATGAATTACAGCAGTCTTTACTA
11241	TAGCAAAAAAATTCACAACTAAACACGATAGAGTAAAATTAGAGAAGCAACGCCTCGCGGTCAGTGAATAGCGTTCCGTTAGAAAACATTCAAAATTACCTAATACTATTCAACAGTTCT
11242	AGAACTGTTGAATAGTATTAGGTAATTTTGAATGTTTTCTAACGGAACGCTATTCACTGACCGCGAGGCGTTGCTTCTCTAATTTTACTCTATCGTGTTTAGTTGTGAATTTTTTTGCTA
11243	TGTCACTACAGCCACAGCAG
11244	TTGGTAAGGCGCCACACTAG
11251	AGAGAAGCGCCACATAGACG
11252	TGCATATGCTACGGGTGACG
11897	CACCCAAGTATGGTGGGTAG
14148	AAGCATCGTCTCATCGGTCTCATATGTCAATTTCAAAGTACTTCACTCCCGTTGCTGAC
14149	TTATGCCGTCTCAGGTCTCAGGATTTAGTTCTGTACAGGCTTCTTC
14150	TTATGCCGTCTCAGGTCTCAAGAATTAGTTCTGTACAGGCTTCTTC
14151	AAGCATCGTCTCATCGGTCTCATATGTCTTTCACTAAAATCGCTGCCTTATTAG
14152	TTATGCCGTCTCAGGTCTCAGGATATCATAAGAGCATAGCAGCGGCACCGGCAATAG
14197	AAGCATCGTCTCATCGGTCTCACAATGAAAGTGATTGAAGAACCCTCAAAC
14198	TTATGCCGTCTCAGGTCTCAAGGGTTAAGCAATTGGATCCTACC
14199	AAGCATCGTCTCATCGGTCTCAGAGTTGCTTAATTAGCTTGTACATGGCTTTG
14200	TTATGCCGTCTCAGGTCTCATCGGGAAGGCCCATATTGAAGACG
14339	CCCAAATCATTTACAATAATGGATCATTTATC
14340	CATGTTATTTATATGAAAGATGATCATTTATC
16366	GTCCCTAGGTTCGTCATT
16367	CAAGATCAATGGTGGCTCTC

Strain construction. The lithium-acetate/polyethylene-glycol method was used for yeast transformation (69). Homologous repair (HR) DNA fragments for markerless CRISPR-Cas9-mediated gene deletions in *S. cerevisiae* were constructed by annealing two 120 bp primers, using primer pairs 11241/11242 and 11233/11234 for deletion of *PDR11* and *AUS1*, respectively. After transformation of *S. cerevisiae* IMX585 with gRNA plasmids pUDE659 and pUDE663 and double-stranded repair fragments, transformants were selected on synthetic medium with acetamide as sole nitrogen source (70). Deletion of *AUS1* and *PDR11* was confirmed by PCR amplification with primer pairs 11243/11244 and 11251/11252, respectively. Loss of gRNA plasmids was induced by cultivation of single-colony isolates on YPD, after which plasmid loss was assessed by absence of growth of single-cell isolates on synthetic medium with acetamide as nitrogen source. An *aus1Δ pdr11Δ* double-deletion strain was similarly constructed by chemical transformation of *S. cerevisiae* IMK802 with pUDE663 and repair DNA. To integrate a *TtSTC1* expression cassette into the *K. marxianus lac4* locus, *K. marxianus* NBRC1777 was transformed using the same lithium-acetate/polyethylene-glycol method with 2 µg DNA *NotI*-digested pUDI246. After centrifugation, cells were resuspended in YPD and incubated at 30 °C for 3 h. Cells were then again centrifuged, resuspended in demineralized water and plated on 200 $\mu g \cdot L^{-1}$ hygromycin B (InvivoGen, Toulouse, France) containing agar with 40 $\mu g \cdot L^{-1}$ X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Fermentas, Waltham, MA). Diagnostic PCR of colonies with primers 16366, 16367 and 11897 indicated that, rather than at the *lac4* locus, genomic integration of *TtSTC1* had occurred elsewhere in the genome. Chromosomal integration outside *lac4* was confirmed for one of the transformants, *K. marxianus* IMX2323 (*TtSTC1*), by short-read Illumina sequencing (see Data availability).

Chemostat cultivation. Chemostat cultures were grown at 30 °C in 2 L bioreactors (Applikon, Delft, the Netherlands) with a stirrer speed of 800 rpm. The dilution rate was set at 0.10 h^{-1} and a constant working volume of 1.2 L was maintained by connecting the effluent pump to a level sensor. Cultures were grown on synthetic medium with vitamins (18). Concentrated glucose solutions were autoclaved separately at 110 °C for 20 min and added to the sterile media along with sterile Pluronic 6100 PE antifoam (BASF, Ludwigshafen, Germany; final concentration 0.2 $g \cdot L^{-1}$). To compensate for a lower biomass yield of fermenting yeast cultures on glucose, media for oxygen-limited chemostat cultivation were supplemented with a higher glucose concentration (25 g L^{-1}) than fully aerobic cultures (7.5 g L^{-1}). Before autoclaving, bioreactors were tested for gas leakage by submerging them in water while applying a 0.3 bar overpressure.

Anaerobic conditions of bioreactor cultivations were maintained by continuous reactor headspace aeration with pure nitrogen gas (≤ 0.5 ppm O_2, HiQ Nitrogen 6.0, Linde AG, Schiedam, the Netherlands) at a flowrate of 500 mL N_2 min^{-1} (2.4 vvm). Gas pressure of 1.2 bar of the reactor headspace was set with a reduction valve (Tescom Europe, Hannover, Germany) and remained constant during cultivation. To prevent oxygen diffusion into the cultivation the bioreactor was equipped with Fluran tubing (14 Barrer O_2, F-5500-A, Saint-Gobain, Cour-

bevoie, France), Viton O-rings (Eriks, Alkmaar, the Netherlands), and no pH probes were mounted. The medium reservoir was deoxygenated by sparge aeration with nitrogen gas (≤ 1 ppm O_2, HiQ Nitrogen 5.0, Linde AG).

For aerobic cultivation the reactor was sparged continuously with dried air at a flowrate of 500 mL air min^{-1} (2.4 vvm). Dissolved oxygen levels were analyzed by Clark electrodes (AppliSens, Applikon) and remained above 40% during the cultivation. For micro-aerobic cultivation, nitrogen (≤ 1 ppm O_2, HiQ Nitrogen 5.0, Linde AG) and air were mixed continuously by controlling the fractions of mass flow rate of the dry gas to a total flow of 500 mL min^{-1} per bioreactor. The mixed gas was distributed to each bioreactor and analyzed separately in real-time. Continuous cultures were assumed to be in steady state when after at least 5 volumes changes, culture dry weight and the specific carbon dioxide production rates changed by less than 10%.

Cell density was routinely measured at a wavelength of 660 nm with spectrophotometer Jenway 7200 (Cole Palmer, Staffordshire, UK). Cell dry weight of the cultures were determined by filtering exactly 10 mL of culture broth over pre-dried and weighed membrane filters (0.45 µm, Thermo Fisher Scientific), which were subsequently washed with demineralized water, dried in a microwave oven (20 min, 350 W) and weighed again (71).

Metabolite analysis. For determination of substrate and extracellular metabolite concentrations, culture supernatants were obtained by centrifugation of culture samples (5 min at 13000 rpm) and analyzed by high-performance liquid chromatography (HPLC) on a Waters Alliance 2690 HPLC (Waters, MA, USA) equipped with a Bio-Rad HPX-87H ion exchange column (BioRad, Veenendaal, the Netherlands) operated at 60 °C with a mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 mL·min^{-1}. Compounds were detected by means of a dual-wavelength absorbance detector (Waters 2487) and a refractive index detector (Waters 2410) and compared to reference compounds (Sigma-Aldrich). Residual glucose concentrations in continuous cultivations were determined by HPLC analysis from rapid quenched culture samples with cold steel beads (72).

Gas analysis. The off-gas from bioreactor cultures was cooled with a condenser (2 °C) and dried with PermaPure Dryer (Inacom Instruments, Veenendaal, the Netherlands) prior to analysis of the carbon dioxide and oxygen fraction with a Rosemount NGA 2000 Analyser (Baar, Switzerland). The Rosemount gas analyzer was calibrated with defined mixtures of 1.98% O_2, 3.01% CO_2 and high-quality nitrogen gas N6 (Linde AG).

Ethanol evaporation rate. To correct for ethanol evaporation in the continuous bioreactor cultivations the ethanol evaporation rate was determined in the same experimental bioreactor set-up without the yeast. To SM glucose media with urea 400 mM of ethanol was added after which the decrease in the ethanol concentration was measured over time by periodic measurements and quantification by HPLC analysis over the course of at least 140 hours. To reflect the media composition used for the different oxygen regimes and anaerobic growth

factor supplementation, the ethanol evaporation was measured for bioreactor sparge aeration with Tween 80, bioreactor head-space aeration both with and without Tween 80. The ethanol evaporation rate was measured for each condition in triplicate.

Lipid extractions & GC analysis. For analysis of triterpene and triterpenoid cell contents biomass was harvested, washed once with demineralized water and stored as pellet at -80 °C before freeze-drying the pellets using an Alpha 1-4 LD Plus (Martin Christ, Osterode am Harz, Germany) at -60 °C and 0.05 mbar. Freeze-dried biomass was saponificated with 2.0 M NaOH (Bio-Ultra, Sigma-Aldrich) in methylation glass tubes (PYREX™ Boroslicate glass, Thermo Fisher Scientific) at 70 °C. As internal standard 5α-cholestane (Sigma-Aldrich) was added to the saponified biomass suspension. Subsequently tert-butyl-methyl-ether (tBME, Sigma-Aldrich) was added for organic phase extraction. Samples were extracted twice using tBME and dried with sodium-sulfate (Merck, Darmstadt, Germany) to remove remaining traces of water. The organic phase was either concentrated by evaporation with N_2 gas aeration or transferred directly to an injection vial (VWR International, Amsterdam, the Netherlands). The contents were measured by GC-FID using Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA) equipped with an Agilent CP9013 column (Agilent). The oven was programmed to start at 80 °C for 1 min, ramp first to 280 °C with 60 °C·min^{-1} and secondly to 320 °C with a rate of 10 °C·min^{-1} with a final temperature hold of 15 min. Spectra were compared to separate calibration lines of squalene, ergosterol, α-cholestane, cholesterol and tetrahymanol as described previously (51).

Sterol uptake assay. Sterol uptake was monitored by the uptake of fluorescently labelled 25-NBD-cholesterol (Avanti Polar Lipids, Alabaster, AL). A stock solution of 25-NBD-cholesterol (NBDC) was prepared in ethanol under an argon atmosphere and stored at -20 °C. Shake flasks with 10 mL SM glucose media were inoculated with yeast strains from a cryo-stock and cultivated aerobically at 200 rpm at 30 °C overnight. The yeast cultures were subsequently diluted to an OD_{660} of 0.2 in 400 mL SM glucose media in 500 mL shake flasks to gradually reduce the availability of oxygen and incubated overnight. Yeast cultures were transferred to fresh SM media with 40 g·L^{-1} glucose and incubated under anaerobic conditions at 30 °C at 200 rpm. After 22 hours of anaerobic incubation 4 µg·L^{-1} NBD-cholesterol with 420 mg·L^{-1} Tween 80 were pulsed to the cultures. Samples were taken and washed with PBS 5 mL·L^{-1} Tergitol NP-40 pH 7.0 (Sigma-Aldrich) twice before resuspension in PBS and subsequent analysis. Propidium Iodide (PI) (Invitrogen) was added to the sample (20 µM) and stained according to the manufacturer's instructions (73). PI intercalates with DNA in cells with a compromised cell membrane, which results in red fluorescence. Samples both unstained and stained with PI were analyzed with Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) with a 488 nm laser and fluorescence was measured with emission filter of 533/30 nm (FL1) for NBD-cholesterol and > 670 nm (FL3) for PI. Cell gating and median fluorescence of cells were determined using FlowJo (v10, BD Bioscience). Cells were gated based on forward side scatter (FSC) and side-scatter (SSC) to exclude potential artifacts or clumping cells. Within this gated population PI positive and negatively stained cells were differentiated based on the

cell fluorescence across a FL3 FL1 dimension. Flow cytometric gates were drafted for each yeast species and used for all samples. The gating strategy is given in Supplementary Fig. 8. Fluorescence of a strain was determined by a sample of cells from independent shake-flask cultures and compared to cells from identical unstained cultures of cells with the exact same chronological age. The staining experiment of the strains IMX585, CBS6556 and NBRC1777 samples was repeated twice for reproducibility, the mean and pooled variance was subsequently calculated from the biological duplicates of the two experiments. The NBDC intensity and cell counts obtained from the NBDC experiments are available for re-analysis in Supplementary Data set 1, and raw flow cytometry plots are depicted in Supplementary Data set 2.

Long read sequencing, assembly, and annotation. Cells were grown overnight in 500-mL shake flasks containing 100 mL liquid YPD medium at 30 °C in an orbital shaker at 200 rpm. After reaching stationary phase the cells were harvested for a total OD_{660} of 600 by centrifugation for 5 min at 4000 g. Genomic DNA of CBS6556 and NBRC1777 was isolated using the Qiagen genomic DNA 100/G kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. MinION genomic libraries were prepared using the 1D Genomic DNA by ligation (SQK-LSK108) for CBS6556, and the 1D native barcoding Genomic DNA (EXP-NBD103 & LSK108) for NBRC1777 according to the manufacturer's instructions with the exception of using 80% EtOH during the 'End Repair/dA-tailing module' step. Flow cell quality was tested by running the MinKNOW platform QC (Oxford Nanopore Technology, Oxford, UK). Flow cells were prepared by removing 20 µL buffer and subsequently primed with priming buffer. The DNA library was loaded dropwise into the flow cell for sequencing. The SQK-LSK108 library was sequenced on a R9 chemistry flow cell (FLO-MIN106) for 48 h. Base-calling was performed using Albacore (v2.3.1, Oxford Nanopore Technologies) for CBS6556, and for NBRC1777 with Guppy (v2.1.3, Oxford Nanopore Technologies) using dna_r9.4.1_450bps_flipflop.cfg. CBS6556 reads were assembled using Canu (v1.8)(74), and NBRC1777 reads were assembled using Flye (v2.7.1-b1673)(75). Assemblies were polished with Pilon (v1.18)(76) using Illumina data available at the Sequence Read Archive under accessions SRX3637961 and SRX3541357. Both *de novo* genome assemblies were annotated using Funannotate (v1.7.1)(77), trained and refined using *de novo* transcriptome assemblies (see below), adding functional annotation with Interproscan (v5.25-64.0)(78).

Illumina sequencing. Plasmids were sequenced on a MiniSeq (Illumina, San Diego, CA) platform. Library preparation was performed with Nextera XT DNA library preparation according to the manufacturer's instructions (Illumina). The library preparation included the MiniSeq Mid Output kit (300 cycles) and the input & final DNA was quantified with the Qubit HS dsDNA kit (Life Technologies, Thermo Fisher Scientific). Nucleotide sequences were assembled with SPAdes (79) and compared to the intended *in silico* DNA construct. For whole-genome sequencing, yeast cells were harvested from overnight cultures and DNA was isolated with the Qiagen genomic DNA 100/G kit (Qiagen) as described earlier. DNA quantity was measured with the QuBit BR dsDNA kit (Thermo Fisher Scientific). 300 bp paired-end libraries were prepared with the TruSeq DNA PCR-free library prep kit (Illumina) according

to the manufacturer's instructions. Short read whole-genome sequencing was performed on a MiSeq platform (Illumina).

RNA isolation, sequencing and transcriptome analysis. Culture broth from chemostat cultures was directly sampled into liquid nitrogen to prevent mRNA turnover. The cell cultures were stored at -80 °C and processed within 10 days after sampling. After thawing on ice, cells were harvested by centrifugation. Total RNA was extracted by a 5 min heatshock at 65 °C with a mix of isoamyl alcohol, phenol and chloroform at a ratio of 125:24:1, respectively (Invitrogen). RNA was extracted from the organic phase with Tris-HCl and subsequently precipitated by the addition of 3 M Nac-acetate and 40% (v/v) ethanol at -20 °C. Precipitated RNA was washed with ethanol, collected and after drying resuspended in RNAse free water. The quantity of total RNA was determined with a Qubit RNA BR assay kit (Thermo Fisher Scientific). RNA quality was determined by the RNA integrity number with RNA screen tape using a Tapestation (Agilent). RNA libraries were prepared with the TruSeq Stranded mRNA LT protocol (Illumina, #15031047) and subjected to paired-end sequencing (151 bp read length, NovaSeq Illumina) by Macrogen (Macrogen Europe, Amsterdam, the Netherlands).

Pooled RNAseq libraries were used to perform *de novo* transcriptome assembly using Trinity (v2.8.3)(80) which was subsequently used as evidence for both CBS6556 and NBRC1777 genome annotations. RNAseq libraries were mapped into the CBS6556 genome assembly described above, using bowtie (v1.2.1.1)(81) with parameters (-v 0 -k 10 --best -M 1) to allow no mismatches, select the best out of 10 possible alignments per read, and for reads having more than one possible alignment randomly report only one. Alignments were filtered and sorted using samtools (v1.3.1)(82). Read counts were obtained with featureCounts (v1.6.0) (83) using parameters (-B -C) to only count reads for which both pairs are aligned into the same chromosome.

Differential gene expression (DGE) analysis was performed using edgeR (v3.28.1)(84). Genes with 0 read counts in all conditions were filtered out from the analysis, same as genes with less than 10 counts per million. Counts were normalized using the trimmed mean of M values (TMM) method (85), and dispersion was estimated using generalized linear models. Differentially expressed genes were then calculated using a log ratio test adjusted with the Benjamini-Hochberg method. Absolute log2 fold-change values > 2, false discovery rate < 0.5, and P value < 0.05 were used as significance cutoffs.

Gene set analysis (GSA) based on gene ontology (GO) terms was used to get a functional interpretation of the DGE analysis. For this purpose, GO terms were first obtained for the *S. cerevisiae* CEN.PK113-7D (GCA_002571405.2) and *K. marxianus* CBS6556 genome annotations using Funannotate and Interproscan as described above. Afterwards, Funannotate compare was used to get (co)ortholog groups of genes generated with ProteinOrtho5 (45) using the following public genome annotations *S. cerevisiae* S288C (GCF_000146045.2), *K. marxianus* NBRC1777 (GCA_001417835.1), *K. marxianus* DMKU3-1042 (GCF_001417885.1), in addition to the new genome annotations generated here for *S. cerevisiae* CEN.PK113-7D, and *K.*

marxianus CBS6556 and NBRC1777. Predicted GO terms for *S. cerevisiae* CEN.PK113-7D and *K. marxianus* CBS6556 were kept and merged with those from corresponding (co)orthologs from *S. cerevisiae* S288C. Genes with term GO:0005840 (ribosome) were not considered for further analyses. GSA was then performed with Piano (v2.4.0)(46). Gene set statistics were first calculated with the Stouffer, Wilcoxon rank-sum test, and reporter methods implemented in Piano. Afterwards, consensus results were derived by p-value and rank aggregation, considered significant if absolute Fold Change values > 1. ComplexHeatmap (v2.4.3)(86) was used to draw GSA results into Fig. 2, highlighting differentially expressed genes found in a previous study (34). DGE and GSA were performed using R (v4.0.2)(87).

Anaerobic growth experiments. Anaerobic shake-flask experiments were performed in a Bactron anaerobic workstation (BACTRON300-2, Sheldon Manufacturing, Cornelius, OR) at 30 °C. The gas atmosphere consisted of 85% N_2, 10% CO_2 and 5% H_2 and was maintained anaerobic by a Pd catalyst. The catalyst was re-generated by heating till 160 °C every week and interchanged by placing it in the airlock whenever the pass-box was used. 50-mL Shake flasks were filled with 40 mL (80% volumetric) media and placed on an orbital shaker (KS 130 basic, IKA, Staufen, Germany) set at 240 rpm inside the anaerobic chamber. Sterile growth media was placed inside the anaerobic chamber 24 h prior to inoculation to ensure complete removal of traces of oxygen.

The anaerobic growth ability of the yeast strains was tested on SMG-urea with 50 $g{\cdot}L^{-1}$ glucose at pH 6.0 with Tween 80 prepared as described earlier. The growth experiments were started from aerobic pre-cultures on SMG-urea media and the anaerobic shake flasks were inoculated at an OD_{660} of 0.2 (corresponding to an OD_{600} of 0.14). In order to minimize opening the anaerobic chamber, culture growth was monitored by optical density measurements inside the chamber using an Ultrospec 10 cell density meter (Biochrom, Cambridge, UK) at a 600 nm wavelength. When the optical density of culture no longer increased or decreased new shake-flask cultures were inoculated by serial transfer at an initial OD_{600} of 0.2.

Laboratory evolution in low oxygen atmosphere. Adaptive laboratory evolution for strict anaerobic growth was performed in a Bactron anaerobic workstation (BACTRON BAC-X-2E, Sheldon Manufacturing) at 30 °C. 50-mL Shake flasks were filled with 40 mL SMG-urea with 50 $g{\cdot}L^{-1}$ glucose and including 420 $mg{\cdot}L^{-1}$ Tween 80. Subsequently the shake-flask media were inoculated with IMX2323 from glycerol cryo-stock at $OD_{660} < 0.01$ and thereafter placed inside the anaerobic chamber. Due to frequent opening of the pass-box and lack of catalyst inside the pass-box oxygen entry was more permissive. After the optical density of the cultures no longer increased, cultures were transferred to new media by 40-50x serial dilution. For IMS1111, IMS1112, IMS1113 three and for IMS1131, IMS1132, IMS1133 four serial transfers in shake-flask media were performed after which single colony isolates were made by plating on YPD agar media with hygromycin antibiotic at 30 °C aerobically. Single colony isolates were subsequently restreaked sequentially for three times on the same media before the isolates were propagated in SM glucose media and glycerol cryo stocked.

To determine if an oxygen-limited pre-culture was required for the strict anaerobic growth of IMX2323 strain a cross-validation experiment was performed. In parallel, yeast strains were cultivated in 50-mL shake-flask cultures with SMG-urea with 50 $g \cdot L^{-1}$ glucose at pH 6.0 with Tween 80 in both the Bactron anaerobic workstation (BACTRON BAC-X-2E, Sheldon Manufacturing) with low levels of oxygen-contamination, and in the Bactron anaerobic workstation (BACTRON300-2, Sheldon Manufacturing) with strict control of oxygen-contamination. After stagnation of growth was observed in the second serial transfer of the shake-flask cultures a 1.5 mL sample of each culture was taken, sealed, and used to inoculate fresh-media in the other Bactron anaerobic workstation. Simultaneously, the original culture was used to inoculate fresh media in the same Bactron anaerobic workstation, thereby resulting in 4 parallel cultures of each strain of which half were derived from the other Bactron anaerobic workstation.

Laboratory evolution in sequential batch reactors. Laboratory evolution for selection of fast growth at high temperatures was performed in 400-mL MultiFors (Infors Benelux, Velp, the Netherlands) bioreactors with a working volume of 100 mL for the *K. marxianus* strain IMS1111 on SMG 20 $g \cdot L^{-1}$ glucose media with Tween 80 in triplicate. Anaerobic conditions were created and maintained by continuous aeration of the cultures with 50 $mL \cdot min^{-1}$ (0.5 vvm) N_2 gas and continuous aeration of the media vessels with N_2 gas. The pH was set at 5.0 and maintained by the continuous addition of sterile 2 M KOH. Growth was monitored by analysis of the CO_2 in the bioreactor off-gas and a new empty-refill cycle was initiated when the batch time had at least elapsed 15 hours and the CO_2 signal dropped to 70% of the maximum reached in each batch. The dilution factor of each empty-refill cycle was 14.3-fold (100 mL working volume, 7 mL residual volume). The first batch fermentation was performed at 30 °C after which in the second batch the temperature was increased to 42 °C and maintained at for 18 consecutive sequential batches. After the 18 batch cycle at 42 °C the culture temperature was again increased to 45 °C and maintained subsequently. Growth rate was calculated based on the CO_2 production as measured by the CO_2 fraction in the culture off-gas in essence as described previously (88). In short, the CO_2 fraction in the off-gas was converted to a CO_2 evolution rate of mmol per hour and subsequently summed over time for each cycle. The corresponding cumulative CO_2 profile was transformed to natural log after which the stepwise slope of the log transformed data was calculated. Subsequently an iterative exclusion of datapoints of the stepwise slope of the log transformed cumulative CO_2 profile was performed with exclusion criteria of more than one standard deviation below the mean.

Variant calling. DNA sequencing reads were aligned into the NBRC1777 described above including an additional sequence with *TtSTC1* construct, and used to detect sequence variants using a method previously reported (89). Briefly, reads were aligned using BWA (v0.7.15-r1142-dirty)(90), alignments were processed using samtools (v1.3.1)(82) and Picard tools (v2.20.2-SNAPSHOT) (http://broadinstitute.github.io/picard), and variants were then called using the Genome Analysis Toolkit (v3.8-1-0-gf15c1c3ef)(91) HaplotypeCaller in DISCOVERY and GVCF modes. Variants were only called at sites with minimum variant confidence

normalized by unfiltered depth of variant samples (QD) of 20, read depth (DP) ≥ 5, and genotype quality (GQ) > 20, excluding a 7.1 kb region in chromosome 5 containing rDNA. Variants were annotated using the genome annotation described above, including the *TtSTC1* construct, with SnpEff (v5.0)(92) and VCFannotator (http://vcfannotator.sourceforge.net).

Statistics. Statistical test performed are given as two sided with unequal variance t-test unless specifically stated otherwise. We denote technical replicates as measurements derived from a single cell culture. Biological replicates are measurements originating from independent cell cultures. Independent experiments are two experiments identical in set-up separated by the difference in execution days. When possible variance from independent experiments with identical setup were pooled together, but independent experiments from time-course experiments (anaerobic growth studies) are reported separately. *p*-values were corrected for multiple-hypothesis testing which is specifically reported each time. No data was excluded based on the resulting data outcome.

Data availability

Data supporting the findings of this work are available within the paper and source data for all figures in this study are available at the data.4TU.nl repository with the doi:10.4121/13265552.

The raw RNA-sequencing data that supports the findings of this study are available from the Genome Expression Omnibus (GEO) website (https://www.ncbi.nlm.nih.gov/geo/) with number GSE164344.

Whole-genome sequencing data of the CBS6556, NBRC1777 and evolved strains were deposited at NCBI (https://www.ncbi.nlm.nih.gov/) under BioProject accession number PRJNA679749.

The code that was used to generate the results obtained in this study are archived in a Gitlab repository (https://gitlab.tudelft.nl/rortizmerino/kmar_anaerobic).

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Chapter 5

Re-oxidation of cytosolic NADH is a major contributor to the high oxygen requirements of the thermotolerant yeast *Ogataea parapolymorpha* in oxygen-limited cultures

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Abstract

Thermotolerance of yeasts is an attractive feature for their application in industrial ethanol production. However, incompletely understood oxygen requirements of known thermotolerant yeasts are incompatible with process requirements. To study the magnitude and molecular basis of these oxygen requirements in the facultatively fermentative, thermotolerant yeast *Ogataea parapolymorpha*, chemostat studies were performed under defined oxygen-sufficient and oxygen-limited cultivation regimes. The minimum oxygen requirements of *O. parapolymorpha* were found to be at least an order of magnitude larger than those of the thermotolerant yeast *Kluyveromyces marxianus*. This high oxygen requirement coincided with absence of glycerol formation, which plays a key role in NADH reoxidation in oxygen-limited cultures of other facultatively fermentative yeasts. Co-feeding of acetoin, whose reduction to 2,3-butanediol can reoxidize cytosolic NADH, supported a 2.5-fold higher biomass concentration in oxygen-limited cultures. The apparent inability of *O. parapolymorpha* to produce glycerol correlated with absence of orthologs of the *S. cerevisiae* genes encoding glycerol-3P phosphatase (Sc*GPP1*, Sc*GPP2*). Glycerol production was observed in aerobic batch cultures of a strain in which genes including key enzymes in mitochondrial reoxidation of NADH were deleted. However, transcriptome analysis did not identify a clear candidate for the responsible phosphatase. Expression of Sc*GPD2*, encoding NAD^+-dependent glycerol-3P dehydrogenase, and Sc*GPP1* in *O. parapolymorpha* resulted in increased glycerol production in oxygen-limited chemostats, but glycerol production rates remained substantially lower than observed in *S. cerevisiae* and *K. marxianus*. These results identify a dependency on aerobic respiration for reoxidation of NADH generated in biosynthesis as a key factor in the unexpectedly high oxygen requirements of *O. parapolymorpha*.

Importance

Thermotolerant yeasts hold great potential for anaerobic fermentation processes but their application is so far hampered by incompletely understood oxygen requirements. Based on quantitative physiological studies in oxygen-limited chemostat cultures, this study shows that the thermotolerant yeast *Ogataea parapolymorpha* has a much higher oxygen requirement than other, previously investigated facultatively fermentative yeasts. The large oxygen requirement of *O. parapolymorpha* was linked to an inability of oxygen-limited cultures to efficiently reoxidize NADH formed in biosynthetic processes by glycerol formation. These results provide a basis for reducing the oxygen requirements of *O. parapolymorpha* by targeted metabolic engineering. In addition, this study shows that diversity of oxygen requirements should be taken into account in selecting yeast species for application in anaerobic or oxygen-limited industrial processes.

Introduction

Microbial biotechnology offers promising options for replacing petrochemically produced chemicals with sustainable bio-based alternatives (1, 2). For example, microbial production of ethanol as a renewable transport fuel, based on plant carbohydrates as renewable feedstocks, is already applied on a large scale. The 2020 annual production volume of 87 Mton ethanol (3) is almost exclusively produced with the yeast *Saccharomyces cerevisiae* (4, 5). In well-established 'first-generation' ethanol processes, this yeast converts readily fermentable sugars, derived from corn starch or sugar cane, at high rates, titers and yields (6). The use of engineered pentose-fermenting *S. cerevisiae* strains for conversion of pentose-containing lignocellulosic hydrolysates, generated from agricultural residues such as corn stover and sugar cane bagasse, is currently explored at industrial scale (4).

Economic viability of the microbial production of ethanol requires minimum processing costs and near-theoretical product yields on carbohydrate feedstocks, which can only be achieved in the absence of respiration (4, 5). In practice, industrial ethanol fermentation is performed in large tanks that readily become and remain anaerobic as a consequence of vigorous carbon dioxide production by fermenting yeast cells. The popularity of *S. cerevisiae* for application in these processes is related to its high fermentation rates under anaerobic conditions, its high innate ethanol tolerance, its tolerance to low pH and increasingly also to its amenability to modern genome-editing techniques (7–10).

S. cerevisiae is a mesophile that grows optimally at temperatures of approximately 35 °C (11). Ethanol fermentation at higher temperatures could reduce cooling costs of large-scale bioreactors and potentially enable higher rates of alcoholic fermentation. An immediate advantage of yeast thermotolerance is related to the integration, in a single unit operation, of enzyme-catalyzed hydrolysis of plant polysaccharides and fermentation of the released monomeric sugars (simultaneous saccharification and fermentation, SSF (12)). This concept could simplify processing and prevent feedback inhibition of polysaccharide hydrolysis by released monosaccharides (12, 13). Use of thermotolerant yeasts could reduce the required dose of fungal hydrolases in SSF processes and thereby further improve process economy. This potential advantage is especially relevant for second-generation bioethanol production, in which physically and/or chemically pre-treated lignocellulosic plant biomass is hydrolyzed to monomeric sugars by hydrolases with temperature optima that are typically between 50 and 80 °C (14, 15). Application of such SSF processes is currently constrained by the limited temperature range of *S. cerevisiae*.

Since supra-optimal temperatures potentially affect all proteins in a cell (16), substantial extension of the temperature range of *S. cerevisiae* by metabolic engineering may prove to be an elusive target. Indeed, elegant adaptive laboratory evolution and metabolic engineering studies aimed at improving the thermotolerance of *S. cerevisiae*, e.g. by engineering its sterol composition, have only enabled modest improvements of its maximum growth temperature (17–19). Exploration of naturally thermotolerant, facultatively fermentative yeasts such as

Ogataea sp. (*Hansenula* sp.)(20) and *Kluyveromyces marxianus*, whose temperature maxima can reach 50 °C (21, 22), appears to offer an attractive alternative approach. As is observed for the large majority of yeast species, these thermotolerant yeasts readily ferment sugars to ethanol under oxygen-limited conditions (23–25). However, with few exceptions, Saccharomycotina yeasts such as *O. parapolymorpha* and *K. marxianus*, whose evolutionary history did not involve the whole-genome duplication event (WGD) that shaped the genomes of *S. cerevisiae* and closely related species (26), cannot grow under strictly anaerobic conditions (23, 27).

Even *S. cerevisiae*, which can grow fast in the absence of oxygen, requires supplementation of anaerobic growth media with sources of sterols and unsaturated fatty acids (UFAs) to do so (28, 29). Supplementation of only a source of sterols leads to a drastic lower growth rate (30). The oxygen requirement of yeasts for UFA synthesis is due to involvement of the cytochrome-b5 Δ9-desaturase Ole1 (31). Similarly, involvement of multiple mono-oxygenases in sterol biosynthesis explains the sterol requirement for anaerobic growth (reviewed in (32)). In practice, anaerobic laboratory cultures of *S. cerevisiae* are routinely supplemented with ergosterol and Tween 80, an oleic acid ester that serves as UFA source (29). In laboratory studies, additional oxygen requirements of *S. cerevisiae* for synthesis of biotin, nicotinate, pantothenate and thiamine (33–35) are generally masked by their routine inclusion in synthetic media for cultivation of yeasts (34).

In most non-*Saccharomyces* yeasts, pyrimidine synthesis depends on a mitochondrial, respiration-coupled Class-II dihydro-orotate dehydrogenase (DHODase, Ura9). In contrast, *S. cerevisiae* only harbors a cytosolic Class-I-A DHODase (Ura1), which couples an oxygen- and respiration-independent dihydro-orotate oxidation to fumarate reduction (26, 36–38). *Kluyveromyces* sp. represent an evolutionary intermediate and contain both Ura1 and Ura9 orthologs, whose expression is regulated in response to oxygen availability (39). Development of metabolic engineering strategies for eliminating oxygen requirements of thermotolerant non-*Saccharomyces* yeasts requires elucidation of the underlying oxygen- and/or respiration-dependent biochemical reactions.

The goal of the present study was to investigate oxygen requirements of the thermotolerant yeast *O. parapolymorpha*. To this end, we first characterized its physiological and transcriptional responses to oxygen limitation in chemostat cultures and compared these to previously reported data from our group for *K. marxianus* and *S. cerevisiae* (39). Co-feeding of acetoin was tested to explore a possible impact of cytosolic NADH oxidation on the observed physiology of *O. parapolymorpha* in oxygen-limited cultures. Subsequently, we analyzed the genome of *O. parapolymorpha* for the presence or absence of orthologs of genes that were implicated in the (in)ability of other yeasts to grow anaerobically and, in particular, in the production of glycerol as 'redox sink' for reoxidation of NADH formed in biosynthetic reactions. Glycerol metabolism in *O. parapolymorpha* was further explored by studying growth of a mutant strain in which key genes involved in mitochondrial respiratory-chain-linked NADH oxidation

were deleted. Based on the results of these analyses, we explored metabolic engineering of redox metabolism in *O. parapolymorpha* by expressing *S. cerevisiae* genes involved in glycerol production.

Results

Oxygen requirements of *O. parapolymorpha* in oxygen-limited chemostat cultures.

Chemostat cultures offer the opportunity to study the impact of different process parameters at a fixed dilution rate, which in ideally mixed, steady-state chemostat cultures equals the specific growth rate. To quantitatively assess oxygen requirements of *O. parapolymorpha*, physiological responses of the wild-type strain CBS11895 (DL-1)(40) were studied under two aeration regimes in chemostat cultures that were grown on glucose at a dilution rate of 0.10 h^{-1} (Table 1, Fig. 2 and 3). Physiological parameters of *O. parapolymorpha* were compared to results obtained under the same cultivation conditions in a recent study of our group on *S. cerevisiae* CEN.PK113-7D and *K. marxianus* CBS6556 (39).

In fully aerobic chemostat cultures sparged with air (0.5 L min^{-1}), growth of *O. parapolymorpha* was glucose limited and sugar dissimilation occurred exclusively via respiration, as indicated by a respiratory quotient (RQ) close to 1 (Table 1). The apparent biomass yield on glucose in these aerobic cultures was approximately 10% higher than previously reported (41) due to co-consumption of ethanol which, because of its use as solvent for ergosterol, was present at a concentration of 0.67 g L^{-1} in all growth media. When oxygen availability was decreased by sparging with a mixture of N_2 and air (0.5 L min^{-1}, oxygen content 840 ppm), the apparent biomass yield on glucose in steady-state cultures was approximately four-fold lower than in fully aerobic cultures (0.15 g g^{-1} and 0.59 g g^{-1}, respectively, Table 1). Moreover, a high residual glucose concentration (15.9 g L^{-1}) indicated that growth was limited by oxygen rather than by glucose. Respiro-fermentative glucose metabolism in the oxygen-limited cultures was evident from a respiratory quotient (RQ) of 10.7 and a specific ethanol-production rate of 4.8 mmol (g biomass)$^{-1}$ h^{-1}. In contrast to results obtained in the same cultivation system with *K. marxianus* and *S. cerevisiae* (39), reduction of the O_2 content of the inlet gas to < 0.5 ppm O_2 led to complete wash-out of the *O. parapolymorpha* chemostat cultures.

S. cerevisiae can grow anaerobically in synthetic media supplemented with sterols, a source of UFA and a standard vitamin solution that is also used for aerobic cultivation (28–30). Absence of a functional sterol-uptake system in *K. marxianus* was recently identified as the key cause of its inability to grow anaerobically on such media (39). Based on UFA and sterol contents of aerobically grown *S. cerevisiae* biomass, the combined oxygen-uptake rates needed for synthesis of these lipids at a specific growth rate of 0.10 h^{-1} corresponds to approximately 0.01 mmol O_2 (g biomass)$^{-1}$ h^{-1} (30). This estimated oxygen-uptake rate was 60-fold lower than the biomass-specific oxygen-consumption rate of 0.60 mmol O_2 (g biomass)$^{-1}$ h^{-1} observed in oxygen-limited cultures of *O. parapolymorpha* (Table 1).

Based on the assumption that oxygen-limited cultures of *O. parapolymorpha* cultures predominantly used oxygen for respiration, a comparison of oxygen-uptake and ethanol-production rates indicated that approximately 3% of the glucose consumed by these cultures was respired. In contrast, under the same oxygen-limitation regime, chemostat cultures of *S. cerevisiae* and *K. marxianus* showed specific oxygen-consumption rates below 0.25 mmol (g biomass)$^{-1}$ h^{-1}, RQ values above 50 and very low residual glucose concentrations (Table 1)(39). For these two yeasts, the estimated fraction of the total consumed glucose that was respired amounted to only approximately 0.5%.

O. parapolymorpha showed an over ten-fold lower biomass-specific rate of glycerol production in oxygen-limited cultures than *K. marxianus* and *S. cerevisiae* (0.02 versus 1.12 and 0.45 mmol g (biomass)$^{-1}$ h^{-1}, respectively, Table 1). Glycerol formation plays an important role in anaerobic and severely oxygen-limited cultures of several yeasts by reoxidizing a surplus of cytosolic NADH formed in biosynthetic reactions (42–45). Under aerobic conditions, this NADH, which is formed as a consequence of carbon dioxide formation during synthesis of biomass components from glucose, is reoxidized by mitochondrial respiration (45). In fully anaerobic cultures of *S. cerevisiae* and in severely oxygen-limited cultures of *K. marxianus*, glycerol formation has been reported to be 7-12 mmol (gram biomass)$^{-1}$ (39). At a dilution rate of 0.10 h^{-1}, reoxidation of an equivalent amount of NADH by aerobic respiration would require an uptake rate of 0.4 to 0.6 mmol O_2 g (biomass)$^{-1}$ h^{-1}, which closely corresponds with the observed oxygen consumption rate of the oxygen-limited *O. parapolymorpha* cultures (Table 1).

In some yeast species, an insufficient capacity for glycerol production has been linked to an inability to grow under severe oxygen limitation. This phenomenon, known as 'Custers effect', can be complemented by supplementation of oxygen-limited cultures with acetoin (42, 46). In *S. cerevisiae*, reduction of acetoin to 2,3-butanediol by its NADH-dependent 2,3-butanediol dehydrogenase Bdh1 (47) can restore fermentation in a glycerol-negative strain (48). The predicted proteome of *O. parapolymorpha* strain CBS11895 was found to harbor an ortholog to ScBdh1 (HPODL_00988) (Supplementary Fig. 1). Addition of acetoin to oxygen-limited chemostat cultures of *O. parapolymorpha* led to an increase of the steady-state biomass concentration from 0.62 to 1.57 g L^{-1}. In addition, metabolism became more fermentative, as indicated by a higher rate of ethanol production, a higher biomass yield on oxygen and a higher RQ (Table 3). Although biomass-specific ethanol production rates in these cultures (5.9 mmol g_x^{-1} h^{-1}, Table 3) approached those of oxygen-limited cultures of *S. cerevisiae* CEN. PK113-7D grown at the same dilution rate (7.5 mmol g_x^{-1} h^{-1}, Table 1), almost half of the glucose in the cultures remained unused. In addition, biomass-specific rates of acetoin consumption (7.5 mmol g_x^{-1} h^{-1}) in the *O. parapolymorpha* cultures were much higher than the rates estimated to be required for reoxidation of NADH generated in biosynthetic reactions. The latter observation suggests that 2,3-butanediol dehydrogenase activity in *O. parapolymorpha* not only reoxidized NADH formed in biosynthetic reactions but also NAD(P)H formed in dissimilatory metabolism, for example by competing for NADH with alcohol dehydrogenase.

Table 1 Physiology of yeast strains in glucose-grown chemostat cultures under different aeration regimes. Cultures were grown at pH 6.0 on synthetic medium with urea as nitrogen source and at a dilution rate of 0.1 h^{-1}. Glucose concentrations in the feed of aerobic ($21 \cdot 10^4$ ppm O_2) and oxygen-limited (840 ppm O_2) cultures were 7.5 and 20 g L^{-1}, respectively. Data for *K. marxianus* and *S. cerevisiae* were obtained from a previous study performed under identical conditions (39). Media were supplemented with the anaerobic growth factors ergosterol and Tween 80, except for aerobic cultures of *O. parapolymorpha*, from which Tween 80 was omitted to prevent excessive foaming. Data are represented as mean ± standard deviation of data obtained from independent chemostat cultures of each strain. Ethanol concentrations were corrected for evaporation (39). Negative and positive biomass-specific conversion rates (q) represent consumption and production rates, respectively; subscript x denotes biomass dry weight. Recoveries of degree of reduction according to (49) based on an assumed biomass composition (50). Calculations not applicable due to co-consumption of ethanol (-) and concentrations below detection limit (B.D., concentrations < 0.01 mM) are indicated.

Yeast strain	*O. parapolymorpha* CBS11895		*K. marxianus* CBS6556		*S. cerevisiae* CEN.PK113-7D	
	Aerobic	O_2-limited	Aerobic	O_2-limited	Aerobic	O_2-limited
O_2 in inlet gas (ppm)	$21 \cdot 10^4$	840	$21 \cdot 10^4$	840	$21 \cdot 10^4$	840
Replicates	2	2	2	5	3	3
Biomass (g_x L^{-1})	4.33 ± 0.06	0.62 ± 0.01	3.79 ± 0.03	1.57 ± 0.22	4.22 ± 0.11	2.29 ± 0.07
Residual glucose (g L^{-1})	B.D.	15.92 ± 0.01	B.D.	0.10 ± 0.03	B.D.	0.07 ± 0.01
Biomass-specific rates						
Specific growth rate (h^{-1})	0.10 ± 0.00	0.10 ± 0.01	0.10 ± 0.00	0.11 ± 0.01	0.10 ± 0.00	0.10 ± 0.00
$q_{glucose}$ (mmol g_x^{-1} h^{-1})	-0.94 ± 0.04	-3.67 ± 0.20	-1.05 ±0.00	-7.46 ± 0.66	-0.95 ± 0.05	-4.59 ± 0.18
$q_{ethanol}$ (mmol g_x^{-1} h^{-1})	-0.48 ± 0.08	4.75 ± 0.33	-0.52 ± 0.00	11.49 ± 0.97	-0.44 ± 0.05	7.48 ± 0.17
$q_{glycerol}$ (mmol g_x^{-1} h^{-1})	B.D.	0.02 ± 0.01	B.D.	1.12 ± 0.12	B.D.	0.45 ± 0.01
$q_{succinate}$ (mmol g_x^{-1} h^{-1})	B.D.	0.00 ± 0.00	B.D.	0.02 ± 0.01	B.D.	0.00 ± 0.00
q_{O2} (mmol g_x h^{-1})	-2.35 ± 0.15	-0.60 ± 001	-3.52 ± 0.07	-0.23 ± 0.05	-2.61 ± 0.20	-0.15 ± 0.01
q_{CO2} (mmol g_x h^{-1})	2.89 ± 0.21	6.42 ± 0.30	3.73 ± 0.04	10.5 ± 1.1	2.82 ± 0.17	7.91 ± 0.91
Stoichiometries						
RQ (qCO_2/qO_2)	1.23 ± 0.15	10.7 ± 0.3	1.06 ± 0.01	49.3 ± 16.8	1.08 ± 0.04	52.2 ± 4.2
$Y_{glycerol/X}$ (mmol g_x^{-1})	B.D.	0.24 ± 0.07	B.D.	10.7 ± 1.5	B.D.	4.66 ± 0.13
$Y_{X/glucose}$ (g_x g^{-1})	0.59 ± 0.01	0.15 ± 0.02	0.53 ± 0.00	0.08 ± 0.01	0.57 ± 0.01	0.12 ± 0.00
$Y_{ethanol/glucose}$ (g g^{-1})	-	0.33 ± 0.04	-	0.39 ± 0.00	-	0.42 ± 0.03
$Y_{X/O2}$ (g_x $mmol^{-1}$)	0.04 ± 0.00	0.17 ± 0.01	0.03 ± 0.01	0.50 ± 0.18	0.04 ± 0.00	0.64 ± 0.06
Recoveries (out/in)						
Carbon (%)	100.3 ± 0.6	94.4 ± 6.2	100.5 ± 0.1	91.1 ± 4.5	99.9 ± 1.1	101.2 ± 5.7
Degree of reduction(%)	97.8 ± 0.8	92.0 ± 6.1	98.8 ± 0.2	94.5 ± 1.0	98.4 ± 1.2	100.1 ± 1.4

This notwithstanding, these results implicated a limited capacity for NADH reoxidation as a key factor in the large oxygen requirements of *O. parapolymorpha*.

Absence of orthologs of *S. cerevisiae* glycerol-3P phosphatase in *Ogataea* species.

To study the molecular basis for the apparent absence of a fully functional glycerol pathway in the oxygen-limited *O. parapolymorpha* cultures, we investigated the presence of orthologs of *S. cerevisiae GPD1/2* and *GPP1/2*. These genes encode isoenzymes catalyzing the two key reactions of the *S. cerevisiae* glycerol pathway, NAD^+-dependent glycerol-3P dehydrogenase and glycerol-3P phosphatase, respectively (51–53). A homology search in translated whole-genome sequences of 16 *Ogataea* species (54) revealed clear orthologs of Gpd, but none were detected for Gpp (Fig. 1). In this respect, *Ogataea* yeasts resembled the phylogenetically related genus *Brettanomyces* (syn. *Dekkera*) (Fig. 1) for which the occurrence of the Custers effect is well documented (55, 56). In the absence of an active glycerol-3P phosphatase, NAD^+-dependent glycerol-3P dehydrogenase can still be involved in glycerolipid synthesis (57) and in the glycerol-3P shuttle, in which glycerol-3P is reoxidized to dihydroxyacetone phosphate by a mitochondrial, respiratory-chain-coupled glycerol-3P dehydrogenase (Gut2 in *S. cerevisiae*, (58–60), Fig. 1).

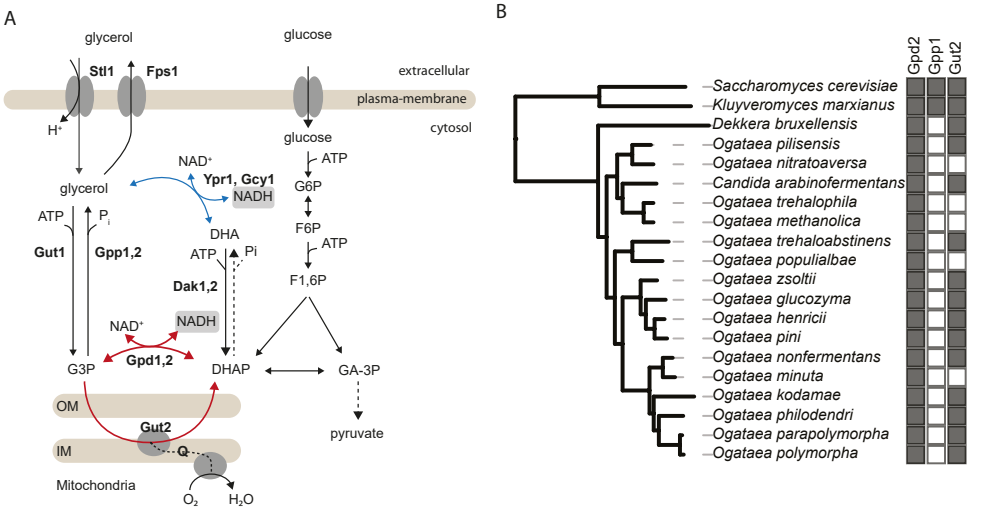

Figure 1. (**A**) Reactions and proteins involved in glycerol metabolism and upper glycolysis in *S. cerevisiae*. Gpd1 is mainly located in peroxisomes and Gpd2 in the cytosol and in mitochondria (61). Red arrows indicate reactions participating in the glycerol-3-phosphate shuttle, the dashed line indicates the theoretical possibility that, in non-*Saccharomyces* yeasts, glycerol may be formed through the activity of a DHAP phosphatase and NAD(P)H dependent DHA reductase (blue arrow)(62). (**B**) Distribution of orthologs of *S. cerevisiae* structural genes encoding glycerol-3P dehydrogenase (Gpd2), glycerol-3P phosphatase (Gpp1) and FAD-dependent mitochondrial glycerol-3P dehydrogenase (Gut2) in *Ogataea* sp., *Brettanomyces* (syn. *Dekkera bruxellensis*), *K. marxianus* and *S. cerevisiae*. Data represent homology search results with *S. cerevisiae* S288C protein sequences as queries against whole-genome translated sequences. Black and white squares indicate presence and absence, respectively, of orthologs. Species are mapped to the phylogenetic tree of *Saccharomycotina* yeasts (54).

Transcriptional responses of *O. parapolymorpha* to oxygen limitation.

To further explore responses of *O. parapolymorpha* to oxygen limitation, transcriptome analyses were performed on chemostat cultures grown under the aeration regimes described above. The resulting transcriptome data were first used to refine the genome annotation of a *de novo* assembled genome sequence of *O. parapolymorpha* CBS11895 obtained from long-read sequence data (see Data Availability).

Transcriptional responses of *O. parapolymorpha* to oxygen limitation were compared with previously obtained transcriptome datasets of *S. cerevisiae* and *K. marxianus* (39), derived from chemostat cultures grown under the same aeration regimes. A global comparison at the level of functional categories indicated large differences in transcriptional responses to oxygen limitation of these three yeasts (Fig. 2A). Also when transcriptional responses were directly compared for genes for which orthologs occurred in *O. parapolymorpha*, *K. marxianus* and *S. cerevisiae*, strikingly different patterns were observed (Fig. 2B), with only very few orthologs showing a consistent transcriptional response to oxygen limitation in all three species (Fig. 2C and 2D).

At first glance, the different global transcriptional responses of the three yeasts appear to indicate a completely different wiring of their oxygen-responsive transcriptional regulation networks. Based on functional categories the only shared global transcriptional responses of *O. parapolymorpha, K. marxianus*, and *S. cerevisiae* were a downregulation, in the oxygen-limited cultures, of genes involved in the metabolism of alternative non-glucose carbon sources (GO categories fatty acid metabolic process, tricarboxylic acid cycle, transmembrane transport, metabolic process, lipid metabolic process; Fig. 2A). These responses are in line with an oxygen dependency for dissimilation of non-fermentable substrates (including fatty acids) and for a key role of the tricarboxylic acid cycle in respiratory glucose metabolism. However, in addition to oxygen availability, different glucose and ethanol concentrations in chemostat cultures of the tested yeast strains (Table 1) may have had a substantial impact on transcript profiles. For example, under aerobic as well as anaerobic conditions, hundreds of *S. cerevisiae* genes were shown to exhibit an at least 2-fold difference in transcript levels in comparisons of glucose-limited and glucose-sufficient chemostat cultivation regimes (63–65). In view of this intrinsic limitation of the chemostat-based transcriptome analysis, we focused further analysis of transcriptome data on genes and pathways that were previously implicated in biosynthetic oxygen requirements of yeasts.

Sterol biosynthesis requires molecular oxygen and, under anaerobic or oxygen-limited conditions, *S. cerevisiae* can acquire ergosterol from the media. In contrast to *S. cerevisiae*, which showed downregulation of genes associated with sterol metabolism in oxygen-limited cultures, *O. parapolymorpha* and *K. marxianus* showed upregulation of genes associated with this GO-term (based on GO-term enrichment analysis) (Fig. 2A). *K. marxianus* and several other pre-WGD yeast species lack a functional sterol-import system (39, 66). The upregulation of sterol synthesis genes in oxygen-limited, sterol-supplemented cultures of *O. parapoly-*

morpha (Fig. 2A) as well as the absence of clear orthologs of the *S. cerevisiae AUS1* and *PDR11* sterol-importer genes in its genome, suggested that this is also the case for this yeast.

A recent study confirmed that Op*URA9*, which encodes the respiratory-chain-linked Class-II dihydroorotate dehydrogenase of *O. parapolymorpha*, complements the uracil auxotrophy of *ura1Δ S. cerevisiae* in aerobic, but not in anaerobic conditions (67). Op*URA9* showed increased transcript levels in oxygen-limited conditions compared to aerobic conditions, while its *K. marxianus* ortholog Km*Ura9* was downregulated (Fig. 3). This response is consistent with the presence and absence of a respiration-independent Class-I-A dihydroorotate dehydrogenase in *K. marxianus* and *O. parapolymorpha*, respectively.

The importance of glycerol production for oxygen-limited growth of *S. cerevisiae* and *K. marxianus* was evident from an upregulation of *GPP1* (Fig. 3), for which no ortholog was found in *O. parapolymorpha* (Fig. 2), in oxygen-limited cultures. This observation and the lack of a transcriptional response of the single *GPD* ortholog in *O. parapolymorpha* to oxygen limitation were in line with the conclusion that this yeast does not use glycerol formation as a redox sink during oxygen-limited growth.

Figure 2 (right page). Genome-wide transcriptional responses of *O. parapolymorpha*, *K. marxianus* and *S. cerevisiae* to oxygen limitation. Chemostat cultures of *O. parapolymorpha* (opar) were grown at pH 6 on synthetic medium with urea as nitrogen source and either aerated with air (aerobic $21{\cdot}10^4$ ppm O_2 in the inlet gas, regime 1) or with a mixture of N_2 and 840 ppm O_2 (micro-aerobic, regime 2). Glucose concentrations in the feed of aerobic and oxygen-limited cultures were 7.5 and 20 g L^{-1}; respectively. Data for *K. marxianus* (kmar) and *S. cerevisiae* (scer) were obtained from a previous study performed under identical conditions (39). (**A**) Comparison of gene-set enrichment analysis using GO-terms for biological processes in response to oxygen limitation (micro-aerobic vs. aerobic, regime 2 to 1) with at least one category overlapping between two of the three yeast strains. Distinct directionalities were calculated with Piano (68) and indicated as distinct directional down (pdddn), mixed directional down (pmddn), non-directional (pnd), mixed directional up (pmdup) and distinct directional up (pddup). Hierarchical clustering was performed based on the frequency of enrichment of GO-terms in the three yeast strains. Data of all enriched GO-terms for biological processes are available in Supplemental Fig. 2, 3 and 4. (**B**) Log fold changes (microaerobic transcript level versus aerobic transcript level) of orthologs in the three yeasts, (**C**) Orthologs showing higher transcript levels in micro-aerobic cultures of all three yeasts. (**D**) Orthologs showing lower transcript levels in micro-aerobic cultures of all three yeasts.

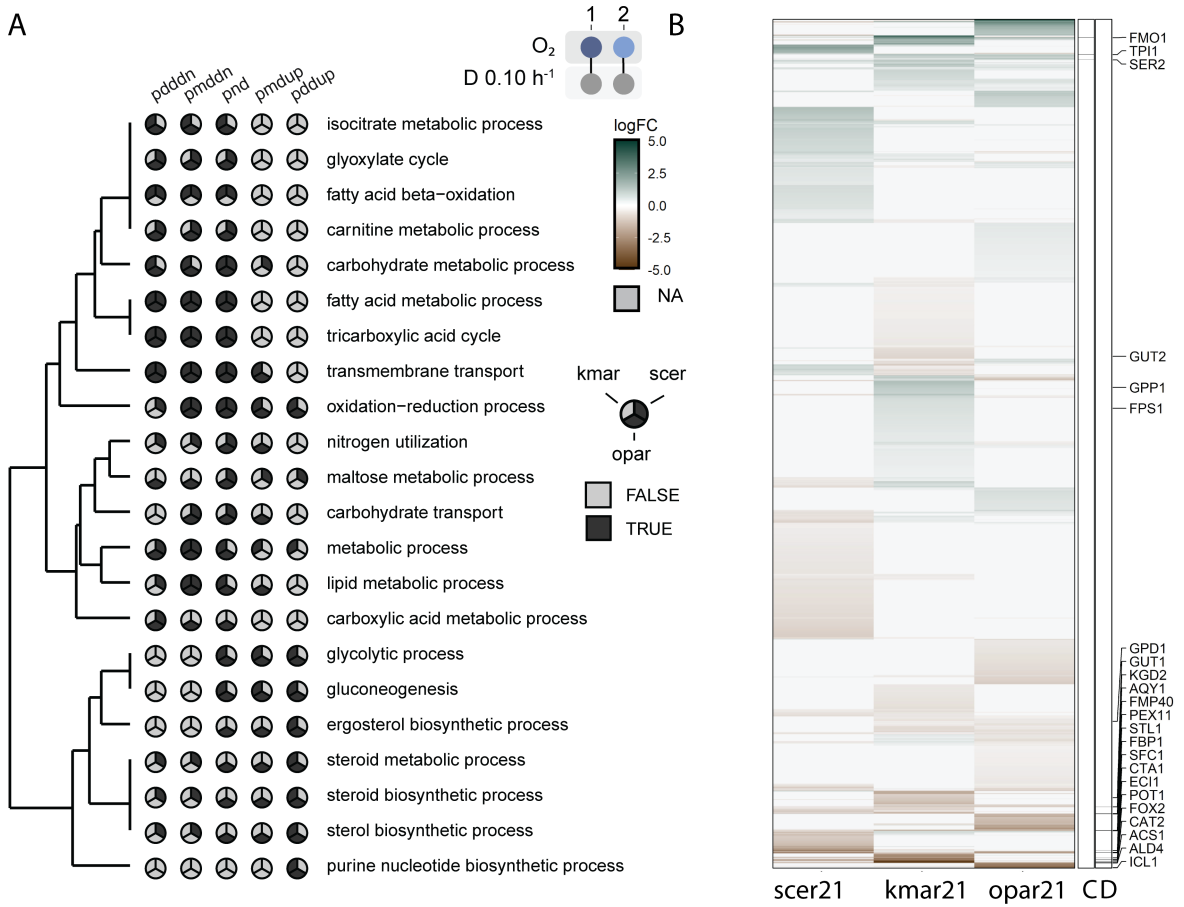

Figure 2. (see description left page)

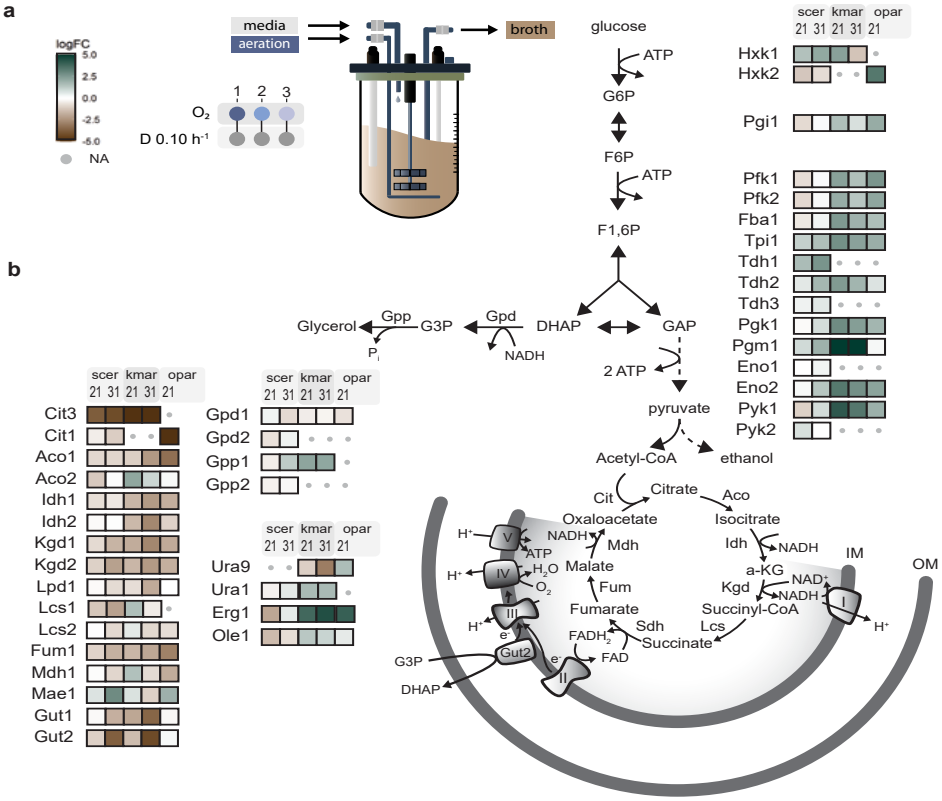

Figure 3. Transcriptional regulation of specific pathways and structural genes in *O. parapolymorpha*, *K. marxianus* and *S. cerevisiae* subjected to different aeration regimes. (**A**) Glucose-grown chemostat cultures (dilution rate of 0.10 h^{-1}) were aerated with air (regime 1), a gas mixture containing 840 ppm O_2 (regime 2), or with nitrogen containing < 0.5 ppm O_2 (regime 3) at a rate of 0.5 L min^{-1}. *O. parapolymorpha* washed out under regime 3. RNAseq data are compared against aerobic, glucose-limited cultures (regime 1) as the reference. Transcriptome data for *K. marxianus* and *S. cerevisiae* were obtained from a previous study (39). (**B**) Biochemical reactions are represented by arrows and multiple reactions by dashed arrows; to facilitate visualization, some reaction products and cofactors are not shown. Boxes with colors indicate gene upregulation (blue-green) or downregulation (brown), intensities indicating the log 2 fold change (logFC) with color range capped to a maximum value of 5. Reactions are annotated with the corresponding *S. cerevisiae* ortholog name; absence of orthologs is indicated by grey dots. Respiratory chain complexes are indicated by Roman numerals. *S. cerevisiae* does not harbor Complex I but the genome of *O. parapolymorpha* genome contains a full complement of genes encoding its subunits (38). Abbreviations used; glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6P), dihydroxyacetone phosphate (DHAP), glycerol-3-phosphate (G3P), glyceraldehyde-3-phosphate (GAP), inner-mitochondrial membrane (IM), outer-mitochondrial membrane (OM).

Glycerol production in aerobic cultures of an *O. parapolymorpha* strain lacking mitochondrial glycerol-3P dehydrogenase and alternative NADH dehydrogenases.

The presence of orthologs of *S. cerevisiae GPD1/2* and *GUT2* in *O. parapolymorpha* suggested possible involvement of a glycerol-3P shuttle (58) in respiratory oxidation of the cytosolic NADH derived from glycolysis and from biosynthetic reactions. To investigate whether elimination of both the glycerol-phosphate shuttle and cytosol-facing mitochondrial NADH dehydrogenases affects glycerol production by *O. parapolymorpha*, we studied growth and product formation in strain IMX2167. In this strain, Op*GUT2* as well as the genes encoding three cytosol- and matrix-facing alternative mitochondrial NADH dehydrogenases were deleted while leaving the Complex-I NADH dehydrogenase complex intact (69).

In aerobic chemostat cultures grown at a dilution rate of 0.10 h^{-1}, conversion rates of strain IMX2167 did not substantially differ from those of the wild-type strain CBS11895 (Table 2). Apparently, as previously observed in aerobic cultures of corresponding mutant strains of *S. cerevisiae* (45), mechanisms such as an ethanol-acetaldehyde shuttle could compensate for the absence of mitochondrial glycerol-3P dehydrogenase and external NADH dehydrogenases. In strain IMX2167, in which also the internal alternative NADH dehydrogenase was absent, such a shuttle mechanism could then couple reoxidation of cytosolic NADH to the *O. parapolymorpha* complex-I NADH dehydrogenase (45).

In contrast to the absence of a clear phenotype in the chemostat cultures, aerobic batch cultures of strain IMX2167 showed a lower specific growth rate than the wild-type strain CBS11895 (0.26 h^{-1} and 0.36 h^{-1}, respectively, Table 2). Glycerol production suggested that, despite the absence of an Sc*GPP1* ortholog, *O. parapolymorpha* contains a phosphatase that can use glycerol-3P as a substrate. In the genome sequence of strain CBS11895, 24 genes were annotated with the GO-term 'phosphatase activity' (GO:0016791). All these genes were transcribed (log Counts per million (CPM) > 3.5). However, none of them showed a significantly higher (log FC > 2) transcript level in aerobic batch or chemostat cultures of in strain IMX2167 than in corresponding cultures of the wild-type strain CBS11895 (Supplemental Table 1, and transcriptome analysis Supplemental Fig. 5 and 6).

Table 2 Physiology of *O. parapolymorpha* wild-type and mutant strains in glucose-grown aerobic batch and chemostat cultures. Aerobic chemostat cultures were grown at a dilution rate of 0.1 h^{-1} on synthetic medium with 20 g L^{-1} glucose as carbon- and energy substrate. Batch cultures were grown on synthetic medium containing 7.5 g L^{-1} glucose. Data on aerobic chemostat cultures of strain IM2167 are derived from a separate study (69). Data are represented as mean ± standard deviation of data obtained from independent chemostat cultures of each strain. Ethanol concentrations were corrected for evaporation (39). Positive and negative biomass-specific conversion rates (q) represent consumption and production rates, respectively; subscript x denotes biomass dry weight. Specific growth rates were determined from samples taken during the exponential growth phase. N.D.: not determined values; and B.D.: compounds below detection limit (concentrations < 0.01 mM).

O. parapolymorpha strain	CBS11895	IMX2167	CBS11895	IMX2167
Relevant genotype	wt	*ndh1-3Δ gut2Δ*	wt	*ndh1-3Δ gut2Δ*
Replicates	2	2	2	2
Cultivation mode	Batch	Batch	Chemostat	Chemostat
Biomass-specific rates				
Specific growth rate (h^{-1})	0.36 ± 0.01	0.26 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
$q_{glucose}$ (mmol g_x h^{-1})	-3.90 ± 0.21	-2.73 ± 0.08	-1.08 ± 0.04	-1.04 ± 0.00
$q_{ethanol}$ (mmol g_x h^{-1})	B.D.	0.22 ± 0.11	B.D.	B.D.
$q_{glycerol}$ (mmol g_x h^{-1})	B.D.	0.34 ± 0.01	B.D.	B.D.
q_{O2} (mmol g_x h^{-1})	N.D.	-3.65 ± 0.07	-2.69 ± 0.07	-2.14 ± 0.00
q_{CO2} (mmol g_x h^{-1})	N.D.	4.34 ± 0.02	2.82 ± 0.04	2.25 ± 0.00
Stoichiometries				
RQ (qCO_2/qO_2)	N.D.	1.19 ± 0.03	1.05 ± 0.01	1.05 ± 0.00
$Y_{X/glucose}$ (g_x g^{-1})	0.52 ± 0.01	0.51 ± 0.01	0.51 ± 0.00	0.52 ± 0.00
$Y_{X/O2}$ (g_x $mmol^{-1}$)	N.D.	0.07 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
Recoveries (out/in)				
Carbon (%)	N.D.	97.8 ± 1.4	99.3 ± 1.7	98.7 ± 0.4

Engineering of glycerol metabolism in *O. parapolymorpha*.

Based on the apparent absence of orthologs of the *S. cerevisiae* glycerol-3P phosphatase ScGpp1 in the genomes of *Ogataea* sp. (Fig. 1B), we investigated whether expression of *ScGPP1* in *O. parapolymorpha* supported glycerol production by oxygen-limited cultures of *O. parapolymorpha*. To this end, an expression cassette in which the coding region of *ScGPP1* was expressed with the *OpPMA1* promoter (70) was integrated into the genome of *O. parapolymorpha* CBS11895. In oxygen-limited chemostat cultures, the resulting engineered strain IMX2119 showed a 9-fold higher biomass-specific rate of glycerol formation than the wild-type strain (0.18 mmol (g biomass)$^{-1}$ h^{-1} and 0.02 mmol (g biomass)$^{-1}$ h^{-1}, respectively, Table 3, Fig. 4A). A further increase of the glycerol production rate to 0.22 mmol (g biomass)$^{-1}$ h^{-1} was observed when, in addition to the expression cassette for Sc*GPP1*, a second expression cassette was integrated in which Sc*GPD2* was expressed from the Op*TEF1* promoter (70) (strain IMX2588, Table 3, Fig. 4A). Integration of only the Sc*GPD2* cassette (strain IMX2587) did not lead to a significantly higher rate of glycerol production in oxygen-limited cultures than observed in the wild-type strain (Table 3).

The higher biomass-specific rates of glycerol production by the Sc*GPP1* and Sc*GPP1*/Sc*GPD2* expressing *O. parapolymorpha* strains enabled higher biomass yields on oxygen under oxygen-limited conditions (0.24 and 0.32 g biomass mmol O_2^{-1}, respectively, versus 0.17 g biomass mmol O_2^{-1} for the wild-type strain, Table 3 and Fig. 4B). A larger contribution of alcoholic fermentation was, in addition, evident from the RQ values of strains IMX2119 and IMX2588 (14.4 and 17.4, respectively), which were significantly higher than those of corresponding cultures of the wild-type strain (RQ of 10.7, Table 3).

For fully anaerobic chemostat cultures of *S. cerevisiae* CEN.PK113-7D grown at a dilution rate of 0.10 h^{-1}, a biomass-specific rate of glycerol production of 0.67 mmol (g biomass)$^{-1}$ h^{-1} was reported (Fig. 4A)(39, 71). An even higher rate of glycerol production (1.1 mmol (g biomass)$^{-1}$ h^{-1}) was reported for strains of another *S. cerevisiae* lineage grown under these conditions (43, 72). Assuming that biomass composition and biosynthetic pathways in *S. cerevisiae* CEN.PK113-7D and *O. parapolymorpha* CBS11895 lead to a similar net generation of NADH from biosynthetic reactions, the glycerol production rate of the Sc*GPP1*/Sc*GPD2* expressing *O. parapolymorpha* strain IMX2588 remained approximately four-fold lower than required for reoxidation of all NADH generated in biosynthetic processes. A limiting capacity of the engineered glycerol pathway was also indicated by residual glucose concentrations in oxygen-limited cultures of strain IMX2588, which were higher than in acetoin-supplemented oxygen-limited cultures of the wild-type strain CBS11895 (Table 3).

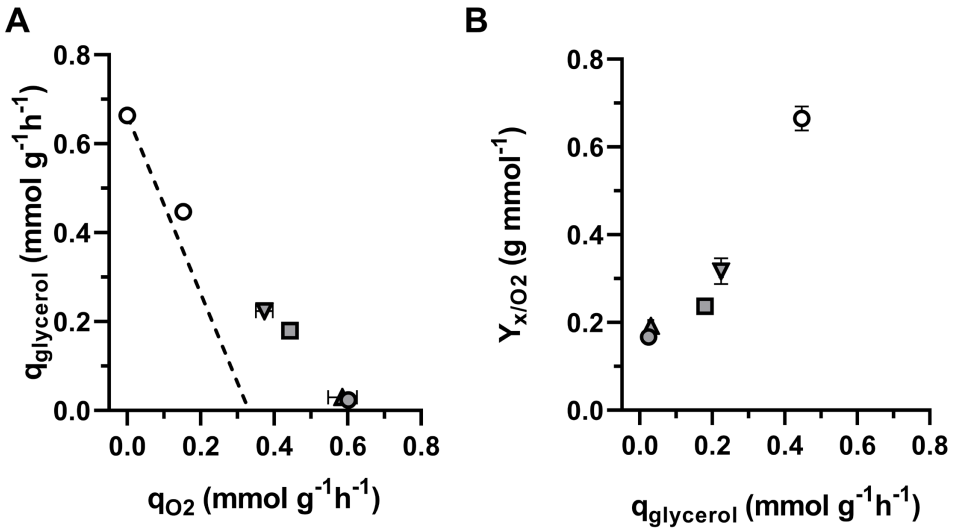

Figure 4. Impact of heterologous expression of Sc*GPP1* and/or Sc*GPD2* expression in *O. parapolymorpha* CBS11895 on glycerol production in oxygen-limited chemostat cultures. Biomass-specific conversion rates (q) of *O. parapolymorpha* strains were measured in oxygen-limited conditions (dilution rate 0.10 h^{-1}), aerated at 500 mL min^{-1} with a gas mixture containing 840 ppm O_2. Data of anaerobic and oxygen-limited *S. cerevisiae* cultures, grown in the same experimental set-up were derived from (39). Symbols: white circle *S. cerevisiae* CEN.PK113-7D, grey circles *O. parapolymorpha* CBS11895, grey box IMX2119 (Sc*GPP1*), grey triangle up IMX2587 (Sc*GPD2*), grey triangle-down IMX2588 (Sc*GPP1* Sc*GPD2*). Data are represented as mean ± standard deviation of data obtained from independent chemostat cultures of each strain. (**A**) Biomass-specific glycerol production rate versus biomass-specific oxygen consumption rate. The dashed line depicts the stoichiometric relationship between glycerol and oxygen interpolated from the anaerobic rate of glycerol production by *S. cerevisiae* under the assumption that, for NADH reoxidation, consumption of one mol O_2 is equivalent to the production of two mol glycerol. (**B**) Biomass yield on oxygen versus biomass- specific rate of glycerol production.

Table 3. Physiology of *O. parapolymorpha* strains expressing *S. cerevisiae* glycerol pathway genes in glucose-grown oxygen-limited chemostat cultures. Cultures were grown at a dilution rate of 0.1 h^{-1} pH 6.0 on synthetic medium with urea as nitrogen source and 20 g L^{-1} glucose as carbon and energy substrate, media contained anaerobic growth factors ergosterol and Tween 80. Where indicated, synthetic medium was supplemented with 85 mM acetoin. * Recoveries for acetoine supplemented cultures assumed a 1-to-1 conversion to 2,3-butanediol. Data are represented as mean ± standard deviation of data obtained from independent chemostat cultures of each strain. Ethanol concentrations were corrected for evaporation (39). Positive and negative biomass-specific conversion rates (q) represent consumption and production rates, respectively; subscript x denotes biomass dry weight. B.D.: concentrations below detection limit (concentration < 0.01 mM). Recoveries of degree of reduction according to (49) based on an assumed biomass composition (50).

Yeast strain	CBS11895	IMX2119	IMX2587	IMX2588	CBS11895
Relevant genotype	wt	*ScGPP1*	*ScGPD2*	*ScGPP1* *ScGPD2*	wt
O_2 in inlet gas (ppm)	840	840	840	840	840
Acetoin in feed (mM)	0	0	0	0	85
Replicates	2	3	2	3	3
Biomass (g_x L^{-1})	0.62 ± 0.01	0.91 ± 0.06	0.66 ± 0.09	0.86 ± 0.09	1.57 ± 0.08
Residual glucose (g L^{-1})	15.92 ± 0.01	14.33 ± 0.61	15.71 ± 0.57	14.98 ± 0.64	9.41 ± 0.41
Biomass-specific rates					
Specific growth rate (h^{-1})	0.10 ± 0.00	0.11 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.00
$q_{glucose}$ (mmol g_x^{-1} h^{-1})	-3.67 ± 0.20	-3.69 ± 0.43	-2.97 ± 0.18	-4.16 ± 0.19	-4.11 ± 0.12
$q_{ethanol}$ (mmol g_x^{-1} h^{-1})	4.75 ± 0.33	4.92 ± 0.52	4.72 ± 0.20	7.26 ± 0.57	5.90 ± 0.40
$q_{glycerol}$ (mmol g_x^{-1} h^{-1})	0.02 ± 0.01	0.18 ± 0.02	0.03 ± 0.01	0.22 ± 0.01	0.03 ± 0.00
$q_{succinate}$ (mmol g_x^{-1} h^{-1})	B.D.	B.D.	0.02 ± 0.02	0.01 ± 0.02	0.02 ± 0.00
$q_{acetoin}$ (mmol g_x^{-1} h^{-1})	-	-	-	-	-7.45 ± 0.32
q_{O2} (mmol g_x^{-1} h^{-1})	-0.60 ± 0.01	-0.44 ± 0.03	-0.59 ± 0.06	-0.37 ± 0.04	-0.29 ± 0.01
q_{CO2} (mmol g_x^{-1} h^{-1})	6.42 ± 0.30	6.41 ± 0.80	5.33 ± 0.37	6.45 ± 0.42	7.00 ± 0.41
Stoichiometries					
RQ (qCO_2/qO_2)	10.7 ± 0.3	14.4 ± 0.9	9.11 ± 0.23	17.4 ± 1.6	24.2 ± 0.9
$Y_{glycerol/X}$ (mmol g_x^{-1})	0.24 ± 0.07	1.71 ± 0.09	0.30 ± 0.12	1.93 ± 0.25	0.32 ± 0.04
$Y_{X/glucose}$ (g_x g^{-1})	0.15 ± 0.02	0.16 ± 0.00	0.19 ± 0.01	0.16 ± 0.01	0.14 ± 0.00
$Y_{ethanol/glucose}$ (g g^{-1})	0.33 ± 0.04	0.34 ± 0.01	0.41 ± 0.00	0.45 ± 0.01	0.37 ± 0.02
$Y_{X/O2}$ (g_x $mmol^{-1}$)	0.17 ± 0.01	0.24 ± 0.01	0.18 ± 0.03	0.32 ± 0.05	0.37 ± 0.00
Recoveries (out/in)					
Carbon (%)	94.4 ± 6.2	98.4 ± 2.2	102.8 ± 1.7	102.4 ± 2.8	96.1 ± 1.8*
Degree of reduction (%)	92.0 ± 6.1	93.5 ± 3.1	102.9 ± 0.4	106.0 ± 3.1	99.5 ± 1.8*

Discussion

In this study, chemostat cultivation with defined aeration regimes revealed surprisingly high oxygen requirements of oxygen-limited cultures of the facultatively fermentative yeast *Ogataea parapolymorpha* (previously *Hansenula polymorpha* (20)) relative to those previously reported for the pre-WGD yeasts *Kluyveromyces marxianus*, *K. lactis* and *Candida utilis* (*Cyberlindnera jadinii*)(43, 73). Very low glycerol-production rates in oxygen-limited cultures and an increase of the biomass yield on oxygen upon acetoin co-feeding identified reoxidation of NADH, formed in biosynthetic reactions, as a key contributor to the large oxygen requirements of *O. parapolymorpha*.

A substantial oxygen requirement for fermentative growth ('Custers effect'; (74)), absence of glycerol production and a stimulating effect of acetoin on oxygen-limited growth were previously observed in *Brettanomyces* (*Dekkera*) yeasts (42, 55, 74, 75). The Custers effect in *B. bruxellensis* was attributed to absence of glycerol-3P phosphatase activity in cell extracts (55) and lack of an ortholog of the *S. cerevisiae GPP1/GPP2* genes (76). The genera *Ogataea* and *Brettanomyces* both belong to the Pichiacaea family (77). Our observations on *O. parapolymorpha*, combined with the apparent absence of Sc*GPP1*/Sc*GPP2* orthologs in other *Ogataea* species, provide an incentive for further studies into the occurrence of this intriguing phenomenon in Pichiacaea. In view of its fast growth in synthetic media (78) and its accessibility to genome-editing techniques (79, 80), *O. parapolymorpha* offers an interesting experimental platform to study the regulatory networks and ecophysiological significance of the Custers effect.

O. parapolymorpha is currently applied in aerobic industrial processes for production of heterologous proteins (81). Based on its thermotolerance and natural ability to metabolize D-xylose, this yeast is also intensively investigated as a potential platform organism for second-generation ethanol production (21). In anaerobic industrial applications of *Saccharomyces* yeasts such as beer fermentation, introduction of a brief aeration phase enables yeast cell to synthesize and intracellularly accumulate sterols and unsaturated fatty acids, which are then used during the subsequent anaerobic fermentation phase (82, 83). The large oxygen requirements of *O. parapolymorpha* observed in this study imply that such a strategy is not feasible for this yeast. Reduction or complete elimination of these requirements is therefore a priority target for development of industrial ethanol-producing strains.

Formation of glycerol in aerobic cultures of strain IMX2167, in which genes encoding key enzymes of respiratory NADH oxidation, including mitochondrial glycerol-3-phosphate dehydrogenase (OpGut2), were deleted, suggested that the *O. parapolymorpha* genome may harbor a gene encoding a glycerol-3-phosphatase. Alternatively, glycerol formation in this strain may reflect activity of an alternative pathway for glycerol production (e.g. involving DHAP phosphatase, Fig. 1). Laboratory evolution of wild-type and engineered *O. parapolymorpha* strains under oxygen-limited conditions and resequencing of evolved strains (84) may contribute to a better understanding of glycerol production in this yeast.

Expression of the *S. cerevisiae GPP1* and *GPD2* genes enabled increased rates of glycerol formation and a higher biomass yield on oxygen in oxygen-limited cultures of *O. parapolymorpha* (Table 3). However, glycerol production rates remained below those observed in anaerobic cultures of *S. cerevisiae* (Fig. 4A) and a large fraction of the glucose fed to the cultures remained unused. These results indicated that the *in vivo* capacity of NADH reoxidation via heterologously expressed Gpp1 and Gpd2 was insufficient to fully replace the role of mitochondrial respiration in reoxidation of NADH generated in biosynthetic reactions. Increased expression of *GPP1* and *GPD2*, possibly combined with expression of a glycerol exporter and/or laboratory evolution under oxygen-limited conditions can be explored to further enhance glycerol production in *O. parapolymorpha*. Alternatively, expression of heterologous pathways for NADH-dependent reduction of acetyl-CoA to ethanol (85) or NADH oxidation via a pathway involving ribulose-1,5-bisphosphatase and phosphoribulokinase (86, 87) can be explored.

In oxygen-limited cultures of *O. parapolymorpha* that were co-fed with acetoin, incomplete glucose consumption occurred despite rates of acetoin conversion that were an order of magnitude higher than glycerol production rates in anaerobic *S. cerevisiae* cultures (Table 3, Fig. 4). This result suggests that, in this yeast, not only a limited capacity for reoxidation of NADH generated in biosynthesis but also for NADH generated in glucose dissimilation may be limited. This hypothesis can be tested by laboratory evolution under oxygen-limited conditions or, alternatively, by overexpression of key enzymes of pyruvate decarboxylase and/or alcohol dehydrogenase.

Predicted stoichiometric oxygen requirements for sterol synthesis and pyrimidine synthesis of *O. parapolymorpha* are small in comparison with those for NADH reoxidation. However, their physiological impacts can be augmented when key enzymes involved in these processes have a low affinity for oxygen. Absence of orthologs of the *S. cerevisiae* Aus1 and Pdr11 sterol transporters indicates that, similar to other pre-WGD yeasts (88), *O. parapolymorpha* is most likely unable to import sterols. Due to the incompletely resolved role of cell wall proteins in sterol import in *S. cerevisiae* (89), functional expression of a heterologous system for sterol import in *O. parapolymorpha* may not be a trivial challenge. We recently showed that, in *S. cerevisiae* and *K. marxianus,* expression of a heterologous squalene-tetrahymanol cyclase enabled synthesis of the sterol surrogate tetrahymanol and sterol-independent anaerobic growth (39, 90). Genome-sequence data indicate that pyrimidine synthesis in *O. parapolymorpha* depends on a respiratory-chain-linked dihydroorate dehydrogenase (OpUra9), thus rendering pyrimidine biosynthesis in this yeast oxygen dependent (91, 92). As previously explored in *Scheffersomyces stipitis* (91), expression of the soluble fumarate-coupled dihydroorotate dehydrogenase from *S. cerevisiae* (Ura1) may be used to bypass this oxygen requirement.

We recently demonstrated that bypassing the small oxygen requirement of *K. marxianus* for sterol synthesis sufficed to enable its anaerobic growth on synthetic medium (39). The present

study shows that oxygen requirements of *O. parapolymorpha* are both larger in magnitude and more complex than those of *K. marxianus*. Comparative physiology studies across strains and species, with rigorous standardization of cultivation conditions to minimize oxygen contamination (93) are required to identify requirements of different species and, thereby, to unlock the industrial potential of non-*Saccharomyces* yeasts for application in anaerobic industrial processes.

Methods

Strain maintenance

Yeast strains used in this study were derived from the wild-type *Ogataea parapolymorpha* strain CBS11895 (DL-1) obtained from CBS-KNAW (Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands). For propagation and strain maintenance, yeast cultures were grown on yeast peptone dextrose (YPD) media (10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone, 7.5 g L^{-1} glucose) in an Innova shaker incubator (New Brunswick Scientific, Edison, NJ, USA) set at 30 °C and 200 rpm. YP medium was heat sterilized (121 °C for 20 minutes). Stock solutions of concentrated D-glucose were autoclaved separately at 110 °C and added to a concentration of 7.5 g L^{-1}. Cryo-stocks were prepared from exponentially growing cultures by addition of glycerol to a final concentration of 30% (v/v) and aseptically stored at -80 °C.

Molecular biology techniques

PCR amplification for cloning was routinely performed with Phusion High Fidelity polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. DreamTaq polymerase (Thermo Fisher Scientific) was used for diagnostic PCR reactions. Oligo-nucleotide primers were ordered either desalted or PAGE-purified (Sigma-Aldrich, St. Louis, MO, USA) and are listed in Table S2. DNA fragments obtained by PCR amplification were analysed by gel electrophoresis, and when required, purified from agarose gels with the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Prior to purification, template plasmid DNA was removed by FastDigest *DpnI* digestion (Thermo Fisher Scientific). Alternatively, DNA fragments were directly purified from the PCR mix using the GenElute PCR Clean-Up Kit (Sigma-Aldrich). Gibson assemblies were performed with the NEBuilder HiFi DNA Assembly Master mix (New England Biolabs, Ipswich, MA, USA) with the total reaction volume downscaled to 5 µL and a total incubation time of 1 h at 50 °C. *Escherichia coli* XL1-Blue cells were used for transformation, amplification and storage of plasmids. Plasmids were isolated from overnight *E. coli* cultures with the GenElute Plasmid Miniprep kit (Sigma-Aldrich). For diagnostic PCR amplifications, yeast colonies were grown overnight in YPD, after which the genomic DNA was extracted using the LiAc/SDS method (94).

Plasmid construction

Plasmids used in this study are described in Table 4**.** *O. parapolymorpha* promoters and terminators of *OpPMA1* and *OpTEF1* (encoding plasma-membrane P2-type H^+-ATPase and trans-

lation elongation factor EF-1α, respectively) were chosen based on high transcript levels of these genes across a range of specific growth rates (70). Promoter and terminator regions were defined as 800 bp upstream and 300 bp downstream of the coding sequence, respectively. For targeted integration into a genetic locus, 500 bp flanking homology regions were designed to partially delete the target region, without altering promoters (800 bp) or terminators (300 bp) of adjacent genes.

Plasmid pUD1069 (Sc*GPP1*) was constructed by Gibson assembly from fragments with 20 bp terminal sequence overlaps. The *GPP1* coding sequence was PCR amplified from genomic DNA of *S. cerevisiae* CEN.PK113-7D with primers 15183 and 15184 thereby introducing 20 bp overlaps. The promoter and terminator of the endogenous *O. parapolymorpha PMA1* were amplified from genomic DNA using primers 15185/15186 and, 15191/15193 respectively. The natNT2 marker (Ag*TEF1*p-nat1-Sc*ADH1*t) from *Streptomyces noursei* (95–97) was PCR amplified from pUD803 (69) with primers 15187 and 15188. Up- and downstream 500 bps homology flanks to the *OpGBU1* locus (98) were PCR amplified from genomic DNA of *O. parapolymorpha* using primers 15192/15196 and 15195/15198 respectively. The pUG6 backbone was PCR amplified using primers 15189 and 15190. Fragments were mixed in equimolar amounts and Gibson assembled resulting in plasmid pUD1069. Correct plasmid assembly was verified by restriction digestion and diagnostic PCR amplification with primers 15224, 15225, 15226, 15227, 15228, 15229, 15230, 15231 and 15232.

For heterologous expression of Sc*GPD2*, its coding sequence was PCR amplified from genomic DNA of CEN.PK113-7D with primers 15745 and 15744, resulting in the addition of 20 bp homology flanks for Gibson assembly. The Op*TEF1* promoter and Op*TEF1* terminator were PCR amplified from genomic DNA of *O. parapolymorpha* CBS11895 with primers 15749/15748 and 15746/15747, respectively. Upstream and downstream 800-bp recombination flanks for integration of the *OpSGA1* integration locus were PCR amplified with primers 15740/15739 and 15735/15736, respectively. A *Klebsiella pneumoniae hph* (Hyg^R) marker cassette, including the Ag*TEF1* promoter and Ag*TEF1* terminator from *Ashbya gossypii,* was PCR amplified from pUDP002 (79) with primers 1312 and 15743. The pUC19 backbone

Table 4. Plasmids used in this study. Superscripts indicate restriction sites; subscripts indicate gRNA target sequences. Sc: *Saccharomyces cerevisiae*; Op: *Ogataea parapolymorpha,* Ag: *Ashbya gossypii*, Aa: *Arxula adeninivorans*, Sp: *Streptococcus pyogenes.*

Plasmid	Characteristics	Source
pUC19	ori ampR	(99)
pUD803	$HR_{NDH2\text{-}3}$ Aa*TEF1*p-nat1-Sc*PHO5*t $HR_{NDH2\text{-}3}$	(69)
pUD1069	ori amp^R *gbu1*(HR) Op*PMA1*p-Sc*GPP1*-Op*PMA1*t-Ag*TEF1*p-nat1-Sc*ADH1*t *gbu1*(HR)	This study
pUD1082	ori ampR *sga1*(HR) Op*TEF1*p-Sc*GPD2*-Op*TEF1*t Ag*TEF1*p-hph-Ag*TEF1*t *sga1*(HR)	This study
pUDP002	panARS Ag*TEF1*p-hph-Ag*TEF1*t Sc*TDH3*p$^{BsaI\ BsaI}$Sc*CYC1*t Aa*TEF1*p-Spcas9-Sc*PHO5*t	(79)
pUG6	ori ampR loxP-kanMX-loxP	(100)

was PCR amplified with primers 15738 and 15737. The resulting 6 fragments were mixed in equimolar amounts and Gibson assembled. The resulting plasmid pUD1082 (Sc*GPD2*) was verified by restriction digestion.

Strain construction

Yeast strains used and constructed in this study are described in Table 5. *O. parapolymorpha* strains were transformed by electroporation of freshly prepared electrocompetent cells (79). Yeast cells were selected after transformation on YPD agar containing hygromycin B (300 μg mL^{-1}) or nourseothricin (100 μg mL^{-1}). Strains IMX2119, IMX2587 and IMX2588 were constructed using the split-marker integration approach (101), with an approximately 480 bp overlapping homology sequences for marker recombination and genome integration. The natNT2 split-marker fragments for integration of a Sc*GPP1* expression cassette into the Op*GBU1* locus were constructed by PCR amplification with primers 15192, 15194, 15196 and 15197 from pUD1069 (Sc*GPP1*) resulting in two integration fragments with a homologous sequence overlap. Similarly, the hph split-marker fragments for integration of the Sc*GPD2* cassette were constructed by PCR amplification of a pUD1082 (Sc*GPD2*) fragment with primers 15740, 15741, 15742 and 15736. The chromosomal integration of the split-marker fragment was verified by diagnostic PCR. Diagnostic PCR of the Op*GBU1* integration locus was performed with primers 15192, 15197, 15233 and 15234, and integration in the Op*SGA1* integration locus was verified with primers 15894, 15748, 15895.

Table 5. Yeast strains used in this study. Sc: *Saccharomyces cerevisiae*; Op: *Ogataea parapolymorpha*

Genus	Strain	Relevant genotype	Reference
S. cerevisiae	CEN.PK113-7D	MATa URA3 HIS3 LEU2 TRP1 MAL2-8c SUC2	(102)
O. parapolymorpha	CBS11895	-	Westerdijk
O. parapolymorpha	IMX2119	*gbu1Δ*::Op*PMA1*p-Sc*GPP1*-OpPMA1t natNT2	This study
O. parapolymorpha	IMX2587	*sga1Δ*::Op*TEF1*p-Sc*GPD2*-Op*TEF1*t-hph	This study
O. parapolymorpha	IMX2588	*gbu1Δ*::Op*PMA1*p-Sc*GPP1*-Op*PMA1*t natNT2 *sga1Δ*::Op*TEF1*p-Sc*GPD2*-Op*TEF1*t-hph	This study
O. parapolymorpha	IMX2167	ndh2-1Δ::kanR ndh2-2Δ::hph ndh2-3::natNT2 gut2Δ::pat	(69)

Bioreactor cultivation

Aerobic batch and chemostat cultures for physiological characterization of *O. parapolymorpha* strains CBS11895 and IMX2167 strains were grown in 2 L bioreactors (Applikon Biotechnology, Delft, the Netherlands) with 1.0 L working volume. Chemostat cultures were subjected to a dilution rate of 0.1 h^{-1}. Cultures were grown on synthetic medium with vitamins (103), supplemented with glucose and with sterile antifoam pluronic acid 6100 PE (0.2 g L^{-1}, BASF, Ludwigshafen, Germany). Mineral salt solutions were sterilized by autoclaving at 121 °C for 20 min. Glucose solutions were prepared and autoclaved separately at 110 °C for 20

min and added to a final concentration of 7.5 g L^{-1}. Where indicated, acetoin was added to a concentration of 85 mM.

Inocula for bioreactor cultures were prepared by harvesting an exponential growing shake-flask culture by centrifugation and washing once with sterile demineralized water. Cultures were continuously stirred at 800 rpm, maintained at 30 °C and sparged with air (O_2 $21{\cdot}10^4$ ppm) at a volumetric rate of 0.5 L min^{-1} (0.5 vvm) to maintain a high dissolved oxygen concentration. After CO_2 emission in the batch cultivation phase had reached a maximum and started to decline sharply, a constant medium feed and continuous effluent removal were initiated to maintain a constant working volume.

Oxygen-limited chemostat cultures of *O. parapolymorpha* strains were grown in 2 L bioreactors (Applikon) with 1.2 L working volume on synthetic medium with vitamins and urea as nitrogen source (104). A stock solution of urea was filter sterilized (0.2 µm) and added to the sterile media. Before autoclaving, bioreactors were checked for gas leakage by submersion in water while applying a 0.3 bar overpressure. Chemostats were started as aerobic batch cultures to obtain a sufficiently high initial cell density. The glucose concentration in the aerobic batch phase was 1.5 g L^{-1} while the chemostat feed media contained 20 g L^{-1} glucose for the oxygen-limited and 7.5 g L^{-1} for the aerobic reference cultures.

An 800-fold concentrated stock solution of the anaerobic growth factors Tween 80 (polyethylene glycol sorbitan monooleate; Merck, Darmstadt, Germany), ergosterol (≥95%; Sigma-Aldrich, St. Louis, MO) was prepared with pure ethanol as solvent as described previously (30), but with a 5-fold lower concentration of Tween 80 to prevent excessive foaming of *O. parapolymorpha* cultures. Addition of this stock solution to sterile media yielded concentrations of Tween 80, ergosterol and ethanol of 84 mg L^{-1}, 10 mg L^{-1} and 0.67 g L^{-1}, respectively. For aerobic cultures, Tween 80 was omitted from media to prevent foaming and the glucose concentration was adjusted to 7.5 g L^{-1}. Dissolved gas concentrations in the bioreactor cultures were maintained by sparging with either air ($21{\cdot}10^4$ ppm O_2) or a gas mixture of N_2/Air (840 ppm O_2) at a volumetric rate of 0.5 L min^{-1} (0.4 vvm). Bioreactors were equipped with Fluran tubing and viton O-rings to minimize oxygen diffusion. The glass medium reservoir was equipped with Norprene tubing and continuously sparged with pure nitrogen gas. Chemostat cultures were assumed to have entered a steady state when, at least 5 volume changes after a change in growth conditions, the biomass concentration and specific carbon dioxide production rate differed by less than 10% over three samples separated by at least one volume change.

Analytical methods

Off-gas analysis, biomass dry weight measurements, optical density measurements, metabolite HPLC analysis of culture supernatants and correction for ethanol evaporation in bioreactor experiments were performed as described previously (39). Rates of substrate consumption and metabolite production were determined from glucose and metabolite concentrations in

steady-state cultures, analyzed after rapid quenching of culture samples (105). Carbon and degree of reduction balances were calculated based on concentrations of relevant components in medium feed, culture samples and in- and out-going gas streams. Calculations were based on an estimated degree of reduction of biomass as described in (50). Recoveries for acetoine supplemented cultures assumed a 1-to-1 conversion of acetoine to 2,3-butanediol.

Genome sequencing and assembly

Cells were harvested from an overnight YPD culture by centrifugation for 5 min at 4000 g and genomic DNA was isolated using the Qiagen genomic DNA 100/G Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. MinION genomic DNA libraries (SQK-LSK108, Oxford Nanopore Technology, Oxford, UK) were prepared using the 1D genomic DNA by ligation and the SQK-LSK108 library was sequenced on an R9 chemistry flow cell (FLO-MIN107). Base calling was performed using Albacore v1.1.5 (Oxford Nanopore Technologies), reads were assembled using Canu v1.4 (106), and the resulting assembly was polished with Pilon v1.18 (107). To perform the genome annotation of *O. parapolymorpha* CBS11895, pooled RNAseq libraries were first used to generate a *de novo* transcriptome assembly using Trinity (v2.8.3)(108) and then used for the PASA pipeline (109) as implemented in funannotate v1.7.7 (110). RNA reads from *O. parapolymorpha* CBS11895 batch and chemostat (70) were downloaded from NCBI (www.ncbi.nlm.nih.gov) with the gene expression omnibus accession GSE140480. Funannotate compare was then used to obtain (co)ortholog groups of genes generated with ProteinOrtho5 (111) using publicly available genome annotations of *S. cerevisiae* S288C (GCF_000146045.2) and a previous version of *O. parapolymorpha* (DL-1; GCF_000187245.1), to import functional annotation, guided by the ortholog assignment, into the new CBS11895.

RNA extraction, isolation, sequencing and transcriptome analysis

Biomass samples from bioreactor batch and chemostat cultures were directly sampled into liquid nitrogen to prevent mRNA turnover (112) and stored at -80 °C before extraction and isolation. Batch cultures were sampled in mid-exponential phase when approximately 75% of the initial glucose concentration was still unused (70). Processing of RNA for long-term storage and isolation was performed as described previously (39). The quality of isolated RNA was analyzed with an Agilent Tapestation (Agilent Technologies, CA, USA) using RNA screen tape (Agilent). RNA concentrations were measured with a Qubit RNA BR assay kit (Thermo Fischer Scientific). The TruSeq Stranded mRNA LT protocol (Illumina, San Diego, CA, USA) was used to generate RNA libraries for paired-end sequencing by Macrogen (Macrogen Europe, Amsterdam, the Netherlands) with a read-length of 151 bp on a NovaSeq sequencer (Illumina).

RNA reads were mapped to the genome of strain CBS11895 (70) using bowtie (v1.2.1.1) (113) and alignments were filtered and sorted using samtools (v1.3.1)(114) as described previously (39). Reads were counted with featureCounts (v1.6.0)(115) of which both pairs of the paired-

end reads were aligned to the same chromosome. edgeR (v3.28.1)(116) was used to perform differential gene expression and genes with fewer than 10 reads per million in all conditions were eliminated from subsequent analysis. Counts were normalized using the trimmed mean of M values (TMM)(117) method and the dispersion was estimated using generalized linear models. Differential expression was calculated using a log ratio test adjusted with the Benjamini-Hochberg method. Absolute log 2 fold-change values (>2), false discovery rate (< 0.5) and *p*-value (<0.05) were used a significance cutoffs.

Gene set analysis (GSA) based on gene ontology (GO) terms with Piano (v2.4.0)(68) was used for functional interpretation of differential gene expression profiles. Interproscan (118) was used to assign GO terms to the genome annotation of *O. parapolymorpha*. Co-ortholog groups of genes were generated with ProteinOrtho5 (111) as implemented in the funannotate pipeline and used to homogenize GO terms for co-ortholog groups as described previously (39). Gene set analysis was performed with Piano (v2.4.0)(68) and gene statistics were calculated with Stouffer, Wilcoxon rank-sum test and reporter methods as implemented in Piano. Consensus gene level statistics were obtained by *p*-value and rank aggregation and considered significant based on absolute log 2 fold-change values > 1. ComplexHeatmap (v2.4.3)(119) was used to visualize the differentially expressed genes, including those shared with a previous study (63).

To interpret the GO-term based gene set analysis between three yeast species in response to oxygen limitation, hierarchical clustering (complete method and Euclidian distance) in R (120) was performed on GO-terms from biological process category. Clustering was based on the number of overlapping distinct directionality *p*-values in the three yeast species with a significance *p*-value cut-off of 0.01.

Sequence homology search

S. cerevisiae protein sequences were used as query for searching whole genome sequences of 16 *Ogataea* species, *K. marxianus*, *Candida arabinofermentans* and *B. bruxellensis* with tblastn (blast.ncbi.nlm.nih.gov)(121). Significance was based on alignment criteria with an e-value of $< 10^{-7}$, alignment coverage >70% and nucleotide percent identity of >50%. Blast results were mapped to a subtree of selected yeast species in the fungal phylum Ascomycota (54) using Treehouse (122) to subset the phylogenetic tree.

Data availability

Data presented in all figures in this work are available at the data.4TU.nl repository doi: 10.4121/14270138. Raw sequencing data that supports this study are available from the NCBI website (www.ncbi.nlm.nih.gov/geo/) with the BioProject PRJNA717220.

The codes that were used to generate the results obtained in this study are archived in a Gitlab repository (gitlab.tudelft.nl/rortizmerino/opar_anaerobic).

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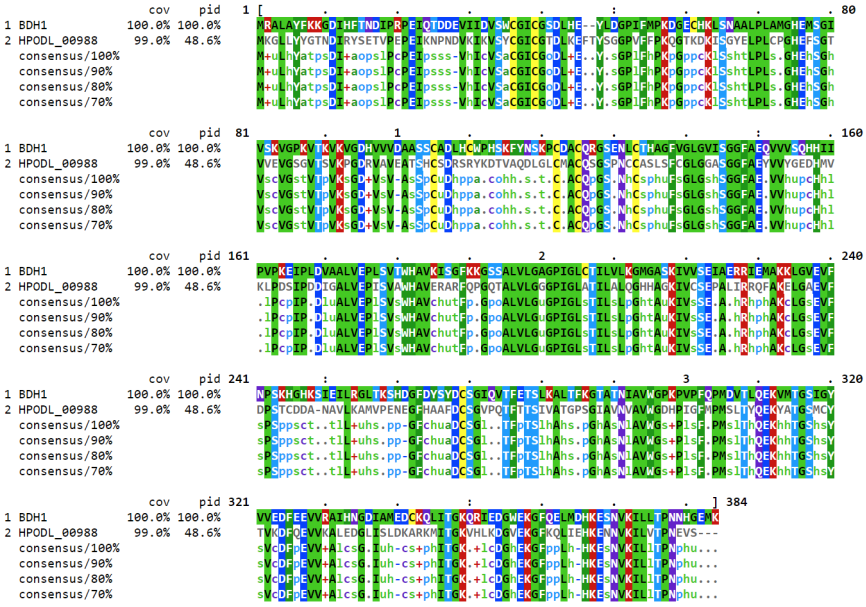
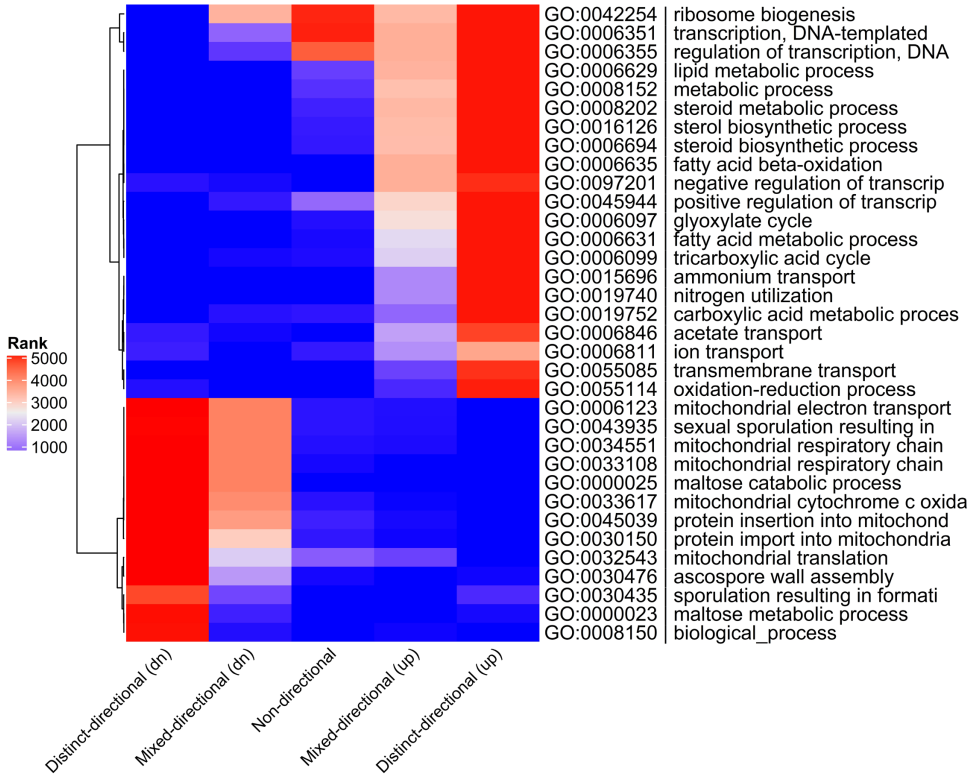
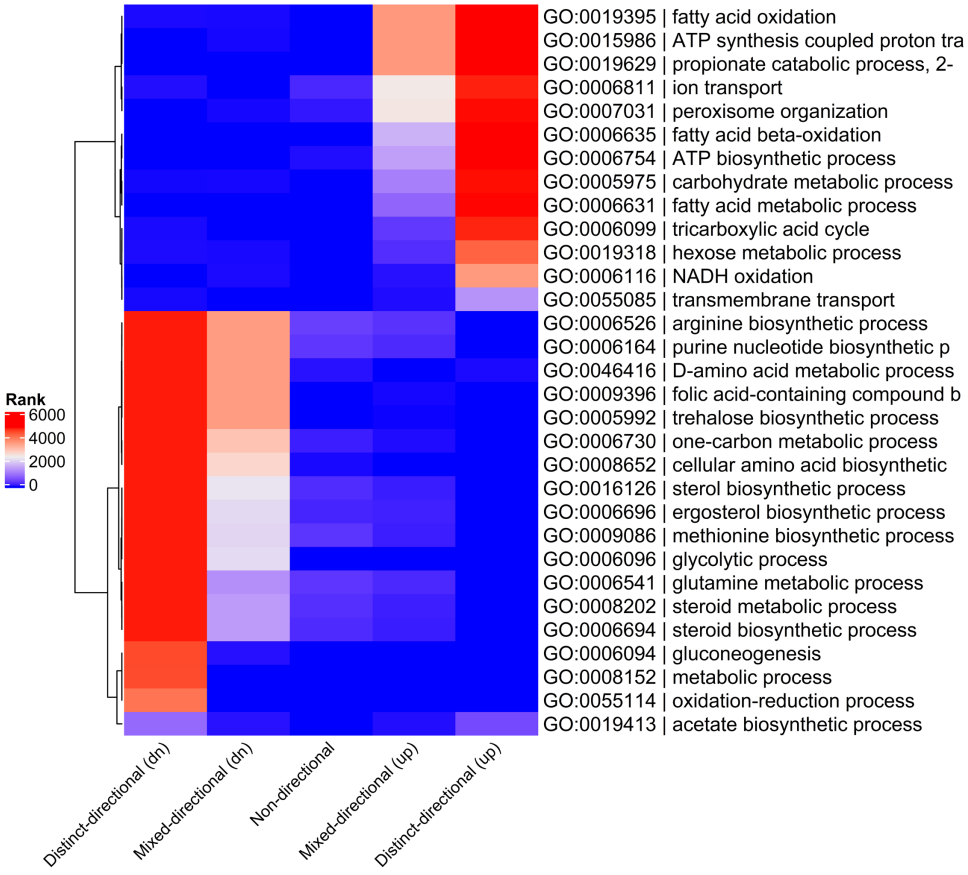
Supplementary Material



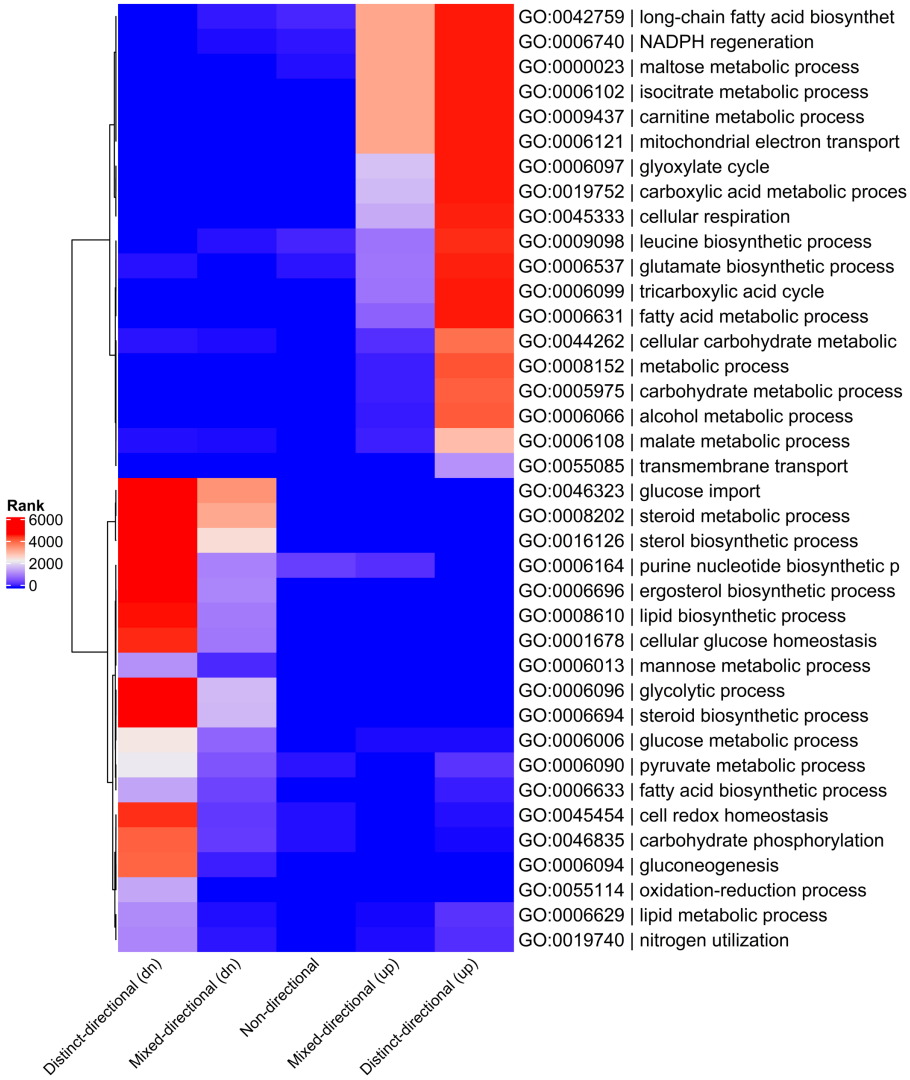
Supplementary Figure 1. Protein sequence alignment of putative *O. parapolymorpha* 2,3-butanediol dehydrogenase (HPODL_00988) and *S. cerevisiae* Bdh1 aligned using Clustal O(1.2.4) and visualized using MView with amino acid identity coloring.



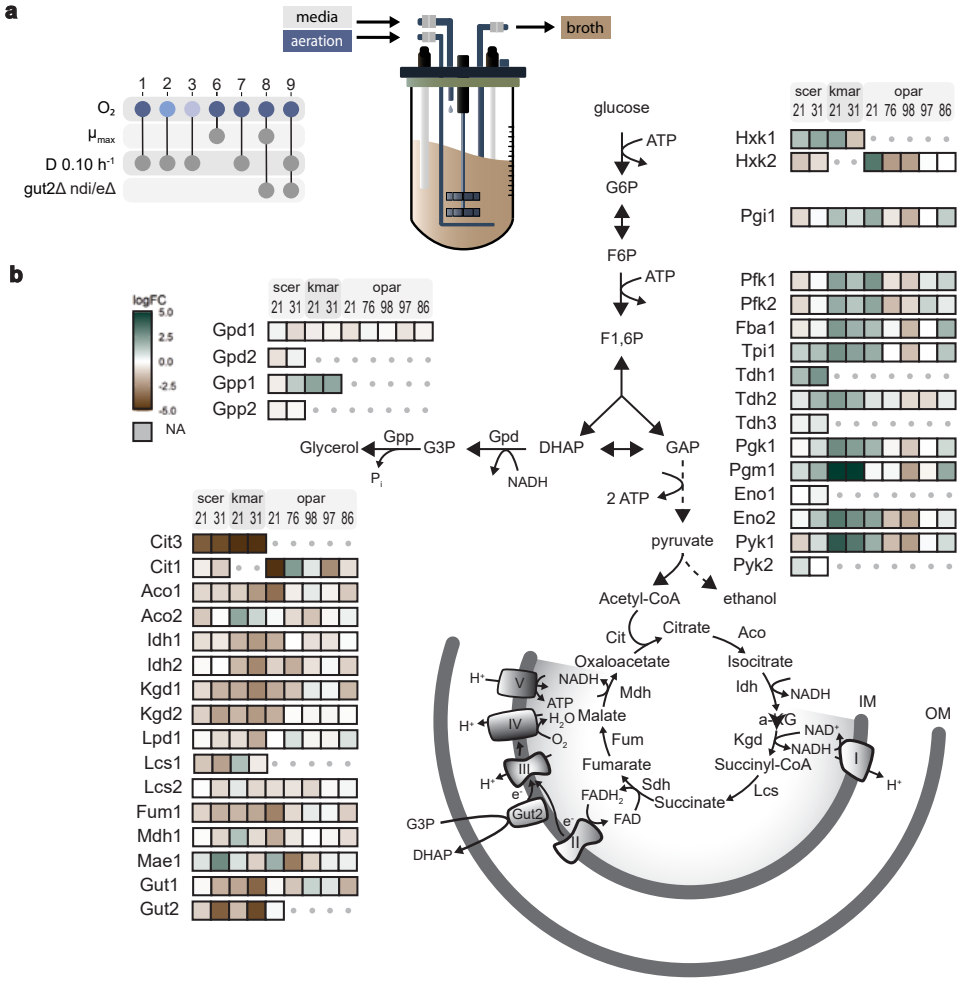
Supplementary Figure 2. Consensus biological process GO term enrichment for *S. cerevisiae* contrast 21 (comparison of cultures with 840 ppm O_2 in the inlet gas to fully aerobic cultures with 21 10^4 ppm O_2 in inlet gas). GO terms are clustered according to their rank. See legend of Fig. 3 for experimental details.



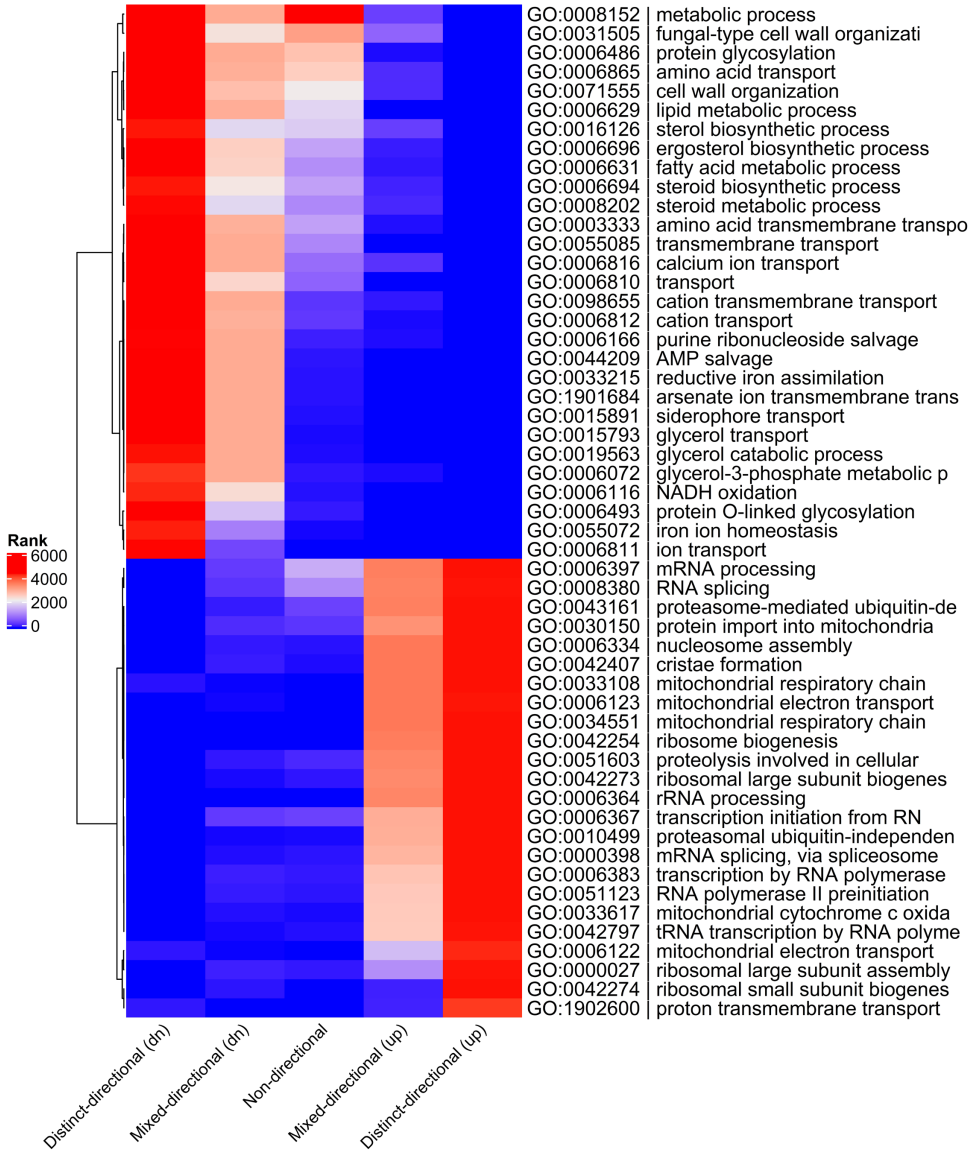
Supplementary Figure 3. Consensus biological process GO term enrichment for *K. marxianus* contrast 21 (comparison of cultures with 840 ppm O_2 in the inlet gas to fully aerobic cultures with 21 10^4 ppm O_2 in inlet gas). GO terms are clustered according to their rank. See legend of Fig. 3 for experimental details.



Supplementary Figure 4. Consensus biological process GO term enrichment for *O. parapolymorpha* contrast 21 (comparison of cultures with 840 ppm O_2 in the inlet gas to fully aerobic cultures with 21 10^4 ppm O_2 in inlet gas). GO terms are clustered according to their rank. See legend of Fig. 3 for experimental details.



Supplementary Figure 5. Different transcriptional regulation of NADH oxidation in *O. parapolymorpha*, *K. marxianus* and *S. cerevisiae*. (**A**) For each aeration regime, RNAseq was performed on samples from chemostat cultures (dilution rate 0.10 h^{-1}) for *O. parapolymorpha* CBS11895 and, *K. marxianus* CBS6556 and *S. cerevisiae* CEN. PK113-7D. The ingoing gas flow of 0.5 L min^{-1} contained either 21 10^4 ppm O_2 (regime 1, 6, 7, 8 and 9), 840 ppm O_2 (regime 2) or < 0.5 ppm O_2 (regime 3, 4 and 5). Chemostat cultures of *O. parapolymorpha* washed out at an oxygen content of < 0.5 ppm O_2 (regime 3) prohibiting a comparison with aerobic conditions (contrast 31). RNAseq data of *S. cerevisiae* and *K. marxianus* (39) were reanalyzed. RNAseq was performed on independent replicate batch and chemostat cultures of *O. parapolymorpha* CBS11895 and IMX2167 (*gut2*Δ *ndi/e*Δ) in aerobic conditions (regime 6 to 9). Regimes were comprised of aerobic batch cultivation of CBS11895 (regime 6), aerobic chemostat cultivation of CBS11895 (regime 7), aerobic batch cultivation of IMX2167 (regime 8) and aerobic chemostat cultivation of IMX2167 (regime 9). (**B**) Biochemical reactions are represented by arrows and multiple reactions by dashed arrows. Boxes with colors indicate up- (blue-green) or downregulation (brown) with color intensity indicating the log 2 fold change (logFC) with color range capped to a maximum value of 5. Reactions are annotated with the corresponding *S. cerevisiae* ortholog name; absence of orthologs is indicated by grey dots. Abbreviations used; pentose-phosphate pathway (PPP), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6P), dihydroxyacetone phosphate (DHAP), glycerol-3-phosphate (G3P),



Supplementary Figure 6. Consensus biological process GO term enrichment for *O. parapolymorpha* contrast 68 (comparison of aerobic batch culture wild-type CBS11895 (regime 6) to aerobic batch culture IMX2167 (regime 8) with both 21 10^4 ppm O_2 in inlet gas). GO terms are clustered according to their rank. See legend of Fig. S4 for experimental details.

(*Supplementary figure 5 continued*)

glyceraldehyde phosphate (GAP), inner-mitochondrial membrane (IM), OM outer-mitochondrial membrane, respiratory chain complexes are indicated with corresponding roman numerals. Complex I is absent from *S. cerevisiae* but genes encoding its subunits are present in the genome of *O. parapolymorpha* (38).

Supplementary Table 1. Comparison of transcript levels of putative phosphatase-encoding genes in *O. parapolymorpha* CBS11895 (wild type) to and the *gut2*Δ *nde/i1-3*Δ knockout deletion strain IMX2167, grown in aerobic batch cultures. Log fold-change (log FC), log counts per million (log CPM), gene identifiers used in the genome annotations (Geneid, DL.1) and *S. cerevisiae* S288C orthologs and three-letter abbreviation (standard name).

Log FC	Log CPM	*p-value*	FDR	Geneid	DL.1	S288C	Standard Name
-1.61	5.74	2.06E-19	1.11E-18	OPAR_004591	HPODL_02465	YGR208W	*SER2*
-1.56	3.52	1.10E-08	2.56E-08	OPAR_004515	HPODL_01991		
1.39	7.90	1.51E-23	1.07E-22	OPAR_002912	HPODL_03896	YKL212W	*SAC1*
-1.37	6.68	2.73E-18	1.36E-17	OPAR_005066	HPODL_02910	YOR283W	
1.36	5.25	9.20E-12	2.80E-11	OPAR_002432	HPODL_03461	YJR110W	*YMR1*
1.29	6.58	6.85E-21	4.09E-20	OPAR_000036	HPODL_00031	YFR028C	*OAF3*
1.19	5.46	2.48E-06	4.74E-06	OPAR_004387	HPODL_01871	YDL230W	*PTP1*
-0.99	7.74	1.28E-12	4.11E-12	OPAR_003314	HPODL_04287	YDL236W	*PHO13*
0.93	5.77	2.55E-09	6.27E-09	OPAR_001025	HPODL_04916	YBR204C	*LDH1*
0.73	6.09	5.08E-06	9.40E-06	OPAR_002585	HPODL_03597	YBR276C	*PPS1*
0.64	5.10	0.011907	0.01586	OPAR_000879	HPODL_04782	YDL057W	
-0.64	6.01	0.000114	0.000186	OPAR_005045	HPODL_02888	YNL128W	*TEP1*
0.63	5.72	0.000104	0.00017	OPAR_001327	HPODL_00279	YDR481C	*PHO8*
0.58	7.20	1.47E-05	2.60E-05	OPAR_001974	HPODL_00894	YBR092C	*PHO3*
0.52	7.69	8.32E-05	0.000139	OPAR_003977	HPODL_01484	YLR019W	*PSR2*
0.48	6.67	0.001628	0.002382	OPAR_002901	HPODL_03887	YOR155C	*ISN1*
0.45	5.71	0.003449	0.004877	OPAR_004178	HPODL_01675	YNL217W	*PPN2*
-0.43	8.41	0.00185	0.002689	OPAR_005130	HPODL_02972	YNL010W	*PYP1*
0.38	6.44	0.004739	0.006577	OPAR_001369	HPODL_00320		
-0.28	4.14	0.256548	0.289337	OPAR_002548	HPODL_03567	YMR087W	*PDL32*
-0.28	6.41	0.066792	0.081355	OPAR_001685	HPODL_00615	YHR004C	*NEM1*
0.18	6.02	0.220151	0.25152	OPAR_000296	HPODL_02098		
0.12	7.52	0.372061	0.408252	OPAR_002280	HPODL_03321	YLR377C	*FBP1*
0.12	6.55	0.366906	0.402931	OPAR_002299	HPODL_03339	YNL108C	

Supplementary Table 2. Oligonucleotide primers used in this study.

Number	Sequence (5'-3')
1312	GACATGGAGGCCCAGAATAC
15183	TGCTAGAATAGAGACTCATCACCATCGCAATGCCTTTGAC
15184	AACCCATTTCAAGTGCTAGCTTACCATTTCAACAAGTCATCCTTAG
15185	CGGTGGATGCGGAATGAATG
15186	GATGAGTCTCTATTCTAGCAATTGAACTGAAAG
15187	TAGTGATCTCCCTCGAGATG
15188	CTATATTCAGCGGTCGGCGGTGCGATGCCAGTTCTTTGAG
15189	GAAGAATCCGCAGTTGGCGTCTGCCGGTCTCCCTATAGTG
15190	CAAACGGTCAGTGGTCCACGCCGCGTTCTATAGTGTCACC
15191	GCTAGCACTTGAAATGGGTTAAT
15192	ACGCCAACTGCGGATTCTTC
15193	CCGCCGACCGCTGAATATAG
15194	GGCCTGTTCACCGTCAGATG
15195	CATCTCGAGGGAGATCACTAATCTCTTTGCCAACGGGAAC
15196	CATTCATTCCGCATCCACCGGAGACGGGCTGGAAAAAAAAAAG
15197	AGTCCCTGGTGATGCTGAAG
15198	CGTGGACCACTGACCGTTTG
15224	GCAACACGTGCAGGCGAAAC
15225	TGTTTCGCCTGCACGTGTTG
15226	ACGGCTGAAACTCGGGATAC
15227	TCGGCAATGTCCCGGTATCC
15228	TTCCTTCGTGCCCACGACAG
15229	CCGCCGACCGCTGAATATAG
15230	GGTGTCGGTGGTGAATGAAC
15231	CCTGATTGAACTGGCGTATG
15232	GCAGATCCGAGCCTTTATCC
15233	GCGGAACTCTTTGAGCACAG
15234	GATGAACCCTGATGGATACG
15735	GTATTCTGGGCCTCCATGTCAGTGCCTGATGGGTTAGAAAGC
15736	GGGAGCCTTGCCCTTGTTTG
15737	CAAACAAGGGCAAGGCTCCCGCCGCGTTCTATAGTGTCACC
15738	TCGCAAGCGTTTGCCGACACTCTGCCGGTCTCCCTATAGTG
15739	GATTTTTTGTGACCTGTTGTTTGACGCGGGCACTATAATTGGG
15740	GTGTCGGCAAACGCTTGCGAAC
15741	GCTGCGCCGATGGTTTCTAC
15742	TTATGCGGCCATTGTCCGTC
15743	AGGGTTCTCGAGAGCTCCAGTATAG
15744	ATTATTCAAAGCTAGCTTGCGGGAGAGTGTCTATTCGTCATC
15745	CAAAGACTGAATACTATAAAATGCTTGCTGTCAGAAGATTAAC
15746	GCAAGCTAGCTTTGAATAATTTC
15747	CTGGAGCTCTCGAGAACCCTGACTGTAAATTCACAAAACACTGTTTC
15748	TTTATAGTATTCAGTCTTTGAAAAAC
15749	CCGTGTACTGTGGTAAATCC
15894	CGCGTGCTCCTTGATAATAG
15895	TCCAGTCACCCTGAAATTCG

Outlook

Exploitation of yeasts as cell factories for a broad range of industrial bio-processes requires a high stress tolerance and extensive remodeling of cellular metabolism for optimal substrate-to-product conversion. Budding yeasts belonging to the Saccharomycotina clade show a broad diversity of stress tolerance characteristics and metabolic capabilities, which allow them to successfully thrive in different ecological habitats. Most yeasts can conserve free energy for growth and cellular maintenance by respiring carbon sources with oxygen or, alternatively, by fermenting sugars to ethanol and carbon dioxide. In microbial metabolism, oxygen is not only important for energy metabolism and redox balancing. In addition, molecular oxygen is directly involved in the biosynthesis of essential lipids and co-factors. Industrial-scale process conditions expose yeasts to various extreme conditions and in large-scale reactors aeration to accurately dose oxygen is complex and expensive. *Saccharomyces* yeasts are capable of fast growth under anaerobic conditions. This ability is a characteristic trait of a small set of Saccharomycotina yeasts that, in their evolutionary history, underwent a whole-genome duplication (WGD) event. The necessity to supply oxygen to cultures of pre-WGD yeasts, to meet their as yet incompletely understood oxygen requirements for biosynthesis, precludes their exploitation in anaerobic bioprocesses. The research described in this thesis explores the molecular origin of these oxygen requirements and provides new methodologies and insights into the impact of oxygen on the physiology of three industrially relevant yeast species.

Saccharomyces sp. are capable of fast anaerobic growth in synthetic media that are only supplemented with B-type vitamins and, as additional, anaerobic growth factors, ergosterol and unsaturated fatty acids. A common challenge for analysis and characterization of microbial performance is to mimic industrial scale conditions in laboratory experiments. In industrial large-scale bio-processes for production of ethanol, dissolved oxygen is quickly consumed and does not enter the reactor through large permeable sensitive points. The challenges involved in minimizing inadvertent entry of oxygen into small-scale laboratory cultures have contributed to conflicting literature reports on the nature and quantity of oxygen-requirements of yeast species. By careful and iterative improvements of culture methods, **Chapter 3** brings a new critical view on oxygen-contamination and documents methods to minimize oxygen entry in anaerobic cultures. Adoption of new guidelines for anaerobic cultures is essential to resolve conflicting reports on anaerobic growth capability of yeasts. By sharing experiences in experimental design of a 5-year research project on anaerobic cultivation of yeasts, it is aimed to aid the interpretation of published studies on anaerobic yeast physiology and to support implementation and further development these processes in other laboratories. New, promising research lines should focus on engineering and characterization of high-throughput, small-volume growth profilers and analytical equipment in anaerobic chambers. Development of such set-ups will not only enable a further exploration of the naturally diversity of anaerobic yeasts and fungi but also increase the capacity for critical analysis of the impact of specific genetic modifications on anaerobic growth. The implementation of process data

systems will enable unbiased learning from process data by tracking materials and process parameters, and linking it experimental data and results. Ease of sharing methods and protocols will lead to increased reproducibility of studies and will promote scientific discussion on methodology and results.

To identify oxygen requirements of non-*Saccharomyces* yeasts, comparative approaches allow for a context-dependent and in-depth analyses. In such studies, it appears logical to include *Saccharomyces cerevisiae* as a reference. However, even for this extremely well-studied 'model yeast', compilation of solid 'baseline' data sprung a surprise. **Chapter 2** demonstrates that, at least for the popular *S. cerevisiae* laboratory strain CEN.PK113-7D, oxygen requirements predominantly originate from sterol biosynthesis, and sterol supplementation to anaerobic cultures on a glucose synthetic medium with vitamins, sufficed to enable anaerobic growth. Supplementation of unsaturated fatty acids, whose biosynthesis requires molecular oxygen, was beneficial for anaerobic growth but, in contrast to many literature reports, was not necessary. The unsaturated-fatty-acid free strain platform described in this thesis provides an excellent basis for further research aimed at resolving the roles of unsaturated fatty acids in anaerobic and aerobic cultures. In addition, it can be used as a metabolic engineering platform for testing strategies to improve membrane robustness under industrially relevant stress conditions. The extensive characterization of anaerobic growth requirements of the *S. cerevisiae* CEN.PK113-7D strain makes it perfectly suited as benchmark and control for anaerobic cultivation systems, thereby facilitating characterization and comparison of other strains and species and improving reproducibility between studies.

Chapter 4 focuses on identification of the oxygen requirements of the facultatively fermentative thermotolerant yeast *Kluyveromyces marxianus*. Considering the many cellular changes of gene expression and metabolic fluxes in *Saccharomyces* upon the limitation or complete absence of oxygen, the physiology and transcriptome of *K. marxianus* and *S. cerevisiae* were characterized in chemostat cultures under different aeration and anaerobic growth factor supplementation regimes. In-depth comparison of the two yeast strains highlighted the importance of oxygen-dependent sterol biosynthesis as the major factor determining the oxygen requirements of *K. marxianus*. Characterization of sterol uptake in *K. marxianus* and *S. cerevisiae* showed that absence, in *K. marxianus*, of a sterol uptake system that is only present in post-WGD yeasts, played a crucial role in its inability to grow under strictly anaerobic conditions. Even in *S. cerevisiae*, the exact mechanism for sterol uptake and, in particular, the role of cell wall proteins, remains to be fully resolved. The industrial relevance of reconstructing such a system in the thermotolerant yeast *K. marxianus* and other pre-WGD yeasts provides a strong incentive for further fundamental studies on yeast sterol transport mechanisms. Bacteria and some anaerobic fungi have adapted to anaerobic conditions and the lack of sterols by synthesizing sterol surrogates in an oxygen-independent manner (tetrahymanol or hopanoids). This thesis showed that introduction of tetrahymanol synthesis in *K. marxianus* was able to replace oxygen-dependent sterol biosynthesis and enabled anaerobic growth of engineered *K. marxianus* strains. Considering the slow growth of the engineered *K.*

marxianus strain in anaerobic conditions the future challenge will include the optimization of the growth rate and cellular robustness in order to enable to exploit its attractive properties for bioethanol production. Considering the increasing number of fungal infections in humans and the dependency of anti-fungal drugs on the disruption of sterol biosynthesis, sterol uptake and the possibility that fungal pathogens acquire the ability to synthesize sterol surrogates will almost certainly gain more attention.

Chapter 5 describes the response of the facultatively fermentative yeast *Ogataea parapolymorpha* to oxygen limitation. In comparison with the minute biosynthetic oxygen requirements for *K. marxianus*, *O. parapolymorpha* required much larger amounts of oxygen to sustain growth in oxygen-limited cultures. The impact of the addition of the electron acceptor acetoin to oxygen-limited cultures showed that the majority of this oxygen requirement resulted from a need to reoxidize cytosolic NADH by mitochondrial respiration. Heterologous expression of key enzymes of the glycerol pathway, which in *S. cerevisiae* enables reoxidation of biosynthetic derived NADH, in *O. parapolymorpha* only led to modest improvements. Considering the low enzymatic activity of the enzymes in the glycerol pathway compared to glycolytic enzymes it remains unclear why glycerol production rates remained low. Disruption of the mitochondrial glycerol-3-phosphate dehydrogenase resulted in glycerol production in batch cultures and combined with the analysis of its transcriptome suggests that rewiring of the regulation of mitochondrial respiration might be necessary for anaerobic growth. These results will have to be explored further, but already highlight the importance of cell regulation of the redox balance in anaerobic cultures. A comparison of the results presented in Chapters 4 and 5 indicates that, at present, *K. marxianus* looks to be a more promising metabolic engineering platform for developing thermotolerant, facultatively anaerobic yeast strains for industrial production of bioethanol than *O. parapolymorpha*.

The role of oxygen in biosynthetic requirements of budding yeasts across the *Saccharomycotina* subphylum remain to be resolved. Comparative genomics approaches have generated a wealth of datasets but the interpretation of multi-loci traits requires extensive metabolic and physiological experimental data integration. The combination of comparative physiology, genomics and transcriptomics to different aeration and oxygen-regimes in bioreactor cultures, in which the dilution rate was maintained constant, was shown to provide an excellent approach to explore the impact of oxygen on cellular metabolism. Computational models have a great role in predicting cellular metabolism but are currently still largely based on the well-studied *S. cerevisiae*. Integration of the oxygen requirements and the diverse metabolism of budding yeasts in these computational models will enable better predictions and strategies to engineer new yeast cell factories. The development of multi-parallel chemostat bioreactors will enable easier physiological characterization of complex phenomena (e.g. the Crabtree, Kluyver or Custer effects) of a multitude of yeast species and combined with the growing availability of genome sequences of Saccharomycotina yeasts will give a tremendous insight into the evolutionary and or genetic origin of these phenomena.

In conclusion, this thesis provides new insights on the diversity of oxygen requirements in yeasts and on the cellular adaptation of budding yeasts required for anaerobic growth but also underlines that many interesting questions remain to be addressed.

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It is awesome that you are reading this thesis. It took a tremendous amount of work that I could not have done all on my own or without support. I had the pleasure to be accompanied on the journey, of which this thesis is the result, by a lot of great people, whom I would like to thank all for the best companionship.

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Life at IMB can be hectic sometimes and accommodating the craziest experimental ideas from PI's, PhD's and students while both keeping the labs running is certainly a big challenge. Thanks, Erik, Marijke, Pilar and Marcel for keeping the excellent lab infrastructure and atmosphere. Erik, I am really grateful for your contribution to developing anaerobic bioreactors which has certainly not gone unnoticed and I am sure you will keep our bioreactors bench (16-24) the most shiny and tidy of all. Pilar thanks a lot for all your work and exploring the sequencing of new yeast strains together with me. It was a lot of fun and don't forget to blow the troubles (soap-bubbles) away. Marijke, your patience, critical analysis and all experimental tricks were crucial for everything what I did in the Mickel's lab, thanks! Marcel thanks a lot for maintaining the IT infrastructure at IMB and for your advice on our transcriptomic work. For many outsiders your work goes unnoticed but without this infrastructure we could not even have started the omics projects.

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Over the course of my PhD I had the opportunity to supervise several bachelor and master students. Thanks, Astrid, Pieter, Frank, Remon, Minke, Julius, Yassine and Lilian for all your work and enthusiasm. I think you learned a lot during your projects but I certainly learned a lot too. Before you started, I had warned you that I could be a joker and yet you still persisted, questionable? Remember to keep smiling while pipetting as your lab-action picture might be there forever! Although some of the research was very challenging it was a lot of fun and your results contributed or were the foundations for the papers in this thesis, thanks.

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Curriculum Vitae

Wijbrand Joannes Cornelis Dekker was born on the 13th of June 1993 in Rotterdam, the Netherlands. In 2011 he finished pre-university education (VWO) at the Marnix Gymnasium in Rotterdam and started the BSc study Life Science and Technology at Leiden University and Delft University of Technology (TU Delft). He performed his BSc thesis research in the Enzymology group (Biocatalysis and organic chemistry) of the Department of Biotechnology of TU Delft. This project, which investigated the role of metalloprotein ZraP in metal homeostasis in *E. coli*, was supervised by dr. Peter-Leon Hagedoorn. In 2014, Wijb started the MSc program in Life Science and Technology at TU Delft and performed his MSc end project in the Industrial Microbiology section (IMB) at TU Delft, under the supervision of dr. Jasmine Bracher and dr.ir. Ton van Maris. This project focused on pentose metabolism of engineered *S. cerevisiae* strains. Wijb performed a six month internship at Evolva (Reinach, Switzerland). With dr. Michael Naesby as supervisor, he worked on development of a production process for the glycosylated aromatic compound salidroside. In 2016, Wijb returned to the Industrial Microbiology section for a PhD research project supervised by prof. dr. Jack Pronk, this project aimed at identifying and eliminating of oxygen requirements of yeasts. During his PhD, Wijb supervised eight student research projects, co-authored various scientific publications and was inventor on a patent application. Results of this project are described in this thesis. As from June 2021 Wijb works as a scientist group coordinator at Eurofins PSS in Leiden the Netherlands.

List of Publications

Dekker, W. J. C., Jürgens, H., Ortiz-Merino, R. A., Mooiman, C., van den Berg, R., Kaljouw, A., Mans, R., Pronk, J. T. (*Manuscript under review*) Identification of inefficient re-oxidation of cytosolic NADH as the major factor in the oxygen requirement of thermotolerant *Ogataea parapolymorpha*. https::://10.1101/2021.04.30.442227.

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