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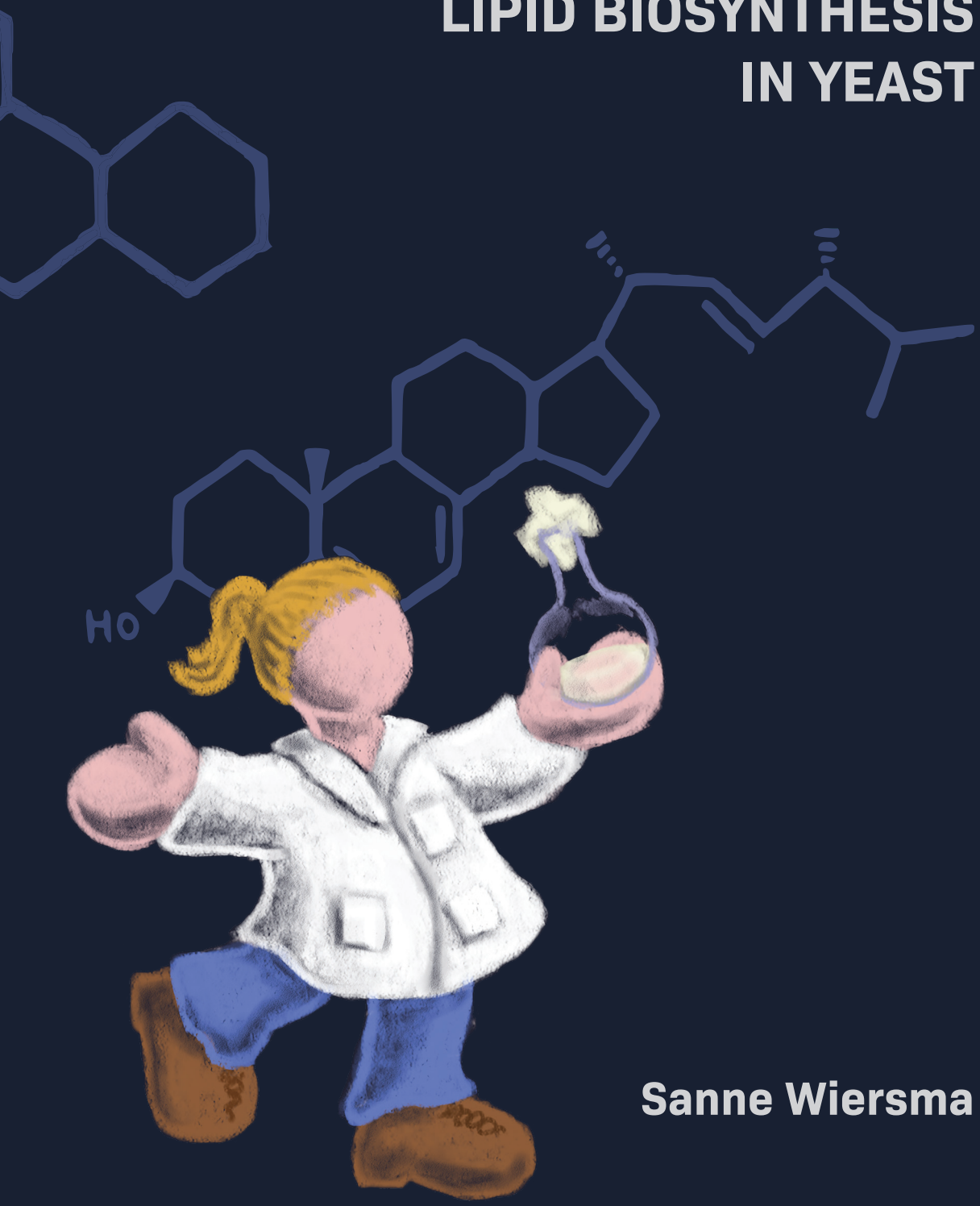
OXYGEN REQUIREMENTS FOR LIPID BIOSYNTHESIS IN YEAST



Sanne Wiersma

Oxygen requirements for lipid biosynthesis in yeast

Proefschrift

ter verkrijging van de graad van doctor

aan de Technische Universiteit Delft,

op gezag van de Rector Magnificus Prof.dr.ir. T.H.J.J. van der Hagen,

voorzitter van het College voor Promoties,

in het openbaar te verdedigen op

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Summary

The yeast *Saccharomyces cerevisiae* has been used by humans for many centuries in microbial fermentation processes for the production of, for example, bread and alcoholic beverages. This long history of use and, in addition, its fast growth, its ability to rapidly convert sugars into ethanol and the ease with which it can be genetically modified, have contributed to this yeast becoming a very popular model organism. *S. cerevisiae* is currently used in large-scale industrial processes for the production of biofuels and a broad range of other chemicals. The ability of *S. cerevisiae* to grow in the absence of oxygen is quite unique among yeasts, and not only important for the production of beer and wine, but also for industrial production of bulk chemicals. The high product yields that are required in these types of processes can in theory only be achieved when sugars are completely converted into product, instead of being partially or completely oxidized to CO_2 via aerobic respiration.

Fast anaerobic growth of *S. cerevisiae* does require that standard synthetic media, that are used to grow this yeast in the laboratory, are supplemented with a number of additional components. These additional nutritional requirements originate from the fact that oxygen is required for biosynthesis of some important components of the yeast cell. While most yeast species are actually able to ferment, they usually cannot grow in the complete absence of oxygen at all, not even when such cell components or their precursors are added to anaerobic growth media. As a consequence, some yeast species that have industrially relevant traits that are absent or less pronounced in *S. cerevisiae*, such as resistance to higher temperatures, cannot at the moment be used in anaerobic industrial processes.

This PhD thesis describes research on oxygen requirements related to membrane synthesis in yeast, using *S. cerevisiae* as the main model organism, with the goal to understand these requirements and eliminate them by genetic modification. Inspiration is obtained from evolutionary adaptations of eukaryotic microorganisms that naturally occur in anaerobic or oxygen-poor environments. Metabolic engineering strategies developed in this way may then possibly be applied to other industrially relevant yeast species and thus aid the elucidation of additional, as yet unknown oxygen requirements, or even enable anaerobic growth of those yeasts as well.

Chapter 1 starts with describing how the gradual rise of atmospheric oxygen levels throughout Earth's history played a role in the development of life on Earth. Initially, Earth's atmosphere was free of oxygen, but this changed when oxygenic photosynthesis developed in an ancestor of modern cyanobacteria. The increasing levels of this very reactive molecule and strong electron acceptor in the biosphere had a strong influence on the evolution of life. In particular, the development of aerobic respiration as new mode of energy metabolism played an important role in the origin of eukaryotic organisms. The acquisition of the mitochondrion, through endosymbiosis of an at least facultatively aerobic, bacterial endosymbiont in an archaeal host, places aerobic respiration at the very beginning of eukaryotic life. In the modern era the majority of eukaryotic organisms, including our own species, still to a greater or lesser extent depends on oxygen. **Chapter 1** next describes how this oxygen dependency manifests itself in yeasts. Oxygen requirements can be found in pathways for *de novo* synthesis of cofactors such as pantothenate, thiamine, biotin and NAD^+. The synthesis of the pyrimidine building blocks of DNA and RNA also requires oxygen in some yeasts, and the balancing of the oxidized and reduced forms of the electron carriers NAD^+/NADH can be dependent on a functional respiratory chain (and therefore, on oxygen as electron acceptor). In *S. cerevisiae*, the synthesis of unsaturated fatty acids and sterols, which are both important constituents of the cell membrane, together account for the bulk of total biosynthetic oxygen requirements. *S. cerevisiae* can only grow anaerobically when an external source of these molecules is present in growth media. To provide context for the following chapters, the most important properties and building blocks of membranes are discussed in **Chapter 1**. Because of the central role that unsaturated fatty acids and sterols play in this dissertation, the pathways for *de novo* synthesis of these molecules are discussed in detail at the end of this introductory chapter.

In *S. cerevisiae* and other yeasts, *de novo* synthesis of unsaturated fatty acids requires oxygen as electron acceptor for the desaturation of saturated fatty acids by the enzyme Ole1. For anaerobic growth of yeasts, it is therefore common practice to add Tween 80, a complex sorbitan-ester of oleic acid, to synthetic media to provide an external source of unsaturated fatty acids. *S. cerevisiae* can store a surplus of fatty acids and/or sterols, either obtained through supplementation or *de novo* synthesis, in lipid droplets in the cell. Studying the influence of biosynthetic oxygen requirements on the physiology of yeast under anaerobic conditions therefore requires the dilution of such intracellular stores of lipids in (sometimes multiple) subsequent anaerobic batch cultures. In **Chapter 2**, it was attempted to restrict anaerobic growth by limiting the availability of unsaturated fatty acids. To this end, multiple consecutive anaerobic batch experiments were performed with the yeast strain *S. cerevisiae* CEN.PK113-7D in bioreactors. These cultures were grown on synthetic media to which ergosterol was added as anaerobic growth factor, but from which a source of unsaturated fatty acids was omitted. Surprisingly, anaerobic growth was consistently observed during these experiments. Analysis

of the fatty acid composition of yeast biomass showed that the levels of palmitoleic acid and oleic acid were considerably lower in these experiments than in aerobic cultures. The presence of small quantities of such unsaturated fatty acids, which was ascribed to *de novo* synthesis, pointed towards an unintended, minimal leakage of oxygen into the bioreactors. To further minimize the effect of oxygen leakage, additional experiments were performed in an anaerobic chamber, and to prevent *de novo* synthesis of unsaturated fatty acids the *OLE1* gene was deleted in *S. cerevisiae* CEN.PK113-7D. After an anaerobic pre-culture without ergosterol or Tween 80, both the *ole1*-deletion mutant and the CEN.PK113-7D strain grew in three consecutive batch cultures on medium to which ergosterol was added, but from which Tween 80 was again omitted. In biomass from these three batch cultures, no unsaturated fatty acids were detected, which demonstrated unsaturated-fatty-acid independent growth of this *S. cerevisiae* strain. The contribution of saturated fatty acids with a shorter chain length of 10 to 14 carbon atoms to the total fatty acid composition was higher in these cultures than in a culture that was supplemented with Tween 80. This shortening of the fatty acid chains was interpreted as a mechanism to maintain membrane fluidity in the absence of unsaturated fatty acids. In contrast to anaerobic growth, aerobic growth of the *ole1*-deletion mutant strictly depended on supplementation with a source of unsaturated fatty acids. This study demonstrated that the requirement of anaerobic *S. cerevisiae* cultures for unsaturated fatty acids is not as strict as has been assumed in scientific literature for a long time. The findings described in **Chapter 2** therefore provide an incentive to investigate the influence of membrane composition on viability and robustness of yeasts in greater detail.

Formation of sterols is strictly dependent on oxygen because of the involvement of multiple complicated demethylation- and desaturation reactions in the strongly conserved pathway for their *de novo* synthesis. Because an oxygen-independent route for sterol synthesis has never been described or demonstrated, sterols need to be replaced by a molecule that can function as 'sterol surrogate' in order to circumvent oxygen requirements for sterol synthesis. Neocallimastigomycetes form a group of strictly anaerobic fungi that demonstrate many adaptations to growth in the absence of oxygen. One example is that they produce the molecule tetrahymanol, which functions as sterol surrogate in these fungi and in some anaerobic protists. Tetrahymanol is a pentacyclic triterpenoid that is directly produced from squalene in a single, oxygen-independent conversion by squalene-tetrahymanol cyclase. **Chapter 3** investigates whether ergosterol can be replaced by tetrahymanol in yeast.

To this end, a gene encoding a squalene-tetrahymanol cyclase from the ciliate *Tetrahymena thermophila* (*TtTHC1*) was expressed in *S. cerevisiae*. Gas-chromatography analysis demonstrated that this modified yeast strain indeed produced tetrahymanol. Tetrahymanol synthesis did not have an effect on the growth rate in anaerobic bioreactor experiments in the presence of Tween 80 and ergosterol. The tetrahymanol-producing *S. cerevisiae* strain

could also grow in absence of ergosterol supplementation but the presence of lanosterol, an intermediate of the sterol-synthesis pathway, in anaerobic biomass indicated a minimal oxygen leakage into the bioreactors. Sterol-free growth was demonstrated under aerobic and anaerobic conditions by blocking native sterol synthesis through deletion of the *ERG1* gene. Anaerobic cultures of the resulting tetrahymanol-producing *S. cerevisiae* strains (either with an intact native sterol synthesis pathway or in a sterol-deficient background) showed lower growth rates and biomass yields in absence of sterol supplementation, but the ethanol yield on sugar was higher. This study demonstrates how the acquisition of a single gene encoding for a cyclase can have conferred an advantage during the evolution of Neocallimastigomycetes. In addition, the results of **Chapter 3** provide a basis to circumvent oxygen requirements related to sterol synthesis in yeast with the aid of genetic modification, which can be important for application of yeasts in anaerobic industrial processes.

The few eukaryotic microorganisms that are described to be naturally able to grow without sterols have all been shown to use tetrahymanol as sterol surrogate. In **Chapter 4**, an earlier report from 1971, which described intriguing observations on the yeast *Schizosaccharomyces japonicus*, was confirmed. Precisely as described by the Dutch scientist Bulder, anaerobic growth of this yeast turned out to be independent of supplementation of sterols or unsaturated fatty acids. Anaerobic biomass of *Sch. japonicus* did not contain sterols, but gas-chromatography analysis detected a number of unidentified compounds in the triterpenoid fraction of the membrane lipids of this yeast. Based on their retention times, none of these molecules could be tetrahymanol. The remainder of this chapter was therefore dedicated to uncovering the identity and origin of these components. The genomes of two *Sch. japonicus* strains (CBS5679 and yFS275) contained a putative squalene-hopene cyclase gene in addition to a putative oxidosqualene cyclase gene. The strong similarity between the former sequences and bacterial squalene-hopene cyclases, in combination with the absence of similar sequences in other yeast species, suggested horizontal gene transfer as probable origin of the putative squalene-hopene cyclase in *Sch. japonicus*. With mass spectrometry, the unknown compounds in *Sch. japonicus* biomass were identified as hopanoids, among which hop-22(29)-ene (diploptene), hop-17(21)-ene, hop-21(22)-ene and hopan-22-ol (diplopterol).

Hopanoids are a class of cyclic triterpenoids that are structurally related to tetrahymanol and mainly occur in bacteria. The hypothetical squalene-hopene cyclase gene from *Sch. japonicus* CBS5679 (*Sjshc1*) was expressed in *S. cerevisiae*, after which the same hopanoid compounds were detected in biomass of anaerobically grown cultures of the modified *S. cerevisiae* strain. Ergosterol-independent growth of this yeast strain was demonstrated in anaerobic shake-flask experiments, showing that at least one of the hopanoid molecules could function as sterol surrogate in anaerobic yeast cultures. The results described in **Chapter 4** demonstrate that hopanoid molecules can function as sterol surrogate in yeast and, therefore, that hopanoid

synthesis represents a previously unidentified adaptation of eukaryotic cells to anaerobic conditions. Furthermore, the fast sterol- and unsaturated fatty acid-independent growth of *Sch. japonicus* is an interesting trait to use as inspiration for the development of robust fungal cell factories for application in anaerobic industrial processes.

Biosynthetic oxygen requirements, as described in **Chapter 2**, **3**, and **4** of this thesis, are very small in comparison to the amount of oxygen used in aerobic respiration. Unless extensive measures are taken to prevent unintended entry of traces of oxygen, they can easily be overlooked in small, laboratory-scale cultivation systems. For this reason, over the course of four years of research on biosynthetic oxygen requirements of yeasts within the ELOXY (eliminating oxygen requirements in yeasts) project, procedures for the cultivation of yeasts in bioreactors and anaerobic chambers were continuously adapted and improved. In **Chapter 5**, crucial considerations for the design of such experiments are described, and protocols for the use of anaerobic chambers and bioreactors are presented. The deliberate and limited use of the air lock of an anaerobic chamber, and minimizing oxygen entry through tubing used in bioreactor set-ups are identified as important points of attention. In addition, the use of multiple consecutive transfers to fresh media to deplete intracellular stores of growth factors and the systematic inclusion of control-strains in experiments are presented as important prerequisites for enabling reliable conclusions about the influence of biosynthetic oxygen requirements in yeasts under anaerobic conditions.

In this thesis, various concepts that can be used to eliminate or circumvent oxygen requirements related to membrane synthesis in yeasts have successfully been applied and studied. These concepts can serve as basis for further research on the influence of membrane composition and function on the robustness of industrial fungal cell factories.

Samenvatting

De gist *Saccharomyces cerevisiae* wordt al eeuwen door mensen gebruikt voor de productie van voedingsmiddelen, zoals brood en alcoholische dranken, in microbiële fermentatieprocessen. Mede dankzij deze lange historie is deze gist nu een zeer populair modelorganisme geworden. Bovendien groeit *S. cerevisiae* snel, zet suikers met hoge snelheid om in alcohol en kan gemakkelijk genetisch worden gemodificeerd. *S. cerevisiae* wordt in grootschalige industriële processen toegepast voor de productie van biobrandstoffen en andere chemicaliën, waaronder ook medicijnen. Het voor gisten vrij unieke vermogen van *S. cerevisiae* om te kunnen groeien in afwezigheid van zuurstof is niet alleen belangrijk voor het maken van bier en wijn maar ook voor industriële productie van bulkchemicaliën. De vereiste hoge productopbrengsten in deze processen zijn in theorie alleen haalbaar als alle suiker wordt omgezet in product door middel van fermentatie, in plaats van dat de suiker geheel of gedeeltelijk wordt verbrand tot CO_2 met behulp van zuurstof.

Voor snelle anaerobe groei van *S. cerevisiae* is het wel nodig om een aantal extra componenten toe te voegen aan de standaard synthetische media waarin *S. cerevisiae* wordt gekweekt in laboratoria. De oorzaak van deze voedingsbehoefte ligt in het feit dat zuurstof nodig is voor biosynthese van een aantal belangrijke componenten van de gistcel. Hoewel de meeste andere gistsoorten ook in staat zijn tot fermentatie, kunnen ze vaak zelfs helemaal niet groeien in afwezigheid van zuurstof, ook niet als deze componenten aan anaerobe kweekmedia worden toegevoegd. Dit heeft tot gevolg dat sommige gisten die vanuit industrieel perspectief interessantere voordelen bieden ten opzichte van *S. cerevisiae*, zoals bijvoorbeeld een tolerantie tegen hogere temperaturen, op dit moment niet kunnen worden ingezet in anaerobe industriële processen.

Dit proefschrift is gewijd aan het bestuderen van de zuurstofbehoeften gerelateerd aan membraansynthese in gist, met als doel om deze behoeften te begrijpen en te omzeilen met behulp van genetische modificatie. Hierbij wordt *S. cerevisiae* als belangrijkste modelorganisme gebruikt, en wordt inspiratie geput uit de evolutionaire aanpassingen van eukaryote micro-organismen die van nature voorkomen in zuurstofloze omgevingen. Lessen die getrokken worden uit dit onderzoek aan *S. cerevisiae* kunnen mogelijk later worden toegepast in andere industrieel relevante gisten om hun verdere zuurstofbehoeften te kunnen bestuderen, of zelfs om anaerobe groei van deze gisten mogelijk te maken.

In **Hoofdstuk 1** wordt, ter introductie, uiteengezet hoe het verloop van het zuurstofgehalte in de atmosfeer in de geschiedenis van de aarde van invloed is geweest op het zich ontwikkelende leven. Bij het ontstaan van de aarde was de atmosfeer vrij van zuurstof, maar dat veranderde toen oxygene fotosynthese ontstond in een verre voorouder van moderne cyanobacteriën. De toenemende aanwezigheid van dit zeer reactieve molecuul en tevens sterke elektronacceptor had een sterke invloed op de evolutie van het leven op aarde. Met name het ontstaan van aerobe ademhaling als vorm van energiemetabolisme is van groot belang geweest voor het ontstaan van eukaryote organismen. Met het verkrijgen van het mitochondrion, via endosymbiose van een op zijn minst facultatief aerobe, bacteriële endosymbiont in een archaeale gastheer, deed ademhaling al aan begin van de eukaryote stamboom haar intrede. In het moderne tijdperk is het grote merendeel van alle eukaryote organismen, waaronder wijzelf, nog steeds in meerdere of mindere mate afhankelijk van zuurstof. In **Hoofdstuk 1** wordt besproken hoe deze zuurstofafhankelijkheid in gisten onder andere terug te zien is in *de novo* synthese van de cofactoren pantothenaat, thiamine en biotine en NAD^+. In sommige gisten is ook voor de synthese van pyrimidine-bouwstenen van DNA en RNA zuurstof nodig, of is een functionele ademhalingsketen (en dus zuurstof als elektronacceptor) een vereiste voor het balanceren van de geoxideerde en gereduceerde vorm van het elektronendragende koppel NAD^+/NADH. In *S. cerevisiae* komt het grootste aandeel van de biosynthetische zuurstofbehoeften voor rekening van de synthese van onverzadigde vetzuren en ergosterol. Deze moleculen zijn belangrijke componenten van het celmembraan, waarvan in **Hoofdstuk 1** de belangrijkste eigenschappen en bouwstenen worden besproken. *S. cerevisiae* kan alleen anaeroob groeien als externe bronnen van deze moleculen aanwezig zijn in het kweekmedium. Vanwege de centrale rol die onverzadigde vetzuren en ergosterol spelen in dit proefschrift, worden aan het slot van **Hoofdstuk 1** de stofwisselingsroutes voor *de novo* synthese van deze verbindingen in detail besproken.

Bij *de novo* synthese van onverzadigde vetzuren door *S. cerevisiae* en andere gisten dient zuurstof als elektronacceptor voor de desaturatie van verzadigde vetzuren door het enzym Ole1. Om deze reden is het gebruikelijk dat Tween 80, een complexe sorbitan-ester van oliezuur, als bron van onverzadigde vetzuren aan synthetische media voor anaerobe groei van gisten wordt toegevoegd. *S. cerevisiae* kan een overschot aan toegevoegde of geproduceerde vetzuren en/of sterolen opslaan als vetdruppels in de cel. Voordat de invloed van biosynthetische zuurstofbehoeften op de fysiologie van gisten kan worden bestudeerd in anaerobe gistcultures, moeten zulke intracellulaire voorraden eerst worden verdund in (soms meerdere) opeenvolgende anaerobe batchcultures. In **Hoofdstuk 2** werd geprobeerd om anaerobe groei te verhinderen door een tekort aan onverzadigde vetzuren te creëren. Hiertoe werden meerdere opeenvolgende anaerobe batchcultures uitgevoerd met de giststam *S. cerevisiae* CEN.PK113-7D in bioreactoren, waarbij synthetische media

werden gebruikt waaraan wel ergosterol als anaerobe groeifactor was toegevoegd, maar waarbij Tween 80 was weggelaten. Verrassenderwijs werd hierbij stelselmatig anaerobe groei waargenomen. Analyse van de vetzuursamenstelling van gistbiomassa liet zien dat gehalten van palmitoleïnezuur en oliezuur aanzienlijk lager waren dan in aerobe cultures. De aanwezigheid van kleine hoeveelheden van deze onverzadigde vetzuren werd toegeschreven aan *de novo* synthese, en dus aan een onbedoeld, miniem zuurstoflek in de bioreactoren. Om de bijdrage van dit zuurstoflek verder te minimaliseren werden aanvullende experimenten verricht in een anaerobe kamer. Bovendien werd, om *de novo* synthese van onverzadigde vetzuren te verhinderen, het *OLE1* gen in de *S. cerevisiae* CEN.PK113-7D stam uitgeschakeld. Na een anaerobe voorculture zonder ergosterol of Tween 80, groeiden deze mutant en de CEN.PK113-7D stam in drie opeenvolgende batch cultures op medium waaraan wederom wel ergosterol, maar geen Tween 80 was toegevoegd. In biomassa afkomstig uit deze drie batch cultures werden geen onverzadigde vetzuren aangetroffen. De bijdrage van verzadigde vetzuren met een kortere ketenlengte van 10 tot 14 koolstofatomen aan de totale vetzuursamenstelling was in deze cultures hoger dan in een cultuur waaraan wel Tween 80 was toegevoegd. Deze ketenverkorting werd geïnterpreteerd als een mechanisme om membraanvloeibaarheid te behouden in afwezigheid van onverzadigde vetzuren. Hoewel anaerobe groei van de *Δole1*-mutant onafhankelijk bleek te zijn van synthese of toevoeging van onverzadigde vetzuren, was aerobe groei van deze giststam wel strikt afhankelijk van de aanwezigheid van een bron van onverzadigde vetzuren in het medium. Deze studie toont aan dat de behoefte van anaerobe cultures van *S. cerevisiae* voor onverzadigde vetzuren niet zo strikt is als heel lang in de wetenschappelijke literatuur is aangenomen. De bevindingen die in **Hoofdstuk 2** zijn beschreven nodigden dan ook uit om de invloed van de membraansamenstelling op de levensvatbaarheid en robuustheid van gisten verder te onderzoeken.

De productie van sterolen is strikt afhankelijk van zuurstof vanwege de vele gecompliceerde demethylerings- en desaturatiereacties in de sterk geconserveerde *de novo* syntheseroute. Omdat er nog nooit een zuurstof-onafhankelijke route voor sterol-synthese is beschreven of aangetoond, moeten sterolen worden vervangen door een zuurstof-onafhankelijk “sterolsurrogaat” om zuurstofbehoeften voor sterolsynthese te omzeilen. Neocallimastigomyceten vormen een groep van strikt anaerobe schimmels die vele aanpassingen vertoont aan groei in afwezigheid van zuurstof. Eén hiervan is dat ze het molecuul tetrahymanol maken, waarvan is aangetoond dat het dient als sterolsurrogaat in deze schimmels en in enkele anaerobe protisten. Tetrahymanol is een pentacyclisch triterpeen dat wordt gevormd door directe, zuurstof-onafhankelijke cyclisatie van squaleen. In **Hoofdstuk 3** wordt onderzocht of ergosterol kan worden vervangen door tetrahymanol in gist. Hiertoe werd een gen uit *Tetrahymena thermophila* dat codeert voor een squaleen- tetrahymanolcyclase

(*TtTHC1*) tot expressie gebracht in *S. cerevisiae*. Met behulp van gaschromatografie werd aangetoond dat de gemodificeerde giststam inderdaad tetrahymanol maakte. Anaerobe bioreactor experimenten wezen uit dat dit geen significant effect had op de groeisnelheid in aanwezigheid van Tween 80 en ergosterol. De tetrahymanol-producerende *S. cerevisiae* stam groeide ook in afwezigheid van ergosterol in het synthetische medium, maar de aanwezigheid van lanosterol, een intermediair uit de sterol-synthese route, wees op een miniem zuurstoflek in de bioreactoren. Om volledig sterol-vrije groei onder aerobe en anaerobe omstandigheden te kunnen bestuderen werd de sterolsynthese in de tetrahymanol-producerende giststam geblokkeerd door deletie van het gen *ERG1*. Anaerobe culturen van de resulterende tetrahymanol-producerende *S. cerevisiae* stammen (zowel met intacte sterol-synthese als de sterolsynthese deficiënte stam) hadden een lagere biomassa-specifieke groeisnelheid en biomassaopbrengst in afwezigheid van ergosterol, maar de ethanolopbrengst op suiker was hoger. Deze studie laat zien hoe het verkrijgen van slechts één enkel gen een direct voordeel biedt voor anaerobe groei van gist in een zuurstof- en sterol-gelimiteerde omgeving. Deze waarneming illustreert hoe het verwerven van een gen dat codeert voor een cyclase een voordeel kan hebben geboden gedurende de evolutie van Neocallimastigomyceten. Bovendien legt het in **Hoofdstuk 3** beschreven onderzoek een basis om zuurstofbehoeften gerelateerd aan sterolsynthese in gisten te omzeilen met behulp van genetische modificatie, wat van belang kan zijn voor het gebruik van gisten in anaerobe industriële processen.

Van het kleine aantal eukaryote microorganismen waarvan sterolonafhankelijke anaerobe groei is vastgesteld, is tevens bekend dat ze het hierboven beschreven molecuul tetrahymanol maken als sterolsurrogaat. In **Hoofdstuk 4** werd in eerste instantie bevestigd dat intrigerende waarnemingen in een publicatie uit 1971, over anaerobe groei van de gist *Schizosaccharomyces japonicus*, klopten. Precies zoals de Nederlandse gistonderzoeker Bulder in deze publicatie rapporteerde, bleek dat anaerobe groei van deze gist onafhankelijk is van een externe bron van sterolen en onverzadigde vetzuren. Anaerobe biomassa van *Sch. japonicus* bevatte geen sterolen, maar met behulp van gaschromatografie werden wel meerdere ongeïdentificeerde moleculen aangetroffen in de triterpeen-fractie van de membraanlipiden van deze gist. Op grond van hun retentietijden konden geen van deze moleculen tetrahymanol zijn. De rest van dit hoofdstuk wijdde zich daarom aan het achterhalen van de identiteit en de herkomst van deze membraancomponenten. De genomen van twee *Sch. japonicus* stammen (CBS5679 en yFS275) bevatten, naast een vermoedelijk oxidosqualeencyclase, elk ook een DNA-sequentie die sterke homologie vertoonde met bacteriële squaleen-hopeencyclases. De bijzonder sterke overeenkomst tussen deze laatste sequenties en die van azijnzuurbacteriën, in combinatie met de afwezigheid van homologe sequenties in andere gisten, wees op horizontale genoverdracht als mogelijke herkomst van deze coderende sequenties in *Sch. japonicus*. Met behulp van massaspectrometrie kon worden aangetoond dat *Sch. japonicus*

hopanoïde moleculen produceert, waaronder hop-22(29)-een, hop-17(21)-een, hop-21(22)-een en hopan-22-ol. Hopanoïden vormen een klasse van cyclische triterpenen die structureel verwant zijn aan tetrahymanol en vooral in bacteriën voorkomen. Het hypothetische squaleen-hopeencyclase gen van *Sch. japonicus* CBS5679 (*Sjshc1*) werd tot expressie gebracht in *S. cerevisiae*, waarna dezelfde hopanoïde moleculen werden aangetroffen in anaeroob gekweekte biomassa van de gemodificeerde *S. cerevisiae*-stam. Ergosterol-onafhankelijke groei van deze stam werd vervolgens aangetoond in anaerobe schudkolfexperimenten, waaruit werd afgeleid dat in ieder geval een van de gevormde hopanoïden kon dienen als sterolsurrogaat in anaerobe gistcultures. De in **Hoofdstuk 4** beschreven resultaten tonen aan dat hopanoide moleculen als sterol-surrogaat kunnen fungeren en, daarmee, dat hopanoïde-synthese een niet eerder gevonden aanpassing van eukaryote cellen aan anaerobe omstandigheden is. Bovendien is de snelle sterol- én onverzadigd vetzuur-onafhankelijke anaerobe groei van *Sch. japonicus* een interessante eigenschap als inspiratiebron voor het ontwikkelen van robuuste gist- en schimmel-celfabrieken voor toepassing in anaerobe industriële processen.

Biosynthetische zuurstofbehoeften, zoals beschreven in **Hoofdstuk 2**, **3** en **4** van dit proefschrift, zijn over het algemeen zeer klein in verhouding tot het gebruik van zuurstof in aerobe ademhaling. Tenzij uitgebreide maatregelen worden genomen om ongewenst binnendringen van sporen zuurstof te voorkomen, worden ze daarom gemakkelijk over het hoofd gezien in kleine, lab-schaal kweeksystemen. Gedurende vier jaar onderzoek naar biosynthetische zuurstofbehoeften in gisten binnen het ELOXY (eliminating oxygen requirements in yeast) project werd daarom voortdurend geprobeerd om protocollen voor het kweken van gisten in anaerobe kamers en bioreactoren aan te scherpen. In **Hoofdstuk 5** worden cruciale punten voor het ontwerp van zulke experimenten beschreven en, op basis hiervan, protocollen voor het gebruik van anaerobe kamers en bioreactoren gepresenteerd. Onder andere het weloverwogen gebruik van de luchtsluis van een anaerobe kamer en het minimaliseren van het binnendringen van zuurstof door slangen en buizen van apparatuur zijn daarbij belangrijke punten van aandacht. Daarnaast worden het opeenvolgend overbrengen van inocula naar vers medium om intracellulaire voorraden van groeifactoren te verdunnen, en het systematisch opnemen van controlestammen in experimenten, aangemerkt als belangrijke voorwaarden om betrouwbare conclusies te kunnen trekken over de invloed van biosynthetische zuurstofbehoeften op de fysiologie van gist onder anaerobe omstandigheden.

In dit proefschrift zijn verscheidene concepten voor het elimineren of vermijden van zuurstofbehoeften gerelateerd aan membraansynthese in gist succesvol toegepast en uitgewerkt. Deze concepten kunnen dienen als basis voor verder onderzoek naar de invloed van membraansamenstelling en -functie op de robuustheid van industriële schimmel-cel fabrieken.

1

Introduction

1 Life and oxygen: an intricate relationship

We intuitively and strongly associate molecular oxygen with respiration, with energy and, indeed, with life. This is not surprising, since approximately 21 % of the air that fills our lungs when we breathe in consists of oxygen. Our dependence on oxygen makes it hard to imagine Earth without O_2, which is one of the first signature molecules we look for when searching for life on other planets[1]. However, several billions of years ago, on a young Earth, oxygen had not yet made its entry into what was to become 'our' world. How the chemical composition of the Earth's atmosphere and oceans gradually changed, and how this may have influenced the diversification of life on our planet, represents an intriguing story of scientific discovery.

1. The rise of oxygen | Planet Earth is estimated to be roughly 4.5 billion years (Gy) old[2]. Studies on the chemical composition of ancient rock formations and sediments, of which the approximate age can be determined with various radiometric and paleomagnetic dating techniques[3,4], have established that the early Earth's atmosphere must have been anoxic and reducing at the start[5,6] (Figure **1.1**). This conclusion is, for example, based on a mass-independent fractionation (MIF) of sulfur isotopes in most sediments that are older than 2.45 Gy. Photolysis of sulfur species in volcanic gases (e.g. SO_2) by UV radiation induced MIF of sulfur isotopes in the early Earth's atmosphere. In an anoxic environment, this UV-induced MIF was not diluted over various pools of oxidized sulfur species but, after precipitation, preserved in the geochemical record. Analyses of such sulfur isotope distributions pose an upper limit for the oxygen concentration in the early Earth's atmosphere that is at least a hundred thousand fold lower ($1{\cdot}10^{-5}$) than present atmospheric levels (PAL)[7,8]. Similarly, oxygen-sensitive detrital grains are common in rock layers dated to until 2.2 Gy ago[9]. Such distinct markers of a virtually oxygen-free atmosphere disappear from the geological record afterwards. Instead, the deposition of iron oxides in banded iron formations that occurred between 2.8 and 1.85 Gy ago[10] and the presence of iron-containing redbeds an manganese- and copper deposits from 2.3 Gy ago onwards[11] indicate increased levels of oceanic and atmospheric oxygen. The geological records of isotopes of carbon, nitrogen and various other elements are also in accordance with a dramatic increase in atmospheric oxygen levels in the period between 2.5 to 2.0 Gy ago. This increase is commonly referred to as the 'Great Oxidation Event' (GOE)[12,13]. The atmospheric oxygen concentration during and after the GOE is hard to constrain and its stability over time is still subject to scientific debate[14,15]. The oceanic oxygen concentration remained too low to assume the presence of modern atmospheric oxygen levels for over a billion years afterwards[16], but there is broad consensus that the atmospheric oxygen concentration did not again decrease to levels allowing for the existence of redox-sensitive grains such as pyrite and uranite. A second surge in atmospheric oxygen

occurred approximately two billion years after the GOE (~0.6 Gy ago) and is referred to as the 'Neoproterozoic Oxidation Event' (NOE)[17–19]. Only at this stage, the deep ocean became oxygenated, as indicated by further changes in carbon, sulfur and other elemental isotopes, and atmospheric oxygen started to increase to its current level.

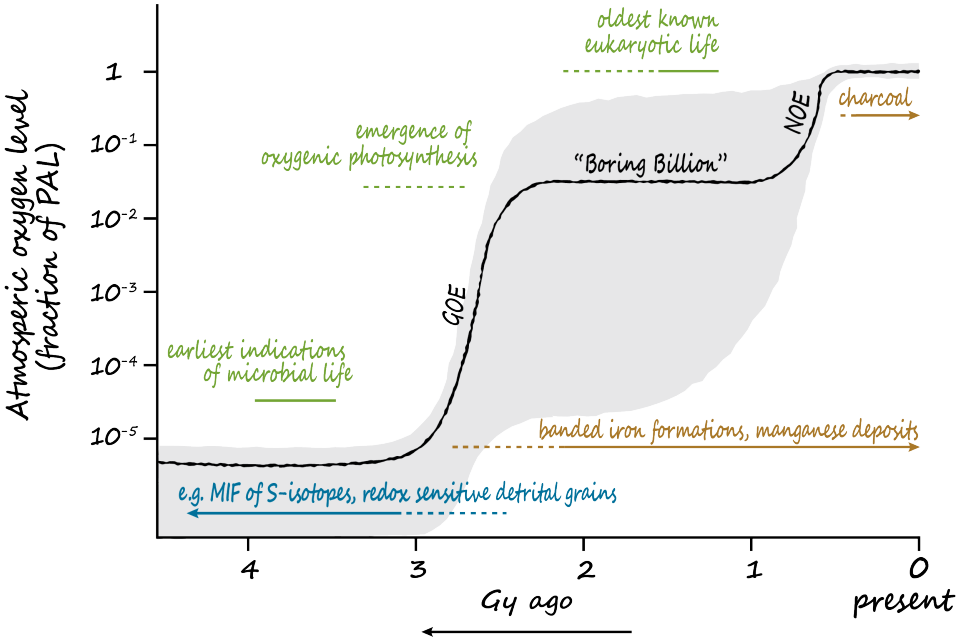

Figure 1.1: Estimated atmospheric oxygen levels throughout Earth's history. Ancient rock formations and sediments have provided geochemical evidence for constraints on atmospheric oxygen levels. A schematic illustration of how the atmospheric oxygen levels changed throughout Earth's history is represented by the black line, and the grey area indicates the uncertainty surrounding the exact oxygen levels. Evidence for a low-oxygen environment is indicated in blue. MIF, 'mass independent fractionation'; S, sulfur. Evidence for increased oxygen levels is indicated in orange. Milestones in the evolution of life on Earth are indicated in green. PAL, 'present atmospheric level'; GOE, 'Great Oxidation Event'; NOE, 'Neoproterozoic Oxidation Event.

2. A biological origin for the Earth's oxygenation | The uninitiated reader may now wonder what triggered these bursts of oxygen throughout Earth's history. The answer is simple: life, and in particular, life's 'invention' of oxygenic photosynthesis[20]. The earliest biological evidence of microbial life in the fossil record predates 3.2 Gy ago[21], and supports that life was present on Earth well before the GOE. In this anoxic environment, anaerobic chemo/photo-lithotrophic microorganisms prevailed[22]. Phototrophy, a mode of energy metabolism in which the energy of photons is converted into chemical energy that can be used by cells, may already be very ancient[23]. Early phototrophic organisms could have relied on various reduced compounds like ferrous iron (Fe^{2+}) or hydrogen gas as electron

donors, and were anoxygenic[20,24]. Primary production by anoxygenic photosynthesis would ultimately have been limited by the availability of electron donors[25]. Light-dependent oxidation of water molecules, leading to the release of oxygen, required a more advanced type of electron chemistry, in which four charge separation events are linked in a single reaction center[24].

Sunlight-driven systems for water oxidation first emerged in an ancestor of current cyanobacteria, which today remain the only class of bacteria capable of oxygenic photosynthesis[20,24]. Access to water as electron donor unlocked an enormous potential for primary productivity. Water oxidation could be used to build the electrochemical gradients across biological membranes that are harnessed to energize biomass formation from CO_2, while releasing O_2 as a by-product. Although a few other examples of biotic[26–28] and abiotic[29,30] sources of oxygen exist, it is generally accepted that only oxygenic photosynthesis can have produced enough oxygen to eventually overcome the pull of Earth's reductive sinks, which mostly consisted of ferrous iron and methane in oceans and in the atmosphere, respectively[5,31]. Oxygenic photosynthesis must therefore have emerged between the appearance of the earliest life forms and the GOE, but the exact timing is still topic of debate[31–35].

When one considers the implications of the increasing levels of oxygen for life, an intriguing paradox arises. Molecular oxygen is highly reactive: it easily accepts an extra electron from a range of electron donors, and thereby forms the highly reactive superoxide radical (O_2^-)[36]. Superoxide can further react with hydrogen to form hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\bullet$), which readily attacks and damages DNA and other cellular biomolecules[37]. In current living organisms that are regularly exposed to air, the enzymes superoxide reductase and superoxide dismutase are responsible for limiting the damaging effects of such reactive oxygen species (ROS)[36]. The H_2O_2 that is produced in this detoxification can be further decomposed to water and oxygen by the enzyme catalase[38]. But how could ancient, anaerobic life forms have survived exposure to ROS? Perhaps early, local occurrence of oxygen from abiotic processes had previously provided a selective pressure for the evolution of 'primitive' enzymatic defences. Alternatively, cells may initially have relied on molecules that originally performed other functions to detoxify ROS[24]. Nevertheless, the dramatic rise in atmospheric oxygen would also have resulted in mass extinction of microbial species that were unable to evade or overcome subsequent oxidative damage.

Early forms of aerobic metabolism may have primarily functioned as a means of scavenging oxygen to prevent oxidative damage to cells that in other respects retained an essentially anaerobic lifestyle. Gradually, the rising availability of molecular oxygen, which is a very potent electron acceptor, further shaped biochemical networks, that thereby became

increasingly complex[39]. Along the way, this allowed for another revolutionary innovation in energy metabolism. Electron transport chains that initially relied on other electron acceptors, such as for example nitrous oxide, evolved to use oxygen instead[40,41]. The ability to use oxygen as terminal electron acceptor in complex, multi-step respiratory chains enabled cells to conserve much more energy from the oxidation of electron donors than was thermodynamically possible with most electron acceptors used in anaerobic respiration processes[42]. This aerobic respiration, because of its high energetic capacity, allowed for the use of a wide range of organic and inorganic compounds as energy sources, and contributed greatly to the further diversification of life on Earth.

3. The origin of eukaryotic life | All ancient life forms that predated and/or caused the Earth's initial oxygenation and the emergence of aerobic respiration, were prokaryotic. The earliest accepted examples of eukaryotic life, in the form of microfossils, stem from 1.5 Gy ago[43,44]. Molecular clock studies, which are based on mutation rates in DNA, estimate an earliest possible origin of eukaryotes ranging between 2.5 and 1.2 Gy ago[45,46]. Therefore, at least in one respect, the 'Boring Billion'[47] years between the GOE and NOE (Figure **1.1**) were ground breaking as this era marks the time of the emergence and early diversification of eukaryotes. Traditionally, the distinction between eukaryotes (eu, "well" or "true", and karyon, "kernel"; 'nucleated') and prokaryotes was made based on the presence of organelles within the cell. Presently, molecular rather than phenotypic characteristics provide the basis for a generally accepted representation of the 'tree of life', in which the three domains of life are defined: Bacteria, Archaea (both prokaryotic) and Eukarya[48].

Protein and DNA sequence data have provided evidence that Eukarya have a chimeric evolutionary origin, with contributions from Bacteria as well as Archaea[49,50]. Rather than representing a single event, the term 'eukaryogenesis' encompasses a series of events, from which a lineage of proto-eukaryotic organisms developed and acquired features that are deemed to be typically eukaryotic. Such features include, but are not limited to, the presence of a nucleus, mitochondria (or related organelles), other membranous organelles such as the endoplasmatic reticulum (ER) and Golgi-system, and a cytoskeleton[51]. The subject of eukaryogenesis is difficult to tackle experimentally due to the current lack of evidence of 'transition forms' between prokaryotes and eukaryotes. The many hypotheses that have been forwarded for this key transition are beyond the scope of this text. However, since aerobic metabolism is considered to have been of vital importance for the origin of the mitochondrion, this important milestone in the evolution of eukaryotes will be briefly discussed.

Although controversial when first postulated, the endosymbiosis theory for the origin of mitochondria is now well accepted[52]. This theory proposes that mitochondria originate from a single type of prokaryote that was, at some point in evolutionary history, engulfed by

another prokaryote, whose survival subsequently became dependent on this 'endosymbiont'. Subsequent evolutionary optimization of this system then led to a (partial) transfer of the genetic material of the endosymbiont to the genome of the host[53,54], thereby converting the endosymbiont to an organelle whose replication fully depended on the host cell.

The endosymbiosis theory is fully consistent with the observation that mitochondrial genomes and a conserved fraction of eukaryotic genomes, which contain information for many processes such as aerobic respiration and the mitochondrial translation machinery, share a common ancestry with genes of modern α-proteobacteria[55–57]. However, the diversity of this class of bacteria allows for diverging theories on the nature of the host-endosymbiont interaction[58]. Endosymbiosis theory as it was first postulated described the interaction between an anaerobic host and an aerobic endosymbiont. In this model, the aerobic endosymbiont conferred the trait of aerobic respiration that resulted in a strong selective advantage in an increasingly oxidative environment, and thereby provided the energy metabolism that was required for further development of eukaryotic features[52,59]. A newer 'hydrogen hypothesis' proposes an initial endosymbiotic relation between a strictly H_2-dependent archaeal host and a facultatively aerobic, H_2-generating bacterium as selection principle for further evolution[60]. While these theories all leave room for interpretation and do not provide watertight argumentation for all aspects of eukaryogenesis, the common denominator is that the endosymbiosis event, that eventually led to the development of the mitochondrion, was essential for the evolution of aerobic respiration in early eukaryotes.

A second endosymbiosis event between an early eukaryote and a cyanobacterial endosymbiont gave rise to photosynthetic eukaryotes[61], the ancestors of land plants. The emergence of those land plants, and their strong oxygenic potential, is associated with the latest great surge in atmospheric oxygen during the NOE (Figure **1.1**)[16,62]. Nowadays, aerobic respiration is the largest sink for oxygen in the biosphere[63] and it balances the production of oxygen by plants in oxygenic photosynthesis. Atmospheric oxygen levels have been fairly constant for the last 400 million years, as is primarily indicated by dating of charcoal deposits which require oxygen levels that are permissive of wildfires (15-35% O_2)[15,64]. As will be discussed later, not all modern eukaryotes possess mitochondria and/or chloroplasts, nor do they all have the ability for aerobic respiration. Some have re-adapted to strictly anaerobic niche environments[65]. Even so, after billions of years oxygen has clearly left its mark, enabling a strong diversification of life on Earth that, eventually, also included our own species.

4. Biotechnology: Domesticating microbial life | Moving from the geological to the archaeological record represents quite a jump forward from the timeline discussed previously. Already early in pre-history, humans started to exploit the enormous diversity of microbial

life for their own benefit. Archaeological evidence for production of fermented beverages, in which microorganisms convert sugars into ethanol, dates back as far as 7000 years before Christ (B.C.)[66]. The roles of microbes in producing bread, dairy products, soy sauce and linen have similar long histories. In these 'classical' biotechnological processes, oxygen (or its absence) can have a crucial impact on product quality. Most early food fermentation processes were performed under anaerobic or severely oxygen-limited conditions and therefore relied on the ability of microorganisms to convert carbon substrates in the absence of oxygen as external electron acceptor. Production of acetic acid by acetic acid bacteria[67] (which make a surprise appearance in **Chapter 4** of this thesis) and microbial leaching of copper from ore[68] are among the few early microbial biotechnology processes that strictly depend on oxygen.

Despite the strong relationship between human civilization and the use of microorganisms, it was not until late in the 17th century that Antonie van Leeuwenhoek first elaborately reported on these 'animalcules', which he observed through one of his now famous microscopes[69,70]. It took another 150 years before Louis Pasteur (1857)[71] finally and convincingly showed that microorganisms, rather than spontaneous decomposition processes, were responsible for fermentation processes. Pasteur also coined the terms 'anaerobic' and 'aerobic', referring to microorganisms that can and cannot, respectively, live without oxygen. Around the start of the 20th century, Sergei Winogradsky[72] and Martinus Beijerinck[73] explored microorganisms and their metabolism within their native ecological contexts and microbial communities. In the same period, the medical microbiologist Robert Koch developed methods for the isolation and studies of 'axenic' cultures, consisting of a single microbial species[74].

The exploration of microbial biodiversity and the availability of aseptic techniques for growing microbial species in axenic cultures gave microbiologists much more control over industrial fermentation processes than was previously possible with undefined mixed cultures. In the 20th century, industrial microbiology started to truly transform. World War I and II accelerated the development of pure-culture biotechnological processes[75] such as acetone production by *Clostridium acetobutylicum* (in the Weizmann process), glycerol production by baker's yeast[76] (Neuberg process) and penicillin production (following its discovery by Alexander Fleming[77]) by *Penicillium* species. The 'DNA revolution' in biotechnology did not start immediately after the determination of the structure of DNA by Watson and Crick in 1953[78]. However, introduction of recombinant-DNA technologies[79], DNA sequencing technologies[80,81] and the polymerase chain reaction (PCR)[82] made it possible to more accurately, efficiently and extensively change ('engineer') microorganisms on a genetic level to improve and extend their industrial applications. The production of human insulin by a genetically modified strain of the bacterium *Escherichia coli*, which rapidly replaced pig insulin isolated from slaughterhouse material, represented an early milestone in this development[83].

The complete sequencing, assembly and annotation of the genomes of model organisms such as *E. coli*[84] and the yeast *Saccharomyces cerevisiae*[85] led to the identification and functional analysis of many previously unidentified genes and proteins. These developments opened up possibilities to study microbial physiology in more detail and to design ever more elaborate genetic modifications. The emergence, over the past 20 years, of the discipline of synthetic biology has provided a range of molecular biology tools that allow for fast and efficient assembly of genetic constructs (both in vitro and in vivo)[86–89], precise genetic editing[90–92] and control of gene expression[93–95]. Originally, synthetic biology toolboxes focused on model organisms such as *E. coli* and *S. cerevisiae*, but they have recently become increasingly available for less conventional microorganisms as well[96–98]. The emergence of CRISPR (clustered regularly interspaced palindromic repeats)-Cas9 technology[99,100] is among the most memorable milestones in modern biotechnology and allows for genome editing with unprecedented accuracy and efficiency. Aided by increasingly advanced computational methods[101–104], such tools are used to increase our fundamental understanding of the complex networks operating in living cells. In addition, in the discipline of metabolic engineering, they are used to optimize and extend applications of microbes for industry.

In other words, within the span of a century, progress in molecular biology has enabled scientists to take micro-organisms out of the natural contexts in which they have evolved over billions of years, to study their genomes and to exploit the resulting knowledge for building high-performance microbial cell factories. Such cell factories are now grown at massive scale in industry to produce a wide variety of products, ranging from bulk chemicals to specialty molecules. This field of research and application, often referred to as industrial biotechnology, offers tremendous opportunities to produce chemicals from renewable feedstocks that can replace their petrochemically or plant-derived counterparts[105–109]. By harnessing the power of synthetic biology, it even enables production of non-natural molecules[107,110–112], that can potentially lead to the sustainable synthesis of a diversity of new materials with different properties, or of superior quality, relative to those based on (petro)chemical synthesis.

5. Oxygen in industrial biotechnology | Based on global product volume, industrial production of bioethanol by microbial fermentation of sugars is currently the single largest biotechnological process[113]. For bulk chemicals such as bioethanol, a large fraction of the product price is generally made up by the cost of the raw material and profit margins are small[114]. To make such processes economically viable, it is therefore crucial to maximize yields that can be obtained from the industrial substrate. Moreover, gross profit margins of biotechnological processes also need to be competitive with those of competing products generated by the petrochemical industry.

In the absence of an external electron acceptor like oxygen, all electrons from a substrate can in theory be transferred into the product, which is a prerequisite for achieving theoretical product yields on substrate[115]. Whenever possible, anaerobic, fermentative metabolic pathways towards biotechnological products are therefore preferable over oxygen-dependent pathways. One crucial factor that determines whether a conversion of substrate into product is feasible under anaerobic conditions, is the change in Gibbs free energy of the reaction under standard biochemical conditions ($\Delta_r G'^0$). $\Delta_r G'^0$ can be determined by comparing the Gibbs free energy density per electron ($\Delta_e G'^0$) of the substrate and that of the product[115]. When $\Delta_r G'^0$ is positive (when $\Delta_e G'^0$substrate < $\Delta_e G'^0$product), this means that the overall conversion is not thermodynamically favourable under standard conditions. An additional dissimilatory pathway is then required to provide the free energy (often in the form of ATP) needed for conversion of product to substrate. In such processes, use of oxygen as external electron acceptor enables cells to harvest much more ATP from substrate dissimilation than would be possible via substrate-level phosphorylation in fermentative pathways[116]. In contrast, when $\Delta_r G'^0$ is negative, conversion of substrate to the desired product can itself yield cellular free energy, and, depending on the metabolic network of the organism, aerobic respiration may not be required[117].

In addition to enabling near-theoretical yields of fermentation products, anaerobic processes offer additional benefits for process economics[116]. Equipment that allows for sufficient oxygenation of large-scale aerobic industrial fermentation tanks is costly and, since aerobic respiration dissipates more energy in the form of heat, the costs associated with cooling of the process to maintain a suitable temperature are higher in aerobic processes. For this reason, the ability of microorganisms to grow in absence of oxygen can be of crucial importance for their application in specific processes.

6. Biosynthetic oxygen requirements in yeast | Micro-organisms isolated from nature rarely harbour all traits required for economically viable, large-scale industrial fermentation processes. This is hardly surprising given the many years of evolution that preceded the industrial application of microorganisms, during which they did not encounter the specific circumstances that are required for the optimal production process. Even micro-organisms such as lactic acid bacteria and brewing yeasts, which are applied on a massive scale in the food and beverages industry and have not been genetically engineered, are the products of centuries of domestication in industrial environments[118,119].

Baker's yeast is a popular industrial production host because of its relative ease of cultivation, tolerance to low pH, fast growth and resistance to phages[120,121]. The word 'yeast' is sometimes used as synonym for 'baker's yeast' *Saccharomyces cerevisiae*. However, there are many other yeast genera and species that are increasingly important in biotechnological applications,

due to industrially relevant traits that are absent or less pronounced in *S. cerevisiae*[122,123]. For example, *Kluyveromyces marxianus* and *Ogataea parapolymorpha* are yeasts with higher temperature optima[124–126], and *Yarrowia lipolytica* naturally produces high levels of lipids[127,128]. But while the large majority of yeast species are able to ferment sugars to ethanol and therefore are able to conserve free energy in the absence of respiration[129], only a few can grow in the complete absence of oxygen[130,131]. This indicates that these yeasts have oxygen requirements that go beyond respiratory dissimilation of sugars. The inability of yeasts, and in particular of non-*Saccharomyces* species, to grow in the absence of oxygen has been investigated for decades[130,131], but the exact role that oxygen plays in their metabolism remains incompletely understood.

S. cerevisiae is one of few yeast species that can grow anaerobically[130,132]. This ability plays a key role in its long history of use in beer brewing, wine fermentation and bioethanol production. Its 'generally regarded as safe' (GRAS) status, the vast body of knowledge about its physiology and its accessibility to genetic modification have contributed to its widespread use in a range of other industrial processes[120]. In addition to being the most widely used eukaryotic industrial microorganism, *S. cerevisiae* is a popular laboratory model eukaryote. Multiple Nobel-prize winning academic studies made use of *S. cerevisiae* as model organism[133].

Although *S. cerevisiae* can grow under anaerobic conditions, this growth does require some additional nutritional factors relative to aerobic growth, due to assimilatory oxygen requirements that are quite well documented[134] (Figure **1.2**). Growth of *S. cerevisiae* in laboratory settings often relies on synthetic defined media containing a carbon- and nitrogen source, mineral salts and a selection of B-type vitamins[135,136]. In the 1950's, Andreasen and Stier demonstrated that *S. cerevisiae* was capable of sustained anaerobic growth on some complex, undefined media, but not on synthetic media[137,138]. They discovered that the crucial missing component was ergosterol, a lipid molecule of which the role in maintaining proper function of typical eukaryotic membranes is now well known[139–142]. The insight that constituents of Tween 80, which the authors used to emulsify the highly hydrophobic ergosterol, also had a growth-promoting effect under anaerobic conditions, followed soon after[143]. These constituents turned out to be unsaturated fatty acids, which are used to build phospholipids. Phospholipids, in turn, are important building blocks of cellular membranes. A source of unsaturated fatty acids and sterols has since been routinely included in synthetic laboratory media for anaerobic growth of yeast, and sterols and unsaturated fatty acids are generally recognized as essential anaerobic growth factors for yeasts (but see **Chapter 2** and **Chapter 4** of this thesis)[130,144]. The role of oxygen in the synthesis of these compounds by yeasts, and the regulation of the responsible biosynthetic pathways, will be discussed in sections **7.1** and **7.2** of this introduction.

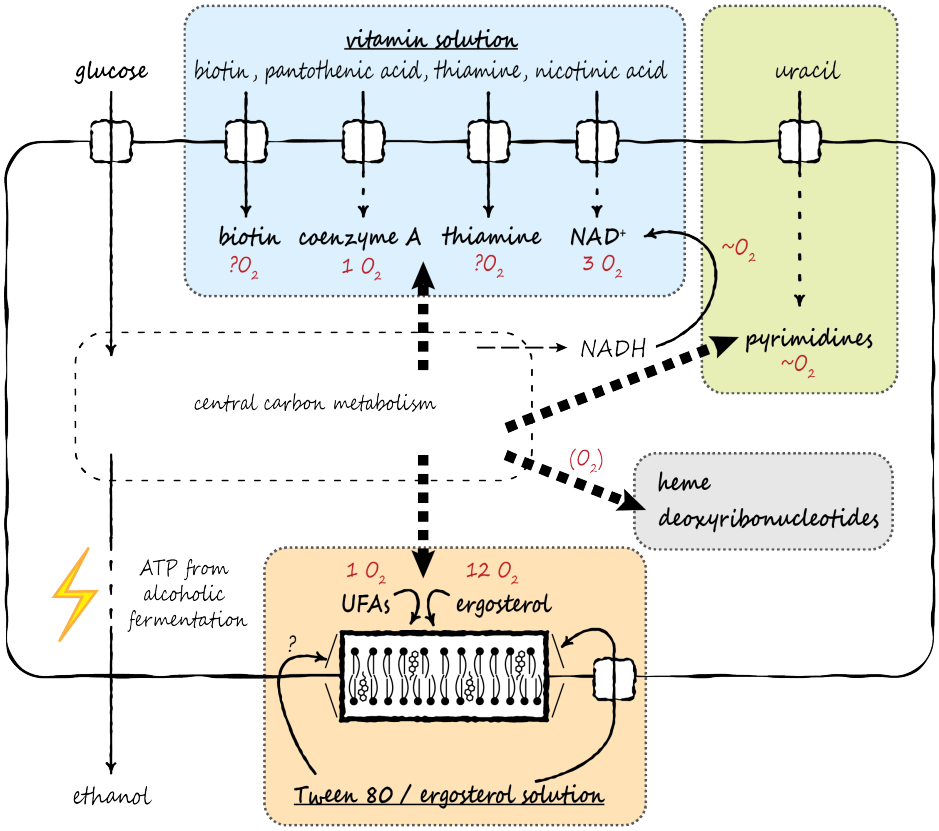

Figure 1.2: Overview of biosynthetic oxygen requirements in yeast, and crucial medium components required for anaerobic growth. This schematic illustration of a yeast cell indicates biosynthetic processes that are oxygen dependent in most yeasts, and indicates exceptions for *S. cerevisiae*. Under anaerobic conditions, fermentative yeasts can gain cellular energy, in the form of ATP, from alcoholic fermentation. Broad black dotted lines indicate *de novo* synthesis. The number of oxygen molecules required for the depicted processes is indicated in red (an unknown stoichiometry for the requirement for oxygen is indicated by $?O_2$). The biosynthetic oxygen requirements for cofactors biotin, coenzyme A, thiamine and NAD^+ (highlighted in blue) and ergosterol and unsaturated fatty acids (highlighted in orange) can be circumvented in *S. cerevisiae* by exogenous supplementation of these molecules or their precursors (indicated by interrupted lines). In *S. cerevisiae*, ergosterol can be transported via ABC (ATP Binding Cassette) type transporters Aus1 and Pdr11, but the mechanism for Tween 80 hydrolysis and/or uptake remains unknown. Pyrimidine synthesis (highlighted in green) does not require oxygen in *S. cerevisiae* (indicated by $\sim O_2$), but is oxygen dependent in many other yeast species. Similarly, *S. cerevisiae* regenerates the NAD^+ cofactor from NADH formed in biosynthetic reactions through glycerol production, but if there is no such oxygen-independent mechanism, NAD^+ needs to be regenerated in aerobic respiration. Heme biosynthesis (highlighted in grey) is oxygen dependent, but is not required under anaerobic conditions (indicated by (O_2)). Synthesis of deoxyribonucleotides presumably requires oxygen, although no clear anaerobic auxotrophy related to this process has thus far been identified.

While sterol and UFA synthesis are major contributors to assimilatory oxygen requirements in yeast, multiple other factors can be involved as well. An elaboration on such additional oxygen requirements follows below, although these do not necessarily apply for all yeast species.

6.1 – Reoxidation of pyrimidine nucleotide cofactors. The pyridine nucleotides NAD^+ and $NADP^+$ and their respective reduced forms NADH and NADPH act as important electron carriers in cellular metabolism, and because they are conserved moieties, reduction of $NAD(P)^+$ and oxidation of NAD(P)H need to be continuously balanced[145]. The oxidative conversion of glucose into pyruvate in glycolysis is accompanied by the generation of two NADH molecules. During alcoholic fermentation in yeast, these two NADH molecules are reoxidized in the conversion of acetaldehyde into ethanol, thus making alcoholic fermentation a redox-neutral process[145]. However, in the assimilation of yeast biomass from glucose and other nutrients, a net generation of NADH occurs, and this 'excess' NADH needs to be reoxidized elsewhere in metabolism[129,144]. In *S. cerevisiae*, the main sink for reducing equivalents under anaerobic conditions is glycerol production, and therefore redox balancing is not oxygen dependent in this yeast[129,145]. When a yeast species is unable to synthesize enough glycerol for redox balancing and does not have alternative oxygen-independent redox sinks, NADH generated in biosynthetic reactions can only be reoxidized through aerobic respiration. In some yeasts this phenomenon, known as the Custers effect, results in oxygen requirements associated to the regeneration of redox cofactors[146].

6.2 – Pyrimidine synthesis. The *de novo* synthesis of the pyrimidine nucleotides cytosine, thymine and uracil can require oxygen depending on the electron acceptor that is used for the conversion of dihydroorotate to orotate[147,148]. In *S. cerevisiae*, a cytosolic dihydroorotate dehydrogenase (DHODase) encoded by *URA1* uses fumarate as preferred electron acceptor, and therefore pyrimidine synthesis is oxygen independent in this yeast. In many other yeast species, the presence of a mitochondrial DHODase that donates electrons to the quinone pool of the mitochondrial respiratory chain causes an oxygen requirement for pyrimidine synthesis[148]. Physiological and comparative genomics studies indicate that soluble DHODases like the one in *S. cerevisiae* only occur in very few yeast species[149,150].

6.3 – Vitamin synthesis. Synthetic media for yeast cultivation are usually supplemented with a mixture of compounds that, in human nutrition, are referred to as B-type vitamins. In the context of yeast cultivation, the word 'vitamin' for compounds such as nicotinic acid, pantothenate, thiamine and biotin is not always correct, as many yeast species are capable of *de novo* synthesis of these molecules[136,151–154]. However, the ability to synthesize such cofactors *de novo* can be strain specific and growth in the absence of supplementation of a 'vitamin' may be much slower than in its presence[136]. For example, growth of the laboratory strain *S. cerevisiae*

CEN.PK113-7D is severely impaired in the absence of biotin supplementation[154]. The addition of these cofactors to synthetic media[135,136,155] can obscure biosynthetic oxygen requirements, as illustrated by the ability of *S. cerevisiae* to grow aerobically, but not anaerobically in the absence of several B-type vitamins[151–154]. For *de novo* synthesis of one mol of the nicotinamide cofactor and, ultimately, NAD^+, three moles of oxygen are required for the reactions catalysed by Bna2, Bna4 and Bna1[151]. Synthesis of pantothenate, a precursor for coenzyme A, requires one mol of oxygen in a reaction catalysed by Fms1[152]. Finally, there is experimental evidence for anaerobic auxotrophy of *S. cerevisiae* for thiamine[153] and an oxygen dependency of the Bio1 enzyme of *Cyberlindera fabianii* upon expression in *S. cerevisiae*[154]. However, the exact origins and stoichiometries of these two oxygen requirements remain to be fully resolved. Summarizing, anaerobic growth of *S. cerevisiae* and, in all likelihood, other yeasts, requires import of these compounds or their precursors from exogenous sources.

6.4 – Other oxygen requirements. Although biosynthesis of heme requires oxygen, anaerobicity does not necessarily appear to cause a heme auxotrophy in *S. cerevisiae*. Cytochromes, desaturases and catalases are examples of heme-containing proteins, but these enzymes either catalyse oxygen-dependent reactions or are otherwise strongly associated with aerobic metabolism[156]. These observations indicate that, at least in *S. cerevisiae*, heme itself is not essential for anaerobic growth. Aerobic cultures of heme-deficient mutants of *S. cerevisiae* share many physiological characteristics of anaerobic cultures of wild-type cells[157]. For example, they become dependent on supplementation of a source of sterols and unsaturated fatty acids and can no longer grow on non-fermentable carbon sources. The construction of *hem1Δ*-mutants in *S. cerevisiae*, in which heme biosynthesis is blocked, has therefore been used as a method to study processes related to anaerobic growth[158–161]. 5-aminolevulinic acid, the product of the reaction catalysed by Hem1, is not only required for oxygen-dependent heme-synthesis, but also for the oxygen-independent synthesis of siroheme. Siroheme, in turn, is an important cofactor for the sulfite reductases that play a role in the early steps of the oxygen independent methionine biosynthesis[162]. Deletion of *HEM1* therefore results in a methionine auxotrophy that is not observed in anaerobic cultures of wild-type cells.

An additional potential oxygen requirement may originate from synthesis of deoxyribonucleotides by ribonucleotide reductases (RNRs). So far, three different classes of RNRs have been identified, of which class II and III are oxygen independent[163]. Fungi and many other eukaryotes possess a class I RNR[164] that requires oxygen for iron binding in diiron-oxygen clusters[165]. The class I RNR of *S. cerevisiae* reportedly displays a unique configuration as compared to other (fungal) class I RNRs. Whereas the typical class I RNR is encoded by two genes whose products form a tetrameric enzyme that consists of two homodimers, each of the four subunits of the *S. cerevisiae* RNR is encoded by a different gene. The subunits involved in the (oxygen dependent) iron binding are configured in a different

manner[166]. No clear anaerobic auxotrophy has been identified in *S. cerevisiae* thus far that can be associated to the inactivity of these enzymes, and it therefore seems probable that the class I RNR of *S. cerevisiae* is not strictly oxygen dependent.

In addition to the oxygen-dependent biosynthetic pathways in yeasts mentioned in this paragraph, anaerobic growth of yeasts other than *S. cerevisiae* may be further restricted by additional, as yet unknown oxygen requirements. Research into biosynthetic oxygen requirements, which are very small in comparison to respiratory oxygen fluxes in yeasts, requires special laboratory experimental set-ups designed to minimize inadvertent oxygen entry into cultures (see **Chapter 5**).

7. Membrane lipid synthesis of yeast | *S. cerevisiae* shows many adaptations to anaerobic growth, as exemplified by the presence of oxygen-independent alternatives to some of the potential oxygen requirements described previously. However, a necessity for the use of oxygen remains in the biosynthesis of important membrane lipids. Membranes define the boundaries of an organism and form a barrier between intracellular metabolism and the extracellular environment. In eukaryotes and a number of prokaryotes[167], lipid membranes also enable compartmentalization, which allows for greater metabolic flexibility. The controlled build-up of concentration gradients of solutes across a lipid bilayer that is virtually impermeable to hydrophilic solutes enables intracellular homeostasis despite fluctuating extracellular conditions and, moreover, is crucial for establishing a proton motive force, which is central to respiratory energy metabolism[168,169]. Lipids are organic substances that are insoluble in water, but can vary from being highly hydrophobic to amphiphilic. In theory, molecules of a single amphiphilic lipid species would suffice to form a bilayer through self-assembly, in which hydrophobic residues face each other to form the core of the bilayer and hydrophilic parts of the molecules face the aqueous phase on either side[170] (Figure **1.3**A). Lipid membranes of living organisms, however, show a remarkable diversity in both amphiphilic and hydrophobic lipid species, which together allow for fine-tuning of important membrane properties under a wide range of extracellular conditions[171]. Typical eukaryotic membranes are comprised of three major types of lipids, and have many proteins with lipophilic domains associated with and inserted into them[172]. The collective chemical behaviour of a mixture of glycerophospholipids (commonly also referred to as phospholipids), sphingolipids and sterols is crucial for the maintenance of important membrane properties such as fluidity, permeability, thickness, packing density and surface charge[173]. In *S. cerevisiae*, phospholipids roughly contribute 60-80 %, and sphingolipids and sterols each contribute roughly 10-20 % of the total of membrane lipids, depending on the specific growth conditions[174].

Phospholipids consist of a glycerol backbone, to which a hydrophilic head group and two fatty-acid tails are esterified[175] (Figure **1.3**B). The phospholipid class is determined by

the hydrophilic residue, and in *S. cerevisiae*, phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant. Phosphatidic acid (PA), phosphatidylserine (PS), diacylglycerol (DAG), phosphatidylglycerol (PG) and cardiolipin (CL) are other contributors to the membrane lipidome[174–176]. The most predominant fatty acids have a length of 16 or 18 carbons and are either fully saturated or mono-unsaturated[177]. The combination of the type of hydrophilic head group and the length and degree of unsaturation of the fatty acid residues can give rise to many phospholipid species[174,176].

In *S. cerevisiae*, the sphingoid-base that forms the backbone of sphingolipids is derived from a saturated fatty acid with a length of 16 or 18 carbon atoms and a serine molecule[178] (Figure **1.3**B). Together with another saturated and very-long-chain (24 or 26 carbons) fatty-acid residue[174,179] it forms the hydrophobic part of the molecule. Both fatty acid residues can be hydroxylated to various degrees[180]. The diversity of sphingolipids in *S. cerevisiae* predominantly stems from their hydrophilic head group that is attached to the serine residue, which can be either just the original hydroxyl group (in ceramides), or a combination of inositol and mannose moieties[181]. In higher eukaryotes, an even larger diversity of sphingolipids is enabled by different modifications of the sphingoid base and more elaborate extensions of the hydrophilic head group[182].

Sterols are rigid, highly hydrophobic molecules that are located deeper in the hydrophobic core of the lipid bilayer and are shielded from the hydrophilic exterior by the surrounding amphiphilic lipids (Figure **1.3**A, see also Figure **1.5**)[142,183]. In the absence of nutritional supplementation with non-native sterols, sterols are the lipid class with the least diversity in *S. cerevisiae*. The predominant sterol in this and other yeasts is ergosterol, which is the final product of the sterol synthesis pathway, with some intermediates constituting a minor fraction of the total sterol pool[184].

Basic membrane properties are determined by the physical shape of phospho- and sphingolipids, the nature of the fatty acid residues, and the presence of sterols[173]. For example, PC and sphingolipids are cylindrical, and tend to form tightly packed and flat membranes by themselves. Lipid species with a different physical shape can distort the optimal packing of fatty acid residues in a lipid bilayer, and thereby cause 'packing defects' and induce curvature[185,186] (Figure **1.3**A). The phospholipid PE has a small head group and therefore a conical shape (Figure **1.3**C), which induces negative curvature in a lipid bilayer, whereas lyso-PL (lacking one fatty acid residue) induces positive curvature[185]. The rigid double bond in an unsaturated fatty acid gives it a bended shape, which reduces lipid packing in the hydrophobic core, and therefore membrane fluidity generally increases with increasing unsaturation of the membrane fatty acids[187]. Conversely, longer acyl chains can form stronger hydrophobic interactions, and thereby reduce membrane fluidity. Sterols rigidify and thicken

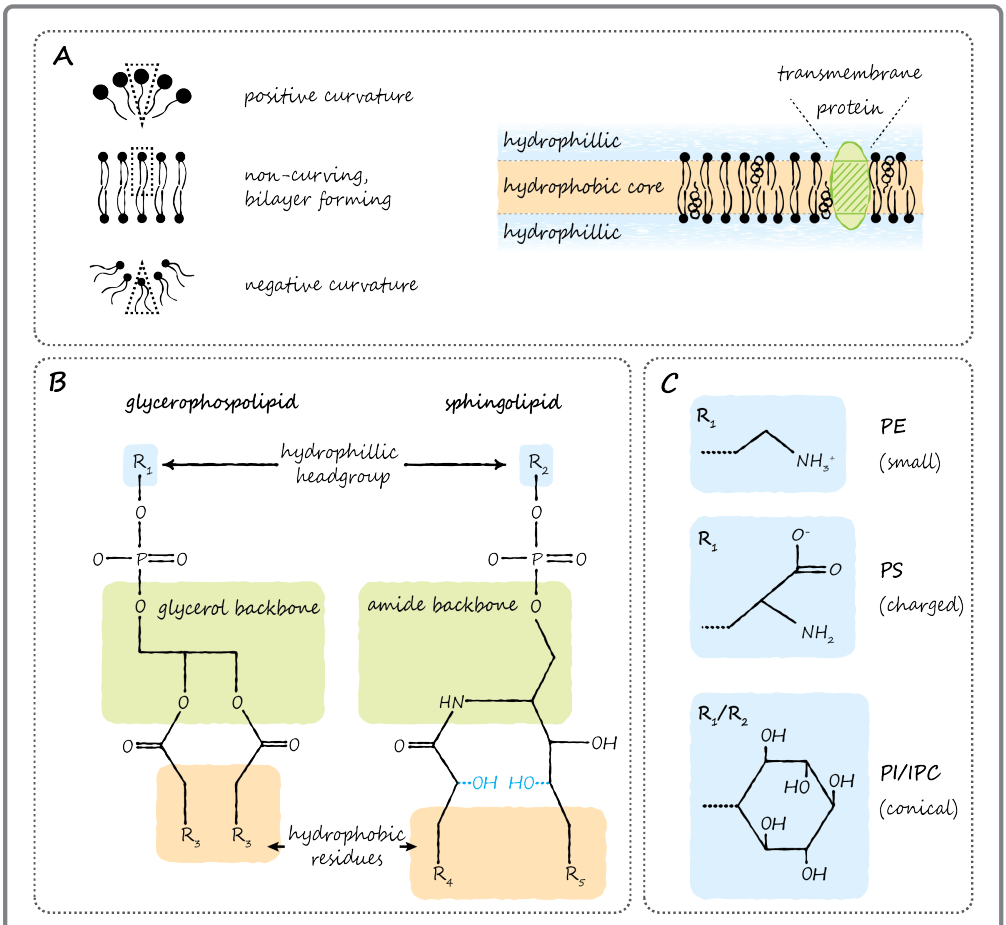

Figure 1.3: Amphiphilic lipids in *S. cerevisiae*. Glycerophospholipids and sphingolipids are amphiphilic and form the basis for lipid membranes. (**A**) Schematic representation of non-bilayer forming and bilayer forming phopsholipids, and of a lipid bilayer with inserted hydrophobic sterols and membrane protein. (**B**) Schematic representation and comparison of the structure of glycerophospholipids and sphingolipids. R_1, the hydrophilic headgroup determines the phospholipid class. This can be a hydroxyl group (PA), a serine (PS), an inositol moiety (PI), a serine residue (PS), a choline-residue (PC) or an ethanolamine group (PE). R_2, hydrophilic headgroups of sphingolipids are simple in *S. cerevisiae* in comparison to higher eukaryotes. It can be a hydroxyl group, an inositol moiety (IPC), a mannose-inositol (MIPC) or a mannose-diinositol ($M(IP)_2C$) group. R_3, fatty acid residues. In *S. cerevisiae*, these are mostly derived from palmitate ($C_{16:0}$), palmitoleate ($C_{16:1}$), stearate ($C_{18:0}$) and oleate ($C_{18:1}$). R_4, fatty acid residue. In *S. cerevisiae*, this is a very-long-chain fatty acid such as lignoceric acid ($C_{24:0}$) or cerotic acid ($C_{26:0}$). R_5, the hydrophobic residue of the sphingoid base. In *S. cerevisiae*, it is mostly derived from palmitate ($C_{16:0}$) or stearate ($C_{18:0}$). (**C**) Illustration of three different hydrophilic headgroups that are encountered in *S. cerevisiae*, and that contribute to important properties of the corresponding lipid class. PE (phosphoethanolamine), lipid class with a tendency to form conical lipids; PS (phosphoserine), establishes negative surface charge; PI (phosphoinositol) or IPC (inositolphosphorylceramide), tendency to form cylindrical and bilayer-forming lipids.

membranes by straightening out neighbouring acyl chains and stabilizing their flexibility through hydrophobic interactions[188,189], while at the same time, they prevent a bilayer from becoming too rigid by interfering with the lipid packing of (longer) acyl chains[190]. Moreover, lipid packing defects and surface charge originating from charged phospholipid head groups (Figure **1.3**C), can promote stable insertion of membrane proteins and enable interactions between membrane proteins and lipid bilayers[186,191].

The properties of the various membranes in yeast cells are different. In general, the membrane of the endoplasmatic reticulum (ER) is thinner than the plasma membrane (PM) and has more packing defects and a lower surface charge[172]. As membranes move towards the PM through the cis- and trans- Golgi, their properties gradually change. The plasma membrane (PM) is thick, rather rigid and impermeable, and has a higher surface charge at the cytoplasmic leaflet[172]. The ER has a higher degree of unsaturation than the PM, and the latter is enriched in sterols and sphingolipids in comparison to other cellular membranes[192]. Cardiolipin (CL), a special, rather bulky type of phospholipid comprised of two phosphatidic acid moieties linked together by another glycerol group[193], predominantly occurs in mitochondria and is thought to be be an important contributor to the formation of cristae in the inner mitochondrial membrane[194]. The two leaflets of a membrane do not necessarily have the same composition[195,196] and, moreover, lateral heterogeneity within the PM at nano-scale ("lipid rafts") is thought to be promoted by preferential interactions between sterols and sphingolipids[197–199]. Such membrane domains are proposed to play a key role in the assembly of membrane-associated protein complexes[200]. Many studies indicate that microorganisms can adapt their membrane composition in response to various environmental signals. For example, *S. cerevisiae* produces more unsaturated and shorter fatty acids at 15°C than at 30°C, most likely to ensure that membranes remain fluid despite the lower temperature[174]. Upon an increase in extracellular ethanol concentration, the ergosterol content of the plasma membrane increases[201], which is consistent with the reduced permeability of sterol rich membranes to polar solutes[202]. *S. cerevisiae* strains that had been subjected to laboratory evolution experiments for growth at temperatures up to 40°C acquired mutations that resulted in the synthesis of fecosterol, which has a more bent shape, as major sterol instead of ergosterol[203].

In addition to their basal role in generating a diffusion barrier, lipids can have other functions in the cell. For example, fatty acids can be used for protein acylation in post-translational modifications[204], sphingolipids have been linked to various signalling pathways in the cell[181], and sterols have been proposed to be involved in cell cycle progression and various transport-related processes[205,206]. Lipid synthesis is energetically expensive and free lipids are in general toxic in high concentrations. Surplus fatty acids and sterols are stored as triacylglycerol (TAG) and steryl ester (SE) molecules[207–209]. Such 'inactive' forms of lipids

cluster together in intracellular lipid droplets, together with enzymes that can catalyse the release of individual lipids when there is a need for them [208,210]. *S. cerevisiae* tends to accumulate TAG and SE towards the end of the exponential growth phase [174,210]. Lipid reservoirs can serve as an energy source through β-oxidation of fatty acids [210], or they can be used as a source for future membrane synthesis [208]. Due to the oxygen dependency of the *de novo* synthesis of unsaturated fatty acids and sterols in yeasts, the ability to store these lipids becomes especially important when aerobically grown cells are exposed to an anaerobic environment. The oxygen requirements for synthesis of unsaturated fatty acids and sterols in *S. cerevisiae* will be discussed in more detail in the following paragraphs.

7.1 – Synthesis of unsaturated fatty acids. The *de novo* synthesis of fatty acids starts with the carboxylation of an acetyl-coenzyme A (acetyl-CoA) with a CO_2 molecule to form malonyl-CoA [204] (Figure **1.4**A). The transacylation of an acetyl-CoA and malonyl-CoA molecule forms the start of a fatty acid elongation cycle (Figure **1.4**B). In each turn of this cycle, a malonyl-CoA molecule is used as donor of two carbon atoms for the growing acyl chain. The transacylation is accompanied by the release of one CO_2 molecule, which makes the overall CO_2 requirement equal to zero. This chain elongation results in a ketoacyl residue, which is subsequently reduced to a hydroxyacyl residue, followed by dehydration to a trans-enoyl residue which is, finally, once more reduced to form a regular acyl-CoA chain that can enter a next elongation cycle (Figure **1.4**A and B).

In living organisms, two enzyme systems can be responsible for fatty acid synthesis [211]. While different in composition and organization, these systems both follow the same reaction scheme as described above. In type I fatty acid synthase (FAS) systems, all catalytic activities required for elongation are present on domains of large multimeric enzymes consisting of one or two subunits [212], while in type II FAS systems each step is catalysed by a separate enzyme [213]. Type II FAS systems use an acyl-carrier protein (ACP) to shuttle the growing acyl-chains between the different enzymes. In general, eukaryotes possess a type I FAS [212,214]. In *S. cerevisiae*, this system is encoded by *FAS1* and *FAS2* [215,216], while *ACC1* [217] and *HFA1* [218] encode cytosolic and mitochondrial isoenzymes, respectively, of acetyl-CoA carboxylase. The FAS complex consists of a hexameric dimer of Fas1 and Fas2 proteins, and is responsible for the synthesis of saturated fatty acids in the cytosol [204]. *S. cerevisiae* additionally harbours a mitochondrial type II FAS system consisting of the enzymes Acp1, Mct1, Cem1, Oar1, Htd2 and Etr1 [219]. Ppt2 is responsible for activating the mitochondrial acyl carrier protein [220]. An established product of mitochondrial type II FAS is octanoic acid, a precursor for lipoic acid which is in turn a cofactor for enzymes such as pyruvate dehydrogenase [221,222].

The two FAS systems in *S. cerevisiae* solely produce saturated fatty acids [204]. Unsaturated fatty acid synthesis in *S. cerevisiae* and many other eukaryotes is oxygen dependent, because

desaturation of one of the double bonds is catalysed by an oxygen-dependent acyl-CoA desaturase[177,223–225]. *S. cerevisiae* Ole1 is a Δ9-desaturase and catalyses the desaturation of the bond between the 9th and 10th carbon atom in the chain, counting from the carboxyl terminus[226,227] (Figure **1.4**C).

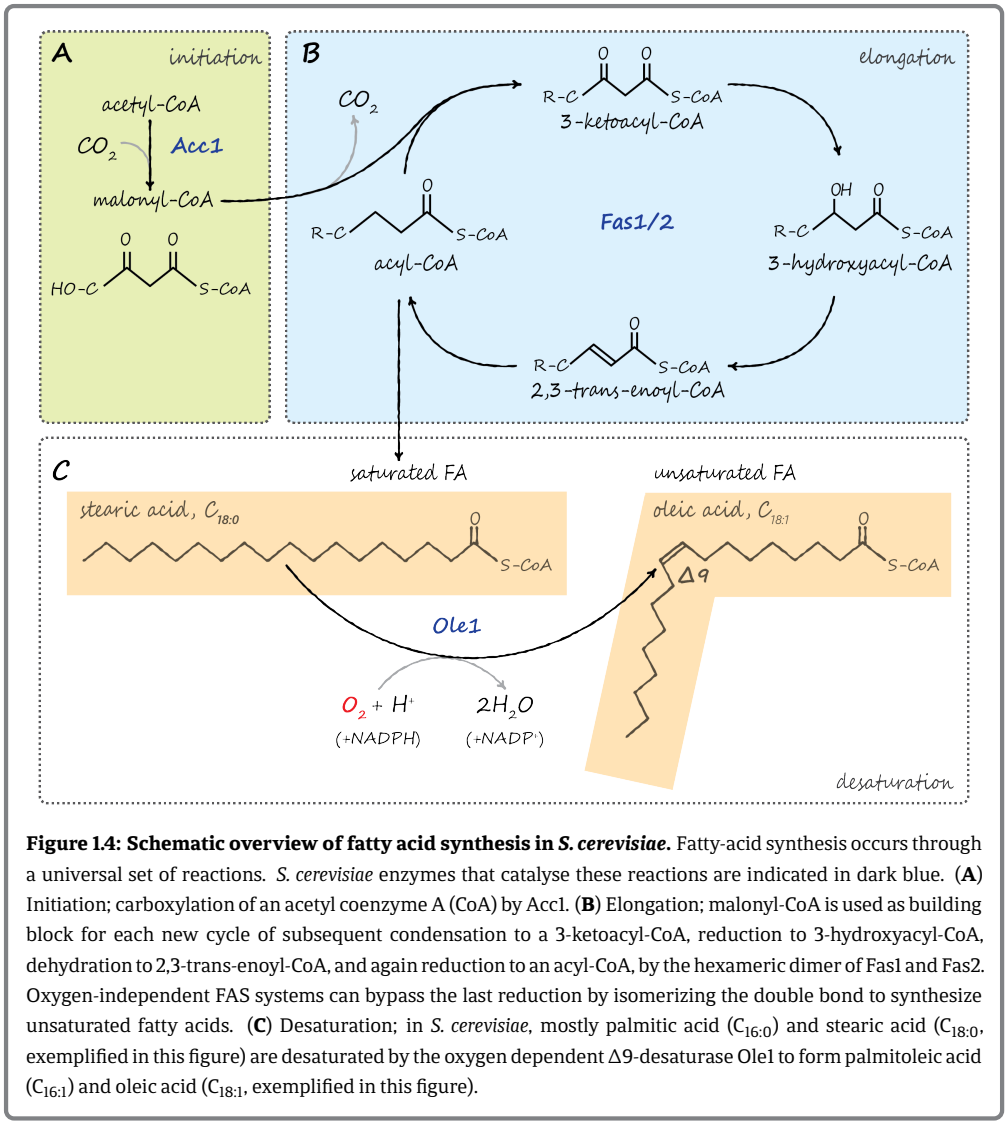

Figure 1.4: Schematic overview of fatty acid synthesis in *S. cerevisiae*. Fatty-acid synthesis occurs through a universal set of reactions. *S. cerevisiae* enzymes that catalyse these reactions are indicated in dark blue. (**A**) Initiation; carboxylation of an acetyl coenzyme A (CoA) by Acc1. (**B**) Elongation; malonyl-CoA is used as building block for each new cycle of subsequent condensation to a 3-ketoacyl-CoA, reduction to 3-hydroxyacyl-CoA, dehydration to 2,3-trans-enoyl-CoA, and again reduction to an acyl-CoA, by the hexameric dimer of Fas1 and Fas2. Oxygen-independent FAS systems can bypass the last reduction by isomerizing the double bond to synthesize unsaturated fatty acids. (**C**) Desaturation; in *S. cerevisiae*, mostly palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$, exemplified in this figure) are desaturated by the oxygen dependent Δ9-desaturase Ole1 to form palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$, exemplified in this figure).

Many other fungi additionally harbour a Δ12- or Δ15-, and even other types of acyl-CoA desaturases, and can therefore synthesize poly-unsaturated fatty acids[177,228,229]. In *S. cerevisiae* such other enzymes have thus far not been reported. Although traces of poly-unsaturated fatty acids have been encountered in a recent and extensive lipidomic study[176], the authors hesitated

to attribute these to the metabolism of *S. cerevisiae*, rather than to contaminants from cultivation media or artifacts from measurement methods. Deletion of *OLE1* renders aerobic cultures of *S. cerevisiae* auxotrophic for (mono)unsaturated fatty acids[226], and unsaturated fatty acids have been assumed to be essential lipids for yeasts ever since this observation.

The membrane-anchored transcription factors Mga2 and Spt23 are required for regulation of *OLE1* expression[230,231]. For Mga2 it has been demonstrated that its transmembrane helix is involved in sensing properties of the ER membrane, and because of sequence similarity a similar mechanism is expected to be relevant for Spt23[231,232]. Upon a specific trigger, which is most likely an increase in lipid packing density that can be caused by for example low levels of unsaturated fatty acids, Mga2 is activated in a proteasome-dependent manner and migrates from the ER membrane to the nucleus to induce *OLE1*-expression[231,232]. *OLE1* expression is increased during growth on non-fermentable carbon sources, at low temperatures, during iron limitation and/or at low oxygen levels[177,233]. The presence of (mono- and/or poly-) unsaturated fatty acids in growth media, which can be readily taken up and incorporated into membranes[234,235], is associated with a decreased Ole1 activity and *OLE1* mRNA levels[236,237]. Although our understanding of the feedback loop via membrane lipid composition through which OLE1-expression is regulated has improved over the past years, it remains a complex process that is not yet completely understood[230].

The oxygen requirement for unsaturated fatty acid synthesis is not universal. For example, the prokaryote *E. coli* harbours a type II FAS system, and synthesizes unsaturated fatty acids by isomerizing the double-bond of the trans-enoyl intermediate, after which this double bond is no longer reduced in the last step of the elongation cycle[213]. This mechanism does not require oxygen as electron acceptor, and therefore allows for the production of unsaturated fatty acids in an oxygen-independent manner. Oxygen independent unsaturated fatty acid synthesis was also reported for one of the type I FAS enzymes of *Brevibacterium ammoniagenes*[238,239], although the mechanism involved in introduction or retention of the double bond has not been elucidated.

7.2 – Sterol biosynthesis and sterol surrogates. Biosynthesis of sterols strictly requires oxygen. Although it has, intriguingly, been postulated that anaerobic sterol synthesis could theoretically be possible based on existing enzymatic mechanisms[240], such a pathway has neither been found in a living organism nor in the geological record[241]. While the dominant sterol species may differ for mammals (cholesterol), fungi (ergosterol) and plants (phytosterols), the biochemical pathways leading to their synthesis are highly conserved[241,242]. Because of their near ubiquitous presence in eukaryotes, the diagenic products of sterols (steranes) have been used as biomarkers for the presence of eukaryotic organisms in rock sediments[241,243]. However, with the discovery that some bacteria can synthesize sterols as

well came the realization that the use of such molecules as biomarkers should be treated with care[244].

In yeasts and other fungi, ergosterol biosynthesis starts with the multistep synthesis of mevalonate from three molecules of acetyl-CoA[245]. In a subsequent series of phosphorylation reactions, farnesyl pyrophosphate (FPP) is produced out of three mevalonate molecules at the cost of three ATP molecules[205]. The condensation of two FPP molecules yields squalene, which is the first triterpenoid molecule in ergosterol synthesis. Up until the synthesis of squalene, no oxygen is required. Afterwards, squalene is epoxidized by squalene monooxygenase Erg1[246], which requires one molecule of oxygen, and subsequently squalene epoxide (or 2,3-oxidosqualene) is the substrate for oxidosqualene cyclase (OSC), encoded by *ERG7*[247] (Figure **1.5**). The cyclization reaction catalysed by this enzyme yields lanosterol, which is the first sterol in the sterol biosynthesis pathway. From lanosterol onwards, a cascade of Erg-proteins is responsible for further modifications of the sterol backbone to ultimately yield ergosterol. First, the methyl group at the C14-position is removed by Erg11, which requires 3 moles of oxygen[248]. Next, the resulting C14 double bond is reduced by Erg24[249]. Subsequently, the two methyl groups at C4 are removed by the concerted actions of Erg25, Erg26 and Erg27, which requires another three moles of oxygen for each methyl group, and yields zymosterol[250–252]. Afterwards, Erg6 can methylate the side chain to yield fecosterol[253,254], and episterol results from isomerization of the double bond at C8 by Erg2[255]. Two more desaturation reactions by Erg3 and Erg5 require one mole of oxygen each[256,257]. Finally, to obtain ergosterol, a double bond in the side chain is reduced by Erg4[258,259].

In *S. cerevisiae*, null mutations in *ERG6*, *ERG2*, *ERG3*, *ERG5* or *ERG4* impair but do not completely prevent aerobic growth on sterol-free synthetic media[253–257,259]. Although presented as a linear pathway in Figure **1.5**, studies on yeast mutants with single or multiple lesions in this final part of the sterol biosynthesis pathway have revealed that the encoded Erg proteins show some substrate promiscuity, and can in principle act on any sterol intermediate downstream of zymosterol[205]. Such mutants then accumulate sterols that lack the modifications that are normally introduced by the missing enzyme. The phenotypes associated with such *erg* mutants range from defects in protein trafficking, mating and proper localization of membrane proteins to alterations in resistance to a wide variety of extracellular stresses including high temperature, osmotic stress, and the presence of toxic compounds in cultivation media[205]. These complex phenotypes illustrate the impact of sterols on a wide range of cellular processes.

In total, 12 molecules of oxygen are required for the *de novo* synthesis of ergosterol. Moreover, at least 24 moles of ATP and 16 moles of NAPDH must be invested, which makes sterol synthesis energetically expensive as well[260]. It is therefore no surprise that sterol synthesis

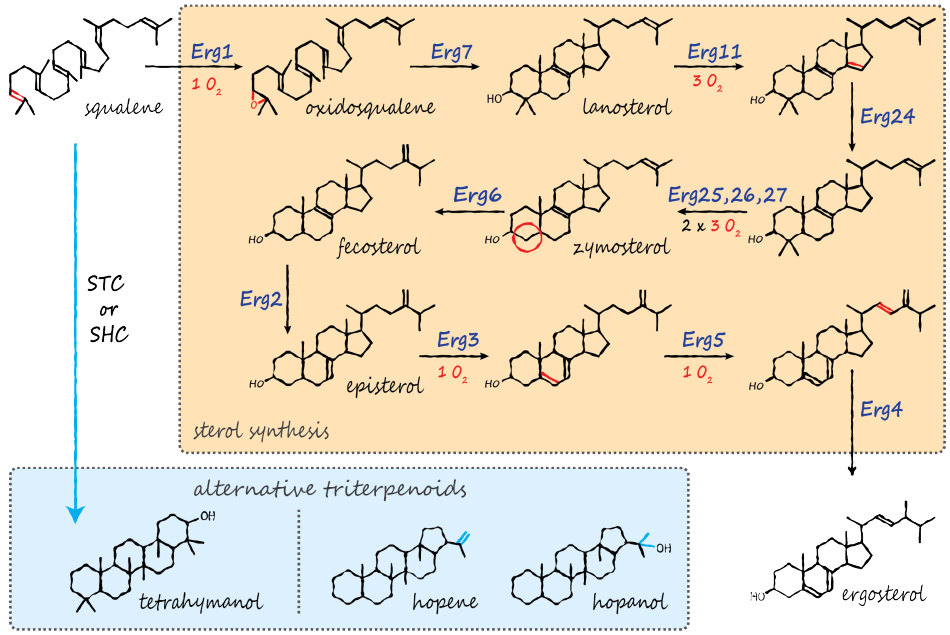

Figure 1.5: Overview of ergosterol synthesis in *S. cerevisiae*, and synthesis of alternative cyclic triterpenoids in other organisms. The orange box highlights the conserved multistep, oxygen-dependent synthesis of ergosterol from squalene. *S. cerevisiae* enzymes are indicated in dark blue and oxygen requirements of individual reactions and the resulting modifications are indicated in red. The light blue box highlights the one-step, oxygen-independent synthesis of alternative polycyclic triterpenoid tetrahymanol by squalene-tetrahymanol cyclase (STC) and that of hopene and hopanol by squalene-hopene cyclase (SHC). The hydroxyl group in these molecules is derived from water. For simplicity, stereochemistry of the polycyclic structures is not indicated in the figure.

is tightly regulated[261]. Multiple negative feedback loops, in which both ergosterol itself and intermediates of sterol biosynthesis play a role, prevent excess sterol synthesis. The transcription factors Ecm22 and Upc2 promote expression of sterol biosynthesis genes upon low sterol levels[262,263]. Upon ergosterol binding in a dedicated hydrophobic pocket, Upc2 is retained in the cytosol which prevents it from activating *ERG*-genes in the nucleus[264]. The synthesis of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), a pre-squalene intermediate of the sterol synthesis pathway, is regulated by availability of geranylgeranyl pyrophosphate (a side product of overactive mevalonate synthesis) and ergosterol. Elevated levels of these molecules reduce translation and induce proteasome-mediated degradation, respectively, of the isoenzymes Hmg1 and Hmg2, which are responsible for HMG-CoA synthesis[265]. Additionally, lanosterol regulates proteasome-dependent degradation of Erg1[266]. Mga2, previously identified as responsible for *OLE1*-expression, also stimulates expression of

ERG1[267]. The dependency of the activity of heme-containing enzymes Erg11 and Erg5, and of di-oxo-iron containing enzymes Erg25 and Erg3 on iron is consistent with indications of regulatory crosstalk between sterol biosynthesis and iron homeostasis[261,268]. Expression of *ERG*-genes is also tightly regulated by the heme-dependent transcriptional activator Hap1 and the repressor Rox1, that act on many oxygen-responsive genes and ensure a stable basal expression of *ERG*-genes under aerobic conditions, but more strongly induce expression upon oxygen-limitation[269,270]. This regulation system may be interpreted as a mechanism to boost the cellular levels of Erg-proteins to scavenge oxygen for sterol synthesis[261,269]. Upc2 is also responsible for activating the ABC-type sterol importers Aus1 and Pdr11 at very low oxygen tensions[269,271,272]. Interestingly, despite the fact that sterol synthesis is energetically expensive, *S. cerevisiae* does not import sterols under aerobic conditions, a phenomenon referred to as 'aerobic sterol exclusion'[273].

Although sterols are considered near-ubiquitous in eukaryotes, some deep-branching anaerobic fungi and protists are known to instead harbour tetrahymanol in their membranes[274–276]. In these organisms, the pentacyclic triterpenoid tetrahymanol acts as a sterol surrogate and is synthesized from squalene in a single, oxygen-independent conversion catalysed by squalene-tetrahymanol cyclase (STC)[277]. For the fungi of the phylum Neocallimastigomycota[274], the acquisition of such a gene by an ancestor through horizontal gene transfer from a prokaryotic donor is considered to have been a key evolutionary adaptation to their strictly anaerobic lifestyle in the gut of large herbivores[278,279]. Sequences of STCs are related to those of squalene-hopene cyclases (SHC) that are frequently encountered in bacteria[280,281]. SHCs catalyse oxygen-independent synthesis of structurally related hopanoid molecules, which are considered to be functional analogues of sterols in bacterial membranes[282].

Scope of this thesis

The research described in this thesis was part of a larger project funded by an ERC Advanced Grant awarded to prof. Jack Pronk. This project, entitled ELOXY (eliminating oxygen requirements in yeast) revolves around studying the anaerobic metabolism of yeasts and fungi, with the aim to eliminate oxygen requirements and, thereby, to enable anaerobic growth of industrially relevant yeasts. Although *Saccharomyces cerevisiae* is one of the few yeast species that can grow under anaerobic conditions, this ability is reported to be strictly dependent on the addition of a source of sterols and unsaturated fatty acids to anaerobic growth media. The research described in this thesis was aimed at developing metabolic engineering strategies to eliminate these oxygen requirements in membrane lipid synthesis. To this end, it was explored whether strategies adopted by anaerobic yeasts and fungi to adapt to strictly anaerobic

conditions can be implemented in *S. cerevisiae* using state-of-the-art techniques for genetic modification. The impact of these modifications on anaerobic physiology of *S. cerevisiae* was then studied in dedicated laboratory set-ups for anaerobic cultivation of yeast. Successful elimination of oxygen requirements in this well-studied model eukaryote can serve as a proof-of-principle for the implementation of such metabolic engineering strategies in other fungal industrial production hosts, and provide new insights into evolutionary adaptations of eukaryotic membrane architecture under oxygen-limited and anaerobic conditions.

The ability of *S. cerevisiae* to store a surplus of membrane lipids in lipid droplets, which can be used when there is a shortage of lipids, can obscure oxygen requirements related to membrane synthesis. In addition, it is notoriously difficult to eliminate minute traces of oxygen from laboratory scale bioreactor set-ups. In initial attempts at depleting intracellular reserves of unsaturated fatty acids through multiple sequential batch-cultivation cycles in anaerobic bioreactors, continued growth of laboratory *S. cerevisiae* strain CEN.PK113-7D was observed in the absence of an exogenous source of unsaturated fatty acids. In **Chapter 2**, this phenomenon was further investigated in shake-flask experiments in an anaerobic chamber. The membrane composition and ability to grow in absence of an unsaturated fatty acid source of reference strain CEN.PK113-7D and a congenic *Δole1*-strain under anaerobic conditions were studied to rigorously test the long-held assumption in the yeast literature that anaerobic growth of *S. cerevisiae* requires supplementation of unsaturated fatty acids.

The ability of Neocallimastigomycota to synthesize tetrahymanol as sterol surrogate is a key feature in the evolutionary adaptation of these deep-branching anaerobic fungi to strictly anaerobic environments. The goal of **Chapter 3** was to investigate whether tetrahymanol could similarly replace sterols in *S. cerevisiae* and thereby eliminate the oxygen requirements associated to sterol synthesis. To this end, a squalene tetrahymanol cyclase of the facultatively anaerobic unicellular eukaryote *Tetrahymena thermophila* was expressed in *S. cerevisiae*. Engineered strains were characterized in anaerobic sequential batch bioreactor experiments, and the fatty acid and triterpenoid composition of anaerobic biomass was studied.

The essentiality of sterols for membrane functionality and growth of yeasts, which can almost be considered a dogma, implies a necessity of exogenous sterol supplementation and sterol transport for anaerobic growth of yeasts. However, a single report from 1971[283] described anaerobic growth of a then little-studied fission yeast in the absence of an exogenous sterol source. **Chapter 4** describes a quest to retest and subsequently uncover the molecular basis underlying this remarkable ability, in search of a better understanding of anaerobic yeast physiology and potential metabolic engineering strategies that could be transferred to other yeasts.

Studying the minute oxygen requirements that are associated with biosynthetic reactions in yeast requires careful consideration of the laboratory set-ups that are used for anaerobic cultivation. In literature, procedures used for the design and execution of anaerobic growth experiments can be unclear, which may lead to contradicting conclusions. **Chapter 5** provides an overview of important design criteria and potential pitfalls for anaerobic cultivation of yeast, which were distilled from four years of expertise of the ELOXY team in designing anaerobic shake-flask and bioreactor experiments.

2

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

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

**These authors have contributed equally to the work presented in this chapter*

Abstract | In *Saccharomyces cerevisiae*, acyl-coenzyme A desaturation by Ole1 requires molecular oxygen. Tween 80, a poly-ethoxylated sorbitan-oleate ester, is therefore routinely included in anaerobic growth media as a source of unsaturated fatty acids (UFAs). During optimization of protocols for anaerobic bioreactor cultivation of this yeast, we consistently observed growth of the laboratory strain *S. cerevisiae* CEN.PK113-7D in media that contained the anaerobic growth factor ergosterol, but lacked UFAs. To minimize oxygen contamination, additional experiments were performed in an anaerobic chamber. After anaerobic precultivation without ergosterol and Tween 80, strain CEN.PK113-7D and a congenic *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 C_{10} to C_{14} 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.

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Introduction

The large majority of known yeast species ferment glucose to ethanol when grown under oxygen limitation[129,131]. 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[130,132]. The molecular basis for the non-dissimilatory oxygen requirements of most facultatively fermentative non-*Saccharomyces* yeasts is still not completely understood[284,285].

Anaerobic growth of *S. cerevisiae* imposes special nutritional requirements. Already in the 1950s, Andreasen and Stier[138,143] reported that strictly anaerobic growth of *S. cerevisiae* required supplementation of media with a sterol and an unsaturated fatty acid (UFA). Ever since these original observations, synthetic laboratory media for anaerobic growth of *S. cerevisiae* are routinely supplemented with a sterol (usually ergosterol) and a UFA source. The latter is generally provided as Tween 80, a poly-ethoxylated sorbitan ester of oleic acid[286–288]. While synthesis of nicotinic acid by *S. cerevisiae* is also oxygen dependent[151], 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[289,290]. In artisanal wine fermentation, *S. cerevisiae* starts to dominate other 'wild' yeast species once oxygen has been depleted during the initial phases of fermentation[291,292].

Sterols and fatty acids are important constituents of cellular membranes. Sterols play a key role in maintenance of membrane integrity and fluidity[206,293], and have also been implicated in specific cellular processes such as endocytosis and nutrient uptake[294,295]. The degree of (un)saturation of the fatty-acyl moieties in phospholipids is an important determinant of membrane fluidity[185]. In addition, fatty acids are involved in energy storage and post-translational modification of proteins[296].

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[241]. The oxygen requirement of *S. cerevisiae* for synthesis of UFAs (mainly palmitoleic acid, $C_{16:1}$, and oleic acid, $C_{18:1}$)[177] originates from the essential role of the Δ9-fatty acid desaturase Ole1. In the presence of ferrocytochrome b5, Ole1 catalyzes the oxygen-dependent introduction of a *cis* double

bond in palmitoyl-CoA and stearoyl-CoA, yielding palmitoleoyl-CoA ($C_{16:1}$-CoA) and oleoyl-CoA ($C_{18:1}$-CoA), respectively[177,204]. The importance of this reaction is illustrated by the strict UFA auxotrophy of *ole1* null mutants in aerobic cultures[226,235,297].

While no indications for oxygen-independent sterol biosynthesis have been found in nature, neither in living organisms nor in the fossil record[241], microbial UFA biosynthesis does not universally require oxygen. For example, during acyl-CoA synthesis by bacterial multicomponent 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[213]. Furthermore, in contrast to the cytosolic *S. cerevisiae* type-I FAS complex that only produces saturated fatty acids[204], some bacterial type-I FAS proteins are capable of oxygen-independent UFA synthesis[298,299].

Based on reported biomass contents, oxygen requirements for UFAs and sterols in *S. cerevisiae* each amount to 0.1 mmol O_2 (g biomass)$^{-1}$[203,287,300]. However, it should be noted that UFA and sterol contents strongly depend on strain background and culture conditions[301]. 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[302]. Minimizing entry of small amounts of oxygen into bench-top laboratory bioreactors is even more challenging and requires use of ultrapure nitrogen gas, applying overpressure and using special materials for tubing and septa[130,303]. Furthermore, as indicated by the practice of aerobically 'loading' brewing yeasts[289,290], 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[304,305], growth was consistently observed in synthetic media that were not supplemented with UFAs, while elimination of both sterols and UFAs almost completely blocked growth. These observations led to the hypothesis that, in contrast to the common assumption in the literature on anaerobic yeast physiology, *S. cerevisiae* does not absolutely require UFAs for anaerobic growth. To test this hypothesis, we analyzed growth of *S. cerevisiae* CEN.PK113-7D and a congenic *ole1* null mutant in cultures grown in an anaerobic chamber and analyzed the lipid composition of anaerobically grown biomass.

Results

Minimal growth in anaerobic 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[130,303], we attempted to eliminate sources of oxygen contamination in bioreactor batch cultures of *S. cerevisiae*. The bioreactor headspace was continuously flushed with ultrapure nitrogen gas and kept under 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 preculture, CO_2 production rapidly increased until, after 17 h, it reached a maximum and subsequently decreased (Figure **2.1**A). At this stage, the biomass concentration in the cultures had increased from 0.21 ± 0.00 to 0.60 ± 0.02 g L^{-1} while the glucose concentration was still above 14 g L^{-1} (Figure S1, Supporting Information). Reactors were then emptied, leaving 25 mL culture broth in the reactor, and refilled with fresh medium without ergosterol and Tween 80. In the subsequent batch culture, CO_2 production was much lower than in the first culture and remained stable for 24 h (Figure **2.1**A). Optical density measurements showed that fewer than two biomass doublings had occurred over this period, leading to a biomass concentration of 0.2 g L^{-1}.

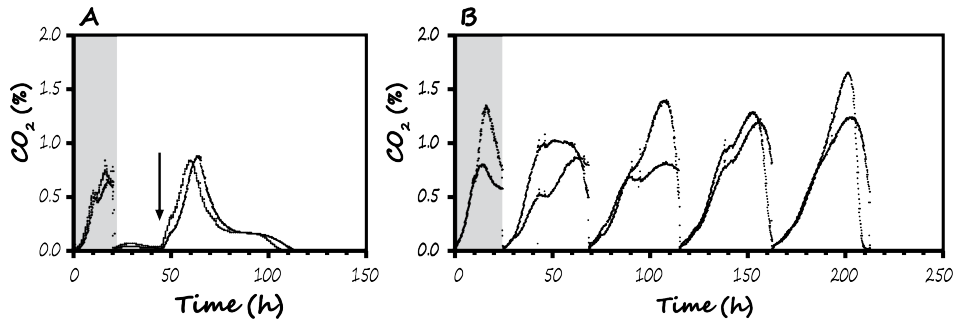

Figure 2.1: CO_2 production profiles of anaerobic bioreactor batch cultures of *S. cerevisiae* CEN.PK113–7D. Each panel shows data from independent duplicate bioreactor cultures grown on synthetic medium. Experiments were started with a 'carry-over' bioreactor batch culture on synthetic medium without 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 SBR cycles on synthetic medium with glucose (40 g L^{-1}), supplemented with 10 mg L^{-1} ergosterol but not with Tween 80.

These results strongly suggested that growth in the first anaerobic batch culture was supported by 'carry over' of anaerobic growth factors from aerobically pregrown inoculum. An initial anaerobic cultivation cycle on medium without ergosterol and Tween 80 ('carry-over culture') was therefore implemented in all subsequent experiments in anaerobic bioreactors, as well as in growth experiments in an anaerobic chamber.

Although the slow increase of the biomass concentration during the second cultivation cycle suggested that oxygen entry had not been completely eliminated, the experimental set-up was considered suitable for further studies on anaerobic growthfactor 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 **2.1**A), indicating that, during the second cultivation cycle, growth in the anaerobic bioreactors was restricted by sterol availability. Since no Tween 80 was added, this observation raised questions about the requirement of these anaerobic cultures for UFAs.

Omission of UFAs does not prevent growth in anaerobic SBR cultures | To further investigate the observed anaerobic growth of *S. cerevisiae* CEN.PK113-7D in synthetic medium supplemented with ergosterol, but not with Tween 80, experiments were performed in SBRs. After an initial carry-over cycle, four consecutive SBR cycles on medium without Tween 80 showed a pronounced CO_2 production and corresponding increase of the biomass concentration (Figure **2.1**B). 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 **2.1**).

Table 2.1: Anaerobic growth of *S. cerevisiae* CEN.PK113-7D in anaerobic 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 **2.1**B), 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

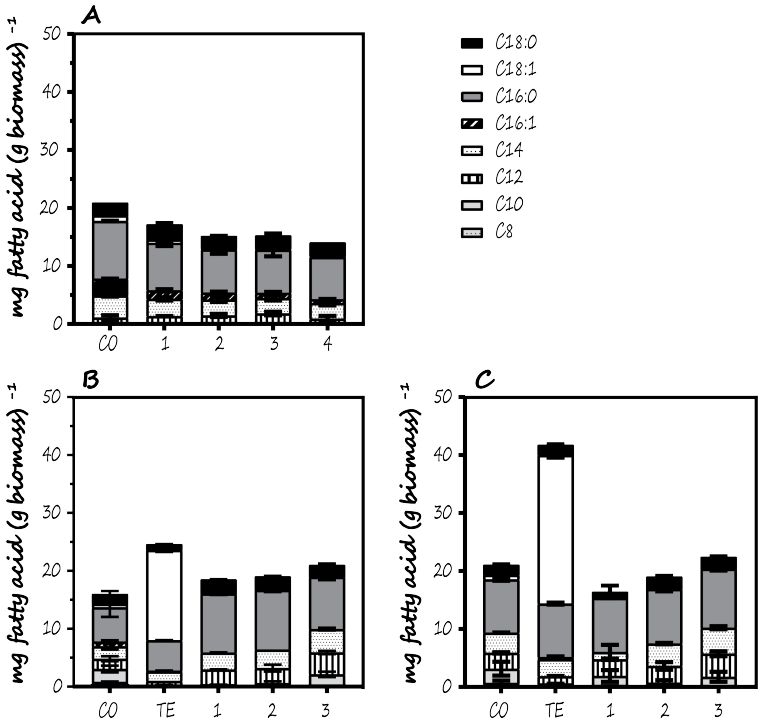

Figure 2.2: Fatty acid composition of anaerobic *S. cerevisiae* cultures. Fatty acid composition, analyzed by GC-FID, of anaerobically grown cultures in SBRs (**A**) and anaerobic-chamber shake-flask cultures (**B**, **C**). (**A**) Fatty acid composition of the reference strain CEN.PK113–7D during anaerobic SBR cultivation; 'CO': carry-over cycle; 1–4: subsequent SBR cycles 1–4 on synthetic medium supplemented with ergosterol but not Tween 80. (**B**) and (**C**) Fatty acid composition of strains IMX585 (CEN.PK113–7D with Cas9 integrated in genome) and its congenic *ole1*Δ mutant IMK861, respectively, during serial-transfer shake-flask cultivation in an anaerobic chamber; 'CO': carry-over culture; 'TE': cultures grown on synthetic medium supplemented with both anaerobic growth factors; 1–3: transfers 1–3, respectively, of cultures grown on synthetic medium supplemented with ergosterol but not Tween 80. Each panel shows data from independent duplicate bioreactor or shake-flask cultures grown on synthetic medium.

To investigate whether growth without Tween 80 supplementation reflected *de novo* UFA biosynthesis, enabled by inadvertent entry of oxygen into the bioreactors, fatty acids were extracted from biomass harvested at the end of each SBR cycle and analyzed by gas chromatography. At the end of the 'carry-over' cycle, but also at the end of the subsequent four cycles on medium from which Tween 80 was omitted, small quantities of palmitoleate ($C_{16:1}$) and oleate ($C_{18:1}$) were detected (Figure **2.2**A; Table S2, Supporting Information). Since the four SBR cycles led to an ~$5{\cdot}10^6$-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[306]) was studied in an anaerobic chamber equipped with a H_2/Pd catalyst system to scavenge traces of oxygen. Since *ole1*Δ strains of *S. cerevisiae* are unable to synthesize UFAs[226,235], growth of the congenic *ole1*Δ 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 **2.3**).

After the initial carry-over culture, both the reference strain and the *ole1*Δ 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 **2.3**).

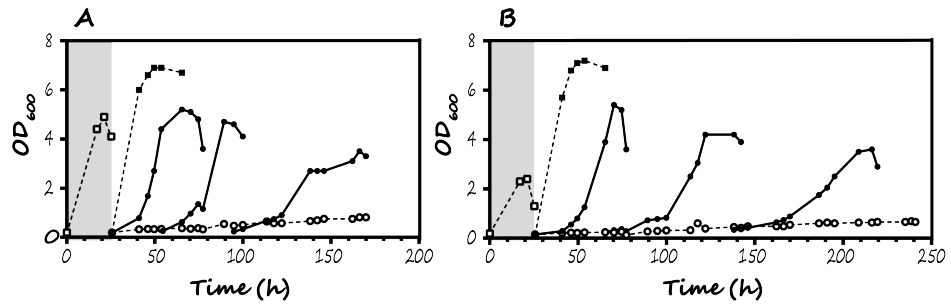

Figure 2.3: UFA-independent anaerobic growth of the reference strain *S. cerevisiae* IMX585 and the *ole1*Δ strain IMK861. Strains IMX585 (**A**) and IMK861 (*ole1*Δ, **B**) were grown in shake-flask cultures placed in an anaerobic chamber. Aerobic precultures were used to inoculate an anaerobic preculture ('carry-over culture', open squares, grey box) on SMD-urea containing 50 g L^{-1} glucose but no anaerobic growth factors. When the OD_{600} no longer increased, cultures were transferred to fresh SMD-urea, supplemented either with Tween 80 and ergosterol (closed squares), only ergosterol (closed circles) or neither (open circles). The initial culture to which only ergosterol was added (closed circles, first line) was sequentially transferred to the same medium (closed circles, second and third line). The data are from a single representative experiment of biological duplicate cultures. Data of the duplicate experiment are shown in Figure S2 (Supporting Information).

These observations suggested that, at least in the CEN.PK genetic background, synthesis or supplementation of UFAs is not required for anaerobic growth of *S. cerevisiae*. This hypothesis was further tested by analyzing the lipid content and composition of yeast biomass in the serial transfer experiments.

At the end of the carry-over cultures, UFAs were detected in both strains (Figure **2.2**B and C). Since growth in the carry-over cultures ceased before glucose was depleted (Table S1, Supporting Information), this observation suggested that depletion of sterols rather than depletion of UFAs caused growth to stop. No UFAs were detected during three subsequent transfers in medium without Tween 80, neither in the reference strain nor in the *ole1*Δ mutant. Instead, contents of palmitic acid ($C_{16:0}$) and short-chain saturated fatty acids (C_{10}-C_{14}) were higher than in cultures supplemented with Tween 80 (Figure **2.2**B and C; Table S2, Supporting Information). When cells from a stationary-phase carry-over culture were instead transferred to medium containing both ergosterol and Tween 80, oleic acid ($C_{18:1}$), which is the main UFA side-chain of Tween 80[287], was the dominant fatty acid in yeast biomass (Figure **2.2**B and C; Table S2, Supporting Information).

UFA synthesis or supplementation is essential for aerobic growth | Several previous studies reported that *ole1*Δ strains constructed in other *S. cerevisiae* genetic backgrounds are unable to grow aerobically without UFA supplementation[226,235,307]. To check if an *ole1* null mutation in the CEN.PK genetic background might have a different phenotype, we investigated aerobic growth of the *ole1*Δ 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 preculture (Figure **2.4**).

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*[226,307], it indicates that, surprisingly, the UFA requirement for anaerobic growth of *S. cerevisiae* is not absolute.

Since nonrespiratory oxygen requirements of *S. cerevisiae* are small[156], interpretation of results can easily be obscured by oxygen contamination and by 'carry-over' of anaerobic growth factors from aerobic or growth-factor supplemented precultures. Indeed, oxygen contamination of bioreactor experiments was evident from synthesis of small amounts of palmitoleic and oleic acid (Figure **2.2**A; Table S1, Supporting Information). This UFA synthesis

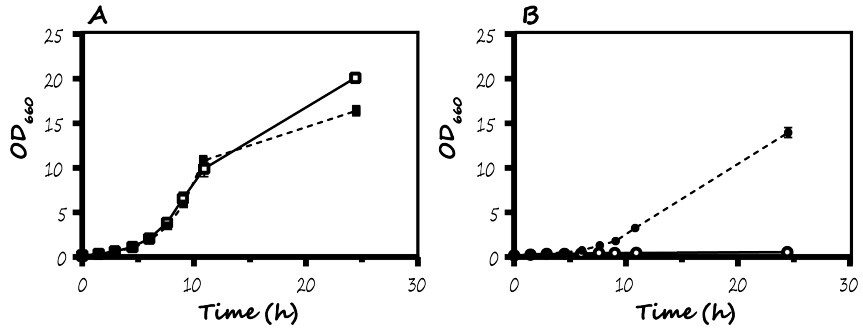

Figure 2.4: Aerobic growth of *S. cerevisiae* strains IMX585 and IMK861 (*ole1*Δ) in the presence and absence of a source of unsaturated fatty acids. Strains IMX585 (**A**) and IMK861 (*ole1*Δ) (**B**) were grown in 100 mL SMD in 500 mL round-bottom shake-flasks at 30 °C and at 200 rpm. Growth was monitored 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.

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[308]. 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 *ole1*Δ mutant continued during three consecutive transfers in UFA-free medium. UFA contents were already below detection limit after the first cycle and, after the second cycle, biomass of the carry-over culture had been diluted by ~500-fold. Although these results do not entirely exclude a minute UFA requirement for anaerobic growth, any remaining UFA levels in the serial batch cultures were too low to account for maintenance of membrane fluidity[309]. Anaerobic cultures in UFA-free medium showed increased contents of medium-chain (C_{10} to C_{14}) fatty acids. This adaptation is in line with the demonstrated flexibility of the yeast lipidome in response to other environmental stresses[174,310]. A similar adaptation was previously observed in promitochondria of cells after anaerobic incubation without a source of UFAs[311] and in a recent chemostat study on severe oxygen limitation in chemostat cultures without UFA or sterol supplementation[308].

Anaerobic growth of *S. cerevisiae* is only rarely studied in media that contain sterols, but not UFAs. In the original work of Andreasen and Stier, cell counts that were 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[143]. One reason for the routine inclusion of Tween 80 is that this surfactant aids distribution of highly hydrophobic sterols in aqueous media[312]. A requirement of anaerobic *S. cerevisiae* cultures for UFA supplementation is often inferred from the well-documented UFA auxotrophy of *ole1* null mutants in aerobic cultures. Stukey *et al.* (1989)[226] showed that aerobic growth of an *ole1* null mutant ceased when the contribution of UFAs decreased below 7.3% mol of the total fatty acid content. The sustained anaerobic growth of an *ole1*Δ mutant in UFA-free media, with undetectable intracellular UFA contents (Figures **2.2** and **2.3**), 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 ($C_{18:1}$), plays a key role in the complex regulation of sterol homeostasis[313] and steryl-ester synthesis has been demonstrated to decrease during anaerobiosis[314]. 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[315]. 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 *ole1*Δ 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 S2, Supporting Information), showed an estimated specific growth rate of 0.14 and 0.20 h^{-1} (Table **2.1**). This value is significantly lower than reported for anaerobic batch cultures of *S. cerevisiae* CEN.PK113-7D supplemented with both Tween 80 and ergosterol[316]. Biomass yields on glucose (0.04 g biomass (g glucose)$^{-1}$, Table **2.1**) were 2-fold lower than in anaerobic chemostat cultures grown with Tween 80 supplementation[317]. 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[318]. 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 **2.3**).

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 [300,319,320], 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 [309,321].

Supplementary Materials

Supporting Information is available at FEMSYR online:
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Materials and methods

Strains, media and maintenance | *S. cerevisiae* strains used and constructed in this study (Table **2.2**) were derived from the CEN.PK lineage [304,305]. Yeast extract peptone dextrose medium (YPD; 10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone, 20 g L^{-1} glucose) was used for making frozen stock cultures. Synthetic medium with 20 g L^{-1} glucose (SMD) was prepared as described previously [135]. 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 [322]. 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 [323]. 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. *Escherichia coli* XL1-Blue was grown in Lysogeny Broth (LB; 10 g L^{-1} Bacto tryptone, 5 g L^{-1} Bacto yeast extract and 5 g L^{-1} NaCl). For selection of transformants, LB was supplemented with 100 mg L^{-1} ampicillin. After addition of sterile glycerol (30% v/v), culture samples were frozen and stored at −80 °C. 'Super optimal broth' (SOB) medium contained 0.5 g L^{-1} yeast extract, 2 g L^{-1} Bacto tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2 \cdot 6H_2O$, 10 mM $MgSO_4 \cdot 7H_2O$, and was autoclaved at 121 °C for 20 min. To prepare 'super optimal broth medium with catabolite repression' (SOC), a concentrated solution of glucose, separately autoclaved at 110 °C for 30 min, was added to SOB to a final concentration of 20 mM.

Table 2.2: Strains used in this study

Name	Revelant genotype	Parental strain	Origin
CEN.PK113-7D	*MATα MAL2-8c SUC2 CAN1*	-	304
IMX585	*MATα MAL2-8c SUC2 can1::cas9-natNT2 URA3 TRP1 LEU2 HIS3*	CEN.PK113-7D	306
IMK861	*MATα MAL2-8c SUC2 can1::cas9-natNT2 URA3 TRP1 LEU2 HIS3 ole1Δ*	IMX585	This study

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 polymerase chain reaction (PCR) was performed with DreamTaq PCR Master Mix (Thermo Scientific), according to the manufacturer's protocol and with desalted oligonucleotide primers (Sigma-Aldrich). PCR-amplified linear integration cassettes were purified from 1% (w/v) agarose gels (TopVision Agarose, Thermo Fisher) with TAE buffer (50x, Thermo Fisher) using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). *E. coli* XL1-Blue competent cells were 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 GenElute Plasmid Miniprep kit (Thermo Fisher Scientific) was used to isolate plasmids from overnight cultures in 15 mL Greiner tubes on selective medium. *S. cerevisiae* was transformed with the lithium-acetate method [324]. 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 [306]. Oligonucleotides and plasmids used in this study are listed in Table **2.3** and Table **2.4**, respectively. A unique guide-RNA (gRNA) sequence targeting *OLE1* was designed using Yeastriction [306] and synthesized as a 103 bp oligonucleotide (Sigma). To construct the *OLE1*-targeting CRISPR plasmid pUDR319, the plasmid backbone of pROS11 was first PCR-amplified

with the double-binding primer 6005. The gRNA-targeting sequence was then introduced as 5' primer overhang with the double-binding primer 11986, using pROS11 as template. Subsequently, both PCR products were gel purified, digested with DpnI (Thermo Scientific) and mixed in equimolar ratio. Gibson assembly was performed in a final volume of 5 µL with the NEBuilder HiFi DNA assembly master mix (NEB, Ipswich, MA), according to 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 [323].

Table 2.3: Primers used in this study

Purpose	Primer nr.	Sequence 5' → 3'
gRNA primer targeting *OLE1*	11231	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAA TGATCCTTTTGTTCTTGTTGAATCAGTTTTAGAGCTAGAAATAGC AAGTTAAAATAAG
Repair fragment, *OLE1* upper strand	11239	CATAGTAATAGATAGTTGTGGTGATCATATTATAAACAGCACTAAA ACATTACAACAAAGGTATCACATTACAATAACAAAACTGCAACTA CCATAAAAAAAAATTGAAAAATCATAAAA
Repair fragment, *OLE1* lower strand	11240	TTTTATGATTTTTCAATTTTTTTTTATGGTAGTTGCAGTTTTGTT ATTGTAATGTGATACCTTTGTTGTAATGTTTTAGTGCTGTTTATA ATATGATCACCACAACTATCTATTACTATG
Diagnostic primer *OLE1* fw	11249	GGTATCCCAGCCTTCTCTGC
Diagnostic primer *OLE1* rv	11250	CTATTGCTCCAGGGCCCAG

Table 2.4: Plasmids used in this study

Name	Relevant characteristics	Origin
pROS11	2 µm ampR *amdSYM* p*SNR52*-gRNA$_{CAN1.Y}$p*SNR52*-gRNA$_{ADE.Y}$	306
pUDR319	2 µm ampR *amdSYM* p*SNR52*-gRNA$_{OLE1}$p*SNR52*-gRNA$_{OLE1}$	This study

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

Anaerobic bioreactor cultivation | Anaerobic bioreactor batch cultivation was performed in 2-L laboratory bioreactors (Applikon, Schiedam, the Netherlands) with a working volume of 1.2 L. Before autoclaving, bioreactors were tested for gas leakage by applying 0.3 bar overpressure while completely submerging them in water. Anaerobic conditions were maintained by continuous flushing of the headspace of bioreactor cultures with 500 mL N_2 min^{-1} (≤0.5 ppm O_2, 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 O2, F-5500-A, Saint-Gobain, Courbevoie, France) and Viton O-rings (Eriks, Alkmaar, the Netherlands). Furthermore, bioreactor cultures were grown on SMD-urea[322] 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 an NGA 2000 Rosemount gas analyzer (Emerson, St. Louis, MO). The gas analyzer was calibrated with reference gas containing 3.03% CO_2 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 preculture on SMD was inoculated at an OD_{660} of 1.0. During the exponential growth phase of this second preculture, 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 CO_2 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 one

h before refilling. Immediately before refilling, ~20 mL medium was purged from the medium inlet line to remove any oxygen contamination in stagnant medium. To further minimize oxygen contamination, gassing was temporarily switched from headspace to sparging during refilling and overpressure (0.2 bar) was applied throughout empty-refill cycles.

Anaerobic growth studies in shake flasks | Anaerobic shake-flask experiments were performed in a Shel Lab Bactron 300 anaerobic workstation (Sheldon Manufacturing Inc., Cornelius, OR) at 30 °C. The anaerobic gas mixture used for flushing the work space and air lock consisted of 85% 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 or 50 g L^{-1} glucose. Concentrated solutions of ergosterol and/or Tween 80 were added as indicated. Sterile growth media were preincubated in the anaerobic chamber for at least 48 h prior to inoculation to allow for complete removal of oxygen. Growth experiments in the anaerobic chamber were started by inoculating anaerobic shake flasks, containing SMD-urea without both ergosterol and Tween 80 and containing 50 g L^{-1} glucose, at an initial OD_{600} of 0.2, from an exponentially growing aerobic preculture 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 preculture 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 analyzed by high-performance liquid chromatography (HPLC) as described previously[325]. Biomass dry weight measurements in SBR cultures were performed at the end of each cultivation cycle, using preweighed nitrocellulose filters (0.45 µm, Gelman Laboratory, Ann Arbor, MI). After filtration of 10 or 20 mL culture samples, filters were washed with demineralized water prior to drying in a microwave oven (20 min at 360 W). Fatty acids in biomass were analyzed as methyl-ester derivatives by gas chromatography with flame-ionization detection (GC-FID). Biomass samples were harvested by centrifuging at least 30 mL of culture broth at 3000 × *g* for 5 min. Pellets were washed once with demineralized water and stored at −80 °C. Frozen samples were lyophilized overnight in a freeze-dryer (Alpha 1–4 LD Plus, Christ, Osterode am Harz, Germany) and 20 to 30 mg of lyophilized material was weighed into glass methylation tubes (Article no. 10044604, PYREX™Borosilicate glass, Thermo Fisher Scientific). After adding 2 mL methanol (Honeywell, Mexico City, Mexico), samples were vortexed thoroughly. 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 (Greiner BioOne, Alphen aan den Rijn, The Netherlands) containing 10–20 mg dried Na_2SO_4 (Merck) to remove remaining traces of water and shaken vigorously. After centrifugation (5 min at 5000 × *g*), the liquid phase was transferred to a GC vial (11 mm crimp-neck vial (10326042) and cap (11821653) with butyl rubber septum (Thermo Fisher Scientific)). The sample was concentrated by evaporating the solvent under a stream of N_2. Fatty acid methyl esters were analyzed on an Agilent Technologies 7890A GCFID system equipped with an FID-1000–220 Gas Station (Parker Balston, Haverhill, MA, USA) and an Agilent Technologies 7693 Autosampler. A VF-5 ms column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness, Agilent part no. CP9013) was used for separation, and nitrogen was used as a carrier gas at a constant flow of 0.4 mL min^{-1}. The oven temperature, which was initially 50 °C, was increased to 220 °C at 60 °C min^{-1}, then kept constant for 3 min, increased to 250 °C at 8 °C min^{-1}, again kept constant for 3 min, and finally increased to 320 °C at 60 °C min^{-1} and kept constant for another 6 min. Inlet temperature was set at 150 °C, and FID temperature at 280 °C. The Supelco FAME mix C8-C24 (Sigma-Aldrich, MO, USA) was used to calibrate the GC-FID system for quantification of individual fatty acid methyl esters. A separate 10-point calibration curve was made with methyl oleate (>99%, Sigma-Aldrich). Data were adjusted for internal standard concentrations and expressed per g of lyophilized biomass.

3

Squalene-tetrahymanol cyclase expression enables sterol-independent growth of *Saccharomyces cerevisiae*

Sanne J. Wiersma, Christiaan Mooiman, Martin Giera and Jack T. Pronk

Abstract | Biosynthesis of sterols, which are considered essential components of virtually all eukaryotic membranes, requires molecular oxygen. Anaerobic growth of the yeast *Saccharomyces cerevisiae* therefore strictly depends on sterol supplementation of synthetic growth media. Neocallimastigomycota are a group of strictly anaerobic fungi which, instead of containing sterols, contain the pentacyclic triterpenoid "sterol surrogate" tetrahymanol, which is formed by cyclization of squalene. Here, we demonstrate that expression of the squalene-tetrahymanol cyclase gene *TtTHC1* from the ciliate *Tetrahymena thermophila* enables synthesis of tetrahymanol by *S. cerevisiae*. Moreover, expression of *TtTHC1* enabled exponential growth of anaerobic *S. cerevisiae* cultures in sterol-free synthetic media. After deletion of the *ERG1* gene from a *TtTHC1*-expressing *S. cerevisiae* strain, native sterol synthesis was abolished and sustained sterol-free growth was demonstrated under anaerobic as well as aerobic conditions. Anaerobic cultures of *TtTHC1*-expressing *S. cerevisiae* on sterol-free medium showed lower specific growth rates and biomass yields than ergosterol-supplemented cultures, while their ethanol yield was higher. This study demonstrated that acquisition of a functional squalene tetrahymanol cyclase gene offers an immediate growth advantage to *S. cerevisiae* under anaerobic, sterol-limited conditions and provides the basis for a metabolic engineering strategy to eliminate the oxygen requirements associated with sterol synthesis in yeasts.

Importance | The laboratory experiments described in this report simulate a proposed horizontal gene transfer event during the evolution of strictly anaerobic fungi. The demonstration that expression of a single heterologous gene sufficed to eliminate anaerobic sterol requirements in the model eukaryote *Saccharomyces cerevisiae* therefore contributes to our understanding of how sterol-independent eukaryotes evolved in anoxic environments. This report provides a proof of principle for a metabolic engineering strategy to eliminate sterol requirements in yeast strains that are applied in large-scale anaerobic industrial processes. The sterol-independent yeast strains described in this report provide a valuable platform for further studies on the physiological roles and impacts of sterols and sterol surrogates in eukaryotic cells.

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Introduction

Sterols are a class of hydrophobic triterpenoid compounds, representatives of which are found in almost all eukaryotic membranes. Sterols affect membrane fluidity and permeability [326,327], and sterol-enriched domains ("lipid rafts") contribute to lateral compartmentalization of eukaryotic plasma membranes by influencing localization of specific membrane proteins [197,328]. Cholesterol is the major sterol in mammals, phytosterol the major sterol in plants, and ergosterol the major sterol in filamentous fungi and yeasts [241].

Saccharomyces cerevisiae is an intensively used model for studying sterol function and biosynthesis in eukaryotes. Analysis of sterol-synthesis mutants of this yeast has revealed a wide range of cellular processes that are influenced by sterol composition (reviewed in references 329 and 205). These include endocytosis [330], intracellular trafficking and excretion of proteins [331], and nutrient uptake [294]. In addition, sterols influence resistance to stresses, such as superoptimal temperature and presence of growth-inhibiting compounds [203,332,333]. Based on the importance of sterols for fungal growth, many fungicides target ergosterol biosynthesis [334–336].

Eukaryotic sterol synthesis starts with the oxygen-independent conversion of acetyl coenzyme A (acetyl-CoA) into squalene via the mevalonate pathway or, in plant plastids, via the 2-C-methyl-D-erythritol 4-phosphate pathway [245]. The subsequent conversion of squalene into specific sterols involves a strongly conserved oxygen-dependent pathway [337], whose activity is initiated by the oxygen-dependent epoxidation of squalene to oxidosqualene, a reaction catalyzed by Erg1 in *S. cerevisiae*. Cyclization of oxidosqualene to lanosterol, which yields the basic tetracyclic sterol backbone structure, is followed by a series of further modifications, several of which require molecular oxygen. The complete synthesis of a single molecule of ergosterol from squalene requires 12 molecules of oxygen (Figure **3.1**).

No evidence is available for anaerobic sterol biosynthesis in either living organisms or the geological record [241]. Consistent with the oxygen dependency of sterol biosynthesis, early yeast research already demonstrated that *S. cerevisiae* is strictly auxotrophic for sterols under anaerobic conditions [138]. *S. cerevisiae* transporters Pdr11 and Aus1, which mediate ATP-dependent import of sterols across the plasma membrane, are expressed only at very low oxygen concentrations [272,273]. While ergosterol is routinely included in synthetic media (SM) for anaerobic growth of *S. cerevisiae*, several other sterols can also complement the sterol auxotrophy of anaerobic cultures and heme-incompetent cells. However, several studies previously reported that sterols with specific structural features are required to successfully complete the yeast cell cycle [206,338,339].

Together with the synthesis of unsaturated fatty acids, which in eukaryotes also requires oxygen [204,226], the oxygen requirement for sterol synthesis strongly affects large-scale,

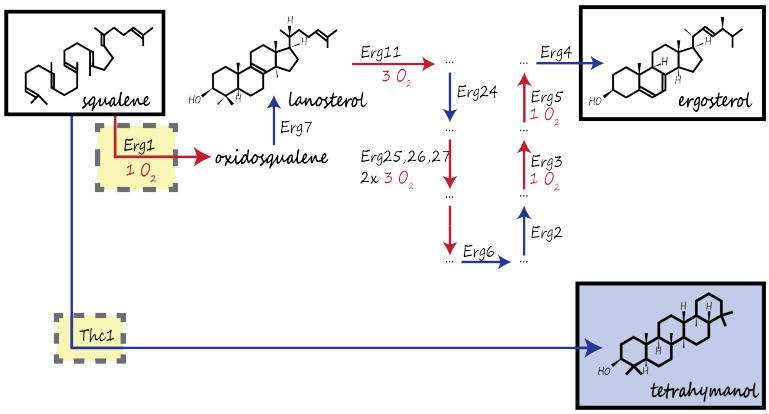

Figure 3.1: Schematic overview of ergosterol and tetrahymanol synthesis pathways. Erg1, Erg7, Erg24, Erg25, Erg26, Erg27, Erg6, Erg2, Erg3, Erg5, and Erg4, native *S. cerevisiae* enzymes involved in the oxygen-dependent synthesis of ergosterol from squalene. Thc1, squalene tetrahymanol cyclase from *Tetrahymena thermophila*. Oxidosqualene, epoxidation product of squalene, formed by Erg1. Lanosterol, first tetracyclic compound in fungal ergosterol synthesis. For oxygen-dependent reactions, the number of moles of oxygen required is indicated. Dashed boxes represent enzymes whose expression levels were modified in this study.

anaerobic industrial applications of *Saccharomyces* yeasts. Anaerobic wine and beer fermentation processes are commonly preceded by a brief aeration phase that enables yeast cells to synthesize and store sterols and unsaturated fatty acids[290,340]. Preliminary arrest of the subsequent anaerobic phase of these processes ("stuck fermentation") is often attributed to premature depletion of the remaining lipid reserves[341,342].

Neocallimastigomycota, a group of obligately anaerobic rumen fungi, lack the genetic information for sterol biosynthesis[279,343,344]. Instead, their membranes contain tetrahymanol, a pentacyclic triterpenoid compound that is considered to act as a sterol surrogate[282,345,346] and whose synthesis by Neocallimastigomycota is seen as a key evolutionary adaptation to their anaerobic lifestyle[278,347]. In contrast to the multistep, oxygen-dependent synthesis of sterols from squalene, tetrahymanol can be produced from this intermediate in a single, oxygen-independent cyclization reaction catalyzed by squalene-tetrahymanol cyclase (STC; EC 4.2.1.123)[277].

Originally discovered in the protozoan *Tetrahymena pyriformis*[276], tetrahymanol also occurs in the fern *Oleandra wallichii*[348] and in several prokaryotes[349–351]. Indeed, DNA sequence analysis indicated that Neocallimastigomycota acquired a prokaryotic STC gene by horizontal gene transfer[279]. However, whether the mere acquisition of a functional STC gene is sufficient to reduce or even eliminate sterol requirements of fungi has not yet been investigated. Addressing this issue may provide not only insight into the roles of sterols and sterol surrogates

in eukaryotes and in the evolution of an anaerobic lifestyle in eukaryotes but also strategies to reduce or eliminate oxygen requirements in anaerobic applications of yeasts and other fungi.

The goals of the present study were to analyze the impact of expression of the STC gene of *T. thermophila* in wild-type and sterol biosynthesis-deficient *S. cerevisiae* strains on triterpenoid and fatty acid composition as well as on sterol requirements and growth rates in anaerobic and aerobic cultures.

Results

Expression of a *Tetrahymena thermophila* squalene-tetrahymanol cyclase gene enables tetrahymanol synthesis in *S. cerevisiae* | The squalene-tetrahymanol cyclase (STC) gene *TtTHC1* of *Tetrahymena thermophila* was codon optimized for expression in *S. cerevisiae* and integrated into the genome of Cas9-expressing reference strain IMX585[306] under the control of the constitutive *TEF1* promoter. To investigate the impact of *TtTHC1* expression under anaerobic, sterol-sufficient conditions, growth of the resulting strain, IMX1438 (*sga1Δ::TtTHC1*), was compared to that of the IMX585 reference strain in anaerobic sequential batch reactor (SBR) cultures grown on SMD-urea (synthetic media supplemented with glucose and urea) supplemented with Tween 80 and ergosterol. Tetrahymanol was detected in cultures of strain IMX1438 (*sga1Δ::TtTHC1*) at cellular contents of 0.47 ± 0.09 mg (g biomass)$^{-1}$ whereas, as anticipated, no tetrahymanol was found in the IMX585 reference strain (Figure **3.2**A; see also Figure S2 and Table S2 in the supplemental material). Neither the cellular levels of squalene, lanosterol, and ergosterol nor the fatty acid levels and compositions of the two strains showed marked differences under these conditions (Figure **3.2**; see also Table S2 and S3).

Strains IMX585 and IMX1438 (*sga1Δ::TtTHC1*) both exhibited fast exponential growth in three subsequent anaerobic SBR cycles on ergosterol-supplemented SMD-urea (Figure **3.3**A and C; see also Figure S4), with glucose being consumed within 26 h (Figure **3.3**B and D; see also Figure S4). The specific growth rates and biomass yields of the two strains differed by less than 10% (Table **3.1**), and no major differences were observed between their extracellular metabolite profiles (Figure **3.3**B and D; see also Table S1). These results indicated that tetrahymanol production by strain IMX1438 did not have a major impact on its physiology in anaerobic ergosterol-supplemented cultures.

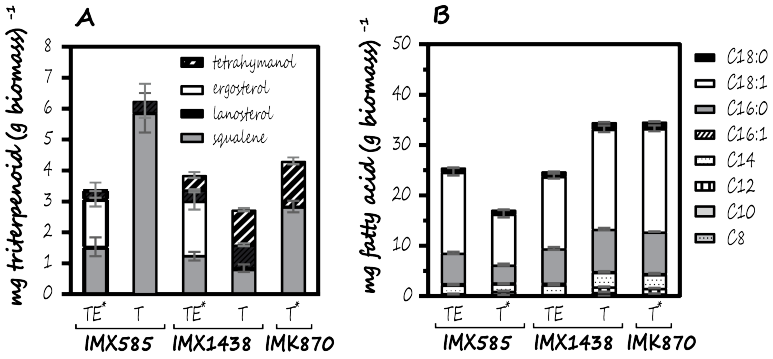

Figure 3.2: Analysis of triterpenoid fraction and total fatty acid composition of anaerobic biomass of *S. cerevisiae* strains IMX585 and IMX1438. Biomass was harvested in the second anaerobic sequential batch reactor (SBR) cultivation cycle after the initial cycle for depletion of anaerobic growth factors. For the IMX585 reference strain, biomass was harvested at the end of this SBR cycle. For IMX1438 (*sga1*Δ*::TtTHC1*) and IMK870 (*sga1*Δ*::TtTHC1 erg1*Δ), biomass was harvested during exponential growth. Unless otherwise indicated, data represent means and standard error of the means of results from three replicate SBR experiments. (**A**) Triterpenoid fraction. (**B**) Fatty acid composition of biomass harvested during anaerobic sequential batch reactor experiments. TE, culture supplemented with both Tween 80 and ergosterol; T, culture supplemented with Tween 80 only; *, data represent results of two replicate SBR experiments.

Table 3.1: Specific growth rates and biomass yields of *S. cerevisiae* strains in anaerobic sequential batch reactor experiments. Strains were grown on glucose synthetic medium (SMD-urea) with Tween 80, with or without supplementation with ergosterol, at 30 °C. Unless otherwise indicated, data are represented as averages ± standard errors of measurements of results from three independent bioreactor experiments. Levels of recovery of glucose carbon in biomass, CO_2, and soluble organic products were between 95% and 105% for all experiments. Growth rate and biomass yield data labeled with matching uppercase letters in parentheses indicate pairs of data sets for which the means were determined to be significantly different using a two-tailed unpaired Student's t-test with a *P* value of 0.05.

S. cerevisiae strain	Relevant genotype	Sterols in medium	Growth rate (h^{-1})	Biomass yield ($g_{biomass}\ g_{glucose}^{-1}$)	Ethanol yield ($g_{ethanol}\ g_{glucose}^{-1}$)
IMX585	*ERG1*	Yes	0.26 ± 0.00	0.086 ± 0.001	0.37 ± 0.00
	(Reference strain)	No[a,b]	~ 0.034 ± 0.000	~ 0.027 ± 0.000	~ 0.38 ± 0.01
IMX1438	*sga1*Δ*::TtTHC1*	Yes	0.24 ± 0.01 (A)	0.082 ± 0.000 (B)	0.37 ± 0.00 (C)
		No	0.15 ± 0.01 (A)	0.052 ± 0.000 (B)	0.39 ± 0.00 (C)
IMK870	*sga1*Δ*::TtTHC1 erg1*Δ	No[b]	0.11 ± 0.01	0.049 ± 0.006	0.39 ± 0.02

[a] Nonexponential growth; estimate based on measurements at start and end of SBR cycles.

[b] Data represent results obtained with two replicates.

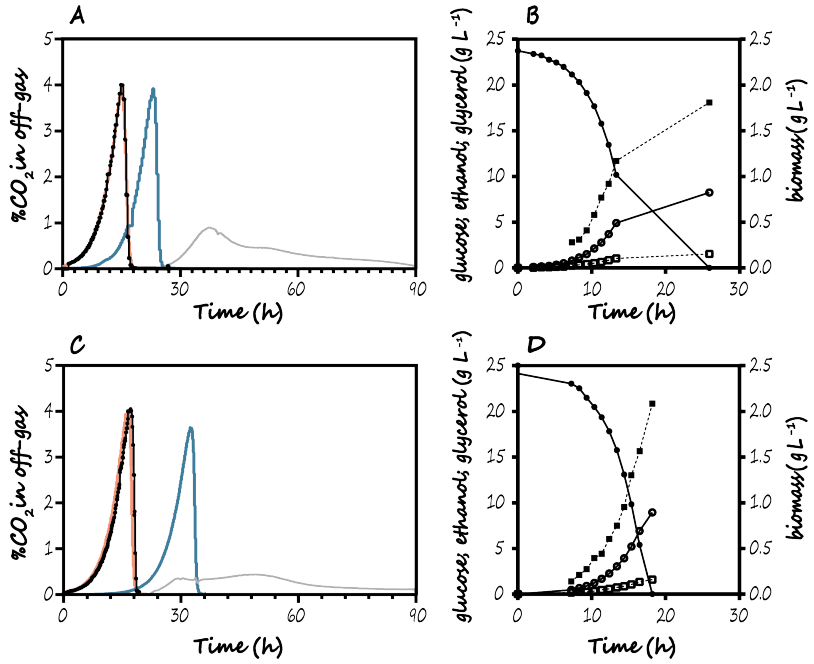

Figure 3.3: Anaerobic sequential batch bioreactor (SBR) cultivation of *S. cerevisiae* strains IMX585 and IMX1438 with sterol supplementation. All panels represent data from a single representative SBR experiment performed at 30 °C on SMD-urea without pH control. Data from replicate experiments are shown in Figure S4. (**A** and **B**) Reference strain IMX585. (**C** and **D**) Strain IMX1438 (*sga1*Δ*::TtTHC1*). (**A** and **C**) Percentages of CO_2 in off-gas during the initial batch cycle without anaerobic growth factors (grey line) and during the first (blue line), second (black line and dots), and third (red line) subsequent SBR cycles on SMD-urea supplemented with Tween 80 and ergosterol. (**B** and **D**) Concentrations of glucose (closed circles), biomass (closed squares), ethanol (open circles), and glycerol (open squares) in the second SBR cycle.

Tetrahymanol synthesis supports anaerobic growth in the absence of sterol supplementation | To investigate whether tetrahymanol can functionally replace ergosterol in anaerobic cultures of *S. cerevisiae*, strains IMX1438 (*sga1*Δ*::TtTHC1*) and IMX585 were tested in anaerobic SBR cultures on SMD-urea without ergosterol. SBR experiments were preceded by a single batch-cultivation cycle on SMD-urea with neither ergosterol nor Tween 80, included to deplete endogenous reserves of sterols and unsaturated fatty acids. This initial culture was followed by three SBR cycles on SMD-urea supplemented with Tween 80 but lacking a source of sterol.

CO_2 off-gas profiles confirmed the inability of *S. cerevisiae* reference strain IMX585 to grow exponentially on SMD-urea without ergosterol (Figure **3.4**A; see also Figure S5). Slow consumption of glucose by this strain took approximately 100 h (Figure **3.4**B). Based on the

initial and final concentrations of glucose and biomass in these experiments (Table S1), the estimated specific growth rate and biomass yield over this period were 0.034 ± 0.000 h^{-1} and 0.027 ± 0.002 g biomass (g glucose)$^{-1}$, respectively (Table **3.1**). These values were 87% and 69% lower, respectively, than those of SBR cultures of this reference strain supplemented with both Tween 80 and ergosterol (Table **3.1**). Similar residual growth rates of *S. cerevisiae* strains in anaerobic bioreactor cultures on synthetic medium without anaerobic growth factors were previously attributed to low levels of contamination with oxygen [308,352].

In contrast to the IMX585 reference strain, strain IMX1438 (*sga1Δ::TtTHC1*) showed exponential anaerobic growth in the absence of sterol supplementation, at a specific growth rate of 0.15 h^{-1} (Figure **3.4**C, Table **3.1**; see also Figure S5). Although its specific growth rate and biomass yield under these conditions were 38% and 37% lower, respectively, than those of corresponding ergosterol-supplemented SBR cultures (Table **3.1**), glucose was completely consumed within 45 h (Figure **3.4**D). Consistent with the observed lower biomass yield on glucose, the ethanol yield of the SBR cultures grown without sterol supplementation was 5.4% higher than that of sterol-supplemented cultures (Table **3.1**).

Squalene contents of biomass from anaerobic SBR cultures of the IMX585 reference strain were 3.8-fold lower in ergosterol-supplemented cultures than in cultures grown on sterol-free SMD-urea, while lanosterol contents were not significantly different (Figure **3.2**A; see also Table S2). This observation is consistent with a previously reported mechanism for ergosterol-induced degradation of β-hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase, leading to lower squalene levels in sterol-supplemented cultures [260,353]. Anaerobic SBR cultures of strain IMX1438 (*sga1Δ::TtTHC1*) grown on sterol-free SMD-urea showed 2.4-fold-higher tetrahymanol levels (1.13 ± 0.05 mg (g biomass)$^{-1}$) than corresponding sterol-supplemented cultures. The squalene levels in these cultures were not substantially different. However, the level of lanosterol was 2.4-fold higher in the cultures grown in the absence of a sterol source. No clear differences in fatty acid chain length or degree of desaturation were observed between strains or sterol-supplementation regimes. However, the IMX585 reference strain showed 20% lower total fatty acid content in the slow-growing sterol-free cultures than in sterol-supplemented cultures. In contrast, strain IMX1438 (*sga1Δ::TtTHC1*) showed 40% higher fatty acid content when grown in sterol-free medium (Figure **3.2**B; see also Table S3).

Sterol-independent anaerobic growth of a tetrahymanol-expressing strain lacking a functional sterol-biosynthesis pathway | Even when extensive measures are implemented to achieve anaerobiosis, it is notoriously difficult to fully eliminate oxygen entry into laboratory bioreactors [130,303,308,352]. Indeed, low levels of residual synthesis of unsaturated fatty acids were observed in the anaerobic SBR setups used in the present study in the absence

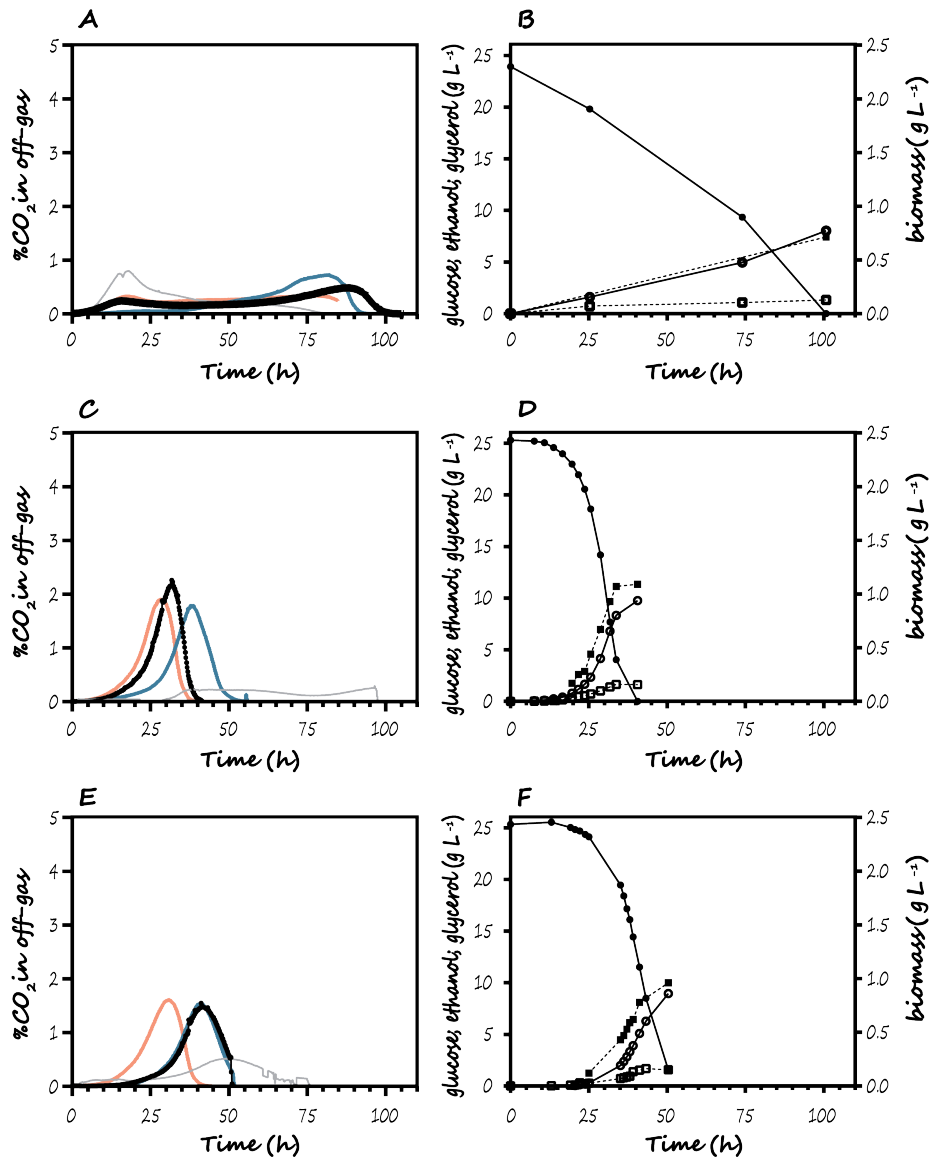

Figure 3.4: Anaerobic sequential batch bioreactor (SBR) cultivation of *S. cerevisiae* strains IMX585, IMX1438, and IMK870 without sterol supplementation. All panels represent data from a single representative SBR experiment performed at 30 °C on SMD-urea without pH control. Data from replicate experiments are shown in Figure S5. (**A** and **B**) Reference strain IMX585. (**C** and **D**) Strain IMX1438 (Δ:*TtTHC1*). (**E** and **F**) Strain IMK870 (*sga1*Δ::*TtTHC1 erg1*Δ). (**A**, **C**, and **E**) Percentages of CO_2 in the off-gas during the initial batch-cultivation cycle on medium without anaerobic growth factors (grey line) and the first (blue line), second (black line and dots), and third (red line) subsequent SBR cycles on medium supplemented only with Tween 80. (**B**, **D**, and **F**) Concentrations of glucose (closed circles), biomass (closed squares), ethanol (open circles), and glycerol (open squares) in the second SBR cycle.

of Tween 80[352]. No ergosterol was detected in biomass of strain IMX1438 (*sga1*Δ::*TtTHC1*) or the IMX585 reference strain, taken from anaerobic SBR cultures grown on SMD-urea without ergosterol (Figure **3.2**A; see also Table S2). However, detection of small amounts of lanosterol, the first cyclic intermediate in the ergosterol biosynthesis pathway (Figure **3.1**), indicated a minor leakage of oxygen into the cultures.

The squalene epoxidase Erg1 catalyzes the first step in sterol synthesis from squalene (Figure **3.1**). To eliminate any residual formation of sterols caused by oxygen entry into the bioreactors, strain IMK870 (*sga1*Δ::*TtTHC1 erg1*Δ) was constructed. Despite the absence of a functional sterol synthesis pathway, strain IMK870 grew exponentially in anaerobic SBR cultures on SMD-urea without ergosterol (Figure **3.4**E; see also Figure S5) and the glucose was completely consumed within 55 h (Figure **3.4**F). The specific growth rate of strain IMK870 in these cultures was 27% lower than that of strain IMX1438 (*sga1*Δ::*TtTHC1 ERG1*), while the biomass and ethanol yields of the two strains were not significantly different (Table **3.1**).

Neither ergosterol nor lanosterol was detected in anaerobically grown biomass of strain IMK870 (Figure **3.2**A; see also Figure S2 and Table S2), while the squalene levels were approximately 2.7-fold higher in strain IMK870 than in strain IMX1438 (*sga1*Δ::*TtTHC1 ERG1*) (Figure **3.2**A) in the absence of sterol supplementation. Fatty acid contents and compositions of anaerobically grown biomass of the two strains did not show marked differences (Figure **3.2**B; see also Table S3).

Aerobic, sterol-free growth of an *erg1* deletion mutant expressing *TtTHC1* | To investigate whether tetrahymanol production would allow aerobic, sterol-independent growth of strain IMK870 (*sga1*Δ::*TtTHC1 erg1*Δ), an anaerobic preculture on sterol-free medium was used to inoculate parallel aerobic cultures on SMD-urea. To investigate the ability of strain IMK870 to grow on nonfermentable carbon sources, additional experiments were performed on synthetic medium supplemented with a mixture of ethanol and glycerol (SMEG-urea). Strains IMX585 and IMX1438 (*sga1*Δ::*TtTHC1*) were included as references.

On SMD-urea, strains IMX585 and IMX1438 rapidly initiated exponential growth (Table **3.2**; see also Figure S3). Strain IMK870 (*sga1*Δ::*TtTHC1 erg1*Δ) showed a lag phase of approximately 20 h, after which its specific growth rate was 0.14 ±0.00 h^{-1}. For over 150 h, strain IMK870 did not show detectable growth on SMEG-urea, suggesting a loss of respiratory capacity. In contrast, both reference strains started growing on these nonfermentable carbon sources after a short lag phase.

To explore whether aerobic growth of strain IMK870 (*sga1*Δ::*TtTHC1 erg1*Δ) was caused by carryover of small amounts of sterols from the preculture, its aerobic growth was further studied in aerobic SBR experiments. Over 10 consecutive SBR cycles, CO_2 profiles indicated an increase of the specific growth rate in sterol-free medium from 0.14 h^{-1} in the first batch to

Table 3.2: Specific growth rates of *S. cerevisiae* strains in aerobic batch cultures. Strains were grown aerobically in a Growth Profiler 960 in 96-well plates on synthetic medium with urea as the nitrogen source, and with either glucose or a mixture of ethanol and glycerol as the carbon source, at 30 °C. Specific growth rates represent averages ± standard errors of the means of measurements of results from 6 individual wells for each combination of medium composition and yeast strain. Since the biomass concentrations were estimated by image analysis (see Materials and Methods), estimated specific growth rates may not precisely match those that were measured in shake-flask cultures.

Strain	Specific growth rate (h^{-1})	
	Glucose	Ethanol/glycerol
IMX585	0.33 ± 0.01	0.19 ± 0.00
IMX1438 (*sga1*Δ::*TtTHC1*)	0.35 ± 0.01	0.17 ± 0.01
IMK870 (*sga1*Δ::*TtTHC1 erg1*Δ)	0.14 ± 0.00	No growth

0.25 h^{-1} in the 10th cycle (Figure **3.5**A and B). Analysis of the triterpenoid fraction of aerobically grown biomass, performed in cycles 2 to 4 and 8 to 10, did not reveal the presence of sterols (Figure **3.5**C). Over the course of the first 4 SBR cycles, squalene content decreased from approximately 45 mg (g biomass)$^{-1}$ to below 10 mg (g biomass)$^{-1}$. The amount of tetrahymanol also decreased slightly but did so to a lesser extent. The fatty acid levels and compositions of the aerobic SBR cultures remained nearly unchanged through the course of the aerobic SBR experiments (Figure **3.5**D).

Discussion

This study demonstrated that expression of a heterologous squalene-tetrahymanol cyclase (STC) in *S. cerevisiae* enabled production of the pentacyclic triterpenoid tetrahymanol and allowed sterol-independent fermentative growth of this yeast under aerobic and anaerobic conditions. The ability of eukaryotes to grow in the absence of sterol synthesis or supplementation is rare, with Neocallimastigomycota as a prominent exception. Horizontal gene transfer of a prokaryotic STC gene into these deep-branching anaerobes has been interpreted as a key evolutionary adaptation to life in the essentially anaerobic environment of the gut of large herbivores[279,347]. Due to the lack of efficient genetic tools[343], the physiological relevance of STC has not yet been experimentally verified in Neocallimastigomycota. By simulating acquisition of an STC gene through a horizontal gene transfer event in a yeast model, this study experimentally demonstrated that acquisition of a functional STC gene by a fermentative eukaryote confers an immediate advantage in anaerobic environments in which sterols are either absent or growth limiting.

In vitro studies demonstrated previously that sterol insertion into phospholipid membranes leads to denser membrane packing and reduced solute permeability[327,354,355]. Increased

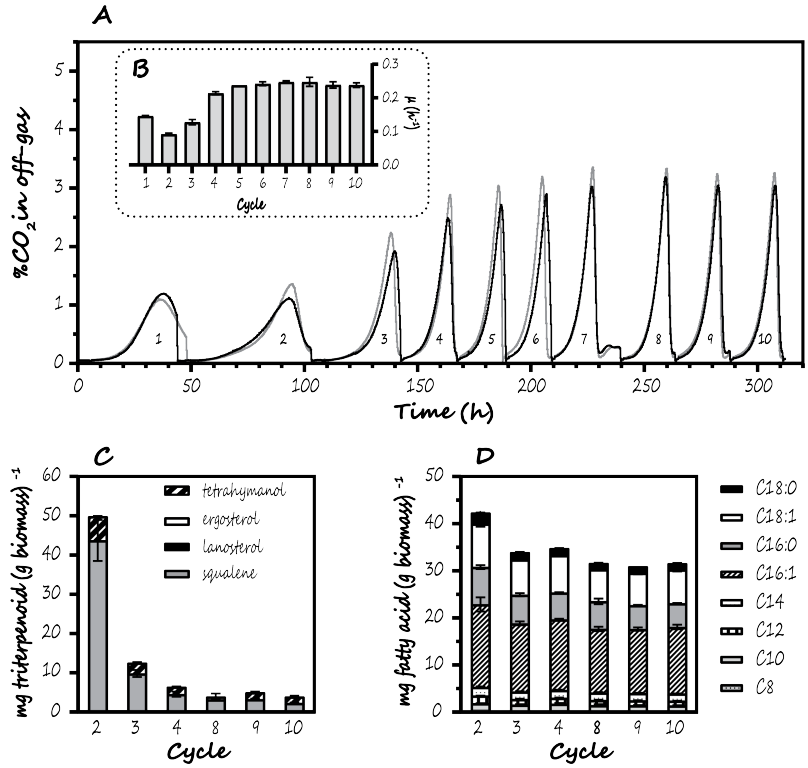

Figure 3.5: Aerobic sequential batch reactor experiments performed with *S. cerevisiae* IMK870 and analysis of triterpenoid fraction and total fatty acid composition of biomass by GC-FID. All panels represent data from two replicate aerobic bioreactor experiments performed at 30°C on SMD with ammonium as the nitrogen source and pH control at 5.0. (**A**) Percentage of CO_2 in the off-gas during 10 subsequent batch cycles of two replicate experiments. (**B**) Specific growth rates estimated from CO_2 production during 10 subsequent batch cycles. (**C**) Composition of the triterpenoid fraction of biomass harvested at the end of the indicated batch cycle. (**D**) Total fatty acid composition of biomass harvested at the end of the indicated batch cycle.

permeability of sterol-depleted, tetrahymanol-containing yeast membranes to protons and/or other solutes may therefore have contributed to the reduced biomass yields and growth rates of *TtTHC1*-expressing *S. cerevisiae* strains in sterol-free media (Table **3.1**). In addition, suboptimal growth characteristics of sterol-free, tetrahymanol-producing yeast cultures may be related to a wide range of other cellular processes that were shown to be affected by sterol composition in experiments performed with yeast sterol-biosynthesis mutants and sterol supplementation[205]. For example, the inability of *S. cerevisiae* IMK870 (*sga1Δ::TtTHC1 erg1Δ*) to grow on the nonfermentable carbon sources ethanol and glycerol is consistent with a reported increased loss of mitochondrial DNA and mitochondrial function in response to reduced ergosterol content[356].

In eukaryotes that acquired a prokaryotic STC gene by horizontal gene transfer, subsequent evolutionary adaptations may have compensated for physiological disadvantages of sterol replacement. *Tetrahymena* species, in which tetrahymanol can be readily replaced by exogenous ergosterol[357], modify their fatty acid composition in response to sterol availability. Tetrahymanol-containing membranes of these protists contain fatty acids with a shorter acyl chain length and a lower degree of unsaturation than ergosterol-containing membranes, with a preference for the $\Delta^{6,9}$ isoform of $C_{18:2}$ over the $\Delta^{9,12}$ isoform[358]. While the total fatty acid content of *TtTHC1*-expressing *S. cerevisiae* was higher during anaerobic growth in sterol-free medium than in sterol-supplemented cultures (Figure **3.2**B; see also Table S3 in the supplemental material), no clear differences in fatty acid composition were observed. However, it should be noted that Tween 80, the source of unsaturated fatty acids in the anaerobic cultures, mainly provides oleic acid ($C_{18:1}$). Sterol-deficient, tetrahymanol-producing *S. cerevisiae* strains provide an interesting platform for further studies on the combined impacts of triterpenoid and lipid compositions of fungal membranes on cellular robustness.

Despite extensive measures to prevent oxygen entry, small amounts of lanosterol, whose synthesis from squalene requires oxygen (Figure **3.1**), were detected in anaerobic SBR cultures of *S. cerevisiae* strains with an intact sterol synthesis pathway (Figure **3.2**; see also Figure S2 and Table S2 in the supplemental material). This observation was in line with literature citing the technical challenges of anaerobic bioreactor cultivation of *S. cerevisiae*[130,308,352] and left the possibility that trace amounts of lanosterol and other sterols, whose levels were below the detection threshold of sterol analysis by gas chromatography (GC), might still be synthesized and contribute to the observed growth. Experiments performed with anaerobic and aerobic sequential batch reactors (SBRs) of a *TtTHC1*-expressing strain in which the *ERG1* gene had been deleted ruled out this possibility. In addition, based on an initial ergosterol level of 1.5 mg (g biomass)$^{-1}$ (Table S2), a total of 3.4×10^{10} cells (g biomass)$^{-1}$[359], and an average of 4.76 generations for each SBR cycle (Table S1), the number of molecules per cell was on the order of 10^3 after three SBR cycles and less than one molecule of ergosterol per cell remained after five SBR cycles (see "Calculations S1" in the supplemental material). Our results therefore demonstrated fully sterol-independent growth of *TtTHC1*-expressing *erg1*Δ *S. cerevisiae*. Early studies in which specific sterols were added to oxygen-deprived *S. cerevisiae* cultures indicated that the hydroxyl group at the C-3 position and the configuration at the C-24 position of sterols were crucial for supporting anaerobic growth[338,360]. However, a large fraction of the required sterols were able to be replaced by cholesterol or any of a variety of other sterols lacking these configurations, as long as small quantities of ergosterol were also added[361]. Similar conclusions were drawn based on experiments performed with aerobic cultures of sterol-auxotrophic *S. cerevisiae* strains[361,362]. These requirements for small amounts of ergosterol or closely related sterols, estimated at 1×10^7 to 2×10^7 molecules

per cell, were proposed to reflect specific sterol-protein interactions[361]. Our results indicate either that production of tetrahymanol can circumvent these requirements or that, in the strain background and under the experimental conditions used in the present study, a strict requirement for specific sterols does not exist. An apparent discrepancy with a previous study[363], which reported that exogenous tetrahymanol did not support anaerobic growth of *S. cerevisiae* on sterol-free medium, is likely to reflect an inability of the *S. cerevisiae* Aus1 and Pdr11 sterol transporters[272,364] to transport tetrahymanol.

The increase, occurring over only 10 cycles of aerobic SBR cultivation, in the specific growth rate of a *TtTHC1*-expressing *erg1*Δ strain (Figure **3.5**) reflects either a physiological adaptation or rapid laboratory evolution. The accompanying decrease of the cellular content of squalene might be related to recently reported negative impacts of squalene accumulation in the *S. cerevisiae* plasma membrane[365]. Dedicated laboratory evolution experiments performed under different environmental stress conditions, followed by whole-genome sequencing[366,367], offer interesting possibilities to explore the genetic requirements for fast, robust growth of sterol-independent strains. In combination with the rapidly increasing knowledge on genome sequences of Neocallimastigomycota, such experiments may further extend our understanding of how anaerobic lifestyles have evolved in naturally occurring anaerobic fungi. In addition, they will provide valuable information for the design and construction of robust, sterol-independent yeast strains for application in anaerobic industrial processes.

Supplementary Materials

Supplemental material is available at AEM online:
https://doi.org/10.1128/AEM.00672-20

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Materials and methods

Strains, media and maintenance | *Saccharomyces cerevisiae* strains used and constructed in this study (Table **3.3**) were derived from the CEN.PK lineage[304,305]. Stock cultures were propagated in synthetic medium (SM[135]) or in complex medium (YP; 10 g L^{-1} Bacto yeast extract (BD Biosciences, Franklin Lakes, NJ), 20 g L^{-1} Bacto peptone (BD Biosciences)). Both types of media were autoclaved at 121 °C, after which they were supplemented with 20 g L^{-1} glucose from a concentrated solution, separately autoclaved at 110 °C, resulting in SMD and YPD respectively. Shake-flask and bioreactor experiments were performed in synthetic medium or in synthetic urea medium (SMD-urea[322]) supplemented with 20 g L^{-1} glucose, or with a mixture of 8.76 g L^{-1} ethanol and 8.76 g L^{-1} glycerol as carbon sources (SMEG-urea). Where indicated, media were supplemented with 10 mg L^{-1} ergosterol (≥ 95% pure; Sigma-Aldrich, St. Louis, MO) and/or 420 mg L^{-1} Tween 80 (polyethylene glycol sorbate monooleate; Merck, Darmstadt, Germany). Concentrated stock solutions of these supplements contained 8.4 g of Tween 80 and/or 0.2 g of ergosterol added to 17 mL ethanol and were heated at 80 °C for 20 min prior to addition to growth media. Gas chromatography analysis with flame-ionization detection (GC-FID) of this concentrated Tween 80 stock solution did not reveal any contamination with sterols (see Figure S1 in supplemental material). Stock cultures of *Escherichia coli* DH5*α* and derived strains were grown in Lysogeny Broth (LB, 10 g L^{-1} Bacto tryptone, 5 g L^{-1} Bacto yeast extract and 5 g L^{-1} NaCl (J.T. Baker, Avantor, Radner, PA)) supplemented with 100 mg L^{-1} ampicillin. After addition of sterile glycerol (30% v/v), samples of *S. cerevisiae* and *E. coli* stock cultures were frozen and stored at -80 °C.

Table 3.3: *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Parental strain	Reference or source
IMX585	*MATα can1Δ::cas9-natNT2*	CEN.PK113-7D	306
IMX1438	*MATα can1Δ::cas9-natNT2 sga1Δ::TtTHC1*	IMX585	This study
IMK870	*MATα can1Δ::cas9-natNT2 sga1Δ::TtTHC1 erg1Δ*	IMX1438	This study

Molecular biology techniques | DNA fragments for construction of plasmids and expression cassettes were amplified with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA), according to the manufacturer's protocol and with PAGE-purified oligonucleotide primers (Sigma-Aldrich, St. Louis, MO). Diagnostic PCR was performed with DreamTaq PCR Master Mix (Thermo Scientific), following the manufacturer's protocol and with desalted oligonucleotide primers (Sigma-Aldrich). PCR-amplified linear integration cassettes were purified from 1% (w/v) agarose gels using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). *E. coli* DH5*α* was transformed by electroporation with a MicroPulser Electroporator (Biorad, Hercules, CA). Plasmids were isolated from overnight *E. coli* cultures on LB with ampicillin with a GenElute Plasmid Miniprep kit (Thermo Scientific). Chemical transformation of *S. cerevisiae* was performed as described by Gietz and Woods[324].

Table 3.4: Plasmids used in this study

Plasmid	Characteristics	Reference or source
pUD696	pMK-RQ GeneArt delivery vector with the squalene-tetrahymanol cyclase gene from *T. thermophila* and PCR flanking regions	GeneArt
p426-TEF	2μ m ori, URA3, *ScTEF1*p-mcs-*ScCYC1*t	368
pUDE666	2μ m ori, URA3, *ScTEF1*p-*TtTHC1-CYC1*t	This study
pUDR119	2μ m ori, *amdSYM*, *SNR52*p-gRNA$_{SGA1}$-*SUP4*t	369
pUG6	Amp^{r,a} Ag*TEF1*p-KanMX-Ag*TEF1*t	370

[a]Amp^r, ampicillin resistance.

Plasmid construction | Plasmids and oligonucleotide primers used and/or constructed in this study are indicated in Table **3.4** and Table **3.5**, respectively. The coding sequence of the *Tetrahymena thermophila* squalene-tetrahymanol-cyclase gene *THC1* (GenBank accession XM_001026696.2) was subjected to codon-optimization for expression in *S. cerevisiae* using the Jcat algorithm[371]. The codon-optimized coding sequence, flanked by 20 bp sequences for PCR amplification, was synthesized by GeneArt (Regensburg, Germany) and delivered in the pMK-RQ vector. Flanking sequences with homology to *TEF1* promoter and *CYC1* terminator sequences were added by PCR with primer pair 10561/10543, using pUD696 as template. p426-TEF was linearized by PCR amplification with primer pair 5921/10547 and the synthetic gene fragment was cloned between the *TEF1* promoter and *CYC1* terminator on this expression plasmid with the Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA) to yield pUDE666.

Strain construction | *S. cerevisiae* IMX1438 (*sga1*Δ::*TtTHC1*) was constructed by Cas9-mediated genome editing[306]. The expression cassette for integration of *TtTHC1* was amplified from pUDE666 using primer pair 9626/10148. Co-transformation of *S. cerevisiae* IMX585 with 400 ng of the expression cassette and 500 ng of pUDR119, followed by curing of pUDR119 with fluoroacetamide[323], yielded strain IMX1438. Correct integration of linear fragments was checked by colony PCR[372] using primer pairs 7298/7479, 7298/11372 and 7479/11371, binding in the regions flanking the integration locus and inside of the linear integration fragment. *S. cerevisiae* IMK870 was constructed by deleting *erg1* in strain IMX1438. A KanMX-expression cassette conferring resistance to G418[370] was amplified from pUG6 using primer pair 11783/11784. Strain IMX1438 was transformed with 1 µg of this fragment, followed by overnight recovery in YPD. Subsequent overnight anaerobic incubation in 20 mL of YPD with Tween 80, ergosterol and 200 mg L^{-1} G418 (Invivogen, Toulouse, France), incubated in an anaerobic chamber for 2 d prior to inoculation, was used to pre-select correct mutants. Cells from these cultures were plated on YPD-Tween 80/ergosterol agar with 200 mg L^{-1} G418 and placed in an anaerobic jar (Article no. SÜ380902, Schütt-biotec, Munich, Germany), together with an Anaerocult A catalyst package (VWR International BV, Amsterdam, The Netherlands) to remove traces of oxygen. Single cell lines were

obtained by restreaking colonies three times on selective media. Correct integration of the KanMX marker in the *ERG1* locus was verified by colony PCR with primer pairs 12183/12184, 12183/3812 and 12184/3811, binding in the regions flanking the integration locus and inside of the KanMX cassette.

Table 3.5: Oligonucleotide primers used in this study

Oligonucelotide	Sequence
3811	CTCGGTGAGTTTTCTCCTTCAT
3812	TAGATTGTCGCACCTGATTG
5921	AAAACTTAGATTAGATTGCTATGCTTTCTTTCTAATGAGC
7298	TTGTTCAATGGATGCGGTTC
7479	GGACGTTCCGACATAGTATC
9626	TTTACAATATAGTGATAATCGTGGACTAGAGCAAGATTTCAAATAAGTAACAGCAGCAAA GCTCATAGCTTCAAAATGTTTCTAC
10148	CTGCAAACGTGGTTGGGCTGGACGTTCCGACATAGTATCTAATCAATTTATAATATCAGA CAAATTAAAGCCTTCGAGCG
10543	GCGTGAATGTAAGCGTGACATAACTAATTACATGATATCGACAAAGGAAAAGGGGCCTGT CGCGCAGATTAGCGAAGC
10547	TCATGTAATTAGTTATGTCACGC
10561	TTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCAATCTAATCTAAGTTTTAATTAC GCGATACCCTGCGATCTTC
11371	TTACCCAGCTTTCGACAAGG
11372	ACCACCTTGAGCAACGATCC
11783	GCATGCCGTGGCTGCTCTCGGTCGGGTATAAGTCTTAGACAATAGTCTTACCTCGCATGT CGACATGGAGGCCCAGAATACC
11784	GGAAGTAATATCGTTAATTGATAACCGAATATGAATCTCAATGCATATTTTGAAGCATAT CGAATCGACAGCAGTATAGC
12183	GTGGTTCAGGGCACTCTACG
12184	CGTTATCACCGTTCCTTTCC

Shake-flask cultivation | Aerobic shake-flask cultures were grown in 500-mL round-bottom shake flasks containing 100 mL of liquid media, in an Innova shaker incubator (New Brunswick Scientific, Edison, NJ) set at 30 °C and 200 rpm. Anaerobic shake-flask experiments were performed in a Shel Lab Bactron BAC X-2E anaerobic workstation (Sheldon Manufacturing Inc, Cornelius, OR). Anaerobic cultures were grown at 30 °C in 50-mL or 100-mL round-bottom shake-flasks containing 40 mL or 80 mL liquid medium, respectively, placed on an IKA KS 260 Basic orbital shaker platform (Dijkstra Vereenigde BV, Lelystad, The Netherlands) set at 200 rpm.

Anaerobic bioreactor cultivation | Anaerobic sequential batch reactor (SBR) experiments were performed as previously described[352] in 2-L bioreactors (Applikon, Delft, the Netherlands) with a working volume of 1.2 L at 30 °C and an initial pH of 6.0. Cultures were stirred at 800 rpm, and to

minimize oxygen contamination, no active pH control was used. Cultures were grown on SMD-urea to minimize changes in culture pH[322]. The outlet gas of the bioreactors was cooled to 4 °C in a condenser and dried with a PermaPure PD-50T-12MPP dryer (Permapure, Lakewood, NJ) prior to analysis with a NGA 2000 Rosemount gas analyser (Emerson, St. Louis, MO). SBR experiments were initiated with a batch-cultivation cycle on medium without ergosterol and Tween 80 to deplete endogenous reserves of these growth factors. On-line measurements of CO_2 concentrations in the outlet gas of reactors were used to monitor growth. When the CO_2 percentage in the off-gas decreased below a level of 0.1%, three consecutive SBR cycles on SMD-urea, containing either both supplements or only Tween 80, were initiated. Each subsequent SBR cycle was initiated when CO_2 concentration in the outlet gas decreased to a level below 0.05%, indicating depletion of the growth-limiting nutrient, by largely emptying the reactor and refilling with fresh medium, resulting in a 48-fold dilution of the original culture. Before refilling, the medium inlet tube was flushed with nitrogen gas to avoid influx of contamination by any oxygen that might have permeated into the tube during the preceding growth phase. The 5-L glass medium reservoir from which the cultures were refilled was kept anaerobic by continuous sparging with N5.5 grade N_2 (Linde Gas Benelux, Schiedam, The Netherlands). Precultures were prepared for anaerobic SBR experiments by inoculation of aerobic shake-flask cultures on SMD with frozen stock cultures of *S. cerevisiae* strain IMX585 or strain IMX1438, or with an anaerobic shake-flask culture of strain IMK870 (80 mL in a 100 mL flask, incubated in an anaerobic chamber) on SMD-urea with Tween 80 and ergosterol. After overnight cultivation at 30 °C, a sample from these cultures was used to inoculate a second pre-culture on the same medium. After at least two biomass doublings, biomass was harvested by centrifugation at 3000 × *g*, washed with sterile demineralised water and used to inoculate anaerobic bioreactors at an initial optical density at 660 nm (OD_{660}) of 0.2.

Aerobic bioreactor cultivation | Aerobic SBR experiments were performed as described for the anaerobic SBR experiments, with the following modifications: cultures were grown on SMD, culture pH was controlled at 5.0 by automated addition of 2 M KOH, aerobic conditions were maintained by sparging with air at 0.5 L min^{-1} and the glass 20-L medium reservoir was not sparged with nitrogen gas. SBR cycles were initiated either manually, or automatically when the CO_2-percentage in the off-gas had decreased below 10% of the maximum value that was measured during exponential phase.

Growth studies in Growth Profiler | Precultures for aerobic growth studies in a Growth Profiler 960 (EnzyScreen BV, Heemstede, The Netherlands) were prepared as aerobic shake-flask cultures on YPD for strains IMX585 and IMX1438 and as anaerobic shake-flask cultures on 80 mL YPD supplemented with Tween 80 and ergosterol for strain IMK870. After overnight cultivation at 30 °C, samples from these cultures were used to inoculate a second shake-flask pre-culture, which contained either 20 mL of SMD-urea for the aerobic pre-cultures, or 40 mL of SMD-urea supplemented with only Tween 80 for the anaerobic preculture. After overnight cultivation at 30 °C, these cultures were washed twice with synthetic medium without a carbon or nitrogen source and were concentrated to an OD_{660} of

10. Aliquots (5 µL) of these suspensions were used to inoculate a 96-well microtiter plate (EnzyScreen, type CR1496dl), with final working volumes of 250 µL and containing either SMD-urea or SMEG-urea, at an initial OD_{660} of 0.2. Microtiter plates were closed with a sandwich cover (EnzyScreen, type CR1296). Growth experiments were performed at 30 °C and at 250 rpm, and images of cultures were made at 30 min intervals. Corrected green-values were obtained by the use of software supplied and installed by the manufacturer, and were directly used for conversion to OD-equivalents based on a 16-point calibration, leading to the following equation:

$$ODequivalent = 0.242 \times (GV(t) - GV_{med})^{0.591} + 8.6 \times 10^{-5} \times (GV(t) - GV_{med})^{2.83} + 5.27 \times 10^{-9} \times (GV(t) - GV_{med})^{4.85}$$

in which GV(t) is the corrected green-value measured in a well at time point 't', and GV_{med} is a green-value obtained through a measurement of a plate filled with medium, before inoculation. Only OD-equivalents between 1.0 and 10 were used to estimate growth rates.

Analytical methods | Metabolite concentrations in culture supernatants were analysed by high-performance liquid chromatography (HPLC) as described previously[325]. HPLC measurements of ethanol concentrations were corrected for ethanol evaporation as described previously[373] using an evaporation coefficient of 0.0062. Biomass dry weight measurements and total fatty-acid contents of freeze dried biomass, as fatty acid methyl esters, were analysed as previously described[352]. Isolation of the triterpenoid fraction of biomass through saponification with NaOH and subsequent extraction with *tert*-butyl-methyl ether (*t*BME) was performed, essentially as described previously[374], with the following modifications. Biomass was harvested at the end of a cultivation cycle and/or during mid-exponential phase by centrifuging 50 mL of culture broth (5 min at 3000 × *g*) and washed once with demineralized water. After the biomass pellets were lyophilized overnight (Alpha 1-4 LD Plus freeze dryer, Christ, Osterode am Harz, Germany), 10 to 30 mg of lyophilized material was weighed and placed into Pyrex borosilicate glass methylation tubes (Article no. 10044604, Thermo Fisher Scientific). Then, 1 mL of 2 M NaOH (Article No. 72068, Sigma-Aldrich) was added and suspensions were heated for 1 h at 70 °C. During incubation, cell suspensions were not sonicated but subjected to vortex mixing for 20 s at 15-min intervals. After cooling to room temperature, the content of the tube was transferred to a 2-mL plastic tube (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) containing 650 µL of *t*BME. Subsequent extraction with *t*BME was done according to the published protocol[374]. After extraction, the dried sterol fraction was dissolved in 100 µL to 1 mL of *t*BME, to obtain a final lipid concentration within the range of 10-500 µg mL^{-1}, and directly used for analysis, without trimethyl-silylation. Sterols were analysed by gas-chromatography with flame-ionization detection (GC-FID) on an Agilent Technologies 7890A GC-FID system equipped with an FID-1000-220 Gas Station (Parker Balston, Haverhill, MA, USA) and an Agilent Technologies 7693 Autosampler. A VF-5ms column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness, Agilent part no. CP9013) was used, with N_2 used as the carrier gas at a constant flow of 1 mL min^{-1}. The initial oven temperature of 80 °C was kept constant for 1 min after sample injection and was then increased to 280 °C at 50 °C min^{-1}, and was finally increased to 320 °C at 6 °C min^{-1} and

kept at 320 °C for a further 15 min. The inlet temperature was set at 150 °C, and the FID temperature at 330 °C. The GC-FID system was calibrated with standards of squalene (≥ 98%, Sigma-Aldrich), ergosterol (≥ 98%, Boom B.V), cholesterol (≥ 99%, Sigma-Aldrich), lanosterol (≥93%, Sigma-Aldrich), 5α-cholestane (internal standard; ≥ 97%, Sigma-Aldrich) and tetrahymanol (≥ 99%, ALB Technologies), using a 10-point calibration curve for all compounds except lanosterol and 5α-cholestane (6-point and 5-point calibration, respectively). Data were adjusted for internal standard concentrations and are expressed as mg sterol per g of lyophilized biomass. Significance of differences between sets of replicate experiments was assessed with unpaired two-tailed Student's t-tests and a threshold (p) value of 0.05.

4

A squalene-hopene cyclase in *Schizosaccharomyces japonicus* represents a eukaryotic adaptation to sterol-independent anaerobic growth

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**These authors have contributed equally to the work presented in this chapter*

Abstract | Biosynthesis of sterols, which are key constituents of canonical eukaryotic membranes, requires molecular oxygen. Anaerobic protists and deep-branching anaerobic fungi are the only eukaryotes in which a mechanism for sterol-independent growth has been elucidated. In these organisms, tetrahymanol, formed through oxygen-independent cyclization of squalene by a squalene-tetrahymanol cyclase, acts as a sterol surrogate. This study confirms an early report (Bulder (1971), Antonie van Leeuwenhoek, **37**, 353–358) that *Schizosaccharomyces japonicus* is exceptional among yeasts in growing anaerobically on synthetic media lacking sterols and unsaturated fatty acids. Mass spectrometry of lipid fractions of anaerobically grown *Sch. japonicus* showed the presence of hopanoids, a class of cyclic triterpenoids not previously detected in yeasts, including hop-22(29)-ene, hop-17(21)-ene, hop-21(22)-ene and hopan-22-ol. A putative gene in *Sch. japonicus* showed high similarity to bacterial squalene-hopene cyclase (SHC) genes and in particular to those of *Acetobacter* species. No orthologs of the putative *Sch. japonicus* SHC were found in other yeast species. Expression of the *Sch. japonicus* SHC gene (*Sjshc1*) in *Saccharomyces cerevisiae* enabled hopanoid synthesis and supported ergosterol-independent anaerobic growth, thus confirming that one or more of the hopanoids produced by SjShc1 can act as ergosterol surrogate in anaerobic yeast cultures. Use of hopanoids as sterol surrogates represents a previously unknown adaptation of eukaryotic cells to anaerobic growth. The fast sterol-independent anaerobic growth of *Sch. japonicus* is an interesting trait for developing robust fungal cell factories for application in anaerobic industrial processes.

Significance statement | Biosynthesis of sterols requires oxygen. This study identifies a previously unknown evolutionary adaptation in a eukaryote, which enables anaerobic growth in absence of exogenous sterols. A squalene-hopene cyclase, proposed to have been acquired by horizontal gene transfer from an acetic acid bacterium, is implicated in a unique ability of the yeast *Schizosaccharomyces japonicus* to synthesize hopanoids and grow in anaerobic, sterol-free media. Expression of this cyclase in *S. cerevisiae* confirmed that at least one of its hopanoid products acts as sterol-surrogate. The involvement of hopanoids in sterol-independent growth of this yeast provides new leads for research into the structure and function of eukaryotic membranes, and into the development of sterol-independent yeast cell factories for application in anaerobic processes.

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Introduction

Sterols are key constituents of canonical eukaryotic membranes, in which they influence integrity, permeability and fluidity[293,329]. The core pathway for sterol biosynthesis is highly conserved but the predominant final products differ for animals (cholesterol), plants (phytosterols) and fungi (ergosterol)[241]. Multiple reactions in sterol biosynthesis require molecular oxygen and no evidence for anaerobic sterol pathways has been found in living organisms or in the geological record[241]. The first oxygen-dependent conversion in sterol synthesis is the epoxidation of squalene to oxidosqualene by squalene monooxygenase. Oxidosqualene is subsequently cyclized to lanosterol, the first tetracyclic intermediate in sterol biosynthesis, in an oxygen-independent conversion catalysed by oxidosqualene cyclase (OSC). Molecular oxygen is also required for multiple subsequent demethylation and desaturation steps[337]. In fungi, synthesis of a single molecule of ergosterol from squalene requires 12 molecules of oxygen.

Deep-branching fungi belonging to the Neocallimastigomycota phylum are the only eukaryotes that have been unequivocally demonstrated to naturally exhibit sterol-independent growth under strictly anaerobic conditions. These anaerobic fungi contain a squalene-tetrahymanol cyclase (STC), which catalyzes oxygen-independent cyclization of squalene to tetrahymanol[274,375]. This pentacyclic triterpenoid acts as a sterol surrogate, and acquisition of a prokaryotic STC gene by horizontal gene transfer is considered a key evolutionary adaptation of Neocalllimastigomycetes to the strictly anaerobic conditions of the gut of large herbivores[279]. The reaction catalyzed by STC resembles oxygen-independent conversion of squalene to hopanol and/or other hopanoids by squalene-hopene cyclases (SHC)[277], which are found in many prokaryotes[282,376]. Some prokaryotes synthesize tetrahymanol by ring expansion of hopanol, in a reaction catalyzed by tetrahymanol synthase (THS) for which the precise mechanism has not yet been resolved[351].

Already in the 1950s, anaerobic growth of the industrial yeast and model eukaryote *Saccharomyces cerevisiae* was shown to strictly depend on sterol supplementation[138] or use of intracellular stores of this anaerobic growth factor[143]. Similarly, fast anaerobic growth of *S. cerevisiae*, which is a key factor in its large-scale application in bioethanol production, wine fermentation and brewing[292,377], requires availability of unsaturated fatty acids (UFAs). Biosynthesis of UFAs by yeasts requires an oxygen-dependent acyl-CoA desaturase[177] and, in anaerobic laboratory studies, the sorbitan oleate ester Tween 80 is commonly used as UFA supplement[143,144].

Per gram of yeast biomass, ergosterol and UFA synthesis require only small amounts of oxygen. Studies on these oxygen requirements therefore require extensive measures to prevent unintended oxygen entry into cultures. Even though most yeast species readily

ferment sugars to ethanol under oxygen-limited conditions, only very few grow anaerobically on sterol- and UFA-supplemented media when such precautions are taken[130,131]. As opposed to Neocallimastigomycetes, no evolutionary adaptations to sterol-independent anaerobic growth have hitherto been reported for yeasts, or for ascomycete and basidiomycete fungi in general.

We recently demonstrated that expression of an STC gene from the ciliate *Tetrahymena thermophila* supported tetrahymanol synthesis and sterol-independent growth of *S. cerevisiae*[378]. This result inspired us to re-examine a 1971 publication in which Bulder[283] reported sterol- and UFA-independent growth of the dimorphic fission yeast *Schizosaccharomyces japonicus*. *Sch. japonicus* was originally isolated from fermented fruit juices[379,380], and its potential for wine fermentation is being explored[381,382]. It shows marked genetic and physiological differences with other fission yeasts[383,384] and has gained interest as a model for studying cell division dynamics and hyphal growth[385–387]. *Sch. japonicus* grows well at elevated temperatures and rapidly ferments glucose to ethanol[388]. A low sterol content, control of membrane fluidity via chain length of saturated fatty acids and respiratory deficiency may all reflect adaptations of *Sch. japonicus* to low-oxygen environments[283,388–390]. However, the report by Bulder[283] stating that *Sch. japonicus* can grow anaerobically without sterol supplementation has not been confirmed or further investigated.

An ability of *Sch. japonicus* to grow in the absence of an exogenous supply of sterols would raise urgent questions on the molecular and evolutionary basis for this trait, which is extremely rare among eukaryotes. Despite the current absence of experimental evidence, it has been proposed that oxygen-independent sterol synthesis is theoretically possible[240]. Alternatively, sterol-independent growth of *Sch. japonicus* might depend on synthesis of an as yet unidentified sterol surrogate, or on membrane adaptations that involve neither sterols nor sterol surrogates. In addition to these fundamental scientific questions, independence of anaerobic growth factors is a relevant trait for large-scale industrial applications of yeasts, as exemplified by 'stuck' brewing fermentations caused by depletion of intracellular sterols and/or UFA reserves[340,341].

The goals of the present study were to reinvestigate the reported ability of *Sch. japonicus* to grow anaerobically without sterol supplementation and to elucidate its molecular basis. In view of reported challenges in avoiding oxygen contamination in laboratory cultures of yeasts[130,308,352,378], we first reassessed anaerobic growth and lipid composition of *Sch. japonicus* in the presence and absence of ergosterol. After identifying a candidate SHC gene in *Sch. japonicus*, we investigated its role in anaerobic growth by its expression in *S. cerevisiae*. In addition, we tested the hypothesis of Bulder[287] that *Sch. japonicus* is able to synthesize UFAs in an oxygen-independent pathway.

Results

Anaerobic growth of *Sch. japonicus* without ergosterol and UFA supplementation | In anaerobic laboratory cultures of yeasts, biosynthetic oxygen requirements are easily obscured by unintended entry of minute amounts of oxygen[130,308,352,378]. Furthermore, upon transfer from aerobic to anaerobic conditions, some yeasts continue growing on media without sterols or UFAs by mobilizing intracellular reserves[352,378]. To check if such complications affected conclusions from an early literature report on sterol- and UFA-independent anaerobic growth of *Sch. japonicus*[283], we performed serial-transfer experiments in an anaerobic chamber[352] using phosphate-buffered synthetic medium with glucose as carbon source (SMPD), with and without supplementation of ergosterol and/or Tween 80 as source of UFAs.

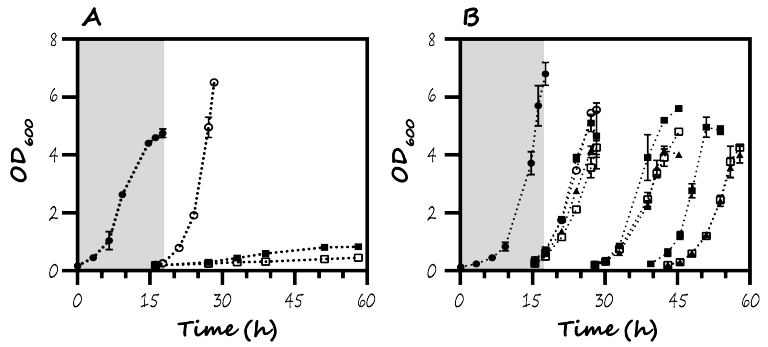


Figure 4.1: Anaerobic growth of *S. cerevisiae* CEN.PK113-7D and *Sch. japonicus* CBS5679 with different ergosterol and UFA (Tween 80) supplementation in a dark anaerobic chamber. Anaerobic pre-cultures on SMPD on 50 g L^{-1} glucose (closed circles, grey shading) were grown until the end of the exponential phase. (**A**) After the anaerobic pre-culture, *S. cerevisiae* was transferred to SMPD (20 g L^{-1} glucose) supplemented with either Tween 80 and ergosterol (open circles), Tween 80 only (closed squares) or neither Tween 80 nor ergosterol (open squares). (**B**) *Sch. japonicus* was grown on the same media as *S. cerevisiae* and additionally on medium containing ergosterol but not Tween 80 (closed triangles). *Sch. japonicus* cultures supplemented with only Tween 80, only ergosterol, and those without supplements were serially transferred to fresh media with the same composition in the anaerobic chamber. Data are represented as average ± SEM of measurements on independent duplicate cultures for each combination of yeast strain and medium composition.

To deplete reserves of sterols and/or UFAs in aerobically grown cells, anaerobic pre-cultures were grown on SMPD with an increased glucose concentration (50 g L^{-1}), and lacking ergosterol and Tween 80. In these pre-cultures, growth of *S. cerevisiae* CEN.PK113-7D ceased after 4.8 doublings (Figure **4.1**A), when less than half of the glucose had been consumed (SI Appendix, Table S1). Under the same conditions, *Sch. japonicus* CBS5679 completed 6.1 doublings (Figure **4.1**B) and, while full glucose depletion was intentionally avoided to prevent excessive

flocculation and sporulation, it had consumed almost 90% of the glucose (SI Appendix, Table S1). Samples from the anaerobic pre-cultures were transferred to SMPD (20 g L^{-1} glucose) supplemented with different combinations of ergosterol and/or Tween 80.

Consistent with earlier reports[352], *S. cerevisiae* showed virtually no anaerobic growth on SMPD without ergosterol and Tween 80 (Figure **4.1**A). In contrast, *Sch. japonicus* showed maximum specific growth rates of 0.26 to 0.30 h^{-1} and reached optical densities of 4 to 5 in all media tested (Figure **4.1**B; SI Appendix, Table S2). This anaerobic growth was sustained upon two consecutive transfers in SMPD lacking either Tween 80, ergosterol, or both (Figure **4.1**B). These results confirmed Bulder's[283] conclusion that *Sch. japonicus* can grow anaerobically in the absence of sterol and UFA supplementation. Remarkably, *Sch. japonicus* grew substantially slower in aerobic cultures (0.19 h^{-1}; SI Appendix, Figure S1) than in anaerobic cultures (Figure **4.1**B; SI Appendix, Table S2).

Absence of ergosterol and UFAs in anaerobically grown *Sch. japonicus* | To further investigate sterol- and UFA-independent anaerobic growth of *Sch. japonicus*, lipid fractions were isolated from anaerobic cultures and analyzed by gas chromatography with flame ionization detection (GC-FID). UFAs were detected in aerobically grown biomass, but not in anaerobic cultures grown on SMPD without Tween 80 supplementation (Figure **4.2**; SI Dataset S01). These results showed that fast anaerobic growth of *Sch. japonicus* on UFA-free medium did not, as suggested by Bulder[287], reflect oxygen-independent UFA synthesis. Total fatty-acid contents of aerobically and anaerobically grown biomass were similar, but anaerobically grown biomass showed higher contents of FA 10:0, FA 16:0 and FA 18:0 and lower contents of FA 26:0. In aerobically grown *Sch. japonicus* biomass, no FA 16:1 was detected and levels of FA 18:1 were higher than in Tween 80 supplemented anaerobic cultures (Figure **4.2**).

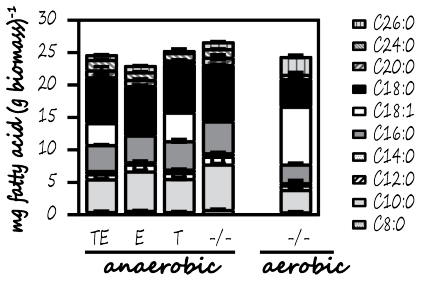


Figure 4.2: Quantification of fatty acids in *Sch. japonicus* CBS5679 biomass. *Sch. japonicus* CBS5679 was grown in SMPD with 20 g L^{-1} glucose. Under anaerobic conditions, cultures were supplemented with Tween 80 and ergosterol (TE), only ergosterol (E), only Tween 80 (T) or neither of those supplements (-/-). Data are shown for the first anaerobic culture following the anaerobic pre-culture. Aerobic cultures of *Sch. japonicus* were grown in SMPD without supplements (-/-). Data are represented as average ± SEM of measurements on independent duplicate cultures for each cultivation condition. Detailed information on data presented in this figure and additional anaerobic transfers are provided in SI Dataset S01.

S. cerevisiae biomass, grown anaerobically on SMPD with Tween 80 and ergosterol, was used as a reference for analysis of triterpenoid compounds and contained squalene, ergosterol and a small amount of lanosterol (Figure **4.3**A). Similarly prepared triterpenoid fractions of anaerobic *Sch. japonicus* cultures that were supplemented with only Tween 80 did not contain detectable amounts of ergosterol or lanosterol. Instead, in addition to squalene, gas chromatography-mass spectrometry (GC-MS) revealed several compounds that were not observed in anaerobically grown *S. cerevisiae* (Figure **4.3**B). We hypothesized that these compounds were as yet unidentified triterpenoids synthesized by *Sch. japonicus* that might serve as sterol surrogates. Tetrahymanol, which has not been found in wild-type yeasts but does occur in several other eukaryotes[274–276], did not match any of the detected peaks based on its relative retention time (RRT, with cholestane as reference[378]).

Predicted *Sch. japonicus* proteins resemble bacterial squalene-hopene cyclases | To identify potential sources of triterpenoids in *Sch. japonicus*, amino acid sequences of three characterized triterpene cyclases were used as queries to search the predicted proteomes of *Sch. japonicus* strains yFS275[383] and CBS5679. Specifically, sequences of an oxidosqualene cyclase (OSC) from Sch. pombe[391] (SpErg7; UniProt accession Q10231), a squalene-hopene cyclase (SHC) from *Acidocaldarius alicyclobacillus*[392] (AaShc; P33247), and a squalene-tetrahymanol cyclase (STC) from *Tetrahymena thermophila*[375,378] (TtThc1; Q24FB1), were used for a HMMER[393] homology search. For each *Sch. japonicus* strain, this search yielded a sequence with significant homology to OSC, and another with significant homology to the SHC (Table **4.1**). Consistent with the lipid analysis results, neither strain yielded a clear STC homolog.

Table 4.1: Homology search results using amino acid sequences of characterized triterpenoid cyclases against *Sch. japonicus* proteomes. Query coverage percentage and E-values were obtained with HMMER3[393], identity percentages were calculated with Clustal[394].

Query	Accession of subject sequence	Query coverage (%)	E-value	Identity (%)
Subject proteome: Sch. japonicus yFS275				
SpErg7[a] (Q10231)	B6JW54	99.7	0.0	65.9
AaShc[b] (P33247)	B6K412	99.2	2.7×10^{-141}	38.1
Subject proteome: Sch. japonicus CBS5679				
SpErg7[a] (Q10231)	SCHJC_A005630 (SjErg7)	99.7	0.0	65.7
AaShc[b] (P33247)	SCHJC_C003990 (SjShc1)	98.9	2.5×10^{-139}	37.8

[a] Protein sequence of Erg7 of *Schizosaccharomyces pombe*
[b] Protein sequence of Shc of *Acidocaldarius alicyclobacillus*

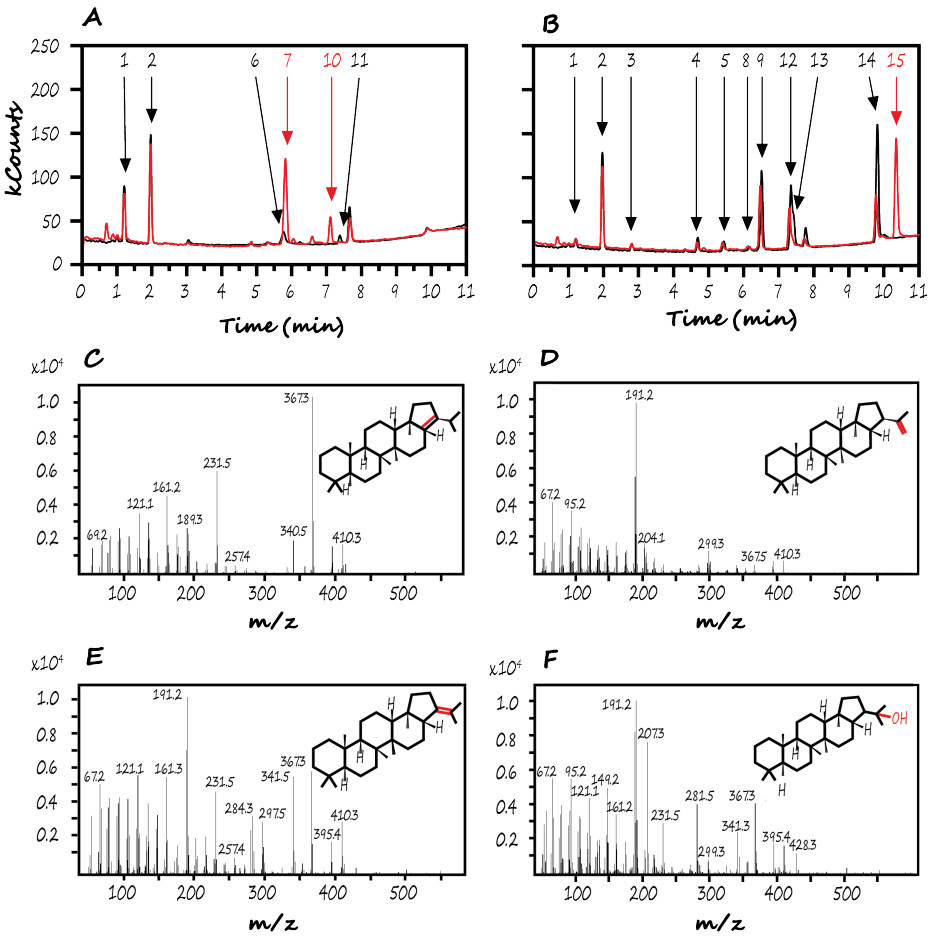
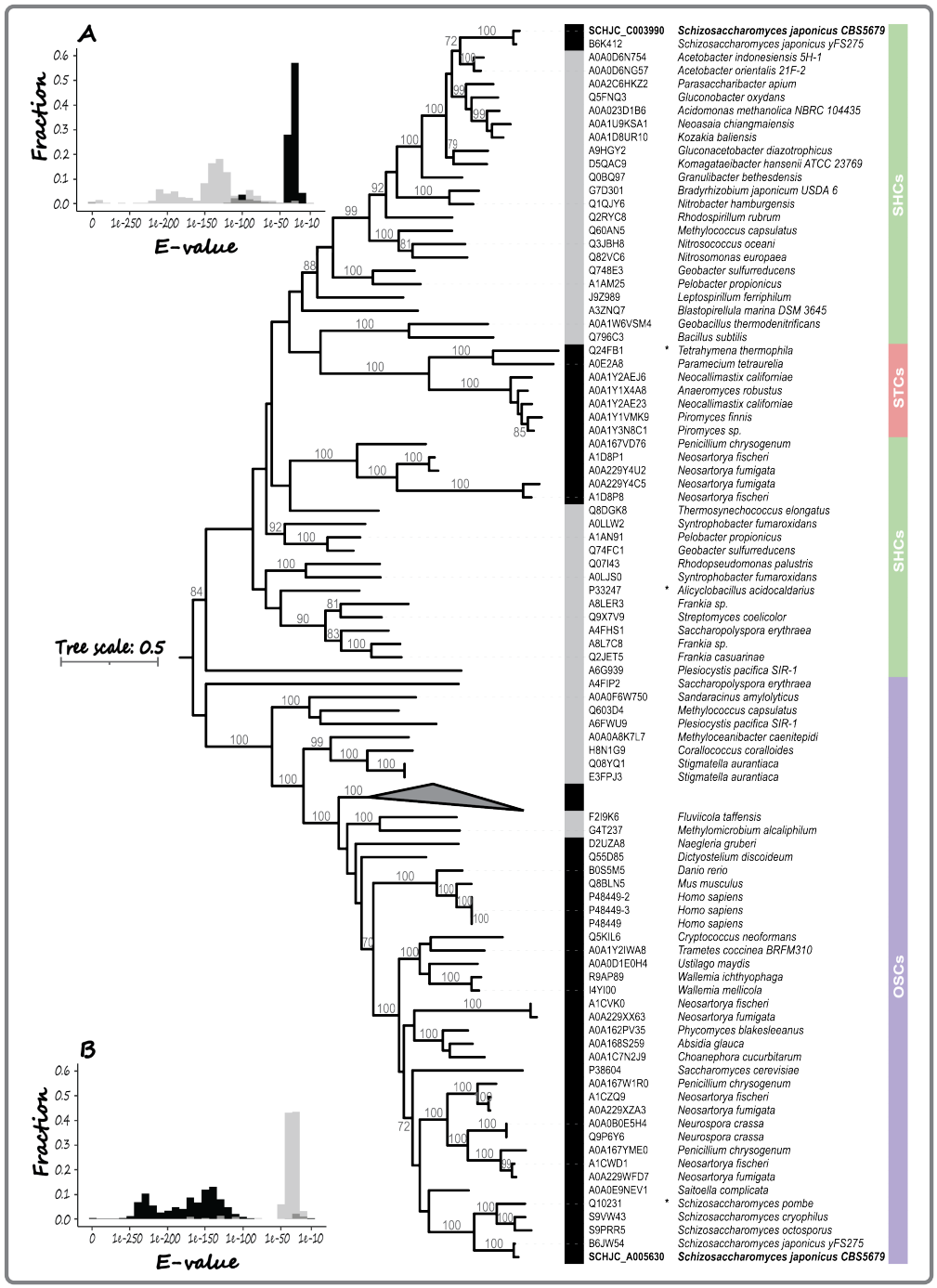


Figure 4.3: Gas chromatography-mass spectrometry (GC-MS) analysis of triterpenoid fractions of anaerobically grown yeast biomass. Anaerobic cultures were harvested in early stationary phase. Triterpenoids were extracted for GC-MS analysis and injected immediately (black lines) or after silylation (red lines). (**A**) *S. cerevisiae* CEN.PK113-7D grown anaerobically on medium supplemented with Tween 80 and ergosterol, (**B**) *Sch. japonicus* CBS5679 was grown on medium supplemented with only Tween 80. Numbers indicate the following compounds: **1**, squalene; **2**, 5α-cholestane (internal standard); **3**, squalene epoxide; **4**, hop-17(22)-ene; **5**, unidentified component; **6**, ergosterol; **7**, ergosterol-TMS-ether; **8**, unidentified component; **9**, unidentified component, possibly a tricyclic intermediate; **10**, lanosterol-TMS-ether; **11**, lanosterol; **12**, hop-22(29)-ene (diploptene); **13**, hop-21(22)-ene; **14**, hopan-22-ol (diplopterol); **15**, hopan-22-ol-TMS-ether. (**C**-**F**) Mass spectra and structures of identified hopanoid compounds in the triterpenoid fraction of anaerobically grown biomass of *Sch. japonicus*. (**C**) Hop-17(21)-ene, (**4**). (**D**) Hop-22(29)-ene, (**12**). (**E**) Hop-21(22)-ene, (**13**). (**F**) Hop-22-anol (diplopterol), (**14**).

To explore the phylogeny of the *Sch. japonicus* OSC and SHC homologs, SpErg7, AaSHC, and TtThc1 were used as queries for HMMER searches against all eukaryotic and all bacterial protein sequences in Universal Protein Resource (UniProt) reference proteomes (see SI Appendix, Table S3 for additional sequences obtained from TrEMBL). This search yielded 1480 eukaryotic and 348 bacterial cyclase homologs from a total number of 764 and 1826 species, respectively (SI Dataset S02). Sequences of cyclase homologs from 28 eukaryotic and 20 bacterial 'species of interest' were further analyzed. These species (Table S3) were either deep-branching anaerobic fungi [395], Schizosaccharomycetes [383] or species included in a previous publication on phylogeny of triterpene cyclases [396]. The selected cyclase homologs (SI Dataset S03) were subjected to multiple sequence alignment and used to generate a maximum-likelihood phylogenetic tree (Figure **4.4**; SI Dataset S04).

The systematic search for triterpene cyclase homologs and subsequent phylogenetic analysis showed that the putative *Sch. japonicus* SHC sequences (SCHJC_C003990 from CBS5679, and B6K412 from yFS275) are related to bacterial SHCs, with sequences from *Acetobacter* spp. (A0A0D6N754 from *A. indonesiensis* 5H-1, and A0A0D6NG57 from *A. orientalis* 21F-2) as closest relatives (Figure **4.4**). To check if this conclusion was biased by the selection of sequences from species of interest, the putative SHC sequence SCHJC_C003990 from *Sch. japonicus* CBS5679 was used as query for a second HMMER search of either the eukaryotic or the bacterial databases described above. The resulting E-values distribution (Figure **4.4**A; SI Dataset S05) showed a strong overrepresentation of low E-values among prokaryotic sequences. Sequences of two *Acetobacter* species, A0A0D6NG57 from *A. orientalis* 21F-2 and A0A0D6N754 from *A. indonesiensis* 5H-1, showed 67.9% and 66.9% sequence identity, respectively, and yielded zero E-values in this search. In contrast, E-value distributions obtained with the putative OSC sequence SCHJC_A005630 from *Sch. japonicus* CBS5679 as query showed an overrepresentation of low E-values among eukaryotic sequences (Figure **4.4**B).

Figure 4.4: Maximum-likelihood phylogenetic tree of selected triterpenoid cyclases. The colored bar indicates different types of cyclases: green, squalene hopene cyclases (SHCs); red, squalene tetrahymanol cyclases (STCs); and purple, oxidosqualene cyclases (OSCs). Sequences were obtained from a systematic homology search using the characterised cyclases marked with an asterisk (*Acidocaldarius alicyclobacillus* AaShc, P33247, SHC; *Tetrahymena thermophila*, TtThc1, Q24FB1, STC; *Schizosaccharomyces pombe* SpErg7, Q10231, OSC) as queries. Eukaryotic and bacterial sequences are indicated by black and grey bars, respectively. The collapsed clade contains 37 leaves from plants and green algae. 100 bootstrap replicates were performed, values above 70 are shown on the corresponding branches. All sequences and the final tree are provided in SI Dataset S02 and SI Dataset S03, respectively. The tree was midrooted, visualized and made available in iTOL (https://itol.embl.de/tree/8384480491291613138765). (**A**) Distribution of HMMER E-values obtained with *Sch. japonicus* SHC (SCHJC_C003990) as query against a bacterial sequence database (grey bars) and a eukaryotic database (black bars). (**B**) Distribution of HMMER E-values obtained with *Sch. japonicus* OSC (SCHJC_A005630) as query against a bacterial sequence database (grey bars) and a eukaryotic database (black bars).


A
Fraction
E-value
B
Fraction
E-value
Tree scale: 0.5
SCHJC_C003990 Schizosaccharomyces japonicus CBS5679
B6K412 Schizosaccharomyces japonicus yFS275
A0A0D6N754 Acetobacter indonesiensis 5H-1
A0A0D6NG57 Acetobacter orientalis 21F-2
A0A2C6HKZ2 Parasaccharibacter apium
Q5FNQ3 Gluconobacter oxydans
A0A023D1B6 Acidomonas methanolica NBRC 104435
A0A1U9KSA1 Neoasaia chiangmaiensis
A0A1D8UR10 Kozakia baliensis
A9HGY2 Gluconacetobacter diazotrophicus
D5QAC9 Komagataeibacter hansenii ATCC 23769
Q0BQ97 Granulibacter bethesdensis
G7D301 Bradyrhizobium japonicum USDA 6
Q1QJY6 Nitrobacter hamburgensis
Q2RYC8 Rhodospirillum rubrum
Q60AN5 Methylococcus capsulatus
Q3JBH8 Nitrosococcus oceani
Q82VC6 Nitrosomonas europaea
Q748E3 Geobacter sulfurreducens
A1AM25 Pelobacter propionicus
J9Z989 Leptospirillum ferriphilum
A3ZNQ7 Blastopirellula marina DSM 3645
A0A1W6VSM4 Geobacillus thermodenitrificans
Q796C3 Bacillus subtilis
Q24FB1 * Tetrahymena thermophila
A0E2A8 Paramecium tetraurelia
A0A1Y2AEJ6 Neocallimastix californiae
A0A1Y1X4A8 Anaeromyces robustus
A0A1Y2AE23 Neocallimastix californiae
A0A1Y1VMK9 Piromyces finnis
A0A1Y3N8C1 Piromyces sp.
A0A167VD76 Penicillium chrysogenum
A1D8P1 Neosartorya fischeri
A0A229Y4U2 Neosartorya fumigata
A0A229Y4C5 Neosartorya fumigata
A1D8P8 Neosartorya fischeri
Q8DGK8 Thermosynechococcus elongatus
A0LLW2 Syntrophobacter fumaroxidans
A1AN91 Pelobacter propionicus
Q74FC1 Geobacter sulfurreducens
Q07I43 Rhodopseudomonas palustris
A0LJS0 Syntrophobacter fumaroxidans
P33247 * Alicyclobacillus acidocaldarius
A8LER3 Frankia sp.
Q9X7V9 Streptomyces coelicolor
A4FHS1 Saccharopolyspora erythraea
A8L7C8 Frankia sp.
Q2JET5 Frankia casuarinae
A6G939 Plesiocystis pacifica SIR-1
A4FIP2 Saccharopolyspora erythraea
A0A0F6W750 Sandaracinus amylolyticus
Q603D4 Methylococcus capsulatus
A6FWU9 Plesiocystis pacifica SIR-1
A0A0A8K7L7 Methyloceanibacter caenitepidi
H8N1G9 Corallococcus coralloides
Q08YQ1 Stigmatella aurantiaca
E3FPJ3 Stigmatella aurantiaca
F2I9K6 Fluviicola taffensis
G4T237 Methylomicrobium alcaliphilum
D2UZA8 Naegleria gruberi
Q55D85 Dictyostelium discoideum
B0S5M5 Danio rerio
Q8BLN5 Mus musculus
P48449-2 Homo sapiens
P48449-3 Homo sapiens
P48449 Homo sapiens
Q5KIL6 Cryptococcus neoformans
A0A1Y2IWA8 Trametes coccinea BRFM310
A0A0D1E0H4 Ustilago maydis
R9AP89 Wallemia ichthyophaga
I4YI00 Wallemia mellicola
A1CVK0 Neosartorya fischeri
A0A229XX63 Neosartorya fumigata
A0A162PV35 Phycomyces blakesleeanus
A0A168S259 Absidia glauca
A0A1C7N2J9 Choanephora cucurbitarum
P38604 Saccharomyces cerevisiae
A0A167W1R0 Penicillium chrysogenum
A1CZQ9 Neosartorya fischeri
A0A229XZA3 Neosartorya fumigata
A0A0B0E5H4 Neurospora crassa
Q9P6Y6 Neurospora crassa
A0A167YME0 Penicillium chrysogenum
A1CWD1 Neosartorya fischeri
A0A229WFD7 Neosartorya fumigata
A0A0E9NEV1 Saitoella complicata
Q10231 * Schizosaccharomyces pombe
S9VW43 Schizosaccharomyces cryophilus
S9PRR5 Schizosaccharomyces octosporus
B6JW54 Schizosaccharomyces japonicus yFS275
SCHJC_A005630 Schizosaccharomyces japonicus CBS5679
SHCs
STCs
SHCs
OSCs

No SHC homologs were found in the predicted proteomes of Schizosaccharomycetes other than *Sch. japonicus*, nor in those of 371 Saccharomycotina yeast species included in the eukaryotic UniProt database. Acquisition of an STC-encoding DNA sequence from bacteria has been proposed as a key event in the evolution of strictly anaerobic fungi[396]. Since the putative *Sch. japonicus* SHC homologs did not show homology with STCs from those deep-branching fungi or from ciliates (Figure **4.4**), acquisition of a bacterial SHC-encoding DNA sequence by an ancestor of *Sch. japonicus* could represent an independent eukaryotic adaptation to an anaerobic lifestyle.

***Sch. japonicus* synthesizes hopanoids |** Since SHCs can catalyze the conversion of squalene to various polycyclic triterpenoids[397,398], we assessed whether the unidentified components in triterpenoid fractions of anaerobically grown *Sch. japonicus* (Figure **4.3**B) were hopanoids. GC-MS analysis of biomass samples (Figure **4.3**C-F; SI Appendix Table S4, SI Dataset S06) yielded 8 distinct analytes that were detected in *Sch. japonicus* (Figure 3B) but not in *S. cerevisiae* CEN.PK113-7D (Figure **4.3**A). Squalene epoxide (compound **3**) was identified based on relative retention time and spectral matching with authentic standard material as previously described[399], and hop-22(29)-ene (diploptene, compound **12**) was identified similarly (Figure **4.3**D). For the remaining six compounds, structures were explored based on published mass spectra of hopanoids[400] (Figure **4.3**; SI Appendix Table S4, SI Dataset S06). A fragment ion with a mass-charge ratio (m/z) of 191, which is present in mass spectra of many hopanoids and frequently as the base peak[400], was detected for compounds **4**, **13** and **14** (Figure **4.3**C, E and F) and comparison with published data putatively identified them as hop-17(21)-ene, hop-21(22)-ene and hopan-22-ol (diplopterol), respectively[351,400]. Mass and retention-time shifts caused by silylation[374] were investigated (Figure **4.3**) and confirmed presence of the hydroxy group of diplopterol (Figure **4.3**B; **14** and **15**) in the *Sch. japonicus* biomass, as well as those of the sterols (Figure **4.3**A; **6** and **7**, **10** and **11**) in the *S. cerevisiae* samples. A small peak at the retention time of unsilylated diplopterol was tentatively attributed to steric hindrance by the tertiary-alcohol context of its hydroxy group. In the chromatograms representing silylated triterpenoids of *S. cerevisiae*, small additional peaks at retention times of 16.0 min and 16.6 min were attributed to ergosta-5,7,22-trien-3β-ol (also detected in the commercial ergosterol preparation used for supplementation of anaerobic growth media; SI Appendix, Figure S2) and fecosterol, an intermediate of ergosterol biosynthesis[401], respectively (Figure **4.3**A).

Compound **5** showed the characteristic base peak of m/z 191 and a molecular ion of m/z 428, pointing towards a hydroxylated structure analogous to diplopterol, but was not readily silylated. For substances **8** and **9**, the ion at m/z 410 suggests an unsubstituted triterpene which is unaffected by silylation, with retention times similar to those of other identified hopenes (Figure **4.3**B). The corresponding base peaks of m/z 259 and 243 were previously reported for

polycyclic triterpenoids with a different backbone configuration than those of substances **4**, **12** and **13**[402]. However, the precise structure of compounds **5**, **8** and **9** could not be identified with a high degree of certainty. All acquired mass spectra are shown in SI Dataset S06.

The newly identified compounds were quantified by GC-FID analysis in biomass samples of anaerobic *Sch. japonicus* cultures supplemented with different combinations of Tween 80 and ergosterol. The detection of ergosterol in biomass from anaerobic cultures grown in the presence of this compound indicated that *Sch. japonicus* is able to import sterols (Figure **4.5**A). Except for the presence and absence of ergosterol, the triterpenoid composition of anaerobically grown biomass was not markedly affected by the supplementation of ergosterol and/or Tween 80. In addition, analyses were performed on aerobically grown *Sch. japonicus* to investigate whether hopanoid synthesis in this yeast is affected by oxygen availability. This experiment confirmed the ability of *Sch. japonicus* strain CBS5679 to synthesize ergosterol (Figure **4.5**A). Aerobically grown biomass showed a 3.5-fold higher squalene content than biomass grown in anaerobic cultures without Tween 80 and ergosterol, while its hopanoid content was 4-fold lower (Figure **4.5**A; SI Dataset S01). These observations suggest that oxygen availability may regulate triterpenoid synthesis in *Sch. japonicus*.

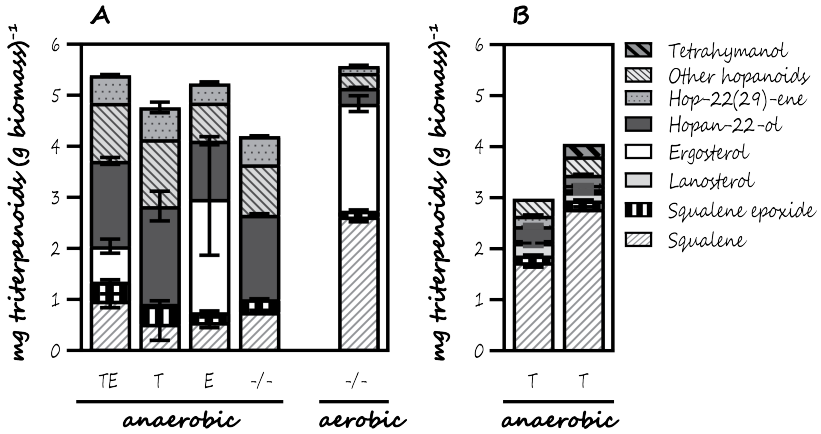


Figure 4.5: Quantification of triterpenoids in yeast biomass. (**A**) Triterpenoid content of cultures of *Sch. japonicus* CBS5679 grown in SMPD with 20 g L^{-1} glucose. Under anaerobic conditions, cultures were supplemented with Tween 80 and ergosterol (TE), only ergosterol (E), only Tween 80 (T) or neither of these supplements (-/-). Data are shown for the first anaerobic culture following the anaerobic pre-culture. Aerobic cultures of *Sch. japonicus* were grown in SMPD without supplements (-/-). (**B**) Triterpenoid composition of anaerobic cultures of *S. cerevisiae* IMX2616 (*sga1Δ::Sjshc1*; left) and IMX2629 (*sga1Δ::Sjshc1 X-2::Maths*; right) grown in SMPD with 20 g L^{-1} glucose and Tween 80 (T) supplementation. Data are represented as average ± SEM of data from two independent duplicate cultures for each cultivation condition. Detailed information on data presented in this figure and additional anaerobic transfers are provided in SI Dataset S01.

Expression of *Sch. japonicus* squalene-hopene cyclase supports sterol -independent anaerobic growth of *S. cerevisiae* | To investigate if the putative squalene-hopene-cyclase gene of *Sch. japonicus* CBS5679 (*Sjshc1*) was responsible for hopanoid synthesis, its coding sequence was codon optimized and expressed in the Cas9-expressing *S. cerevisiae* strain IMX2600. Growth and triterpenoid production of the resulting strain IMX2616 (*sga1Δ::Sjshc1*; SI Appendix, Table S6) was studied in anaerobic shake-flask cultures. After an anaerobic pre-culture for depletion of cellular reserves of sterols and/or hopanoids, neither the reference strain *S. cerevisiae* CEN.PK113-7D nor the strain carrying the *Sjshc1* expression cassette grew on SMPD without ergosterol and Tween 80 (Figure **4.6**). On SMPD with only Tween 80, *S. cerevisiae* CEN.PK113-7D reached an optical density of 0.7 after 33 h (Figure **4.6**A), at which point approximately 70% of the initially present glucose remained unused (SI Appendix; Table S5). In contrast, *S. cerevisiae* IMX2616 (*sga1Δ::Sjshc1*) reached an optical density of 2.1 after the same time period (Figure **4.6**B), at which point 98% of the initially added glucose had been consumed (SI Appendix; Table S5), and showed sustained anaerobic growth upon transfer to a second flask containing the same medium (Figure **4.6**B). Upon termination of the experiments after 58 h, the OD_{600} of the *S. cerevisiae* CEN.PK113-7D cultures had increased to 1.1. Similar very slow growth in sterol-free media was previously attributed to minute oxygen leakage[378].

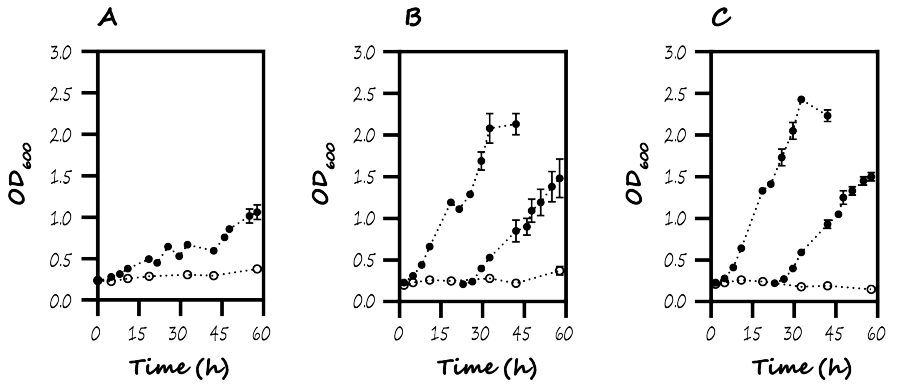


Figure 4.6: Anaerobic growth of *S. cerevisiae* strains in sterol-free media. *S. cerevisiae* cultures were inoculated from an anaerobic pre-culture on SMPD (50 g L^{-1} glucose) to fresh SMPD (20 g L^{-1} glucose), either supplemented with Tween 80 (closed circles), or lacking unsaturated fatty acids and sterols (open circles). (**A**) Reference strain CEN.PK113-7D. (**B**) *S. cerevisiae* strain IMX2616 (*sga1Δ::Sjshc1*). (**C**) *S. cerevisiae* strain IMX2629 (*sga1Δ::Sjshc1 X-2::Maths*). Cultures supplemented with Tween 80 represented in panel (**B**) and (**C**) were transferred to fresh medium of the same composition (closed circles) during exponential phase. Data are represented as average ± SEM of measurements on independent duplicate cultures for each yeast strain.

To investigate whether *S. cerevisiae* IMX2616 (*sga1Δ::Sjshc1*) produced the same hopanoid compounds as *Sch. japonicus* CBS5679, biomass was harvested from anaerobic shake-flask cultures grown on SMDP with Tween 80. Analysis of the triterpenoid fraction by GC-MS and GC-FID showed the same hopanoids that were detected and identified in *Sch. japonicus* strain CBS5679 (SI Appendix; Figure S3), albeit in smaller amounts (Figure **4.5**B; SI Dataset S01). The only sterol identified in these samples was lanosterol. Synthesis of small quantities of this first tetracyclic intermediate of ergosterol biosynthesis by *S. cerevisiae* strains has been attributed to small oxygen leakages into anaerobic cultivation systems[378].

In some prokaryotes, SHC is involved in a pathway for tetrahymanol production, in which a tetrahymanol synthase converts hopene into tetrahymanol[351]. To investigate whether such a two-step pathway for tetrahymanol synthesis can be engineered in *S. cerevisiae*, a codon-optimized expression cassette for the *Methylomicrobium alcaliphilum*[351] gene encoding THS (locus tag MEALZ_1626; referred to as *Maths*) was integrated at the X-2 locus[403] in strain IMX2616 (*sga1Δ::Sjshc1*), yielding strain IMX2629 (*sga1Δ::Sjshc1 X-2::Maths*; SI Appendix, Table S6). Anaerobic growth and sugar consumption rates of these two *S. cerevisiae* strains were similar (Figure **4.6**B and C; SI Appendix, Table S5). Tetrahymanol was detected in anaerobically grown biomass of strain IMX2629, but not of strain IMX2616 (Figure **4.5**C; SI Appendix, Figure S4). Together, these results confirm that *Sjshc1* encodes a bona fide SHC, at least one of whose hopanoid products can act as sterol surrogate in anaerobic yeast cultures.

Discussion

Research on obligately anaerobic fungi belonging to the phylum Neocallimastigomycota has dispelled the notion that sterols are indispensable components of all eukaryotic membranes. Growth studies linked the ability of these fungi to maintain proper membrane function in the absence of oxygen or exogenous sterols to their ability to synthesize the triterpenoid sterol surrogate tetrahymanol[275,396]. A recent study on expression of squalene-tetrahymanol cyclase in the model eukaryote *S. cerevisiae*[378] confirmed that tetrahymanol synthesis is not only required but also sufficient for sterol-independent growth. Inspired by a half-century old, intriguing publication by Bulder[283] on the yeast *Sch. japonicus*, the present study uncovered a different eukaryotic adaptation to circumvent oxygen requirements for sterol biosynthesis.

GC-MS analysis identified several hopanoids in anaerobically grown *Sch. japonicus* CBS5679 (Figure **4.3**; SI Appendix, Table S4) that were subsequently also detected in an *S. cerevisiae* strain expressing a codon-optimized *Sch. japonicus* ORF with sequence homology to known prokaryotic squalene hopene cyclase (SHC) genes (Figure **4.6**; SI Appendix, Figure S4). These

results indicate that product diversity originated from the *Sch. japonicus Sjshc1*-encoded SHC, rather than from additional enzyme-catalyzed modifications. Formation of multiple products is consistent with reports on triterpenoid extracts of bacterial hopanoid producers and product spectra of purified bacterial SHCs[404,405]. For example, analysis of triterpenoids in *Zymomonas mobilis* biomass revealed a number of minor hopene variants, in addition to diploptene and diplopterol, whose synthesis was attributed to deviation from the regular cyclization process[406].

Functional SHC enzymes and hopanoid synthesis have been found in ferns[407,408] and putative SHC proteins have been identified in several filamentous fungi[280,409] (Figure **4.4**). Although *Sch. japonicus* is therefore not unique among eukaryotes in containing an SHC, hopanoid synthesis has not previously been found in yeasts or associated with sterol-independent anaerobic growth of eukaryotes. No putative SHC genes were found in other *Schizosaccharomyces* species, nor in more distantly related yeasts. Confinement to a single yeast species and a strong similarity with putative SHC sequences from *Acetobacter* species identifies horizontal gene transfer (HGT) from acetic acid bacteria as a highly plausible evolutionary origin of *Sjshc1*[280]. Similar HGT events have been implicated in acquisition of squalene-tetrahymanol cyclase during transitions of eukaryotic lineages from aerobic to anaerobic lifestyles[280,396]. The independent acquisition of different squalene-cyclase genes by phylogenetically distant eukaryotes represents a remarkable case of convergent evolution towards an anaerobic eukaryotic lifestyle.

In the absence of efficient procedures for genetic modification of *Sch. japonicus* CBS5679[410], the role of one or more hopanoids as sterol surrogates was confirmed by heterologous expression of *Sjshc1* in *S. cerevisiae*, which enabled anaerobic growth under ergosterol-depleted conditions (Figure **4.6**). This positive impact on growth of a yeast in an anaerobic, sterol-free environment illustrates how the mere acquisition of an SHC gene by HGT may have benefited an ancestor of *Sch. japonicus*. However, ergosterol-independent anaerobic growth of the *Sjshc1*-expressing *S. cerevisiae* strain, as well as that of a strain that co-expressed *Sjshc1* with a bacterial tetrahymanol synthase from *M. alkaliphilum*[351] was much slower than that of *Sch. japonicus* and of ergosterol-supplemented *S. cerevisiae* cultures[352] (Figure **4.6**). A reduced specific growth rate in sterol-free media was previously also observed for *S. cerevisiae* and *Kluyveromyces marxianus* strains expressing a squalene-tetrahymanol cyclase gene from the ciliate *T. thermophila*[378,411].

Our results confirm the report by Bulder[283] that *Sch. japonicus* does not require supplementation of unsaturated fatty acids (UFAs) for anaerobic growth. However, we did not find evidence for his hypothesis that this yeast is capable of oxygen-independent UFA synthesis[287]. Instead, in comparison with UFA-supplemented cultures, cultivation in UFA-free medium led to a reduction of the average chain length of saturated fatty acids (SFA; Figure **4.2**).

A similar shift in SFA composition has been observed during slow UFA-independent anaerobic growth of *S. cerevisiae* CEN.PK113-7D [352] and in membranes of anaerobically grown cultures of the dimorphic fungus *Mucor rouxii* [412].

Our results indicate that its membrane composition and/or membrane architecture enable *Sch. japonicus* to maintain a much faster growth rate than *S. cerevisiae* upon sterol replacement by hopanoids, as well as in the absence of UFAs. A recent study on membrane properties of *Sch. japonicus* and the more closely related yeast *Sch. pombe* showed a higher membrane stiffness and lipid packing in *Sch. japonicus* as well as generally shorter and more saturated fatty acid residues than found in *Sch. pombe* [390]. Further research should resolve the impact of hopanoids on membrane properties of *Sch. japonicus*. In bacteria, hopanoids have been implicated in tolerance to external stresses such as non-optimal temperature and pH, and the presence of antimicrobials [413–415]. Elucidating how sterol surrogates interact with other membrane components, including proteins, and thereby influence membrane functionality can contribute to a deeper insight in microbial adaptation to anaerobic environments and to physicochemical stress factors. In addition, such studies will contribute to the design of membrane engineering strategies aimed at the construction of robust industrial strains of *S. cerevisiae* and other yeasts for application in anaerobic fermentation processes.

Supplementary Materials

Supplementary Information is available at BioRXiv online:
https://doi.org/10.1101/2021.03.17.435848

Data availability

Whole-genome sequencing data for *Sch. japonicus* CBS5679 have been deposited under the BioProject accession PRJNA698797 in NCBI.

Acknowledgements

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Materials and methods

Strains, media and maintenance | *Schizosaccharomyces japonicus* CBS5679 was obtained from the Westerdijk Institute (Utrecht, The Netherlands). *Saccharomyces cerevisiae* strains used and constructed in this study belonged to the CEN.PK lineage[304,305] (SI Appendix, Table S5). Yeast strains were propagated in YPD and stored at -80 °C after addition of 30% sterile glycerol[378]. To avoid sexual co-flocculation[416] of *Sch. japonicus* CBS5679, carbon source depletion in pre-cultures was prevented and buffered synthetic media were used in all growth studies. Yeasts were grown on synthetic medium with ammonium as nitrogen source[135] with an increased concentration of KH_2PO_4 (14.4 g L^{-1}, SMP[417]). Unless otherwise indicated, glucose was added from a concentrated stock solution, separately autoclaved at 110 °C, to a concentration of 20 g L^{-1} (SMPD). Where indicated, SMPD was supplemented with Tween 80 (polyethylene glycol sorbitan monooleate; Merck, Darmstadt, Germany) and/or ergosterol (≥95%; Sigma-Aldrich, St. Louis, MO) at concentrations of 420 mg L^{-1} and 10 mg L^{-1}, respectively, from a sterile 800-fold concentrated stock solution[352]. Bacto Agar (BD Biosciences, 20 g L^{-1}) was added to prepare solid media. The counter-selectable *amdS*-marker was used as described previously[323]. Strains with geneticin, hygromycin or nourseothricin resistance were selected by supplementing YPD with 200 mg L^{-1} geneticin (G418), 100 mg L^{-1} hygromycin B (hygB) or 100 mg L^{-1} nourseothricin (CloNAT), respectively.

Molecular biology techniques | Open-reading frames of a putative squalene-hopene-cyclase gene (SHC; SCHJC_C003990) from *Sch. japonicus* CBS5679 and of a *Methylomicrobium alkaliphilum* tetrahymanol synthase gene (Genbank Accession number CCE23313) were codon-optimized for use in *S. cerevisiae* with the GeneOptimizer algorithm (GeneArt, Regensburg, Germany)[418]. DNA fragments for plasmid construction were amplified with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA) as specified by the manufacturer, using PAGE-purified oligonucleotide primers (Sigma-Aldrich). Diagnostic PCR was performed with DreamTaq PCR Master Mix (Thermo Scientific), according to the manufacturer's protocol using desalted oligonucleotides (Sigma-Aldrich). PCR-amplified linear integration cassettes were purified from 1% (w/v) agarose gels (TopVision Agarose, Thermo Fisher) with TAE buffer (Thermo Fisher) using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Assembly of DNA fragments was performed with Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA). *E. coli* XL-1 Blue cells (Agilent Technologies, Santa Clara, CA) were transformed with assembled plasmids according to the provider's instructions and stored at -80 °C. *S. cerevisiae* was transformed with the lithium-acetate method[324] and marker-less CRISPR/Cas9-based genome editing of *S. cerevisiae* was performed as described previously[306]. Transformants were selected on YPD-CloNAT, YPD-G418 or YPD-hygB agar for IMX2600, IMX2616 and IMX2629 respectively with subsequent isolation of single-cell lines by three consecutive re-streaks. Plasmids and oligonucleotides used are listed in SI Appendix, Tables S7 and S8, respectively.

Plasmid and strain construction | All *S. cerevisiae* strains that were used and constructed in this study are listed in SI Appendix, Table S6. A *S. cerevisiae* strain suitable for marker-less genome editing[306] was constructed by integrating of expression cassettes for *Spcas9* and the natNT2 marker gene in the *CAN1* locus of *S. cerevisiae* CEN.PK113-7D. Transformation of CEN.PK113-7D with 2000 ng and 900 ng of the *Spcas9* and *natNT2* cassettes respectively, yielded strain IMX2600. These integration cassettes were amplified from p414-TEF1p-cas9-CYC1t[99] and pUG-natNT2[419], respectively, using primer pairs 2873/4653 and 3093/5542. Expression cassettes for the putative SHC-gene of *Sch. japonicus* (*Sjshc1*) and the THS-gene from *M. alcaliphilum* (locus tag MEALZ_1626; referred to as *Maths*) used the *S. cerevisiae TEF1* promoter and *CYC1* terminator, or *TDH3* promoter and *ADH1* terminator sequences, respectively. Codon-optimized coding sequences were first amplified from plasmids pUD1151 and pUD1150 using primer pairs 15100/15101 and 17519/17520, respectively. The p426TEF and pUD63 backbones were linearized using primer pairs 5921/10547 and 10546/3903, respectively. Gibson assembly of the linearized p426TEF-backbone and *Sjshc1*-insert resulted in plasmid pUD1059, and assembly of the pUD63-backbone and *Maths*-insert resulted in plasmid pUDE1060. The expression cassettes for the *Sjshc1*-gene and *Maths*-gene were then amplified from these plasmids using primer pairs 15002/15003 and 9034/17521, respectively. The *Sjshc1*-construct was integrated in the *SGA1* locus of *S. cerevisiae* IMX2600 by co-transformation with 500 ng of the linear fragment and 500 ng of the *SGA1*-targeting plasmid pUDR119, resulting in *S. cerevisiae* IMX2616. Correct integration was verified by diagnostic PCR using primers 7298/7479 (both binding outside the *SGA1* locus) and 7298/15103 and 7479/15102 (binding outside of the integration locus and inside of the heterologous SHC-gene). To target the X-2 locus, pUDR538 was constructed by Gibson assembly of the backbone of pROS12, linearized with primer 6005, and a 2µm cassette amplified from pROS12 with primer 10866. The *Maths*-gene was subsequently integrated in the X-2 locus[403] of *S. cerevisiae* strain IMX2616 by co-transformation with 500 ng of the linear cassette and 500 ng of the X-2 targeting plasmid pUDR538, resulting in strain IMX2629. Correct integration was verified by diagnostic PCR using primers 7376/7377 (both binding outside the X-2 locus) and 7376/17523 and 7377/17522 (binding outside of the integration locus and inside of the heterologous THS gene).

Aerobic shake-flask cultivation | Aerobic cultivation on SMPD was performed in 500-mL shake flasks with a working volume of 100 mL. A pre-culture was inoculated from a frozen stock culture and, after overnight incubation, transferred to a second pre-culture. Upon reaching mid-exponential phase, cells were transferred to fresh SMPD and optical density was monitored at 660 nm. All experiments were performed in duplicate. Light-induced flocculation of *Sch. japonicus*[420] was prevented by wrapping flasks in aluminium foil.

Anaerobic shake-flask cultivation | Anaerobic chamber experiments were performed as previously described[352], using 100-mL shake flasks containing 80 mL of medium. The anaerobic chamber was placed in a mobile darkroom, which was only illuminated with red LEDs[421] during sampling. An aerobic pre-culture on SMPD was used to inoculate an anaerobic pre-culture on SMPD with an increased

glucose concentration of 50 g L^{-1}, which was grown until the end of the exponential phase. Samples from these pre-cultures were then used to inoculate experiments on regular SMPD supplemented with either Tween 80 and ergosterol, only Tween 80 or ergosterol, or neither. All growth experiments were performed by monitoring optical density at 600 nm in independent duplicate cultures.

Analytical methods | High-performance liquid chromatography was used to analyze extracellular metabolite concentrations as described previously[325]. Extraction of fatty acids and triterpenoids and quantitative analysis by gas-chromatography with flame ionization detection (GC-FID) was performed as previously described[352,378]. The GC-FID system was calibrated for hop-22(29)-ene (Sigma Aldrich, 0.1 mg mL^{-1} in isooctane) using a 6-point calibration, and this calibration curve was additionally used for quantification of other detected hopanoid compounds. For anaerobic cultures, optical density at 600 nm was measured with an Ultrospec 10 cell density meter (Biochrom, Harvard Biosience, Holliston, MA) placed in the anaerobic chamber. In aerobic cultures, optical density at 660 nm was measured on a Jenway 7200 spectrophotometer (Bibby Scientific, Staffordshire, UK). For gas chromatography-mass spectrometry (GC-MS) analysis, a Varian 3800 gas chromatograph with a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland) was coupled with a Saturn 2200 ion trap MS with a Varian 1177 injector set in 1:3 split mode (Varian, Darmstadt, Germany) controlled by Varian Workstation 6.9 SP 1 software. An Agilent VF 5ms (Agilent Technologies) capillary column of 30 m length with a 10 m EZ Guard column (0.25 mm internal diameter and 0.25 µm film thickness) was used with an inlet temperature of 250 °C, and an injection volume of 1 µL (splitless time 1.0 min). Helium (99.999%; Air Liquide, Düsseldorf, Germany) was used as carrier gas at a constant flow rate of 1.4 mL min^{-1}. The GC oven started at 55 °C (1.0 min hold), ramped up to 260 °C at 50 °C min^{-1}, followed by a gradient of 4 °C min^{-1} up to 320 °C (hold time 3.9 min). The total run time was 24.0 min. The transfer line temperature was set at 270 °C and the ion-trap temperature was 200 °C. The ion trap was operated in two segments. The mass spectrometer (MS) was switched off (solvent delay) for the first 10 min, and from 10 to 24 min the MS scanned at a mass range from 50 to 600 m/z (EI, 70 eV). Data analyses were carried out with the Agilent MassHunter Workstation Software package B.08.00 (Agilent Technologies). Triterpenoid compounds were identified by comparison with commercial references[374,378] or literature data[351,400]. Autosampler vials and caps and the silylation reagents N-trimethylsilylimidazole (TSIM) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were purchased from Macherey-Nagel (Düren, Germany). The primary secondary amine (PSA) for dispersive solid phase extraction (dSPE) was acquired from Agilent Technologies. Ergosterol (≥ 95.0%), 5α-cholestane (≥ 97.0%) and hop-22(29)-ene (0.1 mg mL^{-1} in isooctane, analytical standard) were obtained from Sigma-Aldrich. Other reagents and solvents were purchased in HPLC-grade or pro-analysis quality from Sigma-Aldrich and all consumables were from VWR (Ismaning, Germany). 8 mg of freeze-dried yeast biomass was mixed with 2 M NaOH to obtain a final concentration of 4 mg biomass mL^{-1}. Subsequent procedures were performed as described previously[374], with slight modifications. After saponification, the suspension was divided in two microcentrifuge tubes (2 × 500 µL). 650 µL of distilled methyl-tert-butylether (MtBE) and 100

µL of internal standard solution (5α-cholestane in MtBE, 10 µg mL-1) were added to each sample. The mixtures were shaken well for 1 min and centrifuged at 9000 × *g* for 5 min. The organic upper layer was transferred into a new 2.0 mL plastic microcentrifuge tube containing 40 ± 2 mg of a mixture (7:1) of anhydrous sodium sulfate and primary secondary amine (PSA). The mixture was extracted a second time in the same manner with another 750 µL of MtBE. The combined organic extracts were vigorously shaken for 1 min, followed by a centrifugation step (5 min, 9000 × *g*). 1 mL of the purified upper layer was then transferred into a brown glass vial and concentrated to dryness under a stream of nitrogen. One residue was dissolved in 700 µL of MtBE and 50 µL of silylation reagent mixture MSTFA/TSIM (9:1) was added. The other residue was dissolved in 750 µL of MtBE before analysis.

Genome sequencing and assembly | The genome of *Sch. japonicus* CBS5679 was sequenced using short-read and long-read sequencing technologies. Genomic DNA was isolated with a Qiagen genomic DNA 100/G kit (Qiagen, Hilden, Germany) with modifications to the 'Part I sample preparation and lysis protocol for yeast' of the manufacturer's instructions. Yeast cells were harvested by centrifugation (10 min at 3700 × *g*) from 100 mL overnight cultures on YPD. Zymolyase incubation was replaced by freezing yeast pellets in liquid nitrogen, manual grinding and resuspension in buffer G2 with RNAse A. Incubation with Proteinase K was extended to 3 h. Further steps were as described in the manufacturer's protocol. DNA quantity was measured on a Qubit Fluorometer (Invitrogen, Carlsbad, CA) using a QuBit BR dsDNA Assay kit (Invitrogen). For short read sequencing, 150 bp paired-end libraries were prepared with a Nextera DNA Flex Library Prep kit (Illumina, San Diego, CA) according to the manufacturer's instructions and whole-genome sequencing was performed on an in-house MiSeq platform (Illumina). For long-read sequencing, genomic libraries were prepared using the 1D Genomic DNA by ligation (SQK-LSK109, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's instructions, with the exception of the 'End Repair/dA-tailing module' step during which the ethanol concentration was increased to 80% (v/v). Quality of flow cells (R9 chemistry flow cell FLO-MIN106, Oxford Nanopore Technologies) was tested with the MinKNOW platform QC (Oxford Nanopore Technologies). Flow cells were prepared by removing 20 µL buffer and subsequently primed with priming buffer. The SQK-LSK109 DNA library was loaded dropwise into the flow cell and sequenced for 48 h. Base-calling was performed using Guppy v2.1.3 (Oxford Nanopore Technologies) using dna_r9.4.1_450bps_flipflop.cfg. Genome assembly was performed using Flye v2.7.1-b167359[422]. Flye contigs were polished using Pilon v1.18[423]. The polished assembly was annotated with Funannotate v1.7.1[424] using RNAseq data from bioprojects PRJNA53947 and PRJEB30918 as evidence of transcription, adding functional information with Interproscan v5.25-64.0[425].

Sequence homology search and phylogenetic analyses | Eukaryotic and bacterial amino acid sequence databases were built from UniProtKB reference proteomes (Release 2019_02) using the taxonomic divisions (taxids) shared with databases from the National Center for Biotechnology Information (NCBI). Only amino acid sequences from reference or representative organisms having

genome assemblies at chromosome or scaffold level according to the NCBI genomes database (Release 2019_03) were included. Table S3 (SI Appendix) indicates proteomes from species of interest, which were included in the corresponding databases obtained from the UniProt TrEMBL division. The *Sch. japonicus* CBS5679 proteome was obtained in this study and consequently not available from UniProt at the time of analysis. Amino acid sequences of an oxidosqualene cyclase (OSC) from Sch. pombe [391] (SpErg7; UniProt accession Q10231), a squalene-hopene cyclase (SHC) from *Acidocaldarius alicyclobacillus* [392] (AaShc; P33247), and a squalene-tetrahymanol cyclase (STC) from *Tetrahymena thermophila* [375,378] (TtThc1; Q24FB1) were used as queries for a HMMER3 [393] homology search. HMMER hits with an E-value below 1×10^{-5} and a total alignment length (query coverage) exceeding 75% of the query sequence were considered significant. The taxids of 28 eukaryotic and 20 bacterial species of interest (SI Appendix, Table S3) were used to select sequences from all significant bacterial and eukaryotic HMMER hits obtained using the three cyclase sequences as queries. This procedure yielded 128 sequences (45 bacterial, 83 eukaryotic) including the 10 HMMER hits with the lowest E-values obtained by using either SjErg7 or SjShc1 as queries against the bacterial and eukaryotic databases (SI Dataset S02). These 128 sequences were subjected to multiple sequence alignment using MAFFT v7.402 [426] in "einsi" mode. Alignments were trimmed using trimAl v1.2 [427] in "gappyout" mode, and used to build a phylogenetic tree with RAxML-NG v0.8.1 [428] using 10 random and 10 parsimony starting trees, 100 Felsestein Bootstrap replicates, and PROTGTR+FO model. The final, midrooted tree provided in SI Dataset S03 was visualized using iTOL [429].

5

Procedures for anaerobic cultivation of yeasts in shake flasks and bioreactors

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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 oxygen entry, 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 pre-grown 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.

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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[129–132]. 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[130,151,177,241,284,378].

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)[135,144,286–288,430]. 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[138,143,177,241,337]. Although the requirement of anaerobic *S. cerevisiae* cultures for UFAs is not absolute, anaerobic growth in the absence of UFA supplementation is slow[352]. 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[144]. This fast anaerobic growth of *Saccharomyces* yeasts is exceptional among yeasts[130,132] and essential for their large-scale industrial application in brewing, wine fermentation and bioethanol production[289–292,431]. 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 synthesis can still negatively affect strain performance during prolonged anaerobic cultivation. For example, 'stuck' brewing fermentations can occur when intracellular sterols and UFAs are prematurely depleted[432,433].

The magnitude and molecular basis of the oxygen requirements of most yeasts other than *Saccharomyces cerevisiae*, remain to be fully elucidated[284,285]. 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[123,434,435].

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 **5.1**). 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 **5.1**) requires an oxygen consumption rate of only 0.08 µmol (g biomass)$^{-1}$ min^{-1} to sustain a specific growth rate of 0.05 h^{-1} (corresponding to a doubling time of 14 h).

In *S. cerevisiae*, biosynthesis of several key cofactors and precursors, including pyridine nucleotides, coenzyme A, biotin and thiamin, involves oxygen-dependent reactions (Table **5.1**). With the exception of pyridine-nucleotide synthesis, these oxygen requirements are at least two orders of magnitude lower than those for sterol and UFA biosynthesis (Table **5.1**). In laboratory studies with synthetic media, they are usually masked by the routine inclusion of a mix of B-type vitamins[136].

In most non-*Saccharomyces* yeasts, pyrimidine metabolism depends on a mitochondrial, respiratory-chain-coupled dihydroorotate dehydrogenase[149,436] and therefore contributes a biosynthetic oxygen requirement similar in magnitude to that for sterol and UFA synthesis (Table **5.1**). Pyrimidine biosynthesis in *S. cerevisiae* does not require oxygen, because its soluble cytosolic dihydroorotate dehydrogenase (Ura1) uses fumarate as electron acceptor[148,150].

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 oxygen entry into yeast cultivation systems to extremely low levels[130,303,352,378]. 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[437–439]. **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[303,440], 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.

Table 5.1: 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*.

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 2.6 3.9	CBS2806, glucose-limited chemostat CEN.PK113-7D, glucose-limited chemostat CEN.PK113-7D, batch culture	201 308 378	30-52
UFA[a] ***Tween 80***	1	21 (1.6 $C_{16:1}$, 19.4 $C_{18:1}$) 103 (44 $C_{16:1}$, 59 $C_{18:1}$) 58 (1.2 $C_{16:1}$, 57 $C_{18:1}$)	CBS2806, glucose-limited chemostat CEN.PK113-7D, glucose-limited chemostat CEN.PK113-7D, batch culture	201 308 378	21-103
Pyridine nucleotides ***Nicotinate[b]***	3	3.8 4.5 2.1-3.9	CEN.PK113-7D, glucose-limited chemostat Strain 210NG, aerobic ethanol-stat vitamin fed-batch CEN.PK113-7D, glucose-limited accellerostat (NAD^+/NADH only)	441 442 443	6.3-14
Biotin ***Biotin***	~ 1[c]	0.002-0.009 0.002-0.008	Industrially produced yeast Strain 1403-7A, aerobic uptake assay	444 445	~ 0.002-0.009
Coenzyme A ***Pantothenate[d]***	1	0.43 ~ 0.38	CEN.PK113-7D, glucose limited chemostat (sum of CoA and acetyl-CoA) CEN.PK2-1C, aerobic shakeflask (only acetyl-CoA)	441 446	~ 0.4
Thiamine ***Thiamine***	~4[c]	0.025-0.22 0.0022-0.0029	Strain 210NG, aerobic ethanol-stat vitamin fed-batch Brewing strain #1007, static and shaken wort cultures	442 447	~ 0.0022-0.22
Pyrimidines[e] ***Uracil***	0.5	179 111	Strain 306, oxygen-limited continuous cultures Biomass equation in genome-scale model	448 449	56-90

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[450]. When bioreactors are operated as fed-batch or chemostat cultures, additional precautions are needed to prevent oxygen entry via the medium feed[130].

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[284,308,437,451–454]. Although these challenges are frequently mentioned in the literature[130,303,455], 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[130,144,352,378], 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 growth factors

Many yeast species accumulate lipids, including UFAs and sterols, in lipid droplets during aerobic growth on glucose[456–458]. Toxic effects of intracellular free fatty acids and sterols are prevented by storage as nonpolar steryl esters (SE) and triacylglycerol (TAG) lipids[459]. Lipid droplet synthesis from extracellular sources of sterols and UFAs has also been observed in heme-deficient cells[460,461] and under anaerobic conditions[314,462]. Such intracellular stores of lipids can be mobilized to supply sources for membrane synthesis[208]. 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[289].

'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[352,437,451]. 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 **5.1**). 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 **5.1**). 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 **5.1**). 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 [411]. 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 [129,285]. 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 [284]. 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 [285]. 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 **5.1**).

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 [130,303,352,378]. 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 [378].

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.

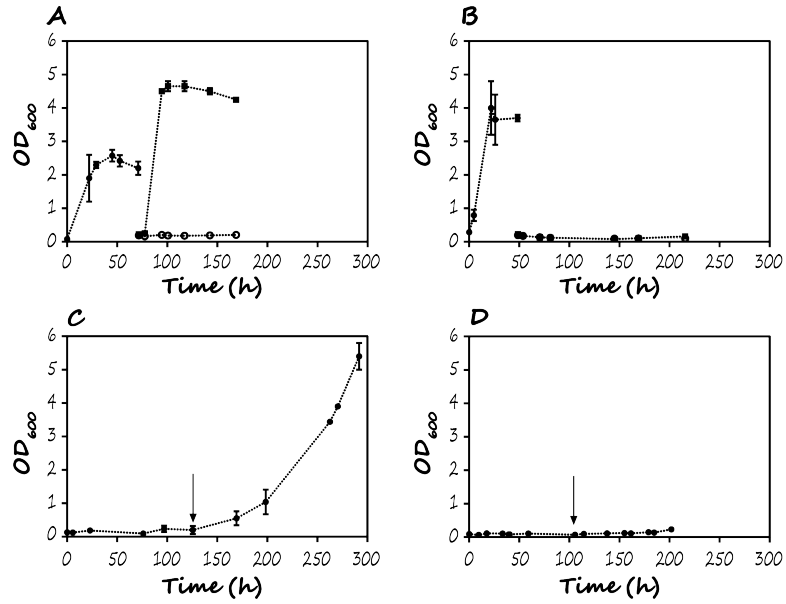

Figure 5.1: Representative growth profiles of four facultatively fermentative yeast species in standardized anaerobic chamber experiments. Anaerobic-chamber experiments were performed as described in **Protocol 1**. The yeasts *S. cerevisiae* (**A**), *K. marxianus* (**B**), *B. bruxellensis* (**C**) and *Tetrapisispora phaffii* (**D**) were grown in 50-mL shake-flasks containing 40 mL synthetic medium with urea as nitrogen source (SMU) [322], 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.

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 followed, use of the air lock can be a significant cause of oxygen entry (Figure **5.2**).

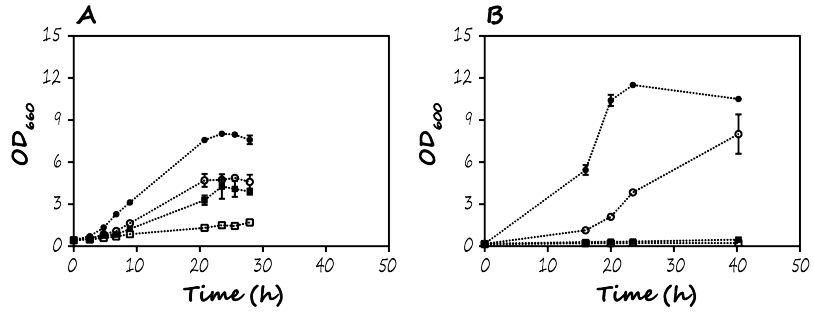

Figure 5.2: Use of the air lock of an anaerobic chamber as main source of oxygen contamination. Anaerobic-chamber experiments were performed as described in **Protocol 1**. *S. cerevisiae* CEN.PK113-7D was grown in 50-mL shake-flasks containing 40 mL synthetic medium with urea as nitrogen source (SMU) [322], 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, and the air lock was not used during the experiment. 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.

5

Reducing the void volume of the air lock by inserting inert solid objects helps to reduce oxygen entry (see **Protocol 1**). 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[144,378,463,464].

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 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 gas, supply through the headspace leads to a lower oxygen transfer to the broth, in which yeast cells maintain a vanishingly low oxygen concentration[129,285,352]. 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 **5.3**).

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[352,378]. 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[322,465]. 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[130,303], 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

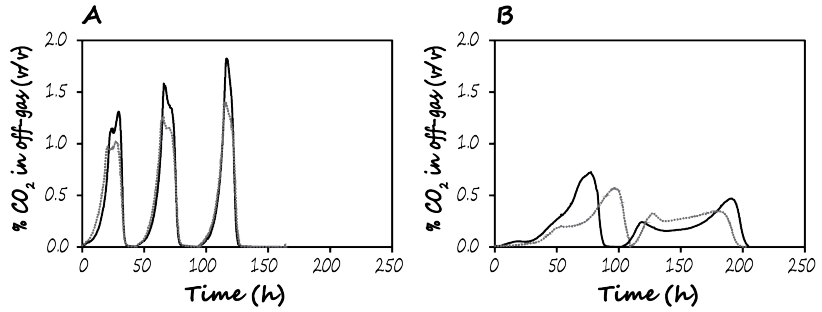

Figure 5.3: Nitrogen sparging versus headspace supply: impact on growth of *S. cerevisiae* in sequential batch reactors (SBR). Bioreactors were assembled according to **Protocol 2**. Anaerobic bioreactors were operated in SBR mode, and the CO_2 content of the off-gas was used to monitor growth of *S. cerevisiae* strain IMX585[306]. Cultures were grown on synthetic medium with urea as nitrogen source (SMU)[322]. 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.

peristaltic pumps need to be considered. For a long time, our group relied on Norprene A-60-G tubing for anaerobic bioreactor set-ups[144]. 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 **5.2**). Masterflex C-Flex Ultra has an even lower oxygen permeability compared 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.

Table 5.2: 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} $cm^3_{STP} \cdot cm \cdot cm^{-2} \cdot s^{-1} \cdot (cm \cdot Hg)^{-1}$), 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	- - -

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.

In SBR cultures, medium in the tubing between the medium reservoir and the reactor is stagnant in between empty-refill cycles, which can lead to slow permeation of oxygen into the liquid. After having observed that this effect is significant, we routinely discard the first 10 mL ('bleed') of sterile medium into a dedicated sample bottle, while using the mild overpressure in the reactor to backflush stagnant medium in the tubing between the reactor and the sampling bottle (Figure **5.4**).

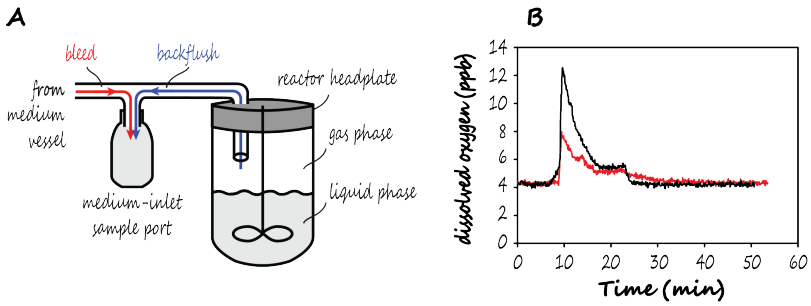

Figure 5.4: Effect of bleed and backflush of the medium inflow on dissolved oxygen concentration in anaerobic bioreactors. (**A**) Schematic representation of 'bleed' and 'backflush' to eliminate stagnant medium in the inlet tubing that had acquired oxygen by permeation through tubing during sequencing-batch 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).

Even when a glass medium reservoir is continuously flushed with high-grade 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) [130] 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[466–468] are extremely efficient, affordable and practical devices for deoxygenating the medium feed of continuous-cultivation systems (Figure **5.5**). When a membrane-contactor module was placed near the medium entry point of bioreactors and connected to a flow of high-purity nitrogen, *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[303,411].

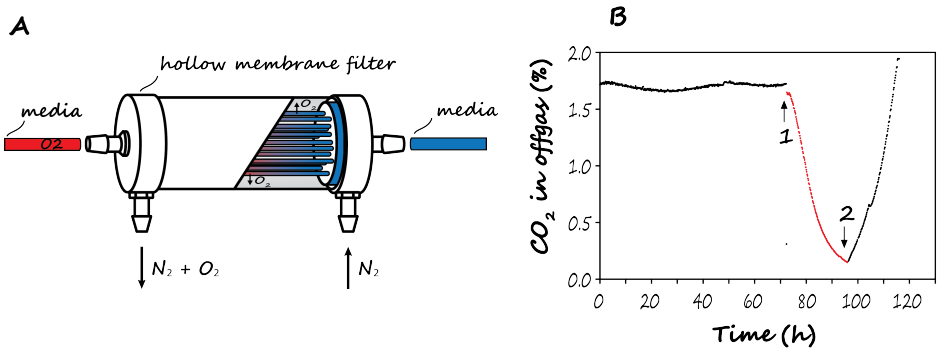

Figure 5.5: The effect of a membrane-contactor removing oxygen from the medium feed on cultures in chemostat. (**A**) Schematic representation of the PDMSXA-1000 membrane module (PermSelect, Ann Arbor, MI, USA). Ingoing medium contaminated with oxygen (red) due to permeation through tubing, is stripped from oxygen with 5.0 quality nitrogen gas (Linde, Schiedam, The Netherlands) and the resulting anaerobic medium (blue) enters the bioreactor. (**B**) *Saccharomyces cerevisiae* CEN.PK113-7D was grown in an anaerobic chemostat culture, as described in **Protocol 2**. Cultures were grown at a dilution rate of 0.10 h^{-1} on synthetic medium with urea as nitrogen source[322] and 20 g L^{-1} glucose without supplementation of a source of either UFAs or sterols. Growth was monitored by on-line analysis of the CO_2 concentration in the off gas. After 70 h, when the steady-state residual glucose concentration was 5.0 mM (indicated by Arrow 1), the medium inlet was rerouted through the membrane module, resulting in a washout (red line). After 96 h, when the residual glucose concentration had increased to 66 mM, the medium flow was restored to the original situation state (Arrow 2).

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 technical grade nitrogen gas and equipped with Norprene tubing[469,470]. 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. Instead, 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*[352].

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 the anaerobic pre-cultures (see Note 5) 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 **5.1**):
    a Growth is observed (Figure **5.1**A 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 **5.1**C 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 **5.1**C; the yeast grows anaerobically when provided with this anaerobic growth factor).
    b No growth is observed (Figure **5.1**D; 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 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 [352,411,471].

Bioreactor batch cultivation | Steps 1–9 describe anaerobic bioreactor batch experiments. Since sequential-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 (grade 6 or above) nitrogen 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.

Bioreactor sequential batch cultivation |

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 high-quality nitrogen 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 **5.4**A).
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.

Bioreactor chemostat cultivation |

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 **5.2**B 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 **5.2**). 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.

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, 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[472].

Note 14. After re-filling of the bioreactor is initiated, we recommend to sparge the broth with nitrogen 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 text and Figure **5.3**).

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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Outlook

Oxygen requirements for biosynthetic processes prevent many yeast species from growing under anaerobic conditions, despite the fact that many are able to ferment sugars to ethanol, and thus conserve free energy, at low oxygen concentrations. The rare ability of *Saccharomyces* yeasts to grow in the complete absence of oxygen has contributed to their status as industrial workhorses, especially in bioethanol production and production of alcoholic beverages. Even so, *Saccharomyces* yeasts still have biosynthetic oxygen requirements, which can lead to problems in anaerobic processes, unless addressed by supplementation of anaerobic growth factors to synthetic media. The research described in this thesis was aimed at investigating and eliminating oxygen requirements for synthesis of unsaturated fatty acids and sterols, which are both important constituents of lipid membranes, in *Saccharomyces cerevisiae*. **Chapter 2** revisited a 70-year old 'dogma' in yeast physiology and shows that, in fact, unsaturated fatty acids are not necessarily an essential growth factor for *S. cerevisiae* cultures. In **Chapter 3** and **Chapter 4**, strategies were investigated to replace sterols by triterpenoids that can be synthesized in an oxygen-independent manner, such as tetrahymanol (**Chapter 3**) and hopanoids (**Chapter 4**). The results of these studies demonstrate that this engineering strategy can indeed circumvent oxygen requirements for sterol synthesis. Finally, technical lessons learned from four years of anaerobic cultivation of yeasts in anaerobic chambers and bioreactor set-ups and studying biosynthetic oxygen requirements in yeasts were summarized in **Chapter 5**.

The demonstration that *S. cerevisiae* CEN.PK113-7D can grow, albeit slowly, under anaerobic conditions in the absence of supplementation or synthesis of unsaturated fatty acids in **Chapter 2** provides an incentive to reinvestigate this ability in other *S. cerevisiae* strains, as well as in other yeast species. A similar absence of unsaturated fatty acids in anaerobic yeast biomass, first reported in a 1971 publication by a Dutch yeast researcher, was demonstrated for the yeast *Schizosaccharomyces japonicus* in **Chapter 4**, highlighting that the findings of **Chapter 2** were not a single occurrence in yeast. While anaerobic growth of *S. cerevisiae* strains was considerably slower when unsaturated fatty acids were omitted from growth media, growth of *Sch. japonicus* was virtually unhindered. Fast and robust anaerobic growth of yeast is essential for their industrial application in anaerobic processes. It is therefore highly interesting to investigate how, in contrast to *S. cerevisiae*, *Sch. japonicus* is able to maintain high growth rates without unsaturated fatty acids in its membrane. Careful analysis of the physiology and membrane architecture of this intriguing yeast species can provide valuable information for the rational design of robust anaerobic cell factories that are not dependent

on supplementation of unsaturated fatty acids. Similar relevant information may be derived from future laboratory evolution experiments, in the absence of an exogenous source of unsaturated fatty acids, of the *S. cerevisiae ole1Δ* strain constructed in **Chapter 2**. In addition, this strain provides a highly useful platform to explore metabolic engineering strategies for enabling oxygen-independent unsaturated fatty acid synthesis by anaerobic yeast cultures. While promising research on this topic was performed during this PhD project, further work is needed before the results can be published.

In **Chapter 3**, expression of an oxygen-independent squalene-tetrahymanol cyclase from *Tetrahymena thermophila* and additional deletion of the squalene epoxidase *ERG1* to abolish native sterol synthesis enabled sustained anaerobic and aerobic sterol-independent growth of *S. cerevisiae*. Multiple examples in literature, especially from brewing processes, describe how lipid metabolism, and sterol homeostasis in particular, can have a strong impact on industrial anaerobic processes. A limited availability of sterols, either caused by depletion of intracellular reserves or by oxygen limitation, can lead to 'stuck fermentations' in which the fermentation rate decreases or the process even completely stops before all fermentable sugars have been consumed. Expression of a heterologous squalene-tetrahymanol cyclase as described in **Chapter 3** (or of a squalene-hopene cyclase, **Chapter 4**) has the potential to resolve such issues by decreasing or eliminating the requirement for sterols. Although sterol-independent growth of yeast is highly promising for anaerobic industrial processes, understanding and resolving the significant reduction in growth rate that was observed for sterol-free, tetrahymanol-producing *S. cerevisiae* strains will be required before the application of this concept can be realized in large-scale industrial processes. Laboratory evolution of sterol-free strains to improve robustness and growth rate may provide valuable leads for further rational design of sterol-free yeast cell factories. The *TtTHC1*-expressing *erg1Δ*-strain constructed in **Chapter 3** provides a useful starting point for such further experiments. Although the introduction of heterologous DNA by genetic modification will most likely limit the use of this approach in the brewing industry, it can also be relevant for anaerobic industrial processes such as bioethanol production. The absence of efficient sterol uptake under anaerobic conditions can be a major contributor to the inability of non-*Saccharomyces* yeast species to grow under anaerobic conditions. The full mechanism through which sterols are taken up from extracellular sources, and in particular the role of cell wall proteins, remains to be elucidated. Sterol uptake is therefore difficult to engineer. Intracellular tetrahymanol synthesis as a bypass for sterol-uptake in such yeasts can be an important step towards enabling anaerobic growth, as was recently demonstrated for the thermotolerant yeast *Kluyveromyces marxianus*[411].

In **Chapter 4**, the unique ability of the yeast *Sch. japonicus* to grow anaerobically in a sterol-independent manner was traced back to its ability to synthesize hopanoids, which

function as sterol-surrogates in many bacteria. A more thorough analysis of membrane architecture and function of *Sch. japonicus* to elucidate its remarkably fast growth in sterol-free media, can provide leads for strategies towards membrane engineering in other yeast species. Additionally, *Sch. japonicus* itself may be an attractive candidate for application in anaerobic industrial processes. While it is already being explored for application in wine making, its remarkable adaptations to anaerobic growth in combination with rapid ethanol production and higher temperature tolerance may make it suitable for bioethanol production as well.

While the pronounced flocculation of *Sch. japonicus* CBS5679 may be relevant for specific industrial applications such as beer-brewing, it complicated the types of experiments that were conducted in this PhD research, for instance by necessitating the use of a mobile darkroom. Eliminating light-induced sexual flocculation in *Sch. japonicus* by deletion of the *WCS1* and *WCS2* genes, which encode for light-responsive white collar complex proteins, has the potential to make experimental design less complicated. Although the isolation and construction of slightly more genetically tractable heterothallic haploid strains has been reported in literature[410], genetic modification of *Sch. japonicus* to date remains laborious in comparison to that of *S. cerevisiae*, for which highly advanced molecular toolboxes are available. The results presented in this thesis provide an incentive to further develop modern genome-editing tools for application in this scientifically intriguing and industrially interesting yeast species.

During the research described in this thesis as well as in other studies that were part of the same ERC-funded ELOXY project, much attention had to be devoted to minimizing oxygen entry into laboratory cultivation systems and, even after four years, completely oxygen-free cultivation in bench-top cultivation systems has not been achieved. Nevertheless, the laboratory set-ups and experimental procedures described in **Chapter 5** of this thesis have successfully been used in studies focused on eliminating biosynthetic oxygen requirements other than those related to membrane synthesis as well. Recently, oxygen-independent synthesis was demonstrated for biotin[473], and for pantothenate and NAD^+ [474] in engineered *S. cerevisiae* strains. The strategies used in these studies can also be harnessed to eliminate remaining oxygen requirements in other yeasts. Future research on remaining, as yet poorly understood or unknown oxygen requirements in yeasts will hopefully benefit from our laboratory set-ups and experimental procedures and lead to further insights and improvements in techniques for anaerobic cultivation of yeast.

The work described in **Chapter 2**, **Chapter 3** and **Chapter 4** of this thesis presents new insights and proof-of-principle concepts for strategies to eliminate oxygen requirements in membrane lipid synthesis in yeasts. However, the reduced growth rates and biomass yields observed for all engineered *S. cerevisiae* strains also emphasize that a deeper understanding of the mechanisms through which membrane composition and function influence overall

physiology is required to optimize membrane engineering strategies that lead to robust cell factories. In laboratory evolution experiments aimed at obtaining unsaturated fatty acid- and sterol-free strains with improved growth characteristics, special attention should be paid to changes in the membrane proteome. Additionally, work described in this thesis was conducted at standard laboratory conditions (30°C, pH 5-6), but it is worth investigating the impact of the altered membrane composition on resistance to various external stresses that microbial production hosts may frequently encounter in industrial processes, such as sub-optimal growth temperatures and pH, or high ethanol concentrations.

Knowledge of the relevance of specific membrane components under different environmental conditions will not only provide a better basis for rational strain design, but it can also lead to new insights into the evolution of eukaryotes and their adaptations to certain natural environments. For example, it could provide context for the evolutionary advantage that was gained by an ancestor of *Sch. japonicus* through the acquisition of a bacterial squalene hopene cyclase gene, presumably from bacteria related to *Acetobacter* species as proposed in **Chapter 4**. Although hopanoid synthesis may have allowed *Sch. japonicus* to adapt to anaerobic conditions, it cannot be excluded that the initial evolutionary pressure was of a different nature. The expression of the squalene hopene cyclase from *Sch. japonicus* in *S. cerevisiae* in **Chapter 4** enabled anaerobic growth in absence of sterol supplementation, but growth was not as pronounced as was previously observed for squalene tetrahymanol expression in **Chapter 3**. This observation poses the intriguing challenge to unravel the mechanisms that underly the differences in the extent to which various polycyclic triterpenoids can replace sterol function in yeast cells. In a similar way, such studies can increase our understanding of the driving forces behind the convergent evolution of bacterial and eukaryotic tetrahymanol synthesis. Furthermore, the presence of alternative triterpenoid cyclases in fungal species can be interesting from a medical perspective as a potential mechanism to escape antifungal drug treatments that target ergosterol synthesis.

Engineered *S. cerevisiae* strains that were constructed in the work presented in this thesis can function as platform strains for future studies on membrane composition, structure and function. Ultimately, the scientific methods described in detail in this thesis will not by themselves be sufficient to come to a full understanding of the inner workings of membranes of living organisms, as insights into specific membrane chemistry and physics will undoubtedly be required. Fathoming the enormous complexity of membrane dynamics and successfully applying such knowledge in the rational design of microbial cell factories will, in my opinion, only be possible through future multidisciplinary research efforts.

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Curriculum vitae

Sanne Jitske Wiersma was born on the 5th of May 1994 in Zoetermeer, The Netherlands. She moved to Leiden when she was 9 years old where, in 2005, she started pre-university education at Stedelijk Gymnasium in Leiden, which she completed in 2011. In the same year, she started the BSc programme in Life Science and Technology, a joint programme of Leiden University and the Delft University of Technology (TU Delft). She spent the fall semester of 2013 at Chalmers University of Technology, Gothenburg, Sweden, as part of a university exchange programme. Upon Sanne's return to the Netherlands, she performed a three-month BSc thesis research project in the group of Dr. Christophe Danelon in the department of Bionanoscience of TU Delft, focused on in vitro synthesis of fluorescently labelled membrane-binding proteins. After obtaining her BSc degree with honours, she immediately continued with the Life Science and Technology MSc programme, with 'Cell Factory' as specialization. Over a two-year period, she chaired a committee of study association LIFE, which organized a well-attended congress with an international list of speakers. Her nine-month MSc project at the Department of Biotechnology of TU Delft, jointly supervised by Dr. Ioannis Papapetridis and Dr. Maarten Verhoeven, focused on forced co-consumption of pentose and hexose sugars by *Saccharomyces cerevisiae*, and the results contributed to a publication. Her MSc studies were completed with a six-month industrial internship at DuPont Industrial Biosciences, during which she developed novel high-throughput molecular biology protocols. After obtaining her MSc degree with honours, and having found a passion for research, she embarked on a PhD project under supervision of Prof. Jack Pronk, in the newly started ERC project ELOXY (eliminating oxygen requirements in yeast). During the subsequent four years, Sanne wrote four papers as first author, which are part of this thesis, and was co-author on one more. Sanne will stay a while longer at TU Delft as a postdoc to explore exciting new topics in yeast metabolic engineering.

List of publications

6. Bouwknegt, J.*, **Wiersma, S.J.***, Ortiz-Merino, R.A., Doornenbal, E.S.R., Buitenhuis, P., Giera, M., Müller, C., Pronk, J.T., A squalene-hopene cyclase in *Schizosaccharomyces japonicus* represents a eukaryotic adaptation to sterol-limited anaerobic environments, *Proceedings of the National Aacademy of Sciences*, **118**(32):e2105225118 (2021).

5. Mooiman, C.*, Bouwknegt, J.*, Dekker, W.J.C.*, **Wiersma, S.J.***, Ortiz-Merino, R.A., de Hulster, E., Pronk, J.T., Critical parameters and procedures for anaerobic cultivation of yeasts in bioreactors and anaerobic chambers, *FEMS Yeast Research*, **21**:foab035 (2021).

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3. **Wiersma, S.J.**, Mooiman, C., Giera, M., Pronk, J.T., Squalene-tetrahymanol cyclase expression enables sterol-independent growth of *Saccharomyces cerevisiae*, *Applied and Environmental Microbiology*, **86**:e00672-20 (2020).

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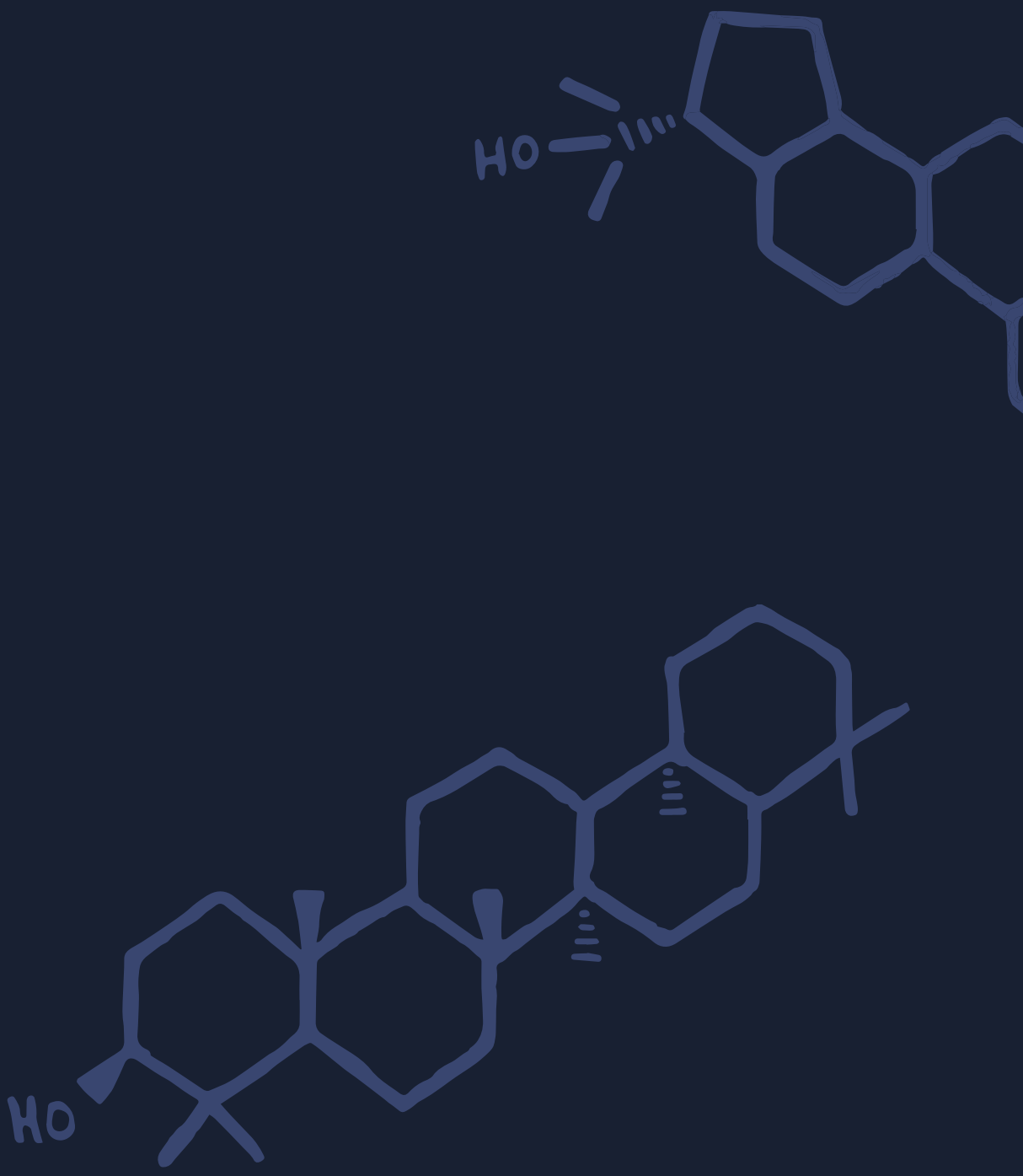
1. Papapetridis, I.*, Verhoeven, M.D.*, **Wiersma, S.J.**, Goudriaan, M., van Maris, A.J.A., Pronk, J.T., Laboratory evolution for forced glucose-xylose co-consumption enables identification of mutations that improve mixed-sugar fermentation by xylose-fermenting *Saccharomyces cerevisiae*, *FEMS Yeast Research*, **18**:foy056 (2018).

**These authors contributed equally to this publication*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