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Anaerobiosis revisited: growth of *Saccharomyces cerevisiae* under extremely low oxygen availability

Bruno Labate Vale da Costa[1,2,*], Thiago Olitta Basso[2,*], Vijayendran Raghavendran[1,3], Andreas Karoly Gombert[1#]

[1]University of Campinas, School of Food Engineering, Rua Monteiro Lobato 80, 13083-862, Campinas-SP, Brazil

[2]University of Sao Paulo, Department of Chemical Engineering, Av. Prof. Luciano Gualberto 380, 05508-010, São Paulo-SP, Brazil

[3]Chalmers University of Technology, Department of Biology and Biological Engineering, Kemivägen 10, Göteborg, SE-412 96, Sweden

*both authors contributed equally to this work

#Corresponding author:

Andreas Karoly Gombert, University of Campinas, School of Food Engineering, Rua Monteiro Lobato 80, 13083-862 Campinas-SP, Brazil, Phone: +55 19 35214031; e-mail: gombert@unicamp.br

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Abstract

The budding yeast *Saccharomyces cerevisiae* plays an important role in biotechnological applications, ranging from fuel ethanol to recombinant protein production. It is also a model organism for studies on cell physiology and genetic regulation. Its ability to grow under anaerobic conditions is of interest in many industrial applications. Unlike industrial bioreactors with their low surface area relative to volume, ensuring a complete anaerobic atmosphere during microbial cultivations in the laboratory is rather difficult. Tiny amounts of O_2 that enter the system can vastly influence product yields and microbial physiology. A common procedure in the laboratory is to sparge the culture vessel with ultrapure N_2 gas; together with the use of butyl rubber stoppers and norprene tubing, O_2 diffusion into the system can be strongly minimised. With insights from some studies conducted in our laboratory, we explore the question 'how anaerobic is anaerobic?'. We briefly discuss the role of O_2 in non-respiratory pathways in *S. cerevisiae* and provide a systematic survey of the attempts made thus far to cultivate yeast under anaerobic conditions. We conclude that very few data exist on the physiology of *S. cerevisiae* under anaerobiosis in the absence of the anaerobic growth factors ergosterol and unsaturated fatty acids. Anaerobicity should be treated as a relative condition since complete anaerobiosis is hardly achievable in the laboratory. Ideally, researchers should provide all the details of their anaerobic setup, to ensure reproducibility of results among different laboratories.

Introduction

During evolution two significant O_2 peaks occurred, at 500 Mya and 2000 Mya ago, which resulted in two distinct growth spurts (Payne et al. 2009). Understandably O_2 is thus the most abundant element on earth (in earth's crust and in air) and is constantly renewed by the photosynthetic action of green plants and algae. Bigger life forms emerged and flourished, owing to the vast amount of energy that was generated in the mitochondria, using O_2 as the terminal electron acceptor in the oxidative phosphorylation via chemiosmotic coupling (Lane 2002; Lane and Martin 2010; David and Alm 2011). To extract the maximum free energy from the energy sources that were consumed, living beings undertook diverse adaptations in O_2 poor environments such as the deep sea (Tyack et al. 2006), high altitudes (Scott 2011; Huerta-Sanchez et al. 2014), or in deep glacial ice crystals (Rohde and Price 2007).

Although unquestionably O_2 has contributed to complexity, microbes from the pre-oxygenation event (ex. obligate anaerobes) are of much industrial relevance for biotechnological processes (Köpke et al. 2014; Hatti-Kaul and Mattiasson 2016). Anaerobic bacteria and archaea are used in the production of thermostable enzymes (amylases, cellulases, lipase, pectinases, proteases, and xylanases) for use as industrial biocatalysts, for the synthesis of chiral compounds for the pharmaceutical industry (Bragger et al. 1989; Littlechild 2015), or for the production of bulk chemicals (acetone-butanol-ethanol, medium-chain carboxylic acids) (Visioli et al. 2014; Jeon et al. 2016).

Anaerobic processes are likely to be more economical than their aerobic counterparts because of the reduced costs in aeration and mixing (de Becze and Liebmann 1944; Curran et al. 1989). Industrial fermenters often have very low surface area compared to their volume[1] resulting in reduced heat and mass transfer rates (Simpson and Sastry 2013). In a typical first-generation sugarcane ethanol plant, as much as 23,000 m^3 of CO_2 is generated in a reactor of 500 m^3. Such large volume of CO_2 not only displaces the dissolved O_2 out of the liquid phase but also ensures a completely anoxic atmosphere.

In contrast to industrial practice, complete anaerobiosis is extremely difficult to establish in the laboratory (where surface area to volume ratios, and thus mass transfer, are high), and it is quite a challenge to exclude O_2

[1] A cylindrical reactor with height (h) and radius (r) has a lateral surface area of $2\pi rh$ and a volume of $\pi r^2 h$. The surface area to volume is inversely proportional to the radius of the reactor; thus, the larger the reactor, smaller the surface area to volume. This has profound consequences for heat and mass transfer. Heat transfer is proportional to the surface area, while the metabolic heat generation is proportional to the culture volume. Thus, at very large volumes (& large radius), the available heat transfer area is insufficient to dissipate the heat that is generated. Unlike laboratory reactors which are well mixed, there will be concentration gradients in large scale reactors affecting the mass transfer of O_2, as well as other nutrients, vastly affecting the cellular physiology.

completely from the cultivation systems. While performing scale-down studies of anaerobic industrial bioprocesses, it is imperative to grow the microorganism under conditions that closely resemble the real industrial conditions, as the presence of even trace amounts of O_2 could affect the overall performance of the microbial cell factories.

In this mini-review, we revisit the much-studied anaerobic fermentation in yeast focusing on the cultivation systems and the role of O_2 in non-respiratory pathways. We illustrate the challenges in mimicking an anaerobic atmosphere in the laboratory and the ways to minimise the leakage of O_2 into the system. We provide a chronological list of all the attempts thus far made to create an anaerobic set-up, as well as the physiology of *Saccharomyces cerevisiae* under anaerobic conditions with emphasis on lipid composition. And we conclude with some perspectives on future research and the need to exercise caution when one declares a set-up as anaerobic.

Discovery of anaerobic life

The discovery of the so-called "anaerobic bacteria" dates back to 1680 when Antonie van Leeuwenhoek observed '*a kind of living animalcules*' in a small heat-sealed glass vial which was previously filled with crushed pepper powder and clear/clean rainwater (Gest 2004). In that condition, the environment inside the glass vessel became anaerobic owing to the depletion of O_2 by the aerobic microorganisms. When the sealed glass vial was opened, an overpressure forced the liquid out (Gest 2004). The pressure inside the vial was due to the formation of CO_2 via fermentation. Leeuwenhoek's experiment was repeated by Martinus Beijerinck in 1913, who identified the predominant microorganism as *Clostridium butyricum*. This species, as most *Clostridium* species, is classified as an obligate anaerobe – absence of growth in the presence of O_2, due to their inability to deactivate the reactive oxygen species (ROS) such as peroxides (O'Brien and Morris 1971). However, obligate anaerobes do tolerate micro-oxic conditions when grown in liquid medium (Kato et al. 1997; Kawasaki et al. 2005; Imlay 2008). Aerobes, on the contrast, produce catalase and superoxide dismutase, two key enzymes that detoxify peroxides and other ROS that cause oxidative damage to DNA, lipid molecules, and proteins (Storz et al. 1990). Anaerobes produce these enzymes to a certain extent, but they possibly have other ways to reduce the oxidative damage. Thus, excluding O_2, either partially or totally, is a necessary requirement for those investigating obligate anaerobes.

O_2 in numbers

Under normal atmospheric conditions, O_2 constitutes 20.948 mole percent in air, which makes the partial pressure[2] of O_2 as 0.20948 atm or 20.67 kPa. Using Henry's law (constant = 756.7 atm L mol^{-1}), the concentration of O_2 in water is ~ 280 µM at 25 °C. Fig. 1 shows some physiological responses to varying levels of O_2 saturation in water. The solubility of O_2 in water increases with decreasing temperature, reaching as high as 400 µM at 0 °C (Weiss 1970; Denny 1993) (Fig. 2).

Permeability of tubing material

The choice of the tubing material used in the cultivation system influences the amount of O_2 that diffuses into the system. Silicone tubing that is regularly used in aerobic cultivations has a permeability coefficient of 800 Barrer[3]. For this reason, anaerobic cultivations are often carried out with norprene tubing which has a 40-fold lower permeability than silicone tubing[4]. Giacobbe (1990) observed that the diffusion of O_2 through a seven meter long coiled teflon tubing was inversely related to the flow rate of N_2 used, as shown in Fig. 3. Visser and co-workers (Visser et al. 1990) reported that the dissolved O_2 concentration had an asymptotic relationship with respect to the N_2 flow rate employed, as depicted in Fig. 4, even after 35 h of continuous flushing. A comparison of the permeability coefficients of various tubing materials is given in Fig. 5; cost is a major factor while performing anaerobic experiments, as the price of N_2 gas increases with increasing purity, as shown in Table 1. The amount of O_2 (present as impurity) entering the system is shown in Table 2.

Anaerobic cultivation systems

Owing to the omnipresence of O_2, ensuring a complete anaerobic atmosphere during microbial cultivation in bioreactors is rather difficult. There are three ways to decrease the O_2 tension during cultivation: firstly, biological reduction using symbionts; secondly, physical reduction by boiling, evacuation or the use of inert

[2] $Partial\ pressure\ of\ O_2 = Total\ pressure * mole\ fraction$ of O_2 *in the gas*

[3] Barrer is a non-SI unit for gas permeability. 1 Barrer = $10^{-10} * \frac{cm^3.\ cm}{cm^2 * s * cmHg}$

[4] For a tubing of 30 cm length, having an internal diameter of 0.31 cm, the diffusion rate of O_2 can be calculated using this relation, for a partial pressure of O_2 of 15.6 cm Hg and molar volume of 22,400 cm^3: $O_2\ diffusion\ rate\ \left(\frac{\mu mol}{h}\right) = \frac{surface\ area\ cm^2 * Permeability * P_{O_2}\ cm\ Hg}{thickness\ cm}$

The rate of O_2 diffusing through a norprene tubing is 1.5 µmol h^{-1}, while it is 59 µmol h^{-1} with a silicone tubing.

gases; thirdly, chemical reduction, such as the catalytic ignition of hydrogen and residual O_2 or using chemicals such as cysteine. However, cysteine is toxic to *S. cerevisiae* as it interferes with its metabolism (Maw 1961). Once the O_2 tension is reduced, it must be maintained low (or absent) during the cultivation by sealing off the medium. For further details, the reader is urged to consult the comprehensive review by Hall (1929). As the solubility of O_2 is lower at high temperatures, boiling is often the quickest way to dispel the O_2 trapped in the liquid. However, this is cumbersome and suitable for complex medium only. Experiments done in a synthetic medium pose additional difficulty, as the addition of filter- sterilized heat labile components inevitably introduce some O_2 into the system. To indicate the O_2 present in the cultivation medium, it is common to use a redox-sensitive dye such as resazurin (Twigg 1945) or methylene blue (Brewer et al. 1966) which turns colourless in the absence of O_2; Wimpenny and Necklen (1971) have used redox potential to measure the O_2 tension in a chemostat cultivation with *Escherichia coli* and *Klebsiella aerogenes* and a redox potential of <0 mV was considered as anaerobic.

Although the development of a reliable and effective system for the cultivation of anaerobes dates to the 1940s, it was only in 1969 that Robert Hungate published what would be the definitive cultivation system for anaerobic organisms. It consisted of a test tube with a thin layer of agar medium uniformly distributed over its internal surface and flushed with an O_2-free gas prior to inoculation (Hungate 1969). Hungate's system was then improved by other groups (Bryant 1972; Balch and Wolfe 1976) and it is still commonly used in laboratory practice for the cultivation of strict anaerobes (Börner 2016). Cultivation systems designed for an O_2-free environment where anaerobes can be handled appropriately has improved thereafter. A commercially available example (since 1969) is the widely used “anaerobic chamber”, with which all laboratory routine and manipulation can be performed in a confined anoxic environment. In this system, O_2 is removed from the chamber by injecting gases such as N_2, H_2, and CO_2 (Thomas et al. 1998) or N_2, Ar, or CO_2. (Plugge 2005; Speers et al. 2009) and any trace of O_2 present is reduced to water on a palladium catalyst. Thus, anaerobic chambers are the system of choice for static-anaerobic cultivations and for O_2-free manipulation in the laboratory. The various systems developed for anaerobic setup in the laboratory are summarized in Table 3.

A common procedure for the anaerobic cultivation of yeast in laboratory bioreactors (in which detailed physiological studies can be carried out) is to constantly flush the culture medium with ultrapure N_2 gas (containing less than 5 ppm O_2), together with the use of Viton O-rings and norprene tubing. In this way, O_2 diffusion into the system is minimised (Boender et al. 2009; de Kok et al. 2011).

The role of O_2 in the metabolism of *S. cerevisiae*

The yeast *Saccharomyces cerevisiae* is classified as a facultative anaerobe (Visser et al. 1990), preferentially-fermenting microorganism (Pronk et al. 1996; Barnett 2003), and is widely used in various biotechnological processes, from fuel ethanol (Della-Bianca et al. 2013) to recombinant protein production (Ceccarelli and Rosano 2014), due to its culture simplicity, rapid growth, safe status and the possibility of achieving a high cell concentration culture (Yin et al. 2007). Some of these applications demand a respiratory metabolism that can be a problem due to the low solubility of O_2 in the culture media (Hanotu et al. 2016). Increasing the stirring speed and the gas flow rate can increase the O_2 transfer but does not prevent O_2 limitation (Garcia-Ochoa et al. 2010), with a concomitant increase in the production costs. The ability of *S. cerevisiae* to grow anaerobically, at similar rates when compared to aerobic growth (~0.3 h^{-1} and ~0.4 h^{-1} respectively) (Verduyn et al. 1992), is of particular interest for applications in which fermentative metabolism is demanded. Moreover, *S. cerevisiae* plays an important role as a model organism for studies on microbial physiology and genetic regulation under various environmental conditions, such as anaerobiosis (Jouhten and Penttilä 2014).

Respiration is an energetically efficient process (in terms of ATP generation) in which O_2 participates as the final electron acceptor. Electrons are transferred from the energy source, via reduced coenzymes such as NADH, to O_2 in the mitochondrial electron transfer chain, generating a proton-motive force that enables ATP synthesis by the enzyme ATP synthase (Jouhten and Penttilä 2014). In the absence of O_2, free energy (in the form of ATP) is exclusively generated from substrate-level phosphorylation, via fermentation. The number of ATP moles generated for each mole of substrate consumed in fermentation is considerably lower than the corresponding yield observed under respiratory metabolism. In response to this lower yield, and to fulfil the cell's energetic requirements, a 7.5 times higher glycolytic flux (in terms of glucose uptake rate) (Jouhten et al. 2008), and consequently a higher ATP production rate through glycolysis, are observed in fermentative metabolism, when compared to respiratory metabolism. In addition, to maintain the redox balance under anaerobic growth, NAD^+ is regenerated via the formation of glycerol.

Bisschops and co-workers (2015) report that anaerobic stationary phase cultures of *S. cerevisiae* had a shortened chronological lifespan and low robustness (assessed through viability and temperature tolerance, and the adenylate energy charge) compared to aerobic stationary phase cultures. This has implications for cell recycling in industry. Merico and others (2007) conducted an exhaustive work on fermentative lifestyle over 40 yeasts belonging to the *Saccharomyces* complex to reflect 150 million years of evolutionary history. Most of the yeasts

exhibited good fermentation ability but only those lineages (*Saccharomyces, Kazachstania, Naumovia, Nakaseomyces* and *Tetrapisispora*) that underwent whole genome duplication could grow in the absence of O_2.

Molecular O_2 is involved in 48 reactions and accounts for 3% of the total reactions collected in the genome-scale model iTO977, proposed by Österlund and co-workers (2013), in the yeast *Saccharomyces cerevisiae*. Two molecules, namely ergosterol and oleic acid, which are essential to provide functional properties of the cell membrane, require O_2 for their biosyntheses, and they must be imported from the extracellular medium to the cell, under full anaerobiosis. Therefore, growth under anaerobic conditions requires the exogenous supply of these two molecules (Andreasen and Stier 1953; Andreasen and Stier 1954). However, there are other non-respiratory pathways in *S. cerevisiae*, that also require molecular oxygen, like biosynthesis of haem, hemoproteins and several other oxidases (Rosenfeld and Beauvoit 2003).

O_2 and Membrane Lipids

Under anaerobic conditions, *S. cerevisiae* grows poorly in culture media containing only water-soluble ingredients. However, the addition of non-saponifiable lipids from edible oils, such as wheat germ, into the culture medium stimulated growth of the yeast (Stier et al. 1950b), and this initial observation prompted the search for anaerobic growth requirements.

Andreasen and Stier (1953) used an anaerobic setup (detailed in Table 3) and a synthetic medium to test the effect of several non-lipid compounds (such as nucleic acids, purines, pyrimidines, amines, simple peptides, vitamin B12, and casein hydrolysates) on the growth of *S. cerevisiae* SC-1 strain under fully anaerobic conditions. Because these compounds did not increase the yield of cells under such conditions, they chose to add ergosterol into the medium, as it was known that ergosterol is an important constituent of yeast biomass. Tween 80, on the other hand, was used only as a surfactant to facilitate solubilisation of ergosterol in the synthetic medium. The effects of Tween 80 alone, and of Tween 80 plus ergosterol additions were assessed, and as a conclusion, ergosterol was found to be an essential anaerobic growth factor for *S. cerevisiae*. It was found later, that the Tween 80 used to solubilise ergosterol, was also supplying another essential requirement for anaerobic growth, oleic acid (Andreasen and Stier 1954). Tween 20 and Tween 40 (sources of saturated fatty acids) were then tested, as well as a non-lipid surfactant to solubilize ergosterol, to clarify whether another lipid ester would promote the same results. As a conclusion, oleic acid and ergosterol, simultaneously, were found to be growth factors for *S. cerevisiae* anaerobic growth. Meanwhile, several groups investigated the effect of trace amounts of

O_2 on the anaerobic growth of *S. cerevisiae* in a chemically-defined medium, without exogenous addition of lipids. Jollow, Wallace and co-workers (Jollow et al. 1968; Wallace et al. 1968) cultivated a diploid strain of *S. cerevisiae* under several different growth conditions, to assess the occurrence of mitochondrial profiles and other cell membrane systems. Their anaerobic setup consisted of a closed flask fitted with a rubber stopper sealed with molten parafilm wax with an airlock. The medium was flushed (after inoculation, for 45 min) with N_2 that has been bubbled through an acidified solution of vanadyl sulphate reduced with amalgamated zinc to provide an O_2-free gas (Meites and Meltes 1948). Even though the yeast extract based medium contains lipids such as ergosterol and UFAs, remarkable differences were observed between cells cultivated in aerobic YPD and anaerobic YPD cultivation (with and without Tween 80 and ergosterol). Although total lipid content varied between the conditions tested, the most pronounced changes were in total fatty acid, sterol fractions, glycerides and phospholipid contents. In general, total lipid, ergosterol, glycerides, and phospholipid contents of cells grown anaerobically were lower, when compared to cells cultivated in aerobiosis. On the other hand, anaerobiosis triggered squalene accumulation, with a reduction in ergosterol levels. Aerobically-grown yeast presented a predominance of mono-UFAs (C16:1 and C18:1). On the other hand, cells cultivated under anaerobiosis without Tween 80 and ergosterol addition presented predominantly saturated fatty acids, comprising: C10:0, C12:0 and C16:0. When ergosterol and Tween 80 were added to the YEG medium, C16:1 and C18:1 species accounted for approximately 70 % of fatty acids, still less than what was synthesized by aerobically grown cells (85%). Thus, it can be observed that the choice of the cultivation medium and conditions are very crucial in understanding microbial physiology.

To expand the range of UFAs that could be incorporated into yeast, Alterthum and Rose (1973) used growth media with different fatty acid compositions (oleic, linoleic, or γ-linolenic acid as a source of UFA) to anaerobically cultivate *S. cerevisiae* NCYC 366. In all the conditions, cells grew to the same extent and did not differ in their content of total lipids or total phospholipids. However, cells were enriched by approximately 60% with the specific fatty acid that was supplemented. Sterol composition changed according to the availability of O_2. These results reveal the flexibility of *S. cerevisiae* to incorporate even polyunsaturated fatty acids such as Omega-6 as building blocks for structural and bulk storage lipids. Anaerobically grown *S. cerevisiae* cells accumulated squalene, which does not require O_2 for its synthesis, which is in accordance with Jollow et al. (1968). The ratio between squalene/ergosterol as a function of O_2 availability, although intriguing, was not correlated to any environmental (dis)advantage.

Watson and Rose (1980) followed the studies conducted by Alterthum and Rose (1973) on the extent to which exogenous FAs are incorporated into lipids in the *S. cerevisiae* NCYC 366 strain. The composition of

aerobically/anaerobically grown *S. cerevisiae* in defined media containing ergosterol and different fatty acid (oleic, linoleic and α-linolenic) was investigated. Fatty-acyl composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and triacylglycerols from anaerobically grown cells showed predominance of C18:1, C18:2, and C18:3 species when the medium was supplemented with oleic (C18:1), linoleic (C18:2), and α-linoleic (C18:3) acids, respectively, followed by C16:0 under all the conditions tested. Jollow et al. (1968) also observed the predominance of C18:1 fatty acid when Tween 80 was added to the anaerobic medium formulation, but rather than C16:0, significant amounts of C16:1 were detected. In aerobically grown cells, C16:0 plus C16:1 accounted for the major fraction of the fatty-acyl composition of PC, PE, PI, PS, and triacylglycerols.

Rosenfeld and Beauvoit (2003) reviewed and compiled data of total FA (saturated, unsaturated, and phospholipid fractions) and sterol (squalene and ergosterol fractions), highlighting the accumulation of squalene over ergosterol in cells cultivated in anaerobic conditions. Klose et al. (2012) examined the lipidome at the level of lipid classes, of aerobically grown *S. cerevisiae* cultivated in complex media with different carbon sources, different temperatures, and growth phases (early and middle logarithmic and early stationary phases) at 95% lipidome coverage. In their study, the flexibility of the yeast's lipidome was confirmed, in face of the variability of triacylglycerol content, and the ratio of unsaturated/saturated glycerophospholipids. On the other hand, a low variability of sphingolipid and ergosterol abundances was observed between the conditions tested. Lindberg et al. (2013) cultivated *S. cerevisiae* CEN.PK 113-7D and *Zygosaccharomyces bailli* under aerobiosis to investigate the high tolerance of *Z. bailli* to acetic acid. Upon exposure of *Z. bailli* cells to acetic acid, they observed an increase of monounsaturated fatty acids, as well as a higher level of complex sphingolipids. This led to a membrane with a lower fluidity that prevented the entry of un-dissociated acetic acid, thereby conferring higher acetic acid tolerance when compared to *S. cerevisiae*.

In wine fermentations, it is common practice to add O_2 in the stationary phase to rescue sluggish fermentations (Valero et al. 2001; Fornairon-Bonnefond et al. 2002). The squalene contents of the cells decreased upon O_2 addition but the ergosterol content did not increase proportionately relative to the total sterol levels. In air saturated beer wort fermentation, the sterol content increased rapidly from 1 mg g_{DCM}^{-1} to 10 mg g_{DCM}^{-1} and then declined rapidly at the end of the fermentation, when O_2 was depleted (Aries and Kirsop 1977).

Most of the investigations mentioned above studied the macromolecular physiology by measuring the lipid composition using a liquid chromatography system and the focus was more on the anaerobic physiology from a process engineering point of view. However, Waldbauer and co-workers (2011) were interested in identifying

the molecular fossil record of archaea and resorted to the microaerobic sterol biosynthesis. They used ^{13}C labelling studies to investigate the role of very low O_2 concentrations (1 nM to 1 µM) on the enzymatic biosynthesis of sterols. Three different O_2 concentrations were tested (6.5 µM, 0.6 µM, and 7 nM), including one anaerobic condition (< 0.7 nM). They used unlabeled ergosterol and ^{13}C glucose and followed the incorporation of carbon label from glucose to steroids using ^{13}C-NMR. Steroid biosynthesis occurred at each of the three dissolved O_2 concentrations tested in their experiments: 6.5 µM, 0.6 µM, and 7 nM, as ^{13}C label was observed in squalene—the last steroid biosynthetic intermediate that can be produced in the absence of O_2, as well as in lanosterol (the first O_2 requiring step in steroid synthesis), demonstrating *de novo* steroid production. However, only under anaerobic conditions (< 0.7 nM), lanosterol was not detected, and only unlabeled ergosterol that was taken up by the cell exogenously could be detected. Thus, yeast can still grow and multiply at such 'anaerobic conditions'.

How anaerobic are our anaerobic laboratory cultivations?

Studies on nutritional requirements of *S. cerevisiae* under anaerobic conditions were initiated in the 1950s, when Andreasen and Stier determined the amount of ergosterol and fatty acid contents necessary in a defined medium to provide for adequate anaerobic growth (Andreasen and Stier 1953; Andreasen and Stier 1954). Their research was based on the evidence that *S. cerevisiae* grown in a complex medium (yeast extract), under anaerobic conditions, were described as "small" (in terms of cell concentration in the culture medium), while under aerobic conditions (in the presence of O_2), the growth was referred to as being "excellent" (Brockmann and Stier 1947). In their studies, N_2 gas with a purity of 99.99 % was employed, and in some cases, the N_2 gas was sparged through a solution of Chromium(II) chloride to further decrease the O_2 concentration to *ca.* 1 ppm. It was concluded that for anaerobic growth of *S. cerevisiae* in a chemically defined medium, ergosterol and a source of UFA (such as Tween 80) must be added to the culture medium. They also concluded that, for aerobic conditions, the addition of both Tween 80 and ergosterol had a negative effect on growth. Their recommendation as regards to the necessity of adding ergosterol and UFAs for anaerobic growth conditions was then corroborated by several authors during the two subsequent decades (Kováč et al. 1967; Paltauf and Schatz 1969; Alterthum and Rose 1973; David and Kirsop 2013).

However, contradicting Andreasen and Stier's recommendations, Macy and Miller (1983), using the Hungate protocol for anaerobic cultivation, did observe *S. cerevisiae* growth after a 12 h lag phase in a defined medium lacking both ergosterol and fatty acids. The authors also emphasized that previous investigations stated as having

been conducted under anaerobic conditions were not carried out under the complete absence of O_2. This observation raised the question: "How anaerobic are our laboratory anaerobic cultivations?", since O_2 is necessary for UFAs and sterols biosyntheses.

The observation made by Macy and Miller prompted Verduyn and co-workers (1990) to investigate whether *S. cerevisiae* was able to grow, and if so, to what extent, in a defined medium under anaerobic conditions. Although they did observe growth in the absence of Tween 80 in anaerobic chemostat cultivations at a dilution rate of 0.1 h^{-1}, the lack of oleic acid supplementation led to a decrease in biomass yield (from 2.6 g_{DCM} L^{-1} with ~420 mg L^{-1} of Tween 80 to 1.8 g_{DCM} L^{-1} without Tween 80 addition) and high levels of residual glucose, suggesting that another substrate other than glucose was acting as the growth-limiting nutrient under this condition. Although special measures to prevent O_2 diffusion into their system were undertaken, the authors claimed that O_2 diffusion was still in the range of 31 µmol h^{-1} and that this flux would be enough for the biosynthesis of all UFAs required for the cell biomass.[5] The growth observed in the absence of added fatty acids did not prove that O_2 was not necessary for the biosynthesis of fatty acids, sterols, and for other biochemical pathways since O_2 could diffuse into the bioreactor and supply the cell requirements. Currently, the chemically defined medium used routinely for anaerobic continuous cultivations with *S. cerevisiae*, containing ergosterol and Tween 80 (10 mg L^{-1} and 420 mg L^{-1} respectively), is the one established by Verduyn et al. (1990).

Thomas et al. (1998), using shake-flasks constantly sparged with pre-purified nitrogen gas (< 5 ppm O_2), reported that when Tween 80 and ergosterol were added to a minimal medium, *S. cerevisiae* NCYC 1324 grew without a lag phase period. However, when the pre-purified nitrogen gas was passed through heated copper turnings (400 °C), and traces of oxygen were removed, the same strain did not present growth within 72 h. After this period, when air was admitted to the flasks, growth was detected within the next 48 h. This observation was in accordance with the oxygen diffusion hypothesis raised by Verduyn et al. (1990). However, the *S. cerevisiae* ATCC 26602 strain, one of the 10 strains tested by Thomas and co-workers (1998), presented growth after a 96 h lag-phase in this setup with Tween 80 and ergosterol supplementation, which was highlighted but not explained by the authors. The growth-promoting effect of purine and pyrimidine bases, as well as amino acids, was also highlighted by the authors, when added to the same defined medium, without Tween 80 and ergosterol,

[5] Oleate requires 1 mol of O_2 and ergosterol requires 12 mol of O_2 to be synthesized. For an ergosterol and an oleate content of 0.2% and 3.5% per dry cell mass, the amount of O_2 needed for their biosynthesis is 185 µmol g_{DCM}^{-1}, assuming the consumed O_2 is utilised only for these two reactions. For a biomass yield of 0.1 g $g_{glucose}^{-1}$, a dilution rate of 0.1 h^{-1}, and a glucose concentration in the feeding medium of 10 g L^{-1}, steady state biomass would be 1 g_{DCM} L^{-1}. Thus, the O_2 demand is $185 \left(\frac{\mu mol\ O_2\ needed}{g_{DCM}\ produced}\right) * 0.10\ \frac{\left(\frac{g_{DCM}\ produced}{g_{DCM}\ present}\right)}{h} * 1\ g_{DCM} = 18.5\ \frac{\mu mol\ O_2}{h}$

which enabled all strains to grow under anaerobiosis. They have ruled out growth due to mitochondrial protein synthesis by adding chloramphenicol to the medium, and hypothesized the generation of molecular or other active oxygen species produced by the decomposition of amino acids and/or nucleic acid bases.

The first *S. cerevisiae* consensus genome-scale metabolic model, created by Herrgård et al. (2008), subsequently reviewed and refined (Dobson et al. 2010; Heavner et al. 2012) was updated by Jouhten et al. (2012) in oxidative phosphorylation and other O_2-dependent and anabolic reactions. This most recent version can simulate the dynamic behaviour of *S. cerevisiae* during the shift from an aerobic glucose-limited steady-state chemostat culture to a fully anaerobic cultivation. However, this model was not able to simulate growth in the absence of O_2, even when the necessary lipid components were present in the culture medium. This is because the model considers the use of molecular O_2 for different reactions other than lipid biosynthesis. On the other hand, in previous chemostat experiments performed by our group (unpublished results), a steady state biomass concentration of 0.3 g_{DCM} L^{-1} (using a glucose concentration of 10 g L^{-1} in the feed as the sole carbon and energy source) was observed, after switching from a fully aerobic steady-state to a fully anaerobic steady-state. In our experimental setup, pure grade N_2 gas (< 3 ppm of O_2) coupled to an O_2 trap (OT3-2, Agilent, USA) was used to deliver an inlet N_2 gas with less than 15 ppb of O_2 (which corresponds to a dissolved O_2 concentration of 20 pM). Also, the feeding medium was not supplemented with Tween 80 or ergosterol, which creates a doubt about the minimal O_2 requirement of *S. cerevisiae* CEN.PK 113-7D strain under anaerobiosis, as previously observed by Verduyn et al. (1990) with another strain (*S. cerevisiae* CBS 8066). Despite the use of norprene tubing, Viton o-rings and high purity nitrogen gas, it is known that O_2 enters the bioreactor, either by diffusion from the surrounding environment or as an impurity in the N_2 gas. Visser *et al.* (1990) calculated the O_2 diffusion into the reactor with all ports closed and report a value of 2 nmol h^{-1} of O_2. However, diffusion is specific for each system. Rodrigues et al. (2001) investigated the O_2 requirements of the food spoilage yeast *Z. bailii* in synthetic and complex media under strictly controlled anaerobic conditions. They observed a linear growth in a synthetic medium as the influx rate of O_2 into the reactor was constant at 0.3 µmol h^{-1}. This value is insignificant when compared to the O_2 that is coming as an impurity at a rate of 6 µmol h^{-1} (considering 5 ppm O_2 in the nitrogen gas stream). However, in a complex medium exponential growth was observed as growth was not limited due to O_2, suggesting that components in YPD contributed the biosynthetic requirements under anaerobic conditions. For our system, the oxygen leakage calculated, with tubings closed, without any N_2 sparging was of 0.04 µmol h^{-1}, an order of magnitude lower than Rodrigues and co-workers.

Although it is reported that *S. cerevisiae* can grow in the absence of O_2 when sterols and fatty acids are supplemented in the culture medium, not all yeast have the same ability. Some strict aerobes, such as

Kluyveromyces lactis, are commonly used as a negative control to ensure that the experimental setup is indeed anaerobic. *K. lactis* lacks a few genes that are present in *S. cerevisiae* (Snoek and Steensma 2006), including genes related to sterol uptake, rendering *K. lactis* cells incapable of growing without O_2, although there are reports that *K. lactis* can grow under very low O_2 tensions (Kiers et al. 1998; Merico et al. 2009). In previous chemostat experiments performed by our group with *K. lactis* CBS 2359 using the defined medium proposed by Kiers et al. (1998), complete wash-out of the cells was observed after switching from an aerobic to an anaerobic steady-state. It is important to mention that, in this experiment, a low-purity N_2 gas (< 50 ppm O_2) was used to sparge the feeding medium and the bioreactor, instead of ultra-pure N_2, as used in the above-mentioned *S. cerevisiae* experiment. This raises questions about the minimum O_2 requirements of different microbial species. Benchmarking a cultivation setup with one strain could not be a suitable way to test whether another strain can or not grow under full anaerobiosis. Also, it makes us think about how close to full anaerobiosis it is possible to get in a laboratory environment. Care should be taken when reading the methodology section of published articles, in which it is described that *S. cerevisiae* was grown under fully anaerobic conditions and that ergosterol and a source of UFAs were added to the medium, in order to sustain growth. It might well be that a fully anaerobic condition was in fact not achieved by the authors. Finally, it is important to remember that establishing fully anaerobic conditions in the laboratory is also important to enable scale-down studies of large-scale anaerobic industrial processes. In the large-scale, contrary to the small-scale, transport phenomena (including O_2 transfer) are hindered by the small surface area to volume ratios, and a fully anaerobic condition is much easier to be achieved.

Genetics and regulation of ergosterol and UFA biosynthesis in *S. cerevisiae*

In this section, we review the molecular aspects and the major physiological roles of the non-respiratory pathways that utilize O_2 in *S. cerevisiae*. In facultative anaerobic yeasts, O_2 is metabolized to fulfil both catabolic and anabolic functions. Molecular O_2 that is not reduced by mitochondrial respiration is used by numerous anabolic pathways, including lipid, amino acid, vitamin, iron, haem and ubiquinone metabolism. The corresponding reactions depend on flavoproteins, hemoproteins and other metalloproteins. However, the respective contribution of these pathways in the overall O_2 consumption is still poorly documented, owing to the very low O_2 quantities involved (Rosenfeld and Beauvoit 2003). Among all the non-respiratory O_2 utilization pathways required for growth, only a few of them may significantly contribute to the O_2 consumption capacity retained by *S. cerevisiae* cells during fermentative metabolism. They are briefly presented in the next paragraphs.

Haem Biosynthesis

Haem is a prosthetic group of hemoproteins and its biosynthesis requires molecular oxygen (O_2). The two O_2 dependent steps involve the cytosolic coproporphyrinogen III oxidase (encoded by the *HEM13* gene) and protoporphyrinogen oxidase (encoded by the *HEM14* gene). Hemoproteins of *S. cerevisiae* include cytochromes involved in respiration (ex. Cytochrome c *CYC1*, *CYC7*), lipid synthesis (e.g. Acyl-CoA desaturase *OLE1*), iron uptake and oxidative stress (e.g. Catalase A *CTA1*). Mitochondrial respiratory cytochromes are the major hemoproteins of wild-type yeast cells grown in the presence of O_2. The synthesis of respiratory cytochromes is optimal under aerobic conditions and becomes limited at O_2 concentrations below 2 to 4 μM (Burke et al. 1997), which is attained when using industrial-grade nitrogen gas (Table 2) during regular chemostat cultivations. This suggests that the decrease in cytochrome synthesis (rather than O_2 availability) can limit growth under O_2-limited conditions. The absence of functional cytochrome aa3 in anaerobic cells is now widely admitted. Dagsgaard et al. (2001) detected almost all the cytochrome c oxidase subunits, except the subunits IV and VIII, which were sufficient to explain the absence of assembled and active cytochrome c oxidase in anaerobic cells. Both microsomal cytochrome b5 (previously named 'b1') and P450 cytochromes are major hemoproteins of anaerobic cells. Only a few genes encoding hemoproteins (*ERG11*, *CYC7*, *OLE1*, and *SCS7*) were found to be upregulated under anaerobic batch culture conditions in galactose medium (Kwast et al. 2002). In contrast, in anaerobic glucose-limited chemostat cultures, upregulation was not observed for *CYC7*, *ERG11* and *OLE1* genes (Linde et al. 1999). Haem biosynthesis in the complete absence of molecular oxygen might occur after oxygen is generated through decomposition of amino acids or nucleic acid bases, as hypothesized by Thomas et al (1998).

Sterol biosynthesis

Sterol biosynthesis represents one of the two major O_2-dependent structural pathways in *S. cerevisiae* (Fig. 6), the other one being UFA biosynthesis (Salmon et al. 1998; Daum et al. 1998; Valachovic et al. 2001). In spite of that, many aspects of its regulation are still unknown, in part, because of the presence of various *ERG* genes. It is known, however, that most of the *ERG* genes are regulated by O_2 and endogenous or exogenous sterols, as well as by the presence of other lipids (Rosenfeld and Beauvoit 2003). The initial steps of the sterol pathway (from acetyl-CoA to squalene) do not require molecular O_2 (Daum et al. 1998). Moreover, the last step of this linear pathway is mediated by the NADPH-dependent condensation of two farnesyl-pyrophosphates into squalene

encoded by *ERG9*. This gene is positively regulated by fatty acids and is negatively regulated by O_2, haem and sterols. Nevertheless, Erg9p activity remains quite high in cells grown anaerobically with ergosterol and oleate in excess. The final steps of the sterol pathway (from squalene to ergosterol), on the other hand, do require molecular O_2 in six enzymatic steps catalyzed by five enzymes: a non-P450 mono-oxygenase (Erg1p), two di-ferric and non-heminic hydroxylases/desaturases (Erg25p and Erg3p) and two cytochromes P450 (Erg11p and Erg5p) (Fig. 6). Although it has been reported that the *ERG2* gene, responsible for the conversion of fecosterol to episterol, is activated under anaerobic batch conditions (Soustre et al. 2000), well-controlled yeast cultivations, such as in anaerobic glucose-limited chemostat cultures, revealed that none of the *ERG* genes were found to be upregulated (at least higher than three-fold) when compared to analogous aerobic cultures (Linde et al. 1999).

UFA biosynthesis

Lipid composition of cytoplasmic membranes is tightly regulated to maintain membrane fluidity, and a key enzyme involved in this process is the membrane-bound acyl-CoA desaturase, encoded by *OLE1*. This enzyme catalyzes the introduction of the initial double bond into palmitoyl-CoA and stearoyl-CoA, resulting in mono-UFA, such as palmitoleate (C16:1) and oleate (C18:1), respectively. Such steps require the presence of molecular O_2 (Nakagawa et al. 2001). Each unsaturation in a UFA molecule requires one NAD(P)H, as an electron donor, and a molecule of O_2 as an acceptor of two electron pairs, one from NAD(P)H and the other from the respective saturated fatty acyl molecule, which is supplied from acetyl-CoA synthesis (Rosenfeld and Beauvoit 2003).

In terms of genetic regulation, *S. cerevisiae* derepresses the expression of *OLE1* under hypoxic conditions to allow more efficient use of limited O_2. *OLE1* transcription is also activated in the presence of saturated fatty acids, and strongly repressed by the presence of UFAs in the growth medium (Rosenfeld and Beauvoit 2003). Although expression of this gene is repressed by dissolved O_2, it is derepressed by this molecule at very low concentrations, in the absence of UFAs (Nakagawa et al. 2001).

Final remarks

Despite the considerable volume of data available so far on the anaerobic growth of *S. cerevisiae*, and of the development of new techniques for lipidomic profiling (Navas-Iglesias et al. 2009), there are still no published lipidomic data obtained from anaerobic chemostat experiments using media without the anaerobic growth factors ergosterol and UFA, and a comparison of these data with those obtained from aerobic cultivations.

It is still not known how O_2 is sensed by *S. cerevisiae*, although differential gene expression has been observed at nano-molar concentrations and traces of O_2 may affect the physiology of yeast dramatically. It is vital to grow the inoculum under anaerobic conditions, in order to eliminate the memory of the preceding aerobic cultivations, while investigating anaerobic physiology.

Most of the studies reported in the literature that define a setup as anaerobic employ butyl rubber stoppers, norprene tubing plus N_2 flushing, with the addition of oleate and ergosterol to the medium. Thus, it is not possible to know whether and how *S. cerevisiae* would grow in these systems, in case the anaerobic growth factors were not supplied. Considering what is discussed in this review, we would like to caution that anaerobicity is a relative, rather than an absolute, term. In this regard, the procedure for cultivating microorganisms anaerobically should be standardized and described in greater detail, to ensure reproducibility across various laboratories and to allow for the proper scale-down and investigation of large-scale anaerobic bioprocesses.

Compliance with Ethical Standards

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Fig. 1 Reported physiological responses to varying O_2 saturation levels (Denny 1993; Waldbauer et al. 2011; Aceituno et al. 2012).

Fig. 2 The dissolved O_2 concentration decreases with increasing temperature, reaching as low as 202 µM at 40 °C (Weiss 1970).

Fig. 3 Oxygen permeation from ambient air through a 7.62 m Teflon-PFA tubing that is flushed with N_2. Open circles refers to a N_2 pressure of 4.4 atm; black triangles refers to a N_2 pressure of 7.8 atm (Giacobbe 1990).

Fig. 4 Effect of N_2 flow rate on the dissolved O_2 in the culture medium. Values are taken from (Visser et al. 1990).

Fig. 5 O_2 permeability coefficients of commonly employed tubing material in Barrer. Silicone has permeability coefficient of 800 Barrer (Masterflex®; Saint-Gobain).

Fig. 6 Lipid biosynthesis in yeast. Figure adapted from (Rosenfeld and Beauvoit 2003).

Table 1

Table 1 The purity of N_2 and its cost.

Product	Volume of the commercially sold cylinder (m^3)	O_2 as impurity (ppm)	Price (Euros)
Nitrogen	10	<50	ca. € 50
Nitrogen 4.6	9	<5	€ 108
Nitrogen 5.2	10	<3	€ 192
Nitrogen 6.0	9*	<0.5	€ 302
Oxitrap*	-	<0.015	€ 120
Gas mix (85% N_2, 10% CO_2, & 5% H_2) for anaerobic chamber	8ᵉ	<5Ω	€ 480

A typical chemostat (in triplicate) at a dilution rate of 0.1 h^{-1} carried out for at least 50 h (five residence times) sparged with 0.5 L of N_2 gas per L of medium per min, would consume 4.5 m^3 of N_2 gas. A batch experiment carried out for 20 h under the same condition would consume 1.8 m^3 of N_2 gas. Prices converted from Brazilian Reais to Euros.

ᵉGas flow in an anaerobic chamber is up to 25 L min^{-1}; H_2 gas greater than 5% poses a severe risk of explosion.

*One Oxitrap (Sigma Aldrich) will be able to capture 5 mL of O_2, equivalent to One N_2 cylinder with 50 ppm O_2 as impurity.

Ω Concentration of O_2 inside the chamber.

Table 2

O_2 in a N_2/O_2 mixture ppm	O_2 entering the reactor[ð] µmol. h^{-1}	O_2 transfer[¤] rate µmol. h^{-1}
210,000 (air)	253,377	99,907
50	60	23.8
10	12	4.8
1	1.2	0.5
0.1	0.12	0.05

Table 2 The amount of O_2 entering the reactor when sparged with 0.5 L per min of N_2 gas of varying purity.

[ð] Calculated using the formula $\frac{f*P*V}{R*T*t}$; where *f* is the fraction of O_2 in the incoming gas, V is the flow rate of the gas in L. min^{-1} at 30 ºC at 1 atm; R is the universal gas constant

[¤]O_2 dissolving into the liquid is calculated using this formula $OTR = k_{la} * C^* * V_l$, assuming a k_La of 0.1 s^{-1}, C^* is the saturation concentration of O_2 at the bubble interface; this can be calculated from Henry's law $p = K_H * c$ where p is the partial pressure of O_2, K_H is Henry's constant, and c is the concentration in water.

Table 3

Table 3: Anaerobic setups reported in the literature.

Anaerobic conditions employed	**Microbial strain (if applicable)**	**Reference**
Excellent review on the various ways to achieve anaerobic conditions	Obligate anaerobic bacteria	(Hall 1929)
A stream of N_2 gas was passed through a solution containing acidified vanadyl sulphate solution and amalgamated Zinc	-	(Meites and Meltes 1948)
Use of 99.99% pure N_2 with 0.01% O_2. All glass with mercury traps and three-way cock for running a chemostat cultivation	*A distillery type yeast; a strain S. cerevisiae* SC-1 (DCL), obtained from Joseph E Seagram and Sons, Inc., Louisville, Kentucky	(Stier et al. 1950a)
Use of 99.99% pure N_2 passed through a solution of chromium chloride to decrease O_2 to ca. 1 ppm	*S. cerevisiae* SC-1 (DCL)	(Andreasen and Stier 1953; Andreasen and Stier 1954)
Closed flask fitted with a rubber stopper sealed with molten parafilm wax with an airlock and medium flushed (for 45 minutes, after inoculation with O_2-free N_2 stream (Meites and Meites 1948)	A locally isolated diploid strain of *Saccharomyces cerevisiae*	(Jollow et al. 1968; Wallace et al. 1968)
Agar medium distributed as a thin layer over the internal surface of test tubes maintained in an anaerobic atmosphere using CO_2 gas	Bacteria	(Hungate 1969)
Use of copper oven to reduce the last traces of O_2	Bacteria	(Gordon and Dubos 1970)
Coy anaerobic chamber (Patent no: US 61000830)	-	(Coy 1969)
2 L round flat-bottomed Pyrex flask with a latex-rubber port on the side and fitted with a water lock allowing CO_2 gas to exit the system. The growth medium was autoclaved and whilst it was warm, flushed with a stream of N_2 passed through an O_2 trap (Nilox scrubber)	*S. cerevisiae* NCYC 366	(Alterthum and Rose 1973)
N_2 flushing during medium preparation; serum bottle closed with a butyl rubber stopper with a crimped metal seal	Bacteria	(Miller and Wolin 1974)
Anaerobic shake flask equipped for continuous gassing and measurement of culture density	Bacteria	(Daniels and Zeikus 1975)
Pressure vessel containing a gas mixture of 80% hydrogen and 20% carbon dioxide at 2 to 3 atm	Bacteria	(Balch and Wolfe 1976)
Bespoke design of serum vial with *in situ* cell measurement, and gassing port; Traces of oxygen are removed from N_2 of 99.995% purity with the help of an Oxisorb cartridge	*S. cerevisiae D 273-10B*	(Bieglmayer and Ruis 1977)
Use of Resazurin, cysteine, boiling, CO_2 flush. Oxygen was removed from the CO_2 by passing it through a vertical Pyrex column packed with copper metal turnings heated electrically to approximately 350 ^oC	The Montrachet strain of wine yeast, UCD enology No. 522	(Macy and Miller 1983)
N_2 gas containing less than 5 ppm O_2 obtained by passing N_2 gas containing less than 100 ppm O_2, through a column filled with copper turnings and heated to 350 ^oC in a bioreactor	*S. cerevisiae* CBS 8066	(Schulze et al. 1996)
N_2 gas (<0.5 ppm) is passed through an Oxyclear O_2 absorber to reduce its residual oxygen level to below 50 ppb in a bioreactor	*Saccharomyces cerevisiae* JM43	(Burke et al. 1998)
Anaerobic chamber, with palladium pellets as a catalyst, charged with a gaseous atmosphere consisting of hydrogen, carbon dioxide, and nitrogen in the ratio	*S. cerevisiae* NCYC 1324 along with several brewing and fuel ethanol industrial	(Thomas et al. 1998)

of 10 :10 : 80. Use of heated (400 °C) copper turnings to reduce residual oxygen levels from pre-purified N_2 gas (< 5 ppm O_2)	strains.	
Use of ultrapure N_2 followed by the O_2 trap. <15 ppb was achieved by passing the N_2 gas through an HPIOT3-2 oxygen trap (Agilent)	*S. cerevisiae* EC1118	(Aceituno et al. 2012)

Figure 1

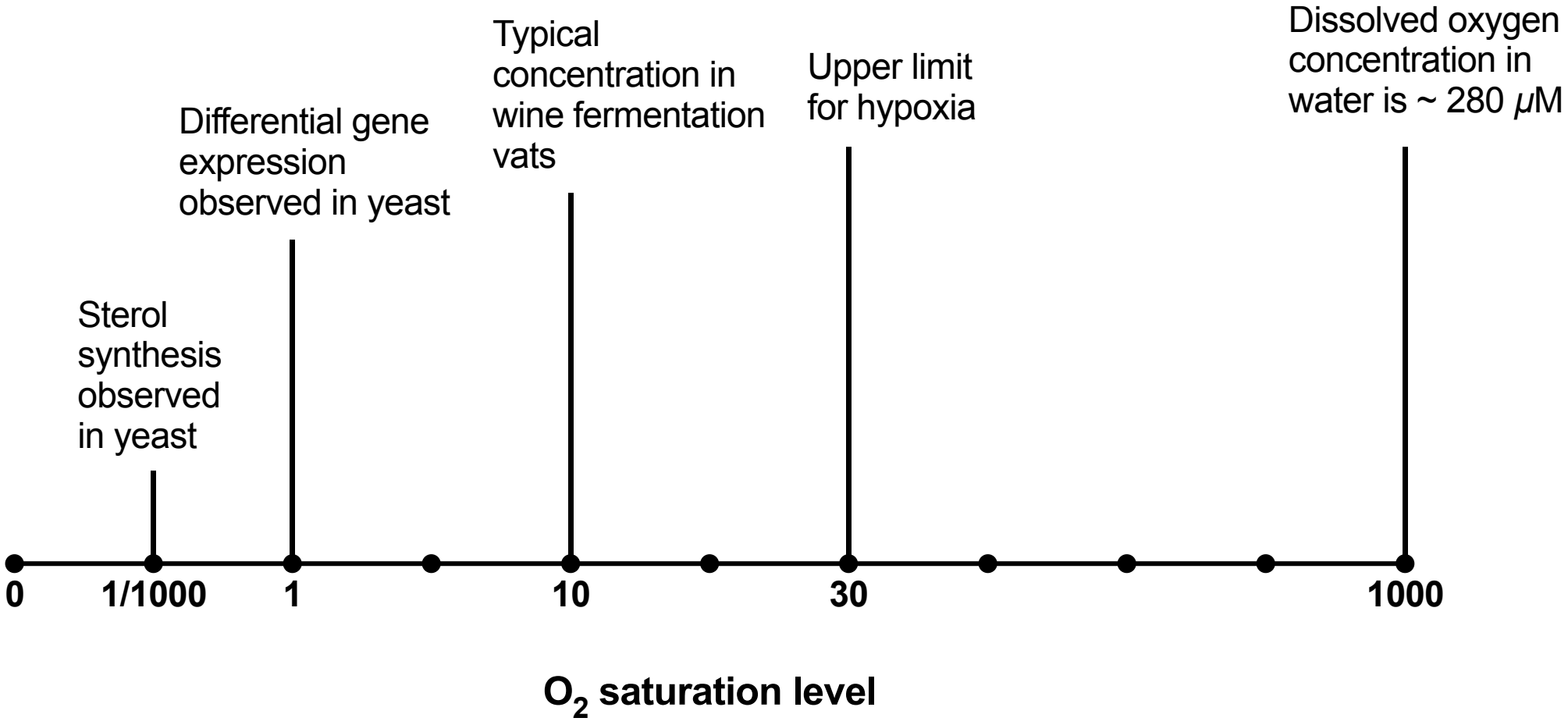

Dissolved oxygen concentration in water is ~ 280 μM
Upper limit for hypoxia
Typical concentration in wine fermentation vats
Differential gene expression observed in yeast
Sterol synthesis observed in yeast
0
1/1000
1
10
30
1000
O2 saturation level

Figure 2

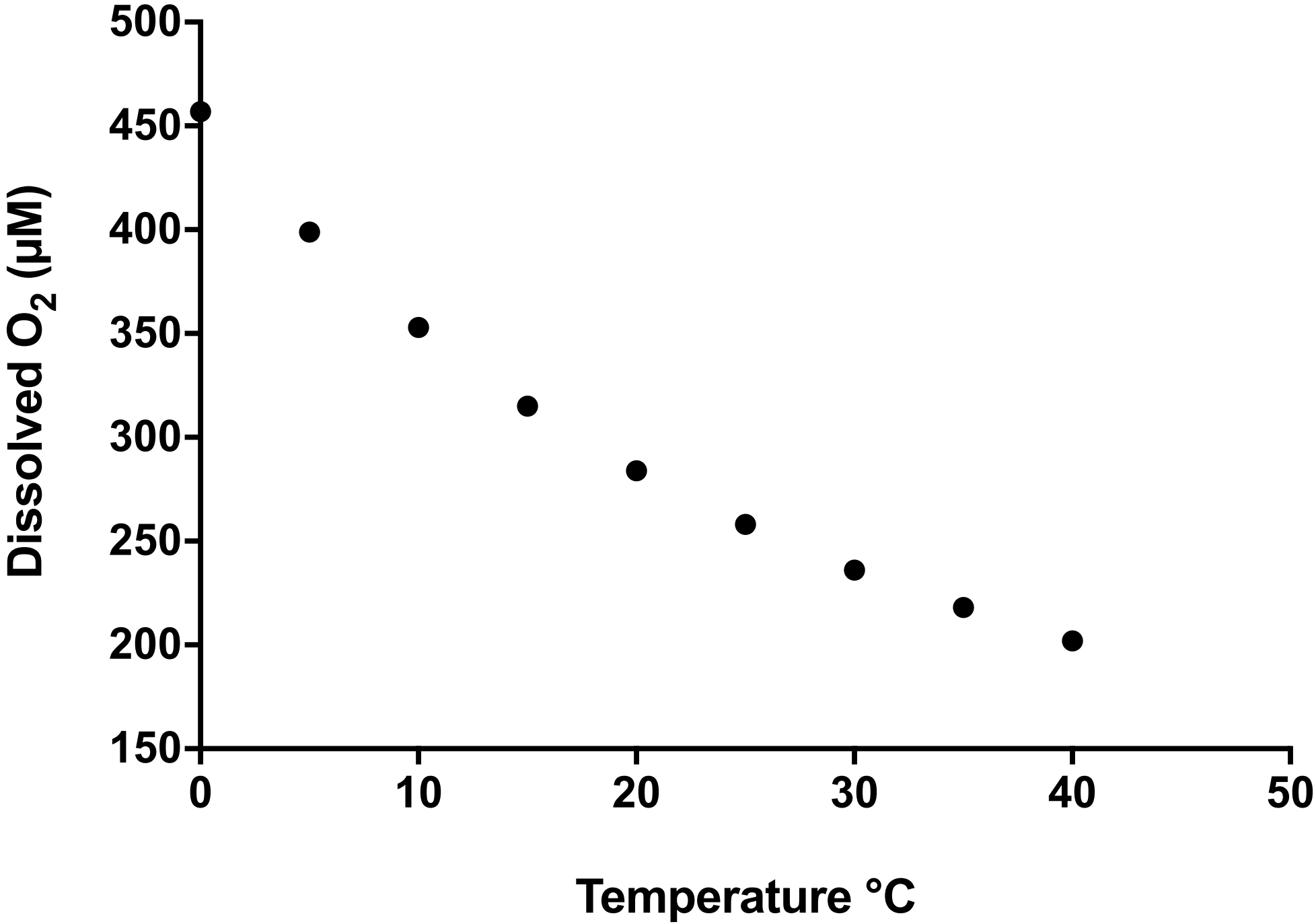

500
450
400
350
300
250
200
150
Dissolved O_2 (µM)
0
10
20
30
40
50
Temperature °C

Figure 3

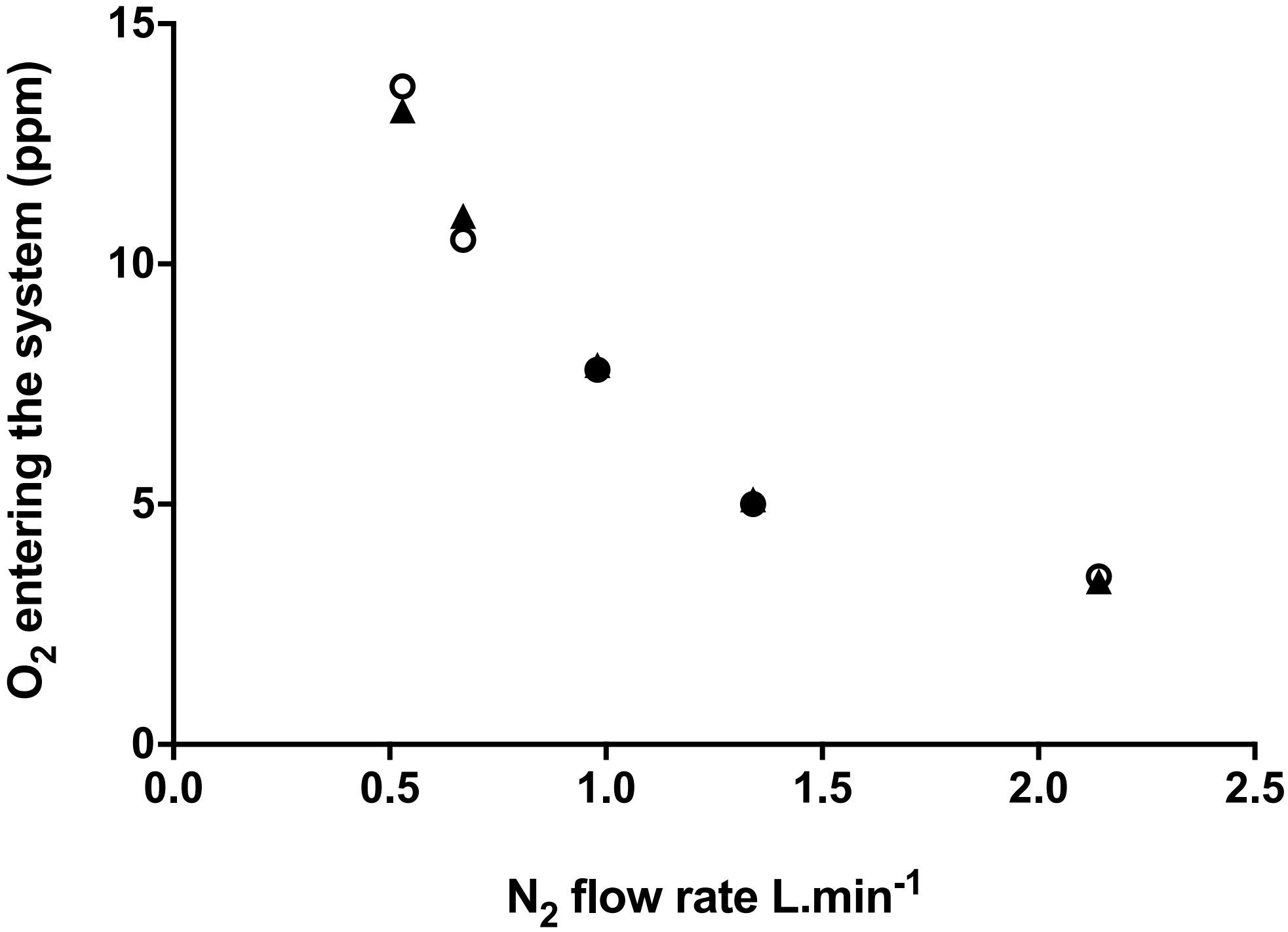

15
10
5
0
0.0
0.5
1.0
1.5
2.0
2.5
O2 entering the system (ppm)
N2 flow rate L.min-1

Figure 4

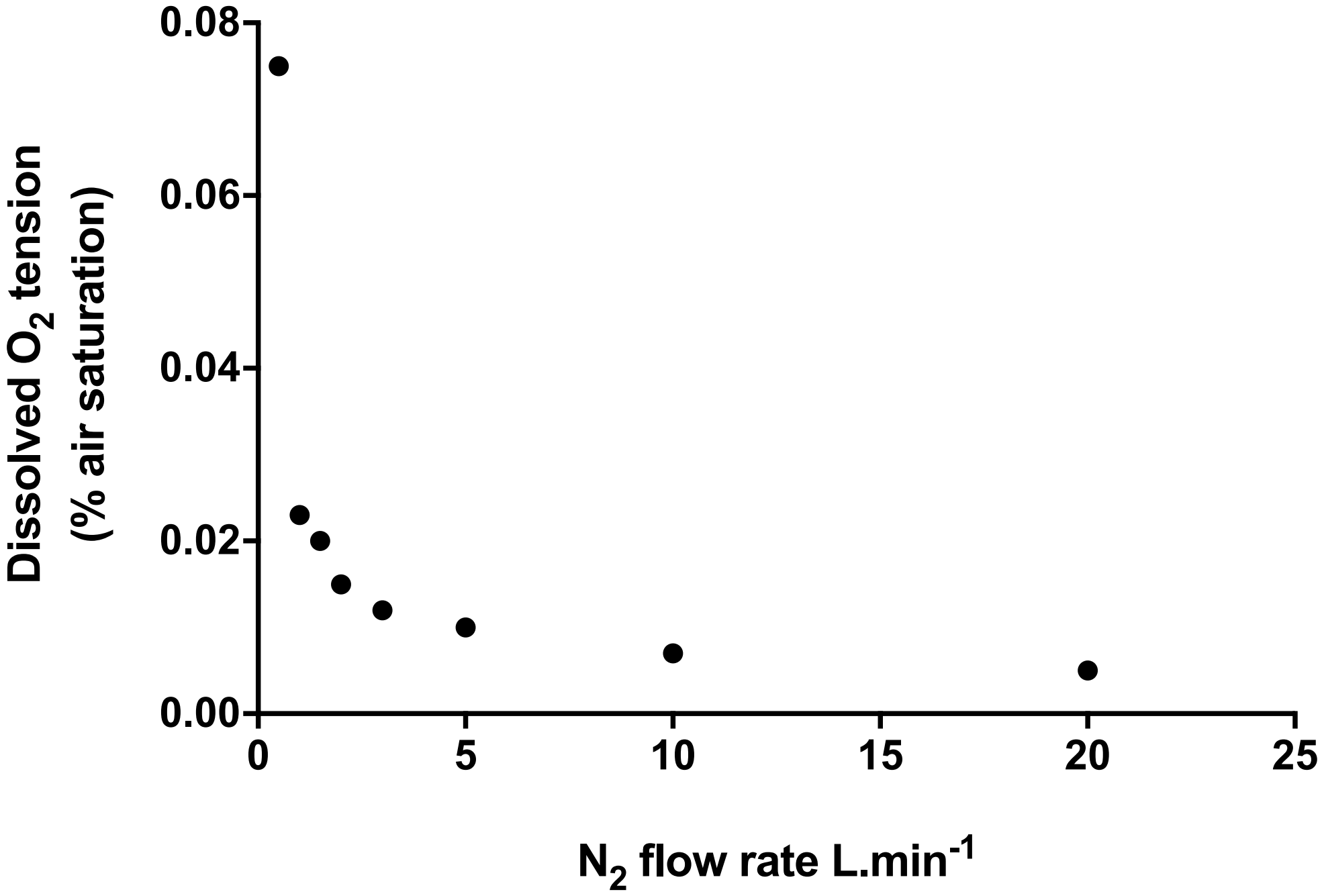

Dissolved O_2 tension
(% air saturation)
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
25
N_2 flow rate L.min^{-1}

Figure 5

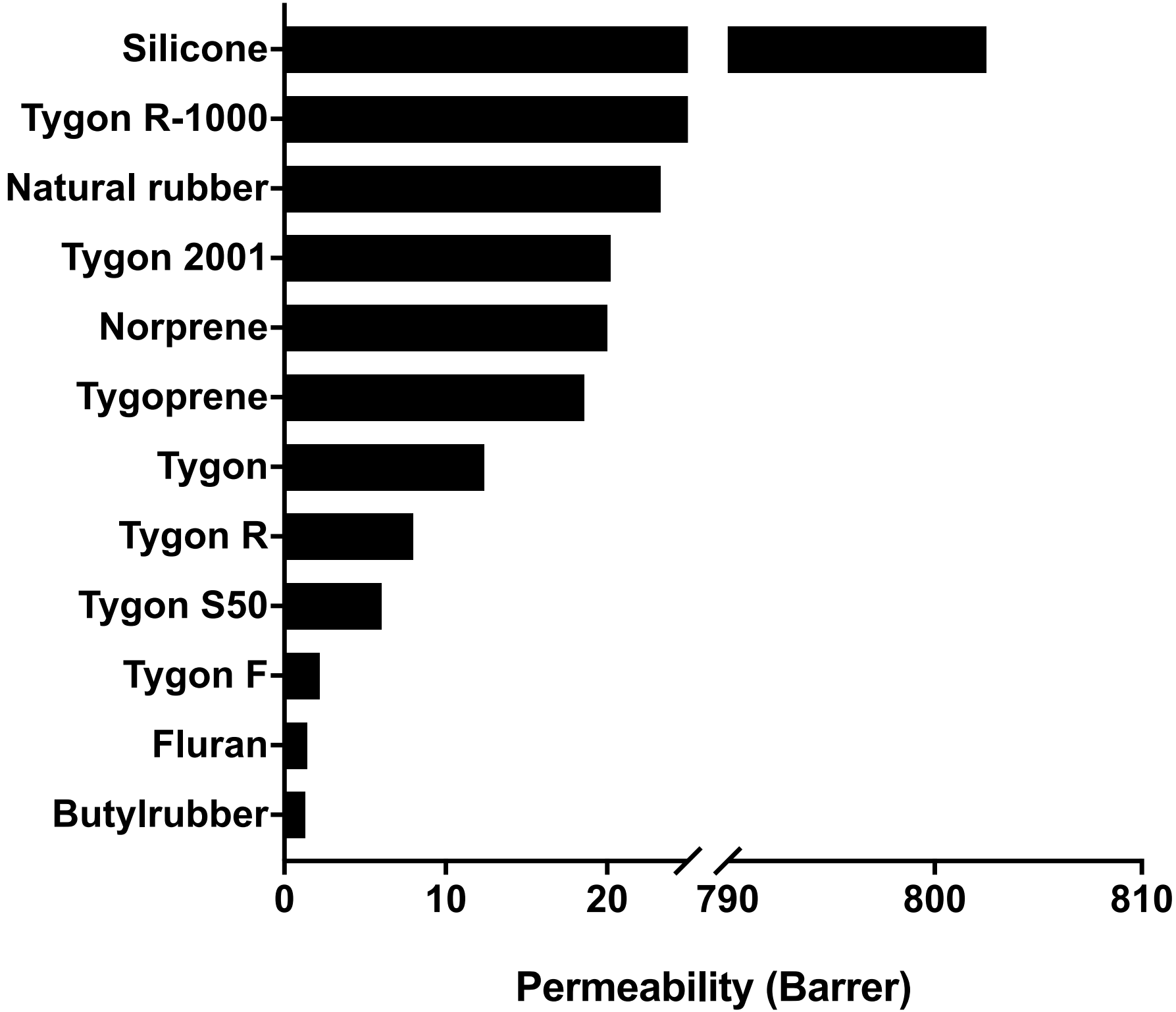
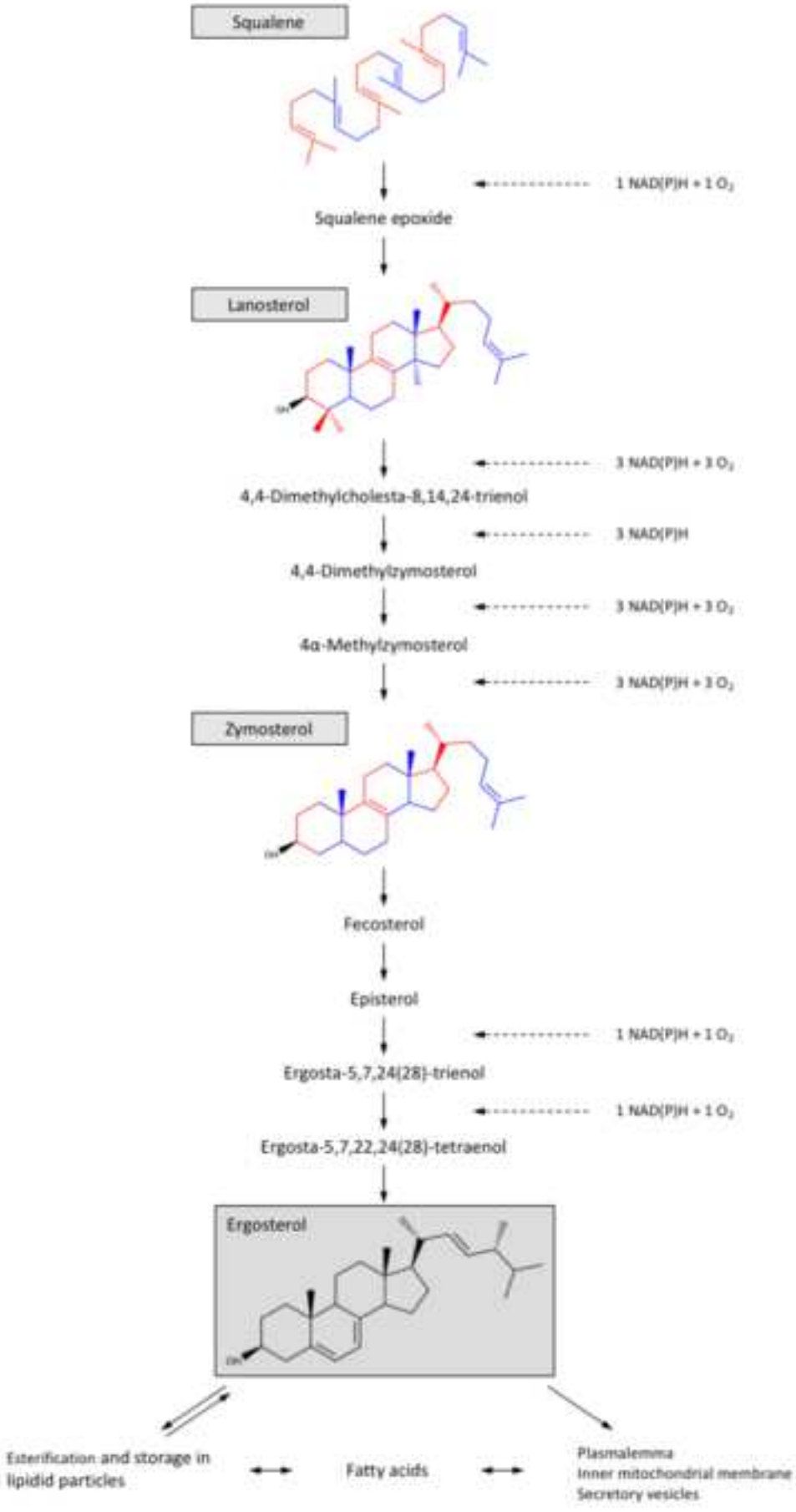

Silicone
Tygon R-1000
Natural rubber
Tygon 2001
Norprene
Tygoprene
Tygon
Tygon R
Tygon S50
Tygon F
Fluran
Butylrubber
0
10
20
790
800
810
Permeability (Barrer)

Figure 6

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