

Wageningen University & Research

MIB - MicFys

Biotechnology MSc

MSc Thesis:

**Understanding Microbial Interactions
between *Geobacter sulfurreducens* and
Saccharomyces cerevisiae in synthetic
cocultures**

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February 2025



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Wageningen University & Research



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ACKNOWLEDGEMENTS

I would like to start by thanking Dr. Martijn Diender and Isabelle Keijsers for their remarkable guidance and support throughout the entire thesis. I am extremely grateful to have had supervisors that created such a welcoming and comfortable environment, where sharing views and ideas was always encouraged. Their mentorship has made this experience truly enriching and has allowed me not only to grow on an academic and personal level, but also to become more eager to continue exploring the world of microbiology.

I would also like to thank Dr. Diana Souza, the group leader, for giving me the opportunity to be a part of such an inspiring team. I am also grateful to the team, for always being willing to offer advice, bake cakes and making my time at the lab so memorable.

Finally, I am deeply grateful to my family for their unwavering love, support and encouragement throughout this whole experience.

ABSTRACT

Microbial communities play crucial roles in the earth's ecosystem and often rely on intricate intra- and interspecies interactions, such as extracellular electron transfer (EET). EET enables microorganisms to receive and donate electrons from and to external compounds, for instance (in)soluble metal ions, electrodes and even other microorganisms. While EET has been widely studied in bacteria, particularly in the *Geobacter* genus, some yeasts, including *Saccharomyces cerevisiae*, have shown EET activity as well. However, little is known about electron exchange between eukaryotes, such as yeast and bacteria. This thesis focuses on the potential interactions in a coculture with the electroactive microorganisms, *G. sulfurreducens* and *S. cerevisiae*. Cocultures were successfully established and proliferated through multiple transfers, confirming an interplay between the microorganisms. Differences in the yeast metabolic activity were observed in coculture compared to the monoculture, including increased malate production, the formation of aggregates and the slowing down of the yeast's metabolism. In coculture, *G. sulfurreducens* was able to grow without the presence of any known electron donor. While the exact mechanism of the interaction remains unclear, experimental results suggest the existence of a parasitic relationship, in which *G. sulfurreducens* exploits *S. cerevisiae*'s metabolic resources. It is proposed that acetate secreted by the yeast becomes the electron donor for *G. sulfurreducens*, to reduce fumarate into succinate. The succinate is hypothesized to subsequently enter the yeast, resulting in the production of more fumarate and malate, a process that would require energy from *S. cerevisiae*. This study provides the foundation for further investigation on the interaction and metabolic interplay between these species and their potential biotechnological applications.

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1. INTRODUCTION

Microbial communities consist of diverse species that coexist in constant communication and interaction with each other. They play critical roles in maintaining the earth's ecosystem, participating in processes such as nutrient cycling, organic matter decomposition or xenobiotic degradation (Mishra et al., 2021). Their adaptability, resilience and diverse metabolic abilities have turned them into valuable assets in many biotechnological processes, such as bioremediation or fermentation (Zhou et al., 2024). For the study and development of these communities, it is essential to be able to culture them in the laboratory. Different methods have been used throughout the years, primarily pure cultures and open mixed cultures. The first, consist of isolated microbial strains, which provide a very controlled environment, highly valuable in areas like genetic engineering. Nonetheless, considering that microorganisms naturally exist in communities, forming different symbiotic relations with other organisms, pure cultures tend to overlook many physiological and metabolic properties. On the other hand, open mixed cultures, consisting of a group of undefined microorganisms, preserve diversity and syntrophic interactions. Under these conditions, organisms behave similarly to how they would in nature, but the large number of interactions taking place makes them challenging to assess, observe and manipulate. (Diender et al., 2021). In recent years, however, a different method has been gaining increasing attention, where two or more strains of interest are grown under controlled, specific conditions. This technique, called "coculturing", allows microorganisms to interact in a defined and controlled environment. In cocultures, microorganisms take advantage of processes like division of labor, metabolic cross talk and compartmentalization of reactions, which provide adaptability and robustness to perturbations (Diender et al., 2021; Xu et al., 2020).

Throughout this study, cocultures will be established to better understand the forms of interaction between a facultative anaerobic yeast, *Saccharomyces cerevisiae*, and an anaerobic bacterium, *Geobacter sulfurreducens*, recognized for its ability to extracellularly exchange electrons (Caccavo et al., 1994; Lovley et al., 2011; Parapouli et al., 2020). This work hypothesizes that these microorganisms might interact by interchanging electrons. It is believed these types of microorganisms could coexist and communicate in specific environments, such as oxic-anoxic interfaces, where oxygen gradients create niches for diverse microbial interactions (Borer et al., 2018). Coculturing these microorganisms could uncover novel interaction mechanisms between eukaryotic and prokaryotic partners.

1.1 Extracellular and Interspecies Electron Transfer (EET, IET)

Microorganisms have developed many forms of interaction, including interspecies signaling and metabolite exchange (Hughes & Sperandio, 2008). However, one that has been broadly researched is the transfer of electrons between microbes, known as Extracellular Electron Transfer (EET). This process allows a distinct group of microorganisms, referred to as Electroactive Microorganisms (EAMs), to exchange electrons with extracellular acceptors or donors (Zhao et al., 2021). This unique characteristic allows them to thrive in environments that can be considered toxic or inhabitable to other microorganisms. For instance, bacteria from the genera *Geobacter* and *Shewanella* are able to grow surrounded by heavy metals, such as iron (Fe(III)), manganese (Mn(IV)) or uranium (U(VI)) (T. Lin et al., 2017; Methé et al., 2003). Additionally, EAMs have been key in the development of microbial fuel cells (MFCs) and other bioelectrochemical systems for their ability to generate an electrical current (Garbini et al., 2023). With EET, microorganisms can gain energy for growth from accepting electrons from an extracellular electron donor or from donating electrons to external acceptors. These EET mechanisms can be mediated (MET) or direct (DET). During MET, microorganisms use endogenous or exogenous redox active compounds or mediators to shuttle electrons from the donor to the acceptor (Figure 1). On the other hand, during DET, microorganisms physically interact with the extracellular donor or acceptor in order to transfer the electrons (Figure 1). DET can be advantageous for the microorganisms since they don't need synthesis or presence of mediators in the environment (Uekia, 2021).

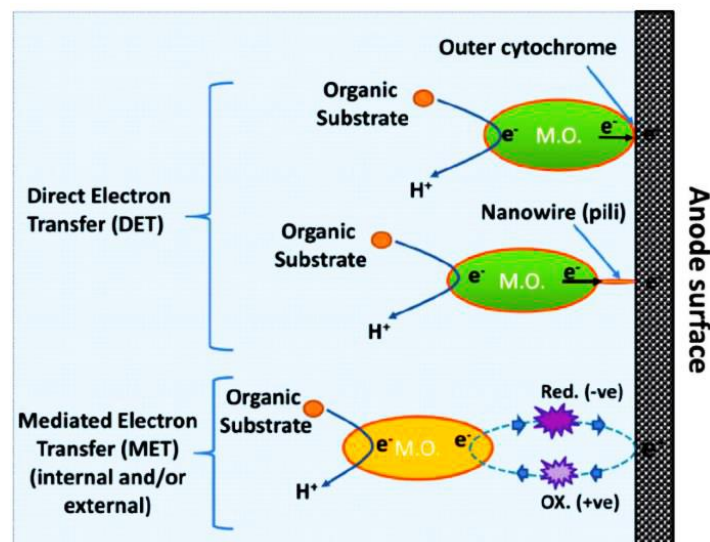


Figure 1. Electron transfer mechanisms. Schematic representation of direct electron transfer (DET) via cytochromes and nanowires and mediated electron transfer (MET) via redox active compounds. M.O: Microorganism. Image from (Sayed et al., 2017)

While much of the research has focused on how these microorganisms exchange electrons with electrodes, soluble metals, and other substrates, recent interest has shifted towards understanding how different EAMs interact with each other (Holmes et al., 2022; Liu et al., 2021; Zhuang et al., 2022). Exchange of electrons between microbial partners is known as interspecies electron transfer (IET), where electrons are also exchanged either directly (DIET) or through mediators (MIET) (Su et al., 2023). In MIET, soluble chemical intermediates like hydrogen (H₂) or formate are used as electron shuttles (Gupta et al., 2023). In these interactions, the donor organism oxidizes an organic substance and reduces, for instance, protons (H⁺) to H₂ or carbon dioxide (CO₂) to formic acid. The reduced H₂ or formate then transfer the electrons to the acceptor organism (Shen et al., 2016). A less explored mechanism of MIET is the quinone-mediated IET (QUIET). In this process, compounds containing quinones, like humic substances, abundant in soils and sediments, become the electron shuttles. A study demonstrated that in a coculture of *Geobacter metallireducens* and *Wolinella succinogenes*, a humic substance analog, Anthraquinone-2,6-disulfonate (AQDS), was able to transport electrons between the two (Lovley et al., 1999). Instead, DIET occurs through direct physical contact between the microorganisms. Two primary mechanisms have been explored. On the one hand, DIET can occur through conductive nanowires or pili, which consist of approximately 3nm long filaments located in the microorganism's membrane, helping transport electrons. On the other hand, electron transfer chain components like flavins, quinones or multi-haem cytochromes can take part in DIET (Gupta et al., 2023). C-type cytochromes are a group of diverse haemoproteins which play a critical role in the electron transport processes in the cell. They are characterized by the presence of a haem group covalently attached to a polypeptide chain through two thioether bonds (Allen et al., 2002). The iron ion in the haem groups alternates between an oxidized (Fe(III)) and a reduced (Fe(II)) state to aid in the electron transport.

1.2 Electroactive Microorganisms: *Geobacter sulfurreducens*

G. sulfurreducens was the second species in the *Geobacter* genus to be isolated, after *Geobacter metallireducens*. The bacterium was found to grow utilizing energy from the complete oxidation of acetate to CO₂ coupled to the reduction of Fe(III) to Fe(II), bringing attention to *Geobacter's* characteristic EET abilities (Figure 2) (Frühauf-Wyllie & Holtmann, 2022). *G. sulfurreducens* became the first *Geobacter* spp. to have a fully sequenced genome and be amenable for genetic manipulation, which has greatly contributed to its use as a model organism for research on *Geobacter* metabolism and EET mechanisms. (Lovley et al., 2011). They are gram-negative, rod-shaped bacteria commonly found in a variety of environments, particularly soils and sediments (Uekia, 2021). They are believed to be anaerobic, non-fermentative and non-motile. However, they are known to tolerate exposure to

atmospheric oxygen and to even use it as an electron acceptor at 10% concentrations (W. C. Lin et al., 2004). *G. sulfurreducens* oxidizes organic compounds via the tricarboxylic acid (TCA) cycle, generating electrons that are then transported to the extracellular electron acceptor (Figure 2) (Uekia, 2021). They are known to reduce compounds like Fe(III), Mn(IV), U(VI), elemental sulfur (S_8), fumarate and malate. However, they can also donate electrons to an anode. Their most commonly used energy source is acetate but can also use H_2 and lactate (Figure 2) (Anna Engel et al., 2020; Coppi et al., 2004; Reguera & Kashefi, 2019).

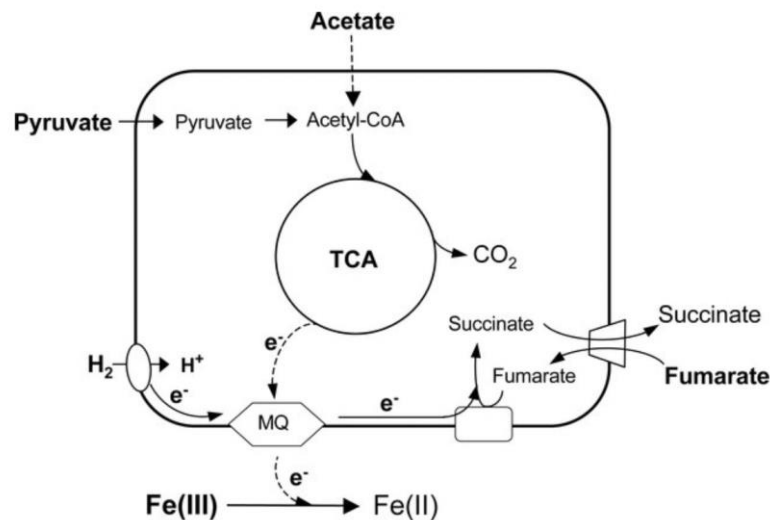


Figure 2. Metabolic pathways involved in EET in *G. sulfurreducens*. Acetate and pyruvate enter the cell and form Acetyl-CoA, which enters the TCA cycle. Electrons are generated during the TCA and are transferred to the menaquinone pool (MQ). Electrons from H_2 oxidation reach the MQ. The MQ passes the electrons to Fe(III) or to Fumarate reductase, which converts fumarate to succinate. Image from (Segura et al., 2008).

According to research, the *Geobacter* species encode many c-type cytochromes. *G. sulfurreducens*, in particular, encodes 111, 78 of which have more than one haem per cytochrome and are located in the inner membrane (e.g, Cbc complexes, ImcH), the periplasm (e.g, PpcA) or the outer membrane (e.g., OmcB, OmcS, OmcZ) (Figure 3) (Uekia, 2021). The haem groups facilitate electron transfer by acting as the electron shuttlers. In the inner membrane, the Cbc complexes are believed to transfer electrons from the menaquinone pool to the periplasmic cytochromes, while generating a proton gradient that allows for ATP synthesis (Figure 3). In the periplasm, PpcA cytochromes, among others, link the electron transfer between the inner and outer membrane (Figure 3). Finally, in the outer membrane, OmcB forms a complex with the porin-like protein OmbB and the periplasmic OmaB protein. This porin-cytochrome complex plays a key role in Fe(III) reduction in *G. sulfurreducens*. One of the most abundant proteins on their outer surface is OmcS, essential for Mn(IV) oxide and Fe(III) oxide reductions. OmcS can also be found along the conductive pili surface (Figure 3).

A final key cytochrome present in the outer cell membrane is OmcZ, which is thought to facilitate electron transfer from biofilms to the anode surface (Uekia, 2021). Regarding the electrically conductive pili or e-pili, shown in figure 3, multiple lines of evidence suggest that *G. sulfurreducens* needs them for long-range EET, which can be critical in environments where the acceptors are dispersed (Liu et al., 2021; Reguera et al., 2005; Wang et al., 2023). In *Geobacter*, the pili are type IV (T4P) and are encoded by the gene *piliA*. Several studies have found that *Geobacter* strains with a mutated *piliA* gene, while still able to reduce soluble electron acceptors like fumarate or Fe(III) citrate, were unable to reduce insoluble electron acceptors like Fe(III) or Mn(IV) oxides, highlighting their importance in the EET process (Tan et al., 2016. Reguera et al., 2005).

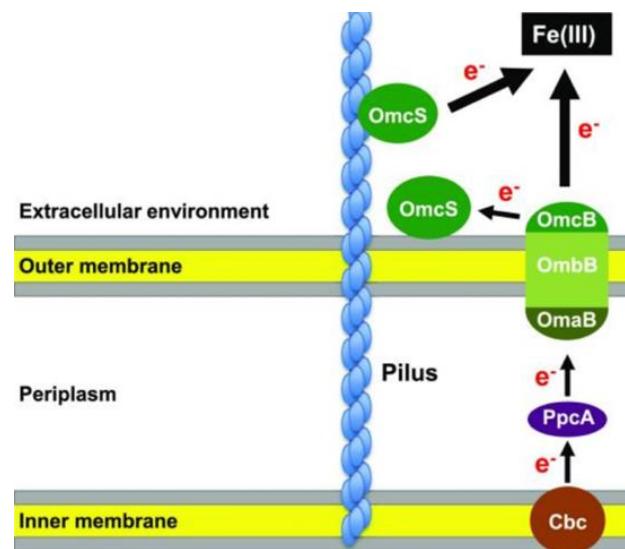


Figure 3. DET mechanism in *G. sulfurreducens*. Schematic representation of the mechanisms employed by *G. sulfurreducens* to transport electrons across the cell membrane to extracellular electron acceptors like Fe (III), including the conductive pili (Pilus) and the c-type cytochromes (Cbc, PpcA, OmaB, OmbB, OmcB and OmcS). Images from (Shen et al., 2016; Uekia, 2021).

G. sulfurreducens is also able to exchange electrons with other symbiotic partners. Studies with *G. metallireducens* and *G. sulfurreducens* cocultures has demonstrated that both H₂ and AQDS can be used as electron shuttles for MIET (Lovley et al., 1999; Summers et al., 2010). However, *G. sulfurreducens* is mostly known for engaging in DIET. As a matter of fact, DIET was first reported in a study where *G. metallireducens* and *G. sulfurreducens* were cocultured in a medium containing ethanol as the electron donor, which *G. sulfurreducens* cannot use, and fumarate as electron acceptor, which *G. metallireducens* cannot use. As a result, the bacteria formed aggregates, establishing direct electrical connections, enabling both species to grow (Summers et al., 2010). It was proposed that e-pili from both organisms come in contact and, with the help of c-type cytochromes, electrons are

transferred (Liu et al., 2018). Since then, studies on the interaction between *Geobacter* with different microorganisms have received an increasing amount of attention (Diender et al., 2021; Rotaru et al., 2014; Wang et al., 2023; Yang et al., 2024).

1.3 Electroactive Microorganisms: *Saccharomyces cerevisiae*

S. cerevisiae, commonly known as baker's yeast, has become one of the most studied eukaryotic model organisms. It is used in many biotechnological applications due to its tolerance to adverse conditions, such as low pH or high osmolarity, high fermentation efficiency and ease of genetic manipulation (Parapouli et al., 2020). In the fermentation process, *S. cerevisiae* mainly produces ethanol and CO₂ (Figure 4). However, glycerol is also a common product, which results from the reoxidation of the NADH generated during the ethanol production (Figure 4), and small amounts of organic acids like acetate or succinate can be secreted under oxygen-limited conditions (Pronk et al., 1996). Additionally, *S. cerevisiae*, like *Geobacter*, possessed the ability transfer electrons to external acceptors. However, the underlying mechanisms have not yet been fully defined (Hubenova & Mitov, 2015).

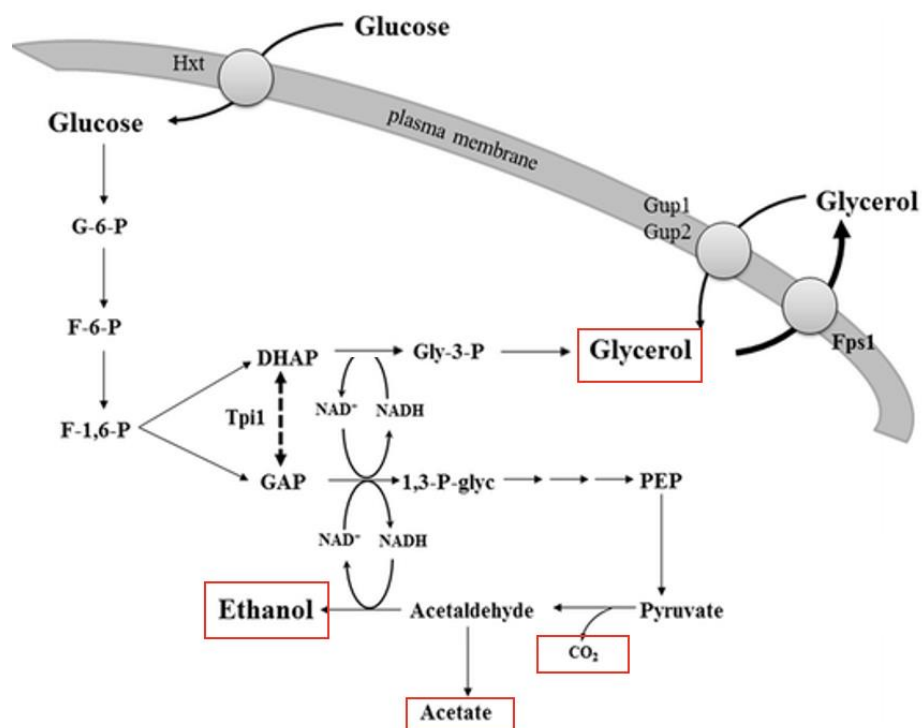


Figure 4. Glucose conversion to Ethanol and Glycerol in *S. cerevisiae*. Schematic overview of the central metabolic pathway in *S. cerevisiae*, highlighting the conversion of glucose to different products, shown in red boxes. Image adapted from (Semkiv et al., 2017).

In recent years, the use of yeasts has become an attractive alternative to the use of bacteria in MFCs due to their broad substrate spectrum, robustness, tolerance, fast growth and easy cultivation. However, much less is known about their EET mechanisms (Hubenova & Mitov, 2015). Several yeast strains, including *Candida melibiosica* 2491, *Arxula adenivorans*, *Hansenula polymorpha*, *Hansenula anomala*, *Kluyveromyces marxianus*, and *S. cerevisiae*, have been studied for their electrogenic capabilities. However, given that *S. cerevisiae* has been the most extensively researched (Verma & Mishra, 2021), this study will focus on the potential interactions of this microorganism with *G. sulfurreducens*.

In yeasts, EET can also be direct or indirect. In the indirect pathway, exogenic mediators, primarily methylene blue, are often used. The use of other organic dyes like methyl orange and methyl red has also been reported (Hubenova & Mitov, 2015). Not much is known about endogenous mediators, but it is believed some yeasts such as *C. melibiosica* 2491 and *A. adenivorans* have the ability to secrete redox active molecules. On the other hand, mediator-free yeast EET has been demonstrated in MFCs, showcasing DET capabilities (Hubenova & Mitov, 2015). While the exact mechanism of this process is unclear, some theories have been proposed. It is believed some yeasts, like *S. cerevisiae* display cytochromes on their surface, involved in the transfer of electrons (Verma & Mishra, 2021). Another study claims that *S. cerevisiae* might secrete flavins that participate in the EET (Christwardana et al., 2018). Some specific enzymes located in the outer membrane that might be involved in the DET in yeasts have also been identified: lactate dehydrogenase (LDH) and ferricyanide reductase (FCR) (Haslett et al., 2011; Prasad et al., 2007). Research has shown that *C. melibiosica*, *A. adenivorans* and *S. cerevisiae* are probably able to produce soluble electroactive compounds, whereas *H. anomala* can participate in mediator-free electron transport, transferring electrons via membrane redox proteins (e.g., FCR and LDH) (Haslett et al., 2011; Sayed et al., 2017). While research has identified potential mechanisms for EET in various yeast species, the precise functioning of EET and MET remains unknown. Further research is necessary to elucidate these processes and their mechanisms.

1.4 Aim of the Thesis

This study aims to investigate the potential interactions between *S. cerevisiae* and *G. sulfurreducens*, with a particular focus on extracellular electron transfer. It is hypothesized that *S. cerevisiae* is able to donate electrons to *G. sulfurreducens*, which could then use them to support its anaerobic respiration processes (Figure 5). Cocultures of these microorganisms will be established to test their co-dependence and ability to thrive in varying conditions, exploring the effects on the microorganisms of exogenic mediators as well as different electron acceptors and donors. Investigating the relationship between these species, of such different natures, could contribute to our understanding of microbial community dynamics and potentially unlock new opportunities for biotechnological applications in the future.

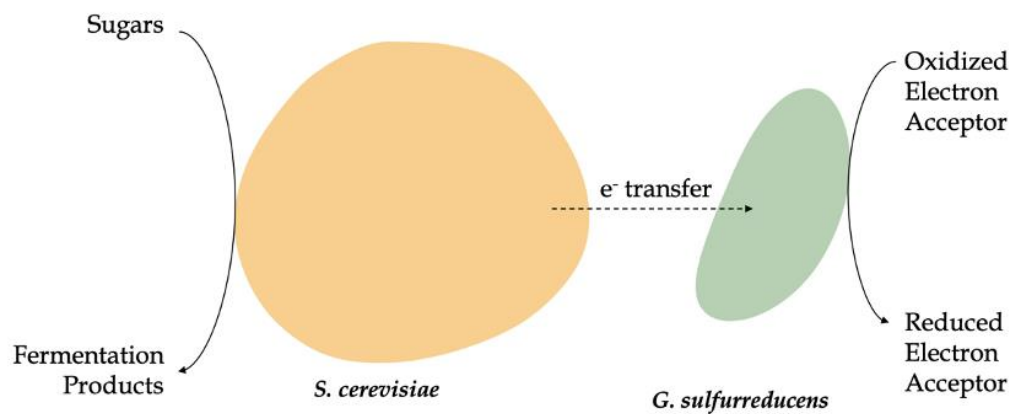


Figure 5. Proposed *G. sulfurreducens* and *S. cerevisiae* interaction. Schematic representation of the hypothetical interaction between the microorganisms. *S. cerevisiae*'s fermentation pathway results in the transfer of electrons to *G. sulfurreducens* in coculture, which can use them for anaerobic respiration.

2. MATERIALS & METHODS

2.1 Strains and Cultivation conditions

G. sulfurreducens (strain ATCC 51573 / DSM 12127 / PCA) and *S. cerevisiae* (strain ATCC 18824 / DSM 70449) were used throughout this study. The experiments were done in CP medium containing (per liter of medium): 0.5 mg resazurin, 0.408 g KH_2PO_4 , 0.534 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.3 g NH_4Cl , 0.3 g NaCl and 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ dissolved in deionized water. It was supplemented with acidic trace elements (0.036 g HCl , 0.062 mg H_3BO_3 , 0.099 mg MnCl_2 , 1.149 mg FeCl_2 , 0.119 mg CoCl_2 , 0.024 mg NiCl_2 , 0.068 mg ZnCl_2 , and 0.017 mg CuCl_2) and alkaline trace elements (0.400 mg NaOH , 0.026 mg Na_2SeO_3 , 0.033 mg Na_2WO_4 , and 0.024 mg Na_2MoO_4). Once all components were mixed, pH was measured and adjusted to 7, when needed, with 1 M NaOH and 1 M HCl . Next, the medium was boiled for 20 sec and immediately cooled, being subjected to N_2 gas flow. It was then distributed in 45 mL aliquots into 120 ml serum bottles, sealed with rubber stoppers and aluminum caps. The headspace of each bottle was filled with the desired gas mixture, 20% CO_2 and 80% N_2 , unless for two cases, the one using 20% CO_2 and 80% H_2 and the other 100% N_2 . Pressure was adjusted to 1.5 atm. Bottles were autoclaved at 121 °C for 20 min.

Afterward, each bottle was enriched with 0.5 ml of a vitamin and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution, previously prepared by adding 10 ml of 0.2 μm filter sterilized vitamin stock solution into 90 ml of a $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.11 g/L medium). The vitamin stock solution contained 20 mg biotin, 200 mg nicotinamid, 100 mg p-aminobenzoic acid, 200 mg thiamin, 100 mg panthothenic acid, 500 mg pyridoxamine, 100 mg cyanocobalamine and 100 mg riboflavin. Thereafter, yeast extract (0.5 g/L) and NaHCO_3 (4 g/L) were added to the medium. The final steps varied according to the experimental set-up. Unless specified otherwise, 5 mM glucose and 50 mM fumarate were added. No reducing agent was added to the cocultures, but 0.5 mM of sodium sulfide was added to *G. sulfurreducens* monocultures. For the maintenance of *G. sulfurreducens* stock cultures, 10 mM acetate, 40 mM fumarate and 0.5 mM sodium sulfide were used. *S. cerevisiae* stock cultures only required 5 mM glucose. All cultures were incubated at 30°C.

2.2 Sample Preparation and Analytical techniques

The pH of each sample was measured, using a calibrate pH meter (Proline pH meter B210). Next, growth of *G. sulfurreducens* and *S. cerevisiae* was followed by measuring the optical density (OD) at 600 nm using the Shimadzu UV-1800 UV/Visible Spectrophotometer (Shimadzu Corporation, Kyoto,

Japan). Before storage, samples were observed under a light microscope at 100 X magnification with oil immersion on a Zeiss microscope AXIO Scope A1. Images were captured using ZEISS AxioCam ICc 5 camera and the Zeiss ZEN 3.7 software, and further analyzed using ImageJ. Finally, samples were centrifuged at 1500 rpm for 2.5 min and stored at -20 °C.

2.2.1 Chromatography techniques

The metabolite concentrations of each sample were measured by High Pressure Liquid Chromatography (HPLC) using Shimadzu LC2030C (Schimazu, The Netherlands) with a MetaCarb 67 H column (Agilent Technologies, Santa Clara, CA). For the measurements, 100 µl of each sample was introduced into a well on a 96-well plate, previously containing 100 µl of Arabinose (0.2 N) or DMSO (0.2 N) as internal standard. For each sample, two different oven temperatures were used, both with a flow rate of 1 ml/min with an eluent of 0.1 N H₂SO₄. Acetate content was quantified at 30 °C and the rest of organic acids at 50 °C.

Gas Chromatography (GC) was used to measure the gas concentrations in the bottles. The Compact GC 4.0 (Global Analyser Solutions, The Netherlands) was used to analyze 0.2 ml gas samples using a thermal conductivity detector. Data acquisition and analysis for both GC and HPLC were performed using the Chromeleon™ data analysis software (Thermo Fisher Scientific), version 7.2.9.

2.2.2 Determination of Fe(II) and Fe(III)

The content of Fe (III) and Fe (II) was measured using the Ferrozine method, based on (Stookey, 1970). In this colorimetric method, 0.2 ml of medium was extracted from the serum bottles and introduced into an Eppendorf tube containing 1.8 ml of HCl (1 M) for extraction. After mixing and waiting for 15 min, the sample was diluted 1:10 in more HCl. Next a cuvette was filled with 100 µl of reagent A (10 mM ferrozine in 0.1 M ammonium acetate), 850 µl MilliQ water and 80 µl of the diluted sample. Absorbance was measured at 562 nm for Fe (II) content. For quantification of Fe (III), 187.4 µl of reagent B (1.4 M hydroxylamine-HCl in 2 M HCl) was added to the cuvette. Finally, after a 20 min of waiting time, the reagent C (10 M ammonium acetate) was added, and another absorbance measurement was carried out at 562 nm. Quantification of iron concentration was done based on the extinction coefficient of ferrozine upon binding of iron, which is 27.900 M⁻¹cm⁻¹.

2.3 Bioreactor operation

Two 1.5-L bioreactors (Applikon) were prepared for the larger-scale cultivation of *S. cerevisiae* monocultures and *G. sulfurreducens* - *S. cerevisiae* cocultures. Both were initiated as batch reactors

but were eventually adapted to work as chemostat bioreactors. In the batch phase, each bioreactor was filled up to 700 ml with CP media, prepared as described above. Once autoclaved, the media were amended with the same proportion of vitamins, yeast extract and carbonate. 10 mM of glucose and 20 mM of fumarate of sterile stock solutions were added. Both systems were equipped with calibrated redox and pH probes. The pH was maintained at 7 using 3 M KOH and 2 M HCl. Both bioreactors were covered by a heating jacket, keeping the temperature at 30°C. Stirring was performed at 50 rpm. Next, the reactors were flushed at 50 ml/min with N₂ to reach reduction values below -300 mV, ensuring anaerobic conditions. Once the anaerobic environment was confirmed, the gas flow was reduced to 20 ml/min and the microorganisms were inoculated: 5 ml of *S. cerevisiae* and for the coculture, an additional 10 ml of *G. sulfurreducens*.

In the chemostat phase, two tanks were connected to the system for the inflow and outflow of the liquid media. The inflow tank was filled with 10 L of fresh media with the same concentration of fumarate and glucose as was initially introduced into the reactors. The inflow tank was autoclaved and acidified with 30 ml of 2 M HCl to avoid contamination. Peristaltic pumps (Masterflex, Gelsenkirchen, Germany) were used to introduce and remove liquid from the bioreactors. Inflow was set at 4.1% for the coculture and 4.4% for the monoculture and outflow at 10% for both. 2 ml samples were extracted and analyzed twice a day. An image of the bioreactor set up can be visualized in the annex (Supplementary Figure 1).

2.4 Data analysis

All data analysis, including statistical calculations and graphical representations, was performed using Microsoft Excel (version 16.45). Significant differences were considered at p value < 0.05 and analyzed by paired two sample T-test.

The percentages of electrons recovered (RE) in each product were calculated by dividing the number of electrons received by each product (E_p) over the number donated by each substrate (E_s). For each compound, the number of electrons was estimated by multiplying their concentrations (mM) by their respective degrees of reduction (Table 1).

$$RE(\%) = \frac{E_p}{E_s} \times 100$$

Table 1. Degree of reduction for substrates and products.

	Compound	Degree of reduction
Substrate	Glucose	24
Product	Ethanol	12
	Glycerol	14
	Acetate	8

3. RESULTS

4.1 Co-culture establishment of *S. cerevisiae* and *G. sulfurreducens*

To explore the interaction between *S. cerevisiae* and *G. sulfurreducens*, it was first determined whether these two microorganisms could be cocultured. *G. sulfurreducens* was cultivated with *S. cerevisiae* using 5 mM of glucose and around 40 mM of fumarate. Given that no known electron donor for *Geobacter* was included in the medium, its growth would showcase its ability to obtain electrons from another source, potentially the yeast. Microscopic observation confirmed the proliferation of both microorganisms within 48 hours (Figure 6a). Additional evidence of both bacteria growing in the medium was visible in the pellet of the samples, where yeast cells appeared white and *Geobacter* acquired a red color, due to the presence of cytochromes (Uekia, 2021).

After proving the microorganisms could be cocultured, further analysis was carried out to evaluate potential changes in their metabolic behavior. To achieve this, both cocultures and *S. cerevisiae* monocultures were prepared following the set up described above. In the monoculture, 4.8 ± 0.23 mM of glucose was consumed, resulting in an increase in ethanol and glycerol production of 7.27 ± 0.22 mM and 0.94 ± 0.07 mM, respectively. In addition, fumarate concentration decreased by 2.59 ± 1.32 mM, while 1.80 ± 0.40 mM of malate was produced (Figure 6b). A similar pattern was observed in the coculture, where 4.69 ± 0.38 mM glucose was consumed, leading to a 7.01 ± 0.22 mM ethanol and 0.88 ± 0.03 mM glycerol increase. While these values resemble those observed in the monoculture, the coculture presented a higher decrease in fumarate (6 ± 2.30 mM) and a higher increase in both malate (3.12 ± 0.18 mM) and succinate (1.54 ± 0.07 mM) (Figure 6c). In both conditions, glucose was fully depleted, and ethanol levels plateaued by the second day. Acetate production was also detected. In the coculture, acetate levels peaked on the second day, reaching up to 0.13 ± 0.02 mM, before decreasing to 0 mM by the last time point (Figure 6c). Instead, in the monoculture, *S. cerevisiae* produced up to 0.42 ± 0.01 mM (Figure 6b).

In the previous experiment, *S. cerevisiae* growth was visible within 24 h, showing a faster growth rate than *Geobacter*, which required 48 h. For a more accurate assessment of the interaction and metabolic changes taking place, it was attempted to synchronize the microbes' growth curves. It was estimated that a prolonged exposure of the yeast cells to the suboptimal, anoxic, conditions in the experiment might slow down their metabolism. Therefore, six consecutive, weekly transfers were made for each replicate: T1, T2, T3, T4, T5 and T6 (Supplementary figures 2,3). The lack of an efficient biomass separation method prevented the generation of separate growth curves for each species.

Nonetheless, the OD measurements of samples from the coculture and monoculture still show a slight inclination towards the synchronization the microbial growth rates (Supplementary figure 4).

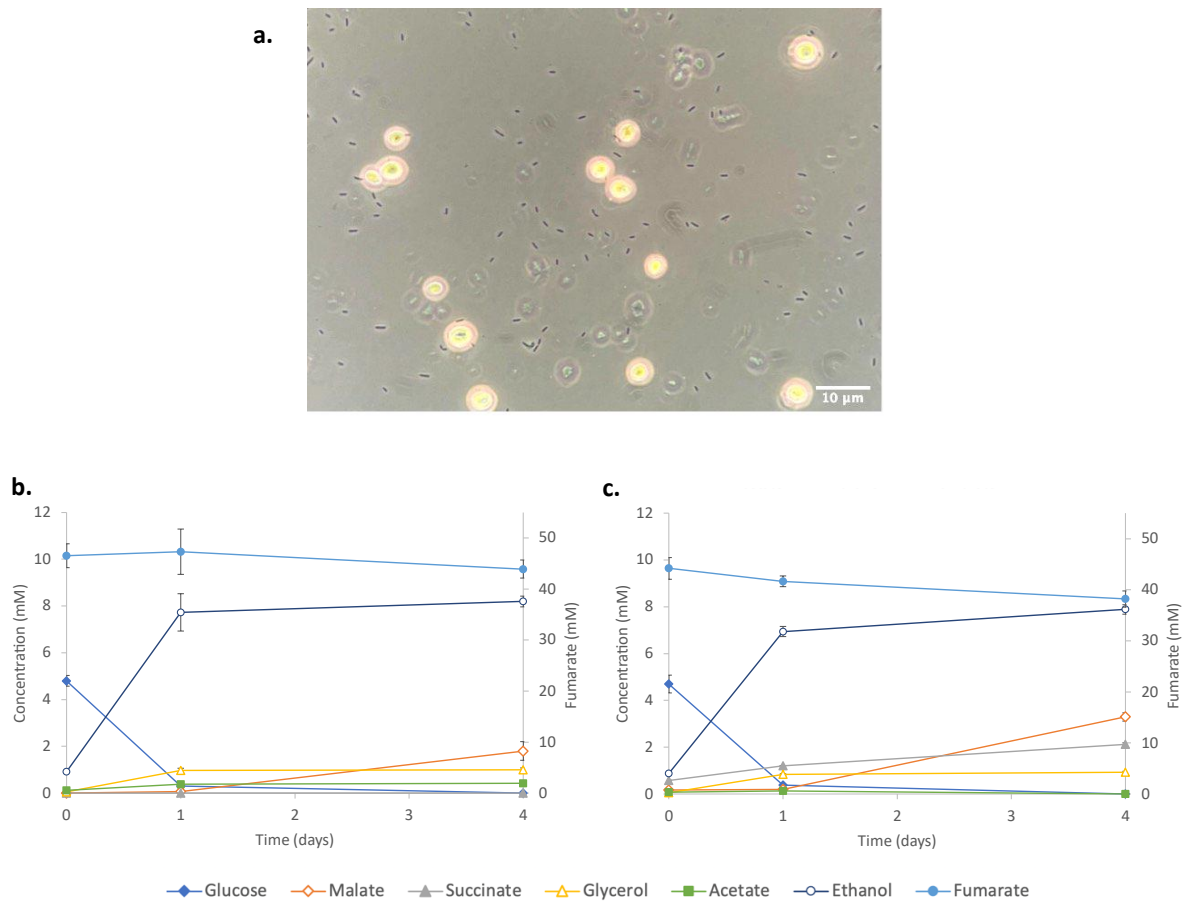


Figure 6. *G. sulfurreducens* and *S. cerevisiae* coculture. **a.** Light microscope image captured at 100X magnification. White cells are *S. cerevisiae*. Rod-shaped bacteria are *G. sulfurreducens*. **b,c.** Linear graphs represent varying metabolite composition in monoculture (**b**) and coculture (**c**) media over time. Data points show average concentrations (\pm SD) of triplicates at each time point.

The transfer experiments show a pattern that had not been observed in the initial experiment (Supplementary figures 2,3). While in the monoculture, *S. cerevisiae* consumes all glucose and ethanol plateaus by the second day, in all cocultures it takes at least 72h. Furthermore, like in the original experiment, in most transfers, the *S. cerevisiae* in monoculture produces acetate up to approximately 0.5 mM, while the coculture shows either no acetate production (T2, T3, T4, T5) or an increase up to 0.3 ± 0.02 mM followed by its complete depletion by the final time point (T0, T1, T6) (Supplementary figures 2,3). Additionally, succinate production tends to begin before glucose depletion in every coculture. Lastly, the electron distribution was assessed for all transfers. The total recovery for the monocultures was 89.35 ± 0.40 % (T0), 95.57 ± 0.41 % (T1), 90.09 ± 0.37 % (T2), 94.36 ± 0.40 % (T3), 97.16 ± 0.41 % (T4), 99.16 ± 0.45 % (T5) and 91.06 ± 0.39 % (T6). In the coculture the values were 88.10

$\pm 0.40\%$ (T0), $95.99 \pm 0.42\%$ (T1), $88.81 \pm 0.39\%$ (T2), $94.79 \pm 0.43\%$ (T3), $103.10 \pm 0.49\%$ (T4), $89.41 \pm 0.42\%$ (T5) and $88.12 \pm 0.40\%$ (T6). Therefore, although not consistently, the tendency seems to incline toward a higher electron recovery in the monoculture (Figure 7).

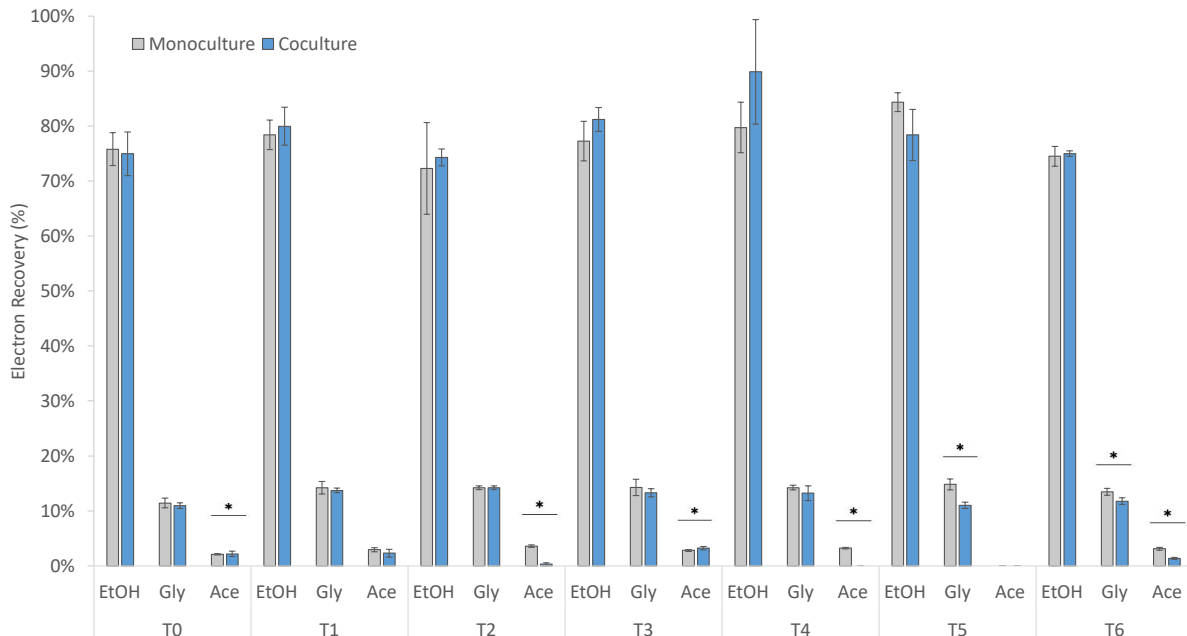


Figure 7. Comparison of electron recovery percentages in *S. cerevisiae* monocultures and cocultures with *G. sulfurreducens* and *S. cerevisiae* cocultures. Data represents electrons recovered by each product, ethanol (EtOH), glycerol (Gly) and acetate (Ace), from the glucose metabolized by *S. cerevisiae*. Asterisks indicate statistical significance ($p < 0.05$).

4.2. Assessing potential IET between *S. cerevisiae* and *G. sulfurreducens*

4.2.1 Growth of *S. cerevisiae* on glycerol

To test whether the *S. cerevisiae* is able to use *G. sulfurreducens* as its electron accepting partner, an experiment was prepared where 10 mM of glycerol was used as the electron and carbon source for the yeast, instead of glucose. Considering *S. cerevisiae*'s ability to exclusively utilize glycerol in presence of an electron acceptor, usually oxygen, it is expected to only see glycerol consumption if the yeast uses *G. sulfurreducens* as its electron acceptor. Cocultures and *S. cerevisiae* monocultures were prepared under these conditions and traced for five days. No growth of either microorganism was observed. Glycerol concentrations remained constant in the media throughout the experiment and no ethanol or acetate production was detected (Supplementary figure 5).

4.2.2 Effects of an exogenic mediator: Methylene Blue

Methylene Blue (MB) has been commonly employed as an exogenic mediator to promote EET in Microbial Fuel Cells (MFCs) using yeast cells, including *S. cerevisiae*. An experiment was conducted to test whether the presence of 0.1 mM of MB resulted in any significant differences in the cocultures. A control consisting of MB, but without cells, was included to ensure no abiotic reactions were taking place. The control showed no metabolite variations in the media. When comparing the cocultures with and without MB, three main differences were revealed. First, malate production levels were much lower in the MB coculture (3.20 ± 0.70 mM) than in the monoculture (7.40 ± 1.14 mM). Second, without MB, glucose was completely consumed by day two, whereas it required three days in presence of MB (Figure 8). Finally, ethanol production was higher with the exogenic mediator (7.39 ± 0.13 mM) than without it (6.74 ± 0.48 mM). Electron distribution showed a tendency toward a higher percentage of electrons recovered in the coculture than in the monoculture (Figure 8). A total recovery of 98.52 ± 8.10 % and 102.77 ± 1.77 % were calculated for the monoculture and coculture, respectively.

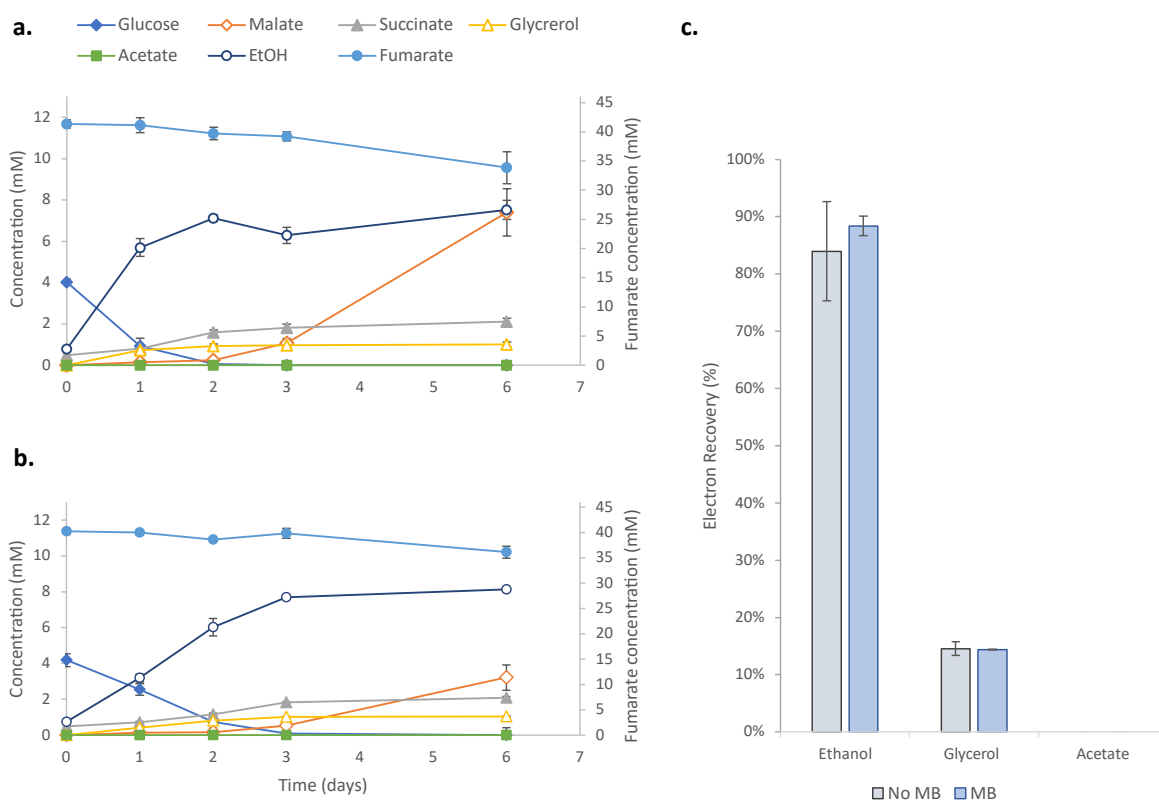


Figure 8. Effect of Methylene Blue *G. sulfurreducens* and *S. cerevisiae* cocultures. *a, b.* Linear graphs show metabolite composition variations in the coculture media without MB (*a*) or with 0.1mM MB (*b*). *c.* Electrons recovered by each product of glucose fermentation in *S. cerevisiae*. Data shows average concentrations (\pm SD) or average percentages (\pm SD) of triplicates. No asterisks indicate lack of statistical significance ($p < 0.05$).

4.2.3 Iron (III) as the electron acceptor

Another approach to test the potential IET activity in the coculture was the use of Fe(III) as *Geobacter's* electron acceptor instead of fumarate. Fe(III) can form insoluble particles when reduced (Fe(II)), which are believed to promote, in some cases, EET (Islam et al., 2005). To test the effects of this other electron acceptor, cocultures and *S. cerevisiae* monocultures were prepared adding 2 mM of glucose and 50 mM of iron citrate, instead of fumarate. In the monoculture, 2.58 ± 0.11 mM ethanol were produced, and plateaued around day three. Glycerol was also produced (0.32 ± 0.01 mM). No succinate or malate were detected. Acetate, however, slightly increased by 0.13 ± 0.09 mM (Figure 9a). Similarly, in the coculture, ethanol increased 2.78 ± 0.82 mM and plateaued by day two (Figure 9b). Nonetheless, the graph shows a slightly slower metabolism in the coculture than in the monoculture (Figure 9a,b). In this experiment, glucose measurements were unreliable due to an overlap in the HPLC citrate and glucose peaks. The machine was run at three different temperatures, but none gave accurate enough measurements. However, glucose was estimated to get fully consumed in both conditions around days two and three.

Regarding the changes in Fe(II) and Fe(III) concentrations, in the monoculture, measurements showed no significant increase in Fe(II) (0.29 ± 0.31 mM), but a slight decrease in Fe(III) of 9.53 ± 1.66 mM (Figure 9a). In contrast, in the coculture, Fe(III) decreased by 12.59 ± 3.22 mM and Fe(II) increased notably by 2.06 ± 0.16 mM, strongly supporting that the coculture can also grow under these conditions (Figure 9b). Citrate, on the other hand, remained constant both in conditions, proving neither the yeast nor the bacteria could uptake it from the environment. While media in the bottles showed only slight differences in color intensity between both conditions, the formation of precipitates was visible in all coculture replicates.

In the electron distribution, a total recovery of only 73.11 ± 4.57 % and 65.03 ± 15.70 % were obtained in the monoculture and coculture, respectively (Figure 9c). Given that iron concentrations were conducted using Ferrozine, a very sensitive technique, the lower electron recovery can be attributed to measurement discrepancies.

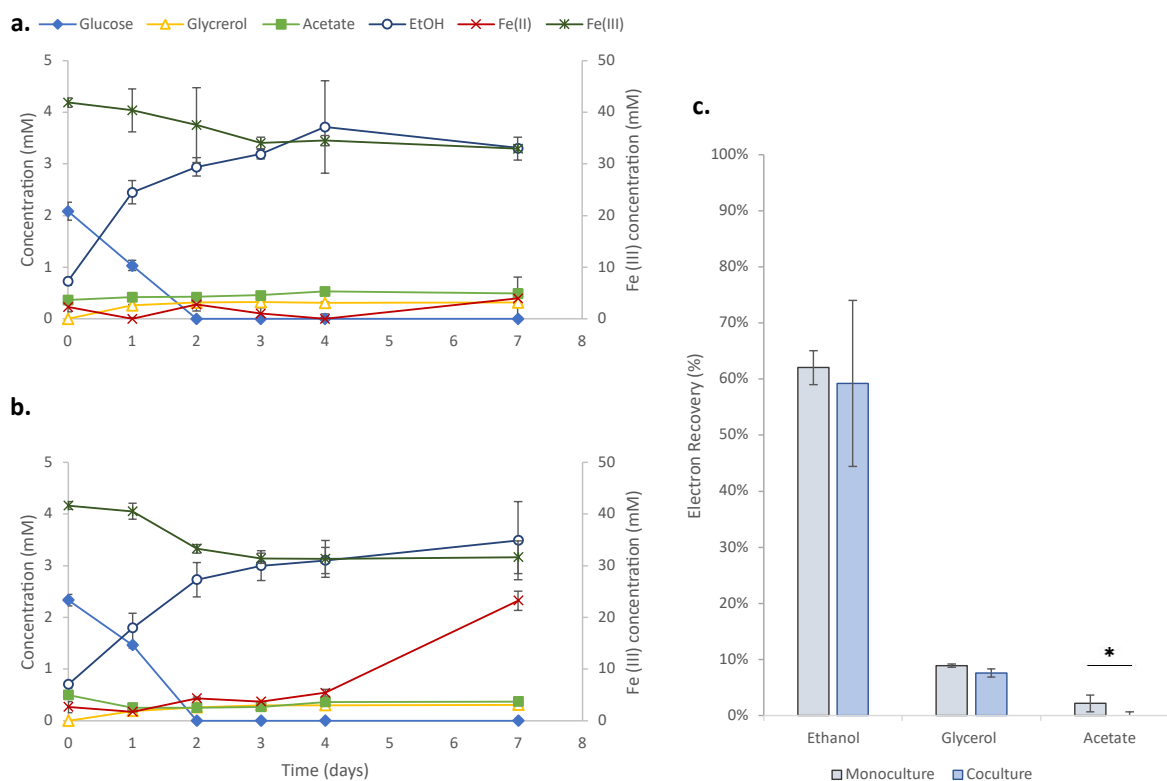


Figure 9. Growth of mono and co-cultures on Iron citrate. a, b. Linear graphs show metabolite composition in the *S. cerevisiae* monoculture (a) and the coculture (b) in presence of ± 50 mM iron citrate. **c.** Electrons recovered by products of glucose fermentation in *S. cerevisiae*. Data shows average concentrations (a,b) (\pm SD) and average percentages (c) (\pm SD) of triplicates. Asterisks show statistical significance ($p < 0.05$).

4.3 Alternative electron acceptors for *G. sulfurreducens*

4.3.1 Growth in absence of fumarate

In previous experimental setups, either fumarate or Fe (III) were introduced as the primary electron acceptors for *G. sulfurreducens*. However, only part of the fumarate was being consumed, while relatively much bacterial growth was being observed under the microscope. It was considered that *Geobacter* might be using an alternative electron acceptor, so growth dependence on fumarate was tested. A new experiment was prepared where all coculture and yeast monoculture replicates were established in absence of fumarate. In the monoculture, 8.21 ± 0.16 mM ethanol was produced, and stabilized after two days. Acetate and glycerol were also increased by 0.29 ± 0.01 mM and 0.87 ± 0.06 mM, respectively. Glucose, on the other hand, was fully depleted within one day (4.48 ± 0.07 mM) (Figure 10a). Instead, the coculture presented a delayed depletion of glucose, taking two days (4.36 ± 0.24 mM). Without fumarate, no malate or succinate were produced. Similar to the monoculture,

ethanol concentrations increased by 7.81 ± 0.29 mM and glycerol levels by 0.88 ± 0.04 mM (Figure 10b). Unlike in previous experiments, 0.25 ± 0.03 mM of acetate was produced, but was not consumed. Fumarate remained 0mM in both conditions.

Despite the lack of fumarate in the media, within 72h, *G. sulfurreducens* growth became clear and abundant under the microscope, comparable to observations made of cultures in fumarate-containing cultures. In addition, when samples were centrifuged before storage, the pellet, while no longer red, was larger in the cocultures. Regarding the electron distribution, no significant difference was observed between the monoculture (105.03 ± 1.94 %) and coculture (103.15 ± 1.95 %) (Figure 10e).

As *G. sulfurreducens* growth in coculture was possible in absence of fumarate, each replicate was subsequently transferred into fresh media, lacking fumarate, once again, to see if growth could continue under these conditions. Similar metabolic activity was observed in the transfer cultures. Again, yeast metabolism seemed slower in the coculture (Figure 10c). In both cases, similar levels of glucose got depleted and of ethanol plateaued on day three. Nonetheless, the values on the second day differed notably, with glucose reaching 0.48 ± 0.50 mM in the monoculture and 1.94 ± 0.48 mM in the coculture (Figure 10c,d). No succinate or malate were produced. In the original experiment (T0), acetate production started on day one, however, in the transfer (T1), the production only began on day three, and was also not consumed (Figure 10b,c). In T1, 0.41 ± 0.03 mM acetate was produced in the monoculture and 0.36 ± 0.03 mM in the coculture, slightly higher values than in T0. In contrast to the initial experiment, microscopy observations did not reveal clear growth of *Geobacter*. Additionally, *S. cerevisiae* growth took longer to become apparent under the microscope, at least three days. In T1, the electron distribution did not show any differences between the percentage of electron recovery in either condition: 96.55 ± 1.23 % in the monoculture and 95.89 ± 1.02 % in the coculture (Figure 10f).

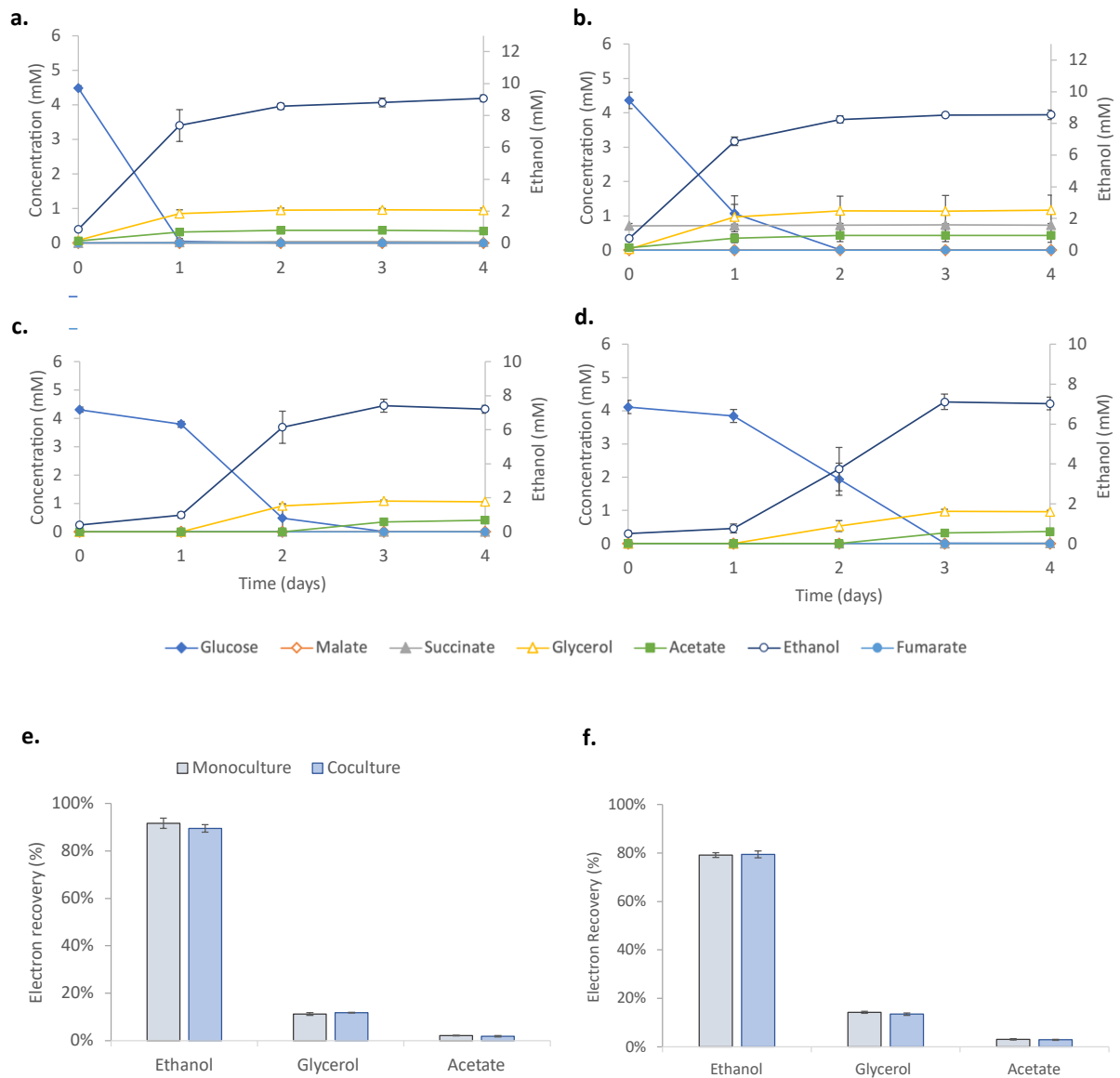


Figure 10. Growth of mono and co-cultures without fumarate. Linear graphs show metabolite changes in the media before (a,b) and after (c,d) the transfer. No fumarate was added to the media. **a.** Monoculture T0. **b.** Coculture T0. **c.** Monoculture T1. **d.** Coculture T1. Bar charts show Electrons recovered by products of glucose fermentation in *S. cerevisiae* in T0 (**e**) and T1 (**f**). Data show average concentrations (a-c) (\pm SD) and average percentages (e,f) (\pm SD) of triplicates. No asterisks indicate no statistical significance ($p < 0.05$).

4.3.2 Dependence of *G. sulfurreducens* growth on CO₂

The potential role of CO₂ as the alternative electron acceptor for *G. sulfurreducens* was assessed by removing it from the headspace. Triplicates of the *S. cerevisiae* monoculture and the coculture were grown in absence of fumarate and containing 100 % N₂ gas in the headspace. No bicarbonate buffer was included in this experimental set-up. However, the complete removal of CO₂ was not possible due to the CO₂ resulting from the glucose fermentation. Both HPLC measurements and microscopy observations confirmed growth of the *G. sulfurreducens* with reduced CO₂ in the headspace. Results were similar to previous experiments. In the monoculture, succinate production was not observed while 0.28 ± 0.03 mM malate was produced. Instead, in the coculture succinate increase (0.68 ± 0.17 mM) and a higher malate production in the cocultures (3.22 ± 1.03 mM) (Supplementary figure 6b).

Contrary to previous observations, fumarate only showed a decrease in concentration in the coculture (5.11 ± 2.44 mM), but remained more constant in the monoculture (1.82 ± 0.52 mM). Ethanol, on the other hand, reached higher values in the monoculture (8.68 ± 1.45 mM) than in the coculture (7.47 ± 1.09 mM) (supplementary figure 6a,b). The electron distribution showed a tendency for a higher percentage of recovery in the monoculture (104.84 ± 24.52 %) than in the coculture (91.65 ± 5.15 %) (supplementary figure 6c).

4.5 Succinate as an intermediate in the coculture

S. cerevisiae's ability to uptake succinate from the media was tested. An experiment was set up comparing the growth of *S. cerevisiae* monocultures with and without succinate. Bottles were prepared containing only 10 mM of succinate and 5 mM of glucose. HPLC measurements revealed no changes in succinate concentrations throughout the experiment. No significant differences were observed in any other metabolites (Supplementary figure 8). However, one of the triplicates showed a 0.5 mM increase in fumarate after four days.

4.6. Comparing alternative *G. sulfurreducens* electron donors

4.6.1 H₂ as the alternative electron donor

G. sulfurreducens is known to use H₂ as an alternative electron donor to acetate (Coppi et al., 2004). To further explore the hypothesis that *S. cerevisiae* provides electrons to *Geobacter* in the

coculture, an experiment was carried out to determine *G. sulfurreducens*' preference between H₂ and yeast cells as electron donors. For this, 100 % H₂ was added to the headspace in monoculture and coculture triplicates, containing glucose (5 mM) and fumarate (40 mM). HPLC measurements showed no succinate production in either condition. However, a significant increase in malate production was observed in the coculture, of 6.26 ± 0.53 mM, while very little was detected in the monoculture, of 0.48 ± 0.48 mM (Figure 11a,b). No significant hydrogen consumption was detected in any of the conditions. More fumarate was depleted in the coculture (4.71 ± 0.64 mM) than in the monoculture (1.81 ± 0.93 mM) (Figure 11a,b). Regarding the electron distribution, the coculture seemed to recover a higher percentage of its electrons than the yeast monoculture, reaching values of 97.98 ± 1.22 % and 93.19 ± 2.43 %, respectively (Figure 11c). Nevertheless, the lack of *G. sulfurreducens* proliferation observed under the microscope suggests the experimental conditions may have been compromised.

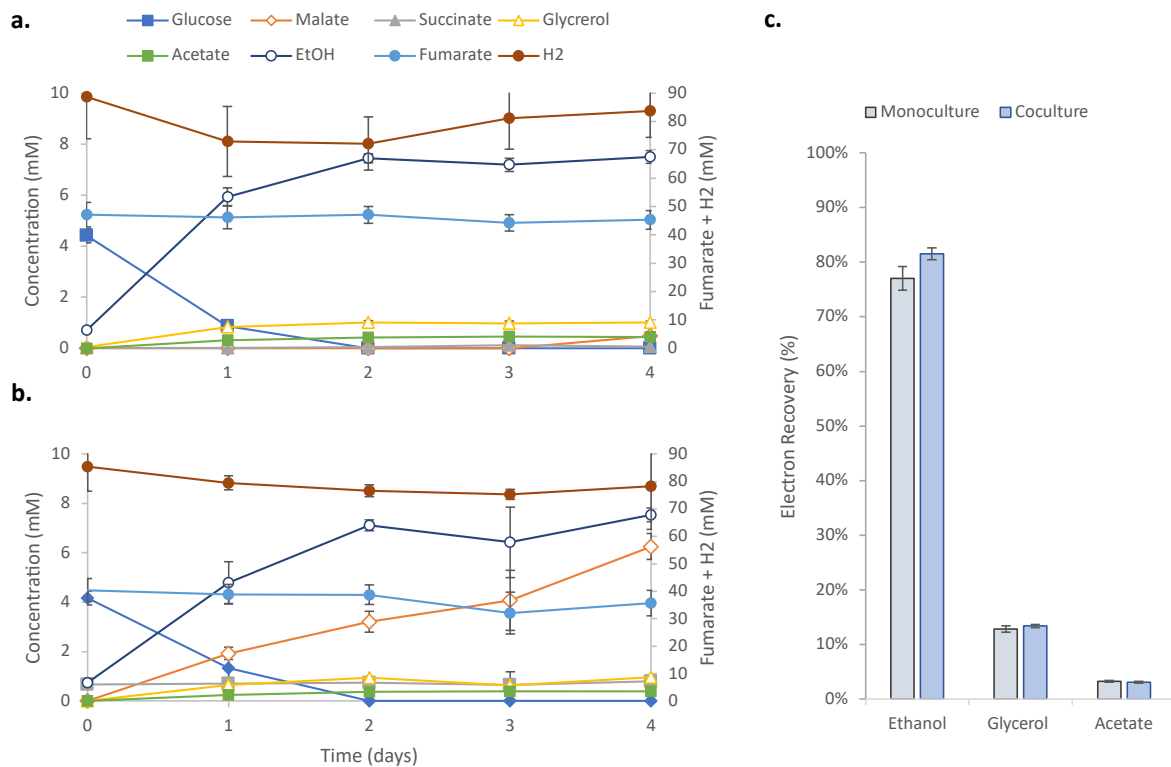


Figure 11. Growth of mono and co-cultures with H₂ in the headspace. a, b. Linear graphs show metabolite composition in the *S. cerevisiae* monoculture (a) and the coculture (b) in presence of H₂. **c.** Electrons recovered by products of glucose fermentation in *S. cerevisiae*. Data shows average concentrations (a,b) (\pm SD) and average percentages (c) (\pm SD) of triplicates. No asterisks indicate no statistical significance ($p < 0.05$).

4.6.2 Acetate as the alternative electron donor

A second experiment to test *G. sulfurreducens'* preference for its electron donor in the coculture was set up introducing 5mM of acetate into a media with 40 mM fumarate and 5 mM glucose. Acetate was not consumed but was produced both in the monoculture (0.33 ± 0.14 mM) and the coculture (0.23 ± 0.16 mM). While no succinate was produced in the monoculture, it increased by 0.31 ± 0.08 mM in the coculture (Figure 12a,b). Malate, on the other hand, while only reaching 0.92 ± 0.13 mM in the monoculture, it increased by 5.93 ± 0.76 mM in the coculture. Ethanol production was similar in both cases, reaching 6.80 ± 0.09 mM in the monoculture and 6.83 ± 0.38 mM in the coculture, but it took longer to plateau in the latter (figure 12a,b). Once again, glucose depletion took longer in the coculture than in the monoculture (three versus two days). The electron distribution, the percentage of electrons destined to each product was very similar in both conditions: 95.56 ± 2.72 % in the monoculture and 92.95 ± 3.26 % in the coculture. (Figure 12c). No significant *G. sulfurreducens* growth was observed under the microscope, which might have been caused by inconsistencies in the experimental set up.

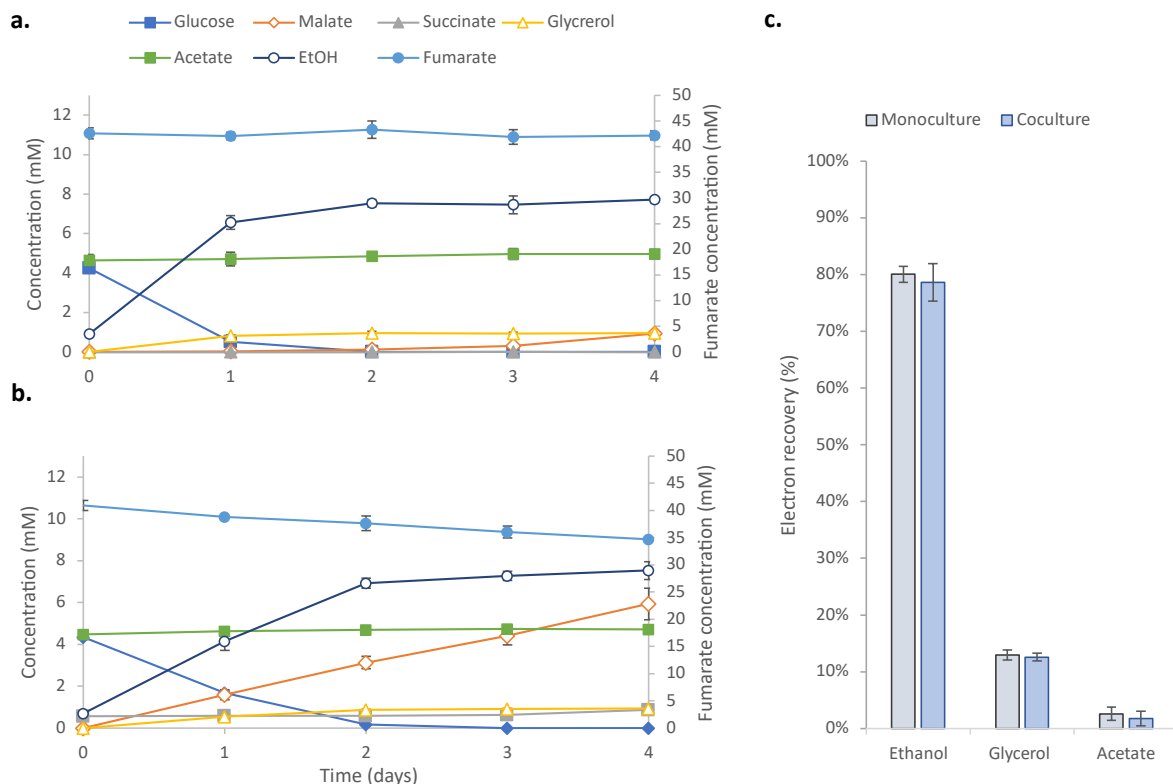


Figure 12. Growth of mono and co-cultures with additional acetate in the medium. a, b. Linear graphs show metabolite composition in the *S. cerevisiae* monoculture (a) and the coculture (b) with an additional ± 5 mM of acetate. **c.** Electrons recovered by products of glucose fermentation in *S. cerevisiae*. Data shows average concentrations (a,b) (\pm SD) and average percentages (c) (\pm SD) of triplicates. No asterisks indicate no statistical significance ($p < 0.05$).

4.4 Bioreactor experiments

4.4.1 Steady state co-cultivation

To more accurately compare the metabolic activity of both microorganisms, an experiment was performed using two bioreactors. A *S. cerevisiae* inoculate was introduced into the first bioreactor. The second was inoculated with *S. cerevisiae* and *G. sulfurreducens*. Next, 20 mM of fumarate and 10 mM of glucose were added to both. At the time of inoculation, bioreactors were built as batch systems. However, after 19 hours, inflow and outflow tanks containing fresh media were included, establishing a new chemostat bioreactor system with 24h turnover times (Figure 14a,b). In the continuous phase, most metabolite concentrations initially peaked, before reaching stability at approximately 120h (steady state). In the monoculture, the concentrations were: 0.04 mM (glucose), 1.03 mM (malate), 0.02 mM (succinate), 1.07 mM (glycerol), 0.16 mM (acetate), 10.10 mM (fumarate) and 8.13 mM (ethanol) (Figure 14a). In contrast, in the coculture, the average concentrations were: 0.11 mM (glucose), 2.68 mM (malate), 3.53 mM (succinate), 1.08 mM (glycerol), 0.00 mM (acetate), 4.18 mM (fumarate) and 8.34 mM (ethanol) (Figure 14b). Therefore, while ethanol and glycerol levels were similar in both conditions, glucose, malate and succinate concentrations were higher in the coculture than in the monoculture, which correlates with the observations made in bottled experiments. On the other hand, fumarate was significantly lower in the coculture than in the monoculture. Acetate was not detected in the coculture, indicating it was probably consumed by *Geobacter*.

Microscopic observations showed significant growth of both species (Figure 10a,b). In several of the bottle experiments, *G. sulfurreducens* showed the ability to aggregate around the yeast cells. However, in the bioreactor, the bacteria formed much larger aggregations or biofilms, both surrounding the yeast cells and in isolation (Figure 13c). In addition, the OD curves for the bioreactor show a much slower increase in biomass in the monoculture than in the coculture (Supplementary figure 7). This slower yeast metabolism was more pronounced in the bioreactors than in the previous bottled experiments.

Production rates for malate, succinate, and ethanol appear higher in the coculture. The yeast monoculture, on the other hand, is not able to produce succinate but does show a higher acetate production rate than the coculture, as was the case in the experiments in bottles (Figure 14c). Electron balances showed 85.41 % recovery in monocultures and 87.52 % in cocultures, indicating a slightly higher recovery tendency in cocultures (Figure 14d).

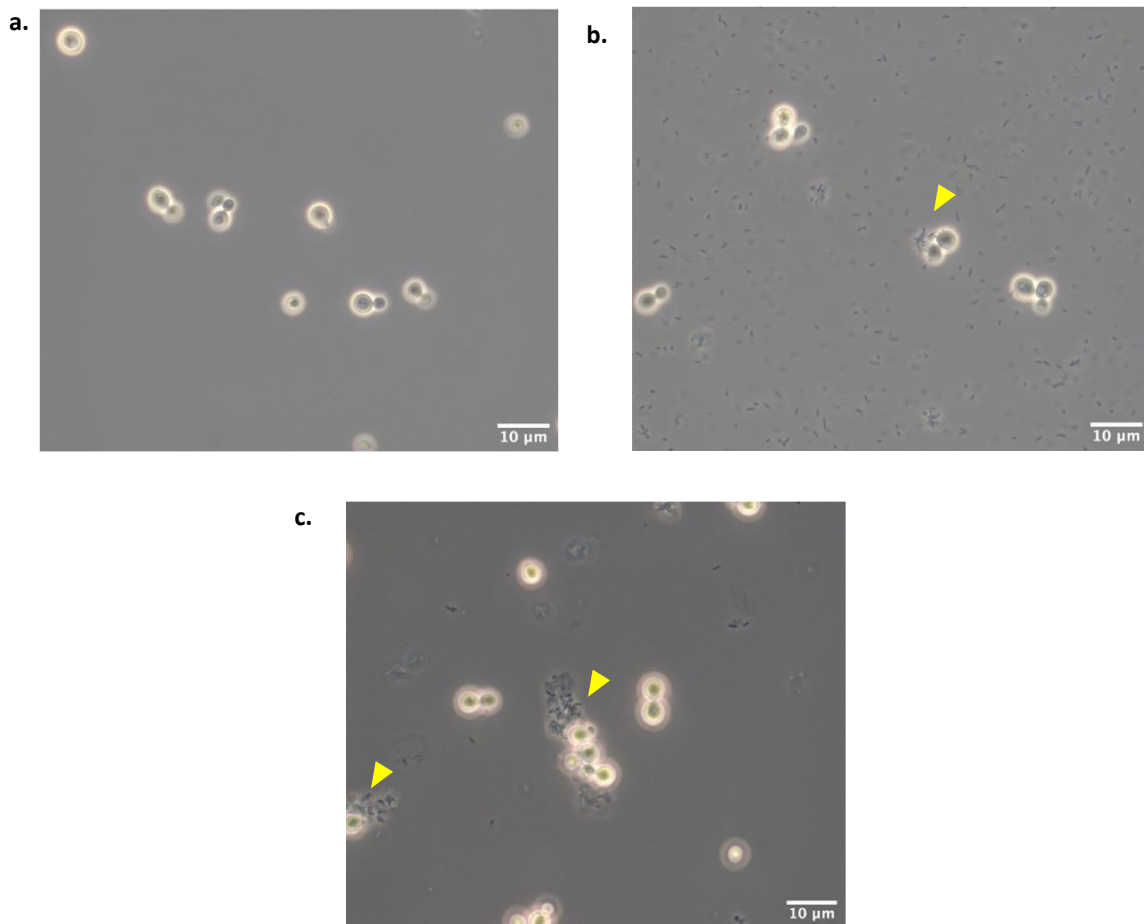


Figure 13. Bioreactor images. Light microscopy images captured at 100 X magnification. **a.** *S. cerevisiae* monoculture. **b,c.** *G. sulfurreducens* coculture with *S. cerevisiae*. Images taken at t19 (a,b) and t120 (c). Yellow arrows show *Geobacter* aggregations.

4.4.1 Absence of Fumarate

Once steady state was reached, an experiment was executed with the already running bioreactors, to test the dependency of the coculture on fumarate under these conditions. The media tank used for the inflow of the bioreactors was replaced by a new tank containing fumarate-free medium. Due to time constraints, steady state was not reached. In the new conditions, ethanol levels continued to increase in both conditions, reaching values of 16.87 mM and 14.40 mM for the monoculture and the coculture, respectively (Figure 14a,b). These higher concentrations were a result of a higher glucose concentration in the fumarate-free media tanks, of 9.32 ± 0.04 mM, instead of the previous 5.6 ± 0.05 mM. While fumarate decreased exponentially in the monoculture, it decreased linearly and much faster in the coculture. Succinate levels, however, took longer than the fumarate to become depleted from the bioreactor and did so at a slower rate, suggesting prolonged production. At the last time point, 1 mM of succinate still remained in the medium (Figure 14b). Malate remained constant in the monoculture but decreased exponentially, similar to fumarate, in the coculture.

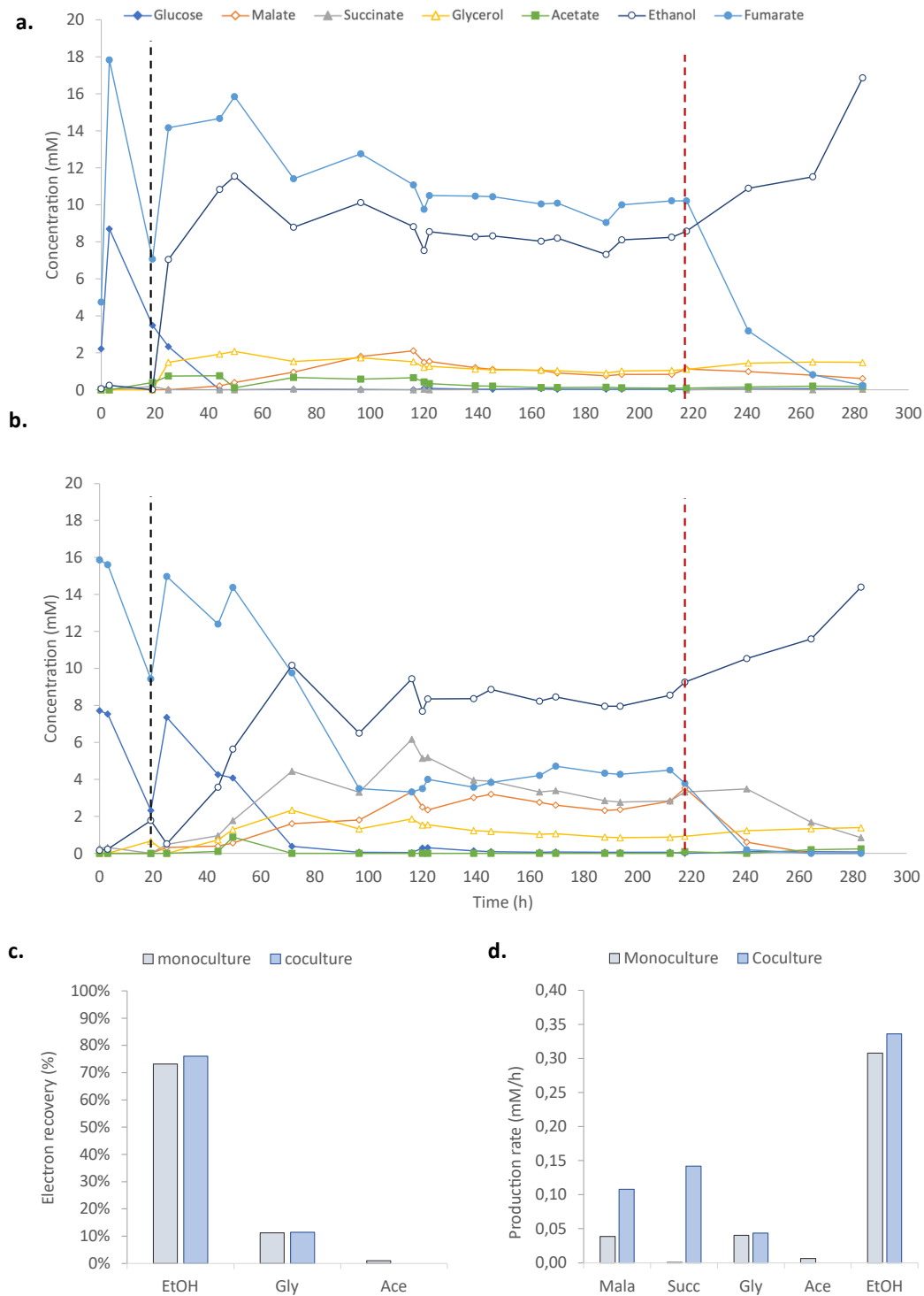


Figure 14. Comparison of growth by mono and co-cultures in bioreactors. a.b. Linear graphs show metabolite changes in the *S. cerevisiae* monoculture (a) and the *G. sulfurreducens* coculture with *S. cerevisiae* (b). Black line shows time of chemostat phase start. Red line indicates moment of fumarate removal from the medium tank. **c.** Electrons recovered by products of glucose fermentation in *S. cerevisiae*. **d.** Production rate of components detected in the bioreactors. Ethanol (EtOH), Glycerol (Gly), Acetate (Ace), Malate (Mala), Succinate (Succ).

4.7 Control experiments

To test whether the viability of the coculture relied on yeast cells, a control experiment was prepared where both 40 mM of fumarate and 1 ml of *G. sulfurreducens* were added to a bottle containing four days old yeast cells. These yeast cells no longer had glucose available, therefore becoming “incative”. Succinate concentrations increased by 1.51 ± 0.23 mM. Significant malate production was detected (5.33 ± 0.31 mM) and fumarate decreased by 5.98 ± 1.76 mM. On the other hand, glycerol and ethanol concentrations remained constant throughout the experiment (supplementary figure 9).

Another control experiment was carried out to assess how much *G. sulfurreducens* could grow on dead *S. cerevisiae* cells. For this, a grown, four-day old *S. cerevisiae* monoculture was autoclaved and subjected to 1 ml of *G. sulfurreducens* and 40 mM of fumarate. Succinate was also produced in this condition, increasing by 1.42 ± 0.07 mM. Malate, on the other hand, barely increased compared to the previous control experiment (0.70 ± 0.05 mM). In this case, fumarate decreased 2.36 ± 0.31 mM. Once again, ethanol and glycerol showed no variation (supplementary figure 9).

To ensure the growth of *Geobacter* was not driven by any components in the media, 1 ml of *Geobacter* was grown on bottles containing only medium. The bottles showed no growth, confirmed by the OD measurements, which remained around 0.02 ± 0.00 mM after five days.

Finally, since *G. sulfurreducens* can potentially perform fumarate disproportionation, via which fumarate becomes both the electron acceptor and the electron donor, an experiment was prepared to test to what extent this process allows its growth (Shi et al., 2022). For this, the *Geobacter* were cultured in medium containing only fumarate (40 mM). In absence of glucose and yeast cells, *Geobacter* was able to increase succinate by 0.75 ± 0.08 mM and to produce 0.70 ± 0.06 mM of malate by day four (supplementary figure 10). It also consumed 1.88 ± 0.43 mM of fumarate. This experiment was compared to a *G. sulfurreducens* monoculture with no fumarate added. However, the first time points show 1.54 ± 0.72 mM, showing the culture was not clean from fumarate. In this case, only 0.10 ± 0.04 mM malate was produced, and succinate increased slightly by 0.55 ± 0.06 mM. (supplementary figure 10). OD measurements showed little cell growth (Supplementary figure 11).

4. DISCUSSION

This study has gathered information on the effect of *G. sulfurreducens* on *S. cerevisiae* in cocultures, under varying conditions, to get insight on their interaction mechanisms. The existence of an interplay between the microorganisms was confirmed with the initial establishment of the coculture (Figure 6). While fumarate disproportionation could have potentially explained the growth of *G. sulfurreducens* in the absence of its electron donor, the control experiment in chapter 4.6 demonstrated that only a portion, 0.75 ± 0.08 mM of succinate and 0.70 ± 0.06 mM of malate, can be attributed to this phenomenon (supplementary figure 10) (Shi et al., 2022). Moreover, *Geobacter* proved to not be able to metabolize any of the medium components, and thus growth of *Geobacter* can be attributed to the presence of *S. cerevisiae*.

The control experiments reveal that *G. sulfurreducens* is able to grow with *S. cerevisiae* that is either dead or no longer has glucose availability. Under these conditions, succinate and malate reach concentrations comparable to other experiments (Supplementary figure 9). Succinate remained stable in both conditions, indicating that the state of the yeast cells might have no effect on *Geobacter's* activity. However, the difference in malate production depicted in each case, might prove otherwise. On the one hand, it is possible that the difference is due to the live yeast cells metabolizing the fumarate, leading to more malate production. On the other hand, it is possible that the increased production is triggered in *Geobacter* in response to the live *S. cerevisiae*. While previous research has proven different inhibitory and stimulatory effects of *S. cerevisiae* on neighboring bacteria, such as lactic acid bacteria, no reports have been made on enhancing malate production (Alexandre et al., 2004). Nonetheless, if *Geobacter* became more active upon *S. cerevisiae's* presence, a higher succinate level would have been expected. In any case, the growth of *Geobacter* on the autoclaved cells shows that at least part of the bacterial growth could be attributed to their ability to directly utilize part of yeast biomass itself.

The main interaction mechanism considered throughout this project was the Interspecies Electron Transfer (IET), with the initial hypothesis being that *S. cerevisiae* transferred electrons to *G. sulfurreducens* either direct or indirectly. The expectation was to observe differences in the electron distribution patterns between the coculture and monocultures, to be precise, a decrease in electrons destined to ethanol in the coculture. Nevertheless, none of the bottle experiments using either fumarate or Fe (III) showed any significant differences in their distributions (figure 7, 9c). In addition, *S. cerevisiae* was not able to grow on glycerol in coculture, which did not support the theory, given it

can only use Glycerol when an electron acceptor is available, usually O₂ (Clomburg & Gonzalez, 2013). (Supplementary figure 5).

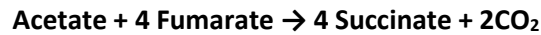
In contrast, possible signs of IET were revealed upon the addition of methylene blue to the cultures, the exogenic mediator commonly used to facilitate electron transfer. Its presence caused the yeast metabolism to slow down and malate production to decrease, showing it has effects on the cocultures (Figure 8). On the one hand, it is possible that the observations were a consequence of potential toxic effects of MB on *S. cerevisiae*, especially considering that succinate levels, produced only by *Geobacter*, did not seem affected. While no toxic effects have been reported on *S. cerevisiae* specifically, MB has shown to affect the biofilm formation and viability of *Candida albicans*, another fungi from the *Saccharomycetaceae* family (de Carvalho Leonel et al., 2019). On the other hand, the slower metabolism observed in presence of MB could be the result of the movement of electrons between the microorganisms, causing a delay in *S. cerevisiae* proliferation due to redox state issues. Additionally, the bioreactor experiments confirmed that there is no difference in the distribution of electrons between the monoculture and the coculture even in the steady state. However, it did reinforce the slower yeast metabolism in the coculture condition (Figure 14c). The aggregations formed between the yeast and the bacterial cells, observed under the microscope, could also be an indicator of IET taking place (Figure 13 b,c) (Summers et al., 2010).

The slower growth rate of *S. cerevisiae* in presence of the bacteria was a recurring event throughout the study. However, while ethanol production and glucose consumption rates decreased, they eventually caught up to the concentrations measured in the monocultures. Given these results, it is proposed that the initial hypothesis was incomplete and instead, the yeast are not only able to directly donate their electrons to the *Geobacter* initially, but that they find a way to recover them later on and continue producing the rest of the ethanol. This movement of electrons would justify both the lack of differences in the electron distributions, since they return to the yeast eventually, and the overall slower rate of ethanol production and glucose consumption. Nonetheless, if this were the case, the addition of MB to the cocultures would still be expected to promote the transfer of electrons toward *G. sulfurreducens*, increasing succinate production, which was not observed (figure 8).

It was confirmed by the bioreactor experiment that the acetate produced by *S. cerevisiae* is consumed by *G. sulfurreducens* in the coculture, potentially serving as the initial source of electrons for the bacteria. According to the stoichiometry in equation 1, the 0.16 mM of acetate produced would only account for 0.64 mM of fumarate consumed and 0.64 mM of succinate formed. However, around a 4 mM difference of each was measured, suggesting that additional reactions are taking place (Figure

14 b,c). CO₂ was proposed as an alternative electron acceptor for *G. sulfurreducens* but did not prove to have a notable effect on the coculture, leaving acetate as the most likely candidate.

Equation 1:



While acetate could be considered the means by which electrons are initially transferred from *S. cerevisiae* to *G. sulfurreducens*, the succinate produced by *Geobacter*, could serve as the vehicle through which the electrons return to *S. cerevisiae* (Figure 15). While not much is known about the yeast's ability to uptake succinate, a study by D. A. Aliverdieva proved the symport of succinate with sodium ion into the cell via a dicarboxylate transporter, located in its plasma membrane (Aliverdieva et al., 2006). The yeasts ability to uptake Succinate was tested under the experimental conditions (Supplementary figure 8). While it did not show great capabilities, a slight indication of succinate consumption and fumarate production was observed in one of the samples. It is possible that the metabolic pathway to uptake this metabolite only becomes active in the yeast under specific conditions, in this case, the presence of *G. sulfurreducens*. As a matter of fact, previous studies have proven that metabolic adaptations and transcriptional changes are induced in *Candida albicans*, also from the Saccharomycetaceae family, in response to the presence of another bacterium, *Lactobacillus rahnosus* (Alonso-Roman et al., 2022). Assuming succinate entered the yeast cells, given it has a higher redox potential than NAD⁺/NADH⁺, it would have to enter a reverse electron transfer to reduce NAD⁺ into NADH. This process, however, requires energy, which is supplied by a proton motive force across the mitochondrial inner membrane. The need for this additional energy would explain *S. cerevisiae*'s delayed metabolic activity, observed in most of the experiments carried out throughout the study (Prag et al., 2023). Therefore, the proposed interaction mechanism between the two microorganisms would result in a parasitic relationship between the species, where *G. sulfurreducens* utilizes *S. cerevisiae*'s resources for its own growth, negatively affecting the yeasts growth and production rates (Figure 15).

Taking the new hypothesis into account, some of the previously discussed observations could have different explanations, for instance, the control experiments comparing the dead to the "inactive" yeast cells. The similar levels of succinate and differences in malate, suggest that the succinate could be absorbed by *S. cerevisiae* and oxidized to form to more malate and fumarate. While malate accumulates in the media, the fumarate could be reused by *Geobacter*, explaining why its concentrations appear similar in both conditions. This new theory would also justify the overall higher malate concentrations detected in the cocultures of most experimental set ups, including the

experiments with H₂ and acetate in the medium. In these last two, where no *Geobacter* growth was observed, there was still a clear difference in malate concentrations (Figures 11, 12). This suggests that *G. sulfurreducens* might not need to be growing to trigger the uptake of C₄ dicarboxylic acids in *S. cerevisiae*. The yeast might respond to cellular components specific to *G. sulfurreducens* such as lipids or outer membrane proteins.

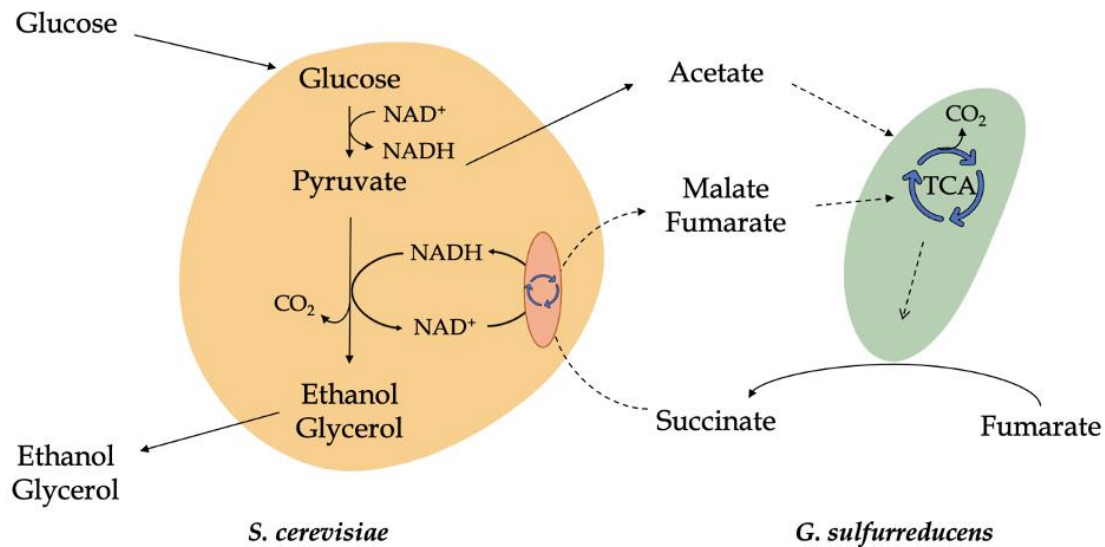


Figure 15. Proposed interaction mechanism. *S. cerevisiae* consumes glucose, which is converted to pyruvate. Pyruvate undergoes fermentation, oxidizing NADH and producing ethanol, glycerol and CO₂. The acetate that is produced by the yeast is taken up by *G. sulfurreducens*, which uses its electrons to reduce fumarate into succinate. Next, succinate re-enters the yeast cell and reaches the mitochondria, where a reverse electron transport takes place. Malate and fumarate are released. Broken lines indicate unproven pathways. Red oval represents mitochondria. This figure is a simplified representation and is not stoichiometrically balanced.

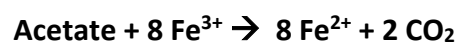
According to the new hypothesis, fumarate might be secreted by the yeast. Therefore, the growth of *Geobacter* without fumarate addition to the media (Figure 10), could support the proposed metabolic model (Figure 15). However, in the bottle experiment cells only thrived in T₀, but not T₁. These results indicate that in order for *S. cerevisiae* to begin producing the fumarate, an initial amount of succinate or fumarate might be needed. As a matter of fact, T₀ replicates presented residual succinate in the media, which could have kick-started the reaction (Figure 10). In contrast, T₁ did not contain succinate, which could explain the lack of bacterial growth. There is a chance that residual fumarate was also present in T₀, undetected by the HPLC, which could have been used by *G. sulfurreducens*, in combination with the acetate, to produce succinate, potentially needed by the

yeast. Given that the *G. sulfurreducens* stock bottle initially contained 40 mM, part of which was probably consumed, and 1 ml of it was introduced into the T0 bottles, a maximum value of 0.8 mM of fumarate could have been present in the bottle. In combination with at least 0.2 mM of acetate, which was detected in the medium, 0.2 mM of succinate could have been formed.

Regarding the elimination of fumarate from the bioreactors, while *Geobacter* cells did not show notable growth and both fumarate and malate decreased exponentially, succinate levels remained in the media longer and did not even reach 0 mM by the last time point (Figure 14). This suggests that succinate production continued after fumarate was removed from the medium. The reason *G. sulfurreducens* probably started to flush out of the system upon fumarate removal was due to a slower growing rate under the new conditions, becoming unable to keep up with the bioreactor turnover time (24h).

G. sulfurreducens growth was also observed with Fe(III) as the electron acceptor. In this case, no fumarate, succinate or malate were detected in the media, but acetate was still produced and probably transferred to *Geobacter*. The 0.17mM of acetate consumed would account for 1.36 mM of Fe (II) production (Equation 2). In the cocultures, 2.06 mM of Fe (II) was detected, most of which could be accounted to the acetate metabolization (Figure 9). It is also possible that other types of cross feeding occurred, such as the bacteria feeding of the yeast biomass. In any case, these results would still be compatible with the proposed model (Figure 13).

Equation 2:



5. FUTURE RECOMMENDATIONS & OUTLOOK

While this study has provided some insights on the possible interactions between *S. cerevisiae* and *G. sulfurreducens*, much is yet to be unraveled. Further research might consider optimizing some of the experiments described in this work, such as extending the duration of experiments, to examine long-term interactions. Likewise, the fumarate-free phase of the bioreactor experiment could be repeated using longer turnover times, to re-evaluate the persistence of the bacteria in the tanks. In addition, the effects of MB on the coculture could be tested at lower concentrations to reduce any potential toxicity (Rahimnejad et al., 2011). To better track the growth of both microorganisms, the use of a method to easily separate the bacterial and yeast biomass would be recommended. Flow cytometry could be considered, possibly using fluorescent dyes for the bacteria to distinguish them from any background noise (Schiavone et al., 2024). An additional experiment that might provide a deeper insight into the relationship between the microorganisms would be the use of different electron acceptors, such as iron oxide, manganese or uranium (T. Lin et al., 2017; Methé et al., 2003). The lack of *Geobacter* growth under these conditions could support the need for succinate shuttling between the species for the coculture to thrive. In later stages, a transcriptomic analysis could also be used to reveal changes in gene expression patterns. The upregulation of c-type cytochromes, type IV pili or other proteins involved in DIET could be key in understanding the mechanisms of interaction (Allen et al., n.d.; Uekia, 2021). Furthermore, to test the viability of the new hypothesis, *S. cerevisiae*'s ability to uptake succinate in presence and absence of *G. sulfurreducens* should be examined. If *G. sulfurreducens* did trigger a response in the yeast, a next step would involve testing whether it requires physical contact. For this, experiments membrane separation experiments could be prepared. These might also be useful to determine whether DIET or MIET mechanisms are taking place. Finally, while the current findings indicate that the relationship between the microbes is of parasitic nature, it is possible that potential beneficial effects on the yeast have not been uncovered in this study and should be further explored.

Some of the findings in this study show the potential future biotechnological applications of the *G. sulfurreducens* and *S. cerevisiae* cocultures. Nowadays, malic acid is widely used in the food, chemical and pharmaceutical industries. However, it is currently primarily obtained from petroleum via an expensive and is non-sustainable process (Wei et al., 2021). Microbial fermentation processes are gaining attention as an alternative, with research focusing strain engineering to increase production rates (Kang et al., 2022; Sun et al., 2023). Nevertheless, the accumulation of malate observed in the cocultures throughout this study illustrates a promising alternative route for malate production, offering potential future industrial applications.

6. CONCLUSION

This study confirmed the existence of an interplay between *S. cerevisiae* and *G. sulfurreducens* by successfully establishing cocultures, which proliferated through multiple transfer rounds. While the exact mechanism driving this interaction remains to be proven and further explored, the experimental results obtained in this work suggest a more complex dynamic between the microorganisms than initially hypothesized. The existence of a direct extracellular electron transfer was not confirmed, but there appeared to be an exchange of organic intermediates between the species. It is proposed that the acetate produced by *S. cerevisiae* is used by *G. sulfurreducens* as the electron donor, converting fumarate to succinate. The presence of *G. sulfurreducens* appears to trigger a response in the yeast, that allows for *S. cerevisiae* to uptake the newly produced succinate from the media, resulting in the continued production of ethanol, malate and fumarate. These findings imply that *G. sulfurreducens* and *S. cerevisiae* could be involved in a parasitic relationship, where *Geobacter* exploits the yeast's resources for growth, causing *S. cerevisiae*'s metabolism to slow down. However, the observations made in this work are very preliminary, and further research is required to gain deeper insight into the mechanisms behind of the interaction between the microorganisms. A deeper understanding of this dynamic could lead to future biotechnological or industrial applications, such as the production of valuable compounds like malic acid.

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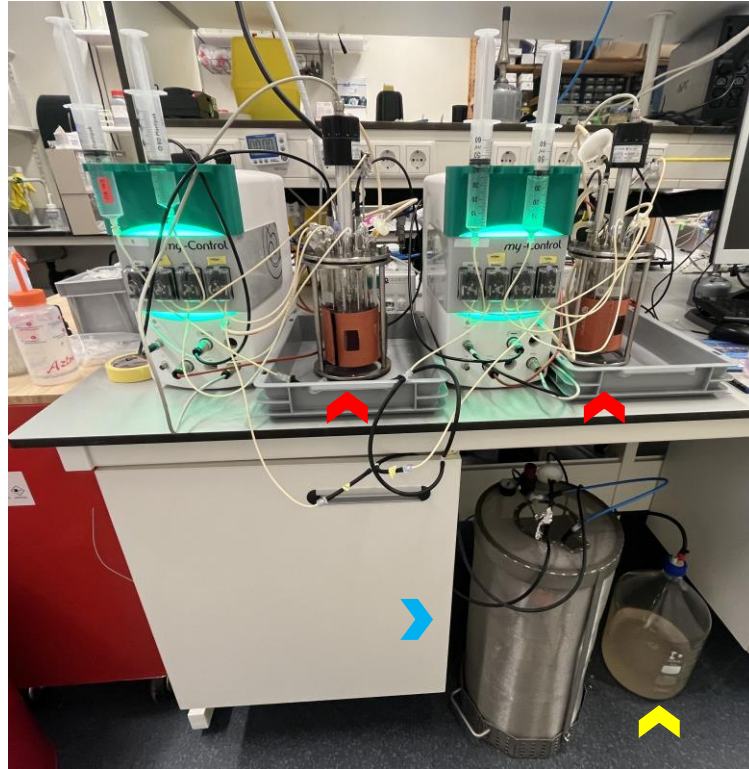
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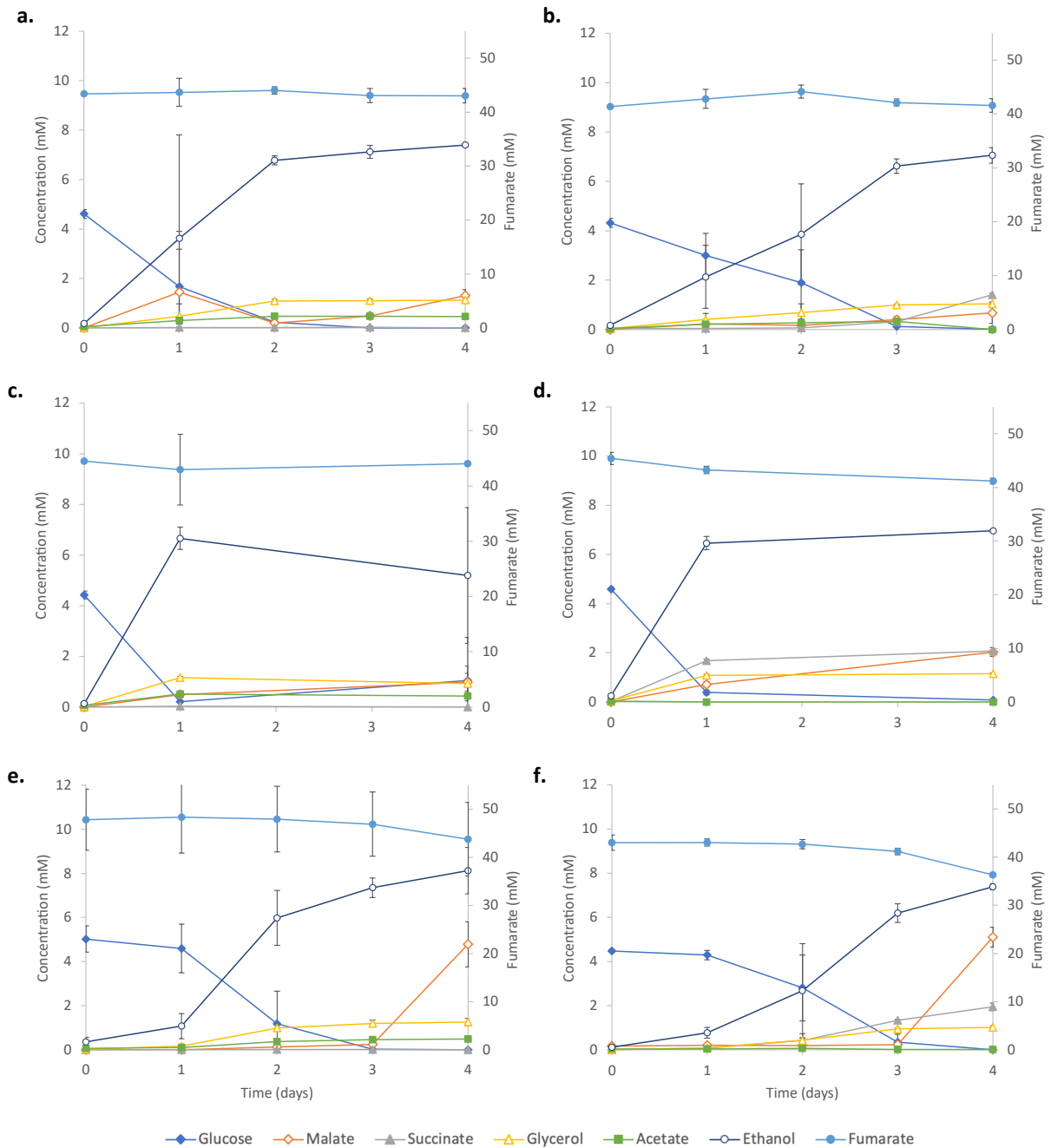
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8. APPENDIX

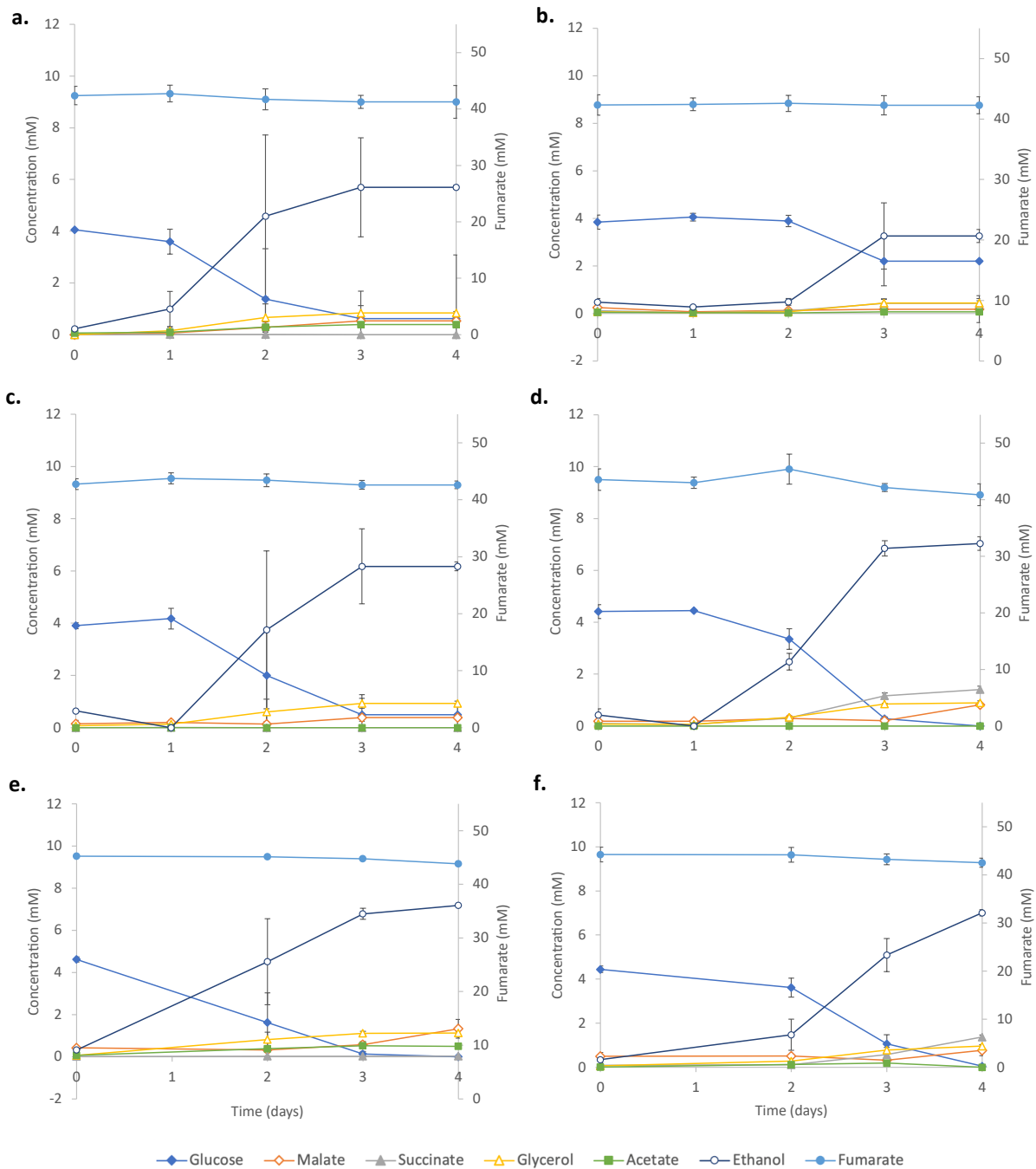
8.1 Supplementary Figures



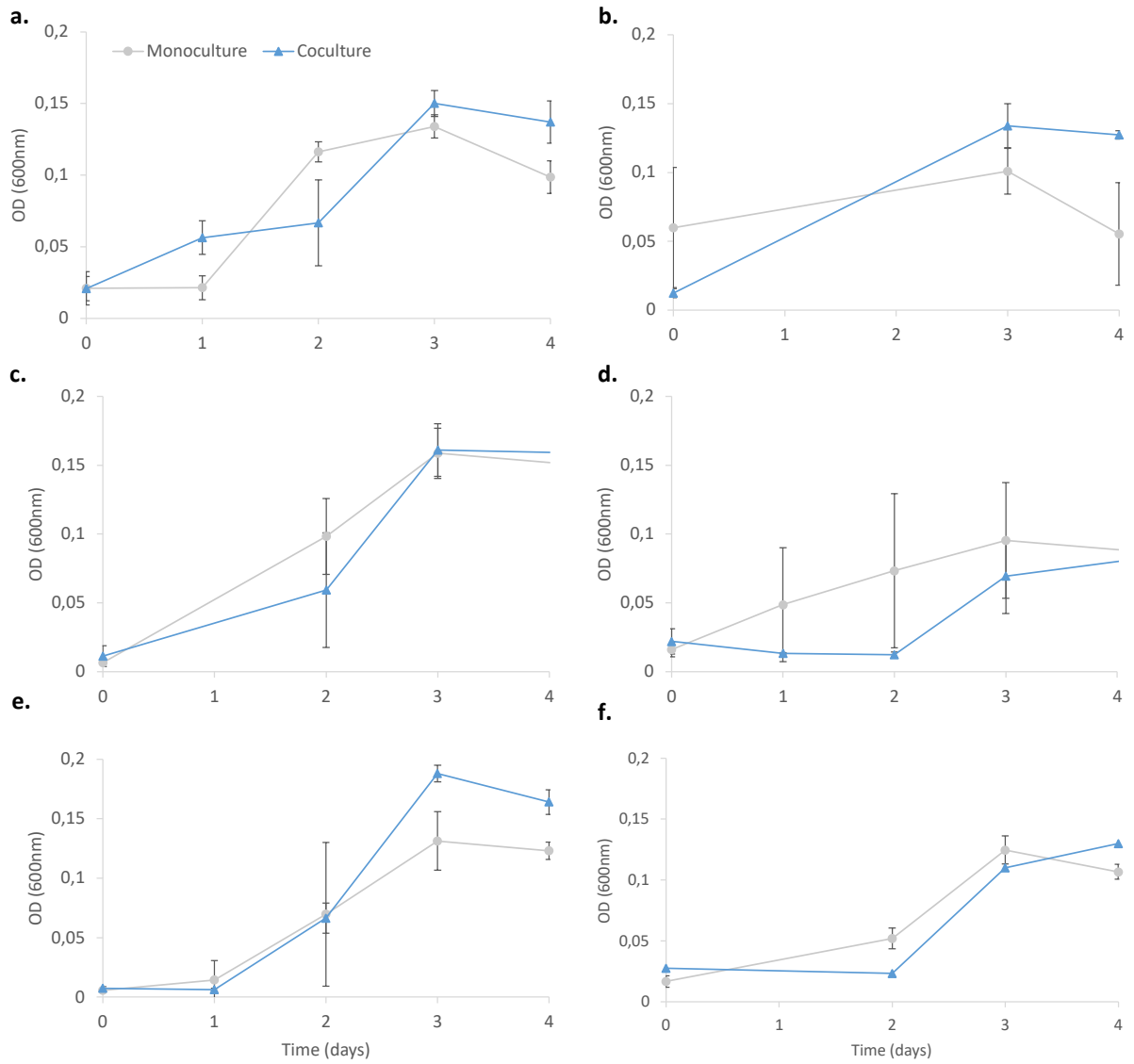
Supplementary figure 1. Bioreactor experimental setup. In red are shown the *S. cerevisiae* monoculture (left) and coculture (right) bioreactors. Blue arrow shows the inflow tank, located inside the metal cage for safety. Yellow arrow indicates the outflow tank.



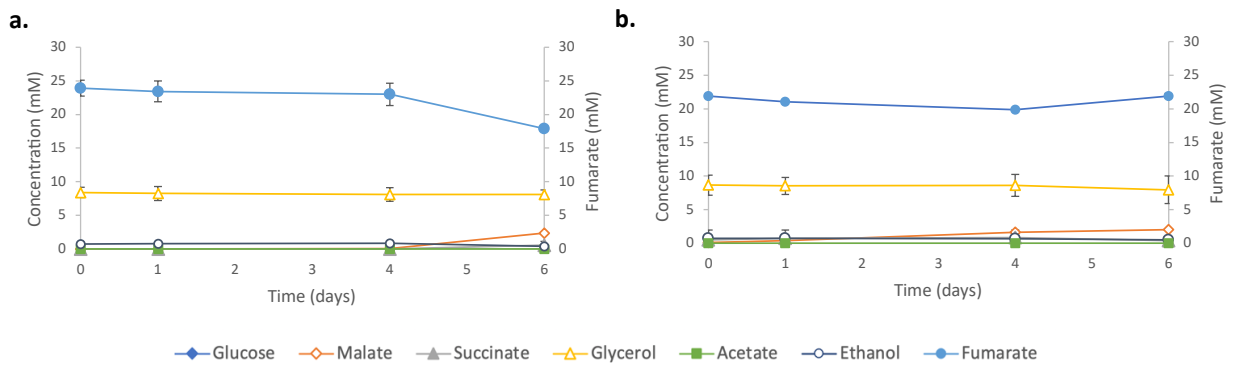
Supplementary figure 2. Metabolite variations in T1 – T3 mono and co-cultures. Linear graphs represent changes in metabolite composition over time in T1 monocultures (a), T1 cocultures (b), T2 monocultures (c), T2 cocultures (d), T3 monocultures (e), T3 cocultures (f). Monocultures contain *S. cerevisiae*. Cocultures contain *S. cerevisiae* and *G. sulfurreducens*. Data points show average concentrations (\pm SD) of triplicates at each time point.



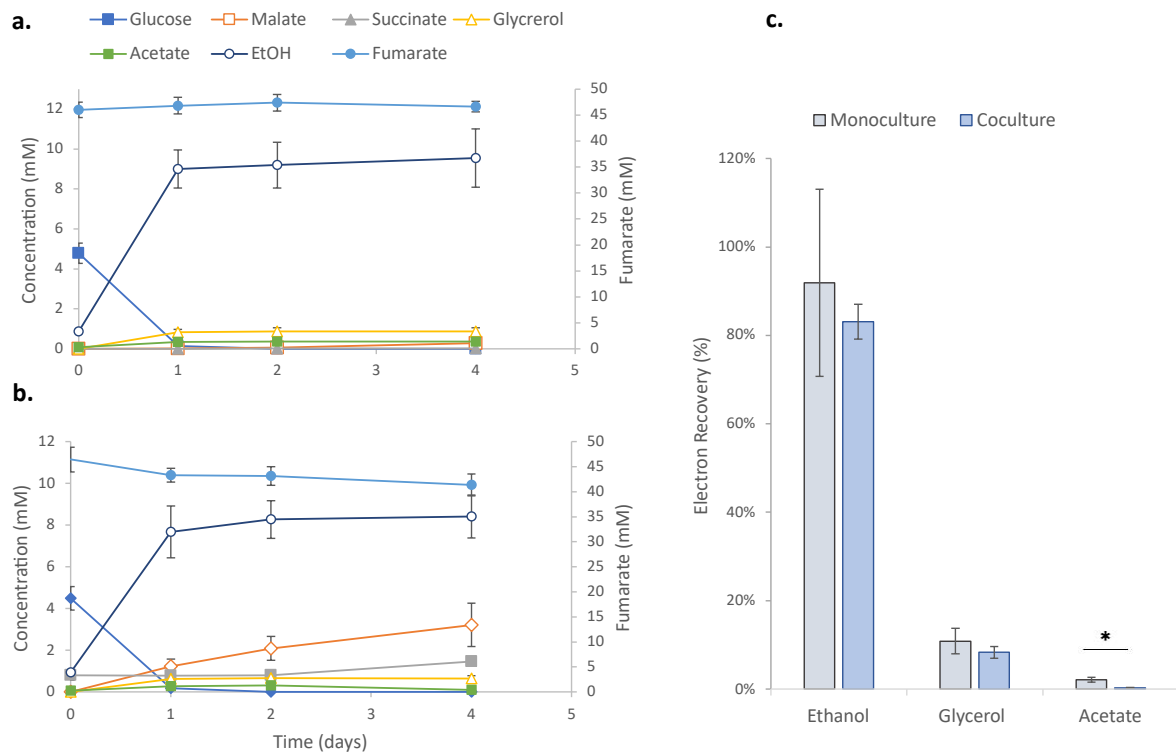
Supplementary figure 3. Metabolite variations in T4 – T6 mono and co-cultures Linear graphs represent changes in metabolite composition over time in T4 monocultures (a), T4 cocultures (b), T5 monocultures (c), T5 cocultures (d), T6 monocultures (e), T6 cocultures (f). Monocultures contain *S. cerevisiae*. Cocultures contain *S. cerevisiae* and *G. sulfurreducens*. Data points show average concentrations (\pm SD) of triplicates at each time point.



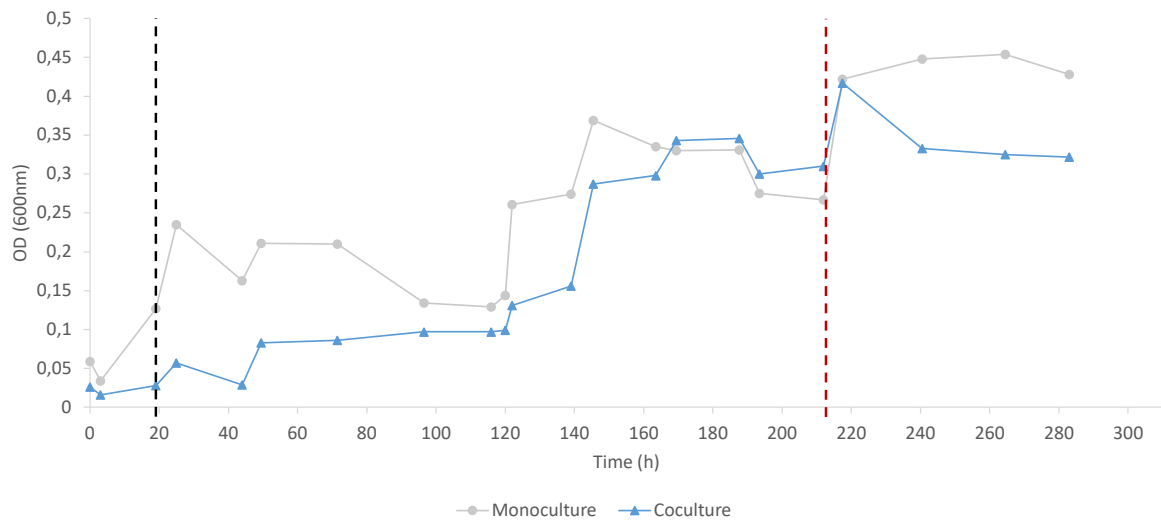
Supplementary figure 4. OD curves for all transfers. T1 (a), T2 (b), T3 (c), T4 (d), T5 (e), T6 (f). Data points show average OD measurements (\pm SD) of triplicates.



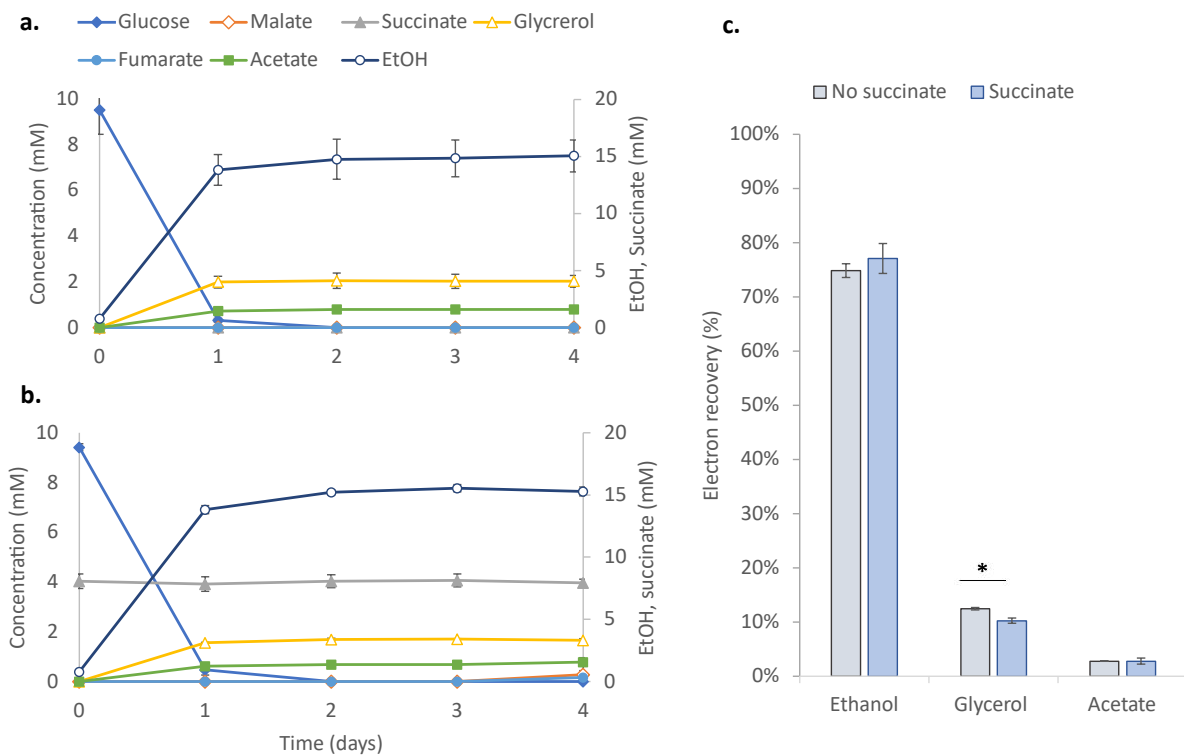
Supplementary figure 5. Growth of cocultures in glycerol. Linear graphs show changes in metabolite composition in the media containing glycerol as substrate. **a.** *S. cerevisiae* monoculture. **b.** *G. sulfurreducens* and *S. cerevisiae* coculture. Data points show average concentrations (\pm SD) of triplicates at different time points.



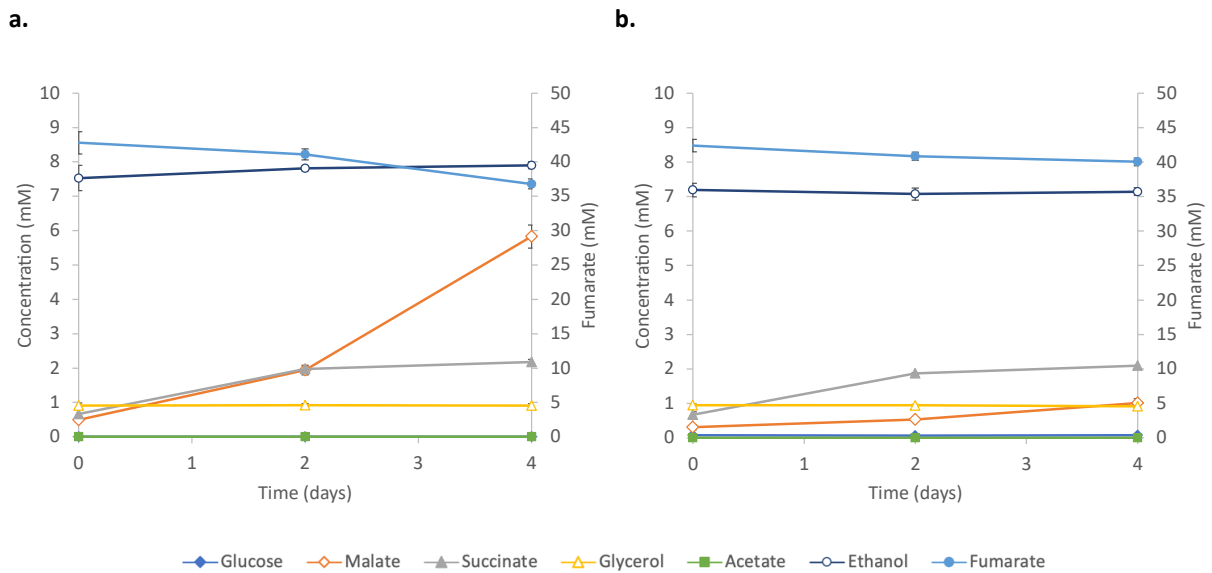
Supplementary figure 6. Growth of the cultures upon removal of CO₂. Linear graphs show changes in metabolite composition of cultures grown under 100% N₂. **a.** *S. cerevisiae* monoculture. **b.** *G. sulfurreducens* and *S. cerevisiae* coculture. **c.** Percentage of electrons recovered by the products. Data points show average concentrations (\pm SD) of triplicates at different time points. Asterisk indicates statistical significance ($p < 0.05$)



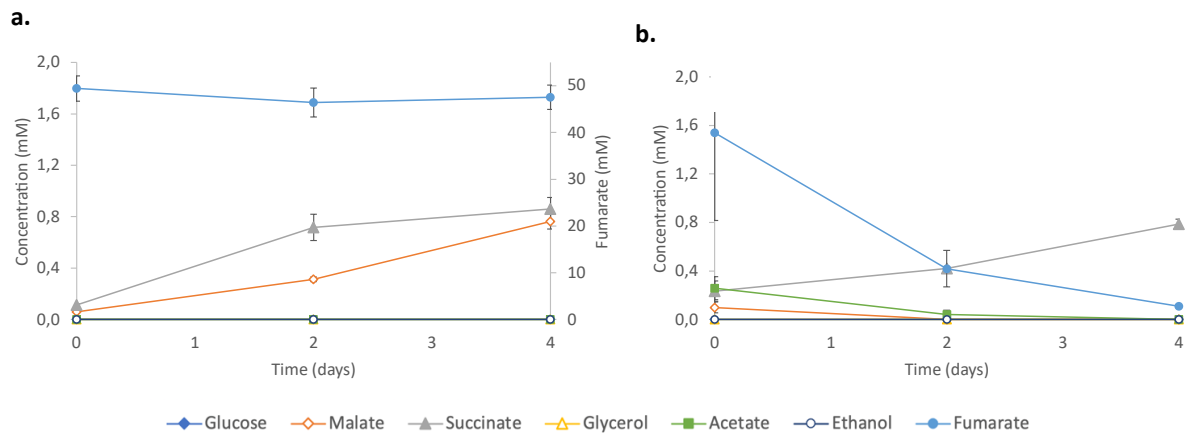
Supplementary figure 7. Bioreactor OD measurements. Linear graph shows OD measurements at 60nm over time of the monoculture and coculture bioreactor samples. Black line indicates beginning of chemostat stage. Red line shows removal of fumarate from the media tank.



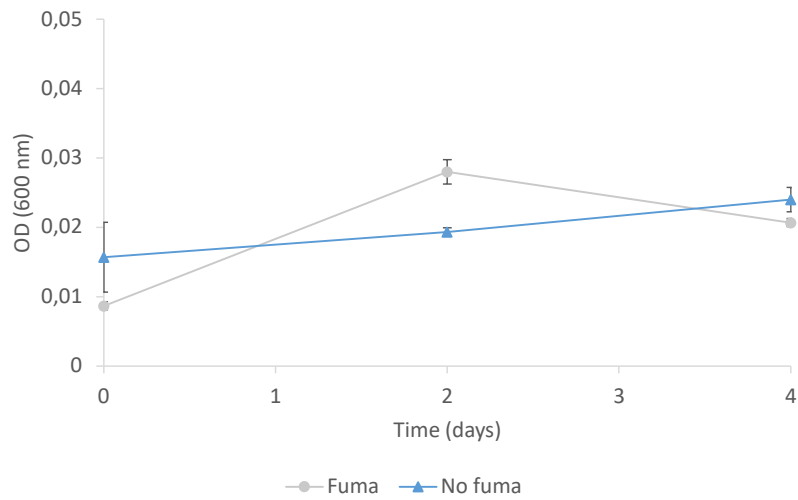
Supplementary figure 8. *S. cerevisiae* monoculture ability to uptake succinate. Linear graphs show changes in metabolite composition in *S. cerevisiae* monocultures containing **a.** no succinate. **b.** 5mM succinate. **c.** Percentage of electrons recovered. Data points show average concentrations (\pm SD) or percentages (\pm SD) of triplicates at different time points. Asterisk indicates statistical significance ($p < 0.05$)



Supplementary figure 9. Coculture growth with dead and inactive *S. cerevisiae* cells. Linear graphs show changes in metabolite composition of the media in cocultures with *G. sulfurreducens* and *S. cerevisiae* either with no glucose available (a), or dead by autoclavation (b). Data points show average concentrations (\pm SD) of triplicates at different time points.



Supplementary figure 10. *G. sulfurreducens* growth on only fumarate. Linear graphs show changes in metabolite composition in the media of *G. sulfurreducens* monocultures containing **a.** 50mM fumarate, or **b.** no fumarate. Data points show average concentrations (\pm SD) of triplicates at different time points.



Supplementary figure 11. OD measurements for *G. sulfurreducens* monocultures with only fumarate. Linear graphs show OD measurements at 600 nm. Growth of *G. sulfurreducens* in monocultures with and without fumarate (40 mM) are compared. Data points show average concentrations (\pm SD) of triplicates at different