Supplementary material

Elucidating the Importance of Numeracy Skills for Undergraduate Students in Life Sciences Using the Oxygen Requirement in Yeast as an Example

1 Contents

1. Motivation

Undergraduate students possess some research experience in the laboratory and this provides a rich opportunity to engage in quantitative reasoning. However, teachers require richer examples in microbial physiology to make the dream of teaching quantitative biology come to fruition. In this regard, a context based mathematical learning is motivating and vital; in asking how many glucose molecules enter a yeast cell, calculations involving nutrient uptake rates presents itself naturally and students might be able to inquire on the various factors that affect nutrient uptake. This 'tips and tools' article is an attempt to provide an example from yeast physiology to bring a quantitative understanding to the anaerobic fermentation process. Examples such as this one could be used in the classroom to foster a stronger and a motivating mathematical basis for students taking undergraduate studies in life sciences.

On the one hand, many laboratory anaerobic setups are not truly anaerobic and some O_2 diffuses into the cultivation system. In this situation, fermentation is not the sole free-energy conserving mechanism used by the cells for maintaining their viability and growth. On the other hand, even when no respiration takes place and fermentation is the sole free-energy conserving mechanism active in the cells, the syntheses of ergosterol and oleic acid – both compounds necessary for *Saccharomyces cerevisiae* to build its cell membrane and to grow – require and consume a certain amount of O_2. For this reason, such compounds are frequently added to the cultivation medium under anaerobic conditions. Misinterpretations about yeast metabolism might have important consequences for medium design and for scaling up bioprocesses. Figure *1* shows the mechanism of transfer of O_2 from gaseous to liquid phase. The array of questions is depicted as a decision flowchart in Figure *2*; students might also ask questions such as '*where do I start, what can I do, and what can I gain by doing so*?' (Polya 1956).

Based on these questions, we envisage a set of learning outcomes (and list some thought problems to consolidate the students' learning, see section ***Error! Reference source not found.***) that can aid the student and the educator to answer if yeast can indeed grow without the exogenous supply of the two growth factors – ergosterol and oleic acid. To facilitate the exercise for students in Life Sciences (Fawcett and Higginson 2012), equations are relegated to the supplementary material and only the key numbers are shown in the main article. The educator and the learner could ask one question at a time, engage in a group discussion, consult the supplementary material from time to time, before arriving at the final solution. The questions can be discussed in the presence of the teacher or a teaching assistant. As George Polya (Polya 1956) writes, "*The*

teacher should put himself in the student's place, he should see the student's case, he should try to understand what is going on in the student's mind, and ask a question or indicate a step that could have occurred to the student himself." We hope that the questions that are posed invite curiosity among the learners. As the concepts used to perform the calculations mostly uses high school physics and chemistry with some basics of microbial physiology, prior exposure to a microbial metabolism course might be beneficial.

2. Step by step calculations for determining the oxygen requirement in yeasts

2.1 O_2 concentration in air

Oxygen is the second most abundant element in earth's atmosphere and accounts for nearly half of the mass of earth's crust in the form of compounds. The O_2 pool in the atmosphere and the superficial waters in the Earth is constantly renewed by the photosynthetic action of bacteria, plants, and algae. At sea level, the relative molar abundance of O_2 in the atmosphere is 20.95 mole percent, and from Dalton's law, the partial pressure of O_2, p_{O2} is 20.67 kPa (thus, the partial pressure will be lower at high altitudes as the ambient pressure would be lower). Concentration (c) relates to the number of molecules and the volume and for a gas, it can be obtained from the ideal gas equation. Strictly speaking, the similarity between O_2 and an ideal gas is only valid at low pressures and high temperatures, but for the purposes of our calculation, we would assume that the gaseous O_2 obeys the ideal gas law under the conditions employed. So:

$$c = \frac{n}{V} = \frac{p_{O_2}}{R * T} \qquad 1$$

where n is the number of moles, V is the volume, p is the partial pressure, R is the universal gas constant and T is the absolute temperature (in Kelvin). Using the universal gas constant (8.314 $J \cdot mol^{-1} \cdot K^{-1}$) and the following conversion factors 1 Pa = 1 $N \cdot m^{-2}$, 1 J = 1 N m, 1 L = 0.001 m^3, we obtain the concentration of O_2 in air at 25°C:

$$Conc.\ of\ O_2\ in\ air\ = \frac{20.67\ kPa}{8.314\left(\frac{J}{mol * K}\right) * 298\ K} = 8{,}343\ \mu M \qquad 2$$

2.2 O_2 solubility in water

Henry's law relates the partial pressure of a gas in the gaseous phase to its solubility in the liquid phase through the proportionality constant K_H, which changes with temperature and composition of the liquid:

$$p = K_H * c \qquad 3$$

Considering that K_H [4] for O_2 in water = 756.7 atm·L·mol^{-1}:

$$Conc.\,of\ O_2\ in\ water\ = \frac{0.2095\ atm}{756.7\ atm*\left(\frac{L}{mol}\right)} = 276.8\ \mu M \qquad 4$$

Thus, the concentration of O_2 in water is 276.8 µM at 25ºC. It is worth noting that the concentration of O_2 in water is nearly **30 times lower than** that in the gaseous phase. The solubility of O_2 in water increases with decreasing temperature, reaching as high as 400 µM at 0ºC and decreases with increased concentration of other dissolved solutes.

2.3 Amount of O_2 entering the reactor with the N_2 gas

It is very common in laboratory microbial cultivations, to continuously sparge the culture medium with N_2 for anaerobic processes. The N_2 used always has traces of O_2 (in our case ~5 ppm, as provided by the supplier). Inflow rate of N_2 is given in L per L of liquid volume per min. For a reactor volume of 1 L, 0.5 L of N_2 could enter the reactor per min. The ideal gas law relates the volume to the number of molecules for a given pressure and temperature. This way:

$$P * V = n * R * T \qquad 5$$

Doing re-arrangements, introducing time (t) at both sides of the equation and introducing the fraction (f = 5/10^6) of O_2 in the compressed gas:

$$\frac{n}{t} = \frac{f*P*\left(\frac{V}{t}\right)}{R*T} \qquad 6$$

Using the gas constant in convenient units (R = 0.08206 atm·L·mol^{-1}·K^{-1}; in equation 2, the value for R is different because it is expressed in a different unit), we can calculate the O_2 entering the reactor by substituting the values for f = 5/10^6, P =1 atm, V/t, = 0.5 L·min^{-1}, R = 0.08206 atm·L·mol^{-1}·K^{-1} and T = 298 K in equation 6

$$\frac{5*10^{-6}*1\ atm*0.5\left(\frac{L}{min}\right)*\left(\frac{60\ min}{h}\right)}{0.08206\left(\frac{L*atm}{mol*K}\right)*298\ K} = 6.13\ \frac{\mu mol\ O_2}{h} \qquad 7$$

2.4 O_2 diffusion through the tubing

In addition to the O_2 coming along with the N_2 sparged into the reactor, one must also calculate the diffusion through the ports and the tubing material used. As the ports are made of stainless steel, we will assume that the diffusion of O_2 through the ports is negligible. For the tubing material, norprene is

commonly employed during anaerobic cultivations as it has 40 times lower permeability to O_2 than silicone tubing, which is most commonly used - in microbial cultivations that do not require O_2 to be excluded. Fick's law relates the flux of O_2 (net movement of molecules per unit time per unit area perpendicular to the direction of flow) to the gradient in concentration through a constant of proportionality (D) that changes with the composition of the solute/solvent and temperature:

$$J_{O2} = -D * \left(\frac{\mathrm{d}c}{\mathrm{d}x}\right) \qquad 8$$

J_{O2} has units of $mol \cdot m^{-2} \cdot s^{-1}$, concentration ($c$) has units of $mol \cdot m^{-3}$ and length (x) has units of m, hence diffusion coefficient D has units of $m^2 \cdot s^{-1}$ (we encourage the students to verify the dimensional consistency of this equation). As the gaseous O_2 is diffusing through a semi-permeable tubing, the constant of proportionality is called permeability coefficient (Denny 1993; Saint-Gobain, n.d.).

As shown above, the concentration of the gases is directly proportional to the partial pressure. Hence, knowing the partial pressure difference of O_2 between the inside of the tube (nearly zero) and the outside air (calculated previously as 20.67 kPa or 15.50 cmHg), and using the information of the characteristics of the tubing material used, we are now able to calculate the flux of O_2 entering the system via the tubing. The smaller the thickness and longer the tubing, the higher is the diffusion of O_2 into it.

For a tubing with a diameter d and length l, the area available for diffusion for a tube of cylindrical shape would be $2 * \pi * 0.5 * d * l$. The molecules of O_2 must diffuse through a tube thickness of x cm. Hence, the rate of diffusion (flux of O_2 multiplied by cross-sectional area) given a tube of d = 0.31 cm, l = 30 cm and x = 0.1 cm and a D = 20 barrer*, can be calculated as follows:

$$\frac{20 * 10^{-10} \left(\frac{cm^3 \cdot cm}{s \cdot cm^2 \cdot cmHg}\right) * 2*\pi*0.5*0.31\ cm * 30\ cm * 15.50\ cmHg}{0.1\ cm} = 9.11 * 10^{-6}\ \frac{cm^3}{s} \qquad 9$$

Converting the volumetric rate to molar rate using molar volume, we obtain the O_2 entering the tubing as:

$$= 9.11 * 10^{-6} \left(\frac{cm^3}{s}\right) * \left(\frac{1\ mol}{22{,}400\ cm^3}\right) * \frac{3600\ s}{h} = 1.47\ \frac{\mu mol\ O_2}{h} \qquad 10$$

* Permeability has units of barrer. 1 barrer = $10^{-10}\ \left(\frac{cm^3 \cdot cm}{s \cdot cm^2 \cdot cmHg}\right)$

O_2 diffusing into the system through the norprene tubing used is ~ **four** times less than the amount entering the reactor via the N_2 gas.

2.5 O_2 transfer from gas to liquid phase

The moles of O_2 transferred into the liquid per unit time (Oxygen Transfer Rate) is given by the expression:

$$OTR = k_{la} * (C^* - C_{bulk}) * V_l \qquad 11$$

Where C^* is the saturation concentration of O_2 in water (mol $O_2 \cdot h^{-1}$) determined using Henry's law, C_{bulk} is concentration in the bulk liquid, k_{la} is the gas-liquid mass transfer coefficient, (s^{-1}), V_l is the liquid volume in the reactor (in our case 1 L). k_{la} is determined by the reactor and impeller geometry, agitation speed, flow rate of gas and the bubble size.

5 ppm of O_2 in the gaseous phase is equivalent to a dissolved O_2 concentration of 6.60 nM[†]. Typical k_{la} value[‡] for laboratory bioreactors is 0.1 s^{-1} (Finn 1954). By substituting these two values and if the bulk concentration is close to zero:

$$k_{la} * C^* * V_l = 0.1 * \left(\frac{1}{s}\right) * 3600 \left(\frac{s}{h}\right) 6.6 * 10^{-9} \left(\frac{mol}{L}\right) * 1\ L = 2.37 \ \frac{\mu mol\ O_2}{h} \qquad 12$$

we get an OTR value of 2.37 µmol $O_2 \cdot h^{-1}$, representing only a **third** of the gaseous phase O_2, the rest of which leaves the bioreactor with the off-gas stream (because there is not enough time for all O_2 molecules to be transferred from the gaseous to the liquid phase).

2.6 O_2 and yeast metabolism

O_2 plays a major role in aerobic respiration as a terminal electron acceptor. In this process, some reduced coenzymes generated both in catabolic and anabolic reactions are re-oxidised, sustaining further growth. The re-oxidation of these coenzymes is usually coupled to processes where a fraction of the chemical potential is conserved through the synthesis of molecules of ATP from ADP and inorganic phosphate via a complex chemo-osmotic process. However, under anaerobiosis, yeast cells mostly conserve a fraction of the free energy coming from the substrate oxidation via a mechanism called substrate-level phosphorylation and re-oxidise the coenzyme NADH via ethanol and glycerol formation.

[†] 209,500 ppm equals 276.8 μM (concentration of O_2) Thus 5 ppm O_2 on gaseous phase amounts to $276.8\ \mu M\ * \frac{5}{209{,}500} = 6.6\ nM$

[‡] For calculating k_{la} for shake flasks, go to https://www.presens.de/support-services/kla-calculator.html

Undergraduate students are more like to rely on textbooks and search engines to obtain information. To find out the number of reactions involving O_2 in *S. cerevisiae*, students could be introduced to databases such as Pubmed, to search for the latest genome-scale model of S. cerevisiae and get a spreadsheet with a list of metabolic reactions. In the latest genome-scale model of yeast (Österlund et al. 2013), the list with O_2 as reactant has 48 reactions accounting for 3% of total reactions (around 1000) and six reactions are involved in oleate and ergosterol synthesis.

2.7 O_2 requirement for anaerobic yeast growth

Facultative anaerobes such as *S. cerevisiae* require the exogenous supply of ergosterol and oleic acid for growth in synthetic defined media, under fully anaerobic conditions. Molecular oxygen is required for the conversion of squalene (linear hydrocarbon) into ergosterol (cyclic sterol), 12 molecules of O_2 are required for the synthesis of one molecule of ergosterol. One O_2 molecule is required for the formation of each double bond from one acyl coenzyme-A in the biosynthesis of oleate (Ishtar Snoek and Yde Steensma 2007). Oleate makes up to 3.5% of dry cell mass (DCM) and ergosterol constitutes 0.2% of DCM (Verduyn et al. 1990).

Therefore, to calculate the requirement of O_2, it is first necessary to know the amount of cell biomass present in the bioreactor. For a glucose concentration of 10 $g \cdot L^{-1}$ in the culture medium, a biomass yield on glucose Y_{xs} of 0.1 $g_{DCM} \cdot g_{glucose}^{-1}$ is expected under anaerobic conditions (Verduyn et al. 1990). Hence, 1 g biomass would have 2 mg of ergosterol and 35 mg of oleate (Verduyn et al. 1990). Knowing the stoichiometric O_2 required, one can calculate the O_2 required for the biosynthesis of these two cofactors.

$$O_2\ for\ ergosterol\ = \frac{2\ mg\ ergosterol}{396.65\left(\frac{g\ ergosterol}{mol\ ergosterol}\right)} * \frac{12\ mol\ O_2}{mol\ ergosterol} = 60.50\ \mu mol \qquad 13$$

$$O_2\ needed\ for\ oleate\ = \frac{35\ mg\ oleate}{282.46\left(\frac{g\ oleate}{mol\ oleate}\right)} * \frac{1\ mol\ O_2}{mol\ oleate} = 123.91\ \mu mol \qquad 14$$

Hence, Y_{O2x} under the anaerobic condition is 184.41 µmol $O_2 \cdot g_{DCM}^{-1}$.

2.8 O_2 demand during exponential growth

The final question one asks is whether the O_2 entering the reactor, both as a contaminant in the N_2 gas and by diffusion through the tubing, can sustain growth in the absence of supplementation of ergosterol and

oleate in the medium. O_2 entering the reactor was already calculated as 7.60[§] µmol·h^{-1} but only a third of this dissolves in the medium ~ 2.5 µmol·h^{-1}. The metabolic demand of O_2 could be calculated knowing the Y_{O2x} (O_2 required per unit biomass), and the specific growth rate (µ, how fast the yeast grows) and the amount of biomass present in the vessel (X). Under anaerobic batch conditions in defined medium, a µ of 0.25 – 0.35 h^{-1} could be expected (Verduyn et al. 1990). The metabolic demand for a µ = 0.30 h^{-1} can be calculated as follows:

$$\frac{Metabolic\ O_2\ demand}{h} = Y_{O2X} * \mu * X \qquad 15$$

$$\frac{Metabolic\ O_2 demand}{h} = 184.41\ \left(\frac{\mu mol\ O_2\ needed}{g_{DCM}\ produced}\right) * 0.30\ \frac{\left(\frac{g_{DCM}\ produced}{g_{DCM}\ present}\right)}{h} * 1\ g_{DCM\ present} = 55.3\ \frac{\mu mol\ O_2}{h} \qquad 16$$

The metabolic O_2 demand (55 µmol O_2·h^{-1}) is not met by its supply (2.5 µmol O_2·h^{-1}). The importance of O_2 during anaerobic cultivation of *S. cerevisiae* in a defined medium in the absence of supplementation with ergosterol and oleic acid was illustrated using basic principles of physical chemistry and some basic biochemistry. The topic allows students to seek out and explore more about the O_2 requirements in other situations (life in deep oceans, in ice crystals for example).

3 References

Denny, Mark M. 1993. *Air and Water: The Biology and Physics of Life's Media*. Princeton University Press.

Fawcett, Tim W, and Andrew D Higginson. 2012. "Heavy Use of Equations Impedes Communication among Biologists." *Proceedings of the National Academy of Sciences of the United States of America* 109 (29). National Academy of Sciences: 11735–39. doi:10.1073/pnas.1205259109.

Finn, R K. 1954. "Agitation and Aeration in the Laboratory." *Bacteriology Reviews* I8 (4): 254–74.

Ishtar Snoek, I S, and H Yde Steensma. 2007. "Factors Involved in Anaerobic Growth of Saccharomyces Cerevisiae." *Yeast (Chichester, England)* 24 (1): 1–10. doi:10.1002/yea.1430.

Österlund, Tobias, Intawat Nookaew, Sergio Bordel, and Jens Nielsen. 2013. "Mapping Condition-Dependent Regulation of Metabolism in Yeast through Genome-Scale Modeling." *BMC Systems Biology* 7 (1): 36. doi:10.1186/1752-0509-7-36.

Polya, George. 1956. "How to Solve It? A New Aspect of Mathematical Method." In , Second edi, 33–36. Princeton University Press.

Saint-Gobain. n.d. "Saint-Gobain Performance Plastics Permeability Coefficients For Peristaltic Pump

[§] O_2 entering (Equation 7) plus O_2 diffusing into the system (Equation 10) equals 6.13 + 1.47 = 7.6 µmol·h^{-1}

Tubings." http://www.processsystems.saint-gobain.com/uploadedFiles/SGPPL-PS/Documents/Peri_Pump/PS-PeriPumps-Permeability(1).pdf.

Verduyn, C, E Postma, W A Scheffers, and J P Van Dijken. 1990. "Physiology of Saccharomyces Cerevisiae in Anerobic Glucose-Limited Chemostats." *Journal of General Microbiology* 136: 395–403.

4 Box

Dalton's law: The total pressure of a gas is equal to the sum of the individual partial pressures of the component gases.

Fick's law: The net flux of solute per unit area (the area perpendicular to the flux direction) is proportional to the gradient in the concentration of solute. The constant of proportionality is called diffusion coefficient denoted D, and it is unique for each pair solute - solvent. D varies with temperature.

Henry's law: The solubility of a gas in a liquid is determined by Henry's law. It states that the concentration of a gas molecule in a liquid phase (in equilibrium with the gas) is proportional to the partial pressure of the gas in the gaseous phase. The constant of proportionality is called Henry's constant (K_H) and is unique for each gas and a given liquid phase. K_H varies with the temperature.

ppm: Parts Per Million. Air has ~21% O_2 which is 21 parts per 100 parts; hence the concentration of O_2 molecules would be 210000 ppm. (in one million parts)

Specific rate (q_i rate): It is calculated from the production or consumption rates. It is defined as the number of species *i* formed or consumed per hour per unit amount of cell biomass present in the cultivation vessel. For a given biomass (x), q_x (denoted μ) is defined as the specific growth rate ($\frac{1}{x} \cdot \frac{dx}{dt}$) and has the unit [time^{-1}]. μ is directly related to the doubling time t_d, thought he relation, $\mu = \frac{ln(2)}{t_d}$.

vvm: Volume per volume per minute. vvm is the common unit used to indicate the gas flow rate into a bioreactor. It refers to the volume of gas sparged per volume of liquid in the reactor per minute.

Yield coefficients Y_{ji}: It is a ratio of change in compound *j* for a unit change in compound *i*.

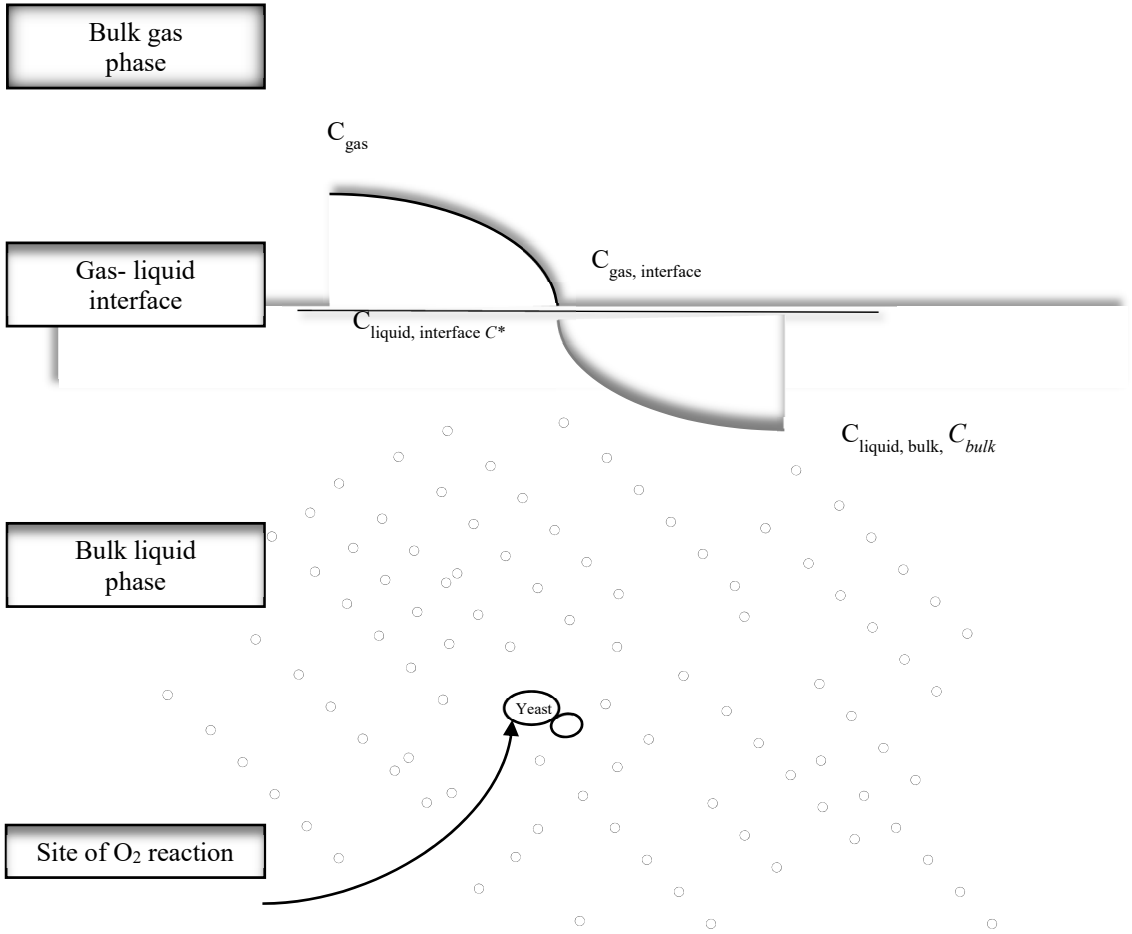

Figure 1. O_2 transfer mechanism. O_2 concentration is at a maximum in the gas phase; the solubility of the gas into the liquid phase occurs at the gas-liquid interface (bubbles) and is determined by two factors: the concentration difference between the outside of the gas-water thin film (C^*) and the bulk liquid (C_{bulk} close to zero), and the gas-liquid mass transfer coefficient, k_{la} (*units of time*$^{-1}$). (compare heat flow and mass flow; heat flows naturally from higher to lower temperature; similarly, mass transfer occurs from a region of higher concentration to a region of lower concentration).

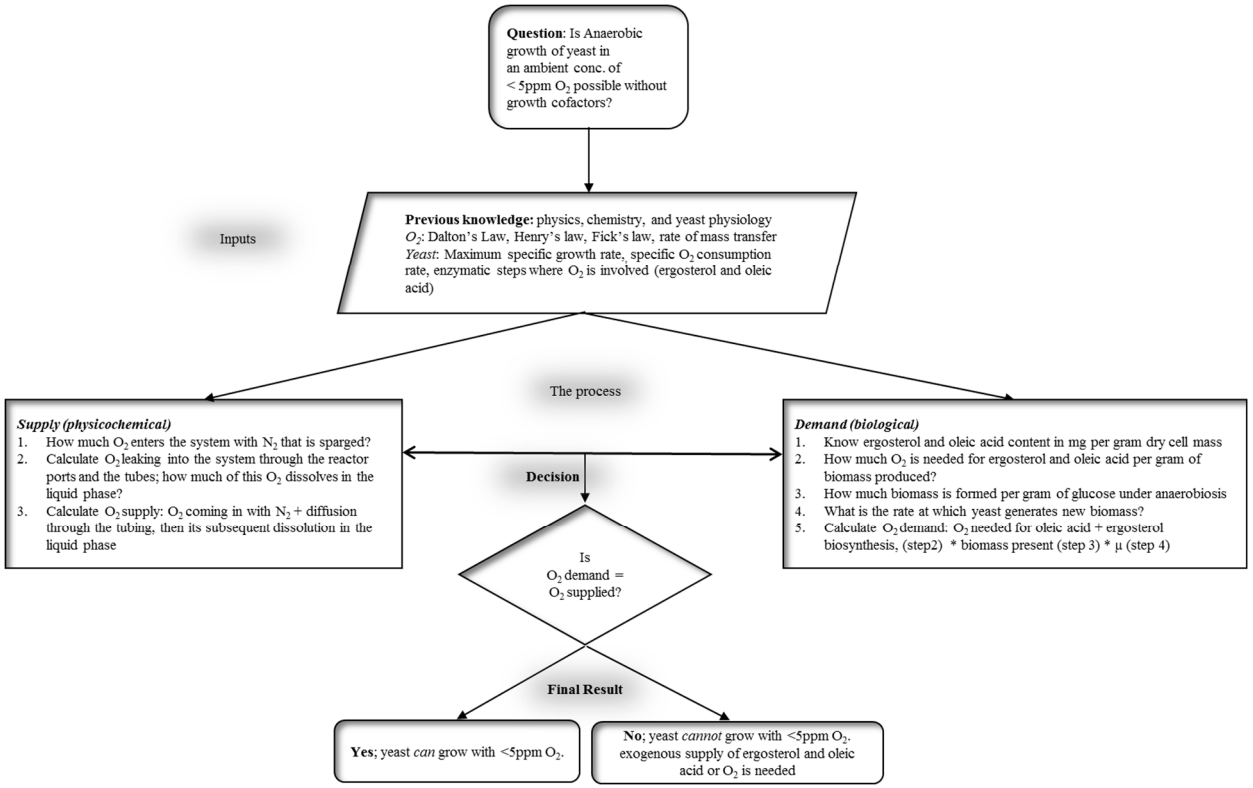

Figure 2. Flowchart depicts the questions that can be formulated with respect to anaerobic cultivations of yeast. The principal question is: does yeast is able to grow under anaerobic conditions in a defined medium (all the components of the medium are usually inorganic compounds, glucose, and vitamins) without the addition of two compounds that require O_2 for their biosyntheses. O_2 enters the system with the N_2 gas as an impurity and by diffusion through the tubing and ports. The key question the learner asks is whether the O_2 supply meets the metabolic demand.