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Process for symbiotic culture of *Saccharomyces cerevisiae* and *Chlorella vulgaris* for *in situ* CO_2 mitigation

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Abstract

Industrial biotechnology relies heavily on fermentation processes that release considerable amounts of CO_2. Apart from the fact that this CO_2 represents a considerable part of the organic substrate, it has a negative impact on the environment. Microalgae cultures have been suggested as potential means of capturing the CO_2 with further applications in high value compounds production or directly for feed applications. We developed a sustainable process based on a mixed co-dominant culture of *Saccharomyces cerevisiae* and *Chlorella vulgaris* where the CO_2 production and utilization controlled the microbial ecology of the culture. By mixing yeast and microalga in the same culture the CO_2 is produced in dissolved form and is available to the microalga avoiding degassing and dissolution phenomena. With this process, the CO_2 production and utilization rates were balanced and a mutual symbiosis between the yeast and the microalga was set-up in the culture. In this study the reutilization of CO_2 and growth of *C. vulgaris* was demonstrated. The two organism populations were balanced at approximately 20×10^6 cells ml^{-1} and almost all the CO_2 produces by yeast was reutilized by microalga within 168 h of culture. The *C. vulgaris* inoculum preparation played a key role in establishing co-dominance of the two organisms. Other key factors in establishing symbiosis were the inoculum ratio of the two organisms and the growth medium design. A new method allowed the independent enumeration of each organism in mixed culture. This study could provide a basis for the development of green processes of low environmental impact.

Keywords

Photo-bioreactor; Flow cytometer; Medium design; Autotrophy; Heterotrophy; Co-dominant culture

Introduction

Industrial biotechnology such as bioethanol production, alcoholic beverage production and liquid effluent treatments form a considerable part of human biological activity. These industries, involving fermentation technology, release large quantities of CO_2. At the same time, the current trend is towards more sustainable industrial processes. Waste recycling is increasingly seen not only as an obligation but an opportunity in industry.

Commercially, the loss of a considerable part of the substrate in the form of CO_2 is an inefficient practice that cannot be avoided with microbial cultures. CO_2 mitigation from exhausts gases could provide an opportunity where the substrate would be entirely used at the same time rendering the process sustainable. To this end, photosynthesis is the best candidate to be associated to the normal production process. This is present in nature, often based on symbiotic relationships between organisms, and in some industrial sectors such as sewage treatment.

Biotransformation can be achieved through the use of GMO organisms. Alternatively, biotransformation can be performed by the use of specific consortia to create the desired microbial ecology. The ability to control mixed cultures is key to the use of consortia for biotransformation.

The term “symbiosis” is credited to Heinrich Anton de Bary who first used and described it as “the living together of unlike named organisms” in 1879 (Oulhen et al. 2016). One of the most studied natural composite organisms, considered as the model of symbiosis, is the lichen. Lichen arises from a symbiotic relationship between a fungi and algae or cyanobacteria (Gargas et al. 1995) and the metabolites obtained from lichens have application in industries such as pharmaceutical industry (Müller 2001).

Co-culture systems based on symbiosis between microbial species have been attempted for biotechnological applications in bioprocess and environmental protection (Santos and Reis 2014; Magdouli et al. 2016). The choice of microbial species (microalgae, bacteria or yeast) depends on the final aims of co-culture: harvesting by bioflocculation (Subashchandrabose et al. 2011; Rai et al. 2012), wastewater treatment (Arumugam et al. 2014), production of extracellular polymeric substances (Haggstrom and Dostalek 1981) or growth promotion and lipid production (Milledge and Heaven 2013; Pragya et al. 2013).

Reports of studies on symbiotic co-cultures of microalgae and yeast have been increasingly appearing in the scientific literature, with the aim of improving biomass and target molecule productivity. These co-cultures fall into two categories: studies with bioreactors in series where the exhaust gases from the heterotrophic culture are fed into the autotrophic culture, and studies where both yeast and microalgae are concomitantly in

the same culture. We have decided to refer to the former as coupled cultures and the latter as mixed cultures (Fig. 1).

Coupled cultures consists of an upstream heterotrophic yeast-culture connected to an autotrophic culture of microalgae in photo-bioreactor through the exhaust gases from yeast culture (Puangbut and Leesing 2012; Santos et al. 2013; Dillschneider et al. 2014; Chagas et al. 2015). Studies on coupled cultures have suggested an increase in the final microalgae biomass and lipid production that is achieved by effectively enriching the air supply to the microalgae cultures with CO_2 from the heterotrophic culture. In a coupled-culture system, the autotrophic organism benefits from the heterotrophic organism with no positive or negative impact on the latter, therefore the symbiosis is commensal.

The mixed culture system of microalgae and yeast focuses on the symbiotic potential of associating both organisms in the same culture. This system has an advantage over coupled-cultures in that it provides an opportunity for direct gaseous exchange in dissolved form bypassing the dissolution and degassing rates of the gas supply. Usually, any gas supplied to a bioreactor has to pass from a gaseous phase into a liquid phase (dissolution) and the gases produced by the culture have to pass from the liquid phase into the gaseous phase (degassing). These transfers are subject to specific surface limitations as well as mixing phenomena that can limit CO_2 supply for the autotroph and O_2 supply for the heterotroph in a coupled culture. In a mixed culture of microalgae and yeast, each organism would use the gas produced by the other organism *in situ* and without passing through a gaseous phase, the organisms would benefit from each other, so the symbiosis based on these gas exchanges would be mutual.

From a CO_2 mitigation viewpoint, as the heterotrophic CO_2 production rate is usually largely superior to it autotrophic consumption, the two populations must be balanced in such way so that the photosynthetic population can cope with the rate of CO_2 production. Hence the heterotrophic activity must be in step with the CO_2 removal rate. This could be achieved though co-dominance of the populations allowing synergy between the two organisms based on gaseous exchange. So far, no scientific studies have been published with the stated aim of developing co-dominant symbiotic mixed cultures.

One of the main challenges for a mixed culture of yeast and microalgae appears to be the dominance of one organism over the other by the end of incubation period. The dominance seems to be due to the use of a culture medium that preferentially promotes either the growth of the yeast or that of microalgae (Dong and Zhao 2004; Cai et al. 2007; Xue et al. 2010; Cheirsilp et al. 2011; Shu et al. 2013; Zhang et al. 2014). In Zhang et al. 2014, a mixed culture of yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* showed a yeast dominance of 88 % of the total population after 2.5 days of growth (18 g l^{-1} of yeast and 2.4 g l^{-1} of microalgae). Additionally, the maximum biomass concentration reached by the yeast in monoculture was 8.5

times higher than of the microalga in monoculture (14.5 and 1.7 g l^{-1} respectively), this suggests that the medium designed for mixed culture was more suitable for yeast than microalgae. Inversely in Cai et al. (2007), the microalga *Isochrysis galbana* was dominant in the mixed culture at the end of the experiment (97 % of the total population). In this study, the medium seems to have been more adapted to the microalgae rather than yeast *Ambrosiozyma cicatricose*, leading to microalgae dominance. In these studies, on mixed cultures, no mention of the enumeration method in the mixed culture is made.

The present study was conducted to develop a co-dominant and symbiotic mixed culture of two model organisms: the yeast *Saccharomyces cerevisiae* and the microalga *Chlorella vulgaris*. In order to promote co-dominance between the two organisms, a new growth medium was specifically designed, and the inoculum ratio was adjusted. The two species were grown in the same medium and in a non-aerated photo-bioreactor fitted with a fermentation lock to prevent gas exchange with the outside atmosphere. The absence of external air supply is intended to force the mutual symbiosis through synergetic effects of *in situ* gas exchange. To monitor the proportion of populations, a flow cytometric method was used to determine the cell concentration of each population in the mixed-culture. Dissolved O_2 and CO_2 were continuously measured in-line to evaluate the *in situ* gas exchange between the two species and to proof the mutual symbiosis.

Through this study, we propose a general methodology for the design of a co-dominant symbiotic mixed culture of a heterotroph and an autotroph in general and assess the success and the challenges of such strategy. The work presented here was performed on well-known model organisms but can provide the basis for more applied studies. The potential advantage of this work is that a symbiotic mixed culture would self-regulate the speed of the bioconversion hence the CO_2-production and -utilization rates; it could potentially eliminate the need for gas supply and can lead to full utilization of the substrate. The potential savings would be those of recovering the cost of the portion of the substrate that is normally lost as CO_2, making considerable savings in terms of gas supply avoidance and reducing environmental CO_2 emissions. In an economical assessment, all these savings would have to be weighed against the losses incurred by moderating the bioconversion speed in step with the photosynthetic rate.

Materials and methods

Strategy of this study

A diagram (Fig. 2) demonstrates the strategy used to establish co-dominant culture of *S. cerevisiae* and *C. vulgaris*.

Microbial strains and their maintenance

S. cerevisiae strain ID YLR249W was supplied by Life Technologies-University of California San Francisco. This clone expresses a cytoplasm fusion protein coupled to a green fluorescent protein (GFP). The protein of interest is the translation elongation factor 3 encoded by the gene YEF3 (Qin et al. 1987). The strain was maintained on YPG agar stock plates incubated at 25°C for 3 days and subsequently stored at 4°C for 3 months before subculture. The YPG agar medium was composed of (g l^{-1}): yeast extract (10), peptone (20) glucose (10) and agar (15) and the stock plates were renewed every three months.

C. vulgaris SAG 211-12 was obtained from the Culture Collection of Algae (SAG), University of Göttingen, Germany. The strain was maintained in liquid culture (50 ml in 250 ml flask) through weekly subculture into fresh medium, incubated at 25°C on an orbital shaker (120 rpm) with continuous lighting at 20 µmol m^{-2} s^{-1} at the surface of the culture and in air enriched with 1.5% (v/v) CO_2. The liquid inorganic medium used was MBM (modified 3N-Bristol medium) (Clément-Larosière et al. 2014), with the following composition (mg l^{-1}): $NaNO_3$ (750); $CaCl_2.2H_2O$ (25); $MgSO_4.7H_2O$ (75); FeEDTA (20); K_2HPO_4 (75); KH_2PO_4 (175); NaCl (20); H_3BO_3 (2.86); $MnCl_2.4H_2O$ (1.81); $ZnSO_4.7H_2O$ (0.22); $CuSO_4.7H_2O$ (0.08); MoO_3 85% (0.036); $CoSO_4.7H_2O$, (0.09).

Design of a specific medium for mixed culture

Monocultures of *S. cerevisiae* and *C. vulgaris* were grown in three different media in order to define a medium suitable for co-dominance of the organisms in mixed culture. The media were based on different combinations of the microalgae growth medium (MBM) (described above) and components from the commonly used yeast growth YPG (yeast extract, peptone and glucose) medium (g l^{-1}):

- MBM-G: MBM medium with glucose (10)
- MBM-GY: MBM medium with glucose (10) and yeast extract (10)
- MBM-GP: MBM medium with glucose (10) and peptone (20)

Erlenmeyer flasks (50 ml working volume; 250 ml total volume) were used for the monoculture of *C. vulgaris* and *S. cerevisiae* in the above media and the inoculation ratio was 1% (v/v) from a fully-grown culture. The flasks were incubated at 25°C on an orbital shaker (120 rpm) with continuous lighting at 80 µmol m^{-2} s^{-1} (LI250A Light Meter; LI-COR, USA) at the surface of the cultures. Yeast monocultures were conducted for 3 days and microalgae monocultures for 5 days.

The medium finally selected and specifically designed for the mixed culture was named MBM-GP and was composed of (mg l^{-1}): $NaNO_3$ (1,500); $CaCl_2.2H_2O$ (50); $MgSO_4.7H_2O$ (150); FeEDTA (40); K_2HPO_4 (75); KH_2PO_4 (175); NaCl (20); H_3BO_3 (2.86); $MnCl_2.4H_2O$ (1.81); $ZnSO_4.7H_2O$ (0.220); $CuSO_4.7H_2O$ (0.08); MoO_3 85% (0.036); $CoSO_4.7H_2O$, (0.09), glucose (10,000); peptone (20,000).

Cultures in photo-bioreactors

All experiments in photo-bioreactor (PBR) were conducted in a stirred bioreactor (5-liter working volume) (BIOSTAT Bplus – 5 L CC; Sartorius Stedim biotech, Göttingen, Germany). The PBR was lit with six LED lamps (Ledare 130 lumen, 2700 Kelvin, 27° dispersion angle, IKEA, Leiden, Netherlands). The light intensity at the inner surface of the bioreactor for each lamp was measured at 1,600 µmol m^{-2} s^{-1} (LI250A Light Meter; LI-COR, USA). The stirring speed was 750 rpm with a 3-blades pitch-blade impeller (UniVessel 5l, Germany), each inclined at 45°from the horizontal axis. The planar diameter of the impellers was 65 mm. The temperature was maintained at 25°C and the pH was controlled at 6.5 with automatic base KOH (1 mol l^{-1}) or acid H_3PO_4 (1 mol l^{-1}) solutions addition based on the continuous measurements made by an internal pH probe (EasyFerm PLUS K8 325, Hamilton, Bonaduz, Switzerland). Dissolved oxygen (pO2) in cultures was measured with an internal probe (VisiFerm DO H2, Hamilton, Bonaduz, Switzerland). The pO2 was expressed in terms of % of O2 partial pressure in the liquid phase of the culture.

The *S. cerevisiae* and *C. vulgaris* specific growth rates (μ) were calculated as the slope of the linear part of the logarithm of cell concentration plotted versus time.

Mixed cultures in PBR

Two non-aerated mixed cultures in PBR were grown using MBM-GP medium. The experimental set up (Fig. 3) involved hermetically isolating the bioreactor to limit the exchange of gases with the atmosphere at the exterior of the bioreactor.

Dissolved CO_2 (pCO_2) was measured only in the mixed culture No. 1 with an external minisensor integrated in a flow cell (CO2 Flow-Through Cell FTC-CD1, PreSens, Regensburg, Germany). The culture was circulated (90 ml min^{-1}) through the flow cell with the aid of a peristaltic pump (520S/R, Watson Marlow) and back into the bioreactor. The flow-through cell was placed as close to the outlet from the bioreactor as possible. The passage of the culture over the sensor in the flow cell allowed the continuous measurement of pCO_2 via an optical fiber. As with the pO_2, the pCO_2 was expressed in % of CO_2 partial pressure in the liquid phase of the culture.

S. cerevisiae inoculum preparation was the same for both mixed cultures*; S. cerevisiae* was grown on MBM-GP medium, at 25°C, for 2 days. The preparation of the *C. vulgaris* inocula for the two mixed cultures differed; for the mixed culture No. 1, the *C. vulgaris* inoculum was grown on autotrophic MBM medium under continuous illumination, for 15 days, at 25°C and for mixed culture No. 2 the *C. vulgaris* inoculum was grown on heterotrophic MBM-GP medium under continuous lighting, for 15 days, at 25°C.

Monoculture of S. cerevisiae in PBR

The monoculture of *S. cerevisiae* was grown in a non-aerated PBR in MBM-GP medium, with culture parameters as described above and the photo-bioreactor configuration was the same as for mixed culture (Fig.

3), there was no aeration and gas outlet was closed as described with a fermentation lock. The culture was lit as for mixed culture. The *S. cerevisiae* inoculum was grown in MBM-GP medium, at 25°C, for 2 days.

Monocultures of C. vulgaris in PBR

Two monocultures of *C. vulgaris* in PBR were grown, one in heterotrophic MBM-GP medium and the other in autotrophic MBM medium. For the first one the inoculum was prepared in MBM-GP medium, the second one using MBM medium and both under continuous light at 25°C for 15 days. Both culture conditions were set up as described above and the photo-bioreactor was continuously aerated with sterile air (Midisart 2000 0.2 µm PTFE, Sartorius, Göttingen, Germany) at 500 ml min^{-1} (0.1 vvm) (1 atm, 25°C).

The impact of ethanol on C. vulgaris growth

C. vulgaris was grown on MBM medium in Erlenmeyer flasks (50 ml working volume; 250 ml total volume) and the flasks were incubated at 25°C on an orbital shaker (120 rpm) with continuous lighting at 20 µmol m^{-2} s^{-1} and in air enriched with CO_2 1.5% (v/v). Four ethanol concentrations were tested (0, 2, 4 and 6 g l^{-1}) (ethanol 96 %).

Analytical methods

Simultaneous enumeration of C. vulgaris and S. cerevisiae by flow cytometry

A flow cytometer (Guava easyCyte™, EMD Millipore, Burlington, United States) was used to simultaneously determine the cell concentrations of *S. cerevisiae* and *C. vulgaris* in mixed culture. The excitation wavelength of the blue laser was 488 nm and detectors separately captured the Forward-scattered light (FSC) that is proportional to cell-surface area (size), the Side-scattered light (SSC) that indicated particles granularity and the fluorescence emitted by the cell (auto-fluorescence). Samples were diluted so that the cell enumeration was always performed at cell concentrations between 1×10^5 and 1×10^6 cells ml^{-1}. The method for cell enumeration by flow cytometer suspensions containing only one of the microorganisms was previously validated against a Thoma counting chamber as the referent method (data not shown). Cell viability of *C. vulgaris* was also determined by flow cytometry using the Guava ViaCount Reagent (EMD Millipore, Burlington, United States).

Glucose and ethanol measurements

Culture supernatants were prepared by sample centrifugation (10 min, 3500 g), filtration (PTFE Syringe Filter 0.2 µm, Fisherbrand, Waltham, United Stated) and High Pressure Liquid Chromatography (HPLC) (Ultimate 3000, Thermo Scientific, United States). A cationic column (Aminex HPX-87H, Bio-Rad, United States) was used with 2 mM sulfuric acid as the mobile phase with a flow rate of 0.5 ml min^{-1}, an injection volume of 10

µl, a temperature of 45°C and a pressure of 60 bar. Detection was by means of a refractive index (RI) detector (RI 101, Shodex, Japan).

Dry weight

The dry weight was determined by sampling and centrifuging 10 ml of culture (10 min and 1,800 g). The pellet was washed with an equal volume of deionized water, and was centrifuged again (10 min, 1800 g) and the final pellet was transferred into a dry pre-weight ceramic cup (24 h, 105 °C). The pellet was dried overnight at 105 °C and cooled in a desiccator containing dry silica gel prior to weighing. A correlation between the dry weight and the cell concentration was established for *S. cerevisiae* and *C. vulgaris*: $DW_{yeast} = 3.25\times10^{-8}\ N_{yeast}$ (9 data points and $R^2=0.91$) and $DW_{algae} = 1.5\times10^{-8}\ N_{algae}$ (13 data points and $R^2=0.96$) with DW the dry weight (g l^{-1}) and N the cell concentration (cells ml^{-1}). The experimental data points for the yeast were obtained from a monoculture in PBR using the MBM-GP medium and for the microalgae from a monoculture in PBR using the autotrophic medium MBM.

CO_2 production and consumption

CO_2 produced by yeast was assumed to be the main cause of culture acidification, resulting in the automatic addition of base (KOH) under the experimental conditions designed to keep the pH at 6.5. Consequently, the quantity of KOH solution is directly proportional to the CO_2 produced and was used to estimate the amount of CO_2 produced by yeast. The difference in the KOH added into the yeast monoculture and the mixed culture indicated the amount of CO_2 used by *C. vulgaris* and was used to perform a carbon mass balance.

Results

Validation of simultaneous enumeration of *C. vulgaris* and *S. cerevisiae* by flow cytometry

A method for separately-enumerating *C. vulgaris* and *S. cerevisiae* populations in a mixed suspension was developed. The two species were distinguished on the basis of their specific auto-fluorescence detected by flow cytometry. *C. vulgaris* cells were distinguished through chlorophyll fluorescence (emission wavelength of 650 nm) and *S. cerevisiae* with the fluorescence of the constitutively expressed GFP protein (emission wavelength of 525 nm) (Fig. S1).

To validate the method, eleven mixed suspensions were prepared over a range of precise microalgae:yeast ratios (reference ratios) and the two populations in the mixed suspensions were measured with flow cytometry (experimental ratios). By plotting the experimental *C. vulgaris* ratio as a function of the referent microalgae ratio (Fig. 4), a linear relationship was obtained with a slope of 1.048 (correlation coefficient of 0.997; 11 data points). A linear relationship was also found for *S. cerevisiae* with a slope of 0.996 and a correlation coefficient of 0.998, validating the method for enumerating microalgae and yeast simultaneously in mixed suspensions.

Strategy for a co-dominance of *C. vulgaris* and *S. cerevisiae* in mixed culture

Design of a specific medium for mixed culture

A growth medium that allowed the growth of both organisms was necessary. According to Fig. 5, MBM-G medium allowed only microalgae growth and *S. cerevisiae* growth was barely detectable. Monocultures of *C. vulgaris* and *S. cerevisiae* in MBM-GY medium showed the opposite results from those in MBM-G medium: MBM-GY allowed good growth of *S. cerevisiae* but not of *C. vulgaris*.

In MBM-GP medium, both *C. vulgaris* and *S. cerevisiae* could grow: the maximum *C. vulgaris* population was 2×10^8 cells ml^{-1} and the maximum yeast population was 10 times lower (2×10^7 cells ml^{-1}).

Definition of parameters for mixed culture in PBR

The temperature and pH in PBR were chosen to favor *C. vulgaris* growth. According to Kumar et al. (2010), temperatures of 15-26°C and neutral pH is optimal for microalgae growth. The form of the dissolved CO_2 concentration and the pH of the culture are directly linked so we chose to control the pH at 6.5 to achieve a good compromise between having a neutral pH and the dissolved CO_2 and bicarbonate species proportioned at around 0.5 at 25°C (Edwards et al. 1978).

The inoculum ratio was set up in way to minimize dominance of yeast and favor microalgae growth:

$$X_{0\ C.\ vulgaris} = \frac{X_{0\ S.\ cerevisiae}\ e^{\mu_{S.\ cerevisiae}\,t}}{e^{\mu_{C.\ vulgaris}\,t}} \quad (1)$$

with:

$X_{0\ C.\ vulgaris}$: initial *C. vulgaris* population

$X_{0\ S.\ cerevisiae}$: initial *S. cerevisiae* population

$\mu_{S.\ cerevisiae}$: *S. cerevisiae* specific growth rate

$\mu_{C.\ vulgaris}$: *C. vulgaris* specific growth rate

t: duration of the *S. cerevisiae* exponential phase.

Monocultures in PBR

Yeast and microalgae monocultures in PBR served as reference cultures for mixed culture. In the case of *C. vulgaris*, two reference conditions were tested: heterotrophic growth in the presence of glucose and, autotrophic growth in the absence of glucose. The behavior of the individual species in mixed cultures would then be compared to the latter reference conditions.

Monoculture of S. cerevisiae in closed and non-aerated PBR

S. cerevisiae was grown on MBM-GP medium in PBR without aeration, exactly under the same conditions as for the subsequent mixed culture.

The yeast exponential growth phase (μ= 0.27 h^{-1}) occurred within the first 24 h of incubation (15 h of exponential phase) and was accompanied with glucose and O_2 consumption. *S. cerevisiae* used all glucose within the first 31 h of incubation reaching a maximum population of 2.2×10^7 cells ml^{-1} (Fig. 6). Within the first 31 h, *S. cerevisiae* also produced ethanol to a peak concentration of 4 g l^{-1}.

Monocultures of C. vulgaris in aerated PBR in mixotrophic conditions

C. vulgaris was grown on MBM-GP medium in PBR in the same way as *S. cerevisiae* in monoculture and as mixed cultures except that the *C. vulgaris* monocultures were continuously aerated. Aeration was mandatory for CO_2 provision to *C. vulgaris* for photosynthesis. The pO_2 in the culture was expected to be stable at 20.9 % in the absence of net production or consumption of O_2 by *C. vulgaris*.

During the first 48 h of *C. vulgaris* growth in MBM-GP (Fig. 7a), the glucose and O_2 concentrations did not decrease while the population increased slightly from 1×10^6 to 1.8×10^6 cells ml^{-1}. From 48 to 116 h of incubation, glucose decreased to complete depletion while the microalgae population increased from 1.8×10^6 to 4×10^8 cells ml^{-1}. During this period, the presumed heterotrophic microalgae growth was exponential with μ=0.09 h^{-1}).

Monocultures of C. vulgaris in aerated PBR in photo-autotrophic conditions

C. vulgaris was grown in autotrophic monoculture using MBM medium in the absence of glucose (photo-autotrophically) and with continuous aeration (Fig. 7b) to supply atmospheric CO_2 as carbon source. The microalgae firstly grew exponentially (μ=0.02 h^{-1}), increasing the population from 9×10^5 to 2×10^7 cells ml^{-1} and producing O_2 via photosynthesis. Starting from a value of 21%, the pO_2 reached 22% at the end of the exponential growth phase (100 h) then it continued to increase up to 22.3% and remained constant at the same level. This is a significant level of O_2 production considering the continuous flow of the air through the photo-bioreactor and the concentration of cells in the culture.

Impact of ethanol on *C. vulgaris* growth

The impact of the ethanol produced by *S. cerevisiae* when grown in MBM-GP medium in photo-bioreactor culture was assessed on *C. vulgaris* growth. Ethanol was added to *C. vulgaris* shake-flask cultures when the population reached 7×10^6 cells ml^{-1} (corresponding to the initial *C. vulgaris* population in the mixed culture). Four ethanol concentrations (2, 4, 6 g l^{-1} and 0 g l^{-1}) were chosen according to the range of ethanol concentrations that could be produced by *S. cerevisiae* in monoculture and mixed culture (Fig. S2). The *C. vulgaris* growth profile was the same in all cultures (with or without the addition of ethanol). Moreover, cell viability of the four cultures remained at approximately 98 %.

Mixed cultures of *S. cerevisiae* and *C. vulgaris* in closed and non-aerated PBR

Mixed culture No. 1

In the first mixed culture, the yeast inoculum was prepared in the newly designed MBM-GP medium while the microalgae inoculum was prepared in autotrophic MBM medium.

The *S. cerevisiae* behavior was similar in both mixed culture No. 1 (Fig. 8) and in the reference yeast monoculture (Fig. 6) (same maximum population, same specific growth rate, and same ethanol productivity). On the other hand, the *C. vulgaris* growth in mixed culture No. 1 was weak compared with the reference photoautotrophic mixed culture. The microalgae population only slightly increased from 7×10^6 to 9×10^6 cells ml^{-1} within the first 13 h and remained mainly constant until the end of incubation (168 h) but the dissolved CO_2 concentration gradually decreased from 16 % to 0 % from 48 h to 168 h at the end of the experiment.

Mixed culture No. 2

For this second mixed culture, both the yeast and the microalgae inocula were prepared in the MBM-GP medium. This was in contrast to the mixed culture No. 1 where the microalgae inoculum was prepared in autotrophic MBM medium. The mixed cultures No. 2 and No. 1 only differed in the microalgae inoculum preparation. All other conditions including the microalgae:yeast inoculum ratio were identical.

The *S. cerevisiae* behavior was similar in both mixed cultures (Fig. 9) and in the reference yeast monoculture (Fig. 6) in terms of maximum population, specific growth rate, and ethanol productivity.

C. vulgaris started to grow from the beginning of incubation period, and without a lag phase, until 24 h and reached a maximum population of 2.4×10^7 cells ml^{-1}, then its population remained stable until the end of the experiment.

CO_2 production by *S. cerevisiae* in monoculture

In monoculture of *S. cerevisiae* using MBM-GP medium, yeast biomass, ethanol and CO_2 were produced during growth, the latter resulting in the acidification of the culture medium. Since a stable pH was specified for the fermentation, the acidification of the culture resulted in the automatic addition of KOH in step with yeast growth during the first 41 h of the culture. Ethanol (3.95 g l^{-1}) was produced (Fig. 6) and CO_2 (3.91 g l^{-1}) was released. The CO_2 concentration was calculated by adopting the stoichiometric fermentation equation 2 (Verduyn et al. 1990) using the ethanol yield (3.95 g l^{-1}) of the yeast monoculture in MBM-GP medium:

$$8.98\ C_6H_{12}O_6 + 0.63\ NH_3 \rightarrow 1\ C_{3.75}H_{6.6}N_{0.63}O_{2.1} + 15.4\ C_2H_6O + 16\ CO_2 + 1.1C_3H_8O_3 + 0.8\ H_2O \quad (2)$$

The CO_2 released into the culture medium reacts with water to form carbonic acid H_2CO_3 and then dissociates into H^+ and HCO_3^- (Peña et al. 2015) acidifying the culture medium. Under the pH-control regime, the KOH

solution is added to maintain the pH at 6.5. The stoichiometry of the reaction between CO_2 and KOH is 1:1. A total KOH volume of 337 ml was added during the yeast growth phase, which corresponded to 0.337 mole of KOH added to the 5-liter culture medium. For ease of the mass balance calculation, the amount of KOH added was expressed as a concentration (6.74×10^{-2} mol l^{-1}):

$$[KOH] = \frac{V_{KOH} \times C_{KOH}}{V} \quad (3)$$

with:

[KOH]: base KOH concentration in the culture medium (mol l^{-1})

V_{KOH}: volume of KOH added to the culture medium (l)

C_{KOH}: concentration of the KOH solution added to the photo-bioreactor (mol l^{-1})

V: working volume (5 l)

Assuming that the KOH reacted exclusively with the H^+ from the hydration of the CO_2 produced, 6.74×10^{-2} mol l^{-1} of KOH was used for pH adjustment:

$$CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^- \quad (4)$$

The CO_2 concentration produced by yeast and neutralized by the KOH was 2.97 g l^{-1} and was calculated as:

$$[CO_2]_{KOH} = [KOH] \times M_{CO2} \quad (5)$$

with:

$[CO_2]_{KOH}$: concentration of CO_2 produced by yeast and reacted with KOH (g l^{-1})

[KOH]: base KOH concentration in the culture medium (mol l^{-1})

M_{CO2}: molar mass of CO_2 (44 g mol^{-1})

From the above calculation, 3.91 g l^{-1} of CO_2 would have been produced during yeast monoculture but only 2.97 g l^{-1} of CO_2 was measured based on the KOH used. This means that 0.94 g l^{-1} of CO_2 remained in solution and/or passed into gaseous phase (Fig. 10b).

CO_2 mass balance for *S. cerevisiae* and *C. vulgaris* in mixed culture No. 2

The CO_2 production and biofixation was studied only in mixed culture No. 2 since the dominance between microalgae and yeast was reached in this mixed culture and not in mixed culture No. 1.

In mixed culture No. 2, the KOH solution was added during the first 39 h of culture corresponding to yeast growth. As explained above, the *S. cerevisiae* behavior was similar in both mixed culture No. 2 and in the

reference yeast monoculture (Fig. 10a); again the assumption was made that the KOH solution was mainly added to the mixed culture No. 2 to compensate for the medium acidification by the CO_2 release by the yeast. KOH (283 ml) was added during the growth phase of the yeast corresponding to 5.7×10^{-2} mol l^{-1} of CO_2 equivalent to 2.49 g l^{-1} of CO_2 (equation 3 and 5).

In the yeast reference monoculture 2.97 g l^{-1} of CO_2 reacted with KOH whereas in mixed culture No. 2 only 2.49 g l^{-1}, of CO_2 reacted with KOH. The difference in CO_2 concentration most likely corresponds to the amount of CO_2 assimilated by microalgae in the mixed culture: 0.48 g l^{-1} of CO_2 i.e. 0.13 g l^{-1} of carbon. This concentration of carbon is coherent with the concentration of carbon required for the *C. vulgaris* biomass measured in mixed culture No. 2; 1.5×10^{7} cells ml^{-1} of *C. vulgaris* was produced corresponding to a dry weight of 0.23 g l^{-1} or 8.8×10^{-3} mol l^{-1} (the microalgae composition is $C_1H_{1.78}N_{0.165}O_{0.495}$ according to Scherholz and Curtis, 2013), and consequently 0.11 g l^{-1} of carbon was required for the microalgae biomass production. Hence, the amount of carbon fixed by microalgae was determined by two different methods; the carbon fixation by *C. vulgaris* calculated from the microalgae biomass concentration corresponded to 85% of that calculated from the KOH consumption.

Discussion

The aim of this work was to establish a symbiotic relationship between a heterotroph organism and an autotroph organism based on gaseous (CO_2) exchange. For this relationship to be useful in terms of in situ CO_2 mitigation, the rates for CO_2 production and consumption must be equal and in order to achieve this neither organisms must dominate, hence a co-dominant culture is necessary.

One of the main challenges in developing a mixed culture of a heterotroph and an autotroph is the selection of medium that would allow the co-dominance of the two species. As the μ of *C. vulgaris* is considerably smaller (slower) than that of *S. cerevisiae*, the growth medium was designed to favor *C. vulgaris* development and to limit *S. cerevisiae* growth. The MBM-GP medium was a good compromise for a co-dominant culture of *C. vulgaris* and *S. cerevisiae* (Fig. 5). Both organisms were able to grow in this medium based on the available nitrogen and carbon for both *C. vulgaris* and *S. cerevisiae*: *C. vulgaris* could obtain nitrogen from nitrate, both organisms could access the short peptides and amino acids in the peptone and *S. cerevisiae* could additionally use the NH_3 supplied by the peptone. Glucose as carbon source would be available to both organisms but CO_2 would be additionally available to *C. vulgaris*. *S. cerevisiae* growth was limited by the availability of assimilable nitrogen to this organism. *S. cerevisiae* did not grow in the absence of peptone, as was the case with MBM-G medium. The addition of yeast extract to MBM-G medium to give MBM-GY medium provided a nitrogen source as well as other nutrients that could be used by the yeast for growth, however, the addition of yeast extract to the growth medium proved toxic to *C. vulgaris*. Finally, the MBM-GP medium allowed growth of both yeast and microalgae and also compensated for the higher μ of yeast by limiting the maximum yeast population at 10% of the microalgae population.

Having designed a medium suitable for the growth of both organisms, three reference cultures were grown in photo-bioreactor: a fermentative culture of *S. cerevisiae* without aeration (MBM-GP medium), a culture of *C. vulgaris* under mixotrophic conditions with continuous aeration (MBM-GP medium) and an autotrophic culture of *C. vulgaris* with continuous aeration and in the absence of glucose (MBM medium). Two mixed cultures were also grown without aeration in MBM-GP medium.

The glucose was mainly fermented by *S. cerevisiae* in monoculture (Fig. 6) although the possibility of some respiration cannot be ruled out. *S. cerevisiae* mixes respiration and fermentation in the presence of O_2 and when external glucose concentration exceeds 0.8 mmol l^{-1} (0.1 g l^{-1}) (Verduyn et al. 1984; Otterstedt et al. 2004). This phenomenon is called the "Crabtree effect" (Verduyn et al. 1984).

Under mixotrophic conditions *C. vulgaris* in monoculture (Fig. 7a), grew without using glucose and O_2 at the beginning of the culture, which, indicated photoautotrophic growth of the organism also reported by Ben Amor-Ben Ayed et al. (2017). After 48 of incubation, *C. vulgaris* started to grow heterotrophically using glucose and O_2. *C. vulgaris* seems to "privilege" autotrophy as long as the microalgae population is small enough to allow satisfactory light penetration into the PBR. After that, *C. vulgaris* seems to have, at least, partly switched to heterotrophic metabolism. Microbial growth leads to an increase in light absorption and auto-shading by the microorganisms (Pfaffinger et al. 2016). The population in the shaded volume (central section of the PBR) may have used glucose and O_2 for growth through respiration, while the population in the lit volume (at the edge of the PBR and closed to the light source) could have grown photoautotrophically. In a well-mixed culture, as employed in this study, this means that as the average amount of light available to each cell decreases, *C. vulgaris* increasingly progresses towards a more heterotrophic metabolism.

In mixed culture, *S. cerevisiae* with its higher μ could be expected to rapidly consume all glucose before *C. vulgaris* would have time to grow heterotrophically. This means that the latter would grow fully photoautotrophically in mixed culture. For this reason, a reference culture of *C. vulgaris* was grown under photoautotrophic conditions in the usual growth medium used for this purpose; MBM medium (Fig. 7b).

For the *C. vulgaris* photoautotrophic monoculture the exponential growth phase was followed by a longer linear growth phase from the point where the population density increased beyond 2×10^7 cells ml^{-1}. This is most likely due to light limitation; once the culture reaches a certain population density that would result in considerable autoshadowing and restricted light penetration into the core of the culture. The growth would then be directly related to the light arrival rate, which is constant resulting in linear growth.

In summary, the *S. cerevisiae* monoculture (Fig. 6) and the microalgae *C. vulgaris* autotrophic monoculture (Fig. 7b) were used as reference cultures to compare with the mixed cultures. The respective μ were used to adjust the microalgae:yeast inoculation ratio to 30:1 (equation 1) in an attempt to minimize the possibility of yeast domination in the mixed culture. Two mixed cultures were grown without aeration where an attempt was made to coordinate the growth of the two organisms by adjusting their respective inoculation rates as described above.

In the first mixed culture (No. 1) (Fig. 8), *S. cerevisiae* consumed the glucose within the first 48 h as had been observed in the reference yeast monoculture (Fig. 6). The *S. cerevisiae* biomass and ethanol production were the same for both the mixed culture and the yeast reference culture. Since no glucose was available for the microalgae, *C. vulgaris* probably grew fully photosynthetically in the mixed culture. This was additionally supported by the observation that in the *C. vulgaris* monoculture in the presence of glucose, the glucose was not consumed during the first 48 h (Fig. 7a). On the other hand, *C. vulgaris* growth in mixed culture was weak with the maximal microalgae population 2.5 times lower than the reference monoculture (9×10^6 and 2×10^7 cells ml^{-1} respectively) (Fig. 7b). Ethanol or CO_2 toxicity can both be excluded as reasons for this low microalgal biomass production, as discussed below. Although the *C. vulgaris* population was weak, the microalgal cells remained active during the entire experiment (168 h). During the latter phases of the experiment, there were instances where the sun shone directly on the PBR; intermittent negative pCO_2 troughs and concomitant positive pO_2 peaks were observed during these transient periods. This can be taken as a strong indicator that both organisms in the mixed culture were metabolically active and that synergy effects between yeast and microalgae occurred. The final pCO_2 concentration reached almost its initial level indicating that in principle, *in situ* CO_2 mitigation in mixed culture is feasible, although the efficiency of the process remains to be improved.

A second mixed culture (No. 2) was grown to increase the microalga population in mixed culture (Fig. 9). The *C. vulgaris* inoculum was prepared in the same medium as used for the mixed culture (MBM-GP) in order to pre-adapt the organism to this medium and promote immediate growth of *C. vulgaris* straight after inoculation into the photo-bioreactor. *C. vulgaris* grew straight away from the start reaching a maximum population 2.7 times higher than that of the first mixed culture. By modifying the preparation of the inoculum, it was possible to achieve the same population concentration for both organisms (2×10^7 cells ml^{-1}). Presumably the pre-adaptation allowed *C. vulgaris* to have the enzymes necessary for the utilization of the amino acids and the small peptides present in the peptone in the photo-bioreactor medium. Like in the first mixed culture, *C. vulgaris* grew on the CO_2 produced by *S. cerevisiae* as there was no other source of CO_2. Of the CO_2 produced by *S. cerevisiae* in mixed culture No. 2, 12% was consumed directly by *C. vulgaris*, and the 64% of CO_2 captured by the KOH was in the HCO_3^- form and still available to the microalgae for utilization (Fig. 10c).

Ethanol is known to exhibit antimicrobial activity by attacking cell membranes (Patra et al. 2006) and it was important to assess the potential toxicity of the ethanol produced by *S. cerevisiae* in mixed culture on *C. vulgaris*. Firstly, the growth of the microalgae in the mixed culture No.2 provided the first indication that at 4 g l^{-1}, ethanol was not toxic to *C. vulgaris*. This observation was further confirmed with a shake-flask experiment were exogenous ethanol (2, 4 and 6 g l^{-1}) was added to growing autotrophic cultures of *C. vulgaris*. The same growth profiles were observed for both control cultures and cultures containing ethanol, even at concentrations higher than those measured in mixed cultures. The cell viabilities of these monocultures were high (approx. 100%) even at the end of the incubation period (411 h).

In conclusion, in order to encourage mutual symbiosis, we developed a mixed culture of *C. vulgaris* and *S. cerevisiae* in PBR in a way that neither organism dominated the other in terms of population concentration. The method developed for simultaneous cell enumeration with flow cytometry permitted to rigorously monitor the two populations in the mixed culture. The dissolved O_2 and CO_2 probes brought relevant measurements that allowed us to follow gas evolution. The results indicated that the medium design, the culture conditions, the inoculum ratio and the *C. vulgaris* inoculum preparation all contributed for co-dominance of the two species. By comparing the physiological behavior of microalgae and yeast in monoculture and mixed culture, co-dominance and a mutual symbiosis based on *in situ* gas exchange were demonstrated. This work opens the perspective for *in situ* CO_2 mitigation, full utilization of the organic substrate and a reduction in aeration costs of biotransformation processes.

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Compliance with ethical standards

- Conflict of Interest Statement

The authors declare no conflict of interest.

- Statement of Informed Consent, Human/Animal Rights

This article does not contain any studies with human participants or animals performed by any of the authors.

- Declaration of authors agreement

The authors declare their agreement to authorship and submission of the manuscript for peer review.

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647

Figure captions

Fig. 1 A coupled culture (a) and a mixed culture (b) of heterotrophic and autotrophic organisms; (a) gases pass from the liquid phase of the heterotrophic culture into a gaseous phase (blue dashed arrows) and they then pass from the gaseous phase into the liquid phase of the photo-bioreactor (red solid arrows); (b) Diagram of a mixed culture of heterotrophic and autotrophic organisms with CO_2 produced by heterotrophic metabolism (the gases are generated and reused in situ)

Fig. 2 Flow chart for the development of a co-dominant mixed culture between *S. cerevisiae* and *C. vulgaris*

Fig. 3 Diagram showing the closed PBR configuration used for the mixed culture of microalgae and yeast. The culture was not aerated, and the bioreactor was fitted with a fermentation lock, by using a U-tube manometer filled with 200 mm of H_2O. The pO_2 was monitored continuously using the immersed optic pO_2 probe and the pCO_2 sensors was placed in a loop that passed culture over it continuously by means of a peristaltic pump

Fig. 4 Validation of simultaneous enumeration of *C. vulgaris* and *S. cerevisiae* by flow cytometer; experimental *versus* reference ratios of *C. vulgaris* in eleven mixed cells suspensions (black circles) at different microalgae:yeast ratios

Fig. 5 Design of a specific medium for mixed culture; maximum population of *S. cerevisiae* GFP (light grey) or *C. vulgaris* (dark grey) in monoculture using three candidate media for mixed culture. Each monoculture of yeast or microalgae was performed in shake-flask and in duplicate

Fig. 6 *S. cerevisiae* monoculture in non-aerated PBR using heterotrophic MBM-GP medium. The yeast population is represented by orange circles. Glucose (filled triangles) and ethanol (empty triangles) are represented by symbols connected by dashed lines. Dissolved O_2 is represented by blue solid line. Error bars represent standard deviations of duplicate analyses of population concentration

Fig. 7 *C. vulgaris* monoculture in aerated PBR using heterotrophic MBM-GP medium (a) and *C. vulgaris* monoculture in aerated PBR using autotrophic MBM medium (without glucose and peptone) (b). The microalgae population is represented by green squares. Dissolved O_2 is represented by blue solid line and glucose (filled triangles) by symbols connected by dashed lines. Error bars represent standard deviations of duplicate analyses of population concentration

Fig. 8 Mixed culture No. 1 of *C. vulgaris* and *S. cerevisiae* in closed and non-aerated PBR using MBM-GP medium. The yeast inoculum was prepared using heterotrophic MBM-GP medium while the microalgae inoculum was prepared in autotrophic MBM medium. The yeast population is represented by orange circles and the microalgae population by green squares. Glucose (filled triangles) and ethanol (empty triangles) are represented by symbols connected by dashed lines. Dissolved O_2 and CO_2 are represented by blue dashed line and red solid line respectively. Error bars represent standard deviations of duplicate analyses

Fig. 9 Mixed culture No.2 of *C. vulgaris* and *S. cerevisiae* in closed and non-aerated PBR using MBM-GP medium. The yeast and microalgae inocula were both prepared in heterotrophic MBM-GP medium. The yeast population is represented by orange circles and the microalgae population by green squares. Glucose (filled triangles) and ethanol (empty triangles) are represented by symbols connected by dashed lines. Dissolved O_2 is represented by blue solid line. Error bars represent standard deviations of duplicate analyses

Fig. 10 Automatic addition of base KOH solution in *S. cerevisiae* monoculture and in mixed culture No. 2 for pH adjustment at 6.5 (a). Repartition of CO_2 produced by *S. cerevisiae* in *S. cerevisiae* monoculture (b) and in mixed culture No. 2 (c). Error bars represent standard deviations of duplicate analyses of yeast population concentration

Figures

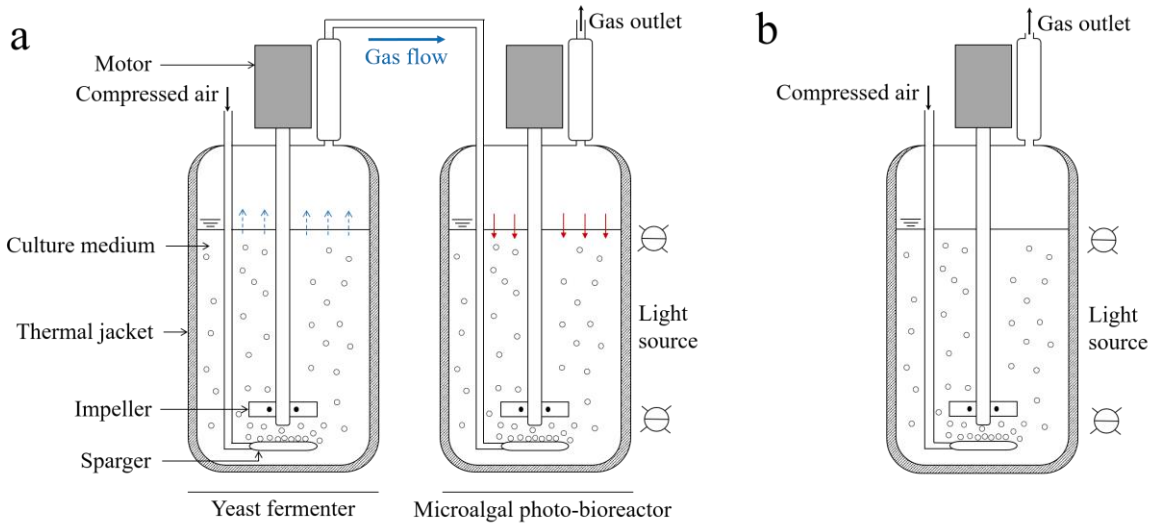
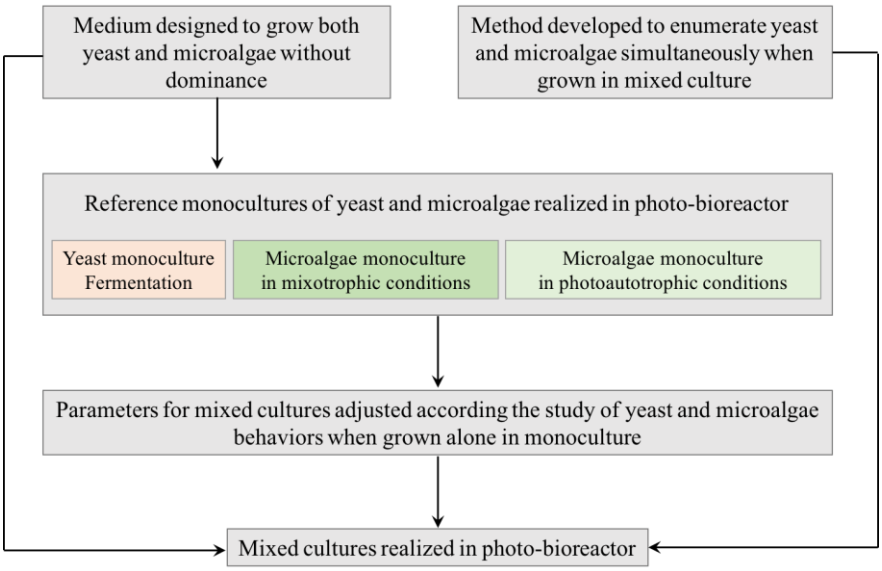


Fig. 1 program: PowerPoint



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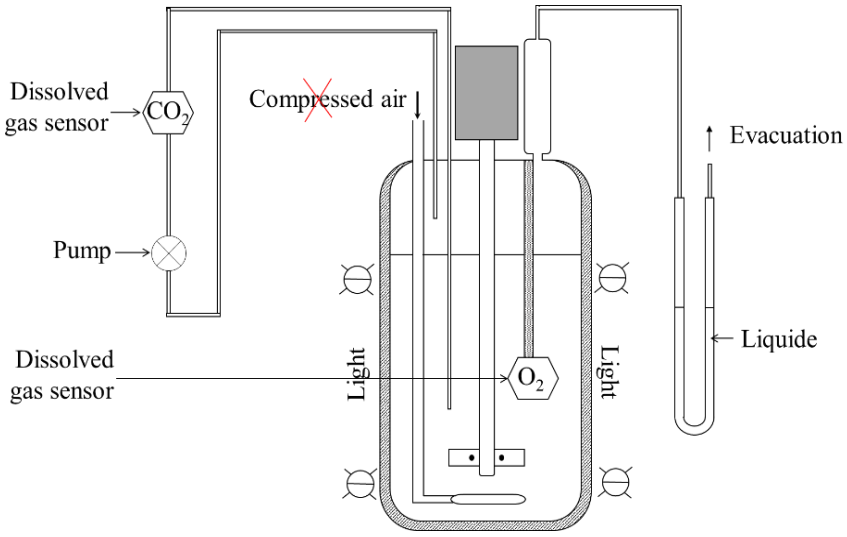

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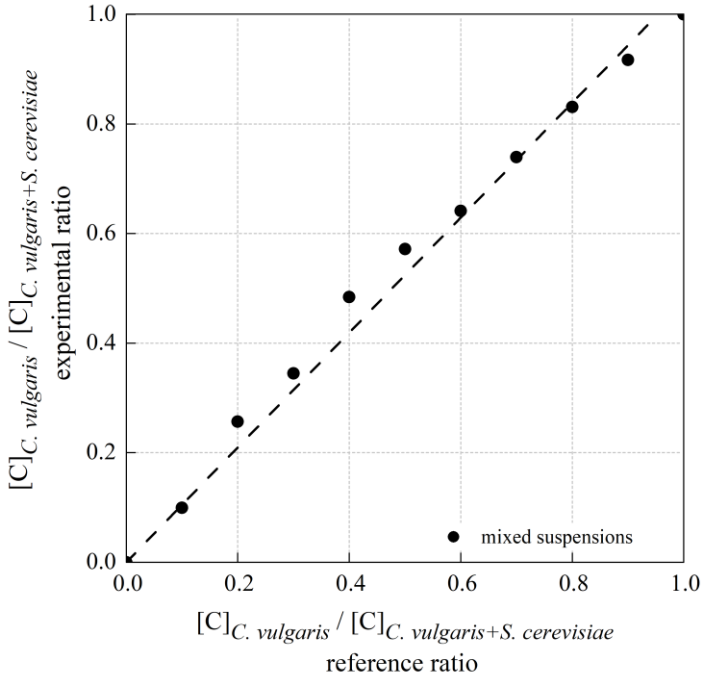

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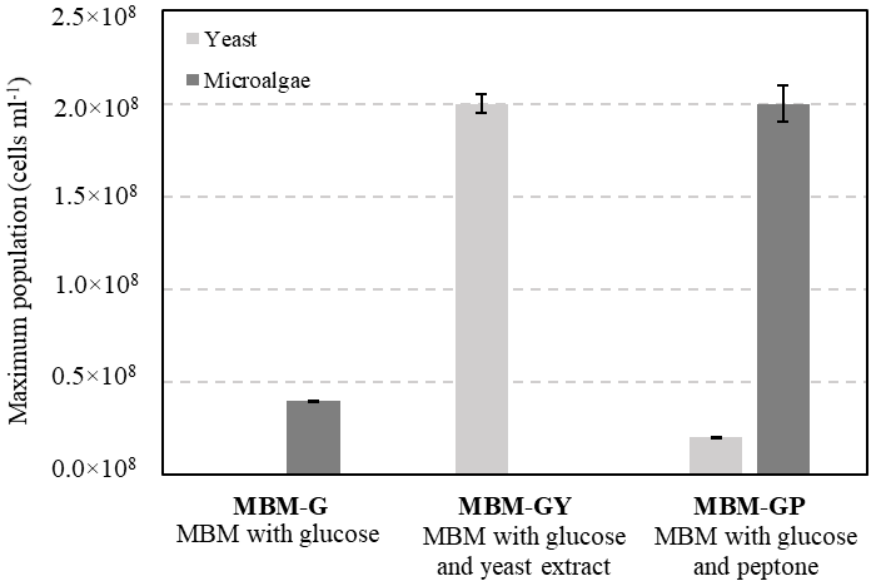

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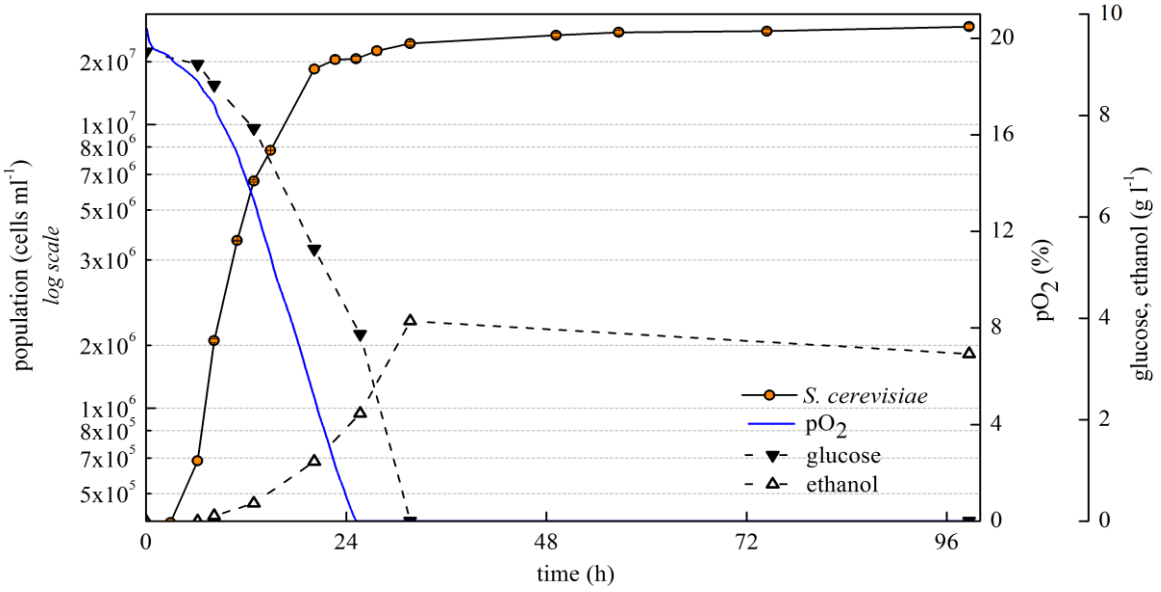

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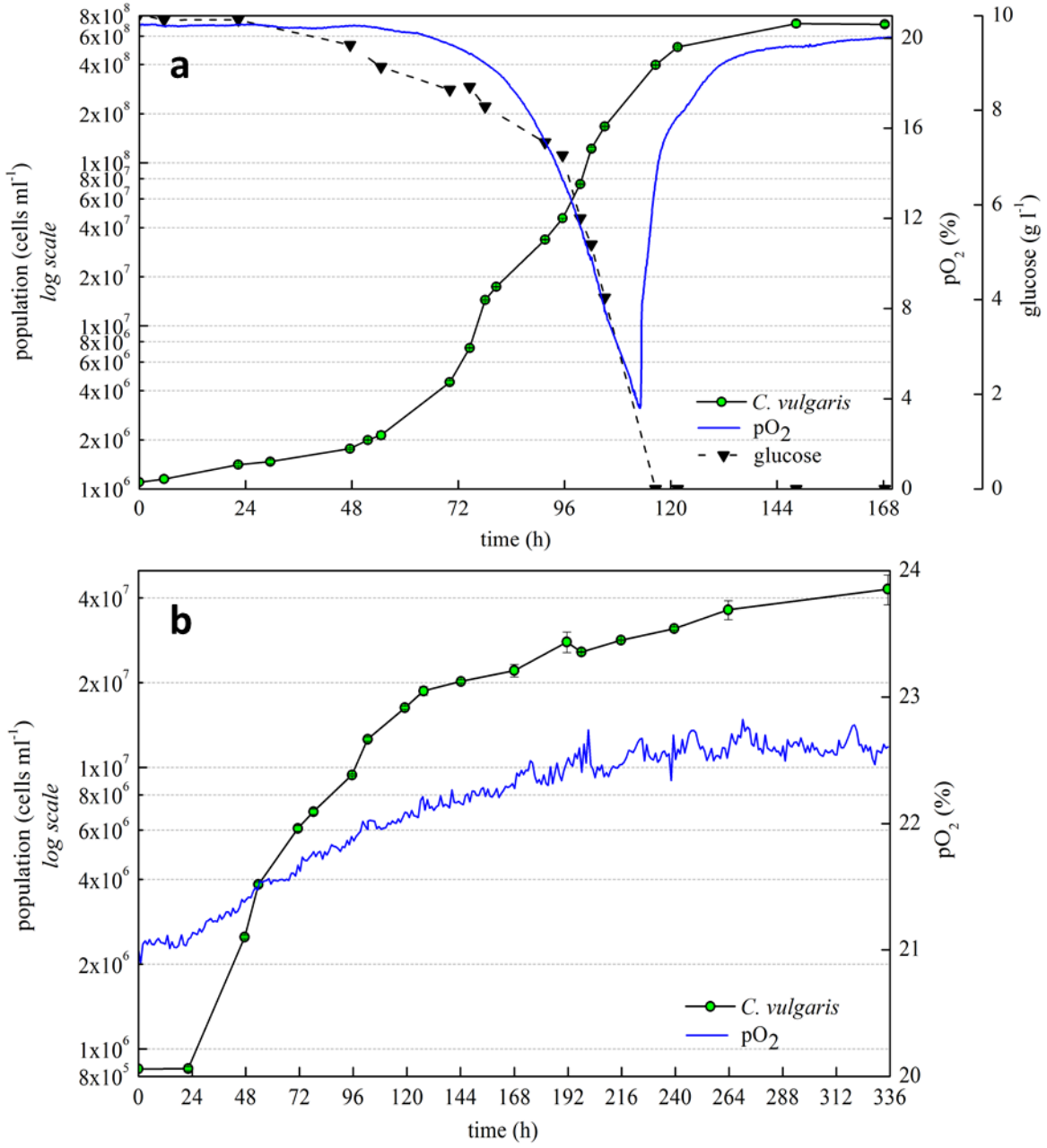

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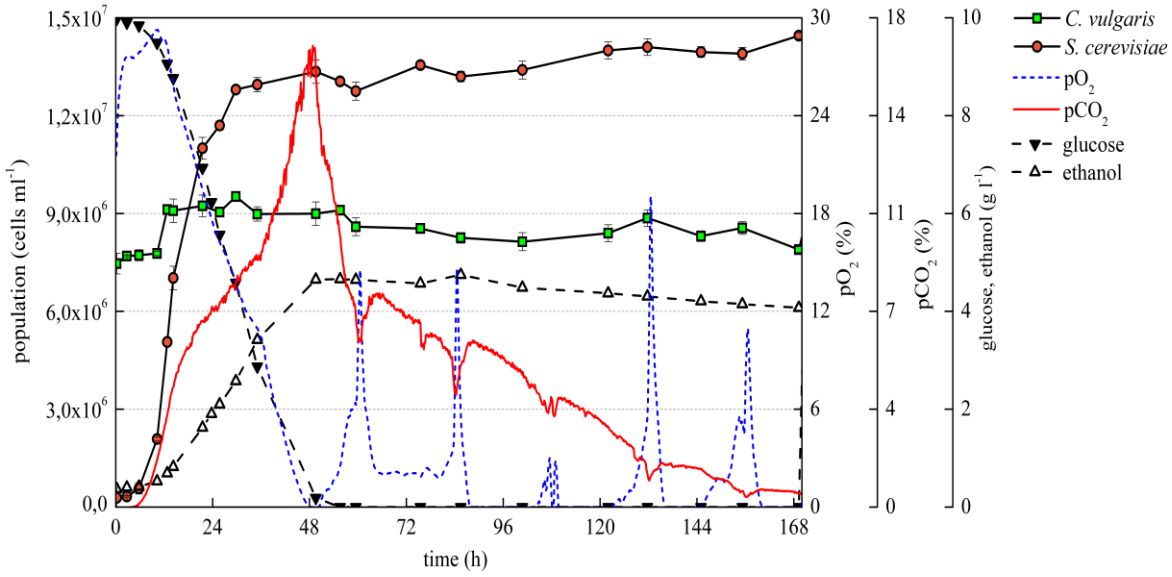

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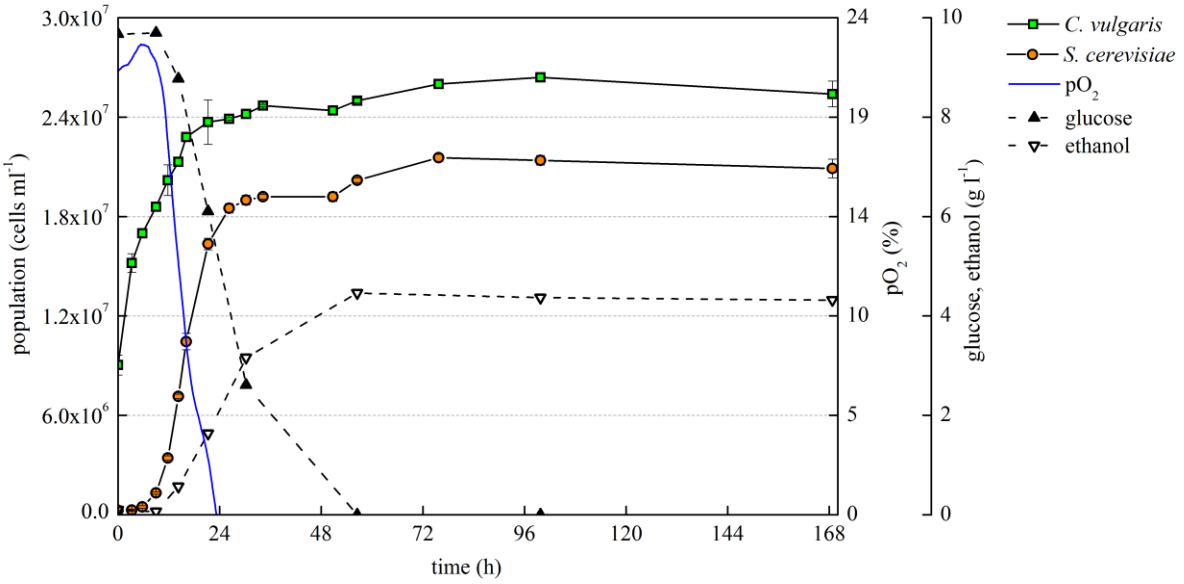

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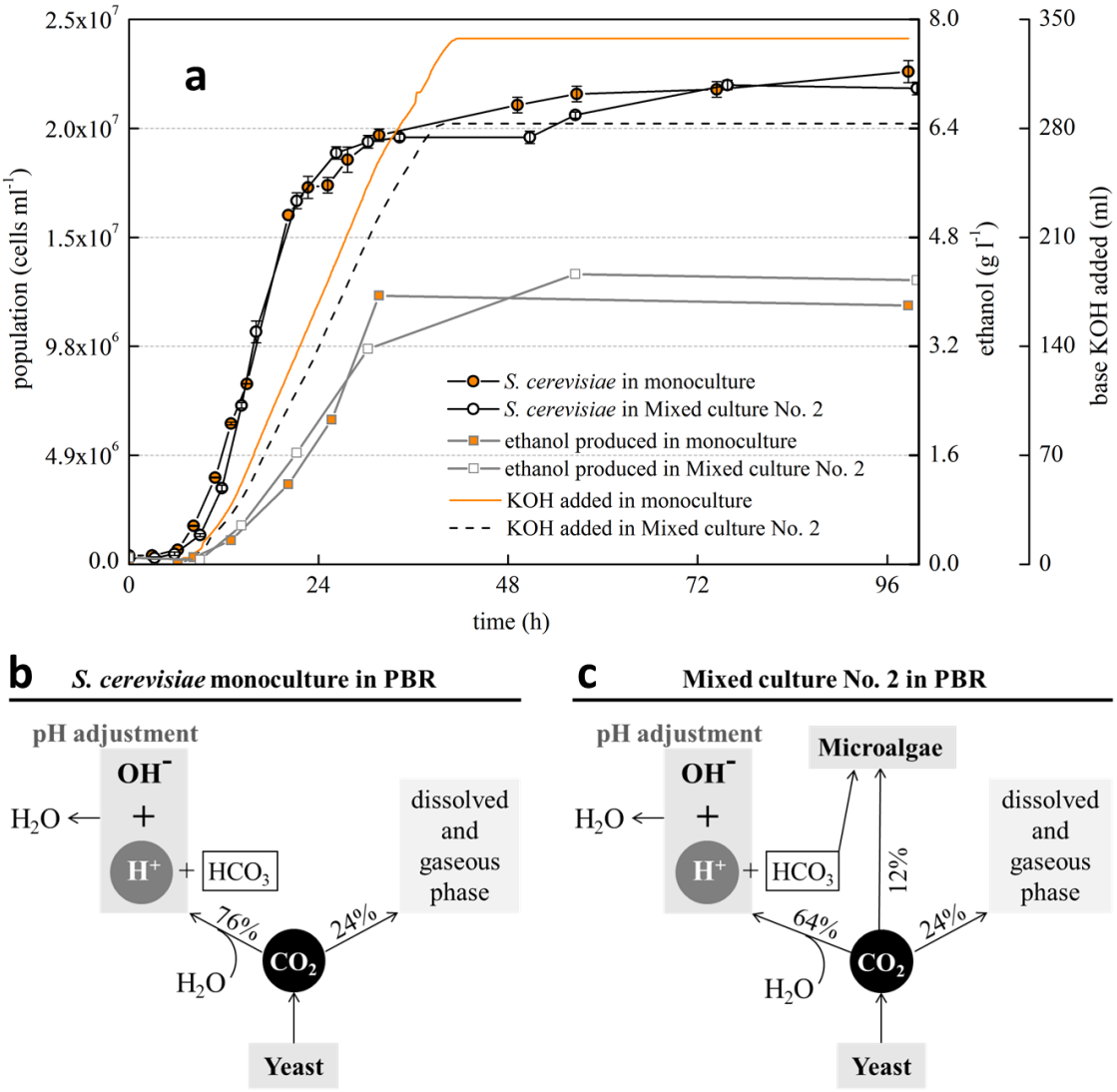

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724 **Fig. 10** program: OriginPro (a) and PowerPoint (b and c)